Utilization of Australian Grain Legumes by Salmonids

by

Mehrdad Farhangi

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M.Sc (Animal Science)

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Abstract

A large range of ingredients have been considered as potential replacements for fish meal but grain legumes have attracted most attention in the past. The main factors that limit the use of grain legumes in animal diets are the number of antinutritional factors (ANFs) that may adversely affect animal performance. Due to the high sensitivity of salmonids to ANFs and the importance of salmonid production in Australia, salmonids have been used as reliable biological tools for screening the most suitable Australian grain legumes.

In this study, the four most abundant Australian grain legumes: chick peas, faba bean, field peas and lupin were targeted as potential alternatives to fish meal in salmonid diets. Chemical, biological and immunological measurements were used to assess their relative performance. The suitability of the four grains (raw and processed) was tested with rainbow trout and the effects of body weight (small, medium and large) and adaptation period on nutrient digestibility were evaluated. Maximum inclusion of the most suitable grain legume (lupin) was determined and the effects of exogenous enzymes on the nutrient utilization and fish performance were also investigated.

The chemical composition and the digestibility of nutrients in lupin were most suited for rainbow trout compared with the other three grains. Also the concentrations of the two most important ANFs (trypsin inhibitor and phytic acid) were lowest in lupin. The concentration of trypsin inhibitor significantly decreased follwing the combination of both soaking and heating the grains. Fish body size did not affect nutrient digestibility of the grains. Over the adaptation period *in vivo* dry matter digestibility improved only for small fish size and *in vivo* crude protein digestibility increased for small and medium fish size. In addition, during the adaptation period *in vivo* crude protein digestibility was improved for the grains that contained higher trypsin inhibition (chick pea and field pea). There was a poor correlation relationship between *in vitro* and *in vivo* digestibility for processed grains, however there was a significant relationship for the raw grains.

Lupin was selected as the most promising grain legume and the subject of further

experiments. Due to the high concentration of neutral detergent fibre (NDF) and relatively low protein content of the whole grain, dehulling was applied to reduce the NDF and increase the protein content. A dose response experiment was conducted to establish the highest possible inclusion level of dehulled lupin (DL) in rainbow trout diets. Fish performed well at up to 40% inclusion of DL in the diet. However, growth and the energy efficiency ratio significantly decreased at 50% inclusion of DL. It was concluded that the fish were unable to effectively utilize the non-protein energy content of DL at this level of inclusion. Supplementation of the diets that contained 50% DL with different exogenous enzymes: Energex™, Bio-feed plus™ and Alpha galactosidase™ (separate or in a mixture) did not improve energy utilization by fish. Surprisingly, the growth performance of the fish that received 50% DL in the diet was marginally better than the group of fish that received a fish meal based diet. This was partly related to higher feed intake (*ad lib vs* set ration), but significantly better energy efficiency ratio suggested improved use of the carbohydrate fraction of the lupin. The DL that was used in the two experiments was from the same source but it had been stored under suitable conditions for about nine months.

Having established the suitability of DL at 50% inclusion level in the rainbow trout diet, the potential of DL as a fish meal replacement was tested for Atlantic salmon. The effect of feeding at the most suitable time, based on the diel rhythm of feed intake on nutrient utilization and growth performance was tested when the fish received the diets that contained 30% DL. Fish were fed with the diets that contained 40% and 45% crude protein both in the morning and in the afternoon or diets that contained 40% crude protein in the morning and 45% crude protein in the afternoon (mixed diets) and vise versa. Results did not show any effect of feeding time on nutrient utilization. However, the growth of fish that received the mixed diets was comparable to groups that received 45% crude protein diets.

The suitability of DL as a fish meal replacement for both rainbow trout and Atlantic salmon was shown in the current study. Additional studies are needed to improve the energy utilization and to minimize the dry matter waste of the diets that contain high levels of DL.

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Abbreviations

ABARE	Australian Bureau of Agricultural & Resources Economics
ABS	Australian Bureau of Statistics
ADC	Apparent digestibility coefficients
ADF	Acid detergent fibre
AMEn	Apparent metabolizable energy
ANF	Antinutritional factors
ANOVA	Analysis of variance
AOAC	Association of Official Analytical Chemists
BAPNA	a-N-Benzoyl-DL-arginine-p-nitroaniliade
BW	Body weight
CF	Crude fibre or condition factor
CL	Crude lipid
CMC	Carboxymethylcellulose
CP	Crude protein
CRC	Cooperative Research Center
CRD	Completely randomized design
CSIRO	Commonwealth Scientific & Industrial Research Organization
CTI	Chymotrypsin inhibitors
CTIA	Chymotrypsin inhibitor activity
DE	Digestible energy
DL	Dehulled lupin
DLWG	Daily live weight gain
DM	Dry matter
DMD	Dry matter digestibility
EAA	Essential amino acids
EAAI	Essential amino acid index
EER	Energy efficiency ratio
FAO	Food & Agriculture Organization of the United Nations
FCR	Feed conversion ratio
FI	Feed intake
FM	Fish meal
FRDC	Fisheries Research & Development Corporation
GE	Gross energy
GI	Gastro intestinal
HPLC	High pressure liquid chromatography
HSI	Hepatosomatic index

ICPAESP	Inductively coupled plasma atomic emission spectrometry
ICPS	Inductively coupled plasma spectrometry
In vitro	In glass
In vivo	In animal
IVCPD	*In vivo* crude protein digestibility
IVDMD	*In vivo* dry matter digestibility
LILI	Large intestine length index
LIWI	Large intestine weight index
LER	Lipid efficiency ratio
LPV	Lipid productive value
LSD	Least significant difference
ME	Metabolisable energy
MJ	Mega joule
NEAA	Non essential amino acids
NDF	Neutral detergent fibre
NRC	National Research Committee
NS	Not significant
NSP	Non-starch polysaccharides
OM	Organic matter
PA	Phytic acid
PCI	Pyloric caeca index
PER	Protein efficiency ratio
PPM	Part per million
PPV	Protein productive value
RO-SBM	Reduced-oligosaccharides soybean
Sed	Standard error of difference
S.E.M	Standard error of mean
SGR	Specific growth rate
SILI	Small intestine length index
SIWI	Small intestine weight index
TI	Trypsin inhibitor
TIA	Trypsin inhibitor activity
µm	Micro meter
WG	Weight gain
Yb_2O_3	Ytterbium oxide
Y_2O_3	Yttrium oxide

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Chapter One

Potential of Australian grain legumes to replace fish meal for expanding global sustainable aquaculture: A review

1.1. Introduction

The world population exceeded 6 billion people in 1999 and is projected to reach approximately 8 billion by 2025 (FAO, 1999). Currently, about 20% of the world population is suffering from malnutrition. If the rate of food production continues at the current rate of population growth, another 600 million people will be undernourished at the end of the first quarter of the new millennium (FAO, 1999). To fill this gap and to improve global food standards, food production must increase at a higher rate. This is especially true for fisheries products (wild fisheries and aquaculture), which are less available in the majority of developing countries. For instance, the annual per capita live fish consumption in Japan was 66.4 kg in 1997, but for most developing countries this figure was below 15 kg in the same year (Laureti, 1999). The annual total wild fisheries catch was 86.3 million tonnes in 1998. This was projected to rise to 92.3 million tonnes in 1999 (FAO, 2000b). It has been suggested that the fisheries sector cannot respond to further demand in the future (Hardy, 1996).

1.2. Opportunities for global expansion of aquaculture

Aquaculture is viewed as the only significant new contributor to food production in the world (Boyd and Schmittou, 1999). Over the last three decades, aquaculture has also been the fastest growing agribusiness sector in the world (FAO, 2000a) and its production has more than doubled in the last 15 years. In contrast, the rate of food production from both crop and animal sectors has slowed down in recent years (FAO, 1999). This situation increases the pressure on aquaculture to respond to further demands for fisheries products in the future. Fisheries products are predicted to fulfill more demands qualitatively in developed countries and quantitatively in developing countries. Aquaculture can play a special role in achieving higher fisheries production for two reasons. Firstly, fish is a highly nutritious source of protein and other essential nutrients such as fatty acids, with no comparable substitute. Secondly, there is great potential for the expansion of

aquaculture as it is a new and underdeveloped production sector compared to agriculture and animal husbandry (FAO, 2000a). Aquaculture production contributed 26.3% of total fisheries products globally in 1999 (FAO, 2000b) and 12.3% in Australia in 1998/1999 (ABARE, 2000b). The ratio of aquaculture to total fisheries production in Australia is significantly lower than the average value in the world. It is projected that aquaculture's share in total fisheries production will be further increased in the future (Stickney, 1995). The aquaculture sector may have to increase its production by 2-3 times to fulfill higher demands for the next generation (New, 1996). Extending the production of non-carnivorous fish in developing countries is predicted to further increase in the future and fulfils extra demand.

1.3. Opportunities for expansion of aquaculture in Australia

Aquaculture remains a relatively underdeveloped agribusiness sector in Australia. It is evident that long distances to population centers (national and international markets), low population and high production of animal products in Australia are the main causes. Furthermore, despite the benefit of the third largest fishing zone in the world (8.94 million km^2) and access to about 37,000 km of coastline, the lack of adequate nutrients in Australian waters has resulted in its fisheries production being ranked only fiftieth in the world (ABARE, 2000a). Furthermore, most Australian fisheries have been fully fished in the past (ABARE, 2000a). This condition has created a suitable opportunity in this country to expand the aquaculture sector over the last 15 years. Currently, aquaculture is one of the fastest growing primary industry sectors in Australia with an annual average increase of 20% (CSIRO, 1999) and total production was valued at over $602 million in 1998/1999 (ABARE, 2000b). The relatively short history of aquaculture in Australia, which has mainly been conducted in less intensive systems, has led to fewer problems in terms of fish diseases, of which only a few are economically important (Munday and Owens, 1998). The geographical isolation of Australia from regions with a high production of fisheries products (South East Asia) may be another important issue that explains lack of development.

Various climatic conditions enable the evaluation of a variety of potential fish species for Australian aquaculture. The number of marine or fresh water fish

species under recent or current investigation for use in aquaculture in Australia is high, despite the constraints that exist in this area. Species include flounder (Carter *et al.*, 1996), barramundi (Williams and Barlow, 1998), silver perch (Allan and Rowland, 1998), southern blue-fin tuna (Carter *et al.*, 1998), black bream (Haddy and Pankhurst, 2000), Murray cod (Ingram, 2000), snapper (Cleary *et al.*, 2000) and striped trumpeter (Morehead *et al.*, 2000) to mention a few.

In recent years, Australia has arranged one of the most efficient infrastructures for research and development of aquaculture development in the world. Research and development organizations include Fisheries Research and Development Corporation (FRDC), Cooperative Research Center (CRC) for Aquaculture (1993-2000 and 2001-2008), Commonwealth Scientific and Industrial Research Organization (CSIRO), state fisheries and universities have been working efficiently together over the last decade to ensure Australia has the potential to upgrade its aquaculture sector, in a way comparable to leading countries in this area.

1.4. Limitations of global expansion of aquaculture

Globally, further expansion of aquaculture is limited mainly by the availability of suitable water and animal proteins (fish meal) that are needed by most aquatic farmed animals (Stickney, 1995). Feed is the single largest cost (Robinson and Li, 1995) in aquaculture. Feed is the main factor that significantly affects the physiological functions of animals including feed intake (appetite) (Forbes, 1995; Lawton *et al.*, 2000), digestion and absorption (Hepher, 1988; McDonald *et al.*, 1995) and metabolism and growth (Fafournoux *et al.*, 2000). Feeds also have the potential to influence immune function (Cunninghamrundles and Lin, 1998), (Suchner *et al.*, 2000), stress resistance (Kolkovski *et al.*, 2000), reproduction (Meinelt *et al.*, 1999; Izquierdo *et al.*, 2001) and product quality (Steffens, 1997). Feeds can also indirectly affect the environment through excretion of unutilized components or end products of metabolism (Cho *et al.*, 1994). The consideration of environmental issues in aquaculture is arguably more important than other animal production systems. This is due to first the lack of control over feeding and uneaten feed, resulting in rapid leaching of several nutrients into the water column, and secondly the extreme difficulty in collecting waste products and uneaten feed (Cho,

1997; Cho and Bureau, 1998). The net retention efficiency for nitrogen in fish (40-50%) is similar to omnivorous birds and mammals. In the case of fish, nearly 60% of assimilated nitrogen is excreted into the surrounding water with the potential for eutrophication (Cowey, 1995). Reduction of waste output is possible through diet formulation and revision of feeding regimes for intact farming condition, improved feed utilization and use of highly digestible ingredients (Cho, 1997).

Currently, aquatic animal feed manufacture is one of the fastest growing agribusiness industries in the world, with an annual growth rate of 30% (Tacon, 1996). Any development in aquaculture should consider the integrated effect of water quality and feeding management, if sustainable aquaculture is to be maintained.

1.5. Fish meal is an indispensable ingredient for most farmed aquatic animals

Fish meal is the main ingredient currently being used in aquafeeds. This is particularly the case for salmonid diets which contain 30 to 70% fish meal in feeds for adults and even higher amounts in starter feeds (Rumsey, 1993). This is because of the unique specifications of fish meal, including high palatability, high protein content, a good balance of both essential amino acids and essential fatty acids and rich energy and mineral sources (Watanabe *et al.*, 1997). Furthermore, the inclusion of unidentified growth factors and essential nutrients such as selenium are other advantages cited in the literature (Rumsey, 1993). Digestibility of all nutrients in fish meal is usually higher than for most other protein sources. The omega-3 fatty acids in both fish meal and extracted fish oil are proposed to contribute to natural immunity that reduce or eliminate the need for medication (Pike and Barlow, 1999). Meanwhile, feed formulation is simpler using fish meal because it more closely matches the nutrient requirements of fish than any other ingredients (Tacon, 1994) and consequently requires less complex formulation using several other ingredients. This is especially true of feed formulation for carnivorous fish.

Total replacement of fish meal has been successfully reported for a few fish species including rainbow trout (Kaushik *et al.*, 1995), catfish and tilapia (Lusas, 1999). However, the only fish species that has shown consistent good growth performance

on a fish meal-free based diet is the channel catfish (Webster *et al.*, 1992; Webster *et al.*, 1995a). It has been shown that soy protein concentrate could totally replace fish meal for rainbow trout (Kaushik *et al.*, 1995), however, use of the same ingredient (more than 50%) in the diet, reduced growth performance in other studies (Stickney *et al.*, 1996). Although recent predictions of fish meal inclusion for other fish species suggest 2.5% fish meal inclusion in the diet of carp (an omnivorous fish species), the figure for catfish is 0% by 2010 (Barlow, 2000).

Catfish and tilapia's ability to be grown on a fish meal-free based diet and their capacity of sparing the protein through using complex carbohydrates (as an energy source) is attributed to their lower position in the food web as omnivorous fish species (Lusas, 1999). However, detailed information for the reasons behind such a phenomenon still needs to be elucidated. Finding fish species that can be grown on fish meal-free based diets may provide great opportunities for expanding the aquaculture industry and may relieve the pressures that the fisheries sector is currently exposed to.

1.5.1. Limitations and increasing concern about using fish meal in aquaculture

Concerns about the use fish meal in the aquaculture industry have increased in recent years (Naylor *et al.*, 2000). Currently, about one third of the total fish catch is converted to fish meal to be used in animal feeds (Naylor *et al.*, 2000). Aquaculture utilizes one-third of total fish meal production (Tacon, 1998a). Considering the rapid expansion of aquaculture, this amount will increase in the future (Stickney, 1995; Barlow, 2000). Despite much lower (2-3%) inclusion of fish meal in pig and poultry diets (Naylor *et al.*, 2000), these animals are the largest consumers of fish meal in the world (Hardy, 1995). Using wild fish as fish meal in aquaculture directly exerts extra pressure on fisheries' resources (Naylor *et al.*, 2000). However, many fish species are considered "industrial" and are not suitable for direct human consumption. It is proposed that uncontrolled fishing diminishes wild fisheries indirectly through habitat modification, the removal of wild seed stock, food web interaction, water pollution and the introduction of exotic species and diseases which endanger wild fish populations (Naylor *et al.*, 2000).

Negative attitudes regarding the use of fish meal in carnivorous fish diets are of more obvious concern. Increasingly, consumers are turning to farmed species instead of wild species, partly because of their concern about the over-fishing of wild species. The following statements illustrate the increasing concern about this issue: "Carnivorous fish are net fish protein reducers rather than protein producers" (Tacon, 1994), and "one man's trash (fish) is another man's meal" (New, 1991). Furthermore, up to 5 kg of wild fish are used to produce 1 kg of good quality farmed fish (Tacon, 1998b). It is also stated that the inclusion of more than 20% fish meal in the diet results in water pollution through excretion of excess phosphorous into the environment (Rumsey, 1993). In addition, the inclusion of a higher protein level in the diet usually results in higher nitrogen excretion into the environment (Rychly, 1980). Finally, it is claimed that fish meal produced with poor technology has the potential for spreading disease (Rumsey, 1993).

1.5.1.1. Necessity of integrating the advantages of different production systems

Intensive fish production systems are less efficient protein producers compared to semi-extensive production systems (Tacon, 1994). In addition, intensive aquaculture has a higher potential for polluting the environment, and expanding disease, and is cost intensive despite its higher productivity. In semi-extensive production systems both omnivorous and herbivorous fish are cultured in polyculture systems with a more efficient use of energy through the use of fertilizers and pond supplementation with low protein feed inputs (Tacon, 1994).

Increased numbers of intensive enterprises due to the limitation of land and suitable freshwater has caused an increased use of fish meal and fish oil in herbivorous and omnivorous fish diets in some Asian countries (Naylor *et al.*, 2000). Meanwhile, due to the rapid global increase in aquaculture, the need to develop diets for particular production conditions is emerging (Cho, 1991). These issues have already exerted an extra demand for fish meal use in this sector as the natural products of ponds cannot support high growth rates at high fish density in more modern systems (Naylor *et al.*, 2000). Suitable fish meal replacements should be identified if the extra pressure of using more fish meal with carnivorous/omnivorous fish is to be minimized in the future. Finding suitable alternative ingredients will allow aquaculturists to integrate the advantages of both

intensive and extensive production systems for establishing a more sustainable aquaculture industry.

Fresh water omnivorous fish have greater potential for using plant protein and oils than carnivorous fish, despite their relatively similar protein requirements (De Silva and Anderson, 1994). Finding suitable candidates, as fish meal replacements in carnivorous fish diets, will also mean the ingredients can be included in other herbivorous and omnivorous fish diets, as these fish seem to be less sensitive to antinutritional factors (ANF's). This may indirectly reduce the increasing pressure on food demand in aquaculture by increasing the future production of non-carnivorous fish species in developing countries. Research findings in salmonids may have application for other valuable fish species. For example, Atlantic salmon have been used as a surrogate for nutritional studies of southern blue-fin tuna in Australia (Carter *et al.*, 1999).

Some new strategies have been developed over the last few years to minimize the detrimental effect of intensive fish culture on the environment. Recently, the incorporation of filter feeders (Troell *et al.*, 1999; Soto and Mena, 1999) and high rate algae ponds (Pagand *et al.*, 2000), in conjunction with intensive fish culture enterprises, has significantly reduced the nutrient pollution load from marine aquaculture enterprises.

1.5.1.2. Necessity of finding alternative protein and energy sources

A large range of potential ingredients has been considered as replacement for fish meal in the past. These ingredients include terrestrial animal by-products, single cell proteins, oilseeds, grain legumes and some plant by-products and protein concentrates (Tacon, 1994). However, only a few animal or plant proteins have shown real potential as fish meal replacements for aquafeed manufacturers (Rumsey, 1993). The rate of production of the selected ingredient should be comparable with the current rate of aquaculture expansion. Meanwhile, in order to be considered as suitable fish meal replacements in aquaculture, alternative proteins should be readily available throughout the year and have reasonably constant and predictable chemical compositions (Tacon, 1994).

There are several potential problems with replacing all the fish meal in aquafeeds. For instance, yellowtail grew normally up to 6-8 weeks on a fish meal-free diet; however, the fish then developed green liver, which could not be entirely attributed to the consumed diet (Watanabe *et al.*, 1992). In another attempt to entirely eliminate fish meal in the diet, rainbow trout grew normally without showing any adverse symptoms for 20 weeks (Watanabe *et al.*, 1997). However, the experimental diets were too expensive to be used commercially. Significantly lower growth, significantly higher feed conversion ratio (FCR) and chicken flavor occurred when eight fish meal-free diets were fed to rainbow trout (Adelizi *et al.*, 1998).

The replacement of fish meal with less expensive plant protein sources has shown variable results and influenced by fish species, fish size and the type of ingredient (Pongmaneerat and Watanabe, 1993). Although reducing the protein cost is the main concern for fish nutritionists, it is preferable that the alternative protein sources have an acceptable level of utilizable energy. This is not an overwhelming concern as there is less competition between the animal production sectors and aquaculture for fish oil. Other animals' diets could be supplemented with alternative energy sources including carbohydrates and other fats (*e.g.* vegetable oil, tallow). High energy levels in alternative protein sources have the extra advantage of a protein sparing effect (Meyers, 1994). Both lipids and carbohydrates are considered as the energy sources, however, lipids are better utilized to produce energy compared with carbohydrates for most aquatic animals. The capacity to spare the protein using lipids and carbohydrates varies greatly among different fish species. Fortunately the price differences between lipids & carbohydrates and protein sources have made the protein sparing effect a cost effective approach in aquaculture nutrition (De Silva and Anderson, 1995). It has been shown that dietary lipid requirements could be partially replaced with carbohydrates in fish to improve productivity as well as carcass quality (Meyers, 1994). However, fish oil contains a fatty acid profile that matches the fish requirements and preserves the palatability of the diet (Meyers, 1994). The global production of vegetable oils in 1996 was more than 90 million tonnes compared to only 1.1 million tonnes of fish oil (Asche *et al.*, 1999). High fish meal inclusion in salmon diets could be partially reduced through a higher inclusion level of lipids or carbohydrates (Hillestad *et al.*, 2001).

There is considerable potential to examine the maximum inclusion level of vegetable oils in fish diet while still preserving the palatability of diets and productivity of fish through fulfiling the minimum requirement for essential fatty acids. The prediction of global fish meal inclusion in farmed aquatic animal diets and its total requirement are shown in Table 1.1. Considering the current limitations on the availability of fish meal to fulfill the aquaculture demand, it is not clear how the further 34% fish meal requirement in 2010 should be provided. This phenomenon maintains the aquatic animal nutrition research in general and fish meal replacement studies in particular, a crucial area of challenge for fish nutritionists in the next decade.

Table 1.1. Prediction of fish meal use in aquaculture in the world (2000/2010)[1]

	% fish meal inclusion		Fish meal required ('000 tonnes)	
Species	2000	2010	2000	2010
Carp	5	2.5	350	675
Catfish	3	-	15	-
Eel	50	40	173	114
Marine fish	100	80	484	931
Milkfish	12	5	36	28
Salmon	40	30	454	377
Shrimp	25	20	372	485
Tilapia	7	3.5	55	74
Trout	30	25	176	147
Total	-	-	2,115	2,831

1. Barlow (2000)

1.5.2. Limitations of using plant meal in aquaculture

A wide variety of plant meals have been considered for fish meal replacement in the last 2 decades. However, soybean meal has been most widely used in animal production systems (Vila and Mascarell, 1999) including aquaculture (Sudaryono *et al.*, 1999c). Soybean is a grain legume but is usually considered as an oilseed. The protein content of soybean is the highest of all plant proteins (Todorov *et al.*, 1996), (Table 1.2).

Table 1.2. Comparative crude protein, total calcium (Ca) and total phosphorus (P) content of major Australian grain legumes and soybean meal (g/kg as is)[1]

	Chick pea	Faba bean	Field pea	Lupin	Soybean meal
Crude protein	201	243	230	322	440
Total Ca	1.8	1.2	0.9	2.2	4.0
Total P	3.6	4.4	3.9	3.0	6.0

1. Petterson *et al.* (1997)

However, the unprocessed soybean is not an ideal ingredient, as it contains the highest number of ANF's and in some cases the highest concentration of antinutrients compared to any other plant protein (Vila and Mascarell, 1999). Unfortunately, despite the extensively searching the literature the authors could not find a reference that has cited all the ANF's in soybean meal to compare these values with the antinutrients of Australian grain legumes (Table 1.3).

Table 1.3. Comparative antinutritional factors of major Australian grain legumes and soybean meal

	Chick pea[1]	Faba bean[1]	Field pea[1]	Lupin[1]	Soybean meal[2]
Alkaloids (%)	-	-	-	0.02	Nd*
CTIA[3] (mg/kg)	7.72	0.40	1.60	0.08	Nd*
Convicine (mg/g)	-	2.22	-	-	Nd*
Lectins (dilut)	-	-	4.0	Nd*	Nd*
Oligosaccahrides[4] (%)	1.65	2.71	3.53	4.07	17.26
Phytate (%)	0.66	0.66	0.59	0.50	Nd*
Saponins (mg/kg)	-	-	-	573	Nd*
Tannins (total, %)	0.52	0.97	0.37	0.29	Nd*
Tannin(condensed, %)	0.04	0.22	0.02	0.01	Nd*
TIA[5] (mg/kg)	3.95	0.14	1.29	0.12	0.6
Vicine (mg/g)	-	4.14	-	-	Nd*

1. Petterson *et al.* (1997); 2. Rumsey *et al.*, (1995); 3. Chymotrypsin inhibitor activity
4. Sum of raffinose, stachyose and verbascose; 5. Trypsin inhibitor activity
* Nd: not determined

The concentration of phosphorus, the main factor causing eutrophication in effluent water (Lall, 1991), is nearly double in soybean compared to other grain legumes (Petterson *et al.*, 1997), (Table 1.2). Almost two thirds of the phosphorus in plant meals is in the form of phytates (Hardy, 1998). The ratio for soybean meal is about 80% (Lusas, 1999). Fish cannot digest the phosphorus that is bound with phytate (Hardy, 1998). It is obvious that the total excretion load of phosphorus in the environment is significantly higher in soybean-based diets compared to any other plant meal based diet. The bitter flavor of unprocessed soybean meal is another factor limiting its use (Rackis *et al.*, 1970). The detrimental effect of the inclusion of soybean meal in salmonid diets on the digestive tract (Hendriks *et al.*, 1990; van den Ingh *et al.*, 1991; Rumsey *et al.*, 1995), digestive enzyme activity (Hendriks *et al.*, 1990; Olli *et al.*, 1994) and feed intake, feed utilization and growth (Refstie *et al.*, 1998; Refstie *et al.*, 2000) has been extensively reported in the past. The inclusion of 27% of dehulled solvent extracted soybean meal (Carter and Hauler, 2000) did not affect the growth rate of Atlantic salmon whereas 34% inclusion of the same ingredient significantly decreased the growth performance of Atlantic salmon (Olli *et al.*, 1995). The inclusion of 62% soy protein concentrate, which totally replaced fish meal, did not affect the growth performance of rainbow trout (Kaushik *et al.*, 1995). However, inclusion of 48% soy protein concentrates (both untreated or treated with phytase) in Atlantic salmon diet, significantly decreased growth performance as compared to the fish group that was fed with a fish meal-based diet (Storebakken *et al.*, 1998b). Additionally, the price of high quality soybean protein concentrate sometimes is comparable to fish meal (van den Ingh *et al.*, 1996).

Suitable conditions for the cultivation of soybean are less available compared to other grain legumes (Nwokolo and Smartt, 1996). Most Australian grain legumes are cultivated in dry-land farming systems. Due to the high water demand of soybean and low price of vegetable oils in international markets, this grain is not currently an attractive crop for Australian farmers (Barry Croker, personal communication). Australia has great potential for producing different sorts of plant and animal proteins (Allan, 1997) and especially grain legumes. Whilst the production of one of the main Australian grain legumes (lupin) was 1,421,000

tonnes in 1998/1999, the total production of soybean was only 107,000 tonnes in the same year (Table 1.4).

Table 1.4. Comparative production of major Australian grain legumes and soybean from 1995-6 to 2001-02 in Australia ('000 tonnes)

	95-96	96-97	97-98	98-99	99-00	00-01	01-02 Estimates
Chick pea[1]	258	278	191	160	187	150	239
Faba bean[1]	119	123	135	133	166	278	260
Field pea[1]	465	466	303	298	357	401	368
Lupin[1]	1,429	1,560	1,381	1,421	1,547	802	1,188
Soybean[2]	-	74	54	107	-	-	-

1. Pulse Australia (2001); 2. ABS (2001)

1.5.3. Advantages of using grain legumes as fish meal replacement in aquaculture

Although both animal (by products) and plant proteins are available in bulk amounts in Australia (Allan, 1997), plant proteins are more globally available compared to animal proteins. Of plant proteins, grain legumes have attracted most attention. This is due to the significantly lower concentration of lysine in cereals, which is normally the first and major limiting amino acid in mono-gastric animal nutrition (Waldroup and Smith, 1989). Meanwhile, crude protein content of grain legumes is considerably higher than for cereals. Furthermore, voluntary feed intake is higher in animals that are fed on grain legumes compared to groups of animals fed on cereals (D'Mello, 1992).

Grain legumes tend to be less expensive than animal proteins and especially fish meal. The cultivation of legumes improves soil fertility and increases the productivity of other crops in the rotation system, resulting in increased sustainability of agricultural production systems (Boddey *et al.*, 1997). Furthermore, the storage, transportation and processing of grains is more convenient compared to animal proteins. Although environmental factors partially affect the chemical composition of grain legumes (Bhardwaj *et al.*, 1998), higher nutrient consistency in plant proteins is probably the major advantage of using plant proteins in fish diets compared to animal proteins as it facilitates feed

formulation. The inclusion of plant meals in rainbow trout diets significantly decreases phosphorus wastage through its increased bioavailability to fish (Riche and Brown, 1999). The production of grain legumes has been significantly increased over the last 25 years in Australia (Siddique and Skyes, 1997). Figures show that approximately 74.2% of the total production of three main produced grain legumes including lupin, field peas and chick pea has been exported in 1999/00 (Pulse Australia, 2001).

Animal by-products (*e.g.* meat meal) are being produced in large quantity in Australia (after Allan, 1997). However their use in aquatic animal nutrition is limited. This is mainly due to the high fat and mineral content (Williams *et al.*, undated) inconsistency in chemical composition (Hardy, 1996) and imbalance of essential amino acids (De Silva and Anderson, 1995). Meanwhile it has been shown that the protein digestibility of most meat meals in aquatic animals (*e.g.* prawns, silver perch and barramundi) is remarkably lower than plant proteins including dehulled lupin (Williams *et al.*, undated).

1.5.4. Limitations of using grain legumes as fish meal replacements in aquaculture

The main factor that limits the use of grain legumes in animal diets is the distribution of a wide range of ANF's with the ability to adversely affect feed intake, nutrient utilization and animal performance (D'Mello, 1992; D'Mello, 1995). The amount of information about the level, distribution, the mode of action and maximum safe inclusion of grain legumes in the diet for different animal species is limited (Batterham and Egan, 1987). The biological effect of antinutrients can range from a slight reduction in performance to mortality, even at low intake levels, according to the species and age of the animals (Melcion and van der Poel, 1993).

Mono-gastric animals are more sensitive to the side effects of ANF's (Batterham and Egan, 1987). In contrast, ruminant animals can modify many ingested ANF's by converting them to less toxic components (Dixon and Hosking, 1992). It is suggested that the antinutritive effect of legume seeds can be influenced by diet composition and feeding methods (D'Mello, 1995). The deleterious effect of grain

legumes has been reduced by gradually increasing them in the diet of some ruminants (D'Mello, 1992). In addition the antinutritional effects of ingredients decrease with age (Fuente *et al.*, 1998). These phenomena have not been studied in fish nutrition to the same extent and information about the adverse effects of ANF's in fish is limited (Tacon, 1995). Recently the effect of different ANF's on fish has been reviewed (Francis *et al.*, 2001). Less agreement exists in terms of the deleterious effect of antinutrients on fish in the literature, as in most studies an ingredient containing a high level of a particular antinutrient has been evaluated and any observed side effects in fish have been attributed to the antinutrient. However, the possible deleterious effect of other antinutrients in the diet or the interaction between various antinutrients in the tested ingredient has been neglected (Francis *et al.*, 2001).

The main body of literature in this area is limited to considering the detrimental effect of protease inhibitors (PI) and phytic acid (PA) on fish physiology and production. It seems carnivorous fish are more sensitive to the side effects of antinutrients, as their natural diet is usually antinutrient free. Carnivorous fish differ in their response to the same amount of antinutrient intake. For instance, Chinook salmon showed a severe response to the inclusion of saponins in their diet compared to rainbow trout (Bureau *et al.*, 1998). In a comparative study, the growth performance of rainbow trout was similar on both a fish meal-based diet and a diet that contained 29.6% soybean meal. In contrast the growth performance of Atlantic salmon that received the same soybean meal-based diet was significantly lower than the group that was fed with fish meal-based diet (Refstie *et al.*, 2000). These results may show the importance of targeting suitable fish species when plant meals that contain ANF's are considered as fish meal replacements.

Some grain legumes contain high levels of non-starch polysaccharides (NSP). However, lupin contains the highest level of NSP (both soluble and insoluble) compared to all other grain legumes (Table 1.5).

Table 1.5. Comparative insoluble, soluble and total non-starch polysaccharides (NSP) of major Australian grain legumes and soybean meal (g/kg 'as is')

	Chick pea[1]	Faba bean[1]	Field pea[1]	Lupin[1]	Soybean meal[2]
Insoluble NSP	84.25	74.87	67.53	216.57	14.1-19.1
Soluble NSP	4.31	3.43	2.82	18.97	1.8-3.2
Total uronic acid	33.89	30.96	16.29	48.70	2.9-4.5
Total NSP	122.45	109.06	86.64	284.24	16.4-22.2

1. Perez-Maldonado *et al.* (1999); 2. Irish and Balnave (1993)

There is extensive information about the antinutritive effect of NSP on mono-gastric animals in the literature. The antinutritive effect of NSP in pigs includes negative influences on the apparent digestibility of protein, fat and some minerals, and increased fermentation in the hindgut (Bakker *et al.*, 1998). Reduced digestibility of protein, starch and fat (Smits and Annison, 1996) and increased fermentation in the small intestine (Choct *et al.*, 1996) are caused by NSP in chickens. Although soluble NSP has been recognized as the antinutrient fraction, some beneficial effects have been attributed to insoluble NSP or "dietary fibre" (Smits and Annison, 1996). Watery faeces and high viscosity of digesta are the main effects of soluble NSP (Graham *et al.*, 1993; Smits and Annison, 1996). Information about the antinutritive effect of NSP in fish is limited and contradictory. Krogdahl *et al.* (1995) reported no antinutritive effect (in terms of nutrient digestibility or growth) of industrial raffinose (soybean meal) when added in a fish meal-based diet in Atlantic salmon. However, negative effect of NSP (oligosaccharides) on fish has been attributed to the adverse effect on diffusion and convective transport of digestive enzyme, nutrients and bile salts resulting in low absorption of fat through impairing emulsification (Storebakken *et al.*, 1998a). If this is correct, fish oil inclusion level should be carefully adjusted in diets that contain a high level of NSP. Although the antinutritive effect of alginates and guar gum on fish has been shown in the literature (Storebakken, 1985; Storebakken and Austreng, 1987), these are not the main NSP in most grain legumes (Vila and Mascarell, 1999). Recently digestion of lupin's NSP has been proved for silver perch (Table 1.6).

Table 1.6. Digestibility (%) of different nutrients of lupin (hulls on or dehulled) at various inclusion levels in the diet for silver perch[1]

	Ingredient							
	Lupinus albus				*Lupinus angustifolius*			
	Dehulled		Hulls on		Dehulled		Hulls on	
	(27.7)	(49.5)	(27.7)	(49.5)	(27.7)	(49.5)	(27.7)	(49.5)
DM	77.8	68.2	64.7	59.4	67.6	68.9	50.3	50.8
GE	85.2	74.7	72.7	67.1	74.0	75.0	59.4	58.4
CP	101.4	97.3	96.1	97.0	100.3	99.1	96.6	95.8
P	73.8	61.0	77.5	67.0	80.1	78.0	71.8	72.0
NSP	28.0	20.8	-29.9	-9.9	21.9	5.1	-37.4	-8.7

1. Evans (1998)
Numbers inside the bracket show the inclusion level (%) of lupin in the diet
DM: Dry matter; GE: Gross energy; CP: Crude protein; P: Phosphorus
NSP: Non-starch polysaccharides

Interestingly a higher inclusion level of NSP in the diet not only decreased the digestibility of lipid but also depressed the digestibility of other nutrients including NSP (Table 1.6) resulting in unbalancing between protein and energy ration in the diet. Meanwhile dehulling significantly improved the digestibility of most nutrients including NSP. However, dehulling significantly decreased the phosphorus digestibility for the diets that contained *Lupinus albus*. It seems total NSP cause the main problem of disturbing the nutrients digestibility in silver perch, as oligosaccharides are mainly concentrated in the kernel of lupin as reported by Evans *et al.* (1993).

The detrimental effect of oligosaccharides on protein utilization was minor in rainbow trout (Arnessen *et al.*, 1993). In a comparative study, the viscosity of intestinal chyme was 2.6-4.2 times lower in Atlantic salmon compared to chickens when different soybean products were included in the diet (Refstie *et al.*, 1999). Digestibility was not affected in Atlantic salmon, whereas digestibility of most nutrients including dry matter, nitrogen, ash and phosphorus significantly decreased in chickens when a soybean meal-based diet was compared with a

reduced oligosaccharide soybean meal (RO- SBM) diet (Refstie *et al.*, 1999). However, it should be borne in mind that the inclusion of soybean was considerably higher in the chicken diet than in the Atlantic salmon diet (67% *vs* 48%). In another study, the soybean products altered the normal morphology of the distal intestine in Atlantic salmon when added at a 26% level in the diet. However, the researchers could not specify whether this problem was related to the presence of oligosaccharides or other antinutrients (van den Ingh *et al.*, 1996). Watery faeces following the use of the grain legumes containing a high level of NSP limit the inclusion of grain legume in chicken diets (Farrell *et al.*, 1999). However, high moisture content alone should not be a big concern in aquaculture if other side effects are not caused by consumed grain. This area certainly has the potential for further investigation in aquaculture.

1.5.4.1. Different approaches to overcoming the limitations of using grain legumes

Different physical (heating, soaking, dehulling, extrusion, irradiation), chemical (solvent extraction, enzyme supplementation) and biological (plant breeding, biotechnology) approaches can be used to eliminate or minimize the detrimental effects of antinutrients. However, effective processing methods need a broad knowledge in terms of type, concentration and distribution of antinutrients in the ingredients (Melcion and van der Poel, 1993) and the sensitivity of the target animals to the concerned antinutrients. The major problems are that antinutrients may all behave differently under a particular processing method and the method selected to optimize the destroying of antinutrients may have negative effects on nutrients. This phenomenon makes selection of the best approach to treating the selected ingredient difficult, especially if the target ingredient contains various ANF's (D'Mello, 1995).

If processing conditions (*e.g.* heating) are not optimized, they could indiscriminately destroy essential nutrients (Cambell and van der Poel, 1998) including vitamins and amino acids (Kwok and Niranjan, 1995). Furthermore, the effect of residual antinutrients after processing on digestive physiology is not clear, due to analytical problems and interactions between antinutrient and non-antinutrient fractions in the diet (Lalles and Jansman, 1998). Although the

concentration of some antinutrients has been effectively decreased using plant-breeding techniques (*e.g.* reducing tannins in lupin), this approach is not desirable in every case. It has been shown that some antinutrients function as a natural defense mechanism against pathogens (Dixon and Hosking, 1992), and some others work as metabolite transporters and play a positive role in cold acclimation and confer desiccation tolerance during seed maturation (Jones *et al.*, 1999). The detoxification of some antinutrients through biotechnology techniques with manipulated bacteria that can be successfully practiced in ruminants (Jones and Megarrity, 1986) is not applicable in aquaculture. New approaches should be considered for the processing of grain legumes if there is an interest to include them in monogastric animal diets including aquatic animals' diets.

It has been shown that salmonids can adapt to the inclusion of grain legumes in their diet. Recently, in a study by Refstie *et al.* (1997) rainbow trout showed similar performance in the second phase of the study (29-56 days) to a high inclusion level of soybean meal in their diet, whereas the performance of rainbow trout receiving a soybean meal-based diet was significantly lower in the first phase (1-28 days) compared to the control group (fish meal-based diet). The same phenomenon was reported for Atlantic salmon (Refstie *et al.*, 1998), when some factors that affect the palatability of soybean meal products were not present in the diet.

1.5.5. Potential of Australian grain legumes for expansion of sustainable aquaculture

It has been claimed that plant proteins have the problem of low protein digestibility and an amino acid profile that does not match the requirements of carnivorous fish (Stickney, 1995). However, this is not the case for some grain legumes. Digestibility of nutrients in different grain legumes varies in various aquatic animals. Crude protein digestibility is higher than the values for gross energy and dry matter in most grain legumes (Table 1.7). Lower digestibility of gross energy is mainly integrated with the low dry matter digestibility, as crude protein digestibility showed less variation in grain legumes. The digestibility of crude protein in lupin and chick pea shows the highest and lowest values respectively. The high protein digestibility of some Australian grain legumes has been shown for Atlantic salmon (Carter, 1998; Carter and Hauler, 2000), barramundi (Williams,

1998), rainbow trout (Gomes *et al.*, 1995; Burel *et al.*, 2000b), turbot (Burel *et al.*, 2000b), Australian silver perch (Allan, 1997; Allan *et al.*, 2000a), Nile tilapia (Fontainhas-Fernandez *et al.*, 1999) and shrimp (Smith, 1998; Sudaryono *et al.*, 1999b) (Table 1.7).

Most processing methods including dehulling, protein concentration and extrusion improve digestibility of different nutrients in various aquatic animals. However, aquatic animals do not show the same response to processing methods. Whilst dehulling improved the digestibility of dry matter, crude protein and gross energy of faba bean and lupin in silver perch and shrimp, it significantly lowered the digestibility of the same nutrients for field pea in silver perch (Table 1.7). In addition, while protein concentration improved nutrient digestibility for faba bean and field pea, it reduced the digestibility of protein for lupin in silver perch (Table 1.7).

Digestibility of all essential amino acids in all tested grain legumes in silver perch was high. The highest and the lowest amino acid digestibility in silver perch were recorded for lupin (both *angustifolius* and *albus*) and chick pea respectively (Table 1.8). Both dehulling and protein concentration, significantly improved the digestibility of all essential amino acids of faba bean and field pea in silver perch. In contrast, protein concentration significantly decreased the essential amino acid digestibility for lupin (*angustifolius*). This is reflected in lower protein digestibility of lupin in silver perch. All this evidence shows the importance of selecting the appropriate processing method for different nutrients when plant proteins are formulated in aquatic animal diets. The suitability of different grain legumes (maximum inclusion levels without side effects) with the exception of chick pea, have been tested in various aquatic animals. However, there is no information in the literature that shows the evaluation of all grain legumes for a fish species under the same experimental conditions. The suitability (maximum inclusion level) of grains have mostly been evaluated by randomly adding the concerned grain legume in the diet rather than finding the maximum inclusion level through running the standard dose response studies (*e.g.* Bransden *et al.*, 2001). Maximum inclusion level of grains in some cases has been compared with a control diet that was mainly based on fish or soybean meal. Because of this, comparison of the suitability of the

maximum inclusion level of a grain for a specific fish species is problematic. However, a maximum inclusion of 70% extruded lupin (*albus*) in rainbow trout and a 71.2% inclusion level of lupin seed meal (unknown species) in shrimp when diets were compared with a fish or soybean meal-based diet (as control diet) have been respectively reported in the literature (Table1.9).

Table 1.7. Apparent digestibility coefficients (%) of different nutrients of various grain legumes in different aquatic animals

Ingredient	Form	Species	Dry matter	Crude protein	Gross energy	Ref
Chick pea	Hulls on (raw)	Silver perch	48.7	84.8	53.6	2
Chick pea	Hulls on (raw)	Silver perch	50.8	79.2	54.8	10
Chick pea	Hulls on (raw)	Silver perch	60.6	79.8	61.3	10
Faba bean	Hulls on (raw)	Silver perch	55.9	91.7	62.2	2
Faba bean	Dehulled (raw)	Silver perch	58.2	96.2	58.8	2
Faba bean	*PC	Silver perch	66.3	95.0	73.4	2
Faba bean	Hulls on (raw)	Silver perch	58.0	90.5	59.2	10
Faba bean	Dehulled (raw)	Silver perch	60.4	96.4	59.6	10
Faba bean	*PC	Silver perch	68.7	94.3	73.0	10
Faba bean	Hulls on (raw)	Rainbow trout	66.1	80.2	60.2	3
Field pea	Hulls on (raw)	Silver perch	62.0	84.0	67.0	2
Field pea	Dehulled (raw)	Silver perch	48.9	88.7	54.5	2
Field pea	*PC	Silver perch	85.9	98.6	91.0	2
Field pea	Hulls on (raw)	Silver perch	51.0	81.0	51.0	10
Field pea	Dehulled (raw)	Silver perch	64.3	88.3	63.2	10
Field pea	*PC	Silver perch	88.5	97.0	90.1	10
Field pea	Hulls on (raw)	Shrimp	72	89	83	4
Field pea	Hulls on (raw)	Rainbow trout	66.1	80.4	59.2	3
Field pea	Hulls on (extruded)	Rainbow trout	66.3	87.9	68.9	5
Field pea	Hulls on (extruded)	Turbot	71.5	92.9	77.7	5
Field pea	Extruded (*PC)	Atlantic salmon	80.8	96.7	84.2	6

Continued over page

Continuation of Table 1.7

Ingredient	Form	Species	Dry matter	Crude protein	Gross energy	Ref
Lupin (*Albus*)	Hulls on (raw)	Silver perch	66.8	95.9	70.1	1
Lupin (*Angustifolius*)	Hulls on (raw)	Silver perch	50.3	96.6	59.4	2
Lupin (*Angustifolius*)	Dehulled (raw)	Silver perch	67.6	100.3	74	2
Lupin (*Angustifolius*)	*PC	Silver perch	78.4	97.4	82	2
Lupin (*Angustifolius*)	Hulls on (raw)	Silver perch	52.4	97.1	51.2	10
Lupin (*Angustifolius*)	Dehulled (raw)	Silver perch	69.8	99.5	70.4	10
Lupin (*Angustifolius*)	*PC	Silver perch	80.8	94.8	80.8	10
Lupin (*Albus*)	Hulls on (raw)	Silver perch	64.6	96.1	72.7	2
Lupin (*Albus*)	Dehulled (raw)	Silver perch	77.8	101.4	85.2	2
Lupin (*Angustifolius*)	Dehulled (raw)	Barramundi	60.6	98.1	61.5	7
Lupin (*Angustifolius*)	Hulls on (raw)	Shrimp	39	88	45	4
Lupin (*Angustifolius*)	Dehulled (raw)	Shrimp	73	95	74	4
Lupin (*Angustifolius*)	Hulls on (?)	Rainbow trout	63.3	85.5	61.2	3
Lupin (*Albus*)	Hulls on (extruded)	Rainbow trout	69.7	96.2	77.0	5
Lupin (*Albus*)	Hulls on (extruded)	Turbot	80.5	97.8	85.1	5
Faba bean	Hulls on (raw)	Nile tilapia	66.8	87.5	68.0	8
Field pea	Hulls on (raw)	Nile tilapia	46.1	77.6	45.2	8
Field pea	(?) Extruded	Nile tilapia	85.5	92.6	89.2	8
Lupin (*Albus*)	Hulls on (raw)	Nile tilapia	72.0	90.2	71.8	8
Lupin (*Luteus*)	Hulls on (raw)	Nile tilapia	57.0	92.7	60.0	8
Lupin (?)	Dehulled (raw)	Atlantic salmon	-	96.7	63.1	9
Lupin (?)	Hulls on (raw)	Atlantic salmon	-	97.4	65.7	9

1. Allan (1997); 2. Allan *et al.* (2000a); 3. Gomes *et al.* (1995); 4. Smith (1998); 5. Burel *et al.* (2000b)
6. Carter (1998); 7. Williams (1998); 8. Fontainhas-Fernandez *et al.* (1999); 9. Carter, unpublished data; 10. Booth *et al.* (2001)

Table 1.8. Apparent digestibility coefficients (%) of different amino acids for Australian grain legumes in silver perch

Ingredient	Form	Arg	His	Ile	Leu	Lys	Met	Phe	Thr	Val	Ref
Chick pea (Desi)	Hulls on	80.6	73.3	60.8	65.8	76.1	75.4	66.0	57.6	58.4	1

Chick pea (Desi)	Dehulled	84.3	83.3	64.8	65.4	77.3	72.4	68.8	69.9	62.2	1
Faba bean (Fijord)	Hulls on	93.5	86.9	83.1	87.4	88.7	81.9	85.4	82.3	82.9	1
Faba bean (Fijord)	Dehulled	95.0	90.9	86.7	87.9	94.0	88.7	88.3	86.8	86.2	1
Faba bean (Fijord)	*PC	98.7	99.6	97.1	98.6	100.1	99.2	97.8	99.0	97.3	1
Field pea (Dunn)	Hulls on	88.9	79.2	78.0	78.8	86.1	67.8	79.8	74.5	75.9	1
Field pea (Dunn)	Dehulled	93.7	89.2	80.0	85.3	88.1	79.3	83.0	81.0	78.3	1
Field pea (Dunn)	*PC	100.1	101.6	98.9	100.0	101.0	100.7	99.6	99.7	99.0	1
Lupin (*Albus*)	Hulls on	99.3	100.3	94.9	95.7	99.2	102.2	96.3	95.4	95.5	2
Lupin (*Angustifolius*)	Hulls on	99.3	102.2	96.7	96.6	99.7	67.4	97.8	98.3	97.0	1
Lupin (*Angustifolius*)	Dehulled	99.9	101.4	97.1	97.4	101.4	103.4	98.5	101.7	98.1	1
Lupin (*Angustifolius*)	*PC	97.3	93.6	95.3	94.7	94.8	84.7	94.1	94.8	93.3	1
Lupin (?)	Dehulled	101	97	97.2	96.3	96.6	108.8	97.3	98.7	97.8	3
Lupin (?)	Hulls on	102.6	99.1	98.	98.3	100.9	100.8	96.7	100.6	98.2	3

1. Booth *et al.* (2001); 2. Allan *et al.* (2000a); 3. Carter, unpublished data; *PC: Protein concentrate

Table 1.9. The maximum inclusion of different grain legumes in various aquatic animal diets without affecting the growth response as compared to the control diets

Ingredient	Form	Species	Maximum inclusion in the diet (%)	Control diet based on	Ref
Lupin (?)	Hulls on (raw)	Atlantic salmon	42.4	Fish meal	1
Lupin (*Angustifolius*)	Protein concentrate	Atlantic salmon	29.2	Fish meal	1
Lupin (*Angustifolius*)	Dehulled (raw)	Atlantic salmon	40	Fish meal	16
Field pea (Dunn)	Protein concentrate	Atlantic salmon	27.6	Fish meal	1
Field pea	Hulls on (raw)	Atlantic salmon	44.9	Fish meal	1
Lupin (*Albus*)	Dehulled (extruded)	Turbot	50	Fish meal	2
Lupin (*Albus*)	Hulls on (extruded)	Rainbow trout	70	Fish meal	3
Lupin (?)	Hulls on (raw)	Rainbow trout	40	Soybean meal	4
Lupin (?)	Dehulled (raw)	Rainbow trout	40	Soybean meal	4
Lupin (*Angustifolius*)	Soaked grain	Gilthead sea bream	34.6	Fish meal	5
Lupin (?)	Hulls on (raw)	Rainbow trout	32.6	Fish meal	6
Lupin (*Angustifolius*)	Dehulled (raw)	Shrimp	36.0	Soybean meal	7
Lupin (*Albus*)	Dehulled (raw)	Shrimp	30.0	Soybean meal	8

Continued over page

Ingredient	Form	Species	Maximum inclusion in the diet (%)	Control diet based on	Ref
Lupin (?)	Hulls on (raw)	Rainbow trout	43.2	Soybean meal	9

Faba bean	Hulls on (raw)	Rainbow trout	26	Fish meal	10
Field pea	Hulls on (raw)	Rainbow trout	38.2	Fish meal	10
Field pea	Hulls on (raw)	European sea bass	40	Fish meal	11
Field pea	Hulls on (raw)	Blue Shrimp	30	Fish meal	12
Field pea	Hulls on (extruded)	Blue Shrimp	30	Fish meal	12
Field pea	Dehulled (raw)	Blue Shrimp	30	Fish meal	12
Field pea	Dehulled (extruded)	Blue Shrimp	30	Fish meal	12
Field pea	Hulls on (micronised)	Blue Shrimp	30	Fish meal	12
Lupin (*Angustifolius*)	Dehulled (raw)	Silver perch	25.5	Fish meal	13
Lupin (*Angustifolius*)	Hulls on (raw)	Carp	45	Fish meal	14
Lupin (?)	Hulls on (cooked)	Shrimp	71.2	Fish & shrimp meal	15

Continuation of Table 1.9

1. Carter (1998); 2. Burel *et al.* (2000a); 3. Burel *et al.* (1998); 4. Hughes (1991); 5. Robaina *et al.* (1995); 6. Moyano *et al.* (1992)
7. Sudaryono *et al.* (1999c); 8. Sudaryono *et al.* (1999a); 9. Morales *et al.* (1994); 10. Gouveia *et al.* (1993); 11. Gouveia and Davies (1998)
12. Cuz-Suarez *et al.* (2001); 13. Allan *et al.* (2000b); 14. Viola *et al.* (1998); 15. Sudaryono *et al.* (1995); 16. Bransden *et al.* (2001)

The evidence presented above shows the potential of grain legumes as fish meal replacement. However, the development of sustainable aquafeed using plant proteins requires further study.

Traditionally, salmonid diets contain high levels of protein simply due to the limited ability of fish to use carbohydrates as energy sources (Pack and Rodehutscord, 1995). Meanwhile, feed manufacturers often over-formulate feed because they lack information about the nutrient requirements of some fish species (Naylor *et al.*, 2000). The formulation of lower protein diets using plant proteins should be carried out after concise determination of amino acid requirements and its availability in feeds (Pack and Rodehutscord, 1995).

1.5.6. Effect of grain legumes in fish diets on organoleptic properties of carcass

This is a complex area of investigation, as the final decision regarding the sensor quality of the carcass is made by a control panel and is subjected to some variation. A fish of medium quality for some people could be highly desirable to others. It seems due to this restriction the amount of research that has investigated the changes in the organoleptic properties of carcass following the inclusion of grain legumes in the diet has been limited in aquatic animals. However, the inclusion of 32.6% lupin seed meal in rainbow trout diet (Moyano *et al.*, 1992) and more than 40% grain legume (25.5% dehulled lupin and 14.9% of field pea) in silver perch diet (Allan *et al.*, 2000b) did not affect the organoleptic properties of the fishes. This evidence emphasizes the potential for grain legumes when they were added at a high percentage in fish diet.

1.5.7. Effect of grain legumes in fish diets on immune function of fish

The relationship between digestive tract and immune response (DeWitt and Kudsk, 1999) and diet and immunity (Chandra, 1999) are well established in the literature. Immunostimulant effects of some complex carbohydrates (Galeotti, 1998) have been reported in fish. However, there is little information regarding immunostimulatory or immunosupressive effects of NSP inclusion in fish diet. Page *et al.* (1999) could find no differences between non-specific immune responses after long term feeding of a high carbohydrate diet (gelatinized starch) in rainbow trout. Also various dietary carbohydrates affected the immune response of

Atlantic salmon to a minor extent in both freshwater and saltwater (Waagbo *et al.*, 1994). In contrast, Hemre *et al.* (1995b) reported a significant decrease of hematocrit and hemoglobin at a higher inclusion of starch in Atlantic salmon. It has been shown that the inclusion of soybean molasses (10.8%) in Atlantic salmon diet could significantly increase the level of both lysozyme and an immunoglobulin M (IgM) in the mucosa of the mid and distal intestine (Krogdahl *et al.*, 2000). However, inclusion of 40% dehulled lupin (*angustifolius*) in Atlantic salmon diet did not affect the immune or blood chemistry responses of fishes (Bransden *et al.*, 2001). It was also found that the mortality rate of fishes that received lupin-based diet did not differ from fish on the control diet when fish were challenged with *Vibrio anguillarum* (Bransden *et al.*, 2001). This data suggests that there should be little concern regarding the immune function of fishes when plant meals are added at low or medium levels to the diet. However, this area deserves further investigation.

1.5.8. Experimental design and data analysis in nutritional studies

In nutrition studies sometimes it is necessary to employ an experimental design using a number of treatments in order to test the experimental hypothesis. In this case due to the available tank numbers, some nutritionists have to employ just two replicates for the treatments. It has been stated that the variation of replicates within treatments in indoor conditions is substantially lower compared to outdoor ones (Knud-Hansen, 1997). It seems that low variation let the scientists to carry out the data analysis by ANOVA without conducting non-parametric data analysis using two replicates with some confidence. As a result a number of well-known nutritionists have conducted various number of studies in this manner and have published their research findings in some peer reviewed international journals (Kaushik *et al.*, 1989; Hemre *et al.*, 1995; Kaushik *et al.*, 1995; Refstie *et al.*, 1997; Xie and Jokumsen, 1997; Small and Soares Jr, 1998; Bjerkeng *et al.*, 1999; Farrell *et al.*, 1999; Refstie *et al.*, 1999; Storebakken *et al.*, 2000; Siddhuraju & Becker, 2001). Due to the required number of treatments in some experiments for testing the experimental hypothesis and the available tank numbers, two replicates (tanks) have been allocated for the treatments in some experiments in the current study.

1.6. Importance of salmonids as a screening tool for selecting the plant proteins

Due to the higher sensitivity of salmonids to ANF's compared to omnivorous fishes and the importance of their production, salmonids can be used as a reliable biological tool for screening the most suitable Australian grain legumes. Salmonid production is the third most highly valued industry in Australian aquaculture after the pearl oyster and tuna industries (ABARE, 2000b). The gross value of salmonid production in 1998/99 reached approximately $79 million (ABARE, 2000b). Salmonid production constituted more than 56% of Australian fish production in terms of quantity. Tasmania was the dominant salmonid producer in Australia producing more than 90% of the salmonids in terms of value in 1998/1999 (ABARE, 2000b). It seems salmonids are more sensitive to the detrimental effects of ANF's in grain legumes compared to other fish species, as their natural diet is antinutrient free. Meanwhile, salmonids were the biggest global users of fish meal, consuming 29.8% of the total fish meal used in the aquaculture sector in 2000 (Barlow, 2000). This proportion is expected to fall to 18.5% by 2010 (the highest reduction of fish meal use in all farmed aquatic animals), as more fish meal is included in diets of other current fast-growing farmed aquatic animals (Table 1.1). Because of these reasons, salmonids were used to screen the most suitable grain legume produced in Australia as a fish meal replacement. The low production of fish meal in Australia may facilitate further research for finding fish meal alternatives in aquaculture. This country imported 32,011 tonnes of fish meal valued at about $ 21.12 million in 1999/2000 (ABARE, 2001). Despite the heavy dependence of the Australian aquaculture feed industry on imported fish meal (90% import) at this stage, simply focusing on fish meal reduction in aquaculture diets without cautiously applying nutritional management tools is not feasible. Currently the total cost involved in fish meal importation is negligible (about $21 million) compared to the value of Australian aquaculture production ($700 million) (Buxton, 2001). Considering the rapid expansion of the aquaculture industry in Australia and the predicted increase in demand for fish meal in the future, fish meal replacement studies are becoming increasingly important.

1.7. Future directions

The biggest soybean producer in the world (United States of America) subsidizes soybean production. That is why the price of soybean meal has been reasonably economic for fish farmers despite some occasional fluctuations in the past. With future limitations on the use of fish meal there is no doubt that fish meal inclusion in fish diet must be decreased globally. Due to some constraints on the use of soybean meal in aquafeeds (high inclusion of antinutrients, cost competition of its use in other animal production sectors), there are benefits to identify replacements for soybean meal as well as for fish meal. Nutritionists, feed technologists and agronomists should work closely together to develop suitable grain legume varieties considering the latest nutritional research findings and using the most efficient technologies. This should be implemented with minimum cost to fulfill the nutrient requirements of cultured fish, considering the variation that exists among fish species (and different stage of growth) and due to the various seasons, to ensure that the highest possible nutrient utilization and growth coincides with the lowest environmental impact.

ABARE projections (ABARE, 2000a) state that Australia's rapidly growing aquaculture industry will be the most efficient in the world with an annual sale capacity of AU$ 2.5 billion in 2010. If this indeed is the case, considering the great potential for grain legume production in Australia, establishing suitable protein sources to replace fish meal will have a substantial effect on expanding aquaculture, not only in Australia but also in the rest of the world. This may be beneficial for both Australian aquaculture and agriculture sectors as the biggest fish-producing region in the world (South East Asia), with a large potential market for suitable grain legumes is located close to Australia (Edwards and van Barneveld, 1998).

1. 8. Scope and aims of research

This study addresses the lack of enough information about the suitability of Australian grain legumes as fish meal replacements for fish in general and for salmonids in particular. The specific aims were as follows:

1. To evaluate the suitability of the four most abundant Australian grain legumes as fish meal replacements based on both chemical analysis and digestibility studies.

2. To examine the effect of various processing methods on nutrient and antinutrient content of the grains and on the digestibility of different nutrients.

3. To test the suitability of an *in vitro* digestibility assay for the prediction of the nutrient digestibility of both raw and processed grains.

4. To study the effect of fish size and the adaptation period on the nutrient digestibility of the diets containing different grain legumes.

5. To screen the most suitable grain legume on the basis of both *in vitro* and *in vivo* studies.

6. To determine the highest possible inclusion level of the screened grain in rainbow trout diet.

7. To investigate the limiting factors of effective utilization of the screened grain at high inclusion levels in the diet.

8. To consider the best possible approaches for improving the utilization of the selected grain legume by rainbow trout.

9. To test the suitability of the best grain as fish meal replacement for Atlantic salmon.

10. To evaluate the effects of feeding time and different crude protein levels of the diets containing the selected grain legume on feed intake, nutrient utilization and growth performance of Atlantic salmon.

Chapter Four of this thesis is published as "Growth, physiological and immunological responses of rainbow trout (*Oncorhynchus mykiss*) to different dietary inclusion levels of dehulled lupin (*Lupinus angustifolius*)" (Farhangi and Carter, 2001). Each chapter, whilst part of a sequence of experiments, was written as a stand alone piece of work to enable publication in peer-reviewed journals. A result of this strategy was that some information is repeated in different chapters.

Chapter Two

Comparative chemical composition of four main Australian grain legumes subjected to various processing methods as fish meal replacement in rainbow trout (*Oncorhynchus mykiss*) feeds

2.1. Introduction

Fish meal closely approximates the chemical composition necessary to fulfill fish nutrient requirements for maintenance and growth. All other protein sources, whether of plant or animal origin, rarely match the nutrient requirements of fish. Successful replacement of fish meal with other protein sources relies on a broad understanding of both nutrient and antinutrient contents of alternative ingredients. Both nutrients and antinutritional factors (ANF's) are unevenly distributed in different fractions of ingredients. For example, some antinutrients including condensed tannin and polyphenol (Deshpande *et al.*, 1982; Alonso *et al.*, 2000) are concentrated in the hull fraction of grains. In contrast, other groups of antinutrients including trypsin inhibitors (TI), chymotrypsin inhibitors (CTI), alpha-amylase inhibitors, phytic acid (PA) (Deshpande *et al.*, 1982, Alonso *et al.*, 2000) and oligosaccahrides (Evans *et al.*, 1993) are mainly located in the kernel of grain legumes. Understanding this issue for each ingredient is of paramount importance, as in many cases an appropriate processing method can be adopted to improve the nutritional value of alternative plant protein sources. It has been shown that significant quantitative and qualitative removal of nutrients may happen if an inappropriate processing method is used (Singh, 1995; Fellows, 2000).

Protein is the most expensive fraction of fish feeds (Robinson and Li, 1995). As fish diets contain 2-4 times more protein compared to the diets of other vertebrates (Wilson, 1994a) the main focus in most fish meal replacement studies has been on proteins. However, in order to obtain a comprehensive picture about the suitability of an ingredient, a broad knowledge regarding all available nutrients in the ingredient is essential. Although the bioavailability of nutrients is the ultimate determinant of optimum metabolism and the subsequent growth performance of animals, chemical analysis is the first stage in the evaluation of the nutritional value of a feedstuff (Pond *et al*, 1995). This is especially true when the suitability of a

wide range of ingredients is tested for a specific animal through a range of screening assays. Lower cost and increased repeatability involved in chemical analyses are the most important factors, resulting in the widespread use of these techniques (Pond *et al*, 1995).

Aquaculture is currently one of the fastest growing primary industry sectors in Australia with an annual average increase of 20% (CSIRO, 1999). To maintain this trend, good quality and sustainable aquafeed should be available in the future. More than 90% of fish meal (approximately 30,000 tonnes) being used in Australia is imported. Meanwhile, the quality of the 10% local production is usually inconsistent (Brumble, personal communication). It has been claimed that alternative protein sources must contain at least 35% protein to be considered as a potential fish meal replacement for salmonids (Hardy, 1995). Much research has been conducted in the last two decades to substitute some part of fish meal in fish diets, without much success (Rumsey, 1993; Smith and Ab, 1995). However, until now soybean has been recognized as the best alternative for fish meal (Lovell, 1988; Tacon, 1994; Webster *et al.*, 1995b). The price of soybean is high due to the lack of suitable conditions for its cultivation in most parts of the world and competition for its use in other animal production systems (Hughes, 1991). The production of soybean in Australia is low due to the lack of suitable growing conditions. Australia produced only 0.072% of the total world production of soybean meal in 1999/2000 (Australian Oilseeds Federation, 2001). Fortunately, Australia has a great potential for production of other plant proteins (grain legumes) (Siddique and Skyes, 1997).

Grain legumes contain different ANF's that may limit their utilization, especially by monogastric animals (Waldroup and Smith, 1989). Of the antinutrients, protease inhibitors (PI) and PA have attracted most attention in the past. PI (especially TI) are groups of proteins that induce their antinutritional effect through competitively binding with protease enzymes (Petterson *et al.*, 1999), resulting in pancreatic hypertrophy/hyperplasia, which impairs growth performance (Liener, 1994). PA and its lower phosphate homologues are the main stored phosphorus fraction of the seed that interfere with the efficient absorption of some minerals including zinc, iron and calcium from the digestive tract of monogastrics (Petterson *et al.*, 1999).

PA has been shown to interfere with the normal hydrolysis of proteins in rainbow trout (Spinelli *et al.*, 1983).

It has been suggested that carnivorous fish are less capable of utilizing diverse diets than omnivorous and herbivorous fish (Walton, 1986). Both lipids and carbohydrates are considered as the energy sources, however, lipids are better utilized to produce energy compared with carbohydrates for most aquatic animals. The capacity to spare the protein using lipids and carbohydrates varies greatly among different fish species. Fortunately the price differences between lipids and carbohydrates and protein sources have made the protein sparing effect a cost effective approach in aquaculture nutrition (De Silva and Anderson, 1995). Warm water fish are able to utilize much higher carbohydrate inclusion in their diet than cold water or marine fish (Wilson, 1994b). Low efficiency of carbohydrate utilization as energy sources by carnivorous fish has been attributed to the natural environment in which they evolved (McGoogan and Reigh, 1996). The reason for such a phenomenon has yet to be elucidated. It has also been shown that cold water carnivorous fish are more efficient in their digestion and metabolism of carbohydrates at higher temperatures (Medale *et al.*, 1991). In addition, the energy derived from gelatinized starch was significantly higher compared to that from raw starch for rainbow trout (Medale *et al.*, 1991). Various processing methods can improve the nutritional value of grain legumes (Gouveia *et al.*, 1993; Melcion and van der Poel, 1993; Fernandez *et al.*, 1996; Nestares *et al.*, 1996; Chau and Cheung, 1997) and most fish species utilize cooked starch more efficiently compared to raw starch (Wilson, 1994b). Information about the suitability of Australian grain legumes for salmonids and the effect of various processing methods on their nutritional value is limited.

The objectives of this research were to evaluate the nutritional potential of the four most abundant grain legumes in Australia for inclusion in salmonid diets, using different chemical analysis techniques. The effect of various processing methods on the chemical composition and some relevant ANF's of the tested grains has also been evaluated. The direct assessment of the nutritional value of the tested grains is presented in Chapter Three.

2.2. Material and methods

2.2.1. Grain preparation and processing

Grain legumes tested in this experiment consisted of chick pea (*Cicer arientinum*), faba bean (*Vicia faba*), field pea (*Pisum sativum*) and sweet lupin (*Lupinus angustifolius*). Grains were obtained from Milne Feeds Company, (Western Australia), and were Australian in origin. Grain legumes were tested as raw (R), soaked (S), heated (H) and soaked & heated (SH). Whole grains were soaked overnight (12h) in distilled water at room temperature (25°C), rinsed with distilled water and air-dried (Soaked grain). Whole grains were dry heated using a pressurized autoclave at 105°C for 10 minutes (Heated grain). After the moisture content of the soaked grains was reduced to about 30%, a batch was heated by pressurized autoclave as described previously (Soaked and heated grains).

2.2.2. Chemical analysis

All samples were milled to pass through a 1 mm screen before analysis, which was carried out in duplicate. Dry matter was determined using a freeze drier (Dynavac). Gross energy was determined by combustion in a bomb calorimeter using benzoic acid as a standard (Galenkamp Auto Bomb). Total lipid was measured by chloroform: methanol (2:1) lipid extraction (AOAC, 1990). Neutral detergent fibre (NDF) was determined using the method described by van Soest *et al.* (1991). Briefly, about 1 g dry sample (W_1) was taken and refluxed for 1 h in NDF detergent solution (pH 7) that included the heat stable amylase. The digested sample then filtered, dried at 105°C for 6 h and weighed (W_2). The dried sample ignited at 550°C for three h (W_3). The percentage of NDF calculated using the following equation (NDF % DM = $100 \times (W_2-W_3)/W_1$. Total starch content was quantified using amiloglucosidase and α-amylase method (Megazyme Kit, Australia). Gelatinized starch was determined by measuring the free glucose using a Sigma Kit (510-A). Total nitrogen was measured using the Kjeldhal method and protein content estimated as N×6.25. The amino acid profile with the exception of tryptophan was quantified using the method described by Elkin and Griffith (1985). Samples were oxidized and hydrolyzed at 110°C for 24 h with 6 N hydrochloric acid under nitrogen. Separation of amino acids was carried out using ion exchange chromatography on water High Pressure Liquid Chromatography (HPLC), (column No. 80002). Amino acids were then quantified at 540 nm with

predetermination at 436 nm after post column reaction with ninhydrin. For measuring the mineral content, samples were first digested with nitric/perchloric acid in a block digestion to a temperature of 200-210°C until white fume of perchloric acid formed. After cooling, the mixture was diluted using deionized water. The mineral content then quantified by Inductively Coupled Plasma Atomic Emission Spectrometry (ICPAES).

TI activity was determined using the procedure described by (Smith et al., 1980) through measuring the loss of activity of added trypsin under standard condition. Briefly, TI extracted from a sample through applying diluted hydrochloric acid. The sub-sample of TI was incubated at 37°C for 10 min. Then the synthetic substrate of a-N-Benzoyl-DL-arginine-p-nitroaniliade (BAPNA) was added and the samples incubated again in the same condition. The reaction then stopped by adding trichloroacetic acid. Due to the presence of BAPNA the remaining trypsin acted on BAPNA and changed the color of the solution to yellowish by releasing p-nitroaniline that subsequently was quantified at 410 nm. The differential of absorbance in the presence of TI was considered as a measure of TI activity. PA quantification was carried out using the method of Latta and Eskin (1980). PA extracted using 2.4% HCl. The extract then neutralized and interfering substances (*e.g.* inorganic phosphorus) were separated from PA using ion-exchange chromatography. The isolated PA then quantified by ICPAES.

2.2.3. Statistical analysis

One-way analysis of variance (ANOVA) was used to compare the chemical composition of raw grains. To evaluate the effect of different processing methods on the chemical composition of the grains, a two-way ANOVA was used, based on a completely randomized design (CRD). Grains and processing methods were chosen as the independent variables and an interaction effect allowed. Type I sums of squares were used because all factors were fixed effects in this experiment (Zar, 1996). The results of the ANOVA were reported in terms of the statistical significance of each main factor and of the interaction. The strength of each factor and of the interaction were also indicated by calculating the proportionate contribution of that factor to the corrected total sum of squares. Significant differences within grains and processing method treatments were explored post hoc

by Tukey test. Differences were regarded as statistically significant at probabilities of 5% or less. Tabulated results were presented as mean ± S.E.M. (standard error of mean). All statistical tests were performed using the SPSS Statistical Analysis Software Program (Version 9.0.1 for Windows, 1999).

2.3. Results

There was wide variation in the chemical composition of the tested grain legumes (Table 2.1).

Table 2.1. Chemical composition of unprocessed Australian grain legumes
Mean (± S.E.M., *n* =2)

	Chick pea	Faba bean	Field pea	Lupin	P
DM (%)	91.71 ± 0.03^{b}	89.71 ± 0.59^{a}	89.60 ± 0.18^{a}	91.85 ± 0.03^{b}	≤ 0.01
CP (%)	22.79 ± 0.70^{a}	26.00 ± 0.56^{b}	26.11 ± 0.37^{b}	31.83 ± 0.05^{c}	≤ 0.001
GE (MJ/kg)	18.80 ± 0.03^{ab}	18.34 ± 0.16^{a}	18.47 ± 0.12^{ab}	19.25 ± 0.33^{b}	≤ 0.05
CL (%)	7.89 ± 0.79^{b}	2.38 ± 0.30^{a}	2.60 ± 0.11^{a}	7.45 ± 0.80^{b}	≤ 0.001
NDF (%)	11.52 ± 0.14^{b}	13.66 ± 0.67^{c}	9.84 ± 0.25^{a}	20.63 ± 0.04^{d}	≤ 0.001
Total starch (%)	36.15 ± 1.52^{b}	35.71 ± 0.01^{b}	39.83 ± 0.47^{c}	0.36 ± 0.01^{a}	≤ 0.001
TIA (mg/g)	4.26 ± 0.33^{c}	1.08 ± 0.02^{b}	1.42 ± 0.03^{b}	0.00 ± 0.00^{a}	≤ 0.001
PA (%)	0.80 ± 0.01^{a}	1.44 ± 0.01^{c}	1.15 ± 0.01^{b}	0.76 ± 0.01^{a}	≤ 0.001

Different superscripts indicate significant differences between grains
DM: Dry matter; CP: Crude protein; GE: Gross energy; CL: Crude lipid
NDF: Neutral detergent fibre; TIA: Trypsin inhibitor activity; PA: Phytic acid

Crude protein content was significantly higher and lower for lupin and chick pea, respectively, compared to the other two grains, which had a similar crude protein content. Gross energy was highest for lupin and was significantly higher than the value obtained for faba bean. The crude lipid content for lupin and chick pea was similar, and was significantly higher than for the two other grain legumes. NDF content was significantly higher and lower for lupin and field pea respectively, compared to the other grains. Total starch content of lupin was significantly lower than for other grain legumes, whereas this nutrient was significantly higher for field pea compared to the other grains. For evaluated ANF's, the TI content of lupin was significantly lower than for all other grains, whereas the TI content of chick pea was significantly higher than for all other grains. Similarly, the PA content of lupin was significantly lower and the PA content of faba bean was significantly higher than for the other grains.

With the exception of Lys and (Met + Cys), that were not significantly different for tested grain legumes, the values for all other amino acids varied widely and were significantly different (Table 2.2). Differences between the Arg and Ile content of grain legumes were more obvious and were significantly higher in lupin. Also the ratio of essential amino acids (EAA) to non-essential amino acids (NEAA) was significantly higher in lupin compared to chick pea.

Table 2.2. Amino acid composition of unprocessed Australian grain legumes. Mean (% / DM ± S.E.M., $n = 2$)

	Chick pea	Faba bean	Field pea	Lupin	P
Arg	2.71 ± 0.08^{a}	3.10 ± 0.07^{ab}	2.69 ± 0.05^{a}	3.60 ± 0.14^{b}	≤ 0.01
His	0.55 ± 0.06^{a}	0.67 ± 0.00^{ab}	0.60 ± 0.04^{ab}	0.83 ± 0.05^{b}	≤ 0.05
Ile	0.99 ± 0.02^{a}	1.15 ± 0.02^{ab}	1.16 ± 0.02^{b}	1.37 ± 0.04^{c}	≤ 0.01
Leu	1.63 ± 0.06^{a}	1.97 ± 0.03^{ab}	1.92 ± 0.06^{ab}	2.18 ± 0.09^{b}	≤ 0.05
Lys	1.37 ± 0.07	1.43 ± 0.14	1.74 ± 0.1	1.43 ± 0.06	NS
Met + Cys	0.34 ± 0.02	0.33 ± 0.01	0.33 ± 0.01	0.40 ± 0.01	NS
Phe + Tyr	1.99 ± 0.07^{a}	2.12 ± 0.03^{a}	2.19 ± 0.06^{ab}	2.53 ± 0.09^{b}	≤ 0.05
Val	0.99 ± 0.04^{a}	1.24 ± 0.01^{b}	1.20 ± 0.04^{ab}	1.30 ± 0.05^{b}	≤ 0.05
EAA/NEAA[1]	49.37 ± 0.15^{a}	50.64 ± 0.37^{ab}	51.58 ± 0.54^{ab}	53.39 ± 0.73^{b}	≤ 0.05
Chemical score	0.60	0.51	0.57	0.61	

Different superscript indicates significant differences between grains
1. Essential amino acids (EAA) /non essential amino acids (NEAA)

Macro-mineral composition of grains varied widely (Table 2.3). The calcium and magnesium content of lupin were significantly higher than for the other grains, whereas the phosphorus content of faba bean was significantly higher than for the other grain legumes. The potassium content of faba bean and field pea was similar, however it was significantly lower in the two other grain legumes. Sodium concentration was very low compared to other macro-elements for all the grain legumes, and was highest in faba bean.

Table 2.3. Macro-mineral composition of unprocessed Australian grain legumes Mean (g/kg DM ± S.E.M, *n* = 3)

	Chick pea	Faba bean	Field pea	Lupin	P
Ca	2.3 ± 0.00^{c}	1.1 ± 0.007^{b}	0.8 ± 0.009^{a}	2.5 ± 0.00^{d}	≤ 0.001
P	4.1 ± 0.04^{a}	5.6 ± 0.06^{c}	5.1 ± 0.08^{b}	4.2 ± 0.04^{a}	≤ 0.001
Mg	1.7 ± 0.007^{b}	1.6 ± 0.01^{a}	1.7 ± 0.02^{b}	2.0 ± 0.01^{c}	≤ 0.001
K	11.6 ± 0.1^{a}	13.27 ± 0.07^{b}	12.9 ± 0.01^{b}	11.1 ± 0.07^{a}	≤ 0.001
Na	0.1 ± 0.00^{c}	0.34 ± 0.007^{d}	0.01 ± 0.00^{a}	0.08 ± 0.00^{b}	≤ 0.001

Different superscript indicates significant differences between grains

Micro-mineral composition of tested grain legumes also showed a wide variation (Table 2.4). While the copper and iron content of lupin were significantly lower, its manganese and zinc content were significantly higher than in the other grain legumes. The iron composition of faba bean was similar to chick pea and significantly higher than for the two other grains. Also the copper composition of faba bean was significantly higher than in all other grains.

Table 2.4. Micro-mineral composition of unprocessed Australian grain legumes Mean (mg/kg DM ± S.E.M, *n* = 3)

	Chick pea	Faba bean	Field pea	Lupin	P
Cu	8.14 ± 0.1^{c}	10.55 ± 0.04^{d}	6.96 ± 0.13^{b}	5.15 ± 0.09^{a}	≤ 0.001
Fe	74.25 ± 0.19^{c}	76.98 ± 0.22^{c}	62.13 ± 0.98^{b}	48.63 ± 0.73^{a}	≤ 0.001
Mn	30.89 ± 0.36^{c}	16.95 ± 0.07^{b}	14.21 ± 0.33^{a}	69.86 ± 0.79^{d}	≤ 0.001
Zn	34.22 ± 0.38^{b}	24.85 ± 0.22^{a}	26.040 ± 0.37^{a}	38.46 ± 0.36^{c}	≤ 0.001

Different superscript indicates significant differences between grains

The great proportion of variation for most nutrients was explained by grain type rather than processing method (Table 2.5). The processing effect was significant for all nutrients with the exception of NDF and crude lipid; however, it was more evident for dry matter, total starch and TI. The interaction of processing and grain was significant for all nutrients with the exception of crude lipid, NDF and total starch. While the combination of soaking and heating significantly decreased the TI, the same procedure significantly increased the PA content of the grains.

Amino acid profiles of the raw and processed (soaked, heated and soaked and heated) grains are shown in Table 2.6. Although a statistical comparison of the effect of processing methods on the amino acid profile of grain was not possible due to the lack of replication, it is unlikely that different processing methods affected the amino acid profile of the grains.

Much of the macro minerals variation was explained by grain type rather than processing method (Table 2.7). However, this value was more evident for potassium. Nevertheless, the effect of both grains and processing methods were significant for all macro minerals. While the interaction of grain and processing was significant for most macro minerals, this value was not significant for magnesium. The great proportion of micro-minerals variation was explained by grain type rather than processing method (Table 2.8). However, the effects of both grains and processing methods were significant for all micro-minerals. While the interaction of grain and processing was significant for most micro-minerals, this value was not significant for copper.

Table 2.5. Mean (± S.E.M., n = 8) of chemical composition of grains explained by grain type (G), processing method (P) or interaction of G and P

	DM (%)	CP (%)	GE (Mj/kg)	CL (%)	NDF (%)	Total starch (%)	G Starch[1] (%)	TI (mg/g)	PA (%)
Grain (combination effect of all processing methods)									
Chick pea	90.59 ± 0.34^{c}	22.71 ± 0.18^{a}	18.93 ± 0.06^{b}	6.92 ± 0.35^{b}	9.89 ± 0.51^{a}	35.98 ± 0.30^{b}	0.07 ± 0.05^{a}	3.75 ± 0.36^{d}	0.82 ± 0.007^{b}
Faba bean	89.38 ± 0.31^{b}	26.20 ± 0.16^{b}	18.39 ± 0.05^{a}	2.58 ± 0.08^{a}	12.69 ± 0.39^{b}	36.17 ± 0.37^{b}	1.13 ± 0.29^{a}	0.91 ± 0.10^{b}	1.45 ± 0.009^{d}
Field pea	89.95 ± 0.36^{a}	25.62 ± 0.34^{b}	18.40 ± 0.05^{a}	2.45 ± 0.10^{a}	9.82 ± 0.20^{a}	40.60 ± 0.20^{c}	0.14 ± 0.09^{a}	1.25 ± 0.10^{c}	1.16 ± 0.008^{c}
Lupin	90.80 ± 0.49^{c}	32.81 ± 0.30^{c}	19.55 ± 0.10^{c}	7.53 ± 0.14^{c}	20.67 ± 0.29^{c}	0.73 ± 0.15^{a}	33.30 ± 8.24^{b}	0.00 ± 0.00^{a}	0.79 ± 0.009^{a}
Processing (combination effect of all grain types)									
Raw	90.72 ± 0.41^{c}	26.68 ± 1.24^{ab}	18.72 ± 0.14^{a}	5.08 ± 0.99	13.91 ± 1.55	28.01 ± 6.07	5.14 ± 3.26^{b}	1.69 ± 0.60^{b}	1.038 ± 0.10^{a}
Soaked	88.30 ± 0.26^{a}	27.39 ± 1.51^{c}	19.00 ± 0.22^{b}	4.87 ± 0.84	13.13 ± 1.88	27.99 ± 6.07	14.22 ± 8.98^{c}	1.70 ± 0.61^{b}	1.068 ± 0.10^{b}
Heated	90.25 ± 0.36^{b}	26.22 ± 1.45^{a}	18.81 ± 0.15^{a}	4.53 ± 0.79	13.32 ± 1.66	28.73 ± 6.01	1.44 ± 0.72^{a}	1.66 ± 0.60^{b}	1.063 ± 0.10^{b}
Soaked & Heated	90.45 ± 0.22^{bc}	27.05 ± 1.42^{bc}	18.74 ± 0.23^{a}	4.99 ± 1.01	12.7 ± 1.72	28.74 ± 6.16	13.83 ± 8.63^{c}	0.85 ± 0.30^{a}	1.061 ± 0.10^{b}
Two-way ANOVA: Proportion of total variation (%) explained by main effects and interaction									
Grain	37.43***	96.66***	86.99***	95.23***	95.42***	99.79***	62.98***	88.16***	99.36***
Processing	55.62***	1.33***	4.76***	0.73	0.93	0.05*	9.49***	5.99***	0.18**
G x P	5.83***	1.171	5.63***	2.65*	1.52	0.07	27.12***	5.46***	0.3*

Different superscript shows significant differences between grain legumes within processing levels. * P ≤ 0.05; ** P ≤ 0.01; *** P ≤ 0.001

Table 2.6. Comparative amino acid profile of raw (R), soaked (S), heated (H) and soaked & heated (SH) for major Australian grain legumes (% DM basis, n = 1)

	Arg	His	Ile	Leu	Lys	Met	Phe	Thr	Val
Chick pea-R	2.72	0.55	1.00	1.64	1.37	0.32	1.30	0.86	0.99
Chick-S	2.86	0.55	1.08	1.72	1.41	0.36	1.35	0.91	1.04
Chick-H	2.70	0.59	0.98	1.64	1.39	0.30	1.29	0.85	1.01
Chick-SH	2.54	0.56	0.96	1.58	1.30	0.30	1.28	0.82	0.97
Faba bean-R	3.10	0.67	1.15	1.97	1.43	0.24	1.18	1.03	1.24
Faba-S	3.09	0.70	1.18	2.00	1.62	0.23	1.20	1.03	1.27
Faba-H	3.03	0.68	1.15	1.94	1.58	0.23	1.17	1.01	1.24
Faba-SH	3.06	0.69	1.16	1.93	1.57	0.23	1.17	1.01	1.24
Field pea-R	2.70	0.60	1.16	1.92	1.74	0.28	1.29	1.02	1.21
Field-S	2.68	0.63	1.16	1.91	1.79	0.29	1.30	1.02	1.22
Field-H	2.62	0.63	1.12	1.88	1.70	0.27	1.26	1.01	1.18
Field-SH	2.64	0.55	1.18	1.93	1.79	0.30	1.29	1.04	1.23
Lupin-R	3.61	0.84	1.37	2.18	1.44	0.26	1.30	1.20	1.30
Lupin-S	3.67	0.85	1.38	2.19	1.46	0.24	1.30	1.17	1.28
Lupin-H	3.74	0.89	1.42	2.26	1.43	0.25	1.32	1.21	1.33
Lupin-SH	4.00	0.90	1.45	2.28	1.52	0.25	1.37	1.23	1.33

Table 2.7. Mean (g/kg DM ± S.E.M., n = 8) of macro-mineral composition of grains explained by grain type (G), processing method (P) or interaction of G and P

	Ca	K	Mg	Na	P
Grain (combination effect of all processing methods)					
Chick pea	2.2 ± 0.02^{c}	11.0 ± 0.1^{a}	1.67 ± 0.01^{b}	0.120 ± 0.006^{c}	4.0 ± 0.03^{a}
Faba bean	1.1 ± 0.01^{b}	12.9 ± 0.1^{c}	1.57 ± 0.01^{a}	0.269 ± 0.009^{d}	5.6 ± 0.04^{d}
Field pea	0.8 ± 0.005^{a}	12.2 ± 0.02^{b}	1.64 ± 0.01^{b}	0.025 ± 0.004^{a}	5.0 ± 0.04^{c}
Lupin	2.5 ± 0.02^{d}	10.9 ± 0.8^{a}	2.05 ± 0.01^{c}	0.093 ± 0.01^{b}	4.2 ± 0.03^{b}
Processing (combination effect of all grain types)					
Raw	1.7 ± 0.2^{b}	12.2 ± 0.3^{c}	1.76 ± 0.06^{b}	0.132 ± 0.04^{b}	4.7 ± 0.2^{bc}
Soaked	1.7 ± 0.2^{b}	11.4 ± 0.02^{b}	1.78 ± 0.06^{b}	0.131 ± 0.03^{b}	4.8 ± 0.2^{c}
Heated	1.6 ± 0.2^{a}	12.1 ± 0.03^{c}	1.71 ± 0.006^{a}	0.180 ± 0.03^{a}	4.7 ± 0.2^{b}
Soaked & Heated	1.6 ± 0.2^{a}	11.2 ± 0.2^{a}	1.69 ± 0.2^{a}	0.152 ± 0.03^{c}	4.6 ± 0.2^{a}
Two way ANOVA: Proportion of total variation (%) explained by main effects and interaction					
Grain	99.58^{***}	75.45^{***}	94.84^{***}	95.94^{***}	96.15^{***}
Processing	0.27^{***}	18.77^{***}	3.23^{***}	1.37^{***}	1.80^{***}
G x P	0.17^{*}	3.05^{***}	0.51	2.63^{***}	0.77^{*}

Different superscript shows significant differences between grain legumes within processing levels. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$

Table 2.8. Mean (mg/kg /DM ± S.E.M., n = 8) of micro-mineral composition of grains explained by grain type (G), processing method (P) or interaction of G and P

	Cu	Fe	Mn	Zn
Grain (combination effect of all processing methods)				
Chick pea	8.28 ± 0.08^{c}	70.27 ± 1.04^{d}	31.099 ± 0.28^{c}	34.92 ± 0.26^{c}
Faba bean	10.29 ± 0.1^{d}	66.54 ± 1.94^{c}	16.40 ± 0.20^{b}	24.56 ± 0.28^{a}
Field pea	6.85 ± 0.1^{b}	60.70 ± 0.77^{b}	14.098 ± 0.18^{a}	25.92 ± 020^{b}
Lupin	5.09 ± 0.08^{a}	48.08 ± 0.54^{a}	71.10 ± 0.58^{d}	39.32 ± 0.45^{d}
Processing (combination effect of all grain types)				
Raw	7.70 ± 0.59^{bc}	65.49 ± 3.40^{d}	32.98 ± 6.70^{a}	30.89 ± 1.71^{a}
Soaked	7.93 ± 0.58^{c}	61.94 ± 2.24^{c}	34.55 ± 7.17^{b}	32.47 ± 2.00^{b}
Haeted	7.39 ± 0.58^{a}	60.46 ± 2.93^{b}	32.68 ± 6.79^{a}	30.55 ± 1.87^{a}
Soaked & Heated	7.50 ± 0.57^{ab}	57.70 ± 1.86^{a}	32.49 ± 6.91^{a}	30.80 ± 1.88^{a}
Two-way ANOVA: proportion of total variation (%) attributed to main effects and interaction				
Grain	97.61^{***}	81.68^{***}	99.74^{***}	97.27^{***}
Processing	1.13^{***}	9.11^{***}	0.126^{***}	1.47^{***}
Grain x Processing	0.47	8.13^{***}	0.079^{***}	0.79^{***}

Different superscript shows significant differences between grain legumes within processing levels. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.0$

2.4. Discussion

Chemical analyses are useful tools for preliminary screening of the wide variety of alternative proteins available as fish meal replacements in aquaculture. Results obtained from various chemical assays provide an efficient means for the selection of the most suitable ingredients with confidence. The data collected in this experiment, suggest that the tested grain legumes have the potential for consideration as fish meal replacements for aquatic animals. However, like most other ingredients, the nutritional characteristics of these grains do not closely match all the nutritional requirements of aquatic animals when compared with fish meal. This is not a serious concern as ingredients are included in diets combined with other ingredients to closely match the nutrient requirement of aquatic animals. Lower protein and higher carbohydrate (crude fibre and total starch) contents and the existence of some ANF's are the main parameters that may limit the effective utilization of grains in fish diets. In order to select the best grain legumes as the fish meal replacement for rainbow trout, grains were evaluated through different perspectives.

2.4.1. Protein and amino acids

Although the protein content of ingredients is an important factor, the concentration and ratios of constituent amino acids determine its nutritional value (Watzke, 1998). Lupin showed the highest protein content and the best amino acid profile, chemical score and EAA/NEAA ratio, compared with the other grains tested in this experiment (Table 2.9). In contrast, chick pea showed the poorest performance for these parameters. Faba bean and chick pea were located in an intermediate position for these nutrients. Results from this experiment show that the amino acid profile of all the grains tested are generally less favorable when compared to the amino acid composition of fish meal. However, there was a wide variation between the tested grains in this regard. Sakamoto *et al.* (1992) suggested that the balance of amino acids in a protein is more important than a single value of a limiting amino acid. In the current experiment, lupin and chick pea showed the best and worst amino acid profiles respectively. In contrast, Wiryawan and Dingle (1995) ranked lupin protein as the worst, and soybean and chick pea protein as the

best for broiler chicken. They concluded that the grain legumes with the higher protein content usually have a lower amino acid profile value. However, this claim does not support their finding, as the protein content of soybean was the highest of all the plant proteins. This contrasts with the present study and with the data reported by Allan *et al.* (2000a) for Australian grain legumes. Cowey (1995) recommends that for maximal growth performance, the ratio of EAA to NEAA should be equal or greater. Our data successively support the better nutritional value of lupin in terms of amino acid requirements for fish. The amino acid profile of the grains tested and the requirement by rainbow trout of these amino acids are shown in Table 2.9.

Table 2.9. Amino acid composition of major Australian grain legumes (%) and rainbow trout requirements (% of diet)*

	Chick pea	Faba bean	Field pea	Lupin	Requirement[1]
Arg	2.49	2.78	2.41	3.31	1.5
Ile	0.91	1.04	1.04	1.26	0.9
HIis	0.50	0.60	0.54	0.77	0.7
Leu	1.50	1.77	1.72	2.01	1.4
Lys	1.26	1.28	1.56	1.32	1.8
Met + Cys	0.31	0.30	0.32	0.37	1
Phe + Tyr	1.83	1.91	1.97	2.32	1.8
Val	0.91	1.12	1.08	1.19	1.2
EAAI[2]	0.65	0.71	0.73	0.82	_
EAA/NEAA[3]	49.37	50.64	51.58	53.39	> 50[4]
Chemical score	0.605	0.515	0.570	0.615	_

1. NRC (1993)
2. Essential amino acid index = $\sqrt[n]{(a_1/a_2)\times(b_1/b_2)\times\ldots\ldots\ldots(z_1/z_2)}$, McDonald *et al.* (1995)
3. Essential amino acids (EAA) / non essential amino acids (NEAA)
4. Cowey (1995)

* The deficient amino acids of grains have been underlined.

The essential amino acids content of all grains, including Arg, Ile, Leu and (Phe & Tyr), were well above the fish requirements. However, all grains were deficient in Lys and (Met & Cys). In terms of His and Val, all grains were deficient with the exception of lupin. When EAAI was considered, lupin and chick pea showed the highest and the lowest values. However, the chemical score showed the highest and lowest values for lupin and faba bean respectively. These phenomena show the different nutritional values when grains are evaluated from various perspectives. It has been suggested that cereals' protein is rich in sulphur amino acids (Cys and Met) but deficient in Lys. In contrast, legumes' protein is higher than cereals' in terms of Lys and poorer regarding to Cys and Met (Juliano, 1999). However, Lys content of legumes is far from the rainbow trout requirement (Table 2.9). This means that the tested grains cannot fulfill the Lys requirement of fish when included in the diet in an unprocessed form. The tested grains must be accompanied with some rich Lys protein sources such as fish meal. A combination of both legumes and cereals could provide a better amino acid mixture, compared to each ingredient alone in a diet (Juliano, 1999). Although grain legumes are deficient in sulphur amino acids, these amino acids are available in feed-grade form to supplement the deficient formulated diets (Batterham and Egan, 1987).

It has been suggested that cereals' protein is rich in sulphur amino acids (Cys and Met) but deficient in Lys. In contrast, legumes' protein is rich in Lys and poor in Cys and Met compared with cereals (Juliano, 1999).

2.4.2. Carbohydrates

Plant proteins contain significantly higher starch and carbohydrates compared to fish meal. The lowest concentration of total starch in lupin could be an advantage, as a high raw starch content in the diet cannot be effectively utilized, and interferes with the normal digestion of other nutrients including fat in carnivorous fish (Hemre *et al.*, 1995a). It was shown that Atlantic salmon can utilize from 5 to 22% dietary starch, however 9% inclusion of starch has been suggested as an optimum level (Hemre *et al.*, 1995a). The lowest starch content in lupin coincides with the highest inclusion of NDF and total non-starch polysaccharides (NSP). This finding

is in agreement with Wiryawan and Dingle (1995) who reported the highest NDF content for lupin among all grain legumes. NSP were not determined in this experiment. However, findings by other researchers (Perez-Maldonado *et al.*, 1999) confirm that lupin contains the highest NSP of all grain legumes (Table 2.10).

Table 2.10. Insoluble, soluble and total non-starch polysaccharides (NSP) of major Australian grain legumes (g/kg 'as is')[1]

	Chick pea	Faba bean	Field pea	Lupin
Insoluble NSP	84.25	74.87	67.53	216.57
Soluble NSP	4.31	3.43	2.82	18.97
Total uronic acid	33.89	30.96	16.29	48.70
Total NSP	122.45	109.06	86.64	284.24

1. Perez-Maldonado *et al.* (1999)

There is some confusion about categorizing oligosaccharides as a main group among carbohydrates. Some people consider the oligosaccharides as soluble NSP (Bakker *et al.*, 1998), whereas others categorize them into a separate group of carbohydrates (Baghurst *et al.*, 1996). There is general agreement that oligosaccharides cannot be effectively utilized by monogastric animals simply due to a lack of suitable enzymes in the digestive tract (Alloui *et al.*, 1994). Recently the antinutritional effect of NSP and oligosaccharides in fish has been reviewed by Francis *et al.* (2001). Information about the utilization of NSP by fish is limited to few fish species (Evans, 1998). It has been claimed that neither NDF (Davies, 1988) nor NSP (Choct, 1997; Danicke *et al.*, 1999) can be effectively utilized by monogastric animals. Interestingly, digestibility of lupin's NSP has been shown for Australian silver perch (Evans, 1998). There have been some arguments, which attribute some advantages of oligosaccharides inclusion in monogastric animal diets (Iji and Tivey, 1998). The oligosaccharide content of lupin shows the highest concentration for all grain legumes (Table 2.11). Despite these issues, the gross energy content for lupin showed the highest value among the tested grains in this experiment.

Table 2.11. Oligosaccharide[1] composition of major Australian grain legumes (%, as is)

Chick pea	Faba bean	Field pea	Lupin	Source
1.65	2.71	3.53	4.07	Petterson *et al.* (1997)
7.5	Na	4.7	19.1	Vila and Mascarell (1999)

1. Sum of raffinose, stachyose and verbascose. Na: not available

2.4.3. Minerals

The macro and micro-mineral concentration of the grains also showed a wide variation. A comparison of mineral content of the grains and fish requirement (Table 2.12 & Table 2.13) revealed that none of the grains have a perfect mineral composition to closely match the fish requirements. This may also cause a higher interaction between minerals that normally exists in an imbalanced diet, resulting in a lower utilization of minerals and other nutrients (Hardy, 1997). This phenomenon is more critical for ingredients that contain a higher PA content (NRC, 1993).

Consideration of the macro and micro-mineral composition of the tested grains with fish requirements reveals that the micro-mineral composition of grain more closely matches the fish's requirement compared with the grains' macro minerals.

Table 2.12. Macro-mineral composition of major Australian grain legumes (g/kg) with rainbow trout requirements (g/kg of diet)

	Chick pea	Faba bean	Field pea	Lupin	Requirement[1]
Ca	2.1	1.0	0.7	2.3	10.0
P	3.7	5.0	4.5	3.8	6.0
Mg	1.6	1.4	1.5	1.9	0.5
K	10.6	11.9	11.6	10.2	7.0
Na	0.1	0.3	0.0	0.1	6.0
Ca:P	0.57:1	0.20:1	0.16:1	0.61:1	2:1

1. NRC (1993)

Table 2.13. Micro-mineral composition of major Australian grain legumes (mg/kg) with rainbow trout requirements (mg/kg of diet)

	Chick pea	Faba bean	Field pea	Lupin	Requirement[1]
Cu	7.47	9.46	6.23	4.73	3
Fe	68.10	69.04	55.67	44.67	60
Mn	28.33	15.20	12.73	64.17	13
Zn	31.36	22.27	23.35	35.33	30

1. NRC (1993)

This is especially true for the Ca:P ratio, as the optimum Ca:P ratio for most terrestrial animals (McDonald *et al.*, 1995) and fish (NRC, 1993) is suggested to be about 2:1 (Table 2.12). In this regard, the ratio of Ca:P of all grains, and especially of chick pea, faba bean and field pea is far beyond the suggested requirement values by NRC (1993). However, it is proposed that as calcium can be readily absorbed through the water column, determination of the Ca:P ratio in fish is less crucial for other animals (Lall, 1991). There is some evidence that no clear relationship between Ca and P requirements exist for salmonids (Davis and Gatlin, 1996). If this is the case, there should be little concern about the out of range low Ca:P ratio of the tested grain legumes.

2.4.4. Antinutritional factors

2.4.4.1. Trypsin inhibitor

The TI content in plant meals varies widely (Petterson *et al.*, 1997). Tested grains in this study also showed the same phenomenon. This could be considered as a suitable tool for ranking the nutritional value of plant meals. Our finding regarding the significantly lower TI content of lupin supports Iji and Tivey's (1997a) comment that suggests lupin contains the lowest level of protease enzyme inhibitors compared with soybean and all other grain legumes. It has been shown that trout protease enzymes are among the most sensitive enzymes to TI compared to terrestrial animals. For instance its inhibition is 15 times higher than human's trypsin enzyme by TI (Krogdahl and Holm, 1983). Salmon have been shown to tolerate up to 5 mg/kg TI in their diet in seawater (Olli *et al.*, 1994). Rainbow trout

showed better tolerance to TI inclusion in the diet in fresh water (Krogdahl *et al.*, 1994). It was not clear whether the higher tolerance of rainbow trout is related to physiological differences between two fish species or to differences in their habitats (fresh water or salt water). The smaller size of Atlantic salmon (180 g) compared with rainbow trout (265 g) might be one of the reasons for lower tolerance of salmon trypsin activity against TI. Trypsin enzyme contains a high level of sulphur amino acids (Liener, 1989). Fish have to produce more trypsin enzyme in order to compensate for the deleterious effect of TI (Liener, 1989; Krogdahl *et al.*, 1994). This causes depletion of endogenous sulphuric amino acids in the body (Liener, 1989), possibly resulting in a lower growth performance. In addition, grain legumes are deficient in sulphur amino acids (Juliano, 1999). It has been suggested that sulphuric amino acids should be supplemented in fish diets if TI activity of the included plant meal in the diets is high (Krogdahl *et al.*, 1994). However, there is some interesting evidence that shows fish may partially compensate for the inclusion of TI in the diet through increased digestibility of protein in the large intestine (Krogdahl *et al.*, 1994). The protein digestibility was 24% for 0% inclusion of TI compared with 37% digestibility value for the highest inclusion level of TI in the diet.

2.4.4.2. Phytic acid

The phosphorus fraction of PA cannot be utilized by animals (Fernandez *et al.*, 1997). Phytate may also interfere with some physiological functions of fish, including the immune function. It has been shown that both macro and micronutrients control immune function (Suchner *et al.*, 2000) through a regulatory effect on the digestive tract (Cunninghamrundles and Lin, 1998). The phosphorus content of all of the tested grain legumes in this experiment was considerably lower than the values previously reported for soybean meal (Arndt *et al.*, 1999). This could be considered an advantage, as inclusion of lower phosphorus in plant meals in fish diet has increased the availability of phosphorus for rainbow trout (Riche and Brown, 1999). It is suggested that the high phosphorus inclusion in fish diet is one of the main factors causing eutrophication in water resources (Lall, 1991).

Our data is in agreement with (Petterson *et al.*, 1997) finding that reported the lowest PA content in lupin compared to other grain legumes. Due to the low PA level in lupin compared to soybean meal, there is no need to add phytase in lupin-based diets. The phytase pre-treatment of a soybean meal based diet did not have a beneficial effect for rainbow trout (Ramseyer *et al.*, 1999). Similarly, phytase supplementation of rainbow trout diet only improved the utilization of protein and minerals in a low ash diet containing soybean meal (50%). In contrast phytase treatment had no effect on a high ash diet containing both fish meal and soybean meal. The researcher did not investigate the effect of phytase supplementation on the growth performance of fish (Sugiura *et al.*, 2001). It should be borne in mind that in practical conditions successful inclusion of 50% soybean meal in rainbow trout diet has not yet been demonstrated. It is suggested that adding phytase is not advisable in the diet of aquatic animals in practice, despite its theoretical justification (Cho, 1997). It is believed phytase can be destroyed during processing (extrusion) and its inclusion in the diet may release more phosphorus into the environment (Cho, 1997).

2.4.5. Processing

The main objective in using different processing methods in this study was to identify the best approach to improving the nutritional value of grains by preserving essential nutrients (amino acids, minerals), increasing gelatinization of starch and minimizing the level of ANF's (TI and PA). Care should be taken to eliminate or minimize deleterious reactions during the storage and processing period, and to optimize the beneficial ones (Freidman, 1996). However, in practice this is very difficult due to the different responses of various nutrients to specific processing methods. A number of methods can be used to inactivate or remove the ANF's in plant meals (D'Mello, 1995). Reduction or inactivation of ANF's using processing methods needs comprehensive information about type and level, distribution, chemical reactivity and sensitivity of antinutrients to heat in the seed matrix (Melcion and van der Poel, 1993). The distribution of several ANF's in a single grain legume makes processing more difficult, especially when some ANF's are heat stable (D'Mello, 1992). The combination of various factors in processing

may result in a very large range of process effects from mild to severe on the final product (Melcion and van der Poel, 1993). Currently, due to the lack of information regarding kinetic reactions of various chemicals during processing, optimization of processing is not completely possible (Kwok and Niranjan, 1995).

The variation of the chemical composition of grains was mainly explained by grain type rather than processing method. However, the proportion of processing effect on some parameters including dry matter, gross energy, gelatinized starch and TI was more obvious. Processing methods significantly influenced most of the nutrients including crude protein, gross energy, total starch, gelatinized starch, TI and PA. However, crude lipid and NDF were not affected. There was no interaction between the processing method and grain for crude protein, NDF and total starch. This means that predicting the behaviour of these nutrients under different processing conditions is easier compared with other nutrients. A combination of soaking and heating was the most effective way of processing and significantly decreased some parameters including trypsin inhibitors. Both soaking and the combination of soaking and heating significantly increased the gelatinization of total starch.

Amino acids in different grains were not affected by the processing methods applied in this experiment. Excessive heat can reduce protein solubility and even damages some amino acids (Wiryawan and Dingle, 1999). Although information is limited about the effect of different processing methods on the utilization of grain legumes by fish, heating lupin (*albus*) seed at 120°C for 30 minutes depressed rainbow trout performance, despite slightly increased feed intake (dela Higuera *et al.*, 1988). This might be related to the loss of some amino acids due to extra heating. It has been shown that even mild heat treatment at 110°C for 15 minutes reduced the lysine availability of field pea despite its acceptable apparent digestion in growing pigs (van Barneveld, 1993). Extra heating could also reduce the digestibility of protein as indicated for blood meal for Chinook salmon (Hajen *et al.*, 1993). Heating lupin seed did not have any beneficial effects for growing pigs (Batterham *et al.*, 1986), however it improved the metabolizable energy for broilers

(Molina *et al.*, 1983). This might be caused by different processing conditions or specific species related responses in the above experiments.

Starch gelatinization was more obvious when water was added during the processing (soaked and soaked and heated condition). Starch was not effectively converted to its gelatinized form in high starch content grains including chick peas, faba bean and field pea. However, it produced a significant amount of gelatinized starch in lupin, despite its significantly lower starch content. This may be related to the different chemical structure of starch in these grains. It has been suggested that the optimum temperature for gelatinization positively correlates with resistant starch (Juliano, 1999). It has been shown that the optimum temperature for starch gelatinization is grain specific and can be determined using differential scanning calorimetry method (Kosson *et al.*, 1994). The effects of the same processing conditions to all legumes in the current experiment on the nutritional composition resulted was variable. Gelatinization of starch improves both the nutritional value and water stability of diets by acting as a natural binder and precludes use of artificial binders in the processed pellets (Niehues, 1993).

TI was only significantly decreased when the combination of both soaking and heating was used. This shows that heating is highly efficient when it coincides with a high moisture condition. The reason for such a phenomenon is not clear. TI, CTI and lectin are categorized as heat sensitive and estrogen, saponin, alfa galactosides, PA, and amino-acid analogs are known to be heat stable antinutrients (Melcion and van der Poel, 1993). The effectiveness of heat treatment depends on temperature, duration of heating, particle size, as well as initial moisture content and extra water added during processing. These factors should be closely monitored during the processing (D'Mello, 1995; Melcion and van der Poel, 1993). Vidalvalverde *et al.* (1997) reported that the TI content decreased slightly in faba bean soaked in water, whereas soaking up to 96 hours did not affect the TI content in *Canavalia braziliensis* (Carlini and Udedibie, 1997). It is suggested that soaking before heat treatment may reduce the level of water soluble or dispersible fractions in the grain, including amino acid analogs, lectins, PA and oligosaccharides (Melcion and van

der Poel, 1993).

Heat treatment of hexane-extracted, defatted soy flour (autoclave, 1.7 atm, 121°C) significantly reduced trypsin activity by inhibiting from 181 to 1.8 unit after 20 min, whereas the same treatment did not affect PA activity (Arndt *et al.*, 1999). Both soaking in water for 12 hours and autoclaving (121° C for 20 min and pressure of 1.05 kg/cm2) treatment significantly decreased PA, tannins, TI and amylase inhibitors for two Chinese indigenous legumes (Chau and Cheung, 1997). The effect of autoclaving however, was more evident. Both methods significantly improved the *in vitro* protein and starch digestibility of legumes seed reflecting the effectiveness of the processing methods used. It is suggested that the efficiency of some processing methods including autoclaving may be legume specific depending on the concentration of different heat-liable ANF's (Wiryawan and Dingle, 1999). Flatulence producing oligosaccharides of grain legumes can be significantly decreased using soaking (Campbell-Platt and Cook, 1991) and other common processing methods (Ruperez, 1998). Soaking in water, and not heating, significantly improved feed intake of the diet that included faba bean for rat (Fernandez *et al.*, 1997).

As PA is categorized as a heat liable antinutrient, it was not expected that employed processing methods would significantly decrease the phytic acid content in the current experiment. Surprisingly, all processing methods significantly increased the PA content when the effect of processing was evaluated alone. This is in contrast with other findings which have shown that PA can be appreciably decreased using different processing methods, including soaking, cooking, milling and fermentation (Campbell-Platt and Cook, 1991; Vijayakumari *et al.*, 1996). There is some evidence, however, that shows heat treatment increases the PA content of soybean meal (Arndt *et al.*, 1999). The reason for such a phenomenon is difficult to explain.

The variation in the macro-mineral composition of the grains was mainly explained by grain type rather than processing method. Although the effect of processing

methods was significant for all macro minerals, this was most evident for potassium. The effect of combination of soaking and heating on macro mineral composition of the grains was more evident compared with other processing methods. The interaction of processing and grain was significant for all macro-minerals, with the exception of magnesium. This might mean that the response of most macro-minerals under different processing conditions is less predictable, compared with that of magnesium. Similarly the mineral content of the grain legume including calcium, magnesium and iron were little affected by all processing methods used by Ruperez (1998).

The variation of the micro-mineral composition of grains was also mainly explained by grain type rather than processing method. Although the effect of processing methods alone was significant for all micro-minerals, this phenomenon was more evident for iron. Also the interaction of processing and grain was significant for all micro minerals, with the exception of copper. This might mean that the response of most micro-minerals to various processing conditions is less predicable compared with that of copper. This evidence shows the complexity of providing an optimum condition for modifying the concentration of nutrients and antinutrients in an ingredient, based on the desired nutrient requirements of target animals. A better understanding of the molecular changes caused by processing is essential to optimize the nutritional quality and safety issues, while preventing the formation of deleterious compounds (Freidman, 1996). Extrusion is a common processing method for pellet making in aquaculture feed manufacturing. However, it may have both beneficial and deleterious effects on the nutritional value and physical properties of food (Pongmaneerat and Watanabe, 1993). Infrared technology has also been shown to be one of the most effective processing methods compared with other heat processing methods (autoclave, microwave, boiling water) for improving the nutritional value of some legumes through reducing most ANF's and increasing the biological value (Kadam *et al.*, 1987).

The data for nutritional changes cannot be directly used by interested individuals due to the differences in processing conditions and ingredients and equipment used by various operators (Fellows, 2000). Collecting information regarding the effect of different processing methods can be used as tools for screening a wide variety of ingredients. However, reliable data regarding the bioavailability of essential nutrients can only be obtained through feeding the processed ingredients to target animals. It has been noted that there is no simple relationship between processing and bioavailability of nutrients (Watzke, 1998). For instance, processing could improve the bioavailability through the enrichment of nutrients by separation and partitioning of minerals, destruction of inhibitors or beneficial complex formation between different nutrients and metal ions. In contrast, it could negatively affect the bioavailability of nutrients by the deactivation of enzymes that degrade inhibitors or by producing insoluble metal compounds (Watzke, 1998).

The best available processing method should be selected after collecting enough information about the nutritional and antinutritional composition and also the distribution and sensitivity of these parameters to different processing methods in tested ingredients. The nutritional requirements of the target animal should always be borne in mind. Successful implementation of processing methods can be carried out after testing the effect of different processing methods on individual nutrient or antinutrients and consideration of the possible effects of other available nutrients or antinutrients. Also the effect of different physical conditions including moisture content and particle size on the response of the nutrient(s) under study should be closely evaluated. A better understanding in this area may provide useful information for food manufacturers to adopt the best possible processing conditions based on the chemical composition of the tested ingredient and the nutritional requirement of the target animals. Gathering universally accepted standard condition protocols for different processing methods into a database would be invaluable for the food industry. It seems only modeling of this information can provide reliable information for food manufacturers as well as animal producers.

2.5. Conclusion

The current study was conducted to screen the 4 main Australian grain legumes as fish meal replacement for salmonids, using various chemical analysis methods. The effect of three different processing methods on both nutrient and antinutrient content of the grains were also evaluated. Preliminary results showed that lupin is the superior grain legume in terms of higher protein, better amino acid balance and lower content of ANF's, followed by field pea and faba bean. Chick pea has a lower chance of being considered as a fish meal replacement. Combination of both soaking and heating was identified as the most effective processing method for lowering ANF's. Screening the most suitable grain legume could be carried out with more confidence after conducting some biological tests such as digestibility and growth trials.

Chapter Three

Growth and nutrient digestibility (*in vivo vs in vitro*) in rainbow trout (*Oncorhynchus mykiss*) of different body size fed raw or processed Australian grain legumes

3.1. Introduction

Chemical analysis is the first stage in the evaluation of the nutritional value of an ingredient. Lower costs and higher repeatability associated with chemical analysis are the most important factors resulting in its widespread use to evaluate feeds and ingredients (Pond *et al.*, 1995). However, a major problem of using chemical analysis is that results do not necessarily show the bioavailability of different nutrients for animals (Pond *et al.*, 1995; Jauncey, 1995; New and Casavas, 1995). This issue is likely to be more problematic when ingredients that contain antinutritional factors (ANF's) are used. Due to the limitations associated with using chemical analysis alone to evaluate the nutritional value of ingredients, they should only be used to complement other *in vivo* techniques for screening a wide range of ingredients (Melcion and van der Poel, 1993).

Digestibility studies are generally the next stage in the evaluation of the nutritional value of ingredients. Results from digestibility studies are of utmost importance as they take into account the direct involvement of animals in the complex digestion process. It has been claimed that there is only a 5% difference between apparent and true digestibility coefficients (Hardy, 1997). Digestibility of ingredients shows the potential availability of energy and other nutrients (Cho, 1991).

Different processing methods have been developed to reduce the deleterious effect of ANF's (Lalles and Jansman, 1998). The best way of assessing the effectiveness of processing methods is through *in vivo* studies, in which animals are actively involved in the digestion process (Melcion and van der Poel, 1993). Unfortunately, the use of *in vivo* digestibility methods is tedious and far more expensive than chemical analysis methods (Dimes *et al.*, 1994). This issue becomes more complicated when digestibility studies are conducted in the aquatic habitat of fish.

Because of these limitations, extensive research was conducted in the past to develop *in vitro* methods for digestibility studies (Hsu *et al.*, 1977; Grabner, 1985; Eid and Matty, 1989; Dimes and Haard, 1994; Bassompierrie *et al.*, 1997; Carter *et al.*, 1999). *In vitro* digestibility methods are not only rapid and cheap but also provide the opportunity to closely observe the dynamic breakdown of nutrients using very small amounts of raw material (Eid and Matty, 1989).

In vitro digestibility methods have not been widely used by the aquaculture industry because they are time consuming, and the results can be variable and inconsistent (Bassompierrie *et al.*, 1997). It has been shown that the results from *in vitro* measurement do not show good agreement with *in vivo* studies for some ingredients (Satterlee *et al.*, 1981; Bodwell, 1977) and for some processed ingredients (Dimes *et al.*, 1994). It has been suggested that *in vitro* studies alone should not be considered a reliable method for the evaluation of feedstuffs (Melcion and van der Poel, 1993). It has also been proposed that more than one *in vitro* test should be performed simultaneously (Melcion and van der Poel, 1993). The number of studies to determine the digestibility value of various grain legumes (raw *vs* processed) using both *in vitro* and *in vivo* studies are limited (Rozan *et al.*, 1997). Finding the factors that may limit the digestibility of ingredients is of paramount importance.

It is proposed that digestibility is mainly affected by the food, however it does not mean that the animal does not affect it (McDonald *et al.*, 1995). The effect of fish size on the digestibility of various nutrients has been demonstrated (Ferraries *et al.*, 1986; Bassompierre *et al.*, 1998). In contrast, there are some reports showing that fish size has no effect on nutrient digestibility (Lee, 1997). However, it is important to find out more information regarding the digestibility of various nutrients by different fish size (age) for the ingredients that contain some ANF's. The normal function of the digestive tract in younger fish may be more severely affected following the ingestion of ingredients that contain ANF's, highlighted by both lower digestibility of nutrients and growth performance. If this is the case, measuring the nutrient digestibility of ingredients will facilitate the best time for their inclusion in fish diet in terms of fish age/or size.

The effect of time (adaptation) on the digestibility of some nutrients has been presented in the literature (Appleford and Anderson, 1997; Carter, 1998). Adaptation is becoming increasingly important when the ingredients that contain some ANF's are considered as fish meal replacement. Fish may show adaptation to some type and/or concentration of ANF's and a deleterious response to others in the diet. The measurement of digestibility is a good index to determine significant alterations that may occur to the normal functioning of the digestive tract.

The objectives of this research were to determine growth performance, feed utilization indices and the digestibility value of both raw and processed grain legumes for juvenile rainbow trout of different body weights. These were the same ingredients described in Chapter Two. In addition, the effect of time (adaptation) on *in vivo* digestibility and the relationship between *in vivo* and *in vitro* digestibility were investigated. The results of the current study can be used to evaluate the suitability of the *in vitro* digestibility methods for ingredients that contain some ANF's.

3.2. Materials and Methods

3.2.1. Experimental animals and culture condition

Rainbow trout were obtained from the National Key Centre for Research and Training for Aquaculture in Australia, where the research was conducted. Experiments were conducted in an indoor recirculation culture system consisting of 24, 300-L polyethylene tanks connected to two 1000-L settlement tanks and a biofilter (Carter and Hauler, 2000). All tanks were aerated and fitted with a settling column for collection of faeces or uneaten pellets. Average water flow in each tank was 11.5 l/min. Water temperature was indirectly controlled using air-conditioning and was constant at 14.5 ± 0.5°C. A light: dark photoperiod of 12:12 hours was used during the experiment. The tanks were covered with 10 mm diameter mesh to minimize stress and to prevent fish escaping. Temperature, dissolved oxygen, total ammonia, nitrite and pH were measured three times weekly, and did not exceed values recommended for rainbow trout (Tarazona and Munoz, 1995).

3.2.2. Feed preparation and processing

The grain legumes tested in this experiment consisted of chick pea (*Cicer arientinum*), faba bean (*Vicia faba*), field pea (*Pisum sativum*), and sweet lupin (*Lupinus angustifolius*). Grains were obtained from Milne Feeds Company (Western Australia) and were Australian in origin. Grain legumes were tested as both raw and processed. As results from chemical analysis studies (Chapter Two) showed that a process of soaking and heating was the most efficient method, grains processed in this way were used in the current experiment. Briefly, whole grains were soaked for 12 h in distilled water at 25°C, rinsed in distilled water and the moisture content reduced to about 30% by aeration. The grains were then heated using a pressurized autoclave at 105°C for 10 minutes, aerated to reduce the moisture content to approximately 10% and milled using a rock crusher. A total of eight experimental diets using four grains legumes, either raw or processed, were cold pelletized (3mm) using a laboratory pellet mill (CPM, California Pellet Mill Co, USA) with the proportion of 25% grain legumes, 1% chromic oxide and 74% reference diet. The reference diet was prepared in the same way. The composition of reference and experimental diets are shown in Tables (3.1 and 3.2).

Table 3.1. Ingredient composition of reference diet[1] used *in vivo* digestibility studies

Ingredient	Source	g/kg
Fish meal*	Triabunna	700
Fish oil	Triabunna	150
Dextrin	-	127
Vitamins[2]	Gibson	7.5
Minerals[2]	Gibson	5
Stay C	Roche	0.5
CMC[3]	Sigma	10
Total (g)	-	1000

1. Cho *et al.* (1982)
2. Added to fulfill in excess of requirements for rainbow trout. NRC (1993)
3. Carboxymethylcellulose

* Proximate composition (DM basis): DM: 87.64%; CP: 71.48%; GE: 20.71% (MJ/kg)

Table 3.2. Chemical composition of experimental diets containing raw (R) or processed (P) grain legumes (% as is)

Grain	Chick pea		Faba bean		Field pea		Lupin	
Processing	R	P	R	P	R	P	R	P
DM (%)	94.4	94.8	95.3	94.1	94.1	93.8	95.2	94.1
CP (%)	40.2	39.7	41.6	41.2	40.9	40.7	42.5	42.1
CL (%)	21.4	21.3	19.5	19.7	20.7	20.2	21.9	20.9
GE (MJ/kg)	21.3	21.3	20.9	21.3	21.1	21.2	21.1	21.5
Chromic oxide (%)	1.18	0.98	1.03	1.06	1.07	1.19	1.02	1.09

DM: Dry matter; CP: Crude protein; CL: Crude lipid; GE: Gross energy

3.2.3. Digestibility

3.2.3.1. *In vivo* digestibility

A completely randomized block design (CRD) was used with fish grouped in three blocks; small (67.8 g ± 0.65 S.E.M.), medium (92.95 g ± 0.50 S.E.M.) or large (114.94 g ± 0.80 S.E.M.) and 40 fish allotted to each experimental tank (3 tanks per treatment). Blocking had the extra advantage of reducing both the variation in fish weight in each tank and the development of hierarchy (McCarthy *et al.*, 1993) and also tested the effect of fish size on the digestibility values of different nutrients. Prior to the experiment, fish were kept in the experimental system for a two-week acclimation to the indoor environment. Fish were fed by hand once a day with a commercial diet (Gibson's Pty Ltd).

Before testing the experimental diets, fish were fed the reference diet twice daily at 09:00 and 17:00 (2.5% of tank biomass per day) for 10 days. Fish were fed by hand in order to observe their feeding behaviour, control hierarchy effects and to measure feed intake. Over this period, daily feed intake was determined by collecting the number of uneaten pellets. Faeces were collected overnight in collection chambers, which were surrounded by ice to minimize the breakdown of nutrients over the collection period. On the morning of the 10^{th} and 11^{th} day after starting feeding with the reference diet, faeces were collected in 9 randomly selected tanks (3 from each block). The faeces from each tank were pooled from the two days collection, freeze-dried and stored at -18°C until analysis.

The apparent digestibility coefficients (ADC) of nutrients in the reference diet was determined using the standard formula (Maynard and Loosli, 1969):

Equation 3.1

$$ADC\ (\%) = 100 - (100 \times (\%M_{diet} / \%M_{faeces}) \times (\%N_{faeces} / \%N_{diet}))$$

Where M is the inert marker and N the nutrient

Upon completion of the reference diet-feeding period, fish were starved for 48 h before determination of tank biomass. The experimental diets then were randomly fed to tanks of each block. Feeding management was exactly the same as during the reference diet-feeding period. Faeces were collected over two intervals on days 9,10 (phase 1) and 18,19 (phase 2) of the experiment. ADC of different nutrients in the ingredients was determined using the following equation:

Equation 3.2

$$ADC_{ing}\ (\%) = (N_{test} \times ADC_{test} - 0.75 \times N_{ref} \times ADC_{ref}) / (0.25 \times N_{ing})$$

Where ADC_{ref} and ADC_{test} were the apparent nutrient digestibility coefficients of the reference and test diets, respectively, and N_{test}, N_{ref} and N_{ing} the nutrient content of the test diet, reference diet, and the ingredient, respectively (Sugiura *et al.*, 1998).

3.2.3.2. *In vitro* digestibility studies

Upon completion of the *in vivo* digestibility trial, five fish were randomly sampled from each tank. They were anaesthetized, dissected and the pyloric caeca removed and stored at -80° C for enzyme extraction. A known weight of pyloric caeca was homogenized in distilled water and then centrifuged (13000g, 20 minutes at 4°C). Supernatants were defatted and stored at -80°C.

The trypsin activity of extracted pyloric caeca for selected fish from each tank was then determined. Buffered enzyme only and buffer plus sample only was prepared as blanks and their pH adjusted to 8.0 using Trizma buffer. A dry sample of tested

grains containing 25 mg nitrogen was then added to 25 ml buffer enzyme solution in conical flasks with foil caps. The buffer included 50 mg thimerosal/l to prevent the growth of microorganisms that might interfere with the digestion procedure (Eid and Matty, 1989). All flasks were incubated in a shaken water bath for 12 hours at 25°C. The flasks were continuously shaken for an extra 20 minutes. Samples were centrifuged (20000g, 5 min, 4°C) and the supernatant discarded. Thirty ml of distilled water was added to each flask, which were then shaken and centrifuged. The washing procedure was repeated four times. Insoluble nitrogen was separated using 1.2 m millipore N free filter, air dried, weighed and quantified by Kjeldahl method. A buffered enzyme extract only sample was used to correct for nitrogen involved in enzyme extract. Apparent *in vitro* protein digestibility was then calculated using the following equation (Carter *et al.*, 1999).

Equation 3.3

$$ADC\ (\%) = 100 \times (N_{diet} - N_{insoluble}/N_{diet})$$

3.2.4. Chemical analysis

Collected digested samples were air dried and milled to 1mm before analysis was carried out in duplicate. Dry matter was determined using a freeze drier (Dynavac). Total nitrogen was measured using the Kjeldahl method and protein content estimated as N × 6.25. Chromic oxide was determined using a process involving perchloric and nitric acid digestion followed by spectrophotometric determination of at 350 (Furukawa and Tsukahara 1966). Trypsin inhibitor (TI) activity was determined using the procedure described by (Smith et al., 1980) through measuring the loss of activity of added trypsin under standard condition. Briefly, TI extracted from a sample using diluted hydrochloric acid. The sub-sample of TI was incubated at 37°C for 10 min. Then the synthetic substrate of a-N-Benzoyl-DL-arginine-p-nitroaniliade (BAPNA) was added and the samples incubated again in the same condition. The reaction then stopped by adding trichloroacetic acid. Due to the presence of BAPNA the remaining trypsin acted on BAPNA and changed the color of the solution to yellowish by releasing p-nitroaniline that subsequently was quantified at 410 nm. The differential of absorbance in the presence of TI was considered as a measure of TI activity.

3.2.5. Statistical analysis

Fish size has been considered as a blocking variable in the current experiment. Blocking has the advantage of minimizing hierarchy problem and also tests the effect of fish size on different biological responses. Grains, processing methods and fish size were chosen as the independent variables and all two-way interaction effects allowed. However, three-way interaction could not be estimated due to resource limitations. Type I sums of squares were used because all factors were fixed effects in this experiment (Zar, 1996). The results of the analysis of variance (ANOVA) were reported in terms of the statistical significance of each main factor and of the interaction. The strength of each factor and the interaction were also indicated by calculating the proportionate contribution of that factor to the corrected total sum of squares. Sokal and Rohlf (1995) stated that arcsine transformation is not necessary for percentage data between 30 to 70%. That is why only the digestibility values for crude protein were arcsine transformed in the current study. Significant differences within grains, processing method treatments and blocks (fish size) were explored post hoc by Tukey test. Differences were regarded as statistically significant at probabilities of 5% or less. Tabulated results were presented as mean ± S.E.M. (standard error of mean). All statistical tests were performed using the SPSS Statistical Analysis Software Program (Version 9.0.1 for Windows, 1999).

3.3. Results

In vivo dry matter digestibility of raw grains was compared (Figure 3.1). This value for lupin was significantly higher than for all other grains. However, *in vivo* dry matter digestibility was similar for chick pea, faba bean and field pea.

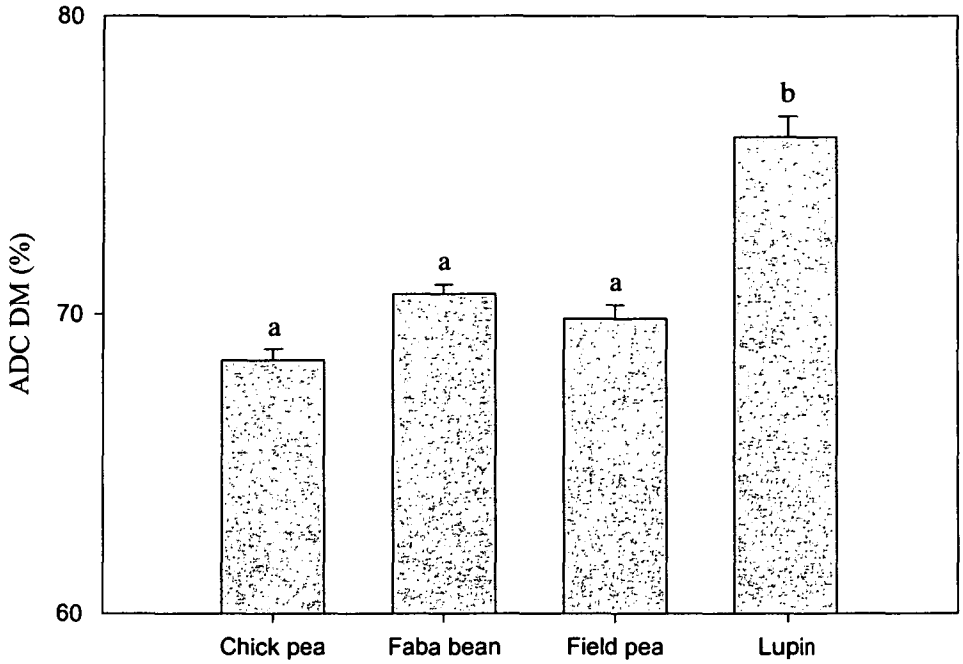

Figure 3.1. Comparative apparent dry matter digestibility of raw grain legumes in rainbow trout

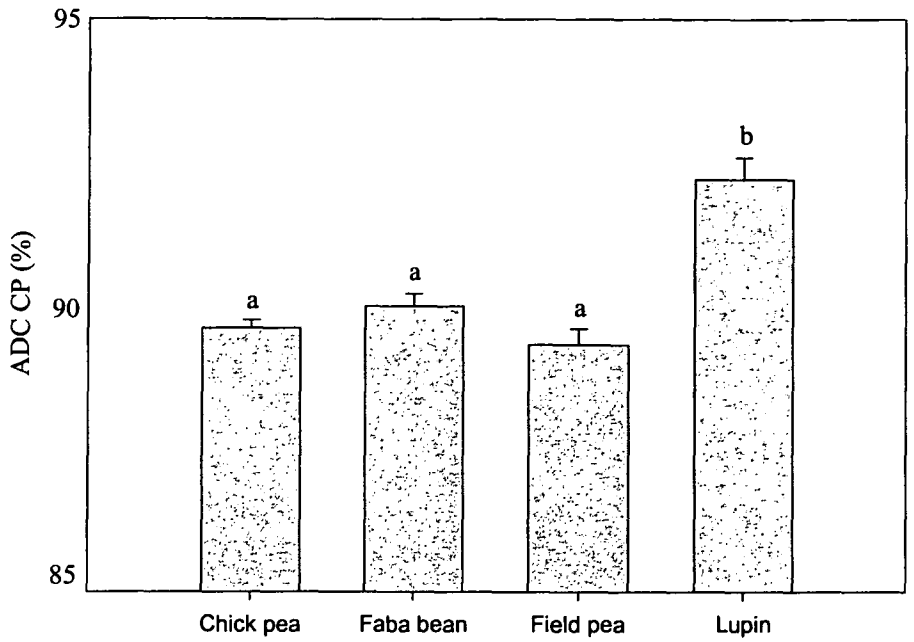

Figure 3.2. Comprative apparent crude protein digestibility of raw grain legumes in rainbow trout

Similarly *in vivo* crude protein digestibility of lupin was significantly higher than for all other grains (Figure 3.2). This value, however, was similar for chick pea, faba bean and field pea.

Digestibility was measured over two periods: phase 1 (days 9 and 10) and phase 2 (days 18 and 19). Results showed that *in vivo* dry matter digestibility (phase 1 and 2), *in vitro* dry matter digestibility, *in vivo* crude protein digestibility (phase 1 and 2) as well as *in vitro* crude protein digestibility, were all affected by grain type (Table 3.3). *In vivo* dry matter digestibility (phase 2) and *in vitro* crude protein digestibility were significantly improved by processing method. In contrast processing significantly decreased *in vivo* crude protein digestibility in both phase 1 and phase 2. Fish size did not affect the digestibility value with the exception of *in vivo* dry matter digestibility (phase 1). The proportion of total variation for dry mater digestibility (*in vivo* and *in vitro*) and crude protein digestibility (*in vivo* and *in vitro*) was mainly explained by grain type rather than processing method.

Table 3.3. Comparison of *in vivo* dry matter digestibility (phase 1, 2), *in vitro* dry matter digestibility, *in vivo* crude protein digestibility (phase 1, 2) and *in vitro* crude protein digestibility for rainbow trout of different body weights when fed with raw or processed grains

	ADC DM *in vivo* 1 (%)	ADC DM *in vivo* 2 (%)	ADC DM *in vitro* (%)	ADC CP *in vivo* 1 (%)	ADC CP *in vivo* 2 (%)	ADC CP *in vitro* (%)
Grain (combination effect of all processing methods)						
Chick pea	71.04^{ab}	71.97^{ab}	30.12^{ab}	89.27^{ab}	90.01^{b}	49.18^{a}
Faba bean	71.51^{b}	71.82^{a}	28.01^{a}	89.70^{b}	89.98^{b}	55.04^{b}
Field pea	69.26^{a}	69.74^{a}	32.17^{b}	88.17^{a}	88.70^{a}	55.56^{b}
Lupin	73.98^{c}	74.17^{b}	60.45^{c}	90.94^{c}	91.26^{c}	91.13^{c}
Processing (combination effect of all grain types)						
Raw	71.19	71.24^{a}	37.65	89.87^{b}	90.27^{b}	60.49^{a}
Processed	71.71	72.61^{b}	37.73	89.18^{a}	89.71^{a}	65.96^{b}
Block (fish size), (combination effect of all grain type and all processing methods)						
Small	70.44^{a}	71.23	37.67	89.19	89.76	64.40
Medium	71.97^{b}	72.01	37.44	89.51	89.97	62.51
Large	71.94^{b}	72.53	37.95	89.87	90.23	62.77
Two way ANOVA: Proportion of total variation (%) explained by main effects and interaction						
Grain	53.21^{**}	32.75^{**}	98.74^{***}	65.81^{**}	60.63^{**}	94.75^{***}
Processing	1.27	6.30^{*}	0.0009	7.97^{*}	5.94^{*}	2.63^{**}
Block	9.59^{*}	3.79	0.025	5.17	2.86	0.247
Grain × Processing	28.54^{**}	49.80^{**}	0.345	8.53	20.32^{*}	1.40^{*}
Grain × Block	1.15	1.53	0.328	2.63	2.61	0.40
Processing × Block	0.03	1.24	0.031	4.54	2.40	1.47

Different superscript shows significant differences between grain legumes within processing levels. *P ≤ 0.05; ** P ≤ 0.01; *** P ≤ 0.001

However, the proportion of none of the digestibility parameters was affected by fish size. While there were no interactions between grain type and fish size and processing method and fish size, the interaction between grain type and processing method was significant for *in vivo* dry matter digestibility (phase 1 and 2), *in vivo* crude protein digestibility (phase 2) and *in vitro* crude protein digestibility. The proportion effect of all digestibility indices was mainly explained by grain type rather than processing method or fish size.

Results from pair comparison data analysis (phase 1 *vs* phase 2 faecal collection) showed that *in vivo* dry matter digestibility was not affected by grain type and processing method over a 9 days adaptation time interval (Table 3.4). Meanwhile, *in vivo* dry matter digestibility significantly improved in the second phase of faecal collection for processed grains and not for raw ones. While *in vivo* dry matter digestibility was not affected by fish size (medium and large groups), it was significantly improved in the second phase of faecal collection for the small fish group.

Table 3.4. Pair-wise comparison of *in vivo* dry matter digestibility (%) measured on two occasions

Grains	*In vivo* ADC DM (Phase 1)	*In vivo* ADC DM (Phase 2)	P
All grains	71.45	71.92	NS
Chick pea	71.04	71.97	NS
Faba bean	71.51	71.81	NS
Field pea	69.26	69.74	NS
Lupin	73.98	74.17	NS
Processing			
Raw	71.19	71.24	NS
Processed	71.71[a]	72.61[b]	≤ 0.05
Block (fish size)			
Small	70.44[a]	71.23[b]	≤ 0.05
Medium	71.97	72	NS
Large	71.94	72.53	NS

In general, *in vivo* crude protein digestibility was affected by grain type (Table 3.5). While time interval did not affect the *in vivo* crude protein digestibility for faba bean and lupin, it significantly improved *in vivo* crude protein digestibility for chick pea and field pea. *In vivo* crude protein digestibility significantly improved for both raw and processed grains in the second faecal collection. *In vivo* crude protein digestibility was not affected in the large fish group, whereas this value was significantly improved in the small and medium fish groups in the second faecal collection.

Table 3.5. Pair-wise comparison of *in vivo* crude protein digestibility (%) measured on two occasions

Grains	*In vivo* ADC CP (Phase 1)	*In vivo* ADC CP (Phase 2)	P
All grains	89.52^{a}	89.99^{b}	≤ 0.001
Chick pea	89.27^{a}	90.01^{b}	≤ 0.05
Faba bean	89.7	89.98	NS
Field pea	88.17^{a}	88.70^{b}	≤ 0.05
Lupin	90.94	91.26	NS
Processing			
Raw	89.87^{a}	90.27^{b}	≤ 0.01
Processed	89.18^{a}	89.71^{b}	≤ 0.05
Block (fish size)			
Small	89.19^{a}	89.76^{b}	≤ 0.001
Medium	89.51^{a}	89.97^{b}	≤ 0.05
Large	89.87	90.24	NS

Regression relationship (for both raw and processed grains) between *in vitro* and *in vivo* dry matter digestibility (Figure 3.3.) and *in vitro* and *in vivo* crude protein digestibility (Figure 3.4) were significant despite their low values.

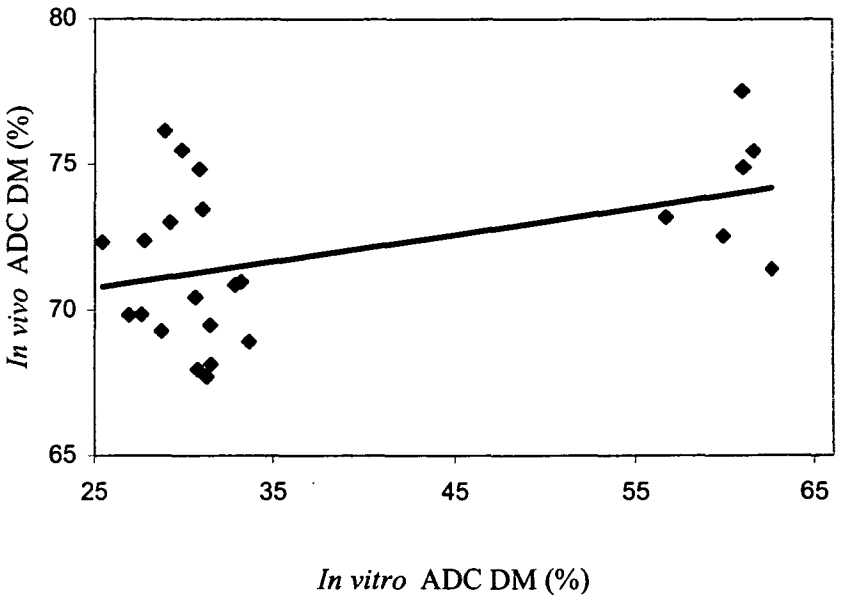

Figure 3.3. Regression relationship between *in vitro* and *in vivo* dry matter digesitbility for all grain legumes (raw and processed)
(R^2 = 0.19; P < 0.05)

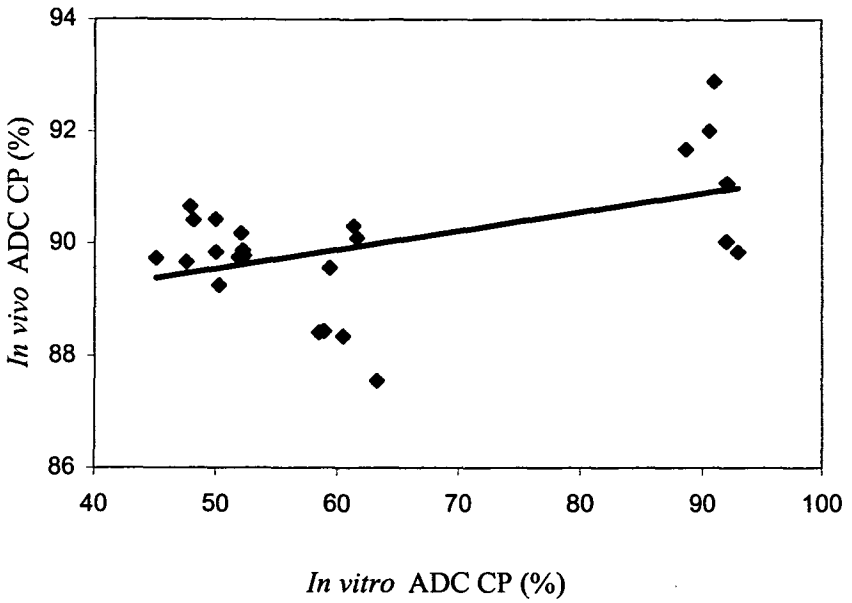

Figure 3.4. Regression relationship between *in vitro* and *in vivo* crude protein digestibility for all grain legumes (raw and processed)
(R^2= 0.24; P < 0.01)

There was a strong and significant regression relationship between *in vitro* and *in vivo* dry matter digestibility for raw grain legumes (Figure 3.5) whereas the regression relationship between *in vitro* and *in vivo* dry matter digestibility for processed grain legumes was low and insignificant (R^2 = 0.11; P > 0.05).

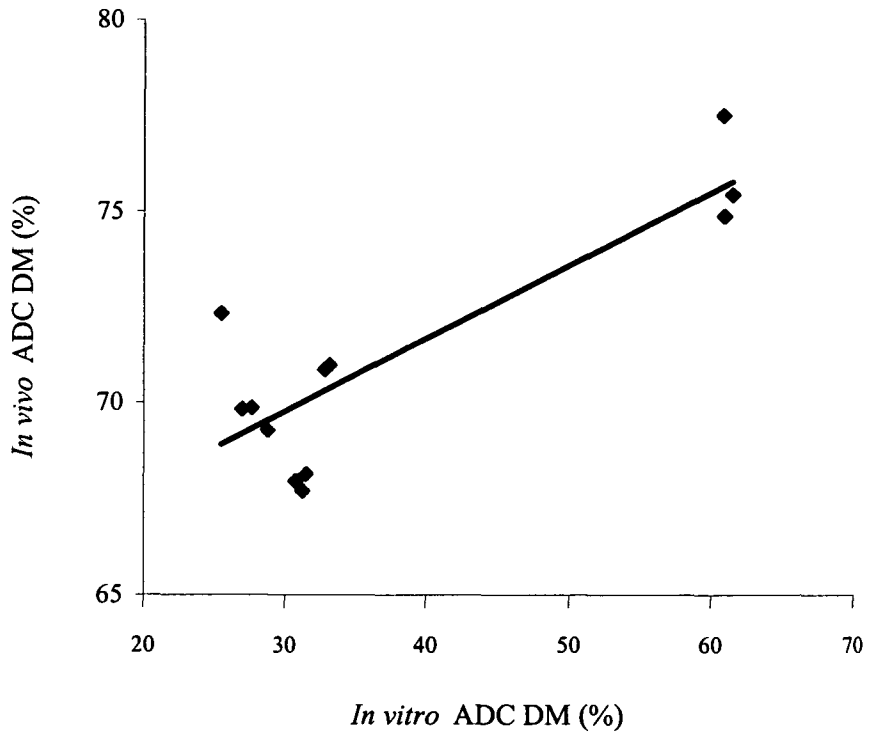

Figure 3.5. Regression relationship between *in vitro* and *in vivo* dry matter digestibility for raw grain legumes
(R^2 = 0.82; P < 0.01)

The same pattern was observed for the regression between *in vitro* and *in vivo* crude protein digestibility. While there was a strong and significant regression relationship between *in vitro* and *in vivo* crude protein digestibility for raw grain legumes (Figure 3.6), the regression relationship between *in vitro* and *in vivo* crude protein digestibility for processed grain legumes was poor and insignificant (R^2 = 0.43; P > 0.05).

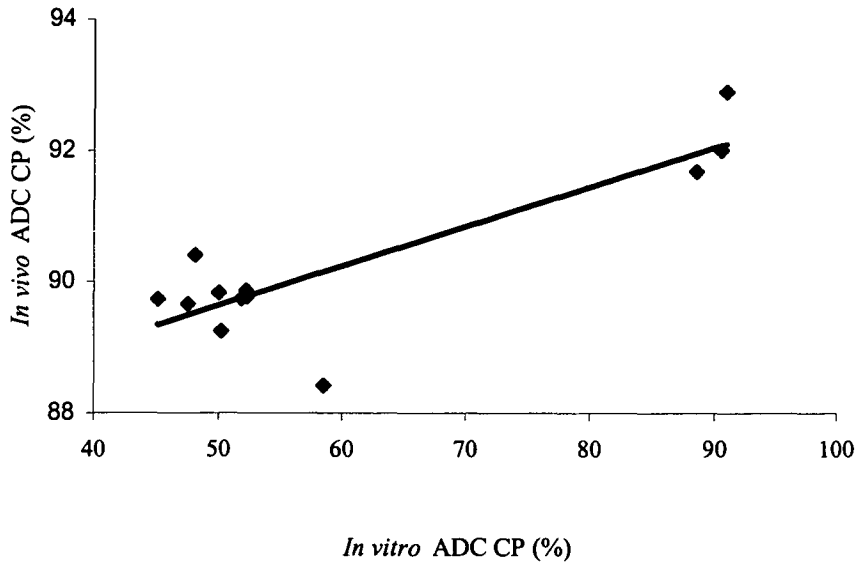

Figure 3.6. Regression relationship between *in vitro* and *in vivo* crude protein digestibility for raw grain legumes

(R^2 = 0.93; P < 0.01)

Regression relationship between trypsin inhibitor content of the diets and dry matter digestibility (*in vivo and in vitro*) and crude protein digestibility (*in vivo and in vitro*) was strong and highly significant (Table 3.6).

Table 3.6. Regression relationship between trypsin inhibitor content of the diets and the digestibility responses in fish fed grain legumes (raw and processed)

Parameter	R^2	P	Y=
In vitro ADC DM	0.88	≤ 0.01	$-281.32X^3 - 490.88X^2 - 299.57X + 58.391$
In vivo ADC DM	0.67	≤ 0.001	$-88.75X^3 + 131.32X^2 - 44.569X + 74.568$
In vitro ADC CP	0.94	≤ 0.001	$-253.1X^3 + 465.86X^2 - 247.43X + 89.734$
In vivo ADC CP	0.52	≤ 0.01	$-31.0254X^3 + 49.617X^2 - 19.081X + 91.25$

ADC DM: apparent dry matter digestibility; ADC CP: apparent crude protein digestibility

3.4. Discussion

Both *in vivo* and *in vitro* dry matter digestibility demonstrated the highest values for lupin, while the lowest values for *in vivo* dry matter digestibility and *in vitro* dry matter digestibility were recorded for field pea and faba bean, respectively. High dry matter (74%) and crude protein (91%) digestibility values for lupin in the current experiment are in agreement with the corresponding data reported for rainbow trout (70% and 96%) (Burel *et al.*, 2000b). *In vivo* protein digestibility of lupin seed in the current study is notably higher than the value reported by Hughes (1988) for lupin (*L. albus)* in rainbow trout (85%). The digestibility data for lupin (*L. angustifolius*) seed (ADC DM 63%, ADC CP 85%), field pea, (ADC DM 66%, ADC CP 80%) and faba bean (ADC DM 66%, ADC CP 80%) reported by Gomes *et al.* (1995) for rainbow trout is also lower than the value obtained in the current experiment. The higher dry matter digestibility of lupin in the current experiment may be related to higher crude protein digestibility as compared with the values reported by Hughes (1988) for lupin. These differences may be related to using different plant cultivars or various experimental conditions in abovementioned experiments and in the current study.

In vivo dry matter digestibility was significantly improved by processing method, while *in vivo* crude protein digestibility was significantly decreased. It has been stated that processing not only improves digestibility by destroying the protease inhibitors but also changes the protein structure through denaturing the protein and making it more susceptible to breakdown by digestive enzymes (Liener, 1976). The processing method used in the current study may have had a deleterious effect on protein structure, resulting in a lower *in vivo* crude protein digestibility value for

processed lupin. However this finding was not supported by *in vitro* crude digestibility that showed significantly higher value for processed grains as compared with the raw counterpart. The negative effect of heat treatment on lupin has already been reported for rainbow trout (de la Higuera *et al.*, 1988). Although, *in vitro* dry matter digestibility was not affected by processing method, *in vitro* crude protein digestibility was significantly increased for processed grains. This can be explained by a significant reduction of trypsin inhibitor concentration in grains as shown in Chapter Two.

With the exception of *in vivo* dry matter digestibility that was lower for the small fish group in the first phase of faecal collection, fish size did not affect digestibility parameters. This is in agreement with findings that suggest fish size does not affect nutrient digestibility (Lee, 1997). The proportion effect of grain type was more evident on digestibility values as compared with the proportion effect of processing method or fish size.

Time (adaptation) had no significant effect on *in vivo* dry matter digestibility when grain type or processing method was evaluated. When fish size was considered *in vivo* dry matter digestibility significantly improved only for the small fish group. This may indicate the gradual adaptation of the small fish group to dietary plant meal. Adaptation of rainbow trout to soybean inclusion was observed by Refstie *et al.* (1997). Specific growth rate (SGR) was significantly lower for the fish that fed on soybean-based diet as compared to a fish meal-based diet in the first phase of the trial day (0-28 days). However, growth performance showed similar value for both diets in the second phase of trial (29-56 days) suggesting the gradual adaptation of fish to soybean meal-based diet (Refstie *et al.*, 1997). It seems gradual inclusion of plant meals in salmonid diets has potential for improving their use. Results from the current experiment show that the grain legumes at the 25% inclusion had no effect on the larger fish.

In vivo crude protein digestibility was generally improved in all the tested grains in the second phase of faecal collection, but it was only significant for chick pea and field pea. *In vivo* crude protein digestibility in the second phase of faecal collection was significantly improved compared to the first faecal collection for both raw

(89.9% *vs* 90.3%) and processed (89.2% *vs* 89.7%) grains. While *in vivo* crude protein digestibility was not affected by time for the large fish group, this value was significantly improved for the small and medium fish groups. This criterion again shows that the time is needed for gradual adaptation of the small fish group for effective utilization of plant meals in their diet. The larger fish had no problem of using the grains in their diet (at 25% inclusion level) with no adaptation required. Similarly, adaptation (day 14 *vs* day 28) significantly improved *in vivo* dry matter and *in vivo* crude protein digestibility for pea protein and soybean meal in Atlantic salmon (Carter, 1998).

To date nearly all digestibility values for different ingredients have been reported by measuring the faecal samples collected overnight and for a short period of time. Recently, there has been some evidence in the literature showing that the digestibility value varies at different times of the day (Percival and Lee, 1998; Bolliet *et al.*, 2000). Digestibility values for different nutrients were also significantly different when faecal samples were collected on different days for Atlantic salmon (Percival and Lee, 1998; Carter, 1998). The discrepancy in the results reported in digestibility experiments findings, illustrates the complexity in selecting the suitable screening tools for finding alternative ingredients for fish meal replacement. This phenomenon suggests that in order to obtain a more precise figure about the digestibility of different nutrients, faecal samples should preferably be collected over a 24 h cycle and for longer period of time when the fish is fully adapted to effectively use the nutrients in the tested ingredient.

An important finding by Refstie *et al.* (2000) showed that while nutrient digestibility was higher in rainbow trout than in Atlantic salmon, utilization of nutrients was actually higher in Atlantic salmon. This phenomenon shows that the determination of the digestibility value is not a reliable tool for screening different ingredients under all conditions. This issue opens a new scenario for considering alternative methods for assessing the nutritional value of ingredients. In the past, emphasis on the importance of digestibility studies has taken precedence.

Studies by Fagbenro (1998) showed that protein digestibility was not significantly different among different grain legumes for Nile tilapia, whereas digestibility for

different amino acids showed significant differences, suggesting the advantage to be gained in measuring the digestibility values of amino acids for feed formulation. At the high inclusion levels of TI in the diet, fish have to use more sulphur amino acids to compensate for lower levels of trypsin activity in the intestine. Therefore, sulphur amino acids should be supplemented in the diet (Krogdahl *et al.*, 1994) as the level of methionine and cysteine is already at a marginal level in plant proteins.

The use of a single enzyme, that may only attack specific peptide bonds, in *in vitro* digestibility studies may show different amino acid digestibility for various protein sources with different amino acid profiles (Hsu *et al.*, 1977). Using the multi-enzyme method should minimize the effect caused by enzyme inhibitors (Hsu *et al.*, 1977). Studies by Dimes and Haard (1994) showed that fish pyloric caeca enzyme(s) better predict protein digestibility than fish purified trypsin. This is the reason the enzyme extracted from the pyloric caeca was used for *in vitro* digestibility studies in the current study.

The pH drop-method was used for *in vitro* digestibility studies in the current experiment. However, the advantage of the pH-stat method over the pH-drop method for predicting the protein digestibility is that the equation can be used for predicting the *in vivo* digestibility values for a wide range of ingredients (Boisen and Eggum, 1991; Dimes and Haard, 1994). However, the pH-stat method was reported to be unsuitable for measuring the digestibility of ingredients that partially hydrolyzed over their preparation (Dimes *et al.*, 1994). The pH-drop method used in this experiment did not show a good regression relationship for processed grain legumes in the current study. Several factors, including concentration of nutrients, temperature, pH stability, activators, as well as inhibitors and incubation time and interaction between the tested nutrients and available compounds in the ingredient itself (e.g. fibre and ANF's), affect enzyme activity (Boisen and Eggum, 1991). It is suggested that the *in vitro* values can be compared with apparent *in vivo* data at the faecal levels, only after correction of endogenous loss (Boisen and Fernandez, 1991).

It seems *in vitro* protein digestibility was found to be markedly decreased in the current study due to the inhibitory effect of end products from grain legumes, as

suggested by Robbins (1978). Unfortunately, trout protease is one of the most sensitive enzymes, as its sensitivity against TI is 15 times higher than human protease (Dimes *et al.*, 1994). A predigesting process with pepsin has been suggested for ingredients containing protease inhibitors in order to improve the prediction value for digestibility measurement (Pedersen and Eggum, 1983). It has been suggested that ANF's mainly affect the digestibility values by increasing the loss of endogenous protein rather than having a directly negative effect on protein digestibility (Boisen and Eggum, 1991). In other words fish have to use more internal sulphur amino acids in order to produce more trypsin enzyme in the digestive tract to partially compensate for the trypsin loss in the digestive tract due to inhibitory effect of TI.

The regression relationship between *in vitro* and *in vivo* dry matter digestibility for raw grains was very high and significant, whereas the regression relationship for processed grain legumes was very low and insignificant. The same pattern was observed for the regression between *in vitro* and *in vivo* crude protein digestibility. While the regression relationship showed a high and significant value for raw grain legumes this value was very low and not significant for processed grain legumes. Similarly, a lower regression relationship between the *in vivo* and *in vitro* digestibility value for processed grains has been reported in other research (Dimes *et al.*, 1994). The reasons for observing such a phenomenon are difficult to explain.

Results from the current experiment show that *in vitro* digestibility could predict the *in vivo* digestibility values for raw grain legumes. This is in agreement with Carter's (1998) finding who reported a reasonably high relationship ($r = 0.77$; $P \leq 0.001$) between the measured and predicted value for crude protein digestibility of different experimental diets containing various plant meals for Atlantic salmon. It should be borne in mind that *in vitro* digestibility methods might not be suitable for predicting the digestibility of all plant proteins. Also under no circumstances should *in vivo* studies be replaced with *in vitro* studies. However, *in vitro* digestibility methods are valuable tools for screening a wide range of raw ingredients for fish feed.

3.5. Conclusion

Results from the current experiment showed that *in vitro* digestibility could predict *in vivo* digestibility values for raw but not processed grain legumes. However, it should be borne in mind that *in vitro* digestibility methods might not be suitable for predicting the digestibility of all plant proteins. Also, under no circumstances should *in vivo* studies be replaced by *in vitro* studies. However, *in vitro* digestibility methods are valuable tools for screening a wide range of raw ingredients for fish feed. Fish size did not affect the dry matter or crude protein digestibility of grain legumes. However, digestibility of grain legumes improved over the adaptation period for smaller fish size suggesting that grain legumes should be gradually included in diets for small fish until full adaptation of digestive system is developed. Considering all data reported in the current experiment, it could be concluded that lupin is the most promising grain legume as fish meal replacement for salmonids. However, field pea and faba bean have also shown some potential as fish meal replacements. Chick pea failed to be considered as a suitable alternative protein meal for fish. Lupin contains the highest complex carbohydrates among all plant protein. Future research should focus for determining the highest inclusion level of lupin in the diet considering all possible methods to improve its utilization as both protein and energy sources.

Chapter Four

Growth, physiological and immunological responses of rainbow trout (*Oncorhynchus mykiss*) to different dietary inclusion levels of dehulled lupin (*Lupinus angustifolius*)*

*Farhangi, M. and Carter, C.G. (2001). Growth, physiological and immunological responses of rainbow trout (*Oncorhynchus mykiss*) to different dietary inclusion levels of dehulled lupin (*Lupinus angustifolius*). *Aquaculture Research*, **32** (Suppl. 1), 329-340.

4.1. Introduction

Finding alternative protein sources has long been a challenge for aquaculture nutritionists. Soybean meal is the most widely used plant protein in animal feeds. However, it contains the highest antinutritional factors (ANF's) compared to any other feedstuff (Vila and Mascarell, 1999). Meanwhile, the lack of suitable environmental conditions for soybean cultivation is of concern in many parts of the world including Australia. Australia has great potential for the production of grain legumes (Siddique and Skyes, 1997). Some of these ingredients have potential as replacements for fish meal. Previous experiments (Chapters Two and Three) have shown that lupin (*L. angustifolius*) is the most promising grain legume in Australia to replace fish meal for rainbow trout. Fortunately, the production of lupin is the highest of the grain legumes produced in Australia (Petterson *et al.*, 1997). Lupin production was 1,421,000 tonne compared to 107,000 tonne for soybean in Australia in 1998/1999 (Pulse Australia, 2001).

Mostly seed of white lupin (*L. albus*) has been evaluated as an alternative protein for aquatic animals, including salmonids (dela Higuera *et al.*, 1988; Burel *et al.*, 1998) gilthead sea bream (Robaina *et al.*, 1995) turbot (Burel *et al.*, 2000a) and prawns (Sudaryono *et al.*, 1999a). Some authors failed to specify which lupin species was used in their research (Hughes, 1991; Moyano *et al.*, 1992; Morales *et al.*, 1994; Sudaryono *et al.*, 1995). The superiority of sweet lupin (*L. angustifolius*) to white lupin (*L. albus*) was shown for aquatic animals in a few comparative studies (Sudaryono *et al.*, 1999c). Generally, the ANF's of lupin (*L. angustifolius*) are similar to or lower than those of lupin (*L. albus*), with the exception of

saponins, which are significantly higher in the former (Petterson *et al.*, 1997). Saponins have the potential to alter the permeability of gut membrane, resulting in the uptake of nutrients that are usually excluded by the gut membrane and interfering with the absorption of essential nutrients (Petterson *et al.*, 1999). While no marked antinutrient effect of saponins in the diet was observed in Atlantic salmon (Krogdahl *et al.*, 1995), some morphological changes in the digestive tract of rainbow trout and Chinook salmon were attributed to their inclusion in the diet (Bureau *et al.*, 1998). However, it is suggested that saponins inclusion in carbohydrate-rich diets could improve digestibility due to detergent like activity that lowers the viscosity in the digestive tract (Francis *et al.*, 2001).

The hull of lupin (*L. angustifolius*) constitutes nearly 25% of the seed and contains approximately 90% dietary fibre (Evans *et al.*, 1993). Dehulling the grain intensifies the protein and effectively dilutes the total non-starch polysaccharides (NSP) content (Evans *et al.*, 1993). However, dehulling significantly increases the concentration of oligosaccharides in the kernel of lupin (Evans *et al.*, 1993). Despite this, dehulling significantly improved the digestibility of dry matter, crude protein, gross energy and most essential amino acids of lupin (both *L. albus* and *L. angustifolius*) for silver perch (Allan, 1997; Booth *et al.*, 2001). However, no advantage in terms of weight gain or feed intake was observed for dehulled lupin (*L. albus*) compared to lupin (hulls on) when added at levels of 40% in rainbow trout diet (Hughes, 1991). In contrast, dehulling significantly reduced dry matter and groos energy digestibility of field pea in silver perch, despite significant improvement in crude protein digestibility (Allan, 1997). This phenomenon clearly shows the different response of various plant meals to particular processing methods. This may be related to the different concentration of complex carbohydrates (NSP) in various fractions of the grains. Although energy should be the first consideration in fish nutrition, in practice protein is usually given the first priority (NRC, 1993).

Similarly mainly the suitability of lupin protein, as an aquaculture feed has been extensively investigated in recent years (Hughes, 1988; dela Higuera *et al.*, 1988; Morales *et al.*, 1994; Robaina *et al.*, 1995; Burel *et al.*, 1998; Burel *et al.*, 2000; Allan *et al.* 2000b). In most of these studies the suitability of lupin protein for

aquatic animals has been shown. Lupin however, contains the highest NSP (van Barneveld, 1999) and the highest oligosaccharide (Vila and Mascarell, 1999) content of all grain legumes. Although dehulling reduces the total NSP content (mostly fibrous fraction) of lupin seed, the NSP content of dehulled lupin is still significantly higher than that of all other grain legumes (Evans, 1993; van Barneveld, 1999). The antinutritive potential of oligosacchrides has been shown in monogastric animals (Annison and Choct, 1991; Choct and Annison, 1992; Annison, 1993; Choct *et al.*, 1996; Bakker *et al.*, 1998; Iji and Tivey, 1998; Simon, 1998). However, evaluation of the suitability of lupin carbohydrates in fish diet has mostly been neglected in the past.

Considering lupin has the lowest starch and highest NSP content compared to other plant proteins, it has been proposed as a unique model for the investigation of carbohydrate utilization in monogastric animals (van Barneveld, 1999). To do this, the first step is to determine the maximum inclusion level of unprocessed dehulled lupin in the fish diet. To the best of our knowledge, the response of rainbow trout to increasing dietary inclusion levels of dehulled lupin (*L. angustifolius*) has not yet been investigated. Therefore, the current study aimed to determine the maximum dietary inclusion level of dehulled lupin as replacement for fish meal in rainbow trout diet, through consideration of the growth, physiological and immunological responses.

4.2. Material and methods

4.2.1. Experimental animals and culture conditions

Rainbow trout were supplied by the School of Aquaculture, University of Tasmania. Fish were kept in 2000-l tanks and fed with a commercial trout diet until required. Experiments were conducted in an indoor air-conditioned recirculation culture system (Carter and Hauler, 2000). Trout were weighed and randomly stocked into 300-l fibreglass tanks in duplicate groups of 38 fish. Tanks were covered with black plastic mesh to minimize disturbance and prevent escape. Tanks were supplied with continuously aerated fresh water (11L/min) at a temperature of 15°C ± 0.5. Temperature, dissolved oxygen, total ammonia, nitrite and pH were measured three times weekly and did not exceed values recommended for rainbow trout (Tarazona and Munoz, 1995). A constant photoperiod of 16L:8D was used.

Prior to the experiment, the fish were fed with a commercial diet for a 2-week adaptation period. At the end of this period, the fish were re-weighed (47.1g ± 0.169 S.E.M.) prior to starting the experiment. The experiment lasted for 8 weeks and experimental diets were fed twice a day at 09:00 and 16:30 at 2% body weight per day. Feed was distributed by hand to control hierarchy and observe feeding behavior. Fish weight was recorded every fortnight and rations adjusted accordingly. Mortality was recorded daily and the weight and number of dead fish noted to adjust feed offered to each tank.

4.2.2. Experimental diets

The suitability of diets with different inclusion levels of dehulled lupin (10, 20, 30, 40 and 50%) was compared with a control diet in which fish meal was the sole protein source (0% diet). Experimental diets were isonitrogenous and isoenergetic. Protein from dehulled lupin replaced 9.9, 19.8, 29.6, 39.5, and 49.4% of protein from fish meal in the diets. Dehulled lupin (*L.angustifolius*) was commercially provided through Milne Feeds Company (Western Australia). The dehulled lupin was finely ground (about 1mm particle size) to prepare a flour. All ingredients were homogenized and mixed, and after adding 10% water, were cold pelleted (pellet diameter, 3 mm). The moisture content of the diets was reduced using a drier (30°C for 24 h). The diets were stored at -20°C until required. The ingredient and chemical composition of the experimental diets are shown in Table 4.1.

4.2.3. Sampling

Prior to starting the experiment, random samples of diets were collected for chemical analysis. Ten fish at the start of the experiment and 3 fish per tank (6 fish per replicate) at the end of the experiment were randomly sampled for carcass analysis. At the end of the experiment, 4 fish per tank were killed, individually weighed, liver and pyloric caeca weights recorded and samples of pyloric caeca frozen in liquid nitrogen and stored at -80°C for digestive enzyme extraction. Samples of pyloric caeca and small intestine were taken, flushed with cold (4°C) phosphate buffered saline (pH 7.2) to remove the faeces and then fixed in 10% buffered formalin at room temperature for histology and image analysis. After about 17 h fasting, another 4 fish per tank were randomly sampled, anesthetized

and their blood collected using heparinized syringe from the caudial vein. Blood samples were stored at -80°C until required.

Table 4.1. Ingredient and chemical composition of experimental diets

	Diet					
Dehulled lupin (%)	0	10	20	30	40	50
Replacement of fish meal crude protein (%)	0	9.9	19.7	29.6	39.5	49.4
Ingredient composition (%)						
Fish meal	61.2	55.1	49	42.9	36.8	30.7
Dehulled lupin	0	10	20	30	40	50
Fish oil	14.4	14.9	14.5	14.2	13.9	13.6
Dextrose	9.8	6.2	3.5	1.7	2.2	2.9
α- cellulose	9.2	7.7	6.2	4.8	3.3	0.7
Bentonite	3.3	3.9	4.5	4.5	1.6	0
CMC[1]	1	1	1	1	1	1
Vitamins & mineral premixes[2]	1	1	1	1	1	1
Choline chloride	0.07	0.07	0.07	0.07	0.07	0.07
Stay C	0.05	0.05	0.05	0.05	0.05	0.05
Chemical composition (% DM basis)						
Dry matter (%)	93.2	94.5	96.8	94.7	93.3	92.4
Crude protein (N x 6.5)	44.1	43.6	43	43.6	43.4	43.4
Crude lipid	22.2	21.5	21	20.8	20.7	21.3
Total NSP[3]	20	18	16.8	16.2	18.5	19.5
Ash	12.8	12.5	11.9	11.8	8.7	6.7
Gross energy (MJ/kg)	23.2	22.7	22.1	22.5	23.1	24
CP/GE[4] (g/MJ)	19	19.2	19.5	19.4	18.8	18.1
EAAI[5]	2	1.9	1.9	1.9	1.9	1.8

1. Carboxymethylcellulose
2. Added to fulfill in excess of vitamin and mineral requirement for rainbow trout (NRC, 1993)
3. Calculated total non-starch polysaccharides
4. Crude protein/gross energy
5. Essential amino acid index = $\sqrt[n]{(a_1/a_2)\times(b_1/b_2)\times\ldots\ldots\ldots(z_1/z_2)}$, McDonald *et al.* (1995)

4.2.4. Analytical methods

All feed samples were milled to pass through a 1 mm screen before analysis that was carried out in duplicate. Whole carcasses were autoclaved in order to break down epithelial tissues, which were then freeze-dried and ground for chemical analysis. Dry matter was determined using a freeze-drier (Dynavac). Gross energy content was determined by combustion in a bomb calorimeter using benzoic acid as a standard (Galenkamp Auto Bomb). Total lipid was measured using the Soxhlet method described by (Faichney and White, 1983). Total nitrogen was quantified using the micro Kjeldhal method and protein content estimated as N × 6.25. Ash was measured following combustion at 600°C for two hours (Windham, 1995). The amino acid profile with the exception of tryptophan was quantified using the method described by Elkin and Griffith (1985). Samples were oxidized and hydrolyzed at 110°C for 24 hours with 6 N hydrochloric acid under nitrogen. Separation of amino acids was carried out using ion exchange chromatography on water High Pressure Liquid Chromatography (HPLC), (column No. 80002). Amino acids were then quantified at 540 nm with predetermination at 436 nm after post column reaction with ninhydrin. For measuring the mineral content, samples were first digested with nitric/perchloric acid in a block digestor to a temperature of 200-210°C until a white fume of perchloric acid formed. After cooling, the mixture was diluted using deionized water. The mineral content then quantified by Inductively Coupled Plasma Atomic Emission Spectrometry (ICPAES).

Plasma was obtained by centrifuging the blood samples at 11700 × g for 5 min at 4°C. Total protein of plasma was quantified using the Lowry method (Lowry *et al.*, 1951). Total plasma immunoglobulin (Ig) was also measured using Lowry method through applying the protocol that explained by Siwicki *et al.* (1994). Hematocrit was determined within two hours after blood sampling following centrifuging for 5 minutes. White blood cell count was completed using the Battlement method. Glucose content of plasma was determined using a Sigma Kit (510-A).

Fixed samples of pyloric caeca and small intestine were dehydrated in an ethanol solution series of 50% to 95%. Tissues were embedded in paraffin and after sectioning (5 μm), stained with haematoxylin and eosin for light microscopic examination. Measuring the height of villi from the pyloric caeca and small

intestine carried out through a morphometric study. Image analysis of villus height was completed using the Scion Image Program for Windows.

Trypsin (EC 3.4.21.4) activity of pyloric caeca was measured using the extract of 400 mg pyloric caeca tissue by applying the method of Preiser *et al.* (1975) modified by Pringle *et al.* (1992). Synthetic trypsin specific substrate, a-N-Benzoyl-D,L-arginine-p-nitroanilide.Hcl (BAPNA) in combination with the Bratton Marshal reaction was used for measuring the trypsin activity. To 700 µl BAPNA-reagent, 50 µl 0.05 M Tris-HCL buffer and 50 µl buffered enzyme were added and incubated 10 min at 37°C. The reaction then stopped by rapidly adding 800 µl 0.2 N HCL. The reaction was completed through adding 0.5 ml of each sodium nitrite, ammonium sulphamate and N-1-naphtylendiamine respectively at 3 min time intervals. Liberation of p-nitroaniline (pNA) occurred as a result of trypsin activity and stained the solution purple over 20 min at room temperature in the presence of N-1-naphtylendiamine. Trypsin activity then was quantified by reading the solution at 550 nm with a spectrophotometer and comparing the results with the standard curve for pNA. Trypsin activity was expressed as µmol pNA/min/g pyloric caeca. Amylase activity was determined using a modification of the method described by Walker and Harmon (1996). Briefly, diluted extracts of pyloric caeca were incubated with potato amylopectine for 15 min at 15°C. Alkaline potassium ferricyanide was added to stop the reaction and the solutions were then boiled for 10 min and diluted after cooling by adding 40 ml distilled water. The amount of end product (reducing sugar) was determined by reading the optical density at 420 nm and comparing the results with the standard curve for maltose. Amylase activity was expressed as µmol maltose/ min/ ml.

4.2.5. Statistical analysis

All statistical tests were performed using the SPSS Statistical Analysis Software Program (version 9.0.1 for Windows, 1999). A general trend effect rather than differences between means of different treatments was the main interest in this study. Thus the regression model was selected as the most powerful design for the current experiment. Pearson's correlation coefficient was used to determine the relation between the different variables. One-way analysis of variance was also conducted to assess the effect of dietary treatments on different responses and,

when appropriate, the differences between means were tested using the Tukey test. Probability values less than 0.05 were considered significant.

4.3. Results

The amino acid composition of lupin (hulls on and dehulled), fish meal and fish requirements were compared (Table 4.2). The amino acid concentration of both dehulled lupin (DL) and hulls on lupin were lower than that of fish meal. However, this value improved for DL compared to hulls on lupin. The amino acid composition of both lupin meals used were well above fish requirements, with the exception of Met & Cys and Lys.

Table 4.2. Selected amino acid composition of lupin (hulls on and dehulled), fish meal (g/kg) and fish requirement (g/kg of diet)

	Lupin (hulls on)	Lupin (dehulled)	Fish meal	Requirement[1]
Arg	33.11	48.70	52.74	15
His	7.70	11.40	23.91	7
Iso	12.60	17.60	30.90	9
Leu	20.00	27.65	50.74	14
Lys	13.20	16.25	63.33	18
Met+Cys	6.20	8.40	27.26	10
Phe+Tyr	23.30	33.35	50.92	18
Val	12.00	16.55	35.89	12
EAAI[2]	1.14	1.58	3.24	-

1. (NRC, 1993)
2. Essential amino acid index = $\sqrt[n]{(a_1/a_2)\times(b_1/b_2)\times\ldots\ldots\ldots(z_1/z_2)}$ McDonald *et al.* (1995)

The amino acid composition of the experimental diets and fish requirements are compared in Table 4.3. Fish requirement for all amino acids was exceeded by all experimental diets. All experimental diets were well accepted and survival was 100% for all treatments (Table 4.4) with the exception of fish that fed with the control diet (0% dehulled lupin).

Table 4.3. Comparison of the amino acid composition of experimental diets (g/kg) and rainbow trout requirement (g/kg of diet)

DL (%)	0	10	20	30	40	50	Requirement[1]
Arg	32.3	33.9	35.6	37.3	38.9	40.6	15
His	14.6	14.3	14.0	13.7	13.4	13.1	7
Iso	18.9	18.8	18.7	18.5	18.4	18.3	9
Leu	31.1	30.7	30.4	30.1	29.8	29.4	14
Lys	38.8	36.5	34.3	32.1	29.8	27.6	18
Met + Cys	16.7	15.9	15.2	14.2	13.4	12.6	10
Phe + Tyr	31.2	31.4	31.6	31.9	32.1	32.3	18
Val	22.0	21.4	20.9	20.4	19.8	19.3	12

1. (NRC, 1993)

Final weight ranged between 125.85 and 139.05g and there was a significant difference between the control diet (0% dehulled lupin) and 50% dehulled lupin diet (Table 4.4). However, there was no significant difference between control and all other treatments. FCR ranged from 0.91 to 1.00 and showed significant differences between the control and 50% dehulled lupin diet. However, this value remained similar for all other groups. Although PER and PPV were lower at higher inclusion levels of dehulled lupin, there were no significant differences between all treatments. The obvious differences occurred in EER, which decreased at higher inclusion levels of dehulled lupin. This value was reasonably similar between the control diet and diets with up to 30% dehulled lupin diet (Table 4.4).

Carcass composition generally showed similar values for all treatments (Table 4.5). However, crude protein composition was significantly higher (16.81%) for the 10% dehulled lupin diet compared with the 20% dehulled lupin diet (16%).

Table 4.4. Growth response and feed utilization of rainbow trout fed diets with increasing dehulled lupin content

	Diet (% dehulled lupin)						
Parameter	0	10	20	30	40	50	P
Initial weight (g)	46.9	47.5	46.85	47.75	47.05	46.5	NS
	0.3	0.6	0.15	0.25	0.25	0.4	
Final weight (g)	139.1^{b}	136.5^{a}	134.2^{a}	133.6^{a}	131.1^{a}	125.8^{a}	≤ 0.05
	0.45	3.4	1.1	0.75	2.8	1.95	
Weight gain (g)	93.9^{b}	89.0^{ab}	87.3^{ab}	85.9^{ab}	84.1^{ab}	79.4^{a}	≤ 0.05
	0.98	2.76	0.97	1.13	2.57	1.6	
CF	1.62	1.58	1.56	1.64	1.52	1.55	NS
	0.05	0.02	0.05	0.1	0.01	0.02	
FCR	0.91^{a}	0.96^{ab}	0.98^{b}	0.98^{b}	0.97^{ab}	1^{b}	≤ 0.05
	0.01	0.01	0.01	0.01	0.01	0.01	
PER	2.49	2.39	2.37	2.34	2.37	2.31	NS
	0.02	0.02	0.03	0.04	0.04	0.02	
PPV	41.37	41.54	38.3	39.74	39.51	38.94	NS
	0.41	0.27	1.24	1.04	0.81	0.76	
LER	4.94	4.85	4.84	4.9	4.98	4.71	NS
	0.03	0.04	0.06	0.08	0.08	0.05	
LPV	76.46	73.48	74.16	71	67.83	68.24	NS
	4.33	1.93	0.72	0.26	2.22	3.21	
EER	47.76^{c}	45.88^{b}	46.12^{b}	45.09^{b}	44.46^{a}	41.81^{a}	≤ 0.01
	0.04	0.35	0.62	0.73	0.71	0.42	
Mortality (%)	1.31	0	0	0	0	0	NS
	1.31						

Each value is the mean (± S.E.M) of two replicates
Means within the same row with unlike superscript letters were significantly different (Tukey's multiple range test)
CF: Condition factor = 100×[(whole live body weight (g)/(fork length (cm^3))]
FCR: Feed conversion ratio = g feed intake /g live weight gain
PER: Protein efficiency ratio = g weight gain /g crude protein intake
PPV: Protein productive value = g protein retained / g protein intake
LER: Lipid efficiency ratio = g weight gain / g lipid intake
LPV: Lipid productive value = g lipid retained /g lipid intake
EER: Energy efficiency ratio = g live weight gain / energy intake (MJ)

Table 4.5. Chemical composition (% wet weight) of rainbow trout fed the diets with increasing dehulled lupin content

	Diet (% dehulled lupin)						
Parameter	0	10	20	30	40	50	P
Dry matter	34.46	34.63	34.31	34.05	33.45	33.86	NS
	0.41	0.31	0.24	0.5	0.37	0.54	
Crude protein	16.31^{ab}	16.81^{b}	16^{a}	16.53^{ab}	16.3^{ab}	16.43^{ab}	≤ 0.05
	0.23	0.18	0.11	0.2	0.06	0.14	
Crude lipid	15.18	14.96	15.1	14.55	14	14.55	NS
	0.49	0.36	0.27	0.58	0.32	0.64	
Ash	2.18	2.23	2.17	2.25	2.18	2.25	NS
	0.03	0.03	0.04	0.02	0.07	0.04	

Each value is the mean (± S.E.M) of two replicates
Means within the same row with unlike superscript letters were not significantly different (Tukey's multiple range test)

There were no significant differences between any of the digestive tract responses and enzyme activities (Table 4.6). Although villus height for both pyloric caeca and small intestine gradually decreased at higher inclusion levels of dehulled lupin, this trend was not significant.

Hematocrit and total Immunoglobulin (Ig) were similar for all treatments (Table 4.7). Total plasma protein was significantly lower in the 30%, 40% and 50% dehulled lupin diets compared to the 10% dehulled lupin diet. White blood cell count also showed similar values for lymphocytes and monocytes. However, the neutrophil count was significantly higher for the group on the 20% dehulled lupin diet compared to the 30% group.

Table 4.6. Digestive enzyme activity and digestive tract characteristics of rainbow trout fed with increasing dehulled lupin content

	Diet (% dehulled lupin)						
Parameter	0	10	20	30	40	50	P
Hepatosomatic index (% BW)	1.62	1.58	1.56	1.64	1.52	1.55	NS
	0.05	0.02	0.05	0.09	0.01	0.02	
Pyloric caeca index (% BW)	5.49	4.82	5.46	5.03	4.61	4.8	NS
	0.12	0.14	0.35	0.28	0.07	0.04	
Trypsin activity µmol pNA/g tissue/min	0.075	0.086	0.08	0.072	0.077	0.076	NS
	0.003	0.002	0.001	0.003	0.004	0.002	
Amylase activity µmol maltose/ml/min	164.1	157.1	160.5	155.9	162.8	156.8	NS
	3.76	5.06	5.6	2.81	4.05	5.73	
Villus height:pyloric caeca (µm)	497.1	472.7	466.6	470.7	519.3	463.7	NS
	48.61	65.58	14.6	68.79	17.1	0.83	
Villus height: small intestine (µm)	578.0	536.8	491.4	516.7	514.1	498.3	NS
	39.63	31.8	77.91	64.62	4.51	28.89	

Each value is the mean (± S.E.M) of two replicates

Table 4.7. Non-specific immune responses of rainbow trout fed with increasing dehulled lupin content

	Diet (% dehulled lupin)						
Parameter	0	10	20	30	40	50	P
Hematocrit (%)	43.9	43.52	43.18	42.27	4.22	41.31	NS
	1.43	1.5	1.52	1.4	1.68	1.72	
Total protein (mg/ml)	46.3^{ab}	48.49	45.63	39.38	38.49	38.32^{a}	≤ 0.01
	1.73	1.13	1.69	1.86	1.76	3.01	
Total Ig (mg/ml)	15.65	15.79	15.39	15.37	15.02	14.66	NS
	0.73	0.73	0.8	0.54	0.71	0.66	
White Blood Cell							
Lymphocyte (%)	91.87	90.75	90.25	93.62	91.37	90.87	NS
	1.31	1.39	0.7	0.86	1.42	1.39	
Monocyte (%)	3.12	3.62	2.25	2.5	2.75	2.87	NS
	0.69	0.75	0.55	0.42	0.88	0.51	
Neutrophils (%)	5^{ab}	5.62^{ab}	7.5^{b}	3.37^{a}	4.87^{ab}	6.25^{ab}	≤ 0.05
	0.77	0.86	0.42	0.59	0.95	1.03	

Each value is the mean (± S.E.M) of two replicates
Means within the same row with unlike superscript letters were not significantly different (Tukey's multiple range test)

Significant relationships between dietary inclusion level of dehulled lupin and feed intake, FCR and growth were described by regression analysis (Table 4.8). There was a strong negative linear relationship between a higher inclusion level of dehulled lupin and growth rate ($R^2 = 0.81$, $P < 0.0001$).

Table 4.8. Regression relationships between dietary inclusion levels of dehulled lupin, feed intake, feed conversion ratio (FCR) and weight gain

Parameter	Equation	R^2	P
Feed intake	$Y= 2885.82 + 757.9X - 2144.36X^2$	0.71	≤ 0.01
FCR	$Y= 0.918 + 0.361X - 0.437X^2$	0.74	≤ 0.01
Weight gain	$Y= 92.96 - 25.47X$	0.81	≤ 0.001

A similar trend relationship (negative) was observed between diets (at higher inclusion level of dehulled lupin) and EER (R^2 = 0.81, $P < 0.0001$). This relationship between different inclusion level of lupin and PER was significant, however it showed a lower value compared to the trend relationship between diets and EER (Figure. 4.1). There was a strong positive correlation between EER and growth ($r = 0.955$, $P < 0.01$) and EER and PER ($r = 0.84$, $P < 0.01$). Although the glucose concentration of plasma was not significantly different between treatments, the total protein concentration of plasma generally decreased at higher inclusion levels of lupin. This parameter was significantly lower in the 30%, 40% and 50% dehulled lupin diets compared to the 10% dehulled lupin diet (Figure. 4.2).

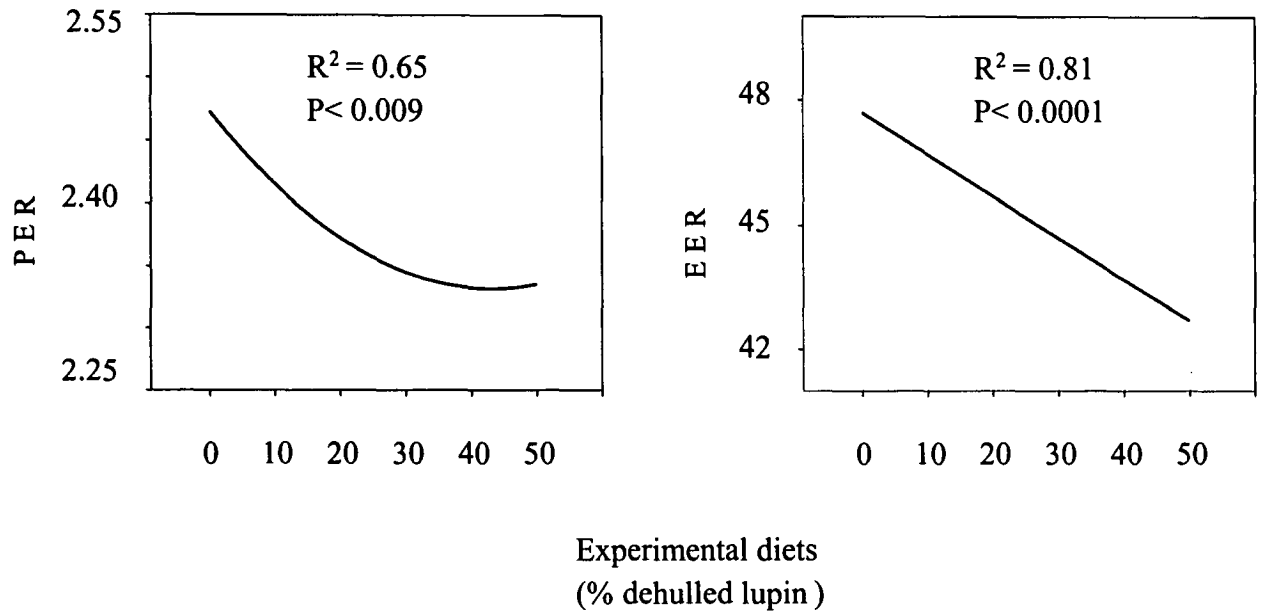

Figure 4.1. Regression analysis of protein efficiency ratio (PER) and energy efficiency ratio (EER) for different experimental diets

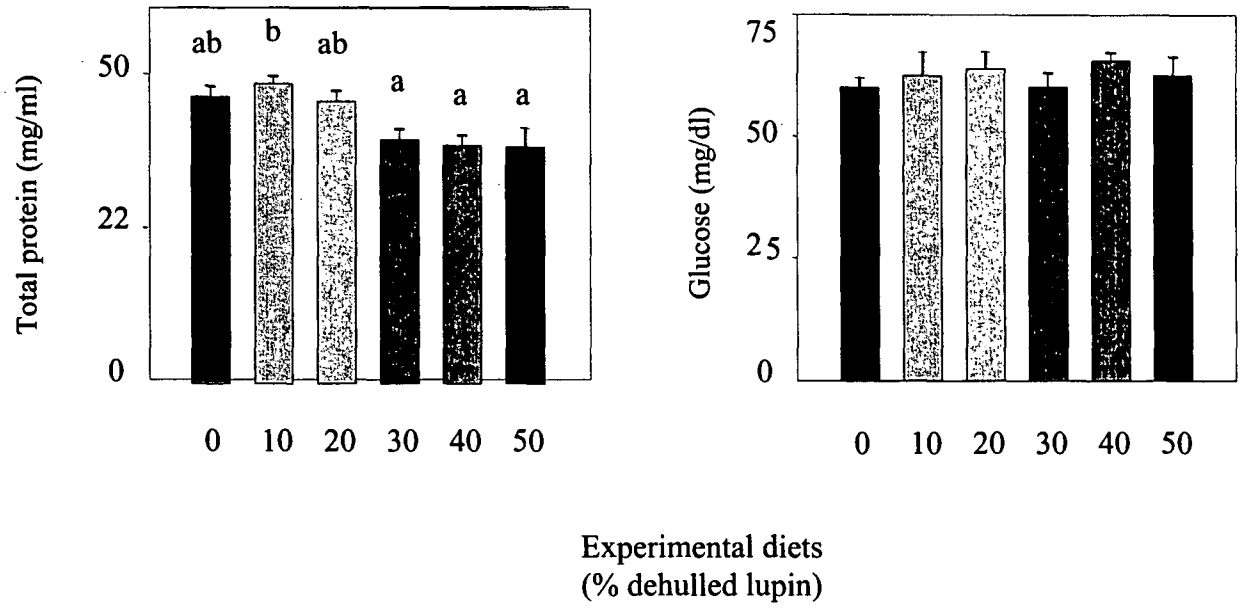

Figure 4.2. Total protein and glucose concentration of plasma for different experimental diets

4.4. Discussion

All diets were well accepted by fish, which is in agreement with the previously reported studies using lupin in salmonids (dela Higuera *et al.*, 1988; Hughes, 1991; Moyano *et al.*, 1992; Morales *et al.*, 1994; Carter, 1998; Burel *et al.*, 1998). However, because of the restricted feeding regime (2% BW) in this experiment, a final conclusion about the palatability of dehulled lupin cannot be made. The restricted feeding regime was applied in order to show the real nutritional value of lupin for rainbow trout. It has been suggested that fish can increase feed intake to fulfill their energy requirements (Tacon and Cowey, 1985). In this case, distinguishing nutrient deficiency in the tested ingredient is not possible due to the compensatory effect of increased feed intake. Morales *et al.* (1994) reported a higher feed intake of lupin by rainbow trout compared to other plant proteins, however they related the fish's higher intake to lower availability of energy rather than higher palatability of lupin. The low digestibility of carbohydrate in lupin compared with other dietary components confirmed their finding. Watanabe *et al.* (1993) formulated a plant-based diet in such a way as to have a higher energy content when compared with fish meal-based diets for rainbow trout, to compensate for the indigestible carbohydrates in plant ingredients. However, a feeding hierarchy can develop in fish fed restricted rations, resulting in considerable individual variability in feed intake and growth (McCarthy *et al.*, 1992). Due to the high fish density in each tank in the present study, hierachy was not developed. However the effect of a restricted feeding regime on individual fish weight variation was not investigated in this study.

Although the weight gain generally decreased at higher inclusion levels of dehulled lupin (Table 4.4), there were no significant differences between treatments up to a 40% inclusion level. Similarly, an inclusion level of 40% dehulled lupin (unknown species) for rainbow trout was demonstrated to have no significant effect on growth performance (Hughes, 1991). However, the researchers supplemented the experimental diets with protein sources other than fish meal. The performance of fish that received the lupin in their diet was compared with a control diet that was based on soybean meal. Inclusion of lupin (*angustifolius*) in gilthead sea bream diet (up to 30%) was

successful when diets were supplemented with Lys and Met (Robaina *et al.*, 1995). In contrast, in the current study the experimental diets were not supplemented with crystalline amino acids. Furthermore, the control diet was based solely on fish meal as the protein source. Weight gain generally decreased with increased diet levels of heat-treated lupin seed (*L. albus*) (supplemented with amino acids) up to 40%. dela Higuera *et al.* (1988) suggested a maximum inclusion level of 30% lupin for rainbow trout. Recently, extruded dehulled lupin (*L. albus*) was included up to 70% in a trout diet without significant effect on growth rate when supplemented with other protein sources and L-methionine (Burel *et al.*, 1998). However, successful incorporation of extruded dehulled lupin (*L. albus*) up to 50% in trout diet was recommended with more confidence (Burel *et al.*, 1998). In all the above-mentioned research projects, experimental diets were offered to fish *ad libitum* and supplemented with other protein sources (Hughes, 1991; Burel *et al.*, 1998), amino acids (dela Higuera *et al.*, 1988; Burel *et al.*, 1998) or poly-unsaturated fatty acids (PUFA) (Robaina *et al.*, 1995). In fact this is the first study in which dehulled lupin (up to 40% inclusion level) successfully supported the growth of rainbow trout similar to the control diet without supplementation with other protein sources (in addition to fish meal) or nutrients under a restricted feeding regime.

In the present study, FCR generally worsen at higher inclusion levels (Table 4.4). However, it was not significantly different up to 40 % inclusion level in the diet. This is in agreement with findings of other researchers (dela Higuera *et al.*, 1988; Robaina *et al.*, 1995; Burel *et al.*, 1998). Although efficient economic return of higher FCR needs more investigation for each experimental diet, generally higher FCR leads to deterioration of water quality (Cho, 1997) and may limit the high inclusion level of lupin in intensive production systems. Regression analysis demonstrated a significant negative relationship between inclusion of lupin and EER (Figure 4.1). In spite of the gradual decrease of EER at higher inclusion levels of dehulled lupin, differences were not significant up to 30% inclusion. Low EER is the main factor limiting efficient utilization of lupin in salmonid diets. This might be caused by a high concentration of NSP due to higher inclusion of lupin in the diet. Digestibility of NSP by silver perch

significantly decreased from 21.9% to 5.1% when the percentage of dehulled lupin (*L. angustifolius*) increased from 30% to 50% respectively (Evans, 1998). PER and PPV were not significantly different among the experimental diets, in spite of their gradual decrease. Low EER in this study is in agreement with Wiryawan and Dingle (1999) who ranked the metabolizable energy of lupin as the second lowest after faba bean for growing chicken, despite its highest gross energy content compared to other grain legumes.

EER showed a significant negative relationship with dehulled lupin inclusion. Although the negative relationship was significant for PER, it showed a lower slope compared to regression value for EER (Figure 4.1). Meanwhile, the glucose concentration was not affected in different experimental diets. On the other hand, plasma total protein significantly decreased at higher inclusion levels (10% *vs* 50% diet) of dehulled lupin (Figure 4.2). This data may indicate gluconeogenesis at higher inclusion levels of dehulled lupin. This is in agreement with the finding by Brauge *et al.* (1994) that attributed the high glucose level of rainbow trout 16 and 30 hours after being fed on low digestible carbohydrates to gluconeogenesis.

Measuring enzyme activity by studying the comparative responses of fish to different diets is a useful tool for evaluating ingredients, and selecting the ingredient pool for feed formulation. A significant reduction in trypsin activity was reported when the levels of lupin seed meal (*L. angustifolius*) was increased from 0% to 30% in gilthead sea bream diet (Robaina *et al.*, 1995). Such an effect was not observed in the present study, even at a 50% inclusion level of dehulled lupin (Table 4.6). This confirms the low concentration of trypsin inhibitor (TI) in dehulled lupin. Wiryawan (1999) and Alonso *et al.* (2000) claimed that dehulling intensifies TI in the cotyledons, however an assay of the TI concentration of cotyledon in this study did not detect such a difference. Torrissen *et al.* (1994) found less trypsin activity in the pyloric caeca than in the small intestine of Atlantic salmon when they tested different dietary proteins. Extraction of the enzyme through the pyloric caeca may have hidden probable significant differences between treatments in the present experiment.

The activity of carbohydrases is more dependent on the type of feeding and food composition than protease activity in fish (Ugolev and Kuzmina, 1994; Kuzmina, 1996). Moullac *et al.* (1996) proposed that protein sources can also affect amylase activity in shrimp and the same phenomenon was shown in rainbow trout (Spannhof and Plantikow, 1983). This means that amylase activity is not specifically determined by carbohydrate. The inhibitory effect of the high inclusion level of NSP on amylase activity was not detected in the present study. The result of this experiment shows that gelatinized starch can be used as a complementary energy source at high inclusion levels of lupin in trout diets. Restricted feeding regimes may have minimized different quantitative responses for enzyme measured in this experiment. It is suggested that restricted feeding regimes can affect enzyme activity, especially of carbohydrase (Ugolev and Kuzmina, 1994).

None of the experimental diets significantly affected the digestive tract (proximal small intestine) morphology in this study (Table 4.6). The deleterious effect of plant meals inclusion and especially of soybean meal in fish diets on both proximal and distal intestine has been reported (van den Ingh *et al.*, 1991; Rumsey *et al.*, 1995). However, morphological changes following the consumption of plant meals has been noticed mainly in the distal intestine (van den Ingh *et al.*, 1996; Refstie, 2000). Despite the various effects produced by using different sources and levels of carbohydrates on growth parameters in trout, Buddington and Hilton (1987) did not observe any histological or morphometric changes in trout digestive tracts. Although liver morphology was not evaluated in this study, no change was reported in liver histology when lupin was included up to 30% in gilthead sea bream diets (Robaina *et al.*, 1995). However, there is some evidence showing that a change of diet from natural to artificial only affects microvillus structure, potentially altering the absorptive efficiency of the pyloric caeca in southern blue-fin tuna (Zarrinkalam *et al.*, 1998). The hepatosomatic index (HSI) was similar in this study for different treatments, which is in agreement with the study of (Robaina *et al.*, 1995). However, Kim and Kaushik (1992) and Brauge *et al.* (1994) reported higher HSI for rainbow trout when fed diets

with higher digestible carbohydrate. It seems there is a correlation between carbohydrate digestibility and higher HSI.

The relationship between the digestive tract and immune response (DeWitt and Kudsk, 1999) and diet and immunity (Chandra, 1999) are well established in the literature. Non-specific immune responses are good indices for evaluating the fish immune response, as they act quickly (Fletcher, 1982). The literature about the influence of more complex carbohydrates such as NSP on non-specific immune response is limited. The immunostimulant effects of some complex carbohydrates have recently been reviewed in fish (Sealey and Gatlin, 1999). It was interesting to find such a phenomenon after consumption of NSP by fish. However, such a response was not observed for those immune responses tested in this study (Table 4.7). With the exception of total protein and neutrophils, none of the non-specific immune responses were affected in the current study. This is in agreement with Page *et al.*'s (1999) findings where non-specific immune responses were not affected by long-term feeding of a high carbohydrate diet (gelatinized starch) in rainbow trout. Also various dietary carbohydrates affected the immune response of Atlantic salmon to a minor extent in both freshwater and saltwater (Waagbo *et al.*, 1994). In contrast, Hemre *et al.* (1995b) reported a significant decrease of hematocrit and haemoglobin at higher inclusions of starch in Atlantic salmon. Recently, it has been shown that the inclusion of soybean molasses in Atlantic salmon diet could significantly increase the level of both lysozyme and Ig in the mucosa of the mid and distal intestine (Krogdahl *et al.*, 2000). Considering all non-specific immune responses, it can be concluded that none of the experimental diets used in this experiment had a suppressive or stimulatory effect on the digestive tract that resulted in abnormal immunological responses. It would be worthwhile investigating the immunostimulant effect of the diets that contain high levels of lupin when fish are challenged by some infectious parameters.

4.5. Conclusion

Up to 40% inclusion of dehulled lupin was successful in this experiment without extra supplementation by amino acid or protein sources other than fish meal. If this were

achieved in a practical feed, the feeding cost would be dramatically decreased. However, further studies are needed to find out the maximum inclusion level of dehulled lupin, using other feeding regimes (e.g. *ad lib* using a different number of feed per day) considering the efficient utilization of essential nutrients. Meanwhile, digestive tract adaptation to high inclusion levels of dehulled lupin should be studied in more detail. The effect of high inclusion levels of dehulled lupin on organoleptic characteristics of carcass and water quality need further study. This information is critical for intensive aquaculture production systems.

Chapter Five

Effect of enzyme supplementation to dehulled lupin-based diets on growth, feed efficiency, nutrient digestibility and carcass composition of rainbow trout (*Oncorhynchus mykiss*)

5.1. Introduction

Australia is in a unique position in terms of grain legume production in the world. The potential of grain legumes for fish meal replacement has been extensively investigated in the past. The superiority of lupin (*L. angustifolius*) over other available grain legumes in Australia has been shown in Chapters Two and Three. Dehulled lupin when added up to 40% in a rainbow trout diet can support growth performance under a restricted feeding regime (Chapter Four). At higher inclusion levels of dehulled lupin, growth performance was significantly decreased, despite there no being obvious influence on physiological or immunological responses of rainbow trout. This might be related to presence of non-starch polysaccharides (NSP) (e.g. oligosaccharides) in lupin. The level of NSP (Perez-Maldonado *et al.*, 1999) and oligosaccharides in lupin is higher than for all other major grain legumes (Petterson *et al.*, 1997). Until recently, oligosaccaharides were classified with other indigestible components as dietary fibre. They are now categorized as NSP, due to their similar response to intestinal enzymes (Iji and Tivey, 1998). NSP comprise of insoluble neutral detergent fibre (NDF) and an soluble fraction. The soluble fraction includes oligosaccharides, pectin and some hemicellulose (Bakker *et al.*, 1998). None or only a small amount of the soluble NSP can be digested by monogastric animals due to a lack of suitable enzymes in the digestive tract (Alloui *et al.*, 1994).

The antinutritive effect of NSP for monogastric animals is well defined in the literature (Annison 1993; Choct *et al.*, 1996; Bakker *et al.*, 1998; Iji and Tivey, 1998; Simon 1998) and mostly attributed to the soluble fraction (Choct, 1997). On the other hand, some beneficial effects of insoluble NSP have been proposed (Iji and Tivey, 1997b; Bakker *et al.*, 1998). It has been suggested that insoluble NSP could regulate the normal motility of the digestive system by absorbing large amounts of water (Smits and Annison, 1996). The major detrimental effects of

soluble NSP are related to a viscous nature and resultant physiological and morphological effects on the digestive tract (Choct, 1997). The viscous properties may impair the diffusion and transport of lipase, oils and bile salt micelles (Smits and Annison, 1996) and inhibit the diffusion of both digestive enzymes and nutrients (Annison *et al.*, 1996). Meanwhile, viscosity increases the weight of digestive tract (Bakker *et al.*, 1998), resulting in higher protein synthesis of gut tissue (Simon, 1998) and increased energy expenditure for maintenance (Simon, 1998; Bakker *et al.*, 1998). Viscosity or the antinutritive effect of soluble NSP could be decreased in aqueous solution by cleaving the polymers using appropriate enzymes (Choct, 1997; Simon, 1998). This procedure has been demonstrated in almost all experiments conducted with pigs and poultry (Simon, 1998). Concomitantly, the same enzymes partially hydrolyse insoluble NSP making them soluble (Simon, 1998). Improving the digestibility by decreasing the viscosity is mainly facilitated through the diffusion of substrate and digestive enzymes, increased gut contraction and improved contact of absorbable nutrients with enterocytes (Simon, 1998).

Applying different enzymes to overcome the antinutritive effect of soluble NSP produces considerable variation between studies, with more consistent results obtained for poultry than pigs (Danicke *et al.*, 1999). The enzyme effect is more obvious if animal oil (high melting point) rather than vegetable oil is used in the diet due to the interference in fat digestion by a viscous ingesta (Danicke *et al.*, 1999). However, using enzymes has not produced results in many cases (Alloui *et al.*, 1994; Annison *et al.*, 1996; Gdala *et al.*, 1997b; Kocher *et al.*, 2000). Significantly lower growth performance of rainbow trout, when dehulled lupin constituted 50% of the diet (Chapter Four), may be related to the antinutritive properties of NSP. It has been shown that dehulling concentrates the soluble NSP (mainly oligosaccarides) in the kernel (Evans *et al.*, 1993). However, there is little evidence to show the antinutritive effect of lupin's highly branched NSP in poultry nutrition (Annison *et al.*, 1996; van Barneveld, 1999), as the antinutritive effect of NSP has been mainly confirmed for cereals (Annison, 1993).

The antinutritive properties of oligosaccaharides have not yet been fully investigated in fish (Francis *et al.*, 2001). The availability of nutrients was reduced when salmonids were fed a diet containing NSP (alginate and guar gum) compared to a NSP-free diet (Storebakken, 1985; Storebakken and Austreng, 1987). However, none of these compounds represent the main NSP that exist in the most widely used ingredients in monogastric nutrition (grain legumes and cereals). Recently Refstie *et al.* (1998) showed that the inclusion of reduced-oligosaccharides soybean (RO-SBM) in Atlantic salmon, results in significantly higher growth performance and digestibility values compared with ordinary soybean meal when both soybean products supplied 40% of the diet protein. However, it was difficult for the authors to attribute the lower growth performance of fish fed with ordinary soybean meal to the existence of oligosaccharides in the diet. The higher growth performance of fish fed by RO-SBM might be related to a lower level of trypsin inhibitor (TI) or lectin in the RO-SBM diet. In another study with Atlantic salmon, the cause of the morphological alterations in distal intestine remained unknown (saponins or oligosacchrides), (van den Ingh *et al.*, 1996). Digestibility of organic matter, nitrogen (significant), fat and starch were reduced when Atlantic salmon were fed with a diet containing soybean meal compared with a diet in which ROM-SBM was included (Refstie *et al.*, 1999).

To the best knowledge of the author, improving the nutrient utilization of lupin using exogenous enzymes has not yet been evaluated for salmonids. The objectives of the current experiment were to determine the effect of commercial enzymes; Energex™ (a multi-component enzyme used for improvement of the digestibility of hemicellulose), Bio-Feed™ Pro (a bacterial protease used for improvement of the digestibility of feed proteins) and Alpha galactosidase™ (used for the hydrolysis of raffinose and stachyose), (separate or in a mixture) on growth performance, feed intake, nutrient digestibility, gastrointestinal response and body composition when rainbow trout were fed at satiation, a diet containing 50% dehulled lupin.

5.2. Materials and methods

5.2.1. Experimental animals and culture conditions

Rainbow trout were supplied by the School of Aquaculture, University of Tasmania. Fish were kept in 2000-l tanks and fed *ad lib* three times a week with

commercial trout diet until required. Experiments were conducted in an outdoor recirculation culture system. Trout were weighed and randomly stocked into 300-l fiberglass tanks in duplicate groups of 38 fish. Tanks were covered with black plastic mesh to minimize disturbance and prevent escape, and supplied with continuously aerated fresh water (11L/min) at a temperature of 15°C ± 0.5. Temperature, dissolved oxygen, total ammonia, nitrite and pH were measured three times weekly and did not exceed values recommended for rainbow trout (Tarazona and Munoz, 1995). A natural photoperiod was used. Prior to the experiment, fish were fed with a commercial diet for a 2-week adaptation period. At the end of this period, fish were re-weighed 47.1g ± 0.169 (S.E.M.) prior to starting the experiment. The experiment lasted for 6 weeks and experimental diets were fed twice a day at 09.00 and 16.30 at satiation level. Feed was distributed by hand to control hierarchy and observe the feeding behaviour. Fish weight was recorded every fortnight and feed intake monitored accordingly. Mortality was recorded daily, and the weight and number of dead fish noted to adjust feed offered to each tank.

5.2.2. Experimental diets

Experimental diets were isonitrogenous and isoenergetic and formulated to fulfill the nutritional requirement of growth out rainbow trout. There were two control diets in the current experiment; a fish meal-based diet (as negative control) without adding any plant protein (FM diet), and a diet, which contained 50% dehulled lupin (Lupin diet) without enzyme supplementation (positive control). Other experimental diets were exactly the same as the positive control diet; however, they included different exogenous enzymes (see Table 5.1). Novo Nordisk Company supplied the exogenous enzymes. Ytterbium oxide (Yb_2O_3) was included at 0.01% level in all diets as an external marker for measuring the digestibility. Yb_2O_3 was used in this experiment as the digestibility marker as its solubility in diet, the precise analytical measurement and absence of toxicity was preferred to other markers including chromium oxide for fish (Williams, 1998). Dehulled lupin was finely ground to prepare homogenized flour.

5.1. Ingredient and chemical composition of experimental diets containing fish meal (FM), lupin and lupin plus enzymes

	FM	Lupin	L (α)	L (B)	L (E)	L (Mix)
Ingredient composition (%)						
Fish meal	65.85	34.14	34.14	34.14	34.14	34.14
Fish oil	12.02	13.09	13.09	13.09	13.09	13.09
Dehulled lupin	0	50	50	50	50	50
Dextrose	10	0	0	0	0	0
A cellulose	10	0.65	0.65	0.65	0.65	0.65
CMC[1]	1	1	1	1	1	1
Vitamins & mineral premixes[2]	1	1	1	1	1	1
Choline chloride	0.07	0.07	0.07	0.07	0.07	0.07
Stay C	0.05	0.05	0.05	0.05	0.05	0.05
Enzyme (in diets, ppm)	-	-	3000	300	1800	@[3]
Chemical composition (% DM basis)						
Dry matter	91.70	92.57	92.31	92.46	92.66	92.18
Crude protein (N x 6.5)	43.04	42.15	41.82	41.74	41.65	41.28
Ash	9.61	6.42	6.37	6.39	6.48	6.30
Gross energy (MJ/kg)	21.44	21.62	21.43	21.66	21.37	21.61

1. Carboxymethylcellulose
2. Added to fulfill in excess of requirements for rainbow trout (NRC, 1993)
3. Combination of all three enzymes (each as the same dosage used for other diets) was added to the mixed diet

The minor ingredients were initially mixed by hand and then gradually mixed with major ingredients. All ingredients were mixed and after adding 10% water, were cold pelleted (pellet size, 3 mm). The moisture content of the diets was reduced using a drier (30°C for 24 h). The diets were stored in thick plastic containers in dark conditions at -20°C until required.

5.2.3. Sampling

Before starting the experiment, random samples of diets were collected for chemical analysis. Ten fish at the start of the experiment and 3 fish per tank (6 fish per treatment) at the end of the experiment were randomly sampled for carcass analysis. One week before finishing the experiment, faeces were collected overnight (two nights) in collection chambers, which were surrounded by ice to minimize the breakdown of nutrients over the collection period. Faeces from each tank were pooled from the two-day collection period, freeze-dried and stored at -18°C until analysis.

At the end of the experiment, four fish were killed, individually weighed and liver and pyloric caeca weights recorded. The weight and length of both small and distal intestine were also recorded. Samples of distal intestine were taken, flushed with cold (4°C) phosphate buffered saline (pH 7.2) to remove the faeces and then fixed in 10% buffered formalin at room temperature for histology work. The effect of different experimental diets was only considered in distal intestine as the previous studies have shown that mainly this location is affected by rich-carbohydrate diets (van den Ingh *et al.,* 1991; van den Ingh *et al.,* 1996; Krogdahl *et al.,* 2000). After about 17 hours fasting, another four fish per tank were randomly sampled, anesthetized and their blood collected from the caudal vein. Blood samples were stored at -80°C until required.

5.2.4. Analytical methods

All feed samples were milled before analysis was carried out in duplicate. Whole carcasses were autoclaved in order to break down epithelial tissues, and then freeze dried and ground for chemical analysis. Dry matter was determined using a freeze drier. Gross energy content was determined by combustion in a bomb calorimeter using benzoic acid as a standard (Galenkamp Auto Bomb). Total lipid was measured using the Soxhelet method as described by Faichney and White (1983). Total nitrogen was measured using the Kjeldhal method and protein content estimated as N×6.25. Ash was measured through burning the samples at 600°C for two hours (Windham, 1995. The protocol dscribed by Refstie *et al.* (1997) was used to quantify Yb_2O_3. Briefly, the homogenized samples of 150-200 mg were freeze dried and then combusted at 550°C overnight in glass scintillation vials. After cooling the vials, 5 ml of HCl:HNO_3 in the proportion of 2:1 was added to

each vial. The samples were boiled until colorless. Then the samples mixed with 1.25 ml concentrate HNO_3 and then were diluted with distilled water to 25 ml. Yb_2O_3 was quantified using an Inductively Coupled Plasma (ICP) spectrometer. Plasma was obtained through centrifuging the blood samples at 11700 × g for 5 min at 4°C. Plasma glucose was determined using a Sigma Kit (510-A). Fixed samples of distal intestine were dehydrated in an ethanol solution series of 50% to 95%. Tissues were then embedded in paraffin and after sectioning (5 μm), stained with haematoxylin and eosin for light microscopic examination.

5.2.5. Statistical analysis

One-way analysis of variance was conducted to assess the effect of dietary treatments on different responses and, when appropriate, the differences between means were tested using the Tukey test. Regression coefficient was used to determine the relation between the different variables. Probability values of less than 0.05 were considered as significant. All statistical tests were performed using the SPSS Statistical Analysis Software Program (version 10 for Windows, 2001).

5.3. Results

The lowest and the highest weight gains were recorded for FM (0% of lupin) and L (E) diets, respectively and showed a significant difference. However, there were no significant differences between groups that were fed lupin-based diets (whether supplemented with enzymes or not). There was a significant difference in terms of feed intake between FM diet and Lupin, Lupin (A) and Lupin (E) diets. However, this value remained similar for all lupin-based diets (whether supplemented with enzymes or not). Protein efficiency ratio (PER) was significantly higher for L (Mix) diet compared with the same value for the groups of fish that were fed FM or Lupin diets. PER showed similar values for all lupin-based diets that were supplemented with enzymes.

Table 5.2. Growth response and feed utilization of rainbow trout fed different experimental diets

Diet	FM	Lupin	L (α)	L (B)	L (E)	L (Mix)
Initial weight (g)	16.74	16.52	16.7	16.22	16.76	16.55
	0.61	0.42	0.15	0.07	0.63	0.3
Final weight (g)	68.45^{a}	75.88^{ab}	78.48^{b}	74.69^{ab}	80.29^{b}	76.72^{ab}
	1.54	1.87	2.23	1.49	0.65	0.87
Weight gain (g)	51.72^{a}	59.36^{ab}	61.78^{b}	58.46^{ab}	63.53^{b}	60.17^{b}
	0.93	1.45	2.38	1.41	0.02	1.17
Feed intake g/fish/day	1.28^{a}	1.51^{b}	1.50^{b}	1.48^{ab}	1.58^{b}	1.45^{ab}
	0.03	0.05	0.05	0	0.01	0.03
SGR	3.35^{a}	3.63^{ab}	3.68^{ab}	3.63^{ab}	3.73^{b}	3.65^{ab}
	0.03	0	0.09	0.03	0.07	0.07
CF	1.49	1.46	1.49	1.47	1.47	1.43
	0.04	0.02	0.04	0.04	0.03	0.04
FCR	1.04	1.07	1.02	1.06	1.04	1.01
	0.01	0.01	0.01	0.02	0.01	0.01
PER	2.43^{a}	2.38^{a}	2.53^{ab}	2.43^{ab}	2.48^{ab}	2.58^{b}
	0.02	0.02	0.01	0.05	0.01	0.01
PPV	38.06	36.79	38.24	36.89	37.57	39.39
	0.28	0.12	0.98	1.6	1.58	0.36
LER	3.56	3.57	3.53	3.52	3.68	3.57
	0.03	0.035	0.015	0.08	0.02	0.01
LPV	0.665	0.74	0.71	0.7	0.765	0.69
	0.03	0	0.01	0.03	0.04	0.03
EER	48.8	46.57	49.39	46.97	48.42	49.47
	0.37	0.48	0.17	1.07	0.27	0.1
Survival (%)	100.0	100.0	100.0	100.0	100.0	100.0
	0	0	0	0	0	0

Each value is the mean (± S.E.M) of two replicates
Means within the same row with unlike superscript letters were significantly different (Tukey Test)
SGR: Specific growth rate (% day $^{-1}$) = $(\log_e W_2 - \log_e W_1)/t \times 100$
CF: Condition factor = 100×[(whole live body weight (g)/(fork length (cm^3)]
FCR: Feed conversion ratio = g feed intake /g live weight gain
PER: Protein efficiency ratio = g weight gain /g crude protein intake
PPV: Protein productive value = g protein retained / g protein intake
LER: Lipid efficiency ratio = g weight gain / g lipid intake
LPV: Lipid productive value = g lipid retained /g lipid intake
EER: Energy efficiency ratio = g live weight gain / energy intake (MJ)

Other parameters including, condition factor (CF), feed conversion ratio (FCR), protein productive value (PPV), lipid efficiency ratio (LER), lipid productive value (LPV) and energy efficiency ratio (EER) showed similar values in different experimental diets. Survival rate was 100% in all the experimental groups (Table

5.2). While dry matter digestibility of Lupin diet was significantly lower compared to the FM diet, Energex addition alone, significantly improved dry matter digestibility for the L (E) diet. Crude protein digestibility for all lupin-based diets was significantly higher than for the FM diet and Energex significantly improved the crude protein digestibility. Growth energy digestibility for all lupin-based diets was significantly lower than for the FM diet. Energex addition significantly improved the energy digestibility of the diet. Crude lipid digestibility in the FM diet was significantly higher than all lupin-based diets. However, none of the enzymes significantly improved the crude lipid digestibility of lupin-based diets (Table 5.3).

Table 5.3. Nutrient digestibility (%) of different experimental diets in rainbow trout

Diet	FM	Lupin	L (α)	L (B)	L (E)	L (Mix)
ADC DM	77.25^{b}	74.84^{a}	75.94^{ab}	74.59^{a}	76.28^{b}	75.83^{ab}
	0.44	0.29	0.57	0.07	0.05	0.07
ADC CP	91.42^{a}	94.37^{b}	94.70^{bc}	94.41^{b}	95.29^{c}	94.65^{bc}
	0.23	0.1	0.24	0.22	0.04	0.07
ADC GE	84.80^{d}	82.20^{ab}	82.64^{abc}	81.48^{a}	83.51^{c}	82.98^{bc}
	0.35	0.27	0.45	0.036	0.14	0.09
ADC CL	97.88^{c}	95.91^{b}	96.22^{b}	94.91^{a}	95.47^{ab}	96.05^{b}
	0.06	0.04	0.15	0.34	0.04	0.17

Each value is the mean (± S.E.M) of two replicates
Means within the same row with unlike superscript letters were significantly different (Tukey Test). ADC: Apparent digestibility coefficient; DM: Dry matter; CP: Crude protein GE: Gross energy; CL: Crude lipid

Plasma glucose content was highest and lowest in L (A) and L (Mix) groups respectively and showed significant differences. However, glucose concentration was similar for FM, Lupin, L (A), L (B), L (E) and L (Mix) groups (Figure 5.1).

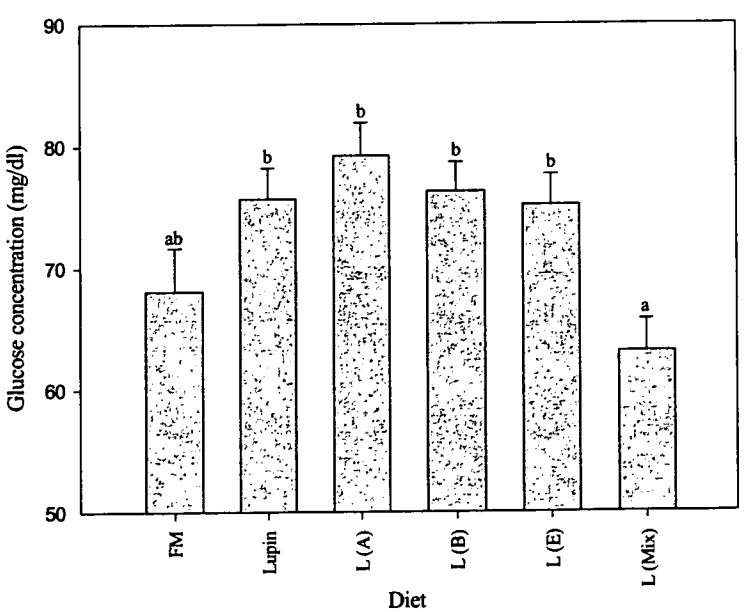

Figure 5.1. Plasma glucose concentration of rainbow trout of using different diets

Digestive tract indices including hepatosomatic index (HSI), pyloric caeca index (PCI), Small intestine weight index (SIWI), large intestine weight index (LIWI), small intestine length index (SILI) and large intestine length index (LILI) showed similar responses for all experimental groups (Table 5.4).

Table 5.4. The digestive tract response of rainbow trout fed different experimental diets

	FM	Lupin	L (α)	L (B)	L (E)	L (Mix)
HSI	1.55	1.45	1.53	1.30	1.43	1.47
	0.04	0.1	0.08	0.07	0.03	0.07
PCI	2.94	2.98	3.4	2.91	3.17	3.33
	0.23	0.09	0.31	0.26	0.25	0.23
SIWI	0.27	0.24	0.27	0.26	0.24	0.24
	0.02	0.03	0.01	0.01	0.01	0.01
LIWI	0.60	0.48	0.59	0.60	0.58	0.58
	0.01	0.01	0.04	0.05	0.02	0.04
SILI	15.33	12.16	15.92	16.36	12.95	12.63
	1.09	1.98	1.06	1.14	1.06	0.89
LILI	17.5	15.56	15.97	17.1	18.12	16.6
	0.56	1.03	0.4	1.18	1.12	0.74

Each value is the mean (± S.E.M.) of two replicates
HSI: Hepatosomatic index; PCI: Pyloric caeca index
SIWI: Small intestine weight index; LIWI: Large intestine weight index
SILI: Small intestine length index; LILI: Large intestine length index

There were no significant differences in whole body chemical composition between lupin based diets whether supplemented with enzymes or not (Table 5.5). The fish meal diet however, resulted in significantly lower dry matter content compared with non-supplemented lupin based diet.

Table 5.5. Chemical composition (% wet weight) of rainbow trout fed different experimental diets

	FM	Lupin	L (α)	L (B)	L (E)	L (Mix)
Dry matter	33.49^{a}	35.14^{b}	34.65^{ab}	34.32^{ab}	35.17^{b}	34.13^{ab}
	0.42	0.16	0.20	0.53	0.28	0.14
Crude protein	47.10^{b}	44.26^{ab}	44.20^{ab}	44.74^{ab}	43.59^{a}	45.22^{ab}
	0.92	0.18	0.41	1.11	1.09	0.40
Crude lipid	45.56^{a}	48.99^{ab}	48.30^{ab}	48.34^{ab}	49.35^{b}	47.43^{ab}
	0.77	0.24	0.22	0.94	1.27	0.67
Ash	6.70^{b}	6.25^{ab}	6.15^{a}	6.57^{ab}	6.29^{ab}	6.12^{a}
	0.09	0.17	0.02	0.16	0.14	0.08

Each value is the mean (± S.E.M) of two replicates
Means within the same row with unlike superscript letters were significantly different (Tukey Test)

5.4. Discussion

In the current experiment inclusion of 50% dehulled lupin (*angustifolius*) in the diet did not affect the fish growth compared to the fish group that was fed a fish meal-based diet. In contrast Roth-Maier and KirchgeBner (1995) found a 35% inclusion of lupin (*albus*) in broiler chicken diet, significantly decreased weight gain by 11% for fresh lupin-based diet and 6.5% for stored lupin-based diet. Enzyme supplementation of lupin-based diets in the current experiment did not significantly improve the growth performance of rainbow trout compared with the group that fed on unsupplemented lupin-based diet (positive control). The current experiment provides some evidence however, that shows some enzyme supplements partially improved growth performance of lupin-fed fish. This is supported by L (α), L (E) and L (Mix) having significantly higher final weights than the FM diet (negative control) although growth was not higher than the control Lupin diet. Advantages of dietary enzymes have been hard to determine in experiments on fish. Enzyme supplementation of the Atlantic salmon (sea water) diet containing 33.9% soybean meal with a mixture of proteolytic enzymes and carbohydrases significantly

improved fish growth compared to fish that received either a fish meal-based diet or a control soybean meal diet without the enzymes (Carter *et al.*, 1994a). However, enzyme supplementation of the salmon (fresh water) diet containing 46.9% soybean meal with a four enzymes batch, including a commercial enzyme designed to be used in aquaculture, phytase, a multi-carbohydrase enzyme and a multi-enzyme containing both carbohydrase and proteolytic enzyme did not affect the growth performance (Carter, 1998). The only enzyme that has shown a more consistent performance is phytase. In another study by Carter (1998) the addition of phytase alone significantly improved the weight gain of Atlantic salmon. It seems a smaller fish size in the later experiment (18.5 g *vs* 63.5 g) was the main reason the enzyme was effective in the second experiment despite a lower phytase addition in the diet (0.6% *vs* 1%).

Enzyme supplementation (Energex, Bio-Feed pro and Novozyme at 0.1% each) of the chicken (layer species) diet containing 50% dehulled lupin (*albus*) significantly improved weight gain. The same enzyme mixture did not affect the same diet when it was autoclaved (Brenes *et al.*,. 1993). Two batch of enzymes (each containing 3 carbohydrases) supplementation of diets that contained 30% dehulled lupin (*angustifolius*) did not improve growth of broiler chicken. This was the case as one of the enzyme mixtures increased the apparent metabolizable energy (AMEn) of lupin by 16.4% (Annison *et al.*, 1996). The reason for no improvement in growth performance despite a remarkable increase in AMEn may be due to the short duration (7 days) of the experiment.

In the current experiment, inclusion of 50% dehulled lupin in the diet did not affect feed efficiency indices compared to a fish meal based diet for trout. However, 35% inclusion of lupin (*albus*) in the broiler chicken diet significantly decreased feed efficiency by 4% in 35% freshly lupin-based diet compared to the stored 35% lupin-based diet. None of the enzymes used in the current experiment improved the feed utilization indices with the exception of mixture of all three enzymes that significantly improved the PER for the L (Mix) diet compared with the Lupin diet. Enzyme supplementation of a soybean based diet (33.9%) with a mixture of proteolytic enzymes and carbohydrases significantly improved the FCR compared with the unsupplemented soybean but not when compared with the control fish meal-based diet. However, enzyme supplementation of the diet containing 46.9% soybean meal with a four enzyme batch (a commercial enzyme designed to be used in aquaculture, phytase, a multi-carbohydrase enzyme and a multi-enzyme containing both carbohydrase and proteinase) did not affect the FCR for salmon reared in fresh water (Carter, 1998).

None of the enzymes affected apparent digestibility coefficient of dry matter, crude protein and gross energy with the exception of Energex that significantly improved all mentioned digestibility indices for the lupin-based diet. It has been suggested that viscous NSP induces endogenous secretion of metabolites, resulting in lower digestibility of nutrients (Smits and Annison, 1996) and mainly impairs fat digestibility (Simon, 1998). In the current study crude lipid digestibility was not affected in non-supplemented lupin-based diet. Similarly enzyme supplementation of a diet containing 33.9% soybean meal for Atlantic salmon reared in seawater (Carter *et al.*, 1994a) or of a diet containing 46.9% soybean meal for Atlantic salmon reared in fresh water (Carter, 1998), did not affect the apparent protein or energy digestibility. Results from the current experiment are in agreement with findings by Alloui *et al.* (1994) who reported a better effect for Energex supplementation over Biofeed for *in vitro* and Energex compared to α-galactosidase for *in vivo* digestibility values for broilers. *In vitro* supplementation of lupin seeds (various cultivars of *angustifolius, albus* and *luteus*) with 1% Energex increased the solubility of nitrogenous components, whereas adding 1% Biofeed had a much lower effect on N solubilization (Alloui *et al.*, 1994). This phenomenon shows that enzymes do not specifically act alone on the target substrate(s), making the selection of a suitable enzyme based just on the chemical composition of the ingredient difficult.

Inclusion of 0.3% of Energex to a diet that contained 50% lupin (*angustifolius*) in broilers, significantly improved digestibility (dry matter, protein and fat), however the supplementation with the same concentration of α-galactosidase did not affect dry matter, protein and fat (Alloui *et al.*, 1994). The addition of α-galactosidase (5 g/kg) to pig diets containing 35% lupin (*angustifolius*) significantly improved the digestibility of dry matter, gross energy and most oligosaccharides. However, despite a significant increase in amino acid digestibility following enzyme addition, total protein digestibility was not affected by this treatment (Gdala *et al.*, 1997a). In broilers diet (soybean and corn based), α-galactosidase supplementation improved AMEn and intestinal nitrogen retention. However, lower supplementation of the enzyme (500 g /tonne) was more effective in improving AMEn and higher supplementation was more effective for the improvement of nitrogen retention (Vila and Mascarell, 1999). It has been noted that although exogenous enzymes do

not target nutrients specifically, an enzyme mixture works more efficiently if it is selected to act on the predominant substrate (Danicke *et al.*, 1999). However this hypothesis was not supported with the findings in the current experiment as mixing of all three enzymes did not produce any advantage over adding each enzyme singly. These phenomena all demonstrate the complexity and variability of enzymatic action on the various nutrients. All lupin-based diets were well accepted and the survival rate was 100%.

The higher growth performance of Atlantic salmon fed by the reduced oligosaccharide-soybean meal (RO-SBM) has been attributed to a lower level of trypsin inhibitor or lectin in the RO-SBM diet (Refstie *et al.*, 1998). In another study with Atlantic salmon, the cause of the morphological alterations in distal intestine remained unknown (saponins or oligosacchrides) (van den Ingh *et al.*, 1996). Similarly the detrimental effect of the alcohol soluble component in soybean and diets containing different soybean products (at 26% level) on the distal intestine of Atlantic salmon has been demonstrated by (van den Ingh *et al.*, 1996). Oligosaccharides and lectins were suspected as the possible candidates. Full fat soybean meal at 30 % inclusion level produced the same problem while a 28% inclusion of soybean protein concentrate did not cause the same phenomenon in Atlantic salmon (van den Ingh *et al.*, 1991). However, van den Ingh *et al.* (1991) suggested that it is unlikely that the oligosaccharides caused the problem and cited protease inhibitors as the possible causal factors. In the current study 50% inclusion of dehulled lupin did not cause morphological changes in the distal intestine. The concentration of oligosaccharides and saponins in lupin is very high whereas the concentration of lectins and protease inhibitors is very low in the grain (Petterson *et al.*, 1997). Considering all the abovementioned evidences it is unlikely that oligosacchrides and saponins are the causative agents. However, the dehulled lupin used in the current experiment has been stored in a suitable condition for more than 6 months. It is not clear at this stage whether the fresh dehulled lupin affects the distal intestine in a similar way compared to a well stored dehulled lupin.

None of the enzyme affected the dry matter, crude protein, crude lipid or ash content of the carcass for lupin- based diets. The same phenomenon was observed when Atlantic salmon were fed different experimental diets supplemented with

different enzyme batches in fresh water (Carter, 1998). While the dry matter content of the carcass was significantly higher for Lupin and L (E) diets than the FM diet, crude protein content of the L (E) diet significantly decreased compared with FM diet. Crude lipid content of the carcass was similar for all the experimental diets with the exception of L (E) diet that was significantly higher for L (E) than the FM diet. Energex supplementation of the lupin-based diet significantly improved the growth performance of the fish compared with the FM diet. However, the higher productivity of the former diet was related to higher feed intake and not to the better feed efficiency.

In the current experiment the fish groups that received lupin-based diets (50% dehulled lupin, whether supplemented with enzymes or not) showed better growth performance (not significant) compared to a group that fed on a fish meal based diet. However, in the previous experiment (Chapter Four) the growth rate decreased significantly when the fish were fed a diet that contained 50% dehulled lupin as compared with a fish meal-based diet. As the conditions between the two experiments were similar the discrepancy was surprising. However, there were two important factors that may have influenced the results. Firstly, in the previous experiment a fresh batch of dehulled lupin was used whereas in the current experiment the same batch of dehulled lupin that had been stored in a suitable condition for at least six months was used. Secondly, in the current experiment fish were fed *ad lib* whereas in the previous experiment (Chapter Four) fish were reared using a restricted feeding regime.

Storage of grains has been found as an efficient method for improving nutrient utilization, as the chemical composition and enzyme activities change over time (Fuente *et al.*, 1998). It is suggested that over the storage time, as long as the activity of endogenous enzyme is high, insoluble carbohydrates are converted to the soluble fraction form with a concomitant decrease in viscosity (Fuente *et al.*, 1998). This phenomenon has been recently investigated in a few detailed studies.

In an experiment with broiler chicken, 35% inclusion of lupin (*albus*) in the diet significantly decreased the weight gain by 11% for fresh lupin-based diet and by 6.5% for a stored lupin-based diet compared to a control diet. Feed intake (6.4%)

and feed efficiency (4%) were also significantly reduced in a 35% freshly lupin-based diet compared to stored 35% lupin-based diet. Enzyme supplementation of Roxazyme (200 mg/kg) significantly increased the weight gain (3%) and reduced the faeces problem (Roth-Maier and KirchgeBner, 1995). They suggested that 20% newly harvested lupin or 30%-stored lupin could be included in the chick diet if the optimal energy-protein ratio was taken into account. In a more detailed study by Fuente *et al.* (1998) a storage time of up to 32 weeks after the harvesting of barley significantly decreased total β-glucan, NSP, *in vitro* viscosity and endogenous enzyme activity of the grain when included at a 50% level in chick diet. These physiologically characteristic changes significantly improved the AMEn ($P< 0.001$) of barley after 32 weeks storage time. Also AMEn was significantly improved by the addition of β-glucanase when used for older chicks (10 days *vs* 30 day old). However, chemical composition of the grain was not changed over the storage period. The effect of storing time on weight gain was not tested in this experiment. The effect of addition of (β-glucanase) on AMEn was higher for fresher barley and for younger birds (Fuente *et al.*, 1998). The same situation has been demonstrated for young broilers (Graham and Balnave, 1996). They concluded that fresher barley, if needed, should be used for older chickens (Fuente *et al.*, 1998). In other words the antinutritive effect of the ingredients is reduced as an animal matures. This may have the same application in fish nutrition.

The effect of different feeding regime on fish growth may be profound. High viscosity ingredients usually suffer from lower metabolizable energy values in all species (Danicke *et al.*, 1999). It has been shown that rainbow trout is continuing to eat until the energy requirement is satisfied (Yamamoto *et al.*, 2000b). It might be difficult for animals to satisfy their energy requirements when they are fed NSP-rich diets under a restricted feeding regime. However results from the current experiment showed that dehulled lupin palatability is very high. It is possible for fish to compensate for the lower metabolizable energy content of lupin by increasing their feed intake. The effect of enzyme supplementation of beta-glucanase and xylanase on layer chickens diets (wheat-rye and barley-rye based diets) was not significant under either *ad lib* or restricted feeding regimes (Oloffs *et al.*, 1998). However, this author suggests that enzyme supplementation studies should be carried out when the animal are under restricted feeding regimes as the

beneficial effect of enzyme addition may be hidden by increased feed intake under an *ad lib* feeding regime.

Supplementation of oligosaccharide-rich ingredients with oligosaccharidase has not shown consistent results (Campbell and Bedford, 1992). This may be related to over formulating the diets above the requirements of animals (Graham and Balnave, 1996) or using the same enzyme batches with completely different enzyme activities. Enzyme activities can be greatly varied for different batch of an enzyme derived from the same microorganism (Graham and Balnave, 1996). Extrusion increased the soluble fibre content and consequently the viscosity of the wheat and barley based diet for broilers compared to pelleting, resulting in lower growth and less efficient feed utilization by birds that received the extruded diet (Vranjes *et al.*, 1994). Post pelleting addition of liquid enzyme has been suggested as a way to avoid this problem when diets are produced by the extrusion method (Danicke *et al.*, 1999). Enzyme addition may have a greater effect on extruded diets due to their increased effect on the soluble fibre fraction of the diet (Danicke *et al.*, 1999).

Using a diet of rich saturated fatty acids with a high melting point (tallow) has a greater detrimental effect on viscosity than diets containing lower melting point oils (Simon, 1998; Danicke *et al.*, 1999). It has been suggested that enzyme supplementation is more efficient for diets that contain fat from animals rather than vegetable oil (Danicke *et al.*, 1999). The enzyme addition effect on fat digestibility is higher than for starch and protein probably due to the relatively larger size of the fat micelles that pass through the viscous digesta in the digestive tract (Graham and Balnave, 1996). Enzyme addition did not improve the crude lipid digestion in the current experiment, suggesting it is unlikely that there is a detrimental effect of fish oil inclusion on NSP rich lupin-based diets.

Although endogenous enzymes work for layer chickens, the results are more pronounced for broilers (Danicke *et al.*, 1999). The effect of enzyme supplementation is greater in younger animals as endogenous enzymes have not yet been developed (Graham and Balnave, 1996). This being so, fish were fed three times a week in order to control weight gain (smaller fish size was preferable in

this study) for the start of the experiment. Remarkably higher SGR in fish fed with unsupplemented lupin diet in the current experiment (about 3.6%/day) compared to other studies suggested there is a compensatory growth phenomenon in fish. Although the experiment lasted for 6 weeks, significant differences were observed between experimental treatments. The benefit of applying the phenomenon of compensatory growth may have the potential for reducing the time needed for nutritional studies in aquaculture.

The additive effect of combining antibiotics and endogenous enzyme is more obvious in poultry than pigs (Danicke *et al.*, 1999). This phenomenon has not yet been tested in fish. A different viscosity problem may occur for similar levels of NSP in diets due to differences between chemical structures of NSP with various molecular weights. High molecular weight NSP usually has a greater antinutritive effect due to its gel-forming properties (Danicke *et al.*, 1999). Further improvement in enzyme technology can be achieved through the future development of superior enzymes, enzyme level optimization, or application systems (Cambell and Poel, 1998). The enzyme must survive low pH, attack by proteolytic enzymes in the digestive tract and the heat and moisture of the environment during processing. The stability of most enzymes is during the hydration state. Fortunately, however, enzymes are kept in a semi-hydrated condition during pelletting (Campbell and Bedford, 1992). Higher enzyme concentration may be added to compensate for probable loss of activity during pelletting, however it might not be economically viable (Danicke *et al.*, 1999).

Enzyme supplementation of fish diets has been mostly carried out without trying to determine the effect on the nutrient digestibility of the treated ingredient. Any alteration in the nutrient digestibility following enzyme supplementation must be taken into account before formulation of the diets that contain the treated ingredients. As significant changes in nutrient digestibility may alter the nutrient balance required for maximum productivity of animals.

5.5. Conclusion

Enzyme supplementation of NSP rich ingredients has the potential of improving the nutrient utilization and growth performance of animals including fish. However, the effect of the enzyme inclusion is not predictable at all times due to the non-specific action of the enzymes on the target substrates. Plant endogenous enzymes can breakdown the NSP into the less complex carbohydrates making them more available to animals. Considering the negative effects on the environment of widely using enzymes, it is suggested that grains be stored for some time in a suitable condition to benefit the natural processing of problematic compounds in the diet. However, addition of enzymes acts more effectively if they are used for younger animals or with fresh NSP rich ingredients. Meanwhile the effect of enzyme addition on the nutrient digestibility of target ingredients should be determined for individual ingredients before the ingredient is included in the diet. The scope of research in this area should be expanded in the future.

Chapter Six

Effect of feeding time and dietary protein level on feed intake, nutrient utilization and growth of Atlantic salmon (*Salmo salar L.*) fed on dehulled lupin

6.1. Introduction

Fish meal constitutes 30 to 70% of the diet for on-growing salmonids and is even higher in starter diets (Rumsey, 1993). Such a high inclusion level of fish meal in salmonid diets will not be possible in the future (Barlow, 2000). Despite the typically higher inclusion levels of fish meal in Atlantic salmon (40%) compared to rainbow trout (30%) diets (Barlow, 2000), fish meal replacement studies have been mainly conducted for rainbow trout. The higher crude protein requirement suggested for Atlantic salmon (45%) compared with 40% for rainbow trout (NRC, 1993) and lower protein content of alternative ingredients, especially of plant meals, limit the formulation of balanced diets using these ingredients for Atlantic salmon. In addition, the inclusion of antinutritional factors (ANF's) (Tacon, 1995; Francis *et al.*, 2001) and the existence of less digestible nutrients in plant meals such as non-starch polysaccharides (NSP) (Alloui *et al.*, 1994; van Barneveld, 1999) further restrict plant meals being considered as fish meal replacements for Atlantic salmon.

It seems rainbow trout can be considered a suitable model for preliminary studies to screen a wide range of plant proteins as alternative to fish meal for Atlantic salmon. Total global fish meal use in Atlantic salmon feeds alone was the highest among all aquacultured fish being nearly 2.5 times higher than the amount used for trout in 2000 (Barlow, 2000). Due to the higher market value of Atlantic salmon and better utilization of nutrients in this species (Refstie *et al.*, 2000), finding a suitable alternative protein source could significantly reduce fish meal use in the salmon industry and improve the sustainability of salmonid production. The fish meal saved by replacing fish meal with other suitable alternative proteins could provide a proportion of the fish meal required for the newly developing marine fish aquaculture industry (Barlow, 2000).

Fresh dehulled lupin was successfully included in rainbow trout diets at up to 40% in a restricted feeding regime (Chapter Four) and up to 50% (well stored dehulled lupin) when fish were fed at apparent satiation (Chapter Five). Fulfilling the protein requirement must provide a sufficient and appropriate balance of amino acids in the diet to support maintenance and growth (Millward, 1989). Grain legumes mainly suffer from the limitation of sulfur amino acids (Petterson *et al.*, 1997; Chapter Two). However, the possible limitation of these amino acids in the diet that could be easily overcome by the addition of crystalline amino acids at a reasonable cost (Bercovici and Fuller, 1996). It might be possible to marginally reduce the protein level of diets by appropriate amino acids balance and suitable adjustment of protein to energy ratio in the diet (Sveier *et al.*, 2000). In this case, the limiting of an energy source by reducing fish meal in the diet should be compensated through the use of other non-protein energy sources, including highly digestible carbohydrates and/or fish oil.

It is suggested that protein synthesis (Carter *et al.*, 1994b; Bolliet *et al.*, 2000) and the efficiency of nutrient utilization in fish varies at different times of the day (Noeske-Hallin *et al.*, 1985; Boujard and Leatherland, 1992b; Boujard *et al.*, 1995; Spieler, 2001). Much research has been conducted to establish the best time for feeding various types of fish by measuring the feed intake using different methods. In most of these reports, the highest feed intake was reported early in the morning (see the review by Spieler, 2001). A similar case is reported for Atlantic salmon (Paspatis and Boujard, 1996). In contrast, in a few other studies, higher feed intake has been noted in the afternoon. Feed intake was higher in the afternoon for greenback flounder when fish were fed once a day either in the morning or in the afternoon at 3% level of their body weight (Verbeeten *et al.*, 1999). However, in most studies, higher feed utilization has been noted in the afternoon, when animals were given the opportunity to feed either in the morning or in the afternoon (Heilman and Spieler 1999; Burel *et al.*, 2000a). In contrast, growth and feed utilization was highest at dawn feeding time when rainbow trout was fed one meal at different times of the day (Boujard *et al.*, 1995). The main reason for this phenomenon is yet to be elucidated (Boujard and Leatherland, 1992b). It is suggested that the best feeding time (circadian rhythms) does not necessarily coincide with the best time for nutrient utilization (Heilman and Spieler, 1999).

Considering this phenomenon, it might be possible to improve nutrient utilization in conjunction with increasing plant proteins inclusion thereby minimizing metabolic wastage. Feeding fish with two different diets that include various nutrient levels over the day could be considered a new feeding strategy in this regard. Fish may better utilize a more nutritious diet in a specific feeding time regardless of feed intake. To the best knowledge of the author, there is no evidence of such an experimental design in the literature.

The aim of this study was to consider the biological responses of Atlantic salmon when two complete diets in terms of essential amino acid content but with varying dietary protein levels supplied from either fish meal or dehulled lupin were fed. The effect of diets with different protein levels (similar dehulled lupin content), fed to the fish in the morning or afternoon, was evalûated in terms of feed intake, nutrients utilization and growth rate. The feed preference of diets including 45% or 40% crude protein was also investigated. The digestibility of crude protein was also determined for experimental diets.

6.2. Material and methods

6.2.1. Experimental animals and culture conditions

Juvenile Atlantic salmon were supplied by a local hatchery (Springfield, Tasmania). Fish were kept in 2000-l tanks and fed with commercial salmon diet until required. Experiments were conducted in an indoor air-conditioned recirculation system (Carter and Hauler, 2000). Fish were weighed and randomly stocked into 300-l fiberglass tanks in duplicate groups of 20 fish (twelve tanks). Tanks were covered with black plastic mesh to minimize disturbance and prevent escape, and supplied with continuously aerated fresh water (9L/min) at a temperature of 14°C ± 0.5. Temperature, dissolved oxygen, total ammonia, nitrite and pH were measured three times weekly, and did not exceed values recommended for Atlantic salmon (Tarazona and Munoz, 1995). A constant photoperiod of 12L/12 D was used. Prior to the experiment, fish were fed with a commercial diet for a ten-day adaptation period, fasted for two days and re-weighed 38.99g ± 0.13 (S.E.M.) g prior to starting the experiment. The experiment lasted for 50 days and experimental diets were fed at satiation level twice a day one hour after light and one hour before dark to follow the preferred feeding time for

salmon as reported by Paspatis and Boujard (1996). Every day feeding started from the next tank to reduce any possible effect of feeding time (within feeding period) on nutrient utilization for each tank. Feed was distributed by hand to control hierarchy and to observe feeding behavior and daily feed intake for each tank was noted.

6.2.2. Experimental diets

Four experimental diets including two fish meal-based diets containing 40% CP (FM 40) and 45% CP (FM 45) and two dehulled lupin-based diets containing 40% CP (DL 40) and 45% CP (DL 45) were formulated. Due to the lower quality of fish meal in this experiment as compared with the previous experiment (Chapter Five) and the higher nutrient requirement of salmon than rainbow trout, dehulled lupin could only be added at 30% level to both DL 40 and DL 45 diets. Commercial dehulled lupin (*L. angustifolius*) was sourced through Milne Feeds Company (Western Australia). Dehulled lupin was finely ground (about 1mm particle size) to prepare a flour. Ytterbium oxide (Yb_2O_3) was used as an external marker in fish meal-based based diets (FM 40 and FM 45). Ytterbium oxide (Yb_2O_3) and yttrium oxide (Y_2O_3) were used as markers in DL 40 and DL 45 diets respectively. Both markers were included at the level of 100 mg/kg in all diets. All ingredients were mixed, and after adding 10% water, were cold pelleted (pellet size, 3 mm). The moisture content of the diets was reduced using a drier (30°C for 24 h). The diets were stored in thick plastic containers in dark conditions at -18°C until required. The ingredient and chemical composition of the experimental diets are shown in Table 6.1.

Two markers were separately used in DL 40 (Yb_2O_3) and DL 45 (Y_2O_3) diets respectively in order to test the feed preference of fish that were previously fed by DL 40 or DL 45 diets. After finishing the growth trial the diets were offered to both groups of fish at the equal ratio of 1:1 for three days. Feed preference then was determined following measuring the markers in the faecal samples that were collected over this period.

Table 6.1. Ingredient and chemical composition of experimental diets

	Diets			
Ingredients (%)	FM 40	DL 40	FM 45	DL 45
Fish meal	56.97	34.78	51.97	32.70
Ddehulled lupin	0.00	30.00	0.00	30.00
Corn gluten	4.68	9.40	18.47	20.00
Fish oil	17.50	17.50	15.00	15.18
Alpha cellulose	10.37	2.37	9.37	0.00
Dextrose	8.36	3.83	3.07	0.00
CMC[1]	1.00	1.00	1.00	1.00
Vitamins & mineral premix[2]	1.00	1.00	1.00	1.00
Stay C	0.05	0.05	0.05	0.05
Choline chloride	0.07	0.07	0.07	0.07
Chemical composition (%)				
Dry matter	91.58	91.86	93.42	92.85
Protein (N X 6.25)	39.86	39.63	45.39	44.53
Gross energy (MJ/kg)	22.12	22.53	22.28	22.13
Ash	9.28	6.02	8.77	6.27
Crude lipid	31.39	30.97	27.11	27.09
DP/DE[3] (g/MJ)	20.23	20.53	22.62	22.71

1. Carboxymethylcellulose
2. Added to fulfill in excess of requirements for Atlantic salmon (NRC, 1993)
3. Digestible protein/digestible energy

Table 6.2. Amino acid composition (g/kg) of experimental diets and fish requirement (g/kg of diet)

	FM 40	DL 40	FM 45	DL 45	Requirement[1]
Arg	29.47	33.95	29.82	35.11	18.4
His	13.62	12.42	14.12	13.21	6.2
Iso	18.47	18.11	20.54	20.24	10.60
Leu	33.27	35.13	45.17	45.17	17.60
Lys	35.8	27.09	33.80	26.65	22.00
Met	12.13	9.23	13.17	10.38	7.40
Phe	17.25	17.86	21.12	21.32	9.6
Val	21.06	19.30	22.64	21.13	12.3

1. (NRC, 1993)

6.2.3. Feeding regime

Six feeding regimes were used in duplicate (Table 6.3). Two groups of fish were fed with FM 40 and FM 45 diets and another two groups were fed with DL 40 and DL 45 respectively both in the morning and in the afternoon. The fifth group was fed with DL 40 in the morning and DL 45 in the afternoon (DL 4045). The diet for the last group was exactly the same as for the fifth group, however DL 45 was fed in the morning and DL 40 was fed in the afternoon (DL 4540).

Table 6.3. Feeding regimes used during growth study

Diet(s) used	AM	PM	Treatment name
FM 40	40% CP	40% CP	FM 40
FM 45	45% CP	45% CP	FM 45
DL 40	40% CP	40% CP	DL 40
DL 45	45% CP	45% CP	DL 45
DL 45, DL 40	45% CP	40% CP	DL 4540
DL 40, DL 45	40% CP	45% CP	DL 4045

6.2.4. Experimental procedure and sampling

Before starting the experiment, random samples of the diets were collected for chemical analysis. The experiment lasted for 50 days. A few days before finishing the experiment, faecal samples were collected for measuring nitrogen digestibility. White muscle samples of four fish per tank were removed and frozen in liquid nitrogen for analysis of nucleic acid and protein content.

After finishing the growth trial a feed preference study was carried out on groups of fish that have already fed by mixed diets (DL 4540 and DL 4045), using the method described by Refstie *et al.* (1997). To do this, fish were starved for three days to ensure that there was no marker left in the digestive tract from previous feedings regime. Then equal mixtures of DL 45 diet (containing Y_2O_3) and DL 40 diet (including Yb_2O_3) were fed to the groups of fish that have been already fed by mixed (DL 4045 or DL 4540) diets for three days at normal feeding times. After each feeding, tanks were flushed to remove uneaten pellets and faeces. Prior to the next feeding, faecal samples were collected and pooled to quantify the preferred diet through the quantification of a related marker. A few days before finishing the growth trial a digestibility study was conducted to determine the apparent digestibility of crude protein for experimental diets (DL 40, FM 40, DL 45 and FM 45). Digestibility was not measured for DL 4045 and DL 4540 treatments.

6.2.5. Analytical methods

All feed samples were milled before analysis, which was carried out in duplicate. Dry matter was determined using a freeze drier (Dynavac). . Total nitrogen was measured using the Kjeldhal method and protein content estimated as N×6.25. Total protein of white muscle was quantified using the Lowry *et al.* (1951) method. The protocol described by Refstie *et al.* (1997) was used to quantify Yb_2O_3 and Y_2O_3. Briefly, the homogenized samples of 150-200 mg were freeze dried and then combusted at 550°C overnight in glass scintillation vials. After cooling the vials, 5 ml of HCl:HNO_3 in the proportion of 2:1 was added to each vial. The samples were boiled until colorless. Then the samples mixed with 1.25 ml concentrate HNO_3 and then were diluted with distilled water to 25 ml. Yb_2O_3 was quantified using an Inductively Coupled Plasma (ICP) spectrometer. Modification of Schmidt-Thannhauser method described by Munro and Fleck (1966) was used to extract

nucleic acids. RNA was measured (mg/g tissue) by dual absorbance method (Ashford and Pain, 1986) following using yeast RNA (Sigma) as the standard.

6.2.6. Calculations

Whole wet body specific growth rates (SGR) were determined using the equation described by Ricker (1979):

Equation 6.1

$$SGR\ (\%\ day^{-1}) = (\log_e W_2 - \log_e W_1)/t \times 100$$

The apparent crude protein digestibility for experimental diets was also calculated using the standard formula (Maynard and Loosli, 1969):

Equation 6.2

$$ADC\ (\%) = 100\ x\ (1-(\%M_{diet}/\ \%M_{faeces}) \times (\%N_{faeces}/\%N_{diet}))$$

Where M is the inert marker and N is the nutrient.

6.2.7. Statistical analysis

Mean values are reported ± (S.E.M.) of each treatment. All statistical tests were performed using the SPSS Statistical Analysis Software Program (version 10 for Windows, 2001). One-way analysis of variance was conducted to assess the effect of dietary treatments on different responses and, when appropriate, the differences between means were tested using Tukey's multiple range test. Paired T test was conducted to find out any possible differences in terms of feed intake between morning and afternoon feeding time and in the feed preference study. Pearson's correlation coefficient was used to determine the relation between the different variables. Probability values of less than 0.05 were considered as significant.

6.3. Results

Weight gain in groups of fish that were fed with 40% crude protein diets (FM 40 and DL 40) was significantly lower than all other groups (Table 6.4). Weight gain, however showed similar values for other groups. Feed intake in those groups that were fed with FM 40 and DL 40 diets was significantly lower than other groups.

Table 6.4. Growth response, feed intake and nutrient utilization of Atlantic salmon fed with different experimental diets

	Diets						
Parameter	FM 40	DL 40	FM 45	DL 45	DL 4540	DL 4045	P
Initial weight (g)	38.53	39.12	38.64	39.23	39.06	39.35	NS
	0.06	0.41	0.17	0.41	0.35	0.29	
Final weight (g)	148.55^{a}	153.00^{a}	167.86^{b}	173.80^{b}	169.35^{b}	174.54^{b}	≤ 0.001
	1.49	1.48	1.48	1.48	2.23	2.22	
Weight gain (g)	110.02^{a}	113.89^{a}	129.21^{b}	134.57^{b}	130.29^{b}	135.19^{b}	≤ 0.001
	1.54	1.07	1.30	1.90	2.58	1.93	
SGR (% day $^{-1}$)	2.70^{a}	2.73^{a}	2.94^{b}	2.97^{b}	2.93^{b}	2.98^{b}	≤ 0.001
	0.02	0.00	0.01	0.03	0.04	0.01	
FI (g/d/fish)	1.89^{a}	1.99^{a}	2.18^{b}	2.31^{b}	2.23^{b}	2.27^{b}	≤ 0.001
	0.04	0.02	0.02	0.01	0.03	0.02	
CF	1.44	1.43	1.48	1.48	1.37	1.40	NS
	0.03	0.00	0.07	0.01	0.02	0.04	
FCR	0.87^{ab}	0.88^{b}	0.85^{ab}	0.86^{ab}	0.86^{ab}	0.84^{a}	≤ 0.05
	0.01	0.00	0.00	0.01	0.00	0.005	
PER	2.88^{b}	2.86^{b}	2.59^{a}	2.61^{a}	2.74^{ab}	2.82^{b}	≤ 0.01
	0.03	0.005	0.01	0.04	0.02	0.05	
EER	51.49	50.42	52.75	52.44	51.67	53.22	NS
	0.15	0.05	0.19	0.77	0.44	0.95	
LER	3.66^{a}	3.66^{a}	4.33^{c}	4.28^{c}	3.96^{b}	4.10^{bc}	≤ 0.001
	0.04	0.005	0.01	0.06	0.03	0.07	

Each value is the mean (± S.E.M) of two replicates
Means within the same row with unlike superscript letters were significantly different (Tukey's multiple range test)
SGR: Specific growth rate (% day $^{-1}$) = $(\log_e W_2 - \log_e W_1)/t \times 100$
FI: Feed intake
CF: Condition factor = 100×[(whole live body weight (g)/(fork length (cm^3)]
FCR: Feed conversion ratio = g feed intake /g live weight gain
PER: Protein efficiency ratio = g live weight gain /g crude protein intake
EER: Energy efficiency ratio = g live weight gain / energy intake (MJ)
LER: Lipid efficiency ratio = g live weight gain / g crude lipid intake

However, feed intake was similar for other experimental diets. FCR was generally better in groups of fish that received fish meal as the main protein source in their diet. Also FCR was better in those groups of fish that received more protein in their diet (45% *vs* 40%). However, these values did not show significant differences.

PER was significantly higher in those groups that received less protein in their diet (40% *vs* 45%). Also PER was significantly higher in DL 4045 group as compared with FM 45 and DL 45 groups. Regardless of the protein source in the diets, LER was significantly lower in groups of fish that received 40% crude protein (FM 40 and DL 40) in their diets as compared with all other groups.

There were no significant differences among different groups in terms of the protein content of white muscle (Table 6.5). However, the concentration of RNA and RNA/protein ratio in FM 40 group showed significantly lower values as compared with all other groups with the exception of DL 40 group.

Table 6.5. Protein and nucleic acid content of white muscle of Atlantic salmon fed on different experimental diets

	Diets						
Parameter	40 FM	DL 40	FM 45	DL 45	DL 4540	DL 4045	P
Protein (mg/g)	181.59	186.18	187.07	181.45	184.72	188.69	NS
	2.60	2.33	3.6	1.04	0.005	3.9	
RNA (mg/g)	1.46^{a}	1.63^{ab}	1.77^{b}	1.70^{b}	1.74^{b}	1.79^{b}	≤ 0.01
	0.06	0.02	0.02	0.04	0.005	0.05	
RNA/Protein (mg/g)	8.05^{a}	8.74^{ab}	9.49^{b}	9.38^{b}	9.44^{b}	9.47^{b}	≤ 0.01
	0.23	0.22	0.05	0.20	0.02	0.07	

Each value is the mean (± S.E.M) of two replicates
Means within the same row with unlike superscript letters were significantly different (Tukey's multiple range test)

There was a strong correlation between SGR and RNA/protein ratio of white muscle of fish that fed different experimental diets (Figure 6.1).

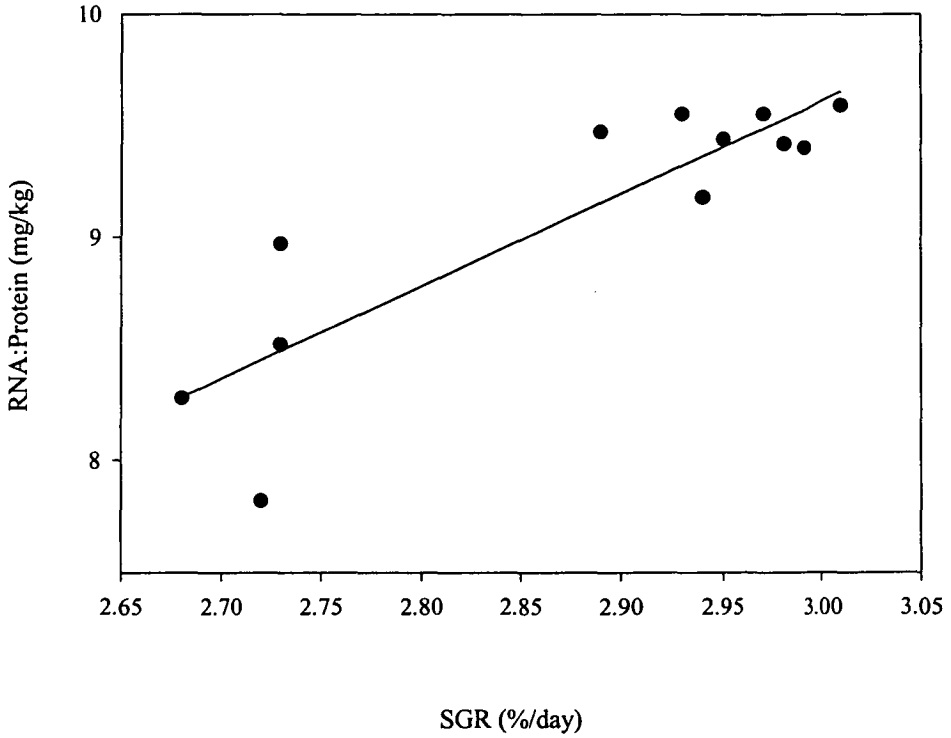

Figure 6.1. Correlation between specific growth rate and RNA:Protein ratio of white muscle for fish fed different experimental diets ($n=12$; $r = +0.88$, $P < 0.01$)

Although feed intake was generally higher in the afternoon, there were no significant differences between feed intake in the morning and in the afternoon (with the exception of FM 40 group) when this parameter was compared for each experimental diet. Feed intake however, was significantly higher in the afternoon when the values for all experimental diets were pooled.

Table 6.6. The comparison of feed intake (%) in the morning and in the afternoon for experimental diets

	Diets						
Feed intake	FM 40	DL 40	FM 45	DL 45	DL 4540	DL 4045	All diets
AM	48.72[a]	47.97	48.00	48.52	48.15	49.38	48.46[a]
PM	51.28[b]	52.03	52.00	51.48	51.85	50.62	51.54[b]
SEM	0.12	0.36	0.67	0.96	0.88	2.5	0.47
P	≤ 0.05	NS	NS	NS	NS	NS	≤ 0.001

Each value is the mean (± S.E.M) of two replicates
Means within the same column with unlike superscript letters were significantly different (Paired t-test)

Feed preference study was only conducted for DL 4540 and DL 4045 experimental diets (Table 6.7). Results showed that fish did not prefer DL 45 to DL 40 or vice versa when the mixture of both diets were fed to those groups of fish that have already experienced DL 4540 or DL 4045 feeding regime.

Table 6.7. Feed preference (%) test of fish received mixed diets

	Preference			
	DL 40 diet	DL 45 diet	SEM	P
DL 4045	49.62	50.38	2.97	NS
DL 4540	50.56	49.35	2.07	NS
All diets	50.09	49.91	1.58	NS

Each value is the mean (± S.E.M) of two replicates
Means within the same row with unlike superscript letters were significantly different (Paired t-test)

Apparent crude protein digestibility of DL 45 diet was significantly higher than for the FM 40 diet (Figure 6.2). There were no other significant differences although apparent crude protein digestibility tended to be higher for diets containing dehulled lupin. The dry matter content of faeces of those fish that fed on dehulled lupin-based diets tended to be lower than those fish that received fish meal based diets (Figure 6.3). However, only FM 45 was significantly different.

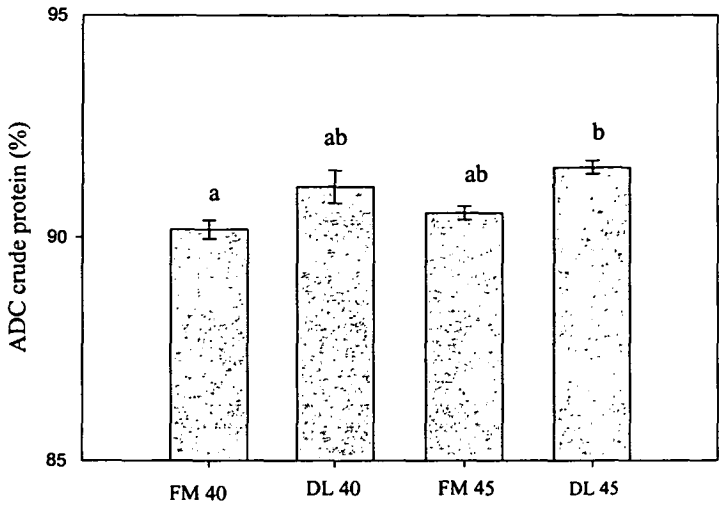

Figure 6.2. Apparent crude protein digestibility in different experimental diets (%)

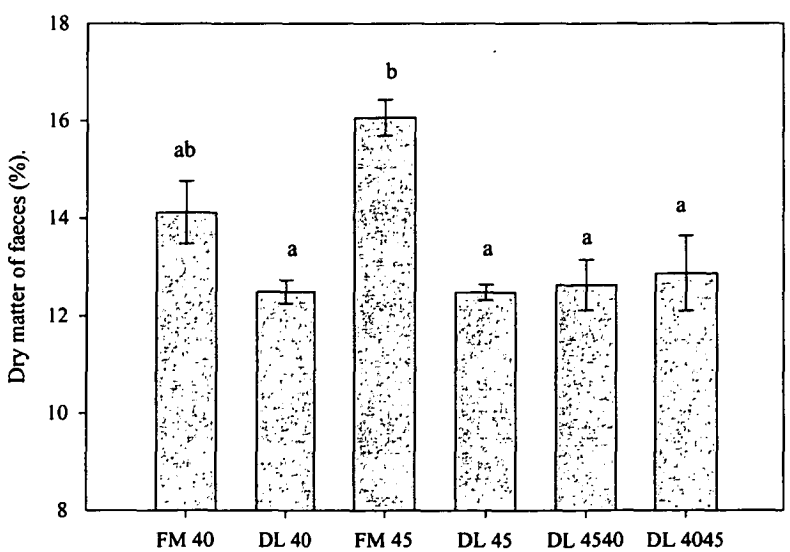

Figure 6.3. Dry matter content of faeces collected from fish fed on different experimental diets

There was a high and significant correlation (negative) between FCR, PER and feed intake. However, the correlation between SGR, EER, LER and feed intake was positively high and significant (Table 6.8).

Table 6.8. The correlation relationship (r) between SGR, FCR, PER, EER and LER for all experimental groups (n = 12)

	FI	P
SGR	+ 0.96	≤ 0.001
FCR	- 0.60	≤ 0.05
PER	- 0.63	≤ 0.05
EER	+ 0.63	≤ 0.05
LER	+ 0.81	≤ 0.001

6.4. Discussion

One of the main goals in the current experiment was to study the effect of reducing the protein content of an Atlantic salmon diet (from 45% to 40%) on the growth performance while fish were fed at satiation level with the diets that fulfilled the amino acid requirements. Protein is the most expensive fraction of each diet. In addition, it has been shown that there is a linear relationship between nitrogen consumption and nitrogen excretion (Rychly, 1980). Regardless of the protein source, those groups of fish that received 40% crude protein diets (FM 40 and DL 40) showed significantly lower growth rate compared to all other groups. This finding is in contrast with the claim by Dabrowski (1993) that suggested fish could grow well with low protein diets when fed under unrestricted feeding regime. Pigs and poultry require individual amino acids rather than crude protein in the diet (Edwards and van Barneveld, 1998). It seems however, that the fulfilling the amino acid requirements without providing optimum amount of protein in the diet is not adequate for good performance in fish. It is suggested that fish are using 50% of crude protein of the diet as energy sources even when balanced amino acids are provided in the diet (Cho, 1991). However, recently Yamamoto *et al.* (2000a) showed that rainbow trout preferred a low protein diet with balanced amino acids to a high protein diet with imbalanced amino acids. Imbalanced protein intake, whether due to feed intake or to dietary protein content will reduce growth performance. For example, feeding brown trout fry at a high ration of a low protein

diet or a low ration of a high protein diet resulted in poorer growth performance that the control, mid-ration of a mid-protein diet (Arzel *et al.*, 1998). A high protein intake requirement of salmonids is necessary to fulfill both the essential and non-essential amino acids requirement and to meet requirements for maintenance and growth. In other words, if low protein diets are fed to fish the limited supply of amino acids is used for maintenance not growth (Cowey, 1995), as reflected by the obligatory nitrogen excretory loss. For maximal growth performance the ratio of essential to non-essential amino acids should be 50:50 or more (Cowey, 1995). It has been suggested that fish have little ability to adjust amino acid deaminating enzymes, that is why obligatory nitrogen loss is high even fish are fed with low protein diets (Cowey and Walton, 1989).

Despite significantly lower growth rate in those groups of fish that received 40% CP in their diets, PER was significantly higher compared with on the 45% CP diets. In other words, lowering protein content in the diet increased retention efficiency. This is in agreement with Nandeesha *et al.*'s (1995) finding that showed higher PER for low protein diets for carp. However, LER in 40% CP diets was significantly lower than for other groups. Results may suggest that lower protein diets have better potential where environmental pollution is a concern, despite significantly lower growth performance of the fish receiving low protein diets.

The main finding in this experiment was the similar growth performance of fish that were fed with DL 4540 and DL 4045 diets as compared with those groups of fish that received 45% CP diets (FM 45 and DL 45). Also FCR, EER and LER did not show significant differences between 45% CP diets (FM 45 and DL 45) and mixed regimes (DL 4540 and DL 4045). Interestingly, PER was significantly higher in those groups of fish that received mixed diets compared with 45% CP diets (FM 45 and DL 45). This phenomenon can be considered as a new approach for better feeding management in aquaculture. Lowering the protein content of a diet even in one feeding time (in the morning or in the afternoon) can save a great amount of protein at farm level. Significantly higher PER in those group of fish that received mixed regimes (DL 4540 and DL 4045) compared with those groups that fed on 45% CP diets (FM 45 and DL 45) may suggest lower nitrogen excretion by fish that received mixed diets. The mechanism by which that fish received

mixed diets (DL 45 40 and DL 4045) can show the similar growth performance comparable with those groups of fish that were fed with 45% CP diets (FM 45 and DL45) open a new area for further research in the future. To the best knowledge of the author there is a lack of similar data series in the literature to compare the data collected in the current experiment.

To ensure that the higher feed intake in the afternoon was entirely related to the feeding time and not to preference of a specific diet, a feed preference test was carried out after finishing the growth trial. Results showed that there was no feed preference when both DL 40 and DL 45 diets were offered in a mixed diet (50%, 50%) twice a day to those groups of fish that have already being fed with DL 4540 and DL 4540 diets. One of the reasons for detecting significant differences between feed intake in the morning and in the afternoon might be related to the feeding level used in the current experiment. In the study conducted by Verbeeten *et al.* (1999) there were no significant differences between feed intake in the morning and in the afternoon for greenback flounder when fish were fed at 1 or 2% of their body weight. However, significantly higher feed intake detected for afternoon feeding time when fish were fed at approximately 3% of their body weight.

Effect of feeding time on growth performance and nutrient utilization in fish (Reddy *et al.*, 1994; Boujard *et al.*, 1995; Bolliet *et al.*, 2000) is extensively described in the literature. This effect attributed to the effect of feeding time on both feed intake and feed utilization (Bolliet *et al.*, 2000). The reason of the effect of feeding time on growth performance is not yet clearly understood (Boujard and Leatherland, 1992a). However, the effect of light on the endocrine regulation is suggested as a major parameter in controlling feeding activity (Murat *et al.*, 1981). This is reflected by different concentrations of metabolites and hormones in various times of the day in fish (Boujard and Leatherland, 1992d; Boujard *et al.*, 1993). In studies with fish a single meal has shown better results both when offered in the morning (dawn) (Boujard *et al.*, 1995) or in the evening (dusk) (Reddy *et al.*, 1994; Verbeeten *et al.*, 1999).

Feeding activity (rhythm) has been extensively reviewed in both fish and other invertebrates (Boujard and Leatherland, 1992a). Some fish have shown more

diurnal feeding activity and others nocturnal feeding activity (Boujard and Leatherland, 1992b). Most of the studies have been conducted when feed was offered freely and continuously to the fish. In most of these cases the highest feed demand has been recorded at dawn with occasional peaks at dusk (Kadri *et al.*, 1991; Boujard and Leatherland, 1992c; Sanchez-Vazquez and Tabata, 1998). However, in practical conditions at farm level, feeding time is likely to be restricted to specific times. Feeding activity may be adjusted when fish are fed at restricted times (Alanara, 1992). This may show the potential of feeding management for controlling feed intake resulting in better performance of fish at specific times. In most studies, time of restricted feeding has been limited to 8 working hours per day (9am to 5pm), (Boujard and Leatherland, 1992a). Fewer attempts have been made to coincide the feeding time with the time that fish is showing the highest feeding activity. In addition the highest feeding activity mostly has been determined in continuous feeding experiment using self-feeding feeders (Boujard *et al.*, 1995; Fast *et al.*, 1997; Sanchez-Vazquez and Tabata, 1998). It has been shown that fish (trout) is capable of synchronizing feeding activity if access to feed is time restricted (Alanara, 1992).

In a few number of studies with salmonids researcher tried to find the best feeding time over a 24 hours cycle. In a study by Boujard *et al.* (1995), rainbow trout showed the best performance when fed a single meal at dawn compared to other feeding times (midday, dusk or midnight). In contrast, growth performance of rainbow trout was lower for post-dawn feeding compared with either at midday or pre-dusk feeding (Reddy *et al.*, 1994). In a recent study by Bolliet *et al.* (2000), rainbow trout showed significantly higher digestion (protein, energy and lipid) as well as significantly higher protein synthesis when fed a single diet once per day in the morning (one hour after lights-on) compared to fish fed in the evening (one hour after light off). However, growth performance did not follow the same trend. The discrepancy in results of the described studies shows the complexity of the phenomenon as well as the possible effect of different experimental conditions on final results. In the current study the correlation between SGR and the capacity for protein synthesis (RNA/protein ratio) was strongly significant ($r = +0.88$, $P < 0.01$). This suggested that, in comparison to Bolliet *et al.* (2000), a more direct relationship between protein synthesis (as indicated by the capacity for protein

synthesis) and growth existed. This is in agreement with the correlation relationship between the same parameters reported in other studies (Houlihan *et al.*, 1995). The strong relationship between SGR and RNA/protein ratio can be used to predict the ongoing growth rate of aquatic animals in both aquaculture and fisheries (Carter *et al.*, 1998). Furthermore, it is suggested that effect of feeding time on growth performance may be further affected by other parameters especially feed intake (Reddy *et al.*, 1994; Verbeeten *et al.*, 1999).

In the current study there was a suggestion of higher protein digestibility for lupin than for fish meal, and is supported by other studies (Allan *et al.*, 2000a). Apparent crude protein digestibility of DL 45 diet was significantly higher than the same value for FM 40 diet. These data are in agreement with Rychly and Spannhof's (1979) finding that showed lowering the protein level and increased carbohydrate in the diet resulted in significantly reduced digestibility of whole diet by rainbow trout. Despite lower crude protein digestibility in 40% CP diets, PER was higher than for 45% CP diets. These data show that the higher nutrient digestibility does not necessarily result in better nutrient utilization. This is in agreement with Refstie *et al.* (2000) finding in which the nutrient utilization in Atlantic salmon was better than rainbow trout despite the higher digestibility of the nutrient in rainbow trout.

The correlation analysis showed that there was a positive relationship between feed intake, and SGR, EER and LER. On the other hand, the correlation between feed intake and both PER and FCR was negative. These data demonstrate that increased feed intake is not always desirable in animal nutrition. In the current study, increased feed intake led to decreased PER. It has been shown that there is a linear relationship between nitrogen consumption and nitrogen excretion (Rychly, 1980). Meanwhile, due to the high and non-adaptive liver deamination enzymes activity (Cowey and Walton, 1989), fish use a high percentage of consumed nitrogen as energy source through the deamination of proteins in the diet. It has been discussed that from the nutritional point of view, optimum nutrients level determined in the diet without measuring the net feed intake has less universal validity (Teshima *et al.*, 2000, Hauler and Carter, 2001). It seems in the future fish nutrient requirements should be closely determined in relation to feed intake. In this case using the new and practical feeding management methods, productivity and

sustainability of aquaculture industry can be improved through reduced nutrient wastage load.

6.5. Conclusion

The influence of feeding time on fish performance has been mainly shown for fish fed once a day. It is obvious that the results obtained through feeding a single daily meal on fish performance have less application at a farm level. The current experiment was the first attempt to determine the effect of feeding time on both growth and nutrient utilization when fish were fed twice a day on two different diets. The results also showed that Atlantic salmon could perform well when the diet contains 30% dehulled lupin. Fish performance using mixed dietary protein regimes (DL 4540 and DL 4045) was comparable to those groups of fish that were fed a higher amount of protein (45% CP: FM 45 and DL 45). Lowering the protein concentration in the diet even at one feeding time has great application at farm level. The effect of feeding time on nutrient utilization could not be totally demonstrated in the current study. Further experiments should examine the effect of various feeding times and feeding levels on fish performance and nutrient utilization, when fish are fed with different diets during the day.

Chapter Seven

General discussion and conclusion

7.1. General discussion

This research has mainly focused on assessing the nutritional potential of sweet lupin (*L. angustifolius*) grain compared with other important Australian grain legumes: chick pea, faba bean and field pea as alternatives for fish meal in salmonid diets. The experiments were conducted in a way that the outcomes from each experiment were used to establish the experimental design for the subsequent experiments. Rainbow trout was the main fish used but results were used to also investigate Atlantic salmon in the last experiment. Chemical analyses were carried out as the first step to assess the nutritional potential of the grains. Results from chemical analysis showed that lupin has the highest crude protein, gross energy, neutral detergent fibre (NDF), and, in particular, a more favourable essential amino acid index/non essential amino acid index compared to other grain legumes. Grain legumes contain different antinutritional factors (ANF's) that may limit their utilization especially by monogastric animals (Waldroup and Smith, 1989). Particular attention was made to quantify the two most important antinutrients: trypsin inhibitor (TI) and phytic acid (PA) in the grains. It is recognized that modern feed manufacture using extruders will destroy considerable ANF activity but this may remain in pressed pellets that will still be used in some operations. Examples are trout farmers in Victoria, Australia (Carter, personal communication) and some countries with developing aquaculture. The TI and PA content of lupin were lower than for other grain legumes. These findings are in agreement with the values reported for the same grains by Petterson *et al.* (1997). Although non-starch polysaccharide (NSP) content of lupin was not determined in this research, the highest NDF and lowest starch content of lupin confirm other findings that reported the highest NSP content for lupin (Perez-Maldonado *et al.*, 1999). This issue is of utmost importance as the antinutritional effect of NSP on monogastric animals has been extensively reported in the literature (Annison 1993; Choct *et al.*, 1996; Bakker *et al.*, 1998; Iji and Tivey 1998; Simon 1998). However, some biological benefits have also been attributed to the inclusion of NSP in monogastric animal diets (Iji and Tivey, 1998).

It has been shown that various processing methods can improve the nutritional value of grain legumes (Gouveia *et al.* 1991; Melcion and van der Poel, 1993; Fernandez *et al.*, 1996; Nestares *et al.*, 1996; Chau and Cheung, 1997). Different processing methods that were employed in Chapter Two did not affect the crude lipid and NDF content of the grains. However, soaking significantly increased the crude protein and the gross energy of the grains. Only soaking and a combination of soaking and heating resulted in starch gelatinization. Also combination of soaking and heating significantly decreased the TI content of the grains. All the employed processing methods increased the concentration of PA in the grains. This was not expected as in other studies the same processing methods significantly decreased the PA content of the grains (Chau and Cheung, 1997). Processing methods significantly but variably affected both macro and micro-mineral content of the grains. These phenomena show the importance of selecting the most suitable processing methods based on comprehensive information about the type and level, distribution, chemical reactivity and sensitivity of antinutrients (Melcion and van der Poel 1993) and nutrients to heat in a seed matrix. The maximum tolerance level of animals to the target antinutrients should also be taken into account when selecting the best processing method. Currently due to a lack of information regarding the kinetics of reactions for various chemicals during processing, the optimization of processing is not completely possible (Kwok and Niranjan, 1995).

Lupin and the combination of soaking and heating were identified as the best grain and processing methods, respectively. The best way of evaluating the effectiveness of a processing method is through *in vivo* studies and *in vitro* laboratory methods should only be used as complementary procedures (Melcion and van der Poel, 1993). In Chapter Three the effect of both fish size and grains (soaked and heated) and adaptation period on the nutrient digestibility was assessed through *in vitro* and *in vivo* tests. Apparent dry matter digestibility (both *in vivo* and *in vitro*) was significantly higher in lupin compared with other grain legumes. Employed processing method significantly improved the apparent *in vivo* dry matter digestibility but not the apparent *in vivo* crude protein digestibility of the grains. In contrast, the processing methods did not affect the apparent *in vitro* dry matter digestibility but they significantly improved the apparent *in vitro* crude protein digestibility of the grains. Fish body size did not affect apparent crude protein and

dry matter digestibility (neither *in vitro* or *in vivo*). This finding is in agreement with Lee's (1997) findings that showed fish size did not affect digestibility. In contrast, some studies have showed fish size affects nutrient digestibility (Ferraries *et al.*, 1986; Bassompierre *et al.*, 1998). This discrepancy may be due to the complexity of interactions between fish size and other environmental factors. The adaptation period did not affect the apparent *in vivo* dry matter digestibility, however it improved the apparent *in vivo* crude protein digestibility of the grains that contained lower concentration of TI (field pea and chick pea). Over the adaptation period of nine days the apparent dry matter digestibility for the small fish size improved. Similarly the apparent crude protein digestibility increased for both the small and the medium fish size. This may explain the lower susceptibility of bigger fish size to the antinutrients included in the diets (Fuente *et al.*, 1998).

A number of *in vitro* digestibility methods have been developed, however, they have not been widely used by the industry due to being time consuming and inconsistent (Bassompierrie *et al.*, 1997). In the present research there was a strong relationship between *in vitro* and *in vivo* (for both dry matter and crude protein) digestibility for raw grains but not for processed grains. This result is in agreement with (Dimes *et al.*, 1994) finding that demonstrated *in vitro* measurement does not show good agreement with *in vivo* studies for some processed ingredients.

The finding through the two screening experiments (Chapters Two and Three) demonstrated the advantage of lupin to other grain legumes for fish meal replacement. However, the relatively low protein content of lupin grain compared with fish meal and the high concentration of NSP in the whole lupin were recognized as the two main limiting factors, causing the less effective utilization of the whole grain by salmonids. It has been shown that dehulling can significantly reduce the NSP content of lupin and increase the protein content of lupin kernel (Evans *et al.*, 1993); therefore it was decided to use the dehulled lupin in the subsequent experiments. Dehulling has also been shown to increase the concentration of TI (Sosulski *et al.* 1982 in Melcion and van der Poel, 1993) and oligosaccharides for some grain legumes (Sosulski *et al.* 1982 in Evans *et al.*, 1993; Melcion and van der Poel, 1993). In the current study the method used to quantify the TI concentration did not distinguish this antinutrient in either the

kernel or the husk of lupin. It was important to find out the highest possible inclusion level of dehulled lupin in rainbow trout diet. A dose response experiment was conducted to assess this issue. Results showed that protein efficiency ratio (PER), protein productive value (PPV), lipid efficiency ratio (LER) and lipid productive value (LPV) were not affected by dehulled lupin inclusion level (up to 50%). Although weight gain was not affected at up to 40% inclusion of the dehulled lupin, it sharply dropped at 50% inclusion level. Energy efficiency ratio (EER) was also significantly decreased at higher than 30% inclusion level of dehulled lupin. The high inclusion level of dehulled lupin in the diets did not affect the digestive tract indices including the digestive enzyme activities. In the current study the enzyme activities were determined using the tissue samples collected from pyloric caeca. Torrissen *et al.* (1994) found less trypsin activity in the pyloric caeca than in the small intestine of Atlantic salmon when they tested different dietary proteins. For comparative studies on fish species it is acceptable to measure the enzyme activities in the pyloric caeca, however if the effect of different diets on enzyme activities is the main concern, it is preferable to determine the enzyme activities on the brush border area of small intestine.

The amount of literature about the influence of the complex carbohydrates (NSP) on non-specific immune response in fish is limited. This information is important for understanding the potential benefits, due to an immunostimulant effect, or avoiding the negative effects, due to immunosuppression, of any new ingredients. With the exception of total protein and neutrophil level, none of the non-specific immune responses were affected in this study. This is in agreement with Page *et al.*'s (1999) findings in which non-specific immune responses were not affected by long term feeding of a high carbohydrate diet (gelatinized starch) in rainbow trout. In contrast, Hemre *et al.* (1995b) reported a significant decrease of hematocrit and haemoglobin at higher inclusion of starch in Atlantic salmon. Considering all non-specific immune responses, it can be concluded that none of experimental diets used in the current study had an adverse effect on digestive tract resulting in abnormal immunological responses. It became clear that the main problem of effectively using dehulled lupin was related to energy utilization. This phenomenon is attributed to the high inclusion level of NSP in 50% dehulled lupin diet.

In Chapter Five the effect of adding exogenous enzymes on energy utilization and growth performance was evaluated for the diets that contained 50% dehulled lupin. The enzymes were added to specifically improve the digestibility of hemicellulose (Energex™) and protein (Bio-Feed™ Pro) or to increase the hydrolysis of oligosaccharides: raffinose and stachyose (Alpha-galactosidase™). Energex increased the dry matter and crude protein digestibility, however feed intake, feed efficiency raio (FCR), LER, EER and LPV were not improved. Surprisingly the performance of the fish that were fed with the control diet (50% inclusion of dehulled lupin) was similar to those fish that received the fish meal based diet. This finding is in contrast with Chapter Four finding in which significantly lower growth rate was noted for those fish that received 50% dehulled lupin diet as compared with the fish that were fed by a fish meal-based diet.

Observing the different responses in fish growth in Chapter Four and Five despite using 50% dehulled lupin in both experiments could be explained by different feed intake and the storing condition of dehulled lupin. In Chapter Four the restricted feeding regime and in Chapter Five an *ad lib* feeding regime were applied respectively. In addition the dehulled lupin used in Chapter Four was fresh whereas the grain used in Chapter Five was stored in a well condition place for about nine months. The comparable EER in groups of fish that received 50% dehulled lupin in Chapter Five as compared with the group that was fed with fish meal-based diets is in contrast with the finding in Chapter Four. It has been suggested that fish can increase feed intake to fulfill their energy requirements (Tacon and Cowey, 1985). It seems fish has compensated the less availability of energy in dehulled lupin-based diet through increased feed intake in Chapter Five. Using well stored dehulled lupin in Chapter Five as compared with the fresh grain in Chapter Four may show some improvement in energy utilization that explain why the exogenous enzymes did not completely affect the 50% dehulled lupin-based diets in Chapter Five.

The dehulled lupin used in Chapter Five was from exactly the same source as in Chapter Four. However, the dehulled lupin used in Chapter Five has been suitably stored for about 9 months before making the diets. It was possible that over this period the NSP were gradually degraded and this meant that enzymes had less

antinutrients to act on. In an experiment with broiler chickens, 35% inclusion of white lupin (*L. albus*) in the diet significantly decreased weight gain by 11% using fresh grain but only 6.5% using stored grain. In a more detailed study by Fuente *et al.* (1998) storage time of 32 weeks of barley, significantly decreased total β-glucan, NSP, *in vitro* viscosity and endogenous enzyme activity of barley when included at 50% level in chick diet. These physiological changes significantly improved apparent metabolisable energy (AMEn) of the barley after 32 weeks storage time. Interestingly, enzyme addition (β-glucanase) only markedly improved AMEn for fresh barley or after 6 weeks storing time. No further improvement in AMEn was recorded after 16 or 32 weeks storage of the barley. One of the reasons for enzyme supplementation having no effect might be related to formulating the diets above the nutrient requirements (Graham and Balnave, 1996). In Chapter Five fish were fed at satiation level but enzyme supplementation may have produced better results under a restricted feeding regime (Oloffs *et al.*, 1998).

It was shown that feeding rainbow with a diet that contained 50% dehulled lupin did not cause any detrimental affect on the fish performance (Chapter Five). The highest acceptable inclusion level of 25% of *L.angustifolius* and *L.albus* has been reported in broiler diets. For pigs, Edwards and van Barneveld (1998) suggested the inclusion level of 10-15% for weaners, 20-25% for growers and 30-35% in finisher diet. Much higher inclusion level of lupin has been reported in fish diet. Recently Burel *et al.* (1998) included up to 70% extruded dehulled *L.albus* in rainbow trout diet (supplemented by L-methionine) without depressing the growth and feed intake. Also whole extruded *L.albus* supported the growth performance of turbot at 50% inclusion level compared to a fish meal-based diet (Burel *et al.*, 2000a). In the current study 40% inclusion of dehulled lupin in rainbow trout diet under restricted feeding regime and 50% inclusion of dehulled lupin using an *ad lib* feeding regime supported the growth performance comparable to a fish meal-based diet. The higher tolerance of fish to such a high inclusion level of lupin compared to terrestrial animals diets is not easy to explain.

The maximum-recorded inclusion level of soybean meal in salmonid diets is appreciably lower than the values that were shown for lupin in the present study. Growth performance of Atlantic salmon that were fed with a soybean meal-based

diet (34% soybean meal) was significantly lower than that of a fish meal-based diet (Olli *et al.*, 1995; Refstie *et al.*, 1998). Possibility of adding significantly higher inclusion level of dehulled lupin as compared with soybean meal in fish diet can be considered a great advantage for lupin. The reason for the significantly lower successful inclusion of soybean meal in fish diets might be related to significantly higher concentration of ANF's in soybean grain after oil extraction. Unfortunately there is a lack of comprehensive information about the concentration of different ANF's in soybean meal in the literature. Having shown the suitability of up to 50% inclusion level of dehulled lupin in rainbow trout diet, the appropriateness of dehulled lupin was tested for Atlantic salmon in Chapter Six. Considering the higher protein requirement of Atlantic salmon compared with rainbow trout (NRC, 1993) and the lower quality of the fish meal used in the final experiment, formulation of a balanced diet was only possible with a maximum inclusion level of 30% dehulled lupin in the diet. Growth performance of fish that were fed with FM 40 and DL 40 diets was significantly lower than other groups, thus supporting the higher protein requirement of Atlantic salmon compared with rainbow trout. However, feeding the fish with mixed diets (DL 4540 and DL 4045) supported the growth performance of fish comparable with 45% CP diets (FM 45 and DL 45). This is a significant finding as lowering the protein concentration, even at one feeding time could save a great amount of protein over the year at farm level and lower the nitrogen excretion in the environment. Better utilization of nutrients in the afternoon feeding time could not be demonstrated in the current study. However, marginally better performance of fish that received a higher protein content diet in the afternoon might be related to the higher feed intake in the afternoon. Apparent crude protein digestibility for DL 45 diet was significantly higher that of FM 40 diet. In contrast, PER in 40% CP diets (FM 40 and DL 40) and DL 4045 diet was significantly higher than for other diets. Similarly, significantly higher PER has been reported for Atlantic salmon when the fish fed the diet with a lower protein (34% vs 43.9%) content (Hillestad *et al.*, 2001). It has been shown even at high protein digestibility level the protein may not be effectively utilized by fish (Dimes *et al.*, 1994). In a comparative study by Refstie *et al.* (2000), nutrient digestibility in rainbow trout was higher than Atlantic salmon, however, nutrient utilization was better in Atlantic salmon. This opens a new scenario for considering the nutritional value of ingredients for different fish

species. As mostly in the past there has been a lot of emphasis on the importance of digestibility studies.

Detrimental effects of extracted soybean meal inclusion at (26 %) of the diet on the distal intestine of Atlantic salmon have been reported in the literature (van den Ingh *et al.*, 1996). Oligosaccharides and lectins were proposed as the possible candidates of causing the changes. Inclusion of 30% full fat soybean meal caused the same problem while 28% inclusion of soybean protein concentrate did not cause these changes in Atlantic salmon (van den Ingh *et al.*, 1991). It is unlikely that oligosaccharides and protease inhibitors caused the morphological changes and lectins were suggested as the possible causative agents (van den Ingh *et al.*, 1991). This hypothesis is supported by the current research, as 30% inclusion of dehulled lupin in the Atlantic salmon diet did not cause any morphological changes in the distal intestine despite the high inclusion of NSP.

The concentration of oligo-sacharides (NSP) in lupin is significantly higher than any other grain legumes (Vila and Mascarell, 1999). The detrimental effect of oligosacchrides is mainly attributed to the soluble fraction (Choct, 1997). It is suggested that extrusion may release the soluble part of NSP (Danicke *et al.*, 1999). This may have contributed to a lower (not significant) growth rate of Atlantic salmon when fed extruded diets containing concentrated lupin *(L.angustifolius)* compared with fish fed protein concentrates from soybean and field peas (Carter and Hauler, 2000). In contrast feeding rainbow trout with an extruded diet that contained 70% *L.albus* supported the growth performance (Burel *et al.*, 1998). The concentration of NSP in kernel of *L.albus* is significantly lower than that of *L.angustifolius* (van Barneveld, 1999). This may explain the detrimental effect of extruded lupin on fish performance by releasing more soluble NSP through *L.angustifolius* compared with *L.albus*.

Results through this research showed that lupin is an excellent fish meal replacement for salmonids. Lupin is a palatable ingredient as the feed intake was increased following its inclusion in fish diet. Concentration of two main antinutrients in lupin is relatively low and there is no need to apply any processing to lower their concentration in the grain. High carbohydrate (NDF) and relatively

lower protein content of the grain compared with fish meal may limit its inclusion in fish diet. Dehulling the grain, however minimize these limitations. Dehulling at the same time intensifies the concentration of oligosaccharides in the kernel of lupin (Evans *et al.*, 1993). This phenomenon should be closely monitored when the high level of dehulled lupin is added in the diet. Higher crude protein and phosphorus digestibility can be considered as the great advantages for lupin compared with fish meal. However, lower dry matter digestibility of lupin may limit its use at large scale. The inclusion level of dehulled lupin in the diet is substantially higher than soybean meal in the diet. However, for the maximum inclusion of dehulled lupin in the diet it is suggested to store the kernel in a suitable condition (for at least six months) and use a high quality fish meal to formulate the diets.

7.2. Future research directions

The following issues identified during the course of the present study that need further research to improve our understanding regarding the potential of plant proteins as fish meal replacement and to improve grain legumes utilization by salmonids.

1. Formulating the balanced diets for aquatic animals is more feasible using both legumes and cereals in the diet. More research is needed to find the best combination of different plant proteins (both legumes and cereals) as fish meal replacement in aquaculture nutrition.

2. It is suggested that adding more fish oil in the diet can compensate the low utilization of carbohydrate. However, the limitation of using fish meal is paralleled with the limitation of fish oil in animal nutrition. Therefore, research focus is needed to improve the utilization of carbohydrates as a dietary energy source in order to minimize the use of fish oil.

3. The maximum tolerance level for different antinutrients should be determined for fish of different species and body size. Meanwhile, the interaction and the additive effect of two or more antinutrients should be more closely evaluated.

4. Research is needed to find the best processing methods for improving the nutrient utilization and to minimize the side effects of antinutrients in the diet. Developing new enzymes to target different nutrients, testing the effect of various chemical, physical and biological methods and ultimately developing some models for selecting the best possible processing methods are strongly suggested for the future research.

5. New feeding management for improving nutrient utilization has utmost importance. More research on the effect of phase feeding, finding the best time of feeding based on the available information on circadian rhythms and formulation of the diets based on the season can be considered as new area of research to improve nutrient utilization.

6. Adaptation to ANF's or high carbohydrate levels maybe achieved by gradual increase of the plant proteins in a diet. This should be investigated further.

7. Initial research based on short-term experiments should be extended to long-term production experiments using bigger fish to ensure that the experimental findings have real applications at farm level.

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