Department of Environment and Agriculture

Dietary organic selenium in yellowtail kingfish *Seriola lalandi* Valenciennes, 1833

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DECLARATION

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgment has been made.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

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Date: 20 February 2015

ABSTRACT

Yellowtail kingfish *Seriola lalandi* has been identified as a potentially valuable aquaculture species. As the aquaculture activity of yellowtail kingfish is expanding, research on nutritional requirements for this species needs intensification. However, there is a lack of information on selenium (Se), an essential nutrient required for normal growth and physiological functions of the fish. Therefore, conducting research on the dietary role of Se in yellowtail kingfish will make significant contributions in modifying its practical diets to enhance its production.

A series of five experiments were conducted to investigate the nutritional role of Se in yellowtail kingfish. In the first experiment, a 3 × 2 factorial design with three levels of Se and two levels of vitamin E was conducted to investigate any interactions between Se and vitamin E in yellowtail kingfish. The second experiment investigated the effects of dietary supplementation of Se on immune competence and resistance of yellowtail kingfish to *Vibrio anguillarum*. In the third experiment, graded levels of Se from Se-yeast were supplemented to a fishmeal-based diet and fed to yellowtail kingfish to estimate the optimal dietary Se requirement and the optimal supplementation level of Se for the fish. The fourth experiment investigated the digestibility and bioavailability of Se from selenomethionine (SeMet), selenocystine (SeCys), Se-yeast, selenite and fishmeal in yellowtail kingfish. In the last experiment, the fish were fed excessive levels of dietary Se to investigate its toxicity, if any.

The results showed positive interactive effects between dietary Se and vitamin E in yellowtail kingfish. They complemented each other to protect the fish from muscle myopathy and increased fish immune responses. The immune responses and resistance of yellowtail kingfish to *V. anguillarum* were improved by feeding Se. Yellowtail kingfish had a requirement for Se that was not met by the fishmeal-based diet, which caused Se deficiency. The optimal level of Se in diets for yellowtail kingfish was estimated to be 5.5 mg/kg and the requirement for the optimal level of Se supplementation in a fishmeal-based diet was approximately 2.2 mg/kg diet. The level of Se from 4.8 mg/kg might be sufficient for the fish. The most digestible sources of Se were from SeMet and Se-yeast, whereas the least was from fishmeal. Selenium from SeMet or Se-yeast was more available for growth and Se accumulation in muscle tissues than Se from SeCys or selenite. The 20.8 mg Se/kg diet resulted in exhibiting

the effects of Se toxicity. Dietary Se between 15.4 and 20.8 mg/kg was a threshold level in juvenile yellowtail kingfish. For enhancement of growth and general health of yellowtail kingfish, the present study suggests using 2 mg/kg of SeMet or Se-yeast as a source of Se supplementation into the diets of yellowtail kingfish.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
BSA	Bovine serum albumin
CARL	Curtin Aquatic Research Laboratories
CFU	Colony-forming unit
DC	Digestibility coefficient
DCS	Digestibility coefficient of Se source
DE	Digestible energy
DI	Digestible Se intake of the fish
DP	Digestible protein
Dw	Dry weight
FCR	Feed conversion ratio
GPx	Glutathione peroxidase
Hb	Haemoglobin
HSI	Hepatosomatic index
Ht	Haematocrit
MS-222	Tricaine methanesulfonate
NRC	National Research Council
PBS	Phosphate buffer saline
RAS	Recirculation aquaculture system
RBC	Red blood cell count
ROS	Reactive oxygen species
S	Sulphur
SD	Standard deviation
Se	Selenium
SE	Standard error
SeCys	selenocysteine
SeMet	Selenomethionine
WBC	White blood cell count
Ww	Wet weight

LIST OF COMMON AND SCIENTIFIC NAMES

Common Name	Scientific Name
African catfish	*Clarias gariepinus*
Atlantic salmon	*Salmo salar*
Beluga	*Huso huso*
Bluegill	*Lepomis macrochirus*
Channel catfish	*Ictalurus punctatus*
Chinook salmon	*Oncorhynchus tshawytscha*
Cobia	*Rachycentron canadum*
Coho salmon	*Oncorhynchus kisutch*
Common carp	*Cyprinus carpio*
Cutthroat trout	*Oncorhynchus clarki bouvieri*
Fathead minnow	*Pimephales promelas*
Giant freshwater prawn	*Macrobrachium rosenbergii*
Gibel carp (Crucian carp)	*Carassius auratus gibelio*
Gilthead seabream	*Sparus aurata*
Goldfish	*Carassius auratus*
Green sunfish	*Lepomis cyanellus*
Grouper	*Epinephelus malabaricus*
Hybrid striped bass	*Morone chrysops* × *M. saxatilis*
Indian major carp	*Labeo rohita*
Japanese yellowtail	*Seriola quinqueradiata*
Lemon shark	*Negaprion brevirostris*
Nile tilapia	*Oreochromis Niloticus*
Northern pike	*Esox lucius*
Perch	*Perca fluviatilis*
Plaice	*Pleuronectes platessa*
Rainbow trout	*Salmo gairdneri*
Rainbow trout	*Oncorhynchus mykiss*
Razorback sucker	*Xyrauchen texanus*
Sacramento splittail	*Pogonichthys macrolepidotus*
Striped bass	*Morone saxitilis*
White sturgeon	*Acipenser transmontanus*
Yellowtail kingfish	*Seriola lalandi*

LIST OF PUBLICATIONS

Journal Articles

1. Le, K.T., and R. Fotedar. 2013. Dietary selenium requirement of yellowtail kingfish (*Seriola lalandi*). *Agricultural Sciences* 4: 68-75.

2. Le, K.T., T.T. Dao, R. Fotedar, and G. Partrigde. 2014. Effects of variation in dietary contents of selenium and vitamin E on growth and physiological and haematological responses of yellowtail kingfish, *Seriola lalandi*. *Aquaculture International* 22: 435-446.

3. Le, K.T., and R. Fotedar. 2014. Bioavailability of selenium from different dietary sources in yellowtail kingfish (*Seriola lalandi*). *Aquaculture* 420–421: 57-62.

4. Le, K.T., and R. Fotedar. 2014. Immune responses to *Vibrio anguillarum* in yellowtail kingfish, *Seriola lalandi*, fed selenium supplementation. *Journal of the World Aquaculture Society* 45: 138-148.

5. Le, K.T., R. Fotedar, and G. Partridge. 2014. Selenium and vitamin E interaction in the nutrition of yellowtail kingfish (*Seriola lalandi*): physiological and immune responses. *Aquaculture Nutrition* 20: 303-313.

6. Le, K.T., and R. Fotedar. 2014. Toxic effects of excessive levels of dietary selenium in juvenile yellowtail kingfish (*Seriola lalandi*). *Aquaculture* 433: 229-234.

Conference Abstracts

1. Le, K.T., and R. Fotedar. 2013. Physiological responses of selenium fed yellowtail kingfish. *Asian-Pacific Aquaculture 2013*, December 10-13, 2013, Ho Chi Minh City, Vietnam.

2. Fotedar, R., and K.T. Le. 2014. Dietary selenium in yellowtail kingfish nutrition. *World Aquaculture Adelaide 2014*, June 7-11, 2014, Adelaide, SA, Australia.

CHAPTER 1
INTRODUCTION

1.1 Background

Yellowtail kingfish *Seriola lalandi* Valenciennes, 1833 is a highly active pelagic marine, carnivorous fish found in sub-tropical and temperate waters throughout the world (Fowler et al., 2003; Kolkovski and Sakakura, 2004). Yellowtail kingfish has been identified as a potentially valuable aquaculture species. In fact, it has excellent attributes for aquaculture including high growth performance, highly accepted taste and market acceptance, and their suitability to be grown in sea cages as well as in inland recirculating systems (Miegel et al., 2010; Abbink et al., 2012). Yellowtail kingfish was first introduced in aquaculture in South Australia in the late 1990s (Miegel et al., 2010) and showed a significant increase in its production over a short time, from 45 tonnes in 1999/2000 (Fowler et al., 2003) to 889 tonnes in 2012/2013 (PIRSA, 2014). They are now farmed commercially in Australia, New Zealand, Japan, Chile and Netherlands (PIRSA, 2002; Garcia et al., 2014; Orellana et al., 2014). They are fed with a range of diets, from trash fish to extruded dry pellets of variable ingredients and nutrient compositions due to the limitation of their nutritional information (Nakada, 2008; Moran et al., 2009; Booth et al., 2010; Orellana et al., 2014). As the expansion and intensity of its aquaculture activity, the research in the area of yellowtail kingfish nutrition has received a considerable attention. However, the research has focused mainly on requirements for protein, lipid and energy (Booth et al., 2010; Bowyer et al., 2012a); and there is a lack of information on the mineral requirements.

One mineral that has been known as an essential trace element for normal growth and physiological function of animals including fish is selenium (Se) (National Research Council, 1993; Watanabe et al., 1997). Selenium is a component of the enzyme glutathione peroxidase (GPx), which plays an important role in protecting cell membranes against oxidative damages (Rotruck et al., 1973). A deficiency of dietary Se has shown to cause reduction in GPx activity in rainbow trout *Salmo gairdneri*, channel catfish *Ictalurus punctatus* and Atlantic salmon *Salmo salar* (Hilton et al., 1980; Gatlin et al., 1986; Bell et al., 1987; Wise et al., 1993a). Selenium 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. Immune-stimulating effects of dietary Se has been reported for channel catfish (Wang et al., 1997). In addition, dietary supplementation of Se has been demonstrated to enhance growth of grouper *Epinephelus malabaricus*, cobia *Rachycentron canadum* and gibel carp *Carassius auratus gibelio* (Lin and Shiau, 2005b; Liu et al., 2010; Han et al., 2011).

Selenium exists in two forms, organic Se and inorganic Se. Organic sources of Se have been known to be more effective than inorganic sources in improving growth, immune responses and maintaining antioxidant defence systems (Mahmoud and Edens, 2003; Kumar et al., 2009). This could be explained by the differences in metabolism of the two sources of Se. Organic Se is readily absorbed through the gut and better absorbed than inorganic Se (Daniels, 1996; Lyons et al., 2007). In fish, higher digestibility and bioavailability of organic over inorganic form has been found in Atlantic salmon and channel catfish (Bell and Cowey, 1989; Wang et al., 1997). Due to its superior bioavailability, organic Se has been chosen as a preferred Se source for supplementation in fish feed (Rider et al., 2010).

The need for nutritional information for yellowtail kingfish has increased and there is no published information on the role of dietary Se for this species. For all these reasons, this research focuses on the dietary organic Se in yellowtail kingfish.

1.2 AIM

The study aimed to improve immune status, disease resistance, growth and survival of yellowtail kingfish *Seriola lalandi* by dietary supplementation of organic Se.

1.3 OBJECTIVES

The above aim of the study can be achieved by meeting the following specific objectives:

1. To investigate the effects of variation in dietary contents of organic Se and vitamin E on physiological responses of yellowtail kingfish, and to examine any interaction of these two micronutrients.

2. To investigate if dietary supplementation of organic Se confers benefits to the health of yellowtail kingfish following bacterial infection.

3. To estimate the optimal dietary Se requirement and the optimal organic Se supplementation for yellowtail kingfish.

4. To investigate digestibility and bioavailability of Se from various dietary sources in yellowtail kingfish.

5. To investigate toxic effects of excessive supplementation of dietary Se in yellowtail kingfish.

1.4 SIGNIFICANCE

The current research makes significant and novel contributions to the field of aquaculture. It provides new information about beneficial use of organic Se for yellowtail kingfish aquaculture industry. Growth and health of yellowtail kingfish can be improved by supplementing organic Se at appropriate levels, which have been demonstrated in this study. In addition, the information of Se bioavailability and interaction between Se and vitamin E in the current study is useful for the refinement of practical diet formulations for yellowtail kingfish. As Se can be toxic at excessive levels of supplementation, the findings of toxic effects of dietary Se in the current study is important in prevention of Se toxicity. The haematological, histopathological, immune and antioxidant data of yellowtail kingfish can be applied in monitoring their health and would assist future research in fish diseases. Other significant contribution is that signs of fish Se toxicity observed in the current study will add to the knowledge of Se toxicity, and by conducting investigations on Se digestibility and bioavailability, the current research contributes to the understanding of Se metabolism. Finally, the levels of dietary Se employed in this study can contribute to the development of a protocol for effective use of Se for aquatic animals.

1.5 STRUCTURE OF THE THESIS

The thesis starts with a literature review in Chapter 2. It examines the status of nutritional research on yellowtail kingfish and its aquaculture activity and explores relevant literature about the importance of Se as an essential nutrient in animals. The first experiment follows in Chapter 3, which deals with the interaction between dietary

Se and vitamin E in yellowtail kingfish. The signs of Se and vitamin E deficiencies in fish are discussed in this chapter.

Chapter 4, in the form of the second experiment, presents benefits of Se supplementation to yellowtail kingfish following a bacterial infection. This experiment proves that the resistance of yellowtail kingfish to pathogen can be improved by dietary addition of Se. Chapter 5, the third experiment on dietary selenium requirement of yellowtail kingfish, suggests the optimal Se supplementation level and the optimal concentration of Se in diets for the fish in normal as well as under infectious conditions.

Chapter 6 (the fourth experiment) provides information on digestibility and bioavailability of dietary Se in yellowtail kingfish. It covers the explanation for the differences in bioavailability between organic and inorganic Se sources. The toxic effects of dietary Se in yellowtail kingfish (the fifth experiment) is presented in Chapter 7. This chapter shows how the fish respond to excessive levels of dietary Se and discusses a threshold level for yellowtail kingfish culture. Chapter 8 discusses and reviews the nutritional role of Se in yellowtail kingfish and combines the research from the previous experiments. It also highlights the main conclusions from the present study and makes recommendations for future research.

CHAPTER 2
LITERATURE REVIEW

2.1 YELLOWTAIL KINGFISH

2.1.1 Yellowtail Kingfish Taxonomy and Biology

Yellowtail kingfish *Seriola lalandi* is also known as goldstriped amberjack in Japan and California yellowtail in USA (Nakada, 2002; Stuart and Drawbridge, 2013). The taxonomic classification of yellowtail kingfish is as follows (Species 2000 & ITIS Catalogue of Life, 2013).

Kingdom	Animalia
Phylum	Chordata
Class	Actinopterygii
Order	Perciformes
Family	Carangidae
Genus	Seriola
Species	*Seriola lalandi* Valenciennes, 1833

Yellowtail kingfish have elongate, fusiform and compressed bodies with very small and smooth scales (McGrouther, 2012). They have a narrow yellowish stripe along middle of the body from snout to near the tail base (Henry and Gillanders, 1999). The upper back of the body is greenish and the sides and belly are silvery white (PIRSA, 2002). The distinguishing characteristic of yellowtail kingfish is their yellow caudal fins, from which the common name for this species comes (PIRSA, 2002).

Yellowtail kingfish are globally distributed in sub-tropical and temperate waters of the Pacific and Indian Oceans with preferred water temperature ranges of 18-24 ºC (Fowler et al., 2003; Kolkovski and Sakakura, 2004). This species inhabits coastal reefs, enters bays and offshore islands and is commonly found in small to large schools in depths up to 50 m (Bray and Gomon, 2011). They feed on small fish, squid and crustaceans during the day (Fielder and Heasman, 2011).

Yellowtail kingfish breed in the spring or summer months (Gillanders et al., 1999; Poortenaar et al., 2001). The size and age of first sexual maturity in yellowtail kingfish vary with gender and geographic location. While in Australia, females first mature at

834 mm and 3+ years, males at 471 mm and 0.9 years (Gillanders et al., 1999), in New Zealand the size and age at maturity are 944 mm and 7-8 years respectively for females and 812 mm and 4 years respectively for males (Poortenaar et al., 2001). The differences in size and age of sexual maturity in yellowtail kingfish populations between Australia and New Zealand could be due to warmer water temperatures in Australia (Poortenaar et al., 2001).

Female yellowtail kingfish are highly fecund, a single spawning can produce between 0.5 and 2 million eggs (PIRSA, 2002). Eggs are spherical and pelagic and 1-1.4 mm in diameter (Henry and Gillanders, 1999). At a water temperature range of 18-20 ºC, they hatch into larvae within 2-3 days (Henry and Gillanders, 1999; PIRSA, 2002). The onset of feeding is estimated to be between 2 and 3 day's post hatch (PIRSA, 2002). Metamorphosis occurs 18 to 22 days post-hatch and is completed 40-50 days after hatching (Fielder and Heasman, 2011).

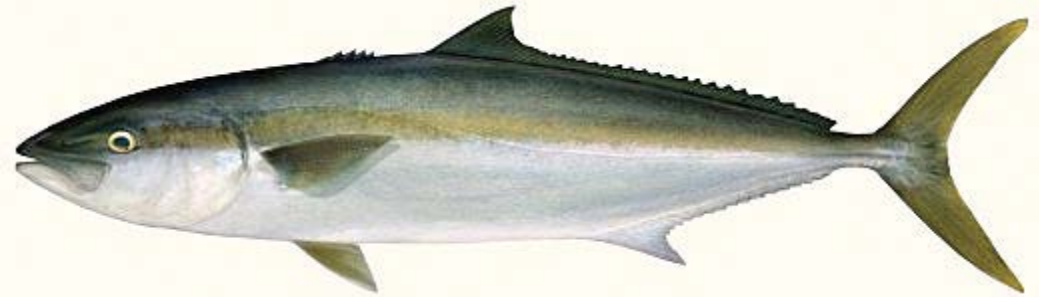

Figure 2.1 Yellowtail kingfish *Seriola lalandi* (Fielder and Heasman, 2011).

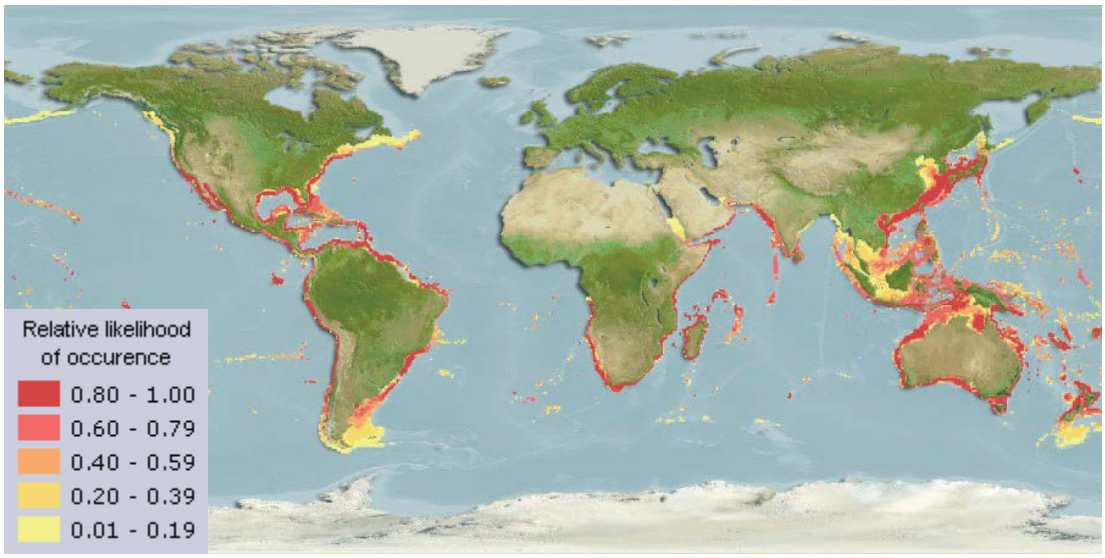

Figure 2.2 Global distribution of yellowtail kingfish (Fielder and Heasman, 2011).

Yellowtail kingfish have an excellent growth rate. They can grow up to 2.5 kg within 12 months (PIRSA, 2002). From the size of 5 g in sea cages, they can attain up to 5 kg in 2 years (Fowler et al., 2003). It is reported that this species can reach a maximum length, weight and age of 250 cm, 96.8 kg and 21 years, respectively (Fielder and Heasman, 2011). Reports on the optimum temperature for growth of yellowtail kingfish are not consistent. While Pirozzi and Booth (2009) suggested the temperature for the optimal metabolic function of this species was 22.8 ºC, Abbink et al. (2012) and Bowyer et al. (2013a) recommended that the optimal temperature for growth was 26.5 ºC and 24 ºC, respectively.

2.1.2 Yellowtail Kingfish Aquaculture

Yellowtail kingfish *Seriola lalandi* are being cultured in Australia, New Zealand, Japan, Chile and Netherlands (PIRSA, 2002; Garcia et al., 2014; Orellana et al., 2014) and are being investigated for culture in USA (Stuart and Drawbridge, 2012, 2013). The aquaculture of yellowtail kingfish in Japan relies on the collection of wild caught juveniles (Nakada, 2008). Wild yellowtail kingfish juveniles of about 700 g are harvested around the Goto Islands and weaned on artificial feed for grow-out phase (Nakada, 2008). On the contrast, the industry of yellowtail kingfish aquaculture in Australia, New Zealand, Chile and Netherlands is based on hatchery-produced seed (PIRSA, 2002; Aguilera et al., 2013; Garcia et al., 2014).

Hatchery protocol for the seed production of yellowtail kingfish is well documented by Fielder and Heasman (2011). Generally, broodstock of 10-40 kg are caught from the wild and maintained in indoor holding facilities (PIRSA, 2002; Benetti et al., 2005). They are usually fed fresh or frozen feed, including chopped fish, squid and mussel (PIRSA, 2002). However, concerns due to vitamin deficiencies in the broodstock, it is recommended that they should be weaned onto a semi-moist or dry pellet supplemented with vitamins and minerals (Benetti et al., 2005). Wild caught yellowtail kingfish spawn naturally in captivity without any use of hormonal induction (PIRSA, 2002). However, to control the reproduction and spawning of the captive broodstock, lighting, temperature and hormone treatments may be used (Poortenaar et al., 2001; Moran et al., 2007). Yellowtail kingfish larvae are reared using standard intensive techniques, which are described in detail by Fielder and Heasman (2011). The optimal *Artemia* feeding regimes for yellowtail kingfish larval rearing can be

found in Woolley et al. (2012). Previously, the hatchery-reared juvenile yellowtail kingfish had a high percentage of body deformity (Benetti et al., 2005). This problem has been attributed to vitamin deficiencies and has been resolved by improving broodstock nutrition (Benetti et al., 2005). When juveniles reach 5 g in weight, they are transferred to grow-out facilities (PIRSA, 2002).

In Australia, New Zealand and Japan, yellowtail kingfish are grown out typically in sea cages (Nakada, 2008; Moran et al., 2009; Booth et al., 2010). Previously, square sea cages ranging from 4 × 4 × 4 m up to 50 × 50 × 50 m were used in Japan (Honma and Kyōkai, 1993). Later, the "polar circle" type cages were introduced, this new sea cage type provides better water exchange, has lower cost of maintenance and is easier to harvest than the traditional one (Nakada, 2000). Similar "polar circle" type cages with size of 25 m in diameter and 4 to 8 m in depth are used in Australia and New Zealand (PIRSA, 2002). A maximum culture density of 10 kg/m^3 is allowable under South Australian aquaculture licence conditions (PIRSA, 2002).

The grow-out phase in Chile and Netherlands focuses in recirculation aquaculture systems (RAS) (Garcia et al., 2014; Orellana et al., 2014). The RAS comprises basically of a drum filter, a protein skimmer, a biological de-nitrification unit, and carbon dioxide removal and oxygenation systems (Garcia et al., 2014; Orellana et al., 2014). Growth performance of yellowtail kingfish in RAC is comparable to that of those cultured in sea cages (Orellana et al., 2014). However, onshore recirculating aquaculture systems would not be economically viable due to low numbers of fish produced (Nakada, 2002; PIRSA, 2002).

While trash fish have been used to feed yellowtail kingfish cultured in Japan, dry pellets from basic to complex formulation have been adapted for yellowtail kingfish grown in Australia, New Zealand, Chile and Netherlands (Moran et al., 2009; Booth et al., 2010; Garcia et al., 2014; Orellana et al., 2014). Yellowtail kingfish that are fed formulated pelleted diets can achieve feed conversion ratio of 1-1.5:1, while using a diet of trash fish, feed conversion ratio would be 8:1 (PIRSA, 2002; Nakada, 2008).

In tropical or subtropical cage culture conditions, yellowtail kingfish can grow to 1.5-3 kg in 12–14 months or 4–5 kg in 18 months (PIRSA, 2002; Love and Langenkamp, 2003; Benetti et al., 2005). Yellowtail kingfish are generally harvested when their whole body weights reach 3-4 kg and are then sold as whole fish, in fillet or cutlet

form (Fernandes and Tanner, 2008; Nakada, 2008). They are also sold for sushi and sashimi as fresh consumption (PIRSA, 2002; Nakada, 2008). Markets for yellowtail kingfish products include Japan, other parts of Asia, USA and the United Kingdom (Love and Langenkamp, 2003; Ottolenghi et al., 2004).

2.1.3 Nutritional Research on Yellowtail Kingfish

A diet deficient in energy will result in the use of dietary protein as an energy source rather than for tissue synthesis, whereas a diet excessive in energy will cause decreased nutrient intake by animals or excessive fat deposition in the body (Masumoto, 2002). Therefore, a balanced energy to protein ratio in a diet is very important for animals. The digestible protein (DP) and digestible energy (DE) requirements of yellowtail kingfish have been reported to be high compared to other carnivorous fish species (Booth et al., 2010). Diets containing 456 g DP/kg and 12 MJ DE/kg would provide the optimum ratio of DP:DE for yellowtail kingfish of 20 to 200 g (Booth et al., 2010). For yellowtail kingfish from 200 to 1000 g, a diet containing 465 g DP/kg and 15 MJ DE/kg would best meet the DP:DE criteria, while a diet containing 432 g DP/kg and 18 MJ DE/kg would be suitable for fish between 1000 and 2000 g (Booth et al., 2010).

There have been attempts to use plant protein sources as alternatives to fishmeal, a scarce and costly protein source in aquaculture (Watanabe et al., 1998; Imanpoor and Bagheri, 2012). The replacement of fishmeal by plant-derived products for yellowtail kingfish diet has been also studied (Bowyer et al., 2013b, 2013c). The fishmeal protein can be replaced up to 20% by soy protein concentrate in the diet of yellowtail kingfish without reducing growth and nutrient utilization of the fish (Bowyer et al., 2013c). However, solvent extracted soybean meal, a less refined soy ingredient than soy protein concentrate, is not a suitable substitute for fishmeal in diets for yellowtail kingfish (Bowyer et al., 2013b). This is explained by the low digestibility of diets containing solvent extracted soybean meal, which in turn is ascribed to anti-nutritional factors and non-protein compounds such as carbohydrates present in the solvent extracted soybean meal (Francis et al., 2001; Bowyer et al., 2013b).

Similar to fishmeal, the substitution of fish oil with alternative sources for yellowtail kingfish has received some research. Up to 50% and 100% of fish oil can be replaced by canola oil and poultry oil, respectively in diets of yellowtail kingfish without detrimental effects on the growth of fish (Bowyer et al., 2012a). The growth of

yellowtail kingfish is improved by the replacement of fish oil with 50% poultry oil, whereas decreased growth and increased expression of glutathione peroxidase 1 gene of the fish are caused by 100% replacement with canola oil (Bowyer et al., 2012b). The reduced growth of yellowtail kingfish as a result of 100% replacement of fish oil by canola oil is also reported by Collins et al. (2012). Concerning about producing high quality fish for the market, Bowyer et al. (2012a) showed that the fatty acid composition of the muscle tissue in yellowtail kingfish changes by replacing fish oil with alternative lipid ingredients, therefore changing the market value of the species. Generally, commercial feeds for yellowtail kingfish contain 15-26% lipid as fish oil or a mixture of fish oil with poultry oil (Moran et al., 2009; Miegel et al., 2010).

Recently, nutritional information of Se for yellowtail kingfish has been published (Le and Fotedar, 2013; Le et al., 2014a; Le and Fotedar, 2014b, 2014c; Le et al., 2014b). This will be presented and discussed in detail in this thesis.

2.2 SELENIUM

2.2.1 Selenium Properties and Sources

Selenium (Se) was discovered by Jöns Jakob Berzelius in 1817 (Lide, 2005). It has an atomic number of 34 and an atomic mass of 78.96 (Lide, 2005). Selenium is classified as a non-metallic element, sharing similar chemical properties with sulphur; consequently it can often substitute sulphur (Barceloux, 1999). Selenium is an essential component to form the active centre (selenol group, SeH) of glutathione peroxidase, thioredoxin reductase and other selenoenzymes (Levander, 1987; Ganther, 1999). Common inorganic forms of Se include selenite and selenate, whereas its organic forms are predominately selenomethionine (SeMet) and selenocysteine (SeCys) (Barceloux, 1999). Selenium species in living organisms are shown in Table 2.1.

In air, most of the Se is presents in the form of elemental Se bound to fly ash and particles, with concentrations average between 0.1-10 ng Se/m^3 (Barceloux, 1999). In water, the primary water-soluble forms of Se are selenate and selenite ions (Tsuji et al., 2012). The typical levels of Se in drinking water range from 0.12 to 0.44 µg/L (Ihnat, 1989). The detectable Se concentrations in sea water are between 0.09

Table 2.1 Selenium species in living organisms (Lobinski et al., 2000)

Selenium in proteins	
Selenoproteins	Selenocysteinyl residues
Se-containing proteins	Selenomethionyl residues
Non-protein selenium species	
Inorganic selenium	Selenite, selenate
Methylated selenium	Monomethylselenol, dimethylselenide, trimethylselenonium ions
Selenoamino acids	Selenocystine, selenomethionine, *Se*-methylselenocysteine, selenoglutathione

and 0.11 µg/L (Valentine, 1997). In the terrestrial environment, the primary sources of Se are rocks and soils, which contain Se at relatively low levels (Tsuji et al., 2012). The major available form of Se is found in grains is selenomethionine (Tsuji et al., 2012). Selenium content in wheat, one of the primary sources of dietary Se, can vary by a factor of 500 due to the differences in geography and fertilization techniques (Wolf and Goldschmidt, 2007). Unless grown on Se-rich soil, Se content in fruit and vegetables is very low, for example, tomatoes, asparagus and lima beans contain < 1, 23 and 72 µg Se/kg, respectively (Tsuji et al., 2012). Fungi, such as mushrooms and yeast, can accumulate Se in substantial amounts and contain more than 20 Se-containing compounds, including organic and inorganic forms (Lobinski et al., 2000). Animal meats contain Se mostly in the form of selenomethionine and selenocysteine (Tsuji et al., 2012). On average, domestic beef, chicken, lamb and pork has 200-350, 100-240, 200-300 and 200-400 µg Se/kg, respectively (Tsuji et al., 2012). Selenomethionine and selenite/selenite are the predominant Se species in fish (Cappon and Smith, 1982; Bierla et al., 2008). The average Se content in fish is 272 µg Se/kg, however, it can vary widely, from 120 µg/kg in freshwater fish to over 700 µg/kg in marine fish (Tsuji et al., 2012).

2.2.2 Selenium Bioavailability and Metabolism

Bioavailability is defined as the percentage of a compound that is absorbed and that reaches the systemic circulation to be distributed to organs and tissues (Di Vincenzo et al., 2004; Thiry et al., 2012). Bioavailability of Se can be estimated by measuring Se levels (in blood and body tissues) and glutathione peroxidase activity (Thiry et al., 2012). The bioavailability of Se is tightly related to its chemical forms, which are absorbed and metabolized differently (Fairweather-Tait et al., 2010).

In fish and other higher vertebrates, ingested Se is absorbed through the membrane of anterior intestine (Daniels, 1996; Finley, 2006). SeMet is actively absorbed through the same pathways as methionine by passing the intestinal barrier using Na^+-dependant process, while uptake of selenite is by passive diffusion (Wolffram et al., 1989; Daniels, 1996; Schrauzer, 2000). Under optimal conditions, the absorption rate is estimated to be 95-98% for SeMet and 62% for selenite (Dreosti, 1986; Thomson and Robinson, 1986). The digestibility of Se from fishmeal, SeCys, selenite and SeMet has been reported in Atlantic salmon *Salmo salar* is 46.6, 52.6, 63.9 and 91.6%, respectively (Bell and Cowey, 1989). The absorption of Se can be affected by the presence of other food components. It may be enhanced by vitamins A, C and E, and total protein; whereas heavy metals (especially mercury, arsenic and cadmium), fibres and high levels of some sulphur compounds tend to reduce it (Greger and Marcus, 1981; Levander, 1991; Fairweather-tait, 1997; Foster and Sumar, 1997; Gropper et al., 2009).

Following the absorption, SeMet can be readily incorporated into protein through the replacement of methionine (Fairweather-Tait et al., 2010). Both ingested inorganic and organic forms of Se can be transformed to the common intermediate, selenide (Suzuki, 2005). Selenite and selenate are reduced simply to selenide, whereas SeCys is directly lysed to selenide, and SeMet is converted to SeCys before lysed to selenide (Suzuki, 2005). After entering the selenide pool, Se is either used for the synthesis of selenoproteins or excreted (Fairweather-Tait et al., 2010). There are two metabolic pools of Se believed to exist (Janghorbani et al., 1990). One termed the exchangeable metabolic pool where all forms of Se are metabolised and utilized for selenoprotein synthesis and the second Se pool comprises of all SeMet containing proteins (Janghorbani et al., 1990). The SeMet pool can contribute to the exchangeable pool, but the exchangeable pool does not contribute to the SeMet pool (Janghorbani et al., 1990).

Another difference in metabolism of organic and inorganic Se has been attributed to the reutilization of organic forms (Swanson et al., 1991). As a result of recycling of organic Se, organic forms of Se appear to be more retained in the body than inorganic forms (Schrauzer, 2000; Burk et al., 2006). The comparative studies of the bioavailability of dietary organic and inorganic Se in fish are shown in Table 2.2.

Table 2.2 Bioavailability of dietary Se from organic and inorganic sources studied in fish

Species	Dosage (mg/kg diet)	Exposure period (weeks)	Organic source	Inorganic source	Comparative effects	References
Atlantic salmon *Salmo salar*	1	4	SeMet, SeCys, fishmeal	Selenite	Digestibility in order of SeMet>selenite> SeCys >fishmeal	Bell and Cowey (1989)
Atlantic salmon *Salmo salar*	1-2	8	SeMet	Selenite	SeMet resulted in higher Se content in muscle and whole body than selenite	Lorentzen et al. (1994)
Channel catfish *Ictalurus punctatus*	0.02, 0.06, 0.20 and 0.40	9	SeMet, Se-yeast	Selenite	Organic Se showed higher bioavailability for growth, GPx activity and Se accumulation in liver and muscle than selenite	Wang and Lovell (1997)
Channel catfish *Ictalurus punctatus*	0.02, 0.06, 0.20 and 0.40	9	SeMet, Se-yeast	Selenite	Organic Se was more effective than inorganic in enhancing macrophage activity and antibody response	Wang et al. (1997)
Channel catfish *Ictalurus punctatus*	0.5	6	Selenium proteinate	Selenite	Absorption of organic Se was higher than that of inorganic Se	Paripatana nont and Lovell (1997)
Crucian carp *Carassius auratus gibelio*	0.5	4	SeMet	Selenite	SeMet produced higher plasma GPx activity than selenite	Wang et al. (2007)
Hybrid striped bass *Morone chrysopx* × *M. saxatilis*	1, 2 and 4	12	SeMet	Selenite	Bioavailability of SeMet was 3.3 fold higher than selenite based on whole-body concentration	Jaramillo et al. (2009)
Rainbow trout *Oncorhynchus mykiss*	0.7	10	Se-yeast	Selenite	Se-yeast was more digestible and efficient in raising whole body Se than selenite	Rider et al. (2010)
Yellowtail kingfish *Seriola lalandi*	2	6	SeMet, SeCys, Se-yeast	Selenite	SeMet and Se-yeast resulted in higher digestibility, weight gain, muscle Se and bactericidal activity than selenite	Le and Fotedar (2014b)

SeMet, selenomethionine; SeCys, selenocystine; GPx, glutathione peroxidase.

2.2.3 Selenium Requirement and Deficiency

Selenium is now known as an essential nutrient required by all living organisms (Schrauzer, 2003b; Kieliszek and Błażejak, 2013). It is present in 25 identified selenoproteins with a variety of biological functions (Kryukov et al., 2003; Fairweather-Tait et al., 2010). The greatest biological significance of Se is its antioxidant properties, which protect the organism from oxidative damage (Rotruck et al., 1973; Arteel and Sies, 2001).

Although fish can uptake Se from the water via their gills, dietary exposure is the dominant pathway of Se uptake (Watanabe et al., 1997; Hamilton, 2004). The requirement of Se has been quantified in some fish species shown in Table 2.3. However, during stress and/or disease exposures, Se requirement of fish may increase (Hjeltnes and Julshamn, 1992; Rider et al., 2009).

Selenium deficiency has been associated with a number of farm animal diseases as well as human diseases. For example, the white muscle disease, a nutritional muscular dystrophy, is the most common Se deficiency disease in livestock animals (Tinggi, 2003). Myxedematous cretinism, Keshan disease and Kashin-Beck disease are observed in humans suffering from severe cases of Se deficiency (Coppinger and Diamond, 2001). Effects of Se deficiency in fish are summarised in Table 2.4.

Table 2.3 Selenium requirements in fish species

Species	Se requirement (mg/kg diet)	Main Se source	References
Rainbow trout *Salmo gairdneri*	0.15 - 0.38	Sodium selenite	Hilton et al. (1980)
Channel catfish *Ictalurus punctatus*	0.25	Sodium selenite	Gatlin and Wilson (1984)
Grouper *Epinephelus malabaricus*	0.70	Selenomethionine	Lin and Shiau (2005b)
Nile tilapia *Oreochromis niloticus*	4.6	Se-yeast	Ahmad et al. (2006)
African catfish *Clarias gariepinus*	3.67	Se-yeast	Abdel-Tawwab et al. (2007)
Cobia *Rachycentron canadum*	0.79 - 0.81	Selenomethionine	Liu et al. (2010)
Gibel carp *Carassius auratus gibelio*	1.18	Selenomethionine	Han et al. (2011)
Yellowtail kingfish *Seriola lalandi*	5.56	Fishmeal and Se-yeast	Le and Fotedar (2013)

Table 2.4 Effects of dietary Se deficiency in fish

Species	Fish size (g)	Se in diet (mg/kg)	Exposure period (weeks)	Deficiency symptoms	References
Atlantic salmon *Salmo salar*	0.1	0.10	4	Increased mortality, nutritional muscular dystrophy, abnormal erythrocytes and reduced GPx activity	Poston et al. (1976)
Atlantic salmon *Salmo salar*	6.0	0.17	28	Reduced growth, abnormal erythrocytes and reduced GPx activity	Bell et al. (1987)
Channel catfish *Ictalurus punctatus*	2.8	0.06	15	Growth depression	Gatlin and Wilson (1984)
Channel catfish *Ictalurus punctatus*	4.7	0.06	26	Suppressed growth, anaemia, severe myopathy, exudative diathesis, increased mortality and reduced GPx activity	Gatlin et al. (1986)
Channel catfish *Ictalurus punctatus*	10.9	0.06	17	Reduced GPx activity	Wise et al. (1993a)
Rainbow trout *Salmo gairdneri*	27.0	0.03	30	Abnormal swimming patterns, reduced packed cell volume and GPx activity, and increased vesiculation and distorted nuclei in hepatocytes	Bell et al. (1986)

GPx, glutathione peroxidase.

2.2.4 Selenium Interaction

Selenium has been found to interact with several compounds in animals including fish; and these interactions can be additive, antagonistic, or synergistic (Poston et al., 1976; Bell et al., 1985; Sorenson, 1991; Le et al., 2014b). The interaction that has received the most attention is the synergism between Se and vitamin E. Selenium and vitamin E perform similar functions as biological antioxidants. Selenium appears to exert its antioxidant effect through its incorporation in the enzyme glutathione peroxidase and vitamin E is a membrane-associated antioxidant of free radicals (Combs and Combs, 1986). The two nutrients complement each other in their activity and protect biological membranes from oxidative damage (Combs and Combs, 1986). In fish, studies of the interactions between Se and vitamin E have shown that diets deficient in both Se and vitamin E result in reduced growth, anaemia, severe myopathy, exudative diathesis and death, but single deficiency of either Se or vitamin E does not cause any of these deficiency signs (Poston et al., 1976; Bell et al., 1985; Bell et al., 1986; Gatlin et al., 1986; Le et al., 2014b). In terrestrial animals, combined supplementation of Se and

vitamin E is more effective than each single nutrient in raising immune responses (Peplowski et al., 1980; Marsh et al., 1981; Baalsrud and ØVernes, 1986).

Many studies have reported the interactions between Se and other trace elements and that Se toxicity is alleviated by antimony, arsenic, bismuth, cadmium, copper, germanium, mercury, silver and tungsten (Levander, 1977; Whanger, 1981; Marier and Jaworski, 1983). The interactions between Se and other trace elements have been also studied in some fish species. For example, the elevated dietary copper reduces the levels of liver Se in Atlantic salmon *Salmo salar*, indicating the antagonistic interaction between Se and copper (Lorentzen et al., 1998; Berntssen et al., 1999). In contrast, a study by Hilton and Hodson (1983) shows a positive correlation between concentrations of Se and copper in liver of rainbow trout *Salmo gairdneri*. While Lorentzen et al. (1998) postulate that the reduced Se concentrations is due to the formation of insoluble copper–Se complexes in the intestinal lumen, resulting in decrease in Se bioavailability, Hilton (1989) suggests that Se and copper bind together to form a Se-copper complex by way of a selenoprotein complex, causing the positive correlation of these minerals in liver. Other studies with northern pike *Esox lucius* and perch *Perca fluviatilis* demonstrated that Se and mercury are antagonistic, Se readily reduces mercury accumulation in the fish (Turner and Rudd, 1983; Turner and Swick, 1983; Paulsson and Lundbergh, 1991). In a dietary study, Hamilton et al. (2001) found that the toxicity of Se was reduced in razorback sucker *Xyrauchen texanus* by exposure to arsenic. Selenium and arsenic can decrease the toxicity of each other by increasing biliary excretion of protein bound Se or arsenic compounds (Marier and Jaworski, 1983).

2.2.5 Selenium and Antioxidant

Selenium is widely studied for its antioxidant properties (Tapiero et al., 2003; Miller et al., 2007; Tinggi, 2008; Atencio et al., 2009). It is an essential component of antioxidant enzymes, such as glutathione peroxidase (GPx) (Tapiero et al., 2003). The major role of GPx in the antioxidant defence system is to maintain appropriately low levels of hydrogen peroxides within the cell, thus decreasing potential damage caused by reactive oxygen species (ROS) (Rotruck et al., 1973; Tinggi, 2008; Brigelius-Flohé and Maiorino, 2013). Reactive oxygen species include superoxide, hydrogen peroxide and hydroxyl radicals, which are induced by the immune system to destroy microbial

pathogens, however excessive production of ROS can damage to the host cells (González-Párraga et al., 2003; Thorpe et al., 2004; Tinggi, 2008). The levels of ROS can be maintained by the activity of the enzymatic antioxidant GPx (Guemouri et al., 1991; Yeh et al., 2009). The activity of GPx, in turn, is dependent on the dietary Se intake (Ganther et al., 1976). In fish, the activity of GPx has been reported to increase with an increase of Se in their diets, whereas dietary deficiency of Se has shown to cause reduction in GPx activity (Hilton et al., 1980; Gatlin et al., 1986; Bell et al., 1987; Wise et al., 1993a; Lin and Shiau, 2005b; Liu et al., 2010; Le and Fotedar, 2014c).

2.2.6 Selenium and Immune Responses

Selenium has been shown to affect the biological function of all components of the immune system, i.e., nonspecific, humoral and cell-mediated responses (Kiremidjian-Schumacher and Stotzky, 1987; Arthur et al., 2003). In nonspecific resistance, cellular and serologic responses such as lysis of invading pathogens and migratory and phagocytic abilities of cells can be modulated by the presence of Se in the diet (Kiremidjian-Schumacher and Stotzky, 1987). Studies with cows and goats showed that diets deficient in Se caused reduced production and activity of the chemotactic factors and migration of white blood cells (Aziz and Klesius, 1985; Droke and Loerch, 1989; Jukola et al., 1996); whereas neutrophils from cows fed Se supplemented diets showed greater phagocytic and bactericidal activities and increased the production of leukotriene (Grasso et al., 1990; Jukola et al., 1996). Similarly, the humoral immune response is impaired by Se deficiency, whereas Se supplementation increases the response and leads to enhanced immunologic competence (Kiremidjian-Schumacher and Stotzky, 1987). Lower antibody titres were observed in dogs fed Se-deficient diets than control dogs when responding to a vaccine (Sheffy and Schultz, 1978); while supplementation of Se resulted in improved antibody responses of lambs and resistance to bacterial infection of mice (Spallholz, 1981; Kumar et al., 2009). Selenium also has the ability to modulate the development of cell-mediated immune responses (Kiremidjian-Schumacher and Stotzky, 1987). Se-deficient mice showed a decrease in the capacity of natural killer lymphocytes to destroy tumour cells, whereas Se supplementation resulted in functional enhancement of natural killer-mediated tumour cyto-destruction (Talcott et al., 1984; Meeker et al., 1985; Koller et al., 1986).

In aquatic animals, the effects of Se on their immune responses have been shown in a number of studies. For example, dietary Se improved resistance of yellowtail kingfish *Seriola lalandi* and channel catfish *Ictalurus punctatus* to bacterial infections (Wang et al., 1997; Le and Fotedar, 2014c). Leukocyte respiratory burst activity, plasma total immunoglobulin concentration and plasma lysozyme activity in grouper *Epinephelus malabaricus* were increased by Se supplementation (Lin and Shiau, 2007). In addition, dietary Se has been reported to increase phagocytic activities and respiratory bursts of the giant freshwater prawn *Macrobrachium rosenbergii* (Chiu et al., 2010).

There are two potential mechanisms of action of Se, which may explain the effects of this micronutrient on the immune system. First, through the activity of antioxidant glutathione peroxidase, Se is likely to protect immune cells such as neutrophils from oxidative damage (Arthur et al., 2003). Second, Se up-regulates the expression of high-affinity interleukin-2 (IL-2) receptors through a posttranscriptional mechanism (Roy et al., 1994). The interaction between IL-2 and its receptor delivers signals for proliferation of the T-cells, which is a key component in providing B-cell help during antibody synthesis (Minami et al., 1993; Brandes et al., 2003). This may explain the stimulatory effect of Se on antibody production. In addition, IL-2 in turn 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).

2.2.7 Selenium Toxicity

At trace concentrations, Se is required for normal growth and development of animals, at moderate concentrations it can be stored and homoeostatic functions are maintained, but at elevated concentrations it can result in toxic effects (Hamilton, 2004). The mechanism for Se toxicity has been attributed to the substitution of Se for sulphur (S) in the process of protein synthesis (Janz et al., 2010). In excessive amounts, Se erroneously replaces S, resulting in the formation of a triselenium linkage (Se-Se-Se) or a selenotrisulphide linkage (S-Se-S), preventing the formation of the necessary disulphide S-S linkages needed for the normal tertiary structure of protein molecules (Diplock and Hoekstra, 1976; Reddy and Massaro, 1983; Sunde, 1984). Therefore, substitution of Se for S could cause improper functioning of proteins (Reddy and Massaro, 1983; Sunde, 1984; Maier and Knight, 1994).

The most well documented toxic effect of Se in fish is reproductive teratogenesis (Lemly, 2002b). Selenium is efficiently transferred from adult fish to larval fish through the eggs (Gillespie and Baumann, 1986; Schultz and Hermanutz, 1990; Coyle et al., 1993). High Se concentrations in the developing fish are believed to cause teratogenic deformities, affecting both hard and soft tissues (Lemly, 1993a).

Other toxic effects of Se in fish include mortality, reduced growth, reproductive failure, haematological changes, and histopathological abnormalities in kidney and liver (Hodson et al., 1980; Sorensen et al., 1984; Hamilton et al., 1990; Coyle et al., 1993). The levels of dietary Se known to be toxic to fish and the tissue Se concentrations associated with toxic effect in fish are reviewed in Table 2.5 and Table 2.6, respectively.

Table 2.5 Levels of dietary Se known to be toxic to fish

Species	Size/ life stage	Se in diet (mg/kg)	Exposure period (weeks)	Toxic effect	References
Rainbow trout *Salmo gairdneri*	1.3 g	13.1	4	Mortality, reduced feed efficiency and growth	Hilton et al. (1980)
	0.1 g	11.8	4	Reduced growth and feed efficiency	Hilton and Hodson (1983)
	0.1 g	11.8	16	Kidney damage	Hilton and Hodson (1983)
Bluegill *Lepomis macrochirus*	3.0 g	13.6	6	Mortality, exophthalmia and loss of equilibrium	Finley (1985)
	5.1 g	30.0	43	Teratogenesis and reduced larval survival	Woock et al. (1987)
	2 years	33.3	20	Decreased larval survival	Coyle et al. (1993)
Fathead minnow *Pimephales promelas*	0.1 g	20.3	8	Reduced growth	Ogle and Knight (1989)
Striped bass *Morone saxatilis*	250.0 g	38.6	11	Mortality, kidney and liver damage	Coughlan and Velte (1989)
Chinook salmon *Oncorhynchus tshawytscha*	Larv-ae	9.6	12	Reduced growth and survival	Hamilton et al. (1990)
Coho salmon *Oncorhynchus kisutch*	4.5 g	13.6	24	Mortality	Felton et al. (1996)
Sacramento splittail *Pogonichthys macrolepidotus*	6.8 g	26.0	20	Mortality and liver lesion	Teh et al. (2004)
Grouper *Epinephelus malabaricus*	1.5 g	4.0	8	Reduced feed efficiency and growth	Lin and Shiau (2005b)
Rainbow trout *Oncorhynchus mykiss*	0.4 g	4.6	12	Reduced growth	Vidal et al. (2005)
White sturgeon *Acipenser transmontanus*	29.8 g	41.7	8	Reduced growth, liver lesion and abnormal behaviour	Tashjian et al. (2006)
Yellowtail kingfish *Seriola lalandi*	19.6 g	20.87	10	Liver lesion and reduced feed intake, growth and haematocrit	Le and Fotedar (2014a)

Table 2.6 Tissue Se concentrations associated with toxic effect in fish

Species	Tissue	Se level (mg/kg)	Toxic effect	References
Rainbow trout	Whole body	0.4[ww]	Blood changes	Hodson et al. (1980)
Oncorhynchus mykiss	Liver	12.7[ww]	Blood changes	Hodson et al. (1980)
	Whole body	1.1[dw]	Mortality	Hunn et al. (1987)
	Whole body	1.2[ww]	Reduced growth	Vidal et al. (2005)
Fathead minnow	Whole body	5.4[dw]	Reduced growth	Ogle and Knight (1989)
Pimephales promelas	Embryos	3.9[ww]	Reproductive failure	Schultz and Hermanutz (1990)
	Ovaries	5.9[ww]	Reproductive failure	Schultz and Hermanutz (1990)
Chinook salmon	Whole body	3.4[dw]	Reduced growth	Hamilton et al. (1990)
Oncorhynchus tshawytscha	Whole body	5.4[dw]	Mortality	Hamilton et al. (1990)
Striped bass *Morone saxatilis*	Muscle	3.5[ww]	Mortality	Coughlan and Velte (1989)
	Whole body	2.0[dw]	Mortality	Saiki et al. (1992)
Green sunfish *Lepomis cyanellus*	Liver	7.0[ww]	Histopathological lesions	Sorensen et al. (1984)
Bluegill	Muscle	5.1[ww]	Mortality	Finley (1985)
Lepomis macrochirus	Liver	8.5[ww]	Mortality	Finley (1985)
	Carcass	5.9[ww]	Reproductive failure	Gillespie and Baumann (1986)
	Ovaries	6.9[ww]	Reproductive failure	Gillespie and Baumann (1986)
	Ovaries	4.5[ww]	Reproductive failure	Hermanutz et al. (1992)
	Muscle	4.2[ww]	Reproductive failure	Hermanutz et al. (1992)
	Liver	7.3[ww]	Reproductive failure	Hermanutz et al. (1992)
	Whole body	4.6[ww]	Reproductive failure	Hermanutz et al. (1992)
	Ovaries	34[dw]	Reproductive failure	Coyle et al. (1993)
	Eggs	42[dw]	Reproductive failure	Coyle et al. (1993)
	Whole body	19[dw]	Reproductive failure	Coyle et al. (1993)
Sacramento splittail	Liver	32.5[dw]	Mortality	Teh et al. (2004)
Pogonichthys macrolepidotus	Muscle	29.4[dw]	Mortality	Teh et al. (2004)
Grouper *Epinephelus malabaricus*	Liver	2.19[ww]	Reduced growth and feed efficiency	Lin and Shiau (2005b)
White sturgeon	Kidney	41.3[dw]	Reduced growth	Tashjian et al. (2006)
Acipenser transmontanus	Liver	26.2[dw]	Reduced growth	Tashjian et al. (2006)
	Muscle	30.0[dw]	Reduced growth	Tashjian et al. (2006)
	Plasma	38.3[dw]	Reduced growth	Tashjian et al. (2006)
	Liver	37.4[dw]	Histopathological lesions	Tashjian et al. (2006)
	Kidney	30.9[dw]	Histopathological lesions	Tashjian et al. (2006)
Yellowtail kingfish *Seriola lalandi*	Liver	6.45[ww]	Histopathological lesions	Le and Fotedar (2014a)

[dw] dry weight;
[ww] wet weight.

CHATER 3
SELENIUM AND VITAMIN E INTERACTIONS IN THE NUTRITION OF YELLOWTAIL KINGFISH

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3.1 INTRODUCTION

The close metabolic interrelation between selenium (Se) and vitamin E was first recognized in mammals in 1957 (Schwarz and Foltz, 1957). Selenium and vitamin E act as biological antioxidants to protect cell membranes from oxidative damage (Rotruck et al., 1973). 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 and Booth, 2009; Abbink et al., 2012), has not been subjected to any research regarding dietary Se and vitamin E. Therefore, obtaining nutritional information of Se and vitamin E in yellowtail kingfish will contribute to the knowledge of nutritional requirements for this species and can be used for modifying diets to improve fish growth and health.

Previous research has shown that diets deficient in Se and vitamin E cause muscle myopathy in some fish species (Poston et al., 1976; Blazer and 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 haematological, immune and antioxidant responses (Hardie et al., 1990; Abdel-Tawwab et al., 2007; Atencio et al., 2009; Rider et al., 2009; Ispır et al., 2011; Betancor et al., 2012).

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. The objectives of this experiment were to investigate the effects of variation in dietary content of Se and vitamin E on growth, haematological, immune and antioxidant responses and histological signs of Se and vitamin E deficiencies, and examination of interactions of these two micronutrients in yellowtail kingfish. Haematocrit, white blood cell count, red blood cell count and haemoglobin concentration were measured as haematological indices. Antibody, lysozyme and bactericidal activities were used as tools to evaluate immune responses. Glutathione peroxidase activity was used as an indicator of antioxidant response.

3.2 MATERIALS AND METHODS

All experimental work in this thesis 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. Fish were anaesthetised or euthanized with tricaine methanesulfonate (MS-222, Sigma-Aldrich, Castle Hill, NSW, Australia) before being sampled. Chemicals used were analytical grade obtained from Thermo Fisher Scientific, Scoresby, VIC, Australia, unless otherwise stated.

3.2.1 Experimental Design and Diets

Three levels of Se and two levels of vitamin E in a two-way factorial experiment was arranged, in which a basal diet was either not supplemented or supplemented with Se (1 or 2 mg/kg) at each of two supplemental levels of vitamin E (40 or 180 mg/kg). A fishmeal-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, Adelaide, SA, Australia. Following extrusion, the pre-determined quantities 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 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 six experimental diets (Table 3.1). 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 *Oncorhynchus mykiss* (Rider et al., 2009). The supplemental levels of vitamin E were 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 came from the same batch and were similar in size. They were individually weighed and stocked into each of 18 experimental 200-L tanks, in a random-block design, at a density of 20 fish/tank. The average of the tank averages was 949.11 ± 0.60 g (mean ± SE), with the average of all the fish of 47.45 ± 0.07 g (mean ± SE). The tanks were supplied with flow-through seawater (35‰ salinity) at a rate of approximately 0.86 L/min producing 600% water exchange/day. 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/^{o}C meter (Cyberscan pH 300, Eutech Instruments, Singapore, Singapore) and an oxygen meter (Handy Polaris, OxyGuard, Birkerød, Denmark), respectively. Total ammonia was monitored daily by an ammonia (NH_3/NH_4^+) test kit (Mars Fishcare, Chalfont, PA, USA). During the trial, water temperature, pH and dissolved oxygen averaged 20.9 ± 0.3 ^{o}C, 7.6 ± 0.1 and 6.5 ± 0.4 mg/L, (mean ± SD), respectively. Total ammonia was always ≤ 1.0 mg/L.

Table 3.1 Experimental diets and their proximate analyses

Diet	Nutrient addition (mg/kg)		Proximate analysis (%)				Gross energy (MJ/kg)
	Se (measured)	Vitamin E (measured)	Crude protein	Crude lipid	Moisture	Ash	
1	0 (3.35±0.01)	40 (41.39±1.19)	46.21±0.90	15.05±0.16	8.52±0.05	9.56±0.16	21.7±0.3
2	1 (4.32±0.06)	40 (41.01±0.87)	46.76±0.17	15.22±0.18	8.74± 0.05	9.59±0.09	22.0±0.3
3	2 (5.39±0.06)	40 (40.35±0.37)	47.28±0.37	15.16±0.17	8.75±0.14	9.65±0.17	21.7±0.2
4	0 (3.36±0.01)	180 (180.07±0.65)	46.90±0.43	15.25±0.20	8.63±0.04	9.72±0.01	21.8±0.1
5	1 (4.36±0.02)	180 (180.41±0.36)	46.07±0.19	15.26±0.20	8.75±0.04	9.64±0.10	21.7±0.1
6	2 (5.34±0.03)	180 (179.23±1.86)	46.81±0.11	15.20±0.19	8.75±0.22	9.82±0.10	21.8±0.1

Values are presented as means ± SD, n=3.

Each of six dietary treatments was randomly assigned to three tanks, making triplicate tanks/diet. The fish were hand fed to satiation once at 10 am daily for six weeks. The fish were fed slowly to ensure no uneaten food. At the end of week 2 and week 4, five fish were randomly removed and sacrificed from each tank to keep acceptable biomass and to be used for measurement of serum agglutinating antibody titre.

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 two weeks and four 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).

3.2.2 Sample Collection

At the start of the experiment, five additional initial fish were filleted, and the muscle was kept at -20 ^{o}C for analyses of Se and vitamin E content and proximate composition. Samples of the rearing water in all tanks during the dietary exposure were taken for Se analysis weekly.

At the end of the feeding trial, three fish from each tank were randomly selected for collection of skin mucus and blood. To collect skin mucus, both sides of a 1-mL inoculation loop were rubbed against the fish in an area located between the vent and the lateral line. The loop then was washed in a pre-weighed centrifuge tube containing 400 µL of PBS (0.1 M, pH 7.2). The tube was reweighed to determine amount of mucus and centrifuged for 10 min at 1500×*g* and 4 ^{o}C using a centrifuge (5804R, Eppendorf, Hamburg, Germany). The supernatant was used to measure mucous lysozyme activity.

Blood was sampled from the caudal vein with a 25-gauge needle attached to a 3-ml syringe. Half of the blood was used for haematological assays. The remaining blood was allowed to clot for 2 h at 4 ^{o}C and serum was separated by centrifugation of the blood at 1500×*g* and 4 ^{o}C for 10 min. Serum was used for agglutinating antibody titre, bactericidal activity and lysozyme assays. The red blood cell pellets were used for glutathione peroxidase assay. Mucous, serum and red blood cell samples were kept at -80 ^{o}C until analysis.

Following the blood sampling, left anterior dorsal muscle and anterior intestine were dissected and fixed in 10% buffered formalin for histopathological examination. The remaining muscles were kept at -20 ºC before being analysed for Se and vitamin E content and proximate composition.

3.2.3 Agglutinating Antibody Titre

At the end of week 4 of the feeding trial, all fish were given an intraperitoneal injection of 0.1 mL phosphate-buffered saline (PBS; 0.1 M, pH 7.2) containing 30 µg of purified bovine serum albumin (BSA; CAS No. 9048-46-8, Sigma-Aldrich, Auckland, 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 titre to BSA was determined for each fish by the method of Chen and 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.

3.2.4 Haematological Assays

***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 (Livingstone, Rosebery, NSW, Australia) and centrifuged at 13000×*g* for 5 min to determine haematocrit (the per cent packed cell volume).

***White blood cell count (WBC) and red blood cell count (RBC)*:** Four blood smears/fish were prepared on microscope slides and stained with May-Grünwald and Giemsa solution. The total WBC and RBC was determined on each blood smear under a light microscope (BX40F4, Olympus, Tokyo, Japan) and the relative percentage was calculated. The absolute values were then obtained by multiplying these percentages by the total number of blood cells counted from a haemocytometer (Improved Neubauer, Superior, Berlin, Germany).

***Haemoglobin*:** Haemoglobin concentration in whole blood from each fish was determined by using the Hb HG-1539 kit (Randox, Crumlin, County Antrim, UK) and a chemistry immune analyser (Olympus AU400, Tokyo, Japan).

3.2.5 Bactericidal Activity

Serum bactericidal activity was performed in duplicate for each fish by the method of Ueda et al. (1999). *Vibrio anguillarum* stock was obtained from Department of Agriculture and Food, Perth, WA, Australia. Fifty microlitres of *V. anguillarum* in PBS (1.6×10^4 CFU/mL) was added to 50 µL serum, and the mixture was reacted for 30 min at 25°C. The same volume of bacteria was added to 50 µL of PBS as control and was 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. Bactericidal activity was calculated as decrease in number of viable *V. anguillarum* cells, that is., $\log_{10}$ CFU/mL in the control minus $\log_{10}$ CFU/mL in serum.

3.2.6 Lysozyme Activities in Skin Mucus and Serum

Lysozyme activities in skin mucus and serum were performed in duplicate for each fish using the turbidimetric assay as described previously (Bowden et al., 2004). Fifty microlitre of sample was pipetted, in duplicate, in a 96-well micro-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, Grödig, Austria). One unit of lysozyme activity was defined as the amount of enzyme resulting in a decrease in absorbance of 0.001/min. The lysozyme activities in mucus and serum were expressed as enzyme units/mg of mucus (U/mg) and enzyme units/mL of serum (U/mL), respectively.

3.2.7 Glutathione Peroxidase Activity

Glutathione peroxidase (GPx) activity in red blood cells from each fish was assayed 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 ºC. The results were expressed as units of GPx/g of haemoglobin (Hb) (U/g Hb). Haemoglobin was measured using the Hb HG-1539 kit (Randox, Crumlin, County Antrim, UK).

3.2.8 Histopathology Examination

The histological samples were routinely processed, 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).

3.2.9 Selenium, Vitamin E and Proximate Analyses

Proximate analyses of the experimental diets were conducted in triplicate. Muscle Se and vitamin E content and proximate composition were analysed for each fish. Gross energies were determined using a bomb calorimeter (C2000, IKA, Staufen, Germany). Protein, lipid, ash and moisture were determined by methods of the Association of Official Analytical Chemists (1990): crude protein by analysis of nitrogen using the Kjeldahl method with Kjeltec Auto 1030 analyser (Foss Tecator, Höganäs, Sweden); lipid by petroleum ether extraction using a Soxtec System (2055 Soxtec Avanti, Foss Tecator, Höganäs, Sweden); moisture by drying at 105 ^{o}C in an oven (Thermotec 2000, Contherm Scientific, Hutt, Newzealand) to a constant weight; and ash by combustion at 550 ^{o}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 in hydrochloric acid (40%) for conversion of Se^{6+} to Se^{4+}. Digested sample was used for the estimation of Se (Association of Official Analytical Chemists, 1990) using an atomic absorption spectrometer equipped with vapour generation assembly (Varian AA280 FS and Varian VGA 77, Mulgrave, VIC, Australia).

Vitamin E was analysed by the liquid chromatographic method (DeVries and Silvera, 2002). Ground sample (1g) was placed into a glass stoppered test tube with 1 mL of ultrapure H_2O. Ten millilitres 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. Five millilitres 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 equipped with a fluorescence detector (Hewlett Packard series 1100 HPLC system, Waldbronn, Germany and HP chemstation software, Avondale, CA, USA) was used for the quantitative determination of extracted α-tocopherol (hereafter referred to as vitamin E).

3.2.10 Data Analysis

Data were analysed using PASW Statistics 18.0 (IBM Corporation, New York, USA). All data were tested for normality and homogeneity of variance using Shapiro-Wilk and Levene's tests, respectively. Data violating the assumptions of parametric tests were transformed prior to analysis. Survival data were arcsine transformed. Data of haemoglobin and mucous lysozyme activity were log transformed. 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. Proximate composition data of the experimental diets 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 ± SE (standard error), unless otherwise stated.

3.3 RESULTS

3.3.1 Water Selenium Analysis and Nutrient Analyses of Experimental Diets

Selenium in the rearing water was undetectable, < 1 µg/L. All nutrients other than Se and vitamin E did not differ significantly ($P > 0.05$) among experimental diets. The diets contained proximately 46.67% protein, 15.19% lipid, 8.69% moisture and 9.66% ash, and provided 21.77 MJ/kg energy (Table 3.1).

3.3.2 Growth Performance

There was no significant difference ($P > 0.05$) in initial weights of fish amongst treatments (Table 3.2). There was no significant effect of dietary Se and vitamin E or their interaction on fish weight gain in the first four weeks of the feeding trial. However, a significant interactive effect ($P < 0.05$) between the two micronutrients was found after being fed for six weeks (Table 3.2). Selenium 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 mg/kg gained significantly ($P < 0.05$) more weight than fish fed diet 1 without Se supplementation, 73.48 and 55.57 g/fish, respectively.

Neither dietary Se nor vitamin E significantly affected ($P > 0.05$) feed intake and feed conversion ratio (Table 3.2). Survival of fish was near 100% and was also unaffected by any dietary treatments (Table 3.2).

3.3.3 Muscle Se, Vitamin E and Proximate Composition

Protein, lipid, moisture, ash and gross energy in muscles of fish were not significantly ($P > 0.05$) influenced by dietary Se, vitamin E or their interaction (Table 3.3). However, muscle Se concentrations significantly ($P < 0.05$) increased by supplemental levels of Se, from 0.35 to 0.51 and 0.65 mg/kg when Se supplemented at 1 and 2 mg/kg diet, respectively (Table 3.3).

Table 3.2 Weight gain, feed intake, feed conversion ratio (FCR) and survival of yellowtail kingfish fed the experimental diets for six weeks[1]

Diet[2]	Initial weight (g/fish)	Weight gain (g/fish)			Feed intake (g/fish)	FCR	Survival (%)
		Week 2	Week 4	Week 6			
1	47.37±0.07	19.46±1.82	42.07±0.82	55.57±2.18^{a}	62.92±5.02	1.13±0.06	95.00±2.89
2	47.52±0.01	19.68±2.30	44.23±0.30	58.08±3.17^{ab}	65.56±4.54	1.13±0.02	96.67±1.67
3	47.49±0.02	21.58±1.35	45.29±0.72	73.48±2.73^{b}	86.86±4.13	1.18±0.04	100.00±0.00
4	47.53±0.06	21.75±0.50	45.29±1.34	72.25±1.39^{ab}	82.97±1.60	1.15±0.03	96.67±1.67
5	47.50±0.06	19.76±1.71	43.17±1.60	61.47±4.79^{ab}	73.75±9.56	1.19±0.07	98.33±1.67
6	47.35±0.13	18.64±0.97	42.87±0.88	62.63±4.92^{ab}	77.01±9.83	1.22±0.06	98.33±1.67
P value	0.311	0.800	0.219	0.022	0.135	0.759	0.488

[1] Values are presented as the mean ± SE of three replicates/dietary treatment.
[2] Diet abbreviations refer to Table 3.1.
Means in the same column with different superscript letters are significantly different ($P < 0.05$, one-way ANOVA).

Table 3.3 Selenium and vitamin E content and proximate composition of muscles of yellowtail kingfish fed the experimental diets for six weeks[1]

Diet[2]	Se (mg/kg)	Vitamin E (mg/kg)	Proximate composition (%)				Gross energy (MJ/kg)
			Crude protein	Crude lipid	Moisture	Ash	
1	0.35±0.00^{a}	3.28±0.15^{a}	20.00±0.05	2.51±0.05	75.48±0.29	1.42±0.02	5.47±0.08
2	0.49±0.01^{b}	3.21±0.02^{a}	20.41±0.14	2.62±0.04	74.88±0.18	1.39±0.02	5.47±0.09
3	0.67±0.04^{c}	3.43±0.10^{a}	20.49±0.19	2.56±0.01	75.27±0.36	1.37±0.02	5.52±0.07
4	0.34±0.02^{a}	5.34±0.15^{b}	20.36±0.11	2.57±0.04	75.07±0.26	1.40±0.02	5.49±0.08
5	0.52±0.02^{b}	5.29±0.15^{b}	20.46±0.10	2.58±0.03	75.47±0.25	1.36±0.03	5.50±0.12
6	0.64±0.02^{c}	5.18 ±0.13^{b}	20.29±0.05	2.58±0.04	75.40±0.16	1.38±0.02	5.47±0.04
P value	<0.001	<0.001	0.110	0.535	0.531	0.411	0.997
Initial	0.35 ± 0.01	3.34 ± 0.06	19.80±0.07	1.93±0.03	76.32±0.12	1.43±0.02	5.08±0.02

[1] Values are presented as the mean ± SE of one determination/fish, three fish/tank and three tanks/treatment.
[2] Diet abbreviations refer to Table 3.1.
Means in the same column with different superscript letters are significantly different ($P < 0.05$, one-way ANOVA). Initial data have been excluded from the ANOVA.

Similarly, vitamin E concentrations in muscles significantly increased with an increase in the dietary level. The diets supplemented with 180 mg vitamin E/kg produced muscle vitamin E of 5.27 mg/kg, significantly ($P < 0.05$) higher than vitamin E content of 3.31 mg/kg in the muscles of fish fed the diets supplemented with 40 mg vitamin E/kg.

3.3.4 Haematological Responses

Haematocrit and white blood cell count (WBC) were not significantly ($P > 0.05$) correlated with the variation in dietary contents of Se and vitamin E (Table 3.4). There was no significant ($P > 0.05$) interactive effect on red blood cell count (RBC) and haemoglobin (Hb) concentration when fish were fed dietary treatments of Se and vitamin E. As dietary Se increased, the mean RBC and Hb concentration increased and was independent of dietary vitamin E (Table 3.4). Vitamin E did not affect RBC and Hb concentrations, but Se did. Supplementation of Se at 2 mg/kg resulted in significantly ($P < 0.05$) higher mean RBC than without Se supplementation, 2.09×10^6/μL (average value of diet 3 and diet 6) and 1.94×10^6/μL (average value of diet 1 and diet 4), respectively. Similarly, there was significantly ($P < 0.05$) higher mean Hb levels in fish fed diets supplemented with 2 mg/kg Se (91.33 g/L; average value of diet 3 and diet 6) than in those given diets without Se supplementation (70.67 g/L, average value of diet 1 and diet 4).

Table 3.4 Haematological responses of yellowtail kingfish fed the experimental diets for six weeks

Diet[1]	Hb[2] (g/L)	Ht[3] (%)	WBC[4] ($\times 10^3$/μL)	RBC[4] ($\times 10^6$/μL)
1	69.00±3.21[a]	35.75±1.46	32.50±4.41	1.92±0.03
2	81.00±5.69[ab]	35.64±1.48	33.46±8.42	2.02±0.04
3	97.33±5.78[b]	38.51±3.00	26.99±4.23	2.10±0.04
4	72.33±4.18[a]	36.27±0.58	29.43±5.41	1.95±0.06
5	78.33±1.86[ab]	38.30±2.96	36.54±2.99	2.07±0.06
6	85.33±3.84[ab]	38.93±1.01	25.55±2.90	2.09±0.04
P value	0.007	0.755	0.652	0.074

Hb, haemoglobin; Ht, haematocrit; WBC, white blood cell count; RBC, red blood cell count.
[1] Diet abbreviations refer to Table 3.1.
[2] Values are presented as the mean ± SE of one determination/fish, three fish/tank and three tanks/treatment.
[3] Values are presented as the mean ± SE of three determinations/fish, three fish/tank and three tanks/treatment.
[4] Values are presented as the mean ± SE of four determinations/fish, three fish/tank and three tanks/treatment.
Means in the same column with different superscript letters are significantly different ($P < 0.05$, one-way ANOVA).

3.3.5 Immune Responses

All sampled fish at week 4 were negative to bovine serum albumin (BSA). Antibody titres to BSA of fish at the end of the trial were presented in Table 3.5. Agglutinating antibody titre to BSA of fish in this study was not significantly ($P > 0.05$) correlated with the variation in dietary contents of Se and vitamin E.

There was significant ($P < 0.05$) interaction between dietary Se and vitamin E with respect to serum bactericidal activity (Table 3.5). Simultaneous supplementation at high levels of both micronutrients (diet 6) significantly ($P < 0.05$) increased the bactericidal activity. In addition, fish fed diets supplemented with Se at 2 mg/kg (diet 3 and 6) had significantly ($P < 0.05$) higher mean bactericidal activity than those fed the un-supplemented Se diets (diet 1 and 4), $\log_{10} = 0.99$ and 0.51, respectively. Likewise, the bactericidal activity was significantly increased by vitamin E, significantly ($P < 0.05$) higher mean bactericidal activity was observed in fish fed high vitamin E diets ($\log_{10} = 0.93$) than those fed low vitamin E diets ($\log_{10} = 0.52$).

Lysozyme activity in skin mucus was not altered by dietary Se, but was by vitamin E (Table 3.5). The mean enzyme activity was five times significantly ($P < 0.05$) higher in fish fed diets high in vitamin E (81.11 units/mg) than in those fed diets low in vitamin E (16.20 units/mg).

Serum lysozyme activity was significantly ($P < 0.05$) affected 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 (Figure 3.1).

Fish fed diets supplemented with Se at 2 mg/kg had significantly ($P < 0.05$) higher mean enzyme activity (192.33 units/mL) than those given diets without Se supplementation (156.66 units/mL). 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 ($P < 0.05$) higher than in those fed low vitamin E, 222.22 and 127.77 units/mL, respectively.

3.3.6 Antioxidant Response

There was no significant ($P > 0.05$) interaction 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 (Figure 3.2). Supplementation of Se at 2 mg/kg resulted in the highest mean GPx activity at 107.48 units/g Hb, while un-supplemented group produced the lowest mean at 72.88 units/g Hb, with significant ($P < 0.05$) difference detected between the treatments.

Table 3.5 Immune responses of yellowtail kingfish fed the experimental diets for six weeks

Diet[1]	Antibody titre[2] (log_{10})	Bactericidal activity[3] (log_{10})	Mucus lysozyme[3] (units/mg)
1	2.21±0.10	0.21±0.05^{a}	5.83±0.42^{a}
2	2.41±0.00	0.57±0.06^{b}	18.93±2.56^{a}
3	2.51±0.10	0.78±0.08^{b}	23.82±7.49^{a}
4	2.41±0.00	0.81±0.04^{b}	75.58±6.00^{b}
5	2.61±0.10	0.79±0.09^{b}	87.27±5.37^{b}
6	2.41±0.17	1.19±0.04^{c}	80.49±6.01^{b}
P value	0.194	<0.001	<0.001

[1] Diet abbreviations refer to Table 3.1.
[2] Values are presented as the mean ± SE of one determination/fish, three fish/tank and three tanks/treatment.
[3] Values are presented as the mean ± SE of two determinations/fish, three fish/tank and three tanks/treatment.
Means in the same column with different superscript letters are significantly different ($P < 0.05$, one-way ANOVA).

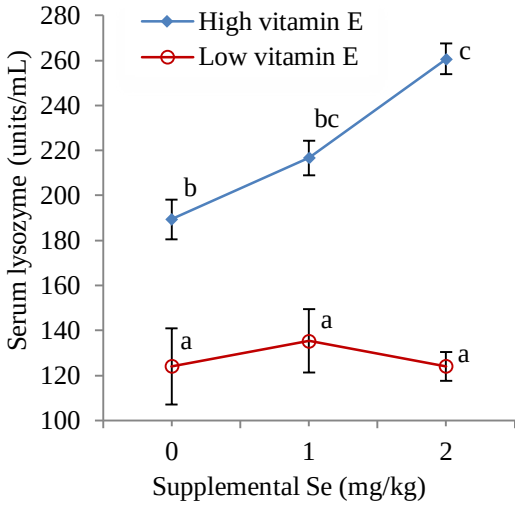

Figure 3.1 Serum lysozyme activity of yellowtail kingfish fed diets containing different levels of Se and vitamin E for six weeks. High vitamin E, 180 mg/kg; low vitamin E, 40 mg/kg. Each point represents the mean of two determinations/fish, three fish/tank and three tanks/treatment. Means with different letters are significantly different ($P < 0.05$, one-way ANOVA).

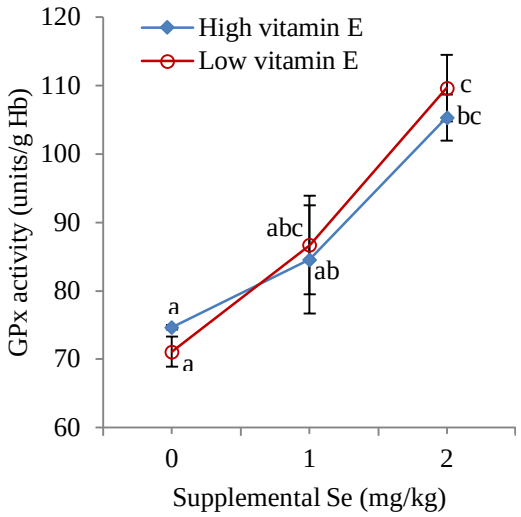

Figure 3.2 Red blood cell glutathione peroxidase (GPx) activity of yellowtail kingfish fed diets containing different levels of Se and vitamin E for six weeks. High vitamin E, 180 mg/kg; low vitamin E, 40 mg/kg. Each point represents the mean of one determination/fish, three fish/tank and three tanks/treatment. Means with different letters are significantly different ($P < 0.05$, one-way ANOVA).

3.3.7 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 the diet 1 without supplementation of Se and low level of vitamin E, whereas those fed the other diets had no myopathy (Figure 3.3). There was no statistical comparison in histology between fish fed different diets due to only one group of fish showed abnormality.

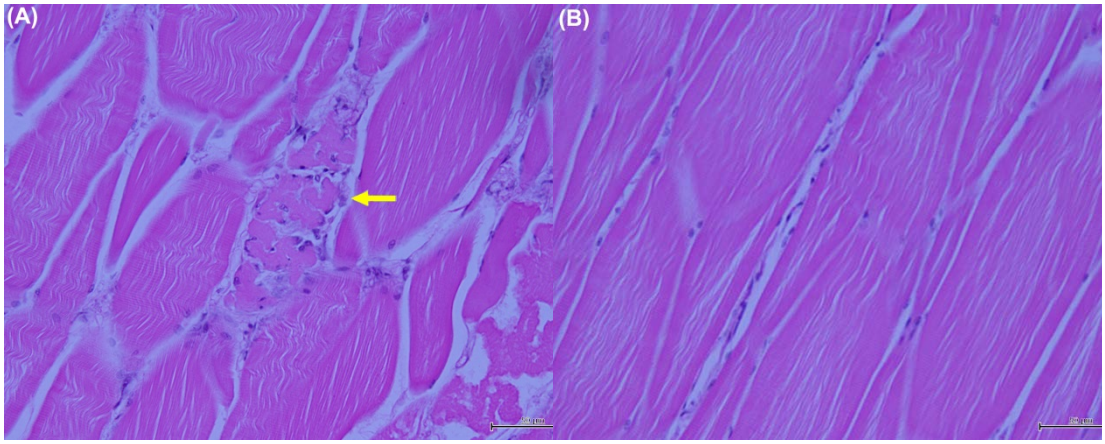

Figure 3.3 Sections of muscles of yellowtail kingfish fed experimental diets for six weeks. (A) depleted of both Se and vitamin E, resulting in necrotic fibres (arrow); (B) supplemented with Se at 2 mg/kg diet, showing healthy cells (Haematoxylin and eosin, scale bar = 50 µm).

3.4 DISCUSSION

Selenium concentrations were undetectable (< 1 μg/L) in all rearing tanks. Therefore, no significant Se leaching from the diets occurred, and effect of waterborne Se on the fish was minimal.

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 four weeks of the feeding trial. However, a significant interactive effect of Se and vitamin E on growth was found after being fed for six 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 and Lim, 2002a).

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 *O. 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* (Ispır et al., 2011), African catfish (Baker and 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, respectively) although these fish were fed for three 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) after eight 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. Selenium 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 Se and/or 52.5 mg/kg 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 and 179.23 mg/kg, 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, 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 and 0.67 mg/kg) in the present study were higher than those of wild yellowtail kingfish (0.33 mg/kg) (Chvojka, 1988) and were shown to increase with increasing dietary Se supplementation levels. In the agreement with the present study, Se concentrations in muscle of channel catfish (Gatlin and Wilson, 1984) and gibel carp *Carassius auratus gibelio* (Han et al., 2011) increased as dietary Se supplementation levels 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 and 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 after being fed for four 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 and 2.50 mg/kg, 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.

Haematological indices are important parameters for evaluation of fish physiological status, indicating how healthy a fish is (Kori-Siakpere et al., 2005). Japanese yellowtail *Seriola quinqueradiata* was considered in an anaemic state when haematocrit was lower than 27.00% and in a healthy status when haematocrit was higher than 38.20% (Watanabe et al., 1998). The average haematocrit values of yellowtail kingfish in the present study ranged from 35.64 to 38.93%, close to the haematocrit of healthy Japanese yellowtail. As sensitive to changes in nutritional conditions, haematological indices have been employed in effectively monitoring physiological responses of fish to nutrients including Se and vitamin E (Baker and Davies, 1996; Abdel-Tawwab et al., 2007). Red blood cell counts and haemoglobin concentrations in African catfish (Abdel-Tawwab et al., 2007) and in yellowtail kingfish increased with the increase of dietary Se intakes, indicating healthier status of fish fed Se supplementation. Haematocrits of African catfish (Baker and Davies, 1996), hybrid striped bass (Kocabas and Gatlin, 1999) and rainbow trout *O. mykiss* (Pearce et al., 2003) were increased by supplemental vitamin E. However, similar to the findings of the present study, vitamin E had no effect on haematocrits and white blood cell counts of beluga *Huso huso* (Amlashi et al., 2011), gilthead seabream *Sparus aurata* (Montero et al., 2001) and Indian major carp *Labeo rohita* (Sahoo and Mukherjee, 2002). While Se increased haematocrit of African catfish (Abdel-Tawwab et al., 2007), it did not affect haematocrits of rainbow trout *O. mykiss* (Rider et al., 2009) and yellowtail kingfish. In contrast to the results of the present study, the significant interactive effect between Se and vitamin E on haematocrit has been found in rainbow trout *S. gairdneri* (Bell et al., 1985). Haematocrits of rainbow trout *S. gairdneri* were reduced significantly when both nutrients were absent, but not when either Se or vitamin E was deficient. The differences in deficient levels of Se and vitamin E between rainbow trout diets (0.06 and 2.05 mg/kg respectively) and yellowtail kingfish diets may have resulted in the differences in interactive effects of Se and vitamin E.

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 and Turner, 1996). As dietary concentration of Se increased, antibody response of channel catfish increased (Wang et al., 1997), while a diet deficient in vitamin E has been reported to suppress antibody response of rainbow trout *S. gairdneri* (Blazer and 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 and ØVernes, 1986). 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 and ØVernes, 1986; Droke and 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 and Shefner, 1966; Trump and Hildemann, 1970). The use of antigen and doses in the present study was based on the previous studies for lemon shark *Negaprion brevirostris* (Clem and Small, 1967), goldfish *Carassius auratus* (Trump and Hildemann, 1970) and rainbow trout *O. mykiss* (Staykov et al., 2007).

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 and 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 and 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). Lysozyme

activities in skin mucus and serum were demonstrated to be stimulated by vitamin E in yellowtail kingfish in the present study. Similarly, stimulating effects of vitamin E on serum lysozyme activity have been reported for rainbow trout *O. mykiss* (Clerton et al., 2001) and Indian major carp (Sahoo and Mukherjee, 2002). The mechanism of action of vitamin E in increasing the activity of lysozyme has not been explained. It was found that Se did not compensate serum lysozyme activity for the lack of vitamin E in yellowtail kingfish. In fish fed diets low in vitamin E, the activities of serum lysozyme were the same, and were not correlated with different levels of Se. At the higher supplemental level of vitamin E, however, the serum enzyme activity was significantly increased by dietary Se.

Another important natural defence factor for protection against invading microorganisms is bactericidal activity, which directly kills bacterial cells and thus reflects the physiological condition of animals (Ueda et al., 1999). The bactericidal activity has been found in serum of fish and reported to be increased by vitamin E supplementation (Sahoo and Mukherjee, 2002). In agreement with this, bactericidal activity in serum of yellowtail kingfish was improved by dietary vitamin E in the present study. Furthermore, Se supplementation also increased serum bactericidal activity, and there was an interactive effect between Se and vitamin E, combined supplementation of both increased the bactericidal activity, indicating better physiological condition of fish being fed Se and vitamin E supplementation.

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). The GPx activity in liver of cobia *Rachycentron canadum* (Liu et al., 2010) and grouper *Epinephelus malabaricus* (Lin and Shiau, 2005b) increased with an increase in the dietary Se intake. Whereas, the GPx activity was shown to decrease in rainbow trout *S. gairdneri* (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. A similar pattern of decreasing red blood cell GPx with decreasing Se was seen in the present study. The previous study on rainbow trout *S. gairdneri* (Cowey et al., 1981) and the present study have revealed that dietary vitamin E had no effect on the GPx activity. In agreement with the findings reported

for rainbow trout *S. gairdneri* (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.

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 and Lim, 2002a). 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. Selenium significantly increased weight gain of fish fed diets low in vitamin E, but not high in vitamin E. Serum lysozyme activity was not improved by dietary Se in vitamin E deficient diets, but was in diets high in vitamin E. Both Se and vitamin E increased serum bactericidal activity, and there was a positive interaction effect between the two micronutrients with respect to the bactericidal activity. 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. Red blood cell counts and haemoglobin concentrations were increased by supplemented level of Se at 2 mg/kg, while vitamin E stimulated lysozyme activity in skin mucus. 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.

CHAPTER 4
SELENIUM SUPPLEMENTATION IMPROVES IMMUNE RESPONSES OF YELLOWTAIL KINGFISH

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4.1 INTRODUCTION

Selenium (Se) is an essential trace element for normal growth and physiological function of animals, including fish (National Research Council, 1993; Watanabe et al., 1997). It is a component of the enzyme glutathione peroxidase (GPx), which plays an important role in protecting cell membranes against oxidative damage (Rotruck et al., 1973). Selenium 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., 1993a) 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).

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 experiment 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.

4.2 MATERIALS AND METHODS

4.2.1 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 46.42% protein, 15.05% lipid, 91.48% dry matter, 9.56% ash and provided 21.68 MJ/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 the 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., 2014b).

4.2.2 Growth Trial

Yellowtail kingfish were supplied by the Australian Centre for Applied Aquaculture Research, Fremantle, WA, Australia and brought to Curtin Aquatic Research Laboratories (CARL), Curtin University. The fish came from the same batch and had similar sizes. They were group weighed and stocked into each of 12 experimental 300-L tanks, in a random-block design, at a density of 15 fish/tank. The average of the tank averages was 208.19 ± 0.50 g (mean ± SE), with the average of all the fish of 13.88 ± 0.03 g (mean ± SE). The tanks were filled with seawater at salinity of 35 ppt and Se

concentration < 1µL, and were supplied with constant aeration and pure oxygen (oxygen compressed, BOC, Perth, WA, Australia). Each tank had an external bio-filter (Fluval 406, Hagen, Rome, Italy) running continuously to create a recirculating system. 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 and dissolved oxygen were recorded at (mean ± SD) 21.4 ± 0.3 °C, 7.4 ± 0.1 and 6.6 ± 0.4 mg/L, respectively. Total ammonia nitrogen was < 1.0 mg/L.

Each dietary treatment was randomly assigned to four tanks. Fish were hand fed to apparent satiation twice a day at 08 am and 04 pm for six weeks. The fish were fed slowly to ensure no uneaten food. 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.

4.2.3 Bacterial Preparation and Challenge

Vibrio anguillarum was obtained from Bacteriology Laboratory, Department of Agriculture and 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 5000×*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 1×10^4 to 1×10^9 CFU/fish with ten fish/dose were conducted. Yellowtail kingfish (51.61 ± 0.93 g, mean ± SE)

provided by the Australian Centre for Applied Aquaculture Research were stocked into each of seven 300-L tanks at a density of ten fish/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* (1×10^4, 1×10^5…or 1×10^9 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 1×10^4, 1×10^5, 1×10^6 or 1×10^7 CFU/fish after two weeks. For 1×10^8 and 1×10^9 CFU/fish, mortalities during two weeks after the injection were 40 and 80%, respectively. The LC_{50} determined by extrapolation from the probit analysis as described by Finney (1971) was 1.7×10^8 CFU/fish, which was used to challenge the experimental fish.

At the end of the growth trial, after three fish/tank were taken for sampling, the remaining fish (12 fish/tank, average individual weight of 48.42 ± 0.79 g, mean ± SE) were challenged with bacteria. The fish were given an intraperitoneal injection of 0.1 mL of *V. anguillarum* suspension in PBS (1.7×10^8 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 weeks 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).

4.2.4 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/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 titre, 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 and livers from the sampled fish (after blood sampling) were dissected and fixed in 10% buffered formalin for histopathological

examination. The remaining fillets were used to estimate Se content and proximate composition.

4.2.5 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).

4.2.6 Red Blood Cell Peroxidation Assay

Sample of whole blood from each fish was washed three times in PBS by centrifugation at 1000×*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. (1993a). The oxidative titre was determined as the highest dilution ($\log_{10}$) of hydrogen peroxide that caused pellet formation due to lysis of red blood cell membranes.

4.2.7 Haematocrit

Haematocrit of each fish was determined in triplicate by the microhaematocrit method (Rey Vázquez and Guerrero, 2007) (see section 3.2.4).

4.2.8 Bactericidal Activity

Serum bactericidal activity was determined according to the method of Ueda et al. (1999) and performed in duplicate for each fish (section 3.2.5).

4.2.9 Lysozyme Assay

Lysozyme activity in serum was performed in duplicate for each fish using the turbidimetric assay as described previously (section 3.2.6).

4.2.10 Glutathione Peroxidase Assay

Glutathione peroxidase activity in red blood cells from each fish was assayed as described previously in section 3.2.7.

4.2.11 Histological Examination

The histological samples were routinely processed, stained and observed as described in section 3.2.8.

4.2.12 Selenium Analysis

Selenium content in muscle of each fish was analysed according to the standard methods of the Association of Official Analytical Chemists (1990) as described previously in section 3.2.9.

4.2.13 Serum Anti-*V. anguillarum* Antibody Titre

V. anguillarum was grown in tryptone soya broth at 25 °C for 24 h and killed in 1% formalin. The cells were centrifuged at 5000×*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-1201spectrophotometer, Shimadzu, Kyoto, Japan) and used as the antigen. Serum agglutinating antibody titre to *V. anguillarum* was determined for each fish 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.

4.2.14 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 ± SE.

4.3 RESULTS

There was no significant difference ($P > 0.05$) in average starting weight of fish amongst dietary treatments (Table 4.1).During six weeks of feeding, dietary Se did not influence feed intake, FCR and survival of the fish, which remained 100% (Table 4.1).

However, weight gain was significantly affected by the dietary treatments (Table 4.1), 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 (Table 4.2). The red blood cell membranes of the post-challenged fish fed the control diet were significantly ($P < 0.05$) more susceptible to peroxidation than the fish fed the supplemented Se diets, while the haematocrits of the post-challenged fish fed Se supplemented diets were significantly ($P < 0.05$) higher 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, whereas 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 ($P < 0.05$) higher Se content in fish muscles (Table 4.2).

The application of bacterial challenge altered immune and antioxidant parameters, except bactericidal activity (Table 4.2). As a result of the challenge, lysozyme and glutathione peroxidase activities were significantly stimulated. The activities of both enzymes were significantly ($P < 0.05$) increased in all treatments. The challenge resulted in a significant ($P < 0.05$) increase in susceptibility of red blood cell membranes to peroxidation, and caused significantly ($P < 0.05$) decreases in haematocrits and muscle Se content.

All serum samples collected at the end of the growth trial, used to measure pre-challenge antibody titres, were negative for *V. anguillarum*. At the end of the challenge, antibody titres against *V. anguillarum* were significantly ($P < 0.05$) increased with dietary Se supplementation of 2 or 4 mg/kg in comparison with the antibody titre of the control group (Table 4.3). The bacterial infection resulted in significantly higher mortalities in control-diet fed-fish than fish fed the Se supplemented diets (Table 4.3). Levels of Se supplementation did not make any difference ($P > 0.05$) in mortality rates.

Table 4.1 Weight gain, feed intake, feed conversion ratio (FCR) and survival of yellowtail kingfish fed the experimental diets for six weeks[1]

Diet	Initial weight (g/fish)	Weight gain (g/fish)	Feed intake (g/fish)	FCR	Survival (%)
Control	13.95±0.06	31.63±1.19[a]	44.05±0.76	1.40±0.06	100
Se 2	13.87±0.06	36.20±0.66[b]	44.70±0.98	1.26±0.04	100
Se 4	13.83±0.05	35.79±1.04[b]	47.06±1.18	1.37±0.02	100
P value	0.352	0.018	0.131	0.118	

[1] Values are presented as the mean ± SE of four replicates/dietary treatment.
Means in the same column with different superscript letters are significantly different ($P < 0.05$, one-way ANOVA).

Table 4.2 Red blood cell peroxidation, haematocrit, lysozyme, bactericidal and glutathione peroxidase activities, and muscle Se of yellowtail kingfish fed the experimental diets for six weeks and subsequently challenged with *V. anguillarum* for two weeks

Diet	Pre-challenge	Post-challenge	Challenge effect (*P* value)[4]
Red blood cell peroxidation (log_{10} titre)[1]			
Control	2.86±0.09	5.12±0.12[a]	<0.001
Se 2	2.64±0.08	4.06±0.09[b]	<0.001
Se 4	2.56±0.09	3.84±0.19[b]	0.001
P value	0.073	<0.001	
Glutathione peroxidase activity (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	<0.001	
Muscle Se (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	<0.001	<0.001	
Lysozyme (units/mL)[2]			
Control	71.50±3.20	134.00±2.94[a]	<0.001
Se 2	64.00±2.94	162.67±1.89[b]	<0.001
Se 4	62.00±5.72	190.67±4.03[c]	<0.001
P value	0.283	<0.001	
Bactericidal activity (log_{10})[2]			
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	<0.001	
Haematocrit (%)[3]			
Control	38.79±0.72	22.77±0.42[a]	<0.001
Se 2	42.00±0.65	31.79±0.65[b]	<0.001
Se 4	41.66±1.21	32.33±0.89[b]	0.001
P value	0.062	<0.001	

[1] Values are presented as the mean ± SE of one determination/fish, three fish/tank and four tanks/treatment.
[2] Values are presented as the mean ± SE of two determinations/fish, three fish/tank and four tanks/treatment.
[3] Values are presented as the mean ± SE of three determinations/fish, three fish/tank and four tanks/treatment.
[4] Pre- and post-challenge data were subjected to a one-way ANOVA.
For each parameter, means in the same column with different superscript letters are significantly different ($P < 0.05$, one-way ANOVA).

Light microscopy analysis of muscle sections revealed that there was multiphasic myopathy in fish fed the control diet (Figure 4.1), whereas 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 (Figure 4.2), but not in the fish fed the Se-supplemented diets. There was no statistical comparison in histology between fish fed different diets due to only one group of fish showed abnormality.

Table 4.3 Accumulative mortality and antibody to *V. anguillarum* of yellowtail kingfish fed the experimental diets for six weeks and subsequently challenged with *V. anguillarum* for two weeks

Diet	Accumulative mortality (%)[1]	Antibody titre (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	<0.001	<0.001

[1] Values are represented as the means ± SE of four replicates/treatment.
[2] Values are represented as the means ± SE of one determination/fish, three fish/tank and four tanks/treatment.
Means in the same column with different superscript letters are significantly different ($P < 0.05$, one-way ANOVA).

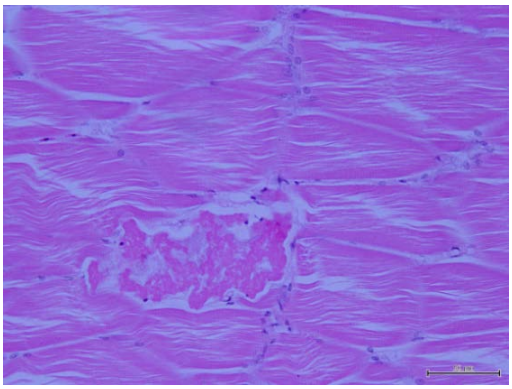

Figure 4.1 Section of muscle of yellowtail kingfish fed the control diet, showing necrotic fibres. Haematoxylin and eosin stain, scale bar = 50 µm.

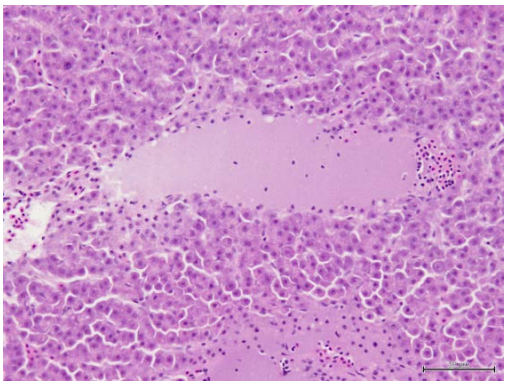

Figure 4.2 Section of liver of yellowtail kingfish fed the control diet, showing necrotic lesion caused by *V. anguillarum*. Haematoxylin and eosin stain, scale bar = 50 µm.

4.4 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 the present 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, 2002a), thus, supplementation of Se in yellowtail kingfish diet is necessary.

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. Selenium 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., 1993a), 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 *S. gairdneri* (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, 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. Selenium 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 *Oncorhynchus mykiss* (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 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.

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 *S. gairdneri*, the fish showed reduced growth and survival, and poor feed efficiency (Hilton et al., 1980). Selenium 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 *O. mykiss* (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 experiment 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.

CHAPTER 5
DIETARY SELENIUM REQUIREMENT OF YELLOWTAIL KINGFISH

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5.1 INTRODUCTION

The need for knowledge of nutritional requirements of yellowtail kingfish *Seriola lalandi* has recently increased as its aquaculture activity is expanding and intensifying. In the last few years, the research in the area of yellowtail kingfish nutrition has been conducted to refine their practical diet formulations. Since selenium (Se) is known as an essential trace element for normal growth and physiological function of fish (Watanabe et al., 1997), its nutritional information in yellowtail kingfish has also been studied (Le and Fotedar, 2014c; Le et al., 2014b). Those studies showed that supplementation of Se brought the beneficial outcomes for yellowtail kingfish, especially it improved immune responses and resistance of the fish to a common serious pathogen affecting cultured marine fish worldwide, *Vibrio anguillarum* (Le and Fotedar, 2014c).

The nutritional requirements of fish have been primarily based on growth and deficiency symptoms (Webster and Lim, 2002b) rather than on health status indicators such as immune responses and disease resistance. This experiment was designed to estimate the optimal Se requirement of juvenile yellowtail kingfish under normal condition as well as after infection with *V. anguillarum*.

5.2 MATERIALS AND METHODS

5.2.1 Experimental Diets

Five experimental dietary treatments were designed. One treatment comprised of the un-supplemented basal diet (Marine CST, Ridley AgriProducts, Melbourne, VIC, Australia) and the others were supplemented with Se at 1.5, 2, 2.5 and 3 mg/kg, based on the recommended supplementation level at 2 mg/kg in the previous study (Le and Fotedar, 2014c). The composition and preparation of experimental diets were as shown in section 4.2.1. The measured Se concentrations in each diet were (mg/kg; mean ± SD, n=3); 3.35 ± 0.01, 4.86 ± 0.02, 5.38 ± 0.03, 5.85 ± 0.03 and 6.38 ± 0.02 for the

un-supplemented basal diet, diets supplemented with Se at 1.5, 2, 2.5 and 3 mg/kg, respectively.

5.2.2 Growth Trial

Yellowtail kingfish, from the same batch and similar in size, were supplied by the Australian Centre for Applied Aquaculture Research, Fremantle, WA, Australia and brought to the Curtin Aquatic Research Laboratory (CARL), Curtin University. The fish were group weighed and stocked into each of 15 experimental 300-L tanks, in a random-block design, at a density of 12 fish/tank. The average of the tank averages was 223.98 ± 0.32 g (mean ± SE), with the average of all the fish of 18.66 ± 0.03 g (mean ± SE). The tanks were filled with seawater at salinity of 35 ppt and Se concentration < 1µL, and were supplied with constant aeration and pure oxygen (oxygen compressed, BOC, Perth, WA, Australia). Each tank had an external bio-filter running continuously. Half of the water was changed twice weekly. 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). During the trial, water temperature, pH and dissolved oxygen averaged (mean ± SD) 21.82 ± 0.80 °C, 7.56 ± 0.24 and 6.56 ± 0.38 mg/L, respectively.

Each dietary treatment was randomly assigned to three replicate tanks. Fish were hand fed to apparent satiation twice a day at 08 am and 04 pm for six weeks. 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).

5.2.3 Bacterial Preparation and Challenge

Vibrio anguillarum was obtained from Bacteriology Laboratory, Department of Agriculture & Food, Perth, WA, Australia. The bacterial concentrations were prepared as described previously in section 4.2.3.

To determine the LC_{50} (concentration lethal to 50% of test fish) to use in the experimental challenge, groups of ten yellowtail kingfish (51.98 ± 3.94 g, mean ± SD) were immersed in seawater (35 ppt) containing suspensions of *V. anguillarum* at 10^4, 10^5, 10^6, 10^7, 10^8 or 10^9 CFU/mL, or without addition of *V. anguillarum* (control) for

1 min. No mortality was observed in the control or in the bacterial suspensions of 10^4, 10^5 or 10^6 CFU/mL after two weeks. For 10^7, 10^8 and 10^9 CFU/mL, mortalities during two weeks after the immersion challenge were 10, 50 and 90%, respectively. The LC_{50} determined by extrapolation from the probit analysis as described by Finney (1971) was 10^8 CFU/mL, which was used to challenge the experimental fish.

At the end of the growth trial, the fish were challenged by immersion in *V. anguillarum* suspension of 10^8 CFU/mL for 1 min. All the challenged fish were returned to their respective rearing tanks and fed twice daily for a further four weeks 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).

5.2.4 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/tank and directly used for measurement of haematocrit. The remaining whole blood was allowed to clot for 2 h at 4°C and serum was separated for agglutinating antibody titre, 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 and spleens from the sampled fish were dissected out and fixed in 10% buffered formalin for histological examination. The remaining muscles were filleted and used to estimate Se contents.

5.2.5 Haematocrit

Haematocrit of each fish was determined in triplicate by the microhaematocrit method (Rey Vázquez and Guerrero, 2007) as in section 3.2.4.

5.2.6 Bactericidal Activity

Serum bactericidal activity was determined according to the method of Ueda et al. (1999) and performed in duplicate for each fish (section 3.2.5).

5.2.7 Lysozyme Assay

Serum lysozyme activity was performed in duplicate for each fish using the turbidimetric assay as described previously (section 3.2.6).

5.2.8 Glutathione Peroxidase Assay

Red blood cell glutathione peroxidase activity was assayed for each fish as described previously in section 3.2.7.

5.2.9 Selenium Analysis

Selenium content of muscle was analysed for each fish as described previously in section 3.2.9. The same method was used to measure Se concentrations in diets.

5.2.10 Serum Anti-*V. anguillarum* Antibody Titre

Serum agglutinating antibody titre to *V. anguillarum* was determined for each fish by the agglutination technique described in section 4.2.13.

5.2.11 Histological Examination

The histological samples were routinely processed, 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 or Perl's Prussian blue (Luna, 1968) and observed under a light microscope (BX40F4, Olympus, Tokyo, Japan). Numbers of macrophage aggregates (MAs) per sections of entire spleens were assessed.

5.2.12 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 ± SE. Second-order regression analysis (Shearer, 2000) was performed on weight gain vs. Se concentrations in diets and supplementation levels of Se to estimate dietary Se requirement for yellowtail kingfish.

5.3 RESULTS

There was no significant difference ($P > 0.05$) in initial weights of fish amongst treatments (Table 5.1). During six weeks of feeding, dietary Se did not influence feed intake, FCR and survival of the fish, which remained 100% (Table 5.1). However, weight gain was significantly ($P < 0.05$) affected by the dietary treatments (Table 5.1), the fish fed the control diet gained significantly ($P < 0.05$) less weight than fish fed the supplemented Se diets, which produced similar weight gains. Second order regression analysis of the levels of Se in diet vs. weight gain (Figure 5.1) indicated the optimal Se level for maximal growth of yellowtail kingfish was 5.56 mg/kg ($y = -1.5579x^2 + 17.328x - 5.039$, $R^2 = 0.989$ and $P < 0.001$). When the relationship between the supplementation levels of Se and weight gain data were analysed by polynomial regression, the maximum of the curve obtained was 2.19 mg/kg ($y = -1.5935x^2 + 6.9733x + 35.523$, $R^2 = 0.9897$ and $P < 0.001$; Figure 5.2).

Se supplementation had no significant effect on haematocrit and lysozyme activity of the pre-challenged fish, but affected the post-challenged fish (Table 5.2). During post-challenge period, the haematocrit and lysozyme activity were significantly higher ($P < 0.05$) in the fish fed Se supplemented diets than the fish fed the diet without supplementation. In both pre- and post-challenged fish, bactericidal and glutathione peroxidase activities were stimulated by Se supplements, fish fed Se supplemented diets had significantly higher ($P < 0.05$) bactericidal and glutathione peroxidase activities than fish fed the un-supplemented diet, while the increases in Se intake by the fish resulted in significant increases ($P < 0.05$) of Se concentrations in muscles (Table 5.2). As a result of the bacterial challenge, lysozyme, bactericidal and glutathione peroxidase activities were significantly elevated ($P < 0.05$). In contrast,

haematocrit and muscle Se decreased significantly ($P < 0.05$) by the challenge (Table 5.2).

The bacterial infection resulted in significantly higher mortalities in control-diet fed-fish than fish fed the diets supplemented with Se at more than 1.5 mg/kg (Table 5.3). Antibody titres against *V. anguillarum* were significantly increased ($P < 0.05$) with dietary Se supplementation of 2 mg/kg or more in comparison with the antibody titre of the control group (Table 5.3).

After the bacterial challenge, the surviving fish fed the un-supplemented diet had significantly higher ($P < 0.05$) numbers of macrophage aggregates (MAs) in spleen than the fish fed the Se-supplemented diets (Table 5.3). Within a MA, melanin, haemosiderin and lipofuscin/ceroid were clearly seen by the Perl's Prussian blue stain (Figure 5.3). The necrotic fibres in muscle were observed in the fish fed the control diet (Figure 5.4), but not in the fish fed the other diets.

Table 5.1 Weight gain, feed intake, feed conversion ratio (FCR) and survival of yellowtail kingfish fed diets containing various inclusion levels of Se for six weeks

Supplemental Se (mg/kg)[1]	Initial weight (g/fish)[2]	Weight gain (g/fish)[2]	Feed intake (g/fish)[2]	FCR[2]	Survival (%)
0.0 (3.35)	18.58±0.03	35.46±1.17[a]	51.43±1.40	1.46±0.05	100
1.5 (4.86)	18.66±0.07	42.72±0.46[b]	55.90±1.16	1.31±0.04	100
2.0 (5.38)	18.73±0.05	43.04±0.36[b]	56.74±1.45	1.32±0.02	100
2.5 (5.85)	18.71±0.06	42.51±0.55[b]	57.37±1.58	1.35±0.03	100
3.0 (6.38)	18.65±0.08	42.38±0.53[b]	56.02±0.52	1.32±0.01	100
P value	0.490	<0.001	0.054	0.065	

[1] Values in parentheses are measured concentrations of Se.
[2] Values are presented as the mean ± SE of three replicates/dietary treatment.
Means in the same column with different superscript letters are significantly different ($P < 0.05$, one-way ANOVA).

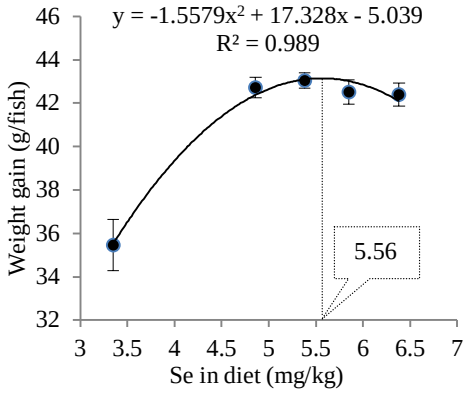

Figure 5.1 Relationship between concentration of dietary Se and weight gain of yellowtail kingfish fed the experimental diets for six weeks. Each point represents the mean ± SE of three replicates of each treatment. The optimal Se level for maximal growth of yellowtail kingfish derived from second order regression method was 5.56 mg/kg.

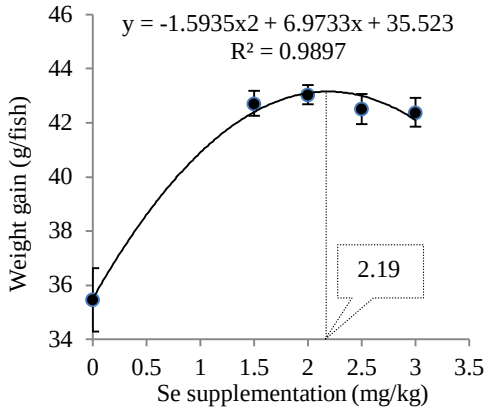

Figure 5.2 Relationship between dietary Se supplementation and weight gain of yellowtail kingfish fed the experimental diets for six weeks. Each point represents the mean ± SE of three replicates of each treatment The optimal Se supplementation level for maximal growth of yellowtail kingfish derived from second order regression method was 2.19 mg/kg.

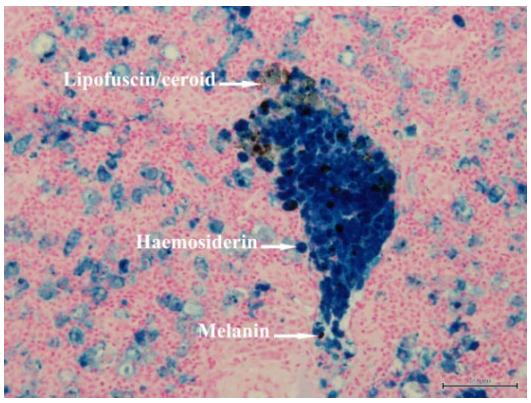

Figure 5.3 A macrophage aggregate in a section of spleen of yellowtail kingfish fed the control diet and subsequently challenged with *V. anguillarum*. Perl's Prussian blue stain, scale bar = 50 µm.

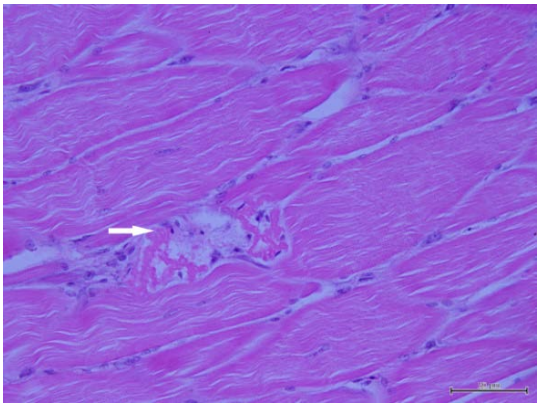

Figure 5.4 Section of muscle of yellowtail kingfish fed the control diet, showing necrotic fibres (arrow). Haematoxylin and eosin stain, scale bar = 50 µm.

Table 5.2 Muscle Se, glutathione peroxidase, lysozyme and bactericidal activities and haematocrit of yellowtail kingfish fed diets containing various inclusion levels of Se and subsequently challenged with *V. anguillarum*

Supplemental Se (mg/kg)[1]	Pre-challenge	Post-challenge	Challenge effect (*P* value)[5]
Muscle Se (mg/kg)[2]			
0.0 (3.35)	0.40±0.01^{a}	0.02±0.01^{a}	0.001
1.5 (4.86)	0.54±0.01^{b}	0.02±0.01^{b}	0.015
2.0 (5.38)	0.65±0.01^{c}	0.01±0.01^{c}	0.039
2.5 (5.85)	0.72±0.01^{d}	0.01±0.01^{d}	0.009
3.0 (6.38)	0.77±0.02^{d}	0.01±0.01^{d}	0.046
P value	<0.001	<0.001	
Glutathione peroxidase activity (units/g Hb)[2]			
0.0 (3.35)	67.97±1.35^{a}	76.80±1.11^{a}	0.007
1.5 (4.86)	82.60±3.20^{b}	91.57±2.29^{ab}	0.085
2.0 (5.38)	87.93±1.07^{b}	96.00±1.15^{b}	0.007
2.5 (5.85)	88.23±1.51^{b}	101.00±2.60^{b}	0.013
3.0 (6.38)	88.20±1.80^{b}	108.37±8.36^{b}	0.078
P value	<0.001	0.003	
Lysozyme (units/mL)[3]			
0.0 (3.35)	64.00±2.31	82.67±5.70^{a}	0.039
1.5 (4.86)	61.33±4.81	106.67±2.40^{b}	0.001
2.0 (5.38)	66.67±3.71	114.67±.69^{b}	0.005
2.5 (5.85)	70.67±5.46	108.00±3.06^{b}	0.004
3.0 (6.38)	70.67±2.91	119.33±4.67^{b}	0.001
P value	0.429	0.004	
Bactericidal activity (log_{10})[3]			
0.0 (3.35)	3.17±0.03^{a}	3.53±0.01^{a}	<0.001
1.5 (4.86)	3.36±0.01^{b}	3.57±0.01^{b}	<0.001
2.0 (5.38)	3.43±0.04^{b}	3.63±0.01^{c}	0.006
2.5 (5.85)	3.45±0.02^{b}	3.64±0.01^{c}	<0.001
3.0 (6.38)	3.49±0.06^{b}	3.66±0.01^{c}	0.052
P value	0.001	<0.001	
Haematocrit (%)[4]			
0.0 (3.35)	38.86±0.68	23.67±0.71^{a}	<0.001
1.5 (4.86)	40.31±0.18	30.25±1.09^{b}	0.001
2.0 (5.38)	40.21±0.28	31.51±1.06^{b}	0.001
2.5 (5.85)	40.09±1.03	32.09±0.72^{b}	0.003
3.0 (6.38)	39.61±0.77	32.15±0.94^{b}	0.004
P value	0.553	<0.001	

[1] Values in parentheses are measured concentrations of Se.
[2] Values are presented as the mean ± SE of one determination/fish, three fish/tank and three tanks/treatment.
[3] Value are presented as the mean ± SE of two determinations/fish, three fish/tank and three tanks/treatment.
[4] Value are presented as the mean ± SE of three determinations/fish, three fish/tank and three tanks/treatment.
[5] Pre- and post-challenge data were subjected to a one-way ANOVA.
For each parameter, means in the same column with different superscript letters are significantly different ($P < 0.05$, one-way ANOVA).

Table 5.3 Accumulative mortality, antibody to *V. anguillarum* and number of macrophage aggregates (MAs) in spleen of yellowtail kingfish fed diets containing various inclusion levels of Se and subsequently challenged with *V. anguillarum*

Supplemental Se (mg/kg)[1]	Accumulative mortality (%)[2]	Antibody titre (log_{10})[3]	Number of MAs per spleen[3]
0.0 (3.35)	48.15±3.70[a]	1.61±0.10[a]	204.33±3.93[a]
1.5 (4.86)	37.04±3.70[ab]	2.21±0.10[ab]	112.00±13.11[b]
2.0 (5.38)	29.63±3.70[b]	2.41±0.17[b]	92.00±2.52[bc]
2.5 (5.85)	29.63±3.70[b]	2.51±0.10[b]	87.00±5.51[bc]
3.0 (6.38)	25.93±3.70[b]	2.41±0.17[b]	77.33±6.17[c]
P value	0.014	0.005	<0.001

[1] Values in parentheses are measured concentrations of Se.
[2] Values are presented as the mean ± SE of three replicates/dietary treatment.
[3] Values are presented as the mean ± SE of one determination/fish, three fish/tank and three tanks/treatment.
Means in the same column with different superscript letters are significantly different ($P < 0.05$, one-way ANOVA).

5.4 DISCUSSION

The biosynthesis of selenoproteins is primarily dependent on Se supply and consequently on the formation of selenocysteine-specific tRNA, which regulates the level of all selenoproteins (Fischer and Pallauf, 2005). In poultry, Se supplementation has been reported to increase expression of genes involved in energy production and protein synthesis pathways (Brennan et al., 2011). Supplementation of Se may increase growth-related gene expression in yellowtail kingfish. Dietary Se and nutrigenomics was reviewed by Fischer and Pallauf (2005). In the present study, the beneficial growth effect of dietary Se for yellowtail kingfish was shown by the weight gain data and this is consistent with the data reported for grouper *Epinephelus malabaricus* (Lin and Shiau, 2005b), cobia *Rachycentron canadum* (Liu et al., 2010) and gibel carp *Carassius auratus gibelio* (Han et al., 2011). The optimal Se requirement for maximal growth of yellowtail kingfish obtained from the present study is higher than that reported for other fish species (Lin and Shiau, 2005b; Liu et al., 2010; Han et al., 2011). Yellowtail kingfish is very active and fast growing and hence may require high Se input (Le et al., 2014b).

Bacterial challenge has been used as a final test to evaluate relationships between nutrients and immune responses of fish (Landolt, 1989). 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. Supplementation of Se at ≥ 2 mg/kg significantly increased survival following infection with *V. anguillarum* and there was a corresponding increase in antibody titre. The same effects of Se

supplementation on survival and antibody response have been reported for channel catfish challenged by immersion with a virulent strain of *Edwardsiella ictaluri* (Wang et al., 1997) and yellowtail kingfish challenged by injection with *V. anguillarum* (Le and Fotedar, 2014c). Similar to the findings of Le and Fotedar (2014c), the immune-stimulating effects of dietary Se were also demonstrated in bactericidal and lysozyme activities, which were increased by the supplementation of Se. Supplementation of Se promotes lymphocyte protein synthesis and may lead to increased immune cell activity and thus results in an improved immune capacity (Pagmantidis et al., 2008). Furthermore, there were significant increases in glutathione peroxidase activities in response to Se supplementation in both pre- and post-challenged fish.

The *V. anguillarum* infection caused haematological changes which included a significant decrease in haematocrit and the presence of abundant macrophage aggregates in spleens of yellowtail kingfish. The haematocrit of the fish fed the control diet decreased to 23.67 %, lower than haematocrit of Japanese yellowtail *Seriola quinqueradiata* considered in an anaemic state, 27.00% (Watanabe et al., 1998). The decrease in haematocrit has also been found in coho salmon *Oncorhynchus kisutch* (Harbell et al., 1979), rainbow trout *Oncorhynchus mykiss* (Lamas et al., 1994) and yellowtail kingfish (Le and Fotedar, 2014c) infected with *V. anguillarum*. Haemolysin produced by *V. anguillarum* is believed to be responsible for the haemolytic anaemia in infected fish (Munn, 1978). The decreases in haematocrits and Se content in fillets and the increases in glutathione peroxidase activities as a result of bacterial infection indicate an increased requirement for Se under infected conditions (Le and Fotedar, 2014c).

The spleen macrophage aggregates play an important role in the storage of damaged cells including red blood cells and they change in number in relation to fish health, fish in poor health or nutritionally deprived tend to have more macrophage aggregates (Wolke, 1992). Thus, they have been suggested as reliable cytological biomarkers for assessment of fish health. In the present study, more macrophage aggregates were observed in the fish fed the control diet deficient in Se than in those fed Se supplemented diets, indicating improved physiological condition of fish being fed Se supplementation. In addition, muscle necrosis observed in the present study confirms the necessity of supplementation of Se for prevention of myopathy in yellowtail kingfish.

These results of the current study indicated that the optimum dietary Se requirement of yellowtail kingfish under normal and susceptible condition was approximately 5.56 mg/kg total Se or 2.19 mg/kg supplemental Se. The level of Se from 4.86 mg/kg might be sufficient for yellowtail kingfish.

CHAPTER 6
BIOAVAILABILITY OF DIETARY SELENIUM IN YELLOWTAIL KINGFISH

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6.1 INTRODUCTION

The nutritional requirement of Se in diet for yellowtail kingfish *Seriola lalandi*, which has excellent attributes for aquaculture, has now been studied (Le and Fotedar, 2013, 2014c; Le et al., 2014b). However, the requirement of dietary Se is not only met by its presence in the diet but also is met by its bioavailability, which in turn depends on various sources of Se in the diet (Fairweather-Tait et al., 2010). Organic sources such as selenomethionine (SeMet) and Se-yeast are generally believed to be more bioavailable than inorganic sources such as selenite. For example, the digestibility of SeMet is higher than selenite in Atlantic salmon *Salmo salar* (Bell and Cowey, 1989). Further, Se derived from SeMet or Se-yeast is more efficiently incorporated into muscle tissues (Wang and Lovell, 1997) and has a greater bioavailability than selenite to provide antibody production and macrophage chemotactic response in channel catfish *Ictalurus punctatus* (Wang et al., 1997).

The information on the bioavailability of Se from different dietary sources to yellowtail kingfish is not known yet; therefore, the aim of this experiment was to select the Se source that is highly bioavailable to juveniles of yellowtail kingfish. The fish were fed various sources of Se and digestibility, tissue accumulation, GPx activity and immune response were measured to assess the bioavailability of Se.

6.2 MATERIALS AND METHODS

6.2.1 Experimental Diets and Design

A fishmeal basal diet (Table 6.1) was supplemented with 2 mg/kg of Se from sodium selenite, DL-selenocystine (SeCys), DL-selenomethionine (SeMet) (Sigma-Aldrich, St. Louis, MO, USA) or Se-yeast (Selplex®, Alltech, Nicholasville, KY, USA). The basal diet contained chromic oxide (0.5%) as a digestibility marker. The pre-determined quantities of chemicals containing Se were dissolved in water and added

Table 6.1 Ingredient formulation and proximate composition of the basal diet

Ingredient[a]	(g/kg)	Proximate composition[c]	(%)
Fishmeal	550	Protein	53.04 ± 0.22
Fish oil	125	Lipid	15.23 ± 0.31
Wheat flour	100	Moisture	7.61 ± 0.24
Wheat gluten	100	Ash	9.64 ± 0.10
Shrimp meal	70	Gross energy (MJ/kg)	22.04 ± 0.10
Starch	40		
Se-free premix[b]	10		
Chromic oxide	5		

[a] Supplied by Specialty Feeds, Perth, WA, Australia, except chromic oxide obtained from Thermo Fisher Scientific, Scoresby, Vic, Australia.
[b] Contains the following (as g/kg of premix): iron, 10; copper, 1.5; iodine, 0.15; manganese, 9.5; zinc, 25; vitamin A retinol, 100 IU; vitamin D3, 100 IU; vitamin E, 6.25; vitamin K, 1.6; vitamin B1, 1; vitamin B2, 2.5; niacin, 20; vitamin B6, 1.5; calcium, 5.5; biotin, 0.1; folic acid, 0.4; inositol, 60; vitamin B12, 0.002; choline, 150 and ethoxyquin, 0.125.
[c] Values are presented as means ± SD, n=3.

to the basal ingredients before pelleting the feeds through a 2.5-mm diameter die. The pellets were then air-dried at room temperature and stored at -20 ^{o}C until used.

The fishmeal contained 5.93 ± 0.12 mg Se/kg (mean ± SD, n=3), which gave a Se concentration in the basal diet of 3.31 ± 0.01 mg/kg (mean ± SD, n=3). The measured Se concentrations in selenite, SeCys, SeMet and Se-yeast supplemented diets were 5.34 ± 0.02, 5.37 ± 0.03, 5.36 ± 0.02 and 5.36 ± 0.02 mg/kg (mean ± SD, n=3), respectively. The selected inclusion of Se was based on the Se requirement of yellowtail kingfish (Le and Fotedar, 2013).

Juveniles of yellowtail kingfish, from the same batch and similar in size, were obtained from the Australian Centre for Applied Aquaculture Research, Fremantle, WA, Australia and brought to the Curtin Aquatic Research Laboratory (CARL), Curtin University. The fish were group weighed and stocked into each of 15 experimental 300-L tanks, in a random-block design, at a density of 15 fish/tank. The average of the tank averages was 146.72 ± 0.31 g (mean ± SE), with the average of all the fish of 9.78 ± 0.02 g (mean ± SE). The tanks were filled with seawater at salinity of 35 ppt and Se concentration < 1µL; and were 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 and an automatic heater (HA-200, Sonpar®, China) to maintain water temperature. Half of the water was changed twice weekly in the first two weeks, and every two days afterwards. 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 was monitored daily by an ammonia (NH_3/NH_4^+) test kit (Mars Fishcare, Chalfont, PA, USA). During the trial, water temperature, pH and dissolved oxygen averaged 21.9 ± 0.8 °C, 7.5 ± 0.2 and 6.6 ± 0.3 mg/L (mean ± SD), respectively. Total ammonia (NH_3/NH_4^+) was always ≤ 1.0 mg/L.

Three tanks of fish were randomly assigned to each dietary treatment. The fish were fed twice daily to satiation for six weeks. The food was proffered by hand to ensure no uneaten food. The amount of feed consumed was recorded daily by calculating the differences in the weight of feed before the first and after the last feeding to estimate feed intake. Mortality was recorded daily to calculate survival. Fish in each tank were group weighed at the end of the 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).

6.2.2 Digestibility Study

Samples of faeces were collected from all fish in each tank at the end of weeks 4 and 5 and at the end of the feeding trial. The fish were anaesthetized with tricaine methanesulfonate (MS-222, Sigma-Aldrich, Castle Hill, NSW, Australia), and faecal samples were collected by stripping from the ventral abdominal region to the anal region. Pooled samples of faeces from each tank were dried at 55 ^oC and kept at -20 ^oC prior to analysis of Se and chromic oxide (Cr_2O_3). Selenium and Cr_2O_3 in collected faeces from each tank were analysed in triplicate.

The Se digestibility coefficients (*DC*) in all diets were calculated by the formula:

$$DC\ (\%)\ = 100\ \times \left[1\ -\ \frac{(\%\ Cr_2O_3\ in\ diet)(\%\ Se\ in\ faeces)}{(\%\ Cr_2O_3\ in\ faeces)(\%\ Se\ in\ diet)}\right]$$

The digestibility coefficients of Se sources (*DCS*) were corrected for residual Se in the basal diet and calculated as follows (Paripatananont and Lovell, 1997):

$$DCS\ (\%)\ = 100\ \times \left[\frac{(DC_{test})(Se_{test})\ -\ (DC_{basal})(Se_{basal})}{amount\ of\ supplemented\ Se}\right]$$

Digestible Se intake of the fish (DI) was calculated as follows:

$$DI\ (\mu g/fish) = Feed\ intake\ \times\ \%\ Se\ in\ diet\ \times DC$$

6.2.3 Collection of Blood and Muscle Samples

After the collection of faecal samples at the end of the feeding trial, three fish from each tank were randomly selected, and blood was sampled from the caudal vein with a 25-gauge needle attached to a 3-ml syringe. The blood was allowed to clot for 2 h at 4°C and serum was separated by centrifugation of whole blood at 1500×g for 10 min at 4 °C using a centrifuge (5804R, Eppendorf, Hamburg, Germany). Serum was used for bactericidal activity assay. 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.

Following the blood sampling, the fish were euthanized with MS-222 and filleted. Muscle Se content and proximate composition was analysed for each fish.

6.2.4 Bactericidal Activity Assay

Serum bactericidal activity was performed in duplicate for each fish by the method of Ueda et al. (1999) as described in section 3.2.5.

6.2.5 Glutathione Peroxidase Assay

Glutathione peroxidase (GPx) activity in red blood cells from each fish was assayed as described previously in section 3.2.7.

6.2.6 Chemical Analysis

Protein, lipid, moisture, ash and Se were analysed according to the standard methods of the Association of Official Analytical Chemists (1990) as described in section 3.2.9. Chromic oxide was measured by the procedure described by Bolin et al. (1952) using a spectrophotometer (UV-1201, Shimadzu, Kyoto, Japan). Gross energy was determined using a bomb calorimeter (C2000, IKA, Staufen, Germany).

6.2.7 Statistical Analysis

Data were analysed using PASW Statistics 18.0 (IBM Corporation, New York, US). All data were subjected to Levene's test for homogeneity of variance and one-way

ANOVA. 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. Linear regression analyses were performed to plot digestible Se intake of the fish against fish weight gain and Se concentration in muscle tissues. The statistical significance was set at $P < 0.05$.

6.3 RESULTS

There was no significant difference ($P > 0.05$) in initial weights of fish amongst treatments (Table 6.2). Dietary Se treatments did not influence feed intake, FCR and survival of yellowtail kingfish (Table 6.2). However, weight gain was affected by the dietary treatments, fish fed the basal diet gained significantly ($P < 0.05$) less weight than fish fed Se supplements (Table 6.2). Weight gains of fish fed SeMet and Se-yeast did not differ but were significantly ($P < 0.05$) higher than that of fish fed selenite. SeMet and Se-yeast resulted in significantly ($P < 0.05$) higher digestible Se intake of the fish than SeCys and selenite (Table 6.2). Linear regression analysis of fish weight gain showed linear response to the digestible Se intake of the fish ($y = 0.0696x + 24.014$, $R^2 = 0.8238$ and $P < 0.001$; Figure 6.1). Similarly, there was a positive linear regression between muscle Se accumulation and the digestible Se intake of the fish ($y = 0.005x - 0.0908$, $R^2 = 0.6394$ and $P < 0.001$; Figure 6.2).

Proximate composition and gross energy of muscles were not affected by the different dietary treatments (Table 6.3). In contrast, the sources of Se had significant effects on Se digestibility, Se concentration in muscle tissues and bactericidal activity (Table 6.4). Selenium derived from SeMet and Se-yeast showed the highest digestibility and bactericidal activities, significantly higher ($P < 0.05$) than Se from selenite and SeCys, whereas Se from the fishmeal (basal diet) was the lowest. Similarly, the highest muscle Se concentrations were in fish fed SeMet and Se-yeast, whereas the lowest was found in fish fed the basal diet. Selenium accumulation in muscle of fish fed SeCys was significantly higher ($P < 0.05$) than fish fed selenite, but significantly lower ($P < 0.05$) than fish fed SeMet or Se-yeast. Red blood cell glutathione peroxidase (GPx) activity was the same for fish fed Se supplemented diets but was significantly higher ($P < 0.05$) than that in fish fed the basal diet (Table 6.4).

Table 6.2 Weight gain, feed intake, digestible Se intake, feed conversion ratio and survival of yellowtail kingfish fed different Se sources[1]

Se source	Initial weight (g/fish)	Weight gain (g/fish)	Feed intake (g/fish)	Digestible Se intake (µg/fish)	FCR	Survival (%)
Basal diet	9.80±0.05	27.46±0.46^{a}	37.7 ±0.68	48.02±0.43^{a}	1.38±0.05	100
Selenite	9.76±0.06	30.24±0.80^{b}	41.12±0.84	100.87±1.37^{b}	1.36±0.04	100
SeCys	9.75±0.04	31.19±0.64^{bc}	40.19±1.35	100.52±2.81^{b}	1.29±0.03	100
SeMet	9.80±0.06	33.02±0.44^{c}	41.19±1.51	126.83±3.92^{c}	1.25±0.05	100
Se-yeast	9.80±0.06	32.95±0.41^{c}	40.33±1.85	123.64±6.96^{c}	1.22±0.05	100
P value	0.939	<0.001	0.394	<0.001	0.138	

SeCys, selenocystine; SeMet, selenomethionine; FCR, feed conversion ratio.
[1] Values are represented as the means ± SE of three replicates/treatment.
Means in the same column with different superscript letters are significantly different ($P < 0.05$, one-way ANOVA).

Table 6.3 Proximate composition of muscles of yellowtail kingfish fed different Se sources

Se source	Protein (%)	Lipid (%)	Moisture (%)	Ash (%)	GE (MJ/kg)
Basal diet	19.88±0.12	2.49±0.06	77.04±0.25	1.32±0.00	5.30±0.11
Selenite	20.04±0.04	2.49±0.04	76.87±0.14	1.35±0.03	5.42±0.02
SeCys	20.17±0.13	2.53±0.04	76.84±0.19	1.34±0.03	5.41±0.08
SeMet	20.17±0.11	2.46±0.04	76.77±0.15	1.34±0.01	5.45±0.09
Se-yeast	20.22±0.15	2.50±0.01	76.61±0.20	1.35±0.02	5.51±0.03
P value	0.318	0.825	0.632	0.887	0.449

SeCys, selenocystine; SeMet, selenomethionine; GE, gross energy.
[1] Values are presented as the means ± SE of one determination/fish, three fish/tank and three tanks/treatment.

Table 6.4 Se digestibility of diets, digestibility of Se sources, muscle Se, glutathione peroxidase and bactericidal activities in yellowtail kingfish fed different Se sources

Se source	Se digestibility of diet (%)[1]	Digestibility of Se source (%)[1]	Muscle Se (mg/kg)[2]	GPx activity (units/g Hb)[2]	Bactericidal activity (log_{10})[3]
Basal diet	38.48±0.82^{a}	38.48±0.82^{a}	0.21±0.01^{a}	67.25±1.72^{a}	3.24±0.01^{a}
Selenite	45.95±0.43^{b}	59.01±1.15^{b}	0.24±0.01^{a}	85.97±1.32^{b}	3.47±0.02^{b}
SeCys	46.56±0.21^{b}	61.41±0.56^{b}	0.35±0.00^{b}	80.80±2.25^{b}	3.46±0.01^{b}
SeMet	57.47±0.43^{c}	90.35±1.16^{c}	0.61±0.01^{c}	91.54±2.34^{b}	3.56±0.01^{c}
Se-yeast	57.12±0.74^{c}	89.48±1.99^{c}	0.62±0.01^{c}	90.71±3.96^{b}	3.56±0.01^{c}
P value	<0.001	<0.001	<0.001	<0.001	<0.001

SeCys, selenocystine; SeMet, selenomethionine; GPx, glutathione peroxidase; Hb, haemoglobin.
[1] Values are presented as the means ± SE of three determinations of pooled samples of 15 fish/tank and three tanks/treatment.
[2] Values are presented as the means ± SE of one determination/fish, three fish/tank and three tanks/treatment.
[3] Values are presented as the means ± SE of two determinations/fish, three fish/tank and three tanks/treatment.
Means in the same column with different superscript letters are significantly different ($P < 0.05$, one-way ANOVA).

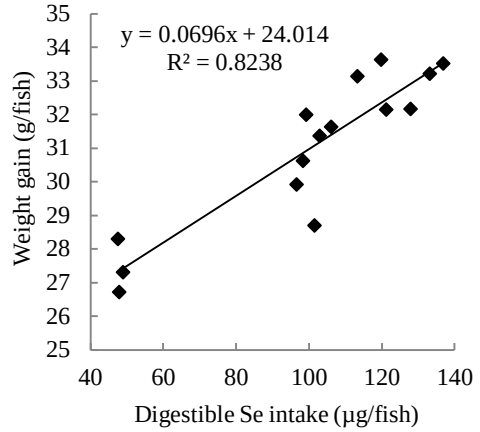

Figure 6.1 Relationship between digestible Se intake of fish and fish weight gain. Each point represents one of three replicates of each treatment.

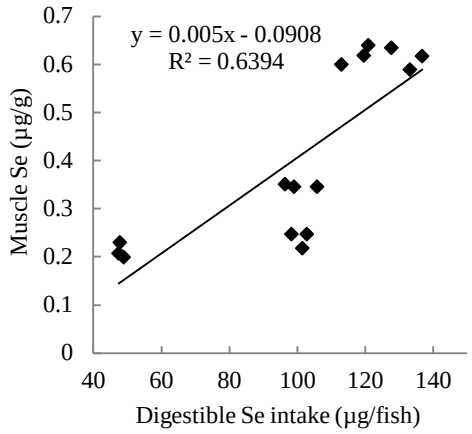

Figure 6.2 Relationship between digestible Se intake of fish and muscle Se accumulation. Each point represents mean of one group of fish with three fish/group and one determination/fish.

6.4 DISCUSSION

A fishmeal-based diet containing 1.2 mg/kg Se has been reported to meet the Se requirement of Atlantic salmon (Lorentzen et al., 1994). This is in contrast to the findings of the present study, in which yellowtail kingfish fed the fishmeal-based unsupplemented Se diet showed lower growth and GPx activity than those fed Se supplemented diets. The reduced growth and GPx activity are signs of Se deficiency (Poston et al., 1976; Bell et al., 1986). This demonstrates that the basal diet was not meeting the Se requirement in yellowtail kingfish. The relatively high Se requirement by yellowtail kingfish was discussed in previous studies (Le and Fotedar, 2013; Le et al., 2014b). The current study was performed to compare the bioavailability of Se from different dietary sources in yellowtail kingfish.

Selenium bioavailability depends on its chemical forms, which are absorbed and metabolized differently (Fairweather-Tait et al., 2010). Organic Se appears to be more bioavailable than inorganic sources to fish (Wang and Lovell, 1997; Jaramillo et al., 2009) as the former is better absorbed (Paripatananont and Lovell, 1997) and has higher retention (Rider et al., 2009). In the present study, the bioavailability of Se from SeMet and Se-yeast was similar for all the tools used to measure physiological performance of yellowtail kingfish. This similarity can be attributed to the fact that Se-yeast contains more than 90% of its Se in the form of SeMet (Schrauzer, 2006). Selenium from both sources, SeMet and Se-yeast, is well digested by yellowtail kingfish. The absorption of Se from these two organic sources was one and a half times more than that of Se from SeCys and selenite, and over twice that of Se from fishmeal. In fish and other higher vertebrates, ingested Se is absorbed in the anterior intestine (Daniels, 1996). The uptake of selenite is by passive diffusion (Daniels, 1996), whereas the absorption of SeMet is more efficient via the Na^+-dependent neutral amino acid transport system (Schrauzer, 2003a). Furthermore, the study on the movement of Se in intestinal sacs of hamsters by McConnell and Cho (1965) showed that there is an active transport of SeMet, but not SeCys or selenite, and that SeMet is transported intact across the intestinal membrane. The absorption of Se from the fishmeal in the basal diet is low as Se is bound to heavy metals (Webster and Lim, 2002a).

Selenium from fishmeal has been reported to have lower absorption than selenite and SeMet in Atlantic salmon (Bell and Cowey, 1989). The absorption of SeMet by Atlantic salmon is similar to the present research; however, the absorption of selenite is higher for Atlantic salmon than yellowtail kingfish. Apart from the dependence on species, different Se absorption could be due to differences in other feed ingredients present in the basal formulated diets. The interaction between minerals and other nutrients in yellowtail kingfish diet may decrease absorption of selenite. The reduced absorption of inorganic minerals by interaction with other nutrients has been reviewed by Paripatananont and Lovell (1997).

Absorption has been used to measure the bioavailability of Se in various food items (Fairweather-Tait et al., 2010). However, absorption alone cannot explain all the differences in the bioavailability of different Se compounds as the metabolism and storage of Se varies depending on its chemical form after being absorbed. SeMet is probably more bioavailable for metabolic processes than other Se forms as it is readily

incorporated into protein in place of methionine (Daniels, 1996). In the present study, more Se from SeCys retained in muscle tissues than from selenite although both Se forms had the same digestibility coefficients. This could be due to the extensive recycling of organic Se (Swanson et al., 1991) and/or the difference in metabolic pathways of different Se compounds in different tissues. The inorganic forms of Se increase Se in liver but not muscle tissues of rainbow trout *Oncorhynchus mykiss*, whereas organic forms can increase both hepatic and muscle Se reserves (Rider et al., 2010). There was almost no increase in Se concentration in muscle tissues of Atlantic salmon (Lorentzen et al., 1994) and yellowtail kingfish when selenite was supplemented to the fish diets. In contrast, muscle Se concentration in channel catfish increased by supplementing the diet with selenite as well as SeMet or Se-yeast; however, SeMet or Se-yeast as a source of Se was more effectively incorporated into muscular tissues of fish than selenite (Wang and Lovell, 1997). The higher bioavailability of SeMet than selenite for whole body Se accumulation was also reported for hybrid striped bass *Morone chrysops* × *M. saxatilis* (Jaramillo et al., 2009). The high muscle Se content in yellowtail kingfish fed SeMet or Se-yeast can be partially attributed to the high absorption of SeMet. In addition, the main protein concentration rests in fish muscle tissues; therefore, when SeMet is incorporated directly into proteins (Waschulewski and Sunde, 1988), it leads to an increase in Se concentration in fish muscles.

GPx is one of the most important antioxidant defence enzymes in fish (Ross et al., 2001), and its activity is dependent on the dietary Se intake (Ganther et al., 1976); thus, the GPx activity is frequently used to estimate Se bioavailability in fish. Organic Se has been reported to be more efficacious than inorganic Se in raising hepatic GPx activity in common carp *Cyprinus carpio* (Jovanovic et al., 1997) and channel catfish (Wang and Lovell, 1997). However, this is not consistent with other studies on other fish species. For example, Cotter et al. (2008) showed that selenite gives higher hepatic GPx activity in hybrid striped bass than Se-yeast when supplemented at 0.4 mg/kg. Another study on Atlantic salmon suggested that selenite or SeCys was a better source of Se for plasma GPx activity than SeMet; more Se from selenite and SeCys was incorporated into plasma GPx than Se from SeMet (Bell and Cowey, 1989). In the present study, GPx activity in red blood cells showed no correlation with the different sources of supplemented Se. This indicates no direct relationship between GPx activity

and Se form, probably because the metabolic role of Se from different forms and sources may be the same in red blood cell GPx.

Selenium exerts its effect on the immune system principally via selenoproteins (Arthur et al., 2003). For example, Se-containing proteins, glutathione peroxidases, protect neutrophils from superoxide- derived radicals, which are produced by neutrophils to kill foreign microbes. Bactericidal activity is a natural defence factor for protection against invading microorganisms, and directly killing bacterial cells (Ueda et al., 1999). The bactericidal activity has been found in serum of fish and is reported to be affected by dietary Se (Le et al., 2014a). Therefore, the measurement of immune competence, such as bactericidal activity, can partially reflect the bioavailability of Se. Unlike GPx activity, serum bactericidal activity in yellowtail kingfish was responsive to the sources of Se. Selenium supplemented as SeMet or Se-yeast was more available for bactericidal activity than Se from selenite or SeCys. This corresponded with the higher absorption of Se from SeMet and Se-yeast in comparison to selenite and SeCys. The higher bioavailability of SeMet and Se-yeast than selenite in improving immune capacity has been also demonstrated in channel catfish (Wang et al., 1997) as channel catfish fed SeMet or Se-yeast had higher antibody production and macrophage chemotactic activity than those fed selenite.

Wang and Lovell (1997) reported that Se from SeMet and Se-yeast had 336% and 269%, respectively, more availability than Se derived from selenite for growth of channel catfish. Similarly, in the present study, SeMet and Se-yeast appeared to be more bioavailable than selenite for the growth of yellowtail kingfish. This could be explained by the higher digestible Se intake of the fish fed SeMet or Se-yeast than those fed selenite.

In conclusion, different forms of Se supplemented to diets are digested and utilized differently by yellowtail kingfish. Selenium supplied as SeMet or Se-yeast was relatively more absorbed and was more bioavailable than SeCys or selenite. As Se-yeast had the same bioavailability as SeMet, it is recommended to use Se-yeast or SeMet as Se supplement in yellowtail kingfish feed.

CHAPTER 7
TOXICITY OF SELENIUM IN THE DIET TO YELLOWTAIL KINGFISH

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7.1 INTRODUCTION

As an essential nutrient in fish diet (National Research Council, 1993; Watanabe et al., 1997), selenium (Se) has gained the attention of many researchers in fish nutrition. This includes research in Se deficiency, requirement and bioavailability of Se in various fish species (Hilton et al., 1980; Gatlin and Wilson, 1984; Bell et al., 1987; Lin and Shiau, 2005b; Liu et al., 2010; Le and Fotedar, 2013). In addition, due to its potential toxicity to terrestrial animals (Halverson et al., 1966; Mézes and Balogh, 2009), the toxic effects of dietary Se in fish has been also of interest. Signs of Se toxicity in fish include high mortalities, histopathological changes in liver tissues, diminished reproductive performance and reduced feed intake, growth response and haematocrit values (Hilton et al., 1980; Gatlin and Wilson, 1984; Sorensen et al., 1984; Lemly, 1997; Tashjian et al., 2006; Jaramillo et al., 2009). However, the toxic levels of dietary Se have been a controversial topic for many years.

Hamilton et al. (1990) proposed that concentrations of dietary Se in the range of 3 to 5 mg/kg are toxic to chinook salmon *Oncorhynchus tshawytscha*. Whereas, Tashjian et al. (2006) suggested the dietary Se toxicity threshold for white sturgeon *Acipenser transmontanus* is between 10 and 20 mg/kg. Interestingly, cutthroat trout *Oncorhynchus clarki bouvieri* fed Se up to 11.2 mg/kg for 2.5 years showed no signs of Se toxicity (Hardy et al., 2010). The authors argued that cutthroat trout can regulate Se through excretion to maintain Se concentrations below toxic levels.

As the difference between beneficial and toxic effects of dietary Se may be narrow (Watanabe et al., 1997), it is essential to map the beneficial and toxic concentrations of dietary Se in order to optimise its dietary inclusion level. The requirement and bioavailability of dietary Se for yellowtail kingfish *Seriola lalandi* has been studied (Le and Fotedar, 2013, 2014b), in which the supplementation of Se from Se-yeast at 2 mg/kg to a fishmeal-based diet containing 3.35 mg Se/kg resulted in the maximal growth, and organic Se such as selenomethionine (SeMet) or Se-yeast appeared to be

more bioavailable than an inorganic form, selenite. However, nothing is reported about its toxic effects to this species. Therefore, this experiment was carried out to investigate physiological responses of yellowtail kingfish to excessive levels of dietary Se and to set a threshold dietary Se level for yellowtail kingfish culture.

7.2 MATERIALS AND METHODS

7.2.1 Experimental Diets and Design

A basal diet (Table 7.1) was supplemented with four graded levels of Se as DL-selenomethionine (SeMet; Sigma-Aldrich, St. Louis, MO, USA). SeMet was chosen as it is the dominant form of Se present in food (Suzuki, 2005) and it has been shown to accumulate in yellowtail kingfish (Le and Fotedar, 2014b). The pre-determined quantities of Se were dissolved in water and added to the basal ingredients before pelleting the feeds through a 3-mm diameter die. The pellets were then air-dried at room temperature and stored at -20 ºC until used. The Se concentrations in the basal diet and the Se supplemented diets were then analysed to be 2.31 and 4.91, 9.58, 15.43 and 20.87 mg/kg, respectively.

Juvenile yellowtail kingfish of similar sizes in the same batch were obtained from the Australian Centre for Applied Aquaculture Research, Fremantle, WA, Australia and brought to the Curtin Aquatic Research Laboratory (CARL), Curtin University. The fish were group weighed and stocked into each of 15 experimental 300-L tanks, in a random-block design, at a density of 12 fish/tank. The average of the tank averages was 234.62 ± 0.53 g (mean ± SE), with the average of all the fish of 19.55 ± 0.04 g (mean ± SE). The tanks were filled with seawater at salinity of 35 ppt and Se concentration < 1µL, and were supplied with constant aeration and pure oxygen. Each tank had an external bio-filter running continuously to create a recirculating system and an automatic heater to maintain water temperature. Faecal matter was removed daily and half of the water was changed every two days. Water temperature, pH and dissolved oxygen were measured daily using digital pH/mV/ºC and dissolved oxygen meters (CyberScan pH 300 and CyberScan DO 300, Eutech Instruments, Singapore). Total ammonia was monitored daily by an ammonia (NH_3/NH_4^+) test kit (Mars Fishcare, Chalfont, PA, USA). During the trial, water temperature, pH and dissolved oxygen averaged 21.7 ± 0.7 ºC, 7.6 ± 0.2, and 6.9 ± 0.4 mg/L (mean ± SD), respectively. Total ammonia (NH_3/NH_4^+) was always ≤ 1.0 mg/L.

Table 7.1 Ingredient formulation and proximate composition of the basal diet

Ingredient[a]	(g/kg)	Proximate composition[c]	(%)
Fishmeal	500	Protein	49.18 ± 0.39
Fish oil	150	Lipid	18.56 ± 0.30
Wheat flour	130	Moisture	8.04 ± 0.03
Wheat gluten	100	Ash	8.27 ± 0.01
Shrimp meal	70	Gross energy (MJ/kg)	21.18 ± 0.16
Starch	40		
Se-free premix[b]	10		

[a] Supplied by Specialty Feeds, Perth, WA, Australia.
[b] Contains the following (as g/kg of premix): iron, 10; copper, 1.5; iodine, 0.15; manganese, 9.5; zinc, 25; vitamin A retinol, 100 IU; vitamin D3, 100 IU; vitamin E, 6.25; vitamin K, 1.6; vitamin B1, 1; vitamin B2, 2.5; niacin, 20; vitamin B6, 1.5; calcium, 5.5; biotin, 0.1; folic acid, 0.4; inositol, 60; vitamin B12, 0.002; choline, 150; and ethoxyquin, 0.125.
[c] Values are presented as means ± SD, n=3.

Each dietary treatment was randomly assigned to three tanks. The fish were hand fed to satiation, twice a day at 08 am and 04 pm. The fish were fed slowly to ensure no uneaten food. The amount of feed consumed was recorded daily to estimate feed intake. All of the fish from each tank were weighed every two weeks to monitor growth. When any Se supplemented diets had resulted in decreases in the growth of fish, considered as a sign of Se toxicity, the experiment stopped in two weeks afterwards.

Total feed intake and weight measurement at the end of the trial were used for the estimation of feed conversion ratio (FCR, feed intake divided by the wet weight gain).

7.2.2 Sample Collection

At the commencement of the trial, 18 additional fish were used to estimate initial Se content in the liver and muscle. Both liver and muscle tissue samples were pooled before the analyses.

At the end of the feeding trial, three fish from each tank were randomly selected and blood was sampled from the caudal vein with syringes and directly used for measurement of haematocrit. The remaining blood was allowed to clot for 2 h at 4 ºC and red blood cell pellets were separated by centrifugation of whole blood at 1500×*g* for 10 min at 4 ºC using a centrifuge (5804R, Eppendorf, Hamburg, Germany). The red blood cell pellets were stored at -80 ºC until used for glutathione peroxidase assay.

Following the blood sampling, liver, spleen, heart, left anterior dorsal muscle and anterior intestine were dissected from each fish and fixed in 10% buffered formalin for

histopathological examination. The remaining muscle tissues from each fish were used for estimation of Se content and proximate composition.

The remaining fish (nine/tank) and their livers were individually weighed to calculate hepatosomatic index $\left(HSI = 100 \times \frac{Liver\ weight}{Body\ weight}\right)$. The livers of the nine fish were pooled for estimation of Se content.

7.2.3 Haematocrit Assay

Haematocrit (Ht) of each fish was determined in triplicate by the microhaematocrit method (Rey Vázquez and Guerrero, 2007) as described in section 3.2.4.

7.2.4. Glutathione Peroxidase Assay

Glutathione peroxidase (GPx) activity in red blood cells from each fish was assayed as described previously in section 3.2.7.

7.2.5 Histopathological Examination

The histological samples were routinely processed as described previously in section 3.2.8 and observed under a light microscope (BX40F4, Olympus, Tokyo, Japan). Numbers of macrophage aggregates (MAs) per sections of entire spleens were counted.

7.2.6 Chemical Analysis

Protein, lipid, moisture, ash, gross energy and Se were analysed as described previously in section 3.2.9.

7.2.7 Statistical Analysis

Data were analysed using PASW Statistics 18.0 (IBM Corporation, New York, US). All data were subjected to Levene's test for homogeneity of variance and one-way ANOVA. Macrophage aggregate data were square-root transformed before analysis. When a significant treatment effect was observed, Tukey's Honest Significant Difference test was used for multiple mean comparisons. Linear regression analyses were performed on tissue Se concentrations against dietary Se concentrations. The statistical significance was set at $P < 0.05$.

7.3 RESULTS

There was no significant difference ($P > 0.05$) in initial weights of fish amongst dietary treatments (Table 7.2). During the first four weeks, no dietary treatment resulted in any significant differences ($P > 0.05$) in fish growth. However, from week 6 the dietary Se supplementations resulted in significantly ($P < 0.05$) higher weight gains than the basal diet (Table 7.2). At week 8, the fish fed 20.87 mg Se/kg diet started to show decrease in weight gain which became similar to the basal diet at week 10, the end of the feeding trial.

Dietary Se had no effects on proximate composition and gross energy of muscle tissues (Table 7.3). Similarly, feed conversion ratio and survival of fish were not affected by dietary Se levels, but feed intakes were significantly influenced by the dietary Se levels (Table 7.4). Significantly ($P < 0.05$) lower feed intakes were found in fish fed the lowest and highest levels of Se.

Initial Se concentrations in liver and muscle were 0.84 and 0.06 mg/kg, respectively. There were significant ($P < 0.05$) increases in yellowtail kingfish liver and muscle Se concentrations which corresponded with increasing dietary Se levels (Table 7.4). Linear regression analysis of tissue Se accumulation showed linear responses to dietary Se levels ($y = 0.2888x - 0.0092$, $R^2 = 0.964$ and $P < 0.001$ for liver; $y = 0.0701x + 0.237$, $R^2 = 0.920$ and $P < 0.001$ for muscle; Figure 7.1).

Significant differences in Ht, HSI and GPx activities between dietary treatments were observed (Table 7.5). Haematocrit values and HSI were significantly ($P < 0.05$) lower in the fish fed the diet containing the highest level of Se. The fish fed the basal diet had significantly ($P < 0.05$) lower GPx activity than other fish. The highest GPx activity was found in fish fed the highest Se level.

Yellowtail kingfish fed different dietary Se concentrations did not show any histopathological lesions or degeneration in heart and intestine tissues. However, the number of splenic macrophage aggregates was four times significantly ($P < 0.05$) higher in fish fed the highest Se diet than those fed the lower Se diets (Table 7.5; Figure 7.2). The highest Se diet also resulted in hepatocyte atrophy (Figure 7.3). In contrast, necrotic muscle tissues were only observed in the fish fed the lowest Se diet, 2.31 mg/kg (Figure 7.4).

Table 7.2 Weight gain of yellowtail kingfish fed different Se levels during the feeding trial[1]

Dietary Se (mg/kg)	Initial weight (g/fish)	Weight gain (g/fish)				
		Week 2	Week 4	Week 6	Week 8	Week 10
2.31	19.61±0.10	8.59±0.33	20.54±0.29	32.95±0.36^{a}	46.06±0.51^{a}	60.04±0.73^{a}
4.91	19.61±0.04	9.48±0.20	22.10±0.38	37.38±0.47^{b}	54.94±0.82^{c}	70.96±0.55^{b}
9.58	19.53±0.04	9.14±0.19	22.28±0.39	37.45±0.62^{b}	52.48±0.81^{bc}	68.64±0.87^{b}
15.43	19.46±0.12	9.30±0.13	21.71±0.38	37.25±0.39^{b}	52.39±0.52^{bc}	68.27±0.97^{b}
20.87	19.54±0.18	9.09±0.40	21.79±0.52	36.31±0.82^{b}	51.04±0.75^{b}	63.12±0.57^{a}
P value	0.859	0.264	0.077	0.001	<0.001	<0.001

[1] Values are presented as the mean ± SE of three replicates/dietary treatment.
In a column, means not sharing a common superscript letter are significantly different ($P < 0.05$).

Table 7.3 Muscle proximate composition of yellowtail kingfish fed graded dietary Se for 10 weeks[1]

Dietary Se (mg/kg)	Protein (%)	Lipid (%)	Moisture (%)	Ash (%)	Gross energy (MJ/kg)
2.31	18.91±0.13	2.15±0.05	77.37±0.14	1.59±0.05	5.04±0.03
4.91	18.60±0.11	2.05±0.12	77.71±0.24	1.43±0.03	4.99±0.06
9.58	18.61±0.22	1.90±0.04	77.73±0.32	1.48±0.05	4.96±0.08
15.43	19.08±0.15	1.96±0.17	77.27±0.27	1.54±0.06	5.09±0.06
20.87	18.98±0.21	2.26±0.15	77.12±0.35	1.47±0.06	5.12±0.10
P value	0.225	0.285	0.471	0.277	0.539

[1] Values are presented as the mean ± SE of one determination/fish, three fish/tank and three tanks/treatment.

Table 7.4 Feed intake, feed conversion ratio, liver Se, muscle Se and survival of yellowtail kingfish fed graded dietary Se for 10 weeks

Dietary Se (mg/kg)	Feed intake (g/fish)[1]	Feed conversion ratio[1]	Liver Se (mg/kg)[2]	Muscle Se (mg/kg)[3]	Survival (%)
2.31	76.33±1.59^{a}	1.27±0.01	0.72±0.06^{a}	0.20±0.01^{a}	100
4.91	88.90±1.33^{b}	1.25±0.01	1.71±0.08^{b}	0.68±0.01^{b}	100
9.58	86.71±0.97^{b}	1.26±0.01	2.48±0.11^{c}	1.10±0.01^{c}	100
15.43	85.65±1.33^{b}	1.25±0.01	3.93±0.02^{d}	1.33± 0.02^{d}	100
20.87	79.60±0.44^{a}	1.26±0.01	6.45±0.17^{e}	1.61±0.03^{e}	100
P value	<0.001	0.793	<0.001	<0.001	

[1] Values are presented as the mean ± SE of three replicates/dietary treatment.
[2] Values are presented as the mean ± SE of pooled samples of nine fish/tank and three tanks/treatment.
[3] Values are presented as the mean ± SE of one determination/fish, three fish/tank and three tanks/treatment.
In a column, means not sharing a common superscript letter are significantly different ($P < 0.05$).

Table 7.5 Glutathione peroxidase activity, splenic macrophage aggregates, hepatosomatic index and haematocrit of yellowtail kingfish fed graded dietary Se for 10 weeks

Dietary Se (mg/kg)	GPx activity (units/g Hb)[1]	Number of MAs per spleen[1]	Hepatosomatic Index (%)[2]	Haematocrit (%)[3]
2.31	50.00±3.25^{a}	24.89±2.41^{a}	0.99±0.04^{a}	38.74±0.61^{a}
4.91	87.17±3.61^{b}	21.44±1.46^{a}	1.01±0.03^{a}	39.89±1.00^{a}
9.58	86.17±3.61^{b}	24.11±1.31^{a}	0.96±0.03^{a}	39.32±0.67^{a}
15.43	98.83±5.92^{bc}	25.89±0.99^{a}	1.01±0.04^{a}	39.93±0.91^{a}
20.87	109.50±1.89^{c}	105.89±4.28^{b}	0.79±0.02^{b}	34.85±0.83^{b}
P value	<0.001	<0.001	0.003	0.007

GPx, glutathione peroxidase; Hb, haemoglobin; MA, macrophage aggregate.
[1] Values are presented as the mean ± SE of one determination/fish, three fish/tank and three tanks/treatment.
[2] Values are presented as the mean ± SE of one determination/fish, nine fish/tank and three tanks/treatment.
[3] Value are presented as the mean ± SE of three determinations/fish, three fish/tank and three tanks/treatment.
In a column, means not sharing a common superscript letter are significantly different ($P < 0.05$).

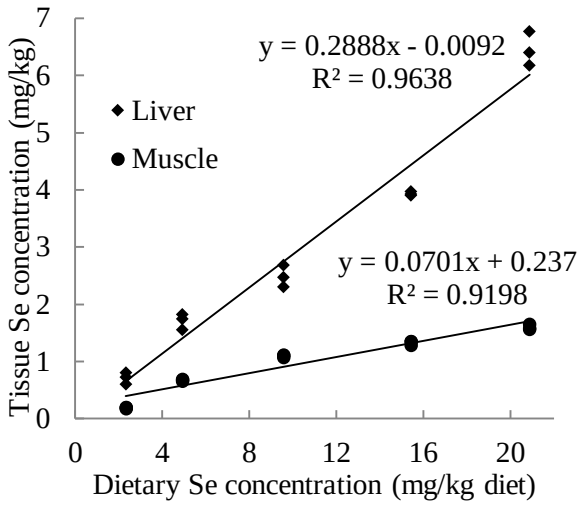

Figure 7.1 Relationship between Se concentrations in diets and tissues. For liver tissues, each point presents mean of pooled samples of nine fish from each replicate group. For muscle tissues, each point represents mean of three fish from each replicate group.

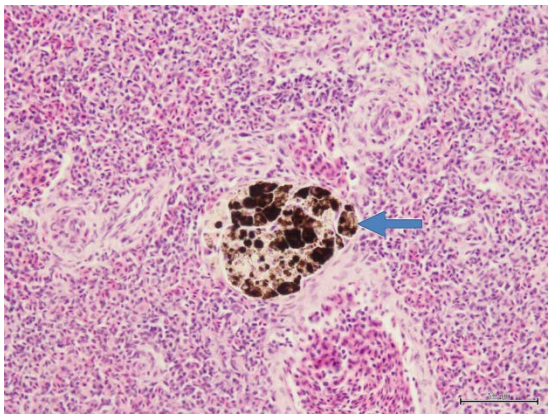

Figure 7.2 A macrophage aggregate (arrow) in a section of spleen of yellowtail kingfish fed the diet containing 20.87 mg/kg Se for 10 weeks. (Haematoxylin and eosin, scale bar = 50 µm).

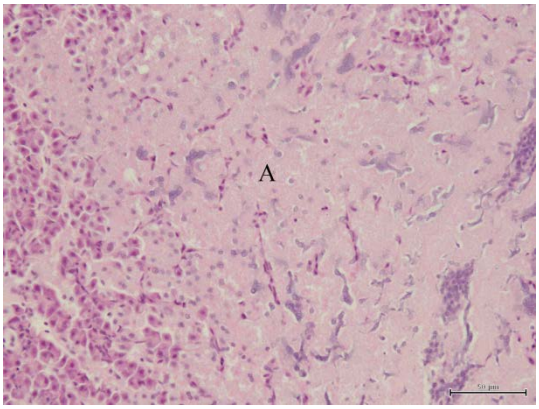

Figure 7.3 Section of liver of yellowtail kingfish fed the diet containing 20.87 mg/kg Se for 10 weeks showing atrophic hepatocytes (A). (Haematoxylin and eosin, scale bar = 50 µm).

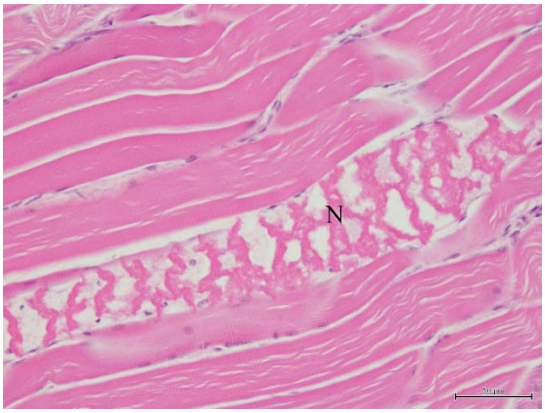

Figure 7.4 Section of muscle of yellowtail kingfish fed the basal diet containing 2.31 mg/kg Se for 10 weeks resulting in necrotic fibres (N). (Haematoxylin and eosin, scale bar = 50 µm).

7.4 DISCUSSION

The dietary Se concentration required to prevent yellowtail kingfish from Se deficiency has been reported between 3.35 and 4.86 mg/kg diet (Le and Fotedar, 2013). In agreement with this, in the present study the basal diet containing 2.31 mg Se/kg resulted in muscle tissue myopathy, reduced feed intake, GPx activity and growth, which are typical Se deficiency symptoms in fish (Poston et al., 1976; Hilton et al., 1980; Gatlin et al., 1986; Le and Fotedar, 2014b), whereas no sign of Se deficiency was observed in the fish fed the diets containing ≥ 4.91 mg Se/kg. On the other hand, the highest dietary Se level of 20.87 mg/kg caused atrophic hepatocytes, increased number of splenic macrophage aggregates, and reduction in feed intake, weight gain, Ht and HSI, which are indications of Se toxicity. The reason for Se toxicity is attributed to indiscriminate substitution of Se for sulphur when present in excessive amounts (Lemly, 2002b). Due to its higher reactivity and lower stability compared to sulphur, Se can cause metabolic problems (Stadtman, 1974; Sunde, 1984).

The toxic dietary Se concentration to fish in the present study is relatively higher than those reported previously. For example, 15.43 mg of dietary Se/kg did not cause any toxic effects in yellowtail kingfish, whereas the diet containing 13 mg Se/kg appeared to be toxic to 1.3-g rainbow trout *Salmo gairdneri* after four weeks of feeding (Hilton et al., 1980). Selenium concentrations of ≥ 4.6 mg/kg in food was toxic to razorback sucker *Xyrauchen texanus* larvae, the mortality occurred after one-week exposure (Hamilton et al., 2005). With regard to survival, yellowtail kingfish are relatively less sensitive to Se toxicity than bluegill *Lepomis macrochirus* and chinook salmon. Dietary Se as SeMet at 6.5 and 9.6 mg/kg caused significant decreases in survival of

3-month-old bluegill (0.2 g) (Cleveland et al., 1993) and 70-mm fingerling chinook salmon (Hamilton et al., 1990) after being fed for 8.6 and 12.8 weeks respectively, whereas the survival of 19.55-g yellowtail kingfish fed up to 20.87 mg/kg of Se remained 100% even after 10 weeks.

As Se concentrations in liver and muscle showed linear response to the dietary Se with no sign of plateauing, the levels of Se in these tissues can be used as bio-indicators of dietary Se exposure. Similarly, Se concentration in kidney, muscle, liver, gill, and plasma tissues of white sturgeon increased as dietary Se (SeMet) increased and no plateau was reached after being fed up to 191.1 mg/kg diet for eight weeks (Tashjian et al., 2006). The histopathological alterations in liver have been reported in green sunfish *Lepomis cyanellus* (Sorensen et al., 1984) and white sturgeon (Tashjian et al., 2006) with liver Se concentrations of 21.4 and $\geq$ 37.4 mg/kg dry weight, respectively. For yellowtail kingfish in the present study, those having liver Se concentration of 6.45 mg/kg wet weight or 20.82 mg/kg dry weight showed histopathological changes in their livers.

Splenic macrophage aggregates in fish play an important role in the storage of damaged cells (Wolke, 1992) and have been used as a bio-indicator for assessment of degraded environments (Fournie et al., 2001). The number of splenic macrophage aggregates may increase as fish are exposed to toxic chemicals. Exposure of plaice *Pleuronectes platessa* to 0.5 mg/L potassium dichromate resulted in an increase in density of splenic macrophage aggregates (Kranz and Gercken, 1987). In the present study, dietary Se at 20.87 mg/kg caused a significant increase in numbers of macrophage aggregates in the spleen, suggesting that splenic macrophage aggregates are sensitive to Se toxicity and can serve as a biomarker for the measurement of toxic effects of high dietary Se concentrations in yellowtail kingfish.

A reduction in Ht caused by waterborne Se poisoning has been reported in green sunfish (Sorensen et al., 1984). The decreased Ht induced by the toxic effect of dietary Se was also seen in yellowtail kingfish fed the 20.87 mg Se/kg diet. Changes in Ht reflect the changes in the overall health of the fish (Watanabe et al., 1998). Reductions in Ht are associated with decreased respiratory capacity, which causes metabolic stress and in turn leads to reduced fish health (Lemly, 1993b).

The results of HSI indicated that the liver of fish fed the highest Se level was smaller compared to fish fed the lower levels. This may be as a result of liver atrophy caused by Se toxicity. Liver necrosis and reduced HSI have also been observed in white sturgeon exposed to 191.1 mg Se/kg diet for eight weeks (Tashjian et al., 2006). However, Sorensen et al. (1984) found that green sunfish with higher Se concentrations in livers (21.4 mg/kg compared to 7.0 mg/kg) had higher HSI. The authors reasoned that larger livers in fish having higher Se levels were due to edema caused by waterborne Se toxicity (Sorensen et al., 1984). Whereas, rainbow trout *S. gairdneri* fed various dietary Se levels from 0.38 to 13.06 mg/kg for four weeks showed no significant differences in HSI (Hilton et al., 1980), probably due to the brief duration of the exposure.

Glutathione peroxidase is considered as an indicator of Se status as its activity is dependent on the dietary Se intake (Ganther et al., 1976). Red blood cell GPx activity in yellowtail kingfish plateaued when dietary Se levels were between 4.91 and 15.43 mg/kg and continued to increase at the toxic dietary Se concentration of 20.87 mg/kg. A similar pattern has been reported for channel catfish *Ictalurus punctatus,* in which plasma GPx activity levelled off above a Se level of 0.56 mg/kg and then increased at a Se level of 15.06 mg/kg, which was recommended as a toxic concentration to the fish (Gatlin and Wilson, 1984). An increase in GPx activity caused by toxic concentrations of Se has been found in algae (Vítová et al., 2011).

This 10-week toxicity experiment showed that there was no detectable toxic effect in the fish fed up to 15.43 mg Se/kg diet. This concentration was more than two times higher than the concentrations of Se in diets in all other experiments in chapter 3, 4, 5 and 6. This supports the designs of six-week experiments in those chapters as it would waste time to continue for longer to identify negative effects of Se at such concentrations.

In summary, Se deficiency symptoms were observed in yellowtail kingfish fed the basal diet containing 2.31 mg Se/kg while fish fed 20.87 mg Se/kg diet showed Se toxicity. Signs of Se toxicity included reduced feed intake, growth, Ht and HIS, increased splenic macrophage aggregates and liver atrophy. The toxic effect threshold of dietary Se for yellowtail kingfish appears to be between 15.43 and 20.87 mg/kg.

CHAPTER 8
GENERAL DISCUSSION AND CONCLUSIONS

8.1 INTRODUCTION

This chapter reviews the present study and discusses the highlights of the research in the light of the existing literature on the nutritional role of Se in aquatic species. Following the review, the main conclusions of the study are highlighted. The chapter ends with recommendations for future research.

8.2 GENERAL DISCUSSION

8.2.1 Selenium and Vitamin E Interactions in Yellowtail Kingfish

The synergism between Se and vitamin E has been studied in mammals since 1957 (Schwarz and Foltz, 1957). The two nutrients function synergistically to form an important antioxidant defence system (Combs and Combs, 1986). Lots of studies have also been conducted on Se and vitamin E interactions in fish (Poston et al., 1976; Bell et al., 1985; Gatlin et al., 1986; Le et al., 2014b). These studies in fish have revealed that diets deficient in both Se and vitamin E result in reduced growth, anaemia, severe myopathy, exudative diathesis and death.

Organic Se has been found to interact with vitamin E in juvenile yellowtail kingfish (Le et al., 2014a; Le et al., 2014b). Selenium and vitamin E complement each other to protect the fish from muscle myopathy (Le et al., 2014b), showing a synergic interaction between the two micronutrients. For Atlantic salmon *Salmo salar*, muscular dystrophy is prevented only when both Se and vitamin E are supplemented (Poston et al., 1976); however the levels of Se and vitamin E in Atlantic salmon diets are unknown. To prevent development of muscle myopathy in yellowtail kingfish, diets are required to contain at least 4.32 mg/kg Se and/or 179.23 mg/kg vitamin E (Le et al., 2014b). These levels are higher than those reported for channel catfish *Ictalurus punctatus* with 0.26 mg/kg Se and 52.5 mg/kg vitamin E (Gatlin et al., 1986). Yellowtail kingfish is a highly active and high-performance, high-energy demanding species (Clark and Seymour, 2006); and thus may require high Se and vitamin E input.

Selenium increases growth of yellowtail kingfish fed diets low in vitamin E, but not high in vitamin E (Le et al., 2014b), indicating an interactive effect of Se and vitamin

E on the fish growth. Vitamin E may act as a partial substitute and/or complement for the low Se by performing a similar function to Se and in maintaining growth (Webster and Lim, 2002a). Another positive interactive effect between Se and vitamin E in yellowtail kingfish is evident in serum bactericidal activity, which is increased by combined supplementation of both micronutrients (Le et al., 2014a). However, Se does not compensate serum lysozyme activity for the lack of vitamin E in yellowtail kingfish. The activities of lysozyme in yellowtail kingfish fed diets low in vitamin E are not correlated with different levels of Se (Le et al., 2014b).

8.2.2 Requirement and Deficiency of Selenium in Yellowtail Kingfish

Although fish can uptake Se from water via their gills, dietary exposure is the dominant pathway of Se uptake (Watanabe et al., 1997; Hamilton, 2004). The requirements of dietary Se have been quantified in some fish species, ranging from 0.25 mg/kg for channel catfish (Gatlin and Wilson, 1984) to 3.67 mg/kg for African catfish *Clarias gariepinus* (Abdel-Tawwab et al., 2007) and 4.6 mg/kg for Nile tilapia *Oreochromis niloticus* (Ahmad et al., 2006). The optimal dietary Se requirement for maximal growth of yellowtail kingfish has been reported to be 5.56 mg/kg (Le and Fotedar, 2013). This level is higher than those reported for other fish species on which Se studies have been conducted.

Juvenile yellowtail kingfish fed a fishmeal-based diet containing 3.35 mg Se/kg exhibit myopathy, reduced glutathione peroxidase activity and growth (Le and Fotedar, 2013; Le et al., 2014b), which are typical Se deficiency symptoms in fish (Poston et al., 1976; Hilton et al., 1980; Gatlin et al., 1986). This indicates that yellowtail kingfish have a requirement for Se that may not be met by the fishmeal-based diet alone. The biological availability of Se from fishmeal is low due to Se being bound to heavy metals (Webster and Lim, 2002a); thus, supplementation of Se to yellowtail kingfish diets is necessary. The beneficial growing effect of Se supplementation has been reported in some fish species (Table 8.1). The effects of Se on fish growth might be associated with its biological functions and probably mediated by selenoproteins (McKenzie et al., 2002).

Table 8.1 Beneficial growing effect of dietary Se supplementation in fish

Species	Se supplementation level (mg/kg)	Se form/source	Exposure period (weeks)	Reference
Grouper *Epinephelus malabaricus*	0.5	Selenomethionine	8	Lin and Shiau (2005b)
African catfish *Clarias gariepinus*	2.6 - 4.5	Se-yeast	12	Abdel-Tawwab et al. (2007)
Crucian carp *Carassius auratus gibelio*	0.5	Selenomethionine	4	Zhou et al. (2009)
Cobia *Rachycentron canadum*	0.4 -1.0	Selenomethionine	10	Liu et al. (2010)
Nile tilapia *Oreochromis niloticus*	4.5	Se-yeast	6	Abdel-Tawwab and Wafeek (2010)
Crucian carp *Carassius auratus gibelio*	1.0 - 5.0	Selenomethionine	14	Han et al. (2011)
Yellowtail kingfish *Seriola lalandi*	1.5 - 3.0	Se-yeast	6	Le and Fotedar (2013)

8.2.3 Bioavailability of Selenium in Yellowtail Kingfish

Bioavailability of Se can be estimated by measuring Se absorption, Se levels in blood and body tissues and glutathione peroxidase (GPx) activity (Fairweather-Tait et al., 2010; Thiry et al., 2012). Absorption of Se in animals depends on its chemical forms, and organic Se appears to be better absorbed than inorganic sources (Paripatananont and Lovell, 1997; Finley, 2006). For example, Selenomethionine (SeMet), an organic Se, is actively absorbed through the same pathways as methionine by passing the intestinal barrier using Na^+-dependant neutral amino acid transport system, whereas uptake of selenite, an inorganic form, is by passive diffusion (Wolffram et al., 1989; Vendeland et al., 1994; Daniels, 1996; Schrauzer, 2000). Under optimal conditions, the absorption rate is estimated to be 95-98% for SeMet and 62% for selenite (Dreosti, 1986; Thomson and Robinson, 1986). The bioavailability of Se depends not only on its absorption by the intestine but also on its metabolic pathways. Ingested SeMet can enter into general proteins as a substitute for methionine or it can be transformed to selenocysteine (SeCys), whereas inorganic forms, selenite and selenate, may be

reduced to selenide and then inter into specific selenoproteins as SeCys (Ganther, 1986).

Absorption of Se in juvenile yellowtail kingfish has been found to be affected by the sources of Se. Selenium derived from SeMet or Se-yeast had higher digestibility than Se from SeCys or selenite, 90.4, 89.5, 61.4 and 59.0%, respectively; whereas Se derived from fishmeal showed the lowest digestibility (38.5%) (Le and Fotedar, 2014b). The lower digestibility of Se from fishmeal than SeMet, SeCys and selenite has been also reported in Atlantic salmon (Bell and Cowey, 1989). Interactions between Se and heavy metals in fishmeal, for example, an insoluble copper–Se compound, may reduce absorption of Se from fishmeal (Lorentzen et al., 1998).

The bioavailability of Se from SeMet and Se-yeast is similar in yellowtail kingfish (Le and Fotedar, 2014b). Se-yeast containing more than 90% of its Se in the form of SeMet (Schrauzer, 2006) can be the reason for the similarity in bioavailability between SeMet and Se-yeast. Both SeMet and Se-yeast are more available than selenite for the growth of yellowtail kingfish (Le and Fotedar, 2014b). This is in agreement with a study on channel catfish (Wang and Lovell, 1997), but differs from those reported for Atlantic salmon (Lorentzen et al., 1994) and grouper (Lin, 2014). In the studies with Atlantic salmon and grouper, SeMet and selenite have a similar bioavailability for fish growth.

SeMet or Se-yeast as a source of Se is more effective in accumulating in muscle tissues of yellowtail kingfish than selenite (Le and Fotedar, 2014b). The higher bioavailability of SeMet than selenite for muscle Se accumulation is also found in channel catfish (Wang and Lovell, 1997) and grouper (Lin, 2014). The higher absorption of SeMet than selenite by the fish can partially contribute to the higher bioavailability of SeMet than selenite for Se accumulation in muscle tissues. In addition, SeMet can be directly incorporated in proteins and stored in large protein masses such as muscle (Sunde, 1984; Waschulewski and Sunde, 1988), whereas inorganic Se is utilized directly for selenoprotein synthesis in the liver and the excess is excreted (Patterson and Zech, 1992).

In yellowtail kingfish, organic and inorganic Se seem equally effective in raising red blood cell GPx activity (Le and Fotedar, 2014b). A similar effect of organic and inorganic Se on GPx activity in red blood cells of domestic animals (Kumar et al., 2009) and GPx activity in liver of rainbow trout *Oncorhynchus mykiss* (Rider et al.,

2010) has been reported. In contrast, organic Se in common carp *Cyprinus carpio* (Jovanovic et al., 1997) and channel catfish (Wang and Lovell, 1997), has shown to be more effective than inorganic Se in raising hepatic GPx activity; whereas in Atlantic salmon, more Se from selenite than from SeMet is incorporated into plasma GPx (Bell and Cowey, 1989). The inconsistency in the bioavailability of organic and inorganic Se for GPx activity may be due to species variation.

8.2.4 Selenium and Antioxidant and Immune Responses in Yellowtail Kingfish

Selenium is widely studied for its antioxidant properties (Tapiero et al., 2003; Miller et al., 2007; Tinggi, 2008; Atencio et al., 2009). It is an essential component of antioxidant enzymes, such as GPx (Tapiero et al., 2003), one of the most important antioxidant defence enzymes in fish (Ross et al., 2001). In yellowtail kingfish, the activity of GPx has been reported to increase with an increase of Se in the fish diets (Le and Fotedar, 2014c). The dependence of the GPx activity on the dietary Se intake has also found in rainbow trout *Salmo gairdneri* (Hilton et al., 1980), channel catfish (Gatlin et al., 1986; Wise et al., 1993a), Atlantic salmon (Bell et al., 1987), grouper (Lin and Shiau, 2005b) and cobia (Liu et al., 2010).

Dietary Se has shown to improve resistance of yellowtail kingfish to bacterial infections (Le and Fotedar, 2014c). It increased antibody response and survival of yellowtail kingfish following the infection with *Vibro anguillarum* (Le and Fotedar, 2014c). Wang et al. (1997) reported the same effect of Se on antibody response and survival of channel catfish being infected with pathogenic bacterium *Edwardsiella ictaluri*. The stimulating effect of Se on antibody response may be explained as Se up-regulates the expression of high-affinity interleukin-2 (IL-2) (Roy et al., 1994). The interaction between IL-2 and its receptor delivers signals to enhance responses of T-cells, which provide B-cell help for antibody synthesis (Minami et al., 1993; Brandes et al., 2003).

The immune-stimulating effects of dietary Se in yellowtail kingfish are also demonstrated in lysozyme and bactericidal activities (Le and Fotedar, 2014c). Dietary Se stimulates the lytic activity of lysozyme in serum of yellowtail kingfish against bacteria; and it increases the bactericidal activity by enhancing the ability of serum to inhibit the growth of *V. anguillarum* (Le and Fotedar, 2014c).

8.2.5 Selenium Toxicity in Yellowtail Kingfish

At trace concentrations, Se is required for normal growth and development of animals (National Research Council, 1993; Watanabe et al., 1997); however, at elevated concentrations, it can become toxic to animals (Hamilton, 2004). The reason for Se toxicity is attributed to indiscriminate substitution of Se for sulphur in the process of protein synthesis (Lemly, 2002b; Janz et al., 2010). In excessive amounts, Se erroneously replaces sulphur, resulting in improper functions of proteins (Reddy and Massaro, 1983; Sunde, 1984; Maier and Knight, 1994). Signs of Se toxicity in fish include high mortalities, histopathological changes in liver, diminished reproductive performance, and reduced feed intake, growth and haematocrit (Hilton et al., 1980; Gatlin and Wilson, 1984; Sorensen et al., 1984; Lemly, 1997; Tashjian et al., 2006; Jaramillo et al., 2009). Toxicity of Se can be affected by the duration of the Se exposure and life stages of the host animal (Lemly, 2002a). The earliest life stages of fish are the most sensitive to Se toxicity (Lemly, 2002b; Teh et al., 2002).

Juvenile yellowtail kingfish of 19.55 g fed a diet containing 20.87 mg Se/kg for 10 weeks showed atrophic hepatocytes, increased number of splenic macrophage aggregates, and reduction in feed intake, weight gain, haematocrit and hepatosomatic index (Le and Fotedar, 2014a), indicating Se toxicity. The toxic effect threshold of dietary Se for yellowtail kingfish is estimated to be between 15.43 and 20.87 mg/kg (Le and Fotedar, 2014a). It is comparable to the dietary Se toxicity threshold for white sturgeon *Acipenser transmontanus*, which is between 10 and 20 mg/kg (Tashjian et al., 2006). However, the toxic dietary Se concentration to yellowtail kingfish is relatively higher than those reported for fingerling chinook salmon *Oncorhynchus tshawytscha* (3 - 5 mg/kg) (Hamilton et al., 1990), rainbow trout *S. gairdneri* (13 mg/kg) (Hilton et al., 1980) and razorback sucker *Xyrauchen texanus* larvae (4.6 mg/kg) (Hamilton et al., 2005). In addition, diets containing 6.5 and 9.6 mg Se/kg caused significant decreases in survival of 0.2-g bluegill *Lepomis macrochirus* (Cleveland et al., 1993) and 70-mm fingerling chinook salmon (Hamilton et al., 1990) after fed for 8.6 and 12.8 weeks respectively, whereas the survival of 19.55-g yellowtail kingfish fed up to 20.87 mg Se/kg remained 100% after 10 weeks (Le and Fotedar, 2014a). The less sensitive to Se toxicity of yellowtail kingfish than other fish species mentioned above could be due to the relatively bigger size of yellowtail kingfish used for the study.

While yellowtail kingfish with liver Se concentration of 3.93 mg/kg wet weight showed no signs of Se toxicity, the fish with livers containing 6.45 mg Se/kg wet weight or 20.82 mg Se/kg dry weight showed histopathological changes in their livers (Le and Fotedar, 2014a). Therefore, it is recommended that the toxic level of Se in liver of yellowtail kingfish is between 3.93 and 6.45 mg Se/kg wet weight. The histopathological changes in the livers have been also observed in green sunfish *Lepomis cyanellus* (Sorensen et al., 1984) and white sturgeon (Tashjian et al., 2006) with liver Se concentrations, respectively, of 7.0 mg/kg wet weight and 37.4 mg/kg dry weight, which is much higher than the toxic level of Se in liver of freshwater and anadromous fish (12 mg/kg dry weight) recommended by Lemly (2002a).

8.3 CONCLUSIONS AND RECOMMENDATIONS

8.3.1 Conclusions

On the basis of the results of the study in this thesis, it can be concluded that:

- The interactions between dietary organic Se and vitamin E exist in juvenile yellowtail kingfish. They compensate for the lack of each other to prevent fish developing muscle myopathy. Vitamin E may complement for the low Se in maintaining fish growth; and there is a positive interactive effect between the two micronutrients with respect to bactericidal activity.

- The fishmeal-based diet does not meet the requirement of dietary Se in juvenile yellowtail kingfish. The fish fed the fishmeal-based diet show common Se deficiency symptoms. The optimal organic Se supplementation for juvenile yellowtail kingfish diet is approximately at 2.2 mg/kg and the optimal level of Se in diets for maximal growth of the fish is estimated to be 5.5 mg/kg. The level of Se from 4.8 mg/kg might be sufficient for the fish.

- SeMet or Se-yeast is more absorbed and more bioavailable than SeCys or selenite; thus, SeMet or Se-yeast is recommended as a source of Se for supplementation to diets of yellowtail kingfish.

- Dietary supplementation of organic Se confers benefits to the health of juvenile yellowtail kingfish under normal and bacterial infectious condition. It increases

activity of an antioxidant enzyme, GPx, and stimulates antibody response, lysozyme and bactericidal activities of the fish.

- Concentrations of Se in liver and muscle tissues can be used as effective indicators of dietary Se exposure in yellowtail kingfish. Juvenile yellowtail kingfish exposed to an excessive level of dietary Se show common signs of Se toxicity in fish. Dietary Se level between 15.4 and 20.8 mg/kg is a threshold level in juvenile yellowtail kingfish.

8.3.2 Recommendations

Further nutritional research on Se needs to be conducted at commercial scales for yellowtail kingfish to ensure that the findings of the present study can be applied under commercial farming conditions. There is need to elucidate a mechanism of action by which Se enhances growth of fish. Further work on the toxic effect of dietary Se at different life stages of yellowtail kingfish is suggested as their thresholds may differ. Also research on Se and nutrigenomics in yellowtail kingfish is recommended.

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APPENDIX A

Raw Data of Fish and Tanks

Initial weights of fish in Chapter 3

Tank No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Diet	1	1	1	2	2	2	3	3	3	4	4	4	5	5	5	6	6	6
No. of fish	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20
Weight (g/tank)	944.5	948.3	949.0	950.6	950.5	950	949.4	949.5	950.4	949.5	952.7	949.2	951.3	950.9	947.5	951.8	942.9	945.9

Initial weights of fish in Chapter 4

Tank No.	1	2	3	4	5	6	7	8	9	10	11	12
Diet	Control	Control	Control	Control	Se 2	Se 2	Se 2	Se 2	Se 4	Se 4	Se 4	Se 4
No. of fish	15	15	15	15	15	15	15	15	15	15	15	15
Weight (g/tank)	209.75	211.46	207.19	208.42	208.09	207.56	210.31	206.05	208.76	206.44	205.89	208.36

Initial weights of fish in Chapter 5

Tank No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Added Se (mg/kg)	0.0	0.0	0.0	1.5	1.5	1.5	2.0	2.0	2.0	2.5	2.5	2.5	3.0	3.0	3.0
No. of fish	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12
Weight (g/tank)	223.66	222.23	222.96	225.56	223.32	222.84	224.79	225.61	223.72	225.92	224.31	223.28	222.19	223.92	225.32

Initial weights of fish in Chapter 6

Tank No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Se source	Basal diet	Basal diet	Basal diet	Selenite	Selenite	Selenite	SeCys	SeCys	SeCys	SeMet	SeMet	SeMet	Se-yeast	Se-yeast	Se-yeast
No. of fish	15	15	15	15	15	15	15	15	15	15	15	15	15	15	15
Weight (g/tank)	146.78	145.74	148.24	146.70	144.79	147.72	146.04	145.37	147.50	145.76	148.67	146.58	147.32	148.25	145.37

Initial weights of fish in Chapter 7

Tank No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Dietary Se (mg/kg)	2.31	2.31	2.31	4.91	4.91	4.91	9.58	9.58	9.58	15.43	15.43	15.43	20.87	20.87	20.87
No. of fish	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12
Weight (g/tank)	235.71	237.39	233.09	236.27	234.43	235.13	234.86	233.56	234.86	232.46	236.43	231.76	236.79	230.16	236.46

APPENDIX B

F rations, mean squares, and degrees of freedom of the ANOVA Analyses

1. F rations, mean squares, and degrees of freedom in Chapter 3

Average Initial Weight of Fish

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.096	5	.019	1.345	.311
Within Groups	.172	12	.014		
Total	.268	17			

Weight Gain Week 2

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	17.833	5	3.567	.458	.800
Within Groups	93.429	12	7.786		
Total	111.262	17			

Weight Gain Week 4

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	26.616	5	5.323	1.657	.219
Within Groups	38.561	12	3.213		
Total	65.177	17			

Total Weight Gain

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	816.804	5	163.361	4.048	.022
Within Groups	484.252	12	40.354		
Total	1301.056	17			

Feed Intake

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1334.318	5	266.864	2.107	.135
Within Groups	1520.203	12	126.684		
Total	2854.522	17			

FCR

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.020	5	.004	.517	.759
Within Groups	.095	12	.008		
Total	.115	17			

Survival

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	45.833	5	9.167	.943	.488
Within Groups	116.667	12	9.722		
Total	162.500	17			

Muscle Se

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.279	5	.056	39.509	.000
Within Groups	.017	12	.001		
Total	.296	17			

Muscle Vitamin E

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	17.484	5	3.497	73.113	.000
Within Groups	.574	12	.048		
Total	18.058	17			

Muscle Protein

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	47.352	5	9.470	2.301	.110
Within Groups	49.394	12	4.116		
Total	96.746	17			

Muscle Lipid

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.909	5	.382	.860	.535
Within Groups	5.326	12	.444		
Total	7.236	17			

Muscle Moisture

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	88.169	5	17.634	.866	.531
Within Groups	244.379	12	20.365		
Total	332.548	17			

Muscle Ash

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.630	5	.126	1.097	.411
Within Groups	1.377	12	.115		
Total	2.006	17			

Muscle Gross Energy

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.006	5	.001	.058	.997
Within Groups	.245	12	.020		
Total	.251	17			

Hb

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1531.778	5	306.356	5.482	.007
Within Groups	670.667	12	55.889		
Total	2202.444	17			

Hematocrit

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	32.933	5	6.587	.522	.755
Within Groups	138.862	11	12.624		
Total	171.795	16			

WBC

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	260.684	5	52.137	.673	.652
Within Groups	929.269	12	77.439		
Total	1189.952	17			

RBC

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.080	5	.016	2.697	.074
Within Groups	.072	12	.006		
Total	.152	17			

Antibody (Log10)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.267	5	.053	1.767	.194
Within Groups	.362	12	.030		
Total	.629	17			

Bac.Act (log10)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.567	5	.313	25.800	.000
Within Groups	.146	12	.012		
Total	1.713	17			

Muscous Lysozyme

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	19690.037	5	3938.007	48.090	.000
Within Groups	982.659	12	81.888		
Total	20672.696	17			

2. F rations, mean squares, and degrees of freedom in Chapter 4

Average Initial Weight of Fish

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.030	2	.015	1.177	.352
Within Groups	.113	9	.013		
Total	.143	11			

Weight Gain

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	51.170	2	25.585	6.517	.018
Within Groups	35.334	9	3.926		
Total	86.504	11			

Feed Intake

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	20.132	2	10.066	2.571	.131
Within Groups	35.233	9	3.915		
Total	55.365	11			

FCR

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.043	2	.021	2.735	.118
Within Groups	.071	9	.008		
Total	.114	11			

Rbc Peroxidation Pre-challenge

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.195	2	.097	3.545	.073
Within Groups	.248	9	.028		
Total	.443	11			

Rbc Peroxidation Post-Challenge

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3.767	2	1.884	24.305	.000
Within Groups	.698	9	.078		
Total	4.465	11			

GPx Pre-Challenge

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1591.544	2	795.772	14.589	.001
Within Groups	490.915	9	54.546		
Total	2082.458	11			

GPx Post-Challenge

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	4324.042	2	2162.021	28.086	.000
Within Groups	692.815	9	76.979		
Total	5016.857	11			

Muscle Se Pre-Challenge

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.288	2	.144	124.169	.000
Within Groups	.010	9	.001		
Total	.299	11			

Muscle Se Post-Challenge

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.329	2	.165	227.959	.000
Within Groups	.006	9	.001		
Total	.336	11			

Lysozyme Pre-Challenge

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	200.667	2	100.333	1.459	.283
Within Groups	619.000	9	68.778		
Total	819.667	11			

Lysozyme Post-Challenge

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	6422.708	2	3211.354	84.674	.000
Within Groups	341.333	9	37.926		
Total	6764.042	11			

Bactericidal .Act Pre-Challenge

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.540	2	.270	9.494	.006
Within Groups	.256	9	.028		
Total	.797	11			

Bactericidal Act. Post-Challenge

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.211	2	.105	214.441	.000
Within Groups	.004	9	.000		
Total	.215	11			

Haematocrit Pre-Challenge

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	24.892	2	12.446	3.861	.062
Within Groups	29.010	9	3.223		
Total	53.902	11			

Haematocrit Post-Challenge

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	230.671	2	115.335	61.964	.000
Within Groups	16.752	9	1.861		
Total	247.423	11			

Mortality Post-Challenge

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1191.912	2	595.956	20.576	.000
Within Groups	260.667	9	28.963		
Total	1452.579	11			

Antibody (Log10)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.455	2	.727	29.100	.000
Within Groups	.225	9	.025		
Total	1.680	11			

3. F rations, mean squares, and degrees of freedom in Chapter 5

Average Initial Weight of Fish

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.040	4	.010	.918	.490
Within Groups	.108	10	.011		
Total	.147	14			

Weight Gain

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	125.161	4	31.290	22.644	.000
Within Groups	13.818	10	1.382		
Total	138.980	14			

Feed Intake

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	65.987	4	16.497	3.376	.054
Within Groups	48.867	10	4.887		
Total	114.854	14			

FCR

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.044	4	.011	3.127	.065
Within Groups	.035	10	.003		
Total	.079	14			

Muscle Se Pre-Challenge

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.260	4	.065	111.131	.000
Within Groups	.006	10	.001		
Total	.266	14			

Muscle Se Post-Challenge

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.332	4	.083	405.728	.000
Within Groups	.002	10	.000		
Total	.334	14			

GPx Pre-Challenge

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	914.777	4	228.694	20.318	.000
Within Groups	112.560	10	11.256		
Total	1027.337	14			

GPx Post-Challenge

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1675.124	4	418.781	8.265	.003
Within Groups	506.673	10	50.667		
Total	2181.797	14			

Lysozyme Pre-Challenge

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	202.667	4	50.667	1.050	.429
Within Groups	482.667	10	48.267		
Total	685.333	14			

Lysozyme Post-Challenge

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2404.267	4	601.067	7.799	.004
Within Groups	770.667	10	77.067		
Total	3174.933	14			

Bactericidal Act. Pre-Challenge

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.189	4	.047	12.311	.001
Within Groups	.038	10	.004		
Total	.227	14			

Bactericidal Act. Post-Challenge

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.034	4	.009	47.278	.000
Within Groups	.002	10	.000		
Total	.036	14			

Ht Pre-Challenge

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	4.288	4	1.072	.799	.553
Within Groups	13.416	10	1.342		
Total	17.703	14			

Ht Post-Challenge

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	153.978	4	38.494	15.244	.000
Within Groups	25.252	10	2.525		
Total	179.230	14			

Mortality Post-Challenge (Asintranform)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.104	4	.026	5.405	.014
Within Groups	.048	10	.005		
Total	.152	14			

Antibody (log10)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.595	4	.399	7.333	.005
Within Groups	.544	10	.054		
Total	2.139	14			

Macrophage Aggregate

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	32160.400	4	8040.100	51.102	.000
Within Groups	1573.333	10	157.333		
Total	33733.733	14			

4. F rations, mean squares, and degrees of freedom in Chapter 6

Average Initial Weight of Fish

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.006	4	.002	.189	.939
Within Groups	.082	10	.008		
Total	.088	14			

Weight Gain

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	63.146	4	15.787	16.298	.000
Within Groups	9.686	10	.969		
Total	72.832	14			

Feed Intake

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	23.692	4	5.923	1.135	.394
Within Groups	52.202	10	5.220		
Total	75.894	14			

FCR

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.054	4	.014	2.234	.138
Within Groups	.061	10	.006		
Total	.115	14			

Se Digestibility Intake

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	5868.786	4	1467.196	320.239	.000
Within Groups	45.816	10	4.582		
Total	5914.601	14			

		Sum of Squares	df	Mean Square	F	Sig.
Muscle Se	Between Groups	.475	4	.119	402.347	.000
	Within Groups	.003	10	.000		
	Total	.478	14			
Muscle Moisture	Between Groups	.287	4	.072	.663	.632
	Within Groups	1.081	10	.108		
	Total	1.368	14			
Muscle Protein	Between Groups	.219	4	.055	1.351	.318
	Within Groups	.405	10	.040		
	Total	.623	14			
Muscle Lipid	Between Groups	.008	4	.002	.369	.825
	Within Groups	.055	10	.005		
	Total	.063	14			
Muscle Energy	Between Groups	.071	4	.018	1.006	.449
	Within Groups	.177	10	.018		
	Total	.248	14			

Muscle Ash

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.001	4	.000	.275	.887
Within Groups	.012	10	.001		
Total	.014	14			

Se Digestibility of Diet (AsinTransform)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.080	4	.020	196.223	.000
Within Groups	.001	10	.000		
Total	.081	14			

Digestibility of Se Source (AsinTransform)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.777	4	.194	187.780	.000
Within Groups	.010	10	.001		
Total	.787	14			

GPx

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1181.230	4	295.307	15.925	.000
Within Groups	185.436	10	18.544		
Total	1366.665	14			

Bactericidal Activity

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.204	4	.051	159.198	.000
Within Groups	.003	10	.000		
Total	.207	14			

5. F rations, mean squares, and degrees of freedom in Chapter 7

Average Initial Weight of Fish

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.047	4	.012	.320	.859
Within Groups	.368	10	.037		
Total	.415	14			

		Sum of Squares	df	Mean Square	F	Sig.
Weight Gain Wk2	Between Groups	1.336	4	.334	1.541	.264
	Within Groups	2.167	10	.217		
	Total	3.503	14			
Weight Gain Wk4	Between Groups	5.593	4	1.398	2.922	.077
	Within Groups	4.784	10	.478		
	Total	10.377	14			
Weight Gain Wk6	Between Groups	43.759	4	10.940	11.664	.001
	Within Groups	9.379	10	.938		
	Total	53.138	14			
Weight Gain Wk8	Between Groups	129.939	4	32.485	22.323	.000
	Within Groups	14.552	10	1.455		
	Total	144.490	14			
Weight Gain Wk10	Between Groups	240.950	4	60.238	35.289	.000
	Within Groups	17.070	10	1.707		
	Total	258.020	14			

		Sum of Squares	df	Mean Square	F	Sig.
Muscle Protein	Between Groups	.581	4	.145	1.704	.225
	Within Groups	.852	10	.085		
	Total	1.432	14			
Muscle Lipid	Between Groups	.251	4	.063	1.460	.285

	Within Groups	.430	10	.043		
	Total	.681	14			
Muscle Moisture	Between Groups	.873	4	.218	.957	.471
	Within Groups	2.280	10	.228		
	Total	3.152	14			
Muscle Ash	Between Groups	.049	4	.012	1.489	.277
	Within Groups	.082	10	.008		
	Total	.130	14			
Muscle Energy	Between Groups	48468.871	4	12117.218	.824	.539
	Within Groups	147063.349	10	14706.335		
	Total	195532.220	14			

		Sum of Squares	df	Mean Square	F	Sig.
Feed Intake	Between Groups	331.884	4	82.971	19.185	.000
	Within Groups	43.247	10	4.325		
	Total	375.131	14			
FCR	Between Groups	.001	4	.000	.417	.793
	Within Groups	.004	10	.000		
	Total	.004	14			

Liver Se

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	59.618	4	14.904	465.474	.000
Within Groups	.320	10	.032		
Total	59.938	14			

Muscle Se

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3.697	4	.924	1038.461	.000
Within Groups	.009	10	.001		
Total	3.705	14			

GPx

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	6041.333	4	1510.333	33.451	.000
Within Groups	451.500	10	45.150		
Total	6492.833	14			

Macrophage aggregate (SquareRoot Transform)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	70.014	4	17.503	194.339	.000
Within Groups	.901	10	.090		
Total	70.915	14			

Hepatosomatic index

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.109	4	.027	8.553	.003
Within Groups	.032	10	.003		
Total	.140	14			

Haematocrit

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	54.061	4	13.515	6.752	.007
Within Groups	20.017	10	2.002		
Total	74.077	14			