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Effects of dietary organic selenium on immune responses, total selenium accumulation and digestive system health of marron, *Cherax cainii* (Austin, 2002)

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Abstract

A 90-day feeding trial was conducted to determine the effects of dietary organic selenium (OS) supplementation on immune responses, total selenium accumulation in various tissues and the digestive system health of marron *Cherax cainii*. A group of marron (initial weight 3.65 ± 0.05 g) were fed 0.2 g kg^{-1} of Sel-Plex® as a source of OS and were compared to a control group of marron that were not fed OS. After 90 days of feeding OS, total haemocyte counts (THC) and the percentages of granular and semigranular cells in marron were significantly higher than in marron fed a control diet. However, dietary OS did not affect the proportion of hyaline cells. The marron fed OS had significantly lower bacteraemia and longer neutral red retention times than the control group. Total selenium levels in the haemolymph, hepatopancreas and muscle tissues of the OS-fed marron group were significantly higher ($P < 0.05$) than in control diet group. The highest total soluble selenium levels were found in the muscle tissues of OS-fed marron on the final day of the feeding trial. Amylase and protease activity, soluble protein, and numbers of microvilli were significantly higher ($P < 0.05$) in marron fed OS. These findings demonstrate the benefits of including 0.2 g kg^{-1} OS in marron diet to enhance immune parameters, increase total selenium accumulation in muscle and improve marron digestive system health.

Keywords: organic selenium, immunity, selenium accumulation, digestive system, marron

Introduction

The use of micronutrients as feed additives has gained popularity and has assisted in minimising the environmental impacts of aquaculture and maximised the health of the farmed animals (Sang, Ky & Fotedar 2009). Considerable attention has been given to organic selenium (OS) as a feed additive, which has been proved more suitable than the inorganic form of selenium (Se). Compared to the inorganic Se, OS 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 & Parrett 1996; Schrauzer 2003; Taylor, Finley & Caton 2005; Wang, Jianzhong, Weifen & Zirong 2007; Kucukbay, Yazlak, Karaca, Sahin, Tuzcu, Cakmak & Sahin 2009). OS produced in this way is known as selenoyeast, is approved by the U.S. Food and Drug Administration and can be used as a feed supplement (Ortman & Pehrson 1999; Chung, Kim, Ko & Jang 2007).

Studies on the use of OS as a dietary supplement have been conducted in red swam crayfish (*Procambarus clarkii*) (Dörr, Pacini, Abete, Prearo & Elia 2008), African catfish (*Clarias gariepinus*) (Schram, Pedrero, Cámara, Van Der Heul & Luten 2008), giant freshwater prawn (*Macrobrachium rosenbergii*) (Chiu, Hsieh, Yeh, Jian, Cheng & Liu 2010) and Pacific white shrimp (*Penaeus vannamei*) (Sritunyalucksana, Intaraprasong, Sa-nguanrut, Filler & Fegan 2011) but have been limited in marron (*Cherax cainii*). Marron are the third largest crayfish in the world and are native to Western

Australia (Rouse & Kartamulia 1992; Morrissy 2000). The marron have become a commercially important aquaculture species 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 showed that marron fed with 0.2 g kg^{-1} Sel-Plex® in the diet showed increased growth performance (Nugroho & Fotedar 2013b) and resistance against *Vibrio mimicus* (Nugroho & Fotedar 2013a). However, additional effects of dietary OS in marron, such as immune responses, Se retention in the various tissues, digestive enzyme activity and midgut profile have remained largely unexplored.

Immune-physiological responses such as alteration of total and differential haemocyte count (THC and DHC), bacteraemia, neutral red retention time (NRRT) (Jussila, Paganini, Mansfield & Evans 1999; Fotedar, Evans & Jones 2006; Sang *et al.* 2009; Sang, Fotedar & Filer 2011b), total selenium accumulation (Elia, Prearo, Pacini, Dorr & Abete 2011) and digestive enzyme activity levels (Pedroza-Islas, Gallardo, Vernon-Carter, Garcia-Galano, Rosas, Pascual & Gaxiola 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, such as midgut section and the microvilli number, were examined to assess marron gut condition.

Materials and methods

Experimental animals

A 90-day feeding experiment was conducted on juvenile marron (mean initial weight 3.65 ± 0.05 g, $n = 100$) supplied by Aquatic Resource Management, Manjimup, Western Australia. We randomly allocated 100 marron into two groups; one was fed basal diet without OS inclusion (Control group), and the other was fed 0.2 g kg^{-1} of Sel-Plex® (OS group).

Diet preparation

A basal diet was formulated (Table 1) using Feed LIVE® software package version 1.52 from Live Informatics Company Limited (Nonthaburi, Thailand). All ingredients of the basal diet were purchased from Specialty Feed (Glen Forrest, Western Australia), except OS, which was provided through a commercial product, Sel-Plex® (Alltech). To make the test diet, 0.2 g kg^{-1} of Sel-Plex®, which is an optimum level for marron (Nugroho & Fotedar 2013a,b), was added to the basal diet containing 0.87 mg kg^{-1} Se, for a total measured Se level in the test diet was 1.3 mg kg^{-1}. According to Burdock and Cousins (2010), Sel-Plex® contains 2 g kg^{-1} OS, mainly selenomethionine. All feed ingredients were passed through a 100-μm mesh sieve and mixed to obtain uniform particle size. The basal (control) and test diet were passed through a mincer to obtain wet strands that were 0.5 mm in diameter. The resulting strands were then air-dried and allowed to cool at room temperature. The dried strands were broken into 3-mm-long pellets and stored in plastic containers at 4°C. Analyses of crude protein, lipid, ash and moisture contents were performed according to the standard methods of the Association of Official Analytical Chemists (AOAC 1995).

Table 1 Ingredient of basal diet (g kg^{-1}) and proximate (% in dry matter) of the diets

Ingredient	(g kg^{-1})
Cod liver oil	32
Wheat bran	545.58
Soybean meal	101.5
Fish meal*	257.14
Calcium carbonate	0.2
Ascorbic acid	0.5
Betaine†	12
Premix‡	1.5
Cholesterol	2.5
Wheat starch	47.08
Proximate composition	(% in dry matter)
Protein	27.05
Lipid	8.02
Moisture content	9.01
Ash	6.56

All ingredients supplied by Specialty Feeds, WA, Australia.
*Peruvian fishmeal, 56% CP.
†Betaine Anhydrous 97%.
‡Commercial vitamin and mineral premix for trout.

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, and 5 randomly selected tanks were fed basal diet and 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, Guangdong, China). Nitrate, nitrite and ammonium tests were performed weekly using chemical test kits (Aquarium Pharmaceuticals™, 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 1997).

Total and differential haemocyte counts

The total haemocyte count (THC) and differential haemocyte count (DHC) of marron from each culture tank were recorded on the final day of the feeding trial. THC was calculated using the method previously used by Fotedar, Tsvetnenko and Evans (2001), with some modification. A 0.2-mL haemolymph sample was obtained from the base of the fifth thoracic leg of marron, and 0.2 mL solution of 1% glutaraldehyde in 0.2 M sodium cacodylate was dispensed into an Eppendorf tube. Total haemocytes were counted in a haemocytometer (Neaubauer, 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 & Stevens 1977). The number and percentages of three major (granular, semigranular and hyaline) marron haemocyte types for each individual marron were counted using a minimum number of 200 cells on each slide. The granular cells had larger cell size, smaller pale nucleus and larger number of eosinophilic granules in the cytoplasm while semi-granular cells had a longer shape, big nucleus and little or no cytoplasm. Hyaline cells can be distinguished as it had round shape, big nucleus and little or no cytoplasm (Sang *et al.* 2009). The DHC was then calculated by using the following equation:

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

Bacteraemia assessment

On the final day of the experiment, we assessed bacteraemia in the haemolymph of marron from each cultured tank. A 0.05-mL sample of haemolymph from each marron was withdrawn into sterile syringe and smeared on a nutrient agar plate. The agar plate was then inverted and put 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).

Neutral red retention time (NRRT)

NRRT assessment was carried out using the procedure described by Hauton, Hawkins and Hutchinson (1998). 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, 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 returned to the incubator. The slide was then 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.

Total soluble Se determination

A sampling procedure similar to midgut micrograph analysis was used on days 0, 45 and 90. The sampled marron from each tank culture were killed to determine the total soluble Se in haemolymph, hepatopancreas and muscle tissues. Total soluble Se was determined using a spectrophotometric method according to Revanasiddappa and Dayananda (2006). Briefly, 4 mL concentrated 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 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 volume was mixed well and allowed to stand for 20 min before absorbance was measured at 615 nm against the reagent blank. The concentration of 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.

Digestive enzyme activity and total soluble protein levels

To evaluate the effects of Sel-Plex® on the activities of digestive enzymes (amylase and protease), hepatopancreata and digestive tracts of marron from each culture tank were sampled at the end of experiment, and crude amylase and protease were extracted (Sang, Fotedar & Filer 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 4500 × *g* for 5 min using Eppendorf centrifuge 5804 R (Eppendorf, 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 & 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).

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 followed 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, Hillsboro, OR, 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, San Jose, CA, USA).

Statistical analysis

The results were expressed as means ± standard error (SE), and data were analysed using SPSS version 17 (SPPS, 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 tests were used to determine significant differences of total soluble Se accumulation in various tissues at days 0, 45 and 90. $P < 0.05$ was considered significant.

Results

Immunological parameters

After 90 days of feeding, THC, granular and semigranular cells of marron fed dietary OS were significantly higher ($P < 0.05$) than marron fed basal diet; however, the proportion of hyaline cells of marron were not affected by the addition of dietary OS. Meanwhile, 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 2).

Total Se accumulation

Total Se accumulation in all sampled tissues of control and OS-fed marron significantly increased ($P < 0.05$) over the feeding trial. At day 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 3).

Table 2 Total haemocyte count ($\times 10^6$ cells mL^{-1}), granular (%), semigranular (%), hyaline (%) cells, bacteraemia (cfu mL^{-1}) and neutral red retention time (min) of OS-fed marron after 90 days of feeding trial

	Groups	
Immune parameters	**Control**	**0.2 g kg^{-1} Sel-Plex®**
THC	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	1.77 ± 0.12[a]	0.91 ± 0.02[b]
NRRT	78.00 ± 3.00[a]	105.00 ± 6.70[b]

Data are given as mean values (mean ± SE). Different superscript (a, b) in the same row indicates significantly different mean values at $P < 0.05$. Sel-Plex® was added to basal diet as a source of organic selenium (OS).
THC, Total haemocyte count; NRRT, neutral red retention time.

Table 3 Total soluble Se in the haemolymph (μg mL^{-1}), hepatopancreas (μg g^{-1}) and muscle (μg g^{-1}) of marron fed OS in the diet for 90 days

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

All data (mean ± SE) in the same row having different superscript (a, b) are significantly different ($P < 0.05$). Different subscript symbol in the same column indicates significantly different mean values ($P < 0.05$). 0.2 g kg^{-1} Sel-Plex® was used as a source of organic selenium (OS).

Digestive enzyme activity and midgut assessment

The digestive enzyme activity results are presented in Fig. 1. At the end of feeding trial, OS-fed

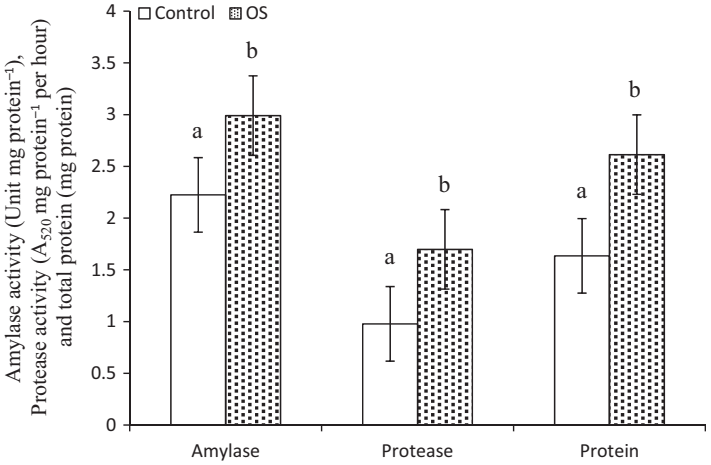

Figure 1 Amylase, protease activity and mg protein content from crude homogenate gut of marron for 90 days. Sel-Plex® was added as a source of organic selenium at level 0.2 g kg^{-1} of basal diet. One unit amylase activity (unit mg protein^{-1}) = 1 mg maltose liberated from starch in 3 min at pH 6.9 at 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 alphabets (a, b) in the same enzyme activity (amylase, protease or protein unit) showing the significant difference in mean value at $P < 0.05$.

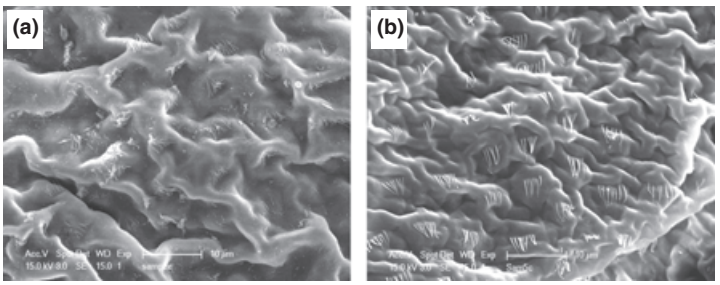

Figure 2 Comparison of midgut micrographic of marron fed basal diet (a = Control) and test diet (b = 0.2 g kg^{-1} Sel-Plex® as a source of organic selenium).

marron had significantly higher ($P < 0.05$) amylase, protease activity and total soluble protein than control. As shown in Fig. 2, SEM of the middle section of the midgut revealed that 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).

Discussion

Crayfish health status can be influenced by food supplementation (Jussila & 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 & Cousins 2010). Selenomethionine incorporates into proteins in place of methionine and can be stored (Suzuki & 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, Charrondiere, Dusemund, Galtier, Gilbert, Gott, Grilli, Guertler, Kass, Koenig, Lambré, Larsen, Leblanc, Mortensen, Parent-Massin, Pratt, Rietjens, Stankovic, Tobback, Verguieva & Woutersen 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, Qie, Wang, Li & Zhao 2010; Han, Xie, Liu, Xiao, Liu, Zhu & Yang 2011), all of which play an important role in enhancing animal immune function (Chiu *et al.* 2010; Smitha & 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, Keyser, Sritunyalucksana & Soderhall 2000). Semigranular cells participate in the encapsulation, recognition and degranulation of foreign molecules or particles (Johansson & Soderhall 1989; Johansson *et al.* 2000), which might reduce the number of bacteraemia. These 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-Szfter & Babula 1996; Soderhall 1997; van de Braak, Botterbloom, Huisman, Rombout & van der Knaap 2002; Sang *et al.* 2009). However, in most crustacean species there is a high inter-individual variation in DHC (Johansson *et al.* 2000). Our previous results indicated that only the hyaline cells of marron were affected by dietary OS (Nugroho & Fotedar 2013b). In addition to nutritional factors, the variation in DHC may be due to intrinsic factors such as moulting. According to Vacca and Fingerman (1993), Sequeira, Vilanova, Lobo-da-Cunha, Baldaia and Arala-Chaves (1995), the hyaline cells of most crustaceans were the dominant population before and soon after moulting, whereas they decreased during 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 & Bindoli 1987). This finding is similar with previous study showing that lysosomal membrane stability increased in marron that were fed BioMOS (Sang *et al.* 2009).

Aquatic animals including salmon (*Salmo salar*) (Lorentzen, Maage & Julshamn 1994, 1998), rainbow trout (*Oncorhynchus mykiss) (Vidal, Bay & Schlenk 2005),* juvenile grouper (*Epinephelus malabaricus*) (Lin & Shiau 2007), medaka (*Oryzias latipes*) (Li, Zhang, Wang, Luo, Zhou & Jiang 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, organic Se binds to protein and transports into plasma to the liver and other tissues via 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, Berger, Grenacher & Scharrer 1989; Wolfram, Berger & Scharrer 1989; Nijhoff & Peters 1992; Yeh, Gu, Beilstein, Forsberg & Whanger 1997; Hamilton 2004; Gropper, Smith & Groff 2008). In addition, adequate Se accumulation in the body of animals also supports optimum immune system function (Arthur, McKenzie & Beckett 2003).

The present results showed that Se is distributed throughout the haemolymph, hepatopancreas and muscles of OS-fed marron at different levels. Furthermore, total Se retention in the various tissues gradually increased throughout the feeding trial. In marron, Se can be retained in the muscle tissues at higher levels than in the hepatopancreas. According to Jacques (2001) selenomethionine is incorporated into protein and mainly accumulates in muscle (Oster, Schmiedel & Prellwitz 1988; EFSA 2009). The turnover of selenomethionine in the peripheral tissue such as muscle is also slower than in the liver and pancreas, indicating that selenomethionine is incorporated into a long-term body pool (EFSA 2009). In contrast to the current results, Waska, Kim, Kim, Kang and Kim (2008) reported that Se levels in the hepatopancreas of squid (*Todarodes pacificus*) were higher than in muscle. The hepatopancreas of marron can be metabolically more active than the squid hepatopancreas, thus consuming OS at a higher rate than in squid. Additionally, the muscle tissue of marron appears to act as a Se storage organ due to the slow turnover of selenomethionine.

Besides 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, protease activity and total soluble protein levels (Fig. 1). The higher digestive enzyme activity contributes to efficient digestion, which can result in increased growth and improved immunity and health (Houlihan, Hall, Gray & Noble 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 distribution that seems more uniform compared to microvilli of marron that were fed the basal diet (Fig. 2). According to Chisaka, Ueno and Futaesaku (1999), increasing the number of microvilli protects the cuticle layer, enhances gut irrigation and facilitates faeces movement. In addition, microvilli enlarge the plasma membrane surface area and secrete brush border enzymes, such as carbohydrate- and protein-digesting enzymes (Tortora & Derrickson 2008).

In 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.

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