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

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Abstract

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 melano-macrophages 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, lctalurus 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/^{o}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

129 concentrations were confirmed by plate-counting on tryptone soya agar (Oxoid, Basingstoke,
130 Hampshire, England).

131 To determine the LC_{50} (concentration lethal to 50% of test fish) to use in the experimental
132 challenge, six different dose regimes from 1×10^4 to 1×10^9 CFU/fish with ten fish per dose
133 were conducted. Yellowtail kingfish (51.61 ± 0.93 g, mean ± SEM) provided by the
134 Australian Centre for Applied Aquaculture Research were stocked into each of seven 300-L
135 tanks at a density of ten fish per tank. The tanks were supplied with aerated seawater (35 ppt)
136 at approximately 21.4 C. The fish from each tank were injected intraperitoneally with 0.1 mL
137 of a suspension of V. anguillarum (1×10^4, 1×10^5…or 1×10^9 CFU/fish) or with 0.1 mL of
138 PBS as a control. No mortality was observed in the control injected with PBS or in the
139 bacterial injection doses of 1×10^4, 1×10^5, 1×10^6 or 1×10^7 CFU/fish after 2 wk. For $1 \times$
140 10^8 and 1×10^9 CFU/fish, mortalities during 2 wk after the injection were 40 and 80%,
141 respectively. The LC_{50} determined by extrapolation from the probit analysis as described by
142 Finney (1971) was 1.7×10^8 CFU/fish, which was used to challenge the experimental fish.

143 At the end of the growth trial, after three fish per tank were taken for sampling, the
144 remaining fish (12 fish per tank, average individual weight of 48.42 ± 0.79 g, mean ± SEM)
145 were challenged with bacteria. The fish were given an intraperitoneal injection of 0.1 mL of
146 V. anguillarum suspension in PBS (1.7×10^8 CFU/fish) using a 1-mL syringe and 27-gauge
147 needle. All the challenged fish were returned to their respective rearing tanks and fed twice
148 daily for a further 2 wk with the same experimental diet that was assigned before the
149 challenge. Mortalities were recorded daily and dead fish were removed.

150 Necropsies of freshly dead fish from the lethal test and the bacterial challenged test were
151 aseptically performed. The kidney and liver tissues were cultured to confirm death as a result
152 of infection with V. anguillarum based on biochemical test methods of Buller (2004).

Sample Collection

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.

Chemical Analysis

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).

Survival and Growth Measurements

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

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

204 Glutathione Peroxidase Assay

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

210 Serum Anti-V. anguillarum Antibody Titer

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

218 Histological Examination

219 The histological samples were prepared according to routine techniques (Refstie et al.
220 2010). The samples were fixed in 10% buffered formalin, dehydrated in ethanol before
221 equilibration in xylene and embedded in paraffin wax. Sections of approximately 5 µm were
222 cut and stained with haematoxylin and eosin, and observed under a light microscope
223 (BX40F4, Olympus, Tokyo, Japan).

224 Data Analysis

225 Data were analysed using PASW Statistics 18.0 (IBM Corporation, New York, US). All
226 data were subjected to a one-way ANOVA. Data were tested for normality and homogeneity
227 of variance using Shapiro-Wilk and Levene's tests, respectively. Where necessary, data were
228 transformed to satisfy the assumptions of ANOVA. All percentage data were arcsine
229 transformed prior to analysis. When a significant treatment effect was observed, Tukey's
230 Honest Significant Difference test was used for multiple mean comparisons. The statistical
231 significance was set at $P < 0.05$ and the results were presented as means ± SEM.

232 **Results**

233 During 6 wk of feeding, dietary Se did not influence feed intake, FCR and survival of the
234 fish, which remained 100% (Table 1). However, weight gain was significantly ($F_{2, 9} = 6.52$,
235 $P = 0.018$) affected by the dietary treatments (Table 1), the fish fed the control diet gained
236 significantly ($P < 0.05$) less weight than fish fed the other two diets, which produced similar
237 weight gains.

238 Se supplementation had no significant effect on red blood cell peroxidation, haematocrit
239 values and lysozyme activity of the pre-challenged fish, but significantly affected the post-
240 challenged fish ($F_{2, 9} = 24.31$, $P = 0.000$; $F_{2, 9} = 61.96$, $P = 0.00$ and $F_{2,9} = 84.67$, $P =$
241 0.000, respectively for red blood cell peroxidation, haematocrit and lysozyme activity) (Table

242 2). The red blood cell membranes of the post-challenged fish fed the control diet were
243 significantly more susceptible ($P < 0.05$) to peroxidation than the fish fed the supplemented
244 Se diets, while the haematocrits of the post-challenged fish fed Se supplemented diets were
245 significantly higher ($P < 0.05$) than the fish fed the diet without supplementation. During
246 post-challenge period, the increase in Se intake by the fish resulted in significant increase (P
247 < 0.05) of lysozyme activity; the lowest mean was in the control group while the highest
248 mean was in the Se 4 group. In both pre- and post-challenged fish, bactericidal and
249 glutathione peroxidase activities were significantly ($P < 0.05$) increased by the
250 supplementation of Se, and higher dietary Se intake produced significantly higher ($P < 0.05$)
251 Se content in fish fillets (Table 2).

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

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

264 Light microscopy analysis of muscle sections revealed that there was multiphasic
265 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

290 bacterium Edwardsiella ictaluri (Wang et al. 1997). Other immune-stimulating effects of Se
291 were evident in bactericidal and lysozyme activities. Serum of yellowtail kingfish had the
292 ability to inhibit the growth of V. anguillarum and this ability was stimulated by dietary Se.
293 Lysozyme in yellowtail kingfish possessed lytic activity against bacteria and this activity was
294 shown to increase as dietary Se increased in post-challenged fish. Se appears to boost
295 immune capacity by the following mechanism. It increases the expression of high affinity IL-
296 2 receptor through a posttranscriptional mechanism (Roy et al. 1994). The interaction of IL-2
297 with its receptor delivers signals for proliferation of T-cells (Minami et al. 1993), which have
298 been shown to provide B-cell help during antibody production (Brandes et al. 2003). In
299 addition, IL-2 regulates multiple biological processes including, enhancement of natural killer
300 cells (Henny et al. 1981) and generation of lymphokine-activated killer cells (Grimm et al.
301 1982). This mechanism may explain the stimulatory effects of Se on antibody and other
302 immune responses in yellowtail kingfish.

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

309 Glutathione peroxidase is one of the most important antioxidant defence enzymes in fish
310 (Filho 1996; Ross et al. 2001) and its activity is dependent on the dietary Se intake (Ganther
311 et al. 1976). The importance of Se to the antioxidant capacity of fish has been well
312 recognized and reported. The glutathione peroxidase activity was shown to decrease in
313 rainbow trout (Hilton et al. 1980), channel catfish (Gatlin et al. 1986; Wise et al. 1993) and

314 Atlantic salmon (Bell et al. 1987) fed diets deficient in Se, whereas the antioxidant capacity
315 of cobia (Liu et al. 2010), grouper (Lin and Shiau 2005b) and yellowtail kingfish in the
316 present study increased with an increase of Se in their diets.

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

322 The average haematocrit values of pre-challenged yellowtail kingfish ranged between
323 38.79 and 42.00%, close to the haematocrit of another species of Seriola, Japanese yellowtail,
324 Seriola quinqueradiata, in a healthy status (Watanabe et al. 1998). However, the haematocrits
325 were significantly decreased by the V. anguillarum infection. When fish were fed the control
326 diet, the haematocrit decreased to 22.77%, which is lower than haematocrit of Japanese
327 yellowtail in an anaemic state, 27.00% (Watanabe et al. 1998). Similar effect of V.
328 anguillarum infection on haematocrits has been found in coho salmon, Oncorhynchus kisutch
329 (Harbell et al. 1979) and rainbow trout (Lamas et al. 1994). The responsibility for the
330 anaemic response in infected fish is haemolysin produced by V. anguillarum (Munn 1978).

331 Dietary Se deficiency has been reported to cause myopathy in Atlantic Salmon (Poston et
332 al. 1976) and channel catfish (Gatlin et al. 1986). Muscle necrosis observed in the present
333 study indicated the necessity of supplementation of Se for prevention of myopathy in
334 yellowtail kingfish. Other histopathological signs were also found in the liver and kidney of
335 post-challenged fish fed the diet deficient in Se. The histology data showed that dietary Se
336 contributed to prevention of liver necrosis in fish. In 1951, Se was recognized as an integral
337 part of Factor 3, an organic Se compound, which can protect rats from liver necrosis

338 (Schwarz and Foltz 1957). Two decades later Moir and Masters (1979) found that liver lesion
339 in pigs can be prevented by providing Se supplements. Liver necrosis in fish infected with V.
340 anguillarum was well manifested by Hjeltnes and Roberts (1993), but no treatment has been
341 described. Another evidence of histological change after the V. anguillarum infection was the
342 occasional occurrence of melano-macrophages in kidney of fish fed the diet deficient in Se.
343 Melano-macrophages occur in association with infectious diseases such as vibriosis (Agius
344 and Roberts 2003) and have been recommended as potential monitors of fish health (Wolke
345 et al. 1985; Wolke 1992).

346 Although Se can be applied to improve immune responses and disease resistance of fish,
347 the use of Se supplements above the optimal requirement level should be avoided as higher
348 levels can be toxic. For example, dietary Se at a level of 13 mg/kg was found to be toxic to
349 rainbow trout, Salmo Gairdneri, the fish showed reduced growth and survival, and poor feed
350 efficiency (Hilton et al. 1980). Se concentrations of more than 4.6 mg/kg in food resulted in
351 rapid mortality of razorback sucker, Xyrauchen texanus larvae (Hamilton et al. 2005) and a
352 sub-lethal toxic effect of Se as selenite at 7 mg/kg was reported in rainbow trout (Rider et al.
353 2009). In the present study, no signs of toxicity were observed for Se supplementation at 4
354 mg/kg. However, two supplementation levels produced no difference. Therefore, Se
355 supplementation at 2 mg/kg could be a preferred choice for yellowtail kingfish.

356 On the basis of the results of this study it may be concluded that growth, immune
357 responses and resistance of yellowtail kingfish to V. anguillarum were improved by feeding
358 with supplementation of Se. Dietary Se significantly increased fish survival, antibody and
359 haematocrit following bacterial infection and as well as stimulated bactericidal and lysozyme
360 activities. During the infectious stage, the role of Se as an antioxidant was demonstrated by
361 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

This research was sponsored by Endeavour Awards. The authors wish to acknowledge the assistance of Dr Fran Stephens for her technical assistance with histology.

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533 TABLE 1. Mean ± SEM weight gain, feed intake, feed conversion ratio (FCR) and survival

534 of yellowtail kingfish fed the experimental diets for 6 wk.[1]

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

535 [1] Values represent means of four replicates per treatment. Means in the same column with

536 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.

Diet	Pre-challenge	Post-challenge	Challenge effect (P value)[4]
RBC peroxidation (log_{10} titer)[1]			
Control	2.86 ± 0.09	5.12 ± 0.12[a]	0.000
Se 2	2.64 ± 0.08	4.06 ± 0.09[b]	0.000
Se 4	2.56 ± 0.09	3.84 ± 0.19[b]	0.001
P value	0.073	0.000	
Haematocrit (%)[2]			
Control	38.79 ± 0.72	22.77 ± 0.42[a]	0.000
Se 2	42.00 ± 0.65	31.79 ± 0.65[b]	0.000
Se 4	41.66 ± 1.21	32.33 ± 0.89[b]	0.001
P value	0.062	0.000	
Lysozyme (units/mL)[3]			
Control	71.50 ± 3.20	134.00 ± 2.94[a]	0.000
Se 2	64.00 ± 2.94	162.67 ± 1.89[b]	0.000
Se 4	62.00 ± 5.72	190.67 ± 4.03[c]	0.000
P value	0.283	0.000	
Bactericidal activity (log_{10})[3]			
Control	2.84 ± 0.11[a]	3.12 ± 0.01[a]	0.054
Se 2	3.24 ± 0.07[b]	3.41 ± 0.01[b]	0.051
Se 4	3.33 ± 0.06[b]	3.38 ± 0.01[b]	0.418
P value	0.006	0.000	
GPx (units/g Hb)[1]			

Control	73.77 ± 1.84[a]	86.33± 3.12[a]	0.013
Se 2	89.40 ± 4.97[b]	115.20 ± 3.55[b]	0.006
Se 4	101.93 ± 3.58[b]	132.33 ± 5.95[b]	0.005
P value	0.001	0.000	
Se in fillet (mg/kg)[1]			
Control	0.50 ± 0.03[a]	0.40 ± 0.01[a]	0.009
Se 2	0.65 ± 0.01[b]	0.61 ± 0.01[b]	0.027
Se 4	0.88 ± 0.01[c]	0.81 ± 0.02[c]	0.016
P value	0.000	0.000	

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.

Diet	Accumulative mortality (%)[1]	Antibody titer (log_{10})[2]
Control	60.42 ± 2.09[a]	1.73 ± 0.08[a]
Se 2	37.50 ± 2.41[b]	2.56 ± 0.09[b]
Se 4	41.67 ± 3.40[b]	2.33 ± 0.08[b]
P value	0.000	0.000

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

[1] Values represent means of four replicates per treatment.

[2] 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.

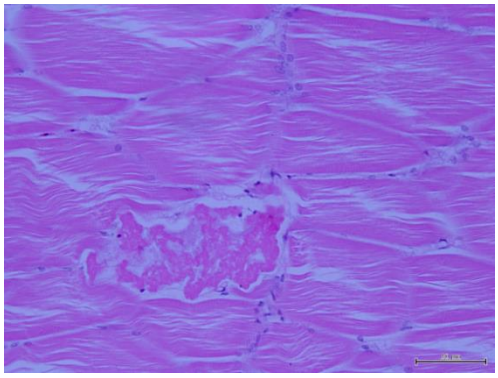
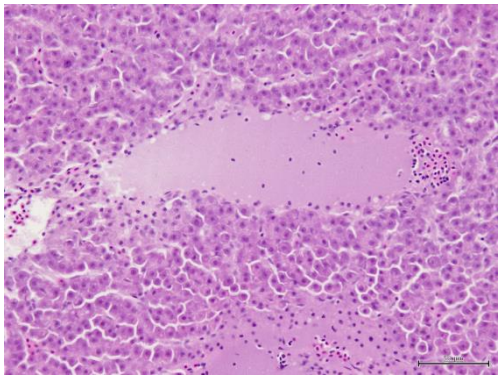
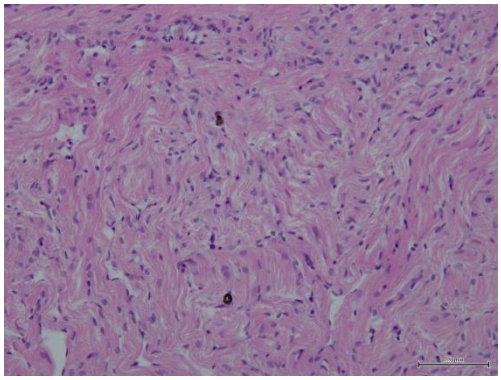


FIGURE 2. Paraffin section of liver of yellowtail kingfish fed the control diet, showing necrotic lesion caused by V. anguillarum. Haematoxylin and eosin stain, scale bar = 50 µm.



568 FIGURE 3. Paraffin section of kidney with melano-macrophages of yellowtail kingfish fed
569 the control diet and subsequently challenged with V. anguillarum. Haematoxylin and eosin
570 stain, scale bar = 50 µm.



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