Dietary organic selenium improves growth, survival and resistance to *Vibrio mimicus* in cultured marron, *Cherax cainii* (Austin, 2002)

Rudy Agung Nugroho, Ravi Fotedar

Sustainable Aquatic Resources and Biotechnology, Department of Environment and Agriculture, School of Science, Curtin University, GPO Box U 1987, Perth, Western Australia, 6845.

Email: rudysatriana@yahoo.com

Abstract

To determine the effects of dietary organic selenium (OS) supplementation on the growth performance and immune competence of marron, *Cherax cainii* (Austin, 2002), a group of marron were fed 0.2 g kg^{-1} of Sel-Plex® supplemented basal diet and then compared with another group (control) of marron fed basal diet without any supplementation. After 90 days of feeding, final weight, average weekly gains (AWG), relative growth rate (RGR), specific growth rate (SGR), survival, total and differential haemocyte count (THC and DHC), were compared between the two groups. Surviving marron from each group were then divided into three sub-groups (three tanks per sub-group with seven marron per tank); (1) first sub-group was injected with 20 µL of 3.24 x 10^6 cfu *Vibrio mimicus*; (2) the second sub-group was injected with 20 µL normal saline and (3) the third sub-group was not subjected to injection and became the control group. THC, DHC, neutral red retention time (NRRT) and *Vibrio* ranks of post-injected marron were evaluated for 96 hours, at every 24-hours interval. The results showed that after 90 days of feeding, final weight, AWG, RGR, SGR, survival, THC, proportion of hyaline cells of OS-fed marron were significantly higher ($P<0.05$) than the control group, whereas proportion of granular and semigranular cells were not affected by dietary OS. After challenging with *V. mimicus*, survival rate of marron without dietary OS significantly decreased ($P<0.05$) as

compared to the control group of marron. THC of marron in all subgroups were significantly reduced ($P<0.05$) after the challenge. However, THC and granular cells of sub-groups fed OS were higher than other sub-groups. *Vibrio* ranks and NRRT of marron fed OS were significantly lower and slower, respectively, than marron fed without OS. These findings demonstrated the benefits of OS inclusion in the marron diet in terms of growth, health and disease resistance.

Keyword: Organic selenium, marron, growth and health, *Vibrio mimicus*

1. Introduction

Vibrio species have been identified as serious pathogens to various aquatic animals [1-6] resulting in mass mortalities to many cultured invertebrates [2, 7, 8]. Among various *Vibrio* species, *Vibrio mimicus* is known to cause disease outbreaks in black tiger shrimp (*Penaeus monodon*) and red claw crayfish (*Cherax quadricarinatus*) [3, 4, 9, 10]. Intramuscular injection of *V. mimicus* can produce virulent reaction and high mortalities in yabbies (*Cherax albidus*) [11]. To overcome high mortalities of cultured animals, some aquaculturist use antibiotics to prevent the virulent reaction of *V. mimicus* infection [12-14].

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 [15, 16]. Thus, various dietary trace elements, such as organic selenium (OS) have been tested and used as an alternative to antibiotics [17-20]. Recently, there has been a surge in the use of organic forms of various trace elements to enhance the productivity of cultured aquatic animals [21-23]. 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 [24]. The dietary OS in channel catfish (*Ictalurus punctatus*) [18], tilapia (*Oreochromis niloticus*) [25] and hybrid striped bass (*Morone chrysopx x M. saxatilis*) [26] have also improved their growth and immunity. Further, OS is reported to be better absorbed, has higher bioavailability and is less toxic than inorganic selenium [18, 27-29]. The dietary inclusion of OS also increases the number of total haemocytes and granular haemocytes in white shrimp [18, 24, 29-31]. In addition, OS as an integral part of selenomethionine and selenoprotein [24, 32, 33], is recognized as a constituent of an antioxidant enzyme responsible for preventing cellular damage and improving immune competence in grouper (*Epinephelus malabaricus*) [34].

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 [35-38], including marron, *Cherax cainii* [39]. The neutral red dye retention technique (NRRT) has also been successfully used as a tool to evaluate the lipid membrane integrity of marron during infection [37]. 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*.

2. Materials and methods

2.1. Preparation of basal diet and test diet

All ingredients of basal diet and test diet, except OS were supplied by Specialty Feeds Pty. Ltd, Western Australia. The source of OS was from Sel-Plex®, which was supplied by Alltech Inc. USA. The basal diet was formulated using Feed LIVE software version 1.52 from Live Informatics Company Limited, Thailand (Table 1). Basal diet pellets (0.5 mm diameter, 1 mm length) were prepared by mixing all ingredients with approximately 1000 mL kg^{-1} distilled water and 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 as a control-basal diet. 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.

2.2. Culture system

The present experiment was carried out in the Curtin Aquatic Research Laboratory (CARL), Technology Park, Curtin University, Western Australia. Eighteen plastic cylindrical culture tanks (800 mm diameter, 500 mm high, 250 L capacity, 70 L of freshwater in each tank) were used. 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 ten PVC pipes (55 mm diameter, 150 mm length) were placed in each tank to provide shelter for marron. To maintain a constant temperature of 20^{o}C in the culture tanks, individual automatic heaters (Sonpar®, Model: HA-100, China) were used.

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).

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 20 µL 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.

115 *2.5. Data collection*

116 *2.5.1. Growth indices, survival and immune responses*

117 Marron were measured for total weight using electronic balance (GX-4000, A&D
118 Company, Ltd., Japan) immediately after acclimation and after 90 days of the feeding trial. The
119 marron weights were used to measure final weight, average weekly gain (AWG) [40], relative
120 gain rate (RGR) and specific growth rate (SGR) [41, 42]. The marron survival was recorded
121 every day and at 0, 24, 48, 72, 96 h post-challenge time and the surviving marron in each sub-
122 group were also analyzed for THC and DHC.

123 To measure THC and DHC, at day 0 and 90 of feeding trial and at 0, 24, 48, 72, 96 h
124 post-challenge, 0.2 mL of haemolymph was collected from each marron represented by each
125 replicate from all treatments. Haemolymph from individual marron was withdrawn from the base
126 of the fifth thoracic leg into a 23-gauge needle containing 0.2 mL solution of 1% glutaraldehyde
127 in 0.2 M sodium cacodylate and dispensed into an Eppendorf tube. [43]. Total haemocytes were
128 counted using a haemocytometer (Neaubauer, Germany) under 100-fold magnification [45]. The
129 haemocytes were counted in both grids and the resulting mean was used as mean THC.

130

131 THC = (cells counted x dilution factor x 1000)/volume of grid (0.1 mm^3)

132

To calculate the DHC, one drop of the mixture of glutaraldehyde in sodium cacodylate and haemolymph was smeared onto a glass slide. After smearing and air-drying, it was fixed in 70% methanol for 10 min. The fixed smear was stained in May-Grünwald and Giemsa stains for 10 min each [44] and then mounted with a coverslip. The number and percentages of three major marron haemocyte types for each individual were counted using a minimum number of 200 cells from each slide. The DHC was then calculated by using the following equation:

DHC = (Number of different haemocyte cell type/Total haemocyte cells counted) x 100

2.5.2. Vibrio ranks

Vibrio rank assessment was done using the procedure used by Sang *et al*. 2009 and Hauton et al. 1998 [45, 46]. 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.

2.5.3. Neutral red retention time assay

Neutral red dye retention time was evaluated using assay based on previous protocol [47]. 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 mL 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.

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.

3. Results

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 2. After 90 days of feeding, final weight, AWG, RGR, SGR and survival were significantly higher (*T-test, P<0.05*) in marron fed dietary OS than marron fed control diet. THC, percentage hyaline cells of marron fed dietary OS 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 OS.

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, was significantly reduced (*P<0.05*), compare to THC before the challenge. However, after 48 hours post-challenge, THCs of marron fed OS supplementation was higher than any other sub-group of marron (Table 3). After 72 h of post-injection the percentage of granular cells of sub-groups with dietary OS 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 OS (Figure 1).

3.2. Vibrio ranks

Vibrio ranks of marron fed OS supplementation were significantly lower than marron fed without OS after being challenged with *V. mimicus* (Figure 2). After 76-h post injection, there was a significant decrease (*P<0.05*) in *Vibrio* ranks in all sub-groups of marron fed OS supplementation. However, any sub-group of marron with OS in their diets showed no significant differences (*P>0.05*) amongst each other.

201 *3.3. Neutral red retention time (NRRT)*

202 NRRT of all marron was significantly reduced 24 h post-challenge, irrespective of dietary
203 OS. However, the NRRT of marron fed OS was significantly longer ($P<0.05$) than marron fed
204 the control diet and continued to remain significantly longer even at 96 h-post challenge (Table
205 3).

206

207 **4. Discussion**

208 Micronutrient, such as selenium (Se), plays a pivotal role in improving aquaculture
209 productivity [17, 20, 48] and in its organic form has proven to enhance the growth and survival
210 [49-51] of aquatic animals. The weight gains of rainbow trout (*Oncorhynchus mykiss*) [28],
211 hybrid striped bass (*Morone chrysops* × *M. saxatilis*) [52] and juvenile grouper (*Epinephelus*
212 *malabaricus*) [49] respond positively to dietary OS supplementations. Current study showed that
213 the application of dietary supplementation of Sel-Plex® as a source of OS, can significantly
214 improve the growth and survival of marron. Sel-Plex® is also known as selenoyeast that contains
215 selenoprotein. It is a baker's yeast dried product, derived from *Saccharomyces cereviceae* strain
216 CNCM I-3060, cultivated in a Se-enriched fermentation medium to provide a high level of
217 selenomethionine [53]. Selenomethionine may be incorporated into proteins in place of
218 methionine or be metabolized to Selenocystein [31, 54, 55]. The present results also showed that
219 0.2 g kg^{-1} of dietary Sel-Plex® can significantly improve marron's survival irrespective of being
220 challenged with *V. mimicus.* Similarly, improved survival of Taura Syndrome Virus (TSV)-
221 infected shrimp (*Penaeus vannamei*) fed Sel-Plex® as a source of OS has also been reported
222 [24].

One kg of Sel-Plex® approximately contains 2 g of OS mainly represented by selenomethionine and has high bioavailability and appears to be 90% absorbed [52, 56]. 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 selenium, 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 [57, 58], 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 [30, 59].

The number of THC decreases due to various stressors including pathogen infections [46, 60]. The decrease in THC is related to defense activities of haemolymph and haemolymph lysis [37, 61, 62]. The present study indicated that THC of both controlled sub-groups and infected-sub-groups were significantly reduced after getting infected with *V. mimicus*. However, the marron fed OS supplementation were healthier as shown by their higher number of THC following 24 h post-challenge. OS supplementation in the diet stabilises the proportion of circulating granular cells which play an important role in defense against bacterial infection [63] of marron through their phagocytic activities. Past research has shown that animals with better phagocytic activity and clearance efficiency have higher disease resistance [46, 64-66]. In this study, the capability to reduce invasive pathogen, *V. mimicus*, were significantly increased following OS supplementation in the diet, which in turn led to increased resistance against *V. mimicus* [22, 67].

The underlying mechanism(s) whereby dietary OS boosts the resistance of marron against *V. mimicus* is not properly understood. However, Alina et al. [68] stated that selenium enriched diet is assimilated into enzymes, such as antioxidant and protein which are important in improving immunity. Selenium, as an active agent plays a role in protecting cell compartments and cell membranes against lipid peroxidation due to pathogen infection [69] and promote antioxidant activity in the body via glutathione peroxides (GPX), a selenium-dependent enzyme which is a primary antioxidant enzyme for cellular defense against oxidative stress [48]. The inclusion of OS in the diet can increase the level of glutathione peroxides (GSH-Px), a main antioxidant enzyme that prevents cellular damage from free radicals [70]. 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 [71]. 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 [72]. A study of amphipods *(Gammarus locusta)* has showed that the high level of lipid peroxidation is triggered by decreasing antioxidant enzyme activity [73]. 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 defense against pathogens [74-76]. 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 [43]. Current results showed that the marron fed OS 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 bacteremia in the haemolymph were also reported in Bio-Mos®-fed infected marron [46] and western king prawn (*Penaeus latisulcatus*) fed a combination of two probiotics, *Pseudomonas synxantha* and *Pseudomonas aeruginosa* [77, 78].

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) [46, 79]. 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 OS. It is possible that dietary selenium can induce the lysosomal membrane stability and reduce the lipid peroxidation [80]. A similar finding was found in marron wherein lysosomal membrane stability increased in Bio-Mos®-fed marron [46].

In conclusion, supplementing 0.2 g kg^{-1} of Sel-Plex®, which equates to approximately 0.4 mg kg^{-1} 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 OS supplementation on antioxidant enzymes activity, such as glutathione peroxide, superoxide dismutase and catalase as well as levels of lipid peroxidase [81, 82] that are related to the health and immunity of marron.

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520 Table 1. Ingredient of basal diet (g kg^{-1}) used for the marron culture

Ingredient	Content (g kg^{-1}) 521
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
Crude protein	27.05
Crude fat	8.02
Crude Fiber	6.39
Moisture content (%)	9.01
Ash (%)	6.56
Dry matter (%)	90.98
Energy (Cal/g)	1,833.249

522 All ingredients supplied by Specialty Feeds Pty Ltd, WA, Australia.[1]Cod liver oil, [2]Peruvian fishmeal,
523 56% CP. [3]Betaine Anhydrous 97%.[4]Commercial vitamin and mineral premix for trout.

524 Table 2. 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}

525 Different alphabets (a, b) indicate significantly different means for different treatments at
526 $P < 0.05$. AWG = average weekly gain; RGR = relative gain rate; SGR = specific growth rate.
527 Sel-Plex® was added to basal diet as a source of organic selenium (OS).

528 Table 3.Total haemocyte count (THC) and Neutral red time retention (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

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

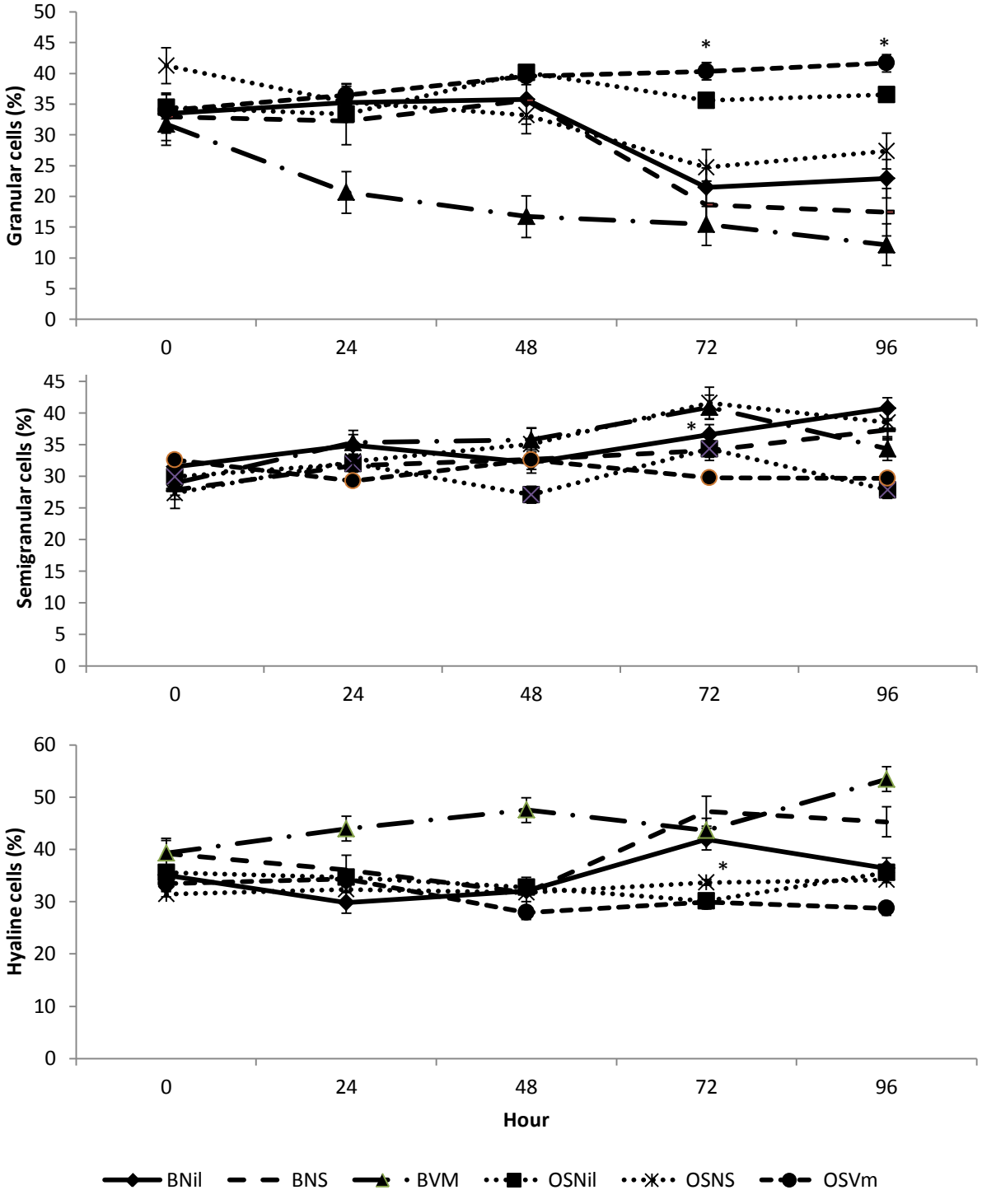

Figure 1. The comparison of differential haemocytes counts (DHC) in the haemolymph of marron. Note: BNil = control with no injection; BNS = control with 20 µL normal saline injection; BVm = control with 20 µL *V. mimicus*; OSNil = 0.2 g kg^{-1} Sel-Plex® supplementation with no challenge; OSNS = 0.2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

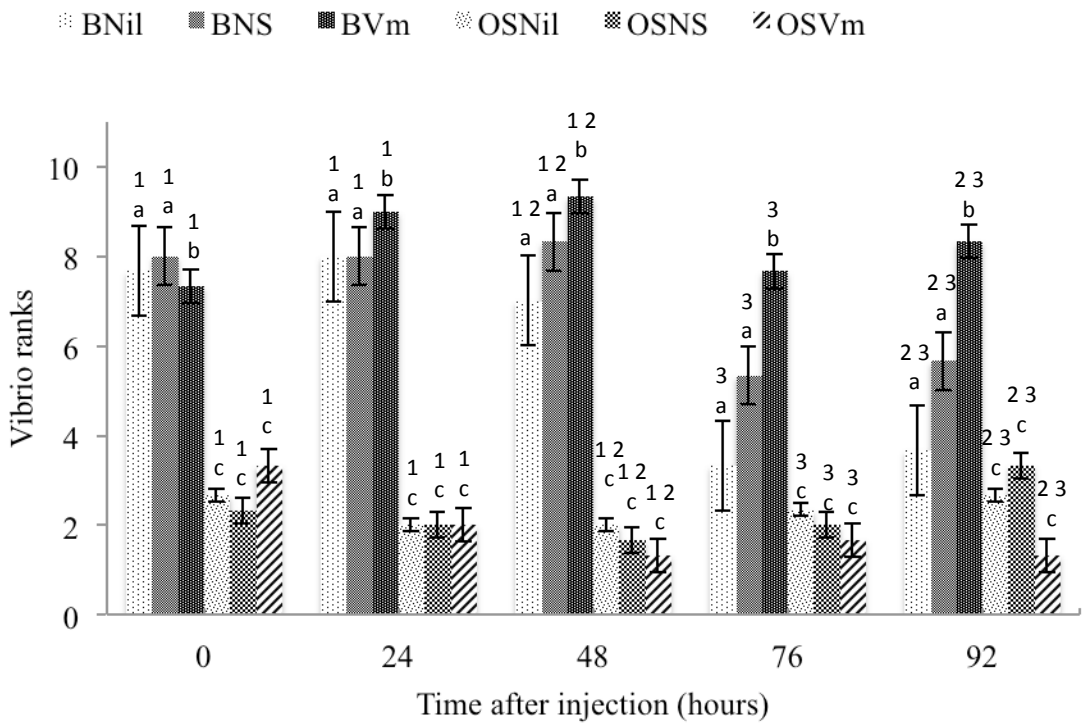

Figure 2. Mean ± SE of *Vibrio* ranks of marron after being challenge with *V. mimicus*. Different alphabets (a, b, c) over bars indicate significantly different means for different treatments at $P < 0.05$. Different numericals (1, 2, 3) over bars indicate significantly different means at different 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.