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Comparison of intestinal gene expression in Atlantic cod (Gadus morhua) fed standard fish meal or soybean meal by means of suppression subtractive hybridization and real-time PCR

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Received 13 November 2006; received in revised form 24 January 2007; accepted 26 January 2007

Abstract

Gene expression was studied in Atlantic cod fed two different diets, fish meal (FM) and dehulled and extracted soybean meal (SBM). RNA was isolated from the distal part of the mid-intestine of Atlantic cod and suppression subtractive hybridization (SSH) was employed to screen for genes that showed changes in expression in response to the two dietary treatments. We made a cDNA subtracted library, isolated and sequenced 192 clones. Identification of 157 clones was predicted by BLAST. Most of the clones were previously unidentified in cod. Expression of 12 selected clones was further studied by quantitative PCR. Expression of four clones showing similarity to aminopeptidase N, transcobalamin I precursor, cytochrome P450 3A40, and ras-related nuclear protein was significantly up regulated in intestine of cod fed SBM compared to cod fed FM. A trend towards up regulation of a clone with similarity to fatty acid binding protein in SBM-fed cod was also observed. No significant differences in expression were observed for: transmembrane 4 superfamily protein member, polypeptide N-acetylgalactosaminyltransferase, glutathione peroxidase, peroxiredoxin 4, SEC61, F-BOX, and 14-3-3.

Keywords: Atlantic cod; Intestine; Real-time PCR; SSH; Expression; Soybean meal

1. Introduction

The global fish farming industry is greatly expanding thus increasing the demand for sustainable protein sources for formulated feeds. Salmon and trout are no longer the only fish species, as a diversity of aquatic species and organisms are being developed for use in aquaculture. To achieve sustainable growth in aquaculture, a replacement of raw materials from marine sources with vegetable sources seems necessary. Extracted soybean meals (SBM) are regarded as potentially very good protein ingredients for fish feeds, and represent the main plant protein source on the world market. However, legumes like soybeans contain high amounts of different bioactive components like protease inhibitors, lipase

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inhibitors, phytic acid, saponins, and lectins (Krogdahl, 1989). Soybean meal has already become an important protein source in fish feeds, but due to a wide range of adverse effects soy products are only included at low levels in diets for farmed salmonids.

The production of farmed Atlantic cod has increased over the last few years in Norway. As wild Atlantic cod is considered a strict carnivorous species, formulated feeds have in general been made from marine protein and lipid sources. Few reports are available studying addition of soybean protein sources to cod diets. However, indications exist that cod is more tolerant to dietary inclusions of soybean protein sources compared to salmonids. In a study by von der Decken and Lied (1993) juvenile cod were fed soybean protein sources compared to salmonids. In this study replacement of 10 and 20% of total protein did not affect feed intake and growth whereas a decrease was observed at 30% replacement of total protein. In the same study, changes in muscle metabolic functions, measured as acid proteinase activity, glycogen and myofibrillar RNA content and myosin heavy chain mRNA levels were altered before measurable changes in growth were observed.

The latter study is in accordance with a pilot study performed prior to the present experiment where Atlantic cod (800 g) were fed diets in which SBM replaced 24 and 48% of total protein in the FM diet (Nylund, 2003). The results showed that lipid and protein digestibility was decreased in the group fed 48% inclusion of SBM but not in the group fed 24% inclusion of SBM.

In a parallel study from the same feeding trial as our study, Atlantic cod were fed either FM, standard SBM or bioprocessed SBM (Refstie et al., 2006a). Both soy products replaced 24% of the total protein in the FM diet and all three diets were fed to small (534 g) and large (1750 g) fish. There was no difference between diets with respect to weight gain within the two size groups, nor any general effect of diet on growth measured as thermal growth coefficient (TGC). The soy products reduced the digestibility of amino acids and lipid in both size groups, but this was, however, compensated for by increased feed intake and gastrointestinal growth. Bioprocessing of the SBM did not improve the nutritional value, indicating that cod has a high tolerance to SBM compared to salmonids.

The pathomorphological changes observed in the distal intestine of Atlantic salmon fed SBM (van den Ingh et al., 1991; Baeverfjord and Krogdahl, 1996; Bakke-Mckellep et al., 2000) has not been observed in the intestine of Atlantic cod fed SBM (Refstie et al., 2006b).

In the present study we used molecular tools like suppression subtractive hybridization (SSH) and real-time PCR to gain information on possible changes in intestinal gene expression in Atlantic cod fed SBM compared to Atlantic cod fed FM. Knowledge of variation in gene expression may become valuable tools for the investigation of intestinal function and regulation in response to novel feedstuffs.

2. Materials and methods

The experiments performed in this report were part of a larger feeding trial in which Atlantic cod (1 and 2 year old) were fed either a fish meal-based diet, or diets in which the fish meal was replaced by either extracted soybean meal (SBM) or a SBM bioprocessed to remove soybean anti-nutritional factors (ANFs). The feeding trial lasted 84 days. The trial was performed at AKVAFORSK’s model sea farm at Ekkilsøy, Norway. Three other reports on feed intake, growth, and utilization of macronutrients and amino acids (Refstie et al., 2006a), as well as digestive capacity, intestinal morphology, and microflora (Refstie et al., 2006b; Ringø et al., 2006) were recently published.

For this molecular gene expression study we studied the 1 year old cod fed either the FM based diet or the extracted SBM diet. The bioprocessed SBM containing diet was not included in this study.

2.1. Experimental animals

Farmed Atlantic cod (Gadus morhua) with an average initial weight of 534 g were obtained from AKVAFORSK Sundalsøra and kept in four separate sea net pens at Averøy, Norway and fed either a standard fish meal diet (two net pens) or a diet in which 24% of the protein was replaced with SBM (two net pens). The average water temperature during the 84 days of feeding was 10.0 °C, with a maximum temperature of 13.7 °C at the start of the trial, and ending at 7.0 °C at the day of sampling. The fish were fed daily for 1 h at dawn. Three randomly selected fish were sampled from each pen (six fish fed FM and six fish fed SBM in total). All fish were handled in accordance with “Regulation on Animal Experimentation” (Norwegian ministry of agriculture). Further details on fish and rearing condition are given in (Refstie et al., 2006a).

2.2. Diets

Three diets were manufactured by high-pressure moist extrusion technology at Nutreco Technology Centre in Stavanger, Norway. Formulation of the two diets used in our studies, are given in Table 1. The FM diet contained 100% crude protein (CP) from low-temperature dried FM (LT-FM, Norse LT-94) obtained from Vedde Herring Oil Factory (Egersund, Norway), whereas in the SBM diet
Table 1
Diet formulations (g/kg)

| Diet          | FM     | SBM     |
|---------------|--------|---------|
| LT-fish meal  | 686.5  | 541.8   |
| Soybean meal  |        | 245.7   |
| Wheat         | 191.8  | 78.6    |
| Fish oil      | 117.9  | 128.3   |
| DL-Methionine |        | 1.6     |
| Constants     | 3.8    | 3.8     |

\[ a \] Norse LT-94 (Vedde Herring Oil Factory, Vedde, Norway).
\[ b \] Dehulled and extracted soybean meal (supplied by Hamlet Protein).
\[ c \] Constant ingredients, g kg\(^{-1}\): 2.3 g vitamin and mineral premix (proprietary composition, Nutreco ARC, Stavanger, Norway), 1 g yttrium premix (100 mg Y\(_2\)O\(_3\) kg\(^{-1}\) diet), 0.4 g Betafin, 0.1 g Lutavit C (BASF, Ludwigshafen, Germany).

24% of crude protein was replaced by dehulled and extracted SBM obtained from Hamlet Protein (Horsens, Denmark). Both diets were formulated to contain 55% CP and 20% lipid (DM basis), and to be iso-energetic on a gross energy basis. The diets were supplemented with D, L-methionine to contain similar amounts of (calculated) methionine. Both diets contained 100 mg yttrium oxide (Y\(_2\)O\(_3\), Sigma, St. Louis, Mo, USA) kg\(^{-1}\) dry mix as an inert marker to permit apparent digestibility measurements. Further details on formulation and chemical composition of the diets are given in (Refstie et al., 2006a).

2.3. Collection of tissue samples

Atlantic cod were anaesthetized in tricain methanesulfonate (MS222, Argent Chemical Laboratories Inc., Redmont, WA, USA), and subsequently killed with a sharp blow to the head. The whole gastro-intestinal tract was then immediately dissected out, cut open and intestinal contents carefully and thoroughly removed. Subsequently the intestine was divided into the following five segments. The intestine between the ring of pyloric caeca and the valve separating the most distal segment was defined as the mid-intestine (MI), and divided into four equal sections (MI1, MI2, MI3 and MI4). The section between the valve separating the most distal segment and anus was defined as the distal chamber (DC). Samples of about 300 mg were taken from MI4, and then quickly rinsed in sterile phosphate-buffered saline (PBS), before immediate transfer to ten times the volume of RNA later\(^{®}\) (Ambion, Inc., Austin, USA), and storage at –20 °C until further analysis.

2.4. RNA isolation

Total RNA was isolated from the 12 (six fish fed FM and six fish fed SBM in total) MI4 tissue samples by use of Trizol (Invitrogen) according to the manufacturer's protocol. High RNA integrity was verified using a 2100 Bioanalyzer from Agilent Technologies, Inc., Palo Alto, USA.

2.5. cDNA synthesis for suppression subtractive hybridization

For the suppression subtractive hybridization, only one fish from each feeding treatment was used in an effort to obtain as many different clones as possible. cDNA was synthesized from 1 μg of total RNA from one fish fed FM and one fish fed SBM by use of the BD Clontech SMART cDNA synthesis kit (BD Clontech, Cat. no. 634902/K1052-1), part VII, according to the manufacturer's protocol (VIIA1-B13). This kit was used to produce high quality cDNA from a small sample. Part VII and VIII of this protocol are designed for synthesizing cDNA for applications such as PCR-Select™ cDNA subtraction or Virtual Northern blots. Briefly, 1 μg of total RNA was combined with 12 μM of the primers 3' BD SMART CDS primer II A (a modified oligo dT primer) and BD SMART II A oligonucleotide, respectively, and incubated at 70 °C for 2 min, before addition of first-strand buffer, DTT, dNTPs, and BD Powerscript Reverse Transcriptase according to the manufacturer's protocol. The RNA was then reversely transcribed for 1 h at 42 °C to synthesize single stranded (ss) cDNA. After this, the ss cDNA was diluted in 40 μl of TE buffer (10 mM Tris (pH 7.6), 1 mM EDTA), and heated at 72 °C for 7 min. Subsequently, 1 μl of the diluted ss cDNA was used in long distance (LD) PCR, while the remaining (49 μl) was stored at –20 °C until further use. LD PCR was performed according to the manufacturer's protocol, and an optimal number of cycles determined for each sample. Each step of the protocol was monitored by gel electrophoresis of aliquots of samples. The LD PCR was terminated by the addition of 5 μl of 20X EDTA/glycogen Mix (0.2 M EDTA; 1 mg/ml glycogen) from the Clontech PCR-Select™ cDNA subtraction kit (BD Clontech, Cat. no. 637401). Instead of performing column chromatography according to part VIII of the SMART cDNA synthesis kit protocol, we performed precipitation of the LD PCR (approximately 86 μl after gel electrophoresis) according to the PCR-Select™ cDNA subtraction kit protocol, part IV D7.

2.6. Suppression subtraction hybridization (SSH)

The terminated LD PCR synthesized by the SMART cDNA synthesis kit was precipitated according to the PCR-Select™ cDNA subtraction kit protocol (BD Clontech, Cat. no. 637401), part IV-D7 by adding 100 μl of
phenol:chloroform:isoamyl alcohol (25:24:1), vortexing thoroughly, and centrifugation at 13,000 × g for 10 min at room temperature. The steps following precipitation were also performed exactly according to the protocol. Briefly, the cDNAs were subsequently digested with Rsa I, precipitated again, and dissolved in H2O, and stored at −20 °C until further use. For the SSH procedure, cDNA from FM-fed fish was defined as the “driver” cDNA, and cDNA from SBM-fed fish defined as the “tester” cDNA. 1 μl of Rsa I-digested tester cDNA (from SBM-fed fish) was then ligated to either adaptor 1 or adaptor 2R at 16 °C overnight (ON), and then terminated by addition of EDTA/glycogen and heating at 72 °C for 5 min. Subsequently the tester cDNA with adaptor 1 was hybridized with an excess of the driver cDNA (from FM-fed fish) in tube 1, while tester cDNA with adaptor 2R was hybridized with an excess of driver cDNA in tube 2 at 68 °C for 8 h (first hybridization), immediately followed by the second hybridization in which tube 1 and 2 from the first hybridization was combined with addition of fresh denatured driver cDNA (subtraction kit protocol, part IV-H), and incubated further at 68 °C ON. The following day 200 μl of dilution buffer was added to the sample, followed by heating to 68 °C for 7 min. The diluted sample was subsequently stored at −20 °C until further analysis. Differentially expressed cDNAs were selectively amplified by two subsequent PCR steps, the first using a PCR primer 1 complementary to both adaptors, and the second using two nested primers complementary to each of the two adaptors (nested PCR primer 1 and nested PCR primer 2R) respectively, according to the manufacturer’s protocol (section IV, I-1 to I-17). In this way the final PCR mixture was enriched for differentially expressed cDNAs. A subtraction efficiency test was performed as a control according to the manufacturer’s protocol.

2.7. Cloning of differentially expressed cDNA from SSH

The enriched PCR product mixture from SSH was ligated into the pCR® 2.1-TOPO® vector using the TOPO TA Cloning™ kit from Invitrogen life technologies, California, USA, according to the manufacturer’s protocol and transformed into chemically competent E. coli. Optimalization of the amount of PCR product ligated with pCR® 2.1-TOPO® vector was performed to ensure an optimal number of transformed colonies. Transformation of E. coli was also performed according to the manufacturer’s protocol. A total number of 192 clones were picked at random and PCR was performed with vector specific primers, M13F and M13R to ensure a single PCR product per clone, and thus one clone picked per well. PCR was performed as follows: 30 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min.

Subsequently the clones were sequenced, using M13R as sequencing primer. Sequences were subsequently trimmed to remove primer- and vector-sequences and 159 successful clones were then subjected to translated BLAST search (blastx or tblastx) at NCBI BLAST. 33 clones failed to pass quality control after trimming, because of sequences either being too short, or consisting of only poly A- or vector-sequences.

On the basis of their possible relevance to changes in amino acid and lipid digestion, some clones were then selected for real-time PCR (qPCR). In addition, some randomly selected clones were included. The selected clones were sequences showing similarity to the following genes: fatty acid binding protein (FABP, clone ID GH4A-F142), putative transmembrane 4 superfAMILY member protein (TM4, clone ID GH4A-F103), polypeptide N-acetylgalactosaminyltransferase (ppGalNtase, clone ID GH4A-F107), Aminopeptidase M (Alanyl aminopeptidase)(CD13)/Aminopeptidase N (APN, clone ID GH4A-F77), transcobalamin I precursor (TCI, clone ID GH4A-F84), Sec61-alpha (SEC61, clone ID GH4A-F137), F-box protein 44 (F-BOX, clone ID GH4A-F138), glutathione peroxidase (GPx, clone ID GH4A-F127), peroxiredoxin 4 (Prx4, clone ID GH4A-F167), cytochrome P450 3A40 (CYP3A40, clone ID GH4A-F166), ras-related nuclear protein (Ran, clone ID GH4A-F159), and 14-3-3B2 protein (14-3-3, clone ID GH4A-F135), polypeptide GH4A-F136, GH4A-F138, GH4A-F127, GH4A-F166, ras-related nuclear protein (Ran, clone ID GH4A-F159).

2.8. DNase treatment

Prior to reverse transcription, total RNA from all samples intended for real-time PCR were subjected to DNase treatment using a TURBO DNA-free™ kit in accordance with the manufacturer’s recommendations (Ambion, Inc., Austin, USA).

2.9. Two-step real-time PCR

Real-time PCR was performed on samples from all twelve individuals sampled (6 fish fed FM and 6 fish fed SBM). Each sample was reversely transcribed in duplicates by PowerScript™ Reverse Transcriptase (BD Biosciences, Franklin Lakes, NJ, USA) (RT) from 740 ng of total RNA isolated from MI4 using a mixture of 250 ng oligo (dT) (Invitrogen Ltd, Paisley, UK) and 25 ng random primers (Invitrogen Ltd, Paisley, UK) according to the manufacturer’s protocol (PowerScript™ Reverse Transcriptase kit, BD Biosciences, Franklin Lakes, NJ, USA).
Real-time PCR amplifications were performed to examine the relative expression of selected genes (Tables 2 and 4) in MI4 in a LightCycler 2.0 (Roche Diagnostics, Mannheim, Germany). Duplicates of 2.5 μl cDNA from each reverse transcription reaction were used as PCR templates. PCR reactions were performed in a total volume of 10 μl using the LightCycler FastStart DNA MasterPLUS SYBR GREEN I kit (Roche Diagnostics, Mannheim, Germany) and a modified protocol with 4.5 μl PCR-grade water, 0.5 μl of each PCR primer (10 μM) and 2 μl master mix. The following LightCycler thermocycling program was used: denaturation program (10 min at 95 °C), amplification and quantification program repeated 40 times (10 s at 95 °C, 15 s at the appropriate annealing temperature for the gene specific primers (Table 2) and 10 s at 72 °C with a single fluorescence measurement), melting curve program (60 °C to 99 °C with a heating rate of 0.1 °C/s) and cooling program down to 40 °C. Fluorescence was monitored (excitation at 470 nm and emission at 530 nm) at the end of the annealing phase (the LightCycler F1 channel). For determination of the crossing point (CP) the “second derivative maximum method” measuring maximum increase rate of newly synthesized DNA per cycle was used on the basis of LightCycler software 4.0 (Roche Diagnostics, Mannheim, Germany). To confirm amplification specificity the PCR products from each primer pair were subjected to melting curve analysis and subsequent agarose gel electrophoresis.

The relative expression ratio of target mRNAs was calculated using the LightCycler software 4.0 (Roche Diagnostics, Mannheim, Germany). The following calculation method was employed: calibrator-normalized relative quantification using PCR efficiency correction based on a linear regression fit. Elongation factor 1 alpha (ELF1a) and 18S rRNA were used as reference genes and cDNA from a FM fed fish was used as the calibrator.

### Table 2
**Primer sequences used for real-time PCR**

| Clone similar to: | GenBank Accession no. | Primer name | Primer sequence | Annealing temp (°C) | PCR product size (bp) |
|------------------|-----------------------|-------------|-----------------|--------------------|-----------------------|
| Fatty acid binding protein (FABP) | EB677063 | FABP2 F1 | TTCCTCAACTACAGCCACAC | 60 | 127 |
| Putative transmembrane 4 superfamily member protein (TM4) | EB677017 | TM4 F1 | CCAACAGAGACACCCAAACAG | 58 | 148 |
| Probable polypeptide N-acetyl galactosaminytransferase 8 (ppGaNTase) | EB677083 | TRANSF F1 | GTCCGTCAAGCAGGCTTAG | 60 | 146 |
| Aminopeptidase M (Alanyl aminopeptidase)(CD13)/Aminopeptidase N (APN) | EB677077 | AMPEP F1 | GCTTTGGCTCAACCGATTT | 58 | 145 |
| Transcobalamin I precursor (TCI) | EB677078 | TCI F1 | TGGTGAGGCTCAACCGGTT | 58 | 145 |
| Sec61-alpha (SEC61) | EB677091 | SEC61 F1 | ATCTCCCTCTTCCTCCTTG | 60 | 194 |
| F-box protein 44 (F-BOX) | EB677092 | F-BOX F1 | CTTCTGTCACCCAACTCCT | 58 | 142 |
| Glutathione peroxidase (GPx) | EB677081 | PEROXI F1 | CCAATTCGGGACATCCAGGA | 57 | 128 |
| Peroxiredoxin 4 (Prx4) | EB677102 | PRX-IV F1 | ATCGTGACACTCTTTGTTT | 56 | 151 |
| Cytochrome P450 3A40 (CYP3A40) | EB677101 | 3A40 F1 | CGGTGTCGTAATCCCAAAG | 56 | 173 |
| Ras-related nuclear protein (RAN) | EB677072 | RAN F1 | AATCTGCACTCTGTTGTT | 56 | 130 |
| 14-3-3B2 protein mRNA (14-3-3) | EB677100 | 14-3-3 F1 | AGAAGGGGGAGGTTTGGTT | 56 | 139 |
| *18S rRNA | AF518205 | 18SrRNA F1 | CTCAACAGGGAACACCTCAC | 60 | 141 |
| *Similar to elongation factor 1-alpha (ELF1a) | CO541820 | ELF1a F1 | CACGTGACTGAACTCTTGTG | 58 | 142 |
| *Gadus morhua* partial mRNA for beta-actin | AJ555463 | bACTIN F1 | TGACCTCAGTACCTCATGACTC | 58 | 162 |

*Housekeeping gene.
| Clone ID | Bp | Similar to BlastX | BlastX E value | GenBank Acc. no. |
|----------|----|------------------|----------------|-----------------|
| GH4A-F3  | 382| Cyclophilin A [Chlamys farreri] | 6.00E-24 | EB677010 |
| GH4A-F179| 171| CypA protein [Xenopus laevis] | 3.00E-13 | EB677011 |
| GH4A-F146| 578| Peptidyl/prolyl isomerase (cyclophilin)-like 3 [Gallus gallus] | 3.00E-50 | EB677012 |
| GH4A-F4  | 261| ATP-binding cassette, sub-family E (OABP), member 1 [Danio rerio] | 8.00E-42 | EB677013 |
| GH4A-F61 | 560| ATP-binding cassette, sub-family E (OABP), member 1 [Danio rerio] | 2.00E-41 | EB677014 |
| GH4A-F5  | 607| Similar to putative transmembrane 4 superfamily member protein [Danio rerio] | 2.00E-22 | EB677015 |
| GH4A-F74 | 532| Similar to putative transmembrane 4 superfamily member protein [Danio rerio] | 8.00E-22 | EB677016 |
| GH4A-F103| 606| Similar to putative transmembrane 4 superfamily member protein [Danio rerio] | 1.00E-19 | EB677017 |
| GH4A-F131| 522| Similar to putative transmembrane 4 superfamily member protein [Danio rerio] | 1.00E-32 | EB677018 |
| GH4A-F133| 321| Similar to putative transmembrane 4 superfamily member protein [Danio rerio] | 2.00E-31 | EB677019 |
| GH4A-F151| 306| Similar to putative transmembrane 4 superfamily member protein [Danio rerio] | 1.00E-10 | EB677020 |
| GH4A-F175| 321| Similar to putative transmembrane 4 superfamily member protein [Danio rerio] | 1.00E-31 | EB677021 |
| GH4A-F186| 321| Similar to putative transmembrane 4 superfamily member protein [Danio rerio] | 2.00E-32 | EB677022 |
| GH4A-F155| 502| Putative transmembrane 4 superfamily member protein [Oncorhynchus mykiss] | 6.00E-18 | EB677023 |
| GH4A-F149| 562| Tetraspanin 1 [Xenopus tropicalis] | 1.00E-25 | EB677024 |
| GH4A-F14 | 428| DCMP deaminase [Danio rerio] | 2.00E-20 | EB677025 |
| GH4A-F19 | 336| DCMP deaminase [Danio rerio] | 2.00E-20 | EB677026 |
| GH4A-F26 | 427| DCMP deaminase [Danio rerio] | 2.00E-20 | EB677027 |
| GH4A-F45 | 402| DCMP deaminase [Danio rerio] | 2.00E-20 | EB677028 |
| GH4A-F147| 427| DCMP deaminase [Danio rerio] | 6.00E-12 | EB677029 |
| GH4A-F161| 413| DCMP deaminase [Danio rerio] | 8.00E-20 | EB677030 |
| GH4A-F165| 426| DCMP deaminase [Danio rerio] | 2.00E-20 | EB677031 |
| GH4A-F171| 429| DCMP deaminase [Danio rerio] | 2.00E-20 | EB677032 |
| GH4A-F95 | 414| Similar to Deoxycytidylate deaminase (DCMP deaminase) isofrom 1 [Canis familiaris] | 2.00E-17 | EB677033 |
| GH4A-F10 | 530| Cytoplasmic actin type 5 [Rana lessonae] | 3.00E-91 | EB677034 |
| GH4A-F33 | 481| Cytoplasmic actin type 5 [Rana lessonae] | 2.00E-78 | EB677035 |
| GH4A-F86 | 477| Cytoplasmic actin type 5 [Rana lessonae] | 5.00E-78 | EB677036 |
| GH4A-F48 | 433| Cytoplasmic actin type 4 [Rana lessonae] | 8.00E-71 | EB677037 |
| GH4A-F102| 397| Beta-actin [Tigriopus japonicus] | 2.00E-16 | EB677038 |
| GH4A-F18 | 397| Actin, alpha 2, smooth muscle, aorta [Danio rerio] | 6.00E-34 | EB677039 |
| GH4A-F20 | 444| Actin, alpha, cardiac muscle like [Danio rerio] | 1.00E-76 | EB677040 |
| GH4A-F42 | 479| Actin, alpha, cardiac muscle like [Danio rerio] | 1.00E-76 | EB677041 |
| GH4A-F96 | 480| Actin, alpha, cardiac muscle like [Danio rerio] | 9.00E-79 | EB677042 |
| GH4A-F130| 467| Actin, alpha, cardiac muscle like [Danio rerio] | 8.00E-79 | EB677043 |
| GH4A-F47 | 328| Alpha-cardiac actin [Mus musculus] | 1.00E-33 | EB677044 |
| GH4A-F160| 271| Skeletal muscle actin mutant [Cyprinus carpio] | 2.00E-23 | EB677045 |
| GH4A-F15 | 550| PAK1 interacting protein 1 [Danio rerio] | 2.00E-37 | EB677046 |
| GH4A-F16 | 499| Putative senescence-associated protein [Pisum sativum] | 4.00E-09 | EB677047 |
| GH4A-F22 | 618| Chymotrypsin B precursor [Gadus morhua] | 1.00E-72 | EB677048 |
| GH4A-F49 | 581| Chymotrypsin B precursor [Gadus morhua] | 3.00E-67 | EB677049 |
| GH4A-F113| 445| Chymotrypsin; prechymotrypsinogen [Gadus morhua] | 3.00E-67 | EB677050 |
| GH4A-F27 | 378| Serum lectin isoform 1 [Verasper variegatus] | 2.00E-12 | EB677051 |
| GH4A-F78 | 278| Serum lectin isoform 1 [Verasper variegatus] | 2.00E-11 | EB677052 |
| GH4A-F99 | 613| Serum lectin isoform 1 [Verasper variegatus] | 4.00E-34 | EB677053 |
| GH4A-F120| 272| Serum lectin isoform 1 [Verasper variegatus] | 4.00E-08 | EB677054 |
| GH4A-F132| 377| Serum lectin isoform 1 [Verasper variegatus] | 1.00E-15 | EB677055 |
| GH4A-F169| 284| Serum lectin isoform 1 [Verasper variegatus] | 1.00E-11 | EB677056 |
| GH4A-F190| 373| Serum lectin isoform 1 [Verasper variegatus] | 2.00E-12 | EB677057 |
| GH4A-F29 | 571| Similar to Ubiquitin-conjugating enzyme E2A [Canis familiaris] | 1.00E-71 | EB677058 |
| GH4A-F81 | 533| Polyubiquitin C [Mus musculus] | 2.00E-87 | EB677059 |
| GH4A-F31 | 545| Fatty acid binding protein 6, ileal (gastrotropin) [Mus musculus] | 2.00E-28 | EB677060 |
| GH4A-F117| 421| Fatty acid binding protein [Epinephelus coioides] | 5.00E-23 | EB677061 |
| GH4A-F122| 439| Fatty acid binding protein [Epinephelus coioides] | 9.00E-46 | EB677062 |
| GH4A-F142| 388| Similar to fatty acid binding protein 6 (bile acid-binding protein) [Gallus gallus] | 1.00E-17 | EB677063 |
| GH4A-F157| 195| Similar to fatty acid binding protein 6 (bile acid-binding protein) [Gallus gallus] | 8.00E-04 | EB677064 |
| GH4A-F181| 272| Fatty acid binding protein 6, ileal (gastrotropin) [Mus musculus] | 6.00E-07 | EB677065 |
| GH4A-F44 | 581| Triosephosphate isomerase B [Xiphophorus maculatus] | 9.00E-68 | EB677066 |
| Clone ID | Bp | Similar to BlastX | BlastX E value | GenBank Acc. no. |
|----------|----|-------------------|----------------|-----------------|
| GH4A-F121 | 520 | Triosephosphate isomerase B [Xiphophorus maculatus] | 9.00E-75 | EB677067 |
| GH4A-F48  | 143 | Cathepsin B [Danio rerio] | 5.00E-19 | EB677068 |
| GH4A-F50  | 431 | Alpha-parvin (actopaxin) [Rattus norvegicus] | 3.00E-34 | EB677066 |
| GH4A-F88  | 545 | Parvin, alpha (actopaxin) [Danio rerio] | 8.00E-84 | EB677070C |
| GH4A-F52  | 141 | Similar to sulfotransferase family 3A, member 1 [Danio rerio] | 3.00E-12 | EB677071 |
| GH4A-F53  | 547 | Ras-related nuclear protein (RAN protein) [Homo sapiens] | 2.00E-74 | EB677072 |
| GH4A-F58  | 506 | Ribosome associated membrane protein 4 [Danio rerio] | 4.00E-27 | EB677073 |
| GH4A-F59  | 461 | Ovomucin alpha-subunit [Gallus gallus] | 1.00E-22 | EB677074 |
| GH4A-F62  | 566 | Ovomucin alpha-subunit [Gallus gallus] | 8.00E-42 | EB677075 |
| GH4A-F67  | 535 | Profilin 2 [Danio rerio] | 2.00E-23 | EB677076 |
| GH4A-F77  | 381 | Aminopeptidase M (Alanine aminopeptidase/C1D13/Aminopeptidase N (APN) | 2.00E-22 | EB677077 |
| GH4A-F84  | 512 | Similar to Transcobalamin I precursor (TCI) [Danio rerio] | 3.00E-08 | EB677078 |
| GH4A-F98  | 358 | Similar to tescalcin [Danio rerio] | 4.00E-22 | EB677075 |
| GH4A-F101 | 491 | Similar to glutathione peroxidase 7 [Bos taurus] | 8.00E-43 | EB67708C |
| GH4A-F127 | 552 | Glutathione peroxidase [Danio rerio] | 4.00E-62 | EB677081 |
| GH4A-F106 | 603 | Similar to FUN14 domain containing 1 [Gallus gallus] | 8.00E-38 | EB677082 |
| GH4A-F107 | 421 | Similar to Probable polypeptide N-acetylgalactosaminyltransferase 8 [Danio rerio] | 7.00E-40 | EB677083 |
| GH4A-F108 | 514 | Proteasome, 26S, non-ATPase regulatory subunit 6 [Danio rerio] | 1.00E-75 | EB677084 |
| GH4A-F189 | 313 | Proteasome 26S ATPase subunit 4 [Mus musculus] | 2.00E-29 | EB677085 |
| GH4A-F111 | 394 | Similar to KAT protein [Danio rerio] | 4.00E-21 | EB677086 |
| GH4A-F112 | 394 | Similar to retinol dehydrogenase 11 (predicted) [Bos taurus] | 5.00E-28 | EB677087 |
| GH4A-F118 | 383 | Similar to beta-hexosaminidase beta chain precursor [Danio rerio] | 2.00E-49 | EB677088 |
| GH4A-F119 | 641 | Annexin A2 [Monopterus albus] | 2.00E-89 | EB677085 |
| GH4A-F136 | 506 | Myosin light chain alkali, smooth-muscle isoform [Danio rerio] | 2.00E-43 | EB67709C |
| GH4A-F137 | 549 | Sec61-alpha [Gadus ogac] | 8.00E-74 | EB677091 |
| GH4A-F138 | 571 | Similar to F-box protein 44 [Danio rerio] | 4.00E-36 | EB677092 |
| GH4A-F140 | 511 | Similar to bA1 globin isoform 4 [Danio rerio] | 4.00E-44 | EB677093 |
| GH4A-F141 | 364 | Gelatinase [Parachthys olivaceus] | 1.00E-09 | EB677094 |
| GH4A-F144 | 489 | Coated vesicle membrane protein [Gallus gallus] | 6.00E-16 | EB677095 |
| GH4A-F154 | 289 | Coated vesicle membrane protein [Danio rerio] | 7.00E-19 | EB677096 |
| GH4A-F150 | 447 | Farnesyl diphosphate synthase (farnesy) pyrophosphate synthetase [Danio rerio] | 4.00E-10 | EB677097 |
| GH4A-F156 | 549 | Prohibitin [Danio rerio] | 3.00E-39 | EB677098 |
| GH4A-F158 | 363 | Bloom's syndrome protein homolog [Gallus gallus] | 5.00E-17 | EB677095 |
| GH4A-F159 | 607 | Oncorhynchus mykiss 14-3-82 protein mRNA, complete cds | 2.00E-35* | EB677100 |
| GH4A-F166 | 498 | Cytochrome P450 3A40 [Oryzias latipes] | 3.00E-47 | EB677101 |
| GH4A-F167 | 533 | Similar to Peroxiredoxin 4 (Prx-IV) (Thioreredoxin peroxidase AO372) [Gallus gallus] | 2.00E-67 | EB677102 |
| GH4A-F177 | 282 | Similar to SAM domain and HD domain-containing protein 1 (Dendritic cell-derived IFNG-inducible protein) (DCIP) (Monocyte protein 5) (MOP-5) [Canis familiaris] | 2.00E-24 | EB677103 |
| GH4A-F182 | 439 | Similar to small nuclear ribonucleoprotein D2 [Danio rerio] | 3.00E-45 | EB677104 |
| GH4A-F185 | 231 | Similar to SH3 domain binding glutamic acid-rich protein like 3 [Danio rerio] | 9.00E-25 | EB677105 |
| GH4A-F188 | 530 | Dendritic cell protein [Danio rerio] | 5.00E-82 | EB677106 |
| GH4A-F60  | 136 | Similar to Mitochondrial 28S ribosomal protein S21 isoform 4 [Bos taurus] | 6.00E-08 | EB677107 |
| GH4A-F163 | 514 | Mitochondrial glycine cleavage system H protein [Danio rerio] | 1.00E-21 | EB677108 |
| GH4A-F152 | 429 | Similar to cytochrome c oxidase subunit VIb precursor [Danio rerio] | 2.00E-21 | EB677105 |
| GH4A-F168 | 303 | NADH dehydrogenase subunit 1 [Theragra chalcogramma] | 7.00E-37 | EB67711C |
| GH4A-F63  | 428 | Similar to cytochrome c oxidase subunit VIb precursor [Danio rerio] | 9.00E-22 | EB677111 |
| GH4A-F100 | 428 | Cytochrome c oxidase subunit I [Gazza squamiventris] | 2.00E-28 | EB677112 |
| GH4A-F187 | 230 | Cytochrome c oxidase subunit VIb precursor [Scombridae gen. sp.] | 1.00E-04 | EB677113 |
| GH4A-F6  | 486 | 60S ribosomal protein L28 [Plectchytus flesus] | 6.00E-48 | EB677114 |
| GH4A-F17  | 197 | Similar to 40S ribosomal protein S26 [Rattus norvegicus] | 4.00E-09 | EB677115 |
| GH4A-F30  | 310 | 40S ribosomal protein S27-1 [Ictalurus punctatus] | 1.00E-29 | EB677116 |
| GH4A-F89  | 534 | 40S ribosomal protein S30 [Hippecampus comeus] | 4.00E-33 | EB677117 |
| GH4A-F123 | 356 | 40S ribosomal protein S10 [Oreochromis mossambicus] | 9.00E-33 | EB677118 |
| GH4A-F145 | 262 | 40S ribosomal protein S28 [Hippecampus comeus] | 5.00E-04 | EB677115 |
| GH4A-F184 | 332 | 40S ribosomal protein S10 [Oreochromis mossambicus] | 2.00E-22 | EB67712C |
| GH4A-F56  | 580 | RpLp0 protein [Danio rerio] | 1.00E-65 | EB677121 |
| GH4A-F134 | 532 | Ribosomal protein large P0-like protein [Sparus aurata] | 4.00E-33 | EB677122 |
Relative standard curves were generated on the basis of cDNA from the calibrator sample diluted in 5-fold or 10-fold dilution steps to cover the expected detection range of target genes and the reference genes. Triplicates of the respective dilution steps were used to determine the standard curves. Results are presented as a normalized calibrated ratio. Hence the concentration ratios for each sample are calibrated to the calibrator sample so that the quantification results are reported as a normalized ratio with the calibrator sample as the denominator:

\[
\text{Relative mRNA level} = \frac{\text{ratio of sample (target/reference)}}{\text{ratio of calibrator (target/reference)}}
\]

Relative mRNA level = ratio of sample (target/reference)/ratio of calibrator (target/reference).

Elongation factor 1 alpha, 18S rRNA and beta-actin were evaluated for use as reference genes. All samples from MI4 were subjected to PCR amplification in the LightCycler with primers specific for the 3 reference genes (Table 2) as described above. By use of the geNorm software (Vandesompele et al., 2002), the most stable reference genes were identified. GeNorm calculates the stability value “M” of the reference genes by

\[M = \sum_{i=1}^{n} \left( \frac{\sigma_i}{\sigma_{max}} \right)^2 \]

where \(\sigma_i\) is the standard deviation for the gene of interest and \(\sigma_{max}\) is the standard deviation of the most unstable gene.
comparing the variation in expression for all the genes. Based on the $M$ value, ELF1a and 18S rRNA were selected as the reference genes in this study.

2.10. Statistics

All tests were carried out two-tailed, with a significance level of 5%. The Shapiro–Wilk $W$ test was used to test conformity with the normal distribution. Differences in the relative expression of selected genes were calculated using the pair wise, student’s $t$-test. Prior to the statistical analyses the relative expression data from each gene was calibrated to the mean of the FM group of the same gene. Hence the mean normalized calibrated ratio of the FM group is 1 for all the genes. Results are presented as mean values with 95% confidence intervals. The statistical analyses were performed using JMP 5.0.1 software package (SAS Institute Inc. Cary, NC, USA). Calculated means±SD for each selected clone is given in Table 4. Fig. 2A and B were made on the basis of statistical reports in the JMP 5.0.1 software package.

3. Results

3.1. Analysis of sequenced clones

Sequencing of 192 randomly picked clones from the Atlantic cod SSH library showed that 33 clones were either too short or consisted of only vector- or poly A-sequences. The remaining 159 clones were subjected to translated blast (NCBI blastx). Of the 159 clones, two virtually translated sequences showed low similarity to other protein sequences, and was shown to be 18S rRNA by nucleotide blast search (NCBI blastn). Another 44 clones gave no or low similarity to other known protein sequences. Virtual translation of the remaining 113 clone sequences showed protein similarities to known sequences from other fish.
species or mammals (Table 3). Blastx E values were between 8.00E−04 and 3.00E−91 as shown in Table 3. The 157 blasted clones were submitted to GenBank, and GenBank accession nos. for the clones are given in Table 3. A grouped overview of the sequenced clones is showed in Fig. 1. The major identified groups were structural proteins, membrane associated proteins, enzymes, and proteins involved in protein metabolism. The majority of these clones (88 out of 113, shaded grey in Table 3) represented new partial sequences that,

![Fig. 2. Relative gene expression of selected clones. Expression of target mRNAs normalized to both 18S rRNA and ELF1a in intestinal tissue of Atlantic cod fed either FM or SBM diets. A representative template from a FM fed fish was chosen as the calibrator. Reverse transcriptase (RT) was performed in duplicates for each individual sample, and template from each RT reaction was run in duplicates in quantitative PCR. An average normalized calibrated ratio for each individual was calculated and is presented as an individual point in the plot. Mean normalized calibrated ratios are presented as the mid-line of each diamond. The vertical range of the diamonds represents 95% confidence intervals. Asterisk * denotes genes that show significantly different expression between dietary treatments (P<0.05), whereas † indicates a trend (P<0.10). APN, aminopeptidase M (Alanyl aminopeptidase)(CD13)/Aminopeptidase N; FABP, fatty acid binding protein; TCI, transcobalamin I precursor; CYP3A40, cytochrome P450 3A40; SEC61, sec61-alpha; F-BOX, F-box protein 44; TM4, putative transmembrane 4 superfamily member protein; RAN, ras-related nuclear protein; ppGaNTase, probable polypeptide N-acetylgalectosaminyltransferase 8; 14-3-3, 14-3-3B2 protein mRNA; GPx, glutathione peroxidise; Prx4, peroxiredoxin 4.](image-url)
to our knowledge, have not previously been described nor annotated for Atlantic cod in GenBank. Predictions of similarity were based on NCBI BLAST searches.

3.2. Gene expression of selected genes analyzed by real-time PCR

Mean normalized calibrated ratios from real-time PCR of 12 clones, selected based on their similarity to genes involved in processes such as protein- and lipid metabolism, growth and antioxidant functions, showed that expression of 4 out of the 12 clones tested were significantly up regulated ($P < 0.05$) in intestine from cod fed SBM compared to intestines from cod fed FM. The 4 clones were: Aminopeptidase M (Alanyl aminopeptidase)(CD13)/Aminopeptidase N (APN, clone ID GH4A-F77, GenBank acc.no. EB677077), transcobalamin I precursor (TCI, clone ID GH4A-F84, GenBank acc.no. EB677078), cytochrome p450 3A40 (CYP3A40, clone ID GH4A-F166, GenBank acc.no. EB677101) and ras-related nuclear protein (RAN, clone ID GH4A-F53, GenBank acc.no. EB677072) (Table 4, Fig. 2Aa, c, d, and Bb). Mean normalized calibrated ratios of TCI, APN, CYP3A40, and RAN were up regulated by factors of 1.48, 2.06, 1.95, and 1.49, respectively, in fish fed SBM compared to fish fed FM (Table 4). Fatty acid
binding protein (FABP, clone ID GH4A-F142, GenBank acc.no. EB677063) showed a trend towards up regulation in SBM fed fish compared to FM fed fish ($P<0.10$) (Table 4 and Fig. 2Ab).

No significant difference in expression was observed for the seven other clones studied: putative transmembrane 4 superfamily member protein (TM4, clone ID GH4A-F103, GenBank acc.no. EB677017), polypeptide N-acetylgalactosaminaltransferase (ppGaNTase, clone ID GH4A-F107, GenBank acc.no. EB677083), Sec61-alpha (SEC61, clone ID GH4A-F137, GenBank acc.no. EB677091), F-box protein 44 (F-BOX, clone ID GH4A-F138, GenBank acc.no. EB677092), glutathione peroxidase (GPx, clone ID GH4A-F127, GenBank acc.no. EB677081), peroxiredoxin 4 (Prx4, clone ID GH4A-F167, GenBank acc.no. EB677102), and 14-3-3B2 protein (14-3-3, clone ID GH4A-F159, GenBank acc.no. EB677100). The average relative expression ratios of all clones evaluated by qPCR, except for GPx, were numerically higher in the SBM group compared with the FM group. For GPx the average relative expression ratio was numerically lower in the SBM group compared to the FM group (Table 4).

4. Discussion

In the present study, we wanted to study the effect of two different diets on gene expression in the intestine of Atlantic cod. One diet was a low-temperature dried (LT) fish meal diet, while in the other diet 24% of crude protein was replaced by SBM. While an inclusion level of 20% have been shown by several studies to induce enteritis in Atlantic salmon distal intestine (van den Ingh et al., 2001; Bakke-McKellep et al., 2000), no such morphological changes have been observed in Atlantic cod fed SBM (Refstie et al., 2006b). However, 24% SBM have been shown to reduce digestibility of both amino acid and lipid metabolism in Atlantic cod (Refstie et al., 2006a).

The clones we obtained from suppression subtractive hybridization (SSH) were derived from a subtraction between two fish fed each of the experimental diets described above. A number of the clones may represent individual differences in gene expression between the two individuals and not differences between dietary groups. Thus 12 clones were selected for real-time PCR analyses of gene expression in six fish from each dietary group to further study if the differential expressed genes could be assigned to dietary treatment. The significantly up regulated clones were APN, TCI, CYP3A40, and RAN.

Aminopeptidase N (APN or CD13) is a transmembrane, zinc-containing ectoenzyme expressed in a wide variety of tissues and cells like brush borders of kidney and small intestine, hepatocytes, osteoclasts, endomtrial cells, fibroblasts, endothelial cells, bone marrow stromal cells, neuronal synaptic membranes, as well as in several cells of the myelomonocytic lineage (Shipp and Look, 1993; Riemann et al., 1993). The function of APN varies with its location. In intestinal brush border APN is involved in terminal degradation of basic or neutral amino acids, as well as processing of the neuropeptides and peptide hormones involved in the regulation of growth and differentiation in the gastrointestinal tract (Riemann et al., 1993; Carl-McGrath et al., 2004). In mammals/humans, expression of APN has been shown to be dys-regulated in inflammatory diseases and cancers, and APN has also been shown to serve as a receptor for coronaviruses (Bauvois and Dauzonne, 2006). In monocytes, expression of APN has been shown to be regulated by cytokines like TGF-β and IL-10 (Kehlen et al., 2004), and IFNγ (Gabrilovac et al., 2005). Likewise, in fibroblasts APN has also been shown to up regulated by both cytokines and glucocorticoids (Sorrell et al., 2003). In comparison, reports on regulation of APN gene expression in intestinal epithelial cells are scarce. Although separate promoters control transcription of APN in myeloid and intestinal epithelial cells, both aminopeptidase N transcripts encode the same polypeptide (Shapiro et al., 1991). In human fetus and children tributyrin and butyric acid have been shown to inhibit the activity of APN (Kushak and Winter, 1999). Butyric acid may be produced by fermentation of dietary fiber by intestinal microflora, and replacing FM with SBM increases the amount of intestinal fiber available to the microflora. The Atlantic cod gut microbiota has been shown to be sensitive to dietary changes (Ringø et al., 2006; Refstie et al., 2006b). This may indicate that mRNA expression of APN is up regulated to compensate for a putative inhibition caused by increased amounts of butyric acid produced by intestinal microflora.

A clone showing similarity to transcobalamin I precursor (TCI) was also significantly up regulated in SBM fed fish compared to FM fed fish. Transcobalamins (I, II, and III) are transporters for cobalamin (cbl)/vitamin B12 in plasma (Hall, 1975). TCI and TCIII are now commonly referred to as haptocorrin (HC) (Afman et al., 2001). In mammals, HC (TCI and TCIII) binds vitamin B12 in saliva, and upon proteolysis in the duodenum vitamin B12 is transferred to intrinsic factor (IF), and this complex is taken up in the distal ileum via the IF-vitamin B12 receptor. Vitamin B12 is subsequently bound to TCI and released into circulation for transport and uptake into various tissues (Afman et al., 2001; Wuerges et al., 2006).
Vitamin B12 is synthesized exclusively by microorganisms in one of the most complex biosynthetic pathways found in nature (Raux et al., 2000), and intestinal absorption, blood transport, and delivery to tissues is therefore essential in mammals. Two derivatives of vitamin B12 (methylcobalamin and adenosylcobalamin) act as coenzymes for methionine synthase and methylmalonyl-CoA mutase, respectively (Wolters et al., 2004; Wuerges et al., 2006). Information about vitamin B12 transport in fish is scarce. In GenBank, partial mRNA sequences for transcobalamin I and II precursors in killifish have been submitted (acc.nos. CV822982, CN984169, and more). In channel catfish, vitamin B12 synthesis and direct absorption from the intestine have been shown (Limsuwan and Lovell, 1981).

As mentioned above, SBM containing diets were shown to change the gut microbiota in Atlantic cod (Ringø et al., 2006; Refstie et al., 2006b). Vitamin B12 is made only by members of the Archea and certain eubacteria (Raux et al., 2000), some of which may be present in the cod intestinal microflora in FM fed fish, and reduced in or absent from the intestine of SBM fed fish, causing a reduction in available vitamin B12.

Cytochrome P450 (CYP) is a superfamily of heme containing monooxygenases which are involved in oxidative metabolism of many drugs, environmental chemicals and endogenous compounds (Guengerich, 1991). In humans, CYP3A isoymes are the most abundant CYP proteins found in liver and the small intestine (Burk and Wojnowski, 2004). These isoforms have been shown to be involved in the termination of the action of steroid hormones, detoxification of bile acids, elimination of xenobiotics and activation of several potent carcinogens (Werck-Reichhart and Feyereisen, 2000). The expression of intestinal CYP3A in fish has been less studied. However, the intestine has been shown to be the major extrahepatic site of expression of various CYP3A isoforms in rainbow trout (Lee et al., 1998, 2001), killifish (Hegelund and Celandier, 2003) and zebrafish (Tseng et al., 2005). The expression of CYP3A40 mRNA has been identified in liver of the fish species medaka (Kullman and Hinton, 2001), but information regarding distribution and induction of this isoform is scarce. Soy contains several phytochemicals such as isoflavons and phytosterols that could potentially induce the expression of CYP3A40 mRNA in the SBM group of the present study. The diet has been indicated to influence the amount of CYP3A-like proteins in the intestine of channel catfish (James et al., 2005) but is not known if this is followed by a concurrent change in mRNA expression. In mouse dexamethasone has been shown to increase the expression of five different isoforms of CYP3A at both the mRNA and protein level (Zhang et al., 2003).

Another clone that was up regulated in cod fed SBM was RAN, a gene encoding a small GTPase which is involved in the control of the cell cycle. The gene exerts its effects through the regulation of nucleocytoplasmic transport, mitotic spindle organization, and nuclear envelope formation (Gruss and Vernos, 2004). Gastrointestinal growth has been reported in cod fed SBM and this could direct the intestine towards higher expression levels of RAN mRNA as indicated in the present study.

Fatty acid binding protein (FABP) showed a trend towards up regulation in SBM fed fish compared to FM fed fish. Fatty acid binding proteins (FABPs) may be divided in cytosolic FAPBs and FABPs associated with the plasma membrane. Cytosolic FABPs (FABPs) can be defined as transport proteins (Storch and Thumser, 2000). FABPs have been shown to facilitate fatty acid (FA) uptake and transport by increasing rate of dissociation from the plasma membrane by enhancing the aqueous solubility of FAs (Vork et al., 1993), or improve transfer to acceptor membranes (Hsu and Storch, 1996) as reviewed by Chmurzynska (2006). In regulation of lipid metabolism, FAs can act as signalling molecules triggering activation of transcription factors. Several FABPs have been shown to interact with members of the peroxisome proliferator-activated receptors (PPAR) family of nuclear receptors (Wolfum et al., 2001; Tan et al., 2002), which are known to regulate the transcription of many genes involved in lipid metabolism (Gregoire et al., 1998; Chmurzynska, 2006). Intestinal FABP (I-FABP) gene expression have been shown to be modulated directly by FA (Halden et al., 1994; Le et al., 1997; Poirier et al., 1997), and by the gut regulatory peptide PYY (Halden and Aponte, 1997; Aponte, 2002). In addition, epidermal growth factor (EGF) has been shown to down regulate expression of I-FABP in Caco2 cells (Darimont et al., 1999). Lipid digestibility was significantly reduced in cod fed the SBM diet (Refstie et al., 2006a). Hence the concentration of FAs in the intestinal content was increased. A higher level of FAs in intestinal content could potentially induce an up regulation of FABP in the intestinal wall.

Both Prx4 and GPx are antioxidant enzymes, protecting against reactive oxidative species (ROS) (Fujii and Ikeda, 2002; Valko et al., 2006). Expression of neither clone (Prx4 AND GPx) was significantly affected in cod fed SBM compared to cod fed FM, indicating no oxidative stress in response to the SBM diet.

In summary, we obtained 159 clones from a SSH subtraction, of which 113 clones showed good similarities with identified sequences from different fish species
or mammals. The majority of the clones represented new sequences in Atlantic cod that to our knowledge have not previously been described or annotated in GenBank. By quantitative real-time PCR we showed a significant up regulation in mRNA expression of four different clones showing similarity to aminopeptidase N (terminal intestinal degradation of amino acids), transcobalamin I precursor (vitamin B12 transport), cytochrome P450 3A40 (oxidative metabolism of drugs, environmental chemicals and endogenous compounds), and ras-related nuclear protein (regulation of cell cycle) in intestine of cod fed SBM compared to cod fed FM. A definite trend towards up regulation of a clone with similarity to fatty acid binding protein (FABP) in cod fed SBM was also observed. No significant difference in expression was observed for the seven other clones studied: transmembrane 4 superfamily protein member (TM4), polypeptide N-acetylgalactoaminotransferase (ppGaNTase), glutathione peroxidase (GPx), peroxiredoxin 4 (Prx4), SEC61, F-BOX, and 14-3-3.

Acknowledgements

The authors want to acknowledge the skilful technical assistance of Ellen Koren Hage and Gunn C. Østby at APC. We are grateful to Hamlet Protein and to Nutreco Aquaculture Research Centre for supplying feed ingredients and manufacturing feeds, and also to AKVAFORSK for making the experimental fish available for our studies. Financial support for the study was provided by the Aquaculture Protein Centre (APC), CoE; grant no 145949/120 from the Norwegian Research Council.

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