Immune Responses to *Vibrio Anguillarum* in Yellowtail Kingfish, *Seriola Lalandi*, Fed

Selenium Supplementation

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Effects of dietary selenium (Se) on immune competence of yellowtail kingfish, *Seriola lalandi*, were investigated. The fish were fed one of three experimental diets including a control diet without Se supplementation and two diets supplemented with Se from Se-yeast (Selplex®) at 2 and 4 mg/kg. After feeding for 6 wk, the fish were challenged by injecting *Vibrio anguillarum* and observed for 2 wk. Dietary Se had no effect on feed intake, feed conversion ratio and survival over the course of 6-wk feeding, however, it significantly increased weight gain and Se content in muscle. Following the bacterial infection, the immune-stimulating effects of Se were observed in antibody, lysozyme and bactericidal responses, and there was a corresponding increase in survival and haematocrit by Se. Under infectious condition, antioxidant capacity of fish as measured in term of resistance of red blood cells to peroxidation and glutathione peroxidase activity also increased by supplementation of Se. Liver necrosis and kidney melanomacrophages were only seen in surviving fish fed the control diet after the challenge. Furthermore, there was evidence of myopathy in fish fed the diet without Se supplementation. This study suggests that Se, supplemented at 2 or 4 mg/kg, can improve growth and health of yellowtail kingfish.
Selenium (Se) is an essential trace element for normal growth and physiological function of animals, including fish (NRC 1993). It is a component of the enzyme glutathione peroxidase, which plays an important role in protecting cell membranes against oxidative damage (Rotruck et al. 1973). Se is also required for the efficient functioning of many components of the immune system (Kiremidjian-Schumacher and Stotzky 1987; Arthur et al. 2003). This is especially important in intensive fish farming as fish often suffer from multiple microbial infections. Dietary supplementation of Se has been found to enhance growth of grouper, *Epinephelus malabaricus* (Lin and Shiau 2005b), cobia, *Rachycentron canadum* (Liu et al. 2010) and gibel carp, *Carassius auratus gibelio* (Han et al. 2011), whereas a deficiency of Se causes reduction in glutathione peroxidase activity in rainbow trout, *Salmo gairdneri* (Hilton et al. 1980), channel catfish, *Ictalurus punctatus* (Gatlin et al. 1986; Wise et al. 1993) and Atlantic salmon, *Salmo salar* (Bell et al. 1987). Immune-stimulating effects of Se and associated increased disease resistance have also been reported for channel catfish (Wang et al. 1997).

Nutritional information of Se on other fish species is available. For example, the requirements of dietary Se for the optimal growth of juvenile grouper (Lin and Shiau 2005b), cobia (Liu et al. 2010) and gibel carp (Han et al. 2011) were 0.7, 0.788 and 1.18 mg/kg, respectively. However, it is unsure whether this information is directly applicable to yellowtail kingfish, *Seriola lalandi*. Yellowtail kingfish is a marine, pelagic and carnivorous fish found globally in sub-tropical and temperate waters of the Pacific and Indian Oceans (Fowler et al. 2003). This species has excellent culture attributes, including high growth rates, good taste and market acceptance, and their suitability to be grown in sea cages and inland recirculating systems (Poortenaar et al. 2001; Chen et al. 2006; Pirozzi and Booth 2009; Abbink et al. 2012).
With the expansion and intensification of fish farming activities, outbreaks of diseases have increased and are being recognized as a significant limitation on sustainable aquaculture (Bondad-Reantaso et al. 2005). One of the primary causes of disease in many aquaculture systems is bacterial infections, vibriosis being the most common in finfish (Rasheed 1989). The most commonly identified aetiological agent of vibriosis in fish is *Vibrio anguillarum* (Vivares et al. 1992). *V. anguillarum* is a primary pathogen of fish, which causes a systemic infection resulting in disease and eventual death (George 1983). Vibriosis of *V. anguillarum* aetiology has been found in over 42 species of fish (Colwell and Grimes 1984) and is described as a serious pathogen affecting cultured marine fish worldwide (Pedersen and Larsen 1993). Therefore, protecting cultured fish from this disease is essential for the expansion and sustainability of the aquaculture industry.

This study was conducted to investigate the effects of dietary addition of Se on immunological and physiological responses and resistance of juvenile yellowtail kingfish to *V. anguillarum*. Resistance of red blood cells to peroxidation and glutathione peroxidase activity were used as indices of antioxidant status. Bactericidal and lysozyme activities and antibody response were used as tools to test the efficacy of Se as an immunostimulant.

**Materials and Methods**

All experimental work was approved by the Curtin University Animal Ethics Committee and performed according to the Australian Code of Practice for the care and use of animals for scientific purposes. Chemicals used were analytical grade obtained from Thermo Fisher Scientific, Scoresby, VIC, Australia, unless otherwise stated.

**Experimental Diets**
Three experimental dietary treatments were designed. One treatment comprised of the un-supplemented basal diet (control) and the others were supplemented with Se at 2 mg/kg (Se 2) and 4 mg/kg (Se 4). A basal mash (fishmeal and fish oil as protein and lipid source, respectively) of a commercially available yellowtail kingfish diet (Marine CST, Ridley AgriProducts, Melbourne, VIC, Australia) without any supplementation of Se was used to prepare the experimental diets. This mash, containing (mean ± SD, n=3) 46.42 ± 1.20% protein, 15.05 ± 1.62% lipid, 91.48 ± 0.05% dry matter, 9.56 ± 0.16% ash and provided 21.68 ± 0.33MJ/kg energy, was extruded into 3 mm pellets. Following extrusion, the necessary quantity of Se from Se-yeast (Selplex®, Alltech, Nicholasville, KY, USA) was top coated to the experimental pellets with gelatin (Davis Gelatine, Christchurch, New Zealand) to form the three experimental diets. The measured Se concentrations in each diet were (mg/kg; mean ± SD, n=3); control (3.35 ± 0.01), Se 2 (5.39 ± 0.06) and Se 4 (7.37 ± 0.03). The selected Se levels were based on our previous study, in which the diet supplemented at 2 mg/kg Se produced the beneficial outcomes for yellowtail kingfish in comparison to un-supplemented diet and supplemented at 1 mg/kg (Le et al. 2013).

Growth Trial

Yellowtail kingfish were supplied by the Australian Centre for Applied Aquaculture Research, Fremantle, WA, Australia and brought to the Sustainable Aquatic Resources and Biotechnology, Curtin University. The fish were group weighed and stocked into each of 12 experimental 300-L tanks at a density of 15 fish per tank. Total weight of fish in each tank was 208.19 ± 0.50 g (mean ± SEM), with an average individual weight of 13.88 ± 0.03 g (mean ± SEM). The tanks were filled with seawater (salinity 35 ppt) and supplied with constant aeration and pure oxygen (oxygen compressed, BOC, Perth, WA, Australia). Each tank had an external bio-filter (Fluval 406, Hagen, Italy) running continuously to create a
recirculating system at a rate of approximately 900 L/h. Half of the water was changed every two days. Water temperature, pH and dissolved oxygen were measured daily using digital pH/mV/°C and dissolved oxygen meters (CyberScan pH 300 and CyberScan DO 300, Eutech Instruments, Singapore). Total ammonia nitrogen was measured before the water change by an ammonia (NH$_3$/NH$_4^+$) test kit (Mars Fishcare, Chalfont, PA, USA). During the trial, water temperature, pH, dissolved oxygen and ammonia nitrogen were maintained at (mean ± SD) 21.4 ± 0.3 °C, 7.4 ± 0.1, 6.6 ± 0.4 mg/L and 0.31 ± 0.11 mg/L, respectively.

Each dietary treatment was randomly assigned to four tanks. Fish were fed to apparent satiation twice a day at 0800 and 1600 h for 6 wk. The amount of feed proffered was recorded daily by calculating the differences in the weight of feed before the first and after the last feeding.

**Bacterial Preparation and Challenge**

*Vibrio anguillarum* was obtained from Bacteriology Laboratory, Department of Agriculture & Food, Perth, WA, Australia. Bacterial preparation followed the previous established method (Lin and Shiau 2005a). The bacteria were cultured in tryptone soya broth (Oxoid, Basingstoke, Hampshire, England) at 25 °C for 24 h and the broth cultures were centrifuged at 5,000 g at 4 °C for 15 min. The supernatant fluids were removed and the bacterial pellets were washed twice in phosphate buffered saline (PBS; 0.1 M, pH 7.2), then the pellets were collected in PBS as a stock bacterial suspension for the injection. The concentration of the culture was adjusted to an optical density of 1.39 at 520 nm using a spectrophotometer (UV-1201, Shimadzu, Kyoto, Japan) to give a *V. anguillarum* concentration of 1 × 10$^{10}$ colony forming units (CFU)/mL. The bacteria were diluted in PBS at 4 °C to obtain desired bacterial concentrations and used to inoculate fish. The bacterial
concentrations were confirmed by plate-counting on tryptone soya agar (Oxoid, Basingstoke, Hampshire, England).

To determine the LC$_{50}$ (concentration lethal to 50% of test fish) to use in the experimental challenge, six different dose regimes from $1 \times 10^4$ to $1 \times 10^9$ CFU/fish with ten fish per dose were conducted. Yellowtail kingfish (51.61 ± 0.93 g, mean ± SEM) provided by the Australian Centre for Applied Aquaculture Research were stocked into each of seven 300-L tanks at a density of ten fish per tank. The tanks were supplied with aerated seawater (35 ppt) at approximately 21.4°C. The fish from each tank were injected intraperitoneally with 0.1 mL of a suspension of _V. anguillarum_ ($1 \times 10^4$, $1 \times 10^5$…or $1 \times 10^9$ CFU/fish) or with 0.1 mL of PBS as a control. No mortality was observed in the control injected with PBS or in the bacterial injection doses of $1 \times 10^4$, $1 \times 10^5$, $1 \times 10^6$ or $1 \times 10^7$ CFU/fish after 2 wk. For $1 \times 10^8$ and $1 \times 10^9$ CFU/fish, mortalities during 2 wk after the injection were 40 and 80%, respectively. The LC$_{50}$ determined by extrapolation from the probit analysis as described by Finney (1971) was $1.7 \times 10^8$ CFU/fish, which was used to challenge the experimental fish.

At the end of the growth trial, after three fish per tank were taken for sampling, the remaining fish (12 fish per tank, average individual weight of 48.42 ± 0.79 g, mean ± SEM) were challenged with bacteria. The fish were given an intraperitoneal injection of 0.1 mL of _V. anguillarum_ suspension in PBS ($1.7 \times 10^9$ CFU/fish) using a 1-mL syringe and 27-gauge needle. All the challenged fish were returned to their respective rearing tanks and fed twice daily for a further 2 wk with the same experimental diet that was assigned before the challenge. Mortalities were recorded daily and dead fish were removed.

Necropsies of freshly dead fish from the lethal test and the bacterial challenged test were aseptically performed. The kidney and liver tissues were cultured to confirm death as a result of infection with _V. anguillarum_ based on biochemical test methods of Buller (2004).
At the end of the growth trial and the end of the bacterial challenge, blood was sampled from the caudal vein of three fish per tank and directly used for red blood cell peroxidation assay and measurement of haematocrit. The remaining whole blood was allowed to clot for 2 h at 4 °C and serum was separated for agglutinating antibody titer, lysozyme and bactericidal activity assays. The red blood cell pellets were used for glutathione peroxidase assay. Serum and red blood cell pellet samples were kept at -80 °C until analysis. Left anterior dorsal muscles, livers and kidneys from the sampled fish (after blood sampling) were dissected out for histological examination and the remaining fillets were used to determine Se contents.

Gross energies were determined using a bomb calorimeter (C2000, IKA, Staufen, Germany). Protein, lipid, dry matter, ash and Se were determined according to the standard methods of the Association of Official Analytical Chemists (1990): crude protein by analysis of nitrogen using the Kjeldahl method; crude lipid by petroleum ether extraction using the Soxhlet method; dry matter by drying at 105 °C to a constant weight and ash by combustion at 550 °C for 24 h. Se was estimated using an atomic absorption spectrometer equipped with vapour generation assembly (AA280 FS and VGA 77, Varian, Mulgrave, VIC, Australia).

Mortality and the amount of feed eaten were recorded daily to calculate survival and feed intake, respectively. Fish in each tank were group weighed at the end of the growth trial to estimate weight gain. Weight measurement and feed intake were used for estimation of feed conversion ratio (FCR, feed intake divided by the wet weight gain).
Red Blood Cell Peroxidation Assay

Sample of whole blood was washed three times in PBS by centrifugation at 1,000 g and 4 C for 5 min, and the supernatant was removed and discarded. The cells were resuspended in PBS to make a 2% red blood cell suspension, which was immediately tested for resistance to oxidative haemolysis as described by Wise et al. (1993). The oxidative titer was determined as the highest dilution ($\log_{10}$) of hydrogen peroxide that caused pellet formation due to lysis of red blood cell membranes.

Haematocrit

Haematocrit of each fish was determined in triplicate by the microhaematocrit method (Rey Vázquez and Guerrero 2007). Blood was collected into heparin-coated microhaematocrit tubes and centrifuged at 13,000 g for 5 min to determine haematocrit (the percent packed cell volume).

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 lysodeiktikus (Sigma-Aldrich, St. Louis, MO, USA) suspended in PBS (0.25 mg/mL). 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) at 25 C. One unit of lysozyme activity was defined as the amount of enzyme resulting in a decrease in absorbance of 0.001/min.

Bactericidal Activity
Bactericidal activity was determined according to the method of Ueda et al. (1999). Fifty microliters of suspension of \textit{V. anguillarum} in PBS ($1.56 \times 10^4$ CFU/mL) were added to 50 µL serum, and the mixture was reacted for 30 min at 25°C. The same volume of bacterial suspension was added to 50 µL of PBS as control, and was also reacted for 30 min at 25°C simultaneously. After reaction, 50 µL from the mixture was plated onto duplicate tryptone soya agar and incubated for 24 h at 25°C. Bacterial activity was calculated as decrease in number of viable \textit{V. anguillarum} cells, i.e. log$_{10}$ CFU/mL in the control minus log$_{10}$ CFU/mL in serum.

**Glutathione Peroxidase Assay**

Glutathione peroxidase (GPx) activity in red blood cells was assayed by the method of Paglia and Valentine (1967) using the Ransel RS-505 kit (Randox, Crumlin, County Antrim, UK) and a chemistry immune analyser (AU400, Olympus, Tokyo, Japan) at 340 nm and 37°C. The results were expressed as units of GPx/g of haemoglobin (Hb). Haemoglobin was measured using the Hb HG-1539 kit (Randox, Crumlin, County Antrim, UK).

**Serum Anti-\textit{V. anguillarum} Antibody Titer**

\textit{V. anguillarum} was grown in tryptone soya broth at 25°C for 24 h and killed in 1% formalin. The cells were centrifuged at 5,000 g for 15 min at 4°C. The resulting cell pellets were washed twice in PBS and suspended in PBS to an optical density of 0.151 at 520 nm (UV-1201 spectrophotometer, Shimadzu, Kyoto, Japan) and used as the antigen. Serum agglutinating antibody titer to \textit{V. anguillarum} was determined with the serum agglutination technique described by Chen and Light (1994) and reported as the last serum dilution which caused clumping of the antigen and transformed to log$_{10}$ values for statistical analysis.

**Histological Examination**
The histological samples were prepared according to routine techniques (Refstie et al. 2010). 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 a light microscope (BX40F4, Olympus, Tokyo, Japan).

Data Analysis

Data were analysed using PASW Statistics 18.0 (IBM Corporation, New York, US). All data were subjected to a one-way ANOVA. Data were tested for normality and homogeneity of variance using Shapiro-Wilk and Levene’s tests, respectively. Where necessary, data were transformed to satisfy the assumptions of ANOVA. All percentage data were arcsine transformed prior to analysis. 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 ± SEM.

Results

During 6 wk of feeding, dietary Se did not influence feed intake, FCR and survival of the fish, which remained 100% (Table 1). However, weight gain was significantly ($F_2, 9 = 6.52, P = 0.018$) affected by the dietary treatments (Table 1), the fish fed the control diet gained significantly ($P < 0.05$) less weight than fish fed the other two diets, which produced similar weight gains.

Se supplementation had no significant effect on red blood cell peroxidation, haematocrit values and lysozyme activity of the pre-challenged fish, but significantly affected the post-challenged fish ($F_2, 9 = 24.31, P = 0.000$; $F_2, 9 = 61.96, P = 0.00$ and $F_2, 9 = 84.67, P = 0.00$, respectively for red blood cell peroxidation, haematocrit and lysozyme activity) (Table
2). The red blood cell membranes of the post-challenged fish fed the control diet were significantly more susceptible ($P < 0.05$) to peroxidation than the fish fed the supplemented Se diets, while the haematocrits of the post-challenged fish fed Se supplemented diets were significantly higher ($P < 0.05$) than the fish fed the diet without supplementation. During post-challenge period, the increase in Se intake by the fish resulted in significant increase ($P < 0.05$) of lysozyme activity; the lowest mean was in the control group while the highest mean was in the Se 4 group. In both pre- and post-challenged fish, bactericidal and glutathione peroxidase activities were significantly ($P < 0.05$) increased by the supplementation of Se, and higher dietary Se intake produced significantly higher ($P < 0.05$) Se content in fish fillets (Table 2).

The application of bacterial challenge altered immune and antioxidant parameters, except bactericidal activity (Table 2). As a result of the challenge, lysozyme and glutathione peroxidase activities were significantly stimulated ($P < 0.05$). The challenge resulted in a significant increase ($P < 0.05$) in susceptibility of red blood cell membranes to peroxidation, and caused significantly decreases ($P < 0.05$) in haematocrits and Se in fillets.

All serum samples collected at the end of the growth trial, used to measure pre-challenge antibody titers, were negative for *V. anguillarum*. At the end of the challenge, antibody titers against *V. anguillarum* were significantly increased ($P < 0.05$) with dietary Se supplementation of 2 or 4 mg/kg in comparison with the antibody titer of the control group (Table 3). The bacterial infection resulted in significantly higher mortalities in control-diet fed-fish than fish fed the Se supplemented diets (Table 3). Level of Se supplementation did not make any difference ($P > 0.05$) in mortality rates.

Light microscopy analysis of muscle sections revealed that there was multiphasic myopathy in fish fed the control diet (Fig. 1), while Se supplementation resulted in no
muscular lesions. Liver with necrotic lesions was observed in the surviving fish fed the un-supplemented diet after the bacterial challenge (Fig. 2), but not in the fish fed the Se-supplemented diets. Following the bacterial infection, occasional melano-macrophage occurred only in kidney of fish fed the control diet (Fig. 3).

**Discussion**

The beneficial growth effect of dietary Se for yellowtail kingfish was shown by the weight gain data and the increased Se accumulation in fish as a result of Se supplementation was seen from the measurement of Se concentration in muscle tissues. These findings are consistent with the data reported for grouper (Lin and Shiau 2005b), cobia (Liu et al. 2010) and gibel carp (Han et al. 2011) fed supplementation of Se in the form of selenomethionine, the main component of Se used in the present study. The supplementation levels of Se in the studies of grouper, cobia and gibel carp are similar to those in the current study; however, the levels of Se in their basal diets are lower. Background Se content in the basal diet in our study may come from fishmeal, but the biological availability of Se from fishmeal is low due to Se being bound to heavy metals (Webster and Lim 2002), thus, supplementation of Se in yellowtail kingfish diet is necessary. Se supplementation has been reported to increase expression of genes involved in energy production and protein synthesis pathways (Brennan et al. 2011). Se may upregulate growth-related gene expression in yellowtail kingfish. Further research is needed to elucidate mechanism of action of Se on fish growth.

The results of bacterial challenge in the present study showed that dietary Se improved immune responses and resistance of yellowtail kingfish to *V. anguillarum* infection. Dietary Se supplementation significantly increased survival following infection with *V. anguillarum* and there was a corresponding increase in antibody response. The same effects of Se on survival and antibody have been reported for channel catfish challenged with pathogenic
bacterium *Edwardsiella ictaluri* (Wang et al. 1997). Other immune-stimulating effects of Se were evident in bactericidal and lysozyme activities. Serum of yellowtail kingfish had the ability to inhibit the growth of *V. anguillarum* and this ability was stimulated by dietary Se. Lysozyme in yellowtail kingfish possessed lytic activity against bacteria and this activity was shown to increase as dietary Se increased in post-challenged fish. Se appears to boost immune capacity by the following mechanism. It increases the expression of high affinity IL-2 receptor through a posttranscriptional mechanism (Roy et al. 1994). The interaction of IL-2 with its receptor delivers signals for proliferation of T-cells (Minami et al. 1993), which have been shown to provide B-cell help during antibody production (Brandes et al. 2003). In addition, IL-2 regulates multiple biological processes including, enhancement of natural killer cells (Henny et al. 1981) and generation of lymphokine-activated killer cells (Grimm et al. 1982). This mechanism may explain the stimulatory effects of Se on antibody and other immune responses in yellowtail kingfish.

In agreement with results of previous study on channel catfish (Wise et al. 1993), resistance of red blood cells of pre-challenged yellowtail kingfish to hydrogen peroxide-induced haemolysis was unaffected by Se supplementation. After being infected with *V. anguillarum*, however, the antioxidant capacity of red blood cells was shown to significantly increase by dietary Se, suggesting the importance of Se in the cell membranes under the condition of infection.

Glutathione peroxidase is one of the most important antioxidant defence enzymes in fish (Filho 1996; Ross et al. 2001) and its activity is dependent on the dietary Se intake (Ganther et al. 1976). The importance of Se to the antioxidant capacity of fish has been well recognized and reported. The glutathione peroxidase activity was shown to decrease in rainbow trout (Hilton et al. 1980), channel catfish (Gatlin et al. 1986; Wise et al. 1993) and
Atlantic salmon (Bell et al. 1987) fed diets deficient in Se, whereas the antioxidant capacity of cobia (Liu et al. 2010), grouper (Lin and Shiau 2005b) and yellowtail kingfish in the present study increased with an increase of Se in their diets.

The loss of muscle tissue Se and the increase of glutathione peroxidase activity as a result of bacterial infection indicate an increased requirement for Se under infected condition. Se from reserves may be mobilized and transferred to synthesize more glutathione peroxidase molecules to meet an increase in demand for protecting fish from oxidative damage during the process of killing invaded microbes.

The average haematocrit values of pre-challenged yellowtail kingfish ranged between 38.79 and 42.00%, close to the haematocrit of another species of Seriola, Japanese yellowtail, Seriola quinqueradiata, in a healthy status (Watanabe et al. 1998). However, the haematocrits were significantly decreased by the V. anguillarum infection. When fish were fed the control diet, the haematocrit decreased to 22.77%, which is lower than haematocrit of Japanese yellowtail in an anaemic state, 27.00% (Watanabe et al. 1998). Similar effect of V. anguillarum infection on haematocrits has been found in coho salmon, Oncorhynchus kisutch (Harbell et al. 1979) and rainbow trout (Lamas et al. 1994). The responsibility for the anaemic response in infected fish is haemolysin produced by V. anguillarum (Munn 1978).

Dietary Se deficiency has been reported to cause myopathy in Atlantic Salmon (Poston et al. 1976) and channel catfish (Gatlin et al. 1986). Muscle necrosis observed in the present study indicated the necessity of supplementation of Se for prevention of myopathy in yellowtail kingfish. Other histopathological signs were also found in the liver and kidney of post-challenged fish fed the diet deficient in Se. The histology data showed that dietary Se contributed to prevention of liver necrosis in fish. In 1951, Se was recognized as an integral part of Factor 3, an organic Se compound, which can protect rats from liver necrosis.
(Schwarz and Foltz 1957). Two decades later Moir and Masters (1979) found that liver lesion in pigs can be prevented by providing Se supplements. Liver necrosis in fish infected with _V. anguillarum_ was well manifested by Hjeltnes and Roberts (1993), but no treatment has been described. Another evidence of histological change after the _V. anguillarum_ infection was the occasional occurrence of melano-macrophages in kidney of fish fed the diet deficient in Se. Melano-macrophages occur in association with infectious diseases such as vibriosis (Agius and Roberts 2003) and have been recommended as potential monitors of fish health (Wolke et al. 1985; Wolke 1992).

Although Se can be applied to improve immune responses and disease resistance of fish, the use of Se supplements above the optimal requirement level should be avoided as higher levels can be toxic. For example, dietary Se at a level of 13 mg/kg was found to be toxic to rainbow trout, _Salmo Gairdneri_, the fish showed reduced growth and survival, and poor feed efficiency (Hilton et al. 1980). Se concentrations of more than 4.6 mg/kg in food resulted in rapid mortality of razorback sucker, _Xyrauchen texanus_ larvae (Hamilton et al. 2005) and a sub-lethal toxic effect of Se as selenite at 7 mg/kg was reported in rainbow trout (Rider et al. 2009). In the present study, no signs of toxicity were observed for Se supplementation at 4 mg/kg. However, two supplementation levels produced no difference. Therefore, Se supplementation at 2 mg/kg could be a preferred choice for yellowtail kingfish.

On the basis of the results of this study it may be concluded that growth, immune responses and resistance of yellowtail kingfish to _V. anguillarum_ were improved by feeding with supplementation of Se. Dietary Se significantly increased fish survival, antibody and haematocrit following bacterial infection and as well as stimulated bactericidal and lysozyme activities. During the infectious stage, the role of Se as an antioxidant was demonstrated by activities such as resistance of red blood cells to peroxidation and glutathione peroxidase. In
addition, myopathy and liver necrosis caused by *V. anguillarum* can be prevented by Se supplementation.

Acknowledgments

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Literature Cited


Fowler, A. J., J. M. Ham, and P. R. Jennings. 2003. Discriminating between cultured and wild yellowtail kingfish (Seriola lalandi) in South Australia. SARDI Aquatic Sciences Publication No. RD03/0159, South Australian Research and Development Institute (Aquatic Sciences), Adelaide, Australia.


TABLE 1. Mean ± SEM weight gain, feed intake, feed conversion ratio (FCR) and survival of yellowtail kingfish fed the experimental diets for 6 wk.¹

<table>
<thead>
<tr>
<th>Diet</th>
<th>Weight gain (g/fish)</th>
<th>Feed intake (g/fish)</th>
<th>FCR</th>
<th>Survival (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>31.63 ± 1.19ᵃ</td>
<td>44.05 ± 0.76</td>
<td>1.40 ± 0.06</td>
<td>100</td>
</tr>
<tr>
<td>Se 2</td>
<td>36.20 ± 0.66ᵇ</td>
<td>44.70 ± 0.98</td>
<td>1.26 ± 0.04</td>
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</tr>
<tr>
<td>Se 4</td>
<td>35.79 ± 1.04ᵇ</td>
<td>47.06 ± 1.18</td>
<td>1.37 ± 0.02</td>
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<tr>
<td>P value</td>
<td>0.018</td>
<td>0.131</td>
<td>0.118</td>
<td></td>
</tr>
</tbody>
</table>

¹ Values represent means of four replicates per treatment. Means in the same column with different superscript letters are significantly different (P < 0.05, one-way ANOVA).
TABLE 2. Mean ± SEM red blood cell (RBC) peroxidation, haematocrit, lysozyme, bactericidal and glutathione peroxidase (GPx) activities, and Se in fillets of yellowtail kingfish fed the experimental diets for 6 wk and subsequently challenged with V. anguillarum for 2 wk.

<table>
<thead>
<tr>
<th>Diet</th>
<th>Pre-challenge</th>
<th>Post-challenge</th>
<th>Challenge effect (P value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC peroxidation (log₁₀ titer)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2.86 ± 0.09</td>
<td>5.12 ± 0.12a</td>
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<tr>
<td>Se 2</td>
<td>2.64 ± 0.08</td>
<td>4.06 ± 0.09b</td>
<td>0.000</td>
</tr>
<tr>
<td>Se 4</td>
<td>2.56 ± 0.09</td>
<td>3.84 ± 0.19b</td>
<td>0.001</td>
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<tr>
<td>P value</td>
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<tr>
<td>Haematocrit (%)</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Control</td>
<td>38.79 ± 0.72</td>
<td>22.77 ± 0.42a</td>
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<tr>
<td>Se 2</td>
<td>42.00 ± 0.65</td>
<td>31.79 ± 0.65b</td>
<td>0.000</td>
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<tr>
<td>Se 4</td>
<td>41.66 ± 1.21</td>
<td>32.33 ± 0.89b</td>
<td>0.001</td>
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<tr>
<td>P value</td>
<td>0.062</td>
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<tr>
<td>Lysozyme (units/mL)</td>
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<tr>
<td>Control</td>
<td>71.50 ± 3.20</td>
<td>134.00 ± 2.94a</td>
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<td>Se 2</td>
<td>64.00 ± 2.94</td>
<td>162.67 ± 1.89b</td>
<td>0.000</td>
</tr>
<tr>
<td>Se 4</td>
<td>62.00 ± 5.72</td>
<td>190.67 ± 4.03c</td>
<td>0.000</td>
</tr>
<tr>
<td>P value</td>
<td>0.283</td>
<td>0.000</td>
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<tr>
<td>Bactericidal activity (log₁₀)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2.84 ± 0.11a</td>
<td>3.12 ± 0.01a</td>
<td>0.054</td>
</tr>
<tr>
<td>Se 2</td>
<td>3.24 ± 0.07b</td>
<td>3.41 ± 0.01b</td>
<td>0.051</td>
</tr>
<tr>
<td>Se 4</td>
<td>3.33 ± 0.06b</td>
<td>3.38 ± 0.01b</td>
<td>0.418</td>
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<tr>
<td>P value</td>
<td>0.006</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>GPx (units/g Hb)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>Se 2</td>
<td>Se 4</td>
</tr>
<tr>
<td>----------</td>
<td>---------------</td>
<td>----------------</td>
<td>----------------</td>
</tr>
<tr>
<td>Se in fillet (mg/kg)¹</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Control</td>
<td>73.77 ± 1.84a</td>
<td>86.33 ± 3.12a</td>
<td>0.013</td>
</tr>
<tr>
<td>Se 2</td>
<td>89.40 ± 4.97b</td>
<td>115.20 ± 3.55b</td>
<td>0.006</td>
</tr>
<tr>
<td>Se 4</td>
<td>101.93 ± 3.58b</td>
<td>132.33 ± 5.95b</td>
<td>0.005</td>
</tr>
<tr>
<td>P value</td>
<td>0.001</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>Se in fillet (mg/kg)²</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.50 ± 0.03a</td>
<td>0.40 ± 0.01a</td>
<td>0.009</td>
</tr>
<tr>
<td>Se 2</td>
<td>0.65 ± 0.01b</td>
<td>0.61 ± 0.01b</td>
<td>0.027</td>
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<tr>
<td>Se 4</td>
<td>0.88 ± 0.01c</td>
<td>0.81 ± 0.02c</td>
<td>0.016</td>
</tr>
<tr>
<td>P value</td>
<td>0.000</td>
<td>0.000</td>
<td></td>
</tr>
</tbody>
</table>

Means in the same column with different superscript letters are significantly different (P < 0.05, one-way ANOVA).

¹ Values are means of one determination per fish, three fish per tank and four tanks per treatment.

² Value are means of three determinations per fish, three fish per tank and four tanks per treatment.

³ Value are means of two determinations per fish, three fish per tank and four tanks per treatment.

⁴ Pre- and post-challenge data were subjected to a one-way ANOVA.
TABLE 3. Mean ± SEM accumulative mortality and antibody to V. anguillarum of yellowtail kingfish fed the experimental diets for 6 wk and subsequently challenged with V. anguillarum for 2 wk.

<table>
<thead>
<tr>
<th>Diet</th>
<th>Accumulative mortality (%)&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Antibody titer (log&lt;sub&gt;10&lt;/sub&gt;)&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>60.42 ± 2.09&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.73 ± 0.08&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Se 2</td>
<td>37.50 ± 2.41&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.56 ± 0.09&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Se 4</td>
<td>41.67 ± 3.40&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.33 ± 0.08&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Means in the same column with different superscript letters are significantly different (P < 0.05, one-way ANOVA).

<sup>1</sup> Values represent means of four replicates per treatment.

<sup>2</sup> Values are means of one determination per fish, three fish per tank and four tanks per treatment.
FIGURE 1. Paraffin section of muscle of yellowtail kingfish fed the control diet, showing necrotic fibres. Haematoxylin and eosin stain, scale bar = 50 µm.
FIGURE 2. Paraffin section of liver of yellowtail kingfish fed the control diet, showing necrotic lesion caused by *V. anguillarum*. Haematoxylin and eosin stain, scale bar = 50 µm.
FIGURE 3. Paraffin section of kidney with melano-macrophages of yellowtail kingfish fed the control diet and subsequently challenged with *V. anguillarum*. Haematoxylin and eosin stain, scale bar = 50 µm.