Effects of replacing fish meal with plant protein in diets for Atlantic cod (*Gadus morhua* L.)

Ann-Cecilie Hansen

Dissertation for the degree philosophiae doctor (PhD)

at the University of Bergen

Bergen, 2009

"Skull torsken oss feile, hva hadde vi da,
hva skulle vi føre til Bergen herfra,
da seilte vist jektene tomme."
Petter Dass

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N I F E S

NATIONAL INSTITUTE
OF NUTRITION AND
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Abstract

In Northern Europe, interest in farming Atlantic cod *Gadus morhua* L. has increased steadily over the past decade stimulated by the decline in landings from fisheries, and the more predictable supply by hatchery reared juveniles for on-growing. From 1999 to 2007 the sale volume of farmed cod has increased from 145 to 10000 tonnes, and in 2008 13500 tonnes were slaughtered. Until recently, the protein in cod diets has been based on expensive, high-quality fish meal. Currently, most marine resources, which are used in production of fish meal, are exploited to the highest maximum level, simultaneously as the global production of farmed fish has increased. Pressure is laid on the farming industry to stop using unsustainable diets, and find solutions that are in agreement with sustainable management. It is therefore essential to evaluate the potential for using plant proteins in diets for Atlantic cod.

Four feeding trials have been conducted to evaluate the use of plant proteins in diets for Atlantic cod. A mixture of soybean concentrate and wheat gluten can replace 58% protein from fish meal, and a mixture of soy protein concentrate, bioprosessed soybean and wheat gluten meal can replace at least 25% of the fish meal protein without reducing growth. Inclusion of plant protein reduces protein utilisation, but can to some extent be compensated for by increased feed intake. Corn gluten is not recommended used as it gave reduced nutrient digestibility and a yellow skin colour. Plant protein inclusion did not affect health negatively, except for severe gut damage when 100% of fish meal was replaced by plant protein (a mixture of protein concentrate, bioprosessed soybean and wheat gluten meal). Gut passage time was not affected by plant protein inclusion. Adding lysine above 1.9% of diet (corresponding to 4.0% of protein) did not improve total growth, but gave reduced lipid storage. Adding methionine above 0.9% of diet (corresponding to 1.8% of protein) did not improve total growth, and did not affect lipid storage.

In conclusion there is a high potential for safe use of plant proteins in diets for Atlantic cod without challenging performance, provided that the plant ingredients are of high quality.

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Abbreviations

ADC	Apparent digestibility coefficient
ALAT	Alanine aminotransferase
ANF	Anti-nutritional factors
ASAT	Aspartate aminotransferase
DAA	Dispensable amino acids
FCR	Feed conversion ratio
FM	Fish meal
G	Corn gluten meal
GDH	Glutamate dehydrogenase
GI	Gastrointestinal
h	Hour
Hb	Haemoglobin
Hct	Haematocrit
HSI	Hepatosomatic index
HSP	Heat shock protein
HPLC	High pressure liquid chromatography
IAA	Indispensable amino acids
LPV	Lipid productive value
MCH	Mean cell haemoglobin
MCHC	Mean cell haemoglobin concentration
MCV	Mean cell volume
mRNA	Messenger ribonucleic acid
PCA	Principal component analysis
PCR	Polymerase chain reaction
PER	Protein efficiency ratio
PP	Plant protein
PPV	Protein productive value
RBC	Red blood cell count
RNA	Ribonucleic acid
S	Soybean meal
SC/WG	Soy protein concentrate/Wheat gluten meal
SD	Standard deviation
S/G	Soybean meal/Corn gluten meal
SGR	Specific growth rate
TAG	Triacylglycerols
Q-PCR	Quantitative polymerase chain reaction

List of publications

Paper I

Hansen, A-C., Karlsen Ø., Rosenlund, G., Rimbach, M. and Hemre, G-I. (2007). Dietary plant-protein utilisation in Atlantic cod, *Gadus morhua* L. *Aquaculture Nutrition* **13**, 200-215.

Paper II

Hansen, A.-C., Rosenlund, G., Karlsen, Ø., Olsvik, P.A. and Hemre, G.-I. (2006). The inclusion of plant protein in cod diets, its effects on macronutrient digestibility, gut and liver histology and heat shock protein transcription. *Aquaculture Research* **37**, 773-784.

Paper III

Hansen, A.-C., Rosenlund, G., Karlsen, Ø., Koppe, W. and Hemre, G.-I. (2007). Total replacement of fish meal with plant proteins in diets for Atlantic cod (*Gadus morhua* L.) I: Effects on growth and protein retention. *Aquaculture* **272**, 599-611.

Paper IV

Olsen, R.E., Hansen, A.-C., Rosenlund, G., Hemre, G.-I., Mayhew, T.W., Knudsen, D.L., Eroldogan, O.T, Myklebust, R. and Karlsen, Ø. (2007). Total replacement of fish meal with plant proteins in diets for Atlantic cod (*Gadus morhua* L.) II: Health aspects. *Aquaculture* **272**, 612-624.

Paper V

Hansen, A.-C., Karlsen, Ø., Koppe, W., Hemre, G.-I. and Rosenlund, G. (2009). Do plant based diets for Atlantic cod need additions of crystalline lysine or methionine? *Aquaculture Nutrition* (submitted).

In the following thesis these five papers are referred to in the text by their Roman numbers.

Introduction

In Northern Europe, interest in farming of Atlantic cod *Gadus morhua* L. has increased steadily over the past decade stimulated by the decline in landings from fisheries and at more predictable supply of hatchery reared juveniles for on-growing (Rosenlund and Skretting, 2006). From 1999 to 2007 the sale volume of farmed Atlantic cod has increased from 145 to 10000 tonnes (Figure 1), and in 2008 13500 tonnes were slaughtered (Lassen, 2009). There is optimism, but the industry faces several biological, technological and financial challenges, necessary to solve to further grow as an industry. The biological problems are related to larvae and juvenile quality, early maturation, disease control and nutrition.
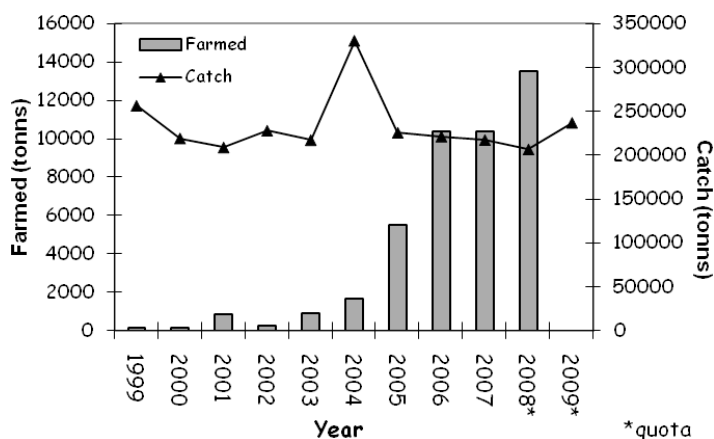
Figure 1. Sale volume of farmed Atlantic cod (bars) and catch of wild Atlantic cod (line) in Norway. The sale volume from 1999-2002 does not distinguish between produced and wild farmed Atlantic cod. The volume for farmed Atlantic cod from 2008 is the production. The catch data from 2008 and 2009 are the quota (Fiskeridirektoratet, 2008; Lassen, 2009).

Atlantic cod is a carnivorous lean fish species that has a relatively low potential to utilize dietary lipid (<20%) and a high protein requirement (>50%; Rosenlund *et al*., 2004). Until recently, the protein in Atlantic cod diets has been based on expensive, high-quality fish meal. Currently, most marine resources, that are used in production

of fish meal, are exploited to the highest maximum level (FAO, 2007) and gives no opportunity to increase the fish meal production, simultaneously as the global production of farmed fish has increased (Figure 2). Pressure is laid on the farming industry to stop using unsustainable diets (World Wildlife Foundation; WWF, 2009), and find solutions that are in agreement with sustainable management. It is therefore essential to evaluate the potential for using more plant proteins in Atlantic cod diets.
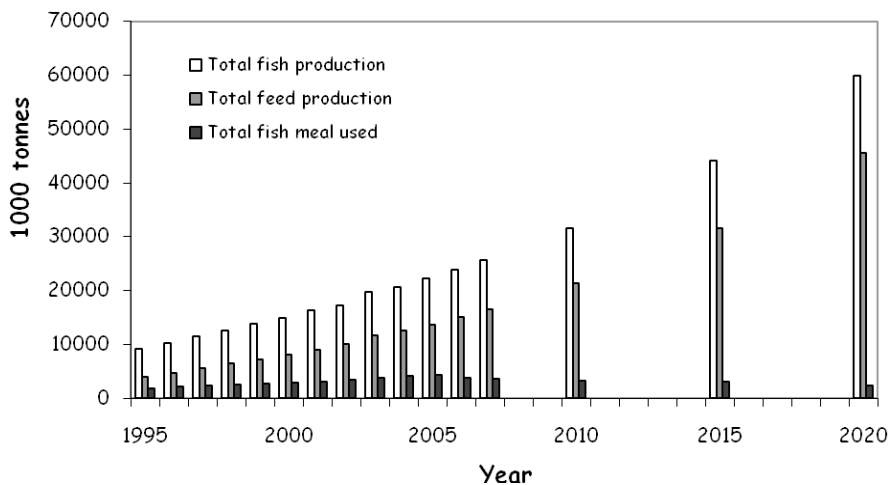
Figure 2. Global production of farmed species (fish and crustaceans) and feed, and the use of fish meal in aqua feeds. The numbers from 2010, 2015 and 2020 are estimates. Modified from Tacon and Metian (2008).

Use of protein rich plants in diets for farmed fish is widely studied, and data suggest that various plant protein sources can be included into fish diets at relatively high levels. When introducing new feed raw materials it is important to secure that they do not compromise fish growth and welfare. Problems with replacing fish meal with plant proteins in fish diets are associated with low levels of protein and minerals, unfavourable amino acid profiles, high levels of fibre and the presence of anti-nutritional factors (The Scientific Committee for Food Safety, Vitenskapskomiteen; Criteria for safe use of plant ingredients in fish diets, 2009; www.vkm.no).

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1. Background

1.1 Plant protein ingredients

There is a wide variety of plant protein ingredients that can be candidates to replace fish meal as a protein source in fish feeds. This includes oilseeds (soybean and canola), legumes (cottonseed, lupin and pea) and cereal grains (corn and wheat). There are several qualities of these plant protein ingredients depending on how they are processed. There are qualities ranging from grinded whole seeds, like full-fat soybean meal, to concentrated protein products, like soy protein concentrate. The plant ingredients with largest world production, and thereby highly available, are soybean (915 million tonnes, 2007), corn (3 678 million tonnes, 2007) and wheat (3 001 million tonnes, 2007) (http://faostat.fao.org). To obtain acceptable growth and feed utilisation, simultaneously avoiding large liver sizes, Atlantic cod will need diets with 50-60% protein, 13-20% lipid and moderate carbohydrate levels (Lied and Braaten, 1984; Jobling, 1988; Lie *et al.*, 1988; Dos Santos *et al.*, 1993; Morais *et al.*, 2001; Rosenlund *et al.*, 2004; Grisdale-Helland *et al.*, 2008; Hansen *et al.*, 2008). Less refined plant ingredients, like solvent extracted-soybean meal, are generally low in protein compared to fish meal (Table 1). To achieve a high dietary protein level in plant based diets (above 50%), processed plant ingredients like wheat and corn gluten and soy protein concentrate needs to be used. Atlantic cod, like other animals, has a requirement for a well balanced mixture of indispensable amino acids, for optimal growth and protein utilisation. The requirement for indispensable amino acids (IAA) has been shown to highly correlate with the amino acid pattern of the fish (Wilson and Poe, 1985; Mambrini and Kaushik, 1995), and this pattern is similar between fish species (Mambrini and Kaushik, 1995). High quality fish meal is therefore regarded a good protein source that covers the need for all amino acids. On the other hand, plant proteins differ from fish meal in several IAAs (Table 2). Soybean is regarded to have

a reasonably balanced amino acid profile, but the IAAs of concern are lysine and methionine that may be limiting in soy-based diets for fish. The same concern also apply for wheat and corn holding 1.5 and 1.8% lysine respectively, compared to 6.1% in soybean and 8.1% in fish meal (Table 2).

Table 1. Typical proximate composition of fish meal and various plant ingredients, given as % of diet, modified from Gatlin et al. (2007).

Ingredient	Dry matter	Protein	Lipid	Ash
Fish meal, herring	92.0	72.0	8.4	10.4
Canola	93.0	38.0	3.8	6.8
Corn	88.0	8.5	3.6	1.3
Corn gluten meal	91.0	60.4	1.8	2.1
Lupin, *Lupious angustifolius*	89.0	39.2	10.3	2.8
Filed peas (whole)	89.0	25.6	1.3	3.4
Soybean meal, de-hulled	90.0	48.5	0.9	5.8
Soy protein concentrate	90.0	64.0	3.0	1.5
Wheat	88.0	12.9	1.7	1.6
Wheat gluten*	--	77.7	1.0	--

*Paper **I**

Table 2. Indispensable amino acid composition (% of protein) of fish meal, Atlantic cod muscle and selected plant ingredients, modified from Waagbø et al. (2001).

	Fish meal	Atlantic cod muscle[a]	Soy-bean	Pea	Rapeseed	Corn gluten	Wheat gluten	Lupin[b]
Arginine	5.9	6.1	7.3	8.0	7.9	3.7	3.6	11.8
Histidine	2.9	5.4	2.8	2.7	3.3	2.9	1.9	3.1
Isoleucine	4.4	4.5	4.7	4.7	4.3	3.5	3.5	3.8
Leucine	7.5	8.3	7.5	7.6	6.1	11.4	7.0	7.2
Lysine	8.1	9.3	6.1	7.8	6.6	1.8	1.5	4.2
Methionine	3.0	3.0	1.4	1.0	2.3	2.3	1.6	0.8
Phenylalanine	4.0	4.2	5.0	5.1	3.8	6.6	5.0	3.5
Threonine	4.3	4.3	4.0	4.0	5.6	3.8	3.7	3.8
Valine	5.4	5.0	4.9	5.3	4.3	4.4	3.9	4.2

[a] Hansen A-C., unpublished results
[b] Burel *et al.* (2000)

Some plant proteins have high levels of undesirable components like anti-nutritional factors (ANFs) and fibre. ANFs have been defined as: "substances which by themselves, or through their metabolic products arising in living systems, interfere with food utilisation and affect the health and production of animals" (Francis *et al.*, 2001). Soybean meal is one of the most studied plant protein ingredient regarding ANFs, and several are identified; saponins, protease inhibitors, lectins and phytic acid (Francis *et al.*, 2001). ANFs are known to affect performance of salmonid fish; altering gut histology (van den Ingh *et al.*, 1991; Sanden *et al.*, 2005), inducing inflammations (Baeverfjord and Krogdahl, 1996; Bakke-McKellep *et al.*, 2000) and decreasing nutrient digestibility (Refstie *et al.*, 2000; Krogdahl *et al.*, 2003). Fibres, also called non-starch polysaccharides (NSP), are non digestible polysaccharides derived from plants, which can be soluble or insoluble. In plants relevant to use in fish feed, soybean has especially high fibre content amounting to approximately 20% in raw beans. Fibres are associated with reduced digestibility and changes in gut passage time (Hilton *et al.*, 1983; Storebakken *et al.*, 1999). Corn and wheat are not known to contain substantial amounts of ANFs or fibres (Francis *et al.*, 2001).

1.2 Growth and feed utilisation of fish fed plant proteins

Specific growth rate (SGR) in Atlantic cod is shown to vary substantially throughout its life cycle, with huge weight increases especially during larval development (Finn *et al.*, 2002), still great during its juvenile stages, and gradually slowing down during the on-growing phase (Rosenlund *et al.*, 2004; Imsland *et al.*, 2005; Björnsson *et al.*, 2007). Several parameters other than fish size determine growth rates, among these feed compositions (Rosenlund *et al.*, 2004), as well as the quality of each ingredient e.g. fish meal quality (Albrektsen *et al.*, 2006). Water temperature and other environmental conditions e.g. light regime, also highly influence growth rates in fish (Hemre *et al.*, 2004b; Björnsson *et al.*, 2007).

Numerous results exist on the replacement of fish meal with various plant proteins at different levels in diets for several fish species (Table 3). The results vary; generally giving reduced growth, feed and nutrient utilization when exceeding a certain level. As far back as in 1974 Cho *et al*. (1974) replaced herring meal with soybean meal in diets for rainbow trout, with reduced growth and feed efficiency as a result. Since then, soybean meal (solvent-extracted and full-fat) is widely tested in several species, and the most dominant result is reduced growth and feed utilisation when exceeding an upper level (Table 3). Use of soy protein concentrate has not shown the same negative effects (Kaushik *et al.*, 1995; Storebakken *et al.*, 1998). Corn and wheat gluten, alone or in combination, have shown higher tolerance levels than soybean meal, regarding growth and feed utilisation (Regost *et al.*, 1999; Pereira and Oliva-Teles, 2003; Fournier *et al.*, 2004; Gòmez-Requeni *et al.*, 2004; Sitja-Bobadilla *et al.*, 2005). One reason for reduced growth with high plant protein inclusions is often caused, at least partly, by a lowered feed intake (Refstie *et al.*, 1998; Espe *et al.*, 2006). This may be due to reduced palatability that might be improved by adding stick water (Kousoulaki *et al.*, 2009), krill (Oikawa and March, 1997; Olsen *et al.*, 2006) and / or hydrolysed fish protein (Espe *et al.*, 1999; Refstie *et al.*, 2004; Hevrøy *et al.*, 2005).

Fish meal has been replaced by plant proteins to some extent in several trials with Atlantic cod (von der Decken and Lied, 1993; Albrektsen *et al.*, 2006; Refstie *et al.*, 2006a; Karalazos *et al.*, 2007). Replacing fish meal protein with 30% protein from full-fat soybean meal in diets for Atlantic cod showed reduced growth (von der Decken and Lied, 1993; Karalazos *et al.*, 2007) possibly due to reduced feed intake. On the other hand, no negative effects on growth and feed intake was observed by Albrektsen *et al*. (2006) using a mixture of full-fat soybean meal and corn gluten up to 54% of total protein. Similarly, Refstie *et al*. (2006a) found no effect on growth using 24% (of total protein) solvent-extracted or bioprocessed soybean meal. This demonstrates a great potential of replacing fish meal in diets for Atlantic cod when feed intake is maintained.

Table 3. An overview of results of selected studies where plant proteins were used to replace fish meal in diets for Salmonids and five marine species.

Species	Plant ingredient	% PP in diet	% FM in diet	Supplemented with amino acids	Growth	Feed efficiency	Protein retention	Reference
Rainbow trout	Soybean meal	51.0	18.0	mixture	reduced	reduced	not reported	(Cho et al., 1974)
	Soy protein concentrate	22.0-62.0	42.0-0.0	met	no difference	no difference	no difference	(Kaushik et al., 1995)
Oncorynchus mykiss	Soyflour meal	24.0-42.0	44.0-30.0	met	no difference	reduced at 30.0% FM	no difference	(Kaushik et al., 1995)
	Defatted soybean meal	29.6	32.0	no	no difference	reduced	no difference	(Refstie et al., 2000)
	Corn gluten + wheat gluten + extruded pea meal + rapeseed meal	69.6	0.0	mixture	reduced	reduced	reduced	(de Francesco et al., 2004)
	Extracted soybean meal	33.9	31.0	met	reduced	reduced	not reported	(Refstie et al., 1998)
	Bio-processed soybean	28.1	31.0	met	no difference	no difference	not reported	(Refstie et al., 1998)
	Extracted soybean meal	20.4-27.3	45.1-40.0	met	no difference	no difference	no difference	(Carter and Hauler, 2000)
	Lupin protein concentrate	21.8-29.2	45.1-40.0	met	no difference	reduced at 40.0% FM	reduced at 40.0% FM	(Carter and Hauler, 2000)
	Pea protein concentrate	20.6-27.6	51.1-40.0	met	no difference	no difference	no difference	(Carter and Hauler, 2000)
Atlantic salmon	Extracted soybean meal	7.6-27.0	49.4-32.4	no	gradually reduced	gradually reduced	no difference	(Krogdahl et al., 2003)
Salmo salar	Full-fat soybean + corn gluten meal	4.0-43.8	51.5-23.2	met, lys	gradually reduced	gradually reduced	gradually reduced	(Opstvedt et al., 2003)
	Wheat gluten+ corn gluten + extracted soybean meal	43.0	12.0	met, lys, his	no difference	no difference	no difference	(Torstensen et al., 2008)
	Wheat gluten+ corn gluten meal	43.4	0.0	mixture	reduced	no difference	no difference	(Espe et al., 2006)
	Wheat gluten+ corn gluten	39.6	5.0	mixture	no difference	no difference	no difference	(Espe et al., 2007)
	Wheat gluten+ corn gluten + full-fat soybean meal	7.0-46.0	48.6-19.1	lys	gradually reduced	gradually reduced	gradually reduced	(Mundheim et al., 2004)

Species	Protein source			Amino acids					Reference
Turbot *Psetta maxima*	Corn gluten meal	20.0-57.0	31.0-0.0	glu, lys, arg	gradually reduced	gradually reduced	gradually reduced	gradually reduced	(Regost *et al.*, 1999)
	Lupin meal	30.0-50.0	44.0-34.5	no	no difference	increased	no difference	increased	(Burel *et al.*, 2000)
	Lupin+ corn gluten+ wheat gluten meal	35.0-80.2	40.0-0.0	mixture	reduced at 0.0% FM	no difference	no difference	no difference	(Fournier *et al.*, 2004)
European seabass *Dicentrarchus labrax*	Corn gluten+ wheat gluten + soybean + rapeseed meal	33-66.9	40.0-5.0	lys	no difference	no difference	no difference	no difference	(Kaushik *et al.*, 2004)
	Soy protein concentrate	45.0	0.0	met	reduced	reduced	reduced	reduced	(Dias *et al.*, 2005)
	Corn gluten meal	45.0	0.0	met, arg, trp	reduced	reduced	reduced	reduced	(Dias *et al.*, 2005)
	Soybean meal	10.1-30.2	69.0-53.6	no	no difference	no difference	no difference	no difference	(Robaina *et al.*, 1995)
	Lupin meal	11.5-34.6	69.0-53.6	no	no difference	no difference	no difference	no difference	(Robaina *et al.*, 1995)
	Rapeseed protein concentrate	20.0-74.5	47.0-0.0	no	gradually reduced	gradually reduced	gradually reduced	no difference	(Kissil *et al.*, 2000)
	Soy protein concentrate	20.0-72.5	47.0-0.0	no	gradually reduced	reduced at 0.0% FM	reduced at 0.0% FM	reduced at 0.0% FM	(Kissil *et al.*, 2000)
Gilthead seabream *Sparus aurata*	Corn gluten meal	13.6-54.2	49.2-12.3	no	reduced at 12.3% FM	reduced at 12.3% FM	reduced at 12.3% FM	no difference	(Pereira and Oliva-Teles, 2003)
	Corn gluten + wheat gluten + extruded peas + rapeseed meal	33.1-65.4	35.2-0.0	mixture	gradually reduced	gradually increased	gradually increased	gradually increased	(Gòmez-Requeni *et al.*, 2004)
	Soy protein concentrate	70.0	0.0	Met	reduced	reduced	reduced	reduced	Kissil and Lupatsch 2004
	Wheat gluten meal	51.0	0.0	mixture	increased	increased	increased	no difference	Kissil and Lupatsch 2004
	Corn gluten meal	65.0	0.0	mixture	reduced	reduced	reduced	reduced	Kissil and Lupatsch 2004
	Corn gluten + wheat gluten + extruded peas + rapeseed meal+ lupin meal	33.0-65.4	35.2-0.0	mixture	gradually reduced	improved up to 17.6% FM	gradually reduced	not reported	(Sitja-Bobadilla *et al.*, 2005)
Atlantic cod *Gadus morhua*	Extracted soybean meal	24.6	54.2	met	no difference	increased	increased	no difference	(Refstie *et al.*, 2006a)
	Full-fat soybean meal	7.9-23.7	22.2-15.7	no	reduced at 15.7% FM	reduced at 15.7% FM	reduced at 15.7% FM	not reported	(von der Decken and Lied, 1993)
	Bioprosessed soybean meal	21.4	53.9	met	no difference	Increased	no difference	no difference	(Refstie *et al.*, 2006a)
	Full-fat soybean meal + corn gluten meal	6.0-42.0	58.8-31.6	lys	no difference	no difference	no difference	no difference	(Albrektsen *et al.*, 2006)
	Full-fat soybean meal	12.0-36.0	55.9-44.5	lys, met	reduced	reduced	reduced	not reported	(Karalazos *et al.*, 2007)

Plant proteins in fish diets may lead to reduced protein retention (Kikuchi, 1999; Regost *et al.*, 1999; Carter and Hauler, 2000; Refstie *et al.*, 2000; Opstvedt *et al.*, 2003; de Francesco *et al.*, 2004; Lim *et al.*, 2004). This is possible due to imbalances in amino acid profile. Protein growth occurs when protein synthesis is larger than protein degradation (Millward and Rivers, 1988), and protein synthesis requires that all amino acids are present at the same time in correct proportions (Geiger, 1947). Shortage of one (or several) IAA(s) from the diet will lead to a shortage of amino acids required for protein synthesis. It is shown that high protein synthesis in muscle is accomplished by high protein degradation, both in rats (Millward *et al.*, 1975) and fish (Houlihan *et al.*, 1988). Apparently, protein degradation regulates the protein turnover rather than the protein synthesis, as malnourished rats maintained growth while protein degradation decreased (Millward *et al.*, 1975). For fish this is shown for European sea bass *Dicentrarchus labrex* by Langar *et al.* (1993) where poor protein quality (imbalanced amino acid composition) resulted in increased protein degradation, and thereby reduced protein retention. Similar results are also found in studies with chicken (Tesseraud *et al.*, 1992) and pig (Fuller *et al.*, 1987). When the dietary protein is composed of the exact amount of each indispensable amino acid required it is called the ideal protein, meaning there will be no amino acids in excess or deficiency. El-Mowafi *et al.* (2009) showed decreased protein productive value (PPV) in Atlantic salmon when the amino acid level was 90% of the ideal protein profile. Also in studies with Korean rockfish *Sebastes schlegli* by Lim *et al.* (2004), protein efficiency ratio (PER) was improved when a plant protein based diet was supplemented with a mixture of crystalline amino acids.

The biological value of plant proteins in fish diets, may vary dependent on water temperature (Lòpez-Urrtia and Acuna, 1999). Along the Norwegian coast there are great variations in sea temperature between winter and summer, and between the Northern and Southern part of the country. Atlantic cod tolerate a wide range of water temperatures and optimal temperature is found to vary with body size, ranging from 14ºC for 50g fish to 6ºC for 5 kg fish (Björnsson and Steinarsson, 2002). It may

therefore be wise to elucidate temperature effects on nutrient utilization to optimize diet composition, e.g. by designing summer and winter specific diets.

1.3 Tissue free amino acid pools

The tissue free pool of amino acids supplies protein synthesis with amino acids. In rats it was found that 70-80% of the pool derived from degradation of endogenous protein (Cowey and Walton, 1988), but in fish endogenous protein only supplied 40-50% of the free amino acid pool (Dabrowski and Guderly, 2002). This may indicate that fish require higher supply of amino acids from the diet when compared to mammals. The concentration of IAAs in tissue free amino acid pools increase with time after feeding (Walton and Wilson, 1986; Carter *et al.*, 2000). In Atlantic cod, concentration of amino acids peak after 12h in plasma and 18h in muscle, when fed whole sandeel *Ammodytes spp.* (Lyndon *et al.*, 1993). DAAs does not vary in the same manner (Walton and Wilson, 1986; Carter *et al.*, 1995). Liver free amino acid levels seemed independent on feeding, showing stable levels after feeding in rainbow trout (Walton and Wilson, 1986; Carter *et al.*, 1995). Feeding hydrolysed protein and / or crystalline free amino acids, gave faster peaks in tissue free pools compared to diets where amino acids were bound to protein (Espe *et al.*, 1993). In Atlantic cod, plasma free lysine peaked 4h after feeding when given as crystalline lysine, compared to 24h when given as protein-bound lysine (Berge *et al.*, 1994). The plasma and muscle free amino acid pool was shown to correlate with the dietary amino acid profile, total amino acids, IAA and single amino acids (Espe *et al.*, 1993; Berge *et al.*, 1994; Carter *et al.*, 2000; Espe *et al.*, 2007). It was therefore suggested that the muscle free amino acid pool can be a tool to determine the adequacy of a dietary amino acid, and to reflect nitrogen balance (Cowey and Walton, 1988; Carter *et al.*, 2000; Mente *et al.*, 2003). Further, the IAA present in the lowest concentration in the muscle free amino acid pool should be considered "the limiting amino acid for protein synthesis and retention" (Carter *et al.*, 2000).

The tissue free amino acids can, besides being used in protein synthesis or metabolic reactions, be transdeaminated to α-ketoglutarate that can enter the energy metabolism, or be transaminated to DAAs (Figure 3). Glutamate dehydrogenase (GDH), alanine aminotransferase (ALAT) and aspartate amino transferase (ASAT) are the most important enzymes in transdeamination. The activity of these enzymes is high in liver, but there are also considerably activity in kidney and muscle tissues (Albrektsen, 1994). Vitamin B_6 in the form pyridoxal-5-phospathe, acts as a cofactor of these enzymes. Decreased transamination activity was observed with low protein intake (Gaye-Siessegger *et al.*, 2006) and liver ASAT activity was found to correlate with protein retention (Gaye-Siessegger *et al.*, 2006; Gaye-Siessegger *et al.*, 2007). Monitoring body reservoirs of vitamin B_6 might therefore give an indication whether the diet levels are sufficient for transaminase activity.

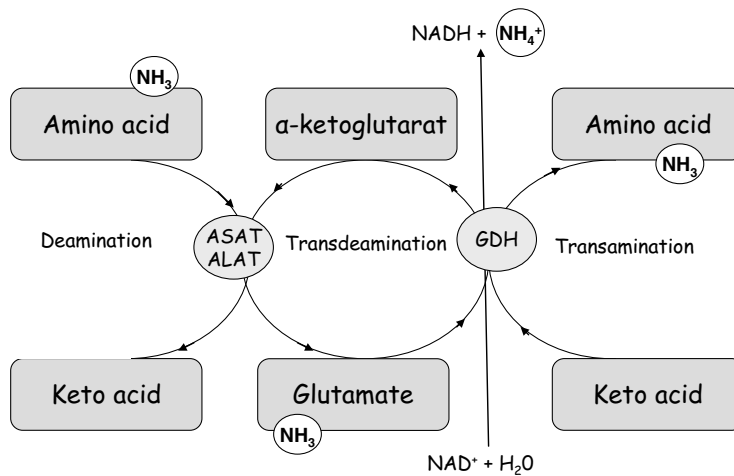*Figure 3. Amino acids are deaminated to its corresponding keto acid, while α-ketoglutarate is transaminated to glutamate by the enzymes ASAT and ALAT. Glutamate is further deaminated to α-ketoglutarat by GDH, which can enter energy metabolism. The amino group (NH_3) from glutamate can be transferred to keto acids creating dispensable amino acids, or enter urea cycle as ammonium (NH_4^+). Modified from Espe et al.(2001).*

1.4 Lysine and methionine

Lysine and methionine are regarded as the first limiting amino acids in most plant proteins (Table 2). Trials have shown large variation in requirement for lysine (3.7-6.2% of dietary protein) and methionine (2.1-3.3% of dietary protein) (Rodehutscord *et al.*, 1995; Rodehutscord *et al.*, 1997; Hauler and Carter, 2001; Sveier *et al.*, 2001; Mai *et al.*, 2006; Espe *et al.*, 2007; Espe *et al.*, 2008). The latter may be due to different species, fish sizes, initial composition of fish, response parameter, experimental conditions e.g. temperature or length of experiment, and/or other unknown factors. The dietary requirements for lysine and methionine are not determined for Atlantic cod. Both SGR and PER were found to be improved when supplementing a plant based diet with lysine and methionine for rohu fingerlings *Labeo rohita* (Mukhopadhyay and Ray, 2001; Sardar *et al.*, 2008) and Korean rockfish (Lim *et al.*, 2004). The same was seen for rainbow trout when supplementing a plant based diet with lysine (Cheng *et al.*, 2003) and for Atlantic salmon when supplementing a plant based diet with methionine (Sveier *et al.*, 2001).

Lysine and methionine are indispensable amino acids with several functions in the body, besides taking part in the synthesis of proteins. Lysine and methionine function as precursors in the biosynthesis of carnitine, where lysine provides the carbon backbone (Horne *et al.*, 1971; Tanphaichitr *et al.*, 1971) and methionine the methyl group (Tanphaichitr and Broquist, 1973). Carnitine is involved in the transport of fatty acids through the outer mitochondria membrane. It is shown in rat that lysine deficiency can lead to reduced β-oxidation and increased storage of fat in the liver (Tanphaichitr *et al.*, 1976). Close interaction between protein retention, lipid deposition, and lysine intake is seen for Channel catfish *Ictalurus punctatus* where whole body lipid content was reduced when supplementing the diet with lysine (Burtle and Liu, 1994), and for Atlantic salmon (Espe *et al.*, 2007) and rainbow trout (Walton *et al.*, 1984) where low levels of dietary lysine gave increased hepatosomatic

index (HSI). Theses interactions are also seen for rainbow trout where supplementing a plant based diet with methionine resulted in decreased intraperitoneal fat deposit (Gibson Gaylor *et al.*, 2007) and for Atlantic salmon where low levels of methionine resulted in increased HSI (Espe *et al.*, 2008).

1.5 Plant proteins may affect the functionality of the gastrointestinal tract

1.5.1 Gut morphology

Morphological changes in the intestine has been shown for salmonids when including plant proteins, especially soybean (van den Ingh *et al.*, 1991; Baeverfjord and Krogdahl, 1996; Refstie *et al.*, 2000; Storebakken *et al.*, 2000; Krogdahl *et al.*, 2003; Sanden *et al.*, 2005). Typically, extensive endocytotic activity and high numbers of intracellular vacuoles are found in cells of the distal part of the gut. Damages are often characterized by increases in number of mucus-producing goblet cells, intracellular absorptive vacuoles, cellular structure of the lamina propria, amount of connective tissue, degree of mucosal folding and infiltration of the epithelium or lamina propria by inflammatory cells (van den Ingh *et al.*, 1991; Baeverfjord and Krogdahl, 1996). In extreme cases, massive necrosis occurs, a condition referred to as soybean-induced enteritis (Baeverfjord and Krogdahl, 1996). One of the ANFs high in soybean is saponin. It is not clear whether saponin alone cause intestinal damage, but Knudsen *et al*. (2008) have shown that saponin, in combination with unknown components in soybean or lupine, induce an inflammatory reaction in the distal intestine of Atlantic salmon. Changes in intestinal morphology is, however, not observed in studies where Atlantic cod was fed diets with 24% solvent-extracted or bioprocessed soybean meal (Førde-Skjærvik *et al.*, 2006; Refstie *et al.*, 2006b). Inflammatory reactions are expected to result in increasing plasma lysozyme activity,

and lysozyme activity may therefore be of diagnostic value in determination of health status of fish (Ingram, 1980).

1.5.2 Nutrient digestibility

Diets with plant proteins are known to reduce digestibility of nutrients (Hilton *et al.*, 1983; Francis *et al.*, 2001), which are associated with soluble fibres, but also anti-nutrients like protease inhibitors (Sandholm *et al.*, 1976; Krogdahl *et al.*, 1994). Krogdahl *et al.* (2003) showed reduced mucosal enzyme activities in salmon fed up to 30% solvent-extracted soybean meal, mirroring reduced macronutrient digestibility. Soluble fibres increase viscosity of gut content, that potentially can reduce digestible enzyme activities, and negatively affect nutrient digestion and absorption (Storebakken, 1985; Leenhouwers *et al.*, 2006). Insoluble fibre do not pose a similar negative effect on digestion (Dias *et al.*, 1998; Hansen and Storebakken, 2007). Tibbets *et al.* (2006) investigated the digestibility of a number of plant protein ingredients in Atlantic cod, and showed apparent digestibility coefficients (ADC) in the same range for plant protein ingredients, like soybean meal, corn gluten meal and wheat gluten meal, as for fish meal. Albrektsen *et al.* (2006) found that the protein digestibility was more affected by the fish meal quality than by plant protein inclusion level, while lipid digestion was highly reduced when including plant proteins (corn gluten meal and full-fat soybean meal). Førde-Skjærvik *et al.* (2006) on the other hand, found both reduced protein and fat digestion when including 24% solvent-extracted or bioprocessed soybean meal.

1.5.3 Gut passage time

It is indicated that gut passage time increases when soy protein is included in salmonid diets (Storebakken *et al.*, 1999) and diets for Catla fingerlings *Catla catla*, Hamilton (Naik and Annappaswamy, 2000). Soybean containing soluble fibres may influence passage time by binding water and increase viscosity of the digesta, on the

other hand insoluble fibre has been shown to decrease passage time in rainbow trout (Hilton *et al.*, 1983). Grove *et al*. (1978) showed that return of appetite for rainbow trout *Oncorhyncus mykiss* was related to gastric emptying (Figure 4). Faster gastric emptying can result in earlier recovery of appetite, and a need for more frequent feeding. Knowledge of this may therefore help in diet planning and feeding.
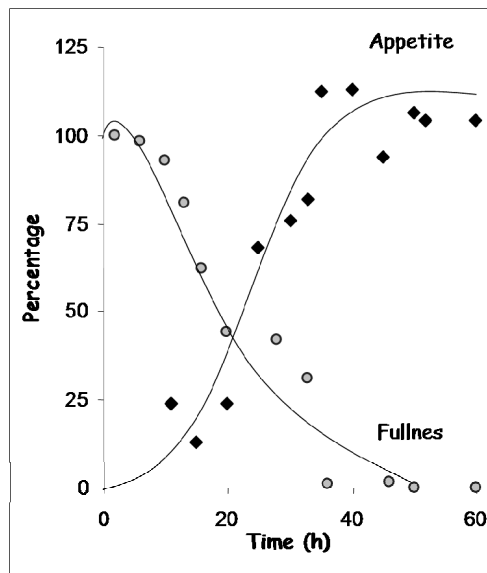
Figure 4. Comparison between the return of appetite (♦) and stomach fullness (○) in rainbow trout Oncorhyncus mykiss, modified from Grove et al. (1978).

1.6 Health parameters

1.6.1 Clinical parameters

Haematology is a useful tool to monitor fish health, and to detect early signs of disease or stress (Waagbø *et al.*, 1988; Hemre *et al.*, 1995). Blood haematocrit (Hct), red blood cell count (RBC) and haemoglobin (Hb) concentration are sensitive to physiological changes in the fish body, and low haematological values can indicate anaemia and / or infectious diseases. Mean cell volume (MCV), mean cell

haemoglobin (MCH) and mean cell haemoglobin concentration (MCHC) are derived relationships between measured Hct, RBC and Hb. If the maturation of the red blood cells is not normal the cells can be either unusually big with abnormal shapes, or smaller and immature, which results in either increases or decreases in MCH and especially MCV, but not in MCHC.

Vitamin B_{12} (cobalamine) is involved as a cofactor in the enzyme methionine synthestase which remethylate homocysteine to methionine in the sulphur metabolism (Hilton, 1989). Since vitamin B_{12} is absent from plant proteins, but present in fish meal, a replacement of fish meal with plant protein might lead to lower diet levels of vitamin B_{12} if not added. Vitamin B_{12} deficiency may result in a megablastic anemia, observed as lowered haemoglobin levels and increased MCV and MCH (Waagbø, 1999). Changing the protein ingredients from fish meal to a high plant protein inclusion, might therefore result in insufficient availability of this cofactor.

ASAT and ALAT are two enzymes involved in amino acid transamination, and the activities of these enzymes are especially high in muscle, liver and kidney. Activity in plasma is at normal conditions low (Sandnes *et al.*, 1988; Lie *et al.*, 1990) and increased leakage of these enzymes into circulation will therefore indicate organ dysfunction (Racicot *et al.*, 1975).

Changes in plasma nutrients (glucose, protein, TAG and cholesterol) levels are often seen when fish are fed inadequate or suboptimal diets. Further, plasma nutrient levels, especially elevated glucose concentrations, can be a sign of stress in Atlantic cod (Hemre *et al.*, 1991; Olsen *et al.*, 2008). Plasma nutrient levels are also affected by feed intake; volumes and whether the animal is in an absorptive or post-absorptive state (Krogdahl *et al.*, 1999). Osmo-regulatory disturbance will give increased plasma protein levels, and decreased plasma protein might indicate disease. Changes in plasma cholesterol level can indicate liver dysfunction, but cholesterol, together with

triglycerides are also shown to be reduced in plasma when fed plant proteins (Regost *et al.*, 1999; Goto *et al.*, 2001). This is possible due to reduced absorption caused by ANFs or increased faecal excretion of steroids (bile acids) (De Schrijver, 1990; Brown *et al.*, 1999; Gallaher *et al.*, 2000) caused by soluble fibre.

1.6.2 Heat shock proteins as stress markers for suboptimal nutrition

Heat shock proteins (HSPs) are highly conserved cellular proteins that exist in all organisms, including fish (Lindquist and Craig, 1988; Iwama *et al.*, 1998). The HSPs are involved in folding of the polypeptide chain, and repair and degradation of altered or denatured proteins. Increased synthesis of these proteins is shown as a response to a variety of stressors (Basu *et al.*, 2002), including as a response to suboptimal diet compositions (Martin *et al.*, 2003; Hemre *et al.*, 2004a). Sagstad *et al.* (2007) found up-regulation of mRNA HSP70 expression in hindgut from Atlantic salmon fed diets with full-fat soybean meal. HSP70 was shown to protect the mucosa against toxins and ulcerogenic conditions in mammals (Otaka *et al.*, 2006). Measurements of gene expression of these proteins may therefore be used as a marker for suboptimal diets.

2. Aims

The aim of the present thesis and its papers was to elucidate the potential for safe use of plant proteins in diets for Atlantic cod, *Gadus morhua* L., without challenging performance, in detail:

- Study the effects on growth, feed and nutrient utilisation including retention of fat and protein, when increasing dietary levels of soybean, corn gluten, wheat gluten or mixtures of these.

- Study the effect on macronutrient digestibility, when increasing dietary levels of soybean, corn gluten, wheat gluten or mixtures of these.

- Study the effects on general indicators of fish health, when increasing the dietary levels of soybean, corn gluten, wheat gluten or mixtures of these.

- Study the effect on gut health when increasing dietary levels of soybean, corn gluten, wheat gluten or mixtures of these.

- Study the effect on gut evacuation time increasing dietary levels of soybean, corn gluten, wheat gluten or mixtures of these.

- Study if adding crystalline lysine or methionine to plant based diets would affect growth and partitioning of growth.

3. Methical considerations

3.1 Fish feeding trials

Four different fish feeding trials are reported in paper **I-V**, and the experimental conditions are summarised in table 4. In paper **I** and **II,** 123±3g and 139±2g fish, respectively, were fed 5 mm pellets at two different temperatures, 11ºC and 6.5ºC. The fish at both temperatures were from broodstock held at Austevoll Aquaculture Research Station (Institute of Marine Research, Austevoll, Norway). The fish reared at 11ºC originated from autumn spawner, while the fish reared at 6.5ºC originated from spring spawners. These trials were carried out in indoor tanks (1700 litres) with waste feed collection, allowing feed intake data to be collected. The trial with the fish reared at 6.5ºC lasted for 20 weeks and the fish reared at 11ºC lasted for 13 weeks.

In paper **III** and **IV** the fish originated from Parisvatnet (Institute of Marine Research, Øygarden, Norway). They were transferred to Austevoll Aquaculture Research Station (Institute of Marine Research, Austevoll, Norway) and kept outside in sea cages (5×5×5 m), from June 2004 until start of the experiment in December 2004. The acclimatisation period lasted for six moths so the fish had reached the wanted weight. During this period the fish were fed a commercial diet. The fish were 1652±6 g at start of experiment and were fed 9 mm pellets. Since the trial was kept outside in sea cages, the temperature fluctuated according to season (3.4-12.9ºC). The trial lasted for 28 weeks. In this trial there was no waste feed collection. The feeding was done by experienced technicians and was based on appetite and in-house feeding tables. Indices where feed intake is a part (FCR, PPV and PER) have therefore been used but with caution.

In paper **V** juvenile Atlantic cod (6 g) produced by Sagafjord Sea Farm (Stord, Norway) was transported to Skretting ARC Fish Trial Station (Lerang, Norway)

where the trial was carried out after 2 months acclimatisation. During the acclimatisation period fish were held at 8-9ºC and fed a commercial diet (Amber Neptun, Skretting, Stavanger, Norway). The fish were 15.4±0.2g at start of experiment, and were kept in indoor tanks (100 litres) with water holding a mean temperature of 8.5ºC. The trial lasted for 15 weeks. This trial was carried out twice, and it is the second trial that is reported in paper **V**. The first time the trial was carried out extra water had to be added to the pellets (2 mm) to get the right technical properties (sinking time), resulting in a moister pellet giving difficulties collecting the waste feed. A recovery test was performed, to test the efficiency of the waste feed collection and thereby the reliability of the feed intake calculations. This was done by adding 40g feed to the tanks (without fish), and then after 3.5, 7 and 24 hours waste feed was collected, and then a formulae to find more exact how much of the feed ended as sampled waste and how large % would be lost in the system. The whole procedure was repeated three times. This test showed a recovery for the lysine diets of 65±15% (mean±SD) and only 19±5% for the methionine diets, and it was decided to design new diets with better technical properties and repeat the trial. A recovery test was performed also on the new diets, giving a recovery for the lysine diets of 80±2% and 71±18% for the methionine diets, which was considered satisfying.

Table 4. Summary of experimental conditions in all papers, given as initial fish size (mean ±SD g), pellet size (mm), temperature (°C), tank size (litres) or cage size (m), trial length (weeks) and if there was waste feed collection.

Paper	Initial fish size (g)	Pellet size (mm)	Temperature (°C)	Tank size (L)	Cage size (m)	Trial length (weeks)	Waste feed collection
I, II	123±3	5	11	1700	--	13	Yes
	139±2	5	6.5	1700	--	20	Yes
III, IV	1652±6	9	3.4-12.9	--	5x5x5	28	No
V	15.4±0.2	2	8.5	100	--	15	Yes

3.2 Diets

The diets were extruded and formulated to contain >50% protein and <18% fat. These levels were reached in all diets in all papers, except the lysine diets in paper **V**. Here the protein level was only 47%, possible due to a mistake in the production of the diets, where wheat gluten may have been replaced with wheat. This assumption is supported by the high starch levels in those diets (15%).

The plant ingredients chosen in the different trials were solvent-extracted soybean meal (paper **I** and **II**), bioprocessed soybean meal (paper **III, IV** and **V**), soy protein concentrate (paper **I, II, III, IV** and **V**), corn gluten meal (paper **I, II** and **V**) and wheat gluten meal (paper **I, II, III, IV** and **V**). Table 5 shows the inclusion of the different plant proteins in the diets used. These plant ingredients were chosen due to high protein content, required to reach the target dietary protein level (>50%). Furthermore, concentrated plant proteins hold low levels of anti-nutrients, and have shown promising results in other species (reviewed by Francis *et al*. 2001). Soybean is complimentary in lysine level with wheat and corn gluten, and corn gluten is complimentary in methionine with wheat gluten and soybean (Table 2 in M&M). Mixtures of these ingredients were therefore chosen in several diets (paper **I** and **II**). The diets in paper **I-IV** had a lysine content ranging from 2.5 to 4.1% of diet (corresponding to 4.6 to 8.0% of dietary protein) and methionine ranging from 0.9 to 1.5% of diet (corresponding to 1.6 to 2.9% of dietary protein). Those values are mostly lower than the fish meal control diet having 3.9% lysine and 1.4% methionine (corresponding to 6.5% lysine of dietary protein and 2.4% methionine of dietary protein). A practical replacement approach was applied in paper **I-IV**, implying that only limiting IAA (lysine, methionine) were added according to requirement levels described for rainbow trout (NRC, 1993). The fish fed the fish meal control diet had slightly better growth than almost all experimental diets possibly because not all IAA was balanced to mirror fish meal as adopted by Espe *et al*. (2006; 2007).

Table 5. Content of the different plant protein ingredients in the different trials, given as % of protein and % of diet. Mean dietary protein content (%) is also given.

Paper	Plant protein ingredient	Dietary protein (%)	% of protein	% of diet
I, II	Solvent-extracted soybean meal	54	4, 7, 11, 15	4, 8,12, 16
	Corn gluten meal	55	7, 15, 23, 31	6, 12, 18, 24
	Solvent-extracted soybean meal + corn gluten meal (2:3)	54	11, 22, 33, 44	10, 20, 30, 40
	Wheat gluten + soy protein concentrate (1:1)	55	32, 58	22, 44
III, IV	Wheat gluten + bioprocessed soybean meal + soy protein concentrate (25:7:18)	53	25, 50, 75, 100	17, 35, 55, 73
V Lys	Wheat gluten + corn gluten (1:1)	47	63	43
V Met	Solvent-extracted soybean meal + soy protein concentrate (3:2)	50	64	42

The trial in paper **V** was designed to evaluate if supplementing a plant based diet with lysine or methionine would be beneficial for fish performance. The lysine diets were designed to contain 2.3-3.8% dietary lysine, and the methionine diets were designed to contain 1.0-1.4% dietary methionine, which is in the lower range of the dietary levels in paper **I**, **II**, **III** and **IV**. The analysed values for lysine were 1.9-3.2% of diet (corresponding to 4.0-6.8% of dietary protein) and 0.9-1.2% of diet for methionine (corresponding to 1.8-2.5% of dietary protein), so the diets were lower in both lysine and methionine than the targeted levels. No diet dependent responses were identified in the methionine study and few in the lysine study (HSI, plasma TAG and LPV). The lack of responses indicates that at least the methionine levels were above requirement, and addition above requirement had no effects on fish performance.

3.3 Sampling

When sampling fish, they were anaesthetized with benzocain or MS222 before killed by a blow to the head. To reduce variance, samples from organs were taken from the same location of the organ each time and by the same person. Blood samples were

held on ice at the sampling site and stored at 4ºC before analysis the following day, except for Hct which was analysed within 10 minutes. The other blood parameters were analysed within three days, to avoid coagulation. All samples were frozen on dry ice and stored at -80ºC, except for samples for RNA that were immediately put in RNA-later (Ambion) (paper **II**) or flash-frozen in liquid nitrogen (paper **IV**), and stored at -20ºC and -80ºC, respectively. Both methods have shown to prevent RNA degradation, but it is found to be better to flash-freeze fish tissue in liquid nitrogen than to use RNA-later (Olsvik *et al.*, 2007). Midgut and hindgut for histological examination were transferred to 4% buffered formalin (paper **II**) or McDowell`s fixative (paper **IV**) and stored at 6ºC until further processing.

Sampling in trials reported in paper **III, IV** and **V** were performed 5 h post feeding to secure comparable results of the plasma and muscle free amino acids (Espe *et al.*, 1993; Lyndon *et al.*, 1993). Sampling in paper **I** and **II** was not timed, and this may be the reason for few correlations between dietary amino acids and muscle free amino acids (plasma free amino acids was not measured).

Measurements of apparent nutrient digestibility coefficients (ADC) were done by adding 0.1% yttrium oxide to the diets as an indigestible marker (papers **II** and **III**). ADC values after collection of faeces by stripping has shown lower values than with faeces collected from the water column, probably due to leakage of some nutrients to the water for the latter method (Vandenberg and De La Noüe, 2001). Faeces collection method (stripping and dissection) has been investigated in Atlantic cod, and no difference between the methods were found (Hemre *et al.*, 2003). Therefore faeces collection was performed by stripping.

Gut evacuation time was measured in paper **II** by following the movement of X-ray dense ballotini glass beads through the gastrointestinal (GI) tract. Fish were fed a diet added ballotini glass beads, and from each tank, the GI tract from 5-7 fish was carefully dissected out 1, 6, 12, 24, 36, 48, 60 and 72h after feeding. Each end of the

GI tract was carefully closed with a string, so that the GI content would not be lost during sampling. The Atlantic cod intestine is highly coiled and an x-ray image of a whole fish makes it difficult to divide the GI tract into different parts and to count the ballotini glass beads. X-ray image was taken of the whole fish in the trial at 11ºC, but the different parts of the intestine could not be separated and these images are not reported in paper **II**. Therefore, the GI tract was stretched and mounted on a board before x-ray images were taken in the trial performed at a water temperature of 6.5ºC. The relative distribution of ballotini glass beads in stomach + pylorus, upper, mid and lower intestine, and hind gut was visually estimated. All assessments were done blind-folded by one person.

3.4 Analytical procedures

Most of the methods used in the present studies, are accredited methods at NIFES according to ISO standard 17025, satisfying high standards regarding precision, reproducibility and uncertainty. All analytical methods have an uncertainty. It is therefore important that significant differences between groups are larger than the analytical uncertainty before considered biological important.

When analysing proximate composition of pooled samples of whole fish, homogenisation is a crucial step in the analysis. If the sample is not homogeneous, it can result in to high/low results i.e. in protein content, resulting in wrong nutrient retention results. It might be suspected that the homogenisation had not been good enough in paper **V**, giving reduced lipid retention not followed by any increased protein retention.

Amino acids in the feed were analysed on HPLC after hydrolysation with 6M hydrochloric acid and derivatisation by phenylisothiocyanat. When analysing amino acids, methionine has relatively high analytical uncertainty (18%) due to rapid oxidation. This makes it difficult to detect small differences in methionine between

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diets, like in paper **V** where a basic diet mix was added four different amounts of crystalline DL-methionine (1, 2, 3 and 4 g/kg). The basic diet was analysed to hold 0.94% dietary methionine (corresponding to 1.82% methionine of dietary protein) and the diets added crystalline methionine 0.98, 1.07, 1.12 and 1.22% dietary methionine (corresponding to 2.01, 2.18, 2.28 and 2.53% methionine of dietary protein).

Analysis of free amino acids in Atlantic cod liver was done using the same method as for the free amino acid analysis of muscle, but gave large deviations between chemical parallels. Lyndon *et al*. (1993) reported the level of free amino acids in several tissues from Atlantic cod, but not for liver because "it was regarded too fatty". Fat was therefore extracted from the liver samples with ethyl acetate, but methionine was destroyed and could not be detected in the samples. Removing fat with a hydrophilic filter (Vivaspin 500, Sartorius Stedim Biotech S.A., Aubagne, France) was also tested with no satisfying results. Free amino acids in liver tissue were therefore not considered reliable and were not reported in any of the papers.

For analysis with Q-PCR a good RNA quality is important (Fleige and Pfaffl, 2006). RNA quality was checked with a UV/VIS spectrophotometer (NanoDrop, ND-1000, NanoDrop Technologies, USA) revealing the absorption at 280, 260 and 230 nm. Nucleic acids absorb light that has a wavelength of 260 nm. Organic contaminants, e.g. trizol and other reagents used in RNA extraction, absorb light at 230 nm, and proteins at 280 nm. Samples with a low 260/280 (<1.8) and 260/230 ratio (<2.0) were considered to have a significant presence of contaminants that may interfere with PCR results. Atlantic cod liver samples often has a low 260/230 ratio, probably due to the high fat content. To solve this problem an extra step after RNA extraction was introduced, precipitating RNA from the solution with 80% ethanol. This gave satisfying 260/230 ratios and thereby increased RNA quality. Another way of validating RNA quality is to check for degradation. This was done by the Agilent 2100 Bioanalyzer (Agilent Technologies, USA) where samples are separated

electrophoretically on a micro-chip, and via laser induced fluorescence, bands of ribosomal RNA was visualized. Acceptable RNA quality is regarded when the 28S/18S rRNA ratio is between 1.8 and 2.0.

3.5 Statistical evaluations

The experimental design for all trials was regression, with dietary plant protein or amino acid as predictor (independent) variable. Regression design is regarded fairly robust, and according to Shearer (2000) replicates are not required if the number of observations are high (number of levels × replicates). In paper **I** and **II** there were 15 dietary levels of the plant protein tested with one replicate for each level of inclusion (15 observations). The data in paper **I** and **II** was also divided into different plant protein sources (soybean, corn gluten and soybean + corn gluten), then giving a lower number of observations (5), reducing the power of this analysis. In paper **III** and **IV** five levels of plant proteins (0-100% PP) were used in duplicate, giving 10 observations, and in paper **V** also five levels of lysine or methionine in duplicate were used, also giving 10 observations. This was regarded as being enough observations to give a satisfying model using the regression analysis.

4. Discussion

4.1 Plant protein affects performance in Atlantic cod

4.1.1 Feed intake

In contrast to what is seen with high plant protein inclusion in diets for Atlantic salmon (Refstie *et al.*, 1998; Espe *et al.*, 2006), Atlantic cod rather increased its feed intake (paper **I** and **III**), agreeing with findings reported also by Albrektsen *et al.* (2006) and Refstie *et al.* (2006a). The exception was for the 100% PP group where reduced appetite was found, which indicated that this inclusion level exceeded the level where the fish accepted the feed, possibly as a result of reduced palatability. In papers **I** and **III**, PP inclusions resulted in a reduced protein utilisation, measured as PPV and PER. The increased feed intake can therefore be a way to compensate for a lower protein quality or availability, as it has been found that Atlantic cod increased its feed intake to meet dietary demands, e.g. for protein and energy (Hemre *et al.*, 1989; Lekva, 2009). Further, there might be problems with the transfer from an all fish meal diet to a plant based diet, as seen in paper **III** where one of the cages given 50% PP had low feed intake in the first period of the trial, which resulted lower final weight in that cage compared with the replicate cage given the same diet.

Measurements of plasma nutrients, confirmed acceptable feed intakes in all diet groups and showing a status consistent with a fish with good nutritional status. The exception were the Atlantic cod given 100% PP (paper **III**) where all plasma nutrients were low, reflecting fish close to a food-deprivation status (Hemre *et al.*, 1993; Krogdahl *et al.*, 1999). Reduced plasma cholesterol due to increased dietary plant proteins was observed in paper **III**, and is similar to results observed also in other fish species given plant based diets (Kaushik *et al.*, 1995; Goto *et al.*, 2001; Kaushik *et*

al., 2004). Kaushik *et al.* (1995) reported reduced plasma cholesterol as dietary fish meal level decreased, and explained this as a consequence of reduced available cholesterol from the diet. Dietary cholesterol was not measured in the present trials, but it is likely that also our plant protein based diets had lower cholesterol concentration than the fish meal reference diet. However, this difference would have been even bigger if parts of the fish oil were replaced by vegetable oil. Further, fibre and ANF reduce absorption of total fat, including cholesterol, when these factors increase in the diet (Krogdahl *et al.*, 2005). Faecal excretion of steroids (bile acids) is the major pathway for elimination of cholesterol from the body. De Schrijver (1990) showed increased faecal excretion of steroids simultaneously as plasma cholesterol decreased, when feeding rats plant protein compared to casein. Further, the reductions in plasma cholesterols can have been caused by changed energy metabolism, due to differences in amino acid profile (Liaset *et al.*, 2009).

4.1.2 Nutrient digestibility

Corn gluten reduced the digestibility of fat, protein and starch (paper **II**), results which agree with findings in turbot (Regost *et al.*, 1999). On the other hand corn gluten is known to be low in fibre (calculated to be between 0.5-3.1% when corn gluten constitutes from 6-24% of diet) (paper **I**) and contain low levels of ANFs. Corn gluten is produced by fractioning shelled corn by wet milling. Following removal of germ, oil, and fibre, starch and gluten are separated by centrifuging the starch-gluten slurry. The digestibility of corn gluten protein is relatively high in fish (~ 86%) (Watanabe *et al.*, 1996; Tibbetts *et al.*, 2006; Aslaksen *et al.*, 2007; Espe *et al.*, 2007), and Pereira and Oliva-Teles (2003) found no effect on digestibility when up to 80% of the protein came from corn gluten. In Atlantic salmon, morphological changes in the stomach, mid and distal intestinal tissues were not observed when using whole corn meal, genetically modified or not, as a carbohydrate source in

fishmeal-based diets for parr (Sanden *et al.*, 2005) nor 20% corn gluten as a protein source in diets for post-smolts (Aslaksen *et al.*, 2007). Even though corn gluten is known to contain low levels of fibre and ANFs, these low levels could still have caused the reduction in digestibility.

Use of solvent-extracted soybean meal resulted in reduced fat digestibility (paper **II**), consistent with results from Førde-Skjærvik *et al.* (2006). The diets used in paper **II** had a higher calculated fibre content (3.9-8.0%) than the fish meal control diets (3.0%). The fibre fraction is shown to disturb fat micelle formation and increase viscosity of gut contents, both factors that can explain the reduced fat digestion. The reduced fat digestibility might also be linked to alcohol-soluble components from soybean, like saponines, that have shown to reduce fat digestibility in salmon (Olli and Krogdahl, 1995). The 50% and 100% PP had a total saponine concentration of 0.42 and 0.86 mg/g (dry weight) respectively, and was not digested (paper **IV**). The mixture of plant proteins in paper **IV** (wheat gluten, bioprosessed soybean meal and soy protein concentrate) did not affect macronutrient ADC results up to 75% PP, but there was a marked drop in dry matter and starch ADC of 12 and 10% respectively, from 75% PP to 100% PP. The soybean ingredients used were highly refined ingredients where the content of ANFs was reduced; in addition, wheat gluten is known to be low in ANFs. Undesirable levels of these components were therefore probably not reached until PP exceeded 75%, consisted with intestinal damages observed in the 100% PP group. In the GI tract you find a high number of bacteria (paper **II**)(Seppola *et al.*, 2006), and Ringø *et al.* (2006) found that the microflora changed in the intestine of Atlantic cod fed soybean meal as compared to fish fed the fish meal diets. So the reduced dry matter and starch digestibility observed may also be a consequence of changed intestinal microflora. However the intestinal microflora was not analysed in the present study.

4.1.3 Gut passage time

The optimal feeding frequency of Atlantic cod has been under debate (Rosenlund *et al.*, 2004; Refstie and Åsgård, 2006). In planning of the feeding frequency of Atlantic cod, data showing at which time after the last feeding the GI tract was ready for a new meal would help. This can be performed e.g. by measuring stomach emptying and then register passage time through the GI tract, being aware of the numerous factors influencing gut evacuation. Gut evacuation has been found to vary according to temperature, meal size, particle size, previous nutritional history, fish size, feeding frequency and feed composition (Flowerdew and Grove, 1979; Talbot *et al.*, 1984; Storebakken, 1985; He and Wurtsbaugh, 1993; Sveier *et al.*, 1999; Boyce *et al.*, 2000). In our study, especially the variable starch fraction, in addition to varying fibre quality between plant protein ingredients, was expected to highly influence gut evacuation, as found with the use of soybean in diets for Atlantic salmon (Storebakken *et al.*, 1999). However, no differences in total gut evacuation time were detected, irrespective of amount and type of plant protein ingredient added to the diet (paper **II**). The pattern of stomach evacuation seemed to vary with dietary plant protein type and amount, but the total time lag in the intestine (after stomach, and before hind gut), where the major part of digestion and absorption takes place, was equal in all fish fed the test diets. This indicates that the differences in macronutrient digestibility observed were not related to differences in gut passage time. This means that feeding frequency can be planned equally independent of the diet being based on pure fish meal, or when 58% of the protein is PP. The total gut evacuation of 72 h, and especially that stomach still held about 50% of the diet after 24 h, indicates that there can be expected no gain if the Atlantic cod is fed more often than once every 24 h at low water temperature (6.5°C).

4.1.4 Growth

The overall growth rates in papers **I, II, III** and **V** was good and in accordance with initial fish size (Björnsson *et al.*, 2007). In our studies the dietary effects on growth, were dependent on the plant ingredients chosen and the inclusion level of plant proteins. Atlantic cod held at 11ºC showed a linearly reduced SGR when 4-15% of protein was solvent-extracted soybean meal, while a mixture of 4-15% solvent-extracted soybean plus 7-31% corn gluten meal resulted in even larger growth depression (paper **I**). This was probably caused by the higher inclusion level. On the other hand using the same diets, but keeping Atlantic cod at 6.5ºC, resulted in no growth differences (Figure 5). Paper **I** has to be considered as a screening of different plant proteins, as a minimum design regarding statistical power was used. Previously solvent-extracted soybean meal alone at concentrations up to 24% of protein has shown no effects on growth in Atlantic cod in sea pens at 7.0-13.7 °C (Refstie *et al.*, 2006a), and the reduced growth in paper **I** at 11ºC is therefore probably influenced by other factors than diet alone. This may be due to Atlantic cod, at this size, thriving better at 6.5ºC than at 11ºC (Björnsson and Steinarsson, 2002). Two different fish groups were used in the two experiments and the quality of the fish may have been different, indicated by higher mortality at 11ºC (9.7%) than at 6.5 ºC (2.8%). However, at 11 ºC the combination of wheat gluten and soy protein concentrate (58% of protein, 22SC/22WG) resulted in the same growth as the fish meal control diet (FM), while all other diets groups grew less than the FM diet.
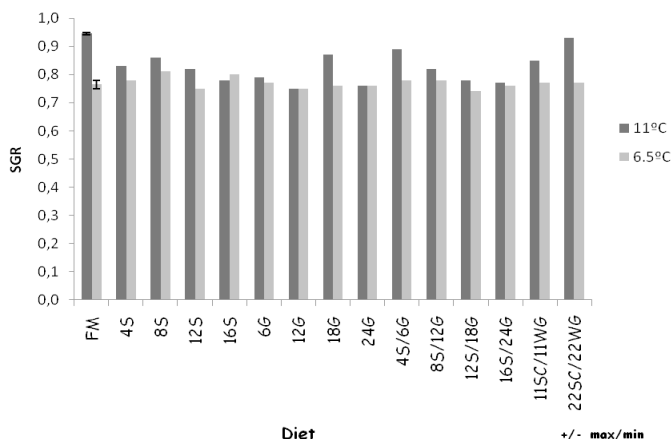
Figure 5. Specific growth rate (SGR) for Atlantic cod fed a fish meal reference diet (FM), increasing level of soybean meal (S), corn gluten (G), a mixture of soybean meal and corn gluten (S/G) or a mixture of soy protein concentrate and wheat gluten (SC/WG), at 11ºC (■) or 6.5ºC (□).

Soy protein concentrate has shown no negative effect on growth in rainbow trout, when supplemented with crystalline methionine (Kaushik *et al.*, 1995). Wheat gluten have also shown promising results in mixtures with other plant proteins in diets for turbot (Fournier *et al.*, 2004). Extreme use of plant proteins (paper **III**), using a regression design from all FM to all PP (50% wheat gluten + 14% bio-processed soybean meal + 36% soy protein concentrate) reduced growth at plant inclusions of 50% and higher, with a mean reduction in SGR of 16% and 43% when given 75% PP and 100% PP. The ingredients used in paper **III**, were chosen based on the good results with soy protein concentrate and wheat gluten in paper **I**, and results from Refstie *et al*. (2006a), showing no effect on growth with use of 24% bio-processed soybean meal. Corn gluten meal was not used in paper **III**, due to yellow skin colour when using this ingredient (paper **I**) (Figure 6). Corn consist the natural pigment zeaxathin, which can be stored in the skin, and to some extent in muscle, and can give an unwanted yellowish colour (Matsuno and Katsuyama, 1982; Li *et al.*, 2007).

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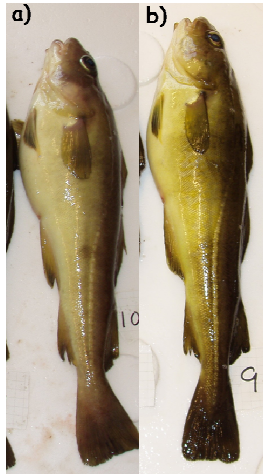
Figure 6. Atlantic cod fed diet without (a) and with (b) corn gluten for 13 weeks. Photo: Grethe Rosenlund.

In paper **III** all diet groups had lower growth in the cold period (Figure 7a), and more pronounced in groups given plant proteins; in the 100PP fed fish group SGR dropped 47% from period 1 (Dec.-Feb., 8ºC) to period 2 (Feb.-April, 5ºC) (Figure 7b). Larger fish has higher optimal temperature than smaller fish (Björnsson *et al.*, 2007), and 8ºC was closer to optimal than 5ºC. Full-fat soybean meal was not used in any of the diets in any of the papers (**I, II, III, IV** or **V**), and it is with the use of full-fat soybean the most pronounced adverse effects are found in Atlantic cod (von der Decken and Lied, 1993; Karalazos *et al.*, 2007). Parts of the referred results might be linked to reduced feed intake, maybe as a consequence of reduced palatability when including full-fat soybean.
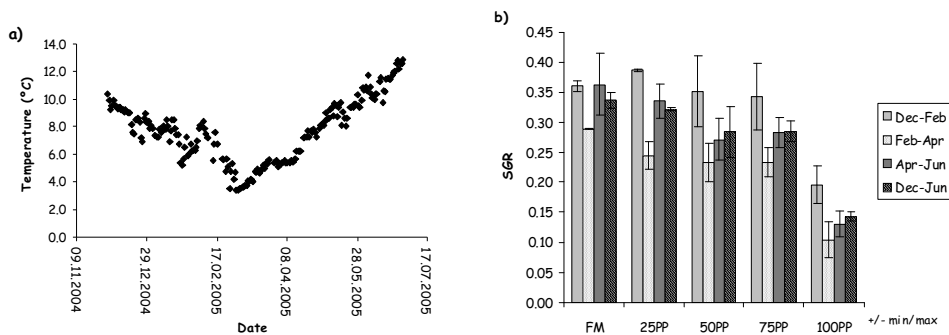
*Figure 7. a) Temperature (°C) in out-door sea cages from 1ˢᵗ December 2003 to 15ᵗʰ June 2004 (paper **III**). b) Specific growth rate (SGR) for Atlantic cod fed a fish meal reference diet (FM) and 25, 50, 75 and 100% plant protein (25PP-100PP) from December to February (mean temperature 8°C), from February to April (mean temperature 5°C), from April to June (mean temperature 9°C) and the whole trial from December to June (paper **III**).*

4.1.5 Protein retention

Reduced protein retention, measured as PER and PPV, can be linked to imbalance in the IAA profile of plant proteins. No attempts (paper **I, II, III** and **IV**) were made to balance the amino acid profiles in the experimental diets beyond supplementation of limiting amino acids (lysine and methionine), so the amino acid content in the experimental diets varied significantly from the fish meal reference diet. This is illustrated in figure 8, showing the amino acid profile of the diets when using different plant proteins (paper **I**). The soybean diets grouped near the fish meal reference diet, only differing slightly in methionine. The diets containing corn gluten, both alone and in mixture with soybean meal, gradually moved away from FM with increased inclusion, this because most of the amino acid concentrations in corn gluten differ from FM. Corn gluten was especially low in lysine and high in proline and leucine. The mixture of soy protein concentrate and wheat gluten differed from all of the other diets, being especially low in lysine, methionine and taurine and high in proline and glutamic acid.
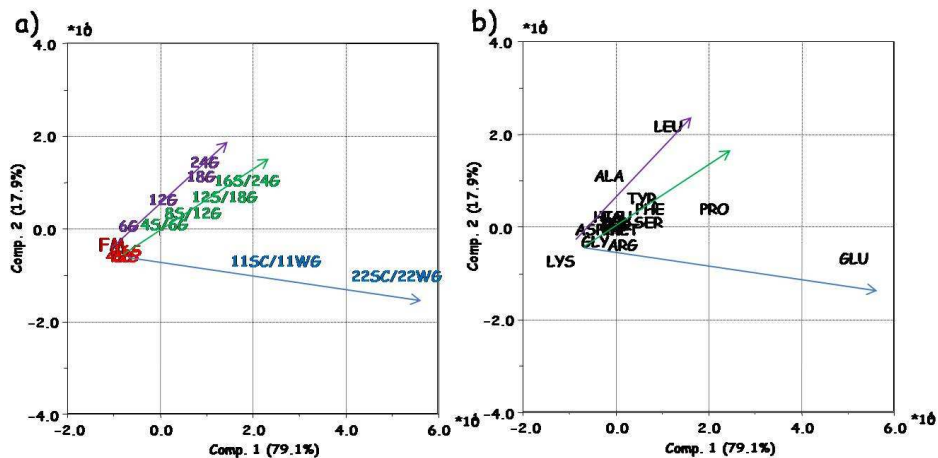
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*Figure 8. PCA scores plot showing the diets (a) and dietary amino acids (b) reported in paper **I**. The fish meal control diet are marked with brown (FM), the corn gluten diets with purple (G), the soybean diets with red (S), the diets with soybean plus corn gluten in green (S/G) and the diets with soy protein concentrate plus wheat gluten in blue (SC/WG). The arrows show the direction the different diets moves from the fish meal reference diet.*

Espe *et al.* (2007) showed that 95% replacement of fish meal with plant protein could be used without compromising growth in diets for Atlantic salmon, provided that the amino acid profile mimicked that of fish meal based control diet and that the feed intake was maintained. This indicates that differences in amino acid profiles may explain the reduction in growth when exceeding 50% PP (paper **III**). In the PP diets methionine was the IAA with the lowest concentration compared to FM, and this was highly reflected in plasma and muscle free methionine concentrations, which significantly correlated with dietary methionine level. At 75% and 100% PP free methionine was not detected in the muscle free pool, showing that the free amino acid pool was emptied of methionine. Further, a decrease in muscle free lysine of 67% was seen when comparing the fish meal group and the 100PP group, correlating well with dietary levels of this amino acid. Thus, methionine and lysine seemed to be limiting amino acids in Atlantic cod diets when these were based on soybean and wheat gluten. In paper **V**, lysine or methionine were added to diets containing 65% PP, however the addition showed no effect on total growth, feed intake or protein retention. This indicates that plant protein based diets for Atlantic cod did not need

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to be added lysine or methionine to maintain total growth, when containing over 4.0% lysine of protein and 1.8% methionine of protein. There was a decrease in lipid retention (LPV) as lysine intake increased, which was not followed by an increase in protein retention (PPV). The IAA/DAA ratio in paper **III** decreased linearly with increased inclusion of plant protein. In pigs it is shown that reduced IAA/DAA ratio reduces the amino acid utilisation and increases the urine production (Lenis *et al.*, 1999). There was a 48% increase in plasma ammonia in fish fed the 100% PP diet compared to fish fed the FM diet. This indicates increased amino acid catabolism, as ammonia in fish is the end product from amino acid catabolism contributing to 60-90% of the nitrogen excreted (Cowey and Walton, 1988).

Vitamin B_6 is an essential co-factor for the enzymes ASAT and ALAT involved in transamination of the amino acids alanine and aspartic acid. The dietary concentration of vitamin B_6 was the same in all diet groups (paper **I** and **III**), as was also the vitamin B_6 concentration in liver (paper **III**) and muscle (results not shown) from all diet groups. However, the liver ASAT activity decreased and there was a tendency to lower liver ALAT activity in fish given diets containing plant proteins compared to the FM control (paper **III**). This indicates a reduction in substrates available for transamination, as a consequence of reduced dietary levels of these amino acids, and not an insufficiency of this vitamin.

4.1.6 Energy metabolism

Imbalanced dietary amino acid profiles influence the energy metabolism in all animals, fish included. In salmonids, increases in whole body lipid content with the use of dietary plant proteins has been reported by several authors (Adelizi *et al.*, 1998; Kaushik *et al.*, 2004). Vilhelmsson *et al*. (2004) reported an up-regulation of several proteins involved in energy metabolism in rainbow trout liver when fed 100% PP compared to 100% FM. However, in paper **III** no increases in lipid deposits were

registered, and almost identical proximate compositions of whole body, liver and muscle were reported in all diet groups, with all values being in the same range as in earlier studies with Atlantic cod fed only fish meal as protein (Rosenlund *et al.*, 2004). On the other hand, in paper **V,** an increase in lysine level did affect lipid storage; reducing LPV, HSI and plasma TAG as lysine intake increased. This is in agreement with findings in several fish species (Rodehutscord *et al.*, 1997; Marcouli *et al.*, 2006; Espe *et al.*, 2007) and mammals (Tanphaichitr *et al.*, 1976; Witte *et al.*, 2000), and can be linked to the role of lysine as a precursor in the biosynthesis of carnitine (Tanphaichitr *et al.*, 1971; Harpaz, 2005). Carnitine is involved in the transport of fatty acids through the outer mitochondria membrane. Imbalanced amino acid profile will also result in excess free amino acids that can not be used in protein synthesis, as all amino acids needed for protein synthesis must be present at the same time and in correct proportions (Geiger, 1947). Excess amino acids can not be stored in the body and are therefore transdeaminated, and used either directly for energy or stored as fat (El-Mowafi *et al.*, 2009). The increased liver size can therefore be explained by amino acids being used for *de novo* fat synthesis or burned for energy "saving" fat for storage, due to imbalances and suboptimal timing of amino acids in the free amino acid pool. A 48% increase in plasma ammonia in fish fed 100% PP compared to 100% FM was observed in paper **III**. Ammonia is the major end product from amino acid transdeamination contributing to 60 to 90% of the nitrogen excreted (Cowey and Walton, 1988). Therefore increased plasma ammonia concentration can be an indicator of increased amino acid transdeamination.

4.1.7 Ash content

The total mineral content of fish meal is higher than in plant ingredients, and the two minerals with highest concentration in fish meal are phosphorus and calcium (Bell and Waagbø, 2008). The phosphorus level in soybean is mostly in the form of phytate which is less bioavailable and is regarded an ANF (Bell and Waagbø, 2008). In

paper **III** ash content decreased from 10% in the FM diet to 5% in the 100% PP diet. High ash levels (18%) from fish bone and crab by-products seems to be beneficial for Atlantic cod, increasing growth with 10% compared to a fish meal reference diet (Toppe *et al.*, 2005). All diets were added a mineral premix, and the diets with the highest PP inclusions were added mono-sodium-phosphate (papers **I** and **III**). Although, the phosphorus and calcium levels of the diets were not measured, the lowered ash levels, and thereby mineral content, may be one of the explanatory factors for the reduced growth.

4.2 Impact of plant proteins on fish health

4.2.1 Clinical markers

The leakage of ASAT and ALAT into plasma were low and comparable to the reference group values, and indicate no organ damage or dysfunction (Racicot *et al.*, 1975). This agrees with previous reports from Atlantic salmon fed 12-17% full-fat soybean meal (Sanden *et al.*, 2006). No detectable levels of plasma lysozyme further confirm no problem with inflammation due to dietary plant proteins (paper **I**).

Blood haematology (Hct, Hb and RBC values) was within normal ranges (Lie *et al.*, 1990) and in agreement with previous reported levels for Atlantic cod (Rosenlund *et al.*, 2004). One of the few effects observed, was a significant reduction of cell size, measured as MCV, as the content of plant proteins increased (paper **I** and **IV**). This has also been observed in some groups of Atlantic salmon fed soybean products (Hemre *et al.*, 2005). As this observation appeared to coincide with increased spleen size, it was suggested that some of the plant ingredients may cause early release of immature erythrocytes. This was not the case in paper **IV** where spleen was of similar size regardless of the diet offered to the fish.

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Increasing plant protein inclusion from 0 to 100% (paper **III**) decreased the dietary level of vitamin B_{12} below what is described as the requirement for salmonids (Woodward, 1994). The 100% PP diet (0.01 mg B_{12}/kg diet) might therefore have been below requirement of vitamin B_{12}, pointing to a need to supply vitamin B_{12} in sustainable Atlantic cod diets based on high levels of plant proteins. Typical vitamin B_{12} deficiency signs such as low blood haemoglobin levels (Waagbø, 1999) were, however, not seen, even in the 100% PP group.

4.2.2 Gut morphology

Some effects on the gut morphology were induced by the diets (paper **IV**) (Figure 9), but the alterations were moderate and involved mostly goblet cells. The incidences of cellular alterations in the gut tended to increase from 1 in every 5 fish in the 25% PP group to around 2 in every 5 fish in the 50% PP and 75% PP groups. The condition would still be considered mild, confirmed by no reductions in nutrient digestibility. Even at 100% PP, most fish were only mildly affected, except for two individuals where elements of a classical enteritis-like condition described in salmonids occurred (van den Ingh *et al.*, 1991; Baeverfjord and Krogdahl, 1996; van den Ingh *et al.*, 1996). In paper **III** and **IV** the main ingredients were bioprocessed soybean meal, soy protein concentrate and wheat gluten, which are highly refined products low in ANFs. Bioprocessed soybean meal has a higher digestibility than ordinary soybean meal due to significantly reduced levels of trypsin inhibitors, indigestible oligosaccharides and has been treated with the phytate lowering enzymes phytase (www.hamletprotein.com). One would therefore not expect to find significant effects of diets with bioprocessed soybean meal in Atlantic cod GI tract. This is supported by Refstie *et al.* (2006b) where an inclusion of 24% bioprocessed soybean meal did not cause any alterations in intestinal morphology.

In salmonids, soybean-induced gut damage are usually related to distal parts of the GI tract affecting cell types with extensive endocytotic activity and high levels of intracellular vacuoles. Further, for salmonids, the proximal part of the gut, where most nutrients are being absorbed, contains different cell types distinguishable from distal cells in not having endocytotic activity and intracellular vacuoles. The only significant effect of soybean diets reported for this region is goblet cell hypertrophy and hyperplasia (van den Ingh *et al.*, 1991). Atlantic cod does not have the same differentiation of cell types (Odense and Bishop, 1966), and most of the intestine contains cells that do not appear to be endocytotic. The different structure of Atlantic cod GI tract compared to Atlantic salmon may have implications for regional sensitivity to dietary ANFs. However, some responsiveness to the diet when passing through the GI-tract from proximal to distal parts was observed. This raises the possibility that some of the effects observed in distal parts of both Atlantic cod and Atlantic salmon GI tract, are related to an up-concentration of ANFs in the undigested digesta, eventually reaching near toxic levels.

As the incidences of enteritis-like conditions were relatively few (paper **IV**), it is also possible that the observed changes in goblet cells are not directly related to this condition. All plant ingredients contain significant amount of fibres, seen in paper **IV** as an increase in "rest" (mainly fibre) from 1% in the FM reference diet to 10% in the 100% PP diet. In mammals, these compounds tend to increase intestinal size in general, and goblet cell volume and numbers in particular (Lundin *et al.*, 1993). Although, no effect of diet on intestinal weight were observed (paper **IV**), previous studies have observed increases in gut weight in Atlantic cod fed soybean meal diets (Refstie *et al.*, 2006a).
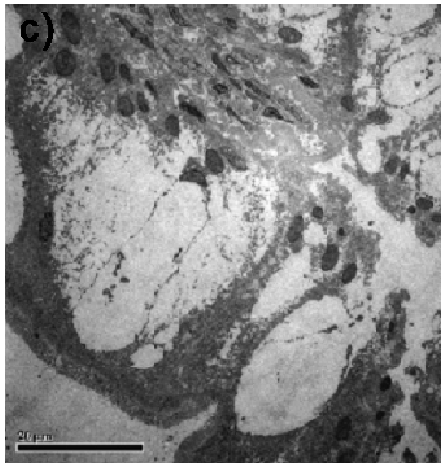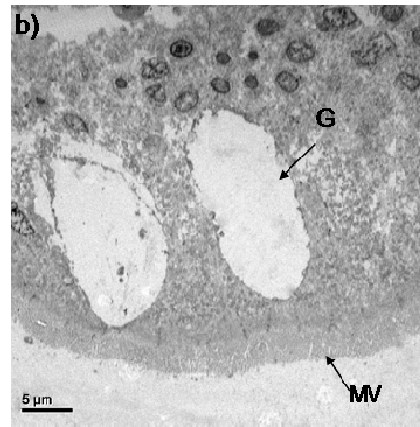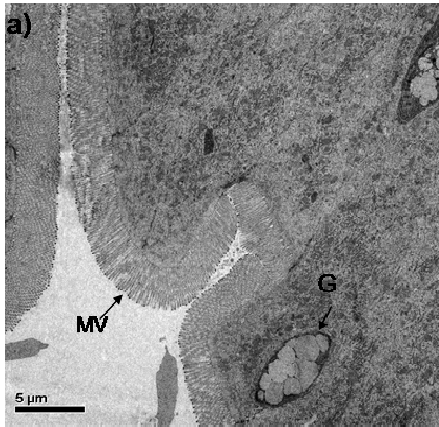
*Figure 9. Histological image of hind gut of Atlantic cod, showing increased number and size of goblet cells (G) and reduced height microvillies (MV) from fish given a 100% PP diet (b) compared to a fish meal reference diet (a) and necrotic tissue from fish fed 100% PP (c) (paper **IV**). Photo: Rolf Erik Olsen.*

4.2.3 Heat shock proteins

Heat shock proteins (HSPs) protect and maintain cell integrity and are expressed when the animal is subjected to various kinds of stress, including hyperthermia, transisted anorexia and nutritional changes, e.g. due to dietary ANFs exposure (Tsukimi and Okabe, 2001; David *et al.*, 2002; Sagstad *et al.*, 2007). A variety of stress proteins have been studied in fish and have shown to be good biomarker proteins of stress induction and environmental pollution (Grosvik and Goksoyr, 1996; Lewis *et al.*, 1999; Ahmad *et al.*, 2000). HSP70 is found to respond to suboptimal

diet composition (Martin *et al.*, 2003; Hemre *et al.*, 2004a), and was a natural choice of an early stress biomarker after feeding Atlantic cod diets with high levels of plant ingredients. In paper **II** no regulation of the transcription levels of HSP70 or HSP90 in liver was detected, which indicates no diet-induced stress response at the sampling time (Iwama *et al.*, 2004). There was an up-regulation of HSP70 in mid-intestine from Atlantic cod fed 100% PP (paper **IV**), indicating a stress response in intestine which also correlated with the histological findings. This was also found in hindgut in Atlantic salmon fed diets with soybean (Sagstad *et al.*, 2007).

5. Conclusions

There is a high potential for safe use of plant proteins in diets for Atlantic cod without challenging performance, provided that the plant ingredients are of high quality.

Effects on growth:

- Solvent extracted soybean meal can at least replace 15% and corn gluten meal at least 31% of the FM protein, without adverse effect on the growth performance. When using a mixture of these two ingredients up to 44% of the protein, a gradual reduction in growth was observed. Corn gluten is not recommended as it leads to reduced nutrient digestibility and coloured the skin yellow.
- Mixture of soybean concentrate and wheat gluten (1:1) can replace up to 58% of the FM protein without resulting in reduced growth in Atlantic cod at a fish size of 140g.
- Mixture of soy protein concentrate, bioprosessed soybean and wheat gluten meal resulted in reduced growth in Atlantic cod with initial weight of 1.5kg gradually up to 75% replacement of FM protein, and dramatically when 100% of the fish meal was replaced.

Effects on feed and protein utilisation:

- Inclusion of plant protein increased the feed conversion ratio (FCR), this could partly be explained by increased mean feed intake possibly as a reaction to the reduced protein retention, measured as PER and PPV. Dietary levels of amino acids were reflected in plasma and muscle free amino acid pools. Especially the level of free methionine was low, and was not detectable in muscle tissue when replacing 75 and 100% of FM protein with plant protein. This indicates that methionine concentration was limiting for maximum protein retention.

Effects on nutrient digestibility:

- A significant lowering of ADC was registered for fat, protein and starch as a consequence of increased corn gluten in diets. No such effects were seen with use of any of the other plant ingredients or blends, except for the fish fed 100% replacement of fish meal, which showed a drop in dry matter and starch digestibility.

Effects on clinical health parameters:

- Low mortality and unaffected haematological values indicated acceptable fish health in all groups in all experiments. Low plasma levels of ASAT and ALAT indicated no liver or kidney dysfunction.

- The measured plasma nutrient levels gave no indications of stress, but increasing plant protein inclusion decreased plasma cholesterol. This can be related to high fibre content or reduced dietary intake of cholesterol. 100% FM replacement gave plasma nutrient values close to starvation, indicating poor feed intake.

Effects on gut health:

- No severe gut damage was seen, except when 100% of fish meal was replaced by plant proteins (protein concentrate, bioprosessed soybean and wheat gluten meal).

Effects on gut passage time:

- Total gut passage time was not effected by plant protein inclusion. The total gut evacuation time at 6.5 ºC of 72h, and especially that stomach still held about 50% of the diet after 24h, indicated that there was no gain when fed more often than once every day.

Effects of adding lysine or methionine:

- Adding lysine above 1.9% of diet (corresponding to 4.0% of protein) or methionine above 0.9% of diet (corresponding to 1.8% of protein) did not improve growth performance. Increased lysine intake reduced lipid storage, HSI, lipid retention and plasma TAG concentration.

6. Future perspectives

6.1.1 How to improve the growth rate when using plant proteins?

The trials in this thesis have shown good opportunities to replace fish meal with plant protein sources in diets for Atlantic cod. Wheat gluten and soy protein concentrate were regarded the best choices of plant proteins, showing no reduction in growth when replacing 58% of the fish meal protein. Although, this result has been difficult to replicate, showing reduced growth when approaching 50% replacement with a mixture of wheat gluten, soy protein concentrate and bioprosessed soybean meal for Atlantic cod with body weight over 1.5 kg. Supplementing diets with crystalline lysine or methionine did not improve the performance which reveals that there is more to plant proteins than protein content. Also the balance between different amino acids is important for growth performance. So for the future balancing all the IAA can be a way to go, and have been promising in trials with Atlantic salmon. When removing fish meal, not only protein and amino acids are removed. Fish meal is also rich in other nitrogen holding compounds and to identifying these and potentially positive effects, can be a way to go. Another observation is that the ash content of plant protein diets is low compared to a fish meal diet. Further research regarding the ash fraction is therefore necessary, to reveal if it is one mineral, several minerals and/or combined effects of lack of minerals/increases in antinutrients that gives the reduced performance.

6.1.2 Can Atlantic cod farming be sustainable?

Sustainable fish farming includes the use of diets formulated using economical, suitable and ethical acceptable feed ingredients. There is increasing public interest in sustainable fish farming and how much wild fish is used in feed for farmed fish, where the criticism has been that the wild fish, used to produce fish meal, could be

used for direct human consumption. In experiments with Atlantic salmon where 80% of the FM and 70% of the fish oil was replaced by plant sources, 1 kg salmon could be produced by only using 1.20kg wild fish (Torstensen *et al.*, 2008). With the diets giving best results tested in the present papers (diet 22SC/22WG and 75PP) around 1.5kg wild fish could be used to produce 1 kg Atlantic cod (Figure 10). 100PP gives the same usage of wild fish as FM, due to poor growth and protein retention. When only focusing the protein part, 3.6 kg protein was needed to produce 1 kg Atlantic cod protein, where as 0.9 kg came from FM and 2.7 from PP (Figure 11). Atlantic cod farming has a potential to be a net producer of fish protein, this provides that the growth rate when including high levels of plant protein is improved, and parts of the fish oil is replaced by plant oil.
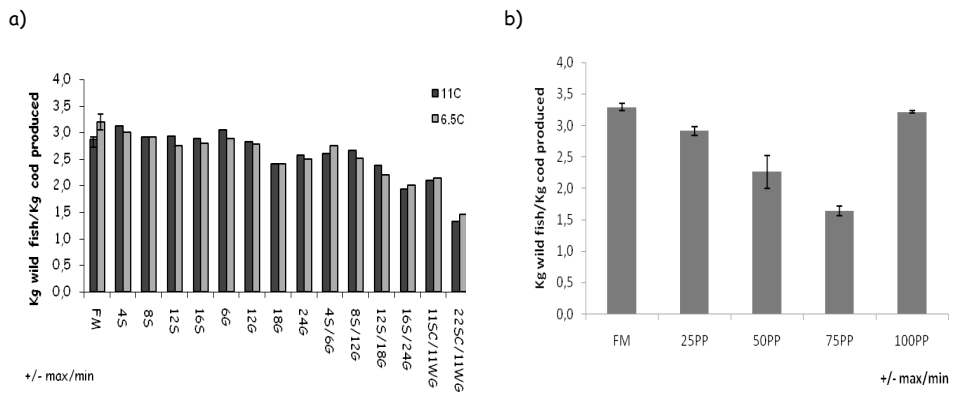
*Figure 10. The figure presents how much wild fish is used to produced 1 kg of farmed Atlantic cod in paper **I** (a) and paper **III** (b).*

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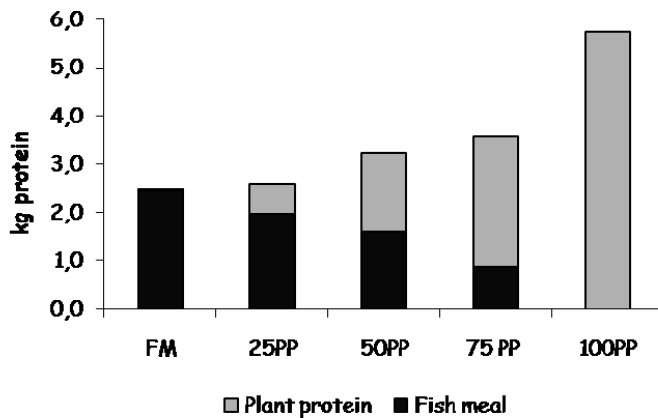
Figure 11. The figure shows how much protein that is used to produce 1 kg Atlantic cod protein when fed diets containing only fish meal (FM) as protein source and from 25 to 100% plant protein (25PP, 50PP, 75PP and 100PP). The black bars shows how much of the protein that comes from fish meal and the grey bar how much that comes from plant protein. The calculation is based on protein productive value (PPV).

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