Department of Environment and Agriculture
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The effects of dietary organic selenium supplementation on physiological status of cultured marron, *Cherax cainii* (Austin, 2002)

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DECLARATION

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Rudy Agung Nugroho **January 2014**

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
AWG	Average weekly gain
CARL	Curtin aquatic research laboratory
cfu mL^{-1}	Colony forming unit per millilitre
cm	Centimetre
DHC	Differential haemocyte count
DO	Dissolve oxygen
DWG	Daily weight gain
g	Gram
GC	Granulocyte(s), granular cell(s)
GPx	Glutathione peroxidase
GST	Glutathione-S-transferase
HM%	Moisture content of hepatopancreas
Hiw	Wet hepatosomatic index
Hid	Dry hepatosomatic index
IS	Inorganic selenium
kg	Kilogram
L	Litre
LPO	Lipid peroxidase
mg kg^{-1}	Milligram per kilogram
min	Minute
mL	Millilitre
NA	Nutrient agar
NRRT	Neutral red retention time
OS	Organic selenium
RGR	Relative growth rate
SE	Standard error
Se	Selenium
SGR	Specific growth rate
SPSS	Statistical package for the social science
THC	Total haemocyte count

TM%	Moisture content of tail muscle
Tiw	Wet tail muscle index
Tid	Dry tail muscle index
WA	Western Australia

LIST OF ANIMALS

African catfish (*Clarias gariepinus*)

Allogynogenetic crucian carp (*Carassius auratus gibelio*)

American lobster (*Homarus americanus*)

Atlantic salmon parr (*Salmo salar*)

Barramundi (*Lates calcarifer*)

Blacklip abalone (*Haliotis rubra*)

Black tiger shrimp (*Penaeus monodon*)

Channel catfish (*Ictalurus punctatus*)

Chinese shrimp (*Fenneropenaeus chinensis*)

Chinook salmon (*Oncorhynchus tshawytscha*)

Clams (*Ruditapes philippinarum*)

Coho salmon (*Oncorhynchus kisutch*)

Common carp (*Cyprinus carpio*)

Crucian carp (*Carassius auratus gibelio*)

European lobster (*Homarus gammarus*)

Fathead minnow (*Pimephales promelas*)

Freshwater characid fish matrinxa (*Brycon cephalus*)

Giant freshwater prawn (*Macrobrachium rosenbergii*)

Grouper (*Ephinephelus malabaricus*)

Hybrid striped bass (*Morone chrysops X M saxatalis*)

Marron (*Cherax cainii*)

Marron (*Cherax tenuimanus)*

Medaka (*Oryzias latipes*)

Pacific white shrimp (*Penaeus vannamei*)

Prawn (*Penaeus vannamei*)

Rainbow trout (*Salmo gairdneri*)

Rainbow trout (*Oncorhynchus mykiss*)

Redclaw crayfish (*Cherax quadricarinatus*)

Red swamp crawfish (*Procambarus clarkii*)

Sea cucumber (*Apostichopus japonicus*)

Shrimp (*Penaeus stylirostris)*

Spiny lobster (*Panulirus interruptus*)

Squid (*Todarodes pacificus*)

Nile tilapia (*Oreochromis niloticus*)

Tropical spiny lobsters (*Panulirus ornatus* Fabricius 1798)

Western king prawn (*Penaeus latisulcatus* Kishinouye)

Western rock lobster (*Panulirus cygnus*)

Yabbies (*Cherax albidus*)

Yabbies (*Cherax destructor* Clark 1936)

PREAMBLE

Aquaculture research, specifically related to decapod crustacean research is widely conducted to increase aquaculture productivity and the sustainability of the aquaculture industry. While most of the research to date has focussed on the use of dietary trace elements to improve productivity of crustaceans, limited information is available on the use of organic form of trace element such as organic selenium (OS). The current research was undertaken to understand the effects of Sel-Plex® as a source of dietary OS on marron's *Cherax cainii* (Austin, 2002) performance and immune competence. The effectiveness of Sel-Plex® supplementation on the performances and immunocompetence of marron was determined by investigating their growth performances, survival rates, physiological and immunological parameters, health of the digestive tract, enzymatic activity of the digestive system, resistant ability to *Vibrio mimicus* infections, activity of antioxidant enzymes and total soluble Se retention in the various tissues.

This thesis consists of ten chapters. Chapter 1 is an introduction that underlines the importance of selenium (Se) as an essential trace element, which plays a pivotal nutritional role in aquatic animals including marron. The aim and objectives of the current research are also present in this chapter.

Chapter 2 reviews the existing literature on the topic related to marron including its biology, taxonomy, market value, and immune system. The overview of the literature available on inorganic and organic form of Se is also described in this chapter. The importance of the use of Se in cultured aquatic animals is also described.

Chapter 3 describes the general materials and methods that are used in conducted the experiments. Chapter 4 to 9 evaluate the effectiveness of dietary Sel-Plex® supplementation on the physiology and/or immunology of marron. The main physiological and/or immunological parameters studied are growth performance indicators, survival rates, haematology, total soluble Se in the various tissues, digestives tract health, digestive and antioxidant enzymes activities. All these chapters are either presented at international conferences or independently submitted to peer-reviewed journals for the publications. Due to this reason, some of the basic

information presented in ‘introduction’ section in these chapters may be repetitive in nature, though care has been taken to avoid the repetition by deleting those sentences without altering the clarity of the contents.

Chapter 4 describes an experiment with the aim of finding the effectiveness of various dietary inclusion levels of Sel-Plex® as a source of OS in marron. The effective inclusion levels of Sel-Plex® supplemented diets are evaluated by comparing growth performance, survival, physiological condition, immunological indices, bacteraemia, digestive enzymes activity and total soluble Se accumulation in the hepatopancreas and muscle tissue of marron. Health status of digestive tract of marron such as, the number of microvilli in the midgut and hepatopancreas histology and comparison of total soluble Se retention in the various tissue of treated marron are described in Chapter 5.

Chapter 6 presents the ability of Sel-Plex®-fed marron to resist bacterial infection. The optimum Sel-Plex® inclusion level determined in the chapter 4 is used as a basis of optimum reference level. Survival, immune responses such as total haemocytes count (THC), different haemocyte count (DHC), *Vibrio* ranks and neutral red retention time (NRRT) are used as tools.

Chapter 7 describes the effects of dietary inclusion of Sel-Plex® on the glutathione S-transferase (GST) and glutathione peroxidase (GPx) activity, lipid peroxidation (LPO), lysosomal membrane stability, NRRT and total soluble Se in the haemolymph of marron. These chapters also compare the effects of inorganic selenium (IS), OS and mannan oligosaccharide (MOS) on the health, immune function and antioxidant enzymes activities of the marron.

In chapter 8, the chronic and acute toxicity of high levels Sel-Plex® in marron are evaluated. The effects of higher levels of Sel-Plex® in the diet and acute toxicity levels of Sel-Plex® in the water are determined in this chapter.

The Chapter 9 investigates the benefits of dietary inclusion of Sel-Plex® in the diet on marron in the outdoor commercial marron ponds and the results are then compared with the benefits of using custom made dietary probiotics. The benefits are

measured in terms of growth indices, enhanced immunological competence and the retention of total soluble Se in the hepatopancreas and muscle tissues of the marron.

Chapter 10 attempts to bring all these results together by discussing the overarching effects of dietary Sel-Plex® in the cultural performance of marron. This chapter includes further statistical analysis by extracting the data from the previous chapters in order to highlight the main conclusions of the research. The Chapter 10 ends presenting some conclusions and recommendations for further research on the topic.

ABSTRACT

Six 90-day feeding experiments including outdoor commercial marron ponds trial were designed and conducted to investigate the effects of dietary Sel-Plex® as a source of organic selenium (OS) supplementation in marron, *Cherax cainii* (Austin, 2002). The effects were evaluated by measuring the growth performances, health indices, immunity responses and antioxidant enzyme activity and finally validated by comparing the marron performance results with other currently employed probiotics under outdoor commercial marron farm environment.

Various marron sizes, mean initial weights of 3.6; 40 and 76 g, were used and reared inder laboratory conditions in the plastic cylindrical tanks with filtration, aeration; automatic heater and PVC pipes for shelter. The marron were fed at a rate of 3% of body weight every second day. Uneaten food and faeces were siphoned out before the next feeding and sufficient freshwater was added to maintain 70 L of water in every tank. During each experiment, water parameter such as temperature, pH, nitrate, nitrite and ammonia levels were measured once a week. To evaluate the effects of Sel-Plex® supplementation, growth and physiology parameters such as, initial and final weight; carapace and total length; specific growth rate (SGR); daily weight gain (DWG); average weekly gain (AWG); relative growth rate (RGR) and organosomatic indices were used as a tool to evaluate of OS supplementation on the marron's growth and health performance. Meanwhile, immune parameters such as total and differential haemocyte count (THC and DHC); neutral red dye retention time (NRRT); lysosomal membrane integrity and bacteraemia were also used as an indicator of the health of marron fed OS. Besides growth, physiological and immune indices, the health of digestive tract such as histology of midgut and hepatopancreas; condition of microvilli; amylase and protease activity of marron fed OS were assessed to examine the effects of OS supplementation. As selenium (Se) can be retained in the various tissues and influence on antioxidant enzymes activity, total Se retention in the hepatopancreas and muscle were determined in the backdrop of antioxidant enzyme activities of glutathione peroxidase (GPx), glutathione-S-transferase (GST) and lipid peroxidase (LPO) measurements.

Sel-Plex® supplementation of 0.1-0.3 g kg^{-1} showed improvements in survival, physiological and immunological responses in the marron. The optimum level of Sel-Plex® supplementation was found at 0.2 g kg^{-1} of feed which improved the survival, health and immune status of the cultured marron. Dietary inclusion of Sel-Plex® also enhanced the health of the digestive system as shown by a healthier midgut and hepatopancreatic condition in term of the number of microvilli and structural improvements, respectively. In addition, marron fed 0.2 g kg^{-1} of Sel-Plex® also improved the digestive enzyme activity and increased resistance ability of marron against *Vibrio mimicus* infections. Further, dietary 0.2 g kg^{-1} Sel-Plex® in the diet showed higher antioxidant enzymatic activity and improved lysosomal membrane integrity, reduced LPO and higher total soluble Se retention in the muscle of marron compared to inorganic form of Se and mannan oligosaccharide (MOS). Higher levels of dietary Sel-Plex®, however, led to increased stress in the cultured marron. The $LC_{50\text{-}96h}$ of Sel-Plex® was calculated at 166.28 g L^{-1}, indicating that natural presence of OS in the water is not harmful to marron. The trial in commercial ponds found that pond-water temperature was significantly higher than the temperature under laboratory conditions resulting in higher growth performances. Further, THC and total soluble Se retention in the muscle of the marron fed any supplement were higher than in marron fed the control diet under outdoor commercial environment. The pond-reared marron fed OS had significantly higher SGR and total Se retention in the muscle tissues but lower THC than laboratory-reared marron. The DHC of marron fed OS did not show significantly difference ($P>0.05$) between pond-reared marron and laboratory-reared marron. The present research concludes that Sel-Plex® as a source of OS can be used as a dietary supplement to improve growth and health of marron. 0.2 g kg^{-1} of Sel-Plex® which approximately equates to 0.4 mg kg^{-1} OS is suggested to be added in the basal diet in order to attain the optimum benefit of OS.

CHAPTER 1: Introduction

As seafood demand continues to grow, the strategies to enhance aquaculture productivity need to develop. The research based on eco-friendly and sustainable commercial aims should also be strategised to assist aquaculturists in increasing aquaculture productivity. To this effect, the use of trace minerals as an alternative to antibiotics and chemicals in production systems to improve productivity and prevent possible outbreaks of disesases has been gaining momentum.

Selenium (Se) is a trace element and occurs in two forms, inorganic selenium (IS) and organic selenium (OS). Selenium plays an important role in enhancing growth, survival and immunity against pathogens in aquatic animals such as rainbow trout (*Salmo gairdneri*), crucian carp (*Carassius auratus gibelio*), hybrid striped bass (*Morone chrysop X M saxatalis*) and prawns (*Macrobrachium rosenbergii*) (Vidal et al., 2005; Wang et al., 2007; Chiu et al., 2010; Jaramillo Jr. et al., 2013). IS is found as selenate and selenite while OS occurs as Se containing proteins such as selenomethionine (Swanson et al., 1991; Barceloux, 1999; Zhan et al., 2010) and selenoprotein (EFSA, 2012).

The use of Se as a dietary supplement has resulted in increased survival of Coho salmon (*Oncorhynchus kisutch*) (Felton et al., 1996) and giant freshwater prawn (*Macrobrachium rosenbergii*) (Chiu et al., 2010) while Elia et al. (2011) found that juvenile carp (*Cyprinus carpio*) fed diet containing 1 mg kg^{-1} Se for 60 days, resulted in higher growth performance than when fed 0.25 mg kg^{-1}. The studies on the dietary OS supplementation has also indicated that OS can improve the growth performance of African catfish (*Clarias gariepinus*) (Abdel-Tawwab et al., 2007), grouper (*Ephinephelus malabaricus*) (Lin and Shiau, 2005) and rainbow trout (*Oncorhynchus mykiss*) (KÜÇÜKbay et al., 2009).

Another positive effect of OS supplementation is enhanced immune performance of shrimp (*Penaeus vannamei*) against Taura syndrome virus (Sritunyalucksana et al., 2011b). Adding OS in the diet of shrimp also improves disease resistance by enhancing antioxidant enzyme, glutathione peroxidase (GPx) activity to protect the

host against the pathogen infection (Rotruck et al., 1973; Sritunyalucksana et al., 2011b).

However, high levels of dietary OS can be harmful and can be potentially toxic to certain species due to its chemical and physical properties (Hamilton, 2004). Vidal et al (2005) found that feeding 4.6 mg kg^{-1} of dietary selenomethionine for 90 days can reduce the growth of rainbow trout (*Oncorhynchus mykiss)*. Similarly, Lee et al. (2010) stated that juvenile olive flounder, (*Paralichthys olivaceus*) when fed diet containing 35.9 mg kg^{-1} of selenomethionine for 10 weeks resulted in 100% mortality. OS from yeast (*Saccharomyces cerevisiae*) is absorbed at a much higher rate, is less toxic and has higher bioavailability than IS (Mahan and Parrett, 1996; Schrauzer, 2003; Taylor et al., 2005; Wang et al., 2007; KÜÇÜKbay et al., 2009). In June 2000, OS from yeast was permitted by US-FDA as a feed supplement (Federal_Register, 2000). Therefore, it is important to understand the role of OS supplementation in the diet of cultured animals, including marron *Cherax cainii*.

Marron is the third largest freshwater crayfish in the world and is successfully farmed commercially with 178 production farms (Anonymous, 2010). Since marron productivity has increased incrementally from 1996, marron has become one of the attractive freshwater decapod crustaceans to be cultured. In 2009/2010, the economic value of marron ($1.4 million) was recorded as the third most valuable industry after barramundi ($4.5 million) and mussels ($1.9 million) in Western Australia (Nobes, 2011). The marron production reached 65 tonnes in 2012 (Nobes, 2011) and may show further increase in 2013 as some marron growers have started producing t more than 2 tonnes/year/Ha (Anonymous, 2012). In addition, there are currently 470 aquaculture licenses holders for marine and inland aquaculture, 39% of which are identified on marron farming that can generate $1.5 million per year (DoF, 2013).

Similar to other cultured crustaceans, the productivity of marron farming can decline due to the possible outbreaks of the diseases, though currently there is no information available on the bacterial infection such as *Vibrio* spp in marron. The application of commercial antibiotics, though not used and recommended can be used to prevent disease due to *Vibrio* spp infections. However, the research from other decapods from Asia has viewed that the application of unmanaged antibiotics can critically

lead to bacterial resistance, antibiotic residues in the final product and damage to the surrounding environment. Thus, investigations need to be performed to find an alternative product(s) The use of dietary OS supplementation for disease prevention and simulataneously maximizing the productivity can be one of the such options. However, there has been limited research and no published information on the effects of dietary OS supplementation and its comparative effectiveness with other feed-additives on the growth and health performance of marron.

1.1 Aim

The aim of this study is to evaluate the effectiveness of dietary organic selenium supplementation in enhancing the growth, survival, physiological responses and immunity of marron against the selected known and/ potential pathogens.

1.2 Objectives

The above aim is achieved by meeting the following specific objectives:

a. To investigate the effects of supplementing varying levels of OS in formulated diet on the growth, survival and physiological, immunological responses and digestive enzymes activity of marron.
b. To investigate the effective optimum level of OS supplementation on growth, survival, physiological, immune responses and Se retention in the various tissues of marron.
c. To assessed the effects of OS supplementation on the resistant capacity of marron against *Vibrio mimicus* infection.
d. To evaluate effectiveness of OS supplementation in the diet on the physiological, immune responses and antioxidant enzymes activity of marron in comparison to inorganic Se and mannan oligosaccharides.
e. To evaluate acute and chronic toxicity of high levels of dietary OS on the growth indices, survival, physiological and immunological condition of marron.
f. To compare the effectiveness of dietary OS and probiotic *B. mycoides* for marron when cultured under laboratory conditions and validate these results under outdoor and commercial environment.

CHAPTER 2: Literature Review

2.1 Background

Aquaculture is one of the fastest growing industries in the world and has continued to rise in the new millennium. In Australia, aquaculture is also steadily growing primary industry that began in the 1980s. The aquaculture production in Australia has almost double from 29,300 tonnes to 58,000 tonnes in 2006-07,continued to rise in 2010 by 69.6 tonnes and can generate $1.5 million per year in 2013 (DoF, 2013). Marron, the world's third largest freshwater crayfish are placed as a valuable and potentially growing aquaculture commodity in 2009/10 after barramundi and mussels. Market demand of marron has also increased gradually and has generated up to $1.5 million per year from approximately 180 marron farms (Lee et al., 2010). Further, the increasing market demand of marron has led to significant boost in research to increase productivity and improve health performance in order to prevent any future disease outbreaks in marron farming.

2.2 Marron Taxonomy

Recently two distinct species of marron *Cherax tenuimanus* also known as hairy marron and *Cherax cainii*, known as smooth marron have been identified from previously known single species called *C. tenuimanus*. The hairy marron are restricted to Margaret River region in WA and the smooth marron have been translocated to wider regions (Merrick and Lambert, 1991; Austin and Knott, 1996; Austin and Ryan, 2002; Bryant and Papas, 2007). Marron has some specific morphological features that can be used to distinguished them with other *Cherax* species. These featues are the presence of five keels on the dorsal surface of their head, two small spines on the telson and narrow pincer like chelipeds (Bryant and Pappas, 2007). A taxonomic classification of *Cherax cainii* is as follows:

Kingdom: Animalia
Phylum: Arthropoda
Subphylum: Crustacea
Class: Malacostraca
Order: Decapoda
Suborder: Pleocyemata
Infraorder: Astacidea
Family: Parastacidae
Genus: *Cherax*
Species: *C. cainii*

According to Austin and Knot (1996), *C. tenuimanus* is considered to be a sub species whereas, allozyme evidence revealed that this species can be distinguished with another *Cherax*, namely *Cherax cainii* (Austin and Ryan, 2002).

2.3 Marron Biology

Marron is an indigenous species in the southwest of Western Australia (Molony et al., 2004). Although marron are endemic species to the region and are threatened by predation, they have been distributed in several part of Western Australia such as Esperance and Geraldton (Lawrence and Jones, Morrissy, 1979; 2002). They naturally live in permanent rivers with high rainfall; clear and deep water. The marron in natural environment use snags and rocks for shelter (Morrissy, 1979; Lawrence and Jones, 2002). Generally, marron are omnivorous scavenger and do not borrow (Morrissy, 1992; Mills et al., 1994; Beatty et al., 2003). However, some reports state that marron can show borrowing habits in the banks of dams where refuge provided habitat is limiting (Clunie et al., Mosig, 1998; 2002). Hence, tyres, PVC piping, rope fiber can be used and added to the dams as artificial refuge to support their growth (Bryant and Papas, 2007).

Marron can grow more than 350 mm in length equivalent to 2.5 kg in weight and generally reach sexual maturity between two to three years under favorable conditions (Merrick and Lambert, 1991; Lawrence and Jones, 2002; Molony et al., 2004). The optimum temperature for marron growth is 24^{o}C with oxygen level > 6 mg L^{-1} provide optimum growth. Temperature of water and day length can be factors to trigger breeding during spring. Majority of females can carry up to 900 eggs underneath its abodomen but normally their number seldom exceed 150 (Merrick

and Lambert, 1991; Bryant and Papas, 2007).

2.4 Marron Anatomy

The body of the marron can be divided into two parts, cephalothorax (commonly termed as head) and abdomen (commonly termed as tail). The head is covered by carapace to protect internal organ. Antenna and antennula are major touch and sensors organs which are used to locate potential food, changes in water temperature and other water quality parameters (DoF, 2011). The dorsal and ventral sides of marron are shown on Figure 2.1.

The tail itself can be divided into six segments that are individually encased in hard sell. Pleopods or swimmerets, known as swimming legs (segment two to five) are located on the ventral side of the abdomen. In sexually mature female marron pleopods have fine hairs in all pleopods which are to to adhere their eggs. Sixth segment of the tail is larger than another segment and is referred as uropods where telson can be found. Telson is tail flap that is used to move quickly through the water (McCormack, 2012).

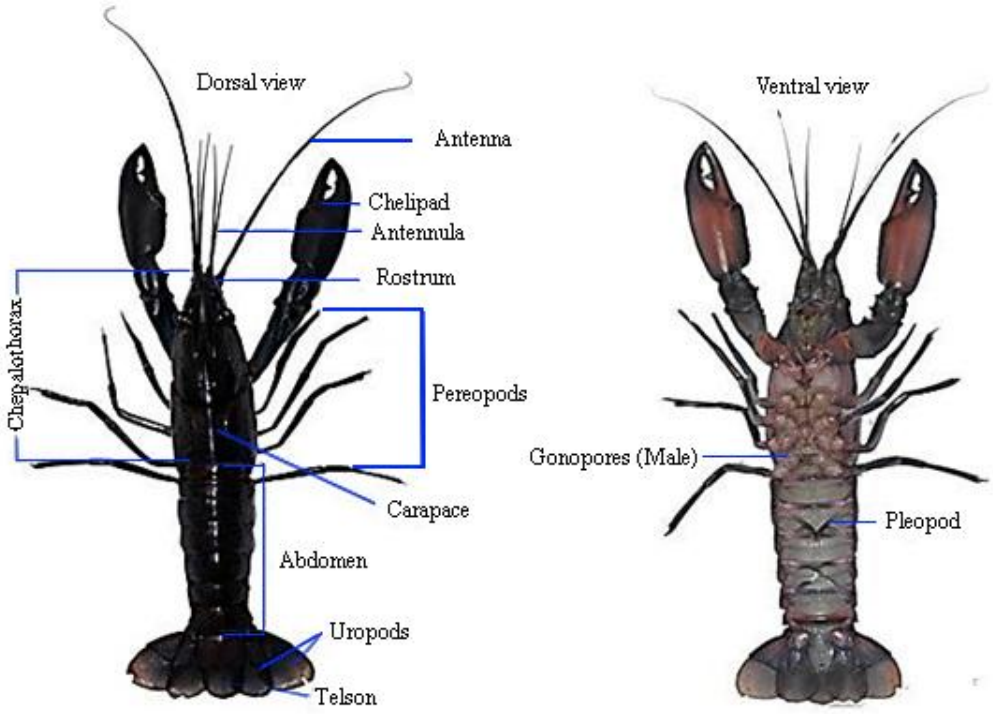

Figure 2.1: Dorsal and ventral view of marron

The marron gendra can be distinguished externally by the position of gonopores (sexual organs) on pereopods (walking legs). The male has sexual organ which is located at the fifth pair of walking legs while the female can be found at the base of the third, or in the middle pair of walking legs (Figure 2.2).

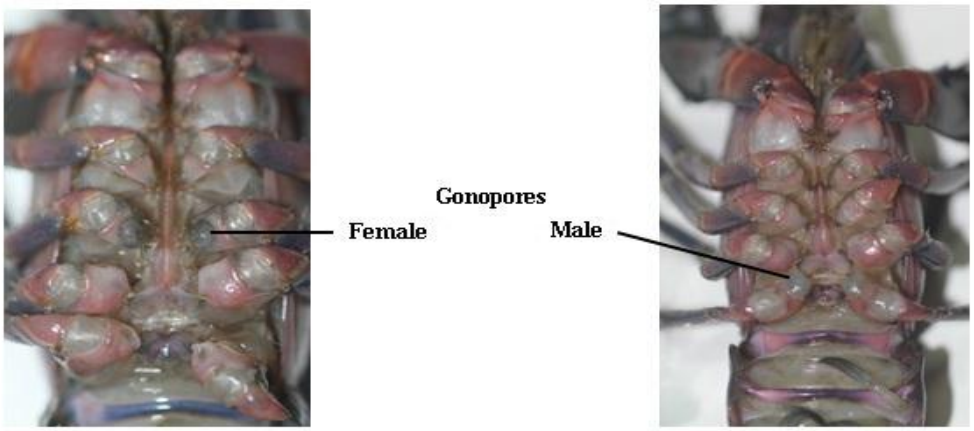

Figure 2.2: Gonopores of marron

2.5 Marron Physiology

Marron grows by moulting. Moulting is the process of discarding and shedding an old body shell in order to grow a new body shell which is a soft texture. The new body shell of marron becomes hard by accumulating calcium which is stored in the body of marron. Marron will then take up water within its body to expand the size and to stretch the new body shell. Moulting frequency is higher in juvenile marron and then decrease as the marron gets older. In a fresh moulted stage, marron are extremely vulnerable to attack from predators (Withnall, 2000; McCormack, 2012).

The entire life cycle of marron is completed in freshwater (Cubitt, 1985). Marron can breed in spring during their second year of life (Safriel and Bruton, 1984; Moor and Bruton, 1988). The females produce eggs numbering from 90-900 per female and is dependent on their body size (Coetzee, 1985). Eggs are carried for twelve to sixteen weeks by female, beneath its pleopod and free swimming larvae are released after that period (Moor and Bruton, 1988)

Marron mate in August - September but on farms in the north region of Western Australia, this can be advanced by at by at least one month. The warmer the temperature, the sooner the mating and hatching. The juveniles are released by middle December. After the marron hatch they moult while they are still attached to the mother's pleopods under the tail. After two months they leave the mother. Some marron mate when reached 18 – 19 months of age, but most marron commence mating in the third spring of their life when they are 30 to 31 months old (DoF, 2011; ACWA, 2013).

The temperature range for optimum growth of marron is achieved between 17^{o}C and 25^{o}C (Merrick and Lambert, 1991; Bryant and Papas, 2007). At temperature below 12 degrees, the growth rate of marron slows down and they become less active. Mortality rate starts to increase when temperature goes above 28^{o}C. Marron have a great tolerance towards salinity which can reach up to 7000 ppm, wider than yabbies (*Cherax destructor*) (Bryant and Papas, 2007).

2.6 Production and Markets Value

Marron farming has started as a valuable aquaculture industry since last 30 years. In Australia commercial marron production exists in Southwest of Western Australia and Kangaroo Islands in South Australia (Piper, 2000). Marron production has started to attract international interest due to their ability to be transported alive to countires such as Zimbabwe, Japan, USA, China and South Africa (Morrissy, 1979, 1990; Rouse and Kartamulia, 1992; Lawrence and Jones, 2002). In 2005-2006 Australian freshwater crayfish farms were counted to 883 farms (ABARE, 2007). The value of marron production in 2009-2010 reached $1.4 million (Nobes, 2011). Marron production reached 53.3 tonnes, with an average price of $27.12 per Kg which is higher unit price than other crustacean such as yabbies and redclaw (Nobes, 2011). The marron farming also has largest number of production licences (182) licences compared to yabbies (*Cherax albidus*), mussel, and barramundi (*Lates calcarifer*).

2.7 Marron Culture

Marron farming generally uses large earthen ponds equipped with aerated water supply and generally are drained for harvesting. As an ideal species for aquaculture, they grow to large size, have a restricted yet simple reproductive cycle, can tolerate reasonably prolonged starvation periods and enjoy niche markets with ever expanding global interest.

Water quality, dissolved oxygen, pH, temperature, food supply, shelter and stocking density are some of the factors which can affect the marron growth under aquaculture environment (Keen et al., 2003). Both water alkalinity and water hardness are important to culture marron. It is recommended that alkalinity and hardness levels should be maintained around 50 to 300 mg L^{-1} which can provide good buffering (stabilizing) effect to pH swings that can occur in pond enviromnent due to the respiration and photosynthesis of aquatic flora and fauna. A lack of calcium in the water can also result in soft shelled marron as they rely on the intake of calcium from the water column to harden their shells after moulting. Beside natural productivity, suspended solids such as clay and silt particles can influence the light penetration and can have indirect effect on water quality. Another water quality parameters that is important to marron culture is ammonia. Ammonia in the ponds is produced from the decomposition of organic wastes resulting in the breakdown of decaying organic matter such as algae, plants, animals and uneaten food. Ammonia is also produced by the marron as an excretory product. Ammonia is present in two forms in water – as a gas NH_3 or as the ammonium ion (NH_4^+). Ammonia is toxic to marron in the gaseous form and can cause gill irritation and respiratory problems. Ammonia form will depend on the temperature pH of the pond's water. For example at a higher temperature and pH, a greater number of ammonium ions are converted into ammonia gas thus causes an increase in toxic ammonia levels within the marron pond (Keen et al., 2003; Anonymous, 2010, 2012; ACWA, 2013).

Oxygen levels in the pond varies during the day and night depending on the water temperatures, stocking rates, and the amount of aquatic vegetation growing in the pond. Although marron can survive in oxygen level as low as 3 ppm for short periods, it is recommended that oxygen levels within a pond be maintained above 6 ppm (Keen et al., 2003). To maintain oxygen level in the pond, artificial pond

aeration is used. Additions oxygen is especially required when ponds are stocked at higher densities of marron and supplementary feeding is provided to maintain the stock. There are some popular methods to aerate the l ponds, such as air-lift pump system, paddle wheels and aspirators (Keen et al., 2003).

Degree of acidity, known as pH, depends on several factors such as the amount of respiration and photosynthesis that occur in the pond. Marron can tolerate pH between 7.0 and 9.0. However, pH between 7.5 and 8.5 are recommended for culturing marron. pH above 9 causes toxicity to marron due to increase in ammonia levels. pH below 7.0 in the pond increases the bioavailability of dissolved metals within the water column and soften the exoskeleton of the marron (Keen et al., 2003).

Beside some water quality parameter as mention above, water supply (Jussila and Evans, 1996), pond design (Keen et al., 2003) and diet (ACWA, 2013) must be considered as an important factor in culturing marron. Culturing marron need plentiful water supply which can be supplied from bore water or run-off from water catchments to the dams (Keen et al., 2003).

Marron are omnivorous and opportunistic feeders (Merrick and Lambert, 1991). However, as marron are unable to digest cellulose, they are not able to obtain sufficient minerals and nutrients from eating these products. The marron show very high degree of cannibalism if they are overcrowded or insufficient food is available. In commercial ponds, marron growers also use other feed supplements such as wheat and barley, lucerne, lupin, vegetable scraps, and processed crayfish pellets manufactured by a feed company. Marron are currently fed every second day or everyday during the summer months which tapers down to around once per fortnight during winter when the activity of the marron slows down. The amount of feed marron are given depends on the temperature and their body weight.

Although all Australian freshwater crayfish are free of major diseases, marron farming can be exposed to potential diseases caused by bacterial, fungal and protozoan (Horwitz, 1990; Merrick and Lambert, 1991; Mosig, 1998). However, the

preventive actions are gaining momentum by the marron industry making it essential to understand the immune mechanisms of marron.

The success and significant increased in marron production has received attention not only from farmers or investor but also researchers who attempts to increase productivity by increasing growth and health performance, especially immune system to protect against pathogen.

2.8 Immune System of Crustaceans

Crustacean immune system depends on the physico-chemical, humoral and cellular systems (Figure 2.3) (Soderhall and Soderhall, 2002). The physico-chemical is the first mechanism of immune system to protect crustacean from pathogens (Soderhall and Cerenius, 1992). The second defence mechanism is humoral and cellular which acts if pathogen successfully infiltrate the body cavity of crustacean (Soderhall and Soderhall, 2002).

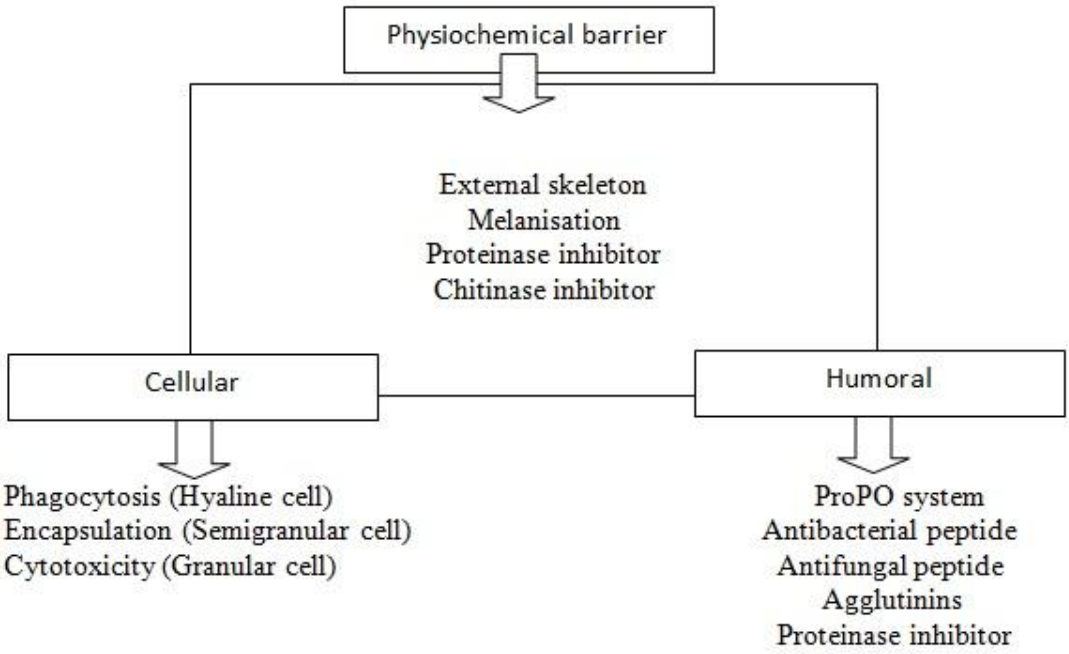

Figure 2.3: Components of crustacean host defence system (Soderhall and Soderhall, 2002).

In cellular and humoral defence mechanisms, haemocytes play a pivotal role in the immune system (Soderhall and Soderhall, 2002; Smith et al., 2003). Phagocytosis, encapsulation, nodule formation, and mediation of cytotoxicity are the main function

of haemocytes which also release microbicidal proteins which kill pathogens (Soderhall and Soderhall, 2002; Smith et al., 2003). Normally, haemocytes are produced in the hematopoietic tissues which are located in the dorsal anterior part of the thorax and on top of the cardiac stomach or the heart (Hose et al., 1992). Basically, there are three types of haemocytes that can be distinguished, based on their morphological and biological functions (Table 2.1 and Figure 2.4) (Bauchau, 1981; Smith et al., 2003). The morphological function of haemocytes is based on their shape, nucleus, endoplasmic reticulum, free ribosome, Golgi, granules, lysosomes and mitochondria (Bauchau, 1981). Different haemocyte types are involved in different biological functions (Soderhall and Cerenius, 1992). There are three types of haemocyte that can be identified as granulocytes, semigranulocytes and hyalinocytes (Cardenas et al., 2000; Johansson et al., 2000).

Granulocytes have specific defence functions such as phagocytic function on bacteria and other small particles, encapsulate mechanism to metazoan parasites, haemolymph clotting system if stimulated by invading microbes and prophenoloxidase activating system (Soderhall et al., 1988; Hose and Martin, 1989; Hose et al., 1990; Soderhall and Cerenius, 1992; Hideaki et al., 1993; Hryniewiecka-Szffter and Babula, 1996; Jussila, 1997a; Al-Mohanna and Subrahmanyam, 2001). Semigranulocytes are also participate in phagocytic activities, especially in encapsulation mechanism and prophenoloxidase (proPO) activating system (Smith and Soderhall, 1983; Persson et al., 1987; Al-Mohanna and Subrahmanyam, 2001). Beside contributing to the formation and hardening of cuticle at moulting, the hyaline cells have activities in haemolymph clotting mechanism and also execute some phagocytosis activity but no prophenoloxidase (proPO) activities are seen in these cells (Vacca and Fingerman, 1993; Sequeira et al., 1995; Al-Mohanna and Subrahmanyam, 2001).

The quality and quantity of granular, semigranular and hyaline cells are affected by several factors, including trace nutrients, to promote crustacean immunity and thereby increase resistance to disease. Trace nutrients such as selenium in the diet of crustacean has been an important trace mineral to boost their immune system.

Table 2.1: Three different haemocyte types in crustaceans

Criteria	Semigranulocytes	Granulocytes	Hyalinocytes
Shape	oval or spindle	oval	round or oval
Nucleus	central or eccentric, oval, lobed	eccentric, kidney shape	central, round, large
Endoplasmic reticulum	smooth, rough, abundant	smooth, rough, moderate	smooth, rough, scarce
Free ribosomes	abundant	moderate	present
Golgi	1 or more	0 or 1	0 or 1
Granules	moderate	abundant	0 or few
Lysosomes	present	present	-
Mitochondria	abundant	abundant	moderate

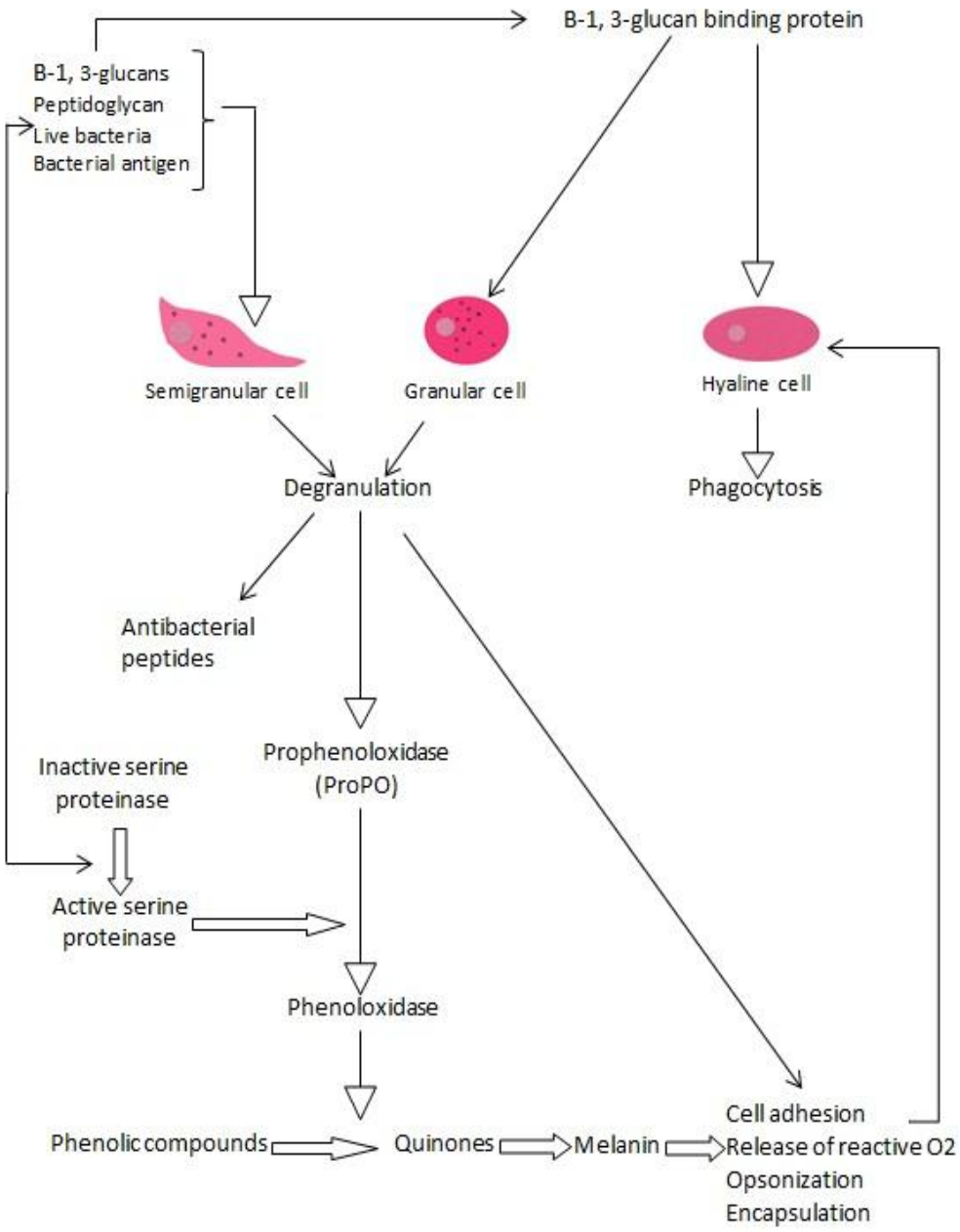

Figure 2.4: Flow diagram of the crustacean defence system (Smith et al., 2003).

2.9 Selenium

Selenium (Se) was discovered in 1817 by a Swedish chemist, Jons Jacob Berzelius and established in 1957 (Schwarz and Foltz, 1957; Tinggi, 2003). Initially Se was thought to be a toxic trace element (Payne and Southern, 2005). Se that belongs to periodic table of elements in-group VIA, has six stable isotopes with atomic number and weight of 34 and 78.96, respectively (USEPA, 2004; Reilly, 2006). Having atomic number of 34, Se presents itself between sulphur and tellurium in Group VIA, arsenic and bromide in period 4 (Foster and Sumar, 1997). Thus, the chemical properties of Se have similarities with sulphur that occurs as oxidized quadrivalent form while Se is a reduced quadrivalent. Both Se and sulphur have similar atomic size, bond energies, ionization potentials, electron affinity and bond length that cannot be distinguished (Foster and Sumar, 1997; Sunde, 1997). Although Se and sulphur have similarities, they are not interchangeable in biological system due to their different strength in the acid form of the elements with hydrogen selenide (H_2Se) that is stronger than hydrogen sulphide (H_2S).

In nature, Se is widely found in soils and waters at levels between 0.01-2 mg kg^{-1} and 0.1-0.4 μg L^{-1} (Mayland, 1994; USEPA, 2004) and can be found in four valence states: selenates (Se^{6+}), selenites (Se^{4+}), selenides (Se^{2+}), and elemental Se (Se) (Goyer, 1991; Chapman, 2000). Se dioxide is a common Se compound that is used in industries which is produced by the oxidation of Se using nitric acid and is followed by burning Se in oxygen or evaporation (Goyer, 1991; HSDB, 1995).

Se can be classified into inorganic Se (IS) and organic Se (OS). In inorganic form, Se is found as selenite and selenite while OS is present as Se containing protein such as selenomethionine (Barceloux, 1999; Zhan et al., 2010). In both animal and plant tissues, selenate is the main inorganic seleno-compound, whereas selenocysteine is the major seleno-amino acid in the tissue when IS is administrated to animals (Guo and Wu, 1998)., The organo-Se compounds play essential roles in the biochemistry processes (Wan, 2007). Mainly, OS is incorporated into amino acids, namely selenomethionine and selenocysteine that are produced from yeast (Wan, 2007). The OS from the yeast is Se that is incorporated in methionine present as amino acid in the yeast protein by culturing the yeast on a specific substrate containing IS (Gerhard, 2001; Jang et al., 2010). Se-yeast can be derived from the fermentation of

specific strains of yeast (*Saccharomyces cerevisiae* strain CNCM I-3060), incubated at high Se levels during their growth phase (Kim and Mahan, 2003). OS from yeast has been proved to be retained at higher levels, is better absorbed at a higher rate, is less toxic and has relatively higher bioavailability (Mahan and Parrett, 1996; Foster and Sumar, 1997; Jacques, 2001; Schrauzer, 2003; Taylor et al., 2005; Wang et al., 2007; KÜÇÜKbay et al., 2009). Se-yeast also has capability to increase the activity of the selenoenzymes and its bioavailability was found to be higher than that of inorganic Se sources (Kim and Mahan, 2003).

In contrast to the OS, inorganic form of Se is poorly absorbed by the intestinal tract and has low retention in the body (Pond et al., 1995). OS compound are mostly found in the common species of bacteria and yeast but majority of animals can not synthesise it. Consequently, Se is transferred to animals via protein bound selenomethionine (Jacques, 2001) which is recognized as a primary source of Se in plant-based and meat-based feed ingredients.

2.9.1 Biological Aspect of Selenium

As an extreme trace element, Se has biological effects on animals which is necessary for appropriate immune function in the muscle tissues, reproductive function and prevention of the tissue damage (Irwin et al., 1997; Rayman, 2000; Burk, 2002). Several experiments have been carried out to evaluate the application and the effects of dietary Se on aquatic animals. In aquatic animals, the uptake of Se can be from water or diet (Figure 2.5) (Tinggi, 2003). For example, fish might absorb water-soluble Se from gills, epidermis or gut. However, the uptake of Se in the diet is the major pathway for animals at higher tropic levels in the aquatic food webs (Hamilton, 2004). The selenomethionine in the diet can be actively transported through intestinal membrane and then actively accumulated in liver and muscle tissues (Navid, 2011).

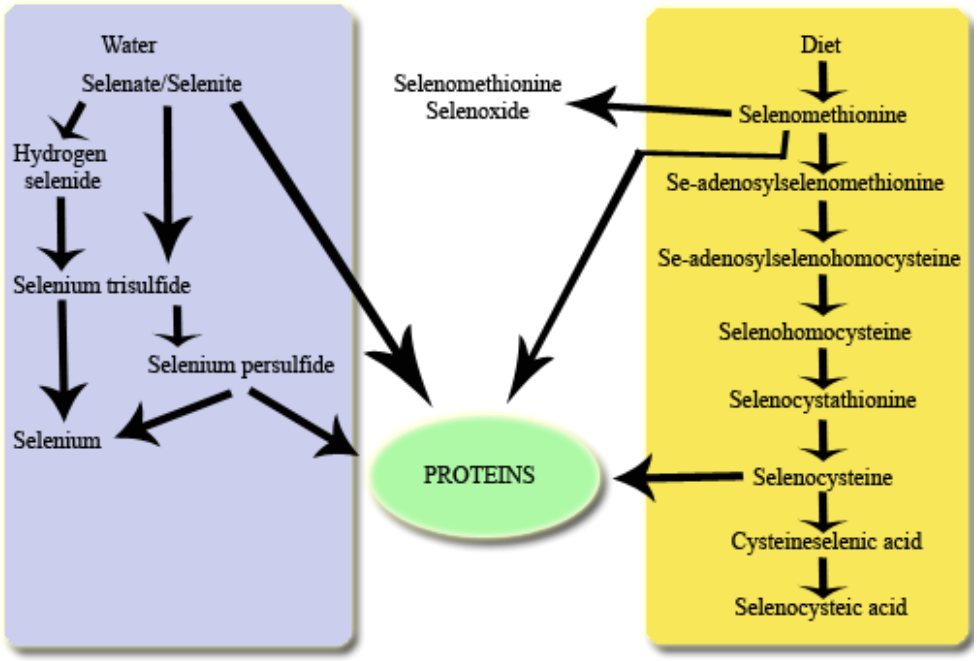

Figure 2.5: The pathway of selenium metabolism in animals (Tinggi, 2003).

There are three levels of pivotal roles of biological activity of Se: (1) trace concentrations are required for normal growth and development; (2) moderate concentrations can be stored for the mainatainence of homeostatic functions and (3) elevated concentrations can result in toxic effects (Hamilton, 2004). It is then suggested that dietary Se intake should be around moderate level but under toxic level (Todd, 2006). As Se requirements for growth and development are different under stressful conditions, the requirements under stressful conditions increase gradually in order to improve the immune system and prevention of chronic diseases (Cotter et al., 2008). However, the level of toxicity is only slightly above the required level of Se. Study in salmonids showed that dietary Se becomes harmful at only 10 times from normal diet (1-2 mg kg^{-1}) resulting in high mortality, reducing feeding rates and slowing growth rates (Hilton et al., 1980, 1982).

Referring to evidence at molecular level, the Se toxicity is caused by substitution sulphur in thiol containing protein. Se may replace sulphur in the amino acids methionine and cysteine, thus impact on structural confirmation and changing their function when incorporated into protein (Stolz and Oremland, 1999; Chapman, 2000). The Other negative impacts of Se toxicity are changes in blood composition,

affecting on hepatopancreatic and gonadal edema (Chapman, 2000; Lohner et al., 2001).

2.9.2 Selenium in Aquatic Animals

Generally, animals uptake OS as the selenoamino acids such as selenomethionine and selenocysteine, both as methylated and non-methylated forms via food while IS can be uptaken through supplementation (Foster and Sumar, 1997). Absorption of dietary OS from food source is approximately 80% via small intestine.

The use of Se supplementation has been applied in some aquatic species (Table 2.2). Chiu et al. (2010) stated that Se has proved to enhance immune function and antioxidant activity which impacts on immunity and disease resistance in giant freshwater prawn (*Macrobrachium rosenbergii*). In term of immune system, Spallholz et al. (1990) stated that Se has three major functions; First, Se reduces organic and inorganic peroxides formed by free radical chain reactions. Second, it modulates hydrogen peroxide and superoxide produced during respiratory burst. Third function is involvement in the lipooxygenase as well as cyclooxygenase pathways of the arachidonic cascade, which produce hydroperoxides that lead to the synthesis of leukotrines, tromboxanes, prostaglandins and lipoxins. Study in catfish (*Ictalurus punctatus*) showed that intracellular macrophage superoxide anion production is affected by Se supplementation in the diet, especially dietary OS increases macrophage chemotaxis with *Escherichia coli* antigen (Wise et al., 1993). In catfish challenged with *E. ictaluri*, mortality was decreased after feeding Se up to 0.4 mg kg^{-1} . The potency to reduce mortality was also increased for OS relative to IS (Wang and Lovell, 1997). However, in tilapia (*Oreochromis* spp) challenged with *E. tarda*, mortality was not affected by 0.2-0.5 mg kg^{-1} IS. In chinook salmon (*Oncorhynchus tshawytscha*), prevalence of *Renibacterium salmoninarum* (bacterial kidney disease) was also not affected by selenite (Thorarinsson et al., 1994). Following thsese studies, it was shown that the effects of Se on immunity was also dependant on the pathogen species, the host species and the level of dietary Se supplementation, as well as source of Se supplementation especially in fish.

Besides its role as an agent to improve immune system, Se supplementation in the diet at recommended level contributes to increase survival rate and growth

performance (Liu et al., 2010). Previous studies on the role of OS in improving growth performances of juveniles sea cucumber (*Apostichopus japonicus*) (Wang et al., 2011) and allogynogenetic crucian carp (*Carassius auratus gibelio*) (Wang et al., 2007) stated that the OS from the diet gets incorporated with protein structure in tissues and then could interact with iodine to prevent abnormal hormonal metabolism (Burk and Hill, 1993; Foster and Sumar, 1997), resulting in higher growth performance. In contrast, the use of high levels of Se in the diet results in decrease in survival, growth and poor feeding efficiency in rainbow trout (*Salmo Gairdneri*) (Hilton et al., 1980). Thus, it is important to establish the appropriate levels Se supplementation in the diet, either inorganic or organic form.

Although, the research on the effects of both inorganic and organic Se supplementations in the diet of crustacean is limited, some attempts have been made on giant freshwater prawn (*Macrobrachium rosenbergii*) (Yeh et al., 2009; Chiu et al., 2010), Pacific white shrimp (*Penaeus vannamei*) (Sritunyalucksana et al., 2011a) and red swamp crawfish (*Procambarus clarkii*) (Dörr et al., 2013). However, the use of Se in the diet of juvenile red swamp crawfish showed no difference on the specific growth rate (Dörr et al., 2013). On the other hand, significant improvements have been found in the immune responses of Pacific white shrimp after supplementing their diet with organic Se (Sritunyalucksana et al., 2011a). Furthermore, the inclusion of Se in the diet has increased the antioxidant activity (Chiu et al., 2010) and improved disease resistance to *Debaryomyces handenii*, a known pathogen, in giant freshwater prawns.

2.9.3 Importance of Selenium in Antioxidant Activity

Se is an immunomodulator that has higher potent antioxidant ability than vitamins E, C A, and beta-carotene, but can be highly toxic (Baraboĭ and Shestakova, 2004). Since it is recognized as an important part of antioxidant enzymes, such as glutathione peroxidase (GPx), there has been an increased interest in the study of Se related antioxidant activity (Tapiero et al., 2003; Tinggi, 2008).

A large quantity of selenoprotein is recognized as cellular antioxidant enzymes (EI-Bayoumy, 2001). Antioxidants, mostly electron donors, suggest as a potential

Table 2.2: Application and the effects of dietary selenium on aquatic animals

Animals	Se level (in the diet µg g^{-1} or water µg L^{-1})	Selenium source	Exposure duration (day)	effects	References
Rainbow trout (*Salmo Gairdneri*)	13	Selenite	80	Mortality and growth	(Hilton et al., 1980)
Chinook salmon (*Oncorhynchus tshawytscha*)	18.2	SEM	90	Growth	(Hamilton et al., 1990)
Fathead minnow (*Pimephales promelas*)	20	Mix	56	Growth	(Ogle and Knight, 1989)
Striped bass (*Morone saxatilis)*	39	Fish	80	Mortality	(Coughlan and Velte, 1989)
Bluegill (*Lepomis macrochirus)*	13	SEM	260	Reproduction	(Woock et al., 1987)
Razorback sucker (*Xyrauchen texanus*)	472	Mix	90	Growth	(Hamilton et al., 2000)
Bonytail (*Gila elegans*)	236	Mix	90	Growth	(Hamilton et al., 2000)
Giant freshwater prawn (*Macrobrachium rosenbergii*)	0.5	Selenate SEM	75	Growth, Mortality, immunity and disease resistance	(Chiu et al., 2010)
African Catfish (*Clarias Gariepinus*)	0.1-0.5	OS	84	Growth and physiological responses	(Abdel-Tawwab et al., 2007)
Common Carp, (*Cyprinus carpio* L.)	0.03	OS	159	Growth and Survival	(Alina et al., 2009)
Red swamp crawfish (*Procambarus Clarkii*)	0.3	OS	50	Growth and Antioxidant enzymes	(Dörr et al., 2008)

indicator of health in marine organism due to their action, scavenging of free radicals and counteracting abilities with oxidative stress to produce innocuous end products such as water (Verlecar et al., 2008). Antioxidants also relate to the level of reactive oxygen species (ROS) and represent cellular mechanism defence to reduce cell damage. It has been established that in order to reduce cellular damage, levels of ROS and free radicals which are resulted from oxidative metabolism can be maintained by increasing the levels of enzymatic antioxidant system such as GST (glutathione-S-transferase) and GPx (Guemouri et al., 1991; Felton, 1995; Felton and Summers, 1995; Yeh et al., 2009).

GST; EC 2.5.1.18 is a multifunctional dimeric protein and an important component that is involved in the detoxification of a wide variety of toxic compounds by conjugating them to glutathione (George, 1994). GST catalyzes the nucleophilic addition of the thiol group of glutathione to form a glutathione-conjugate, then transformed to more water soluble for elimination or excretion (Al-Ghais and Ali, 1995; Gadagbui and James, 2000; Huang et al., 2008).

The activity of GST is affected by Se inclusion in the diet as proved in the study using giant freshwater prawn, *Macrobrachium rosenbergii* in which GST activity significantly improved after receiving Se in the diet (Chiu et al., 2010). Some GSTs also have glutathione peroxidase activity, thus providing an oxygen-detoxification function, for example reduction of lipid peroxides (LPO). This activity is particularly important in invertebrates because they are deficient in the other vertebrate-type selenium-dependent GPx.

The first activity of GPx (EC 1.11.1.9) was described by Mill, 1957 and hypothesized that it was related to oxidative hemolysis of red blood cells. GPx is a collective enzyme which protects the body of animals from harmful peroxide which are often produces from respiration process (Arthur, 2001). The main function of GPx as also known as glutatione peroxidase (GSH) is to catalyze a reaction that removes hydrogen peroxide via reduced glutathione. Hydrogen peroxides can be harmful to the body of animals as they can lead and increase free radicals that can damage cells (Arthur, 2001). GSH is also an important antioxidant molecule, which protects biomembranes and other cellular components from oxidative damage by

catalyzing the reduction of a variety of hydroperoxides (ROOH), using GSH as the reducing substrate during phagocytosis and/or physiological metabolism (Speier et al., 1985; Liu et al., 2004). Rotruck et al. (1973) described the general reaction for GSH, as shown below:

$$2GSH + H_2O_2 \rightarrow GSSG + 2H_2O$$

GSH is glutathione in the reduced form and GSSG is oxidized glutathione (Rotruck et al., 1973; Sunde, 1997). To maintain proper function of GSH activity, GSH must be collaborated with Se (Rotruck et al., 1973).

2.9.4 Sel-Plex® as a Source of Organic Selenium

Sel-Plex® is a commercial name of selenium yeast, a dried baker's yeast. It is manufactured by Alltech Inc., USA using yeast, *Saccharomyces cerevisiae* strain CNCM I-3060 which is cultivated in a selenium-enriched fermentation medium. The manufacturing process of Sel-Plex® is presented in Figure 2.6 (Burdock and Cousins, 2010).

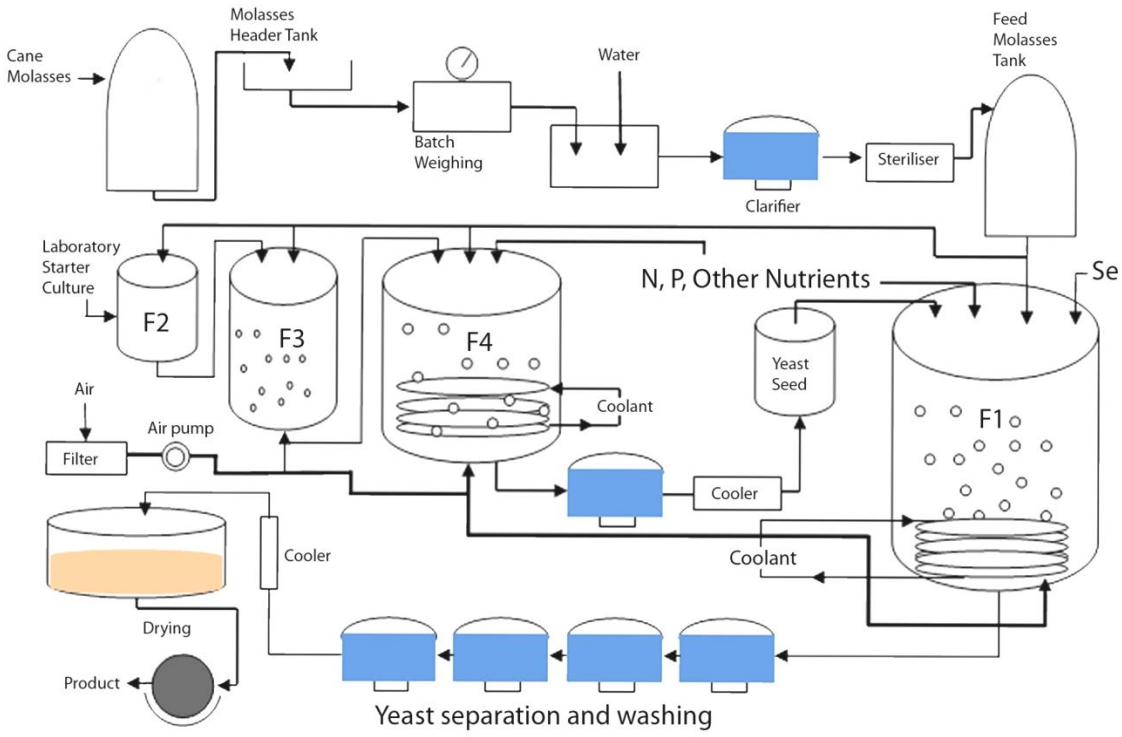

Figure 2.6: Schematic diagram of the Sel-plex® manufacturing process (Burdock and Cousins, 2010)

During the manufacturing process of Sel-Plex®, the IS is converted into organic form which is approximately 2000 mg kg^{-1} of total Se. There is no specific formula for this product which is composed of dried yeast containing selenomethionine (SeMet), selenocysteine (SeCys) and selenoprotein. According to a regression analysis, Sel-plex® is stable for 24 months at room temperature after the manufacturing date with SeMet content found to remains stable as well. The physical, chemical properties and specification of Sel-Plex® is provided in Table 2.3.

Table 2.3: Specification of Sel-Plex®

Analysis	Method	Specification	Average Batch analysis Results (n=5)
Physical aspect	N/A	Light to dark brown free-flowing	
Yeast strain	N/A	powder with a slight yeast aroma	
Loss on drying	2nd Directive 71/393/EC	*Saccharomyces cerevisiae* strain	5.53 ± 0.35
pH	-	CNCM I-3060	6.39 ± 0.18
Ash (total %)	NF V 18-101	Not more than 7.0%	6.55 ± 0.9
Crude protein	NF V 18-100		46.66 ± 0.93
Mesophilic aerobic microorganism	NF V 18-051	- Maximum of 8%	2.5×10^4 cfu g^{-1}
Coliforms	NF V 18-050	Minimum of 45%	<5 cfu g^{-1}
Salmonella (search on 25 g)	NF V 18-052	Less than 0.5×10^4 cfu g^{-1} Less than 10 cfu g^{-1} Negative	Negative
Heavy metals (mg kg^{-1})			
Arsenic	AAS	<3 mg Kg^{-1}	0.79 ±0.08
Lead	AAS	<1 mg Kg^{-1}	0.07 ± 0.03
Cadmium	AAS	<3 mg Kg^{-1}	0.022 ± 0.005
Mercury	AAS	<0.5 mg Kg^{-1}	<0.005
Total Selenium (mg kg^{-1})		Not less than 2000 mg Kg^{-1}	2,275 ± 148.5
Total organic selenium	ICP-MS	≥ 98%	99.6 – 99.63%
Selenomethionine	ICP-MS	65% (62-68%)	63.4 – 66.6%
Other organic selenium Compound	ICP-MS	33% (32-38%)	33 – 36.2 %
Inorganic selenium as Se(IV)	ICP-MS	≤ 2%	0.37 – 0.4 %
Inorganic selenium as Se(VI)	ICP-MS	≤ 2% (Combined with Se(IV))	Below detection limit

AAS = atomic absorption spectrometry; CFU colony forming unit; ICP-MS = inductively coupled plasma mass spectrometry; N/A = not applicable; mg g^{-1} = milligram per gram

2.10 Approaches to Evaluate Physiological Status of Marron

2.10.1 Growth Indices

Weight gains, relative growth rate (RGR) and specific growth rate (SGR) are the simplest parameters to evaluate the growth indices of experimental animals over the trial periods (Busacher et al., 1990). Growth is an important final indicator to assess crustacean health that is the final resultant of of all biotic and abiotic variables acting on aquatic animals (Fotedar, 1998). Previous experiments have conducted in aquaria to intensive crayfish culture system, recorded growth of marron is between 0.5 to 1.1% day-^{1} (Morrissy, 1976, 1979, 1990; Morrissy et al., 1990; Jussila and Evans, 1996; Fotedar et al., 1997; Morrissy, 2002; Fotedar, 2004). Besides RGR and SGR, harvest biomass is also used as a tool to evaluate the effectiveness of the diets by measuring survival of marron (Fotedar, 1998; Verhoef and Austin, 1999).

2.10.2. Physiological Indicator

Hepatosomatic indices and tail muscle indices

Hepatopancreas of crayfish is the main energy reserve organ that can be used as an indicator for chronic stress due to changes in relat it shows in its water content and dry mass (Holdich and Reeve, 1988; Haefner and Spaargaren, 1993). The change in relative weights, both wet and dry weights of of hepatopancreas is known as hepatosomatic indices that have been established as a tool to evaluate physiological condition and the effect of stressor in crustacean (Haefner and Spaargaren, 1993; Lignot et al., 2000; Sang and Fotedar, 2004; Prangnell and Fotedar, 2006). The hepatosomatic indices can be affected by nutrient status and developmental stage and are expressed as the ratio of wet and dry hepatopancreas weight to whole body weight (Hernández-Vergara et al., 2003). Moreover, Hepatosomatic indices together with moisture content of hepatopancreas are used to describe the condition of crustacean and to estimate thir growth rates under difference feeding regimes and treatments (Cabib et al., 1982; Cockcroft, 1987; Jussila and Mannonen, 1997; Musgrove, 1997).

Tail muscles to body weight ratios and tail moisture levels

Besides hepatopancreas, tail muscles of crustacean acts as a energy storage. The tail muscles to are considered as storage site of energy reserves, and tail muscles to body weight ratio and tail moisture level can be used as condition indices (Fotedar, 1998).

Starvation increased the whole body moisture content of redclaw crayfish (*Cherax quadricarinatus*) juveniles. The water content in the crayfish deceased when the feeding rate is increased and during starvation they catabolized tissue protein to meet their metabolic requirements. Tail moisture content of western king prawn (*Penaeus latisulcatus*) decreases with the increase in rearing salinity (Sang and Fotedar, 2004).

2.10.3 Immunological Indicators

Total haemocyte count (THC)

Immune performance of crustacean can be assessed using a common indicator such as THC. Nutrition status, rearing condition and the occurrence of pathogen have been shown to affect marron THC as shown in Table 2.4. The changes in the THC relates to stress conditions and could be used to evaluate physiological performance, health and immune status in crustacean including marron (Durliat, 1985; Smith et al., 1995; Jussila and Evans, 1997; Evans et al., 1999; Jussila et al., 1999). Higher THC was observed in marron fed beta-1,3-glucan or mannan oligosaccharide (MOS) dietary supplementation (Sang et al., 2009; Sang and Fotedar, 2010a). Meanwhile, lower THC was reported in marron reared in intensive culture system than in earthen ponds and in communal tanks (Evans et al., 1999; Jussila et al., 1999). Pathogen infection such as *Vibrio mimicus* was also resulted to cause a decrease in marron THC (Sang et al., 2009).

Table 2.4: THC of marron in different nutrient status, rearing condition and pathogen infection

Factors	Marron THC (x 10^6 cells mL^{-1})	References
Nutrition		
Beta 1, 3-Glucan	5.5 – 17.6	(Sang and Fotedar, 2010a)
MOS	1.4 – 4.5	(Sang et al., 2011a)
Rearing condition		
Farmed and wild population	2.3 - 5.3	(Evans et al., 1992)
Semi intensive farm pond	6.5 - 8.5	(Jussila, 1997b)
Intensive crayfish culture system	2.3 – 5.3	(Jussila, 1997b)
Farmed and acclimated for 30 days in aquarium	4.3	(Jussila et al., 1999)
Communal tanks	9.7 - 10.7	(Jussila, 1997b)
***Vibrio* spp infection**		
24 h post infection	3.6 – 4.5	(Sang et al., 2009)
96 h post infection	2.9 - 3.9	(Sang et al., 2009)

Differential haemocyte count (DHC)

Proportion of haemocytes cells in the haemolymph known as differential haemocytes count (DHC) has been used to evaluate immune response of crustacean (Jussila et al., 1997; Fotedar et al., 2001; Fotedar et al., 2006). The proportion of different haemocytes is affected by several factors such as environmental stressor (Fotedar et al., 2001), animal conditions, sex (Sequeira et al., 1995), dietary supplementation (Sang and Fotedar, 2010a; Sang et al., 2011a), holding duration (Fotedar et al., 2006) and disturbance (Jussila et al., 2001). Jussila et al (1997) reported that the proportion of granular cells in western rock lobster (*Panulirus cygnus*) ranged from 5.1% – 13.1% whereas Fotedar et al (2006) found 11.4%. However, semigranular haemocytes were 51.1% - 62.9% and hyaline cells were 29.1% – 37% of the THC of the western rock lobster (Jussila et al., 1997). In comparison, Sang et al. (2011b) stated the proportion of semigranular were 3.49% and hyaline cells were 90.44% in yabbies (*Cherax destructor*).

Vibrio rank and bacteraemia

Both *V*ibrio rank and bacteraemia have been used as an health indicator, stress status and immune response of crustacean, as bacteria can be found in marron haemolymph (Fotedar et al., 2001; Fotedar et al., 2006; Sang and Fotedar, 2010a; Sang et al., 2011a). Marron with Low *Vibrio* rank or less number of bacteraemia indicates healthier than either high *V*ibrio rank or the number of bacteraemia (Sang and Fotedar, 2010a; Sang et al., 2011a).

Neutral red retention time (NRRT)

NRRT have been previously used as a tool to assess immune performance of European lobster (*Homarus gammarus)* (Hauton and Smith, 2004), Chinese shrimp *Fenneropenaeus chinensis* (Yao et al., 2008), blacklip abalone (*Haliotis rubra*) (Song et al., 2007) and marron (Sang et al., 2009). Neutral red, 3-amino-7-dimethylamino-2-methylphenazine hydrochloride), is a week cytotoxic that is used as a biomarker to assess either survival or cell viability and structural integrity of lysosomal membranes (DeRenzis and Schechtman, 1973; Borenfreund and Puerner, 1985; Weyermann et al., 2005). Lysosomes, released by degranulation process of haemocytes during immune response, can be found both in the semigranular and granular cells of invertebrates (Ratcliffe et al., 1985; Sung and Sun, 1999). Once lysosomal membranes are damaged, proteolytic enzymes are released and neutral red colour disappears. Hence, the time that neutral red retained in the lysosome could be used as a marker of immunological and stress status (Lowe and Pipe, 1994).

2.10.4 Biochemical Status

Total Se retention

As an important trace element, the availability of Se in the body of animal is an important immune indicator. Se can be accumulated in the haemolymph, hepatopancreas and muscle tissues of majoriry of aquatic animals. Study with adult crayfish (*Procambarus clarkii*) revealed that Se-treated crayfish had higher Se retention in their hepatopancreas than control group. Se retention in the hepatopancreas showed positive correlation with the antioxidant enzyme activity (Dörr et al., 2008). In contrast, low retention of Se in the body of rainbow trout (*Salmo gairdneri*) and Atlantic salmon parr (*Salmo salar*) reduced the activity of antioxidant system (Hilton et al., 1980; Bell et al., 1987). The high Se treatment in

animal can also be harmful, as high Se accumulation in the body shows toxic effects and results in high mortality.

Antioxidant status

Most organism have a mechanism to protect against the effects of oxidative stress by using detoxifying enzymes such as GST and GPx (Bhargavan, 2008). Both GST and GPx can be antioxidant agent to reduce LPO level in the haemolymph of marron. Thus, GST, GPx and LPO level in the haemolymph can be used as a tool to measure oxidative stress and health of animals.

LPO has been known as a mechanism of cellular injury both in vertebrate and invertebrate. Malonaldehyde (MDA) is a naturally occurring product of lipid peroxidation and prostaglandin biosynthesis, which is mutagenic and carcinogenic. It reacts with DNA to form DNA adducts (Marnett, 1999). Therefore, measurement of MDA is widely used as an indicator of lipid peroxidation.

CHAPTER 3: General Materials and Methods

This chapter describes general materials and methods that are used in this research. The specific materials and methods are described in individual chapters.

3.1 Basal and Test Diet Preparation

All ingredients of the basal and test diets except OS, were supplied by Specialty Feeds Pty. Ltd, Great Eastern Highy way, Western Australia. The source of OS was from Sel-Plex®, which was donated by Alltech Inc. USA. The basal diet was formulated (Table 3.1) using Feed LIVE software version 1.52 from Live Informatics Company Limited, Thailand. The basal diet pellets of 0.5 mm diameter and 1 mm to 3 mm length were prepared by mixing all ingredients with Sel-Plex® at the selected levels for the specific trial. One kg of Sel-Plex® approximately contains 2 g of OS, represented mainly by selenomethionine (Burdock and Cousins, 2010). All diets were pelletized using a mincer and then dried under direct sunlight. Dried pellets were then allowed to cool at room temperature, packed and stored in a dark room before being used.

3.2. Culture System

All experiments, except one described in Chapter 9, were carried out in Curtin Aquatic Research Laboratory (CARL), Technology Park, Curtin University, Western Australia. Plastic cylindrical culture tanks (800 mm diameter, 500 mm high, 250 L capacity, 70 L of freshwater per tank) were used for laboratory experiments. Freshwater in each tank was continuously filtrated using fluval 205 filters (Hagen, USA) at a rate of approximately 2 L min^{-1}. Each tank was provided with aeration and PVC pipes (55 mm diameter, 150 mm length) to provide shelter for each marron. To maintain a constant temperature of 20^{o}C in the culture tanks, individual automatic heaters (Sonpar®, Model: HA-100, China) were used in each tank.

Table 3.1: Composition of basal diet (g kg^{-1}) used for the marron culture

Ingredient	Content (g kg^{-1})
Fish oil[1]	32
Wheat bran	545.59
Soybean meal	101.5
Fish meal[2]	257.14
Calcium carbonate	0.2
Ascorbic acid	0.5
Betaine[3]	12
Premix[4]	1.5
Cholesterol	2.5
Wheat starch	47.08

All ingredients were supplied by Specialty Feeds Pty Ltd, WA, Australia.
[1]Cod liver oil, [2]Peruvian fishmeal, 56% CP. [3]Betaine Anhydrous 97%.
[4]Commercial vitamin and mineral premix for trout

3.2 Growth Indices

For measuring marron weights, an electronic balance (GX-4000, A&D Company, Ltd., Japan) was used. Both initial and final weights of marron were used to calculate average weekly gains (AWG), daily weight gains (DWG) relative growth rates (RGR) and specific growth rates (SGR). The AWG, DWG and RGR were calculated as follows:

$$\text{AWG (g week}^{-1}) = (Wt - Wo)/wk$$

$$\text{DWG (g day}^{-1}) = \frac{Wt - Wo}{days}$$

$$\text{RGR (\%)} = \frac{Wt - Wo}{Wo} \times 100\%$$

Where Wf is the final weight (g) and Wo (g) is the weights of the marron at the commencement of the growth period, respectively, and wk is the number of weeks during the growth period.

Specific growth rate (SGR % g day-^{1}) was calculated using the following equation (Hopkins, 1992):

$$SGR = 100 \times (\ln(Wt) - \ln(Wo))/d$$

Where Wt and Wo are the weights of the marron at current time (t) and at the commencement of the experiment (0) respectively, d = culture period (days).

3.4 Survival

Survival was calculated using the following equation:

Survival (S) =100 x (n_t/n_0)

Where: S is the survival; n_t is the number of marron at time t and n_o is the initial number of marron at the commencement of the experiment.

3.3 Physiological Parameters

To measure physiological parameters, all hepatopancreatic lobes and complete mass of muscle tissues from marron abdomen were used and weighed to determine organosomatic indices (Fotedar, 1998). The percentage moisture of hepatopancreas (HM %) and tail (TM %), dry hepatopancreas index (H_{id}), wet hepatosomatic index (H_{iw}), dry tail muscles to wet body weight ratios (T/B_d), and tail muscles to wet body weight ratios (T/B_w) were calculated using the following equations as previously described by Jussila and Mannonen (1997); Fotedar (1998, 2004):

$HM\% = (WH_{wet} - WH_{dry}) \times 100/WH_{wet}$

$TM\% = (WT_{wet} - WT_{dry}) \times 100/WT_{wet}$

$H_{id} = WH_{dry} \times 100/W_t$

$H_{iw} = WH_{wet} \times 100/W_t$

$T/B_d = WT_{dry} \times 100/W_t$

$T/B_w = WT_{wet} \times 100/W_t$

Where, WHwet = weight of wet hepatopancreas (g); WH_{dry} = weight of dry hepatopancreas (g); WT_{wet} = weight of wet tail muscles (g) WT_{dry} = weight of dry tail muscles (g) and W_t = total weight of marron (g)

3.4 Immunological Indices

To provide haemolymph from individual marron, haemolymph was withdrawn from the base of the fifth thoracic leg into a 23-gauge needle containing 0.2 mL solution of 1% glutaraldehyde in 0.2 M sodium cacodylate and dispensed into an Eppendorf

tube.(Fotedar et al., 2001). This mixture was used to calculate total and differential haemocyte count.

Total haemocytes count (THC) of marron was calculated using previous method described by Fotedar *et al*. (2001) with some modifications. Total haemocytes were counted in a haemocytometer (Neubauer, Munich, Germany) under 100× magnification. Haemocytes were counted in both grids, and the mean was used as the THC. The total haemocyte value was calculated as THC using the following equation: THC = (cells counted × dilution factor × 1000)/grid volume (0.1 mm^3).

To calculate DHC, one drop of the mixture of glutaraldehyde in sodium cacodylate and haemolymph were smeared onto a glass microscope slide. It was then air dried and fixed in 70% methanol for 10 min. The fixed smears were stained with routine May-Grunwald and Giemsa stains for 10 min each and then mounted with coverslips (Bancroft and Stevens, 1977). The number and percentages of three major marron haemocyte types for each individual marron were counted using a minimum number of 200 cells on each slide. The DHC was then calculated by using the following equation:

$$\text{DHC} = \frac{\text{Number of different haemocytes cell type}}{\text{Total haemocytes cells counted}} \times 100$$

3.5 Total Soluble Selenium Determination

Total soluble Se was determined using a spectrophotometric method according to Revanasiddappa and Dayananda (2006). Briefly, 4 mL conc. HNO_3 was added to all samples and heated at 80°C for 1 h, then another 3 mL HNO_3 was added to the solution. Heating was continued for an additional 3 h until the samples were completely mineralised. The solution was then cooled and diluted to 10 mL with distilled water. Next, 1 mL of the resulting solution was transferred into a test tube and 1 mL concentrated HCl was added to reduce Se^{6+} to Se^{4+}. This solution was then heated at 100°C for 10 min in a thermostat bath and diluted to 10 mL with a 2% HCl solution after it had cooled to room temperature. The final solution was used to analyse total soluble Se levels.

To measure total soluble Se, 1 mL final solution was transferred into a 10 mL flask, and 1 mL 1% potassium iodide and 0.5 mL 1 M HCl were then added, and the mixture was shaken. Next, 0.5 mL 0.05% Leuco malachite green (LMG) was added and shaken gently. After 2 min, 3 mL of acetate buffer (pH 4.5) was added, and the reaction mixture was kept in a water bath at 40°C for 3 min. The solution was then cooled to room temperature and diluted to 10 mL with distilled water. The final 10 mL solution was mixed well and allowed to stand for 20 min before absorbance was measured at 615 nm against the blank reagent. The concentration of the total soluble Se was established by reference to the calibration graph, which was generated with nine levels of standard solution (0.04, 0.08, 0.12, 0.16, 0.24, 0.28, 0.32, 0.36, and 0.4 µg g^{-1} sodium selenate). Each standard solution dilution was determined using 0.05% LMG and the spectrophotometric method as described above.

CHAPTER 4: Growth, Survival and Physiological Condition of Cultured Marron, *Cherax cainii* (Austin, 2002) Fed Different Levels of Organic Selenium

4.1 Introduction

Selenium (Se) is an important trace element required for growth, survival and immune function in rainbow trout (*Oncorhynchus mykiss*) (Vidal et al., 2005; Kucukbay et al., 2009), Atlantic salmon (*Salmo salar*) (Lorentzen et al., 1994), juvenile grouper (*Epinephelus malabaricus*) (Lin and Shiau, 2005), crucian carp (*Carassius auratus gibelio*) (Wang et al., 2007; Zhou et al., 2009), prawn (*Macrobrachium rosenbergii*) (Chiu et al., 2010) and coho salmon (*Oncorhynchus kisutch*) (Felton et al., 1996). Se has also been used to increase the total protein content in African catfish (*Clarias gariepinus*) (Abdel-Tawwab et al., 2007) and augment pancreatic activities of amylase, trypsin, and lipase that are closely related to the growth performance (Zhan et al., 2010).

In nature, Se can be categorized into two forms, inorganic and organic Se (OS) (Swanson et al., 1991). Inorganic Se is found as selenate while OS is present in Se containing amino acid such as selenomethionine (Barceloux, 1999; Zhan et al., 2010). OS from yeast can be retained and absorbed at a higher rate, is less toxic and has relatively higher bioavailability than inorganic Se (Mahan and Parrett, 1996; Schrauzer, 2003; Taylor et al., 2005; Wang et al., 2007; Kucukbay et al., 2009). Hilton et al. (1980) and DeForest et al. (1999) suggested that a detary Se limit is 11 mg kg^{-1} for coldwater fish and 10 mg kg^{-1} for warmwater. However, there is no information available on the effects of dietary of OS in the health performance of marron *Cherax cainii* (Austin, 2002).

OS, for instance Sel-Plex®, is an important trace element that has been used as a food supplement in the diet of aquatic animals such as shrimp (Sritunyalucksana et al., 2011b) and hybrid striped bass (Cotter et al., 2008). Sel-Plex® is in a form of dried cellular product from yeast containing at least 98% of OS in which 62-68% are selenomethionine (Burdock and Cousins, 2010). As Se-containing amino acid, selenomethionine can incorporate into structural proteins and acts as a biological reserve in the body of animals (Suzuki and Ogra, 2002). Selenomethionine then enters into the selenide pool and is used for selenoprotein synthesis and can then also

be incorporated directly and non-specifically into protein through sulphur metabolism of methionine (Susan et al., 2010; Zhan et al., 2010). The evidence from past research has shown that selenomethionine interacts with iodine to prevent abnormal hormonal metabolism (Burk and Hill, 1993; Foster and Sumar, 1997). Selenomethionine can also be deposited and retained as selenoprotein in the muscle and hepatopancreatic or liver tissues of animals for approximately three years where it can be extensively utilized and re-utilized to maintain Se levels in the body (Aguilar et al., 2009). Selenomethionine in the body of animals has been identified as a trace element that can trigger and promote the activation of antioxidant enzymes such as Glutathione reduced (GSH), Superoxide dismutase (SOD) and catalase (Zhan et al., 2010; Han et al., 2011) which play an important role in enhancing growth performance and survival, boosting immune activities and disease resistance, increasing digestive enzymes activities such as amylase and protease (Chiu et al., 2010; Smitha and Rao, 2010; Han et al., 2011).

In this study, Sel-Plex® as a source of OS is also known as selenoyeast that contains selenoprotein. It is a dried product (non-viable) baker's yeast, derived from the *Saccharomyces cerevisiae* strain CNCM I-3060, cultivated in a Se-enriched fermentation medium to provide a high level of selenomethionine (Burdock and Cousins, 2010). Selenomethionine may be incorporated into proteins in place of methionine or be metabolized to Selenocysteine, which can be catabolized into hydrogen selenide (H_2Se) by a P-lyase enzyme (Esaki et al., 1982; Schrauzer, 2000; Combs Jr et al., 2001). In contrast to selenomethionine, inorganic Se such as selenate (Na_2Se0_4) and selenite (Na_2Se0_3) is metabolized to H_2Se through reduction and subsequently incorporated into selenoproteins including GSH-PX, iodothyronine 5'-deiodinases (TDI), thioredoxin reductases (TR), selenophosphate synthetase (SePsyn), plasma Selenoprotein P (Se-P), and muscle selenoprotein W (Se-W) (Combs Jr et al., 2001). Some of these enzymes are related to antioxidant activity such as GSH-Px (Diwadkar-Navsariwala and Diamond, 2004; Ebert et al., 2006).

The marron is one of the ecologically and commercially important native freshwater crayfish species in Western Australia (Lawrence and Morrissy, 2000; Lawrence and Jones, 2002). It is known as the third largest freshwater crayfish in the world, an indigenous species in the southwest of Western Australia and potential to be farming.

As a result of the interest in marron farming, the need for knowledge in the area of marron nutrition and the use of supplemented microelement to improve productivity has also increased significantly.

To evaluate health and productivity of marron, there are some tools that can be used to determine performance of marron. Previous research stated that organosomatic indices such as hepatosomatic indices were important tools to evaluate the health status of marron (Jussila and Mannonen, 1997). Further, the positive physiological performance can finally be manifested by high growth and survival rates of marron (Wang et al., 2007; Sang et al., 2011b). Furthermore, growth performance and health of marron can also be assessed using various physiological tools such as, daily weight gain (DWG), relative growth rate (RGR), specific growth rate (SGR), biomass increment, survival rate, the digestive enzyme activity, total haemocytes count (THC), differential haemocytes count (DHC) and bacteraemia (Fotedar et al., 2006; Wang and Zirong, 2006; Wang, 2007; Sang et al., 2009; Sang et al., 2011a).

However, there is no information available on the growth and health performance of cultured marron fed OS. Thus, this experiment was aimed to investigate the effects of OS sourced from Sel-Plex® on the growth performances, survival, hepatosomatic indices, haematological indices, the number of bacteraemia in the haemolymph, digestive enzyme activity (amylase and protease), total soluble protein of digestive tract and total Se accumulation in the hepatopancreas and the tail muscle tissues of laboratory-reared marron.

4.2 Materials and Methods

4.2.1 Test diets preparation

A basal diet mixture was formulated and prepared referring to the methods that described on chapter 3. To the basal diet, 0.1; 0.2 or 0.3 g kg^{-1} of Sel-Plex® was added to obtain the test diets. Each test diet was prepared using the same method of basal diet. Actual Se levels in the diet were measured based on the spectrophotometry methods (Revanasiddappa and Dayananda, 2006).

4.2.2 Experimental design

The experiment was conducted in sixteen blue plastic cylindrical tanks with seven PVC pipes (55 mm diameter, 150 mm length) to provide shelter for each marron. Marron of mean initial body weight of 39.43 ± 0.55 g, (n=112) supplied by Aquatic Resource Management Pty Ltd, Western Australia were used for the 90 days of the experiment. After transportation and placing in the blue cylindrical tanks, marron were acclimated to the culture conditions for one week.

During the acclimation period, the marron were fed the basal diet at a rate of 3% of body weight every second day. The marron were then randomly distributed to sixteen culture tanks at a density of seven marron per tank. Randomized blocks of four tanks were used as an experimental design, with each block fed one of the test diets so that each diet was represented by four replicates. The marron from each tank were fed the test diet at a rate of 3% of body every second day. This feeding rate was determined by previous experiments (Sang et al., 2009). Uneaten food and faeces were siphoned out before feeding and sufficient freshwater was added to maintain 70 L of water in each tank.

4.2.3 Data collection

Temperature, pH and dissolved oxygen in the water were recorded weekly using a Cyberscan pH 300, Eutech Instruments, Singapore. Nitrate, nitrite and ammonium were monitored and recorded weekly using chemical test kits (Aquarium Pharmaceuticals^{TM}, Inc., USA). Nitrate and nitrite level were monitored to not exceed than 0.1 mg L^{-1} whereas total ammonium was maintained lower than 0.2 mg L^{-1} to provide optimum water quality for cultured marron (Jussila, 1997b).

Growth and Survival

The marron were counted everyday to calculate the survival. Meanwhile, the weight of each marron from each tank was measured at the day 0 and 90 of the feeding trial to calculate the DWG, RGR and SGR.

Moisture contents and hepatosomatic indices

To measure moisture levels and hepatosomatic indices, four marron from each treatment group were dissected at the end of the feeding trial. The moisture content and hepatosomatic indices were measured following equations as previously described by Jussila (1997a).

Total and Differential haemocytes count (THC and DHC)

To determine THC and DHC, four marron from each treatment group were used. The THC and DHC of the marron were counted on the day 0, 45 and 90 of feeding trial. The THC of the marron was determined using procedure as described in chapter 3.

Bacteraemia

Haemolymph from four marron from each treatment was used to evaluate the bacteraemia levels. A-0.05 mL of haemolymph from each marron was withdrawn from the base of the fifth thoracic leg into sterile syringe using 23-gauge needle, placed and smeared on a nutrient agar plate. The agar plate was then inverted and put into incubator at temperature 25^{o}C for 24 h. Colony forming units (cfu) were determined for each plate as cfu mL^{-1} and counted on the basis of a total volume of 0.05 mL plate^{-1} (Fotedar et al., 2001; Sang et al., 2009).

Digestive enzyme activity and total soluble proteins

In order to evaluate the effects of Sel-Plex® on the activities of digestive enzymes (amylase and protease), hepatopancrease and digestive tract from one marron from each culture tank were sampled at the end of experiment, and crude amylase and protease were extracted (Sang et al., 2011a). The hepatopancreas and digestive tract of each marron were homogenised and placed in ice in 5 mL 0.1 M citrate phosphate buffer (pH 5.5). The resulting homogenates were then centrifuged at 5000 rpm for 5 min using Eppendorf centrifuge 5804 R (Eppendorf Inc., Hamburg, Germany), and the supernatants (solution) were used directly for enzyme assays.

Amylase activity was assessed following the methods proposed by Bernfeld (1955) and Biesiot and Capuzzo (1990). Amylase activity in solution was assayed on the basis of maltose liberated per mg protein per hour using soluble starch (15 mg mL^{-1}) as substrate in 0.1 M phosphate buffer (pH 6.5) with 0.05 M NaCl at 37°C.

Meanwhile, protease activity was measured using Azocoll (10 mg mL^{-1}) in 0.1 M citrate phosphate buffer (pH 5.5) at 37°C and determined as the increase in absorbance unit (A_{520}) per mg protein per hour (Biesiot and Capuzzo, 1990). The protein content in solution was assayed using the Folin phenol method, and bovine serum albumin was used as a standard for protein determination (Hartree, 1972).

Determination of Se

At the day 90 of the culture period, four marron from each treatment group, one from each tank, were sacrificed to determine total Se concentrations in hepatopancreas and tail muscle tissues. Total Se was determined by spectrophotometric method according to the procedure described by Revanasiddappa and Dayananda (2006).

4.2.4 Statistical analysis

Statistical analysis was performed using SPSS software version 17. All data were expressed as mean ± SE (standard error). The data of growth, survival, hepatosomatic indices, total Se in the diet and marron tissues (hepatopancreas and muscle) were subjected to one way ANOVA followed by Tukey's *post hoc* to evaluate significant differences among the group of organic Se supplementation. Percent data of survival was transformed to arcsine values before statistical analyses. All significant tests were at $P<0.05$ levels.

4.3 Results

4.3.1 Water quality parameters

During the feeding trial, the temperature, pH and dissolved oxygen were found within accepted optimum ranges of 20-23^{o}C, 7.16-7.48, and 5.55-5.81 mg L^{-1} respectively, over the experimental period (Morrissy, 1990; Rouse and Kartamulia, 1992; Sang and Fotedar, 2010a).

4.3.2 Selenium levels in the diet

The actual levels of Se in the diet showed significantly differences ($P<0.05$) among any levels of the Sel-Plex® supplements in the diet (Figure 4.1).

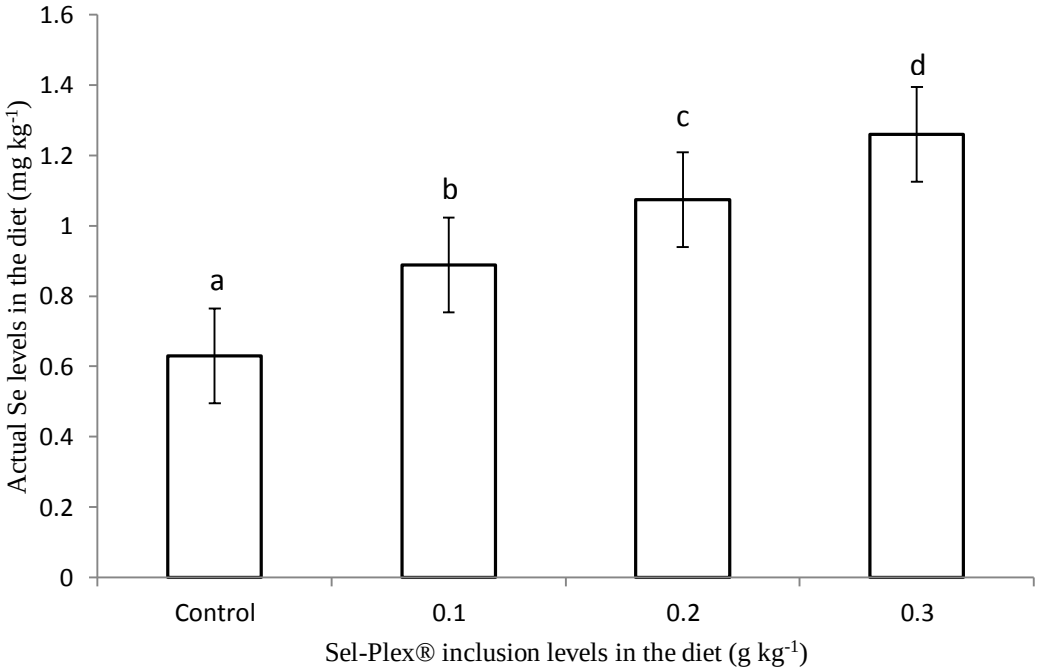

Figure 4. 1: Actual selenium levels in g kg^{-1} (mean ± S.E) in the diet after Sel-Plex® inclusion. Different letters indicate significantly different (*P<0.05*) concentration.

4.3.3 Growth and Survival

After 90 days of feeding trial, the inclusion Sel-Plex® in the diet higher than 0.1 g kg^{-1} improved final weight, RGR and SGR of marron. Meanwhile, the highest DWG was achieved when the marron were fed 0.3 g kg^{-1} Sel-Plex® in the diet (Table 4.1). Any levels of OS supplemented diet also resulted in higher (*P<0.05*) survival rates of the marron. The highest marron survival was achieved at level 0.2 g kg^{-1} Sel-Plex® supplemented diet (Figure 4.2).

Table 4.1: Growth indices of marron fed different levels Sel-Plex® (g kg^{-1}) in the diets for 90 days of feeding trial.

Parameters	Control	0.1	0.2	0.3
Initial weight (g)	39.48 ± 0.27^{a}	39.54 ± 0.16^{a}	39.48 ± 0.14^{a}	39.21 ± 0.32^{a}
Final weight (g)	40.21 ± 0.23^{a}	42.29 ± 0.56^{ab}	44.67 ± 0.85^{b}	44.70 ± 0.67^{b}
DWG (g day^{-1})	0.0080 ± 0.0028^{a}	0.0299 ± 0.0055^{a}	0.0576 ± 0.0106^{ab}	0.0669 ± 0.0091^{b}
RGR	1.89 ± 0.65^{a}	4.72 ± 0.57^{a}	13.71 ± 1.14^{b}	16.69 ± 2.75^{b}
SGR (%)	0.023 ± 0.004^{a}	0.052 ± 0.005^{a}	0.139 ± 0.008^{b}	0.163 ± 0.024^{b}

Different superscripts alphabet in the same row indicate significantly different (*P<0.05*). Sel-Plex® (Alltech Inc. USA) was used as a source of organic selenium (OS).

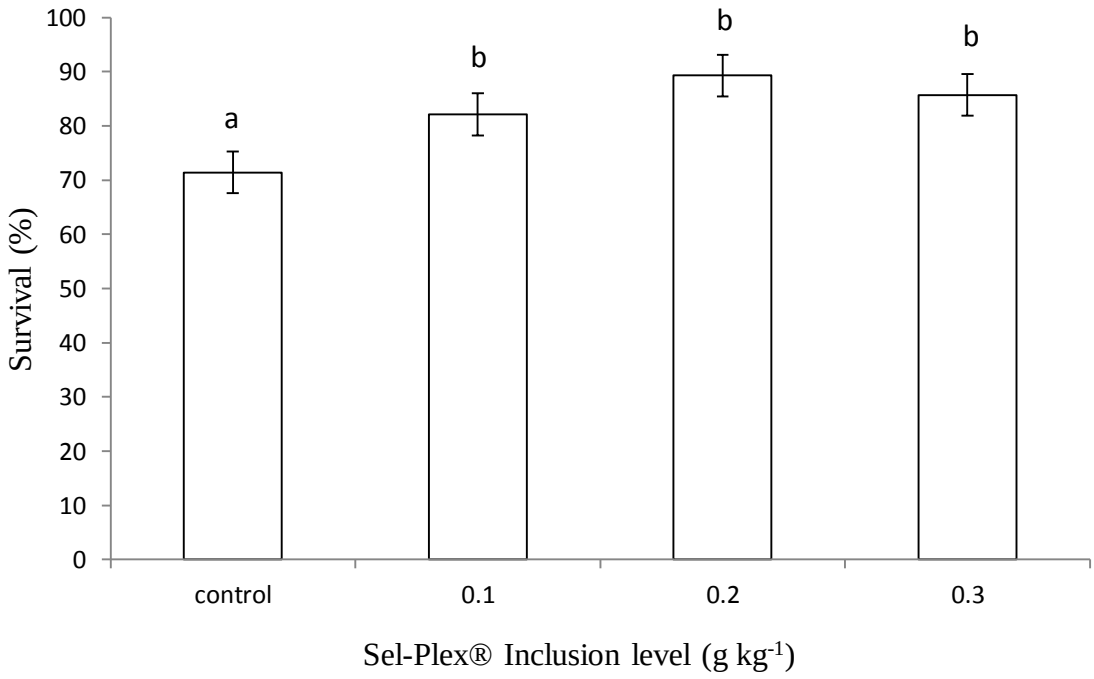

Figure 4.2: Survival of marron fed different levels of Sel-Plex® for 90 days. Different letters indicate significantly different (*P<0.05*).

4.3.4 Moisture and hepatosomatic indices

After 90 days of feeding, HM% of the marron fed 0.2 g kg^{-1} Sel-Plex® was significantly lower (*P<0.05*) than the marron fed the control diet. The inclusion of 0.3 g kg^{-1} Sel-Plex® in the diet resulted in significantly higher Hiw (*P<0.05*) while the Hid of marron fed more than 0.1 g kg^{-1} of Sel-Plex® was significantly higher (*P<0.05*) than the control (Figure 4.3).

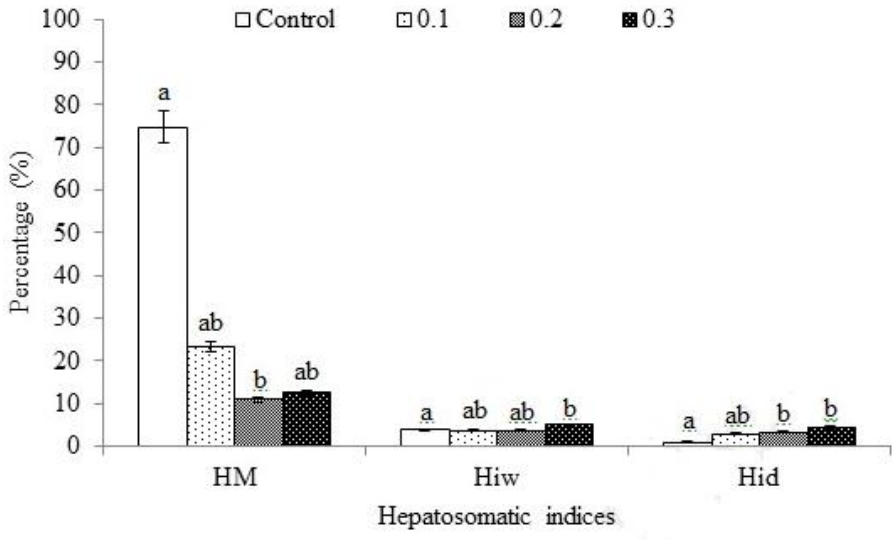

Figure 4.3: The mean values of hepatosomatic indices of marron fed different levels of Sel-Plex® (g kg^{-1}) for 90 days. Different alphabet (a, b) above the bars at the same parameters denote significant differences at P<0.05 in mean values. HM %= the percentage of hepatopancreas moisture, Hiw % = the percentage of wet hepatosomatic indices, Hid % = the percentage of dry hepatosomatic indices.

At the end of the experiment, the mean THC and the proportions of granular cells of the marron fed Sel-Plex® more than 0.1 g kg^{-1} were significantly higher (*P<0.05*) than the control and the highest THCs and granular cells were found in marron fed 0.2 g kg^{-1} Sel-Plex®. Meanwhile, semigranular cells were not affected by any dietary Sel-Plex® levels and the percentage of hyaline cells were significantly lower (*P<0.05*) when marron were fed 0.2 g kg^{-1} of Sel-Plex® (Table 4.2).

Table 4.2: The immune responses (means ± SE) of marron fed different levels of Sel-Plex® inclusion in the diets for 90 days.

Parameters	Day	Sel-Plex® inclusion level (g kg^{-1})			
		Control	0.1	0.2	0.3
THC (x10^6 cells mL^{-1})	0	$_{1}1.75\pm0.20^{a}$	$_{1}1.67\pm0.26^{a}$	$_{1}1.48\pm0.05^{a}$	$_{1}1.52\pm0.14^{a}$
	45	$_{1}1.87\pm0.08^{a}$	$_{1}1.62\pm0.20^{a}$	$_{1}1.69\pm0.25^{a}$	$_{1}2.12\pm0.24^{a}$
	90	$_{2}1.30\pm0.07^{a}$	$_{2}3.31\pm0.21^{b}$	$_{2}3.53\pm0.09^{b}$	$_{2}2.50\pm0.10^{c}$
Granular (%)	0	$_{1}42.00\pm6.64^{a}$	$_{1}20.50\pm5.89^{ab}$	$_{1}56.75\pm4.04^{bc}$	$_{1}60.75\pm5.31^{c}$
	45	$_{1}35.50\pm7.81^{a}$	$_{1}57.50\pm9.35^{ab}$	$_{1}66.75\pm3.94^{bc}$	$_{1}40.00\pm9.30^{c}$
	90	$_{2}31.25\pm5.35^{a}$	$_{2}49.50\pm4.50^{ab}$	$_{2}63.25\pm3.35^{bc}$	$_{2}58.50\pm4.42^{c}$
Semigranular (%)	0	$_{1}11.00\pm2.67^{a}$	$_{1}37.50\pm10.37^{a}$	$_{1}19.25\pm7.48^{a}$	$_{1}8.00\pm2.12^{a}$
	45	$_{1,2}18.00\pm2.61^{a}$	$_{1,2}12.00\pm1.77^{a}$	$_{1,2}14.50\pm2.36^{a}$	$_{1,2}17.75\pm4.23^{a}$
	90	$_{2}35.25\pm1.54^{a}$	$_{2}23.50\pm1.93^{a}$	$_{2}25.00\pm3.24^{a}$	$_{2}18.25\pm3.25^{a}$
Hyaline (%)	0	$_{1}46.75\pm7.87^{a}$	$_{1}42.00\pm10.83^{ab}$	$_{1}24.00\pm3.43^{b}$	$_{1}31.25\pm6.79^{ab}$
	45	$_{1,2}47.00\pm6.32^{a}$	$_{1,2}30.50\pm9.20^{ab}$	$_{1,2}18.50\pm3.96^{b}$	$_{1,2}42.25\pm11.29^{ab}$
	90	$_{2}33.75\pm5.03^{a}$	$_{2}27.00\pm5.87^{ab}$	$_{2}11.75\pm1.10^{b}$	$_{2}23.25\pm2.49^{ab}$

All data in the same column within the same parameter having different subscript numerical (1, 2) are significantly different ($P<0.05$). Different superscript alphabet (a, b, c) in the same row indicate significantly different means ($P<0.05$). Sel-Plex® was used as a source of organic selenium.

After 90 days, marron fed without Sel-Plex® in the diet had higher bacteraemia than marron fed Sel-Plex®. Among marron fed Sel-Plex® in the diets, the lowest bacteraemia was observed in marron fed 0.3 g kg^{-1} Sel-Plex® which was significantly lower ($P < 0.05$) than marron fed no Sel-Plex® (Figure 4.4).

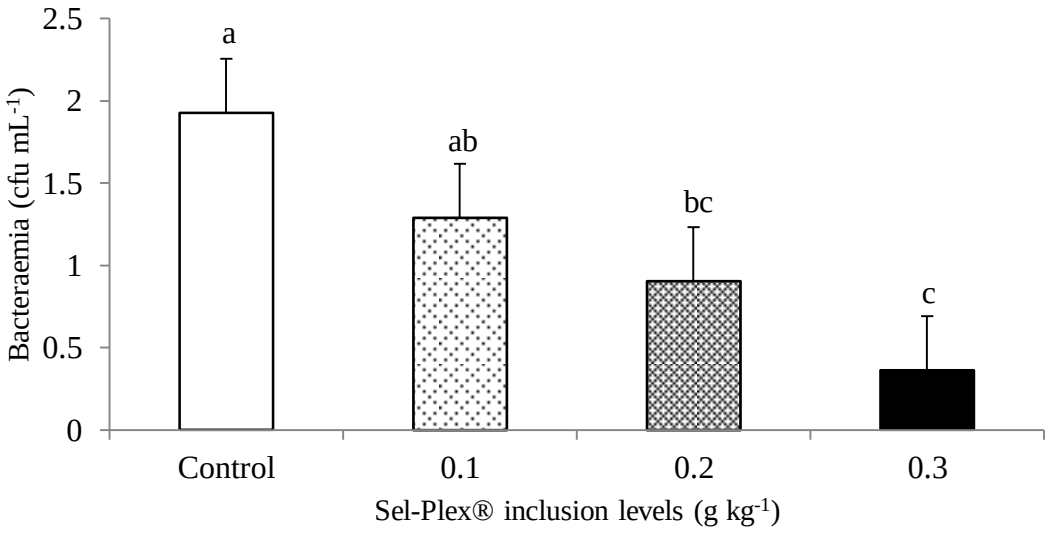

Figure 4.4: Bacteraemia of marron fed different levels of organic selenium for 90 days of feeding trial. Different alphabets (a, b, c) showing the significant difference in mean value at $P< 0.05$.

The mean amylase, protease activities and protein content of all marron fed more than 0.1 g kg^{-1} Sel-Plex® were significantly higher ($P<0.05$) than the control At the day 90. The highest mean value of amylase activity and soluble protein content in the digestive track were achieved in marron fed 0.2 g kg^{-1} Sel-Plex® while the highest activity of protease activity was at 0.3 g kg^{-1} (Figure 4.5).

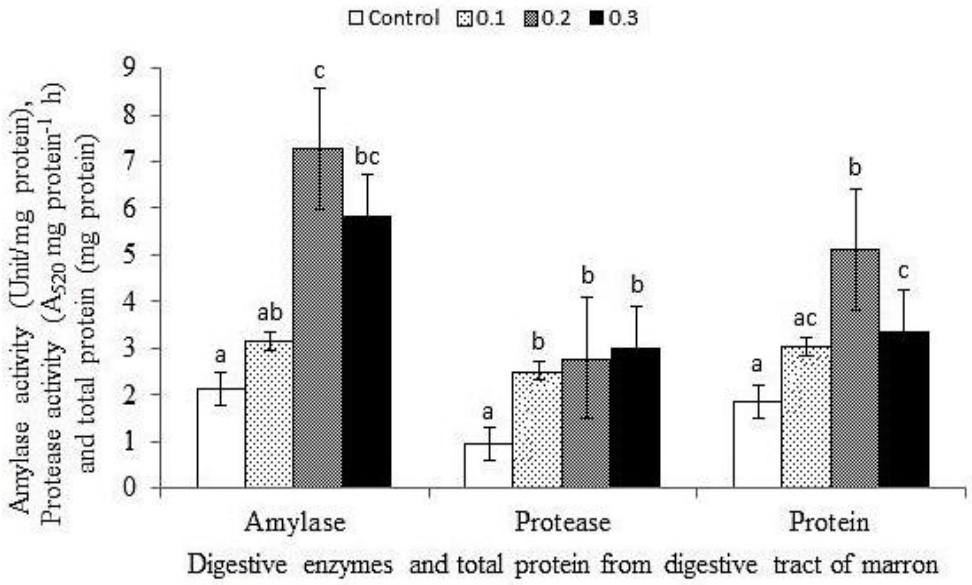

Figure 4.5: Amylase, protease activity and mg protein content from crude homogenate gut of marron. Sel-Plex® was added as a source of organic selenium at levels 0.1; 0.2; 0.3 g kg-1 of basal diet. One unit amylase activity (unit mg protein-1) =1 mg maltose liberated from starch in 3 min at pH 6.9 at 200C. Reaction condition for protease activity (A520 mg protein-1 h) = 37oC and 0.1 M Citrate phosphate buffer at pH 5.5. Different alphabets (a, b, c) in the same enzyme activity (amylase, protease or protein unit) showing the significant difference in mean value at $P< 0.05$.

4.3.5 Total selenium levels

After 90 days of feeding, marron fed the basal diet showed significantly lower total Se levels both in the hepatopancreas ($P<0.05$) and in muscle ($P<0.05$) tissues than marron fed Sel-Plex® supplementation (Figure 4.6). The marron fed 0.3 g kg^{-1} Sel-Plex® accumulated the highest levels of the total Se in their muscle tissues while the highest level of total Se was in the hepatopancreas of marron fed 0.2 g kg^{-1} Sel-Plex®.

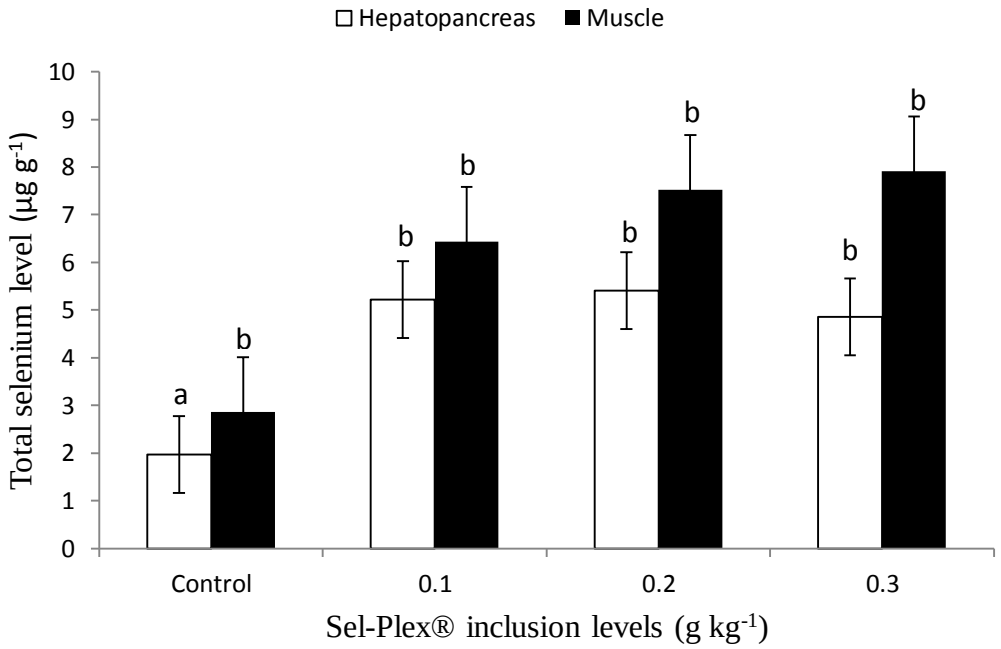

Figure 4.6: The mean values of total selenium level on the hepatopancreas and muscles tissue of marron fed different levels of Sel-Plex® for 90 days. Different alphabets at the same colour bars denote significant differences (*P< 0.05*) in mean value.

4.4 Discussion

Current research is the first attempt to determine the effects of Se supplementation in the diet of marron that given levels 0.1; 0.2 and 0.3 g kg^{-1} Sel-Plex® supplements in the diet of marron. .

4.4.1 Growth and survival

Crayfish growth can be influenced by age, seasons, stocking density, type of food and supplementation of dietary immunostimulants (Lowery, 1988; Morrissy, 1990, 2002; Rider and Sweetman, 2008; Sang and Fotedar, 2010a). Research on OS supplementations in the diet to enhance growth performance and survival has been performed in some aquatic animals such as Chinook salmon (*Oncorhynchus tshawytscha*) (Hamilton and Buhl, 1990), African Catfish (*Clarias Gariepinus*) (Abdel-Tawwab et al., 2007) and common Carp (*Cyprinus carpio* L.) (Alina et al., 2009). Current results showed that DWG, RGR, SGR, final weight, biomass increment and survival of marron increases by adding dietary OS supplements. These

results are similar to the previous research on sea cucumber (*Apostichopus japonicas*) (Wang et al., 2011) and allogynogenetic crucian carp (*Carassius auratus gibelio*) (Wang et al., 2007) that adding OS in the diet improves their growth performance.

The OS in the diet is incorporated with protein structure in the marron tissues and then could interact with iodine to prevent abnormal hormonal metabolism (Burk and Hill, 1993; Foster and Sumar, 1997), resulting in higher growth performance. In addition, OS can be deposited and retained as seleno protein in the muscle and liver tissues of marron for approximately three years where it can be extensively utilized and re-utilized (Aguilar et al., 2009). Higher marron growth performance can also be mediated through the activation of antioxidant enzymes such as GPx (Glutathione peroxide), SOD (Superoxide dismutase) and catalase which are responsible for the improvement in the growth performance, survival and disease resistance (Leclère et al., 2004; Zhan et al., 2006; Zhang et al., 2011).

4.4.2 Moisture and hepatosomatic indices

Moisture level and hepatopancreas indices have been widely used as tools to evaluate the condition of marron (Jussila and Mannonen, 1997; Sang and Fotedar, 2004; Prangnell and Fotedar, 2006; Tantulo and Fotedar, 2006). These indices are also affected by the nutrient profile and supplementation in the diet (Jussila and Mannonen, 1997; Sang and Fotedar, 2004). The lower moisture and higher weight of hepatopancreas indicate higher total energy reserved reflecting improved health condition (McClain, 1995a, b; Morrissy et al., 1995).

The present study showed that after 90 days of feeding trial, the marron fed 0.2 g kg^{-1} Sel-Plex® had HM% lower than control groups. These conditions were indicated that the marron fed Sel-Plex® supplementation were nutritionally healthier by having higher energy reserves in hepatopancreas. In addition Hiw and Hid of marron fed Sel-Plex® diet also indicated a greater ability of marron to distribute and reserve minerals, organic substances such as lipid, carbohydrate and protein (Dall and Moriarty, 1983; Lin and Shiau, 2007). Similarly, the moisture content of hepatopancreas of tropical spiny lobsters juvenile (*Panulirus ornatus*, Fabricius 1798) fed mannan oligosaccharide (MOS) indicated improved physiological

condition by showing lower HM% and higher Hiw than control (Sang and Fotedar, 2010a).

4.4.3 Immunocompetence

The incorporation of OS in the diet may stimulate and increase the proliferation rate of marron haemocytes and the subsequent higher THCs in marron haemolymph indicates the elevated immune competence of marron. Similar outcomes were also suggested in freshwater characid fish matrinxa (*Brycon cephalus*) and giant freshwater prawn (*Macrobrachium rosenbergii*) when fed Se enriched-diets (Oster et al., 1988; Chiu et al., 2010). Meanwhile, several types of haemocytes such as hyaline cells, semi-granular cells and granular cells have been recognized in crustaceans which play an important role in the host immune response (Smith and Soderhall, 1983; Soderhall and Cerenius, 1992; Bachère, 2003). The quality and quantity of three types of haemocytes in crustaceans can be affected by extrinsic factors such as food intake (Durliat and Vranckx, 1983; Jussila and Evans, 1998; Fotedar et al., 2001; Jussila et al., 2001). The hyaline cells of crayfish are involved in phagocytosis while semigranular cells are responsible in encapsulation and granular cells are recognized as participating in prophenoloxidase (proPO) and cytotoxicity (Johansson et al., 2000). Current results have shown that the percentage of granular cells of marron increases when fed OS. These findings are in line with previous studies showing that OS supplementation enhances immune system of freshwater characid fish matrinxa and giant freshwater prawn (Monteiro et al., 2009; Chiu et al., 2010). Further, Alina et al. (2009) stated that Se enriched diet is assimilated into enzymes and antioxidant which are important in the body development and immunity. Se appears to promote antioxidant activity in the body via glutathione peroxides (GPX), a Se-dependent enzyme which is a primary antioxidant enzyme for cellular defences against oxidative stress (Han et al., 2011).

The number of bacteria in the haemolymph termed as bacteraemia can be an important indicator to evaluate the immunity of marron (Fotedar et al., 2006; Sang and Fotedar, 2010a). A high level of bacteraemia indicates a decline in the immune system which in turn is related to the THC, granular cells and thus could increase risk of infection (van de Braak et al., 2002b; Cui-Luan et al., 2008; Sang and Fotedar, 2010b). Bacteraemia of cultured crayfish is also affected by the stocking

density, water supply, and sterility of the equipment and feed (Nylund and Westman, 1992; Sritunyalucksana et al., 2005). The current study showed that OS in the diet can reduce bacteraemia levels but the underlying mechanisms are unknown.

Besides improving growth, survival, immune competence and reducing bacteraemia, OS supplementation in the diet of animals including mammals and crustaceans have also been responsible to enhance the digestive enzyme activities (Wang, 2007; Zhan et al., 2010). The activities of amylase and protease are closely associated with the digestion and absorption of nutrients caused by selenomethionine intake (Zhan et al., 2010). As amylase and protease are major digestive enzymes in digestibility of starch and protein (Houlihan et al., 1988; Ceccaldi, 1989; Pavasovic, 2004), their activities have also increased significantly after OS supplementation.

4.4.3 Total selenium deposition in marron tissues

Se bioaccumulation has been studied in several aquatic animals, including salmon (Lorentzen et al., 1994), rainbow trout (Vidal et al., 2005), juvenile grouper (Lin and Shiau, 2005), medaka (*Oryzias latipes*) (Li et al., 2008) and adult crayfish (*Procambarus clarkii*) (Dörr et al., 2008). The uptake of Se can be from water or diet and the uptake of water-soluble Se by fish can be from gills, epidermis or gut (Hamilton, 2004). In the current experiment, total Se accumulation in hepatopancreas and muscle of marron increased as Sel-Plex® supplementation level in the diet increased. Similarly, Mahan and Parrett (1996) and Wang et al.(2007) reported that the Se supplemented diet resulted in Se accumulation in various tissues of channel catfish (*Ictalurus punctatus)* and can vary in their tissues and organs (Rainbow, 2002). Particularly in the case of the hepatopancreas, a main digestive gland that responsible for absorption of nutrients from the digestive track, a high level of Se may occur in this organ. However, at higher levels of Sel-Plex® (0.3 g kg^{-1}) in the diet, Se can be deposited in the muscle tissue of marron. Studies in hybrid striped bass (*Morone saxatilis* x *M. chrysops*), channel catfish and Atlantic salmon revealed that high Se concentration occurs in muscle tissues when Se was supplemented in their diets (Maher et al., 1992; Lorentzen et al., 1994; Wang and Lovell, 1997). The ingested OS has higher absorption, availability and retention than inorganic Se (Wang and Lovell, 1997) and is known to be incorporated into selenoprotein that can be accumulated in the body of animals (Kyriakopoulos and Behne, 2002; Kucukbay

et al., 2009). Se in organic form such as selenomethionine that binds to amino acid can efficiently be incorporated into animal tissue and has greater retention in muscles than the inorganic form (Waschulewski and Sunde, 1988b; Lorentzen et al., 1994; Hamilton, 2004), although the absorption, availability and tissue accumulation of Se in the body organs can be affected by dietary ingredients, hepatic metabolism and prevailing gut pH (Meltzer et al., 1993; Suzuki and Ogra, 2002; Fox et al., 2004).

4.5 Conclusions

The present study suggests that dietary supplementation of OS can be used to improve the physiological condition and health of marron as indicated by growth, survival, hepatosomatic indices, digestive enzymes activity, and acceptable levels of total Se in the hepatopancreas and muscle. It is also suggested that 0.2–0.3 g kg^{-1} Sel-Plex® should be added in the diet of marron. Further research need to be conducted to evaluate the direct effects of Sel-Plex® supplementation on the digestive enzymes profile and antioxidant enzymes activity.

CHAPTER 5. Effects of Dietary Organic Selenium on the Health Status of Digestive System and Total Selenium Accumulation on Marron, *Cherax cainii* (Austin, 2002)

5.1 Introduction

The use of micronutrients as feed additives has gained popularity and has assisted in minimising the environmental impacts of aquaculture and maximising the health of the farmed animals (Sang et al., 2009). Considerable attention has been paid to organic selenium (OS) as a feed additive, which has proved to be more suitable than the inorganic form of selenium (Se). Compared to the inorganic Se, OS extracted from yeast (*Saccharomyces cerevisiae*) is absorbed at a higher rate, has greater bioavailability, is better retained in the body of animals, and is less toxic than inorganic Se (Mahan and Parrett, 1996; Schrauzer, 2003; Taylor et al., 2005; Wang et al., 2007; Kucukbay et al., 2009). OS produced in this way (known as selenoyeast) is approved by the U.S. Food and Drug Administration, and can be used as a feed supplement (Ortman and Pehrson, 1999; Chung et al., 2007).

Studies on the use of OS as a dietary supplement have been conducted in red swamp crawfish (*Procambarus clarkii*) (Dörr et al., 2008), African catfish (*Clarias gariepinus*) (Schram et al., 2008), giant freshwater prawn (*Macrobrachium rosenbergii*) (Chiu et al., 2010), and Pacific white shrimp (*Penaeus vannamei*) (Sritunyalucksana et al., 2011b), but have been limited in marron (*Cherax cainii*). Marron are the third largest crayfish in the world and are native to Western Australia (Rouse and Kartamulia, 1992; Morrissy, 2002). Production of marron has become a commercially important aquaculture industry in Western Australia (Nobes, 2011); there are currently 470 aquaculture licenses holders for marine and inland aquaculture, and 39% of these are identified as marron farming, which can generate $1.5 million per year (DoF, 2013).

Recent research has demonstrated that marron fed with 0.2 g kg^{-1} Sel-Plex® in the diet show increased growth performance (Nugroho and Fotedar, 2013b) and resistance against *Vibrio mimicus* (Nugroho and Fotedar, 2013a). However, additional effects of dietary OS in marron, such as immune responses, Se retention in various tissues, digestive enzyme activity, and midgut profile have remained largely unexplored.

Immune-physiological responses, such as an alteration in total and differential haemocyte counts (THC and DHC), bacteraemia, neutral red retention time (NRRT) (Jussila et al., 1999; Sang and Fotedar, 2004; Fotedar et al., 2006; Sang et al., 2009), total selenium accumulation (Elia et al., 2011), and digestive enzyme activity levels (Pedroza-Islas et al., 2004; Sang et al., 2011b), have been successfully used as indicators of the immune and health status of several crustacean species. Thus, this study aimed to evaluate the effects of 0.2 g kg^{-1} dietary OS (Sel-Plex®, Alltech, Nicholasville, KY, USA) supplementation on immunity; total Se accumulation in various tissues, such as haemolymph, hepatopancreas and muscle; and digestive enzyme activity of OS-fed marron under laboratory conditions. Marron gut parameters, in particular midgut physiology, such as midgut section and the microvilli number were examined to assess marron gut condition.

5.2 Materials and Methods

5.2.1 Animal and diets

The 90-day experiment was performed on juvenile marron (mean initial weight 3.65 ± 0.05 g, n = 100) supplied by Aquatic Resource Management Pty Ltd, Western Australia. One hundred marron were randomly allocated into two groups: a control group (fed without Sel-Plex® inclusion) and an OS group (fed 0.2 g kg^{-1} Sel-Plex® inclusion level in the basal diet) with five replicates within each group:

5.2.2 The basal and test diets preparation

A basal diet mixture was formulated using a procedure as previously describe in chapter 3. To the basal diet, 0.2 g kg^{-1} of Sel-Plex® (approximately 0.4 mg kg^{-1} OS in the diet of marron) was added to obtain the test diet. Test diet was prepared using same method of basal diet.

5.2.3 Experimental design

The experiment was conducted in 10 blue plastic cylindrical tanks (800 mm diameter, 500 mm high, 250 L capacity, 70 L freshwater/tank) at the Curtin Aquatic Research Laboratory (CARL), Curtin University, Western Australia. Mechanical filtration (fluval 205 filters, Hagen, Mansfield, MA, USA) at a rate of approximately 2 L min^{-1} was used in each tank. Ten PVC pipes (55 mm diameter, 150 mm length)

were also placed in each tank to provide shelter for each marron. After transportation and placement in the blue cylindrical tanks, marron were acclimated to the culture conditions for 1 week. During the acclimation period, the marron were fed the basal diet at a rate of 3% of body weight every second day. This feeding rate was determined by our previous experiments and is considered to be above the satiation feeding level for marron (Sang et al., 2009). The marron were then randomly distributed to 10 culture tanks at a density of 10 marron per tank. Those in 5 randomly selected tanks were fed the basal diet, whereas those in the remaining 5 tanks were fed test diet at the same rate. Uneaten food and faeces were siphoned out before each feeding, and sufficient freshwater was added to maintain 70 L water in each tank. Temperature was maintained at 20°C by using automatic heaters (Sonpar®, Model: HA-100; Yong Cheng Aquarium Co., Ltd., China). Nitrate, nitrite, and ammonium tests were performed weekly using chemical test kits (Aquarium Pharmaceuticals^{TM}, Inc., McLean, VA, USA). To provide optimum water quality for cultured marron, nitrate and nitrite levels were monitored to not exceed 0.1 mg L^{-1}, whereas total ammonium was maintained below 0.2 mg L^{-1} (Jussila, 1997b).

5.2.4 Total and differential haemocytes count

The total haemocytes count (THC) and differential haemocytes count (DHC) of marron were counted on the day 90 of the feeding trial. THC of marron was calculated using previous method from Fotedar et al. (2001) with some modification.

5.2.5 Bacteraemia assessment

On the final day of the experiment, we assessed bacteraemia in the haemolymph of marron from each culture tank. A 0.05-mL sample of haemolymph from each marron was withdrawn from the base of the fifth thoracic leg into a sterile syringe and smeared on a nutrient agar plate. The agar plate was then inverted and placed into an incubator at 25°C for 24 h. Colony-forming units (cfu) were determined for each plate as cfu mL^{-1} and counted on the basis of the total volume of a 0.05 mL $plate^{-1}$ (Fotedar et al., 2001; Sang et al., 2009).

5.2.6 Neutral red retention time

NRRT assessment was performed using the procedure described by Hauton et al. (1998). A stock solution containing 10 mg neutral red dye powder was dissolved in 1 mL dimethyl sulphoxide. To prepare the working solution, a dye concentration of 0.02 mg mL^{-1} was mixed with 10 mL stock solution and then diluted with 5 mL saline water. On day 90, a 0.2-mL marron haemolymph sample was transferred into an Eppendorf tube containing 0.2 mL saline water and gently mixed. The mixture of haemolymph sample was placed onto a microscope slide treated with a poly L-lysine solution to enhance cell adhesion. The slide was immediately placed in a 10°C incubator for 15 min to allow the haemocytes to attach to the slide. The excess haemolymph was then removed. Next, 40 µL neutral red working solution was added to the slide and covered with a coverslip. The slide was then returned to the incubator and subsequently taken out every 15 min to examine the sample under a microscope. The time at which 50% of the haemocytes had begun to lose dye from their lysosomes was noted as the NRRT of the marron lysosomal membrane.

5.2.7 Midgut micrograph assessment

Three marron from three randomly selected tanks within each group, were dissected on day 90, and the midgut were prepared for scanning electron microscopy (SEM) using the method proposed by Dunlap and Adaskaveg (1997). Marron midguts were immersed in 3% glutaraldehyde in 0.1 M cacodylate buffer overnight. The midguts were then washed in three changes of cacodylate buffer and three changes in distilled water for 5 min per change. The midguts were immersed in 2% OsO_4 for 2 h following by three washes in distilled water for 5 min per wash, then dehydrated using a series of 50%, 75%, and 95% ethanol solutions for 5 min before three final washes in 100% ethanol for 5 min per change. The samples were then chemically dried by washing in a series of 50%, 75% and 100% (twice) hexamethyldisilizane (HMDS) in ethanol solutions for 5 min per change. Finally, the samples were dried at room temperature and mounted on a stub using carbon tape, coated with gold, and viewed under a pressure scanning electron microscope (SEM, model Phillips XL 30, FEI, USA). The images obtained from SEM were used to calculate the number of midgut microvilli by counting and averaging microvilli on each slide (n = 3) using Adobe Photoshop CS6 (Adobe, Inc., San Jose, CA, USA).

5.2.8 Histological analysis

Same sampling method with midgut micrograph assessment, sampled marron were sacrificed at the end of feeding period to perform histological analysis of midgut and hepatopancreas following the method described by Genc et al. (2007). Midgut and hepatopancreas were removed from the marron, and fixed in 4% buffer formalin for 24 hours. The tissues were then dehydrated by passing them through a series of alcohol solutions of 70%, 85%, and 98%. After dehydration, the samples were vacuum embedded in paraffin. The histological sections of 4–5 μm thickness were then stained using hematoxylin and eosin. The samples were photographically analyzed and documented using an Olympus SC30 BH2 microscope at 400 X magnification.

5.2.9 Total soluble selenium determination

At the day 90, three marron (same sampling method with midgut micrograph assessment as described above) were sacrificed to determine total soluble Se in haemolymph, hepatopancreas and muscle tissues. Total soluble Se was determined using spectrophotometric method according to Revanasiddappa and Dayananda (2006).

5.2.10 Statistical analysis

The results were expressed as means ± SE (standard error), and data were analysed using SPSS version 17 (SPPS Inc., Chicago, IL, USA). Student's t-tests were performed to compare THC, DHC, bacteraemia, NRRT, amylase, protease, and total protein and the number of microvilli in marron midgut between the two groups. Multiple comparisons followed by Tukey's post hoc test were used to determine significant differences in total soluble Se accumulation in various tissues at days 0, 45, and 90. $P < 0.05$ was considered significant.

5.3 Results

After 90 days of feeding, the THC, granular cells, and semigranular cells of marron fed dietary OS were significantly higher ($P < 0.05$) than those of marron fed a basal diet; however, the proportion of hyaline cells in marron were not affected by the addition of dietary OS. Further, the marron fed 0.2 g kg^{-1} Sel-Plex® had significantly

lower bacteraemia and longer NRRT than marron that were fed the basal diet (Table 5.1).

Table 5.1: Immunological parameters of OS-fed marron (mean ± SE) after 90 days of feeding trial

Immune parameters	Groups	
	Control	0.2 g kg^{-1} Sel-Plex®
THC (× 10^6 cells mL^{-1})	2.25 ± 0.05^a	2.68 ± 0.06^b
Granular cells (%)	34.00 ± 1.70^a	38.00 ± 2.16^b
Semigranular cells (%)	30.80 ± 1.88^a	25.80 ± 1.77^b
Hyaline (%)	35.40 ± 0.81^a	36.00 ± 1.70^a
Bacteraemia (cfu mL^{-1})	1.77 ± 0.12^a	0.91 ± 0.02^b
NRRT (min)	78.00 ± 3.00^a	105.00 ± 6.70^a

THC = Total haemocyte count. Different alphabets (a, b) in the same row indicate significantly different means at $P < 0.05$. Sel-Plex® was added to basal diet as a source of organic selenium (OS).

Total Se accumulation in all sampled tissues of the control and OS-fed marron increased significantly ($P < 0.05$) over the feeding trial. At days 45 and 90, marron that were fed dietary OS had significantly higher ($P < 0.05$) total soluble Se in all sampled tissues compared to marron fed the basal diet without OS. The highest total soluble Se level was measured in the muscle of OS-fed marron on day 90, followed by levels in the hepatopancreas (Table 5.2).

Table 5.2: Total soluble selenium (mean ± SE) in the various tissues of marron fed OS in the diet for 90 days

Tissue	Day	Groups	
		Control	0.2 g kg^{-1} OS
Haemolymph (μg mL^{-1})	0	$_1$0.0446 ± 0.0009^a	$_1$0.0459 ± 0.0010^a
	45	$_2$0.0476 ± 0.0015^a	$_2$0.0532 ± 0.0047^b
	90	$_3$0.0492 ± 0.001^a	$_3$0.0719 ± 0.0152^b
Hepatopancreas (μg g^{-1})	0	$_1$0.0435 ± 0.0001^a	$_1$0.0450 ± 0.0010^a
	45	$_2$0.0469 ± 0.0045^a	$_2$0.2066 ± 0.0323^b
	90	$_3$0.0594 ± 0.0028^a	$_3$0.3340 ± 0.0255^b
Muscle (μg g^{-1})	0	$_1$0.0501 ± 0.0099^a	$_1$0.0544 ± 0.0030^a
	45	$_2$0.0510 ± 0.0051^a	$_2$0.3123 ± 0.0319^b
	90	$_3$0.1014 ± 0.0079^a	$_3$0.7442 ± 0.1240^b

All data in the same column within the same parameter having a different numerical subscript (1, 2, 3) are significantly different ($P < 0.05$). Different superscript letters (a, b) in the same row indicate significantly different means ($P < 0.05$). 0.2 g kg^{-1} Sel-Plex® was used as a source of organic selenium (OS).

The digestive enzyme activity results are presented in Figure 5.1. At the end of the feeding trial, OS-fed marron had significantly higher ($P< 0.05$) amylase and protease activity and total soluble protein than the control. As shown in Figure 5.2, SEM of the middle section of the midgut revealed that the microvilli in marron that were fed OS were longer, more numerous, and more concentrated towards the inner surface of the midgut than marron fed without OS. The number of microvilli in OS-fed marron (21.93 ± 1.00) was significantly higher ($P < 0.05$) than that in marron fed the basal diet (17.53 ± 1.03).

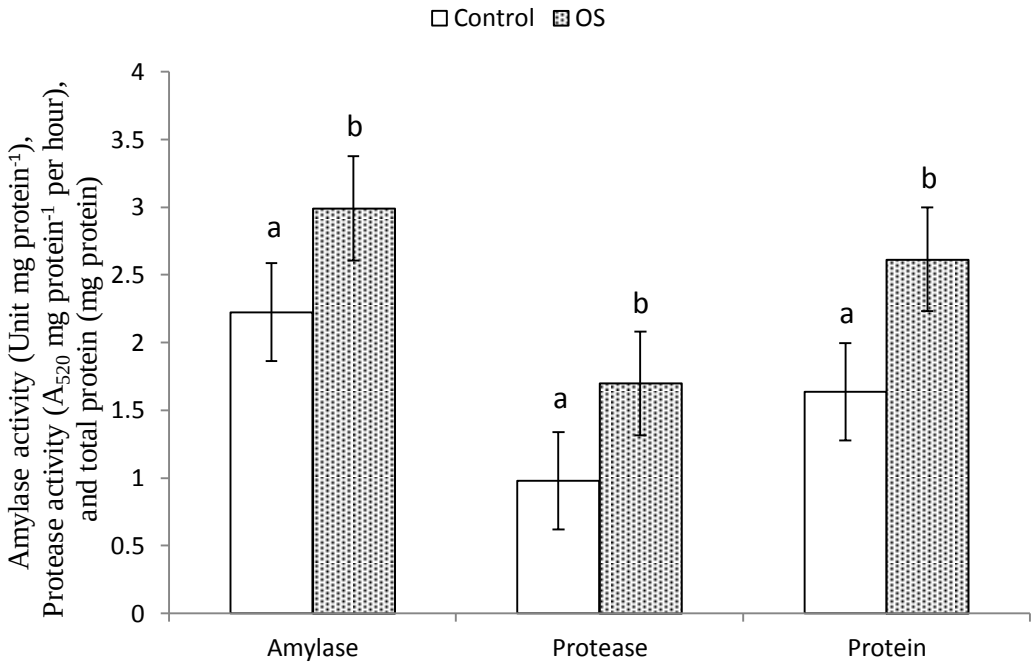

Figure 5.1: Amylase and protease activity and mg protein content from crude marron gut homogenate after 90 days of feeding on organic selenium. Sel-Plex® was added as a source of organic selenium at a level of 0.2 g kg^{-1} of basal diet. One unit of amylase activity (unit mg $protein^{-1}$) = 1 mg maltose liberated from starch in 3 min at pH 6.9 at 20°C. Reaction condition for protease activity (A_{520} mg $protein^{-1}$ h) = 37°C and 0.1 M citrate phosphate buffer at pH 5.5. Different letters (a, b) for the same enzyme activity (amylase, protease, or protein unit) indicate a significant difference in the mean value at $P< 0.05$.

Microvilli of marron fed Sel-Plex® are longer, numerous and more distributed towards the inner surface of the midgut than marron fed without Sel-Plex®. Meanwhile, marron fed Sel-Plex® had a thinner midgut epithelial layer and hepatocytes than marron fed basal diet (Figure 5.3).

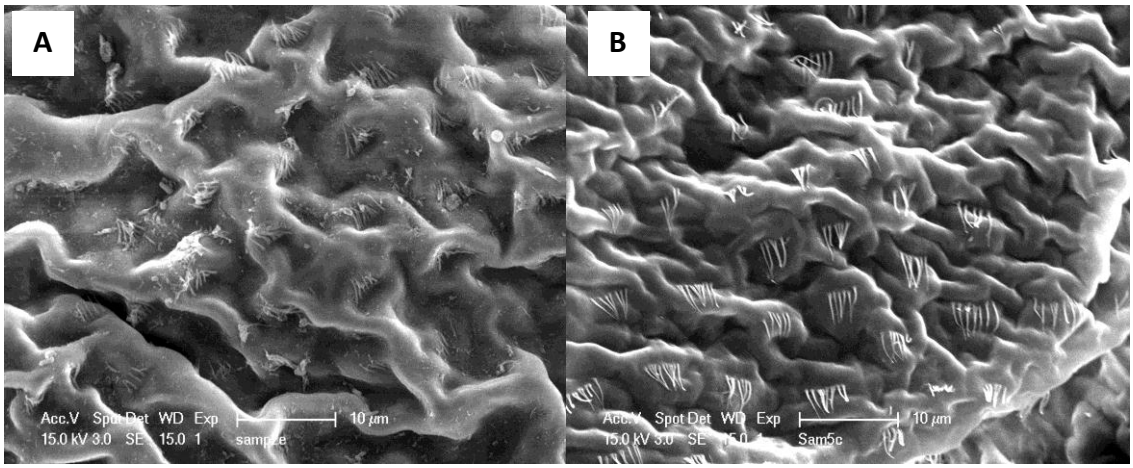

Figure 5.2: Comparison of midgut micrographic of marron fed basal diet (A = Control) and test diet (B= 0.2 g kg-^{1} Sel-Plex® as a source of organic selenium).

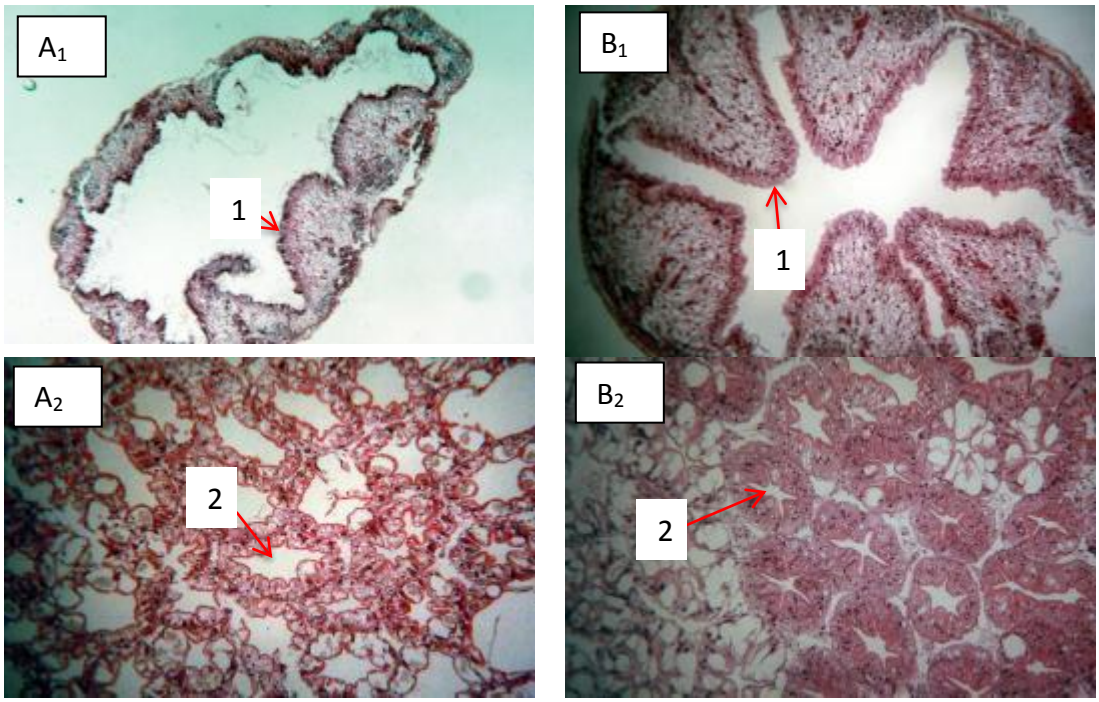

Figure 5.3: Photomicrograph (400x LM magnification) of an H and E stained section of gut (A_1 and B_1) and hepatopancreas (B_1 and B_2) of marron. A_1 and A_2 = midgut and hepatopancreas of marron fed basal diet; B_1 and B_2 = midgut and hepatopancreas of marron fed 0.2 g kg^{-1} Sel-Plex® in the diet. The arrow points (1) the epithelium layer of the inner midgut lining; (2) Lumen of hepatocellular.

5.4 Discussion

Crayfish health status can be influenced by food supplementation (Jussila and Evans, 1996, 1998; Sang et al., 2011a). Sel-Plex® is a source of OS that mainly contains selenomethionine, a dried product extracted from the baker's yeast *Saccharomyces cerevisiae* strain CNCM I-3060 (Burdock and Cousins, 2010). Selenomethionine incorporates into proteins in place of methionine and can be stored (Suzuki and Ogra, 2002) as a reserve in muscle tissues and/or the hepatopancreata of animals and can be extensively utilised and re-utilised to maintain appropriate Se levels in the body (Aguilar et al., 2009). Selenomethionine in the body of animals can trigger and promote the activation of antioxidant enzymes, such as glutathione peroxidase, superoxide dismutase, and catalase (Zhan et al., 2010; Han et al., 2011), all of which play an important role in enhancing animal immune function (Chiu et al., 2010; Smitha and Rao, 2010; Han et al., 2011).

The current results suggest that the supplementation of Sel-Plex® in the marron diet can enhance immune responses by increasing THC and the percentages of granular and semigranular cells. Greater THC could contribute to immune system function at the cellular level (Sang et al., 2011b), while granular cells are chiefly involved in the storage and release of the prophenoloxidase (proPO) system (Johansson et al., 2000). Semigranular cells participate in the encapsulation, recognition, and degranulation of foreign molecules or particles (Johansson and Soderhall, 1989; Johansson et al., 2000), which might reduce the number of bacteraemia. This findings are consistent with previous reports that animals with higher THC and increased percentages of granular and semigranular cells have better defences against pathogens (Hryniewiecka-Szffter and Babula, 1996; Soderhall, 1997; van de Braak et al., 2002a; Sang et al., 2009). However, variation in DHC of most crustacean species is high between individual animals (Johansson et al., 2000). Our previous results found that only hyaline cells of marron were affected by the dietary OS (Nugroho and Fotedar, 2013b). Besides nutritional factor, the variation of DHC may be due to intrinsic factor such as moulting. According to Vacca and Fingerman (1993); Hose et al. (1992); Sequiera et al. (1995) stated that the hyaline cells of most crustacean were seen to be the dominant population before and soon after the moult, whereas they decreased over the intermoult.

The present results also imply that inclusion of 0.2 g kg^{-1} Sel-Plex® in the diet increases NRRT. OS inclusion in the marron diet might reduce lipid peroxidation and stabilise lysosomal membrane integrity by protecting against oxidative damage (Ursini and Bindoli, 1987). This finding is similar to that of previous studies showing that lysosomal membrane stability increases in marron fed BioMOS (Sang et al., 2009). Lysosomal membrane stability of BioMOS-fed marron, infected with *Vibrio* spp, also was significantly more stable than the control (Sang et al., 2009).

Aquatic animals, including salmon (*Salmo salar*) (Lorentzen et al., 1994; EFSA, 2009), rainbow trout (*Oncorhynchus mykiss)* (Vidal et al., 2005), juvenile grouper (*Epinephelus malabaricus*) (Lin and Shiau, 2007), medaka (*Oryzias latipes*) (Li et al., 2008), and adult crayfish (*Procambarus clarkii*) (Dörr et al., 2008), are able to accumulate Se from dietary sources. After Se dietary intake and small intestine absorption, dietary organic Se binds to protein and is transported from the plasma to the liver and other tissues via the hepatic portal circulation. Se can also be transported via intestinal mucosal cells through the amino acid transport mechanism into all tissues, including haemolymph, hepatopancreas, and muscle, and finally accumulates as a component of selenoproteins (Wolfram et al., 1989a; Wolfram et al., 1989b; Nijhoff and Peters, 1992; Yeh et al., 1997; Hamilton, 2004; Gropper et al., 2008). In addition, adequate Se accumulation in the body of animals also supports optimum immune system function (Arthur et al., 2003).

The present results showed that Se is distributed throughout the haemolymph, hepatopancreas, and muscle of OS-fed marron at different levels. Meanwhile, total Se retention in the various tissues gradually increased throughout the feeding trial. Se can be retained in the muscle tissues at higher levels than in the hepatopancreata of marron. According to Jacques (2001) selenomethionine incorporates into protein and mainly storage in the muscle (Oster et al., 1988; EFSA, 2009). The turnover of selenomethionine in the peripheral tissue such as muscle is also slower than in liver and pancreas, indicating that selenomethionine is incorporated into a long-term body pool (EFSA, 2009). In contrast with current results, Waska et al. (2008) reported that Se levels in the hepatopancreas of squid (*Todarodes pacificus*) were higher in than in muscle. Hepatopancreas of marron can be metabolically more active than squid hepatopancreas thus consuming OS at a higher rate than squid. In addition, muscle of

marron appears to act as a Se storage organ due to slow turnover of selenomethionine.

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In addition to increasing total Se retention, the present results imply that inclusion of 0.2 g kg^{-1} Sel-Plex® in the diet for 90 days improves digestive system health by enhancing amylase and protease activity and total soluble protein levels. The higher digestive enzyme activity contributes to efficient digestion, which can result in increased growth and improved immunity and health (Houlihan et al., 1988; Sang et al., 2011b). According to Zhan et al. (2010), the activities of amylase and protease are closely associated with the digestion and absorption of nutrients and may be affected by selenomethionine intake. However, the mechanism by which selenomethionine stimulates digestive enzyme activity has not been investigated. The inclusion of OS in the marron diet also results in higher numbers of longer microvilli and a distribution of microvilli that appears more uniform compared to the microvilli of marron that were fed the basal diet. According to Chisaka et al. (1999), increasing the number of microvilli protects the cuticle layer, enhances gut irrigation, and facilitates faecal movement. In addition, microvilli enlarge the plasma membrane surface area and secrete brush border enzymes, such as carbohydrate- and protein-digesting enzymes (Tortora and Derrickson, 2008).

5.5 Conclusion

The results of this experiment have extended the current knowledge on the effects of dietary 0.2 g kg^{-1} of Sel-Plex® on immune responses, total Se accumulation, and digestive system health. Further research needs to be conducted to determine antioxidant activity in the tissues of OS-fed marron.

CHAPTER 6: Dietary Organic Selenium Improves Growth, Survival and Resistance to *Vibrio mimicus* in Cultured Marron, *Cherax cainii* (Austin, 2002)

6.1. Introduction

Vibrio species have been identified as serious pathogens to various aquatic animals (Sudheesh and Xu, 2001; Austin and Austin, 2007; Chrisolite et al., 2008; Won and Park, 2008; Austin, 2010; Pengsuk et al., 2010) resulting in mass mortalities to many cultured invertebrates (Karunasagar et al., 1994; Prayitno and Latchford, 1995; Won and Park, 2008). Among various *Vibrio* species, *Vibrio mimicus* is known to cause disease outbreaks in black tiger shrimp (*Penaeus monodon*) and red claw crayfish (*Cherax quadricarinatus*) (Eaves and Ketterer, 1994; Payne et al., 2004; Austin and Austin, 2007; Austin, 2010). Intramuscular injection of *V. mimicus* can produce virulent reaction and high mortalities in yabbies (*Cherax albidus*) (Eaves and Ketterer, 1994). To overcome high mortalities of cultured animals, some aquaculturists use antibiotics to prevent the virulent reaction of *V. mimicus* infection (Liao, 1996; Pathak et al., 1996; Supriyadi and Rukyani, 1996).

However, the increasing global demand for safe seafood and the need to preserve an eco-friendly environment, the application of antibiotics, notorious for creating antibiotic-resistant pathogens and environmental deterioration has been questioned (Capone et al., 1996; Kesarcodi-Watson et al., 2008). Thus, various dietary trace elements, such as organic selenium (OS) have been tested and used as an alternative to antibiotics (Wang and Lovell, 1997; Wang, 2007; Dörr et al., 2008; Chiu et al., 2010). There has been a surge in the use of organic forms of various trace elements to enhance the productivity of cultured aquatic animals (Campa-Córdova et al., 2002; Bachère, 2003; Soltanian et al., 2007). OS has been tested to improve growth and resistance to Tara syndrome virus (TSV) in white shrimp (*Penaeus vannamei*). Five weeks of feeding 0.3 mg kg^{-1} of OS to shrimp resulted in higher survival (Sritunyalucksana et al., 2011b). The dietary OS in channel catfish (*Ictalurus punctatus*) (Wang and Lovell, 1997), nile tilapia (*Oreochromis niloticus*) (Abdel-Tawwab and Wafeek, 2010) and hybrid striped bass (*Morone chrysopx x M. saxatilis*) (Cotter et al., 2008) has also improved their growth and immunity. Further, OS is reported to be better absorbed, has higher bioavailability and is less toxic than inorganic selenium (Wang and Lovell, 1997; Taylor et al., 2005; Gajčević et al.,

2009; Kucukbay et al., 2009). The dietary inclusion of OS also increases the number of total haemocytes and granular haemocytes in white shrimp (Bell et al., 1987; Wang and Lovell, 1997; Schrauzer, 2003; Taylor et al., 2005; Sritunyalucksana et al., 2011b). In addition, OS as an integral part of selenomethionine and selenoprotein (Barceloux, 1999; Zhan et al., 2010; Sritunyalucksana et al., 2011b), is recognized as a constituent of an antioxidant enzyme responsible for preventing cellular damage and improving immune competence in grouper (*Epinephelus malabaricus*) (Lin and Shiau, 2007).

The immunity related physiological responses measured by alterations in total haemocytes counts (THC), differential haemocyte counts (DHC) and *Vibrio* ranks can be used as indicators of immune competence and health status of several crustaceans (Jussila and Mannonen, 1997; Morrissy, 2002; Sang and Fotedar, 2004; Sang et al., 2009), including marron, *Cherax cainii* (Sang and Fotedar, 2010a). The neutral red dye retention time (NRRT) technique has also been successfully used as a tool to evaluate the lipid membrane integrity of marron during infection (Sang et al., 2009). However, the effect of dietary OS on the growth performance and immune competence of marron when challenged with *V. mimicus* is unknown. The aim of this experiment was to evaluate the effects of dietary OS on the growth performance, survival, various immune responses and *Vibrio* ranks in OS fed-marron when challenged with *V. mimicus*.

6.2 Materials and Methods

6.2.1 Preparation of basal diet and test diet

All ingredients of basal diet and test diet, except OS (donated by Alltech Inc. USA) were supplied by Specialty Feeds Pty. Ltd, Western Australia. The basal diet was formulated using Feed LIVE software version 1.52 from Live Informatics Company Limited, Thailand. To prepare a test diet, 0.2 g kg^{-1} of Sel-Plex® was added and mixed with the basal diet ingredients and then constituted into the pelleted form similar to the basal diet.

6.2.2. Culture system

The present experiment was carried out in Curtin Aquatic Research Laboratory (CARL), Technology Park, Curtin University, Western Australia. Eighteen plastic

cylindrical culture tanks were used and each tank was provided with aeration and ten PVC pipes (55 mm diameter, 150 mm length) were placed in each tank to provide shelter for marron.

6.2.3 Animals

A total of 180 marron (average initial weight 3.29 ± 0.08 g),purchased from Aquatic Resource Management Pty. Ltd., Western Australia were used for 90 days feeding trial followed by a challenge-test. All marron, after transportation were placed in the cylindrical experimental tanks for 1 week for acclimation to the culture conditions. During the acclimation period, the marron were fed the basal diet at a rate of 3% of body weight every two days. The marron after acclimation were randomly distributed into two groups (nine tanks per group with ten marron per tank). First group were fed the basal diet and the second group were fed 0.2 g kg^{-1} of Sel-Plex® supplemented test diet. The marron in every tank were fed the diets at a rate of 3% of their body weight every second day. Before every feeding, uneaten food and faeces were siphoned out and sufficient freshwater was added to maintain a constant water level of 70 L in each tank. Water quality parameters, such as temperature, pH and dissolve oxygen were monitor weekly using Cyberscan pH 300, Eutech Instruments, Singapore. Nitrate, nitrite and ammonium were measured and recorded weekly using chemical test kits (Aquarium Pharmaceuticals™, Inc., USA).

6.2.4. Challenge test

At the end of the trial, both groups of marron were further divided into three sub-groups each (three tanks per sub-groups, seven marron per tank). Two sub-group, one from each group were injected with 20 µL of 3.24 x 10^6 cfu *V. Mimicus* stock suspension that was obtained from the Department of Agriculture, Western Australia; two sub-groups from each group were injected with 20µL normal saline solution and; the third and final two sub-groups from each group were not subjected to injections (controlled sub-group). All injections were performed through the base of the fifth thoracic leg. All marron were then monitored for survival, THC and DHC, *Vibrio* ranks and NRRT at 0, 24, 48, 76 and 96 h post-injection time.

6.2.5 Data collection

Growth indices, survival and immune responses

Marron were measured for total weight immediately after acclimation and after 90 days of the feeding trial. The marron weights were used to determine final weight, daily weight gain (DWG), relative gain rate (RGR) and specific growth rate (SGR) (Wang et al., 2007; Sang et al., 2011a). The marron survival was recorded every day and at 0, 24, 48, 72, 96 h post-challenge time and the surviving marron in each sub-group were also analysed for THC and DHC.

To measure THC and DHC, at day 0 and 90 of feeding trial and at 0, 24, 48, 72, 96 h post-challenge, 0.2 mL of haemolymph was collected from each marron represented by each replicate from all three treatments. Haemolymph from individual marron was withdrawn from the base of the fifth thoracic leg into a 23-gauge needle containing 0.2 mL solution of 1% glutaraldehyde in 0.2 M sodium cacodylate and dispensed into an Eppendorf tube.(Fotedar et al., 2001). Total haemocytes were counted using a haemocytometer (Neaubauer, Germany) under 100-fold magnification. The haemocytes were counted in both grids and the resulting mean was used as mean THC. DHC was calculated following the procedure as describe in the chapter 3.

Vibrio ranks

Vibrio rank assessment was done using the procedure used by Sang et al. 2009 and Hauton et al. 1998 (Hauton et al., 1998; Sang et al., 2009). 0.1 mL of haemolymph was withdrawn into sterile syringe and then smeared onto a nutrient agar plate. The plate was then inverted and placed in an incubator at 25^0C for 24 h. Each plate was examined for colony forming units (cfu) and cfu mL^{-1} were counted based on the total volume of 0.1 mL $plate^{-1}$. The cfu mL^{-1} was ranked 1 (1–399 cfu mL^1) to 10 (3600–3999 cfu mL^{-1}). A final rank of 11 was assigned as too numerous for an accurate count.

Neutral red retention time assay

Neutral red dye retention time was evaluated using assay based on previous protocol (Hauton and Smith, 2004). To prepare a stock solution, 10 mg of neutral red dye powder was dissolved in 1 mL of dimethyl sulphoxide. A working solution (dye

concentration 0.02 mg mL^{-1}) was prepare by mixing 10 mL of stock solution and then diluted with 5 mL of saline water. 0.2 mL of marron haemolymph sample was transferred into an eppendorf tube containing 0.2 mL saline water and gently mixed. The mixture of haemolymph sample was placed onto a microscope slide treated with a poly-L-lysine solution to enhance cell adhesion. The slide was immediately placed in a $10^{o}C$ incubator for 15 min to allow the haemocytes to attach to the slide. The slide was removed from the incubator and the excess haemolymph was removed. A 40 µL of neutral red working solution was added to the slide and then covered with a coverslip. The slide was then returned to the incubator. Every 15 min the slide was taken out and the sample was examined using a microscope. The time at which 50% of the haemocytes had started to lose dye from their lysosomes was recorded as the neutral red retention time of the marron lysosomal membrane.

6.2.6 Statistical analysis

All data were represented as mean ± standard error (SE). A student t-test was performed to compare the growth indices, survival and immune responses of marron between two treatment groups. Percent data of survival were normalized using an arcsine transformation before performing significant differences analysis. Multiple comparison and post hoc test (Tukey's) were performed to determine significant differences of survival, immune responses including THC, DHC, *Vibrio* ranks and NRRT after being challenge with *V. mimicus*. All statistical analysis were made using SPSS for Microsoft software version 18 (SPSS, Inc., USA). Significance at $p < 0.05$ was used.

6.3 Results

6.3.1 Growth indices, survival and immune responses

Growth indices, survival and immune response parameters of the marron fed two different diets are presented in the Table 6.1. After 90 days of feeding, final weight, AWG, RGR, SGR and survival were significantly higher (*T-test, P<0.05*) in marron fed dietary Sel-Plex® than marron fed control diet. THC, percentage hyaline cells of marron fed dietary Sel-Plex® were significantly higher (*P<0.05*) than control group, whereas the proportion of granular and semigranular haemocytes of marron were not affected by the dietary Sel-Plex®.

After being challenged with *V. mimicus*, survival rate of marron fed only the basal diet was significantly lower (*P<0.05*) than the other marron. THC in 24 h post-challenged marron, were significantly reduced (*P<0.05*), compared to the THC before the challenge. However, after 48 hours post-challenge, THCs of marron fed OS supplementation were higher than any other sub-group of marron (Table 6.2). After 72 h of post-injection the percentage of granular cells of sub-groups with dietary Sel-Plex® was also higher (*P<0.05*) than sub-groups of marron fed only basal diets, whereas the percentage of semigranular and hyaline cells, of marron fed the control diet was significantly reduced (*P<0.05*) compared to the sub-group of marron fed the dietary Sel-Plex® (Figure 6.1).

Table 6.1: Growth indices, survival and immune responses of marron after 90 days of feeding

Parameters	Groups	
	Control	0.2 g kg^{-1} Sel-Plex®
Growth indices		
Final weight (g)	3.92 ± 0.05^{a}	4.20 ± 0.05^{b}
AWG (g/week)	0.049 ± 0.002^{a}	0.072 ± 0.007^{b}
RGR (%)	20.255 ± 2.26^{a}	29.69 ± 2.85^{b}
SGR (%)	0.19 ± 0.01^{a}	0.27 ± 0.02^{b}
Survival (%)	77.77 ± 3.64^{a}	94.44 ± 2.42^{b}
Immune competence		
THC (x10^{6} cells/mL)	2.47 ± 0.30^{a}	3.75 ± 0.15^{b}
Granular (%)	32.66 ± 1.45^{a}	36.54 ± 1.32^{a}
Semigranular (%)	29.44 ± 1.29^{a}	29.85 ± 1.51^{a}
Hyaline (%)	37.77 ± 1.07^{a}	33.49 ± 1.79^{b}

Different alphabets (a, b) indicate significantly different means for different treatments at *P <0.05*. AWG = average weekly gain; RGR = relative gain rate; SGR = specific growth rate. Sel-Plex® was added to basal diet as a source of organic selenium (OS).

6.3.2 Vibrio ranks

Vibrio ranks of marron fed Sel-Plex® supplementation were significantly lower than marron fed without Sel-Plex® after being challenged with *V. mimicus* (Figure 6.2). After 76-h post injection, there was a significant decrease ($P<0.05$) in *Vibrio* ranks in all sub-groups of marron fed Sel-Plex® supplementation. However, any sub-group of marron with Sel-Plex® in their diets showed no significant differences ($P>0.05$) amongst each other.

6.3.3 Neutral red retention time

NRRT of all marron was significantly reduced 24 h post-challenge, irrespective of dietary Sel-Plex®.However, the NRRT of marron fed Sel-Plex® was significantly longer ($P<0.05$) than marron fed the control diet and continued to remain significantly longer even at 96 h-post challenge (Table 6.2).

Table 6.2: Total haemocyte count (THC) and Neutral red retention time (NRRT) of marron challenge with *V. mimicus.*

Parameters	Hour	Groups					
		Control diet			0.2 g kg^{-1} Sel-Plex®		
		BNil	BNs	BVm	OSNil	OSNs	OSVm
THCs (x 10^6 mL^{-1})	0	$_1$2.47±0.03^a	$_1$2.44±0.06^a	$_1$2.51±0.06^b	$_1$3.87±0.07^c	$_1$4.06±0.24^c	$_1$4.29±0.33^d
	24	$_2$2.61±0.14^a	$_2$2.04±0.09^a	$_2$1.21±0.03^b	$_2$3.81±0.04^c	$_2$3.69±0.04^c	$_2$3.52±0.13^d
	48	$_2$2.54±0.10^a	$_2$2.11±0.04^a	$_2$1.25±0.00^b	$_2$3.57±0.32^c	$_2$3.77±0.09^c	$_2$3.66±0.10^d
	72	$_2$2.48±0.09^a	$_2$2.41±0.12^a	$_2$1.17±0.04^b	$_2$3.67±0.09^c	$_2$3.40±0.17^c	$_2$4.35±0.26^d
	96	$_2$2.43±0.11^a	$_2$2.48±0.18^a	$_2$1.04±0.03^b	$_2$3.55±0.15^c	$_2$3.12±0.11^c	$_2$4.45±0.15^d
NRRT (mins)	0	$_1$85.00 ± 3.16^a	$_1$92.50 ± 4.60^a	$_1$82.00 ± 6.78^b	$_1$130.50 ± 11.40^c	$_1$145.50 ± 8.36^d	$_1$125.50 ± 5.00^e
	24	$_2$95.00 ± 5.00^a	$_2$85.00 ± 3.16^a	$_2$22.50 ± 3.35^b	$_2$122.50 ± 7.15^c	$_2$79.00 ± 2.13^d	$_2$45.00 ± 3.87^e
	48	$_2$97.50 ± 3.35^a	$_2$92.5 ± 2.49^a	$_2$30.00 ± 4.74^b	$_2$122.50 ± 6.02^c	$_2$115.00 ± 3.16^d	$_2$52.50 ± 3.35^e
	72	$_2$87.50 ± 2.49^a	$_2$95.00 ± 3.16^a	$_2$33.75 ± 3.75^b	$_2$115.00 ± 3.16^c	$_2$100.00 ± 3.16^d	$_2$52.50 ± 3.35^e
	96	$_2$95.00 ± 3.16^a	$_2$85.00 ± 3.16^a	$_2$22.50 ± 7.49^b	$_2$110.00 ± 5.00^c	$_2$92.50 ± 2.49^d	$_2$57.50 ± 7.15^e

Different alphabets (a, b, c, d, e) indicate significantly different means for different treatments at $P < 0.05$. Different numericals (1, 2) indicate significantly different means at different times at $P < 0.05$. Note: BNil = control with no injection; BNS = control with 20 µL normal saline injection; BVm = control with 20 µL *V. mimicus*; OSNil = 0.2 g kg^{-1}Sel-Plex® supplementation with no challenge; OSNS = 0.2 g kg^{-1}Sel-Plex® supplementation with 20 µL normal saline injection; OSVm = 0.2 g kg^{-1} Sel-Plex® supplementation with 0.2 µL *V. mimicus* injection.

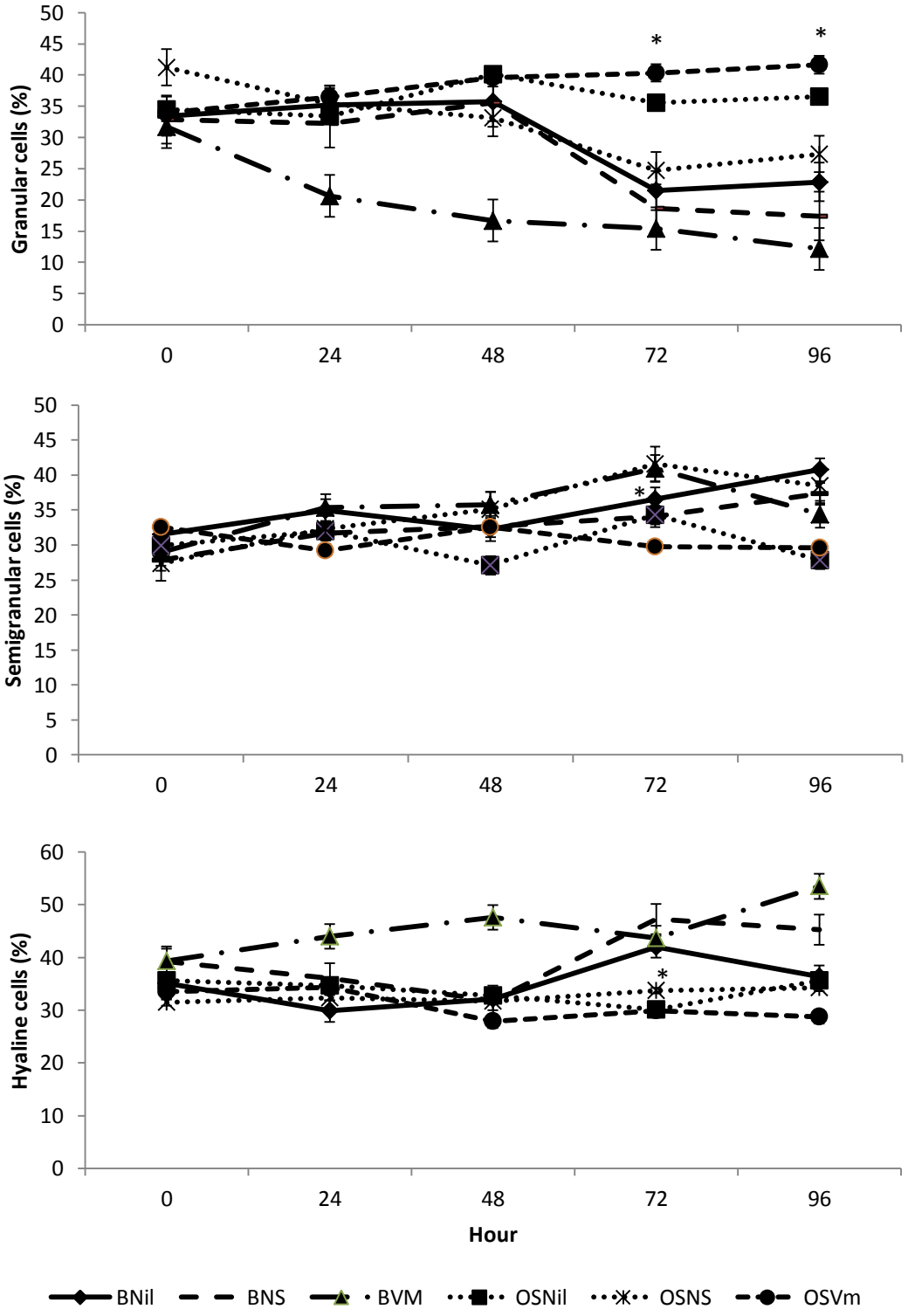

Figure 6.1: The comparison of differential haemocyte counts (DHC) in the haemolymph of marron. Note: BNil = control with no injection; BNS = control with 20 µL normal saline injection; BVm = control with 20 µL *V. mimicus*; OSNil = 0.2 g kg^{-1} Sel-Plex® supplementation with no challenge; OSNS = 0.2 g kg^{-1} Sel-Plex® supplementation with 20 µL normal saline injection; OSVm = 0.2 g kg^{-1} Sel-Plex® supplementation with 20 µL *V. mimicus* injection. * = significantly difference at $P<0.05$.

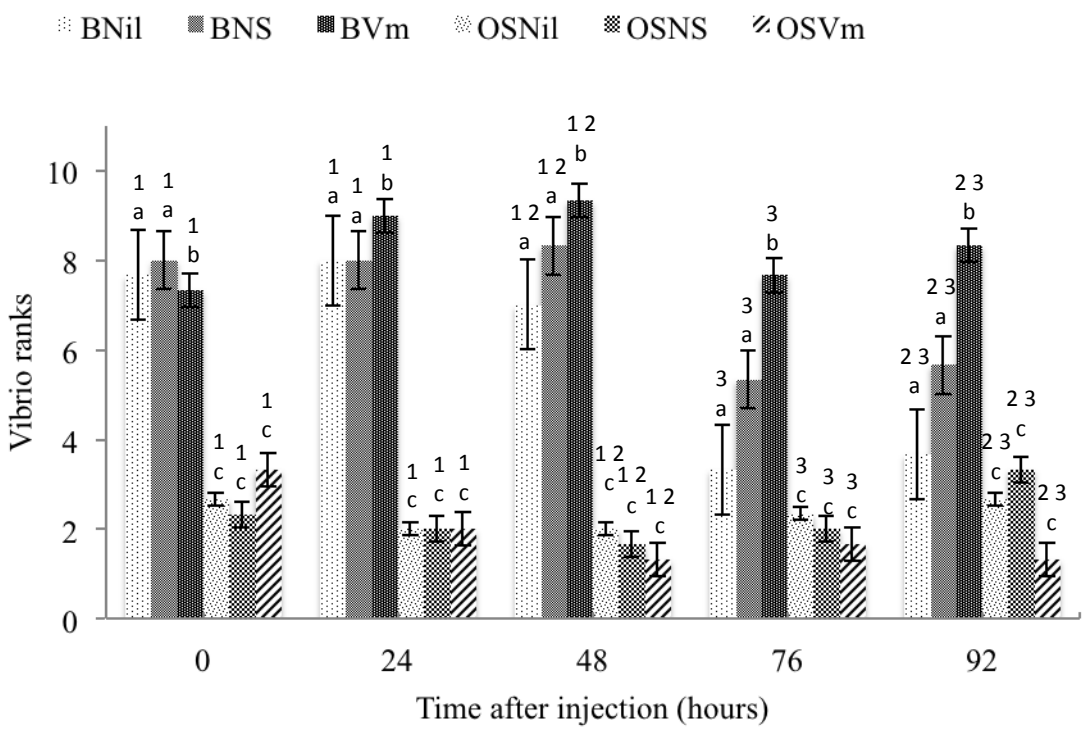

Figure 6.2: Mean ± SE of *Vibrio* ranks of marron after being challenge with *V. mimicus*. Different alphabets (a, b, c) over bars indicate significantly different means for different treatments at *P <0.05*. Different numericals (1, 2, 3) over bars indicate significantly different means at different times at *P* <0.05. Note: BNil = control with no injection; BNS = control with 20 µL normal saline injection; BVm = control with 20 µL *V. mimicus*; OSNil = 0.2 g kg^{-1} Sel-Plex® supplementation with no challenge; OSNS = 0.2 g kg^{-1} Sel-Plex® supplementation with 20 µL normal saline injection; OSVm = 0.2 g kg^{-1} Sel-Plex® supplementation with 20 µL *V. mimicus* injection

6.4 Discussion

Micronutrient, such Se, plays a pivotal role in improving aquaculture productivity (Dörr et al., 2008; Chiu et al., 2010; Han et al., 2011) and in its organic form has proven to enhance the growth and survival (Lorentzen et al., 1994; Lin and Shiau, 2005; Wang et al., 2006) of aquatic animals. The weight gains of rainbow trout (*Oncorhynchus mykiss*) (Kucukbay et al., 2009), hybrid striped bass (*Morone chrysops* × *M. saxatilis*) (DoF, 2013) and juvenile grouper (*Epinephelus malabaricus*) (Lin and Shiau, 2005)respond positively to dietary OS supplementations. Current study showed that the application of dietary supplementation of Sel-Plex® as a source of OS, can significantly improve the growth and survival of marron. Sel-Plex® is also known as selenoyeast that contains selenoprotein. It is a baker's yeast dried product, derived from *Saccharomyces cerevisiae* strain CNCM I-3060, cultivated in a Se-enriched fermentation medium to

provide a high level of selenomethionine (Burdock and Cousins, 2010). Selenomethionine may be incorporated into proteins in place of methionine or be metabolized to Selenocysteine (Esaki et al., 1982; Schrauzer, 2003; Fox et al., 2004). The present results also showed that 0.2 g kg^{-1} of dietary Sel-Plex® can significantly improve marron's survival irrespective of being challenged with *V. mimicus.* Similarly, improved survival of Taura Syndrome Virus (TSV)-infected shrimp (*P. vannamei*) fed Sel-Plex® as a source of OS has also been reported (Sritunyalucksana et al., 2011b).

Sel-Plex® mainly contains selenomethionine and has high bioavailability and appears to be 90% absorbed (Robouch et al., 2011; DoF, 2013). Following absorption, selenomethionine is metabolized to other forms of selenium, such as hydrogen selenide, which is the key metabolite derived from the inorganic form of Se, selenite or selenate, and/or is diverted into pathways of methionine metabolism and finally stored as selenoprotein. Active selenoprotein as a type 1 iodothyronine 5′-deiodinase interacts with iodine and prevents abnormal hormone metabolism (Burk and Hill, 1993; Foster and Sumar, 1997), which can be reflected in higher growth. In addition, OS can be deposited in muscle tissues longer than inorganic selenium and retained in muscles and hepatopancreas as selenoprotein for about three years. OS is extensively utilized and re-utilized to maintain *status quo* of selenium in animals to sustain growth performance and boost immune competence (Bell et al., 1987; Aguilar et al., 2009).

The number of THC decreases due to various stressors including pathogen infections(Sahul Hameed et al., 2006; Sang et al., 2009). The decrease in THC is related to defence activities of haemolymph and haemolymph lysis (Omori et al., 1989; van de Braak et al., 2002b; Sang et al., 2009). The present study indicatedthat THC of both controlled sub-groups and infected-sub-groups were significantly reduced after getting infected with *V. mimicus*. However, the marron fed Sel-Plex® supplementation were healthier as shown by their higher number of THC following 24 h post-challenge. Sel-Plex® supplementation in the diet stabilizes the proportion of circulating granular cells which play an important role in defence against bacterial infection (Hryniewiecka-Szyfter and Babula, 1996) of marron through their phagocytic activities. Past research has shown that animals with better phagocytic

activity and clearance efficiency have higher disease resistance (Ratcliffe et al., 1985; Alday-Sanz et al., 2002; van de Braak et al., 2002c; Sang et al., 2009). In this study, the capability to reduce invasive pathogen, *V. mimicus*, were significantly increased following supplementation of the Sel-Plex® in the diet, which in turn led to increased resistance against *V. mimicus* (Soderhall, 1997; Bachère, 2003).

The underlying mechanism(s) whereby dietary Sel-Plex® boosts the resistance of marron against *V. mimicus* is not properly understood. However, Alina et al. (2009) stated that Se enriched diet is assimilated into enzymes, such as antioxidant and protein which are important in improving immunity. Se, as an active agent plays a role in protecting cell compartments and cell membranes against lipid peroxidation due to pathogen infection (Rameshthangam and Ramasamy, 2006) and promote antioxidant activity in the body via glutathione peroxides (GPX), a Se-dependent enzyme which is a primary antioxidant enzyme for cellular defence against oxidative stress (Han et al., 2011). The inclusion of Sel-Plex® in the diet can increase the level of glutathione peroxides (GSH-Px), a main antioxidant enzyme that prevents cellular damage from free radicals (Sritunyalucksana et al., 2011b). GSH-Px is also associated with increasing cellular membrane stability and is linked with phospholipids hydroperoxide (PHGSH-Px), associated with the plasma membrane. In Addition, GSH-Px plays a main role in the protection of biological membrane integrity, especially during bacterial infection (Winston and Di Giulio, 1991). During bacterial or viral infection in the haemolymph, the level of lipid peroxidation is increased, due to the increased oxidative stress and induced peroxidation of membrane lipids. The increased level of lipid peroxidation can lead to a decreased membrane fluidity and membrane disorganization (Mohankumar and Ramasamy, 2006). A study of amphipods (*Gammarus locusta*) has showed that the high level of lipid peroxidation is triggered by decreasing antioxidant enzyme activity (Correia et al., 2003). Thus, it is possible that adding selenium in the diet may induce the antioxidant activity, in order to reduce the lipid peroxidation and enhance the lysosomal membrane stability during the bacterial invasion.

It is widely accepted that haemolymph of crustacean is the main internal defence against pathogens (Itami et al., 1989; Shiu-Nan, 1992; Bachère et al., 1995). Thus, the number of bacteria in the haemolymph can be used as an indicator to evaluate the

health of the animal. A low number of bacteraemia levels in the haemolymph indicates an improvement in the immune system, health status and possibly decreased susceptibility to infections (Fotedar et al., 2001). Current results showed that the marron fed Sel-Plex® in the diet pre and post-24 h challenge with *V. mimicus*, had lower *Vibrio* ranks than marron fed without OS. Similarly, low levels of bacteraemia in the haemolymph were also reported in Bio-Mos®-fed infected marron (Sang et al., 2009) and western king prawn (*Penaeus latisulcatus*) fed a combination of two probiotics, *Pseudomonas synxantha* and *Pseudomonas aeruginosa* (Hai et al., 2007; Hai and Fotedar, 2009).

Bacterial infections may alter the stability of the lysosomal membrane of marron and Chinese shrimp (*Fenneropenaeus chinensis*) and can be evaluated by using the neutral red retention time (NRRT) (Cui-Luan et al., 2008; Sang et al., 2009). The unhealthy cells, caused by decreasing lipid membrane integrity due to bacterial infection lose neutral red dye at a faster rate than healthy cells. In this study, lysosomal membrane integrity was affected by the injection of *V. mimicus*, as indicated by longer NRRT on 72-hour post-challenged marron fed Sel-Plex®. It is possible that dietary selenium can induce the lysosomal membrane stability and reduce the lipid peroxidation (Ursini and Bindoli, 1987). A similar finding was found in marron wherein lysosomal membrane stability increased in Bio-Mos®-fed marron (Sang et al., 2009).

6.5 Conclusions

Supplementing 0.2 g kg^{-1} of Sel-Plex®, which equates to approximately 0.4 mg kg^{-1} OS in the diet of marron, is recommended to enhance growth performance, survival and disease resistance against *V. mimicus*. Further research needs to be conducted to validate the effects of Sel-Plex® supplementation on antioxidant enzymes activity, such as glutathione peroxide, superoxide dismutase and catalase as well as levels of lipid peroxidase (Watanabe et al., 1997; Marnett, 1999) that are related to the health and immunity of marron.

CHAPTER 7: Comparing the Effects of Dietary Selenium and Mannan Oligosaccharide on the Health, Immune Function and Antioxidant Enzyme Activity in Cultured Marron *Cherax cainii* (Austin, 2002)

7.1 Introduction

Many diets enriched with various supplements have been used in aquaculture to improve the growth and health of cultured animals (Dörr et al., 2008; Chiu et al., 2010; Sang and Fotedar, 2010b). Selenium (Se) and mannan oligosaccharide (MOS) are common dietary supplements that have been used in cultured prawns (*Macrobrachium rosenbergii*) (Chiu et al., 2010), marron (*Cherax tenuimanus*) (Sang et al., 2011b), and rainbow trout (*Oncorhynchus mykiss*) (Kucukbay et al., 2009). In nature, Se is present in both inorganic and organic forms. In the inorganic form, Se is present as selenate or selenite, whereas in the organic form, it is present as selenocysteine and selenomethionine (Barceloux, 1999). Both inorganic and organic forms of Se have a pivotal role in the normal metabolism that enhances health, immune function, and antioxidant enzyme activity (Abdel-Tawwab et al., 2007; Han et al., 2011). Another effective supplement, MOS, derived from the cell wall of the yeast, *Saccharomyces cerevisiae*, is used as an effective dietary immunostimulant for improving growth in yabbies (*C. destructor*) (Sang et al., 2011b) and survival in rainbow trout (Dimitroglou et al., 2008). In addition, dietary MOS amplifies antioxidant enzyme activity in sea cucumbers (*Apostichopus japonicus)* (Gu et al., 2011).

Antioxidant enzymes, either glutathione S-transferase (GST) or glutathione peroxidase (GPx), protect cellular tissues and membranes against oxidative damage caused by free radicals (Michiels et al., 1994; Felton, 1995; Felton and Summers, 1995). GST and GPx have also been identified as antioxidant agents to reduce lipid peroxidase (LPO) generated by oxidative metabolism (Monteiro et al., 2009; Lavarías et al., 2011); therefore, it is imperative to maintain the highest possible levels of GST and GPx activity in the haemolymph to counteract the damaging impacts of free radicals. Both GST and GPx convert hydrogen peroxide and fatty acid hydroperoxides into water and fatty acid alcohol through the reduced glutathione (GSH) pathway (Watanabe et al., 1997). The GST and GPx activities can

be quantified as GSH, which is consumed per minute during the free-radical deactivation process.

LPO is known as an indicator of oxidative stress in cells and tissues. Malondialdehyde (MDA) naturally occurs during the lipid peroxidation process; therefore, MDA levels can also be used to monitor LPO (Marnett, 1999). Increasing levels of MDA are associated with various conditions and pathological states of animals; therefore, MDA levels can be measured to quantify LPO and can be used as a tool to monitor the health of an animal. In addition to MDA, neutral-red retention time (NRRT) has been used to evaluate lysosomal membrane stability and, in turn, membrane stress levels (Lowe and Pipe, 1994; Dailianis et al., 2003). The stability of animal cell membranes decreases as the level of LPO increases (Ochoa et al., 2003; Catalá, 2006). In addition to LPO, total and differential haemocyte counts (THC and DHC) are common tools that can be used to monitor the health of cultured animals such as the marron, *C. cainii* (Sang et al., 2009).

As an important cultured species in Western Australia, marron, the third largest crayfish in the world, is recognized as a valuable aquaculture commodity (Nobes, 2011). Increasing market demand of the marron has led to a significant boost in research to improve the health performance of the animal; however, the information regarding the effects of Se and MOS dietary supplements on the growth, survival, immunocompetence, and antioxidant enzyme activity of marron is limited. Thus, the current study was designed to evaluate growth, survival rate, GST, GPx activity, LPO, NRRT, and total Se levels in the haemolymph of marron fed both inorganic and organic forms of Se and MOS.

7.2 Materials and Methods

7.2.1 Experimental diets

Three different types of supplements were added to the basal diet of cultured marron. Either 0.4 mg kg^{-1} sodium selenate as a source of inorganic Se (IS), or 0.2 g kg^{-1} (approximately equates to 0.4 mg kg^{-1} OS) Sel-Plex® as a source of organic Se (OS) that contains at least 98% OS, of which 62–68% is selenomethionine (Burdock and Cousins, 2010), and 0.4% Bio-MOS® (MOS) was added to the basal diet. All levels of supplementation were selected as described in previous experiments (Sang and

Fotedar, 2010b; Nugroho and Fotedar, 2013a). Dietary ingredients and the composition of the basal diet were formulated using Feed LIVE® version 1.52, Live Informatics Company Limited, Nonthaburi, Thailand (Table 7.1). All chemicals and reagents were purchased from Sigma-Aldrich Chemical Company, St Louis, USA, while the ingredients for the basal diet were supplied from Specialty Feed, Pty. Ltd. (Glen Forrest, Western Australia), except for Sel-Plex® and Bio-MOS®, which were donated by Alltech, Nicholasville-USA. All ingredients were thoroughly mixed with sodium selenate, Sel-Plex®, or Bio-Mos® to prepare the test diets. Each test diet or basal diet was mechanically minced to obtain a uniform particle size (0.5 mm in diameter). The resulting strands were then dried in direct sunlight for 6 h and allowed to cool at room temperature. The dried strands were broken into 3 mm lengths and stored in a dark room.

Table 7.1: Ingredients of basal diet (g kg^{-1}) and proximate composition of the diets

Ingredients	Content (g kg^{-1})
Fish oil[1]	32
Wheat bran	545.59
Soybean meal	101.5
Fish meal[2]	257.14
Calcium carbonate	0.2
Ascorbic acid	0.5
Betaine[3]	12
Premix[4]	1.5
Cholesterol	2.5
Wheat starch	47.07
Proximate composition	**Percentage (%)**
Crude protein	27.05
Crude fat	8.02
Crude Fibre	6.39
Moisture content	9.01
Ash	6.56
Energy (cal/g)	1,833.249

All ingredients supplied by Specialty Feeds Pty Ltd, WA, Australia. [1]Cod liver oil, [2]Peruvian fishmeal, 56% CP. [3]Betaine Anhydrous 97%. [4]Commercial vitamin and mineral premix for trout. Actual Se in the basal diet = 0.89 mg kg^{-1} Se.

7.2.2 Animals and experimental setup

Marron were purchased from Blue Ridge Marron Farm, Manjimup Western Australia, and acclimated at Curtin Aquatic Research Laboratory (CARL), Curtin University, Western Australia, for 1 week. Marron were then randomly distributed into 4 triplicate groups (n = 12) consisting of 7 marron each. Each group was then placed in blue plastic cylindrical tanks (800 mm in diameter, 500 mm high, 250-L capacity, 70 L freshwater in each tank). Mechanical filtration (fluvial 205 filters; Hagen, Mansfield, Massachusetts, USA) by filtering at a rate of approximately 2 L min^{-1} was used in each tank. Seven PVC pipes (55 mm in diameter, 150 mm long) were also placed in each tank to provide shelter for each marron. The water temperature was maintained at 24°C–25°C by using automatic heaters (Sonpar®, Model: HA-100; Yong Cheng Aquarium Co., Ltd., China). Dissolved oxygen was monitored and maintained at 6 ppm every 2 days using a CyberScan pH 300 (Eutech Instruments, Singapore, China). NO_2-N, NO_3-N, NH_3/NH^{+4}, PO_4-P, pH, were monitored every 2 days using chemical test kits (Aquarium Pharmaceuticals™, Inc., McLean, Virginia, USA). The marron in each tank were fed either the test or basal diet at a proportion of 3% of its body weight every other day. This feeding rate was determined by previous experiments (Sang and Fotedar, 2010b; Nugroho and Fotedar, 2013a). Uneaten food and faeces were siphoned out before the next feeding and sufficient fresh water was added to maintain 70 L of water in each tank.

7.2.3 Sampling and analytical procedure

Surviving marron from each tank were counted every 15 days until the end of the feeding trial. The marron haemolymph was taken from the base of the fifth thoracic leg of each marron at day 90. Following collection of haemolymph samples, THC, DHC, GST, GPx, LPO, and NRRT were determined. Total soluble Se levels in the marron haemolymph was also analysed after 90 days of feeding.

The weight of each marron from each tank was measured at days 0 and 90 of the feeding trial to calculate initial weight, final weight, and specific growth rate (SGR) by using the following equation:

$$SGR = 100 \times (\ln(Wt) - \ln(Wo))/(d)$$

Where Wt and Wo are the weight of the marron at current time (t) and at the commencement of the experiment (0), d = culture period (day), respectively.

7.2.4 Total and differential haemocytes counts

Total haemocyte counts (THC) and differential haemocyte counts (DHC) were determined using Rose Bengal to stain haemocytes (Sritunyalucksana et al., 2005). One-tenth of a millilitre of haemolymph was withdrawn into a syringe containing 0.1 mL fixative (10% formalin in 0.45 M NaCl) and transferred into an Eppendorf tube. After 10 min, the mixture was fixed using 20 µL Rose Bengal solution (1.2% Rose Bengal in 50% ethanol). The mixture was incubated at room temperature for 20 min and was used for determining THC with an improved Neubauer Bright-Line Haemocytometer, Sigma Aldrich, USA and to prepare smears on microscope slides for determining the DHC. THCs were determined and expressed as cell $\times 10^6$ cells mL^{-1} haemolymph. To calculate DHCs, smears were completely dried before counterstaining with hematoxylin solution for 8 min. The slide was then rinsed with tap water for 10 min, followed by dehydration with 95% ethanol (10 dips) and 100% ethanol (10 dips). After dehydration, the slide was submerged in xylene (thrice for 3 min each) before it was mounted with albumin and covered with a cover slip. The proportions of granular, semigranular, and hyaline cells from 200 haemocytes were recorded (Sritunyalucksana et al., 2011b).

7.2.5 Antioxidant enzyme activity and LPO assay

The haemolymph from individual marron was diluted with physiological saline at a ratio of 1:1 and stored at 4°C until used for determining GST, GPx, and LPO. GST was measured using an UV-visible spectrophotometer (Habig et al., 1974). A mixture containing 200 µL phosphate buffer (pH 6.5), 20 µL 1-chloro-2,4 dinitrobenzene (CDNB) in 95% ethanol, and 730 µL distilled water was placed in a control tube, and 200 µL 0.5 M phosphate buffer, 20 µL of 25 mM CDNB, and 680 µL distilled water were placed in test tubes. Both the control and test tubes were incubated at 37°C for 10 min. After incubation, 50 µL 20 mM GSH were added to both sets of tubes and mixed. After mixing, 50 µL haemolymph was added to the test tubes. An increase in absorbance was noted at 340 nm for 5 min. Values were

expressed in nanomoles of CDNB conjugated per minute per milligram of protein. Protein was estimated using the method described by Lowry et al. (1951).

To calculate GPx activity, 0.2 mL of 0.4 M phosphate buffer (pH 7.0), 0.1 mL 10 mM sodium azide, 0.2 mL 0.2 mM reduced glutathione, and 0.1 mL 0.2 mM hydrogen peroxide were mixed. The mixtures were then incubated for 10 min at 37°C after which 0.4 mL of 10% trichloroacetic acid (TCA) was added to stop the reaction, and the mixtures were centrifuged at 3200 rpm for 20 min. The supernatant was assayed for glutathione content using Ellman's reagent (19.8 mg 5,5′-dithiobis-[2-nitrobenzoic acid] [DTNB] in 100 mL 0.1% sodium nitrate). The GPx activity was expressed as micrograms of GSH consumed per minute per milligram of protein (Rotruck et al., 1973; Lawrence et al., 1974).

LPO of marron haemolymph was determined by measuring the concentration of thiobarbituric acid reacting substances (Buege and Aust, 1978). One hundred microliters of marron haemolymph were mixed with 500 μL mixture solution containing 15% (w/v) TCA, 0.375% (w/v) thiobarbituric acid, and 80% (v/v) hydrochloric acid 0.25 N. The mixture was heated to 100°C for 15 min and cooled at room temperature. The mixture was then centrifuged at 1500 rpm for 10 min. Absorbance in the supernatant was measured at 535 nm. The level of LPO was expressed as nmol malondialdehyde (MDA) per mg protein. Protein in the haemolymph was estimated by the method of Lowry et al (1951).

7.2.6 Neutral red retention time

NRRT was determined using assays based on previous protocols (Hauton and Smith 2004). To prepare a stock solution, 10 mg neutral-red powder was dissolved in 1 mL dimethyl sulphoxide. A working solution (dye concentration 0.02 mg mL^{-1}) was prepared by mixing 10 mL stock solution and diluting with 5 mL artificial saline water. Then, 0.2 mL marron haemolymph sample was transferred to an Eppendorf tube containing 0.2 mL artificial saline water and gently mixed. A sample of the mixture was placed onto a microscope slide treated with a poly-L-lysine solution to increase cell adhesion. The slide was immediately placed in a 10°C incubator for 15 min to allow the haemocytes to attach to the slide. The slide was then immediately

removed from the incubator and the excess haemolymph was removed. Forty microlitres neutral-red working solution was added to the slide and covered with a cover slip. The slide was then returned to the incubator. The slide was removed from the incubator every 15 min and the sample was examined under a light microscope (400× magnification, Olympus SC30 BH2; Olympus, UK) for 2 min. The time at which 50% of the haemocytes had begun to lose dye from their lysosomes was recorded as the NRRT of the marron lysosomal membrane.

7.2.7 Determination of total soluble selenium

At the end of the trial, three marron from each group were sacrificed to determine the total soluble Se in haemolymph. Total Se was determined by spectrophotometric method according to Revanasiddappa and Dayananda (2006).

7.2.8 Statistical analysis

Results are expressed as means ± standard error (SE), and data were analysed using SPSS version 17. All percent data of survival was transformed to arcsine before prior to one way ANOVA The data of initial weight, final weight, SGR, THC, DHC, antioxidant enzymes activity (GSH and GPx), LPO, NRRT, and total soluble Se accumulation on day 90 of feeding were subjected to one-way ANOVA, followed by the Duncan post-hoc test to evaluate significant differences among the groups of supplements. All significant tests were at $P < 0.05$ levels.

7.3 Results

All dietary supplements significantly improved ($P < 0.05$) the final weight, SGR, and survival, and resulted in significant differences in mean THCs and the mean proportion of hyaline cells in the marron haemolymph. Supplementing with dietary MOS resulted in higher final weight and SGR of marron than any other supplement (Table 7.2). The highest survival (Figure 7.1) and THC (Table 7.3) were found in marron fed dietary Sel-Plex®, whereas the lowest mean hyaline cells were found in the marron fed MOS.

Table 7.2: Mean ± SE growth performance of marron fed different supplements

Parameters	Types of supplements			
	BD	IS	OS	MOS
Initial weight (g)	38.68 ± 0.18^{a}	43.17 ± 2.13^{a}	39.57 ± 1.72^{a}	38.80 ± 0.99^{a}
Final weight (g)	40.54 ± 0.28^{a}	45.90 ± 0.82^{b}	45.50 ± 0.91^{b}	47.33 ± 0.89^{b}
SGR (% day^{-1})	0.052 ± 0.006^{a}	0.070 ± 0.003^{ab}	0.157 ± 0.025^{bc}	0.22 ± 0.008^{c}

Different superscript alphabets in the same row indicate significantly different means $P < 0.05$. BD = basal diet; IS = 0.4 mg kg^{-1} Se (sodium selenate was used as a source of inorganic Se); OS = 0.2 g kg^{-1} Sel-Plex®, approximately equates to 0.4 mg kg^{-1} of organic Se; MOS = mannan oligosaccharide (0.4% of Bio-MOS®).

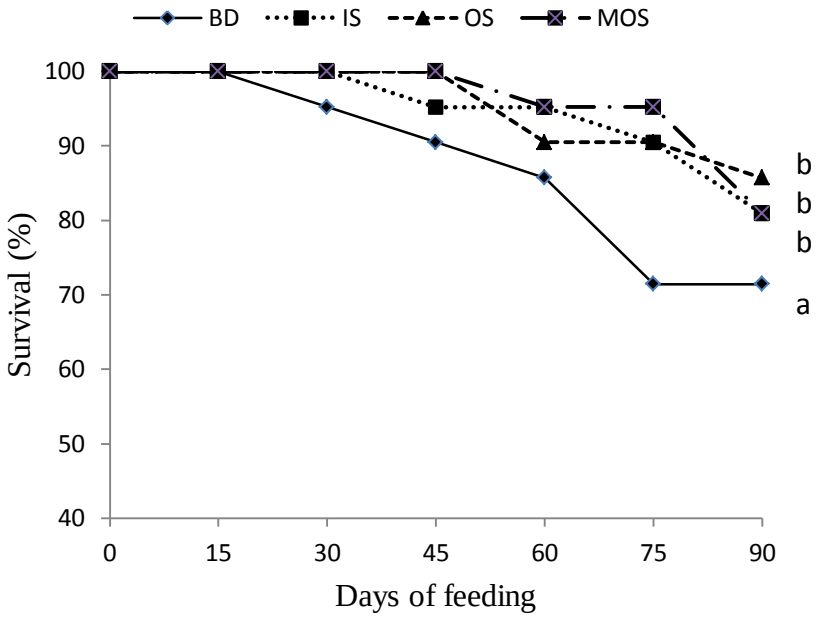

Figure 7.1: Survival of marron fed different diet supplementations. BD = basal diet; IS = inorganic selenium (0.4 mg Se kg^{-1} of sodium selenate); OS = organic selenium (0.2 g kg^{-1} of Sel-Plex®); MOS = mannan oligosaccharide (0.4% of Bio-MOS®). Different alphabets denote significant difference ($P < 0.05$).

After 90 days of feeding, the mean proportion of granular cells was significantly higher ($P < 0.05$) in the marron fed MOS, but there were no significant differences ($P > 0.05$) in the marron fed either the control diet or any source of selenium. The proportion of semigranular cells were significantly different between the control group and marron fed either IS or MOS (Table 7.3).

Table 7.3: Mean ± SE immunocompetence of marron fed different supplements

Parameters	Types of supplements			
	BD	IS	OS	MOS
THC	1.76 ± 0.06^{a}	2.41 ± 0.05^{b}	3.13 ± 0.08^{c}	2.28 ± 0.03^{b}
Granular (%)	34.33 ± 2.02^{a}	23.66 ± 0.33^{a}	23.00 ± 1.52^{a}	59.00 ± 1.15^{b}
Semigranular (%)	35.00 ± 3.05^{a}	20.66 ± 1.76^{b}	27.33 ± 2.02^{ab}	19.66 ± 0.66^{b}
Hyaline (%)	34.33 ± 1.45^{a}	55.66 ± 1.45^{b}	50.33 ± 1.85^{b}	20.66 ± 0.88^{c}

THC = Total haemocyte count (cells x 10^6 mL). Different superscript alphabets in the same row indicate significantly different means $P < 0.05$. BD = basal diet; IS = 0.4 mg kg^{-1} Se (sodium selenate was used as a source of inorganic Se); OS = 0.2 g kg^{-1} Sel-Plex®, approximately equates to 0.4 mg kg^{-1} of organic Se; MOS = mannan oligosaccharide (0.4% of Bio-MOS®).

Inclusion of any types of feed supplement increased the antioxidant activity in the marron. No significant difference in GST activity was evident among any marron fed with supplements. Adding Sel-Plex® to the diet resulted in significantly higher ($P < 0.05$) GPx activity, reduction in LPO levels, and improvement in NRRT in the marron haemolymph (Table 7.4). Marron fed IS or Sel-Plex® supplements showed significantly higher ($P < 0.05$) total soluble Se in the haemolymph than those fed the control and MOS supplements. The highest total Se in the haemolymph was found in marron fed Sel-Plex® (Table 7.4).

Table 7.4: Antioxidant enzymes activity, LPO activity, NRRT and total soluble Se in the haemolymph of marron fed different supplementation for 90 days of feeding trial

Parameters	Types of supplements			
	BD	IS	OS	MOS
GST	159.70 ± 49.46^{a}	319.55 ± 7.64^{b}	329.49 ± 15.24^{b}	277.22 ± 47.01^{b}
GPx	198.04 ± 65.35^{a}	408.34 ± 8.62^{b}	616.61 ± 18.76^{c}	213.48 ± 9.74^{d}
LPO	0.126 ± 0.002^{a}	0.095 ± 0.006^{b}	0.067 ± 0.004^{c}	0.110 ± 0.004^{ab}
NRRT	65.00 ± 5.00^{a}	115.00 ± 5.00^{b}	150.00 ± 8.66^{c}	115.00 ± 5.00^{b}
Total soluble selenium	0.08 ± 0.01^{a}	0.41 ± 0.04^{b}	0.54 ± 0.05^{c}	0.17 ± 0.05^{a}

Different superscript numericals in the same row indicate significantly different means $P<0.05$. BD = basal diet; IS = 0.4 mg kg^{-1} Se (sodium selenate was used as a source of inorganic Se); OS = 0.2 g kg^{-1} Sel-Plex, approximately equates to 0.4 mg kg^{-1} of organic Se; MOS = mannan oligosaccharide (0.4% of Bio-MOS®). GST = Glutathione-S-transferase (nanomoles of CDNB conjugated min^{-1} mg $protein^{-1}$); GPx = Glutathione peroxidase (µg GSH consumed min^{-1} mg $protein^{-1}$); LPO = Lipid Peroxidase (nmol malondialdehyde-MDA mg^{-1} of protein); NRRT = Neutral red retention time (min). Total selenium soluble in the haemolymph expressed as µg mL^{-1}.

7.4 Discussion

The present study indicated that the basal diet supplemented with either sodium selenate, Sel-Plex®, or Bio-Mos® could equally improve SGR (Table 7.2) and marron survival (Figure 7.1). Current research also indicated that marron fed a supplement of MOS showed significantly higher SGR than those fed other supplements. This finding was in line with previous research that MOS inclusion in the diet significantly improved marron SGR (Sang and Fotedar, 2010b). According to Sang and Fotedar (2010b), MOS can improve marron SGR by improving digestive-tract health, such as increasing the number of beneficial bacteria and villi density in the gut as well as enhancing epithelium thickness.

In addition to improving SGR, the current study showed that any source of Se supplementation and MOS inclusion in the diet enhanced marron survival rate; however, there was no differential effect of Se and MOS supplementation on survival. These results were concomitant with previous research on prawns fed an Se-enriched diet (Torrecillas et al., 2007) and cobia (*Rachycentron canadum*) larvae fed MOS that showed similar increases in survival; however, organic Se had a greater impact than inorganic Se on the survival of cultured channel catfish (*Ictalurus punctatus*) (Wang and Lovell, 1997) and rainbow trout (Kucukbay et al., 2009).

The results of the present study indicate that, because of their positive influence on the immune system, supplementation of diet with both IS and Sel-Plex® can increase THCs in marron. Supplementing the diet with both Se forms can affect the proportion of circulating haemocytes without affecting the proportions of granular cells. The mechanism by which dietary inclusion of Se alters the haemocyte profile of marron is not yet fully understood; however, Alina et al. (2009) stated that an Se-enriched diet can be assimilated into enzymes and antioxidants, which are important in body development and immunity. Meanwhile, Bio-Mos®, which is derived from the outer wall of *S. cerevisiae,* has also been proven to enhance an animal's performance, reinforce the natural defences, and stimulate immune response (Sang et al., 2011b). Current results showed that MOS supplementation increases THCs and the mean proportion of granular cells of marron, but it reduces the proportion of hyaline cells. Dietary MOS can increase phagocytic cell activity (Yoshida et al.,

1995; Savage et al., 1996) and inhibit the attachment and penetration of bacteria into cells (Swanson et al., 2002), thereby confirming the mode of action to improve immune function and antioxidant activity in marron.

In addition to enhancing immunity, inclusion of any type of dietary supplement in the marron's diet resulted in higher GST and GPx. Present findings showed that supplementing the diet with Sel-Plex® had the highest impact on the GPx activity. Dietary Sel-Plex® also had a beneficial effect on membrane stability compared to IS or MOS supplements. These findings were in agreement with a previous study that Sel-Plex® has more positive effects on GPx activity than does IS (Cotter, 2006). GPx makes cellular and subcellular membranes less sensitive to oxidative damage and reduces free radicals by scavenging, binding, and inactivating free radicals to prevent damage to the cells (Winston and Di Giulio, 1991; Michiels et al., 1994; Felton et al., 1996). Furthermore, GPx makes cellular and subcellular membranes less sensitive to oxidative damage, which is related to the LPO level.

The present results showed that Sel-Plex®-treated marron had significantly lower LPO than any other marron group. This result was supported by Han et al. (2011), who stated that GPx activities of fish fed diets supplemented with OS were significantly higher than fish fed the control diet. In contrast, Chiu et al. (2010) revealed that GPx activities of prawns fed OS-enriched diets did not significantly differ from prawns fed the control diet. The activity of GPx of marron fed Se could contribute to the maintenance of LPO levels (Monteiro et al., 2009). LPO, specifically polyunsaturated fatty acid (PUFA) oxidation, is highly unstable and can cause damage to the cellular biomembranes as a consequence of oxidative deterioration of bipolar lipid membranes (Kanazawa, 1991, 1993). Furthermore, increased levels of LPO may lead to decreased membrane fluidity and membrane disorganization (Mohankumar and Ramasamy, 2006).

It is possible that adding Se to the diet might induce the lysosomal membrane stability and minimize the number of unhealthy cells (Ursini and Bindoli, 1987). The unhealthy cells, caused by decreasing lipid membrane integrity, can lose neutral-red dye at a faster rate than healthy cells. Results of the present study show that lysosomal membrane integrity was affected by both forms of Se supplementation and

Bio-Mos®, as shown by the increase in NRRT. Furthermore, marron fed OS had the longest NRRT compared to those fed IS or MOS supplements.

The ability of animals to accumulate Se from dietary sources has been studied in several aquatic animals such as rainbow trout (Vidal et al., 2005) and juvenile grouper (*Epinephelus malabaricus*) (Lin and Shiau, 2007). Current research showed that both organic and inorganic forms of Se in the test diet significantly increased the total soluble Se in marron haemolymph; however, total soluble Se in the haemolymph of OS-fed marron was significantly higher than total soluble Se in the haemolymph of IS- and MOS-fed marron. Similar research stated that selenomethionine has been proved to have a higher bioavailability than sodium selenite in Atlantic salmon (*Salmo gairdneri*) (Lorentzen et al., 1994) and channel catfish (Wang and Lovell, 1997) and can be deposited as selenoprotein in the muscle and hepatopancreatic tissues of animals (Aguilar et al., 2009).

7.5 Conclusion

Supplementing the marron's basal diet with IS and Sel-Plex® along with MOS has beneficial effects on growth performance, survival, THC, DHC, and antioxidant activity. Further research needs to be conducted to trace the biological pathways of Se and MOS absorption into the marron's body tissues.

CHAPTER 8: Chronic and Acute Toxicity of High Levels Organic Selenium in Marron C*herax cainii* (Austin, 2002)

8.1 Introduction

Selenium (Se) is an essential trace element not only essential for human health but also required for the wellbeing of aquatic animals (Wang et al., 2006; Dörr et al., 2008). In aquatic animals, the Se uptake can be directly from water or diet but the dietary uptake constitutes the major pathway (Tinggi, 2003; Hamilton, 2004). Further, organic form of Se in the diet can be actively transported through intestinal membrane and then accumulated in various tissues including muscles (Navid, 2011).

As Se can be found in organic form, organic selenium (OS) is mainly approved to be used as a dietary supplementation in animals (Swanson et al., 1991; Barceloux, 1999; Zhan et al., 2010) as it shows higher absorbance, higher retention, lower toxicity and is more easily bioavailable than the inorganic form (Mahan and Parrett, 1996; Schrauzer, 2003; Taylor et al., 2005; Wang et al., 2007; Kucukbay et al., 2009).

Se plays an important role in growth and immune functions of aquatic animals such as, rainbow trout (*Salmo gairdneri*) (Hilton et al., 1980, 1982; Vidal et al., 2005), catfish ((*Ictalurus punctatus*) (Gatlin and Wilson, 1984), Atlantic salmon (*Salmo salar*) (Bell et al., 1987), juvenile grouper (*Ephinephelus malabaricus*) (Lin and Shiau, 2005), crucian carp (*Carassius auratus gibelio*) (Wang et al., 2007) and freshwater prawn (*Macrobrachium rosenbergii*) (Chiu et al., 2010). However, the presence of higher than required Se in the diet can be toxic to prawns and can reduce the growth in fish (Hilton et al., 1980; Wang et al., 2006). The high Se results in malfunctioning of protein synthesis, compromises the functionality of various proteins, reduces growth and increases mortality (Hamilton, 2004; Han et al., 2011). Besides this the natural availability of Se in the water and its toxicity effects are largely unexplored.

The studies on the effects of Se on the physiological and immune responses in grouper (Lin and Shiau, 2007), rainbow trout (Kucukbay et al., 2009) and gibel carp (Han et al., 2011) have been investigated by using various tools such as growth indices, Se retention, organosomatic indices, moisture content, total haemocyte count

(THC), differential haemocyte count (DHC) and osmoregulatory capacity (OC) (Jussila and Mannonen, 1997; Morrissy, 2002; Sang and Fotedar, 2004; Sang et al., 2009). However, there is no published research on the effects of high levels of dietary Sel-Plex® and acute toxicity level of Sel-Plex® in the culture medium on the smooth marron *Cherax cainii* (Austin, 2002), a commercial crayfish species cultured in Western Australia (Morrissy, 2002). Therefore, the aim of this experiment was to evaluate the effect of dietary high levels of Sel-Plex® on the growth, survival, physiological and immunological responses of marron and also to assess an acute toxicity level of Sel-Plex® from the water.

8.2 Materials and Methods

8.2.1 Basal and test diet preparation

To prepare a marron basal diet, commercial marron-diet was used which was supplied by West Feeds Pty Ltd, Western Australia. The proximate composition of the basal diet was: 8% moisture, 26% crude protein, 9% crude fat and 5% crude ash. The test diet was prepared using basal diet that was re-pelleted by mixing with 500 mL distilled water per kg of basal diet. To this mixture, 1; 2 and 3 g kg^{-1} of Sel-Plex®, as a source of OS, donated by Alltech Inc. USA was added to obtain three different test diets and in one portion no Sel-Plex® was used as a control diet. Each test diet was passed through a mincer to obtain strands of approximately 0.5 mm diameter. The resulting strands were then dried in direct sunlight for six hours and allowed to cool at room temperature. The dried strands were broken into pellets of 3 mm length and then stored in a dark room for further use.

8.2.2 Experimental animals and culture system design

In the first trial, the experiment was conducted in sixteen plastic cylindrical tanks (800 mm diameter, 500 mm high, 250 L capacity, 70 L of freshwater in each tank) at Curtin Aquatic Research Laboratory (CARL), Curtin University, Western Australia. The freshwater was filtered at a rate of approximately 2 L min^{-1} with a filter (fluval 205 filters, Hagen, USA), placed in each tank. Eight PVC pipes (55 mm diameter, 150 mm length) were placed in each tank to provide shelter for marron. One hundred twenty eight marron with an average initial body weight of 76.00 ± 0.58 g were supplied by Aquatic Resource Management Pty Ltd, Western Australia, and were

used for the 90 days of the feeding experiment. Marron were acclimated to the culture conditions for 1 week and were then randomly distributed to sixteen culture tanks (randomized blocks of four tanks with four replicates for each diet) at a density of eight marron per tank. Marron were fed every second day at a rate of 3% of their body weight. Any remaining uneaten test diet and faeces were siphoned out before the next feeding commenced. The required quantity of freshwater was added to maintain 70 L of water in every tank. Temperature was maintained at 20^{o}C by using automatic heaters (Sonpar®, Model: HA-100, China). The water quality parameters, such as temperature, pH and dissolved oxygen were monitored weekly using Cyberscan pH 300, Eutech Instruments, Singapore. Nitrate, nitrite and ammonium were also recorded weekly using chemical test kits (Aquarium Pharmaceuticals^{TM}, Inc., USA). Number of surviving marron were recorded daily to calculate the survival rate. The weight of each marron from each tank was measured using electronic balance (GX-4000, A&D Company, Ltd., Japan).

The second trial, a static bioassay acute toxicity experiment, was conducted using forty glass tanks (60x30x30 cm, 15 L of freshwater in each tank). Six PVC pipes (50 mm diameter, 140 mm length) were placed in each tank to provide shelter for marron. Nine freshwater solutions with 9 concentrations of Sel-Plex®, *viz.* 3; 6; 12; 24; 48; 96; 192; 384 and 768 g L^{-1} were prepared. Two hundred and forty marron with an average initial weight of 67.87 ± 0.55 g supplied by Aquatic Resource Management Pty Ltd, Western Australia were used for the 96-h of the acute toxicity test. Marron were acclimated to the culture conditions for 1 week before the commencement of the experiment and then were randomly assigned to each dilution of Sel-Plex® at a density of six marron per tank. The water quality parameters, such as temperature, pH and dissolve oxygen were monitored every 24-h using Cyberscan pH 300, Eutech Instruments, Singapore. Nitrate, nitrite and ammonium were also recorded using chemical test kits (Aquarium Pharmaceuticals^{TM}, Inc., USA). Surviving marron were recorded at 12; 24; 48; 72; 96-h to determine acute toxicity. The endpoint of this test was death which was defined as the cessation of the movement of all appendages as well as no heartbeat. Verification of the death was also performed by dissecting the marron.

8.2.3 Data collection

Growth and survival rates

In the trial one, all the marron were measured for total weight (W), carapace length (CL) and total length (TL) immediately after acclimation and 90 days after commencement of the experiment. Survival rates were calculated using equation in chapter 3.

Total and differential haemocyte count

The THC of marron were measured according to the procedure of Fotedar (2004). To estimate THC, haemocytometer (Neaubauer, Germany) under 100-fold magnification was used and haemocytes was counted in both grids. The mean was used as a total haemocyte count (THC). DHC was calculated using the procedure as describe in chapter 3,

Moisture contents and organosomatic indices

The wet hepatosomatic index (H_{iw}) and tail muscles to wet body weight ratios (T/B_w) were calculated according to the equations used by Fotedar (2004).

Haemolymph osmolality

The haemolymph osmolality (HO) of the marron was determined using a cryoscopy osmometer (Gonotec-osmomat 030-D, Gonotec GmbH, Germany) (Sang and Fotedar, 2004). To measure HO, 0.5 mL of haemolymph from the intersegmental membrane between the cephalothorax and the first abdominal segment from one marron from each tank (n=3) was extracted using a 0.5 mL hypodermic 23-gauge needle and syringe and syringe containing anticoagulant (0.1% glutaraldehyde in 0.2 M sodium cacodylate, pH 7.0). The haemolymph and blank anticoagulants osmolality were calculated using an equation as described by Sang and Fotedar (2004).

HO = 3 x osmolality of mix – 2 x osmolality of anticoagulant

The osmoregulatory capacity (OC) was measured based on the difference between the osmotic pressures of the haemolymph and of the external medium (Lignot et al., 2000).

Determination of total soluble Se

At the day 0 and 90 of the culture period, three marron (n=3) from each group of feeding experiment were sacrificed and used for analysing the total soluble Se from the hepatopancreas and the muscle tissues. Total soluble Se in hepatopancreas and muscles of marron were measured spectrophotometrically (Revanasiddappa and Dayananda, 2006).

Determination of LC_{50}

In the trial two, the concentration of OS which results in 50% of the marron mortality during 96 h of static bioassay test (known as LC_{50}), percentage of mortality, control mortality (percentage of mortality at zero concentration of Sel-Plex®) and probit value were calculated based on probit model proposed by Finney (1971). The corrected mortality was performed using Abbott's correction (Abbott, 1925). The purpose of the control mortality calculation was to determine whether any marron died due to factors other than Sel-Plex® exposure. Control mortality was then used to calculate corrected mortality using Abbott's equation:

$$\text{Corrected mortality (\%)} = \frac{\text{M(obs)-M(control)}}{\text{100-M(Control)}} \text{ x } 100$$

Where: M(obs) = the number mortality at the concentration test; M(control) = control mortality

Probit value was determined using probit table, referring to corrected mortality. LC_{50} was then estimated using regression of Log_{10} concentration against probit value.

8.2.4 Statistical analysis

Statistical analysis was performed using SPSS software version 17. All data were expressed as means ± SE (standard error). Analysis of variance (ANOVA) followed by Duncan's *post hoc* analysis was used to determine significant differences in growth, survival, physiological and immunological parameters of the marron fed different levels of Sel-Plex® supplemented diets . Differences among means were considered significant at $P<0.05$.

8.3 Results

During the 90 days of the feeding high levels of Sel-Plex® and 96-h of acute exposure to Sel-Plex®, water parameters such as nitrite, nitrate, and total ammonia-N were within the normal range for marron's growth. The average temperature was recorded within optimum ranges of 19-22.5^{o}C and dissolve oxygen were never below 5.50 mg L^{-1}.

In trial one, after 90 days of the feeding, there were no significant differences (*P>0.05*) either in total body weight, total length, or carapace length and survival of marron among any feeding groups of marron (Table 8.1). Marron when fed any levels of Sel-Plex® in the diet showed the reduction in the final weight and total length. The lowest final marron weight (75.95 ± 1.27 g) was when marron were fed 3 g kg^{-1} of Sel-Plex®, whereas the lowest total length (140.05 ± 1.93 mm) was of the marron fed 2 g kg^{-1} of Sel-Plex®. Feeding 1 g kg^{-1} of Sel-Plex® to marron resulted in lowest carapace length (59.33 ± 1.52 mm).

Table 8.1: Final body weight (g), total length (mm), and carapace length (mm) of marron fed different diets supplemented Sel-Plex®, respectively for 90 days (Mean ± SE)

Parameters	Sel-Plex® inclusion levels (g kg^{-1})			
	Control	1	2	3
Final body weight	77.46±3.68^{a}	77.72±1.88^{a}	78.09±0.15^{a}	75.95±1.27^{a}
Total length	143.25±0.25^{a}	141.31±1.56^{a}	140.05±1.93^{a}	141.06±1.03^{a}
Carapace length	60.33±1.15^{a}	59.33±1.52^{a}	60.33±1.52^{a}	60.66±0.57^{a}
Survival	84.37±3.12^{a}	81.25±3.60^{a}	81.25±3.60^{a}	78.12±3.12^{a}

Mean in the same row within the same parameter (Final body weight, total length, carapace length) sharing a common alphabetical superscript letter are not significantly different at *P<0.05*.

The moisture contents, organosomatic indices, osmoregulatory capacity and haemolymph osmolality of marron fed the four diets are shown in Table 8.2. No significant differences in the HM%, Hiw, Hid, Tid, OC, and HO in the marron fed any levels of Sel-Plex® was observed. At the end of the culture period, there was a

significant increase (*P<0.05*) in the TM% of the marron fed Sel-Plex® and the highest Tiw (18.16 ± 0.48) was achieved in marron fed 2 g kg^{-1} of Sel-Plex®.

The THC and the percentage of granular cell were not affected by the dietary high levels of Sel-Plex®. However, the proportion of semigranular cells was significantly lower in marron fed Sel-Plex® higher than 1 g kg^{-1}. Meanwhile, the highest proportion of hyaline cells was found in the marron fed 2 g $kg^{-1}$of Sel-Plex® (Table 8.3).

Table 8.2: Physiological responses of marron fed different diets supplemented with Sel-Plex® for 90 days.

Parameters	Sel-Plex® inclusion levels (g kg^{-1})			
	Control	1	2	3
HM%	27.91±5,63[a]	41.46±6,30[a]	44.50±6,31[a]	40.28±3,67[a]
TM%	72.45±0.59[a]	75.66±1.48[b]	77.24±0.51[b]	78.61±0.30[c]
Hiw	5.62±0.23[a]	5.49±0.89[a]	6.24±0.38[a]	7.22±0.53[a]
Hid	4.07±0.41[a]	3.09±0.30[a]	3.47±0.47[a]	4.29±0.33[a]
Tiw	15.10±1.23[a]	14.85±0.67[a]	18.16±0.48[b]	17.28±1.11[ab]
Tid	4.16±0.37[a]	3.58±0.12[a]	4.13±0.07[a]	3.70±0.28[a]
OC	0.374.75±9.38[a]	0.365.00±6.05[a]	0.365.25±9.19[a]	0.364.75±6.25[a]
HO	385.50±10.26[a]	379.00±6.01[a]	373.00±7.42[a]	366.25±6.08[a]

Data in the same row sharing a common superscript are not significantly different at *P<0.05*. HM%=hepatopancreas moisture content; TM%=Tail muscle moisture content; Hiw=wet hepatosomatic index; Hid=dry hepatosomatic index; Tiw=wet tail muscle index; Tid=dry tail muscle index; OC=osmoregulatory capacity (mOsM kg^{-1}); HO=haemolymph osmolality (mOsM kg^{-1}).

Table 8.3: Immunological parameters (mean ± SE) of marron fed without (control) or with diets supplemented with Sel-Plex®, respectively for 90 days.

Parameters	Sel-Plex® inclusion levels (g kg^{-1})			
	Control	1	2	3
THC (x10^6 cells/ml)	1.83±0.21[a]	2.50±1.59[a]	3.21±1.03[a]	2.65±0.32[a]
Granular cell (%)	65.33±0.69[a]	67.66±0.40[a]	59.00±0.68[a]	63.00±0.29[a]
Semi-granular cell (%)	7.66±0.17[a]	8.00±0.92[a]	4.00±0.57[b]	5.33±0.66[b]
Hyaline cell (%)	27±0.96[a]	24.33±0.88[ab]	37.00±0.52[b]	31.67±0.29[ab]

Data in the same row sharing a common superscript are not significantly different at *P<0.05*. Means in the same row having different superscript letters (a, b, c) are significantly different at α level of 0.05.

After 90 days of feeding, the Sel-Plex® inclusion in the diets increased the total Se levels in hepatopancreas and muscle tissues. The total soluble Se in the hepatopancreas showed higher accumulation (*P<0.05*) when marron were fed diets with 3 g kg^{-1} of Sel-Plex® than the rest. Meanwhile, the total soluble Se in the muscle of marron fed any level of Sel-Plex® found no significant differences (Figure 8.1).

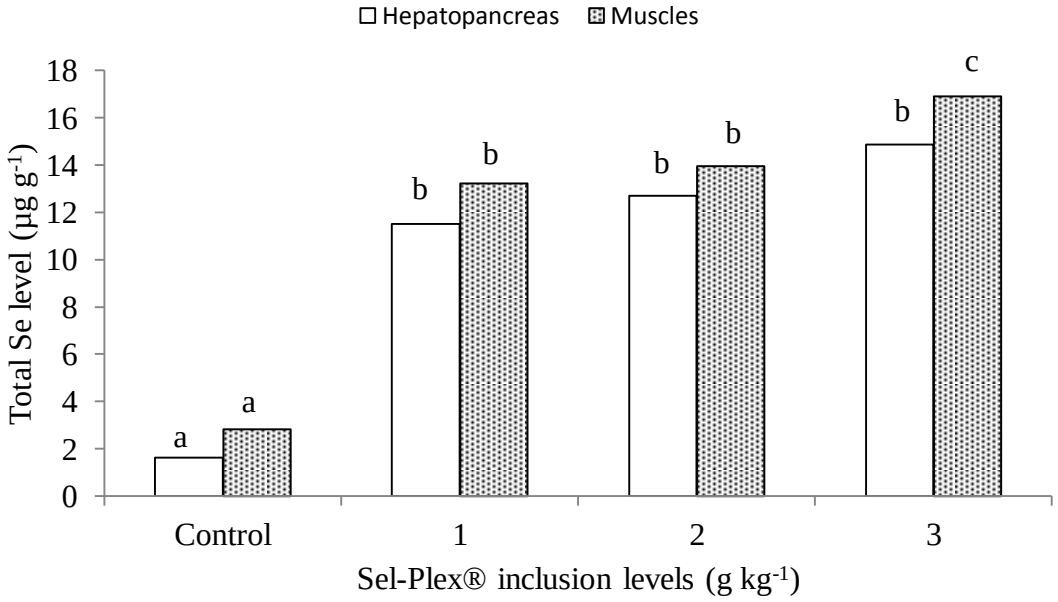

Figure 8.1: Total selenium level in the hepatopancreas and muscle of marron fed different high levels Sel-Plex® in the diet for 90 days.

In trial two, LC_{50} 96-h of Sel-Plex® was 166.28 g L^{-1} (Figure 8.2). The highest mortality (83.33%) occurred in a group where marron were exposed to 786 g L^{-1} (Table 8.4).

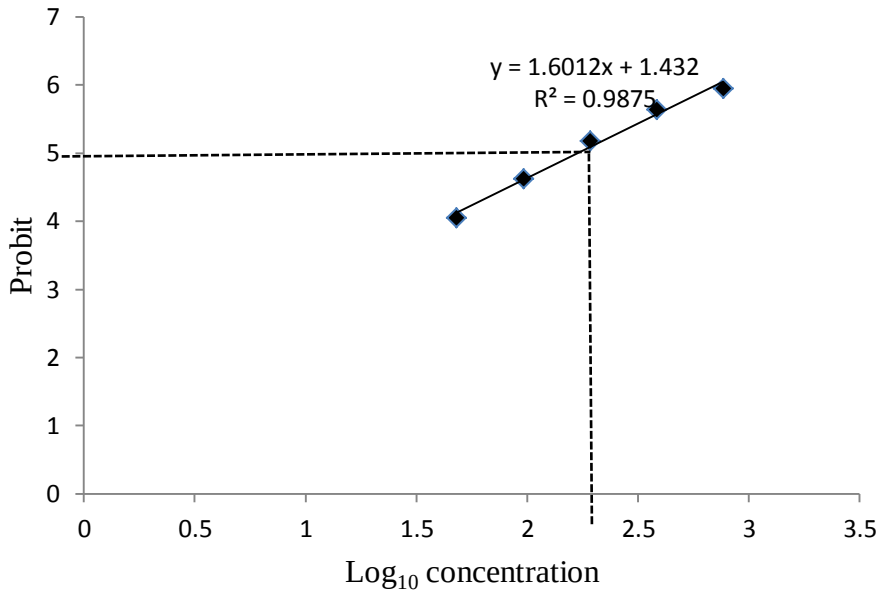

Figure 8.2: Determination of $LC_{50\ 96\text{-}h}$ of Sel-Plex®. $LC_{50\ 96\text{-}h}$ was found at 166.28 g L^{-1} of Sel-Plex®.

Table 8.4: Probit analysis calculation of acute toxicity test of Sel-Plex® in marron

Concentration (mg L^{-1})	Log_{10} Concentration	Total Marron	Number of mortality	Mortality (%)	Corrected mortality (%)	Probit
0	-	24	1	4.16	-	-
3	0.4771	24	0	0	0	-
6	0.7781	24	0	0	0	-
12	1.0791	24	0	0	0	-
24	1.3802	24	1	4.16	0	-
48	1.6812	24	5	20.83	17.39	4.05
96	1.9822	24	9	37.5	34.78	4.62
192	2.2833	24	14	58.33	56.52	5.18
384	2.5843	24	18	75	73.91	5.64
768	2.8853	24	20	83.33	82.60	5.95

8.4 Discussion

The effects of dietary Se have been studied intensively in aquatic animals (Wang and Lovell, 1997; Alina et al., 2009; Thomas and Janz, 2011). The current experiment is the first attempt to investigate the effects of high levels of Sel-Plex® on the

physiological responses and the immunological parameters of marron. Results showed that the growth performance and survival of marron were independent of dietary Sel-Plex® levels, which is consistent with the previous research that high dietary Se had no effect on the length and weight of prawns (*Penaeus vannamei)* (Wang et al., 2006). However, the negative impact of high Se level (15 µg g^{-1}) causes a reduction in growth performance of rainbow trout (*Salmo gairdneri*) due to the toxic physiochemical properties of Se (Hilton et al., 1980; Wang et al., 2006; Kucukbay et al., 2009), especially inorganic Se. The dietary inorganic Se has similar chemical and physical properties as sulphur-containing enzymes that can interfere with sulphur metabolism causing Se toxicity. The Se toxicity can also be caused by substituting sulphur with Se in thiol containing protein. Se may also replace sulphur in the amino acids methionine and cysteine and thus can impact on the structural confirmation and changes in the functional properties of the proteins (Stolz and Oremland, 1999; Chapman, 2000). In addition, it is known that an excessive amount of Se is erroneously substituted for sulphur which resulting a tri-selenium linkage (Se-Se-Se) or a selenotrisulfide linkage (S-Se-S). Either tri Se or selenotrisulfide prevents the formation of the necessary disulphide chemical bonds (S-S) causing a distorted, dysfunctional enzymes and protein molecules, which can impact on cellular biochemistry (Ambas et al.; Stadtman, 1990; Lee et al., 2010).

The other reported negative impacts of Se toxicity are changes in blood composition, affecting the hepatopancreatic and gonadal edema in sunfish (*Lepomis* sp.) (Chapman, 2000; Lohner et al., 2001). Similar to fish, changes in total number of haemocytes and the proportion of haemocyte types can be used as indicators of crustaceans' health (Fotedar et al., 2001; Fotedar et al., 2006). In the current experiment, semigranular cells of marron fed higher than 1 g kg^{-1} of Sel-Plex® resulted in reduced ($P<0.05$) proportion of DHC. However, there is no information about the mechanism by which the changes in the number of haemocyte type of marron is induced by different dietary levels of Se.

Tail muscle is a main energy reserve in crustacean and has been successfully used as an indicator of chronic stress (Jussila, 1997b; Sang and Fotedar, 2004). The present study showed that there is a positive correlation ($r^2 = 0.97$) between tail moisture and dietary Sel-Plex®-supplemented levels, indicating that energy reserves got depleted

in the tail tissues. Therefore, stress level of marron was relatively higher when fed higher levels of Sel-Plex®.

The Se supplemented diet can also result in Se accumulation in various tissues of the aquatic animals, including hepatopancreas and muscle tissues (Wang et al., 2007; Zhan et al., 2010). The present study showed that high levels of Sel-Plex® supplementation results in different levels of Se retentions in body tissues. The diet supplementation with high levels of Sel-Plex® increased the total soluble Se levels in hepatopancreas and muscles. Similarly in Atlantic salmon (*Salmo salar*), Se levels in whole body including muscle tissues were significantly higher when fed selenomethionine (Lorentzen et al., 1994). The total soluble Se in the muscle tissues was higher than the hepatopancreas in all groups of marron, similar to the channel catfish (*Ictalurus punctatus*) which showed that the Se concentration in the muscle tissues increased linearly with increasing levels of dietary selenite (Gatlin and Wilson, 1984). Hefnawy and Tortora-Perez (2010) also showed that the muscle tissues can also act as a depository for Se either from direct uptake from water or through feed.

The toxicity impacts on aquatic animals from high levels of Se from water largely remain unexplored. Sensitivity to chemical test, for instance, Se dioxide is affected by the size and the age of the test animals (Chisaka et al., 1999) and is species dependent (Pedroza-Islas et al., 2004). Acute Se dioxide toxicity tests on bluegill (*Lepomis macrochirus*), conducted for 96 to 336 h in intermittent-flow bioassay systems showed that bluegill is less sensitive to Se (Chisaka et al., 1999). Similarly, salmon (*Oncorhynchus kisutch*) showed no mortalities at any concentrations up to 21.6 mg L^{-1} in water with seleno-DL-methionine (Hamilton, 2004). However, study of selenate on amphipod (*Hyalella Azteca*) resulted in LC_{50} value of 760 µg L^{-1} (Brix et al., 2001). The $LC_{50\text{-}96\text{-}h}$ of sodium selenate on chinook salmon (*Oncorhynchus tshawytscha*) was 121 mg L^{-1} but no mortalities occurred in any other concentrations of seleno-DL-methionine as OS is less toxic than the inorganic Se, namely sodium selenite (Hamilton and Buhl, 1990).

8.5 Conclusion

Dietary high levels of Sel-Plex® supplementation can lead to stress indicated by the nutrient deprivation from the tail muscle tissues without compromising on their survival. Further research needs to be conducted to evaluate the effects of higher doses of Sel-Plex® on marron survival when feeding continues for more than 90 days. The total Se can be retained at higher concentrations in the muscles than the hepatopancreas of marron, which subsequently can have concerns for human health. The $LC_{50\text{-}96\text{-}h}$ value of Sel-Plex® in the water for marron is 166.28 g L^{-1}.

CHAPTER 9: The Effects of Dietary Organic Selenium and a Probiotic, *Bacillus mycoides* on the Growth Performance and Immune Competence of Marron *Cherax cainii* (Austin, 2002) Under Outdoor Environment

9.1 Introduction

Marron, *Cherax cainii* (Austin, 2002), is one of the most popular species in the Western Australia and its farming is considered to be economically viable (Wingfield, 1997). The retail price for marron ranges from $16 to $32 per kg, indicating one of the high-valued species in Australia (Lawrence, 2007). Previously, marron were only stocked into farm dams and waterways from Hutt River. However, currently the marron are successfully farmed by approximately 176 licensed marron-growers in purpose-built ponds in Western Australia to supply both domestic and international markets (DoF, 2011) which generates $1.5 million per annum (DoF, 2013)

To support increasing market demand, marron farmers need to increase their farm productivity. Although there is a published information available on feeding and nutritional requirement of marron (Morrissy, 1989; Fotedar, 2004), additional research, especially on feed supplement is necessary to boost the marron farm productivity. Further, in farm based production system, the presence of pathogens can constitute a serious threat. The past methods to combat disease with antibiotics in other decapod crustaceans have been questioned and alternatives have been sought (Bermúdez-Almada and Espinosa-Plascencia, 2012), As an alternative to antibiotics, OS and various selective probiotic are being tested (Rengpipat et al., 2000; Li et al., 2006; Chiu et al., 2010; Sritunyalucksana et al., 2011b).

According to Higgs (2004), Se deficiency is widespread throughout Australia and is seen mainly in areas receiving annual rainfall more than 410 mm particularly in the south-west of Western Australia. Northcliff, a town in the south-west of Western Australia has an average mean rainfall of 1200 mm. Thus, there is a possibility of marron farmed in Northcliff and surrounding area may encounter Se deficiency and therefore, Se enriched marron diets should be tested to improve marron productivity.

The uses of dietary supplementation of OS and probiotics have been proven to improve growth performance and provide protection against the selected pathogens in *Penaeus monodon* (Rengpipat et al., 2000), *Penaeus latisulcatus* (Hai et al., 2009) *Penaeus vannamei* (Sritunyalucksana et al., 2011b). Various studies have been undertaken on dietary supplementation of OS (Vidal et al., 2005; Kucukbay et al., 2009; Abdel-Tawwab and Wafeek, 2010) and non-pathogenic strains of Vibrio, *Aeromonas*, *Pseudomonas* and *Alteromonas* (Irianto and Austin, 2002; Moriarty, 2003) in Atlantic salmon (*Salmo salar*) (Bell et al., 1987), juvenile grouper (*Ephinephelus malabaricus*) (Lin and Shiau, 2005), western king prawn (*Penaeus latisulcatus*) (Hai et al., 2007) but limited research has been conducted on the marron. To evaluate the effectiveness of dietary OS supplementation or probiotics, specific growth rate (SGR), final weight, THC and DHC can be used as tools. The present study was undertaken to evaluate growth, haematological responses and total Se retention status in the hepatopancreas and muscle of marron fed OS and a preselected probiotics *Bacillus mycoides* under an outdoor commercial farming pond environment. The study was also used to validate the previous laboratory-based findings on the use of 0.2 g kg^{-1} Sel-Plex® as a dietary supplementation for marron. The study was conducted on an existing commercially operated marron farm where marron were cultured in earthen ponds. The data was collected for 90 days when marron diet was switched to experimental diets.

9.2 Materials and Methods

9.2.1 Study period and farm site

A feeding trial was conducted in nine earthen ponds of about 900 m^2 each in 432 Boorara Rd Boorara Brook, Northcliffe WA 6262 (Latitude -34.66001 N; Longitude 116° 9' 49.644 W). The depth of the ponds was from 1.6 to 1.7 m. The experiment was conducted for 90 days from October to December 2012. The trial was conducted on the existing and operational marron farm and it was not feasible to drain the marron ponds and commence the trial on newly stocked marron due to commercial reality. Therefore, the start of trial was marked by switching of the marron existing diet to three (3) experimental diets (basal, probiotic-supplemented and OS–supplemented).

9.2.2 Basal and test diets

The first experimental diet (the basal diet) in the form of a commercial marron diet was supplied by Specialty feeds, Glen Forrest Western Australia. The proximate composition of the basal diet was: 26% crude protein, 9% crude fat and 5% crude ash. The second experimental diet was prepared by using basal diet which was mixed with 0.2 g kg^{-1} of Sel-Plex®, as a source of OS, donated by Alltech Inc. USA. The third experimental diet with OS supplementation was also prepared by Specialty feed, where basal diet was sprayed with *Bacillus mycoides.* The isolated, *B. mycoides* from stock cultured was re-grown onto a blood agar plate. After overnight incubation at 25^{o}C, the number of colonies of the inoculum was diluted into 100 mL of sterilized normal saline. Before getting sprayed onto the basal diet, the feed was coated with fish oil blend (Bait mate®, Western Australia) at 20 mL per kg of the basal diet. Subsequently, the probiotic *B. mycoides* was sprayed onto 1 kg of basal diet at a concentration of 10^{7} cfu g^{-1} of feed and then immediately covered with aluminium foil and stored in a refrigerator at 4^{o}C to avoid any bacterial growth. The density of each probiotic bacteria sprayed onto the feed was determined by producing a standard curve using optical density (Spectrophotometer, BOECO S-20, Hamburg, Germany) and then grown on blood agar plates to obtain the actual density.

9.2.3 Experimental design

Nine ponds were used to test three different experimental diets, providing three replicates for each diet. Each pond was marked as control (marron fed basal diet); probiotic (marron fed probiotic supplemented diet) and OS (marron fed 0.2 g kg^{-1} Sel-Plex in the diet) (Figure 9.1).

Figure 9.1: Pond location and experimental design. OS = organic Se (Sel-Plex® supplemented diet); Pro = *B. Mycoides* supplemented diet; C = control (basal diet).

Marron were fed every day at a rate 2.3% (October); 3.6% (November) and 2.7% (December) based on mean demand feeding rates described by the second Pemberton Grow out data set 1990-1993 from Department of Fisheries Western Australia. Marron were randomly sampled from each pond at the start of the experiment, and at the end of experiment to determine the growth performance and total soluble Se in the hepatopancreas and muscle tissues of marron. THC and DHC of sampled marron from each pond were measured at the commencement of the experiment, at day 45 and at the end of the experiment, referring to the method as described in chapter 3.

Temperature, pH and dissolve oxygen of pond's water (Cyberscan pH 300, Eutech Instruments, Singapore), nitrate, nitrite and ammonium (Chemical test kits, Aquarium Pharmaceuticals^{TM}, Inc., USA) were recorded at the sampling days.

9.2.4 Data collection

Growth performance

The weights, carapace length and total length of ten sampled marron from each pond (n=90 per sampling day) were measured at the commencement day and day 90. Initial weight and final weight were measured to calculate specific growth rate (SGR % g day-^{1}) (Aiken, 1980).

Total and Differential haemocytes count (THC and DHC)

The THC and DHC of marron (n=45 per sampling day; 15 marron per treatment) were calculated on the day 0, 45 and 90 of feeding trial using procedure as described in Chapter 3.

Determination of Se

At the commencement of the experiment and the day 90 of the feeding, in total 27 marron per sampling day were sacrificed to determine the total soluble Se levels in hepatopancreas and in tail muscle tissues. Total Se was spectrophotometrically determined according to the procedure described by Revanasiddappa and Dayananda (2006) as mention in the chapter 3. The total actual Se in the basal and test diets were also analysed by using the same method used for analysing Se in hepatopancreas and muscles of marron.

9.2.5 Statistical analysis

Statistical analysis was performed using SPSS software version 17. All data were expressed as mean ± SE (standard error). The data of growth, total Se in the diet and marron tissues (hepatopancreas and muscle) were subjected to one way ANOVA. Meanwhile THC and DHC were performed using two ways ANOVA. All significant differences was analysed using Tukey's *post hoc* to evaluate significant differences. All significant tests were at $P<0.05$ levels.

9.3 Results

9.3.1 Environment and water quality

Based on the secondary data (Bonzle (2013), Northcliff, WA has rainfall and humidity levels above average. The highest mean rainfall was recorded at 169.4 mm in September and gradually decreased until 34.5 mm on December. Temperature was recorded around 20^{o}C in September and reached up to 25^{o}C in December (Bonzle, 2013). Daytime and nighttime temperatures were below average. Summer in Northcliff is between December and February with maximum temperature averages between 23.8-26.3^{o}C and overnight minimum between 11.9 and 13.5. The highest temperature in the summer days is around 26.3^{o}C.

During the feeding trial, the temperature, pH and dissolved oxygen viz. 26-28^{o}C, 7.00-7.50, and 4.7-4.8 mg L^{-1} respectively were measured and found within the acceptable ranges for marron survival (Morrissy, 1990; Sang and Fotedar, 2010a). Nitrate, nitrite and ammonia were also recorded within the acceptable levels. Nitrate and nitrite levels were monitored as less than 0.1 mg L^{-1} whereas total ammonium was observed at no more than 0.2 mg L^{-1}, providing optimum water quality for culturing marron (Jussila, 1997b).

8.3.2 Total soluble selenium levels in the diet

The total soluble Se analysis showed that OS-supplemented diet had a significantly higher (*P<0.05*) actual total Se level than the basal or probiotic-supplemented diet (Figure 9.2).

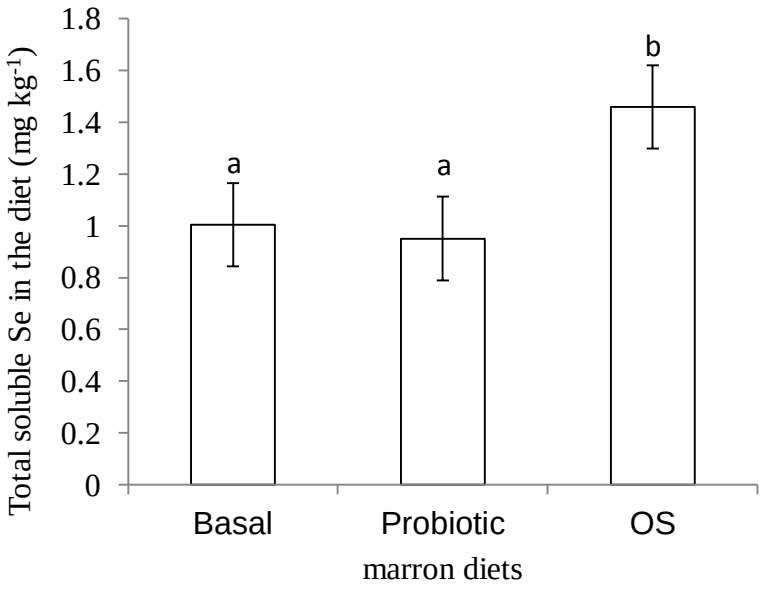

Figure 9.2: Actual total soluble selenium in the diet of marron

9.3.3 Growth indices

After 90 days of feeding, the marron fed OS or probiotic supplemented diet had significantly higher (*P<0.05*) final weight, carapace length and total length than control. The highest final weight was achieved when marron were fed probiotics. The juvenile marron fed OS resulted significant higher (*P<0.05*) SGR than control (Table 9.1).

Table 9.1: Growth indices (mean ± SE) of juvenile marron fed different diets for 90 days in commercial ponds.

Growth parameters	Supplements		
	Control	Probiotic	OS
Initial weight (g)	11.69 ± 0.55^{a}	12.28 ± 0.52^{a}	11.26 ± 0.96^{a}
final weight (g)	12.77 ± 0.63^{a}	16.52 ± 0.63^{b}	15.46 ± 0.83^{b}
final carapace length (mm)	37.38 ± 0.58^{a}	41.78 ± 0.67^{b}	41.18 ± 1.13^{b}
final total length (mm)	83.80 ± 0.79^{a}	92.14 ± 1.46^{b}	93.51 ± 1.39^{b}
SGR (g day $\%^{-1}$)	0.281 ± 0.04^{a}	$0.381. \pm 0.06^{ab}$	0.483 ± 0.06^{b}

All data are given as mean ± SE (n = 90 marron at sampling day). Different superscript alphabets in the same row indicate significantly different means $P<0.05$. Control = Basal diet; Probiotic = Basal diet supplemented with isolated *B. mycoides*. OS = organic selenium (0.2 g kg^{-1} of Sel-Plex®). SGR = Specific growth rate.

After 45 days of feeding, the mean THC of marron fed any supplements were significantly higher ($P<0.05$) than control, The highest THC was in marron fed OS (Figure 9.3) and had significantly higher percentage of granular cells than marron fed probiotic after 45 days. In contrast, the percentage of hyaline cells of marron fed OS was significantly reduced during the feeding trial. The percentage semi-granular cells were not affected by any supplements over the sampling periods (Table 9.2).

After 90 days of feeding, there was no significant difference ($P>0.05$) in the total Se levels in hepatopancreas of marron fed any diet. However, the marron fed OS in the diet had higher total Se soluble in the muscle than marron fed probiotic or control (Table 9.3).

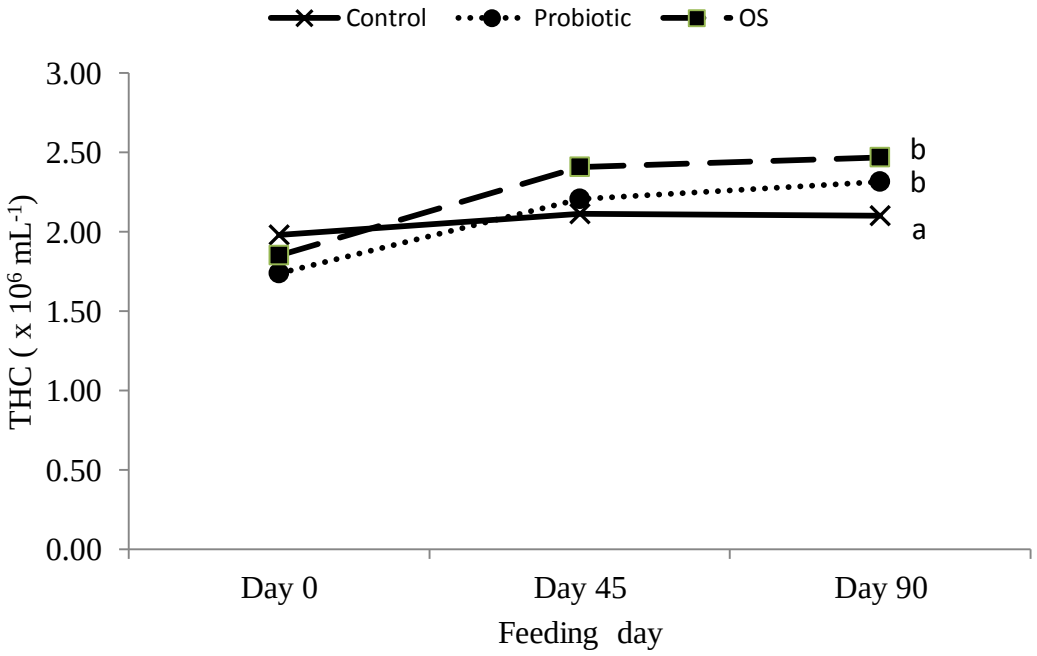

Figure 9.3: Total haemocyte count of pond cultured marron (n = 45 marron per sampling day) fed different diet for 90 days. Control = Basal diet; Probiotic = Basal diet supplemented with isolated *B. mycoides*. OS = organic selenium (0.2 g kg^{-1} of Sel-Plex®). Different alphabets (a, b) denote significant difference (*P < 0.05*).

Table 9.2: Percentage of DHC (mean ± SE) of marron fed different diets for 90 days in commercial ponds.

Haemocytes types	Day	Types of diet		
		Control	Probiotic	OS
Granular (%)	0	$_{1}31.67 \pm 0.36^{a}$	$_{1}24.84 \pm 0.23^{b}$	$_{1}30.86 \pm 0.53^{c}$
	45	$_{1}31.21 \pm 0.40^{a}$	$_{2}36.25 \pm 1.06^{b}$	$_{2}56.73 \pm 0.37^{c}$
	90	$_{2}37.23 \pm 0.68^{a}$	$_{2}34.46 \pm 0.52^{b}$	$_{2}57.80 \pm 0.89^{c}$
Semigranular (%)	0	$_{1}31.10 \pm 0.52^{a}$	$_{1}31.44 \pm 0.16^{a}$	$_{1}30.93 \pm 0.51^{a}$
	45	$_{1}31.05 \pm 0.20^{a}$	$_{1}30.57 \pm 1.48^{a}$	$_{1}29.71 \pm 0.48^{a}$
	90	$_{1}31.88 \pm 0.37^{a}$	$_{1}30.01 \pm 0.74^{a}$	$_{1}30.19 \pm 0.95^{a}$
Hyaline (%)	0	$_{1}37.21 \pm 0.74^{a}$	$_{1}43.70 \pm 0.30^{b}$	$_{1}38.19 \pm 0.92^{c}$
	45	$_{1}37.73 \pm 0.29^{a}$	$_{2}33.17 \pm 0.84^{b}$	$_{2}13.55 \pm 0.45^{c}$
	90	$_{2}30.87 \pm 0.70^{a}$	$_{3}35.52 \pm 0.40^{b}$	$_{3}11.99 \pm 0.65^{c}$

All data are given as mean ± SE (n = 27 marron per sampling day). Different superscript alphabets in the same row indicate significantly different . Different subscript numericals in the same column indicate significantly different (*P<0.05*). Control = Basal diet; Probiotic = Basal diet supplemented with isolated *B. mycoides*. OS = organic selenium (0.2 g kg^{-1} of Sel-Plex®).

Table 9.3: Total soluble selenium (mean ± SE) in the hepatopancreas and muscle of pond cultured marron fed different diets for 90 days.

Total soluble Se ($\mu g\ g^{-1}$)	Types of diet		
	Control	Probiotic	OS
Hepatopancreas			
Day 0	$_1$0.053 ± 0.004^a	$_1$0.050 ± 0.006^a	$_1$0.059 ± 0.006^a
Day 90	$_2$0.066 ± 0.002^a	$_2$0.060± 0.005^a	$_2$0.228 ± 0.006^b
Muscle			
Day 0	$_1$0.064 ± 0.007^a	$_1$0.060 ± 0.009^a	$_1$0.069 ± 0.006^a
Day 90	$_2$0.061 ± 0.006^a	$_2$0.067 ± 0.009^a	$_2$0.892 ± 0.056^b

All data are given as mean ± SE (n = 27 marron per sampling day). Different superscript alphabets (a, b) in the same row indicate significantly different means *P<0.05*. Different subscript numericals in the same column indicate significantly different means *P<0.05*. Total soluble Se in the hepatopancreas and muscle expressed as $\mu g\ g^{-1}$ sample. Control = Basal diet; Probiotic = Basal diet supplemented with isolated *B. mycoides*. OS = organic selenium (0.2 g kg^{-1} of Sel-Plex®). n = 27 marron per sampling day).

9.4 Discussion

Optimum water temperature is an important physical factor for culturing marron. Marron is categorized as temperate species, having optimum growth temperature between 17.5- 24.5^{o}C (Bryant and Papas, 2007). However, Lawrence (1998) and Morrissy et al (1990) found that marron can tolerate water temperature up to 30^{o}C. Temperature below 17.5^{o}C can reduce marron's growth and mortalities can occur when temperature reach above 30^{o}C. Current field experiment recorded mean temperature to be 27.64^{o}C, which is within the tolerance range for marron but only for a brief duration (Bryant and Papas, 2007). Water temperature in the pond can be high due to thermal stratification, which can be broken down by mechanical aeration (Keen et al., 2003).

The productivity of cultured animals in the pond can be improved by adding specific biologically active compounds such as OS extracted from the yeast and from other live organisms. The benefits of using OS from Sel-Plex® (Sritunyalucksana et al., 2011b) and probiotics from other live organisms (Moriarty, 1998; Hai and Fotedar, 2009) have been described by several authors. Beside improving growth

performance, OS and probiotic are known to act as immunostimulants (Smith et al., 2003; Chiu et al., 2010; Sang et al., 2011a) and therefore are extensively investigated as dietary supplements in animals (Dörr et al., 2008; Alina et al., 2009; Kucukbay et al., 2009; Sang et al., 2011a). The present results showed that the marron fed either OS or probiotic had higher final weight and carapace length than the controlled group which is similar to the previous studies in juveniles sea cucumber (*Apostichopus japonicus*) (Wang et al., 2011) and juveniles European sea bass (*Dicentrarchus labrax*, L.) (Carnevali et al., 2006). However, growth of African catfish farmed was not affected by any dietary Se enriched garlic (Luten and Schram, 2006).

The OS in the diet gets incorporated into protein structure in the marron tissues and then interacts with iodine to prevent abnormal hormonal metabolism (Burk and Hill, 1993; Foster and Sumar, 1997), resulting in higher growth performance . Probiotics can induce growth performances by enhancing beneficial gut micro floral populations, this in turn can enhance the absorption of nutrients which finally results in boosted immune competence of the host (Bomba et al., 2002). The past research has also stated that the use of probiotic on cultured aquatic animals can have a positive effect on the digestive system (Reid, 2008) by increasing the surface area of the intestine in prawns (*Penaeus latisulcatus* Kishinouye, 1896), resulting in better nutrient absorption (Hai and Fotedar, 2009). The use of *Bacillus* spp, has also increased the productivity of black tiger prawn's (*Penaeus monodon)* farming and improved the water quality by decreasing the concentrations of ammonia and nitrites in water (Porubcan, 1991b, a).

Present results showed that SGR of marron fed OS is significantly higher than the marron fed basal diet. However, there was no impact of probiotics on marron SGR measured on the last day of the experiment. A study with pollack (*Pollachius pollachius*) showed that a combination of two probiotics Bactocell (*Pediococcus acidilactici*) and Levucell (*Saccharomyces cerevisiae*) can improved the SGR (Gatesoupe, 2002). According to Mohanty et al. (1996) the use of combined probiotics in a diet is more effective in growth promotion than single probiotic in Indian major carp, *Catla catla*. In addition, using probiotic and prebiotic, known as synbiotics, is also suggested due to the fact that a probiotic, without its prebiotic food

ingredient, does not survive well in the digestive tracts (Sekhon and Jairath, 2010). Currently, my colleague is continuing this trial to evaluate the long term effects of OS and probiotic supplementation on harvestable marron.

Besides improving SGR, the incorporation of OS in the diet may also stimulate and increase the proliferation rate of marron haemocytes which indicates the elevated immune competence of marron. The increased THC in OS-fed marron in the current trial are in line with previous studies showing that OS supplementation enhances immune system of freshwater characid fish matrinxa (*Brycon cephalus*) and giant freshwater prawn (*Macrobrachium rosenbergii*) (Monteiro et al., 2009; Chiu et al., 2010). The role and mechanism of OS and/or probiotic in increasing THC in marron haemolymph is not clearly understood, however Alina et al. (2009) postulated that Se enriched diet is assimilated into antioxidant enzymes and protein which are important in improving immunity (Rameshthangam and Ramasamy, 2006). The probiotic supplementation may also provide immugens which may elicit an immune function (Itami et al., 1998; Rengpipat et al., 2000) and enhance immunomodulatory capacity of haemolymph (Waldroup et al., 2003).

Crustacean's haemocytes such as hyaline cells, semi-granular cells and granular cells are part of crustacean immunity which plays an important role in the host immune response (Smith and Soderhall, 1983; Soderhall and Cerenius, 1992; Bachère, 2003). The quality and quantity of three types of haemocytes in crustaceans can be affected by extrinsic factors such as food intake (Durliat and Vranckx, 1983; Jussila et al., 1999; Fotedar et al., 2001; Jussila et al., 2001). Current experiment have shown that percentage of granular cells of marron fed OS in the diet was significantly higher ($P<0.05$) than marron fed either probiotic supplemented diet or basal diet after 45 days of feeding. The higher granular cells of OS-fed marron in the current results indicate the higher ability of the marron haemocyte in degranulation process to defeat against the foreign substances (Sang et al., 2011b). Present results also showed that the percentage of semigranular cells was not affected by any kind of diet. However, the percentage of hyaline cells in marron fed any diet was significantly reduced during feeding period. The proportion of haemocytes can vary time to time depends on several factors such as temperature elevation (Hopkins, 1992), existing pathogen (Sang et al., 2009) and food intake (Sang and Fotedar, 2010a). In addition,

the variation of haemocytes proportion is regulated by haematopoetic tissue both releasing and storage process of haemocytes (Johansson et al., 2000).

The ability of animals to accumulate Se from dietary sources has been studied in several aquatic animals, such as salmon (*Salmo salar*) (Lorentzen et al., 1994; Lorentzen et al., 1998), rainbow trout (*Oncorhynchus mykiss*) (Vidal et al., 2005), juvenile grouper (*Epinephelus malabaricus*) (Lin and Shiau, 2007), medaka (*Oryzias latipes*) (Li et al., 2008) and adult crayfish (*Procambarus clarkii*) (Dörr et al., 2008). Current research showed that OS and probiotic supplementation in the basal diet for 90 days significantly increased the total soluble Se in the muscle of marron. Similarly, Luten and Schram (2006), stated that the Se content in the muscle of farmed African catfish increased linearly with increasing dietary selenomethionine or Se enriched garlic.. However, selenomethione supplemented diet showed significantly higher Se content than Se enriched garlic supplemented diet.

It has been known that probiotic supplementation in the diet can increase total bacterial count in the intestine. The increased bacterial load in the intestine may contribute a positive effect on the absorption and digestion of nutrients (Hai and Fotedar, 2009) including Se, resulting higher total retention Se in the muscle of probiotic-fed marron than control. The total soluble Se in the muscle of marron fed OS showed significantly higher than control or probiotic supplementation. Similarly, Wang and Lovell (1997) reported that Se can accumulate from the diet into various tissues of channel catfish (*Ictalurus punctatus)* and can be retained at a higher level in the muscles. The retention of Se in the muscle tissues of marron has a positive effect in preventing future (if any) Se deficiency. Although, the marron did not show any symptoms of Se deficiency, Se enriched diet can be seen as a preventive effort to avoid Se deficiency during the prolonged periods of marron farming at the same site. Studies in some aquatic animals have shown that Se deficiency can reduce the growth in common carp (*Cyprinus carpio*) and channel catfish (*Ictalurus punctatus*); can increase the mortality and muscular dystrophy in Atlantic salmon (*Salmo salar*) (Tacon, 1987).

9.5 Conclusion

Overall, there were some advantages on the physiological and haematological responses of the marron fed either probiotic or OS. This experiment validated laboratory findings that OS can be used in cultured marron and applied in commercial pond situations in order to boost growth performance, enhance immunity and Se retention in the muscle tissue of marron. Therefore, the use of probiotic or OS in the diet of cultured marron in the pond environment is suggested.

CHAPTER 10: Discussion, Conclusions and Recommendations

10.1 General Discussion

Due to the health benefits of organic trace elements coupled with their eco-friendly role, this research was focused on the evaluation of dietary OS on the growth and health performance of cultured marron. Previous research on OS has been concentrated on cattles (Nicholson et al., 1991), horses (Jackson and Pagan, 1996), ewes (Steen et al., 2008) and pigs (Jang et al., 2010). However, OS has been currently tested as a dietary supplement on the cultured aquatic animals such as gibel carp (*Carassius auratus gibelio*) (Han et al., 2011), rainbow trout (*Oncorhynchus mykiss*) (Misra et al., 2012) and largemouth bass (*Micropterus salmoide*) (Zhu et al., 2012). OS supplementation is also more effective than inorganic Se. For example, if selenomethionine is not immediately broken down into selenocysteine for selenoprotein synthesis, it can be incorporated into various organs, whereas if inorganic Se is not immediately utilized, it is quickly eliminated (Jacques, 2001).

The reasons that organic form of Se is trialled as a dietary supplement for aquatic animals is based on the fact that 1) Se cannot be synthesised *in vivo* in cultured animals as inorganic Se naturally, occurs in low amount in geological raw materials such as rocks, soils and sediments (Ralston et al., 2009) and can be found as selenite, selenate or selenide (Rotruck et al., 1973; Cotter et al., 2008). On the other hand, selenomethionine can be produced from yeast and is commercially available in the market (Burdock and Cousins, 2010). 2). Se plays a crucial role in various bodily functions including growth.

Although, Se is a trace element, the occurrence of Se in the body is essential as Se deficiency may reduce growth and health status (Gatlin and Wilson, 1984; Hamilton, 2004). 3). Dietary Se is required to combat stress caused by intensive farming (Pickering, 1998). It is also suggested that dietary Se should be supplemented above the required level in order to maintain adequate defence during the stress conditions (Cotter, 2006). To achieve desired outcomes, the dosage of Se supplementation in the diet needs to be considered carefully as the gap between the requirement and its toxic threshold is very narrow. It has been reported that the supplementation of Se in the diet of cultured aquatic animals is 0.35 mg kg^{-1} in rainbow trout (*Salmo gairdneri*)

(Hilton et al., 1980) ; up to 1 mg kg^{-1} in gibel (*Carassius auratus gibelio*) (Han et al., 2011) and 0.3 mg kg^{-1} in *Penaeus vannamei* (Sritunyalucksana et al., 2011b).

The current research suggested that 0.2 g kg^{-1} of Sel-Plex® which is equivalent to 0.4 mg kg^{-1} OS in the diet is sufficient to improve growth and health performance of marron. The final weight, RGR, SGR, digestive enzymes activity and the total Se retention in hepatopancreas and muscle tissues of marron improved significantly when marron were fed higher than 0.1 g kg^{-1} of Sel-Plex® (Chapter 4 and 5). Meanwhile, the highest DWG was achieved when marron were fed Sel-Plex® higher than 0.2 g kg^{-1} whereas survival of marron improved with all levels of dietary Sel-Plex®.

Results under laboratory and pond experiments demonstrated that OS through dietary supplementation of Sel-Plex® improves the SGR of marron (Figure 10.1). These results are consistent with the previous findings on the role of OS in improving growth performances of several fish species (Wang et al., 2007; Zhou et al., 2009; Han et al., 2011; DoF, 2013) and crustacean species (Sritunyalucksana et al., 2011b). Higher SGR of marron fed OS can be due to increase in antioxidant enzyme activity which is demonstrated in allogynogenetic crucian carp (*Carassius auratus gibelio*) (Wang et al., 2007) and nile tilapia (*Oreochromis niloticus* L.) (Abdel-Tawwab and Wafeek, 2010) too.

Besides, improving SGR, supplementing 0.2 g kg^{-1} of Sel-Plex® to marron diet also enhanced its disease resistance against *V. mimicus* (Chapter 6) as OS-fed marron showed significantly lower *Vibrio* ranks, longer NRRT, higher THC and higher granular cells after 96h post-injection with *V. mimicus.*

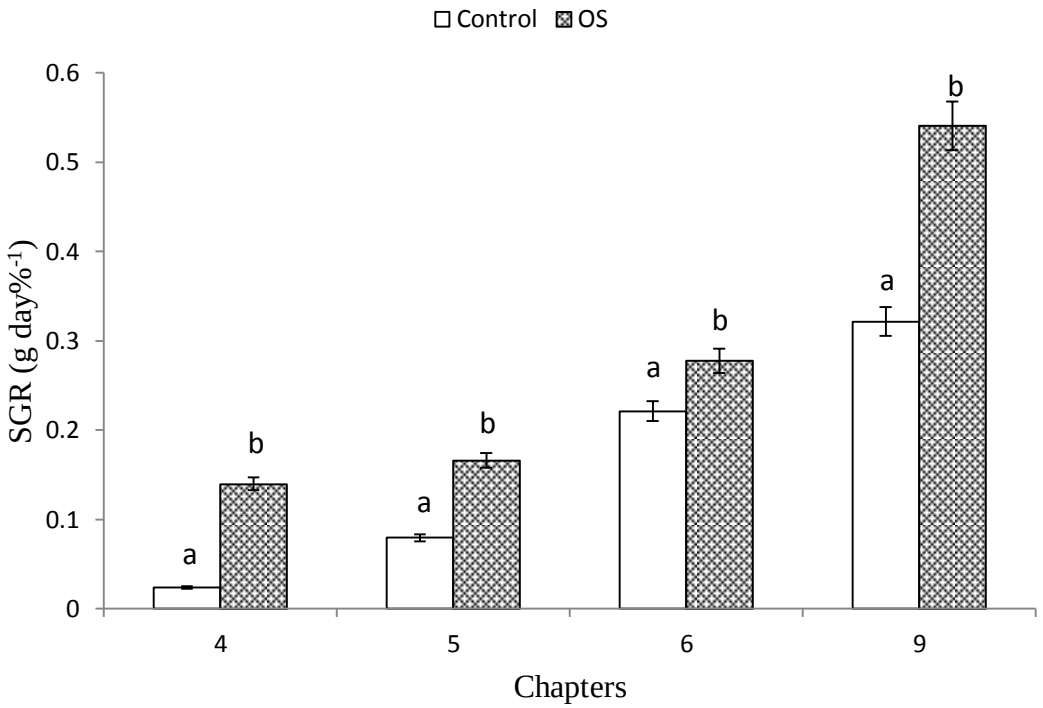

Figure 10.1: Specific growth rate of marron fed 0.2 g kg^{-1} organic selenium (OS) in the diet for 90 days of feeding trial. Sel-Plex® was used as a source of OS. Different alphabets (a, b) above the bars in the same chapter data series indicate significantly different (*P<0.05*).

It was also shown that OS-fed marron had higher survival, THC, antioxidant enzymes activity and membrane cell stability than IS-fed marron. OS-fed marron also had higher percentage of hyaline cells, GST, GPx activity, total Se retention in the haemolymph and longer NRRT but lower granular cells and LPO than MOS-fed marron (Chapter 7).

Though, higher than 0.2 g kg^{-1} of Sel-Plex® in the diet showed higher DWG, Se retention in hepatopancreas and muscles and lower bacteraemia levels (Chapter 4), yet the use of OS at high levels must be considered cautiously due to the reported negative effects on the growth (Watanabe et al., 1997), increase mortality and severe haematological changes (Hilton et al., 1980; Hicks et al., 1984). The current research (Chapter 8) showed that up to 3 g kg^{-1} of Sel-Plex® had a positive correlation with the TM% of marron indicating constant increase in stress levels. At high levels, the Se compounds can disrupt intracellular calcium homeostasis and induce enzymatic aponecrosis leading to cell death (Misra, 2011). The occurrence of The $LC_{50\text{-}96\text{-}h}$

value of Sel-Plex® in the water for marron was 166.28 g L^{-1}. In addition, Sel-Plex® in the water up to 768 g L^{-1} had negative effect on the survival of marron where over 80% mortality was obsereved. According to Misra (2011) animals exposed to high Se levels can lead to organ damage due to Se toxicity which may vary depending on the species (Hamilton, 2004) and its chemical form (Misra, 2011).

To validate laboratory findings, the use of 0.2 g kg^{-1} Sel-Plex® in the diet of marron were also tested in a field-based commercial marron ponds where , probiotic *Bacillus* was also used to get some comparisons with the most commonly used probiotics. The use of *Bacillus*-based probiotic products is one of the main success in probiotic applications in aquaculture industry (Gatesoupe, 2005). Currently, probiotic *B. mycoides* has also been identified as a potential probiotic that can be applied in a basal diet to enhance growth and health status of marron (Personal communication, Irfan 2013). Due to the time limitation, the data was collected only for 90 days. However, my colleague, Irfan is still continuing this trial until all marron stocks are harvested for commercial sale.

Decapod crustaceans have capabilities to accumulate Se in hepatopancreas and muscle tissues (Hosseini et al., 2012). Current research showed that the marron fed Sel-Plex® accumulate Se in the muscle tissues at a higher concentration than in their hepatopancreas (Figure 10.2). Similarly, Se is retained at a higher level in the muscle tissues of adult white sucker (*Catostomus commersoni*) than the hepatopancreas (Muscatello et al., 2008).

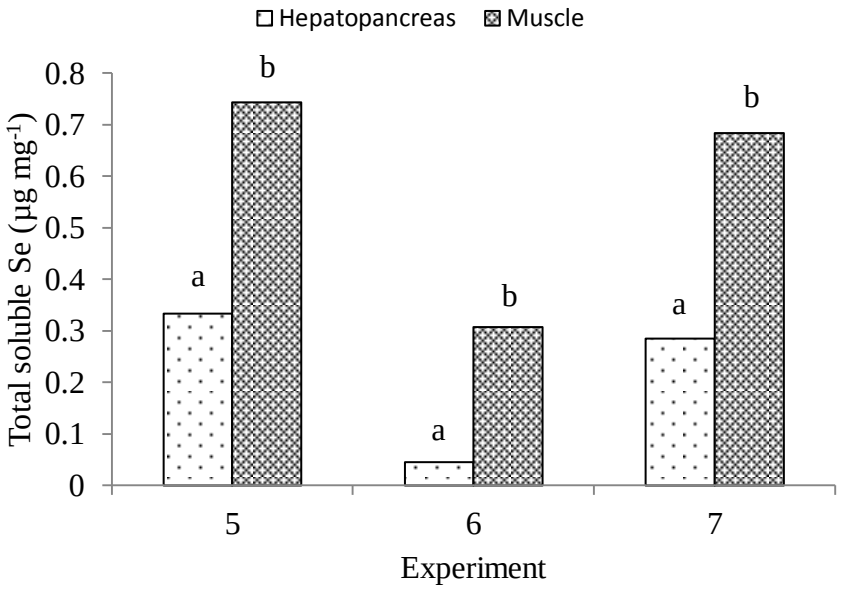

Figure 10.2: Total soluble selenium in the hepatopancreas and muscle of marron fed 0.2 g kg^{-1} OS in the diet for 90 days. Sel-Plex® was used as a source of OS. Different alphabets (a, b) above the bars in the same chapter data series indicate significantly different (*P<0.05*). (Mean value of total soluble Se from each experiment described in chapters 5, 6 and 7).

It is known that organic Se is absorbed by a different mechanism than inorganic Se. Selenomethionine for instance, is absorbed in small intestine by using Na^+ dependent neutral amino acid transport system (Wolfram et al., 1989a; Wolfram, 1999). Once OS is absorbed and not directly used, OS can be incorporated into non-active selenoproteins and stored in muscle tissues which is one of main site for Se metabolism reserve pools (Bell and Cowey, 1989; Wang and Lovell, 1997; Schrauzer, 2000). In general, there are two reserve pools of Se that can be found in animals (Waschulewski and Sunde, 1988a). In the first pool, up to 50% of Se in the body is stored in the muscle while the other organs such as kidney and liver have also relative high levels of Se (Burk and Levander, 2002). OS which is being incorporated in the muscle tissue has also known to show long term body reserves (Lorentzen et al., 1994; Hinojosa et al., 2006). The second pool is related to liver glutathione peroxidase and the Se availability for synthesis of selenoproteins. Study in rat stated that only 25% of total Se in the body can be found in the second pool (Behne and Wolters, 1983).

The total retained Se in the muscle can support growth (Zhu et al., 2012). Similarly, the current research showed that total soluble Se in the muscle of marron had a positive correlation with SGR ($y = 76.115x - 10.521$; $r^2 = 0.81$, regression generated using data which taken from experiment described in chapter 5, 6 and 7). As discussed earlier, OS containing selenomethionine is absorbed from the intestine by the same mechanism as methionine which is quite efficient process (>80%). Selenomethionine, finally builds up in body reserves and is further converted into general proteins including muscle proteins and selenoenzymes such as GPx which in turn benefits for growth and the health status of the host (Figure 10.3) (Weiss, 2010).

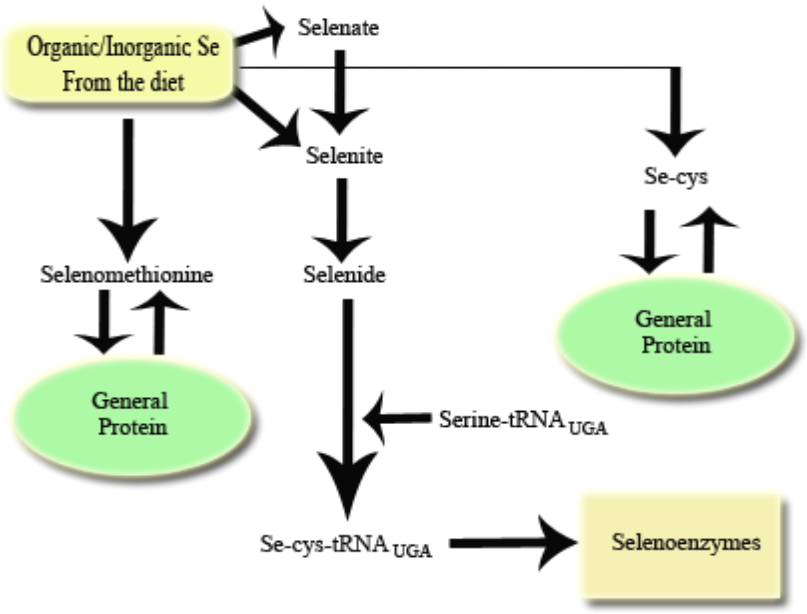

Figure 10.3: Pathway of selenium metabolism (Weiss, 2010)

In general, dietary supplementation of OS has significant impact on THC. Positively, THC of marron also influenced by rearing period (time) during dietary supplementation of OS (Figure 10.4). Se retention in the hepatopancreas of OS-fed marron significantly increased and showed a positive correlation with percentage of granular cells ($y = 0.153x - 4.559$; $r^2 = 0.84$). Crustaceans including marron must rely on non-specific immunity to ensure efficient defense responses against infectious pathogens that continually threaten their survival.

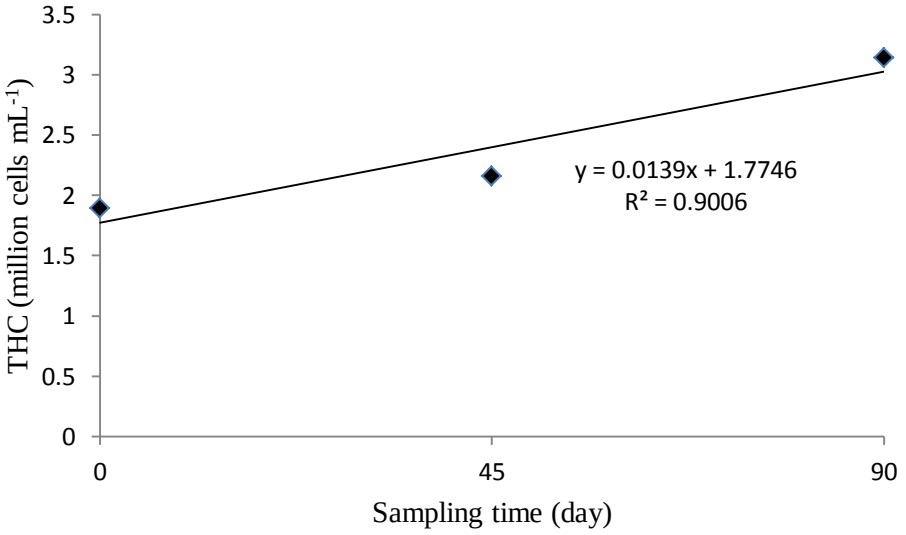

Figure 10.4: A positive correlation between total haemocyte count (THC) of marron and rearing period (day) during dietary supplementation of 0.2 g $kg^{-1}$of OS. Sel-Plex® was used as a source of organic selenium (OS). (Data in the graph were developed using data from chapters , 4, 5, 6 and 9).

The availability of Se in the hepatopancreas may support the proliferation of granular cells which play an important role in the immune response including recognition (Lee et al., 2000; Cheng et al., 2005), phagocytosis, melanization, cytotoxicity (Johansson and Soderhall, 1989; Johansson et al., 1995) and cell to cell communication (Johansson and Soderhall, 1988; Thornqvist et al., 1994; Liu et al., 2005). The numbers of haemocytes, especially the granular cells, have been used as a measure for shrimp health although number of granular cells do vary in cultured animals (Sritunyalucksana et al., 2011b). The granular cells are filled with large granules storing the prophenol oxidase enzyme which plays a pivotal role in the defense mechanism of crustaceans. The activity of granular cells can be easily triggered to undergo exocytosis and release of the prophenol oxidase enzyme. The prophenol oxidase system may be activated by ß-1.3-glucans, peptidoglycans and lipopolysaccharides. The activation by ß-1.3-glucans is very specific. In its active form the phenol oxidase catalyzes the oxidation of phenols to semiquinones and quinones (Barracco et al., 1991; Soderhall et al., 1994).

There apparently, is a further biochemical link between Se availability in the hepatopancreas and granular production. The haemocytes and hepatopancreas play

an important roles for the immune system in crustaceans as hepatopancreas is the main production site for immune recognition molecules, initiates the humoral reaction, and takes part in the cellular reaction by some specialized cells and phagocytes (Gross et al., 2001). Hepatopancreas is a sensitive indicator for storage of inorganic and organic reserves (Dunlap and Adaskaveg, 1997). The hepatopancreas also secretes antioxidant enzymes which are involved in antioxidant responses and oxidative stress including catalase (CAT), superoxide dismutase, and glutathione S-transferase (GST) (Borkovic et al., 2008). Most of the biologically active selenium in hepatopancreas is in the mitochondrial fraction (Taylor and Maher, 2012). Further, the hepatopancreas can synthesize and excrete numerous proteins involved in immune defense, such as antibacterial peptides (AMPs) (Ried et al., 1996) and β-1,3-glucan binding proteins (Roux et al., 2002).

Comparison between laboratory and commercial pond-based experiments

Water temperature is one of the crucial physical parameters that effects growth and physiological status of cultured marron. The average temperature of water in commercial ponds during the feeding trial was significantly higher than the water in the experimental tanks under the laboratory conditions (Figure 10.5).

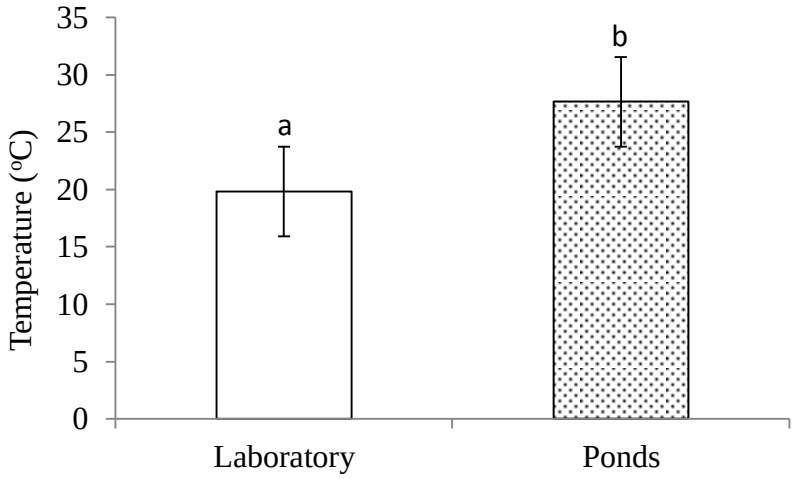

Figure 10.5: Comparison between mean water temperature in experimental tanks under laboratory condition and experimental ponds under outdoor field conditions. (Bars in the graph were developed using data from chapters 4, 5, 6 and 9).

According to Keen et al. (2003), the water temperature is affected by the diurnal temperature changes and light penetration. During the sampling times the mid-day average water temperature was 27.6^{o}C which was within the acceptable range (Morrissy, 1990; Lawrence, 1998). The marron is reported to tolerate water temperature up to 30^{o}C (Cubitt, 1985). Mortalities occur when temperature reaches above 30^{o}C and growth rate starts to decline when temperature drops below 12.5^{o}C (Keen et al., 2003). Present results showed that SGR of marron reared in outdoor ponds was significantly higher (*P>0.05*) than SGR of marron reared in the laboratory based tanks (Figure 10.6) as growth rate of marron is predominantly affected by water temperature (Keen et al., 2003; Bryant and Papas, 2007).

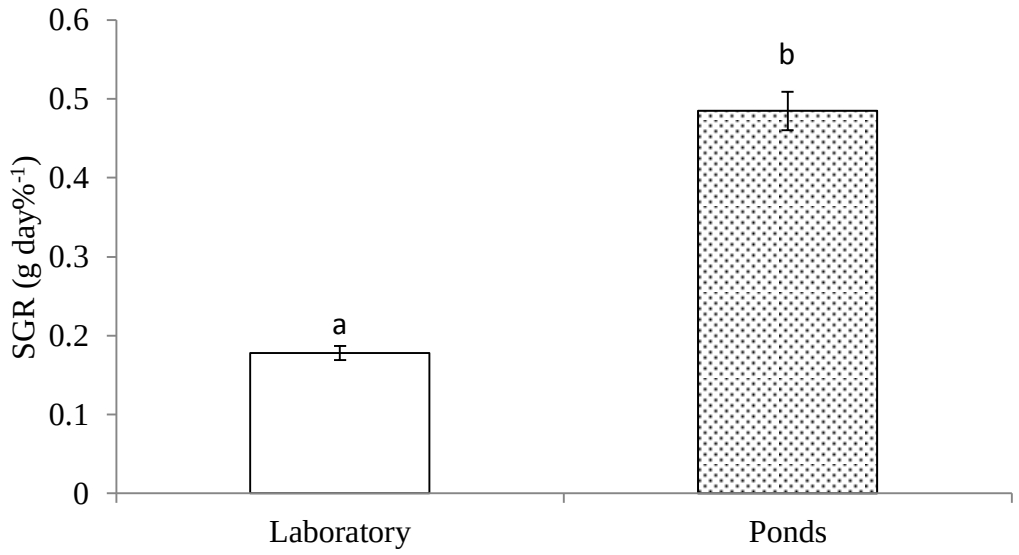

Figure 10.6: Comparison specific growth rate of OS-fed marron reared in the laboratory and outdoor ponds. 0.2 g kg^{-1} of Sel-Plex® was used as a source of organic selenium (OS). Feeding period was for 90 days. (Bars in the graph were developed using data from chapters , 4, 5, 6 and 9).

Besides growth, immune parameters are also influenced by the rearing environment. Jussila et al. (1997) stated that, immune parameters such as THC may vary depend on rearing condition. THC of Sel-Plex®-fed marron reared under laboratory conditions was significantly greater than marron reared under outdoor pond condition (Figure 10.7). Similarly, THC of marron reared in outdoor communal tanks is higher than marron reared in earthen ponds (Jussila et al., 1997). THC can be

declined in the presence of bacteria (Stewart et al., 1967) and water pollutants (Smith et al., 1995).

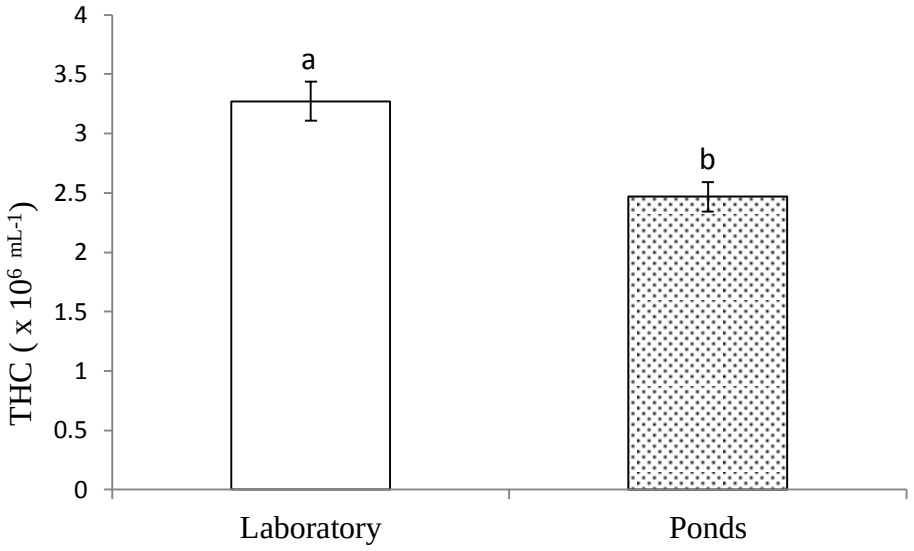

Figure 10.7: Comparison between total haemocyte count of OS-fed marron reared under the laboratory and outdoor pond environment. 0.2 g kg^{-1} of Sel-Plex® was used as a source of organic selenium (OS). Feeding period was for 90 days. (Bars in the graph were developed using data from chapters 4, 5, 6 and 9).

Though, DHC can show high degree of variations due to environmental factors such as temperature (Flye-Sainte-Marie et al., 2009) and feed intake (Sritunyalucksana et al., 2011b), yet in the present study, DHC of OS-fed marron were not affected by any rearing environment (Figure 10.8). Study in clams (*Ruditapes philippinarum*) found that temperature has a high correlation coefficient with granulocytes (Flye-Sainte-Marie et al., 2009). Besides temperature, feed intake such as OS supplementation has some correlation with granular cells of prawn (*Penaeus vannamei*) (Sritunyalucksana et al., 2011b). In contrast, probiotic supplementation in the diet of prawns (*Penaeus monodon*) resulted in no variations in DHC (Balasundaram et al., 2013). Similarly, the proportion of granular cells in marron did not change after stress induction (Sang et al., 2009).

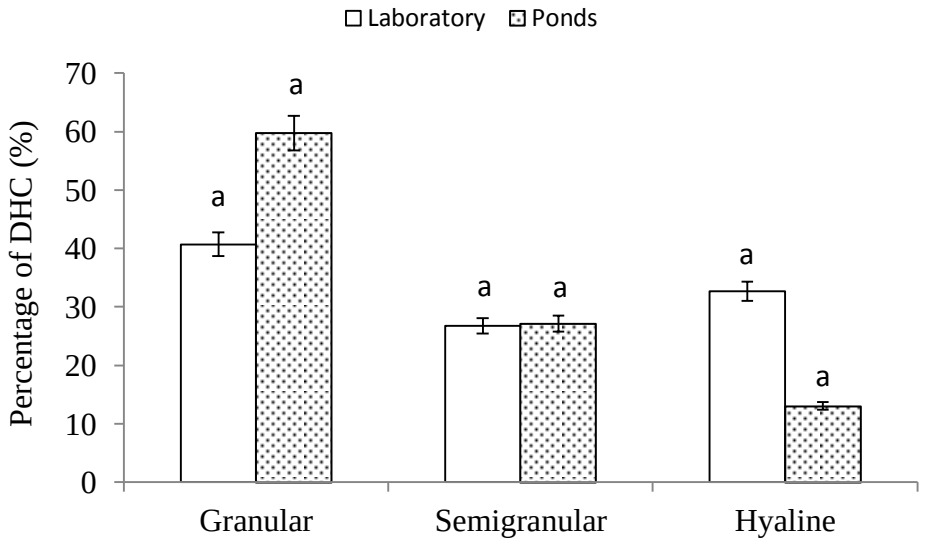

Figure 10.8: Comparison between percentage differential haemocyte count of OS-fed marron reared under laboratory and outdoor pond conditions. 0.2 g kg^{-1} of Sel-Plex was used as a source of organic selenium (OS). Feeding period was for 90 days**.** (Bars in the graph were developed using data from chapters 4, 5, 6 and 9).

Compared to laboratory-reared marron, total Se retention in the muscle of OS-fed marron in the outdoor ponds was significantly higher (Figure 10.9). This is possible as pond-reared marron can uptake additional Se from the pond sediments, biota and water in the pond environment (Lemly, 1987). Hence, the natural availability of Se in pond environment can result in marron having higher accumulation of Se in the muscle tissues. The Se retention in the hepatopancreas of OS-fed marron was not influenced by the rearing conditions as Se metabolic cycle in the hepatopancreas requires a shorter period as transport of Se via hepatopancreatic system into and out of liver or pancreatic compartment is very rapid (Patterson et al., 1989). Thus, Se in organic form then can be deposited into the muscle tissue for a longer peroiods instead of hepatopancreas (Maher, 1985; Pacini et al., 2012).

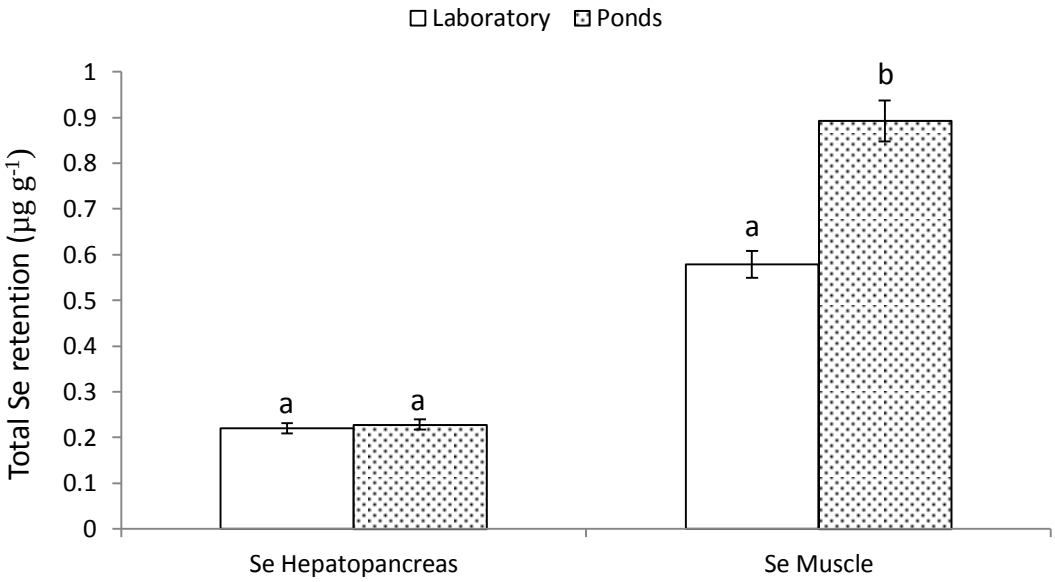

Figure 10.9: Comparison between total selenium retention (hepatopancreas and muscle) of OS-fed marron rearing in the laboratory and outdoor ponds. 0.2 g kg^{-1} of Sel-Plex® was used as a source of organic selenium (OS). Feeding period was for 90 days. Different alphabets above the bars at the same parameters showing the significant difference in mean value at $P< 0.05$. (Bars in the graph were developed using data from chapters 4, 5, 6 and 9).

10.2 Conclusions

The conclusions have been summarised and stated in this chapter. However, following list is the summary of conclusions drawn:

a. During dietary administration of Sel-Plex®, rearing period (time) positively influenced the THC of marron.
b. Sel-Plex® inclusion level of 0.2 g kg^{-1} in the diet increases THC of marron and increases total Se retention in the muscle tissues.
c. Microvilli of marron fed Sel-Plex® are longer, numerous and more distributed towards the inner surface of the midgut than marron fed without Sel-Plex® while marron fed Sel-Plex® had thinner midgut epithelial layer and hepatocytes than marron fed basal diet.
d. Dietary supplementation of 0.2 g kg^{-1} of Sel-Plex® improves marron resistance against *Vibrio mimicus* and increases THC at 24 post-challenge with *Vibrio mimicus.*
e. Sel-Plex® inclusion in the diet optimizes antioxidant enzyme activity such as GST, GPx and LPO.

f. High dietary level of 3 g kg^{-1} Sel-Plex® can cause stress in marron.
g. The $LC_{50\text{-}96}$ value of Sel-Plex® in the water is 166.28 g L^{-1}.
h. Dietary 0.2 g kg^{-1} Sel-Plex® supplementation is suggested for marron reared in the outdoor ponds as it leads to increase in SGR, final weight, total carapace length, THC and total Se retention in the muscles.
i. Dietary 0.2 g kg^{-1} Sel-Plex® supplementation in marron results in a positive correlation ($y = 76.115x - 10.521$; $r^2 = 0.81$) between SGR and total Se retention in the muscle of marron as well as between Se retention in the hepatopancreas and percentage of granular cells of marron ($y = 0.153x - 4.559$; $r^2 = 0.84$).

10.3 Recommendations

Based on current findings, following recommendations are made in terms of further research and usage of OS:

a. Determination on the growth and health performance of marron when OS is applied for a longer duration and preferably throughout the whole culture period.
b. Challenge the marron immune system with other commonly encountered physical stressors such as various temperature regimes, nitrate levels and air exposure to evaluate the effectiveness of OS supplementation in the diet of marron.
c. Study into the effects of dietary OS on gene expression of marron.
d. Other antioxidant activity such as superoxide dismutase (SOD), catalase indicators, ROS and superoxide anion production are important to evaluate the immune response of marron. Thus, future research should incorporate all these parameters to evaluate the OS efficiency in marron.
e. Study comparison the effectiveness of OS, MOS and probiotic-treated marron in the commercial ponds should be evaluated in the same time and incorporated all parameters such as growth indices, immune competence, and antioxidant enzymes activity.
f. Finally, 0.2 g kg^{-1} of Sel-Plex® in the diet should be supplemented to maintain higher growth performance and health of marron.

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APPENDIX

1. List of publications

a. Nugroho, R. A and Fotedar, R. (2013). Growth, Survival and Physiological Condition of Cultured marron, *Cherax tenuimanus* (Smith, 1912) Fed Different Levels of Organic Selenium. Journal of Agricultural Science and Technology B 3:125-135.

b. Nugroho, R. A and Fotedar, R. (2013). Dietary Organic Selenium Improves Growth, Survival and Resistance to *Vibrio mimicus* in Cultured Marron, *Cherax cainii* (Austin, 2002). Fish Shellfish Immunology. 35:79-85

c. Nugroho, R. A and Fotedar, R. (2013). Comparing the Effects of Dietary Selenium and Mannan Oligosaccharide on the Health, Immune Function and Antioxidant Enzyme Activity in Cultured Marron *Cherax cainii* (Austin, 2002). Aquaculture International. Accepted 31 July 2013; DOI 10.1007/s10499-013-9682-1

d. Nugroho, R. A and Fotedar, R. (2013). Effects of dietary organic selenium on immune responses, total selenium accumulation, and digestive system health of marron, *Cherax cainii* (Austin, 2002). Aquaculture International. Acceptable to be published (Aquaculture Research). 8 August 2013.

2. List of conference abstracts

a. Nugroho, R. A. and Fotedar R. Does dietary organic selenium improve physiological condition of cultured marron *Cherax tenuimanus* (Smith 1912)?. In Skretting Australasian Aquaculture 2012 International Conference and Trade Show. Melbourne-Australia, 2012.

b. Nugroho, R. A and Fotedar, R. Dietary organic selenium improves marron, *Cherax tenuimanus* (Smith, 1912) growth, survival rate and resistance to *Vibrio mimicus*. In World Aquaculture Society Conference, September 1-4 September, 2012, Prague, Czech Republic.