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Effects of organic selenium supplementation on growth, glutathione peroxidase activity and histopathology in juvenile barramundi (*Lates calcarifer* Bloch 1970) fed high lupin meal-based diets

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ABSTRACT

Very limited information is available on the relationship between dietary selenium (Se) and plant protein (PP) sources in carnivorous marine aquaculture species. Therefore, this study employed a 2 × 3 experimental layout to investigate the effects of lupin meal (LM) protein inclusion levels (0, 25 and 75%) and organic selenium (OS) levels (0 or 2 g kg^{-1}) on the growth, physiology and histopathology of juvenile barramundi (*Lates calcarifer*). The experimental diets (LM_0, LM_{0+OS}, LM_{25}, LM_{25+OS}, LM_{75} and LM_{75+OS}) were formulated on an isonitrogenous (48.8% crude protein) and isocalorific (20.6 MJ kg^{-1} gross energy) basis. In the 60-day feeding experiment, final weight (FW), specific growth rate (SGR) and weight gain (WG) were improved by the supplementation of Se in LM-based diets. Fish fed diets containing Se had higher FW, SGR and WG compared with those fed diets lacking Se supplementation ($P < 0.05$). Both LM inclusion levels and Se supplementation levels affected the apparent digestibility coefficient of protein (ADC-P). Meanwhile, survival and the thermal growth coefficient (TGC) were not significantly different among all dietary treatments. The inclusion of a high LM level resulted in decreased glutathione peroxidase (GPx) activities, but this effect was not observed when Se was supplemented in the diets. Furthermore, there was a linear relationship between muscle Se level and Se concentration of the experimental diets. Se-induced myopathy was observed in skeletal muscles of fish fed LM diets without Se supplementation. In addition, structural alteration was found in the liver; however, the kidney, spleen and intestine were histologically normal. Overall, these results suggest that high LM diets supplemented with organic selenium can enhance growth, physiological and histological performances of juvenile barramundi.

Statement of relevance: While plant-based feed sources such as lupin meal have the potential to reduce the reliance on unsustainable wild fishmeal in aquaculture, such products may reduce the feed availability of selenium, an essential element for aquatic animals. We believe that the findings of this study are relevant to the general field of commercial aquaculture.

1. Introduction

Fishmeal (FM), the primary protein source in aquaculture feeds, is fabricated from either whole fishes, fish cut-offs or fish processing by-products (Shepherd and Jackson, 2013). Demand for FM increases as aquaculture production intensifies, resulting in exorbitant prices for the commodity (OECD-FAO, 2014). In addition, there is also concern over the continuity of FM supply along with the finite nature of wild fisheries (Delgado et al., 2003). These economic and ecological sustainability issues are putting more pressures on the aquaculture industry to lower the levels of FM in aquaculture feeds (Gallagher, 1994; Hardy, 2010; Hua and Bureau, 2012). Nevertheless, the sustainability of the aquaculture industry fundamentally relies on the reduction of FM in feed composition (Bostock et al., 2010; Bulbul et al., 2013) or the shift

Abbreviations: AA, amino acid; AAC, amino acid chelates; ADCs, apparent digestibility coefficients; ANF, antinutritional factors; ANOVA, analysis of variance; CARL, Curtin Aquatic Research Laboratory; CRM, certified reference material; CP, crude protein; Cr_2O_3, chromic oxide; Cu, copper; DM, dry matter; FCR, feed conversion ratio; Fe, iron; FI, feed intake; FM, fishmeal; FW, final weight; GE, gross energy; GI, gastro-intestinal; GPx, glutathione peroxidase; Hb, haemoglobin; HCl, hydrochloric acid; ICP-MS, inductively coupled plasma-mass spectrometry; LOD, limit of detection; LM, lupin meal; Mg, magnesium; Mn, manganese; N, nitrogen; Ni, nickel; HNO_3, nitric acid; OS, organic selenium; P, phosphorus; PA, phytic acid; PP, plant protein; RBCs, red blood cells; S, survival; SBM, soybean meal; Se, selenium; SGR, specific growth rate; Se-Met, selenomethionine; SDS, sudden death syndrome; SPSS, statistical package for Social Sciences; TGC, thermal growth coefficient; WA, Western Australia; Zn, zinc.

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from FM to non-FM as the major protein source used in aquaculture feeds (Hardy, 2010; Huntington and Hasan, 2009). Therefore, numerous studies embodying a wide variety of ingredients, feed formulations and experimental systems have been undertaken to investigate the inclusion of a non-FM dietary component derived from plant protein (PP) sources, which are considered to be more cost-effective and more ecologically friendly.

Among PP sources, lupin meal (LM, *Lupinus angustifolius*) has received considerable attention as a potential alternative to FM because of its comparatively balanced nutritional profile, desirable palatability, high digestibility, cheaper price and reliable supply (Gatlin et al., 2007). The LM obtained after the dehulling process is a favourable protein source with protein content ranging between 350 g kg^{-1} and 500 g kg^{-1} dry matter (DM) (Drew et al., 2007). Several studies have shown considerable success in low replacement of FM with LM (≤30%) in diets for a variety of fish species (Glencross and Hawkins, 2004; Glencross et al., 2008; Omnes et al., 2015; Pereira and Oliva-Teles, 2004; Refstie et al., 2006; Zhang et al., 2012). However, a number of studies show that partial or high replacement of FM with LM (≥50%) is conceivable at least in rainbow trout *(Oncorhynchus mykiss)* (Borquez et al., 2011; Burel et al., 1998; Farhangi and Carter, 2007; Glencross et al., 2004). The reasons for a discrepancy among aquaculture nutritionists on the utilisation of LM as a protein source for fish might be associated with a number of factors including product quality, treatment and inclusion levels of LM, feed formulation, culture condition, fish size and variations in fish species.

For carnivorous species, problems occur when FM is highly or fully replaced with PP ingredients in the diet. These include reduced fish performance and health caused by poor palatability, amino acid (AA) deficiency, lower nutrient digestibility, decreased energy content as well as the manifestation of particular compounds in plants that are unfavourable to fish, identified as anti-nutritional factors (ANF) (Bonaldo et al., 2011; Farhangi and Carter, 2001; Francis et al., 2001; Krogdahl et al., 2010; NRC, 2011). Moreover, certain ANF such as phytic acid may reduce the bioavailability of minerals such as copper (Cu), zinc (Zn), nickel (Ni), cobalt (Co), manganese (Mn), iron (Fe), magnesium (Mg), calcium (Ca) and selenium (Se) (Connelly, 2011). The complexity of mineral chelation and subsequent deficiencies can impair growth and health of fish fed diets containing high PP ingredients. Therefore, the use of mineral-derived feed additive in the low FM diet is an alternative approach to diminish the adverse effects of PP sources and may help improve fish growth and health performance.

Se is a trace mineral essential for fish cellular metabolism. However, it becomes poisonous for aquatic organisms at high concentrations. Se serves as an integral structural element of the active core of glutathione peroxidase (GPx) enzymes in red blood cells (RBCs) (Rotruck et al., 1973). The beneficial effects of supplementing diets of a variety of fish species with Se additives have been well documented in previous studies (Arshad et al., 2011; Bell et al., 1987; Elia et al., 2011; Gatlin and Wilson, 1984; Hardy et al., 2010; Jaramillo et al., 2009; Kucukbay et al., 2009; Le and Fotedar, 2014a; Lin and Shiau, 2005; Liu et al., 2010; Lorentzen et al., 1994; Rider et al., 2009). However, none of those studies utilised PP ingredients as the protein source in the diet. Instead, FM was used as the main input for dietary protein. The importance of dietary Se in PP-derived ingredients such as LM remains a lacuna in fish-nutrition studies (Prabhu et al., 2014), including those applied for marine carnivorous finfish species.

Barramundi (*Lates calcarifer*), also known as Asian seabass, is a carnivorous fish that is widely distributed throughout the Asia–Pacific region. The species has been an economically important species in Australia and Asian countries (Paul et al., 2013). Although barramundi are fed with commercial diets in Australia, feeding with trash fish is very common in several Asian countries (Job, 2011; Rimmer and John Russell, 1998; Tantikitti et al., 2005). Nutritional requirements of barramundi have been extensively studied; however, less attention has been paid to trace element nutrition of the species. Therefore, this study was carried out to investigate the effect of organic selenium (OS) supplementation in barramundi diets containing LM as the major dietary protein source. Growth performance, feed utilisation, blood chemistry and histopathology were particularly examined.

2. Materials and methods

2.1. Diets and experimental design

Three isonitrogenous (48.8% crude protein, CP) and isoenergetic (20.6 MJ kg^{-1} gross energy, GE) experimental diets were prepared and formulated, as LM_0, LM_{25}, and LM_{75}. Diets were supplemented with 0 and 2 g OS kg^{-1} dry matter (DM). Thus, there were two control diets in the feeding experiment: an FM-based diet without OS supplement (LM_0) and an FM-based diet with OS supplement (LM_{0+OS}). FM, LM, casein and gluten were used as protein sources in the diets. Formulation and proximate composition of the experimental diets are presented in Table 1. Water (50 g kg^{-1}) was added prior to pelleting. All ingredients were ground to pass through a 1 mm mesh screen, pelleted in a mixer, crumbled to the desired size, air-dried, and stored at 4 °C until feeding. Cr_2O_3 was included in all diets at 0.5% as an inert, indigestible marker to determine apparent digestibility coefficient of protein (ADC-P). AA profiles of the experimental diets are shown in Table 2.

2.2. Fish, experimental conditions and feeding

Three hundred and sixty healthy barramundi juveniles of average weight, 5.38 ± 0.16 g (mean ± SE), were supplied by the Australian Centre for Applied Aquaculture Research (Fremantle, Western Australia). Prior to the feeding trial, fish were acclimated and fed with a commercial diet twice daily for two weeks until fully acclimated to the rearing conditions. At the commencement of the feeding trial, 18 groups with 20 fish each were bulk weighed and then randomly stocked into 18 tanks. Each experimental diet was triplicated.

Table 1
Formulation and proximate composition of the experimental diets.

Ingredients[a] (g kg^{-1} DM)	Diets					
	LM_0	LM_{0+OS}	LM_{25}	LM_{25+OS}	LM_{75}	LM_{75+OS}
Fishmeal	460	460	340	340	150	150
Lupin kernel meal[b]	–	–	185	185	510	510
Wheat gluten	100	100	100	100	100	100
Wheat flour	80	80	40	40	–	–
Casein	120	120	120	120	120	120
Fish oil	100	100	100	100	80	80
Wheat starch	65	63	50	53	15	13
Cellulose	50	48	40	38	–	–
Se-free premix[c]	20	20	20	20	20	20
Organic selenium[d]	–	2	–	2	–	2
Chromic oxide	5	5	5	5	5	5
Proximate content (%)						
Dry matter	86.20	86.35	90.15	90.25	90.18	90.23
Ash	8.13	8.37	5.82	5.88	5.35	5.61
Protein	49.33	49.50	48.75	48.81	48.25	48.16
Lipid	15.41	15.40	15.07	15.05	14.52	14.55
Gross energy (MJ/kg)	20.59	20.41	20.71	20.45	20.75	20.69
Se (mg kg^{-1})	3.11	5.08	2.53	4.51	1.58	3.56

[a] Supplied by Specialty Feeds, Perth, WA, except for Sel-Plex® and chromic oxide, obtained from Alltech Inc., Lexington, Kentucky, USA and Thermo Fisher Scientific, Scoresby, Vic., Australia, respectively.
[b] Australian sweet lupin, *Lupinus angustifolius*.
[c] Contains the following (as g kg^{-1} of premix): iron, 10; copper, 1.5; iodine, 0.15; manganese, 9.5; zinc, 25; vitamin A retinol, 100 IU; vitamin D3, 100 IU; vitamin E, 6.25; vitamin K, 1.6; vitamin B1, 1; vitamin B2, 2.5; niacin, 20; vitamin B6, 1.5; calcium, 5.5; biotin, 0.1; folic acid, 0.4; inositol, 60; vitamin B12, 0.002; choline, 150; ethoxyquin, 0.125.
[d] Sel-Plex® (Alltech Inc., Lexington, Kentucky, USA).

Table 2
Hydrolysed amino acid composition of FM, LM, wheat gluten and casein (g 100 g^{-1} protein).

Amino acid	FM	LM	Wheat gluten	Casein
Essential				
Arginine	4.35	4.67	1.50	3.32
Histidine	2.09	1.11	0.96	2.73
Isoleucine	3.04	1.80	2.35	4.70
Leucine	5.17	2.90	9.75	8.70
Lysine	4.77	1.84	0.80	7.46
Methionine	1.86	0.31	1.05	2.19
Phenylalanine	2.87	1.78	3.47	4.67
Threonine	3.19	1.58	1.76	3.71
Valine	3.26	1.70	2.58	5.75
Non-essential				
Alanine	4.42	1.52	NA	2.56
Aspartic acid	6.20	4.15	NA	6.24
Glutamic acid	8.25	8.84	NA	18.60
Glycine	4.73	1.75	NA	2.38
Proline	3.81	2.12	NA	8.80
Serine	3.05	2.14	NA	5.36

NA: not analysed.

The feeding trial was conducted at the Curtin Aquatic Research Laboratory (Technology Park Bentley, Western Australia). The experimental system consisted of 18 circular, 300 L fibreglass tanks. Each tank received recirculated water from an external biofilter (Fluval 406, Hagen, Italy) at 10 L min^{-1}. All experimental tanks were supplied with constant aeration and pure oxygen (compressed oxygen, BOC, Perth, WA).

The feeding trial lasted for 60 days, during which fish were hand-fed with the experimental diets to satiation twice daily at 0900 h and 1500 h. Throughout the experiment, the water-quality parameters were maintained at a temperature between 27 °C and 29 °C, dissolved oxygen >5 mg/L, and salinity 32–34 ppt. Dead fish were weighed and recorded for adjusting the calculation of feed conversion ratio (FCR) and survival. Fish-handling procedures, care and facilities complied with the guidelines of the Animal Ethics Committee of Curtin University and followed the Australian Code of Practice for the care and use of animals for scientific purposes.

2.3. Protein digestibility

To investigate the effect of dietary treatment on protein digestibility, faecal matter was collected immediately prior to the morning feeding by stripping techniques (Austreng, 1978) one week before the end of the feeding experiment. Faecal collections from individuals were pooled by tank and quickly stored at −20 °C. Prior to analysis, the faecal samples were dried to a constant weight at 105 °C. ADC-P was measured using the indirect method (Cr_2O_3), as suggested by Cho et al. (1982).

2.4. Sampling and analytical methods

At the measurement of the terminal body weight, all fish were starved for 24 h prior to final sampling to achieve a basic metabolite state. The fish were then anaesthetised with tricaine methanesulfonate (MS-222, Sigma-Aldrich, Castle Hill, NSW, Australia) at 100 mg L^{-1} and weighed individually. Blood samples from three fish in each tank were then withdrawn by caudal vein puncture with a 1 mL plastic syringe. The extracted blood was transferred to a heparinised tube for haematology. Haematocrit and leucocrit were determined by centrifugation of capillary glass tubes, according to the method of McLeay and Gordon (1977). An Hb kit (Randox Laboratories, Antrim, United Kingdom) was used to measure the haemoglobin (Hb) content. Erythrocyte (red blood cell) glutathione peroxidase (GPx) activity was quantitatively assayed using the Randox Laboratories test combination (Ransel, Antrim, United Kingdom).

The proximal compositions of the diet and faecal samples were determined based on Association of Official Analytical Chemists procedures (AOAC, 1990). Dry matter was determined by oven drying to constant weight at 105 °C; crude ash by combustion at 550 °C; crude protein content (N × 6.25) by the Kjeldahl digestion method; crude lipid content by the Soxhlet technique; and gross energy content by an IKA oxygen bomb calorimeter (Heitersheim, Germany). AA content of the diets was determined after samples were hydrolysed in HCl (Barkholt and Jensen, 1989; Rayner, 1985). Analyses were performed on an Agilent 1100 series high-performance liquid chromatography (HPLC, Agilent Technologies, Germany) system using conditions similar to those described by Gratzfeld-Huesgen (1998).

2.5. Se determination

Se was analysed at the Intertek Genalysis Laboratory (Perth, Australia) using inductively coupled plasma-mass spectrometry instrument (ICP-MS, 7500 series, Agilent Technologies, Australia). The total Se in diet and muscle tissue samples was determined using an aqua regia digestion with reduction precipitation. 0.5 g sample was dissolved in aqua regia, filtered, and the selenium reduced to the zero oxidation state where it precipitated. The precipitate was collected using filtration, dissolved in acid and read on an ICP-MS. A mass of 5 g was catch-weighed in a beaker. Nitric acid (HNO_3) and hydrochloric acid (HCl) were added sequentially to make aqua regia. The excess acid was boiled off and the digested sample was leached in HCl. The sample was filtered, the residue and beaker were thoroughly washed, and the filtrate was collected. The selenium was precipitated using a Cu solution in a mixture of citric and ascorbic acids at a low temperature. The selenium was filtered off and dissolved in nitric acid. HCl and deionised water were added and the solution was mixed and read on a calibrated ICP-MS. Selected certified reference material (CRM) for the analysis was OREAS 97.01, which was prepared from OREAS 97 (Ore Research and Exploration Pty Ltd., Victoria, Australia) by diluting this 100× in high purity silica. The secondary CRM OREAS 97.01 had an aqua regia extractable value of 0.673 ± 0.063 mg Se kg^{-1}. Recovery of the CRM was 98–99% and the limit of detection (LOD) was 0.01 mg Se kg^{-1}. Se concentration was reported as dry weight.

2.6. Histopathology

At the end of the trial, segments of muscle, liver, kidney, spleen and intestine from three fish in each tank were fixed in 10% buffered formalin, dehydrated in ethanol before equilibration in xylene and embedding in paraffin wax. Sections of approximately 5 μm were cut and stained with haematoxylin and eosin for histological observation under an Olympus BX40F4 light microscope. Light microscopy samples were prepared according to standard histological techniques (Luna, 1968).

2.7. Calculation

Growth and feeding performances were measured using the calculated parameters below:

$$\text{Specific growth rate}\left(\text{SGR}, \%\text{day}^{-1}\right) = 100 \times \left[\frac{\ln \text{final weight} - \ln \text{initial weight}}{\text{days}}\right]$$

$$\text{Weight gain } (\%) = 100 \times \left[\frac{\text{final weight} - \text{initial weight}}{\text{initial weight}}\right]$$

$$\text{Feed intake}\left(\text{FI}, \text{g fish}^{-1}\ \text{days}^{-1}\right) = \left[\frac{\text{dry diet given} - \text{dry remaining diet recovered}}{\text{number of fish}}\right]/\text{days}$$

$$\text{Feed conversion ratio (FCR)} = \frac{\text{feed intake}}{\text{weight gain}}$$

$$\text{Survival (S, \%)} = 100 \times \left[\frac{\text{final number of fish}}{\text{initial number of fish}}\right]$$

$$\text{ADC—P (\%)} = 100 - \left[\left(100 - \frac{\%\text{Cr2O3 in diet}}{\%\text{Cr2O3 in faeces}} \times \frac{\%\text{protein in faeces}}{\%\text{protein in diet}}\right)\right]$$

$$\text{Thermal growth coefficient (TGC)} = \left[\frac{\left(\text{final weight}^{1/3} - \text{initial weight}^{1/3}\right)}{\text{temperature } \text{Å} \times \text{days}}\right] \times 1000.$$

2.8. Statistical analysis

All data regarding the effects of LM level, Se level and their interactions on growth, feeding, haematological and enzymatic GPx responses, as well as muscle Se content were subjected to two-way analysis of variance (ANOVA). Assumptions of homogeneity of variances were checked using Levene's equal variance test. In the present study, because significant interaction was not found between the two factors, the main effect was examined for both factors using Duncan's test to check all differences among the dietary treatments. All differences were considered significant when P value < 0.05. Percentage data were computed using arcsine transformations. All statistical analyses were performed using SPSS (version 22, IBM Inc., Australia).

3. Results

3.1. Growth, survival and ADC-P

Data for FW, SGR, FI, WG, FCR, ADC-P, survival and TGC are presented in Table 3. All experimental diets were voluntarily ingested by juvenile barramundi over the course of the feeding experiment and, thus, no significant differences were observed in FI. OS supplementation levels influenced FW, SGR and WG. Fish fed diets containing OS had higher FW, SGR and WG compared with those fed diets lacking OS supplementation ($P < 0.05$). LM inclusion levels and OS supplementation levels affected ADC-P. Fish fed diets containing OS supplement attained higher ADC-P in comparison with those fed OS-deficient diets. In addition, irrespective of OS supplementation level, ADC-P decreased as dietary LM increased. Survival exceeded 98% and did not differ significantly among dietary treatments. Meanwhile, LM inclusion levels, OS supplementation levels, or the interaction of these factors, did not affect TGC.

3.2. Blood physiology and muscle Se level

There was no synergistic effect between OS supplementation and LM inclusion levels on the haematocrit, leucocrit, GPx activity and muscle Se concentration (Table 4). No significant effect was observed on haematocrit ($P > 0.05$). However, increased leucocrit was found in fish fed OS-containing diets. OS supplementation levels significantly affected GPx activity. GPx activity of fish in OS-supplemented group ranged from 197 to 205 (U^{-1} g Hb), higher than those without OS supplementation. Muscle Se concentration was influenced by both LM inclusion and OS supplementation levels and muscle Se contents were significantly affected by both LM inclusion and OS supplementation levels ($P < 0.05$). In addition, muscle Se concentration was related to the Se level in the diets (Fig. 1).

3.3. Histopathological evaluation

As observed in the tissue of fish fed diets supplemented with OS, the normal structure of skeletal muscle in cross-section was characterised by rounded, densely packed, and uniformly identical muscle fibres (Fig. 2a and c). However, severe histopathological alterations were observed in muscle tissue of fish fed LM_{25} and LM_{75} diets. The skeletal muscle exhibited signs typical of nutritional muscular dystrophy attributable to Se deficiency, including falsification, disconnection, and longitudinal rupture of muscle fibres (Fig. 2b and d). Also, LM diets lacking OS supplementation caused notable morphological changes in the liver of fish, which were characterised by variations in hepatocyte vacuolation, with an increased amount of lipid droplets found in the liver of fish fed the LM_{75} diets (Fig. 3). However, all tissues of the gastrointestinal tract were histologically normal, as were the kidney and spleen.

4. Discussion

The inclusion level of FM in compounded aquaculture feed has been substantially reduced, and PP materials have been, and will possibly remain, the foremost alternative when substituting FM in fish diets. In recent years, aquaculture feed development has been directed towards further reduction of FM as the main protein source in fish feeds without hampering growth performance and nutrient utilisation (Olsen and Hasan, 2012). To achieve this, nutritional strategies should include not only finding other PP sources as alternatives to FM but also improving the quality of the already existing PP materials through innovative approaches, such as proper raw material processing and nutrient enrichment. For instance, Krogdahl et al. (2010) suggested that the presence of phytic acid ANF in PP ingredients might be counteracted by mineral supplementation. Indeed, effects of Se supplementation on performance

Table 3
Performance of juvenile barramundi fed different LM levels with and without OS supplementation for 60 days.

Parameters	0 g OS kg^{-1}			2 g OS kg^{-1}			Analysis of variance (ANOVA)				
	LM_0	LM_{25}	LM_{75}	LM_0	LM_{25}	LM_{75}	OS (g kg^{-1})	LM level (%)			Interaction
							0 *vs* 2	0	25	75	
FW	31.6	30.2	29.8	34.4	35.4	33.3	<	ns			ns
SGR	3.17	3.13	3.04	3.30	3.32	3.26	<	ns			ns
WG	490.3	477.5	449.5	533.6	543.7	522	<	ns			ns
FI	0.63	0.64	0.61	0.64	0.62	0.62	ns	ns			ns
FCR	1.26	1.24	1.30	1.23	1.22	1.29	ns	ns			ns
ADC-P	90.4	92.8	94.3	93.4	94.8	94.7	<	a	b	b	ns
S	98.3	96.7	98.3	100	98.3	98.3	ns	ns			ns
TGC	0.851	0.844	0.802	0.840	0.886	0.837	ns	ns			ns

LM_0, LM_{25}, LM_{75} (FM protein replaced by LM protein with 0%, 25% and 75%, respectively).
FW (final weight, g); SGR (specific growth rate, % body weight day^{-1}); FI (feed intake, g $fish^{-1}$ day^{-1}); WG (weight gain, %); FCR (feed conversion ratio); ADC-P (apparent digestibility coefficient of protein, %); S (survival, %); TGC (thermal growth coefficient, day 1–60; 28.5 °C).
a, b: For variables with a significant effect of LM level, values without a common letter are different (b indicated the highest value; $P < 0.05$).
< or >: For variables with a significant effect of OS level ($P < 0.05$), < or > indicates whether the values measured at 0 g OS kg^{-1} supplementation level were less than or greater than those measured at 2 g OS kg^{-1} supplementation level.
ns: non-significant ($P > 0.05$).

Table 4
Haematocrit, leucocrit, GPx activities and muscle Se concentration of barramundi fed different LM levels with and without OS supplementation for 60 days.

Parameters	0 g OS kg^{-1}			2 g OS kg^{-1}			Analysis of variance (ANOVA)				
	LM_0	LM_{25}	LM_{75}	LM_0	LM_{25}	LM_{75}	OS (g kg^{-1})	LM level (%)			Interaction
							0 *vs* 2	0	25	75	
Haematocrit	33.30	32.76	33.27	32.30	34.73	33.07	ns	ns			ns
Leucocrit	1.14	1.05	1.01	1.42	1.46	1.18	<	ns			ns
GPx	195.7	194.4	193.3	205.3	199.7	197	<	ns			ns
Muscle Se	0.48	0.38	0.37	0.58	0.59	0.50	<	c	b	a	ns

LM_0, LM_{25}, LM_{75} (FM protein replaced by LM protein with 0%, 25% and 75%, respectively).
Haematocrit (%); Leucocrit (%), GPx (U g^{-1} Hb); Muscle Se (μg g^{-1}).
a, b, c: For variables with a significant effect of LM level, values without a common letter are different (c indicated the highest value; $P < 0.05$).
< or >: For variables with a significant effect of OS level ($P < 0.05$), < or > indicates whether the values measured at 0 g OS kg^{-1} supplementation level were less than or greater than those measured at 2 g OS kg^{-1} supplementation level.
ns: non-significant ($P > 0.05$).

of fish fed PP-based protein sources remain unstudied (Prabhu et al., 2014).

One of the undesirable features of PP-based diets has been related to low FI. Several authors have reported the suppression of FI with high proportion of PP sources (Alexis, 1990; Espe et al., 2006; Gomes et al., 1995; Refstie et al., 1998; Torstensen et al., 2008). In the case of LM, a reduction in FI was attributable to alkaloid content (de la Higuera et al., 1988), high-fibre substance (Bangoula et al., 1993), AA deficiency (Jobling et al., 2007) and the presence of ANF (Francis et al., 2001) in the diets. The effects on FI are commonly manifested in nutrient intake and thus growth and health performance. In the current experiment, the fish promptly accepted all experimental diets, and the FI was not then an inducer in the resulting growth indices. This might show that the level of detractive compounds in PP-based diets employed in the current study was inadequate to deteriorate FI. Moreover, Australian sweet lupin *L. angustifolius*, used in the present study, is minimal in alkaloid contents, and thus palatability problems due to elevated alkaloids, as reported by Glencross et al. (2006), were not found.

To our knowledge, this is the first study to have investigated the effects of OS as feed supplement in LM diets on the growth, feed utilisation, blood physiology and histopathology of marine carnivorous finfish species. In the present experiment, the FW, SGR and WG of juvenile barramundi fed high LM diets were significantly lower than those fed OS-supplemented LM diets. The finding that high PP inclusion level (≥50%) resulted in unfavourable growth performance agrees with the results reported for similar species (Tantikitti et al., 2005) and other marine carnivorous finfish species (Chou et al., 2004; Farhangi and Carter, 2001; Glencross et al., 2004; Hernández et al., 2007; Peng et al., 2013; Wang et al., 2006; Zhou et al., 2005). In contrast, several authors have reported that 50–70% LM can be incorporated in the marine carnivorous finfish diets (Borquez et al., 2011; Burel et al., 1998). In addition, with reference to growth performance, Gallagher (1994) found that soybean meal (SBM) could replace 75% FM protein in hybrid striped bass (*Morone saxatilis*) feeds. The depression in overall growth indicators in fish fed OS-deficient diets may be associated with the existence of phytic acid (PA) in LM. PA, or phytate in salt form, is the primary phosphorus (P) storage compound in seeds, typically representing 50–80% of the total P in plant meals and providing around 1.5% to the meal dry weight (Kumar et al., 2012). It has been a general consensus that most finfish and land monogastric animals cannot utilise the P bound in PA since they lack the enzyme (phytase) that is needed to mortify phytate (Robaina et al., 1995). Inclusion of phytate (18 g kg^{-1}) in diets for Atlantic salmon (*Salmo salar*) caused a substantial reduction in growth, feed utilisation and mineral bioavailability (Storebakken et al., 1998). Retarded growth and reduced mineral absorption was obvious in striped bass (*M. saxatilis*) when served diets containing high phytate content (Papatryphon et al., 1999). Furthermore, in the presence of high phytate with low Zn diet, feed utilisation and growth of Chinook salmon (*Oncorhynchus tshawytscha*) were depressed, resulting in high mortality and irregularities in pyloric caecal formation (Richardson et al., 1985).

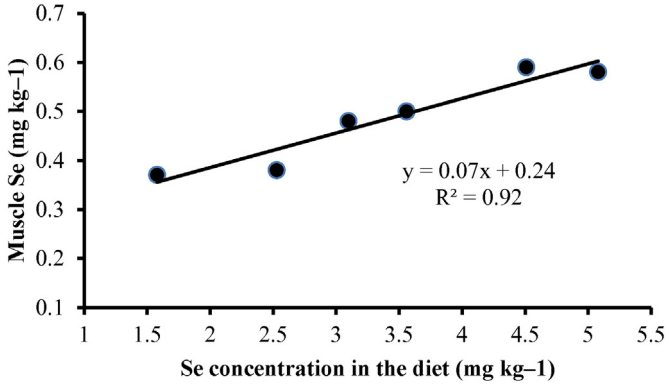

Fig. 1. Relationship between Se concentration in the diets and muscle Se level of juvenile barramundi after 60-day feeding trial.

However, the results of the present experiment indicate that, when supplemented with 2 mg kg^{-1} OS, 75% FM protein could be replaced with LM protein, without triggering a considerable adverse implication on growth, feed utilisation, blood chemistry or histology of juvenile barramundi. This is in accordance with results from our earlier studies (unpublished), in which supplementation of 2 mg kg^{-1} OS in high SBM-based diets also resulted in improved fish performances; FW and SGR were slightly lower when barramundi were fed high SBM diets, but similar if not better growth performances were attained in fermented SBM fed to barramundi. A synergistic effect between dietary vitamin E and Se concentration in the diets reportedly modified the dietary Se requirement (Jaramillo et al., 2009; Le et al., 2014). However, the required vitamin E level of barramundi was assumed to be satisfied in the present experiment with the supplementation of 130 mg α-tocopherol kg^{-1} DM. Table 5 shows a range of FM-based studies confirming the significance of dietary Se in various fish species. However, only a few adhere to European Union (EU) feed legislation that has set an upper limit of 0.5 mg Se kg^{-1} for animal including fish (EU, 2015). Indeed, in most finfish species, dietary Se requirement of fish varies with species, age, the form of Se ingested, vitamin E level of the diet and pathway and duration of exposure to Se (NRC, 2011). Further, high replacement level of FM with PP ingredients in aquaculture feeds might affect the supply of minerals including P, Zn and Se (Prabhu et al., 2014). In the present study, although the dietary concentrations of Se were relatively high (1.58–3.11 mg Se kg^{-1}), the inhibitory effect of PA could have depressed the absorption of Se, and therefore elevated Se levels were required in the diets for juvenile barramundi.

The OS used in this experiment was in the form of selenised yeast (Sel-Plex®, Alltech Inc., Lexington, Kentucky, USA) that contains a mixture of selenoamino acids with Se-Met representing more than 50% of the total Se (Lyons et al., 2007). Trace element amino acid chelates

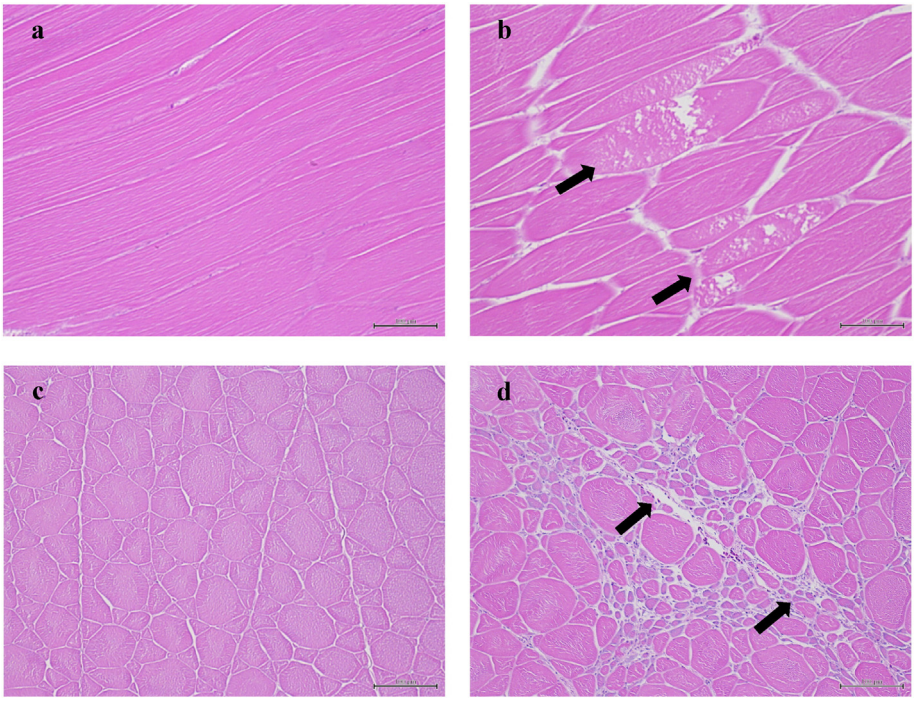

Fig. 2. Longitudinal and transverse-section of the muscle of barramundi diets showing normal histological structures (a, c) observed in Se-supplemented dietary group, and Se-induced myopathy (b, d) found in Se-deficient dietary group. Note severe muscle degeneration and hypercontraction of the surrounding muscular fibres (arrows). Scale bar = 100 μm.

(AAC) have been widely used in animal nutrition. They have shown to improve mineral bioavailability and affect growth and tissue mineral deposition in several terrestrial and aquatic animals (Sarker et al., 2007; Satoh, 2007). Owing to protection of the structural alignment of chelates, it is likely that, when ingested as AAC, there is a minor chance of the complexing action of phytate on mineral cations occurring (Apines-Amar et al., 2004). Interestingly, this may explain, indirectly, the increased ADC-P observed in fish fed the OS-containing LM diets, as revealed in the present experiment. As OS was supplemented in the diets, high absorption of AA-chelated Se into the mucosal tissues may promote an increased source of essential trace element that was later utilised as a co-factor in the production of hydrolytic enzymes, such as gastro-intestinal (GI) GPx, in the mucosal tissues. Moreover, GI-GPx is the most influential selenoprotein antioxidant in preserving the intestinal mucosal integrity (Lindh, 2013). A substantial quantity of digestive enzymes in the gut would presumably instigate the enhancement of feed digestion.

Nevertheless, regardless of OS supplementation, LM inclusion levels in the diets influenced the ADC-P in the present experiment. The ADC-P was shown to increase with increasing LM inclusion levels. At 75% LM protein inclusion level, the ADC-P was around 94.3–94.7%, significantly higher than FM-based diets (90.4–93.4%). This finding was in agreement with the previous studies using similar species (Glencross, 2006, 2011; Tabrett et al., 2012), which suggested that LM protein was typically better digested than animal-derived protein. In addition, Boonyaratpalin et al. (1998) found that, when fed to barramundi, the ADC-P of SBM products (94% in average) were higher than FM (92%), with the exception of raw SBM (73%). High ADC-P of LM-based diets has also been reported in diets for rainbow trout (*O. mykiss*) (Borquez et al., 2011; Burel et al., 1998; Glencross et al., 2007; Glencross et al., 2010). In comparison, the ADC-P of LM ingredients was reported to be identical to that of FM in diets for rainbow trout (*O. mykiss*) (de la Higuera et al., 1988) and gilthead seabream (*Sparus auruta*) (Robaina et al., 1995). Perhaps differences in ADC-P are related to variations in product quality, diet composition and species being examined.

FM-based diets, in general, represent a major source of minerals and trace elements to satisfy the nutritional prerequisites of fish. However, FM-based diets without Se supplement have been reported to generate reduced antioxidant capacity when fed to several fish species (Kucukbay et al., 2009; Le and Fotedar, 2014a; Liu et al., 2010; Pacini

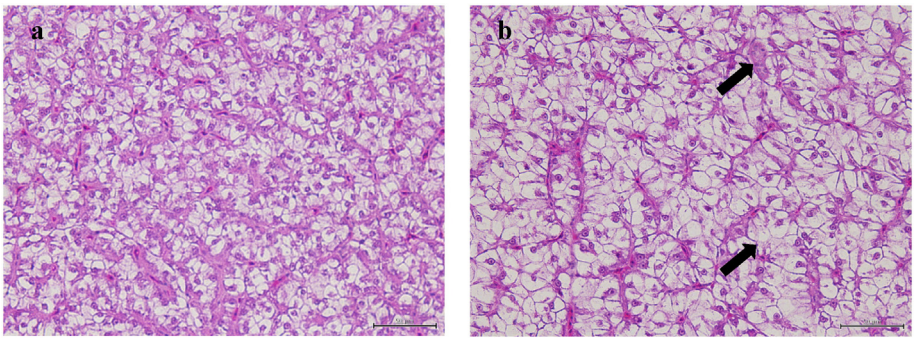

Fig. 3. (a) Section of liver of barramundi fed Se-supplemented diets showing normal liver containing predominantly glycogen vacuoles, (b) fatty liver of barramundi fed diets lacking Se supplementation. Note the extension the hepatocytes and the generation of apparent hepatic steatosis, with intense vacuoles in the hepatocytes resemble lipids (arrows). Scale bar = 50 μm.

Table 5
Dietary Se source, level and requirement (mg kg^{-1}) in various fish species.

No	Species	Se source	Se level (mg kg^{-1})	Requirement (mg kg^{-1})	Reference
1	Atlantic salmon (*S. salar*)	Se-Met	2.1–3.1	<2.1	Lorentzen et al. (1994)
2	Beluga sturgeon (*Huso huso*)	Se-Met	1.26–20.3	11.56	Arshad et al. (2011)
3	Channel catfish (*Ictalurus punctatus*)	Na_2SeO_3	0.06–5.0	0.25	Gatlin and Wilson (1984)
4	Channel catfish (*I. punctatus*)	Se-Met	0.02–0.4	0.12	Wang and Lovell (1997)
5	Cobia (*Rachycentron canadum*)	Se-Met	0.21–1.4	0.8	Liu et al. (2010)
6	Common carp (*Cyprinus carpio*)	Nano-Se	0.43–2.51	1.46	Ashouri et al. (2015)
7	Cutthroat trout (*Oncorhynchus clarkii*)	Se-Met	1.2–11.2	9.2	Hardy et al. (2010)
8	Gibel carp (*Carassius auratus gibelio*)	Se-Met	0.34–5.13	1.18	Han et al. (2011)
9	Grouper (*Epinephelus malabaricus*)	Se-Met	0.17–4.0	0.7	Lin and Shiau (2005)
10	Hybrid striped bass (*Morone chrysops* × *M. saxatilis*)	Na_2SeO_3	1.19–21.23	1.19	Jaramillo et al. (2009)
11	Hybrid striped bass (*M. chrysops* × *M. saxatilis*)	Na_2SeO_3	1.22–4.42	0.4	Cotter et al. (2008)
12	Largemouth bass (*Micropterus salmoide*)	Na_2SeO_3	0.97–2.06	1.60–1.85	Zhu et al. (2012)
13	Yellowtail kingfish (*Seriola lalandi*)	Organic (Sel-Plex®)	4.86–6.38	5.56	Le and Fotedar (2013)

et al., 2013; Rider et al., 2009). The most important biological significance of Se is its antioxidant capacity mediated through GPx that provides cellular protection against toxic peroxides (Arthur et al., 2003; Watanabe et al., 1997). Thus, insufficient intake of Se induces low GPx activity (Dhur et al., 1990). However, based on molecular genetics, Penglase et al. (2014) observed that whole body GPx activity and expression was at the minimum at maximum growth rate in zebrafish (*Danio rerio*), thus the relationship between Se requirement and GPx activity is not straightforward in fish. Because muscle tissue is a strong Se accumulator, muscle Se level has been associated with GPx activity in a variety of fish species (Bell et al., 1985; Han et al., 2011; Hilton et al., 1980; Kucukbay et al., 2009; Le and Fotedar, 2014b; Wang et al., 2007; Zhou et al., 2009; Zhu et al., 2012). However, there was a dose-dependent relationship between muscle Se concentration and dietary Se level (Le and Fotedar, 2014c). Further, Fontagne-Dicharry et al. (2015) investigated the effects of dietary Se enrichment on the antioxidant status of rainbow trout (*O. mykiss*) fed diets containing 75% PP mixtures (wheat gluten, corn gluten meal, soybean protein concentrate, soya meal, rapeseed meal, white LM and dehulled pea meal). This is so far the only information available regarding the impacts of Se inclusion in PP-based diets. While there was no significant differences in growth performances among dietary treatments, the lowest whole-body Se level as well as whole-body Se-GPx activity was observed in fish fed diets without Se supplement, indicating a nutritional Se deficiency. These results, aside from growth outcomes, are in line with the findings of the current experiment. Increased dietary Se level led to the augmentation of both muscle Se level and plasma GPx activity. Also, a linearity between muscle Se concentration and dietary Se level did exist over the course of the feeding experiment. These findings may suggest that both muscle Se level and plasma GPx activity can be used as a biological indicator in delineating Se status in marine carnivorous finfish species, particularly when fed PP-based diets. The implications of OS supplementation on growth indices of barramundi might be attributed to the antioxidant capacity of Se.

Haematological assays are useful diagnostic tools for examining physiological conditions of the fish (Silkin and Silkina, 2005). In the present experiment, no differences were observed for haematocrit, and the haematocrit values in the current experiment (32.40–34.73%) were within the normal range (30–45%), as suggested by Adams et al. (1993). Furthermore, leucocrit values may reflect the health condition of fish (Wedemeyer et al., 1983), thus malnutrition and increased susceptibility to disease are ascribed to low levels of leucocrit (Bandyopadhyay and Das Mohapatra, 2009; El-Asely et al., 2014). In this study, significant increases in leucocrit were observed in barramundi fed OS-supplemented diets, suggesting that OS may include particular compounds that stimulate leucocrit production. Leucocrit assessment is considered a rapid and inexpensive tool for evaluating fish health status (Adams et al., 1993).

Histologically, signs of Se deficiency include the proliferation of lipid deposition in the liver (Burk et al., 1995), as well as myopathy in skeletal muscle (Le and Fotedar, 2013). It is thus interesting to note that the disruption in growth, tissue Se content and GPx activity corroborate the histological findings observed in the current experiment, where muscle damage and fatty liver were obvious in fish fed LM-based diets lacking OS supplementation. While fish fed LM_{25} diets exhibited moderate multifocal necrosis that was characterised by degenerating muscle bundles, those fed LM_{75} diets displayed even more severe necrosis of muscle fibres. Of additional note in this experiment is that at LM_{75} dietary group, where juvenile barramundi had the highest PP levels, there was the largest proportion of fish displaying skeletal disorder, and moreover their lesions were more intense. Previously, some authors have reported histological abnormalities in the muscle of marine finfish due to Se deficiency as a distortion of muscle fibres (Le and Fotedar, 2014a) and a variation in size of degenerating muscle fibres (Poston et al., 1976). Rodger et al. (1991) suggested that accumulation of lactic acid in the muscle fibres were likely to appear when muscle fibres were damaged. This muscle-affecting acidosis might induce the reduction in blood oxygen carrying capacity, as suggested by Root (1931). Sudden death syndrome (SDS) of Atlantic salmon (*S. salar*) was associated with mortality that could be the result of either malfunction of essential muscles or the anoxic condition from lactic acidosis, or an interaction of both (Rodger et al., 1991). Therefore, skeletal muscle integrity is used as an important indicator in fish health histological-based assessment.

Furthermore, GPx activity is also associated with the configuration and function of cell membranes (Wang et al., 2013). In the current study, the level of GPx activity in plasma significantly reduced with the decreasing level of OS supplementation in LM diets. The reduction in antioxidant capacity would be presumed to trigger a substantial implication on the tissue cellular structure of the studied fish. Thus, lipid droplet congregation in hepatocytes could be expected. Similarly, Wang et al. (2013) reported an accumulation of lipid peroxidation in the liver of fish fed Se-deficient diets. Atencio et al. (2009) demonstrated that the recovery of the cyanobacterial-induced histopathological changes primarily occurred with the maximum level of Se supplementation in most fish organs. Obviously, in the present study, the OS supplementation conferred protection against cellular damages when fish were fed with PP-based diets. Although needed in trace amounts, Se plays an important function as the constraint in the antioxidant enzyme biosynthesis. Under Se-deficient conditions, there would be a higher incidence of pro-inflammation that might expose fish to severe diseases.

5. Conclusion

On the basis of the results from the present study, it can be concluded that feeding juvenile barramundi with high LM-based diets without Se supplementation might be unfavourable to the growth, physiology and histology of fish. The present findings also confirm that Se in FM-based diets is inadequate to achieve maximal growth and GPx antioxidant enzymatic capacity of fish. However, it is not clear whether Se

supplementation is still needed if the LM is further processed into more refined products, for instance through the fermentation process. Therefore, the effects of fermented LM with and without Se supplementation in the diets for barramundi should be further investigated.

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