The inclusion of plant protein in cod diets, its effects on macronutrient digestibility, gut and liver histology and heat shock protein transcription

Ann-Cecilie Hansen1, Grethe Rosenlund2, Orjan Karlsen3, Pål A Olsvik1 & Gro-Ingunn Hemre1
1NIFES, National Institute of Nutrition and Seafood Research, Nordnes, Bergen, Norway
2Nutreco ARC, Stavanger, Norway
3Institute of Marine Research, Austevoll, Storebø, Norway

Abstract
This paper reports on the use of plant protein in cod diets, and where corn gluten meal, soybean meal, a mixture of these, or a mixture of wheat gluten meal and soy protein concentrate, substituted fish meal in a regression design up to 440 g kg$^{-1}$ plant ingredients. Feeding lasted for a period of 20 weeks. High growth rates were obtained, and fish were able to maintain growth in all groups by increasing feed intake when plant proteins exerted high amounts of the protein fraction. This was confirmed by increased feed conversion ratio (FCR) values. The apparent digestibility measured by means of faecal stripping, showed high apparent digestibility coefficients (ADC) for fat, starch and protein. Small decreases in protein ADC and larger decreases in fat ADC were observed with high levels of plant protein ingredients. No histopathological changes were found, neither in liver nor in the different sections of the gastrointestinal (GI) tract, for any of the diet groups. Expression of stress genes (heat shock protein 70 and 90 (HSP70 and HSP90)) in liver showed no response to high levels of plant protein. Invasion of gut-bacteria in the distal part of the GI tract was substantial, but independent of diet level of plant ingredients. Gut evacuation analysis showed that the time for a meal to pass through the stomach and the GI tract was more than 72 h, with no variation dependent on diet plant protein level.

The major conclusion is that cod shows a high tolerance to the plant protein sources investigated in this experiment, and consequently that Atlantic cod safely can be fed diets holding up to 440 g kg$^{-1}$ of the present investigated plant protein ingredients without any adverse effects on intestinal or liver function. There seems to be no gain if feeding frequency exceeds more than one large meal every 24 h at 6–7°C.

Keywords: Atlantic cod, alternative plant proteins, digestibility, stress gene expression and gut histology

Introduction
The demand for feed in the aquaculture industry of carnivorous species has increased over recent years in parallel with increases in total production (Watanabe 2002), simultaneously as traditional marine resources are exploited to the highest possible level (FAO 2002). The most viable alternatives to fish meal and -oil are plant protein and oil resources. Although data suggest that these can be included into fish diets at relatively high levels, there is still some concern that they may compromise fish welfare. Plant ingredients contain varying levels of anti-nutritional factors, such as protease inhibitors, lectins, antigenic proteins, phenolic compounds, oligosaccharides and phytates (Davies & Morris 1997; Francis, Makkar & Becker 2001). These anti-nutritional factors are known to affect performance of salmonid fish, altering gut histology (van den Ingh, Krogdahl, Olli, Hendriks & Koninkx 1991; Sanden 2004), inducing inflammations (Baeverfjord & Krogdahl 1996; Bakke-Mckellep, McI Press, Baeverfjord, Krogdahl & Landsverk 2000), leading to...
Plant proteins in cod diets  A-C Hansen et al.

Aquaculture Research, 2006, 37, 773–784

decreased digestion (Kaushik, Cravedi, Sumpfer, Lauconneau & Larroche 1995; Refstie, Korssen, Storebakken, Baeverfjord, Lein & Roem 2000; Krogdahl, Bakke-McKellep & Baeverfjord 2003) and reduced utilization of proteins (Krogdahl, Lea & Olli 1994; Vielma, Makinen, Ekholm & Koskela 2000). No data on this matter have so far been published on cod.

Heat shock proteins (HSPs) are highly conserved cellular proteins that exist in all kinds of organisms, including fish (Iwama, Thomas, Forsyth & Vijayan 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 also shown as a response to a variety of stressors, both biotic and abiotic (Basu, Todgham, Ackerman, Bibeau, Nakano, Schulte & Iwama 2002). Also dietary factors like fatty acids (Samples, Pool & Lum 1999) and vitamin A (Hemre, Deng, Wilson & Berntssen 2004) have shown to affect the HSP expression. In rainbow trout several changes in gene expression of the HSPs in liver were identified when fed diets with soybean meal (Martin, Vilhelms-son, Médale, Watt, Kaushik & Houlihan 2003; Vilhelmsson, Martin, Médale, Kaushik & Houlihan 2004).

Both moderate and high levels of plant protein in diets for different fish species have resulted in altered gut functionality, e.g. it is indicated that gut passage time increases when soy protein is included in salmonid diets (Storebakken, Kven, Shearer, Grisdale-Helland & Helland 1999) and diets for Catla (Catla catla, Hamilton) fingerlings (Naik & Annappaswamy 2000). Increased passage time can affect feeding frequency in a negative manner (López-Urtúa & Acuna 1999; Boyce, Murray & Peck 2000; Naik & Annappaswamy 2000), and knowledge of this may help in diet planning and feeding.

The aim of the present study was to elucidate how the use of plant protein in diets for Atlantic cod influenced macronutrient digestibility, gut evacuation and fish health. Protein ingredients evaluated were corn gluten meal, soybean meal, a mixture of these and a mixture of wheat gluten meal and soy protein concentrate. Measurements of macronutrient digestibility, gut evacuation, liver and gut histology and liver HSP70 and HSP90 expressions were undertaken and are discussed in relation to macronutrient digestibility and fish health when using plant protein ingredients in cod feeds.

Materials and methods

Fish experiment and design

Atlantic cod were fed 15 different dry extruded diets, produced as 7 and 9 mm pellets by Nutreco Technology Centre (Stavanger, Norway). The design was a regression design with one control fish meal (LT quality) group tested in duplicate, and four increasing levels (4–16%) of solvent-extracted soybean meal (named S together with a number that indicates the level of inclusion) and four increasing levels (6–24%) of corn gluten meal (named G together with a number that indicates the level of inclusion). The increasing levels of plant protein replaced LT fish meal on a protein basis. There were also four diets with a mixture of solvent-extracted soybean meal and corn gluten meal (named S/G together with a number that indicates the level of inclusion). Two diets with a mixture of soy protein concentrate (SC) and wheat gluten (WG) were also included. Diet composition is given in Table 1 (to be published in A.-C. Hansen, G. Rosen-lund, Ø. Karlsen, M. Rimbach & G.I. Hemre, accepted for publication, 2006). The composition of all diets was similar with 53–56% protein, 14–16% lipid and 9–15% starch (impossible to avoid variable starch due to ingredient composition), vitamins and minerals according to NRC (1993). DL-methionine was added to meet requirement levels (NRC 1993) when needed. The diets with the lowest levels of fish meal were added mono-calcium-phosphate (MCP). To all diets yttrium oxide was added as inert marker for measurements of apparent digestibility coefficients (ADC) of protein, fat and starch.

Atlantic cod with an initial body weight of 139 ± 2 g were randomly distributed into 16 tanks (100 in each). The tanks, diameter 1.5 m and water depth 1 m, were supplied with running sea water (40 L min⁻¹) and exposed to light continuously. The fish were fed using automatic disc feeders (Storvik AS, Sunndalsora, Norway) over a period of 1.5 h every day and mean water temperature was 6.5 °C. To a batch of each diet was added 1% X-ray dense ballotini glass beads (British Optical, Aldridge, UK) for investigation of gut evacuation. The ballotini feed was offered in one single meal without pre- and post-feeding starvation (Talbot & Higgins 1983; Talbot, Higgins & Shanks 1984).

Sampling

After a feeding period of 20 weeks, faecal matter was stripped from 10 fish using the stripping method
described by Hemre, Karlsen, Mangor-Jensen and Rosenlund (2003). The faeces samples were pooled, homogenized and kept frozen at −20 °C until analyses were performed. Samples from proximal and distal intestine and liver were taken from 5 fish-tanks, and preserved in 4% buffered formalin for histological examinations. Furthermore, about 100 mg of liver tissue from three additional fish from the two fish meal groups (FM), the 16% solvent-extracted soybean group (16S), the 24% corn-gluten group (24G) and the 16% solvent-extracted soybean plus 24% corn-gluten group (16S/24G), were sampled to obtain RNA for HSP70 and HSP90 transcription analysis. The tissue samples were immediately immersed in RNA−later (Ambion, Austin, TX, USA), held on ice and thereafter stored at −20 °C until RNA extraction.

Five to seven fish from each diet group were killed with an overdose of benzocain (Sigma-Aldrich, St. Louis, MO, USA) 1, 6, 12, 24, 36, 48, 60 and 72 h after feeding the ballotini feed, for determination of gut evacuation. The gastrointestinal (GI) tract was removed from the fish carefully after each end of the GI tract was closed with a string, and immediately frozen and stored at −20 °C before further processing.

Table 1: Feed ingredients given as g kg−1 and analysed feed composition

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Fish meal (70.2/10.6)</td>
<td>740</td>
<td>713</td>
<td>686</td>
<td>659</td>
<td>632</td>
<td>686</td>
<td>632</td>
<td>577</td>
<td>523</td>
<td>659</td>
<td>578</td>
<td>496</td>
<td>416</td>
<td>496</td>
<td>300</td>
<td></td>
</tr>
<tr>
<td>Solvent-extracted Soya (48.8/2.7)</td>
<td>40</td>
<td>80</td>
<td>120</td>
<td>160</td>
<td>40</td>
<td>80</td>
<td>120</td>
<td>160</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corn gluten (62.4/1.0)</td>
<td>60</td>
<td>120</td>
<td>180</td>
<td>240</td>
<td>60</td>
<td>120</td>
<td>180</td>
<td>240</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soya concentrate (65.0/0.6)</td>
<td>114</td>
<td>106</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wheat gluten (77.7/1.0)</td>
<td>186</td>
<td>170</td>
<td>155</td>
<td>139</td>
<td>124</td>
<td>180</td>
<td>170</td>
<td>163</td>
<td>155</td>
<td>116</td>
<td>89</td>
<td>178</td>
<td>154</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fish oil</td>
<td>69</td>
<td>72</td>
<td>75</td>
<td>77</td>
<td>80</td>
<td>71</td>
<td>73</td>
<td>75</td>
<td>76</td>
<td>74</td>
<td>78</td>
<td>82</td>
<td>86</td>
<td>93</td>
<td>113</td>
<td></td>
</tr>
<tr>
<td>Premix</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Yttrium oxide</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>α-Methionine</td>
<td>0.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCP§</td>
<td>3.4</td>
<td>14.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Analysed feed composition</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dry matter (%)</td>
<td>94.1</td>
<td>93.9</td>
<td>93.4</td>
<td>91.4</td>
<td>92.6</td>
<td>94.1</td>
<td>93.3</td>
<td>93.4</td>
<td>92.7</td>
<td>92.8</td>
<td>93.3</td>
<td>92.4</td>
<td>95.7</td>
<td>93.3</td>
<td>92.4</td>
<td></td>
</tr>
<tr>
<td>Protein (%)</td>
<td>55.6</td>
<td>54.5</td>
<td>55.7</td>
<td>53.3</td>
<td>56.2</td>
<td>55.5</td>
<td>55.3</td>
<td>53.9</td>
<td>53.4</td>
<td>55.1</td>
<td>53.8</td>
<td>55.2</td>
<td>55.2</td>
<td>54.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fat (%)</td>
<td>15.9</td>
<td>15.4</td>
<td>15.6</td>
<td>16.2</td>
<td>14.3</td>
<td>15.9</td>
<td>14.2</td>
<td>15.4</td>
<td>14.2</td>
<td>13.4</td>
<td>14.2</td>
<td>13.5</td>
<td>16.6</td>
<td>15.8</td>
<td>15.3</td>
<td></td>
</tr>
<tr>
<td>Starch (%)</td>
<td>11.4</td>
<td>11.8</td>
<td>10.8</td>
<td>10.1</td>
<td>9.0</td>
<td>13.0</td>
<td>13.7</td>
<td>14.4</td>
<td>15.1</td>
<td>15.1</td>
<td>12.7</td>
<td>11.9</td>
<td>12.0</td>
<td>10.7</td>
<td>12.6</td>
<td>12.9</td>
</tr>
<tr>
<td>Ash (%)</td>
<td>8.2</td>
<td>8.3</td>
<td>8.8</td>
<td>6.5</td>
<td>7.6</td>
<td>8.8</td>
<td>7.7</td>
<td>8.2</td>
<td>6.4</td>
<td>6.5</td>
<td>7.0</td>
<td>6.4</td>
<td>6.4</td>
<td>8.6</td>
<td>8.8</td>
<td></td>
</tr>
<tr>
<td>Residue (%) **</td>
<td>3.0</td>
<td>3.9</td>
<td>2.5</td>
<td>5.3</td>
<td>8.0</td>
<td>0.2</td>
<td>2.2</td>
<td>0.1</td>
<td>3.1</td>
<td>6.8</td>
<td>5.1</td>
<td>6.7</td>
<td>6.8</td>
<td>1.1</td>
<td>3.6</td>
<td></td>
</tr>
<tr>
<td>Gross energy (MJ Kg−1)</td>
<td></td>
<td>21.5</td>
<td>21.1</td>
<td>21.3</td>
<td>20.8</td>
<td>20.0</td>
<td>20.0</td>
<td>21.9</td>
<td>21.2</td>
<td>22.0</td>
<td>21.0</td>
<td>20.2</td>
<td>20.8</td>
<td>20.2</td>
<td>21.5</td>
<td>21.5</td>
</tr>
</tbody>
</table>

* Nordsild mel (Fyllingsdalen, Norway).
† Cargill (Minneapolis, MN, USA).
‡ ADM (Europport, Rotterdam, the Netherlands).
§ Amylum (Ghent, Belgium).
* Star korn (Oslo, Norway).
†† Denofa (Fredrikstad, Norway).
** Vitamin and mineral premix according to NRC (1993), Trouw Nutrition (Boxmeer, the Netherlands).
† † Treibacher Auermet (Althogen, Austria).
†† Trouw Nutrition (Boxmeer, the Netherlands).
§§ Mono-calcium-phosphate, Trouw Nutrition.
** Residue calculated as follows: 100% − % protein − % fat − % starch − % ash − % water.
||| Estimated using the following caloric values: starch 17 MJ kg−1, fat 39 MJ kg−1 and protein 24 MJ kg−1.
FM, fish meal control diet; S, soybean; G, corn gluten; SC, soya concentrate; WG, wheat gluten. Protein/fat contents in protein raw materials are given in brackets.
Analytical procedures

Digestibility determinations

Feeds were analysed for proximate composition; dry matter was determined by differences in weight after drying at 104 °C for 24 h. Total nitrogen was determined with a nitrogen element analyser (LECO, FP-428; system 601-700-500, Perkin Elmer, Wellesley, CT, USA), and crude protein calculated as N × 6.25. Fat was determined gravimetrically after acid hydrolysis and extraction with di-ethyl ether and ash gravimetrically after combustion at 540 °C for 16 h (EU commission directive 84/4 EUF, the European Union Publication, No. L15/28, 18.1.84, part B, and EU commission directive 98/64/EF, the European Union Publication No. L257/23, 19.9.98, part B). Starch was analysed using an enzymatic method as described by Hemre, Lie, Lied and Lambertsen (1989).

For digestibility determination, faeces (fresh) were thoroughly homogenized before measurements of nitrogen (N × 6.25 = protein), lipid and starch as described above for feed. Also, yttrium concentration was determined in feeds and faeces using ICP-MS after complete digestion of homogenized and freeze-dried (104 °C for 24 h) samples in nitric acid after cooking in a microwave oven for 1 h. This method shows high reproducibility, linearity and recovery.

Histological examinations

Formalin preserved samples of intestines and liver were processed and stained with haematoxylin and eosin (H&E) and examined histologically for any signs of enteritis (Krogdahl et al. 2003) at the Institute of Aquaculture, University of Stirling, Scotland, in particular intestines were examined for enteritis.

RNA extraction

Total RNA was extracted from liver slices using Trizol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. RNA was isolated with phenol–chloroform extraction as described by Chomczynski and Sacchi (1987), and stored in 100 μL RNase-free MilliQ H₂O. Genomic DNA was eliminated from the samples by DNase treatment according to the manufacturer description (Ambion). The RNA was then stored at −80 °C before further processing. The quality of the RNA was assessed with the NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). A 260/280 nm absorbance ratio of 1.8–2.0 indicates a pure RNA sample. The RNA 6000 Nano LabChip® kit (Agilent Technologies) was used to evaluate the integrity of the RNA.

Design of PCR primers and TaqMan probes

Polymerase chain reaction primers for amplification of HSP70 and HSP90, in addition to the control gene 18S rRNA, were designed using the Primer Express 2.0 software (Applied Biosystems, Foster City, CA, USA). The PCR primers and TaqMan probe sequences used to quantify the mRNA levels of the genes of interests are given in Table 2. The RNA sequences of HSP70 and HSP90 were obtained from two Atlantic salmon ESTs (GenBank Accession numbers: BG933934 and BQ035751 respectively), and designed to align with conserved regions of these genes in other fish species. For 18S rRNA the PCR primers and probe were designed from Atlantic cod AF518205, and placed in conserved regions of this gene based on comparisons with fish sequences.

The PCR primers for HSP70, HSP90 and 18S rRNA were not designed to span exon–exon borders, as they were made from mRNA sequences. Instead, the extracted RNA samples were subjected to DNase treatment to avoid genomic DNA contamination, and amplified PCR products of all three genes were sequenced and BLASTed to ensure that the correct mRNA sequences were quantified. The genes were sequenced with the BigDye version 3.1 sequencing

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>TaqMan MGB probe</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S rRNA</td>
<td>ATTGGGCCTGTAGAGGTTGAA</td>
<td>CGAACCTCCGAACCTTCT</td>
<td>TCTTGGACCAGGGGCAA</td>
<td>105</td>
</tr>
<tr>
<td>HSP70</td>
<td>CCCCCCGCTGGGTTGATTTAG</td>
<td>CACCAGGGTGTTGTGTCCAGTG</td>
<td>CCGTGGAGGTGCATG</td>
<td>121</td>
</tr>
<tr>
<td>HSP90</td>
<td>TCTGGGGAATGAGGTCCTCAACA</td>
<td>CTTTGGACCTGGGAGAACAAGAA</td>
<td>TGTCATGAGAACGACC</td>
<td>98</td>
</tr>
</tbody>
</table>
kit (Applied Biosystems), using an ABI PRISM® 377 DNA Sequencer (Applied Biosystems) at the University of Bergen Sequencing Facility. For assay verification, a one-step RT-PCR protocol was used to amplify the genes (Qiagen OneStep RT-PCR kit; Qiagen, Crawley, UK). The PCR products were run on a 2% agarose gel, and subsequently sequenced as described above.

Real-time quantitative RT-PCR

A semi-quantitative two-step real-time RT-PCR protocol was developed to measure the mRNA levels of HSP70, HSP90 and 18S rRNA in liver cells of Atlantic cod. Methods for cDNA synthesis and quantitative RTPCR have been described earlier (Olsvik, Kristen- sen, Waagbo, Rosseland, Baeverfjord & Berntssen 2005). Twofold serial dilutions of total RNA were used for RT and PCR efficiency evaluation. Two hundred and fifty nanograms of total RNA was used in each reaction. Mean normalized expression was calculated with the Microsoft Excel-based software Q-Gene BioTechniques Software Library (Mul- ler, Janovjak, Miserez & Dobbie 2002). 18S rRNA was used as an endogenous control in the final calculations of mean normalized expression.

Gut evacuation

Gut evacuation was measured by following the movement of the X-ray dense ballotini glass beads through the GI tract. The cod intestine is highly coiled and X-ray images of whole fish make it difficult to divide the GI tract into different parts and to count the ballotini glass beads. Therefore, the GI tract was stretched and mounted on a board before X-ray images was performed at Teknologisk Institut (Stavanger, Norway). X-ray images from each fish were analysed on a light box (Kowolux 3, Inspection Equipment, Oxford, UK) and the relative distribution of ballotini glass beads in the stomach + pylorus, upper, mid and lower intestine, and hind gut was vis- ually estimated. All assessments were done blindfolded by one person.

Calculations

Residue was calculated as follows:

\[
100\% - \%\text{Protein} - \%\text{Fat} - \%\text{Starch} - \%\text{Ash} - \%\text{Water}
\]

Gross energy (MJ kg\(^{-1}\)) was calculated used the following caloric values:

- 17 MJ kg\(^{-1}\) for starch,
- 39 MJ kg\(^{-1}\) for fat and
- 24 MJ kg\(^{-1}\) for protein.

Apparent digestibility coefficient was calculated as follows:

\[
100 - 100 \times \frac{\% \text{ yttrium oxide in feed}}{\% \text{ yttrium oxide in faces}} \times \frac{\% \text{ nutrient in faeces}}{\% \text{ nutrient in feed}}
\]

Evacuation time of diet in the different parts of the intestine was calculated as:

\[
\% \text{ diet in } X \text{ at time } t = \frac{\text{sum number ballotini glass beads in } X \text{ from } n \text{ fish at time } t}{\text{total number ballotini glass beads in } Y \text{ from } n \text{ fish at time } t} \times 100
\]

Where \(X\) are the different parts of intestine (stomach + pylorus, upper intestine, mid intestine, lower intestine or hind gut) and \(Y\) are the whole intestine.

Statistics

Linear regression was used to evaluate gut evacuation time, ADC and nutrient amount in diet, using diet plant protein source levels as independent variable. Spearman’s rank order was used to evaluate nonparametric correlation between ADC and diet compositions, and multiple comparisons among slopes with Tukey test was used to evaluate difference between slopes in regression models. All statistical analysis was performed using STATISTICA™ 7D software program (STATSOFT, Tulsa, OK, USA, 2005).

Results

Diets

Analysed dietary levels of fat and protein were nearly identical, and close to the targeted levels of 55% protein and 15% fat in all diets (Table 1), while increased inclusion of corn gluten resulted in increased starch concentrations from 107 to 151 g kg\(^{-1}\) (starch = 117.0 + 1.16 × % corn gluten meal in diet, \(R^2 = 0.96, P < 0.001\)). Increasing the inclusion of soybean meal decreased dietary starch levels from 140 to 90 g kg\(^{-1}\) (starch = 117.0 – 1.49 × % soybean meal in diet, \(R^2 = 0.86, P < 0.01\)). These variations were not intended but impossible to avoid. There was no increase in residue level in diets, in spite of increased inclusion of plant protein, reminding on the variable plant mixtures used in the different diets evaluated.
Digestibility

Protein digestibility (ADCP) for the fish meal diets was 81%, when using soybean meal ADCP ranged between 77.3% and 83.0%. Corn gluten meal gave an ADCP of 70.5–81.4%, the mixture of soybean and corn gluten 76.4–83.0% and the SC/WG diets had an ADCP of 85.4 (11SC/11WG) and 87.3% (22SC/22WG). A linear reduction in protein digestibility with increasing level of corn gluten meal was identified (Fig. 1a). Fat digestibility (ADCF) ranged from 88.2% to 98.1%, and was linearly reduced due to increased soybean and corn gluten inclusions (Fig. 1b). Regression analysis showed no significant reduction in ADCF when using the mixed diets with soybean plus corn gluten, but the two diets with highest inclusion (12S/18G; 88.7 and 16S/24G; 91.3) showed lower ADCF compared with the two diets with lower inclusions (4S/6G; 96.0 and 8S/12G; 93.6). Starch digestibility (ADCS) ranged from 79.7% to 93.4%, and was significantly reduced by dietary corn gluten (Fig. 1c).

Growth and feed utilization

The fish grew from 139 ± 25 to 532 ± 125 g in the FM groups. Percent live weight gain ranged from 269% to 296% for fish given soybean meal, from 263% to 276% for fish given corn gluten, from 260% to 283% for fish given the S/G mixture and from 280% to 282% for the SC/WG groups. Growth was not significantly influenced by dietary treatment, and the control groups (LT-fish meal protein only) showed the same live weight gain (from 268% to 288% live weight increase), as all other diet groups (total range from 260% to 296%), with no regression due to variable inclusion of plant protein.

The fish meal diets gave a feed conversion ratio (FCR) after the whole trial of 0.94 and 1.03. The FCR range was 0.96–1.01 for the soybean diets, 0.95–1.09 for the corn gluten diets, 0.95–1.10 for the S/G diets and 0.98 and 1.11 for the SC/WG diet. Regression analysis of all diets together showed a general increase in FCR with increasing levels of plant protein in diets (FCR = 0.95 + 0.01 × % plant protein in diet, $R^2 = 0.59$, $P < 0.001$). Daily amount of feed consumed also increased with increasing levels of plant protein in the diet if the SC/WG diets was excluded from the regression (daily feed intake = 154 g + 0.45 × % plant protein in diet, $R^2 = 0.34$, $P < 0.05$).

Histological findings

Normal histological appearance was observed in sections of mucosa from all diet groups, and with no detectable enteritis-type changes, independent of dietary plant protein resource exerting up to

**Figure 1** Apparent digestibility coefficient (ADC) for protein (a), fat (b) and starch (c) for cod fed different inclusions (%) of soybean meal, corn gluten meal, mixture of soybean and corn gluten meal and a mixture of soyprotein concentrate and wheat gluten. Linear regression lines: ADCP = 82.4–0.4 × % corn gluten meal in diet ($R^2 = 0.82$, $P < 0.01$) (a), ADCF = 96–0.4 × % soybean meal in diet ($R^2 = 0.75$, $P < 0.05$), ADCF = 95.2–0.1 % corn gluten meal in diet ($R^2 = 0.91$, $P < 0.01$) (b), ADCS = 899–0.4 × % corn gluten meal in diet ($R^2 = 0.76$, $P < 0.05$) (c).
440 g kg\(^{-1}\) of the feed. Many lower intestines contained columns of bacteria, arranged along and extending outwardly from the mucosal brush border. Some fish had also clusters of these exfoliated into the gut lumen, or other clusters of long-rod or small, shorter rod bacteria within the lumen. Also noted among many lower intestines were nucleated cell structures of small and moderate size either loosely adherent to the brush border, or clustered near to, or sometimes more numerous throughout the gut lumen (Fig. 2). No histopathological findings were detected in livers in any diet group.

**Expression of HSP70 and HSP90**

No up- or down-regulation of the transcription levels of HSP70 and HSP90 was identified in fish fed the 16S diet, the 24G diet or the 16S/24G diet compared with the fish meal control diet (Fig. 3a and b). The other diet groups were therefore not included in these measurements.

**Gut evacuation**

Stomach and pylorus evacuation started 6–12 h after feeding for all diets except diets 16S, 24G and 16S/24G where stomach evacuation started just after 1 h. For both diets containing soy protein concentrate and wheat gluten (11SC/11WG and 22SC/22WG) stomach evacuation started after 12 h. All diets gave a steady evacuation up to 48–60 h after feeding. At 72 h evacuation from the stomach levelled off. When the food evacuated from the stomach it was close to evenly distributed along the intestine, peaking in the
upper intestine after 36 h for diet FM2, 24G, 8S/12G and 12S/18G, after 48 h for diets 4S, 8S, 6G, 18G, 12G, 4S/6G, 16S/24G, 11SC/11WG and 22SC/22WG and 60 h for FM1, 12S and 16S. In the mid-intestine diet 16S, 12S/18G and 16S/24G had a top after 48 h, diet FM2, 6S, 12S, 12G, 18G, 6G/12S and 22SC/22WG after 60 h, and diet FM1, 4S, 24G, 4S/6G and 11SC/11WG increased up to 72 h. In hind gut there was an increase in ballotini density towards the end of sampling (72 h).

Evacuation in almost all parts of the GI tract and of all diets fitted a linear regression model, except all diets in the upper-intestine, diet 8S and 16S/24G in the mid-intestine, diet 24G in the lower intestine, diet 16S and 6G in the hind-gut. Multiple comparison among slopes with a Turkey test gave no difference between slopes in any part of the intestine. All diets in each part of the GI tract is therefore pooled to one regression line (Y = Relative ballotini density (%), and t = time after feeding; stomach + pylorus: Y = 100.9 − 1.3 t, R² = 0.88, mid-intestine: Y = − 0.28 + 0.34 t, R² = 0.64, lower intestine: Y = − 2.51 + 0.41 t, R² = 0.74 and hind-gut: Y = − 2.49 + 0.31 t, R² = 0.60). In Fig. 4 the results are visualized in a bar chart.

Discussion

Digestibility

Despite very high growth rates obtained in the present study (Hansen, Rosenlund, Karlsen, Rimbach & Hemre 2006), protein digestibility was in the lower range when compared with earlier cod studies, while fat and starch digestibilities were in the upper expected ranges (Holdway & Beamish 1984; Lie, Lied & Lambertsen 1987; Hemre et al. 1989, 2003; Toppe, Aksnes, Hope & Albrektsen 2005). Still, a significant lowering effect on ADC results were registered for all macronutrients as a consequence of increased corn gluten in the diets, results which agrees with findings on Atlantic salmon stating that the starch and/or fibre fraction, unavoidable in cost-efficient plant proteins, will affect intestinal function in Atlantic salmon, Salmo salar (Krogdahl, Hemre & Mommsen 2005), rainbow trout, Oncorhynchus mykiss (Cheng & Hardy 2003), gilthead seabream, Sparus aurata (Pereira & Oliva-Teles 2003) and turbot, Psetta maxima (Regost, Artzel & Kaushik 1999). The fibre fraction (residue calculated, as well as undigested starch) is shown to disturb micelle formation, to result in adherence between fibre components and enzymes essential in protein and starch hydrolyzes, and thickening of the unstirred water layer at the mucosal surface (Krogdahl et al. 2005). For fat digestibility only, solvent-extracted soy ingredients affected ADC results, but with no effect on ADC for protein or starch, results which indicate that the level of the different fibre types most probably varied in our diets. Undesirable saponins, which are found in solvent-extracted soybean in a higher level than in corn gluten, have been found to reduce feed intake and growth in studies with Chinook salmon (O. tshawytscha) and rainbow trout (O. mykiss) (Bureau, Harris & Young Cho 1998), and to reduce fat digestibility in Atlantic salmon (Olli & Krogdahl 1995), and macronutrient digestibilities in several species (Kaushik et al. 1995;
In fish and have shown to be good biomarker proteins of stress induction and environmental pollution (Grosvik & Goksoyr 1996; Lewis, Handy, Cordi, Billinghamurst, & Depledge 1999; Ahmad, Hamid, Fatima, Chand, Jain, Athar & Raisuddin 2000). Heat shock proteins 70 is found to respond to suboptimal diet compositions (Martin et al. 2003; Hemre et al. 2004), and was a natural choice of an early stress biomarker when using extremely high levels of plant ingredients to the strict carnivorous cod. The lack of any intestinal injury, maintenance of growth, as well as other health parameters within normal ranges (Hansen et al. 2006), add to the arguments that cod can both tolerate and utilize sustainable plant ingredients from soy, wheat and corn gluten.

### Gut evacuation

The optimal feeding frequency of cod is under debate (Rosenlund, Karlsen, Tveit, Magnor-Jensen & Hemre 2004). One tool that might be applied is planning of feeding frequency based on data showing at which time after feeding the GI tract might be ready for a new meal. This can be done e.g. by stomach emptying and register of passage time through the GI tract, being aware of the numerous factors influencing gut evacuation. Gut evacuation is found to vary according to temperature, meal size, particle size, previous nutritional history, fish size, feeding frequency and feed composition (Flowerdew & Grove 1979; Talbot et al. 1984; Storebakken 1985; He & Wurtsbaugh 1993; Sveier, Wathne & Lied 1999; Boyce et al. 2000). In our study, especially the variable starch fraction, in addition to varying fibre quality between plant ingredients, was expected to highly influence gut evacuation, as found with use of soybean in diets for Atlantic salmon (Storebakken et al. 1999). However, no consequences regarding total gut evacuation time was detected, irrespective of amount and type of plant protein ingredient added to the diet, although the pattern of stomach evacuation seemed to vary with dietary plant protein type and amount, 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 for all diet groups. This means that feeding frequency can be planned equally in cod reared at 6.5 °C, size 100–600 g, independent of the diet being based on pure fish meal, or holding up to 440 g kg⁻¹ plant protein ingredients. The total evacuation of 72 h, and especially that stomach still held about 50% of the diet after 24 h, indicates that there is no gain when fed more often

### Histological findings

The non detectable enteritis-type changes in the intestine of cod, as found here, and also reported by Refstie and Morkore (2005), are in strong contrast to what have been shown in Atlantic salmon (van den Ingh et al. 1991; Baeverfjord & Krogdal 1996; Storebakken et al. 2000; Krogdal et al. 2003) and rainbow trout (Refstie et al. 2000) in response to plant proteins, especially soybean meal, in the feed. Cod have a great number of pyloric ceca that increase the total length and area of the intestine compared with salmonids, e.g. rainbow trout (Buddington & Diamond 1987). Also, a significant number of bacteria were observed in the lower intestinal tract in the present study and it is speculated whether they may have a digestive function, thus explaining the absence of visible enteritis changes.

### Expression of HSP70 and HSP90

The lack of up- or down-regulation responses of HSP70 or HSP90 mRNA expressions, indicates no diet-induced stress response at the sampling time (Iwama, Aono, Todgham, Ackerman & Nakano 2004). 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 & Goksoyr 1996; Lewis, Handy, Cordi, Billinghamurst, & Depledge 1999; Ahmad, Hamid, Fatima, Chand, Jain, Athar & Raisuddin 2000). Heat shock proteins 70 is found to respond to suboptimal diet compositions (Martin et al. 2003; Hemre et al. 2004), and was a natural choice of an early stress biomarker when using extremely high levels of plant ingredients to the strict carnivorous cod. The lack of any intestinal injury, maintenance of growth, as well as other health parameters within normal ranges (Hansen et al. 2006), add to the arguments that cod can both tolerate and utilize sustainable plant ingredients from soy, wheat and corn gluten.

© 2006 Blackwell Publishing Ltd. No claim to original NIFES work. Aquaculture Research, 37, 773–784
than once every 24 h, or there is no capacity in the GI tract to take care of additional nutrients supplied. These results are in line with results from Lie (1991), who concluded that cod should be fed every, or every second day, one large meal, and that a higher feeding frequency resulted in enlarged livers without extra muscle growth.

**Conclusion**

Overall results showed no adverse effects from high levels of plant protein ingredients in cod diets on the digestive system. Digestibility was affected only to a minor extent, and no histopathological changes were observed in intestine or liver even at inclusions up to 440 g kg⁻¹ plant ingredients. No diet-induced expression of stress genes further confirmed a high tolerance to the present diet variations. Although stomach evacuation varied, total time lag in the GI tract was equal in all groups, and indicated no gain in feeding more frequent than every 24 h, or there is no capacity in the GI tract to take care of additional nutrients supplied.

**Acknowledgments**

This work was supported by the Research Council of Norway (grant # 156195/120) and by Nutreco ARC. The authors wish to thank Johnny Elvrum for technical assistance with the x-ray images, Prof. Richard Collins for valuable comments on the histological findings and Jacob Wessels for help with the chemical analyses of feed and faeces.

**References**


