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**Selenium and vitamin E interaction in the nutrition of yellowtail kingfish (*Seriola lalandi*):**

**Physiological and immune responses**

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Running title: Dietary selenium and vitamin E in yellowtail kingfish

**KEY WORDS:** Yellowtail kingfish, selenium, vitamin E, interaction, deficiency, physiological responses

## **Abstract**

Six dietary treatments were prepared in a  $3 \times 2$  factorial design (un-supplemented or supplemented with Se at 1 or 2 mg kg<sup>-1</sup> × supplemented with vitamin E at 40 or 180 mg kg<sup>-1</sup>) and fed to yellowtail kingfish (*Seriola lalandi*) for 6 weeks to evaluate the effects of dietary Se and vitamin E on growth performance, immune and antioxidant responses and to investigate the potential interaction between these two micronutrients in this species. The results have revealed significantly interactive effects with positive physiological responses. Se significantly increased weight gain of fish fed diets low in vitamin E, but not high in vitamin E; whereas serum lysozyme activity was significantly improved by Se in diets high in vitamin E, but not low in vitamin E. Moreover, there was evidence of myopathy in fish deficient in both Se and vitamin E, but not single deficiency. There was no significant effect of dietary Se, vitamin E or their interaction on survival, antibody titer, feed intake, feed conversion ratio and fillet proximate composition; however, fillet Se and vitamin E were significantly responsive to dietary Se and vitamin E respectively, and higher dietary Se intakes produced significantly higher red blood cell glutathione peroxidase activity.

## **Introduction**

The close metabolic interrelation between selenium (Se) and vitamin E was first recognized in mammals in 1957 (Schwarz & Foltz 1957). Se and vitamin E act as biological antioxidants to protect cell membranes from oxidative damage (Rotruck *et al.* 1973). Se is an essential trace element required by all animals including fish (NRC, 1993). In fish, the interaction of dietary Se and vitamin E was first studied in Atlantic salmon (*Salmo salar*) in 1976 (Poston *et al.* 1976). Since then more studies on the effects of Se and vitamin E deficiency on fish production and the benefits of dietary supplementation of both these micronutrients have been reported (Hilton *et al.* 1980; Bell *et al.* 1985; Gatlin *et al.* 1986; Wise *et al.* 1993a; Jaramillo *et al.* 2009). These studies have revealed mixed and varied responses to Se and vitamin E among various fish species (Bell *et al.* 1985; Wise *et al.* 1993a; Jaramillo *et al.* 2009). However, yellowtail kingfish (*Seriola lalandi*), one of the commercially important fish species, which can be grown in sea cages as well as on-shore recirculation systems

(Poortenaar *et al.* 2001; Chen *et al.* 2006; Pirozzi & Booth 2009; Abbink *et al.* 2012), has not been subjected to any research regarding dietary Se and vitamin E. Se requirement for this fish is not known and there is no published information on the interrelationships between Se and vitamin E in yellowtail kingfish nutrition.

Previous research has shown that diets deficient in Se and vitamin E cause muscle myopathy in some fish species (Poston *et al.* 1976; Blazer & Wolke 1984; Bell *et al.* 1985; Gatlin *et al.* 1986; Bell *et al.* 1987), thus it is worthwhile examining histological signs of Se and vitamin E deficiencies in yellowtail kingfish. Other important parameters, which can assist in understanding the effects of Se and vitamin E on fish health, are immune and antioxidant responses (Hardie *et al.* 1990; Atencio *et al.* 2009; Rider *et al.* 2009; Betancor *et al.* 2012).

Antibody response is sensitive to changes in Se and/or vitamin E status and has been used as a tool to evaluate the effect of these two micronutrients on immune status (Finch & Turner 1996). As dietary concentration of Se increased, antibody response of channel catfish (*Ictalurus punctatus*) increased (Wang *et al.* 1997), while a diet deficient in vitamin E has been reported to suppress antibody response of rainbow trout (*Salmo gairdneri*) (Blazer & Wolke 1984). Furthermore, supplementation with both nutrients was more effective than each single micronutrient in raising the antibody responses of chickens (Marsh *et al.* 1981), pigs (Peplowski *et al.* 1980) and horses (Baalsrud & ØVernes 1986).

Another tool that has been used as an indicator of immune status is lysozyme activity (Paulsen *et al.* 2001; Bowden *et al.* 2004; Staykov *et al.* 2007; Rider *et al.* 2009). Lysozyme has been detected in mucus, serum, organs and ova of fish (Murray & Fletcher 1976; Ourth 1980; Yousif *et al.* 1991). It plays an important role in the non-specific immune response in fish as a natural defence against microorganisms, particularly bacteria (Fletcher & White 1976; Yousif *et al.* 1991; Paulsen *et al.* 2001). The biological function of this enzyme is to attack the peptidoglycan layer of bacterial cell walls, resulting in bacterial cell lysis (Bachali *et al.* 2002). In fish, the effect of Se on lysozyme activity has been studied in rainbow trout (*Oncorhynchus mykiss*) (Rider *et al.* 2009).

Glutathione peroxidase (GPx) is one of the most important antioxidant defence enzymes in fish (Filho 1996; Ross *et al.* 2001) and its activity is dependent on dietary Se intake (Ganther *et al.* 1976). Therefore, GPx activity has been used to study antioxidative effects of dietary Se in fish (Poston *et al.* 1976; Hilton *et al.* 1980; Bell *et al.* 1987; Wise *et al.* 1993a; Atencio *et al.* 2009).

Selenium exists in two forms, organic Se and inorganic Se. Organic sources have been found to be more effective than inorganic sources in improving growth and immune responses (Kumar *et al.* 2009) and in maintaining antioxidant defence systems more efficiently than inorganic sources (Mahmoud & Edens 2003). This could be explained by differences in metabolism of the two sources of Se. Organic Se is readily absorbed through the gut (Lyons *et al.* 2007) and better absorbed than inorganic Se (Daniels 1996). In fish, superior bioavailability of organic over inorganic sources of Se has been reported for Atlantic salmon (Lorentzen *et al.* 1994), channel catfish (Wang & Lovell 1997), crucian carp (*Carassius auratus gibelio*) (Wang *et al.* 2007) and hybrid striped bass (*Morone chrysops* × *M. saxatilis*) (Jaramillo *et al.* 2009). Recently, therefore, organic Se has been chosen as the preferred source of Se supplementation in fish feed (Rider *et al.* 2009).

Nutritional information of Se and vitamin E on other fish species is available (Poston *et al.* 1976; Hilton *et al.* 1980; Cowey *et al.* 1981; Bell *et al.* 1985; Gatlin *et al.* 1986; Hardie *et al.* 1990; Wise *et al.* 1993a; Wise *et al.* 1993b; Jaramillo *et al.* 2009), but it is unsure whether this information is directly applicable to yellowtail kingfish. Yellowtail kingfish are relatively faster growing than other fish species on which Se and vitamin E studies have been conducted, therefore they subsequently may have a higher requirement. The objectives of this study were to investigate the effects of variation in dietary content of organic Se and vitamin E on growth, immune and antioxidant responses and histological signs of Se and vitamin E deficiencies, and examination of interactions of these two micronutrients in yellowtail kingfish. Antibody and lysozyme activity were used as tools to evaluate immune responses; and GPx activity was used as an indicator of antioxidant response of yellowtail kingfish.

## Materials and methods

### *Chemicals*

Chemicals used were analytical grade obtained from Thermo Fisher Scientific, Scoresby, VIC, Australia, unless otherwise stated.

### *Experimental diets and design*

All experimental work was performed according to the Australian Code of Practice for the care and use of animals for scientific purposes. A  $3 \times 2$  factorial experiment was arranged, in which a basal diet was either supplemented or not supplemented with Se (0 or 1 or 2 mg kg<sup>-1</sup>) at each of two supplemental levels of vitamin E (40 or 180 mg kg<sup>-1</sup>). A basal mash of a commercially available yellowtail kingfish diet (Marine CST, Ridley AgriProducts, Melbourne, VIC, Australia) without addition of any vitamin or mineral premix was used to prepare the experimental diets. This mash was extruded into 3 mm pellets at the Australasian Experimental Stockfeed Extrusion Centre (AESEC), Adelaide, SA, Australia. Following extrusion, the necessary quantity of organic Se from Se-yeast (Selplex®, Alltech, Nicholasville, KY, USA), vitamin E (Vitamin E, Animal Health Solution, Perth, WA, Australia) and 10 g kg<sup>-1</sup> of a vitamin mineral premix without Se and vitamin E (Specialty Feeds, Perth, WA, Australia) were top coated to the experimental pellets with gelatine (Davis Gelatine, Christchurch, New Zealand) to form the 6 experimental diets (Table 1). The supplemental Se levels were based on the benefit of organic Se for African catfish (*Clarias gariepinus*) (Abdel-Tawwab *et al.* 2007) and rainbow trout (Rider *et al.* 2009). The supplemental levels of vitamin E was based on established requirement for other *Seriola* species, Japanese yellowtail (*Seriola quinqueradiata*) (Masumoto 2002).

Yellowtail kingfish were hatched and reared at the Australian Centre for Applied Aquaculture Research, Fremantle, WA, Australia, where the experiment was conducted. The fish were individually weighed and stocked into each of 18 experimental 200 L-tanks at a density of 20 fish tank<sup>-1</sup>. Total weight of fish in each tank was  $949.11 \pm 0.60$  g (mean  $\pm$  SE), with an average individual weight of

47.45 ± 0.07 g (mean ± SE). The tanks were supplied with flow-through seawater (35 g L<sup>-1</sup>) at a rate of approximately 0.86 L min<sup>-1</sup> producing 600% water exchange day<sup>-1</sup>. The water was continuously aerated and supplied with pure oxygen using an oxygen generator (Oxair Gases, Oxair, Perth, WA, Australia). Water temperature, pH and dissolved oxygen were measured daily using a digital pH/mV/°C meter (Cyberscan pH 300, Eutech Instruments, Singapore) and an oxygen meter (Handy Polaris, OxyGuard, Birkerød, Denmark), respectively. During the trial, water temperature, pH and dissolved oxygen were maintained at (mean ± SD) 20.9 ± 0.3 °C, 7.6 ± 0.1 and 6.5 ± 0.4 mg L<sup>-1</sup>, respectively.

Each of 6 dietary treatments was randomly assigned to 3 tanks, making triplicate tanks diet<sup>-1</sup>. Fish were fed to apparent satiation once at 10 am daily for 6 weeks. At the end of week 2 and week 4, 5 fish were randomly removed and sacrificed from each tank to keep acceptable biomass in each tank and to be used for measurement of serum agglutinating antibody titer.

#### *Proximate analyses of experimental diets and fish fillets*

At the end of the feeding trial, three euthanized fish from each tank were filleted and the muscle was kept at – 20 °C before being assayed for Se and vitamin E accumulation and proximate composition. Proximate analyses of the experimental diets were conducted in triplicate. Gross energies were determined using a bomb calorimeter (C2000, IKA, Staufen, Germany). Protein, lipid, ash and dry matter were determined according to the standard methods of the AOAC (1990): crude protein (Method No. 954.01) by analysis of nitrogen using the Kjeldahl method with Kjeltac Auto 1030 analyser (Tecator, Höganäs, Sweden); lipid (954.02) by petroleum ether extraction using a Soxtec System (2055 Soxtec Avanti, Foss Tecator, Höganäs, Sweden), dry matter (934.01) by drying at 105 °C in an oven (Thermotec 2000, Contherm Scientific, Hutt, Newzealand) to a constant weight and ash (942.05) by combustion at 550 °C for 24 h in an electric furnace (Carbolite, Sheffield, UK).

For the analysis of Se, sample was digested in mixture of nitric and perchloric acid (10:3) using a block digestion system (AIM 500-C, A.I. Scientific, Sydney, NSW, Australia). The digest was then

further extracted into water and acid (40% HCl, for conversion of Se<sup>6+</sup> to Se<sup>4+</sup>). Digested sample was used for the estimation of Se (986.15) (AOAC, 1990) using an atomic absorption spectrometer (AAS) (Varian AA280 FS) equipped with vapour generation assembly (Varian VGA 77) (Mulgrave, VIC, Australia).

Vitamin E was analysed by the liquid chromatographic method (DeVries & Silvera 2002). Ground sample (1g) was placed into a glass stoppered test tube with 1 mL of ultrapure H<sub>2</sub>O. 10 mL of 6% pyrogallol in ethanol was added into the sample tube. The sample was dispersed by an Ultra-turrax homogeniser (T-25, IKA, Staufen, Germany). It was then saponified at 70 °C using KOH (60%) and extracted by vortexing after the addition of 20 mL of Hexane. 5 mL of the Hexane extraction was concentrated by evaporation and reconstituted in 0.5 mL of 0.1% butylated hydroxyl toluene in methanol giving a final dilution factor of 80. The automated high performance liquid chromatography unit (Hewlett Packard series 1100 HPLC system, Waldbronn, Germany and HP chemstation software, Avondale, CA, USA) equipped with a fluorescence detector was used for the quantitative determination of vitamin E (extracted  $\alpha$ -tocopherol).

#### *Survival, feed conversion ratio and growth measurement*

Mortality and the amount of feed eaten were recorded daily to calculate survival and feed intake, respectively. Fish in each tank were group weighed after 2 weeks and 4 weeks of the feeding trial and individually weighed at the end of the experiment to estimate weight gain. Weight measurement and feed intake were used for estimation of feed conversion ratio (FCR, feed intake divided by wet weight gain).

#### *Blood and serum collection*

At the end of the feeding trial, 3 fish from each of the three replicate tanks were randomly selected and anaesthetized with tricaine methanesulfonate (MS-222, Sigma-Aldrich, Castle Hill, NSW, Australia) at 100 mg L<sup>-1</sup>. Blood was sampled from the caudal vein and allowed to clot for 2 h at 4 °C. Serum was collected by centrifugation of whole blood at 1,500 × g for 10 min at 4 °C using a



centrifuge (5804R, Eppendorf, Hamburg, Germany). Serum was used for agglutinating antibody titer and lysozyme assays. The red blood cell pellet was used for glutathione peroxidase assay. All samples were kept at -80 °C until analysis.

#### *Agglutinating antibody titer assay*

At the end of week 4 of the feeding trial, all fish were given an intraperitoneal injection of 0.1 mL sterile phosphate buffer saline (PBS, pH 7.2) containing 30 µg of purified bovine serum albumin (BSA, CAS No. 9048-46-8, Sigma-Aldrich, New Zealand) as an antigen. Fish sera collected at week 4 and at the end of the trial were used for antibody assay. Agglutinating antibody titer to BSA was determined by the method of Chen & Light (1994) and reported as the last dilution of the serum which caused clumping of the antigen and transformed to  $\log_{10}$  values for statistical analysis.

#### *Lysozyme assay*

Lysozyme activity was measured using the turbidimetric assay in a 96-well micro-plate as described previously (Bowden *et al.* 2004). Briefly, 50 µL of serum was pipetted, in duplicate, in a 96-well plate (Iwaki, Tokyo, Japan). To each well was added 50 µL of *Micrococcus lysodeikticus* (Sigma-Aldrich, St. Louis, MO, USA) suspended in PBS (0.25 mg mL<sup>-1</sup>). The plate was monitored for absorbance at 450 nm every 2 min for a total of 20 min with a MS212 reader (Titertek Plus, Tecan, Austria). One unit of lysozyme activity is defined as the amount of enzyme resulting in a decrease in absorbance of 0.001 min<sup>-1</sup>.

#### *Red blood cell glutathione peroxidase assay*

This assay was based on that of Paglia and Valentine (1967). Glutathione peroxidase (GPx) catalyses the oxidation of glutathione by cumene hydroperoxide. In the presence of glutathione reductase and NADPH, the oxidized form of glutathione is immediately converted to the reduced form with the concomitant oxidation of NADPH to NADP<sup>+</sup>. The decrease in absorbance at 340 nm is then measured at 37 °C. GPx activity in red blood cell pellet was analysed by using the Ransel RS-505 kit (Randox, Crumlin, County Antrim, UK) and an Olympus AU400 chemistry immune analyser (Tokyo, Japan).

The results were expressed as units of GPx g<sup>-1</sup> of haemoglobin (Hb). Haemoglobin was measured using the Hb HG-1539 kit (Randox, Crumlin, County Antrim, UK).

#### *Histopathology examination*

At the end of the trial, left anterior dorsal muscle and anterior intestine from 3 euthanized fish tank<sup>-1</sup> (after blood collection) were dissected out and prepared according to routine techniques (Refstie *et al.* 2010) for histological examination. The samples were fixed in 10% buffered formalin, dehydrated in ethanol before equilibration in xylene and embedded in paraffin wax. Sections of approximately 5 µm were cut and stained with haematoxylin and eosin, and observed under an Olympus (BX40F4, Tokyo, Japan) light microscope.

#### *Data analysis*

Data were analysed using PASW Statistics 18.0 (IBM Corporation, New York, USA). Survival data were arcsine transformed prior to analysis. A two-way ANOVA was carried out to test the dietary effects of Se and vitamin E on growth and a number of other indices and to elucidate any interrelation between them. When a significant interaction was detected between the main effects, the variable was analysed using a one-factor ANOVA. Nutrient data were subjected to a one-way ANOVA. When a significant treatment effect was observed, Tukey's honest significant difference test was used for multiple mean comparisons. The statistical significance was set at  $P < 0.05$  and the results were presented as means  $\pm$  SE (standard error).

## **Results**

#### *Nutrient analyses of the experimental diets*

The proximate compositions of the experimental diets contained proximately 466.72 g kg<sup>-1</sup> crude protein, 151.90 g kg<sup>-1</sup> crude lipid, 913.08 g kg<sup>-1</sup> dry matter, 96.63 g kg<sup>-1</sup> ash and provided 21.77 MJ kg<sup>-1</sup> energy (Table 1). All nutrients other than Se and vitamin E did not differ significantly ( $P > 0.05$ ) among diets.

### *Agglutinating antibody titer to bovine serum albumin and growth performance*

All sampled fish at week 4 were negative to bovine serum albumin (BSA). Antibody titers to BSA of fish at the end of the trial were presented in Table 2. Agglutinating antibody titer to BSA of fish in this study was not significantly correlated ( $P > 0.05$ ) with the variation in dietary contents of Se and vitamin E.

There was no significant effect of dietary Se and vitamin E or their interaction on fish weight gain in the first 4 weeks of the feeding trial. However, a significant interactive effect ( $P < 0.05$ ) between the two micronutrients was found after being fed for 6 weeks (Table 2). Se significantly increased weight gain of fish fed diets low in vitamin E, but not high in vitamin E. Fish fed diet 3 supplemented with Se at  $2 \text{ mg kg}^{-1}$  gained significantly ( $P < 0.05$ ) more weight than fish fed diet 1 without Se supplementation,  $73.48 \pm 2.73$  and  $55.57 \pm 2.18 \text{ g fish}^{-1}$ , respectively.

Neither dietary Se nor vitamin E significantly affected ( $P > 0.05$ ) feed intake and feed conversion ratio (FCR) (Table 2). Survival of fish was near 100% and was also unaffected by any dietary treatments (Table 2).

Fish fillet protein, lipid, dry matter, ash and gross energy were not significantly ( $P > 0.05$ ) influenced by dietary Se, vitamin E or their interaction (Table 3). However, fillet Se concentrations significantly increased ( $P < 0.05$ ) by supplemental levels of Se (Table 3), from  $0.35 \pm 0.01$  to  $0.51 \pm 0.01$  and  $0.65 \pm 0.02 \text{ mg kg}^{-1}$  when Se supplemented at 1 and 2  $\text{mg kg}^{-1}$  diet, respectively. Similarly, vitamin E concentrations in fillet significantly increased with an increase in the dietary level (Table 3). The diets supplemented with 180  $\text{mg vitamin E kg}^{-1}$  produced fillet vitamin E of  $5.27 \pm 0.08 \text{ mg kg}^{-1}$ , significantly higher ( $P < 0.05$ ) than vitamin E content of  $3.31 \pm 0.06 \text{ mg kg}^{-1}$  in the fillet of fish fed the diets supplemented with 40  $\text{mg vitamin E kg}^{-1}$ .

### *Lysozyme activity*

Serum lysozyme activity was significantly affected ( $P < 0.05$ ) by dietary Se, vitamin E and the interaction of these two factors. As Se increased, the lysozyme activities of fish fed diets high in vitamin E increased, but the lysozyme activities of fish fed diets low in vitamin E remained unchanged (Fig. 1).

Fish fed diets supplemented with Se at  $2 \text{ mg kg}^{-1}$  had significantly higher ( $P < 0.05$ ) mean enzyme activity ( $192.33 \pm 30.84 \text{ enzyme units mL}^{-1}$ ) than those given diets without Se supplementation ( $156.66 \pm 16.92 \text{ enzyme units mL}^{-1}$ ). However, it should be noted that this was due to the lysozyme activity of fish fed diets high in vitamin E. In low vitamin E diets, the mean lysozyme activities of fish fed different levels of Se were the same.

The mean enzyme activity in fish fed high vitamin E supplementation was significantly higher ( $P < 0.05$ ) than in those fed low vitamin E,  $222.22 \pm 11.09$  and  $127.77 \pm 6.90 \text{ enzyme units mL}^{-1}$ , respectively.

### *Red blood cell glutathione peroxidase activity*

There was no significant interaction ( $P > 0.05$ ) between dietary Se and vitamin E with respect to red blood cell glutathione peroxidase (GPx) activity. As dietary Se increased, the mean activity of GPx increased and was independent of vitamin E (Fig. 2). Supplementation of Se at  $2 \text{ mg kg}^{-1}$  resulted in the highest mean GPx activity at  $107.48 \pm 2.82 \text{ units g}^{-1} \text{ Hb}$ , while un-supplemented group produced the lowest mean at  $72.88 \pm 1.27 \text{ units g}^{-1} \text{ Hb}$ , with significant difference detected between the treatments ( $P < 0.05$ ).

### *Histopathology*

Histological examination by light microscopy showed that the dietary treatments had no effect on the anterior intestinal region of fish. All sections of intestine had large numbers of mucus cells and basophilic droplets in the lumen but there was no obvious difference between the treatments.

However, analysis of muscle sections revealed that there was multiphasic myopathy in fish fed diet 1, while those fed the other diets had no myopathy (Fig. 3).

## Discussion

The dietary effects of Se and vitamin E have been studied in other fish species and a complex range of interactive responses described in these target species (Poston *et al.* 1976; Bell *et al.* 1985; Gatlin *et al.* 1986; Wise *et al.* 1993a; Jaramillo *et al.* 2009). Some of these interactive effects were observed in yellowtail kingfish in the present study.

Zhou *et al.* (2009) reported a 30 day-feeding trial in which dietary Se positively affected growth of crucian carp (*Carassius auratus gibelio*). In the present study, neither Se nor vitamin E supplementation significantly affected growth of yellowtail kingfish in the first 4 weeks of the feeding trial. However, a significant interactive effect of Se and vitamin E on growth was found after being fed for 6 weeks. Growth of fish was improved by Se in the diets low in vitamin E, but not high in vitamin E. Vitamin E may act as a partial substitute and/or complement for the low Se by performing the similar function to Se and in maintaining growth (Webster & Lim 2002). In contrast, Se did not compensate lysozyme activity for the lack of vitamin E. In fish fed diets low in vitamin E, the activities of lysozyme were the same, and were not correlated with different levels of Se. At the higher supplemental level of vitamin E, however, the enzyme activity was significantly increased by dietary Se. These findings confirm the necessity of the two micronutrients in yellowtail kingfish diet. For another species of *Seriola*, Japanese yellowtail (*Seriola quinqueradiata*), vitamin E requirement for maximum growth is 119 mg kg<sup>-1</sup> (Masumoto 2002).

Similar to the finding of the present study, the interaction between dietary Se and vitamin E on growth has been reported for channel catfish (*Ictalurus punctatus*) (Gatlin *et al.* 1986) and rainbow trout (*Salmo gairdneri*) (Bell *et al.* 1985) in a 2 × 2 factorial study. However, this interactive effect on growth was not found for hybrid striped bass (*Morone chrysops* × *M. saxatilis*) in a similar 2 × 2 factorial arrangement (Jaramillo *et al.* 2009). This could be due to species-specific responses to Se

and vitamin E in growth performance. Growth of rainbow trout (*Oncorhynchus mykiss*) was not affected by dietary Se (Rider *et al.* 2009), but growth of crucian carp (Wang *et al.* 2007; Zhou *et al.* 2009) and African catfish (*Clarias gariepinus*) (Abdel-Tawwab *et al.* 2007) was increased by Se supplementation. Similarly, different fish species responded differently to dietary vitamin E in growth performance. Growth of Nile tilapia (*Oreochromis Niloticus*) (Ispir *et al.* 2011), African catfish (Baker & Davies 1996) and Atlantic salmon (*Salmo salar*) (Hardie *et al.* 1990) was independent of vitamin E (ranging from 0 to 240, 500 and 800 mg kg<sup>-1</sup>, respectively) although these fish were fed for 3 months, 70 days and 30 weeks respectively, growth of beluga (*Huso huso*), however, was significantly increased by supplemental vitamin E (from 25 to 200 mg kg<sup>-1</sup>) after 8 weeks of feeding (Amlashi *et al.* 2011).

Another interaction between dietary Se and vitamin E in yellowtail kingfish in this study was evident in histopathological sign of disease. Se and vitamin E may compensate for the lack of each other to prevent muscle myopathy. Myopathy occurred in the diet deficient in both Se and vitamin E, but not in single deficiency. Similarly, diets deficient in both Se and vitamin E caused severe myopathy in channel catfish, but single deficiencies did not (Gatlin *et al.* 1986). Diets with 0.26 mg kg<sup>-1</sup> Se and/or 52.5 mg kg<sup>-1</sup> vitamin E can help to prevent channel catfish from myopathy (Gatlin *et al.* 1986), but the required levels of Se and vitamin E were much higher for yellowtail kingfish to prevent myopathy in the present study, 4.32 mg kg<sup>-1</sup> and 179.23 mg kg<sup>-1</sup> respectively. Yellowtail kingfish may be more active and relatively faster growing than channel catfish and hence require higher Se and vitamin E input. For Atlantic salmon (*Salmo salar*) muscular dystrophy was prevented only when both Se and vitamin E were supplemented (Poston *et al.* 1976), however the levels of Se and vitamin E in Atlantic salmon diets were not measured. The reasons for the differing responses of different fish species to Se and vitamin E deficiency are unexplained.

Muscle Se concentrations of cultured yellowtail kingfish (between 0.34 ± 0.02 and 0.67 ± 0.04 mg kg<sup>-1</sup>) in the present study were higher than those of wild yellowtail kingfish (0.33 ± 0.01 mg kg<sup>-1</sup>, mean ± SE) (Chvojka 1988) and were shown to increase with increasing dietary Se supplementation level. In

the agreement with our results, Se concentrations in muscle of channel catfish (Gatlin & Wilson 1984) and gibel carp (*Carassius auratus gibelio*) (Han *et al.* 2011) increased as dietary Se supplementation level increased. Likewise, a similar pattern of increasing muscle vitamin E with increasing dietary vitamin E was seen in yellowtail kingfish and other fish species (Boggio *et al.* 1985; Frigg *et al.* 1990; Gatlin *et al.* 1992; Baker & Davies 1996).

Previous research has shown that Se and vitamin E affect fish survival, with combined deficiencies of both micronutrients causing high mortality in channel catfish after 26 weeks of feeding (Gatlin *et al.* 1986) and simultaneous supplementation of both significantly reduced the mortality of Atlantic salmon (*Salmo salar*) after being fed for 4 weeks (Poston *et al.* 1976). Conversely, survival of yellowtail kingfish was not affected by dietary Se and vitamin E in the present study. However, the deficient levels of Se and vitamin E in the channel catfish study were respectively 0.06 mg kg<sup>-1</sup> and 2.50 mg kg<sup>-1</sup>, which were much lower than those in the current study, while the actual levels of Se and vitamin E in the study of Atlantic salmon are unknown.

Se has been reported to affect the activity of glutathione peroxidase (GPx) in most fish species. The GPx activity in liver of cobia (*Rachycentron canadum*) (Liu *et al.* 2010) and grouper (*Epinephelus malabaricus*) (Lin & Shiau 2005) increased with an increase in the dietary Se intake. In addition, the GPx activity was shown to decrease in rainbow trout (Hilton *et al.* 1980), channel catfish (Gatlin *et al.* 1986; Wise *et al.* 1993a) and Atlantic salmon (Bell *et al.* 1987) fed diets deficient in Se. The study by Rider *et al.* (2009) showed that supplementation of Se did not affect liver GPx activity in un-stressed rainbow trout (*Oncorhynchus mykiss*), but did in stressed fish. A similar pattern of decreasing red blood cell GPx with decreasing Se was seen in the present study. The previous study on rainbow trout (Cowey *et al.* 1981) and our study have revealed that dietary vitamin E had no effect on the GPx activity. In agreement with the findings reported for rainbow trout (Bell *et al.* 1985) and channel catfish (Wise *et al.* 1993a), there was no interactive effect on GPx activity between Se and vitamin E in yellowtail kingfish.

Yellowtail kingfish in this study responded in the same way to the variation in dietary content of Se and vitamin E with respect to their antibody. Previous research has shown that simultaneous supplementation of Se and vitamin E increased the antibody responses of animals which were deficient in both nutrients (Peplowski *et al.* 1980; Marsh *et al.* 1981; Baalsrud & ØVernes 1986; Droke & Loerch 1989), but were less effective in animals which had previously been fed sufficient amounts of one or both nutrients (Hayek *et al.* 1989). The fish in the present study may have already received sufficient nutrients before the experiment and this can result in the same antibody responses of fish to antigen. Besides receiving sufficient nutrients, there are two possible things that can affect the efficiency of Se and vitamin E on antibody responses of an animal; the nature and concentrations of antigens used to stimulate the immune system. For example, supplementation of vitamin E improved the antibody responses of lambs to *Brucella ovis* but not to keyhole limpet hemocyanin (Ritacco *et al.* 1986). Dietary Se and vitamin E affected the immune response of chickens at antigenic doses of 1% and 10% sheep red blood cells, but not at 20% (Marsh *et al.* 1981). Antibody response of yellowtail kingfish in this study was measured in terms of the ability of serum to agglutinate bovine serum albumin, a soluble immunogen. As bovine serum albumin is relatively easier to prepare than viral and bacterial antigens, it has been used in fish antibody studies since 1966 (Everhart & Shefner 1966; Trump & Hildemann 1970). The use of antigen and doses in the present study was based on the previous studies for lemon shark (*Negaprion brevirostris*) (Clem & Small 1967), goldfish (*Carassius auratus*) (Trump & Hildemann 1970) and rainbow trout (*Oncorhynchus mykiss*) (Staykov *et al.* 2007). Background Se content in the basal diet may come from fishmeal, but the biological availability of Se from fishmeal is low due to Se being bound to heavy metals (Webster & Lim 2002). Thus, for optimal growth, prevention of deficiency signs and maintaining normal immune response, Se supplementation may be necessary for yellowtail kingfish.

In conclusion, this study has shown that interactions between dietary Se and vitamin E exist in yellowtail kingfish. Se significantly increased weight gain of fish fed diets low in vitamin E, but not high in vitamin E. Lysozyme activity was not improved by dietary Se in vitamin E deficient diets, but



was in diets high in vitamin E. The concentrations of Se and vitamin E in muscle of fish were increased by supplementation of Se and vitamin E respectively, and they may compensate for the lack of each other to prevent fish developing muscle myopathy. The increase in dietary Se intake resulted in increase of red blood cell GPx activity. Therefore, it is necessary to supplement both Se and vitamin E into yellowtail kingfish diets for enhancement of growth and general fish health.

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**Figure legends:**

Figure 1 Mean ( $\pm$  SE) serum lysozyme activity of yellowtail kingfish fed diets containing different inclusion levels of Se and vitamin E for 6 weeks. High vitamin E, 180 mg kg<sup>-1</sup>; low vitamin E, 40 mg kg<sup>-1</sup>; means with different letters are significantly different ( $P < 0.05$ ).

Figure 2 Mean ( $\pm$  SE) red blood cell glutathione peroxidase (GPx) activity of yellowtail kingfish fed diets containing different inclusion levels of Se and vitamin E for 6 weeks. High vitamin E, 180 mg kg<sup>-1</sup>; low vitamin E, 40 mg kg<sup>-1</sup>; means with different letters are significantly different ( $P < 0.05$ ).

Figure 3 Sections of muscles of yellowtail kingfish fed experimental diets for 6 weeks: (A) depleted of both Se and vitamin E, resulting in necrotic fibres (arrow); (B) supplemented with Se at 2 mg kg<sup>-1</sup> diet, showing healthy cells (Haematoxylin and eosin, scale bar = 50  $\mu$ m).

Table 1 Experimental diets and their proximate analyses (mean  $\pm$  SE)

Diet	Additions (mg kg <sup>-1</sup> )		Proximate analysis (g kg <sup>-1</sup> )				Gross energy (MJ kg <sup>-1</sup> )
	Se (measured)	Vitamin E (measured)	Crude protein	Crude lipid	Dry matter	Ash	
1	0 (3.35 $\pm$ 0.00)	40 (41.39 $\pm$ 0.69)	462.07 $\pm$ 5.21	150.53 $\pm$ 0.94	914.77 $\pm$ 0.28	95.60 $\pm$ 0.92	21.68 $\pm$ 0.19
2	1 (4.32 $\pm$ 0.03)	40 (41.01 $\pm$ 0.50)	467.57 $\pm$ 0.99	152.17 $\pm$ 1.02	912.58 $\pm$ 0.29	95.93 $\pm$ 0.52	21.97 $\pm$ 0.18
3	2 (5.39 $\pm$ 0.04)	40 (40.35 $\pm$ 0.21)	472.80 $\pm$ 2.11	151.63 $\pm$ 0.96	912.48 $\pm$ 0.79	96.47 $\pm$ 0.95	21.68 $\pm$ 0.11
4	0 (3.36 $\pm$ 0.00)	180 (180.07 $\pm$ 0.37)	469.03 $\pm$ 2.47	152.53 $\pm$ 1.17	913.67 $\pm$ 0.21	97.17 $\pm$ 0.07	21.80 $\pm$ 0.06
5	1 (4.36 $\pm$ 0.01)	180 (180.41 $\pm$ 0.20)	460.73 $\pm$ 1.07	152.57 $\pm$ 1.13	912.51 $\pm$ 0.21	96.43 $\pm$ 0.57	21.69 $\pm$ 0.04
6	2 (5.34 $\pm$ 0.02)	180 (179.23 $\pm$ 1.08)	468.13 $\pm$ 6.42	151.97 $\pm$ 1.10	912.49 $\pm$ 1.24	98.17 $\pm$ 0.58	21.78 $\pm$ 0.04

Table 2 Mean ( $\pm$  SE) weight gain, feed intake, FCR (feed conversion ratio), survival and antibody titer to bovine serum albumin of yellowtail kingfish fed experimental diets for 6 weeks

Diet <sup>1</sup>	Weight gain (g fish <sup>-1</sup> )			Feed intake			Antibody titre
	2 wk	4 wk	6 wk	(g fish <sup>-1</sup> )	FCR	Survival (%)	(log <sub>10</sub> )
1	19.46 $\pm$ 1.82	42.07 $\pm$ 0.82	55.57 $\pm$ 2.18 <sup>a</sup>	62.92 $\pm$ 5.02	1.13 $\pm$ 0.06	95.00 $\pm$ 2.89	2.21 $\pm$ 0.10
2	19.68 $\pm$ 2.30	44.23 $\pm$ 0.30	58.08 $\pm$ 3.17 <sup>ab</sup>	65.56 $\pm$ 4.54	1.13 $\pm$ 0.02	96.67 $\pm$ 1.67	2.41 $\pm$ 0.00
3	21.58 $\pm$ 1.35	45.29 $\pm$ 0.72	73.48 $\pm$ 2.73 <sup>b</sup>	86.86 $\pm$ 4.13	1.18 $\pm$ 0.04	100.00 $\pm$ 0.00	2.51 $\pm$ 0.10
4	21.75 $\pm$ 0.50	45.29 $\pm$ 1.34	72.25 $\pm$ 1.39 <sup>ab</sup>	82.97 $\pm$ 1.60	1.15 $\pm$ 0.03	96.67 $\pm$ 1.67	2.41 $\pm$ 0.00
5	19.76 $\pm$ 1.71	43.17 $\pm$ 1.60	61.47 $\pm$ 4.79 <sup>ab</sup>	73.75 $\pm$ 9.56	1.19 $\pm$ 0.07	98.33 $\pm$ 1.67	2.61 $\pm$ 0.10
6	18.64 $\pm$ 0.97	42.87 $\pm$ 0.88	62.63 $\pm$ 4.92 <sup>ab</sup>	77.01 $\pm$ 9.83	1.22 $\pm$ 0.06	98.33 $\pm$ 1.67	2.41 $\pm$ 0.17

<sup>1</sup> Diet abbreviations refer to Table 1.

Means in the same column with different superscript letters are significantly different ( $P < 0.05$ ).

Table 3 Mean ( $\pm$  SE) proximate composition, and Se and vitamin E accumulation in fillets of yellowtail kingfish fed experimental diets for 6 weeks

Diet <sup>1</sup>	Protein (g kg <sup>-1</sup> )	Lipid (g kg <sup>-1</sup> )	DM (g kg <sup>-1</sup> )	Ash (g kg <sup>-1</sup> )	GE (MJ kg <sup>-1</sup> )	Se (mg kg <sup>-1</sup> )	Vit. E (mg kg <sup>-1</sup> )
1	200.03 $\pm$ 0.45	25.10 $\pm$ 0.50	245.22 $\pm$ 2.93	14.15 $\pm$ 0.23	5.47 $\pm$ 0.08	0.35 $\pm$ 0.00 <sup>a</sup>	3.28 $\pm$ 0.15 <sup>a</sup>
2	204.08 $\pm$ 1.41	26.21 $\pm$ 0.44	251.23 $\pm$ 1.83	13.91 $\pm$ 0.18	5.47 $\pm$ 0.09	0.49 $\pm$ 0.01 <sup>b</sup>	3.21 $\pm$ 0.02 <sup>a</sup>
3	204.86 $\pm$ 1.88	25.62 $\pm$ 0.10	247.26 $\pm$ 3.60	13.67 $\pm$ 0.16	5.52 $\pm$ 0.07	0.67 $\pm$ 0.04 <sup>c</sup>	3.43 $\pm$ 0.10 <sup>a</sup>
4	203.63 $\pm$ 1.10	25.68 $\pm$ 0.37	249.27 $\pm$ 2.64	13.98 $\pm$ 0.17	5.49 $\pm$ 0.08	0.34 $\pm$ 0.02 <sup>a</sup>	5.34 $\pm$ 0.15 <sup>b</sup>
5	204.63 $\pm$ 1.03	25.76 $\pm$ 0.32	245.3 $\pm$ 2.50	13.60 $\pm$ 0.26	5.50 $\pm$ 0.12	0.52 $\pm$ 0.02 <sup>b</sup>	5.29 $\pm$ 0.15 <sup>b</sup>
6	202.90 $\pm$ 0.49	25.75 $\pm$ 0.44	245.97 $\pm$ 1.62	13.80 $\pm$ 0.15	5.47 $\pm$ 0.04	0.64 $\pm$ 0.02 <sup>c</sup>	5.18 $\pm$ 0.13 <sup>b</sup>

<sup>1</sup> Diet abbreviations refer to Table 1.

DM, dry matter; GE, gross energy; Vit. E, vitamin E.

Means in the same column with different superscript letters are significantly different ( $P < 0.05$ ).

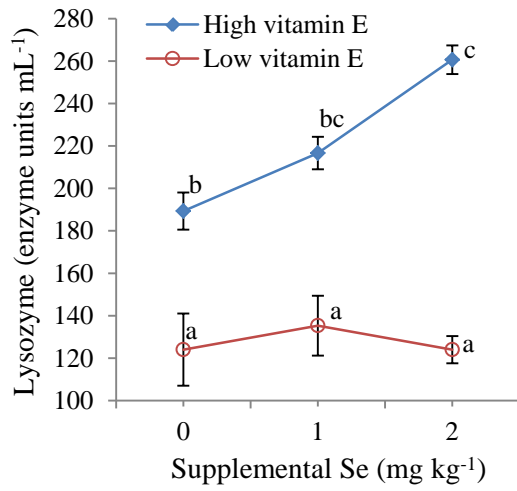


Figure 1 Mean ( $\pm$  SE) serum lysozyme activity of yellowtail kingfish fed diets containing different inclusion levels of Se and vitamin E for 6 weeks. High vitamin E, 180 mg kg<sup>-1</sup>; low vitamin E, 40 mg kg<sup>-1</sup>; means with different letters are significantly different ( $P < 0.05$ ).

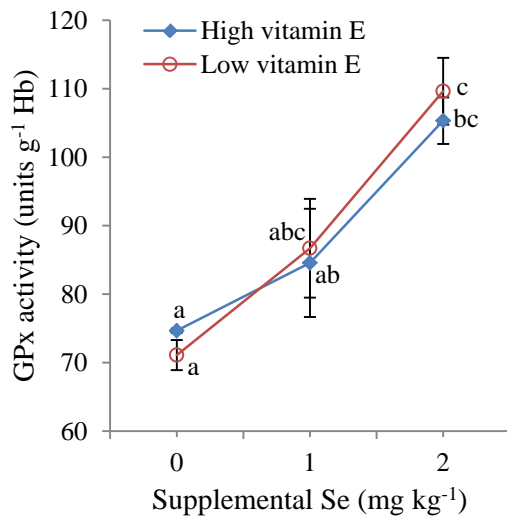


Figure 2 Mean ( $\pm$  SE) red blood cell glutathione peroxidase (GPx) activity of yellowtail kingfish fed diets containing different inclusion levels of Se and vitamin E for 6 weeks. High vitamin E, 180 mg kg<sup>-1</sup>; low vitamin E, 40 mg kg<sup>-1</sup>; means with different letters are significantly different ( $P < 0.05$ ).

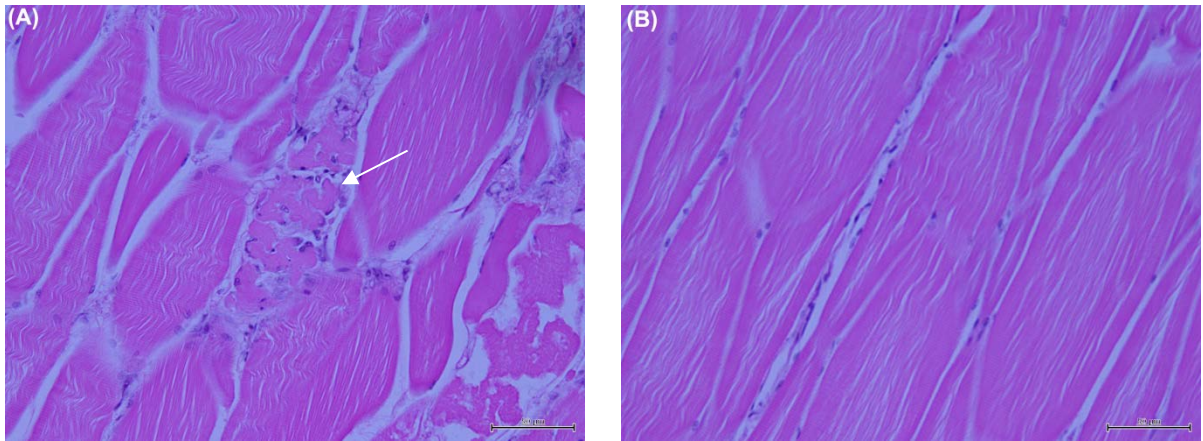


Figure 3 Sections of muscles of yellowtail kingfish fed experimental diets for 6 weeks: (A) depleted of both Se and vitamin E, resulting in necrotic fibres (arrow); (B) supplemented with Se at 2 mg kg<sup>-1</sup> diet, showing healthy cells (Haematoxylin and eosin, scale bar = 50 µm).