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Effects of canola meal on growth, feed utilisation, plasma biochemistry, histology of digestive organs and hepatic gene expression of barramundi (Asian seabass; *Lates calcarifer*)

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Abstract

The serial replacement of fish meal (anchovetta) by canola meal (CM) (100, 200, 300 g kg^{-1} as either solvent extracted (SE) CM or expeller extracted (EX) CM was undertaken to investigate the effects of increasing dietary CM levels on feed intake, growth, protein and energy retention, plasma biochemistry and the expression of a suite of hepatic genes in barramundi (Asian seabass; *Lates calcarifer*) over an eight week feeding trial. An additional diet using lupin kernel meal (LM) to replace the fish meal was also included as a comparative reference. Eight iso-digestible nitrogenous (423 ± 29 g kg^{-1}) and iso-digestible energetic (14.6 ± 8 MJ kg^{-1} DM) diets were formulated. Each diet was randomly allocated to triplicate groups of fish in seawater tanks (600L), and each tank was stocked with 15 fish (53.4 ± 7.0 g). Fish were fed once daily (9:00-10:00) to apparent satiation, and uneaten feed was collected to determine feed consumption. The results showed that the survival, feed intake, growth, FCR, energy and protein retention of fish fed the diet containing SE CM were similar or even higher to those of fish fed the fish meal reference diet (FM) and the LM diet. Fish fed with the diet containing 300 g kg^{-1} SE CM did not show any changes in biochemistry and gene expression in a suite of detoxification genes. However, the diet with 300 g kg^{-1} EX CM depressed feed intake, growth performance and increased feed conversion ratio (FCR).

Transcription of genes involving in fatty acid synthesis and the TCA cycle were not changed by different diets. The down regulation of gene expression in certain detoxification genes (*Lc CYP1A1, Lc CYP3A, Lc CYP2N* and *Lc GST*) was observed in fish fed with the diet containing 300 g kg^{-1} EX CM compared to the FM control diet and other experimental diets. In general, the SE CM can be used up to 300 g kg^{-1} diet without negative performance effects or signs of clinical plasma biochemistry. By contrast the maximum acceptable level of the EX CM for barramundi was only 200 g kg^{-1}. Higher inclusion level of the EX CM induced negative effects on growth performance, feed utilisation, plasma biochemistry and gene expression in relation to detoxification.

Key words: barramundi, canola meal, growth, fish meal replacement, plant protein

1. Highlights

- 300 g kg−1 solvent (SE) extracted canola meal (CM) and 200 g kg−1 expeller (EX) extracted CM can be used in barramundi's diet without depression in growth performance
- There were minor changes in plasma biochemistry but not in digestive histology of barramundi fed CM levels
- Down-regulation of several genes in detoxification system in barramundi fed with diet containing 300 g kg−1 EX CM

2. Introduction

Canola meal (CM) is considered a potentially useful plant protein source for fish meal replacement in diets for aquaculture species (Burel and Kaushik, 2008). Canola is the second biggest oilseed product with production around 59 million tons in 2010, in which the production of CM was 32 million tons (Enami, 2011). It has high nutrition value with protein content varying between 320 and 450 g kg^{-1} of dry matter (Burel *et al.*, 2000a) and favorable amino acid compared to other available plant proteins (Friedman, 1996), and it is also the source of mineral, vitamin and other microelement. Many fish species have been shown to have good growth performance when fed with diets containing CMs. These include rainbow trout (Gomes *et al.*, 1993; Hardy and Sullivan, 1983; Leatherland *et al.*, 1987; McCurdy and March, 1992; Yurkowski *et al.*, 1978), juvenile Chinook salmon (Higgs *et al.*, 1982), gilthead seabream (Kissil *et al.*, 2000), red seabream (Glencross *et al.*, 2004a), channel catfish (Lim *et al.*, 1998; Webster *et al.*, 1997), and tilapia (Zhou and Yue, 2010). However, CM also

contains many anti-nutritional factors (ANFs) which limit its utilisation. A decrease in growth performance has been reported when fish were fed with high levels of CM in their diets (Burel *et al.*, 2000c; Cheng *et al.*, 2010; Luo *et al.*, 2012; Satoh, 1998; Webster *et al.*, 1997).

Using plant ingredients has raised considerations of the effects of ANFs on the growth performance and health status of fish (Francis *et al.*, 2001). As with other plant ingredients, CM contains many ANFs including fibre, oligosaccharides, phenolic compounds, tannins, phytic acid, glucosinolates (GSL) and their derivatives (Bell, 1993; Higgs *et al.*, 1995). Rapeseed meal/CM and ANFs caused goitrogenicity and internal organ abnormalities in animals (Mawson *et al.*, 1994). In fish, although the GSL content in most of commercial CMs is considerably reduced compared to earlier varieties of rapeseed, there are still concerns about the effect of these compounds on thyroid function, such as thyroid hypertrophy or a reduction in the plasma thyroid hormone levels triiodothyronine (T3) and thyroxine (T4) (Burel *et al.*, 2000c; Burel *et al.*, 2001; Hilton and Slinger, 1986; Yurkowski *et al.*, 1978). In addition, the activities of some protein metabolism enzymes in liver (e.g. asparate aminotransferase (AST), alanine aminotransferase (ALAT)) have been reduced with increasing dietary CM levels (Cheng *et al.*, 2010; Luo *et al.*, 2012).

Understanding the molecular pathways that regulate the utilisation of dietary nutrients and energy are additional elements to understanding the feeding and growth response in fish when fed with a particular diet. It is generally assumed that the replacement of fish meal by plant materials is likely to change the biological values of diets thereby also likely affecting molecular metabolism in certain pathways (Panserat *et al.*, 2008; Panserat *et al.*, 2009). Detoxification plays an important role in the protection of the body against the damage of toxic compounds from endo- and exogenous sources (Xu *et al.*, 2005). The detoxifying mechanisms in the liver rely on the involvement of phase 1 and 2 biotransformation enzymes. Phase 1 (cytochrome P450-CYP450) involves in oxidation, reduction and hydrolysis reactions to produce polar metabolites and if they are sufficiently polar they may be readily excreted at this point (Parkinson, 2001). However, most phase 1 products are not eliminated rapidly and undergo subsequent reactions. Phase 2 (such as glutathione group - GSH) comprises conjugation reactions with phase 1 metabolites to produce more polar metabolites that are readily excreted (Parkinson, 2001). The ingestion of GSLs has shown to not only inhibit catalyst activity of CYP1A1 but also decrease transcriptional level of this gene via modification of Aryl hydrocarbon receptor (AhR) (Wang *et al.*, 1997). Meanwhile, GSLs and their derivatives are known as inducers of up-regulation of phase II enzymes including GST and GPx (Nho and Jeffery, 2001).

Barramundi (or Asian Seabass; *Lates calcarifer*), is a commercially important species in Australia and Southeast Asia (Tucker *et al.*, 2002). Barramundi are a fast growing species, with a growth rate of approximately 1 kg/year and can reach a marketable size (350 g – 5 kg) in 6 – 24 months (Boonyaratpalin, 1997; Rajaguru, 2002; Yue *et al.*, 2009). Like other marine carnivorous species, barramundi require a relatively high dietary protein intake. The few studies on fish meal replacement with barramundi using plant protein sources suggest that different raw materials can be effectively used with as little as 15% fish meal remaining in diet (Glencross *et al.*, 2011b). The few available studies on CM use in the diet for juvenile barramundi indicate that the introduction of CM into diets for barramundi have been acceptable (Glencross *et al.*, 2011b). However, in that study only one type at a single inclusion level of expeller extracted CM was evaluated. Therefore, this study used a serial inclusion experiment to study nutrient utilisation and the inclusion level limitations of two canola meals from solvent (SE) and expeller (EX) extraction. The utility of these ingredients was based on examining the growth and feed utilisation parameters such as weight gain, daily growth coefficient, feed intake, feed conversion ratio (FCR), protein and energy retention. The alternations of plasma biochemistry, histology and hepatic gene expression in relation to fatty acid synthesis, energy production and detoxification were also studied.

3. Materials and methods

3.1. Experimental diets

The experiment included eight diets. Six diets were used to generate a serial inclusion level design (100, 200 and 300 g kg^{-1}) of each of SE CM and EX CM. These diets were compared to two reference diets (a fish meal (FM) based diet and a lupin kernel meal (LM) diet with 300 g kg^{-1} of LM). Diets were formulated to iso-digestible nitrogenous (423 ± 29 g kg^{-1}) and iso-digestible energetic (14.6 ± 8 MJ kg^{-1} DM) specifications, based on previous digestibility data (Blyth *et al.*, 2015; Ngo *et al.*, 2015). The two CMs selected to use in the growth experiment were SE CM (Numurkah, Vic, Australia) and EX CM (Pinjarra, WA, Australia). Chemical composition of each ingredient is described in Table 1.

Feed manufacturing

After the various diets were prepared, each mash was mixed by using a 60L upright Hobart mixer (HL600, Hobart, Pinkenba, QLD, Australia). The mash was then made into pellets using a laboratory-scale, twin-screw extruder with intermeshing, co-rotating screws (MPF24:25, Baker Perkins, Peterborough, United Kingdom). All diets were extruded through a 4 mm die at the same parameters for consistency. Pellets were cut into 6 mm to 8 mm lengths using two-bladed variable speed cutter and collected on an aluminium tray and dried

at 65 ºC for 12 h in a fan-forced drying oven. After the pellets were dried the oil allocation of each diet was vacuum infused using methods described previously (Glencross *et al.*, 2010). The pellets were then stored at -20 ºC for later use. The formulation and composition of the test and basal diets are presented in Table 2 and Table 3 respectively.

3.2. Fish handling and experiment management

The experiment was carried out at CSIRO's Bribie Island Research Centre in a flow-through seawater array of tanks. The culture system was designed with flow-through sea water at a rate of 3 L min^{-1}. During the experiment the water temperature was monitored at 29 ± 0.1 ºC and oxygen concentration was maintained 4.8 ± 0.21 mg L^{-1} (mean ± SD). Photoperiod was held to a constant 12:12 h light-dark cycle.

Barramundi (Asian seabass; *Lates calcarifer*) for this experiment were obtained from the Gladstone Area Water Board hatchery (Gladstone, QLD, Australia) and grown to 53.4 ± 7.0 g (mean ± SD, n = 360) for the experiment. Fish were randomly assigned across 24 tanks (600 L), with each dietary treatment having three replicates. Fish density was 15 fish/tank.

Fish were fed once daily, between 9:00 am and 10:00 am to slight excess to ensure fish were fed to satiation. For each feeding event, the feed was weighed, and one hour after feeding the uneaten feed from each tank was collected. Uneaten feed was collected from the culture tanks using a Guelph style system collector (Cho and Slinger, 1979). The drainpipe and the collection column of each tank were brushed out to remove waste and faecal residues from the system before each feeding. The uneaten feed was dried in an oven at 105 ºC for 24 h and then weighed. Factors to account for the leaching loss of material from the feed over one hour were applied to the dry weight of uneaten feed to enable determination of feed consumption within each tank.

Five fish were randomly selected at the beginning of the experiment, and three fish from each tank were randomly sampled at the end of experiment after eight weeks and stored at –20 ºC until used for analysis of body composition.

3.3. Proximate analysis of diets and fish

Whole fish (initial and final fish samples) were separately minced and homogenised. A subsample of the homogenate was allocated for dry matter determination while another sub-sample was freeze-dried for chemical composition analysis.

Ingredient, diet and fish samples were analysed for dry matter, ash, total lipid, nitrogen and gross energy content. Dry matter was calculated by gravimetric analysis following oven drying at 105 ºC for 24 h. Protein levels were calculated from the determination of total nitrogen by organic elemental analyser (Flash 2000, Thermo Fishery Scientific), based on N × 6.25. Total lipid content was determined gravimetrically following extraction of the lipids

using chloroform: methanol (2:1), based on method of Folch *et al.* (1957). Gross ash content was determined gravimetrically following loss of mass after combustion of a sample in a muffle furnace at 550 ºC for 12 h. Gross energy was determined using a ballistic bomb calorimeter (PARR 6200, USA)

3.4. Plasma analysis

For sampling, fish were euthanized by placing them in seawater containing an overdose of 0.2 ml L^{-1} AQUI-S (AQUI-S New Zealand Ltd). Blood samples were collected from three fish from each tank at 24 h post last feeding using a 1 mL heparinised syringe and 18G needle via caudal vein puncture. Blood from fish within the same tank were pooled in a lithium heparin vacutainer. The blood was then centrifuged at 1000 × g for 5 minutes to separate plasma from erythrocytes. The plasma was then transferred to a new Eppendorf ™ tube before it was frozen at –80 ºC and sent to the Western Australian Animal Health Laboratories (Western Australia) for plasma clinical panel analysis. Plasma enzymes and metabolites included on the clinical panel included alanine aminotransferase (ALAT), creatinine kinase (CK), glutamate dehydrogenase (GDH), total protein, creatinine, alkaline phosphatase, glucose, urea and haem (haemoglobin in total). The plasma samples were analysed by automatic chemistry analyser (Olympus A400) using a standard kit method for each assay. Trace elements were determined by inductively coupled plasma atomic emission spectroscopy (ICP-MS) after samples were prepared using a mixed acid digestion. The thyroid hormones tri-iodothyronine (T3) and thyroxine (T4) were determined by a competitive immunoassay method using chemiluminescence detection as described by (Fisher, 1996).

3.5. Histology analysis

Head kidney, liver, stomach, distal intestine and pyloric caeca from three fish of each tank were dissected following blood sampling. The samples from each fish were fixed in 10% neutralized, buffered formalin for 72 hours. Tissue samples were then cleared by soaking in ethanol prior to being embedded in paraffin, sectioned at 5 µm and stained in haematoxylin and eosin. Samples were examined under light microscope (Zeius, Auxoviet 25) at 100, 200 and 400x magnification. For liver, the area of 10 hepatocytes per section was measured (in 3 fish × 3 replicates, n = 90) and evaluated for the degree of vacuolization and steatosis status. For each liver section a semi quantitative histological assessment (grade 1-none, grade 2-mild, grade 3- moderate and grade 4-severe) was used. For caeca and distal intestine analysis, goblet cells were estimated per each 100 µm mucosal fold (2 folds × 3 fish × 3 replicates, n = 18). The length of villi was also measured (2 folds × 3 fish × 3 replicates, n = 18). The density of melano macrophage centres (MMC) and pigment deposits in kidney was

determined on three fields considered to be representative of the whole section (3 fields x 3 fish × 3 replicates, n = 27). The area of MMC in each of these fields was measured and then an average area of MMC was calculated as percentage of total kidney area.

3.6. Gene expression analysis

RNA extraction and normalization

Liver samples dissected from the three fish in each tank were examined from four dietary treatments 300SE-CM, 300EX-CM, LM and FM at 24 h post last meal. Samples were stored at -80 ºC until analysis. The total RNA was isolated from the liver tissues of seven individuals per experimental treatment. Tissues were homogenised in Trizol (Invitrogen) using a Precellys 24 (Bertin Technologies), and RNA was separated in the chloroform layer. RNA was precipitated by isopropanol and RNA precipitation solution (1.2 M sodium chloride, 0.8 M sodium citrate) at a ratio of 1:1. The RNA pellet was washed in 950 µL 85% ethanol, and air-dried before being resuspended in RNase-free water. DNA contamination of RNA samples was removed using TURBO DNA^{TM} –free kit (Applied Biosystems) according to the manufacturers instructions. The concentration of the RNA was quantified by spectrophotometry (Nano Drop Technologies, Wilmington, DE, USA) and all RNA samples were normalised by dilution to 200 ng µl^{-1}. Finally, the integrity of RNA was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) and the RNA 6000 Nano Kit (Agilent). The RNA was stored at -80 °C until required for cDNA synthesis.

RT-qPCR

Expression of selected genes was determined by quantitative reverse transcription polymerase chain-reaction (qRT-PCR). For reverse transcription, 1µg of total purified RNA of each sample was reverse transcribed into cDNA using SuperScript. III First-Strand Synthesis System for RT-PCR (Invitrogen^{TM}), including 25 µM oligo(dT), 25 µM random hexamers and 400 pg of internal non-endogenous control Luciferase RNA (Promega L4561). Primers used in real-time PCR were specific to each gene (Table 4), and designed by PerlPrimer V1.1.17. The amplification efficiency of each primer pair was optimized to be between 95 and 105% using the slope of a standard curve over 5-fold serial dilutions of the pooled cDNA sample containing all samples. The qPCR amplifications were carried out in triplicate on a ViiA7 real-time PCR system (Applied Biosystems) in a final volume of 10 µL containing 1X SYBR master mix, the equivalent of 7.5 pg of cDNA and 0.2 µM of each primer. The thermal cycle profile of the qPCR included incubation stage at 95^{o}C for 10 minutes followed by 40 cycles: 15 s at 95^{o}C and 1 min at 60^{o}C. After amplification phase, a melting curve was performed, enabling confirmation of amplification of a single product in each reaction. Negative controls were performed using an equivalent amount of a pool of all

RNA samples to check for DNA contamination or contamination of reagents. The positive control contained an equivalent amount of cDNA pooled from all samples and was used to normalise across plates and treatments. Normalisation was performed using the ΔCq method (where Cq is the qualification cycle) as it was considered the least biased approach (De Santis *et al.*, 2011). The relative expression level was determined by normalising the cycle threshold values for each gene to that obtained for the reference gene elongation factor 1 alpha (Ef1-α), then to the cycle threshold of each gene in the FM control treatment. The gene EF1-α has been routinely used as reference gene for gene expression analysis in barramundi (De Santis *et al.*, 2011; Wade et al 2014) and for postprandial metabolic gene expression analysis in other species (Enes *et al.*, 2013; Mennigen *et al.*, 2012; Olsvik *et al.*, 2005; Skiba-Cassy *et al.*, 2009). PCR efficiency was assumed 100% in relative qualification analysis (Livak and Schmittgen, 2001). To confirm that the correct fragment had been amplified, PCR products were purified and then sequenced by Sanger sequencing using BigDye V3.1 and a 3130xl Genetic Analyser (Hitachi) according to established methods. Sequencing PCR reactions were cleaned with Agencourt CleanSEQ Sequencing Reaction Clean-Up system utilizing Agencourt's patented SPRI® paramagnetic bead technology (Beckman Coulter, Beverly, MA, USA). All sequences were confirmed by using NCBI nucleotide BLAST software. The barramundi sequences of genes in this study used raw sequence reads available through the CSIRO Data Access Portal (CSIRO. Data Collection. 102.100.100/13190).

3.7. Performance indices

$$Feed\ intake\ =\ Total\ feed\ consumed\ per\ tank/total\ fish\ per\ tank$$

$$Weight\ gain\ =\ W_f\ -W_i$$

where W_f: final weight of fish; W_i: initial weight of fish

$$Daily\ growth\ coefficient\ (DGC)(\%)\ =\left(W_f^{1/3}-\ W_i^{1/3}\right)/t\ \times 100$$

Where W_f is the mean final weigh (g), W_i is mean initial weigh (g) and t is time (days).

$$FCR\ =\ (feed\ consumed/weight\ gain)$$

$$Survival\ (\%)=(Final\ number\ of\ fish/Initial\ number\ of\ fish)\ \times 100$$

$$Protein\ retention\ \ (\%)\ =\ \left(P_f\ -\ P_i\right)/P_c\times 100$$

where P_i is protein content of the fish at initial, P_f is protein content of fish at the end of experiment and P_c is the total amount of protein consumed by fish over the experiment.

$$Energy\ retention\ (\%)=\left(E_f-E_i\right)/E_c\times 100$$

where E_i is energy content of the fish at initial, E_f is energy content of fish at the end of experiment and E_c is the total amount of energy consumed by fish over the experiment.

3.8. Statistical analysis

All data are presented as mean ± SEM. Data were subjected to one-way analysis of variance (ANOVA) followed by Duncan's multiple range tests. Levene's test for homogeneity of variances was used before ANOVA analysis. All percentage data were arcsine-transformed prior to being analysed. RT-qPCR data were presented as Log_2 transformed fold changes (treatments)/FM control diet. A significance level of $P < 0.05$ was used for all comparisons. Once equal variances were not assumed, Game-Howell's post-hoc test was used (ALAT, GDH, Urea, Mg, Haem). The variation in vacuolization degree and steatosis status in the liver between the treatments were analysed using Kruskal-Wallis Test. The effect of CM inclusion levels on fish productivity, feed intake, protein and energy retention, biochemical and histological parameters by fish were subsequently examined using regression analysis according to the best relative fit using linear or quadratic models (Shearer, 2000). All statistical analysis was performed using SPSS 11.0 for Windows.

4. Results

4.1. Growth performance

The growth performance of barramundi fed the different experimental diets is reported in Table 5. All dietary treatments that contained the CMs and LM had growth performance as good as or better than that of the FM based control diet, with the exception of the 300EX-CM diet (containing 300 g kg^{-1} EX CM) (Table 5). Fish fed the diets containing 200-300 g kg^{-1} SE CM (200SE-CM and 300SE-CM diets) and the LM diet grew significantly better than fish fed the FM based diet (mentioned as weight gain and DGC). The weight gain and DGC of fish fed other diets that contained 100 g kg^{-1} SE CM (100SE-CM diet), 100-200 g kg^{-1} EX CM (100EX-CM and 200EX-CM diets) was similar to that of fish fed the FM diet. However, a significant reduction in weight gain and DGC of fish fed the 300EX-CM diet (300 g kg^{-1} EX CM) diet compared to the FM control diet and other test diets was observed over the eight week culture period.

There was no negative effect on feed intake with increasing inclusion levels of the SE CM (Fig. 1). Feed intake was significantly greater for fish fed the diets containing 200 g kg^{-1} SE CM compared to that observed for the FM control diet but 300 g kg^{-1} SE CM in the diet showed similar feed intake to the FM based diet (Table 5). The feed intake of diets with substitution of any SE CM levels was also similar to that of the LM diet. Feed intake of the diet containing 100 g kg^{-1} SE CM was similar to that of the FM diet. For the EX CM, the second-degree regression analysis indicated that when substitution level of the EX CM was 123 g kg^{-1}, feed intake had the maximum value (Fig. 2). A significant improvement in feed

intake was observed by fish fed diets with 100 g, 200 g compared to the FM control diet (without inclusion of CM). When more than 200 g kg^{-1} EX CM was included in the diet, feed intake significantly decreased and was the lowest intake among all the treatments. Digestible protein and energy intake was higher in of the diets containing 200 to 300 g kg^{-1} SE CM or LM than that of the FM control diet and other test diets. However, the digestible protein and energy intake of diets containing 100 to 200 g kg^{-1} EX CM was similar to that of the FM control diet. Digestible protein and energy intake of the 300EX-CM diet was lowest among the diets. There was a strong correlation between feed intake and weight gain (Fig. 2).

There was no significant difference in protein retention (31.9% to 36.3%) and energy retention (39% to 42.5%) among dietary treatments. FCR was similar among almost all of the treatment diets and the LM and the FM control diets (ranging from 1.15 to 1.24) but greater FCR in the 300EX-CM diet (1.38).

The survival of fish in the experiment was high (97% to 100%) and not affected by the dietary treatments.

3.2. Plasma chemistry

The concentration of Fe was lower in fish fed the 200EX-CM and 300EX-CM diets compared to the FM reference diet (5.2 and 7.4 mmol L^{-1} against 17 mmol L^{-1}) while no differences among other test diet were observed compared to the FM diet. Other parameters (i.e. plasma enzymes: ALAT, CK, GDH; metabolites and electrolytes: total protein, glucose, haem, urea, creatinine, Mg, Ca, phosphate; and plasma thyroid hormones: T3 and T4) were not significantly different among different dietary treatments. The details of plasma biochemical parameters are presented in Table 6.

3.3. Histology

No changes in lipid droplet accumulation were observed in the pyloric caeca of fish in the experimental treatments. There were also no significant differences in the number of goblet cells in pyloric caeca among fish in different treatments. A number of these cells in the caeca varied from 1.2 to 2.2 cells/100 µm mucosal fold. These cells were more abundant in the distal intestine, ranging from 9.8 to 12.9 cells/100 µm but no significant differences were observed among the dietary treatments. The length of villi in the pyloric caeca and distal intestine were also unchanged among treatments. No inflammatory changes were found in the lamina propria of intestine.

Histological examination of the liver samples showed normal glycogen and lipid content (grade 1 or 2) but only few liver samples showed moderate steatosis (grade 3) (Table 7) with an elevated number of lipid droplets. However, this pattern only occurred in random individual fish fed the experimental diets (one sample in each of the diets with 100, 200, 300

g SE CM, 100 g EX CM and two samples in the LM diet). There was no significant variation in scores in the steatosis degree in the liver of fish among treatments ($P > 0.05$, 2 *df*, Kruskal-Wallis test statistic = 3.347). There were also no significant differences in hepatocyte area in fish fed different levels of CMs compared to the LM and FM control diet.

With regard to kidney histological investigation, there were no alterations observed in kidney structure of fish fed either of the CM or the lupin diet compared to the FM control diet. Kidney samples were also examined for the presence of MMC and results showed that MMC area comprised of 3% - 4% kidney area. The density of MMC in kidneys was not changed among fish fed any of the experimental diets.

3.3. Gene expression

Details of the relative quantification of the expression of specific genes from fish fed different diets are presented in Fig. 3. The relative expression of farnesoid X receptor (*Lc FXR*) in the liver of fish fed the 300EX-CM was less abundant than that of fish fed the FM diet. There were no differences in the expression levels of the genes that regulate fatty acid metabolism (*Lc FAS* and *Lc SCD*). The expression levels of the gene *Lc CS* and *Lc PDK* in the liver of fish were also not affected by different diets. However, a large degree of variability was seen in relative expression of *Lc FAS* of fish fed the FM control diet that it was impossible to detect any significant differences in gene expression of *Lc FAS* from fish fed other test diets.

Among the 7 genes in xenobiotic metabolism the expression of all CYP genes (*Lc CYP1A1, Lc CYP3A, Lc CYP2N)* and *Lc GST* were down-regulated in fish fed the 300EX-CM compared to that of fish fed the FM reference diet. In particular, the expression of *Lc CYP3A* was significantly lower in fish fed all the diets containing plant ingredients (CMs and LM) than the expression observed in fish fed the fish meal diet. However, for other genes involved in xenobiotic metabolism (*Lc GR, Lc GPx* and *Lc GHGPx*), expression levels were similar among the different dietary treatments.

5. Discussion

In this study we examined the effects of a serial inclusion level (100, 200, 300 g kg^{-1}) of either SE CM or EX CM in diets for barramundi with comparison to a FM based diet and a LM diet (300 g kg^{-1} of lupin kernel meal). To assess this, a suite of performance parameters, changes in plasma biochemistry and gastrointestinal histology and hepatic gene expression were examined.

4.1. Performance parameters

In our study, barramundi fed the diets with a serial inclusion level of 100 to 300 g kg^{-1} SE CM had similar or greater weight gain compared with that of the FM control diet and was comparable to that of the LM diet. This supports that the SE CM could be used at an inclusion level of up to 300 g kg^{-1} in diet without having any negative effect on the growth performance and feed utilisation of barramundi over an eight week period. The present results are consistent with those of previous studies which showed that CM can be used at fairly high inclusion levels in diets for some species, without adverse effects on the growth performance, such as rainbow trout (30% of inclusion level) (Shafaeipour *et al.*, 2008), channel catfish (31%) (Lim *et al.*, 1998), red seabream (60%) (Glencross *et al.*, 2004a). Our observations are supported by study of Glencross *et al.* (2011b) which indicated that 30% CM can be accepted in the diet by juvenile barramundi without any deleterious effect on growth performance, feed utilisation and plasma biochemistry. However, for the EX CM, while the inclusion of 100 to 200 g kg^{-1} in the diet was acceptable, a higher level (300 g kg^{-1}) of this EX CM led to a decrease in growth performance. A similar depression in growth has been reported when 30% or even less SE CM or rapeseed meal was used in diets for rainbow trout (Burel *et al.*, 2000c; Hilton and Slinger, 1986; McCurdy and March, 1992), turbot (McCurdy and March, 1992), Chinook salmon (Hajen *et al.*, 1993; McCurdy and March, 1992; Satoh, 1998), Japanese seabass (Cheng *et al.*, 2010) and cobia (Luo *et al.*, 2012). In contrast, EX CM could be used up to 60% in diet for red seabream without any negative effects on growth performance and other fish productivity (Glencross *et al.*, 2004a).

There was a significantly greater feed intake and digestible protein intake by fish fed with the diets containing 200 to 300 g kg^{-1} SE CM, 100 to 200 g kg^{-1} EX CM and 300 g kg^{-1} LM than the FM based diet. This suggests that to some extent these inclusion levels of the CMs and the LM improved the palatability of diets for barramundi. This result is supported by the findings of Glencross *et al.* (2011b), who reported that greater feed intake was obtained with barramundi when fed with a series of plant protein containing diets. Cheng *et al.* (2010) also indicated that feed intake by Japanese seabass increased with increasing CM inclusion levels but the higher feed intake in that study was due to the compensation for the loss of digestible

energy of diet with the increasing CM levels in diets. In the present study, there was a positive correlation between feed intake and weight gain (Table 5). Indeed, growth performance of barramundi substantially improved with an increased level of feed intake observed in some diets (the 200SE-CM, 300SE-CM and LM diets) relative to the FM control diet. However it is worth mentioning that although digestible protein and energy intake of several diets (100EX-CM, 200EX-CM and 300EX-CM) were similar to that of the FM diet, the improvement in performance of fish was obtained in the fish fed those diets. Therefore, it is suggested that the improvement in growth performance of fish in the present study was due to enhancements in both feed intake and non-additive effects between the digestibility of key raw materials in terms of increases to digestible protein and energy value of the diets. This is supported by the previous report of Glencross *et al.* (2011a) which indicated that improvements in feed intake and digestible protein and energy values of diets fed to rainbow trout when those diets were also initially formulated to be isonitrogenous and isoenergenic based on a digestible nutrient basis.

Feed intake of the 300EX-CM diet was least among diets although dietary digestible protein and energy specifications were similar to those of the 200SE-CM diet. Hilton and Slinger (1986) suggested that suppression of feed intake could be the main reason for reduced growth of rainbow trout as dietary CM level increased. Burel *et al.* (2000b) also demonstrated that lower growth performance of turbot fed with CM containing diets was a result of the decrease in feed intake compared a FM control diet. Hence, it could be concluded that suppression of feed intake due to decreased palatability significantly influenced the growth performance of barramundi fed the 300EX-CM in the present study. However, in the case of our study, it is not clear why the feed intake decreased in the 300EX-CM diet but a higher concentration of phytic acid was found in the EX CM (44 g kg^{-1} DM) than that in the SE CM (24 g kg^{-1} DM). With the increasing inclusion levels of EX CM, the phytic acid content in the diets ranged from 4.4 to 13.2 g kg^{-1}, and the concentration of phytic acid (13.2 g kg^{-1}) at the highest inclusion level (300 g kg^{-1} EX CM) probably exceeds the tolerance of barramundi with this compound. Sajjadi and Carter (2004) reported that feed intake decreased when salmon were fed with diet containing above 10 g kg^{-1} phytic acid. Whether or not phytic acid caused the decrease in appetite or changes in the physiological properties of fish remains to be elucidated.

There was no significant difference in protein retention and energy retention by fish among the different treatments. This implies that the biological protein and energy values of the two types of CM were not different and similar to that of FM and/or that formulating the diets to be relatively similar in digestible protein and energy could minimize the differences

in nutritional values of ingredients contributing into diets. Protein (31.9-35.7%) and energy efficiency (39-42.5%) in the present study for barramundi is similar or higher than those in studies on CM for other species (27.1-37.5% for protein and 23.9-38.9% for energy efficiency) (reviewed by Burel and Kaushik (2008)). Compared with the same species, the present results for protein retention were less than that for barramundi in a previous study (48.8%) (Katersky and Carter, 2007) although energy retention was similar. This might be explained by different diet formulation, fish species or genetic quality of different strains for same species.

4.2. Biochemistry effects

In the present work, the majority of the plasma chemistry parameters did not show differences among the dietary treatments. An exception to this was for iron content. The plasma iron concentration in all plant containing diets was lower than in the FM control diet although some differences were not significant. This suggests that phytic acid in plant ingredients might have effect on iron absorption (Hurrell *et al.*, 1992). Indeed, the plasma iron concentration significantly declined in fish fed the diets (200EX-CM and 300EX-CM) containing high phytic acid content compared to that of fish fed the FM control diet (5.2 to 7.4 vs. 17.0 mmol L^{-1}). The lack of differences in plasma CK (used as a biochemical marker of both smooth and striated muscle damage (Chen *et al.*, 2003)) suggests that the inclusion of either SE CM or EX CM did not cause any muscle-related dysfunction in this study. Similarly, high levels of ALAT and GDH enzymes are associated with liver damage (Chen *et al.*, 2003; O'Brien *et al.*, 2002), but there were no significant differences in these enzyme levels among the fish fed CM containing diets relative to the FM control diet. These findings are similar to the observations of Glencross *et al.* (2011b) which denoted that the inclusion of 300 g kg^{-1} CM in diet for juvenile barramundi did not cause any alteration in plasma enzymes. Both studies suggest that CM can be incorporated up to 300 g kg^{-1} without any implications of liver or muscle damage.

One of the considerations when feeding fish with diets containing CM is disturbance to thyroid function and/or changes in the regulation of plasma thyroid hormones (Burel *et al.*, 2000c; Burel *et al.*, 2001; Higgs *et al.*, 1982). In the present study, fish fed different dietary CM levels did not show any changes in T3 and T4 level in plasma compared to that in FM control diet. The levels of thyroid hormones were consistent with the growth performance, demonstrating that the GSLs in the tested CMs in the present study were not a factor contributing to the decreased growth performance when barramundi were fed the 300 g kg^{-1} EX CM diet. It is plausible that the GSL content (0.6 - 1.8 μmol g^{-1}) present in the diets in

the present study was not sufficient to cause a reduction in plasma thyroid hormone. These results were similar to studies on red seabream (Glencross *et al.*, 2004b) and rainbow trout (Shafaeipour *et al.*, 2008) that plasma T3 and T4 level in fish were not influenced by dietary CM. However, these observations contrast the findings of the previous studies (Burel *et al.*, 2000c; Burel *et al.*, 2001), which reported a decrease in T3 and T4 when rainbow trout were fed with diets containing 30% European CM even at very low GSL content (1.4 μmol g^{-1}). In the present case, the observations could be explained due to lack of breakdown of GSLs into toxic by-products in Australian and Iranian CMs compared to those of European (French) canola/rapeseed meals. Difference in country of origin regarding different growing conditions (weather, soil), cultivars and processing conditions are known to affect GSL content and their breakdown products in CMs. The measurement of the breakdown products of GSLs could provide a more comprehensive understanding of the effects of CM rather than the intact GSLs. However, it is noticeable that even though the plasma thyroid hormones did not show differences, in some case the hyperplasia and/or hypertrophy of the thyroid have been found in rainbow trout and salmon as the result of ingestion of GSLs (Hardy and Sullivan, 1983; Yurkowski *et al.*, 1978).

4.3. Histological effects

Plant protein sources contain many different ANFs, in which some are toxic and can influence fish health if they are fed with diets containing those ingredients (Francis *et al.*, 2001). GSLs are major toxic compounds in CM or rapeseed meal which induce negative effects of feeding high GSLs on animal's health or impair function of organs (Mawson *et al.*, 1994; Papas *et al.*, 1979; Tripathi *et al.*, 2010; Yamashiro *et al.*, 1975). In fish, many studies reported changes in thyroid histology at high level of dietary GSLs or even at low content (Burel *et al.*, 2000c; Higgs *et al.*, 1982; Yurkowski *et al.*, 1978). However, there is limit on investigation of effects of GSLs and other ANFs in CM on digestive organs in fish. In the present study major digestive organs (kidney, liver, pyloric caeca, distal intestine and stomach) were examined for changes in histology when barramundi was fed dietary CM levels. Our results indicate that there were no changes in histological index of these organs associated with the CM supplemented in diets relative to the FM based diet and LM diet. The results support that Australian CMs containing low GSL content are potential plant protein sources for fish meal replacement in barramundi without impairing fish health at up to 300 g kg^{-1} inclusion level.

4.4. Gene expression effects

The expression levels of a range of genes that regulate different metabolic pathways were measured. Hepatic expression levels of genes involved in fatty acid synthesis (*Lc FAS*, *Lc*

SCD, *Lc FXR*) or energy production derived from carbohydrates and amino acid metabolism via the TCA cycle (*Lc PDK* and *Lc CS*) were unaffected by dietary inclusion of CM or LM. This included analysis of a nuclear receptor that modulates a range of downstream targets in the lipogenic pathway, that is known to directly reduce lipogenesis via inhibition of sterol-regulatory element-binding protein 1C (SREP1C) and fatty acid synthase, and indirectly reduce glycogenesis (Calkin and Tontonoz, 2012; Kalaany and Mangelsdorf, 2006). Substantial post-prandial modulation occurs in the expression level of most hepatic metabolism genes in barramundi over a 24 h period after feeding (Wade *et al.*, 2014). A time series analysis of expression of metabolism genes may highlight other general metabolic changes in response to dietary CM.

In the present study, a decrease in hepatic gene expression of all the targeted CYP genes (*Lc CYP1A1*, *Lc CYP3A*, *Lc CYP2N*) and *Lc GST* was observed in fish fed the 300EX-CM diet, but not in fish fed the 300SE-CM. Although the lack of *CYP* expression may theoretically underlie the poor growth performance observed in EX CM fed fish, the poorest performance or CYP gene expression levels were not correlated with the highest GSL content recorded in the 300SE-CM diet. This suggests that GSLs were not directly inducing the expression of the detoxification enzymes in the present study. In terms of *Lc CYP3A*, the expression of this gene was down regulated in all the plant protein containing diets. The reduced expression of *Lc GST* may indicate reduced production of reactive oxygen species in fish fed the diet with 300 g kg^{-1} EX CM, but how this may link to less feed intake or metabolism is unknown. Although the key factor in CM products that influenced the expression of these genes is not clear, and that there is very little understanding of the regulatory mechanisms controlling the expression of *CYP* genes (Uno *et al.*, 2012), these results suggest that several anti-nutritional factors in these plant ingredients might be affecting the expression of members of the CYP gene family in different ways.

Conclusions

Overall, this study has identified that SE CM can be utilised at up to a 300 g kg^{-1} inclusion level in the diet for barramundi without any deleterious effects on the growth performance and other performance parameters. The inclusion level of 200 g kg^{-1} is acceptable for the EX CM but higher levels of EX CM (300 g kg^{-1}) resulted in significant impairment in growth performance and the down regulation of expression level of some genes involving in phase 1 (*Lc CYP1A1*, *CYP2N* and *CYP3A*) and phase 2 (*Lc GST*) of detoxification. Limited effects of either ingredient type on gastrointestinal histology or plasma biochemistry were observed.

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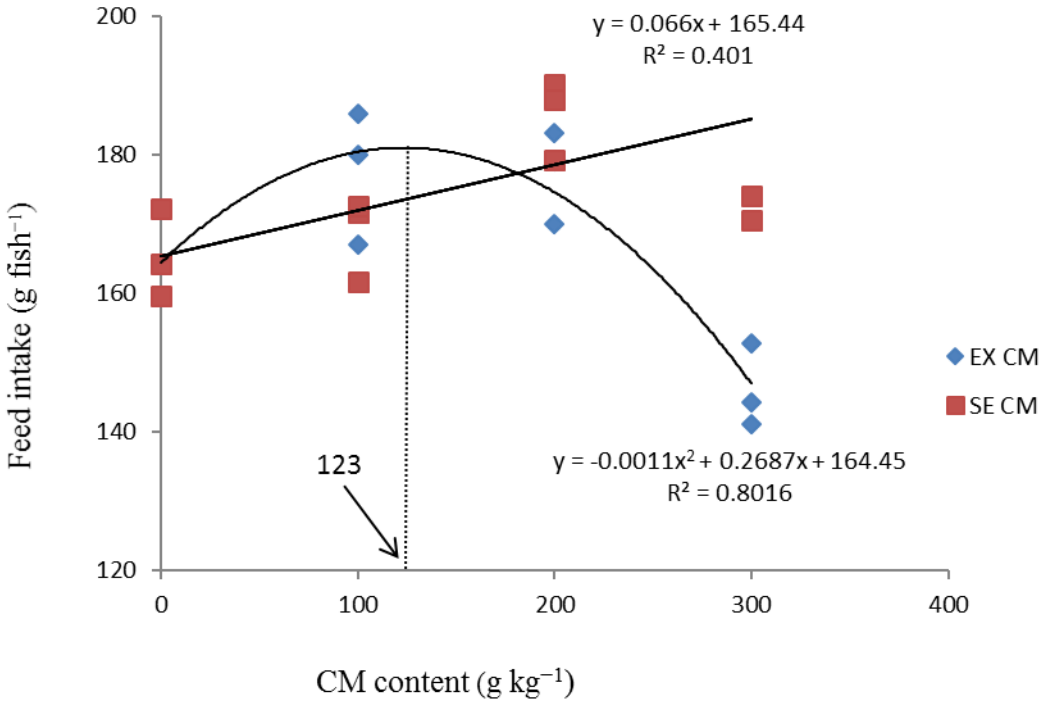


Fig. 1. Feed intake (g fish^{-1}) of barramundi fed with varying SE-CM (solvent extracted canola meal), EX-CM (expeller extracted canola meal)

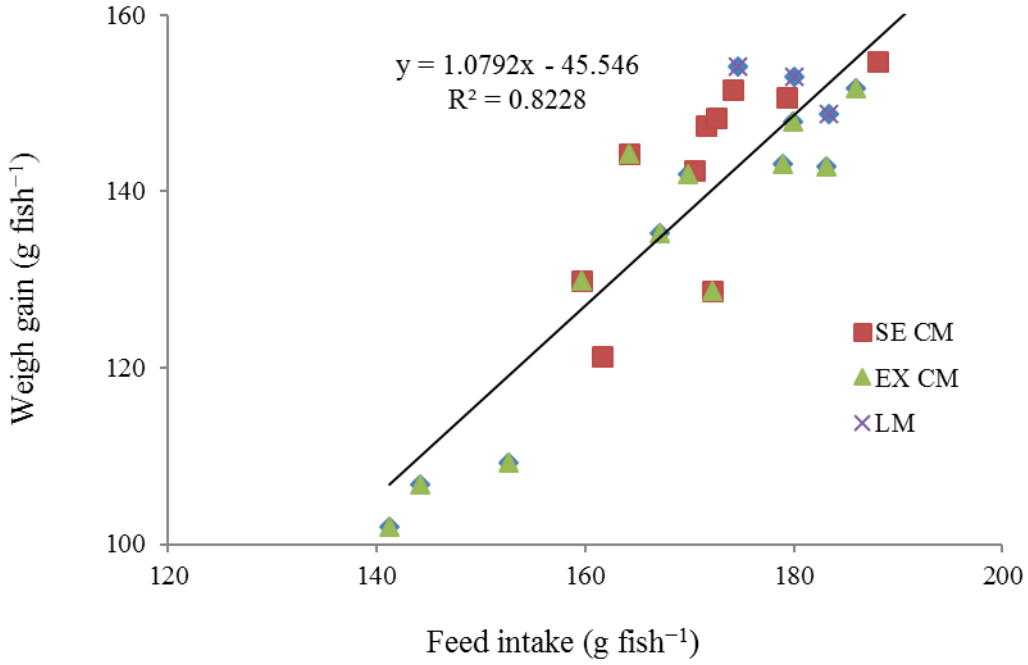
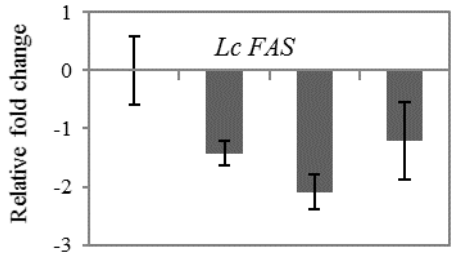
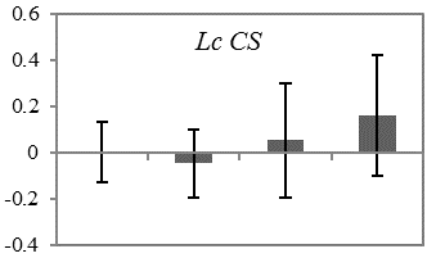


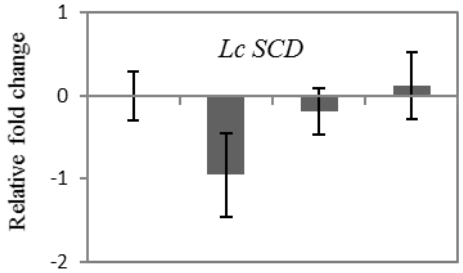
Fig. 2. Common regression of feed intake and weight gain of barramundi

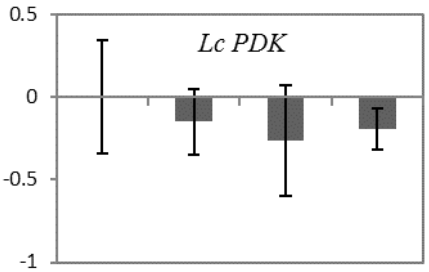
Fatty acid synthesis



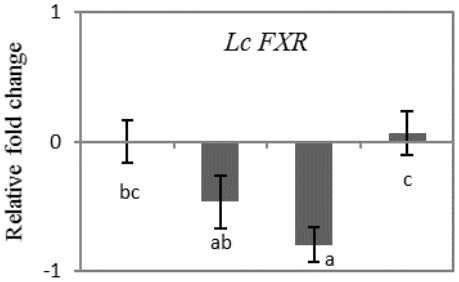
TCA cycle



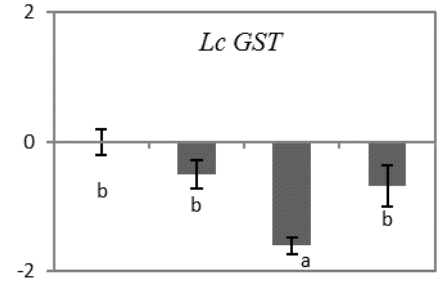




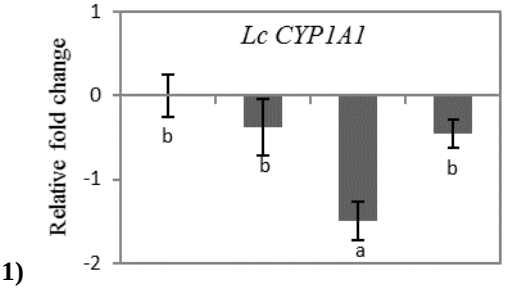
Nuclear receptor

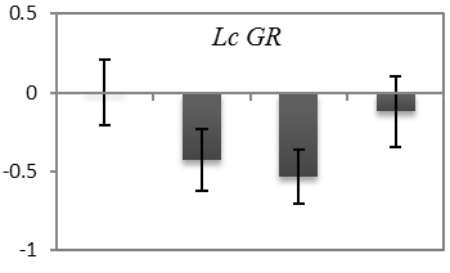


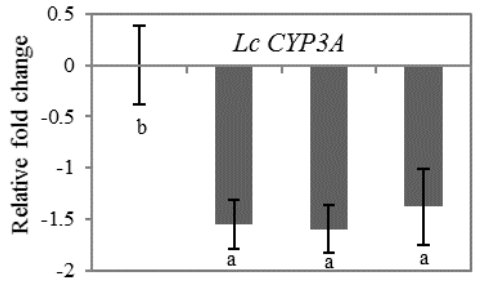
Detoxification (phase 2)

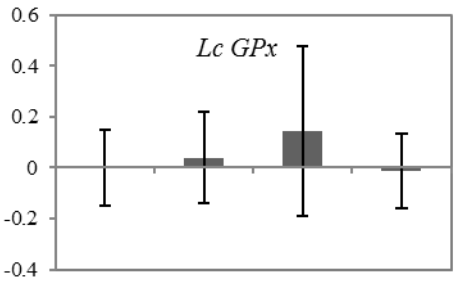


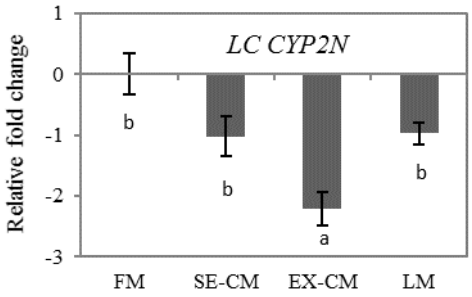
Detoxification (Phase 1)

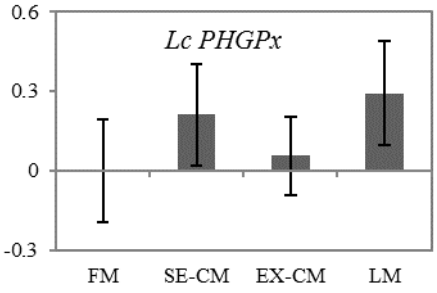












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770 **Table 1**. The ingredient formulation and nutritional composition of experimental diets
771 (g/kg DM), otherwise as indicated

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Ingredient	FM	LM	10SE-CM	20SE-CM	30SE-CM	10EX-CM	20EX-CM	30EX-CM
Fish meal	600	386	540	480	420	526	451	377
SE-CM	0	0	100	200	300	0	0	0
EX-CM	0	0	0	0	0	100	200	300
Lupin kernel meal	0	300	0	0	0	0	0	0
Wheat gluten	89	120	108	128	147	99	110	120
Pregelled starch	50	60	50	50	50	50	50	50
Cellulose	200	53	134	68	2	160	119	79
Fish oil	55	61	56	58	59	54	53	52
$Ca_3(PO_4)_2$	0.0	10.0	3.3	6.7	10.0	3.3	6.7	10.0
Pre-mix vitamins	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
L-Histidine	0.0	2.0	1.0	2.0	3.0	1.0	2.0	3.0
DL-Methionine	0.0	2.0	0.0	0.0	0.0	0.3	0.7	1.0
L-Lysine	0.0	0.0	1.0	2.0	3.0	0.7	1.3	2.0
Yttrium oxide	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Diet composition								
Dry matter (g/kg)*	966	964	966	963	967	957	960	962
Protein*	487[c]	506[d]	492[c]	507[d]	522[e]	473[b]	467[a]	460[a]
Digestible protein	410	435	429	439	448	395	387	379
Lipid*	144	150	147	146	142	139	142	143
Carbohydrate	222	230	218	207	207	249	265	278
Phosphorus	15	14	16	16	16	15	15	14
Ash*	147	114	143	140	129	139	126	119
Gross Energy*	21.1	22.0	21.3	21.3	21.5	20.8	21.3	19.8
Digestible Energy	13.4	14.5	14.1	14.4	14.8	13.5	13.6	13.7
DP : DE (g/MJ)	30.6	30.0	30.5	30.4	30.4	29.2	28.5	27.8
Total tannins (units?)	n/a	<0.3	0.6	1.2	1.8	0.4	1.8	1.2
Phytic acid (units?)	n/a	2.7	2.4	4.8	7.2	4.4	8.8	13.2
Glucosinolates (µmol/g)	n/a	n/a	0.6	1.2	1.8	0.3	0.6	0.9
Lysine	33	27	33	32	32	31	30	29
Threonine	18	16	18	19	19	18	17	17
Methionine	12	10	12	12	11	11	11	11
Isoleucine	21	19	21	21	21	20	19	19
Leucine	35	32	35	36	36	34	33	32
Tryptophan	5	4	5	5	5	5	5	5
Valine	23	21	24	24	24	23	22	22
Phenylalanine	19	18	20	20	21	19	19	19
Histidine	10	11	11	12	12	11	11	12
Arginine	33	34	32	32	31	30	29	28

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776 **Table 4** Target genes and primer sequences

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Target gene	Gene abbreviation	EC number	Primer sequence	Length	Tm
TCA cycle					
Pyruvate dehydrogenase kinase	*Lc PDK*	EC 2.7.11.2	(F)GAAAGAACGCACAGTTTGTC (R)GAATTGCTTCATGGATAAGGG	20 21	53.6 52.6
Citrate synthase	*Lc CS*	EC 2.3.3.12	(F)TTTCATATTTCCACCTCCTCCC (R)AGATGGACTGATGACACTGG	22 20	56.0 55.0
Fatty acid synthesis					
Fatty acid synthase	*Lc FAS*	EC 2.3.1.85	(F)TCCCTGGCAGCCTACTATGT (R)CTGGTCGGGTTGAATATGCT	20 20	59.4 56.2
Stearoyl CoA Desaturase	*Lc SCD*	EC 1.14.19.1	(F)CCTGGTACTTCTGGGGTGAA (R)AAGGGGAATGTGTGGTGGTA	20 20	58.0 57.3
Nuclear receptor					
Farnesoid X receptor	*Lc FXR*	n/a	(F)CTTCAAGGTCAGGCAAACAG (R)AGGAGAAGGGAAGAAAGTGG	20 20	55.2 55.5
Detoxification					
Cytochrome P450, family 1, subfamily A, polypeptide 1	*Lc CYP1A1*	EC 1.14.14.1	(F)ATCCCTGTTCTTCAATACCT (R)ATCCAGCTTTCTGTCTTCAC	20 20	51.2 53.5
Cytochrome P450, family 2, subfamily N	*Lc CYP2N*	EC 1.14.14.1	(F)TCAGACAGATACTTCAGCGT (R)CAGGAGGAGATAGAGAAGGA	20 20	54.0 53.7
Cytochrome P450, family 3, subfamily A	*Lc CYP3A*	EC 1.14.14.1	(F)GGGAGAGGAACAGGATAAAGG (R)GTAAGCCAGGAAACACAGAG	21 20	56.4 54.6
Glutathionine peroxidase	*Lc GPx*	EC 1.11.1.9	(F)CTAAGATCTCTGAAGTATGTCCGT (R)GCATCATCACTGGGAAATGG	24 20	54.5 55.4
Glutathionine Reductase	*Lc GR*	EC 1.8.1.7	(F)TCACAAGCAGGAAGAGTCAG (R)GGTCGTATAGGGAAGTAGGG	20 20	55.7 55.5
Glutathione S-transferase	*Lc GST*	EC 2.5.1.18	(F)GTAATTCAAGATCGCCTTTGTC (R)TTAACAGTTGCAGAAGTGGAG	22 21	53.2 53.6
Phospholipid hydroperoxidase	*Lc PHGPx*	EC 1.11.1.12	(F)CACACCAAACCCTATCAGAC (R)CACTTAACATTCAGAAAGGACAGG	20 24	54.2 54.7
Control genes					
Elongation factor 1 alpha	*Lc EF1α*	n/a	(F)AAATTGGCGGTATTGGAAC (R)GGGAGCAAAGGTGACGAC	19 18	52.0 58.2
Luciferase	*Luc*	n/a	(F)GGTGTTGGGCGCGTTATTTA (R)CGGTAGGCTGCGAAATGC	20 18	57.7 59.1

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Table 5 Growth, feed intake, survival rate of experimental diets in feeding trials (n = 3 tanks/treatment)

	FM	LM	10SE-CM	20SE-CM	30SE-CM	10EX-CM	20EX-CM	30EX-CM	Pooled S.E.M
Intial weight (g/fish)	53.5^{abc}	51.8^{a}	52.2^{a}	53.1^{abc}	54.3^{abc}	54.7^{bc}	55.5^{c}	52.6^{ab}	0.34
Final weight (g/fish)	187.4^{b}	203.8^{bcd}	191.2^{bc}	209.5^{d}	205.8^{cd}	199.7^{bcd}	198.0^{bcd}	158.6^{a}	3.51
Weight gain (g/fish)	134.2^{b}	151.9^{cd}	139.0^{bc}	156.5^{d}	151.6^{cd}	145.0^{bcd}	142.6^{bcd}	106.0^{a}	3.40
Feed conversion ratio	1.24^{a}	1.18^{a}	1.22^{a}	1.19^{a}	1.15^{a}	1.23^{a}	1.24^{a}	1.38^{b}	0.02
Feed intake (g/fish)	165.4^{b}	179.4^{cd}	168.6^{bc}	185.8^{d}	181.7^{bcd}	177.7^{cd}	177.3^{cd}	146.0^{a}	2.60
Survival (%)	100.0	100.0	100.0	100.0	100.0	97.8	97.8	97.8	0.37
Protein retention (%)	34.4	35.5	33.6	35.7	35.7	36.3	35.7	31.9	0.54
Energy retention (%)	39.8	39.9	39.2	42.3	42.5	42.0	41.9	39.0	0.42

787 **Table 6** Plasma chemistry of fish in each of the experimental diets.

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	FM	100SE-CM	200SE-CM	300SE-CM	100EX-CM	200EX-CM	300EX-CM	LM	Pooled SEM
ALAT (U L^{-1})	15.3	4.7	23.8	24.0	13.0	11.0	14.2	17.3	2.49
CK (U L^{-1})	2821.0	2357.5	2581.2	2282.0	2286.3	2677.5	2392.8	3368.0	269.77
GDH (U L^{-1})	7.7	5.2	8.5	9.0	5.7	5.8	3.8	6.7	0.60
Total protein (g L^{-1})	45.4	42.5	49.3	46.9	42.3	40.3	43.8	46.7	1.23
Glucose (mmol L^{-1})	6.7	9.7	5.0	6.1	5.1	4.2	3.9	5.2	0.53
Heam (mg/dL^{-1})	20.0	17.6	18.5	15.7	34.3	11.5	16.2	10.7	2.88
Mg (mmol L^{-1})	1.4	1.1	1.1	1.3	1.0	1.0	1.1	1.1	0.06
Ca (mmol L^{-1})	3.0	2.8	3.2	3.1	2.6	2.7	2.8	2.9	0.06
Phosphate (mmol L^{-1})	3.0	2.8	3.1	3.0	2.5	2.8	3.0	2.9	0.06
Fe (mmol L^{-1})	17.0^{b}	11.2^{ab}	8.6^{ab}	11.0^{ab}	12.2^{ab}	5.2^{a}	7.4^{a}	10.3^{ab}	0.88
Urea (mmol L^{-1})	1.6	2.2	1.9	1.7	1.8	2.0	2.1	2.4	0.09
Creatinine (µmol L^{-1})	66.3	91.5	81.6	56.7	51.0	54.4	45.4	39.3	7.15
Tri-iodothyronine (pmol L^{-1})	51.1^{ab}	63.7^{ab}	87.2^{b}	66.7^{ab}	56.3^{ab}	39.4^{ab}	32.7^{a}	54.9^{ab}	4.59
Thyroxine (pmol L^{-1})	11.3	16.9	18.7	15.2	12.3	10.1	8.0	12.0	1.06

789 Different superscripts within rows indicate significant differences between means among dietary treatments but not between
790 parameters ($P < 0.05$). Lack of any superscripts within a row indicates that there were no significant different among any of
791 those treatments for that parameter.

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