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JAMES COOK UNIVERSITY AUSTRALIA

EVALUATION OF CANOLA MEAL AS AN AQUAFEED INGREDIENT FOR BARRAMUNDI (ASIAN SEABASS; *LATES CALCARIFER*)

Thesis submitted by

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In December 2014

For the degree of Master of Philosophy in Aquaculture

Within the College of Marine and Environmental Sciences

James Cook University

Acknowledgement

First I would like to thank my Primary Supervisor Dr Igor Pirozzi at James Cook University and Associate Supervisor Dr Brett Glencross at CSIRO for their support, inspiration and guidance throughout my candidature. To Dr Nick Wade from CSIRO who advise me in molecular work and interpretation of my data in this field. Secondly I must thank staff in JCU and CSIRO including Nicholas Bourne, David Blyth, Michael Salini, Hiep Le Huu and Steve Gamble, who contributed excellent technical assistance in this research.

I would like to thank ACIAR for financial support (under John Allright Fellowship) for this research. I want to thank JCU and CSIRO for providing facility and infrastructure for this project.

Finally I must thank my family who provided emotional support and encouragement throughout my candidature.

Statement on the contribution of others

Research funding:

John Allright Followship (stipend, research and other)	$135773
Graduate Research School	$2000

Supervision

Dr Igor Pirozzi, JCU

Dr Brett Glencross, CSIRO

Research assistance

Dr Nick Wade, CSIRO

Nicholas Bourne, CSIRO

David Blyth, CSIRO

Hiep Le Huu, JCU

Steve Gamble, JCU

Additional assistance

Dr Brett Glencross advised on interpretations of digestibility, growth, histology and gene expression data, co-author on Aquaculture publication and Abstract at Conference; edited draft versions

Dr Igor Pirozzi advised on interpretations of digestibility, growth data; co-author on Aquaculture publication and Abstract at Conference; edited draft versions

Dr Nick Wade advised on interpretation of gene expression data; co-author on Abstract at conference

Statement on sources

I declare that this thesis is my own work and had not been submitted in any form for another degree or diploma at any university or other institution or tertiary education. Information derived from the published or unpublished work of others has been acknowledged in the text and a list of reference is given

Diu Thi Ngo

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Declaration on animal ethics

This research presented and reported in this thesis was conducted in compliance with the National Health and Medical Research Council (NHMRC) Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, 7th Edition, 2004 and Qld Animal Care and Protection Act, 2001. The proposed research study received animal ethics approval from the JCU Animal Ethics Committee Approval Number A2028 and A1652 and CSIRO Brisbane Animal Ethics Committee, Approval Number A4/2013.

Abstract

Canola meal (CM) is one of many potential plant ingredients for fishmeal replacement in fish diets. Many fish species have performed good growth when fed with dietary CM. However, there is limited information for using this ingredient in barramundi. In order to use this ingredient for aquaculture feeds, the information such as nutritional value, nutrient digestibility and ingredient utilisation have to be provided. Therefore, the present study described in this thesis was carried out to: (1) characterise nutritional composition and determine nutrient and energy digestibility of four Australian CMs with respect to different origin and processing method; (2) assess effects of diets with serial inclusion levels of two different CMs regarding different processing methods (expeller and solvent extraction) on growth performance and feed utilisation; (3) examine effects of CMs on changes in plasma chemistry, histology of digestive, metabolic organs and hepatic gene expression.

To achieve the above objectives, two experiments were undertaken. The first experiment (digestibility experiment) was designed with six diets (four CMs: three solvent extracted (SE) CMs from Newcastle, Footscray, Numurkah and one expeller extracted (EX) CM from Pinjarra), a diet with fishmeal (FM) as the sole protein and a diet based on lupin kernel meal (LM) were included as reference diets. Each CM test diet and LM diet were made by incorporation of 30 % of test ingredient and 70 % of basal mash (FM reference). Dry matter, protein, energy, amino acid and yttrium content of the diets, ingredients and faeces were analysed to enable the determination of the apparent digestibility of corresponding parameters. The second experiment (growth experiment) included eight dietary treatments each with three replicates, one FM reference diet (sole protein as fishmeal) (FM), one lupin (LM) diet (300 g/kg LM) and the CM diets (100, 200, 300 g/kg as either SE CM or EX CM). Performance indices such as feed intake, weight gain, DGC, FCR, protein and energy retention were determined. Following, an examination of the health effects and molecular responses of fish fed the CM containing diets compared to the FM and LM diets were also carried out. Plasma samples were analysed for biochemical parameters. The liver, kidney, caeca, distal intestine and stomach were used for histological analysis. For molecular expression, genes involved in fatty acid metabolism (*FAS, SCD* and *FXR)* and energy production pathways *(CS* and *PDK*) and others involved in detoxification (*CYP1A1,*

CYP3A, CYP2N, GST, GHGPx and *GPx*) were examined using RT-qPCR. The relative expression level of each gene in each sample was determined by normalising the cycle threshold values for each gene to Ef1-α.

Compositional analysis of the ingredients showed that the protein content of the SE CMs (370 to 423 g/kg DM) was higher than that of the EX CM (348 g/kg DM), but the lipid content was lower than that of the EX CM. Among the SE CMs, the protein digestibility of the CMs from Numurkah and Newcastle was similar (84.1 % and 86.6 % respectively), corresponding to that of the LM but significantly higher than that of the CM Footscray (74.5 %). The protein digestibility was the lowest (63.1 %) for the EX CM. The energy digestibility of the CMs (43.1 % to 52.5 %) was similar to that of the LM (54.8 %) except for the lower of the SE CM Footscray (32.4 %). The SE CMs provide 276 to 366 g/kg DM of digestible protein while that of the EX CM is only 220 g/kg DM. The digestible energy content of the SE CM Footscray (6.5 MJ/kg) was significantly lower than that of other CMs (8.7 to 10.6 MJ/kg DM).

After an eight week culture period the feed intake, growth performance, and protein retention efficiency of fish fed with dietary CM levels were similar or even higher to those of fish fed the FM and the LM diets. The FCR is also similar or better than the control diets. The exception to this was for fish fed with the 300 g/kg EX CM diet. The diet containing 300 g/kg EX CM depressed growth performance, feed intake, and increased FCR. In general, the SE CM can be used up to 300 g/kg diet without negative growth effects while 200 g/kg is the maximum acceptable level of the EX CM for barramundi.

Plasma biochemistry parameters were fairly similar among each of the dietary treatments. There were no modifications in the morphology of the liver, kidney, caeca, distal intestine or stomach of fish caused by any of the experimental diets. The expression of genes involved in fatty acid metabolism and TCA cycle was not influenced by fish fed with CM containing diets relative to the FM control and LM diets. However, fish fed with the diet containing 300 g/kg EX CM were shown to down-regulate the expression of some genes acting in detoxification pathways (*Lc CYP1A1, Lc CYP3A, Lc CYP2N* and *Lc GST*), but not *Lc GPx, Lc PHGPx* and *Lc GR.*

Overall, this study demonstrates that CM is a promising plant ingredient for FM replacement in barramundi based on determined digestible values and feed utilisation.

However, implications regarding different origin and processing method importantly affect CM utilisation for barramundi.

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Abbreviation list

ADC	Apparent digestibility coefficient
ADF	Acid detergent fibre
ANOVA	One-way analysis of variance
CS	Citrate synthase
CYP	Cytochrome P450
DGC	Daily growth coefficient
DM	Dry matter
DNA	Deoxyribonucleic acid
EX CM	Expeller extracted canola meal
FAS	Fatty acid synthase
FCR	Feed conversion ratio
FXR	Farnesoid X receptor
IF1α	Elongation factor 1 alpha
Luc	Luciferase
NDF	Neutral detergent fibre
PDK	Pyruvate dehydrogenase kinase
RNA	Ribonucleic acid
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
SCD	Stearoyl CoA desaturase
SE CM	Solvent extracted canola meal
SEM	Standard error of mean
TCA	Tricarboxylic acid

CHAPTER 1 . GENERAL INTRODUCTION

The rapid growth of aquaculture production throughout the world, especially the significant increase in the production of farmed carnivorous species, has increased the demand for fishmeal (Tacon and Metian, 2008). This increased demand coupled with static supply has seen a 300 % increase in the price of fishmeal over the past decade. The heavy reliance on fishmeal as the main protein source for aquaculture feeds has no longer ensured the sustainability of aquaculture. It is therefore critical to evaluate alternative protein sources suitable for fishmeal replacement. One of the options is to increase the utilisation of plant protein sources. Numerous studies have been conducted on a range of plant ingredients with promising results (Gatlin *et al.*, 2007; Hardy, 2010).

Among plant protein sources, canola meal (CM) appears to be one of the most promising ingredients for fishmeal replacement and the use of CM has been studied for many aquaculture species (Higgs *et al.*, 1995; Burel and Kaushik, 2008). However there is little information on using this ingredient for barramundi, *Lates calcarifer* (Glencross, 2011; Glencross *et al.*, 2011c).

This introduction will describe the nutritional composition of CM, review studies on digestibility and utilisation of CM for fish species, and consider what is presently known regarding using alternative ingredients for fishmeal replacement in barramundi. The strategies in ingredient evaluation for aquaculture feeds are also discussed.

1.1. Canola meal and its utilisation in fish diet

1.1.1. Chemical composition of canola meal

The chemical composition of CM has been generally reported based on the residual oil and protein content. The variation in quality of CM depends on origin (with regard to weather and soil condition) and processing (solvent oil extraction or expeller oil extraction). The protein content of CM varies from 34 % to 48 % of dry matter. Lipid content varies from 1 % to 4 % for solvent extracted (SE) CM and 10% to 12% for expeller (EX) CM (reviewed by Burel and Kaushik (2008)). It is noted that the balance of amino acids of canola protein is one of the best among commercial vegetable protein sources. The complementarity of the amino acid profile of canola protein is similar to that of fishmeal but better than that of soybean meal as it has a higher content of sulphur containing amino acids (methionine and cystine) (Higgs *et al.*, 1996). However, canola protein has a deficiency in lysine which is relatively abundant in soybean meal. CM is also recognized as high essential mineral sources such as selenium and phosphorus. These minerals are relatively higher in CM than that of other oilseeds and also of fishmeal in terms of magnesium, phosphorus, though at lower level in calcium (Burel and Kaushik, 2008).

1.1.2. Digestibility of nutrients and energy of canola meal for fish

Protein digestibility of CM has been determined for many fish species. Results have shown that there is a great variation among CM products for different fish species. Protein digestibility of CM ranges from 23 % to 94 % despite most values over 80 % (Burel and Kaushik, 2008). The variation can be due to different quality of CM, different fish species and/or different methodologies employed in the determination of digestibility in the different studies. Protein digestibility of CM can be improved by some treatments to reduce anti-nutritional factors (ANFs) in the meal. Protein digestibility of European CMs for rainbow trout (89 % to 93 %) (Burel *et al.*, 2000c) were higher than that of Canadian CMs (Cho and Slinger, 1979; Higgs *et al.*, 1996; Mwachireya *et al.*, 1999) because these European CMs were dehulled to reduce fibre content. Temperature also has important effects on the quality of CM. Heat treatment substantially improved protein digestibility of CM for turbot due to a decrease in the level of glucosinolates (26 μmol/g in heat-treated CM compared to 40 μmol/g in untreated meal) (Burel *et al.*, 2000c). However, this heat-treated CM did not change digestible values for rainbow trout. It is suggested that different species have different

tolerance to ANFs, and in case of this study turbot is likely more sensitive to glucosinolates than trout. In contrast, Glencross *et al.* (2004a) indicated that heat treatment of EX CM at 120 ^{o}C and 150 ^{o}C had negative effects on digestibility for red seabream. Indeed, protein digestibility of heat treated meal (51 % and 23 % respectively) was much lower than that of untreated meal (94 %). In this case, CM protein was likely degraded by excessive heat.

Amino acid digestibility of CM on salmonids has shown to be fairly higher, varying 73.4 % to 90.5 % (Hilton and Slinger, 1986; Anderson *et al.*, 1992). Allan *et al.* (2000) indicated apparent digestibility coefficients (ADCs) of amino acids of CM were over 80 % for silver perch, and CM amino acids were more digestible than those of the whole canola seed meal. Allan *et al.* (2000) also demonstrated that ADCs of some amino acids such as sulphur containing amino acids were lower than others. High amino acid availability was also obtained for cobia (Zhou *et al.*, 2004). In contrast, very poor amino acid digestibility of CM was determined for hybrid striped bass (Gaylord *et al.*, 2004), which reported that average digestibility of amino acids was only 48.1 %, much lower than that in other studies, and it also showed that lowest values were for histidine and valine (42 % and 37 % respectively). However, ANFs were not characterized in that study and may have contributed to the poor digestibility. Effects of temperature on amino acid bioavailability were assessed by Newkirk *et al.* (2003), who indicated that excessive heat in processing was the main reason for lower digestible amino acids in CM.

For energy digestibility, it has been shown that energy digestibility varies greatly from 21 % to 83 %, in which the lowest values have been observed in early studies for rainbow trout with 21 % to 45 % (Cho and Slinger, 1979; Hilton and Slinger, 1986). A great variation of energy digestibility among different species has been reported for Atlantic salmon (62 % to 73 %), rainbow trout (39 % to 83 %), Chinook salmon (51 % to 71 %), gilthead seabream (79 %), silver perch (58 %), red seabream (30 % to 62 %) (reviewed by Burel and Kaushik (2008)). As protein is a main source of energy in CM, any improvement in protein digestibility will also lead to an increase in energy digestibility. Heat treatment improved protein digestibility of CM for turbot; therefore, energy digestibility was also improved (Burel *et al.*, 2000c). In the study by Glencross *et al.* (2004a), poor protein digestibility in CM with heat treatment at 130 ^{o}C and 150 ^{o}C was also consistent with poor energy digestibility (33 % and 30 % respectively). In

general, low energy digestibility is due to low dry matter digestibility of CM as carbohydrates in CM is less digestbible (Van Barneveld, 1998).

ANFs presenting in CM include sinapine, tannins, glucosinolates and phytic acids. Each of these are elements associated with decreasing protein digestibility (McCurdy and March, 1992; Mwachireya *et al.*, 1999). CM also contains a high fibre content which leads to low digestibility of dry matter as it appears to be less digestible in monogastric species such as fish. Moreover, the presence of fibre can decrease the absorption of other nutrients. Therefore, a reduction in fibre and other ANFs can improve CM nutrient and energy digestibility for fish.

1.1.3. Utilisation of canola meal in fish diets

Among studies on the utilization of canola protein products for fish species, salmonids have accounted for most studies. The studies have reported different results of CM use on salmonids. CM is considered as a good protein source for coho and chinook salmon, which can be included with 16 % to 20% in diets for these species (Higgs *et al.*, 1982; Higgs *et al.*, 1983). For rainbow trout, Shafaeipour *et al.* (2008) reported that Iranian CM could be incorporated in diets for rainbow trout at 30 % without any adverse effects on growth performance, feed intake, feed utilisation, physiological and biochemical parameters. However, the previous study of Hilton and Slinger (1986) reported that CM could not replace soybean meal or fishmeal in diet for fry rainbow trout even at low inclusion level (13.5 %). In general, most salmonids cannot use CM in diets with inclusion exceeding 25 % to 30 % without deleterious effects or compromising growth (Hilton and Slinger, 1986; Hajen *et al.*, 1993; Burel *et al.*, 2000a).

Assessment of CM utilisation for non-salmonid species is limited in amount of research; however reported results from some non-salmonids indicate that CM can be used more efficiently than in salmonids. Glencross *et al.* (2004b) demonstrated that CM can be incorporated in a diet at up to 60 % for red seabream without any deleterious effects on growth performance, other growth parameters or plasma thyroid hormone content. Catfish can use up to 31 % CM (replacement of 75 % soybean meal) in diets and a decrease in feed intake and weight gain was only observed at an inclusion level of 46 % (Lim *et al.*, 1998). Webster *et al.* (1997) also reported that 36 % CM was used effectively in practical diets for channel catfish but exceeding this level resulted in poorer weigh gain and a higher FCR which might be due to reduced palatability.

However, recent research with some marine species, such as Japanese seabass (Cheng *et al.*, 2010) and cobia (Luo *et al.*, 2012) have shown that the incorporation of CM in diets for these species was limited to 10 % and 12.5 % respectively. Higher inclusion levels resulted in the decrease in growth performance of both species. Clearly, the acceptance of CM in diets for fish depends on the quality of CM and/or fish species. In some cases, the reduction in growth performance of fish fed with CM is generally a result of reduced palatability as presenting ANFs in diets (Hilton and Slinger, 1986; McCurdy and March, 1992; Webster *et al.*, 1997; Burel *et al.*, 2000b; Kissil *et al.*, 2000; Luo *et al.*, 2012). In addition, a reduced growth performance can be caused by low feed utilisation efficiency associated with the decrease in the nutrient and energy digestibility (Cheng *et al.*, 2010).

Processing methods may have influence on ingredient quality by reducing ANFs and improving the nutritional value of CM. Growth performance of rainbow trout fed a diet containing up to 40 % of protein (26 % – 38 % of feed) from upgraded CMs that were treated by acid- or solvent-wash and reduced in fibre content, is similar to that of rainbow trout fed a fishmeal based diet (McCurdy and March, 1992). In that study a normal growth performance was also obtained when Chinook salmon were fed with the diet containing 25 % of the dietary protein from CM. In this case the growth performance was improved in fish fed CM containing diets due to the reduction of ANFs in CM by solvent washing process which reduced 40 % to 90 % glucosinolate content. Extrusion processing of CM at 90 ^{o}C and 150 ^{o}C has subjected favourable effects for chinook salmon as the phytic acid content is reduced by 10 % to 30 % respectively (Satoh, 1998). As a result CM could incorporate in diet with 240 g/kg without any decrease in growth performance of this species.

1.1.4. Effects of diets containing canola meal on fish health: changes in biochemistry, histology and molecular response

ANFs in plant ingredients not only cause decreased growth performance but also affect fish health (Francis *et al.*, 2001). Higgs *et al.* (1995) showed that the elevated levels of phytic acid has contributed to an increased incidence of structural changes in the pyloric caeca. In addition, glucosinolates are bioactive compounds affecting thyroid function, such as thyroid hypotrophy or decrease in thyroid hormone (Yurkowski *et al.*, 1978; Leatherland *et al.*, 1987; Hossain and Jauncey, 1988; Teskeredžić *et al.*, 1995;

Webster *et al.*, 1997; Burel *et al.*, 2000a; Burel *et al.*, 2001). Thyroid abnormalities were found in carp fed a diet containing 3.3 g purified glucosinolates/kg (Hossain and Jauncey, 1988). Although commercial CMs contain less glucosinolates than early rapeseed meals, some studies still report effects of these compounds on thyroid function. Burel *et al.* (2000a) reported that the decrease in plasma thyroid hormone was observed in rainbow trout even at low content of glucosinolates in the diet (1.5 μmol/g). In contrast, Shafaeipour *et al.* (2008) demonstrated that fish fed CM did not cause any changes in thyroid hormone albeit higher glucosinolate content (3 μmol/g diet) than in Burel's study. This suggests that glucosinolate breakdown products of these CMs are different. In fact, intact glucosinolates are not active compounds but their hydrolysis products are. Likewise, the ingestion of 60 % CM in the diet (2.1 μmol glucosinolates/g diet) did not affect plasma thyroid hormone (T3 and T4) concentration in red seabream (Glencross *et al.*, 2004a). It is clear that effects on thyroid function such as plasma thyroid hormone content vary greatly depending on concentration of glucosinolates, their breakdown products and/or fish species. In some studies the effects of CM containing diets have been shown to decrease metabolic enzymes in the liver in Japanese seabass and cobia (Cheng *et al.*, 2010; Luo *et al.*, 2012). However, Glencross *et al.* (2011c) indicated that Australian CM did not cause any changes in plasma chemistry and metabolic enzymes in juvenile barramundi. Clearly, effects of CM use greatly depend on the quality of CM and/or fish species.

CM contains glucosinolates which are known bioactive compounds inducing liver detoxification enzymes in mammals. The ingestion of breakdown products of glucosinolates has been shown to not only inhibit catalytic activity of the cytochrome P450 1A1 (CYP1A1) but also decrease the transcriptional level of this gene via modification of the aryl hydrocarbon receptor (AhR) which regulates some CYPs genes such as CYP1A1 (Wang *et al.*, 1997). Meanwhile, glucosinolates and their derivatives are also known as inducers of up-regulation of detoxification of phase II enzymes including GST and GPx (Nho and Jeffery, 2001). However, it is mentioned that the effects of glucosinolate breakdown products depend on the dose, time of treatment, type of glucosinolate breakdown products and the tested tissue. As for other ingredients, CM also contains relatively high levels of phenolic compounds. These compounds also have potent antioxidant activities associated with beneficial health effects (Adom and Liu, 2002; Bub *et al.*, 2003)). In fish, the information regarding the relationship between fish

nutrition and the antioxidant status is limited. A previous study on gilthead seabream has shown that activities of antioxidant enzymes glutathionie (GSH), glutathionine reducase (GR) and ɤ-Glutamyl transferase (cGT) increased with the increased inclusion levels of plant protein for fish meal replacement in diet (Sitjà-Bobadilla *et al.*, 2005). Currently, there have been no such studies undertaken on barramundi.

1.2. Barramundi and fishmeal replacement in diet

Barramundi (or Asian Seabass; *Lates calcarifer*), is a commercially important species in Australia and Southeast Asia (Tucker *et al.*, 2002), and has recently become one of the fastest growing finfish aquaculture industries worldwide with recent expansions and market development in Australia, Southeast Asia, Middle-east, USA and Europe (Palmer *et al.*, 1993). Global production of barramundi in 2012 reached over 70,000 tons, in which Australia produced approximately 5000 tons (Nystoyl, 2013).

Barramundi are an ideal tropical finfish species for aquaculture in many culture systems. This species is hardy, tolerates crowded conditions and handling, and thrives in a wide range of physiological and environmental conditions including high turbidity, and varying salinities and temperatures. Barramundi are also 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 very high dietary protein and lipid level. Protein demands by juvenile barramundi have been estimated to range from 45 % to 55 % of the diet (subject to dietary energy density) and optimum dietary lipid level for fingerlings is 15 % and 18 % at protein levels of 50 % and 45 % respectively (Williams *et al.*, 2003). Barramundi has limited ability to digest and utilise carbohydrates (Boonyaratpalin *et al.*, 1998). Studies with a range of starch sources show that increasing the level of starch in the diet results in a dramatic decline of the ability of animal to digest this nutrient (Glencross *et al.*, 2012a).

Barramundi can efficiently utilise plant protein sources for partial fishmeal replacement. Williams (1998) suggested that approximately 30 % soybean meal can be used for barramundi. Lupin has been also successfully replaced for fishmeal at an inclusion level of up to 45 % without negatively affecting growth performance of barramundi (Katersky and Carter, 2009). Plant protein sources can be efficiently used in diets for barramundi but it was suggested that at least 15 % fishmeal needs to be

retained in the diet to maintain acceptable palatability (Glencross *et al.*, 2011c). CM appears to be a favourable ingredient for fishmeal replacement, which can be included at 30% in barramundi juvenile diet (Glencross *et al.*, 2011c). However, this previous study evaluated only one type of CM (expeller extracted) and at a single inclusion level. Clearly, there is still limited information on using this ingredient for barramundi.

1.3. Strategy of ingredient evaluation for aquaculture feeds

In order to utilise any ingredient for aquaculture feeds, it is necessary to understand the implications such as nutritional value, nutrient digestibility and feed utilisation of the selected ingredient (Glencross *et al.*, 2007).

First, the characterization of ingredient needs to be done, and through this step the variables of nutritional composition, processing, origin and variation in quality are documented. Glencross *et al.* (2003) showed that there was a substantial variation in nutrient parameters such as protein and energy among lupin cultivars. The source of the ingredient is an important element, especially for plant ingredients as it is well known that soil type and weather condition can affect nutritional composition of ingredient (Hickling, 2001). In terms of processing method, there are clear differences in both quality and nutritional composition of CMs produced by different oil extraction methods (expeller extraction and solvent extraction) (Glencross *et al.*, 2004b,a). The quality of protein can be affected during processing. For example the loss of some amino acids can occur though heat damage by the interaction of components in the ingredient such as protein, carbohydrates and moisture in Maillard reactions (Carpenter and Booth, 1973; Anderson *et al.*, 1993).

Formulation of diets based on digestible energy and nutrients are more practical than on crude values (Cho and Kaushik, 1990). Therefore, importantly nutrient digestibility needs to be determined for the ingredient. This determines the proportion of energy and nutrients digested and absorbed by fish. Methods used for digestibility assessment include direct and indirect methods; however indirect method is more practical. In this method, the preparation of the diet with addition of indigestible marker and representative samples of both feed and faeces are required. The ratio of marker in feed and faeces is used to calculate apparent digestibility coefficient (ADC) for nutrients and energy of the ingredient (Glencross *et al.*, 2007).

Another important aspect is determination of ingredient palatability as indicated by feed intake. This step is important because irrespective of how nutritionally

appropriate the ingredient might be, if the ingredient reduces feed intake, it will have limited value. Subsequently, nutrient utilisation which is based on the capacity of the animal to utilise the digested nutrients for growth needs to be assessed. This assessment process includes the measurement of growth performance, feed efficiency, survival, energy and nutrient retention. The efficiency by nutrients and energy are retained from the feed provides a useful assessment of the efficiency of nutrient utilization form diets (Cho and Kaushik, 1990; Booth and Allan, 2003; Glencross *et al.*, 2004b,a). These are determined though a feeding trial which is designed with different models, in which a serial inclusion trial of the selected ingredients is useful to provide substantially more information than the single inclusion level trial. Typically, such trials have used series of inclusion levels, three or more, such as 0%, 10%, 20, 30%, 40 and 50% inclusion of the test ingredient into a reference diet (0%). All diets in this model are formulated to relevant practical protein and energy levels (usually on digestible basis) for use with respective aquaculture species (Glencross *et al.*, 2007).

Once ingredients are utilised in the diet they may concurrently introduce unexpected compounds such as ANFs into feed, which have the capacity to cause deleterious effects on fish health. Therefore, these assessments should be considered in the ingredient evaluation. The alternatives in biochemical parameters such as changes in blood glucose levels, enzyme activities or thyroid hormone levels were indicated when rainbow trout fed with CM containing diets (Burel *et al.*, 2001). In addition assessment of tissue histology has been useful in examining some of long term effects of ANFs in ingredients for fish diets (Krogdahl *et al.*, 2000; Krogdahl *et al.*, 2003). Recently, significant advancement of molecular techniques has been applied to assess the influence on hepatic metabolism in response to specific diets (Vilhelmsson *et al.*, 2004). It is clear that there are a range of additional variables that can be included in ingredient evaluation criteria. However, the selected variables depend on the specific objectives of the study.

Finally physical function of finished feeds can be included in the assessment. These properties include aspects such as sink rates, pellet stability, starch gelatinization and oil absorption capacity. However whether this facet needs to be mentioned in ingredient evaluation also depends on the objectives of the study (Glencross *et al.*, 2007).

1.4. Research aims

The major aim of this project was to evaluate CM as an ingredient for partial replacement of fishmeal in barramundi diet. This was done by examining the apparent digestibility of dry matter, protein, energy and amino acids of four Australian CMs of different origin and processing techniques compared to reference plant meal (lupin kernel meal) and fishmeal ingredients. Subsequently, a feeding trial including a serial inclusion design was undertaken to study ingredient utilisation and maximum threshold for CM use in diet for barramundi though examination of growth and feed utilisation parameters. Further the health effects of CM use were examined though plasma biochemical alterations, histology of the gastrointestinal tracts, liver, kidney and hepatic gene expression level. The results of this study provide practical information relating to canola utilisation in barramundi diets. To accomplish this major goal, the following specific objectives were set to:

- Characterise the nutritional values and determine the nutrient and energy digestibility of four different CMs with respect to different origins and processing methods

- Assess the effects of diets with serial inclusion levels of two different CMs with regard to different processing methods (expeller and solvent extraction) on feed intake, growth performance, feed conversion, protein and energy retention

- Examine effects of CMs on plasma chemistry, histology of digestive organs and hepatic gene expression

CHAPTER 2 . DIGESTIBILITY OF CANOLA MEAL IN BARRAMUNDI

The following Chapter is published as:
Ngo, D.T., Pirozzi, I., Glencross, B. (2015). Digestibility of canola meals in barramundi (Asian seabass; *Lates calcarifer*). Aquaculture 435, 442-449. doi: http://dx.doi.org/10.1016/j.aquaculture.2014.10.031

2.1. Introduction

Canola (rapeseed) meals (*Brassica spp*.) (CM) have considerable potential for fishmeal replacement in fish diets as they contain a relatively high protein content, varying from 34 % to 48 % dry matter with a good amino acid profile, notably higher in lysine and sulphur containing amino acids (methionine and cysteine) compared to soybean meal, and are also a source of some minerals and vitamins (Burel and Kaushik, 2008). Canola protein has been shown to be well digested by a number of species (Cho and Slinger, 1979; Hilton and Slinger, 1986; Anderson *et al.*, 1992; Hajen *et al.*, 1993; Higgs *et al.*, 1995; Higgs *et al.*, 1996; Mwachireya *et al.*, 1999; Allan *et al.*, 2000; Burel *et al.*, 2000c; Glencross *et al.*, 2004a). Many fish species have been also shown to have good growth and feed utilisation efficiency when fed diets containing CM (Yurkowski *et al.*, 1978; Higgs *et al.*, 1982; Hardy and Sullivan, 1983; McCurdy and March, 1992; Gomes *et al.*, 1993; Webster *et al.*, 1997; Kissil *et al.*, 2000; Glencross *et al.*, 2004b; Shafaeipour *et al.*, 2008; Cheng *et al.*, 2010; Luo *et al.*, 2012).

Like other tropical species, there has been relatively little effort carried out for barramundi in seeking a replacement of fishmeal for this species. The limited studies on replacement of fishmeal by plant protein sources such as soybean meal and lupin meal suggested that different raw materials can be effectively used with as little as 15 % fishmeal remaining in the diet (Glencross *et al.*, 2011c). The few available studies on CM use in the diet for barramundi indicate that the introduction of CM into diets for barramundi have been acceptable (Glencross, 2011; Glencross *et al.*, 2011c). However, there is limited information on the nutritional value of CM for barramundi. Therefore a comprehensive study is suggested to provide clear data and guidelines for the use of this ingredient in diets for barramundi.

The nutritional value of CM varies according to the amount of residual oil content, which is a direct consequence of the oil extraction technique used. Solvent

extraction and expeller pressing are the two main canola oil extraction methods used which produce different qualities of canola meals (Glencross *et al.*, 2004b,a). Other aspects, such as different growing conditions (e.g. weather and soil type), are also able to influence the nutrient composition of CM (Hickling, 2001). Therefore, a comprehensive assessment of this ingredient should include an examination of the variation in nutritional value of CM based on different processing methods and origin.

There are several key steps to effectively assess a raw material for aquafeeds. Initially, the raw material needs to be comprehensively characterised, so the composition and history of raw material are documented in order to allow a meaningful comparison with other raw materials. Secondly, the digestible values of the ingredient needs to be measured so as to allow for an understanding of the nutritional values of the ingredient via digestible values for a species rather than crude values; then the formulation of diets based on digestible values will be more nutritionally appropriate and economical. Once these fundamental assessments have been made then the acceptable levels of inclusion of the ingredient in the fish diets can be investigated by conducting feeding trials through the assessment of feed palatability, intake, growth performance and effects of replaced diets on fish health or any biochemical, physical changes as well (Glencross *et al.*, 2007).

This study therefore aims to assess the variation of the nutritive composition of the four CMs (from four crushing plants in four different regions in Australia - Newcastle, Footscray, Pinjarra and Numurkah, which are produced from the two different oil extraction techniques (solvent and expeller). Further to this the apparent digestibility of dry matter, protein, amino acids and energy of each of the four CMs were determined when fed to barramundi (*Lates calcarifer*).

2.2. Materials and Methods

2.1.1 Ingredient preparation and characterisation

Four samples of canola meal produced from mixed genotypes were used in this experiment (including three solvent-extracted (SE) CMs and one expeller (EX) CM) were obtained from four different crushing plants (Newcastle, New South Wales; Footscray, Victoria; Pinjarra, Western Australia; Numurkah, Victoria) (Figure 2.1), and a lupin kernel meal (*Lupinus anguitifolius* cv. Coromup) used as a plant reference ingredient. These ingredients were ground to pass through a 750 μm screen prior to

being included in a series of experimental diets. The chemical composition of four CMs and reference ingredients are described in Table 2.1 and Table 2.2.

Figure 2.1. Origin of the four canola meal samples used in this study

Table 2.1 Chemical composition of ingredients (values are g/kg DM unless otherwise indicated)

	FM [a]	LM [b]	CM					
			SE-CM Footscray[c]	SE-CM Newcastle[d]	SE-CM Numurkah[e]	EX-CM Pinjarra[f]	Mean ±SD[g]	CV (%)
Dry matter (g/kg)	925	906	900	908	903	974	921±35.3	3.8
Crude protein	721	408	370	423	381	348	381±31.5	8.3
Total lipid	91	64	57	44	56	92	62±20.7	33.2
Total ash	175	31	67	69	78	70	71±4.8	6.8
Gross energy (MJ/kg DM)	20.6	21.1	20.1	20.2	20.3	20.6	20±0.2	1.1
NDF	n/a	n/a	250	240	249	310	262±32.1	12.3
ADF	n/a	n/a	191	182	196	216	196±14.4	7.3
Lignin	n/a	n/a	94	95	111	134	109±18.7	17.2
Total poly-phenolics	n/a	3.3	15.6	14.3	19.9	16.4	16.6±2.4	14.6
Total tannins	n/a	<1.1	4.4	3.3	6.6	4.1	4.6±1.4	30.9
Phytic acid	n/a	9.9	44.4	35.2	26.6	45.2	37.9±8.8	23.2
Glucosinolates (μmol/g DM)	n/a	n/a	<3.3	3.3	6.6	3.1	4.3±2.0	45.4

[a] Peruvian fishmeal, supplied by Ridley Aquafeeds, Narangba, QLD, Australia

[b] Lupin kernel meal, supplied by Coorow Seed Cleaners Pty Ltd, Coorow, WA, Australia

[c] Solvent extracted canola meal, supplied by Cargill, Footscray, Victoria, Australia

[d] Solvent extracted canola meal, supplied by Cargill, Newcastle, New South Wales, Australia

[e] Solvent extracted canola meal, supplied by Riverland Oilseeds, Numurkah, Victoria, Australia

[f] Expeller extracted canola meal, supplied by Riverland Oilseeds, Pinjarra, WA, Australia

[g] Mean of values of four canola meals

Table 2.2 Amino acid composition of ingredients (values indicated as g/kg DM, number in parenthesis presents g amino acid/kg protein)

	LM[b]	CM					
		SE-CM Footscray[c]	SE-CM Newcastle[d]	SE-CM Numurkah[e]	EX-CM Pinjarra[f]	Mean ±SD[g]	CV (%)
Aspartic acid	41.8 (102.5)	29.8 (80.5)	29.8 (70.4)	28.1 (73.8)	25.7 (73.9)	28±1.9	6.8
Glutamic acid	87.5 (214.5)	72.1 (194.9)	77.0 (182.0)	68.5 (179.8)	61.8 (177.6)	70±6.4	9.2
Serine	21.3 (52.2)	18.6 (50.3)	19.1 (45.2)	17.9 (47.0)	16.2 (46.6)	18±1.3	7.1
Histidine	10.0 (24.5)	11.2 (30.3)	11.6 (27.4)	10.0 (26.2)	9.5 (27.3)	11±1.0	9.3
Glycine	14.9 (36.5)	18.1 (48.9)	18.6 (44.0)	17.8 (46.7)	16.1 (46.3)	18±1.1	6.1
Threonine	14.3 (35.0)	18.1 (48.9)	18.3 (43.3)	17.8 (46.7)	16.1 (46.3)	18±1.0	5.7
Cysteine-X	5.5 (13.5)	10.7 (28.9)	11.3 (26.7)	10.8 (28.3)	9.2 (26.4)	11±0.9	8.6
Arginine	45.7 (112.0)	24.7 (66.8)	25.6 (60.5)	24.8 (65.1)	21.3 (61.2)	24±1.9	7.9
Alanine	13.9 (34.1)	18.1 (48.9)	18.8 (44.4)	17.6 (46.2)	16.1 (46.3)	18±1.1	6.5
Tyrosine	16.6 (40.7)	13.1 (35.4)	13.0 (30.7)	12.9 (33.9)	11.7 (33.6)	13±0.7	5.2
Valine	16.5 (40.4)	21.0 (56.8)	20.8 (49.2)	20.0 (52.5)	18.8 (54.0)	20±1.0	5.0
Methionine	2.6 (6.4)	7.5 (20.3)	8.5 (20.1)	7.7 (20.2)	6.8 (19.5)	8±0.7	9.2
Phenylalanine	17.1 (41.9)	16.7 (45.1)	17.4 (41.1)	16.8 (44.1)	14.9 (42.8)	16±1.1	6.6
Isoleucine	16.5 (40.4)	15.7 (42.4)	16.0 (37.8)	15.2 (39.9)	14.1 (40.5)	15±0.8	5.5
Leucine	28.6 (70.1)	29.0 (78.4)	30.1 (71.2)	28.3 (74.3)	25.7 (73.9)	28±1.9	6.6
Lysine	14.6 (35.8)	17.3 (46.8)	17.4 (41.1)	17.7 (46.5)	12.3 (35.3)	16±2.6	16.0
Proline	18.0 (44.1)	20.1 (54.3)	30.8 (72.8)	25.7 (67.5)	23.6 (67.8)	25±4.5	17.9

Ingredient description as per footnote in Table 2.1

2.1.2 Diet and experiment design

The experiment design was based on a strategy that allowed for the diet-substitution digestibility method to be used (Glencross *et al.*, 2007). For this method, a basal diet was formulated and prepared with the composition of approximately 530 g/kg DM protein, 100 g/kg DM fat and an inert marker (yttrium oxide at 1 g/kg) (Table 2.3). Initially a basal mash was prepared and thoroughly mixed, forming the basis for all diets used in this study. Each canola meal was supplemented at a ratio of 30 %: 70 % to the basal mash to prepare each of the test diets; the reference diet was made from 100 % of basal mash, without addition of any other ingredients.

After the various diets were prepared, each mash was mixed by using a 60L upright Hobart mixer (HL 600, 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 operational through a 4 mm Ø 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 ^{o}C for 12 h in a fan-forced drying oven. The pellets were then stored frozen for later use. The formulation and composition of the test and basal diets are presented in Table 2.3 and Table 2.4

Table 2.3. Diet formulation (g/kg)

	FM	LM	SE-CM Footscray	SE-CM Newcastle	SE-CM Numurkah	EX-CM Pinjarra
Fishmeal	740	518	518	518	518	518
Fish oil [1]	20	14	14	14	14	14
Wheat flour [2]	133.0	93.1	93.1	93.1	93.1	93.1
SE CM Newcastle	-	-	300	-	-	-
SE CM Footscray	-	-	-	300	-	-
SE CM Numurkah	-	-	-	-	300	-
EX CM Pinjarra	-	-	-	-	-	300
Lupin kernel meal	-	300	-	-	-	-
Cellulose	101.0	70.7	70.7	70.7	70.7	70.7
Vitamin and mineral premix [3]	5.0	3.5	3.5	3.5	3.5	3.5
Yttrium oxide [4]	1.0	0.7	0.7	0.7	0.7	0.7

[1] Sourced from Ridley Aquafeed, Narangba, QLD, Australia

[2] Sourced from Manildra, Auburn, NSW, Australia

[3] Sourced from Rabor, Beaudesert, QLD, Australia. Includes vitamin and mineral premix includes (IU/kg or g/kg of premix): Vitamin A, 2.5MIU; Vitamin D3, 0.25 MIU; Vitamin E, 16.7 g; Vitamin K,3, 1.7 g; Vitamin B1, 2.5 g; Vitamin B2, 4.2 g; Vitamin B3, 25 g; Vitamin B5, 8.3; Vitamin B6, 2.0 g; Vitamin B9, 0.8; Vitamin B12, 0.005 g; Biotin, 0.17 g; Vitamin C, 75 g; Choline, 166.7 g; Inositol, 58.3 g; Ethoxyquin, 20.8 g; Copper, 2.5 g; Ferrous iron, 10.0 g; Magnesium, 16.6 g; Manganese, 15.0 g; Zinc, 25.0 g.

[4] Sourced from Stanford Materials, Alisa, Viejo, CA, USA

Table 2.4. Chemical composition of diets (g/kg dry matter otherwise indicated)

	FM	LM	SE-CM Footscray	SE-CM Newcastle	SE-CM Numurkah	EX-CM Pinjarra
Dry matter (g/kg)	968	976	975	960	971	975
Protein	536	505	496	516	500	486
Total lipid	92	89	81	79	74	98
Ash	138	106	118	113	119	113
Carbohydrate (*)	203	275	280	253	277	278
Energy (MJ/kg DM)	20.4	20.7	20.0	20.5	20.5	20.8
Aspartic acid	47.4	46.4	43.1	45.0	41.5	40.5
Glutamic acid	71.0	76.2	71.2	78.0	69.2	67.7
Serine	21.8	21.9	21.0	22.6	20.5	20.0
Histidine	16.0	14.8	13.7	15.34	14.4	13.3
Glycine	29.3	25.5	26.8	28.1	25.7	25.0
Threonine	22.2	20.2	21.5	22.8	20.8	20.3
Cysteine-X	6.1	5.7	7.2	9.1	7.0	6.8
Arginine	29.7	34.7	28.8	30.7	28.5	27.1
Alanine	32.5	27.5	28.7	30.3	28.0	27.6
Taurine	5.3	4.0	3.8	4.1	3.8	3.8
Tyrosine	16.9	17.0	16.0	16.8	15.5	15.3
Valine	26.5	24.0	25.6	26.7	24.4	23.9
Methionine	15.4	11.8	13.1	14.2	12.7	12.4
Phenylalanine	22.1	21.2	21.1	22.2	19.9	19.4
Isoleucine	21.6	20.5	20.3	21.2	19.5	19.0
Leucine	38.0	35.9	35.9	37.9	35.2	34.4
Lysine	31.5	27.5	28.2	29.6	27.8	25.1
Proline	19.5	23.8	26.6	28.7	25.4	24.7

(*) Determined as DM – (protein + lipid + ash)

2.1.3 Fish handling and faecal collection

Hatchery produced barramundi (Gladstone, Queensland) were reared in a stock holding tank on a commercial pellet (Ridley Aquafeeds, Narangba, Australia) before being used in this experiment. Fish were acclimatised to their dietary treatment for one week prior to faecal collection which has been shown to be adequate for establishing an equilibrium in digestibility values (Blyth *et al.*, 2014).

The experiment included 6 treatments, with each treatment having 4 replicates. Each of the 24 cages was stocked with 5 fish of 390 ± 85 g (mean ± SD, n = 120). Treatments were randomly allocated and replicates evenly distributed across 6 x 2500 L tanks each with four HDPE mesh cages (300 L) per tank. No replicate cage of the same treatment occurred more than once per tank. Cages were rotated once per week across tanks after stripping events. This removed potential confounding effects due to tank effects. Tanks were supplied with aeration and temperature controlled recirculated freshwater. Water quality data was monitored on a daily basis during the experiment. Mean ± SD of water temperature, pH, NO_2, NH_3 were 29.8 ± 0.3 ºC, 7.3 ± 0.1 units**,** 0.5 ± 0.3 mg L^{-1} and 0.3 ± 0.2 mg L^{-1} respectively over the 30 day experiment duration.

Barramundi were manually fed once daily to apparent satiety, as determined over three separate feeding events between 1600 and 1700 each day. The experiment was designed with two blocks over time, with 12 cages for each block. The fish within the same block had their faeces collected on the same day. Faeces were collected in the following morning (0800 – 0900) from each fish within each tank using stripping techniques based on those reported by Glencross *et al.* (2011a) and Blyth *et al.* (2014). Fish were anesthetised using AQUI-S (20 ppm) in a small oxygenated tank (120 L). Once loss of equilibrium was observed, close attention was paid to the relaxation of the ventral abdominal muscles of the fish to ensure the fish were removed from the water before they defecated in the anaesthetic tank. The faeces were then expelled from the distal intestine using gentle abdominal pressure. Faecal samples were expelled into small plastic jars (70 mL) and stored in a freezer at -20 °C. To ensure accuracy for determination of digestion values, faecal collection was carefully handled to avoid contaminating the faeces with mucus and urine. No fish were stripped on consecutive days in order to minimise stress on the animal and maximise feed intake prior to faecal collection. Faeces were collected until sufficient sample for chemical analysis (over a twenty-day period of faeces collection for this experiment), with each fish being

stripped six times, once every second day. Faecal samples from different stripping days from each tank were pooled within replicate, and kept frozen at –20 °C before being freeze-dried in preparation for analysis.

2.1.4 Chemical analyses

Diets, ingredients and faecal samples were analysed for dry matter, yttrium, ash, total lipid, nitrogen, amino acids and gross energy content. CMs were also analysed for neutral detergent fibre (NDF), acid detergent fibre (ADF), lignin, phytic acid, tannins, polyphenolic compounds and glucosinolates.

Dry matter was calculated by gravimetric analysis following oven drying at 105 ºC for 24 h.

Total yttrium concentration was determined after mixed acid digestion using inductively coupled plasma mass spectrometry (ICP-MS: ELAN DRC II, Perkin Elmer) based on the method described by (McQuaker *et al.*, 1979).

Protein levels were calculated from the determination of total nitrogen by organic elemental analyser (Flash 2000, Thermo Fishery Scientific), based on N × 6.25.

Amino acid composition of samples, except for tryptophan, was determined by an acid hydrolysis (HCl) at 110 ^{o}C for 24 h prior to separation via HPLC.

Total lipid content of the diets and ingredients 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 °C for 12 h.

Gross energy was determined using a ballistic bomb calorimeter (PARR 6200, USA).

Total glucosinolate content in four CMs were determined according to method AOF4-1.22 of AOF (2007). On the basis of this method, CMs were heated to destroy the natural myrosinase enzyme in these meals. Glucosinolates were then extracted by water onto a solid phase extraction column. Myrosinase was then added and the samples were incubated to allow the myrosinase enzyme to cleave the glucose molecules from the glucosinolate moleculars. The glucose molecules were washed off the solid phase

extraction and the concentration determined by calorimetric reaction. A calculation was then used to determine glucosinolate concentration.

Total poly phenolics and total tannins were assayed based on the method of Makkar *et al.* (1993). Briefly, phenolic compounds from canola meals and lupin were extracted in ethanol solution with the Folin Ciocalteu reagent and sodium carbonate added. The supernatant containing phenols was measured at 725 nm using Merck standard tannic acid solution for calibration. Then tannins from phenol containing extract were precipitated using insoluble polyvinyl pyrrolidone (polyvinyl polypyrrolidone, PVPP), and the second supernatant containing simple phenols was measured as above method. Total tannins were determined by difference between the total phenolic content and the single phenolic content.

Phytic acid in samples were separated and concentrated by ion-exchange chromatography. The phytic acid concentrate is then quantitatively determined as phosphorus by inductively coupled plasma atomic emission spectrometry (ICP-AES).

NDF content was determined by using FibreCap^{TM} 2021/2023 following to the method described in the standard of EN ISO 16472. This method is based on the principle that a neutral detergent solution, with a heat-stable alpha amylase, is used to dissolve the easily-digested proteins, lipids, sugars, starches and pectins in samples, leaving fibrous residue (aNDF). ADF and Lignin were determined following the standard of EN ISO 13906: 2008.

2.1.5 Digestibility analysis

Apparent digestibility coefficients (ADCs) of dry matter, protein, amino acids and gross energy for reference and test diets were calculated by following formula (Maynard and Loosli, 1969):

$$\text{ADC of diet (\%)} = (1 - (Y_2O_{3\ diet} / Y_2O_{3\ faecal}) \times (Nutr_{faeces} / Nutr_{diet})) \times 100$$

where Y_2O_{3diet} and $Y_2O_{3faeces}$ are the yttrium content of the diet and faeces respectively, and $Nutr_{diet}$ and $Nutr_{faeces}$ are the nutritional parameters (dry matter, protein, amino acid or energy) of the diets and faeces respectively. Then, the ADCs of ingredients were determined according to the formula:

$$\text{ADC of ingredient (\%)} = (ADC_{test} \times Nutr_{test} - ADC_{basal} \times Nutr_{basal} \times 0.7) / (0.3 \times Nutr_{ing})$$

where ADC_{test} and ADC_{basal} are apparent digestibility of test diet and basal (reference) diet respectively; $Nutr_{test}$, $Nutr_{basal}$ and $Nutr_{ing}$ represent the nutritional parameters (dry matter, protein, amino acids and energy) of test diet, basal diet and ingredient respectively. All raw material inclusion levels were corrected on dry matter basis and an actual ratio of basal diet to test ingredient was used for digestibility calculation of test ingredient (Bureau and Hua, 2006).

Digestibility values calculated exceeding 100 % were not corrected because they indicate potential effects of interaction between diet and test ingredient and are reported as determined. However, for practical reasons, only digestibility values in a range of 0 % to 100 % were used for calculation of digestible nutrients and energy as per recommendations from Glencross *et al.* (2007).

2.1.6 Statistical analysis

All figures are mean ± SEM. Data were analysed for homogeneity of variation by Levene's test before being analysed with a one-way analysis of variance (ANOVA) using SPSS 11.0 for Windows. Differences among the means were tested by Duncan's multiple range tests with the level of significance $P < 0.05$. All percentage data were arcsine-transformed prior to analysed. Three outliers of homogeneity of variances were identified and removed from data set with degrees of freedom adjusted accordingly for subsequent statistical analyses (Table 2.5 and Table 2.6). These outliers were dietary ADCs of proline in the SE-CM Newcastle and EX-CM Pinjarra diets and one ingredient ADC of histidine for SE CM Newcastle.

2.3. Results

2.3.1 Variation in raw materials

The chemical composition of the ingredients is presented in Table 2.1 and Table 2.2. The difference in nutrient composition of canola meals was mainly observed in protein and lipid content. The crude protein content of solvent extracted (SE) CMs varied from 370 to 423 g/kg DM, and was higher compared to that of the expeller CM (348 g/kg DM). However, lipid content of the SE CMs was lower (44 g to 56 g/kg DM) compared to that of expeller extracted (EX) CM 92 g/kg DM). There was also a variation in the chemical composition among the SE CMs. The CM from Newcastle had higher protein content than the CM from Footscray and Numurkah. Energy values were relatively consistent among the different CMs, range of from 20.1 to 20.6 MJ/kg DM. The lupin kernel meal had a relatively similar composition to SE CMs (Table 2.1) but was lower in ash content (31 g/kg DM) compared to CMs (67 to 70 g/kg DM).

Similar to protein, amino acid content was fairly consistent among solvent CMs, while lower content of almost all amino acids of EX compared to SE were observed. Lysine content was significantly lower in the EX. In general, although some lower amino acid content was recorded for CMs, sulfur containing amino acids and lysine were higher in the CMs than in the lupin meal (Table 2.2).

In addition to the nutritive values, anti-nutritional factors were also characterised in this study. These include phenolic compounds (14.3 to19.9 g/kg DM), tannins (3.3 to 6.6 g/kg DM), phytic acid (26.6 to 45.2 g/kg DM) and glucosinolates (3.1 to 6.6 μmol/g DM). In comparison with the lupin meal, all antinutritional compounds presented in the CMs were consistently higher (Table 2.1). Fibre (reported as NDF, ADF and lignin) content was higher in the EX CM than in the SE CMs (NDF: 310 vs. 240 and 250 g/kg DM respectively).

2.3.2 Dietary digestibility

Dietary ADCs of protein were virtually identical (82.0 % to 83.8 %) among the different SE CM diets and were higher than that of EX CM diet (79.7 %). Overall, the dietary protein digestibility of SE CM diets was relatively similar to the reference diet (85.7 %) but less than that of the lupin diet (86.3 %). The same trend was seen for amino acid digestibilities (Table 2.5). Lower dietary amino acid digestibilities were recorded for the EX CM than for the SE CMs. The amino acid ADCs of the SE CMs were similar to those of the lupin meal except for those of the SE CM from Footscray.

The digestibility values of the test diets were consistent for both dry matter and energy (except for lower values of the SE-CM Footscray diet), and were lower than those of the reference diet (detailed in Table 2.5).

Table 2.5. Diet apparent digestibility coefficients (%) (n = 4)

Nutrient	Reference	LM	SE-CM Footscray	SE-CM Newcastle	SE-CM Numurkah	EX-CM Pinjarra	Pooled SEM
Dry matter	66.1^{b}	58.5^{a}	54.8^{a}	58.9^{a}	57.7^{a}	55.7^{a}	0.99
Protein	85.7^{cd}	86.3^{d}	82.0^{b}	83.8^{bc}	83.8^{bc}	79.7^{a}	0.53
Energy	78.3^{c}	71.2^{b}	66.4^{a}	70.6^{b}	68.0^{ab}	67.6^{ab}	0.92
Amino acids							
Aspartic acid	82.5^{b}	83.3^{b}	79.7^{b}	81.7^{b}	80.9^{b}	76.2^{a}	0.64
Glutamic acid	93.0^{c}	92.9^{c}	90.6^{b}	91.8^{bc}	91.5^{bc}	88.6^{a}	0.37
Serine	88.1^{c}	87.6^{c}	83.2^{ab}	85.0^{bc}	84.8^{bc}	80.4^{a}	0.68
Histidine	89.5^{c}	88.5^{bc}	81.3^{a}	86.8$^{b\,*}$	86.1^{b}	79.6^{a}	1.07
Glycine	84.2	83.6	80.6	82.5	83.0	77.1	0.65
Threonine	90.7^{d}	89.6^{cd}	86.0^{b}	87.9^{bc}	87.3^{bc}	83.4^{a}	0.58
Cysteine-X	73.8^{c}	69.4^{bc}	64.7^{b}	74.8^{c}	67.7^{bc}	56.6^{a}	1.51
Arginine	93.1^{cd}	94.4^{d}	90.8^{ab}	92.1^{bc}	92.0^{bc}	90.1^{a}	0.36
Alanine	92.3^{c}	91.6^{c}	89.6^{ab}	90.7^{bc}	90.5^{bc}	88.1^{a}	0.35
Taurine	79.6^{b}	72.3^{ab}	63.8^{a}	69.6^{ab}	70.5^{ab}	69.3^{ab}	1.59
Tyrosine	91.4^{c}	91.1^{c}	86.5^{ab}	88.2^{b}	87.8^{ab}	85.4^{a}	0.56
Valine	91.8^{c}	91.0^{c}	88.1^{ab}	89.3^{bc}	88.2^{ab}	85.7^{a}	0.52
Methionine	91.5^{c}	89.9^{bc}	89.0^{ab}	90.7^{bc}	90.3^{bc}	87.6^{a}	0.36
Phenylalanine	92.2^{b}	92.1^{b}	90.7^{ab}	91.1^{ab}	89.6^{a}	89.2^{a}	0.32
Isoleucine	92.7^{d}	91.8^{cd}	89.0^{ab}	90.0^{bc}	89.4^{ab}	87.3^{a}	0.46
Leucine	94.1^{d}	93.6^{cd}	91.5^{ab}	92.3^{bc}	92.2^{ab}	90.3^{a}	0.33
Lysine	92.4^{d}	91.0^{cd}	87.2^{ab}	89.3^{bc}	90.1^{cd}	86.2^{a}	0.52
Proline	81.8^{a}	82.3^{a}	87.0^{bc}	88.8$^{c\,*}$	85.7^{b}	81.4^{a*}	0.64

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

(*) mean for three replicates after removal of extreme outlier

2.3.3 Ingredient digestibility

The findings from the present study indicate that there is an influence of oil extraction methods on the ingredient protein digestibility of CMs. Protein digestibility of EX CM was significantly lower than that of SE CMs (63.1 % vs. a range of 74.5 % to 84.1 %). Furthermore, there was also a difference in protein digestibility Among SE CMs. Protein digestibility of CM Footscray was lower than those of CM Newcastle and Numurkah. There were no significant differences among protein digestibility values of CM Footscray, CM Numurkah and lupin meal; however a higher value was still recorded for the lupin meal (92.7 %).

There was no significant difference in the ADCs of dry matter among the different CMs, although the lower value was still seen for SE CM Footscray (29.9 %). The results showed that dry matter digestibility did not exceed 50 % for any of the CMs or the lupin meal.

There was a correlation between DM digestibility and energy digestibility (Figure 2.2), therefore low DM digestibility reflected poor energy digestibility of CMs and lupin, except for EX (poor DM digestibility but high energy digestibility). Energy digestibility of the SE CMs and EX CM was similar and equivalent to that of lupin, excluding a significant lower value (32.4 %) recorded for solvent CM Footscray.

Table 2.6. Ingredient apparent digestibility coefficients (n = 4) and digestible nutrient and energy values of test ingredients

Nutrient	LM	SE-CM Footscray	SE-CM Newcastle	SE-CM Numurkah	EX-CM Pinjarra	Pooled SEM
Dry matter	44.2	29.9	42.2	40.1	32.9	2.98
Protein	92.7^{c}	74.5^{b}	86.6^{c}	84.1^{bc}	63.1^{a}	2.78
Energy	54.8^{b}	32.4^{a}	52.5^{b}	43.1^{ab}	46.9^{b}	2.42
Amino acids						
Aspartic acid	89.3^{bc}	78.0^{b}	104.6^{c}	73.3^{b}	44.8^{a}	5.28
Glutamic acid	93.7^{bc}	84.8^{b}	110.0^{c}	83.3^{b}	74.3^{a}	2.92
Serine	89.6^{c}	71.7^{b}	99.8^{c}	73.3^{b}	53.5^{a}	4.18
Histidine	101.0^{c}	34.5^{a}	93.5$^{c\,*}$	77.9^{b}	24.0^{a}	7.92
Glycine	90.8^{bc}	79.2^{b}	105.6^{d}	76.3^{b}	42.0^{a}	5.62
Threonine	94.2^{c}	81.3^{b}	108.1^{d}	75.4^{b}	58.7^{a}	4.18
Cysteine-X	50.4^{b}	47.1^{b}	107.4^{c}	48.6^{b}	24.0^{a}	6.86
Arginine	97.9^{b}	90.9^{b}	115.7^{d}	92.7^{b}	79.5^{a}	2.92
Alanine	101.6^{c}	88.2^{b}	116.5^{d}	82.5^{b}	68.7^{a}	4.08
Taurine	-	-	-	-	-	-
Tyrosine	94.2^{b}	76.6^{a}	102.1^{b}	73.3^{a}	63.9^{a}	3.67
Valine	97.0^{cd}	87.9^{d}	109.0^{c}	73.9^{b}	60.3^{a}	4.26
Methionine	88.5^{c}	77.9^{bc}	118.2^{d}	66.7^{ab}	48.1^{a}	5.90
Phenylalanine	101.6^{b}	97.5^{b}	114.9^{c}	70.2^{a}	67.8^{a}	4.39
Isoleucine	96.4^{cd}	86.5^{c}	105.8^{d}	74.3^{b}	60.5^{a}	3.95
Leucine	100.3^{c}	90.1^{b}	110.4^{d}	87.7^{b}	78.9^{a}	2.73
Lysine	106.5^{c}	80.6^{b}	115.9^{c}	87.6^{b}	34.8^{a}	6.67
Proline	155.7^{c}	198.5^{d}	154.3$^{c\,*}$	137.5^{b}	127.0$^{a\,*}$	6.83
Digestible nutrients						
DM (g/kg)	401	269	383	362	320	
Protein (g/kg DM)	378	276	366	320	220	
Energy (MJ/kg DM)	11.5	6.5	10.6	8.7	9.7	

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

(*) mean for three replicates after removal of extreme outlier

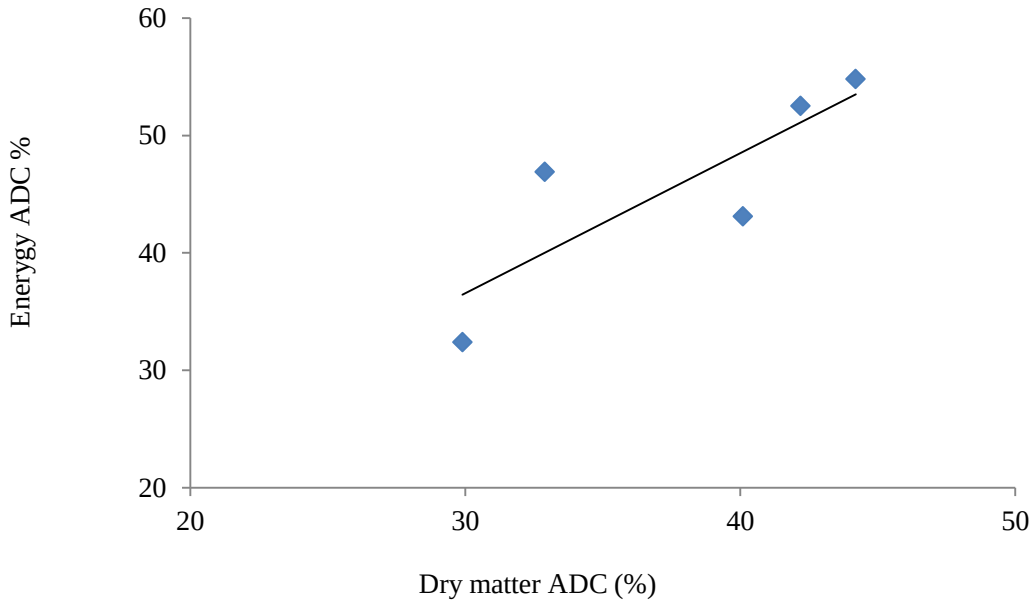

Figure 2.2. Correlation between dry matter ADC and energy ADC values across all test ingredients ($y = 1.1927x + 0.786$, $R^2 = 0.6889$)

In general, amino acid availability reflected protein digestibility (Table 2.6). Indeed, many amino acid digestibility values were recorded exceeding 70 % for CMs which were similar to protein values; however, for some amino acids, very low digestibility values were observed (some below 50 %), such as for histidine, cysteine, methionine and lysine in expeller meal. There was substantial variation in amino acid digestibility among ingredients, and a significant decrease in digestibility of almost all amino acids was reported for EX CM compared to other ingredients. In some cases digestibility values over 100 % were recorded, such as for proline in all ingredients, and some other amino acids in the SE CM Newcastle.

2.4. Discussion

The findings of this study provide a comprehensive assessment of the influence of oil extraction methods on the bioavailability of nutrients from various Australian CMs when fed to barramundi. These ingredient digestibility values were compared to a lupin kernel meal which have previously been shown to have good acceptability as a plant protein ingredient for use in barramundi (Glencross *et al.*, 2011c).

2.4.1. Variation in raw materials

Results of the present study showed that the processing method applied in canola oil extraction process affects the nutritional composition of the canola meals and their subsequent digestibility by barramundi. Indeed, a 61 % to 109% higher level of oil, accompanied with a reduction of 6 % to 22% of protein content, was observed in the expeller-extracted meal compared with the solvent-extracted meals. In terms of "protein quality", the loss of lysine content in expeller canola meal was probably due to heat damage in canola processing (Carpenter and Booth, 1973).

The variation in composition of the four canola meals from different regions suggests that growing conditions (e.g. weather, soil quality) may also affect quality of canola meal. Furthermore, canola meal crushers probably also influence the quality of produced canola meal by adjusting quality parameters in processing (Clandinin *et al.*, 1959; Bell, 1993; Hickling, 2001). Moreover, different cultivars which were not identified in this study may be a reason for dissimilarity in the qualities of the canola meals. In general, the Australian SE CMs characterised in our study had protein (370 - 423 g/kg DM) equivalent to European meals and Canadian meals, but were higher in lipid content (40 – 57 g/kg DM) compared to European meal (French Feed Database, 2005) and the Australian meal in the study of Glencross *et al.* (2004a). For the EX meal, the protein content reported in this study was consistent with European and Canadian expeller meals' but the lipid content was lower (French Feed Database, 2005). For amino acids, the greatest differences were seen for lysine. The lysine content of the EX CM in this study (12.3 g/kg DM) was lower than that of other EX Australian meals (17.7 - 21.1 g/kg wet basis) in report of (Spragg and Mailer, 2007), that of Australian EX meal (20 g/kg DM) (Glencross *et al.*, 2004a) that of European (39 g/kg DM) (French Feed Database, 2005), despite having similar protein levels.

2.4.2. Variation in ingredient digestibility

The findings of the current study indicate that the processes applied in oil extraction to canola seed have affected not only their composition but also the digestibility of the meals when fed to barramundi. Indeed, protein digestibility of the EX meal was lower than that of SE meals (63.1 % vs. 74.5 % to 86.6 %). The results of our study were dissimilar to the results of (Glencross *et al.*, 2004a) where protein digestibility of Australian canola meals was determined for red seabream. In that work, there were no significant differences in protein digestibility between expeller and solvent meals but a higher value was still seen for expeller (93.6 % for expeller meal vs. 83.2 % for solvent meal. However, heat treatment of this EX CM at 130 ^{o}C and 150 ^{o}C substantially depressed its digestible protein to 51.3 % and 23.1 % respectively. In the present study, although operation temperature in oil processing of the CMs was not described, substantial depletion of protein digestibility of the EX CM suggests that high temperature was probably applied in the processing which might have caused Maillard reactions leading to a modification of protein quality due to cross-linkages of amino acids (Carpenter and Booth, 1973). Spragg and Mailer (2007) described that in some canola oil extraction plants the temperature can be increased up to 135 ^{o}C to increase oil production. However, there are also other reasons which can explain a decrease of 10 % in protein ADC of EX meal. The higher phytic acid content together with higher fibre (expressed as ADF and NDF content) presented in the EX CM than in the SE meals could adversely affect protein digestion of barramundi. Mwachireya *et al.* (1999) reported that high levels of fibre either alone or together with phytate adversely impacted the digestibility of CM for rainbow trout. In terms of fibre (reported as non-starch polysaccharides (NSP)), a certain decrease in protein digestibility was observed when fish fed increased dietary NSP classes (Glencross, 2009; Glencross *et al.*, 2012b). The effect of fibre on nutrient digestibility is thought to interfere with the transport of nutrients along the gastrointestinal tract and consequently the efficiency of nutrient absorption is limited. In that study, the glucosinolate content was reported to be higher in the expeller meal, but might not compromise its protein digestibility. In the present study, glucosinolate content in the EX was similar or lower compared to those in the SE CMs; however, protein digestibility of the EX CM was still much lower. This suggests that in our study with barramundi, glucosinolates were not a factor depressing protein digestibility of the CMs.

The current results of digestibility from the two SE CM samples (Newcastle and Numurkah) were consistent with the digestibility results reported for solvent-extracted canola meal fed to Chinook salmon (Hajen *et al.*, 1993), Atlantic salmon (Higgs *et al.*, 1996) rainbow trout (Mwachireya *et al.*, 1999), turbot (Burel *et al.*, 2000c), silver perch (Allan *et al.*, 2000) and red seabream (Glencross *et al.*, 2004a). Compared to results of Burel *et al.* (2000c), the protein digestibility of Australian CM for barramundi (74.5 % to 86.6 %) was lower than that of European solvent-extracted rapeseed meal for trout (89 % to 91%); however, in that study, the CM was dehulled to reduce fibre content of the ingredient. In the present study, the protein digestibility of the SE CM Footscray was lower than that of SE CM Newcastle, which indicated that there was a certain variation in digestibility of the CMs from different growing regions and different plants. These comparisons suggest that the different CMs significantly affect the digestible values determined for each species. In regards to the expeller meal, the protein digestibility determined for barramundi in this study was much lower than that reported for both silver perch (Allan *et al.*, 2000) and red seabream (Glencross *et al.*, 2004a).

While amino acid digestibility generally reflects protein digestibility, in some cases, there were some major differences in amino acid digestibility (Table 2.6). In terms of different types of processing, amino acid ADCs of the EX CM was significantly lower than those of the SE CMs. In case of the EX CM, many amino acid ADCs were below 50 % which were far lower than those of the SE CMs in this study for barramundi and those of different solvent meals for other species (Hilton and Slinger, 1986; Anderson *et al.*, 1992; Allan *et al.*, 2000). Maillard reactions could also occur during the expeller processing resulting in cross-linkages of amino acids, typically with lysine, leading to its limited digestibility value (34.8 % for the EX meal compared to >80.6 % for the SE meals). Newkirk *et al.* (2003) also showed that high temperature decreased digestible amino acids of canola meal in broiler chickens. In our results, several digestibility values of amino acids were calculated exceeding 100 % (Table 2.6). In several previous studies, unusual observations for digestibility parameters were also reported (Allan *et al.*, 2000; Glencross *et al.*, 2004c; Glencross *et al.*, 2012a). These could be explained through errors relating to measurement or interactions among ingredients. Glencross *et al.* (2007) recommended that these values should be reported but values rounded 0 % to 100 % used to formulate diets on digestible nutrient basis.

In general, carnivorous species tend to ineffectively utilise dry matter and energy from plant ingredients (Cho *et al.*, 1982; Sullivan and Reigh, 1995). In the present study, the low DM digestibility was determined for both the EX and SE meals (29.9 % to 40.1 %), and they were much lower than that of European meals (46 % to 71 %) (Burel *et al.*, 2000c) and still less that than of Canadian meals (38 % to 60 %) (Cho and Slinger, 1979; Hajen *et al.*, 1993; Higgs *et al.*, 1996; Mwachireya *et al.*, 1999; Allan *et al.*, 2000). As with to DM digestibility, the energy ADCs of the Australian CMs were also lower for barramundi (32.4 % to 52.5 %) than those of other CMs for other fish species such as Chinook salmon (51 % to 71 %) , Atlantic salmon (62 % to 73 %), turbot (69 % to 81 %), gilthead seabream (79 %) silver perch (58 %), red seabream (62 %) (reviewed of Burel and Kaushik (2008)) and snakehead (57.2 %) (Yu *et al.*, 2013). Low ADC values of dry matter and energy suggests that carbohydrates in canola meals are poorly digestible. This is consistent with a previous report regarding the composition of carbohydrates, which indicated that carbohydrates in CM appear to be predominant by non-starch polysaccharides (NSPs) (Van Barneveld, 1998). A number of studies have reported effects of NSPs or their classes on digestible values and in most cases NSPs have negative effects on DM and energy digestibility of ingredients or diets (Hansen and Storebakken, 2007; Glencross, 2009; Glencross *et al.*, 2012b). The low digestible energy of CMs may limit their inclusion in diets as the critical specification of a diet is to meet the energy requirement for an animal. Further work is suggested to focus on the reduction of fibre and anti-nutritional compounds to maximise digestible nutrients and energy of Australian CMs for barramundi.

In the present digestibility study, the digestibility data of raw materials was determined at one inclusion level (300 g/kg of CM). Formulations of practical diets can include CM at different levels (such as in a growth trial in Chapter 3) and it is suggested that if interactive effects of ingredients within a feed formulation occur, true digestible values of diets can be different when those diets were initially formulated on equivalent nutrients based on digestible basis.

In conclusion, although low protein and amino acid digestibility of the EX CM were observed for barramundi, other SE CMs were fairly well digested, and similar to that seen for lupin meal. The digestibility profiles of nutrients and energy in this study provide useful information for the formulation of nutritionally balanced diets for barramundi.

CHAPTER 3 . EFFECTS OF CANOLA MEAL ON GROWTH PERFORMANCE OF BARRAMUNDI

3.1 Introduction

Canola meal (CM) is considered an important plant protein source for fishmeal (FM) replacement in diets for both terrestrial animals and aquaculture species. Many fish species have been shown to have good growth performance when fed with diets containing CMs. These include rainbow trout (Yurkowski *et al.*, 1978; Hardy and Sullivan, 1983; Leatherland *et al.*, 1987; McCurdy and March, 1992; Gomes *et al.*, 1993), juvenile Chinook salmon (Higgs *et al.*, 1982), gilthead seabream (Kissil *et al.*, 2000), red seabream (Glencross *et al.*, 2004b), channel catfish (Webster *et al.*, 1997; Lim *et al.*, 1998), tilapia (Zhou and Yue, 2010). However, a decrease in growth performance has been reported when fish were fed with high levels of CM in their diets. The reduced growth of fish fed with high dietary CM can be associated with low feed efficiency or reduced palatability, direct or indirect effects of ANFs (Hardy and Sullivan, 1983; Hilton and Slinger, 1986; McCurdy and March, 1992; Burel *et al.*, 2000a; Burel *et al.*, 2000b; Cheng *et al.*, 2010; Zhou and Yue, 2010; Luo *et al.*, 2012).

The limited studies on FM replacement with plant protein sources in barramundi such as soybean meal and lupin meal in suggested that different raw materials can be effectively used with as little as 15 % FM remaining in diets for (Glencross *et al*. 2011). 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.*, 2011c). However, in that study only one CM type at a single inclusion level was evaluated. A study with serial inclusion levels evaluated provides more capacity for extrapolation of effects of using novel ingredients (Glencross *et al.*, 2007).

Solvent and expeller extraction are two different canola oil processing methods which can produce CM types with different quality. Results from Chapter 2 indicated that the chemical composition and digestible values, especially in terms of digestible protein of the two CMs (SE and EX), were significantly different. Hence, these CMs may have different effects on growth performance, feed utilisation efficiency and capacity in fish meal replacement if they are used in diets for barramundi. It is worth to include both CMs in this growth study to evaluate them though growth performance and feed utilisation specifications.

This study included a serial inclusion experiment (100, 200 and 300 g/kg of each of the CMs) to study nutrient utilisation and inclusion level limitations of the two CMs (SE and EX) based on examining growth and feed utilisation parameters such as weight gain, daily growth coefficient, feed intake, feed conversion ratio (FCR), protein and energy retention.

3.2 Materials and methods

3.2.1 Experimental diets

This experiment was conducted to define the threshold inclusion level of CM in diets for barramundi based on the examination of feed intake, growth performance, and feed utilisation efficiency. The experiment included eight diets. Six diets were used to generate a serial inclusion level design (100, 200 and 300 g/kg) of each of SE CM and EX CM. These diets were compared to two reference diets (a fishmeal (FM) based diet and a lupin kernel meal (LM) diet with 300 g/kg of LM). Diets were formulated to iso-digestible protein (420 g/kg) and iso-energetic (14 MJ/kg) specifications and DP:DE ratio (30 g/MJ), based on digestive nutrient basis (Glencross, 2008). Digestibility coefficient values for key ingredient were based on those reported from Chapter 2 (section 2.3) and unpublished data (Glencross). The two CMs selected to use in the growth experiment were SE CM (Numurkah, Vic) and EX CM (Pinjarra, WA). Crystalline amino acids were added to CM diets and LM diet to achieve an equivalent essential amino acid profile.

Diets were formulated by Excel.

The origin of ingredients was described in chapter 2 (Table 2.1). Ingredient preparation and diet manufacture followed the procedure described in Chapter 2 (section 2.2). Chemical composition of ingredients, diet formulations and chemical composition of diets was presented in Table 3.1, Table 3.2 and Table 3.3 respectively.

Table 3.1. Chemical composition of ingredients (values are g/kg DM unless otherwise indicated)

	FM	LM	SE CM	EX CM	Wheat gluten[1]	Pregelled starch	Fish oil
Dry matter	929	906	903	974	900	950	990
Protein	642	408	381	348	848	1	0
Lipid	117	64	56	92	9	1	985
Carbohydrate	4	497	485	490	120	993	0
Ash	237	31	78	70	23	5	5
Gross energy (MJ/kg)	20.4	21.1	20.3	20.6	22.9	17.9	38.4
Essential amino acids							
Lysine	49	15	18	12	15		
Threonine	25	14	18	16	20		
Methionine	17	3	8	7	10		
Isoleucine	28	17	15	14	27		
Leucine	46	29	28	25	49		
Tryptophan	8	3	3	3	2		
Valine	32	17	20	19	30		
Phenylalanine	24	17	17	15	34		
Histidine	15	10	10	10	1		
Arginine	46	25	25	21	27		

[1] Sourced from Manildra, Auburn, NSW, Australia.

Table 3.2. The diet formulations (g/kg)

Ingredient	FM	LM	100SE-CM	200SE-CM	300SE-CM	100EX-CM	200EX-CM	300EX-CM
Fishmeal	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
Dicalcium phosphate	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

Table 3.3. Chemical composition of experimental diets as measured (g/kg DM otherwise as indicated)

Ingredient	FM	LM	100S E-CM	200S E-CM	300S E-CM	100EX -CM	200E X -CM	300EX -CM	Require ment
Drymatter (g/kg)*	966	964	966	963	967	957	960	962	
Protein*	487	506	492	507	522	473	467	460	400-550 [e]
Digestible protein[a]	425	454	431	444	458	402	391	380	
Lipid*	144	150	147	146	142	139	142	143	
Carbohydrates[b]	222	230	218	207	207	249	265	278	
Ash*	147	114	143	140	129	139	126	119	
Gross energy* (MJ/kg DM)	21.1	22.0	21.3	21.3	21.5	20.8	21.3	19.8	
Digestible energy[c] (MJ/kg DM)	14.2	15.7	14.8	15.1	15.5	14.0	14.3	13.2	
DP: DE[(d)] (g/MJ)	29.9	28.9	29.0	29.4	29.6	28.8	27.4	28.7	25-30 [e]
Total tannins	n/a	<0.3	0.6	1.2	1.8	0.4	1.8	1.2	
Phytic acid	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	34.1	27.7	32.3	32.2	32.0	30.6	28.9	27.2	20 [f]
Threonine	18.6	16.9	18.1	18.3	18.5	18.0	17.7	17.4	12 [f]
Methionine	12.5	10.4	11.5	11.2	11.0	11.6	11.2	10.7	7 [f]
Isoleucine	21.4	19.7	20.4	20.5	20.5	20.2	19.7	19.1	11 [f]
Leucine	36.0	33.4	35.3	35.8	36.4	34.3	33.6	32.8	19 [f]
Tryptophan	5.2	4.5	5.0	4.9	4.9	4.9	4.7	4.5	2 [f]
Valine	24.1	21.4	23.2	23.4	23.6	23.2	22.7	22.3	12 [f]
Phenylalanine	19.7	18.9	19.4	19.8	20.1	19.2	19.0	18.8	12 [f]
Histidine	10.3	11.1	10.9	11.6	12.2	11.1	11.5	11.8	8 [f]
Arginine	33.8	35.1	32.2	32.3	32.3	32.7	32.2	31.6	23 [f]

(*) values determined by analysis, other values were based on calculation of values from ingredients through diet formulation.

[a,c] Values were calculated based on the digestible protein and energy data of ingredients in Chapter 2 and unpublished data of Glencross for of other ingredients or assumed digestibility for nonprotein feed ingredients. Protein ADC for wheat gluten and pregelled starch was 100% and energy ADCs for wheat gluten and pregelled starch were 90% and 75% respectively.

[b] Carbohydrates = DM – (protein + lipid + ash).

[d] Digestible protein: digestible energy.

[e] According to the review of (Glencross *et al.*, 2007).

[f] Amino acid requirement was determined based on native of amino acid composition of European seabream (Kaushik, 1998) and required ratio of Lys:CP of rainbow trout (NRC, 1993)

3.2.2 Fish handling and experiment management

The experiment was carried out at the Bribie Island Research Centre, CSIRO in a flow-through seawater array of tanks. Fish for this experiment were obtained from the GAWB (Gladstone Area Water Board) hatchery in Gladstone, QLD and grown up to 51.0 ± 11.6 g (mean ± SD, *n* = 40 from a representative sample of the population) for the experiment. Fish were randomly assigned across 24 cylindrical tanks (600 L), with each dietary treatment having three replicates. Fish density was 15 fish/tank. Culture system was designed with flow-through sea water at a rate of 3 L/min. During the experiment the water temperature was monitored at 29 ± 0.1 ^{o}C (optimal level for feed intake, growth and feed efficiency (Katersky and Carter, 2007)) and oxygen concentration were maintained 4.8 ± 0.21 mg/L (mean ± SD). Photoperiod was held to a constant 12:12 h light-dark cycle.

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. This uneaten feed was dried in oven at 105 °C 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. Fish were weighed at the beginning, after four weeks and at the end of experiment (eight weeks), for each handling the fish were anesthetised to reduce stress using AQUI-S^{TM,} at concentration of 20 ppm.

At the beginning of the experiment, five fish at random and at the end of experiment (eight weeks) three fish from each tank were withdrawn and stored at –20 ^{o}C until used for analysis of body composition.

3.2.3 Chemical analysis

Whole fish (initial and final fish samples) were minced and the initial moisture content of the fish was determined by drying a sub-sample in an oven at 105 ^{o}C for 24 h, while another sub-sample was freeze-dried for chemical composition analysis.

All ingredients, feeds and fish were analysed for dry matter, protein, lipid, ash and gross energy according to the procedure described in Chapter 2 (section 2.2)

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

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

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

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

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

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

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 digestible protein consumed by fish over the experiment.

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

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 digestible energy consumed by fish over the experiment.

3.2.4 Statistical analysis

All data are presented as mean ± SEM. Data were subjected to one-way analysis of variance (ANOVA) using SPSS 11.0 for Windows 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 analysed. A significance level of $P < 0.05$ was used for all comparisons. Linear analysis was performed on feed intake of SE CM against inclusion levels of CM and weight gain against feed intake. A second-degree polynomial regression analysis was performed on feed intake of EX CM against CM levels.

3.3 Results

Details on the growth performance of barramundi fed the different experimental diets are reported in Table 3.3. All dietary treatments containing the CMs and LM were

shown to have growth performance that was 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 EX CM) (Table 3.3). Fish fed the diets containing 200-300 g/kg 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 containing 100 g/kg SE CM (100SE-CM diet), 100-200 g/kg 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 EX CM) diet compared to the FM control diet and other test diets was observed over the eight week culture period.

There was an increased trend in feed intake with increasing inclusion levels of the SE CM (linear regression in Figure 3.1). Feed intake was significantly greater for fish fed the diets containing either 200 to 300 g/kg SE CM and compared to that observed for the FM control diet but similar to the LM diet. Feed intake of the diet containing 100 g/kg 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 123g/kg, feed intake had the maximum value (Figure 3.1). A significant improvement in feed intake was observed by fish fed diets with 100g, 200g compared to the FM control diet (without inclusion of EX CM). When replace more than 200g/kg EX CM, feed intake significantly decreased and was the least among all the treatments. In terms of digestible protein and energy intake, there was still higher in these values of the diets containing 200 to 300 g/kg SE CM and the 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 EX CM was similar to that of the

FM control diet. Digestible protein and energy intake of the 300EX-CM diet was the lowest among diets. There was a strong correlation between feed intake and weight gain (Figure 3.2).

There were no significant differences in protein retention among dietary treatments (31.9 % to 36.3 %). However, energy retention by fish fed the diet with 300 g/kg EX CM (the 300EX-CM diet) (54.2 %) was significantly lower than that of the FM diet (60.5 %). FCR was similar among diets containing 100 to 300 g/kg SE CM, 100 to 200 g/kg EX CM, 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.

The initial weight of fish were statistically different; however, these differences were numerically very small (51.8 - 55.5 g) and therefore unlikely to impact on final results.

Table 3.4. Growth and feed utilisation parameters of fish fed experimental diets (n = 3 tanks/treatment)

	FM	LM	100SE-CM	200SE-CM	300SE-CM	100EX-CM	200EX-CM	300EX-CM	Pooled S.E.M
Initial 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
DGC (%/day)	3.49^{b}	3.85^{d}	3.61^{bc}	3.89^{d}	3.78^{cd}	3.66^{bcd}	3.60^{bc}	2.98^{a}	0.06
FCR	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^{bc}	177.7^{cd}	177.3^{cd}	146.0^{a}	2.60
Digestible protein intake (g/fish)	80.5^{b}	90.8^{cd}	82.9^{b}	94.2^{d}	94.8^{d}	84^{bc}	82.8^{b}	67.2^{a}	1.9
Digestible energy intake (MJ/fish)	2.3^{b}	2.8^{c}	2.5^{b}	2.8^{c}	2.8^{c}	2.5^{b}	2.5^{b}	1.9^{a}	0.1
Protein retention (%)	39.5	39.9	37.3	38.3	38.6	41.6	41.8	37.5	0.53
Energy retention (%)	60.5^{bc}	58.2^{abc}	57.1^{ab}	61.4^{bc}	59.7^{bc}	62.0^{bc}	63.1^{c}	54.2^{a}	0.83
Survival (%)	100.0	100.0	100.0	100.0	100.0	97.8	97.8	97.8	0.37

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

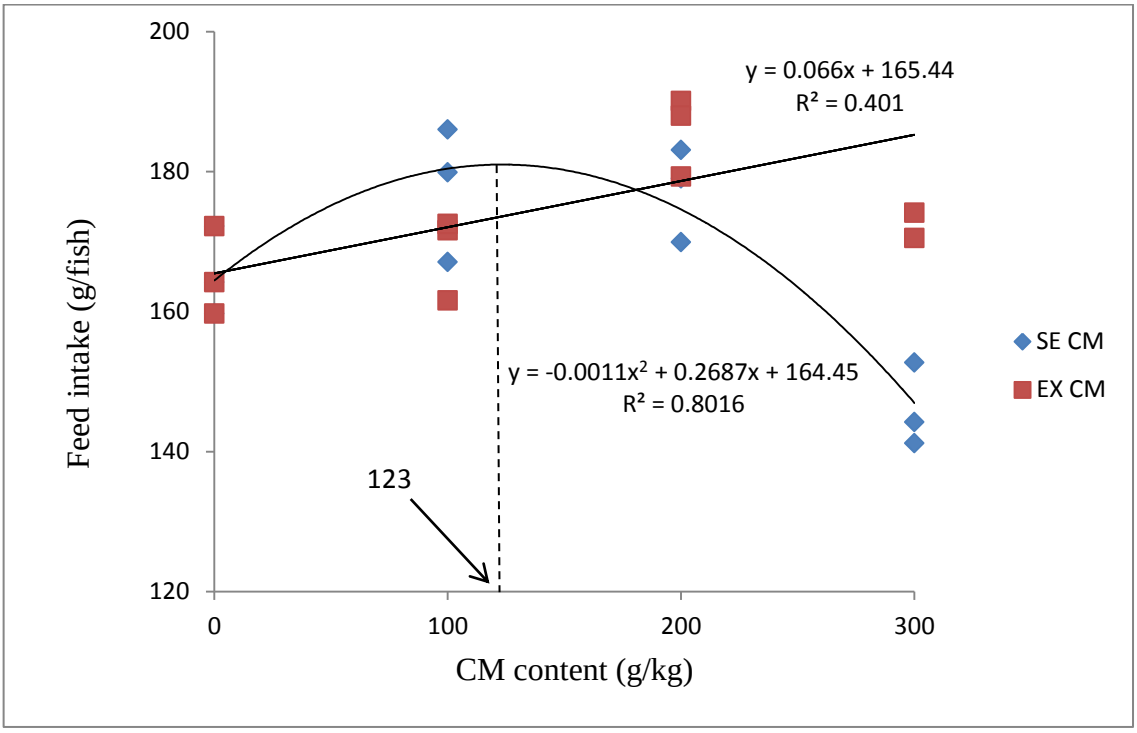

Figure 3.1. Feed intake (g/fish) of barramundi fed with varying SE-CM (solvent extracted canola meal), EX-CM (expeller extracted canola meal)

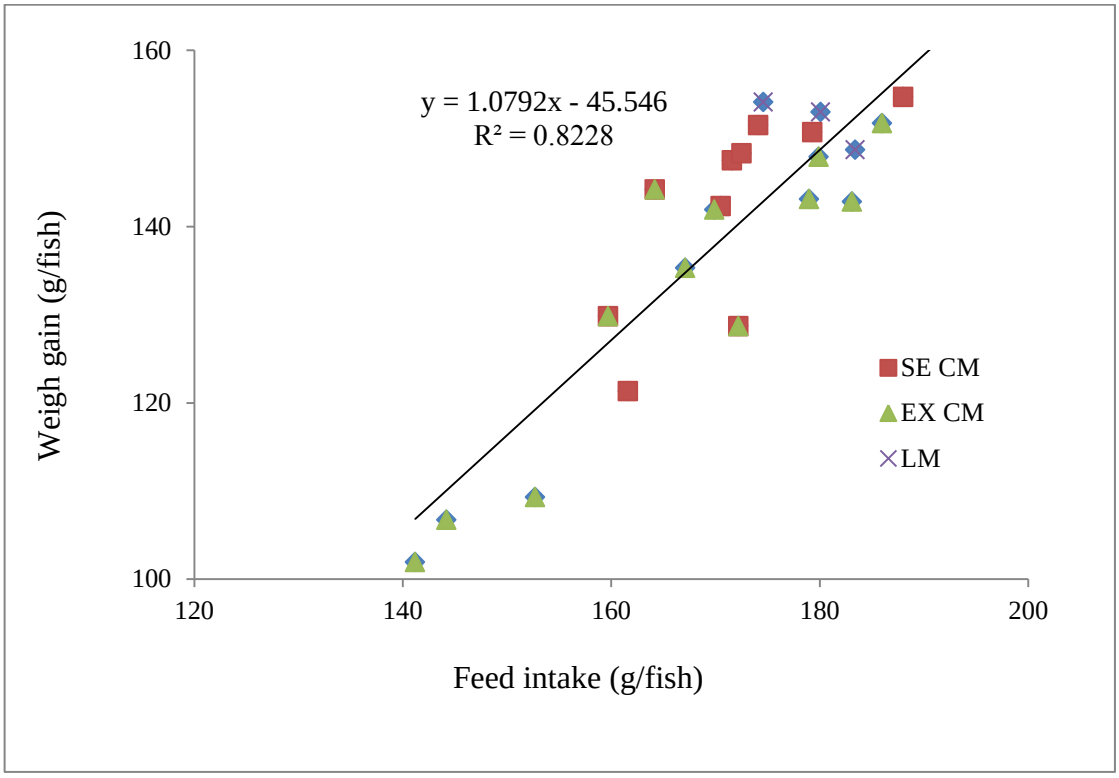

Figure 3.2. Regression of feed intake and weight gain of barramundi. Common regression equation is: y = 1.0792*x* – 45.546, R^2 = 0.8228

3.4 Discussion

In our study, barramundi fed the diets with a serial inclusion level of 100 to 300 g/kg 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. It indicated that the SE CM could be used at 300 g/kg in diet without having any negative effect on the growth performance and feed utilisation of barramundi over and 8 week period. The results of the present study 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 %) (Shafaeipour *et al.*, 2008), channel catfish (31 % of inclusion level) (Lim *et al.*, 1998), red seabream (60 %) (Glencross *et al.*, 2004b). Our observations are supported by study of Glencross *et al.* (2011c) 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. Nevertheless, for the EX CM, while the inclusion of 100 to 200 g/kg in the diet was acceptable, a higher level (300 g/kg) of this EX CM led to a decrease in growth performance. A similar depression in growth has been reported when 30 % or even less CM was used in diets for rainbow trout (Hilton and Slinger, 1986; McCurdy and March, 1992; Burel *et al.*, 2000a), turbot (McCurdy and March, 1992), Chinook salmon (McCurdy and March, 1992; Hajen *et al.*, 1993; Satoh, 1998), Japanese seabass (Cheng *et al.*, 2010) and cobia (Luo *et al.*, 2012).

There was a significantly greater feed intake and digestible protein intake by fish fed with the diets containing 200 to 300 g/kg SE CM, 100 to 200 g/kg EX CM and 300 g/kg 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.* (2011c), 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 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 (Figure 3.2). Indeed, growth performance of barramundi substantially increased with improvement of feed intake in some diets (the 200SE-CM, 300SE-CM and LM diets) relative to the FM control diet. However it is worth to mention that although digestible protein and energy intake of several diets (100EX-CM, 200EX-CM,

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-addictive 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.* (2011b) 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.

However 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 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 DM) than that in the SE CM (24 g/kg 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, and the concentration of phytic acid (13.2 g/kg) at the highest inclusion level (300 g/kg EX CM) probably exceeds the tolerance of barramundi with this compound. However, it is unclear whether higher phytic acid content in the 300EX-CM diet caused the decrease in appetite or changes in the physiological properties of fish.

In this study the protein and energy varied between some of the diets which were probably due to a mismatch between chemical composition of ingredients in analysis and in formulation. However, the protein specification in diets for barramundi (size > 50g) at 30 ^{o}C varying from 400 – 500 g/kg with constant DP: DP (30 g/MJ) ratio can produce similar growth (Glencross, 2008) although better FCR can be observed in the high protein diet. This difference may be due to feed intake as fish tend to eat more in lower protein diet. In the present study, there was a variation in protein content between some of diets (higher digestible protein of the LM, 200SE-CM, 300SE-

CM diets than the FM diet; and lower digestible protein of the 100EX-CM, 200EX-CM, 300EX-CM diets than the FM diet) but not much variation was seen in DP:DE ratio and therefore unlikely to cause differences in growth.

There were no significant differences in protein retention by fish among the different treatments. This implies that the biological protein values of the alternative ingredients (CMs) were similar to the 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. However, energy retention decreased in the diet containing 300 g/kg EX CM. Lower feed intake could be a reason for declining energy retention because of higher proportion of energy intake used for maintenance.

The growth performance of fish can be depressed by plant protein containing diets due to ANFs presenting in plant ingredients (Francis *et al.*, 2001). Previous studies indicated that the presence of phytic acid in diets has negative effects on the digestibility of protein, bioavailability of trace and consequently decreases growth performance of fish (Spinelli *et al.*, 1983; Satoh *et al.*, 1989; Forster *et al.*, 1999). In our study, the diet with 300 g/kg EX-CM contained a higher phytic acid level (13.2 g/kg DM) than that of the 300SE-CM diet (7.2 g/kg DM), which might be another reason for the reduced growth performance in fish fed with the 300EX-CM diet. In addition, one of the considerations regarding the use of CM in aquaculture feeds is the detrimental effects of glucosinolates and their derivatives, leading to a decrease in feed intake and the thyroid hormone T3 and T4, or causing abnormal thyroid follicle development and subsequently decreasing feed efficiency and growth performance of fish (Yurkowski *et al.*, 1978; Higgs *et al.*, 1982; Hilton and Slinger, 1986; Leatherland *et al.*, 1987; Burel *et al.*, 2000a; Burel *et al.*, 2000b; Burel *et al.*, 2001). In the present study, although higher glucosinolate content was observed in the SE CM than in the EX CM, the SE CM did not have any adverse effect on feed intake, growth performance and FCR when fed to barramundi at an inclusion level up to 300 g/kg. Moreover, levels of plasma thyroid hormone T3 and T4 (presented in Chapter 4) were unchanged among fish fed the CM diets compared to the LM and the FM diets. It could be concluded that glucosinolates in the present study were not a limited factor for using CMs in diets of barramundi. This observation is supported by a previous research on red seabream (Glencross *et al.*, 2004b). Both these two studies reported that there were lower glucosinolate content in

Australian CMs (3.3 to 6.6 µmol/g) in the present study (Chapter 2) and in the previous study (1.1 to 3.6 µmol/g) (Glencross *et al.*, 2004a) compared with Canadian meal (8.8 to 19.7 µmol/g) and European meal (5.2 to 40.7 µmol/g) (reviewed by (Burel and Kaushik, 2008)). The results of our study also agree with the report of Spragg and Mailer (2007), which showed that there have been marginally improvement in varieties of canola in terms of decrease in glucosinolate content.

In our study, the EX CM had low lysine content and low digestibility of some amino acids (lysine, histidine and cysteine) (Chapter 2). Crystalline amino acids were supplemented to meet the essential amino acid requirement of barramundi, but it is possible that these supplemented crystalline amino acids could not be efficiently utilised as absorption of crystalline amino acids and protein amino acids was different resulting in essential amino acid imbalance. The imbalance in dietary essential amino acids can cause greater oxidation of amino acids and decrease growth efficiency in fish (Williams *et al.*, 2001; Conceição *et al.*, 2003).

In general, the SE CM can be utilised at a 300 g/kg inclusion level in the diet for barramundi without any deleterious effects on the growth performance and other growth parameters. The inclusion level of 200 g/kg is acceptable for the EX CM but higher levels of EX CM (300 g/kg) resulted in significant impairment in performance. The study suggests that though CMs appear to be potential ingredient in diets for barramundi, considerations on reduction of ANFs such as phytic acid or fibre and balancing indispensable amino acids when formulating diets will improve nutritive value of CMs for using in fish diet.

CHAPTER 4 . BIOCHEMICAL, HISTOLOGICAL AND MOLECULAR EFFECTS OF CANOLA MEAL ON BARRAMUNDI

4.1. Introduction

Plant protein sources have been widely utilised as a potential ingredient for fishmeal replacement (Gatlin *et al.*, 2007; Hardy, 2010). However using plant ingredients has raised considerations of the effects of anti-nutritional factors (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 and their derivatives (Bell, 1993; Higgs *et al.*, 1995). In some cases, phytic acid is associated with some incidences of cataracts, vacuolization and hypertrophy of cytoplasm of epithelium in the pyloric caeca and intestine (Richardson *et al.*, 1985; Hossain and Jauncey, 1993). Although the glucosinolate content in most of commercial CMs is considerably reduced compared to earlier varieties of rapeseed, there are still concerns about the disturbance of these compounds on thyroid function, such as thyroid hypertrophy or decrease in the plasma thyroid hormone levels tri-iodothyronine (T3) and thyroxine (T4) (Yurkowski *et al.*, 1978; Hilton and Slinger, 1986; Burel *et al.*, 2000a; Burel *et al.*, 2001). In addition, the activities of some enzymes in liver (e.g. asparate aminotransferase (AST), alanine aminotransferase (ALT)) have been affected by fish fed CM containing diets (Cheng *et al.*, 2010; Luo *et al.*, 2012).

Understanding the molecular pathways that regulate the utilisation of dietary nutrients and energy are critical elements to understanding the feeding and growth response in fish when fed with a particular diet. It is generally assumed that the replacement of fishmeal by plant materials is likely to change the biological values of diets, so it is hypothesised that molecular metabolism is also likely to be affected in certain pathways. Some earlier studies have shown that replacement of fish meal by plant proteins leads to decreased growth of rainbow trout (*Onchorynchus mykiss*) possibly linked to a modification of a number of hepatic metabolic pathways (Vilhelmsson *et al.*, 2004; Panserat *et al.*, 2009). In addition, results from Chapter 3 demonstrated that there was a decrease in growth performance and energy retention of the fish fed the diet with 300 g/kg inclusion of expeller extracted canola meal (EX CM). This observation raises a question as to which molecular mechanism or metabolic

pathways, such as those involved in energy production or fatty acid metabolism, are affected and how these effects are mediated.

Detoxification plays an important role in the protection of the body against the damage of toxic compounds from endo- and exogenous sources. The detoxification mechanism that underlies the activities of biotransformation enzymes includes: Phase 1 (mono-oxidisation), phase 2 (conjugation) and phase 3 enzymes (defluxion). In mammals, cytochrome P450 genes are dominant in activities of phase 1 while the activation of phase 2 is primarily activated by transferase enzymes such as glutathione S transfease (GST). Bioactive compounds in plants such as glucosinolates and phenolic compounds, have potent antioxidant activities associated with beneficial health effects, which induce hepatic detoxification enzymes activities in mammals (Rabot *et al.*, 1993; Wang *et al.*, 1997; Adom and Liu, 2002; Bub *et al.*, 2003). In fish, information regarding the relationship between fish nutrition and antioxidant status is limited. A previous study on gilthead seabream has shown that levels of antioxidant enzymes glutathionie (GSH), glutathionine reducase (GR) and ɤ-Glutamyl transferase (cGT) increased with the increased inclusion levels of plant protein for fish meal replacement in diet (Sitjà-Bobadilla *et al.*, 2005).

The present study was undertaken to examine the influences of diets containing different CMs on the alternation of plasma biochemistry and histology of the gastrointestinal tract, kidney and liver. Moreover, changes in the function of cells in detoxification were investigated by the determination of expression levels of genes involved in detoxification including cytochrome P450 (*CYP1A1* and *CYP3A*) and glutathionine-S-transferase (*GST*) and also some other targets which are generally less considered including cytochrome P450 family 2 subfamily N (*CYP2N*), glutathionine peroxidase (*GPx*), phospholipid hydroperoxidase (*GHGPx*) and glutathionine reductase (*GR*) were also sighted. In addition, expression levels of key genes involved in fatty acid metabolism (fatty acid synthesis gene (*FAS)*, stearoyl CoA desaturase (*SCD)*, Farnesoid X receptor (*FXR*), in TCA cycle (pyruvate dehydrogenase kinase (*PDK*) and citrate synthase (*CS*)) were also determined to understand metabolic response to CM containing diets in relation to the decrease in energy retention and growth performance in the diet containing 300g/kg EX CM in Chapter 3. The results of this study provide valuable information as implications in terms of health effects of using CMs for barramundi.

4.2. Materials and methods

At the end of the growth experiment (after week 8) (in Chapter 3), 24 hours after the last meal, three fish from each tank were sampled for analysis in this study.

4.2.1. 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). Instaneously, blood samples were collected from three fish from each tank using a 1 mL syringe and 18G needle via caudal tail vein puncture. Blood from fish within the same tank were pooled in an Eppendorf^{TM} tube. 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 ^{TM} tube, before it was frozen at –80 ^{o}C and sent to Western Australian Animal Health Laboratories (Western Australia) for plasma clinical panel analysis. Samples were always kept in dry-ice box when carried to the laboratory.

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. The plasma samples were analysed by automatic chemistry analyser (Olympus A400). A standard kit was used for each assay. Trace elements were determined by inductively coupled plasma atomic emission spectroscopy after samples were prepared using a mixed acid digestion. The thyroid hormones T3 and T4 were determined by a competitive immunoassay method using chemiluminescence detection as described by (Fisher, 1996).

4.2.2. 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. Then these tissue samples were 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 evaluation of vacuolization degree and steatosis status in liver sections was made using a semi quantitative histological assessment (grade 1-none, grade 2-mild, grade 3-moderate and grade 4-severe). 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 and pigment deposits in kidney was determined on three fields to be representative of the whole section (3 fish × 3 replicates, n = 9). The area of melano-macrophage centres (MMC) in each of these fields was measured and then an average area of MMC was calculated as percentage of total kidney area.

4.2.3. Gene expression analysis

Expression of selected genes was determined by quantitative reverse transcription polymerase chain-reaction (RT-qPCR)

Liver samples dissected from the seven fish in each treatment were examined from four dietary treatments 300SE-CM, 300EX-CM, LM and FM from the study in Chapter 3. Samples were stored at – 80 ^{o}C until analysis. The total RNA was isolated from the liver tissues of seven individuals per experimental treatment. The RNA was extracted from each sample through several steps.

4.2.3.1 RNA extraction and normalization

First, Trizol reagent (Invitrogen) was used to dissolve components in the tissue, and RNA was separated in the chloroform layer. Following this the 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. Then, the pellet containing the RNA was washed in 950 μL 85 % ethanol, and air-dried before being resuspended in RNase-free water. DNA contamination was minimized by treatment with TURBO DNA^{TM} –free kit (Applied Biosystems) to produce purified RNA. The concentration of the RNA was tested 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 by using Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). The RNA was stored at -80 °C until required for cDNA synthesis.

4.2.3.2 RT-qPCR

Expression of selected genes was determined by quantitative reverse transcription polymerase chain-reaction (RT-qPCR). 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, and designed by PerlPrimer V1.1.17. Details on the target qPCR primer sequences are given in Table 4.1

In RT-qPCR, a negative control containing an equivalent amount of a pool of all RNA samples was performed to check DNA contamination, and a positive control containing an equivalent amount of cDNA pooled from all samples was also used to normalise across plates and treatments. RT-qPCR analysis used relative qualification with amplification efficiency of primer pairs being optimised and assessed by serial dilutions of the pooled cDNA. 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 the equivalent of 7.5 pg of cDNA, 1X SYBR Green PCR Master Mix (Applied Biosystems) 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. Normalisation was performed using the ΔCq method (where Cq is qualification cycle). 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) and the internal Luciferase (Luc) control. 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 (http://hdl.handle.net/ 102.100.100/13190?index=1).

Table 4.1. Target genes and primer sequences

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

4.2.4. Statistical analysis

All figures are mean ± SEM. Data were analysed for homogeneity of variation by Levene's test before being analysed with a one-way analysis of variance (ANOVA) using SPSS 11.0 for Windows. Differences among the means were tested by Duncan's multiple range tests with the level of significance $P < 0.05$. Once equal variances were not assumed, Game-Howell's post-hoc test was used (ALAT, GDH, ure, Mg, heam).

4.3. Results

4.3.1 Plasma chemistry

There were no significant differences in any of the plasma chemistry parameters among the different diets except for plasma Fe level and thyroid hormone T3. The concentration of Fe was lower in fish fed the 200EX-CM and 300EX-CM diets compared to the fishmeal 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. The concentration of the hormone T3 in fish fed the 300EX-CM diet substantially decreased relative to fish fed the 200SE-CM diet but not significantly different to the FM control diet and other test diets. Other parameter were not significant different among different dietary treatments. The details of plasma metabolic enzymes and metabolites are presented in Table 4.2.

Table 4.2. Plasma chemistry (n = 3) of fish in each of the experimental diets

	FM	LM	100SE-CM	200SE-CM	300SE-CM	100EX-CM	200EX-CM	300EX-CM	Pooled SEM
ALAT (U L^{-1})*	15.3	17.3	4.7	23.8	24.0	13.0	11.0	14.2	
	±2.40	±8.35	±4.67	±9.23	±14.19	±4.36	±4.02	±2.14	
CK (U L^{-1})	2821.0	3368.0	2357.5	2581.2	2282.0	2286.3	2677.5	2392.8	269.77
GDH (U L^{-1})*	7.7	6.7	5.2	8.5	9.0	5.7	5.8	3.8	
	±0.88	±0.60	±0.60	±1.89	±3.51	±1.67	±1.18	±0.60	
Total protein (g L^{-1})	45.4	46.7	42.5	49.3	46.9	42.3	40.3	43.8	1.23
Glucose (mmol L^{-1})	6.7	5.2	9.7	5.0	6.1	5.1	4.2	3.9	0.53
Heam (mg/dL^{-1})*	20.0	10.7	17.6	18.5	15.7	34.3	11.5	16.2	
	±2.00	±4.06	±5.00	±9.09	±6.23	±16.97	±4.07	±10.21	
Mg (mmol L^{-1})*	1.4	1.1	1.1	1.1	1.3	1.0	1.0	1.1	
	±0.39	±0.15	±0.15	±0.10	±0.20	±0.09	±0.06	±0.15	
Ca (mmol L^{-1})	3.0	2.9	2.8	3.2	3.1	2.6	2.7	2.8	0.06
Phosphate (mmol L^{-1})	3.0	2.9	2.8	3.1	3.0	2.5	2.8	3.0	0.06
Fe (mmol L^{-1})	17.0^{b}	10.3^{ab}	11.2^{ab}	8.6^{ab}	11.0^{ab}	12.2^{ab}	5.2^{a}	7.4^{a}	0.88
Urea (mmol L^{-1})*	1.6	2.4	2.2	1.9	1.7	1.8	2.0	2.1	
	±0.03	±0.20	±0.28	±0.47	±0.07	±0.12	±0.22	±0.17	
Creatinine (µmol L^{-1})	66.3	39.3	91.5	81.6	56.7	51.0	54.4	45.4	7.15
T3 (pmol L^{-1})	51.1^{ab}	54.9^{ab}	63.7^{ab}	87.2^{b}	66.7^{ab}	56.3^{ab}	39.4^{ab}	32.7^{a}	4.59
T4 (pmol L^{-1})	11.3	12.0	16.9	18.7	15.2	12.3	10.1	8.0	1.06

Different superscripts within rows indicate significant differences between means among dietary treatments but not between parameters ($P < 0.05$).

* Non-homogenous variances amongst treatments

4.3.2 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 1/2) but only few liver samples showed moderate steatosis (grade 3) 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 were no significant differences in hepatocyte area in fish fed different levels of CMs compared to the LM and FM control diet (Table 4.3).

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.

The structure of pyloric caeca, distal intestine, stomach, liver and kidney were described in Figure 4.1 to 4.5.

Table 4.3. Histological parameters in caeca, distal intestine, liver and kidney of barramundi fed the experimental diets

	FM	LM	100SE-CM	200SE-CM	300SE-CM	100EX-CM	200EX-CM	300EX-CM	Pooled SEM
Number of goblet cell/100 µm villus in pyloric caeca	11.0	10.1	10.4	12.1	12.0	12.9	9.3	12.8	0.33
Number of goblet cell/100 µm villus in distal intestine	2.4	2.2	1.6	1.6	2.1	1.2	1.2	1.8	0.13
Length of villus in caeca (µm)	1247	1130	1087	1263	1100	1177	1152	1083	20.6
Length of villus in distal intestine (µm)	912	955	957	907	881	941	937	941	11.0
Hepatocyte area (μm^2)	212	247	200	228	211	218	250	254	5.0
MMC area (% kidney area)	3.40	3.63	3.61	3.57	3.63	3.61	3.58	3.59	0.03

Lack of superscripts within a row indicates that there were no significant differences among treatments (P>0.05)

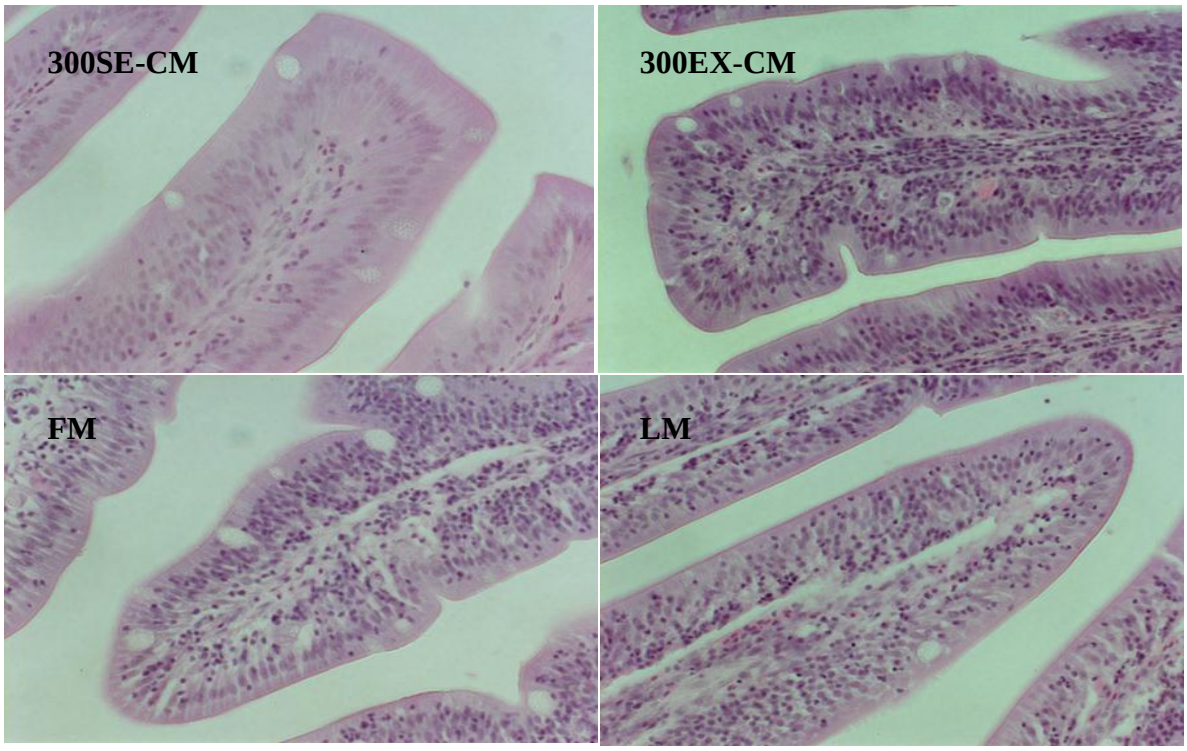

Figure 4.1. Pyloric caeca at 400X

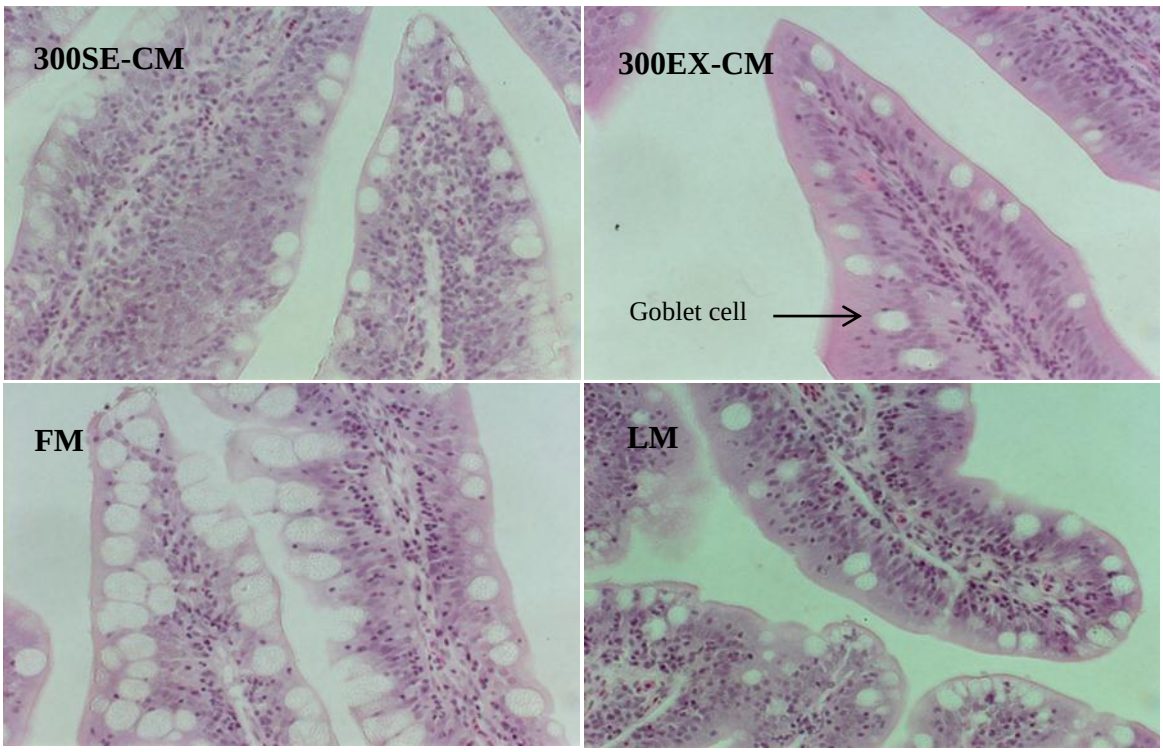

Figure 4.2. Distal intestine at 400X

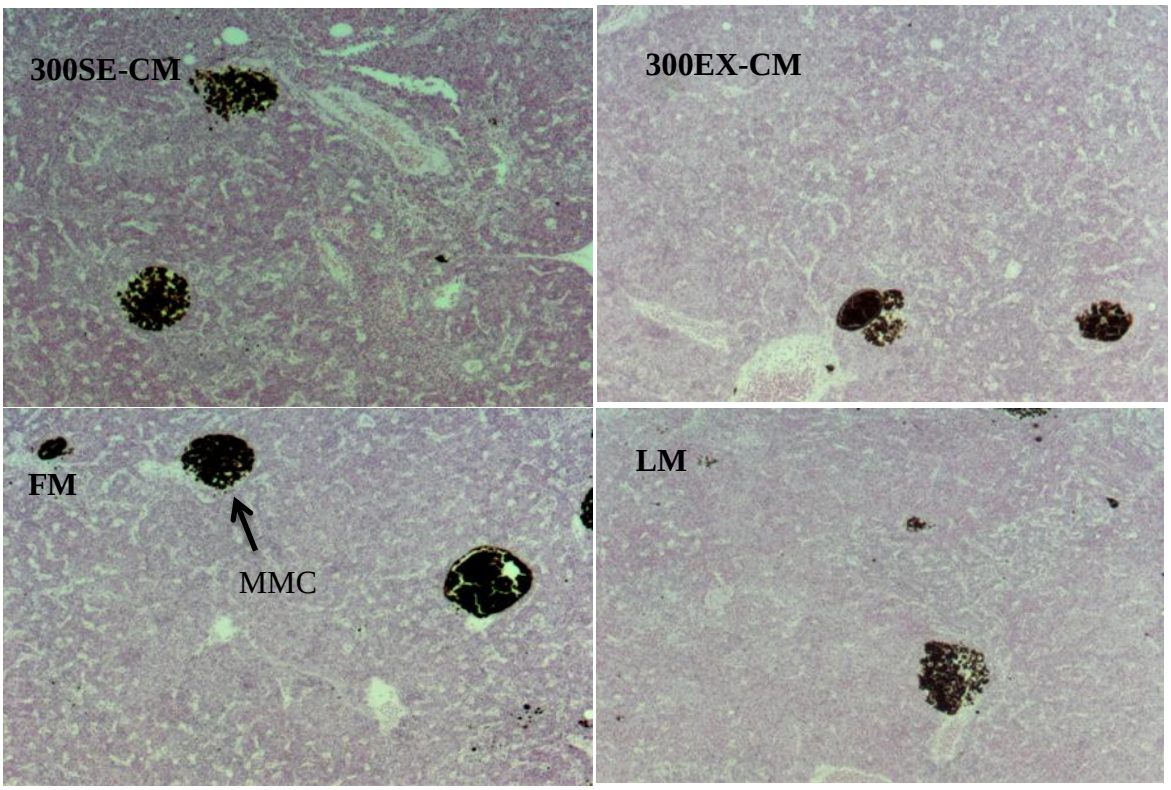

Figure 4.3. Kidney at 200X

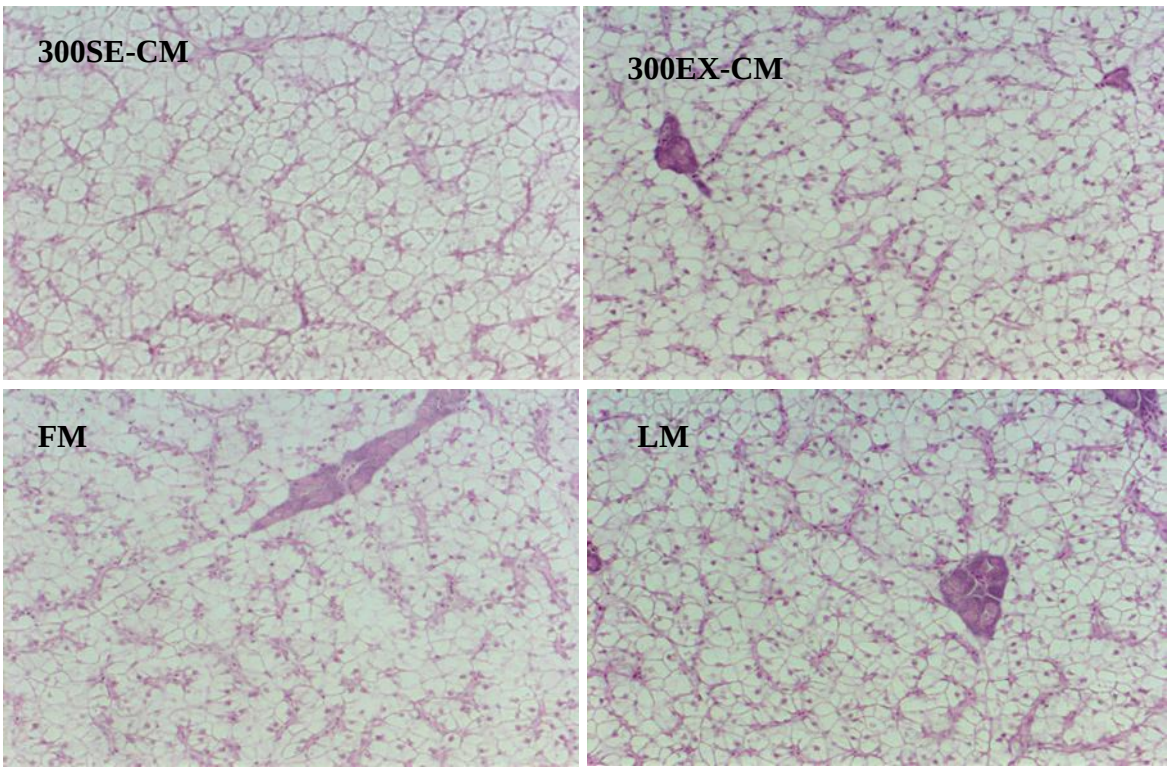

Figure 4.4. Liver at 200X

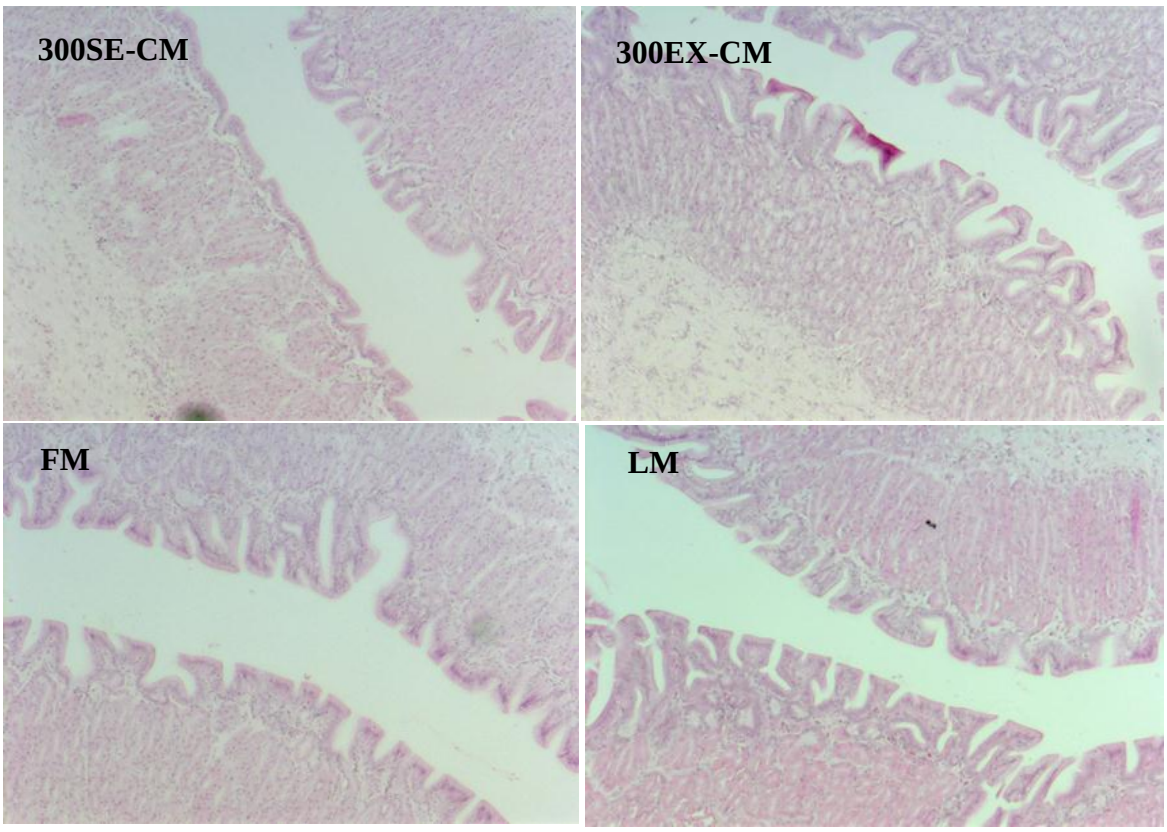

Figure 4.5. Stomach at 100X

4.3.3 Gene expression

Details of the relative qualification of genes of fish fed different diets are presented in Figure 4.6. Among the 12 genes analysed in this study, the expression of some genes involved in xenobiotic metabolism (*Lc CYP1A1*, *Lc CYP3A*, *Lc CYP2N* and *Lc GST*) was down-regulated in fish fed the 300EX-CM compared to that of fish fed the FM reference diet. Particularly in the expression of *Lc CYP3A*, a lower expression level was observed in fish fed all the diets containing plant ingredients (CMs and LM) than that observed in fish fed the fishmeal diet. However, for other genes involved in xenobiotic metabolism (*Lc GR*, *Lc GPx* and *Lc GHGPx*) had the similar expression among the different dietary treatments. 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, it is worth to mention that 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.

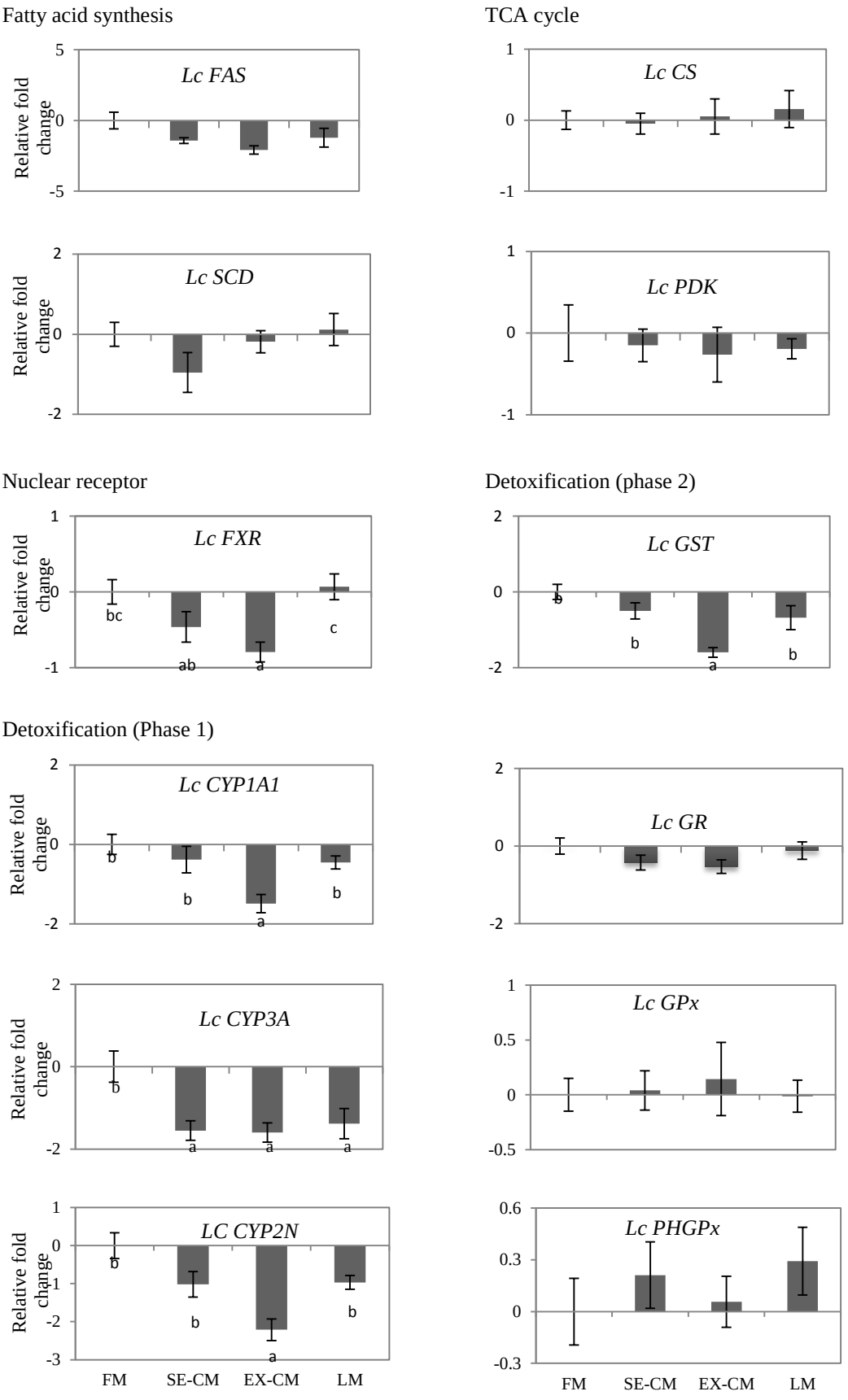

Figure 4.6. Liver gene expression of barramundi from each of the different treatments. Values shown are fold change relative to the FM control diet. Values are mean ±SEM (n = 7); values bearing different superscripts differ significantly ($P < 0.05$).

4.4. Discussion

This study assessed the effects of diets containing CMs (100 - 300 g/kg SE CM or EX CM) on the biochemistry and tissue histology of barramundi, when compared to that of barramundi fed a FM based diet and a LM diet (300 g/kg). Molecular effects of the diets at high inclusion levels (300 g/kg SE CM and 300 g/kg EX CM) in comparison with the FM control diet and the LM diet were assessed through the examination of the expression levels of some genes involved in particular metabolic pathways and detoxification.

4.4.1. Biochemistry effects

There are few published studies on blood chemistry/enzymology in barramundi by which to compare the present work. One of the few other examinations of a suite of blood plasma parameters was reported by (Glencross *et al.*, 2011c). In the present work, the range of biochemical markers were assessed for fishmeal, lupin and CM was similar to that of the previous study. For present observations, almost all the plasma chemistry parameters did not show any differences among the dietary treatments. An exception to this was for iron content. The plasma iron concentration declined in fish fed the diets containing 200 - 300 g/kg EX CM compared to that of the fish fed the control diet (5.2 to 7.4 vs. 17.0 mmol L^{-1}), but was not different among the other diets. It is not clear why the iron concentration was depressed in this study. With regard to the plasma enzymes, plasma CK activities are used as a biochemical marker of both smooth and striated muscle damage (Chen *et al.*, 2003). In the present study, the concentration of CK was not significantly different among the treatments. This suggests that the inclusion of either SE CM or EX CM did not cause any muscle-related dysfunction in this study. The high level of ALT and GDH enzymes are associated with liver damage (O'Brien *et al.*, 2002; Chen *et al.*, 2003). Again the results of the present study supported that 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.* (2011c) which denoted that the inclusion of 300 g/kg 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 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 (Higgs *et al.*, 1982; Burel *et al.*, 2000a; Burel *et al.*, 2001). 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. In this study, it is likely that the glucosinolate content (0.6 - 1.8 μmol/g) present in the diets in the present study was not sufficient to cause a reduction in plasma thyroid hormone. These results were similar to reports of Glencross *et al.* (2004a) on red seabream and Shafaeipour *et al.* (2008) on rainbow trout that plasma T3 and T4 level in fish were not influenced by dietary CM. These observations contrast the findings of the previous studies (Burel *et al.*, 2000a; 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 glucosinolate content (1.4 μmol/g). In the present case, the observations could be explained due to lack of breakdown of glucosinolates 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 in growing condition (weather, soil) and cultivars might affect glucosinolate content and their breakdown products in CMs. The measurement of the breakdown products of glucosinolates could provide a more comprehensive understanding of the effects of CM rather than the intact glucosinolates. The results of the thyroid hormones were in accordance with the growth performance in chapter 3, demonstrating that the glucosinolates 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 EX CM diet. 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 glucosinolates (Yurkowski *et al.*, 1978; Hardy and Sullivan, 1983).

4.4.2. 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). Soybean meal (SBM) has been suggested as one of the best alternatives to replace fishmeal for salmonid diets. However, the inclusion of this ingredient induces enteritis in Atlantic salmon (*Salmo salar* L.). The pathological changes seem to be particularly present on the distal intestinal segment rather than on the proximal as reported in several studies on salmonids (Van den Ingh *et al.*, 1991; Burrells *et al.*, 1999; Nordrum *et al.*, 2000; Buttle *et al.*, 2001). The symptoms that define the condition

are: a shortening of the mucosal folds; a loss of the normal supranuclear vacuolization of the absorptive cells in the intestinal epithelium; a widening of the central stroma within the mucosal folding, with increased amounts of connective tissue; a pro-found infiltration of inflammatory cells in the lamina propria (Van den Ingh *et al.*, 1991; Krogdahl *et al.*, 2000; Buttle *et al.*, 2001); an increased amount of goblet cells in the epithelium, as well as a decreased height of the microvilli together with increased microvillar vesicle formation (Van den Ingh *et al.*, 1991).

In the present study there were no changes in histology or histological parameters of kidney, liver, pyloric caeca, distal intestine and stomach of Barramundi fed the CM diets relative to the FM based diet and LM diet. This suggests that diets with CM inclusion levels in this study did not cause any adverse effects on cell structure of the organs of the Barramundi over 8 weeks of the study. However, it may be possible that longer effects of CM use may have effects, and this could be considered.

4.4.3. Gene expression effects

Research on animal nutrition has demonstrated that gene expression can be altered by dietary components (Zduńczyk and Pareek, 2009). From the point of view of nutrigenomics, nutrients are dietary signals, detected by the cellular systems that have influences on gene and protein expression and subsequently on metabolite production.

In the present study, the results of hepatic gene expression revealed that genes involved in fatty acid synthesis (*Lc FAS*, *Lc SCD*) and energy derived from carbohydrates and amino acid metabolism into TCA cycle (*Lc PDK* and *Lc CS*) were unchanged among the dietary treatments. In terms of FXR, this is a nuclear receptor which has a crucial role in regulation of bile acid homeostasis. This nuclear receptor also modulates lipogenic pathway by reducing lipogenesis via inhibition of sterol-regulatory element-binding protein 1C (SREP1C) and fatty acid synthase, and indirectly reduce glycogenesis as well (Kalaany and Mangelsdorf, 2006; Calkin and Tontonoz, 2012). Similar to *Lc FAS* and *Lc SCD* expression, transcription levels of *Lc FXR* gene in the current study were unchanged among the experimental diets. It is worth mentioning in the present study that liver samples were taken at 24h after feeding while it has been demonstrated that there was a substantial variation in the expression level of most hepatic metabolism genes in barramundi over a 24 h period after feeding (Wade *et al.*, 2014). In that study, the author also reported that gene expression level of *FAS* and *SCD* were most variable during 2 to 12 h after feeding and *FXR* decreased after 2 to 8h but at

24 h after feeding the expression of these genes were similar to prior feeding. This suggests that understanding molecular response to intake nutrients of a diet over serial time events is primarily critical then comparison of effects of different diets on gene expression should be carried out at a particular time event when influence of diets on gene expression can be clearly seen. However, in the present study, a variation of gene expression over a time course after feeding was not investigated. Therefore, further study is required to investigate the variation of postprandial gene expression of barramundi when fed diets such as that used in the present study. Moreover, in the present study, only a few genes were investigated and thus make the assumption that those genes chosen in the present study were sensitive indicators.

The detoxifying mechanism in the liver relies on the involvement of phase 1 and 2 biotransformation enzymes. Phase 1 involves in oxidation, reduction and hydrolysis reactions and results in making the xenobiotics are more hydrophilic which can result in inhibition of activity and increase in solubility of the compounds and subsequently increased excretion rates but can also produce xenobiotics which are more toxic if other phases of detoxification are obstructed. The most important enzymes in phase 1 are cytochrome P450 enzymes. Phase 2 comprises conjugation reactions with phase 1 metabolites to produce metabolites more polar and readily excreted. Dominant enzymes in phase 2 include glutathione S-transferases (GST). The ingestion of glucosinolates has been 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, glucosinolates and their derivatives are known as inducers of up-regulation of phase II enzymes including GST and GPx (Nho and Jeffery, 2001). However, it is mentioned that the effects of glucosinolate breakdown products depend on the dose, time of treatment, type of glucosinolate breakdown products and the tested tissue.

In the present study, the was a decrease in hepatic gene expression of all the CYP genes (*Lc CYP1A1*, *Lc CYP3A*, *Lc CYP2N*) was observed in fish fed the 300EX-CM diet but not in fish fed the 300SE-CM, despite higher glucosinolate content in the latter. There was no increase in the expression level of phase 2 enzyme in diets containing plant ingredient compared to the FM control diet, even *Lc GST* was down regulated in the 300EX-CM. This suggests that bioactive compounds such as glucosinolates or phenolic compounds in this study were not factors affecting the

expression of the detoxification enzymes in the present study. This contrasts the report of Sitjà-Bobadilla *et al.* (2005) that plant ingredients in diets for gilthead seabream enhance activities of antioxidant enzymes. In the present work, it is difficult to explain the down regulation of above genes in the 300EX-CM but it is known that oxidative activities of mitochondria are a primary endogenous source of the reactive oxygen species (ROS). So, it is predicted that the ROS generation in fish fed the diet with 300 g/kg EX CM decreased as fish ate less resulting in the decrease in the rate of metabolism. In fish, the information regarding the relationship between fish nutrition and the antioxidant status is limited. In a previous study in rainbow trout, total hepatic glutathione was higher in fish fed a high protein diet comparison to those fed a low protein diet (Yokoyama and Jun-Ichi, 1991). In terms of *Lc CYP3A*, the expression of this gene was down regulated in all the plant protein containing diets. It is not clear what the key factor in those products was that influenced the expression of this gene, but it is suggested that one or more of other ANFs in these plant ingredients might be affecting the expression of *Lc CYP3A.* Although there were not much understanding mechanism of down regulation of *CYP* genes and *Lc GST*, the lack of *CYP* expression may underlie the poor growth performance observed in EX CM fed fish.

As far as we know, there is little understanding of function of *CYP* enzymes in fish (Uno *et al.*, 2012). Therefore, whether function of *CYP* genes is conserved between fish such as barramundi and mammals requires clarification. Moreover, there was a significant variation in transcriptional levels of many metabolic genes in barramundi after feeding (Wade *et al.*, 2014) but there is no information on the variation of detoxification genes over postprandial feeding time events. Hence, it is important that this is identified in further research. In the current circumstance, there is limited published molecular data on many of the nutrigenomic responses in barramundi. Clearly, further studies are required to discover new potential target genes for understanding the molecular response of barramundi to variations in their diet and what the implications of these changes are. These may include broader approaches to identify novel genes affected by feeding CM or LM, as well as investigation of other tissues. Moreover, to improve our understanding of implications of changes in gene expression, an examination on enzyme activities of those gene targets is also desirable to validate the gene expression data. From our results, the assessment of effects the CMs based on expression of the detoxification regulatory genes was shown to be a complementary tool

to examine the nutritional value of these alternative protein ingredients of FM for barramundi.

In general, using CMs up to 300 g/kg inclusion level in diets for barramundi did not cause any changes in plasma biochemical parameters and structure of digestive organs when compared to fish fed either the LM or FM diets. However, the ingestion of 300 g/kg EX CM in diet resulted in the down regulation of expression level of some genes involved in phase 1 (*Lc CYP1A1*, *CYP2N* and *CYP3A*) and phase 2 (*Lc GST*) of detoxification.

CHAPTER 5 . GENERAL DISCUSSION

The present study evaluated CM as a feed ingredient for barramundi based on criteria such as nutritional composition, digestible value, feed utilisation and fish health effects. Four Australian CMs with respect to different origin and processing method (solvent and expeller extraction) were described for nutritional values and determined for digestibility of nutrients and energy. The two of these ingredients were then chosen to study effects of a serial inclusion level (100, 200, 300 g/kg) of either SE CM or EX CM on growth performance and feed utilisation. Further examination including changes in plasma biochemistry, histology and hepatic gene expression was carried out to investigate effects of CM regarding fish health.

The nutritional composition of CMs was shown to have variability depending on processing method. The SE CMs had higher protein content but lower lipid content than the EX CM. Glencross *et al.* (2004a) demonstrated a significant difference in nutritional composition of Australian SE CM and EX CM. The present study also indicated the variation in nutritional composition of CMs from different growing regions. The previous study reported a great variation in nutritional composition of various Australian CMs from main growing regions (Spragg and Mailer, 2007). It is well understood that the different origin regarding the different growing conditions such as weather and soil quality may affect nutritional composition of canola seed (Hickling, 2001). These results suggest the origin of ingredients (CM) need to be documented for ingredient characterisation and ingredient management strategy.

The study also examined apparent digestibility coefficient (ADC) of nutrients (dry matter, protein) and energy of all four CMs in barramundi. Protein of the SE CMs is fairly well digested by barramundi (74.5 – 86.6%) which is consistent with protein digestibility of the lupin kernel meal. However significantly lower digestibility of protein (63.1 %) and some amino acids such as lysine, were observed for the EX CM. This suggests that processing method affected digestible protein of CMs. These results differs from findings of Glencross *et al.* (2004a) who reported heat treatment of CM at 120 ^{o}C and 150 ^{o}C severely influenced protein digestibility of this ingredient for red seabream. Excessive heat during processing can damage protein, especially lysine (Carpenter and Booth, 1973). Generally, temperature is consistent in canola crushing plants but to some extent temperature can be increased to improve oil production. In addition, the high phytic acid and fibre content in the EX CM could partly contribute to

the depression in protein digestibility. The differences in the digestibility of some amino acids such as lysine and sulphur containing amino acids within a single CM, indicate the need for amino acid availability data such the data provided in this study when formulating diets contains CMs. In general, the determined protein and amino acid digestibilities were relatively difference among CMs; therefore, this digestibility data permit to formulate diets based on digestible protein and amino acids rather than crude values.

CMs had low dry mater and energy digestibility (< 50 %), which suggests that carbohydrates in CMs are poorly digestible. Van Barneveld (1998) reported that dominant carbohydrates in CMs are non-starch polysaccharides. In our study CMs have fairly high fibre content (presented as NDF and ADF). The results of present study on CMs also agree with results of Allan *et al.* (2000) that dry mater and energy digestibility are low in most plant ingredients with high carbohydrate content. This may be a challenge for formulating diets containing plant ingredients such CM because of requirement of high energy density in diets of most marine fish species.

The growth study assessed effects of a range of inclusion level (100, 200, 300 g/kg) in diets for barramundi. The formulated diets satisfied requirement of DP (420g/kg) and DP:DE (30g/MJ) for barramundi (>50g) at 30 ^{o}C (Glencross *et al.*, 2007; Glencross, 2008). For almost inclusion of CMs (all inclusion levels of SE CM: 100, 200, 300 g/kg and 100-200 g/kg of SE CM) and LM, there was an improvement or at least similar feed intake and growth performance of CM containing diets compared to the FM diet. Other specifications such as FCR, PR, ER were no significantly different.. The results of the SE CM in this study agree with previous studies that CM is a favourable ingredient for several species, which can be included in diets at approximately 30% of higher (Webster *et al.*, 1997; Lim *et al.*, 1998; Glencross *et al.*, 2004b; Shafaeipour *et al.*, 2008; Glencross *et al.*, 2011c). However, there was a limitation on the use of the EX CM. For the EX CM, limitation was observed in fish fed 300 g/kg EX CM. Feed intake, growth performance and FCR was inferior to the FM control diet. It is unclear what contributed for this depression in fish fed the 300 EX CM but high phytic acid probably affected digestibility and/or caused the decrease in appetite or changes in the physiological properties of fish. In addition the balance of amino acid profile might be not reached as low amino acid content and low ADC of some amino acids in the EX CM despite of crystal amino acid supplemented in the

300EX CM diet. It is concluded that a inclusion level of 300g/kg SE CM can be effectively utilised for barramundi but lower acceptance was given for EX CM (200g).

In early studies glucosinolates as known compounds limit the acceptability of CM for fish diets because of disturbance of thyroid function such as thyroid hypotrophy or decrease in thyroid hormone T3 and T4 (Hardy and Sullivan, 1983; Hilton and Slinger, 1986; Leatherland *et al.*, 1987; Burel *et al.*, 2000a; Burel *et al.*, 2001). In the present study, no significant differences in T3 and T4 were indicated between fish fed the CM containing diets and the FM control diet. This suggests that glucosinolates presenting in these CMs with low content might not exceed the threshold of these compounds in barramundi. Both the present study and the previous study of Glencross *et al.* (2004b) indicated that glucosinolates were not a factor limiting the inclusion of Australian CMs in fish diets. In addition, no changes in other biochemical parameters such as plasma enzymes and histology of digestive and metabolic organs were observed in the present study. However, it is suggested that long effects of CM use on histology should be included in further examination. These observations suggest that the CMs in the present study can be included (300 g/kg) in fish diet without any health effects. These ingredients can be considered as an ingredient for partial fishmeal replacement at single plant ingredient or combination with other ingredients. In fact a combination of different plant ingredients for fishmeal replacement may have benefit as in some case a single plant supplementation can cause detrimental effects on fish health such as enteritis status in salmonids causing by ingestion of high soybean meal level in diet (Van den Ingh *et al.*, 1991; Krogdahl *et al.*, 2000).

Molecular mechanism of nutrient metabolism is response to dietary intake. Studying transcriptional level of some genes involved in fatty acid synthesis (*Lc FAS, Lc SCD and Lc FXR*) and energy production (*Lc CS and Lc PDK*) indicated that the expression levels of these genes did not depend on the experimental diets although the decrease in energy retention and growth performance by fish fed the 300EX-CM diet was seen in fish fed this diet. However, in this study gene expression was analysed at only one time event (24 h post feeding) while a great variation in expression of various metabolic genes in barramundi was observed over a time course after feeding (Wade *et al.*, 2014). Further study requires including an examination of variation in gene expression over time events after feeding.

Glucosinolates in bassica plants have been known as bioactive compounds that have affected activity of enzymes in the antioxidation (detoxification) of mammals (Wang *et al.*, 1997; Nho and Jeffery, 2001). Other bioactive constituents such as phenolic compounds have been known to enhance the activity of oxidative enzymes belonging to glutathione groups (Sitjà-Bobadilla *et al.*, 2005). The present study indicated that there was a down regulation in expression level of cytochrome P450s (*Lc CYP1A1*, *Lc CYP3A*, *Lc CYP 2N*) and *Lc GST* in fish fed with the 300EX-CM diet but not in the 300SE-CM. This suggests that glucosinolates or phenolic compounds in this study did not cause such changes in expression of those genes. It is suggested that the decrease in ROS (endogenous reactive oxygen species) generation as lower metabolic rate of fish fed with the 300EX-CM could be a reason for this lower expression level. In addition, a lower protein intake might lead to a lower expression of these gene as the suggestion of Yokoyama and Jun-Ichi (1991). The reduction in *Lc CYP3A* transcription in all plant ingredient containing diets suggests that to some extent the regulation in expression of this gene was probably associated with ANFs in plant ingredients but needs to be clarified in further work. Whether the lack of these genes underlies the reduction in detoxifying activities of barramundi requires further data. The current work may suggest significant changes in detoxification genes (*Lc CYPs gene* and *Lc GST*) associated with nutritional status.

Conclusion

This study provided critical information relating to the nutritional composition, digestibility, nutrient utilisation and also implications of health effects of some commercial CMs from two processing methods and different origin using in barramundi. The findings of this study confirm that

- CM is a promising plant ingredient for a partial fishmeal replacement in aquaculture diets as their protein content is fairly high and protein digestibility is equivalent to fish meal and lupin meal. However, different origin and processing method suggest variability in nutritional composition and digestibility.
- An inclusion level of 300 g/kg the SE CM can be used in diets for barramundi without any negative effects on growth performance and other aspects. The maximum inclusion level for the EX CM is 200 g/kg. The high CM inclusion level (300g/kg) in the diet suggests the potential depression in palatability, FCR, growth performance of barramundi. Further work should consider broader

inclusion levels for the SE CM to define the limitation of this ingredient for barramundi.

- CMs can be replaced for fishmeal up to 300 g/kg in diet for barramundi without adverse effect on fish health status but long effects should be considered.
- There were no changes in expression levels of genes involving in fatty acid synthesis and energy production pathways. The down expression level of several genes in detoxification in the present study regarded dietary nutrition. However, the study examined expression of genes only at a one-time event post feeding and at different single genes. Further work requires the examination in variation of gene expression over time events after feeding in fish fed with CM diets. Moreover, to understand the implications of down regulation of genes in detoxification needs more effort in approaching new target genes, investigating gene function in barramundi and measuring enzyme activities.

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Appendix

This Appendix contains copies of papers which have been published based on results reported in this thesis

Ngo, D. T., Pirozzi, I., & Glencross, B. (2015). Digestibility of canola meals in barramundi (Asian seabass; Lates calcarifer). *Aquaculture, 435*(0), 442-449. doi: http://dx.doi.org/10.1016/j.aquaculture.2014.10.031 (Chapter 2)

Conference Presentation

Ngo, D. T., Glencross, B., & Pirozzi, I. (2014). *Evaluation of canola meal as feed ingredient for barramundi (Lates calcarifer).* Abstract presented at the 16th International Sympossium on Fish Nutrion and Feeding, Cairns, Australia)

Aquaculture 435 (2015) 442–449

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aqua-online

Digestibility of canola meals in barramundi (Asian seabass; *Lates calcarifer*)

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ARTICLE INFO

Article history:
Received 27 July 2014
Received in revised form 15 October 2014
Accepted 18 October 2014
Available online 28 October 2014

Keywords:
Barramundi
Canola meal
Rapeseed meal
Digestibility
Plant protein

ABSTRACT

The influence of two different oil processing methods and four different meal origins on the digestibility of canola meals when fed to barramundi (*Lates calcarifer*) was examined in this study. The apparent digestibility coefficients were determined using the diet-substitution method with faeces collected from fish using stripping techniques. The protein content of the solvent extracted (SE) canola meals (370–423 g/kg DM) was higher than that of the expeller extracted (EX) canola meal (348 g/kg DM), but the lipid content was lower than that of the expeller extracted canola meal. Among the SE canola meals, the protein digestibility of the canola meals from Numurkah and Newcastle was similar (84.1% and 86.6% respectively), but significantly higher than that of the canola meal from Footscray (74.5%). The protein digestibility was lowest (63.1%) for the EX canola meal. The energy digestibility of the canola meals (43.1–52.5%) was similar to that of the lupin (54.8%) except for the lower of SE canola from Footscray (32.4%). The SE canola meals provide 276–366 g/kg DM of protein while that of the EX is only 220 g/kg DM. The digestible energy content of the SE canola meal Footscray (6.5 MJ/kg) was lower than the other canola meals (8.7–10.6 MJ/kg DM). This study shows that there can be significant variability in the digestibility of canola meals subject to potential processing and sourcing variables.

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http://dx.doi.org/10.1016/j.aquaculture.2014.10.031
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POSTER 103

EVALUATION OF CANOLA MEAL AS A FEED INGREDIENT FOR BARRAMUNDI (LATES CALCARIFER)

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Two experiments were conducted to determine the digestible nutritive value of Australian canola meals, their utilization and examination of effects on biochemistry, histology and gene expression when fed to barramundi. In the first experiment, the digestibility of four Australian canola meals (three solvent-extracted meals and one expeller extracted meal) from four crushing plants in different regions, were determined. Diets were formulated from 70% of a basal mash with a 30% inclusion of each test ingredient with another diet including (also at 30%) a lupin kernel meal as a reference ingredient and another diet as a basal reference diet. Barramundi (~390 g) were assigned to 24 mesh cages (300L) placed in 6 x 2500L tanks. Fish were acclimatized to the diets for one week before faeces were collected by stripping techniques over a three week period. Faeces and diet samples were analysed for yttrium, dry matter, protein, amino acids and energy. The second experiment was designed to determine the optimal canola meal inclusion level in diets for barramundi. A serial inclusion (0%, 10%, 20%, 30%) of two of the canola meals in diets (solvent and expeller) was formulated to the same digestible protein and energy specification for barramundi, with an eighth diet including 30% lupin kernel meal as a reference. Fish (~50 g) were allocated across 24 tanks (300 L) with three replicates per treatment and at a density of 15 fish per tank. Fish were fed to satiation once per day, and uneaten feed was collected to calculate feed intake. After 8 weeks fish were weighed and three fish from each tank were sampled for chemical composition analysis while another three fish were used for taking blood, kidney, liver, pyloric caeca, distal intestine and stomach for biochemical, histology and gene expression analysis.

The results of this study revealed that there was a variation in protein digestibility amongst canola meals. Protein digestibility of expeller canola meal (63.1%) was significantly lower than that of solvent canola meals (74.5% to 86.6%) and lupin (92.7%) while energy and dry matter digestibility were fairly consistent among canola meals and similar to that of the lupin kernel meal. Amino acid digestibilities closely followed a similar trend to that of protein digestibility. The results of the growth experiment showed that 30% inclusion of solvent canola meal in diet did not negatively affect feed intake, growth performance, and feed efficiency of barramundi; however 30% inclusion of the expeller canola meal diet caused a reduction in feed intake, poorer growth and poorer feed efficiency compared to the control and other test diets.