Impact of Camelina-Containing Diets on Hepatic Transcript Expression of Atlantic Cod and Atlantic Salmon: Functional Genomics Approaches

by

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ABSTRACT

The finfish aquaculture industry relies heavily on fish oil (FO) and fish meal (FM), two marine-derived ingredients, for the production of aquafeeds. The demands for FO and FM to meet the expansion of the aquaculture industry will exceed the supply from forage fisheries (e.g. herring, anchovies, capelin) which are at a static phase. Therefore, there is a need to find alternatives such as plant-derived oils and proteins that might replace FO and FM, respectively, in aquafeeds. The products from *Camelina sativa*, camelina oil (CO) and camelina meal (CM), are currently considered viable options for sustainable aquafeed ingredients.

Two feeding experiments were conducted to determine the effectiveness of CO as an alternative to FO in the diets for two economically important aquaculture species: Atlantic cod (*Gadus morhua*) and Atlantic salmon (*Salmo salar*). The effect of a low level inclusion of CM combined with CO in the salmon diets was also evaluated. Atlantic cod growth (e.g. weight gain) was significantly reduced by replacing 100% FO with CO (100CO) in the diet after a 13-week feeding trial. In contrast, a 100CO diet did not significantly alter the growth performance of Atlantic salmon after a 16-week feeding trial. However, salmon fed the other three camelina-containing diets [100CO + solvent-extracted FM (100COSEFM), 100CO + 10% CM (100CO10CM), or 100CO + SEFM + 10CM (100COSEFM10CM)], exhibited significantly lower weight gain or weight-specific growth rate (SGR) compared to control fish.

In order to study the effect of replacing FO with CO in diets for farmed Atlantic cod and Atlantic salmon on long chain polyunsaturated fatty acids (LC-PUFA)

biosynthesis, Atlantic cod *fatty acyl elongase* (*elovl*) gene family members were characterized at the cDNA level and QPCR assays for genes involved in LC-PUFA biosynthesis for both species were developed. The QPCR-based transcript expression analyses (Chapters 2 and 3) suggest that low LC-PUFA accompanied with high C_{18} PUFA in the diet may cause the up-regulation of *fatty acyl elongase* and *desaturase* genes, critical for controlling the LC-PUFA biosynthetic pathway, in Atlantic cod (*elovl5* and *fadsd6*) and Atlantic salmon (*elovl2*, *fadsd5* and *fadsd6a*). In addition, a 44K salmonid microarray was used to study the impact of a camelina-containing diet on salmon liver global gene expression, and a set of microarray-identified camelina-responsive biomarker genes was validated using QPCR (Chapter 3). These functional genomics studies have contributed significant resources (e.g. cDNA sequences and QPCR assays) to the cod and salmonid research communities, and provide valuable information for the development of novel aquafeeds using camelina products.

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LIST OF ABBREVIATIONS

Δ	Delta
°C	Degree centigrade
µL	Microlitre
ω	Omega
AA	Amino acid
ACOA	Atlantic Canada Opportunities Agency
aRNA	Anti-sense amplified RNA
actb	*β-actin*
AFI	Apparent feed intake
AIF	Atlantic Innovation Fund
ALA	α-linolenic acid (18:3ω3)
ANOVA	Analysis of variance
ARA	Arachidonic acid (20:4ω6)
bar	Bile acid receptor
BLAST	Basic local alignment search tool
bp	Base pair
btg1	*B-cell translocation gene 1-like*
CF	Condition factor
CGP	Atlantic Cod genomics and Broodstock Development Project
cGRASP	consortium for Genomic Research on All Salmonids Project
clra	*C type lectin receptor A*
CM	Camelina meal
CO	Camelina oil
contig	Contiguous sequence
cpt1	*Carnitine palmitoyltransferase 1*
C_T	Threshold cycle
DHA	Docosahexaenoic acid (22:6ω3)
dnph1	*2'-deoxynucleoside 5'-phosphate N-hydrolase 1*
eef1α	*Eukaryotic elongation factor 1α*
elovl	*Elongation of very long fatty acids/fatty acyl elongase*
EPA	Eicosapentaenoic acid (20:5ω3)
EST	Expressed sequence tag
EtBr	Ethidium bromide

facl4	*Long chain fatty acyl-CoA ligase 4*
fadsd	*Fatty acyl deseaturase*
FCR	Feed conversion ratio
FDR	False discovery rate
Fig	Figure
FM	Fish meal
FO	Fish oil
g	Grams
g	Gravitational force
GaP	Genomics and Proteomics Facility
GO	Gene ontology
GOI	Gene of interest
GSP	Gene-specific primer
HIS	Hepatosomatic index
Hr	Hour
ID	Identity
IGF	Insulin-like growth factor
IGFBP	Insulin-like growth factor binding protein
JBARB	Dr. Joe Brown Aquatic Research Building
klf9	*kruppel-like factor 9*
L	Litre
LB	Luria broth
LC-PUFA	Long chain polyunsaturated fatty acid
lect-2	*Leukocyte cell-derived chemotaxin 2 precursor*
LNA	Linoleic acid (18:2ω6)
m	Metre
mg	Milligram
min	Minute
mL	Millilitre
M-MLV	Moloney murine leukemia virus
mRNA	Messenger RNA
mTOR	Mammalian target of rapamycin
MUFA	Monounsaturated fatty acid
MUN	Memorial University of Newfoundland
NCBI	National Center for Biotechnology Information
NTC	Non-template control

oligo	Oligonucleotide
ORF	Open reading frame
OSC	Ocean Sciences Centre
p	P-value
pcb	*Pyruvate carboxylase*
PCR	Polymerase chain reaction
PMT	Photomultiplier tube
QC	Quality check
QPCR	Quantitative reverse transcription-polymerase chain reaction
r^2	Correlation coefficient
RACE	Rapid amplification of cDNA ends
rpl32	*60S ribosomal protein 32*
rplp1	*60S acidic ribosomal protein P1*
RQ	Relative quantity
RT-PCR	Reverse transcription-polymerase chain reaction
SAM	Significance analysis of microarrays
SD	Standard deviation of the mean
SE	Standard error of the mean
SEFM	Solvent extracted fish meal
SGR	Specific growth rate
TMS	Tricaine-methane-sulfonate
U	Units
UTR	Untranslated region
VLC-FA	Very long chain fatty acid
VSI	Visceral somatic index

LIST OF APPENDICES

CO-AUTHORSHIP STATEMENT

The research described in this thesis was carried out by Xi Xue, with guidance from Dr. Matthew L. Rise. Xi Xue was responsible for conducting experiments, data collection and analysis, and thesis writing. In addition, the following people have contributed to the research with intellectual and labour inputs.

Charles Y. Feng[a] provided various aspect of training through the study including RNA extraction and purification, RACE PCR, cloning, sequencing, sequence assembly, sequence analysis and QPCR experimental procedures; in addition, he edited and provided comments on Chapter 2 of the thesis. Dr. Marije Booman[a] and Dr. Tiago S. Hori[a] provided training in microarray experimental procedures and bioinformatics, and edited and provided comments on Chapter 3 of the thesis. Dr. Stefanie M. Hixson[a] conducted multivariate statistical analyses to compare hepatic fatty acyl desaturase and elongase transcript expression data with fatty acid data from the same fish involved in Chapter 2 and 3, and wrote the corresponding sections in two manuscripts that will be submitted to peer-reviewed journals; these statistical analyses are not included in the current thesis. Dr. Kim Johnstone[b] was involved in the design of all fish feeding trials, provided training on constructing molecular phylogenetic trees, and edited and provided comments on Chapter 2 of the thesis. Dr. Christopher C. Parrish[a] contributed to the fish feeding experimental design, edited and provided comments on the thesis, and also provided valuable suggestions/comments on the multivariate statistics that Stefanie conducted. Dr. Derek M. Anderson[c] contributed to the fish feeding experimental design, and the formulation and production of the diets used in all feeding experiments. Dr.

Matthew L. Rise[a]: supervised the entire study; and edited and provided comments on the thesis.

Authorship for a future publication arising from **Chapter 2** is Xi Xue, Charles Y. Feng, Stefanie M. Hixson, Kim Johnstone, Derek M. Anderson, Christopher C. Parrish, Matthew L. Rise. This manuscript has been submitted to the journal *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*. Authorship for a future publication arising from **Chapter 3** is Xi Xue, Stefanie M. Hixson, Tiago S. Hori, Marije Booman, Christopher C. Parrish, Derek M. Anderson, Matthew L. Rise. This manuscript is being prepared for submission to the journal *Physiological Genomics*.

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CHAPTER 1: General Introduction

1.1 Introduction

The flesh of finfish provides important components of human diets (Morais et al., 2011). It can provide not only high quality proteins, vitamins, and minerals, but is also the main source of essential ω3 long chain polyunsaturated fatty acids ($\geq C_{20}$; LC-PUFA) in human diets (Morais et al., 2011). Dietary LC-PUFA, including eicosapentaenoic acid (EPA; 20:5ω3) and docosahexaenoic acid (DHA; 22:6ω3), can benefit human health by preventing a number of cardiovascular and inflammatory diseases (Calder and Yaqoob, 2009). Worldwide demand for seafood for human consumption with a large contribution from aquaculture (~50% in 2008), continues to climb (FAO, 2009; Bell et al., 2010). Consequently, there is great potential for the aquaculture industry to expand.

The finfish aquaculture industry (e.g. salmonids and cod) relies heavily on fish meal (FM) and fish oil (FO), two marine-derived ingredients, for the production of aquafeeds (Torstensen et al., 2008; Morais et al., 2011). The demands for FM and FO to meet the expansion of the aquaculture industry will exceed the supply from traditional fisheries (e.g. herring, anchovies, capelin) which are at a static phase (FAO, 2009). This will result in increased prices of FM and FO, and potentially threaten the sustainability of fish farming (Espe et al., 2006; Tacon and Metian, 2008). Looking for plant-derived proteins and oils that might replace FM and FO, respectively, would be a way to make the aquaculture industry more sustainable.

The products from *Camelina sativa* are currently considered a viable option as aquafeed components due to the relatively high seed oil content and high crude protein in the meal (Acamovic et al., 1999). However, many aspects of the potential use of camelina

products in fish diet formulations must be examined. My research, as part of a collaborative project - Developing Camelina as the Next Canadian Oilseed (referred henceforth as The Camelina Project; http://www.genomeatlantic.ca/), aims to determine nutritional value of camelina-containing diets in terms of fish growth and to assess the effects of camelina-containing diets on the hepatic gene expression responses of two economically important aquaculture species: Atlantic cod (*Gadus morhua*) and Atlantic salmon (*Salmo salar*), using functional genomics tools such as DNA microarrays and quantitative reverse transcription - polymerase chain reaction (QPCR). Furthermore, the data provided in this research could potentially guide future work on the development of optimal fish diet formulations including camelina products.

1.2 Demand for FO and FM in aquafeeds

The aquaculture of finfish, especially carnivorous fish (e.g. Atlantic cod and Atlantic salmon), has traditionally relied heavily on FM and FO in diet formulation (Espe et al., 2006; Torstensen et al., 2008; Bell et al., 2010). The high quality (e.g. essential amino acids and fatty acids) and palatability make FM and FO the most preferred ingredients in aquafeed (Thiessen et al., 2003; Espe et al., 2006). On the other hand, due to the increasing demand for seafood (largely contributed from fish farming), aquaculture production has dramatically increased in the past few years (Bell et al., 2010). For example, the production of farmed Atlantic salmon, a commonly cultured species, increased from approximately 0.9 million metric tonnes in 2000 to 1.5 million metric tonnes in 2009 (FAO, 2012). Collectively, this evidence suggests that finfish aquaculture production has great potential to expand in the future. However, global yields of FM and

FO from traditional fisheries are not likely to increase (Bell et al., 2010). The excessive demand on these ingredients by the aquaculture industry will result in increased prices of FM and FO and is a major concern about fish farming sustainability (Espe et al., 2006; Tacon and Metian, 2008). Therefore, the need to find alternatives to FM and FO for aquafeeds has been recognized as one of the most important areas of research in aquaculture (Bell et al., 2010).

1.3 *Camelina sativa* and its products

One of the plants that could potentially provide an opportunity to meet the demand for plant-based ingredients in the aquaculture feed industry is *Camelina sativa*. Camelina, also known by its common name "false flax", is an ancient member of the Brassicaceae family and has been grown extensively in parts of Europe (e.g. France, Belgium, Holland, Russia and Sweden) for human consumption and fuel oil (Zubr, 1997; Zubr, 2003). Commercial cultivation of camelina in Canada has occurred since the 1990s (Francis and Warwick, 2009).

As an oilseed crop, camelina has an oil content of about 40%, which is especially rich in ω3 fatty acids (Acamovic et al., 1999). The levels of PUFA such as linoleic acid (LNA; 18:2ω6) and α-linolenic acid (ALA; 18:3ω3) in camelina oil (CO) are about 15% and 40% respectively (Zubr, 1997). The fatty acid profile of CO is distinct from that of rapeseed, soybean and flaxseed oils. CO has the highest ALA content among those vegetable oils (Zubr, 2003). Compared to FO, however, vegetable oil including CO lacks the highly bioactive LC-PUFA, such as DHA and EPA (Glencross, 2009).

Camelina has great potential in the bioenergy sector since it can be utilized as a green fuel for jets and other engines (Fröhlich and Rice, 2005; Shonnard et al., 2010). Some by-products of camelina from the oil extraction process, such as the seed meal, may be used ultimately in the aquaculture and livestock (e.g. cow, pig, chicken) feed industries. Camelina meal (CM) has a crude protein level of approximately 45%, which is similar to canola and other rapeseed meal (Acamovic et al., 1999; Frame et al., 2007). Essential amino acids (i.e. amino acids cannot be synthesized *de novo*) are particularly important when plant meals are used as fish feed ingredients. There are at least 18 amino acids found in camelina seed, and 9 of them are essential (Zubr, 2003). The most dominant essential amino acid in camelina seed is arginine (8.2%), while the content of some other essential amino acids (e.g. glycine, proline, and valine) exceed 5.0%. The amino acid profile of camelina seed is very similar to that of rapeseed and soybean (Zubr, 2003). The plant meals are naturally rich in carbohydrates such as oligosaccharides and non-starch polysaccharides (Hemre et al., 2002; Leaver et al., 2008a; Panserat and Kaushik, 2010). Previous study reported that the carbohydrate content in camelina seed; the mean fructose content was 0.04%, stachyose 0.36%, glucose 0.42%, raffinose 0.64%, pectin 0.96%, starch 1.21%, sucrose 5.5%, mucilage 6.7%, lignin 7.4%, and crude fibre 12.8% (Zubr, 2010). In defatted soybean meal, the content of the three main oligosaccharides (i.e. stachyose, raffinose and sucrose) were 5-6%, 1-2% and 6-7%, respectively (Francis et al., 2001). It is worth noting that a disadvantage for the use of most *brassica* crops (e.g. camelina and rapeseed) as animal feed ingredients is the content of glucosinolates (Acamovic et al., 1999; Francis et al., 2001). Glucosinolates may negatively affect birds; decreases in weight gain and poor feed conversion were observed

in one study of young turkey as the amount of CM increased in the diets (Frame et al., 2007). In another study, the ingestion of very low amounts of glucosinolates (1.4 μmol/g diet) led to a depression of growth in rainbow trout (*Oncorhynchus mykiss*) (Francis et al., 2001).

1.4 FO and FM substitutions in aquafeeds

Fish nutritionists have tried to incorporate different kinds of terrestrial plant proteins and oils to substitute FM and FO due to the relatively low cost and sustainability of these plant products compared to traditional ingredients such as FM and FO (Gomes et al., 1995; Bell et al., 2001; Thiessen et al., 2003; Espe et al., 2006; Torstensen et al., 2008; Bell et al., 2010). A number of researchers have made progress on FM substitutions with soybean meal (Vilhelmsson et al., 2004; Torstensen et al., 2008), canola meal (Thiessen et al., 2003), rapeseed meal (Vilhelmsson et al., 2004), and FO replacement with linseed oil (Torstensen et al., 2008), canola oil (Miller et al., 2007) and rapeseed oil (Jordal et al., 2005) for a range of fish species including Atlantic cod and Atlantic salmon.

1.4.1 The effect of plant oils on fish growth, physiology and gene expression

Dietary lipid and fatty acids are the predominant source of energy for teleost fish (Leaver et al., 2008a). Also, essential fatty acids including LC-PUFA (e.g. DHA and EPA) in dietary oils play important roles in several physiological functions in fish including cell synthesis, neural development, immune function and reproduction (Glencross, 2009). Hence, any dietary alternative to FO must satisfy the nutritional needs of the fish (e.g. supplying lipids and essential fatty acids used for optimal growth) without

having a negative impact on the fish health or quality of the flesh. Studies have demonstrated that vegetable oil (either singly or as blends) which is low in LC-PUFA and high in C_{18} PUFA such as ALA and LNA, can be used to replace up to 100% of FO without negatively influencing growth in salmonids and marine fish including cod (Bell et al., 2001; Torstensen et al., 2005; Bell et al., 2010; Morais et al., 2012; Hixson et al., 2013). However, as a result of replacing FO, the flesh ω3 LC-PUFA content in fish can be reduced significantly if FO is replaced by vegetable oil completely (Bell et al., 2010; Morais et al., 2012).

Replacement of FO with vegetable oil in fish diets has been suggested to affect lipogenesis in teleosts (Leaver et al., 2008b). For example, Atlantic salmon fed a FO-based diet showed lower triacylglycerol levels in liver compared to vegetable oil-fed fish (Jordal et al., 2007). Vegetable oil inclusion in gilthead seabream (*Sparus aurata*) diets resulted in significantly increased hepatic lipid deposition, compared to fish fed control diet (reviewed in Leaver et al., 2008a). Other lipid metabolic processes in salmonids, for example, fatty acid β-oxidation, cholesterol and fatty acid biosyntheses, were also linked with the intake of the plant oil based diets (Leaver et al., 2008b; Panserat et al., 2009). Beta-oxidation of fatty acids for energy production can be achieved in two different organelles in the cells, mitochondria and peroxisomes. The carnitine palmitoyltransferase (CPT) system is a limiting step in the mitochondrial β-oxidation of fatty acids (reviewed in Leaver et al., 2008a). The transcript encoding Cpt2 (also known as Cpt-II) was shown to be significantly down-regulated in the liver of Atlantic salmon fed rapeseed oil diet compared to FO-based diet, indicating lower β-oxidation in fish fed plant oil diet (Jordal et al., 2005). Moreover, plant oil-containing diets induced several genes (e.g. *squalene*

epoxidase and *lathosterol oxidase*) involved in the cholesterol biosynthetic pathway (Leaver et al., 2008b).

In vertebrates, biosynthesis of LC-PUFA can be achieved by sequential desaturation and elongation of FA precursors such as LNA and ALA (reviewed in Leaver et al., 2008a). Genes involved in the pathway are known to be regulated by vegetable oil (reviewed in Leaver et al., 2008a). Particularly, *delta-5 fatty acyl desaturase* (*fadsd5*) and *fatty acyl elongase* [*elongation of very long chain fatty acids* (*elovl*); e.g. *elovl2* and *elovl5*] genes are often up-regulated in the liver of Atlantic salmon fed vegetable oil (e.g. rapeseed oil), and often down-regulated with high levels of ω3 LC-PUFA content in the diets (reviewed in Leaver et al., 2008a). To what extent fish can convert LNA and ALA into LC-PUFA varies when fed diets containing plant oil. In general, freshwater fish are capable of synthesizing DHA and EPA from ALA (Santigosa et al., 2011). In contrast, most marine fish including Atlantic cod are inefficient in the desaturation/elongation of FA precursors that are essential for producing EPA and DHA (Santigosa et al., 2011). This may be due to apparent deficiencies in one or more enzymes in the biosynthesis pathway (Ghioni et al., 1999; Tocher et al., 2006). Hence, there was particular interest in this project to study the expression of genes involved in fatty acid biosynthesis in Atlantic cod and Atlantic salmon fed CO-containing diets. Prior to the current study, the only fully characterized Atlantic cod fatty acyl elongase had been Elovl5, and this enzyme showed low elongase activity (Agaba et al., 2005). In contrast, in Atlantic salmon, *elovl2*, *elovl4*, two *elovl5* paralogues were functionally characterized previously; all of these genes except *elovl4* have been studied at the transcript expression level in response to different

dietary treatments containing plant oils (Agaba et al., 2005; Morais et al., 2009; Carmona-Antoñanzas et al., 2011).

The changes in the fatty acid profiles of diets due to the replacement of FO by vegetable oils may alter fish metabolism, which could potentially affect fish health, immune function and pathogen resistance (Montero et al., 2003; Mourente et al., 2005). In addition, the inclusion of vegetable oils often alters the ω3/ω6 fatty acid ratio in the diet. A well-balanced ω3/ω6 ratio is required for the good health of animals as it will affect animal immune cell function and structure, cell signalling and eicosanoid production (Yaqoob and Calder, 2007; Montero et al., 2010). There is a large body of research on the effect of dietary vegetable oils on fish immunity at different levels including the fatty acid composition of immune cells (Thompson et al., 1996; Farndale et al., 1999), immune cell function (Sheldon and Blazer, 1991; Kiron et al., 1995), alternative complement activity (Montero et al., 2003) and eicosanoid production (Mourente et al., 2007). However, the mechanisms involved in the modulation of fish immune systems by variations in dietary fatty acids, and the genes involved in these mechanisms, are poorly understood. Recently, the transcript expression of genes involved in stress response (*steroidogenic acute regulatory protein*, *glucocorticoid receptor* and *phosphoenolpyruvate carboxykinase*) were reported to be significantly reduced in gilthead seabream larvae fed arachidonic acid (ARA; 20:4ω6) containing diets with ARA level above 0.4% (Alves Martins et al., 2012). Another study directly measured the effect of dietary vegetable oils on the transcript expression of genes related to inflammation (*tumor necrosis factor-α* and *interleukin 1-β*) in gilthead seabream after exposure to a bacterial pathegen (*Photobacterium damselae sp. piscicida*) (Montero et al., 2010). Both

of the pro-inflammatory genes studied by Montero and colleagues (2010) were up-regulated in the intestine and head kidney of fish fed complete replacement diets.

1.4.2 The effect of plant meals on fish growth, physiology and gene expression

Sustainable alternative protein sources with crude protein content of 20 to 50%, for replacement of FM, have been incorporated in aquafeeds for finfish aquaculture (Torstensen et al., 2008). However, there are several potential problems associated with using such plant protein-based diets (Torstensen et al., 2008). For example, the essential amino acid profile in plant protein sources differs from FM, resulting in the need to mix different protein sources or add certain crystalline amino acids not represented in plant-based sources (or present in inadequate amounts), to meet the nutritional requirements of fish fed plant-based diets (Espe et al., 2006; Torstensen et al., 2008). The carbohydrate fraction of plant meal, as well as anti-nutritional factors, are additional constraints that may affect digestion and utilization of nutrients by fish when plant protein sources are included in their diets (Francis et al., 2001; Hemre et al., 2002; Krogdahl et al., 2005). While some early work in rainbow trout reported that no reduction of fish growth was found for diet consisting of 100% plant proteins when replacing fish meal with the addition of selected amino acids (Rodehutscord et al., 1995), other studies showed that the replacement of fish meal with plant meals (> 50%) reduced the growth of trout (Gomes et al., 1995; Kaushik et al., 1995). High plant protein (> 60% replacement of FM) diets affected growth performance of Atlantic salmon; however, this may have been caused by reduced feed intake and intestinal disorders (Baeverfjord and Krogdahl, 1996; Espe et al., 2006; Hevrøy et al., 2008).

The role of amino acids in the regulation of genes such as those involved in the control of growth, amino acid metabolism and protein turnover has been recognized especially in humans (Kimball and Jefferson, 2006). For example, the mammalian target of rapamycin (mTOR), involved in the regulation of initiation of translation and protein synthesis, is modulated by dietary amino acids either directly or indirectly through insulin in mammals (Kimball and Jefferson, 2006; Panserat and Kaushik, 2010). In rainbow trout hepatocytes, the branched-chain amino acid leucine combined with insulin can activate the TOR pathway, and reduce *glucose-6-phosphatase* gene expression which is involved in gluconeogenesis (Lansard et al., 2011). FM substitution by plant protein is known to affect the somatotropic axis in gilthead seabream, which plays a key role in the control of the regulation of metabolism and growth (Gómez-Requeni et al., 2004). In vertebrates, the somatotropic axis consists of growth hormone, insulin-like growth factors, their associated carrier proteins, and receptors (Renaville et al., 2002; Wood et al., 2005). Gilthead seabream fed a plant-based diet experienced decreased growth, as well as the down-regulation of hepatic transcripts encoding growth hormone receptor and insulin-like growth factor 1 (Gómez-Requeni et al., 2004).

Plant protein sources are naturally rich in carbohydrates, however, the utilization of dietary carbohydrates by fish seems limited, particularly in carnivorous species (Hemre et al., 2002). A large number of studies have been focused on the impact of plant-containing diets on mechanisms involved in glucose homeostasis in finfish. Only low levels of carbohydrates in fish diets showed improved feed utilization and protein retention in rainbow trout, Atlantic cod, European eel (*Anguilla anguilla*) and Atlantic salmon, resulting from a protein sparing effect (Hemre et al., 2002). The enhanced growth

and protein sparing are thought to be due to the use of glucose as an oxidative substrate for nervous tissue and blood cells, and the diversion of amino acids away from oxidative pathways (Hemre et al., 2002).

Last but not least, the presence of anti-nutritional factors in plant materials, such as protease inhibitors, lectins, oligosaccharides, phytates and glucosinolates, is another challenge in the development of plant-based aquafeeds (Vilhelmsson et al., 2004). Some fish species are known to be sensitive to the presence of anti-nutrients. For example, diets containing 10 to 30% of dietary soybean meal caused inflammation and morphological changes in distal intestine of Atlantic salmon in a 6-week feeding experiment (Baeverfjord and Krogdahl, 1996). In more recent studies, the expression of some inflammatory biomarker genes was investigated during the development of soybean meal-induced enteritis in Atlantic salmon (Lilleeng et al., 2009; Skugor et al., 2011). For instance, both *transforming growth factor β* and *interferon-γ inducible lysosomal thiol reductase* transcripts were significantly down-regulated in the distal intestine of salmon fed a soybean-containing diet after 3 days feeding (Lilleeng et al., 2009). Consequently, an inflammatory response in fish fed soybean meal-containing diets may result in a higher susceptibility to bacterial diseases (Krogdahl et al., 2000).

1.5 Nutrigenomic approach to study hepatic gene expression response to plant-based diets

DNA microarrays have been shown to be useful tools for the identification of genes that are differentially expressed in fish fed altered diets, for example those with FO or FM replaced by plant-based ingredients (Jordal et al., 2005; Leaver et al., 2008b;

Panserat et al., 2008; Morais et al., 2011). All of these studies focused on the gene expression changes in fish liver since this is the main organ involved in metabolizing carbohydrates, lipids, and proteins into biologically useful materials in animals, as well as other functions such as detoxification and immunity (Vilhelmsson et al., 2004; Panserat et al., 2009). Furthermore, QPCR is often used as a more sensitive technique to measure changes in the expression of selected transcripts (Pfaffl, 2001), and also to validate the results of microarray experiments (Booman et al., 2011; Hori et al., 2012). Therefore, functional genomics techniques (e.g. microarray hybridizations, QPCR) are powerful tools in the genome-wide characterization of gene expression changes in fish subjected to the changes in protein and/or oil sources in their diets. The availability of genomic resources for Atlantic cod and Atlantic salmon are discussed in the following section.

1.5.1 Atlantic cod functional genomics resources

Two major projects have contributed to the creation of Atlantic cod functional genomics resources: the Atlantic Cod Genomics and Broodstock Development Project (CGP; a Canadian project; http://codgene.ca/) and the cod genome project (a Norwegian project; http://codgenome.no/). Out of the 229,094 Atlantic cod expressed sequence tags (ESTs; represent portions of transcribed cDNA sequence) currently in the GenBank EST database (http://www.ncbi.nlm.nih.gov/dbEST/), 158,877 ESTs were generated by the CGP (Bowman et al., 2011). Additional cod genome resources are made available from the Norwegian cod project, which recently published the first draft of the Atlantic cod genome (Star et al., 2011). The cod EST database and genome draft, as well as other fish genome databases such as zebrafish (e.g. *Danio rerio*) available in Ensembl

(www.ensembl.org), are valuable resources for the discovery and characterization of novel genes and gene families in Atlantic cod.

1.5.2 Atlantic salmon functional genomics resources

The Atlantic salmon has been extensively examined at the genomic level and, as for Atlantic cod genomic resources, the Atlantic salmon resources are also available in public databases (Rise et al., 2004b; Koop et al., 2008; Jantzen et al., 2011). At present, the Atlantic salmon genome is being fully sequenced through an international collaborative project including researchers from Canada, Chile and Norway (Davidson et al., 2010). Furthermore, a number of salmonid DNA microarray platforms have been generated through different projects for use in global gene expression studies (Rise et al., 2004a; Rise et al., 2004b; von Schalburg et al., 2005; Rise et al., 2006; Koop et al., 2008; Jantzen et al., 2011). The 44K salmonid oligonucleotide (oligo) microarray (GEO accession # GPL11299) from Agilent Technologies (Santa Clara, CA) with each slide containing four ~44,000-feature (44K) arrays is a newly developed platform by Dr. Ben F. Koop's research team (Jantzen et al., 2011). This array comprises about 22,000 60-mer oligos that are similar (95%) between rainbow trout and Atlantic salmon, plus 14,866 additional Atlantic salmon-specific sequences and 5,661 additional rainbow trout-specific sequences, resulting in a broad representation of transcripts in salmonids with low redundancy (Jantzen et al., 2011). The details on the sequence selection for oligo probe design and platform construction have been described previously (Koop et al., 2008; Jantzen et al., 2011). This newly developed 44K microarray has been used in salmonid gene expression studies (Jantzen et al., 2011; Sahlmann et al., 2013).

1.6 Overall objectives

The overall objectives of the present studies were to: 1) characterize *elovl* family member transcripts as well as measure their basal expression in various tissues in Atlantic cod; 2) investigate the effect of changes in the FA composition of diets containing different levels of CO on the growth performances and hepatic transcript expression of *fatty acyl desaturase* (*fadsd*) and *fatty acyl elongase* (*elovl*) genes in Atlantic cod and Atlantic salmon; and 3) use functional genomics tools to assess the impacts of CO and/or CM containing diets on liver of Atlantic salmon at the transcriptome level, and to identify candidate molecular biomarkers of responses to camelina-containing diets. I anticipate that the molecular biomarkers (i.e. camelina product-responsive genes) identified in this study will be useful in the future development of camelina-containing diets.

In Chapter 2, a variety of molecular tools and techniques [e.g. bi-directional rapid amplification of cDNA ends (RACE), TA cloning, DNA sequencing, and sequence assembly] were utilized to characterize Atlantic cod *elovl* family members at the cDNA level, and I examined how dietary CO influenced the cod growth performance and the transcript expression of genes that regulate LC-PUFA biosynthesis. In Chapter 3, the cGRASP-designed Agilent 44K salmonid oligonucleotide microarray followed by QPCR were used to examine how camelina product-containing diets affected the liver transcriptome and gene expression of potential biomarkers that were microarray-identified. I also investigated the effect of diets containing different levels of CO on the salmon growth performance and the transcript expression of genes involved in LC-PUFA biosynthesis.

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CHAPTER 2: Characterization of fatty acyl elongase (*elovl*) gene family, and hepatic *elovl* and delta-6 fatty acyl desaturase (*fadsd6*) transcript expression responses to diets containing camelina oil in Atlantic cod (*Gadus morhua*)

2.1 Abstract

For aquaculture to become sustainable, there is a need to substitute fish oil [FO, rich in ω3 long chain polyunsaturated fatty acids (LC-PUFA) such as 22:6ω3 (DHA) and 22:6ω3 (EPA)] in aquafeed with plant oils such as camelina oil [CO, rich in C_{18} PUFAs such as 18:3ω3 (ALA) and 18:2ω6 (LNA)]. The LC-PUFA are essential components in fish diets for maintaining optimal health, physiology and growth. However, most marine fish including Atlantic cod are inefficient at producing LC-PUFA from precursors. Since *fatty acyl elongase* (*elovl*) genes encode enzymes that play key roles in fatty acid biosynthesis, it was hypothesized that they may be involved in Atlantic cod responses to diets rich in ALA and LNA. Ten members of the cod *elovl* gene family were characterized at the mRNA level. RT-PCR was used to study constitutive expression of *elovl* transcripts in fifteen tissues. Some transcripts (e.g. *elovl5*) were ubiquitously expressed, while others had tissue-specific expression (e.g. *elovl4a* in brain and eye). Cod fed a CO-containing diet (100% CO replacement of FO and including solvent-extracted fish meal, 100COSEFM) showed significantly lower weight gain, with significant up-regulation of *elovl5* and *delta-6 fatty acyl desaturase* (*fadsd6*) transcripts in liver, compared with cod on a FO diet after a 13-week trial. The high ALA and/or low ω3 LC-PUFA levels in the diet may associate with the up-regulation of *elovl5* and *fadsd6*, which are involved in LC-PUFA biosynthesis in farmed cod.

2.2 Introduction

Dietary long chain polyunsaturated fatty acids (LC-PUFA) [also referred to as highly unsaturated fatty acids (HUFA)], including eicosapentaenoic acid (EPA, 20:5ω3) and docosahexaenoic acid (DHA, 22:6ω3), are crucial to maintaining various biological processes including development, immunity, and reproduction in vertebrates (Agaba et al., 2005). In humans, DHA and EPA are known to benefit health by preventing a number of cardiovascular and inflammatory diseases (Calder and Yaqoob, 2009). The very long chain fatty acids (VLC-FAs) consist of a group of fatty acids with chain lengths >24 carbons (Monroig et al., 2010), and are present in various tissues in most animals (e.g. saturated VLC-FA in skin; VLC-PUFA in retina, brain, and testis) (Brush et al., 2010; Monroig et al., 2010; Carmona-Antoñanzas et al., 2011). In mammals, previous studies have shown that VLC-FA play key roles in phototransduction, skin permeability and fertility (Agbaga et al., 2010; Monroig et al., 2011).

The biosynthesis of LC-PUFA, VLC-FA, and VLC-PUFA in mammals is thought to be achieved by the elongation and desaturation of fatty acids with chain lengths > 16 carbons (Jakobsson et al., 2006). The elongation process is catalyzed by fatty acyl elongases (ELOVL; Elongation of Very Long chain fatty acids), a family of membrane-bound enzymes that are predominantly located in the endoplasmic reticulum (ER) (Nugteren, 1965; Morais et al., 2009). These enzymes are believed to play a role in the first step (condensation of fatty acids) of the elongation pathway of fatty acids. Seven fatty acyl elongase family members (ELOVL1 to ELOVL7), with characteristic fatty acid substrate specificity, have been identified in human and mouse (Jakobsson et al., 2006;

Kitazawa et al., 2009; Monroig et al., 2010). In general, ELOVL1, ELOVL3, ELOVL6 and ELOVL7 prefer saturated and monounsaturated fatty acids (MUFA) as substrate, while ELOVL2, ELOVL4 and ELOVL5 use PUFA as substrate (Monroig et al., 2010). In vertebrates, the source of LC-PUFA can be derived directly from the diet or through the biosynthesis of LC-PUFA from precursors such as α-linolenic acid (ALA, 18:3ω3) and linoleic acid (LNA, 18:2ω6) (Wang et al., 2005).

Fish products are a major source of LC-PUFA in human diets (Tocher et al., 2006). With flat or declining global wild fisheries, there is increasing demand for farmed fish for human consumption (Agaba et al., 2005; Tocher et al., 2006). Consequently, there is great potential for the aquaculture industry to expand. However, the current high demand for fish oil (FO) from wild stocks for the production of aquaculture feeds, particularly for carnivorous fish, threatens the sustainability of the fisheries and aquaculture industries (Tocher et al., 2006). Hence, there is a need to substitute FO, which is rich in LC-PUFA, in aquaculture feeds with oils from plants that have abundant ALA and LNA (Bell et al., 2010). As an oilseed crop, camelina (*Camelina sativa*), has several characteristics that make it desirable for the aquaculture feed industry. The oil content of camelina seed is about 40%, and camelina oil (CO) is especially rich in LC-PUFA precursors ALA and LNA; the levels of these fatty acids in CO are approximately 40% and 15%, respectively (Zubr, 1997; Hixson et al., 2013).

Fish species differ in the extent to which they can tolerate diets high in PUFA and low in LC-PUFA, and this trait appears to be evolutionarily related to the fatty acid profiles of their natural diets (Agaba et al., 2005). For example, most marine fish, including Atlantic cod (*Gadus morhua*), are inefficient at producing LC-PUFA from

shorter chain precursors as the LC-PUFA are abundant in their natural habitat (Agaba et al., 2005; Tocher et al., 2006). This may be due to limited elongation of C_{18} to C_{20} fatty acids as indicated by an *in vitro* study of the LC-PUFA synthetic pathway in turbot (*Scophthalmus maximus*) cell lines (Ghioni et al., 1999). Prior to the current study, the only Atlantic cod *elovl* transcript to be fully characterized was *elovl5*; the cod Elovl5 protein showed the lowest elongase activity (i.e. 7.4% and 0.8% conversion of 18:4ω3 to 20:4ω3 and 20:5ω3 to 22:5ω3, respectively) compared with freshwater fish, salmonids, and other marine species studied therein (Agaba et al., 2005). *Elovl4* transcripts have been studied in zebrafish (*Danio rerio*) (Monroig et al., 2010), Atlantic salmon (*Salmo salar*) (Carmona-Antoñanzas et al., 2011) and cobia (*Rachycentron canadum*) (Monroig et al., 2011), and the encoding enzymes demonstrated capacity to convert C_{20} LC-PUFA to longer products including DHA and EPA.

The publicly available Atlantic cod genomic resources, including a draft genome assembly (gadMor1 v73.1) and transcriptome (Star et al., 2011), as well as over 150,000 expressed sequence tags (ESTs) arising from cDNA libraries representing various tissues and developmental stages (Bowman et al., 2011), allowed me to use bioinformatic techniques to mine sequence databases to identify partial sequences for the following Atlantic cod *fatty acyl elongase* transcripts: *elovl1a*, *elovl1b*, *elovl4a*, *elovl4b*, *elovl4c-1*, *elovl4c-2*, *elovl5*, *elovl6a*, *elovl6b*, and *elovl7*. The objective of the study was to use bi-directional rapid amplification of cDNA ends (RACE), TA cloning, DNA sequencing, and sequence assembly to characterize Atlantic cod *elovl* family member transcripts in order to study their evolutionary relationships and expression profiles. Also, as part of a large nutritional feeding trial, this study evaluated the effect of changes in the FA

composition of diets containing different levels of CO on the hepatic transcript expression of *elovl* family members and *delta-6 fatty acyl desaturase* (*fadsd6*, also involved in LC-PUFA biosynthesis).

2.3 Materials and methods

2.3.1 Experimental animals

This research project was conducted through a large collaborative project (The Camelina Project; http://www.genomeatlantic.ca/), and protocols were approved by the Institutional Animal Care Committee of Memorial University of Newfoundland, protocol number 12-50-MR. The tissue panel used for *elovl* transcript characterization and tissue distribution studies (described in detail below) was collected from two juvenile Atlantic cod (~13 g, ~7 months old) reared at the Dr. Joe Brown Aquatic Research Building (JBARB) at the Ocean Sciences Centre (Memorial University of Newfoundland, Canada). Fish were kept in a 6,000 L tank with flow-through seawater supply (~10°C, dissolved oxygen ≥ 90% saturation). Fifteen tissues (skeletal muscle, skin, eye, brain, head kidney, posterior kidney, spleen, pyloric caecum, midgut, hindgut, stomach, liver, blood, heart, and gill; 50-100 mg tissue^{-1}) were rapidly dissected from each fish, placed in RNase-free 1.5 mL tubes, flash-frozen in liquid nitrogen, and stored at -80°C until RNA extraction. Fish used in the feeding trial (see section 2.3.2) were obtained from the same group as the fish used for the tissue panel.

2.3.2 Experimental design and sampling: cod fed diets containing camelina oil

All diets were formulated as isonitrogenous and isolipidic practical diets according to the nutritional requirements of marine fish (National Research Council, 2011). The current study involved a control diet with FO, 100% FO replacement with CO (100CO), and 100% FO replacement with CO and including solvent extracted fish meal (100COSEFM) to remove residual FO from the meal (Table 2-1); an additional experimental diet was included in this feeding trial (Hixson and Parrish, 2014), but was not part of the current transcript expression study. The experimental diets involved in this study were tested on Atlantic cod to determine the nutritional value of CO in terms of fish growth, and to assess the effect of CO-containing diets on the hepatic transcript expression responses of four *elovl* genes and *fadsd6* (see section 2.3.8). Further details on formulation, proximate and fatty acid compositions of the diets are described elsewhere (Hixson and Parrish, 2014).

Juvenile Atlantic cod (14.4 ± 1.6 g) were randomly distributed among twelve 500 L experimental tanks (70 fish $tank^{-1}$) supplied with flow-through seawater (~10°C, dissolved oxygen ≥ 90% saturation). The fish were gradually weaned from a commercial diet (Europa 15, 2 mm, Skretting Canada) to the experimental diets for one week before the feeding trial started. Triplicate tanks of fish were fed experimental or control diets to apparent satiety, twice each day for a period of 13 weeks. At the starting point (i.e. when fish were first fed an experimental diet), week 1, week 6 and week 13 of the feeding trial, seven fish from each tank at each time point were euthanized with a lethal dose (400 mg L^{-1}) of tricaine-methane-sulfonate (TMS; Syndel Laboratories, Vancouver, BC) after 24h

Table 2-1. Composition of experimental diets used in juvenile cod feeding trial (percentage of total weight as fed)[1].

Ingredient	**FO (control)**	**100CO**	**100COSEFM**
Fish oil	5.43	-	-
Camelina oil	-	5.43	9.7
Herring meal	50.5	50.5	-
SEFM[2]	-	-	47.6
Wheat middlings	27.1	27.1	25.7
Wheat gluten meal	5	5	5
Whey powder	5	5	5
Krill hydrolysate	2.5	2.5	2.5
Corn starch (pre-gelatized)	0.25	0.25	0.25
Vitamin mixture[3]	1.95	1.95	1.95
Mineral mixture[4]	1.95	1.95	1.95
Choline chloride	0.3	0.3	0.3
Total	100	100	100
Proximate composition analyzed, as-fed basis[5]			
Moisture	10.9 ± 0.02	10.7 ± 0.03	10.2 ± 0.02
Ash	2.8 ± 0.1	2.9 ± 0.2	3.1 ± 0.6
Protein	46.8 ± 0.1	47.3 ± 0.04	46.1 ± 0.01
Lipid	8.8 ± 0.2	9.1 ± 1.3	9.9 ± 1.4

[1]Note that the diet composition data presented here were previously published (Hixson and Parrish, 2014); we include them herein as they pertain to the current study as well.
[2]SEFM = Solvent-extracted fish meal (herring meal).
[3]Vitamin Premix contains per kg: Vitamin A 0.9 g, Vitamin D3 8.0 g, Vitamin E 50.0 g, Vitamin K 3.0 g, Thiamin 2.8 g, Riboflavin 4.0 g, Pantothenic acid 24.0 g, Biotin 0.1 g, Folic acid 26.7 g, Vitamin B12 0.03 g, Niacin 15.1 g, Pyridixine 3.3 g, Ascorbic acid 10.8 g, Wheat middlings (carrier) 851.3 g.
[4]Mineral Premix contains per kg: Manganese oxide 12.3 g, Zinc oxide 20.6 g, Copper sulphate 6.1 g, Iodine 15.8 g, Wheat middlings (carrier) 954.2 g.
[5]Proximate composition are percentage of total weight and are means ± standard deviation (n = 3).

of fasting. Body weight, total length and liver weight of fish as well as feed intake were measured. Liver samples (50-100 mg tissue^{-1}) were rapidly dissected from the fish, placed in RNase-free 1.5 mL tubes, flash-frozen in liquid nitrogen, and stored at -80°C until RNA extraction. Further details on fish rearing conditions and sampling for lipid analysis are described elsewhere (Hixson and Parrish, 2014).

2.3.3 RNA extraction and column purification

Tissue samples representing the cod tissue panel, and liver samples collected from the feeding experiment, were homogenized in 2.0 mL tubes containing TRIzol reagent (Invitrogen, Carlsbad, CA) with a volume of 0.8 mL sample^{-1} and a stainless steel bead (2.5 mm; QIAGEN, Mississauga, ON) using a TissueLyser (QIAGEN) at 25 Hz for 3 min, further disrupted using QIAshredder spin columns (QIAGEN), and subjected to RNA extraction according to the manufacturers' instructions. After extraction, the quality of total RNA was examined by 1% agarose/ethidium bromide gel electrophoresis, and RNA quantity and purity were measured by NanoDrop spectrophotometry (ThermoFisher, Mississauga, ON). Total RNA of each sample was treated with DNase I (QIAGEN) to degrade residual genomic DNA, and all samples were purified from salts, proteins and nucleotides using the RNeasy Mini Kit (QIAGEN) following the manufacturer's protocols. The quantity and quality of cleaned RNA samples were assessed as previously described. Only high quality (260/280 ratio > 2.0, 260/230 > 1.9, with tight 18S and 28S ribosomal RNA bands) total RNA samples were used for transcript characterization and expression studies.

2.3.4 Genomic screening for members of Atlantic cod *elovl* gene family

In order to identify putative Atlantic cod Elovl-encoding transcripts, zebrafish Elovl protein sequences obtained from NCBI (see Appendix I for accession numbers) were used to tBLASTn query the Atlantic cod genome (gadMor1 v73.1) or the cDNA collection predicted based on their genomic sequences (i.e. GeneScaffolds; available through Ensembl website: http://www.ensembl.org) and transcriptome (available through the ViroBlast server at http://www.codgenome.no/viroblast/viroblast.php) databases (Star et al., 2011). This approach allowed the identification of sequence fragments that represent putative Atlantic cod Elovl-encoding transcripts. To obtain full-length Atlantic cod Elovl-encoding transcripts, 5' and 3' RACE reactions (see section 2.3.5) were performed using gene-specific primers designed based on these sequences (Appendix II).

2.3.5 *Elovl* cDNA cloning, sequencing, and sequence assembly

The full-length cDNA sequences of cod *elovl* transcripts were obtained using RACE. Full-length 5' and 3' RACE cDNAs were synthesized using the SMARTer RACE cDNA Amplification Kit following the manufacturer's instructions (Clontech, Mountain View, CA). The poly(A)$^+$ RNA (i.e. mRNA) was isolated from an RNA pool that included an equal quantity of each cleaned total RNA from 15 different tissues of two juvenile Atlantic cod, using the MicroPurist mRNA isolation kit (Ambion, Austin, TX) and following the manufacturer's instruction. This mRNA template was used for the RACE cDNA synthesis. Ten μL of 5' and 3' RACE cDNA (corresponding to 1 μg of input mRNA) was diluted into a final volume of 260 μL with nuclease-free water (Invitrogen). Based on sequence information obtained from public databases, gene specific primers

(GSPs) for each cod *elovl* transcript were designed using the Primer3 program (available at http://frodo.wi.mit.edu) (Rozen and Skaletsky, 2000). RACE involved a touch-down PCR followed by a nested PCR. Using 2.5 μL of the diluted cDNA (representing ~ 4 ng of input mRNA) as template, both 5' and 3' RACE PCRs (including first-round and nested PCR) were performed using the Advantage 2 Polymerase (Clontech) following the manufacturer's instructions. The cycling parameters for touch-down PCR consisted of an initial denaturation period of 1 min at 95°C, followed by 5 cycles of (95°C for 30 s, 72°C for 30 s, 72°C for 3 min), 5 cycles of (95°C for 30 s, 70°C for 30 s, 72°C for 3 min), 20 cycles of (95°C for 30 s, 68°C for 30 s, 72°C for 3 min), and 1 cycle at 68°C for 10 min. For the nested RACE PCR, cycling parameters consisted of 1 min at 95°C, followed by 20 cycles of (95°C for 30 s, 68°C for 30 s, 72°C for 3 min), and 1 cycle at 68°C for 10 min.

All PCR products were gel-extracted from 1% agarose gel stained with ethidium bromide, using the QIAQuick Gel Extraction kit (QIAGEN) following the manufacturer's protocol. The extracted products were then ethanol precipitated, washed, and TA cloned into a pGEM-T-Easy vector (Promega, Madison, WI) at 4°C overnight using standard techniques. The recombinant plasmids were transformed into Subcloning Efficiency DH5α Competent Cells (Invitrogen) following the manufacturer's instructions. After transformation, 300 μL of SOC medium (Invitrogen) were added to the ligation reaction and incubated for 1 h at 37°C with shaking (~225 rpm), and the cells were grown for 16 h at 37°C on Luria broth (LB)/agar with 100 μg mL^{-1} ampicillin. Individual colonies were cultured for 16 h at 37°C in LB/ampicillin (100 μg mL^{-1}), and the plasmid DNA was

purified and isolated in the 96-well format using standard molecular techniques. Prior to sequencing, the insert sizes of recombinant plasmids were estimated either by *Eco*RI (Invitrogen) digestion followed by agarose gel electrophoresis, or by PCR using TopTaq DNA Polymerase (QIAGEN) with M13 primers (forward and reverse) following the manufacturer's instructions. For each PCR product, three individual clones were sequenced [by the ABI 3730 DNA Analyzer using the BigDye Terminator chemistry (Applied Biosystems, Foster City, CA)] at the Genomics and Proteomics facility of Memorial University; clones were sequenced as many times as required to give at least 6-fold coverage for *elovl* sequences.

Overlapping sequence fragments obtained from 5' and 3' RACE were assembled using Lasergene 7.20 software (DNASTAR, Madison, WI). To verify each sequence assembly, GSPs flanking the overlapping region of 5' and 3' RACE products [and designed to flank the entire open reading frame (ORF) if possible] were used to amplify each *elovl*. PCR was carried out using the TopTaq polymerase kit (QIAGEN) in a 25 μL reaction volume that contained 2 μL of 5' RACE cDNA (representing ~3.2 ng of input mRNA), 0.5 μM each of forward and reverse GSP (Appendix II), 0.625 U of TopTaq DNA Polymerase, 2.5 μL of TopTaq PCR buffer and 100 μM of each dNTP. The cycling parameters for PCR consisted of an initial denaturation period of 3 min at 94°C, followed by 30 cycles of (94°C for 30 s, 60°C for 30 s, 72°C for 3 min), and 1 cycle at 72°C for 10 min. The resultant PCR products were analyzed by agarose gel electrophoresis to verify if the sequences were correctly assembled, and to confirm that the assembled cDNAs represented bona fide transcripts.

2.3.6 Sequence analysis

The amino acid (AA) sequences of putative Atlantic cod Elovls were predicted based on full-length *elovl* cDNA sequences using the SeqBuilder function of Lasergene software (DNASTAR). Using the same software, the cDNA sequences obtained from RACE were mapped to the Atlantic cod genomic sequence downloaded from the Ensembl website (http://www.ensembl.org) to determine gene structure. By using the AA sequences of zebrafish Elovls, homologous Elovl sequences from other fish species [e.g. Atlantic salmon and pufferfish (*Takifugu rubripes*)] were collected from the NCBI database using the tBLASTx alignment search tool. The predicted AA sequences of cod Elovls were aligned with homologous Elovl sequences using the MUSCLE function of MEGA5 software (Edgar, 2004; Tamura et al., 2011). Pair-wise sequence comparisons were carried out with the MegAlign function of Lasergene software (DNASTAR). An unrooted phylogenetic tree was constructed based on the alignment results of the deduced amino acid sequences, using the Maximum Likelihood method implemented in MEGA5, bootstrapped 1000 times.

2.3.7 Tissue distribution of cod *elovl* gene family transcripts

Two sets of GSPs for each *elovl* transcript [except *elovl5*, which had been previously characterized (Agaba et al., 2005)] were designed using the Primer3 program based on the sequence information arising from the RACE studies (Appendix II). For each individual sample, 1 μg of DNase I-treated and column purified total RNA was reverse-transcribed using random primers (250 ng, Invitrogen) and Moloney murine leukemia virus (M-MLV) reverse transcriptase (200 U, Invitrogen) at 37°C for 50 min in

a 20 µL reaction volume, which also contains 10 mM of each dNTPs, 0.2 M DTT, and 4 µL 5× first-strand buffer, following manufacturer's instructions. The resulting cDNA was diluted with nuclease-free water (Invitrogen) to a volume of 200 µL. Reverse transcription - polymerase chain reaction (RT-PCR) amplifications were carried out using the same chemistry as ORF PCR, but with a set of templates from the cod tissue panel (for complete list of tissues see section 2.3.1; 2 µL of diluted cDNA representing 10 ng of input total RNA for each tissue sample) and RT-PCR GSPs (Appendix II). The cycling parameters for RT-PCR were also identical to ORF PCR except for the extension period, which was decreased to 1 min. The reference gene used in this study was *elongation factor 1α* (*eef1α*), and was included in the same PCR run as the GOI. Negative ("no-template") controls were also included for each gene of interest (GOI) to confirm the absence of template contamination. PCR products were electrophoretically separated in 1.5% agarose/ethidium bromide gels along with a 1Kb plus ladder (Invitrogen), and visualized under UV light in a G:BOX gel imaging system (Syngene, Frederick, MD).

2.3.8 QPCR: hepatic transcript expression responses of four *elovls* and *fadsd6* to diets containing camelina oil

The transcript expression responses of *elovl1b*, *elovl4c-2*, *elovl5*, *elovl6a* and *fadsd6* to the three diets (Table 2-1; FO, 100CO and 100COSEFM) were studied in juvenile cod liver tissue at week 13 of the feeding trial, using quantitative reverse transcription - polymerase chain reaction (QPCR). QPCR primers for the five GOIs, and the normalizer gene *60S acidic ribosomal protein P1* [*rplp1*; 20K cod microarray probe identifier (ID) #35667 (Booman et al., 2011)], are shown in Appendix II. All QPCR

primer sets were assessed for quality using dissociation curves to ensure that the primer pairs amplified single products with no primer dimers. The amplification efficiencies of primer pairs (Pfaffl, 2001) were determined using a 5-point 1:5 (*rplp1*), 1:3 (*elovl1b*, *elovl4c-2*, and *elovl5*), or 1:2 (*elovl6a* and *fadsd6*) dilution series starting with pooled cDNA (corresponding to 10 ng of input total RNA) with equal contributions of all individuals involved in the QPCR study. For a given primer pair, quality control was performed in triplicate.

For each experimental treatment, nine individuals (three from each triplicate tank) were involved in the QPCR study. For each liver template, 1 μg of DNase I-treated and column purified total RNA was reverse-transcribed using random primers (250 ng, Invitrogen) and Superscript II reverse transcriptase (200 U, Invitrogen) at 42°C for 50 min in a 20 μL reaction volume following the manufacturer's instructions. The resulting cDNA was diluted with nuclease-free water (Invitrogen) to a volume of 200 μL. QPCR reactions were performed using Power SYBR Green I dye chemistry on the ViiA™ 7 Real-Time PCR System (Applied Biosystems, Foster City, CA). The PCR reactions contained 2 μL of diluted cDNA (10 ng input total RNA), 50 nM each of forward and reverse primer, and 1× Power SYBR Green PCR Master Mix (Applied Biosystems) in a final volume of 13 μL. The PCR program consisted of 1 cycle of 50°C for 2 min, 1 cycle of 95°C for 10 min, followed by 40 cycles of (95°C for 15 s and 60°C for 1 min), with the fluorescent signal data collection after each 60°C step. GOI and normalizer (*rplp1*) for a given template were run in triplicate on the same 96-well plate. *Rplp1* was chosen as the normalizer gene due to its stable transcript expression in other cod liver microarray experiments (data not shown). In addition, *rplp1* was tested with QPCR in a subset of

individuals (three from each condition) and had the lowest threshold cycle (C_T) range (0.7) compared to two other candidate normalizers [*eef1α* and *tubulin α*]. In every multi-plate study, a linker control (a pooled cDNA sample from all samples involved in the study) was used to check the variability between plates. All thresholds were set automatically, and relative quantity (RQ) of each QPCR target transcript for each individual was calculated using actual amplification efficiencies (see Supplemental Table 2) for GOI and normalizer primer pairs using the the ViiA™ 7 Software v1.2 (Applied Biosystems). The individual with the lowest GOI expression regardless of treatment was used as the calibrator sample (i.e. RQ = 1) for each GOI study. The underlying algorithm for calculating RQ (i.e. the $2^{-\Delta\Delta CT}$ method for relative quantification) was described previously (Livak and Schmittgen, 2001).

2.3.9 Statistical analyses of growth and QPCR data

All statistical analyses of growth-relevant and QPCR data were performed using Minitab (v16; State College, PA) with one-way ANOVA, followed by the Tukey post-hoc test for multiple comparisons at the 5% level of significance, to detect differences between dietary treatments. All data were subjected to normality testing using the Anderson-Darling method. The growth-relevant data (as shown in Table 2-3) were presented as mean ± standard deviation (SD). RQ data were $\log_2$ transformed in Excel in order to meet with statistical assumptions (i.e. normality), and were presented as mean ± standard error (SE). However, such data transformation on some data sets (e.g. *elovl5* of FO) was still not able to meet the normality assumption ($p < 0.01$; Anderson-Darling method). The transformed RQ data were also subjected to outlier tests (i.e. Grubbs' test;

available at http://graphpad.com/quickcalcs/grubbs1/) to identify potential outliers. Two outliers, one fish of *elovl5* from FO (4.22× higher than the group average) and one fish of *elovl4c-2* from 100COSEFM (4.42× lower than the group average), were identified ($p < 0.01$; Grubbs' test); thus they were excluded from the data analyses. For QPCR fold-change calculation, overall fold up-regulation was calculated as 2^{A-B}, where A is the mean of $\log_2$ transformed RQ from an experimental group (i.e. 100CO or 100COSEFM), and B is the mean of $\log_2$ transformed RQ from the FO group (Cui and Churchill, 2003).

2.4 Results

2.4.1 *Elovl* gene family characterization and molecular phylogenetics

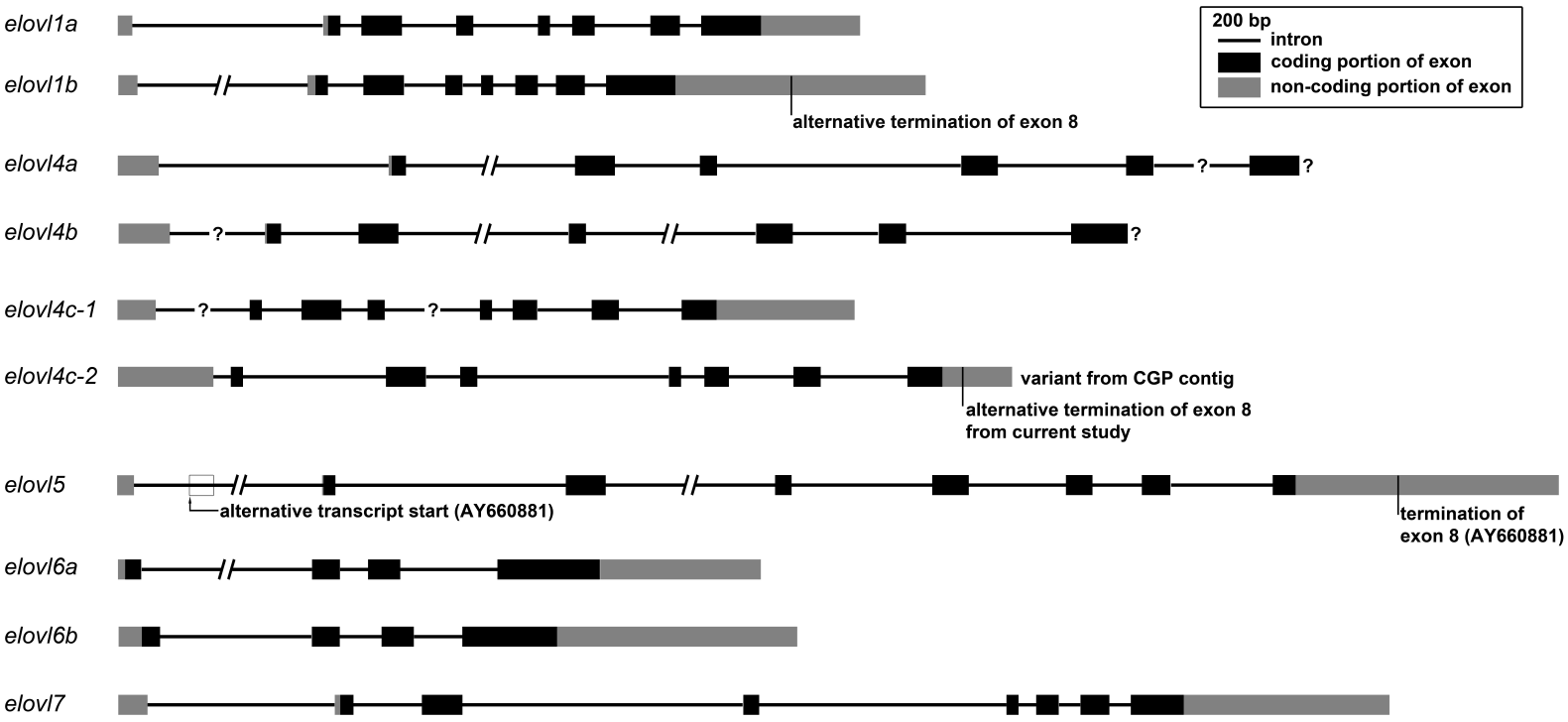
In this study, bioinformatic and molecular techniques (mining sequence databases, RACE, and sequencing) were used to characterize ten members of the *elovl* gene family (*elovl1a*, *elovl1b*, *elovl4a*, *elovl4b*, *elovl4c-1*, *elovl4c-2*, *elovl5*, *elovl6a*, *elovl6b*, and *elovl7*) in Atlantic cod. All cDNA sequences of the cod *elovl* gene family obtained through the current study were deposited in GenBank under the accession numbers KF964005 - KF964015 (Table 2-2). The details of transcript structure, including untranslated regions (UTRs), open reading frames (ORFs) and sequence lengths, as well as associated genomic sequences (i.e. GeneScaffolds), are shown in Table 2-2 and Appendices IV-XIII. In addition, the full-length *elovl* cDNA sequences obtained from RACE were mapped to the corresponding Atlantic cod genomic sequences to determine the genomic organization of the *elovl* gene family members as shown in Fig. 2-1.

Table 2-2. Atlantic cod *elovl* transcript analyses and associated genomic sequences.

Gene[1]	Transcript	Genomic sequences[2]	5' UTR (nt)	ORF (nt/AA)[3]	3' UTR (nt)	Sequence length (bp)
elovl1a	KF964005	GeneScaffold_4551	92	921/306	459	1472
elovl1b	KF964007 or KF964006	GeneScaffold_1484; contig 52498	129	960/319	552 or 1178	1641 or 2267
elovl4a	KF964008	GeneScaffold_3464; contig 129825	209	*810/270*	-	*1019*
elovl4b	KF964009	GeneScaffold_2661	252	*903/301*	-	*1155*
elovl4c-1	KF964010	GeneScaffold_1484; contig 70127; contig 70123	180	795/264	651	1626
elovl4c-2	KF964011	GeneScaffold_1484	449	795/264	96	1340
elovl5	KF964012	GeneScaffold_1260	86	867/288	1240	2193
elovl6a	KF964013	GeneScaffold_3464	34	846/281	757	1637
elovl6b	KF964014	GeneScaffold_2788	109	822/273	1129	2060
elovl7	KF964015	GeneScaffold_1288	164	882/293	996	2012

[1]All genes including transcript variants presented here were identified through the current RACE studies. Numbers in italics represent incomplete sequences. *Elovl1b* has two full-length cDNA variants (GenBank accession numbers KF964007 and KF964006) as shown in the table; transcript variant KF964006 has a longer 3' UTR.
[2]Putative cod *elovl* transcript-associated genomic sequences (i.e. GeneScaffolds and contigs; available through Ensembl website: http://www.ensembl.org) identified using BLASTn with cod *elovl* transcript sequences.
[3]Nucleotide and amino acid lengths of open reading frame of the transcript.

Fig. 2-1. Schematic representation of gene organisation for the Atlantic cod *elovl* gene family. Boxes represent exons, while lines represent introns. The grey and black colours are used to distinguish non-coding and coding portion of exons, respectively. The gene structure and full-length cDNA for cod *elovl4a* and *elovl4b* have not yet been fully determined. The alignment of the current RACE-identified *elovl4c-2* cDNA (GenBank accession number KF964011) and CGP contig all_v2.0.1432.C1, represented by ESTs ES779567 and FG329933 (Bowman et al. 2011), suggest the existence of an *elovl4c-2* transcript variant with a longer 3' UTR. The alignment of *elovl5* cDNA (KF964012) and the previously identified *elovl5* (AY660881) showed some differences in both 5' and 3' UTR sequences as demonstrated in this figure. The open box within *elovl5* shows the first exon (111 bp) of the transcript variant represented by AY660881. A question mark within an intron indicates that the entire intron was not identified due to the lack of, or discontinuous, genomic sequences.


elovl1a
elovl1b
alternative termination of exon 8
elovl4a
elovl4b
elovl4c-1
elovl4c-2
variant from CGP contig
alternative termination of exon 8
from current study
elovl5
alternative transcript start (AY660881)
termination of
exon 8 (AY660881)
elovl6a
elovl6b
elovl7
200 bp
intron
coding portion of exon
non-coding portion of exon

Full-length cDNA sequences including the 5' and 3' UTR regions were identified for two *elovl1* paralogues (*elovl1a*, *elovl1b*). *Elovl1a* and *elovl1b* encode 306 and 319 AA proteins, respectively (Table 2-2). *Elovl1b* demonstrated two full-length cDNA variants (1641 and 2267 bp) that showed 100% identity over the 1639 bp aligned at the 5' end (Fig. 2-1, Table 2-2, and Appendix V). The longer variant had an additional sequence of 628 bp excluding the poly (A) tail in the 3' UTR (Appendix V). Both *elovl1a* and *elovl1b* genes consist of 8 exons and 7 introns based on the genome assembly (Fig. 2-1, Appendices IV and V). The overall similarity between these two paralogues (i.e. Elovl1a and Elovl1b) is 55% at the amino acid level based on sequence alignment (Appendix III).

Four Atlantic cod *elovl4* paralogues were identified in publically available sequence databases and further characterized by RACE and sequencing. Of these, only *elovl4c-1* and *elovl4c-2* transcripts were fully characterized. *Elovl4c-1* and *elovl4c-2* are 1626 and 1340 bp long, respectively, and both encode 264 AA proteins which share 86% identity (Table 2-2, Appendices III, VIII, and IX). Both of these cod genes consist of 8 exons and 7 introns, and are located in the same GeneScaffold (1484) (Table 2-2 and Fig. 2-1). In addition, a contig (all_v2.0.1432.C1, represented by ESTs with GenBank accession numbers ES779567 and FG329933) from the Atlantic Cod Genomics and Broodstock Development Project (CGP, http://codgene.ca) (Bowman et al., 2011) revealed a second *elovl4c-2* transcript variant with a longer 3' UTR compared with the *elovl4c-2* transcript variant characterized in the current study (Fig. 2-1). Partial coding sequences (containing incomplete ORFs) for two additional *elovl4* paralogues (*elovl4a* and *elovl4b*) were obtained in the current study (Fig. 2-1). The partial sequences of *elovl4a* and *elovl4b* are 1019 and 1155 bp in length, encoding partial predicted proteins of

270 and 301 amino acid residues, respectively (Table 2-2, Appendices VI and VII). The analysis of *elovl4a* and *elovl4b* gene structure showed at least 7 exons and 6 introns (Fig. 2-1). Elovl4a and Elovl4b are 70% identical at the predicted amino acid level (Appendix III). Attempts to extend the 3' RACE sequences of *elovl4a* and *elovl4b* with various primer sets (data not shown) were not successful.

The cod *elovl5* cDNA consensus sequence obtained from the current RACE-based research and the full-length Atlantic cod *elovl5* cDNA sequence in GenBank (accession number AY660881) are different at their 5' and 3' ends. While both variants have 8 exons and 7 introns according to genome sequence mapping (Fig. 2-1 and Appendix X), the first exon (79 bp) in the current RACE-identified *elovl5* cDNA sequence is 262 bp upstream of the first exon (111 bp) of the cod *elovl5* transcript variant represented by GenBank accession number AY660881; this results in a longer intron 1 (i.e. between exon 1 and exon 2) by 373 bp in the current RACE-identified *elovl5* cDNA sequence (Fig. 2-1). Both of the *elovl5* variants' intron 1 have a classic "GT-AG" intron splicing motif (Appendix X). The coding sequences (288 AA) for both *elovl5* variants are 100% identical at the nucleotides level. However, the last exon (i.e. exon 8) from the current RACE study possessed an additional sequence of 753 bp in the 3' UTR compared with AY660881 (Fig. 2-1).

Complete cDNA sequences were determined for Atlantic cod *elovl6a*, *elovl6b*, and *elovl7*. *Elovl6a* and *elovl6b* had similar cDNA and protein lengths (2060 vs. 2012 bp; 281 vs. 273 AA, respectively) (Table 2-2). Interestingly, both *elovl6a* and *elovl6b* were encoded by 4 exons and 3 introns, which is different from the other members of the Atlantic cod *elovl* family (Fig. 2-1, Appendices XI and XII). The overall similarity

between these two paralogues based on the aligned predicted protein sequences is 70% (Appendix III). *Elovl7* cDNA is 2012 bp long excluding the poly(A) tail, and encodes 264 AA protein (Table 2-2 and Appendix XIII). The analysis of *elovl7* transcript and genomic sequences revealed a gene structure including 8 exons and 7 introns (Fig. 2-1).

Multiple alignment of Atlantic cod Elovl amino acid sequences with putative orthologous sequences from zebrafish using the MUSCLE algorithm built into MEGA5 (Tamura et al., 2011) revealed four different conserved motifs (KXXEXXDT, QXXFLHXXHH, NXXXHXXMYXYY, and TXXQXXQ) in all cod and zebrafish Elovls [as shown in (Agaba et al., 2005)]. A single histidine box motif (contained within the QXXFLHXXHH motif), five putative transmembrane domains [as predicted by (Zhang et al., 2003)], and C-terminal lysine or arginine residues (i.e. KXKXX, KXRXX, or KKXX) were found in most Elovls that were included in the multiple sequence alignment (Fig. 2-2). An unrooted phylogenetic tree was constructed based on the putative protein sequences of cod Elovls, and the Elovls from other fish species (i.e. zebrafish, pufferfish, and Atlantic salmon), which were aligned using MUSCLE. In the molecular phylogenetic tree, all fish Elovl proteins were classified into three major clusters (Fig. 2-3). All Elovl2, Elovl4 and Elovl5 sequences were grouped together sharing a single branch, while all Elovl7 and Elovl1 sequences grouped together and shared a single branch in the tree (Fig. 2-3). Elovl6 proteins were grouped separately from all other Elovl family members for fish species included in the tree (Fig. 2-3). Moreover, all cod Elovls cluster with their putative orthologues of other fish species (Fig. 2-3). It is noteworthy that all cod, zebrafish, and pufferfish Elovl4c proteins were separated from Elovl4a and Elovl4b from these species in the phylogenetic tree, with cod *elovl4c-1* and

Fig. 2-2. Multiple alignment of predicted Elovl proteins of Atlantic cod with orthologous sequences from zebrafish retrieved from the NCBI protein database. Black shading with white font is used to denote identical residues. For residues with conservative substitution (i.e. 80% conserved as defined by GeneDoc), grey shading with white font is used. Light grey with black font specifies that semi-conservative substitutions (i.e. 60% conserved as defined by GeneDoc) have been observed. Four different motifs (KXXEXXDT, QXXFLHXXHH, NXXXHXXMYXYY, and TXXQXXQ) are highly conserved in all Elovls presented in this figure (as shown in Agaba et al., 2005). A single histidine box which is contained within the QXXFLHXXHH motif, and five putative transmembrane domains (solid underlines) predicted by Zhang and colleagues (2003), were indicated for most of the Elovls.

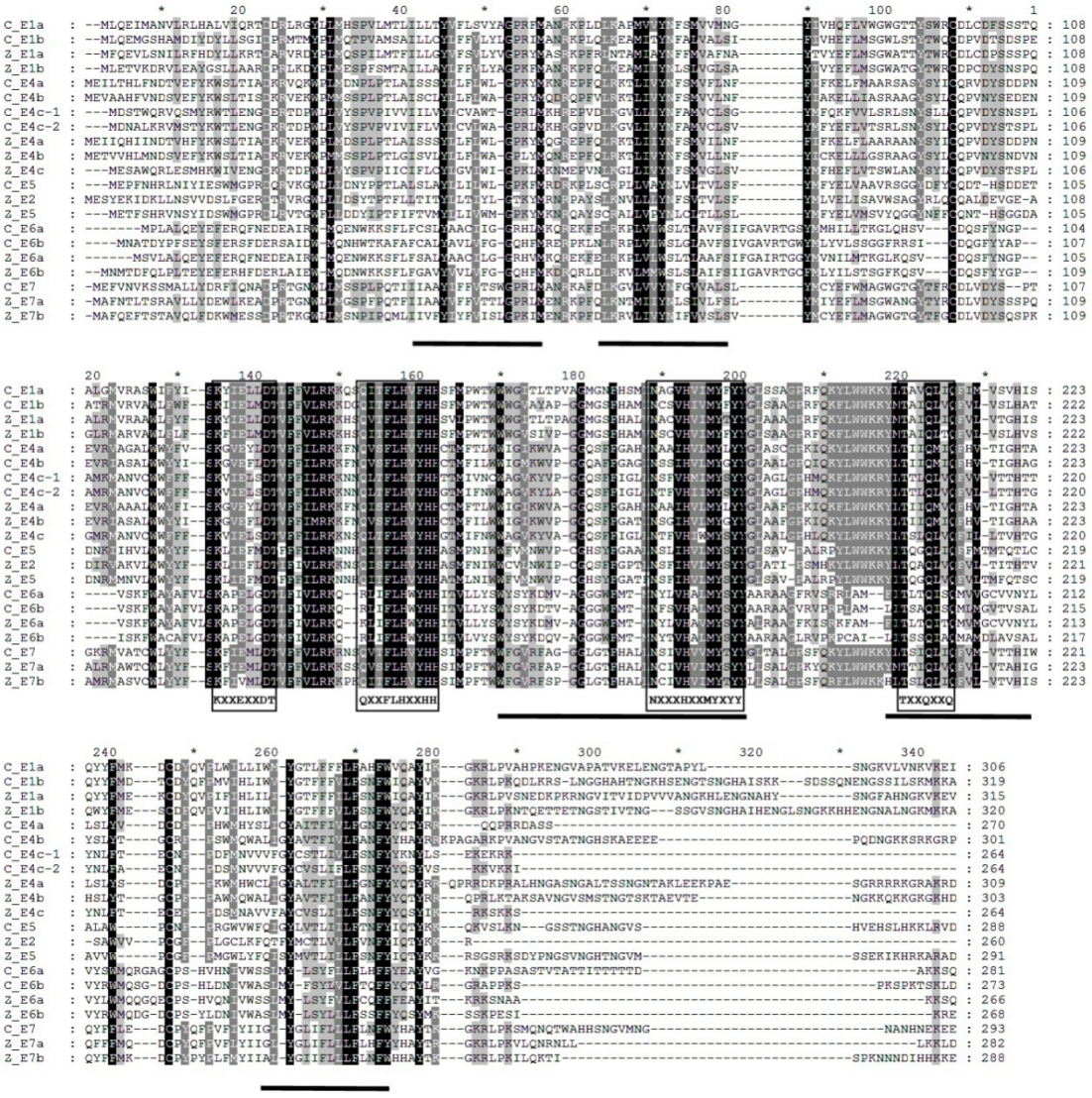
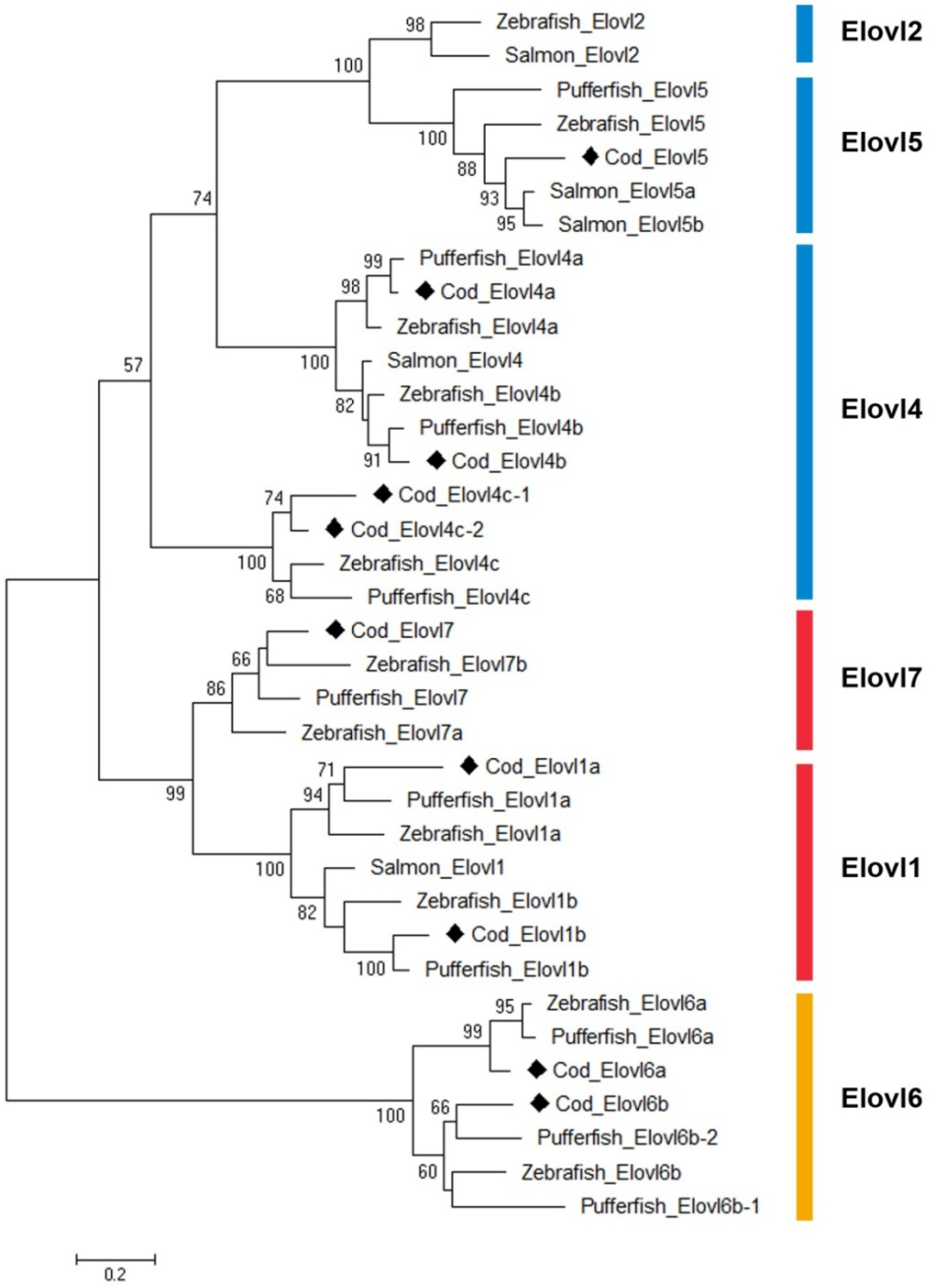

KXXEXXDT
QXXFLHXXHH
NXXXHXXMYXYY
TXXQXXQ

Fig. 2-3. Phylogenetic analysis of the Atlantic cod Elovl family. The predicted proteins of cod Elovls were aligned against homologous proteins from other fish species [i.e. zebrafish (*Danio rerio*), pufferfish (*Takifugu rubripes*), and Atlantic salmon (*Salmo salar*)] using MEGA5 (v5.10). Based on the multiple sequence alignment, all sequences used in this figure were trimmed in order to eliminate technical bias, and an unrooted phylogenetic tree was constructed by the Maximum likelihood method. The tree was bootstrapped 1,000 times, and the bootstrap values are shown at the branch points. Any branches that present in less than 50% of bootstrap replicates are not shown. Zebrafish Elovl1a (NP_001005989), Elovl1b (NP_998581), Elovl2 (NP_001035452), Elovl4a (NP_957090), Elovl4b (NP_001191453), Elovl4c (AAH60897), Elovl5 (NP_956747), Elovl6a (NP_955826), Elovl6b (AAH46901), Elovl7a (AAH46901) and Elovl7b (AAH45481); Salmon Elovl1 (NP_001139865), Elovl2 (NP_001130025), Elovl4 (NP_001182481), Elovl5a (NP_001117039) and Elovl5b (NP_001130024); Pufferfish Elovl1a (XP_003975604), Elovl1b (XP_003974086), Elovl4a (XP_003966009), Elovl4b (XP_003971605), Elovl4c (XP_003974148), Elovl5 (XP_003964216), Elovl6a (XP_003970691), Elovl6b-1 (XP_003976166), Elovl6b-2 (XP_003961164) and Elovl7 (XP_003974898).


Zebrafish_Elovl2
Salmon_Elovl2
Pufferfish_Elovl5
Zebrafish_Elovl5
Cod_Elovl5
Salmon_Elovl5a
Salmon_Elovl5b
Pufferfish_Elovl4a
Cod_Elovl4a
Zebrafish_Elovl4a
Salmon_Elovl4
Zebrafish_Elovl4b
Pufferfish_Elovl4b
Cod_Elovl4b
Cod_Elovl4c-1
Cod_Elovl4c-2
Zebrafish_Elovl4c
Pufferfish_Elovl4c
Cod_Elovl7
Zebrafish_Elovl7b
Pufferfish_Elovl7
Zebrafish_Elovl7a
Cod_Elovl1a
Pufferfish_Elovl1a
Zebrafish_Elovl1a
Salmon_Elovl1
Zebrafish_Elovl1b
Cod_Elovl1b
Pufferfish_Elovl1b
Zebrafish_Elovl6a
Pufferfish_Elovl6a
Cod_Elovl6a
Cod_Elovl6b
Pufferfish_Elovl6b-2
Zebrafish_Elovl6b
Pufferfish_Elovl6b-1
0.2
Elovl2
Elovl5
Elovl4
Elovl7
Elovl1
Elovl6

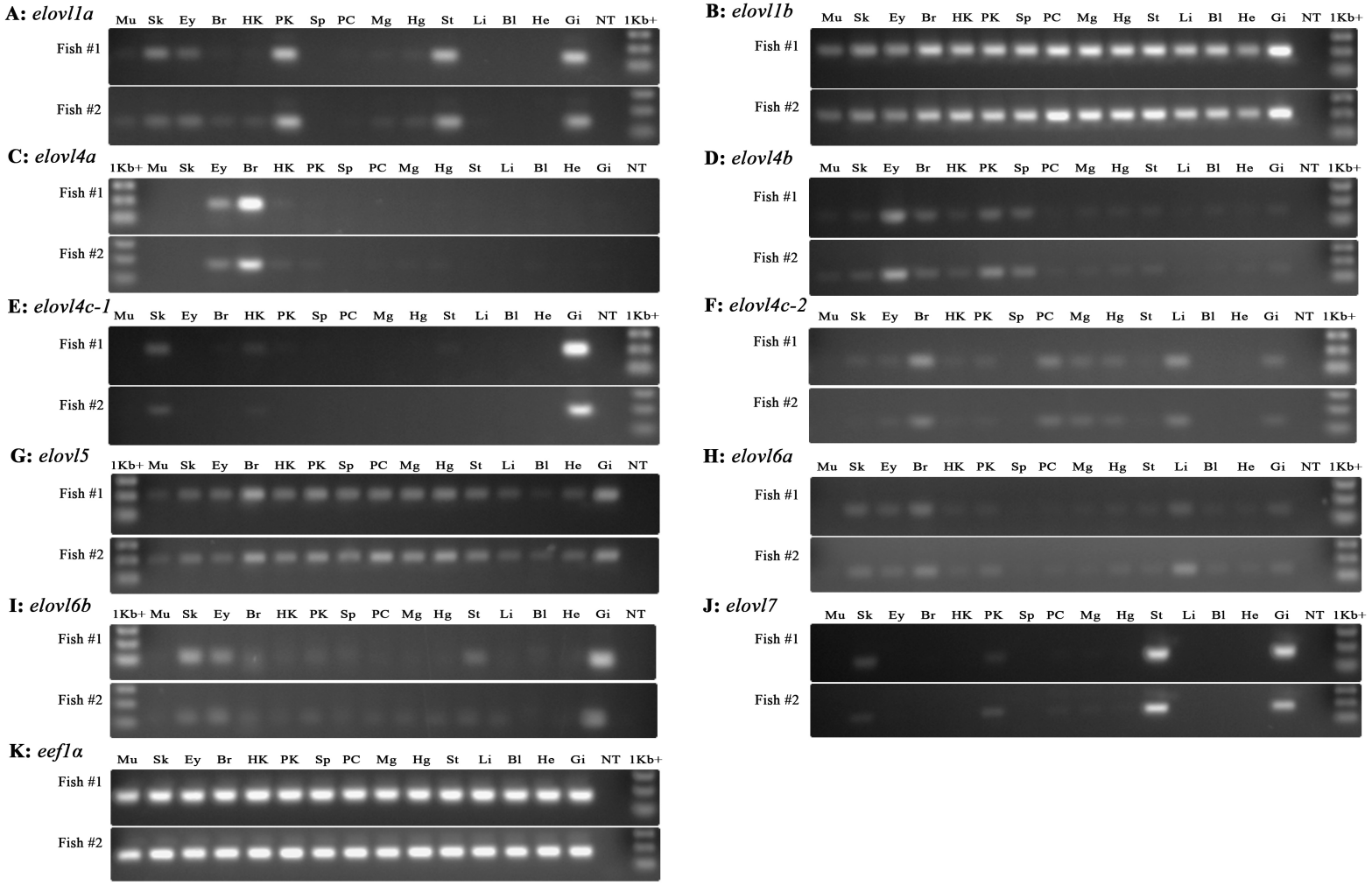
elovl4c-2 appearing to have arisen from a gene duplication event in the cod lineage (Fig. 2-3). Based on the phylogenetic tree, *elovl1* and *elovl6* gene duplication events preceded the divergence of cod, pufferfish, and zebrafish (Fig. 2-3).

2.4.2 *Elovl* gene family constitutive transcript expression

Qualitative RT-PCR was used to study constitutive expression of *elovl* transcripts in fifteen tissues (skeletal muscle, skin, eye, brain, head kidney, posterior kidney, spleen, pyloric caecum, midgut, hindgut, stomach, liver, blood, heart, and gill; Fig. 2-4). All PCR products ranged between 100 to 200 bp (Appendix II). There was no amplification in the "no-template controls" for all GSP pairs, and all individual samples exhibited bands of 170 bp with comparable intensity for the reference gene, *eef1α*. For each gene except *elovl5* and *eef1α*, two primer pairs were used to examine the *elovl* gene family constitutive transcript expression; the results using the first primer pair per gene are shown in Fig. 2-4, and the results for the second primer pair per gene are shown in Appendix XIV.

For each *elovl* family member, constitutive transcript expression profiles across the 15-tissue panel were very similar for the two juvenile cod included in the study (Fig. 2-4 and Appendix XIV). *Elovl1a* and *elovl1b* constitutive transcript expression profiles were very different from one another; *elovl1b* was ubiquitously expressed in the 15 tissues tested, whereas *elovl1a* expression appeared to be more tissue-specific with highest transcript levels observed in posterior kidney, stomach gill, skin, and eye (Fig. 2-4A,B). The constitutive transcript expression of the four cod *elovl4* paralogues (*elovl4a*, *elovl4b*, *elovl4c-1* and *elovl4c-2*) exhibited various profiles across the tissues examined

Fig. 2-4. RT-PCR assessment of tissue distribution of *elovl1a*, *elovl1b*, *elovl4a*, *elovl4b*, *elovl4c-1*, *elovl4c-2*, *elovl5*, *elovl6a*, *elovl6b* and *elovl7* transcripts in Atlantic cod for various tissues including skeletal muscle (Mu), skin (Sk), eye (Ey), brain (Br), head kidney (HK), posterior kidney (PK), spleen (Sp), pyloric caecum (PC), midgut (Mg), hindgut (Hg), stomach (St), liver (Li), blood (Bl), heart (He), and gill (Gi). These tissues were collected from two juvenile cod. Expression of the reference gene, *elongation factor 1α* (*eef1α*) is shown in the bottom row. NT, no-template control. 1Kb plus ladder (Invitrogen) was included in each panel to show the size of the PCR amplicon. The primer pairs used in these RT-PCRs were *elovl1a* (f5, r5), *elovl1b* (f4, r4), *elovl4a* (f5, r5), *elovl4b* (f4, r4), *elovl4c-1* (f5, r5), *elovl4c-2* (f5, r5), *elovl5* (f4, r4), *elovl6a* (f4, r4), *elovl6b* (f5, r5) and *elovl7* (f4, r4) as listed in Appendix II.


A: elovl1a
Mu Sk Ey Br HK PK Sp PC Mg Hg St Li Bl He Gi NT 1Kb+
Fish #1
Fish #2
B: elovl1b
Mu Sk Ey Br HK PK Sp PC Mg Hg St Li Bl He Gi NT 1Kb+
Fish #1
Fish #2
C: elovl4a
1Kb+ Mu Sk Ey Br HK PK Sp PC Mg Hg St Li Bl He Gi NT
Fish #1
Fish #2
D: elovl4b
Mu Sk Ey Br HK PK Sp PC Mg Hg St Li Bl He Gi NT 1Kb+
Fish #1
Fish #2
E: elovl4c-1
Mu Sk Ey Br HK PK Sp PC Mg Hg St Li Bl He Gi NT 1Kb+
Fish #1
Fish #2
F: elovl4c-2
Mu Sk Ey Br HK PK Sp PC Mg Hg St Li Bl He Gi NT 1Kb+
Fish #1
Fish #2
G: elovl5
1Kb+ Mu Sk Ey Br HK PK Sp PC Mg Hg St Li Bl He Gi NT
Fish #1
Fish #2
H: elovl6a
Mu Sk Ey Br HK PK Sp PC Mg Hg St Li Bl He Gi NT 1Kb+
Fish #1
Fish #2
I: elovl6b
1Kb+ Mu Sk Ey Br HK PK Sp PC Mg Hg St Li Bl He Gi NT
Fish #1
Fish #2
J: elovl7
Mu Sk Ey Br HK PK Sp PC Mg Hg St Li Bl He Gi NT 1Kb+
Fish #1
Fish #2
K: eef1α
Mu Sk Ey Br HK PK Sp PC Mg Hg St Li Bl He Gi NT 1Kb+
Fish #1
Fish #2

(Fig. 2-4C,D,E,F). For example, the transcript expression of *elovl4a* was tissue-specific (eye and brain; Fig. 2-4C). In contrast, the expression of *elovl4b* was not limited to eye and brain, but was also seen in skin, head kidney, posterior kidney and spleen (Fig. 2-4D). *Elovl4c-1* transcript expression appeared to be tissue-specific (gill and skin, with relatively higher expression in gill; Fig. 2-4E); in contrast, *elovl4c-2* transcript expression was detected in several tissues (brain, pyloric caecum, liver, posterior kidney, midgut, hindgut and gill; Fig. 2-4F). *Elovl5* transcript was found to be expressed in all tissues tested although with relatively low levels in muscle and blood (Fig. 2-4G). The constitutive transcript expression profiles of *elovl6a* and *elovl6b* were somewhat similar, with both transcripts expressed in skin, eye, brain and gill; however, of these two paralogous transcripts only *elovl6a* had detectable expression in liver tissue (Fig. 2-4H), and only *elovl6b* had detectable expression in stomach tissue (Fig. 2-4I). The transcript expression of *elovl7* appeared to be tissue-specific (stomach, gill, skin, and posterior kidney, with highest expression in stomach and gill; Fig. 2-4J). The RT-PCR qualitative transcript expression study showed that *elovl1b*, *elovl4c-2*, *elovl5* and *elovl6a* were expressed in juvenile cod liver, which is functionally important for fatty acid metabolism. Hence, these four *elovl* transcripts were selected for the QPCR experiment designed to study hepatic transcript expression responses to diets containing CO.

2.4.3 Growth performance of cod fed CO-containing diets versus FO control diet

The growth performance and fatty acid data for this feeding trial were reported in Hixson and Parrish (2014). However, since the growth data are also relevant to the current study, they are briefly described and included in this chapter (Table 2-3).

Table 2-3. Growth performance of juvenile Atlantic cod (week 13)[1].

	FO (control)	**100CO**	**100COSEFM**
Initial weight (g)	14.3 ± 1.2	14.6 ± 1.0	14.5 ± 1.4
Final weight (g)	50.8 ± 10.3[a]	43.6 ± 8.9[b]	46.6 ± 10.7[b]
Weight gain (g)	36.6 ± 1.4[a]	29.0 ± 1.2[b]	32.1 ± 1.0[b]
Initial length (cm)	11.3 ± 0.3	11.2 ± 0.3	11.2 ± 0.4
Final length (cm)	17.0 ± 1.1[a]	16.4 ± 1.0[b]	16.8 ± 1.1[a]
SGR[2]	1.31 ± 0.03[a]	1.13 ± 0.05[b]	1.21 ± 0.10[ab]
Condition factor[3]	1.01 ± 0.1[a]	0.98 ± 0.1[ab]	0.97 ± 0.1[b]
HSI[4] (%)	6.8 ± 1.5	6.9 ± 1.4	7.6 ± 1.5
AFI[5]	33.0 ± 1.8[a]	28.2 ± 1.4[b]	29.3 ± 1.0[b]
FCR[6]	0.90 ± 0.03	0.97 ± 0.03	0.91 ± 0.05

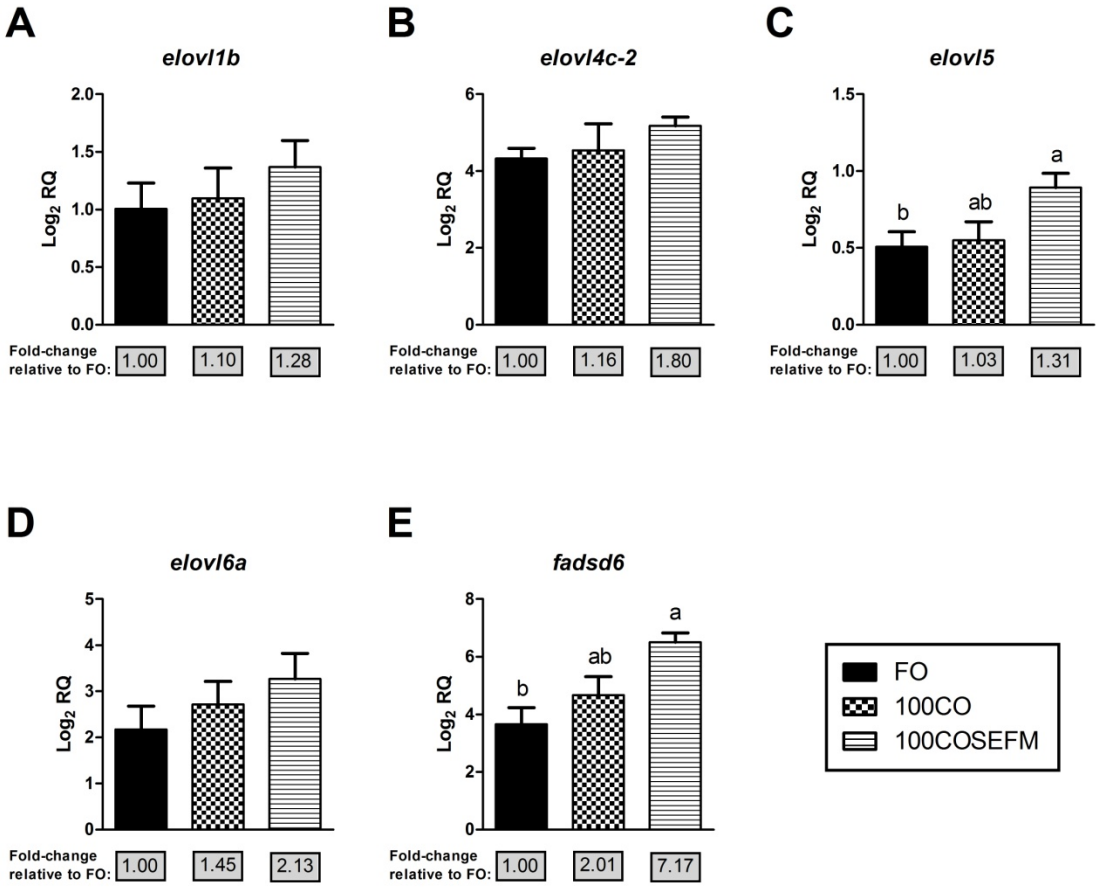
[1]Data are presented as mean ± standard deviation. Means with different letters are significantly different ($p < 0.05$), determined by one-way ANOVA (Minitab v16). Initial measurements, n = 9. n is variable depending on number of fish remaining in tank for final measurements: FO = 99, 100CO = 104, 100COSEFM = 105. Note that all data here were presented in Hixson and Parrish (2014), however, all statistics were re-run due to the exclusion of a dietary treatment in the current study.
[2]SGR, Specific growth rate (%/day) = 100*[ln(final weight) - ln(initial weight)]/days.
[3]Condition factor = 100*final weight/final length^3.
[4]HSI, Hepatosomatic index = 100*(liver weight/body weight).
[5]AFI, Apparent feed intake (g/fish).
[6]FCR, Feed conversion ratio = AFI/weight gain.

Initially, cod were 14.4 ± 1.6 g and grew to 43.6 to 50.8 g (average final weight in different diet groups) after 13 weeks of feeding experimental diets (Table 2-3). Initially, there were no significant differences in weights or lengths of fish in the three diet groups (Table 2-3). Cod fed either 100CO or 100COSEFM had significantly lower final weight than cod fed the FO control diet (Table 2-3). The same result was observed in weight gain. In terms of changes in the length of fish, cod fed FO and 100COSEFM were significantly longer than cod fed 100CO. The apparent feed intake (AFI) per fish through the feeding trial was affected by diet, with fish fed the FO diet consuming more than fish fed either of the CO-containing diets (Table 2-3). Furthermore, cod fed 100CO had a significantly lower specific growth rate (SGR; 1.13% day^{-1}) than FO fed cod (1.31% day^{-1}). However, fish fed all three diets had comparable feed conversion ratios (FCR) after 13 weeks of feeding, and the hepatosomatic index (HSI) of cod was not significantly affected by diet (6.0 to 7.6%) (Table 2-3).

2.4.4 Hepatic transcript expression responses to diets containing camelina oil

The QPCR experiment included four *elovl* transcripts shown from the tissue panel RT-PCR to be expressed in liver tissue (*elovl1b*, *elovl4c-2*, *elovl5*, and *elovl6a*). An additional transcript, *fadsd6*, also involved in LC-PUFA biosynthesis, was also evaluated with Atlantic cod liver templates from fish fed three diets (FO, 100CO and 100COSEFM) from week 13 of the feeding trial. The transcript expression of *elovl1b* (p = 0.552), *elovl4c-2* (p = 0.426), and *elovl6a* (p = 0.349) in cod liver was not significantly affected by feeding either of the CO-containing diets (i.e. 100CO and 100COSEFM) compared with the FO diet (Fig. 2-5A,B,D). While the overall fold-change values of *elovl4c-2* and

Fig. 2-5. QPCR analysis of *elovl1b*, *elovl4c-2*, *elovl5*, *elovl6a* and *fadsd6* transcript expression in liver of cod fed 100CO, 100COSEFM, or FO diets at week 13. Gene expression data, presented as mean $\log_2$ transformed relative quantity (RQ) ± standard error, was normalized to *60S acidic ribosomal protein P1* (*rplp1*). RQ values were calibrated to the individual with the lowest normalized gene expression. Within a given gene of interest study, different letters indicate significant difference ($p < 0.05$) between groups. For each condition (i.e. diet), fold up-regulation was calculated as 2^{A-B}, where A is the mean $\log_2$ transformed RQ from an experimental group (i.e. 100CO or 100COSEFM), and B is the mean $\log_2$ transformed RQ from the FO group (Cui and Churchill, 2003). As noted in the Methods, two outliers (one 100COSEFM fish for elovl4c-2, and one FO fish for elovl5) from were identified ($p < 0.01$; Grubbs' test) and excluded from the data analyses.


A
elovl1b
Log2 RQ
2.0
1.5
1.0
0.5
0.0
Fold-change relative to FO:
1.00
1.10
1.28
B
elovl4c-2
Log2 RQ
6
4
2
0
Fold-change relative to FO:
1.00
1.16
1.80
C
elovl5
Log2 RQ
1.5
1.0
0.5
0.0
b
ab
a
Fold-change relative to FO:
1.00
1.03
1.31
D
elovl6a
Log2 RQ
5
4
3
2
1
0
Fold-change relative to FO:
1.00
1.45
2.13
E
fadsd6
Log2 RQ
8
6
4
2
0
b
ab
a
Fold-change relative to FO:
1.00
2.01
7.17
FO
100CO
100COSEFM

elovl6a were 1.80 and 2.13, respectively, in 100COSEFM compared with FO fed cod, this up-regulation was not statistically significant (Fig. 2-5B,D). However, *elovl5* transcript was significantly up-regulated (1.31-fold) in the 100COSEFM group compared with the FO group, with no difference between 100CO and FO groups (Fig. 2-5C). *Fadsd6* transcript was significantly up-regulated (7.17-fold) in cod fed 100COSEFM compared with cod fed the FO diet, with no significant different between 100CO and FO groups (Fig. 2-5E).

2.5 Discussion

In this study, ten putative Elovl-coding transcripts were identified in Atlantic cod by mining the Atlantic cod genome database with the aid of other databases such as the cod transcriptome assembly, CGP EST, and NCBI nr databases. For cod *elovl1a*, *elovl1b*, *elovl4c-1*, *elovl4c-2*, *elovl5*, *elovl6a*, *elovl6b*, and *elovl7*, I sequenced the full-length cDNA and resolved the gene structure. I also obtained the partial cDNA sequences and partially resolved the gene structures of Atlantic cod *elovl4a* and *elovl4b* (Fig. 2-1). In addition, I studied the constitutive expression of all ten *elovl* transcripts across fifteen tissue types from juvenile cod. Furthermore, I investigated growth performance and hepatic transcript expression of four *elovls* and *fadsd6* in a metabolically important tissue (liver) of cod fed CO-containing diets (i.e. 100CO and 100COSEFM) versus cod fed a FO-based diet.

2.5.1 *Elovl* gene family characterization and molecular phylogenetics

The full-length cDNAs of cod *elovl1a*, *elovl1b*, *elovl4c-1*, *elovl4c-2*, *elovl5* and *elovl7* each include eight exons, while the full-length cDNA of cod *elovl6a* and *elovl6b* each include four exons. Zebrafish gene structure of both *elovl6a* and *elovl6b* matches the pattern observed in cod. From the analysis of cod *elovl* transcript sequences (Fig. 2-1), both *elovl1b* and *elovl4c-2* have two transcript variants that only differ at the 3' UTR, resulting from alternative polyadenylation. Interestingly, the cod *elovl5* transcript sequence obtained from the current RACE study is different at the 5' and 3' ends from the cod *elovl5* cDNA sequence (GenBank accession number AY660881) that was previously characterized (see section 2.4.1) (Agaba et al., 2005). The two different *elovl5* transcript variants may arise from different transcript splicing events.

All cod Elovl members exhibit characteristic features of microsomal-bound enzymes from other systems (Agaba et al., 2005), including four different conserved motifs (i.e. KXXEXXDT, QXXFLHXXHH, the tyrosine box NXXXHXXMYXYY, and TXXQXXQ), several predicted transmembrane domains and a single histidine box contained within the QXXFLHXXHH motif. In addition, the C-terminal lysine or arginine residues (i.e. KXKXX, KXRXX, or KKXX) found in most cod Elovl members, as well as in zebrafish Elovl proteins, may function as ER retention signals (Jakobsson et al., 2006; Morais et al., 2009). It is worth noting that both zebrafish and cod Elovl6 proteins are very different from other Elovl family members based on the multiple sequence alignment (Fig. 2-2). This is also seen in the molecular phylogenetic analysis since Elovl6 proteins were grouped separately from any other Elovl family members for

all fish species included in the tree (Fig. 2-3). Overall, however, the conservation of protein sequence across the Elovl family is remarkably high (Appendix III).

The phylogenetic tree indicated that the Elovl proteins can be grouped into three subfamilies (Fig. 2-3). In terms of one of the PUFA-responsive elongases Elovl2, which has shown capacity in elongating C_{20} and C_{22} PUFA with low activity towards C_{18} as seen in Atlantic salmon and zebrafish (Morais et al., 2009), a tBLASTn search of the cod genome assembly (gadMor1 v73.1) in the Ensembl database using the AA sequence of zebrafish Elovl2 as query showed hits [e.g. 70.7% identity over 58 aligned AA with E-value of 2.0e-60 (most significant)] against the regions of GeneScaffold 1260 which encode *elovl5*, rather than a potential *elovl2* family member. Moreover, Morais et al. (2009) previously performed searches of other marine fish genomes such as pufferfish, stickleback (*Gasterosteus aculeatus*), and medaka (*Oryzias latipes*) (all of which are members of superorder Acanthopterygii) for evidence of *elovl2* genes with negative results. Hence, it is reasonable to speculate that the *elovl2* gene might have been lost (e.g. silenced) in Atlantic cod (superorder Paracanthopterygii) as well as in fish belonging to superorder Acanthopterygii (Morais et al., 2009; Monroig et al., 2011). In contrast, *elovl2* genes are present in Atlantic salmon (superorder Protacanthopterygii) and zebrafish (superorder Ostariophysi) (Morais et al., 2009). Previous studies reported that Elovl4 acts in the biosynthesis of LC-PUFA in cobia and Atlantic salmon (Carmona-Antoñanzas et al., 2011; Monroig et al., 2011). It may be speculated that the loss of *elovl2* from the cod genome may have altered LC-PUFA biosynthetic capacity, and thereby influenced the evolution of cod *elovl4* paralogues. The molecular phylogenetic analysis revealed that Atlantic cod *elovl4* has expanded into 4 different paralogues (Fig. 2-3); the cod *elovl4c-1*

and *elovl4c-2*, which encode proteins that clustered along with their homologue from zebrafish (i.e. Elovl4c), appears to have arisen from a gene duplication event in the cod lineage.

2.5.2 *Elovl* gene family constitutive transcript expression

Qualitative RT-PCR analysis revealed that *elovl1b* transcript is ubiquitously expressed in all tissues tested. Unfortunately, there is no available tissue expression distribution data on *elovl1b* transcript in any other teleost species. However, in agreement with the current cod *elovl1b* transcript expression results, a previous study on *ELOVL1* in humans also exhibited a broad range of transcript expression including 16 different tissues (Ohno et al., 2010). This evidence supports the hypothesis that cod *elovl1b* is an orthologue of human *ELOVL1*. In *in vitro* studies, human ELOVL1 protein showed high activity toward both saturated and monounsaturated C_{20} and C_{22} acyl-CoAs. These fatty acyl-CoAs are important for the production of C_{24} sphingolipids in mammals (Ohno et al., 2010). The ubiquitous expression of cod *elovl1b* transcript suggests that the encoded protein may be required as a 'housekeeping elongase' to prevent the fluctuation of specific fatty acids as proposed in mammals (Jakobsson et al., 2006; Guillou et al., 2010). In contrast to cod *elovl1b*, *elovl1a* transcript was expressed in a more narrow range of tissues (posterior kidney, stomach, gill, skin, eye). Since *elovl1* is a single copy gene in mouse (Asadi et al., 2002), whereas there are two *elovl1* paralogues in evolutionarily diverged fish species (e.g. Atlantic cod, pufferfish, zebrafish; Fig. 2-3), the *elovl1* gene likely duplicated early in the teleost lineage. The different constitutive transcript expression profiles of cod *elovl1a* and *elovl1b* suggest that these paralogues have

undergone regulatory (and potentially functional) divergence. However, the physiological roles of these cod *elovl1* paralogues are not known and require further investigation.

The four paralogous cod *elovl4* transcripts (i.e. *elovl4a*, *elovl4b*, *elovl4c-1* and *elovl4c-2*) displayed very different constitutive transcript expression profiles across the fifteen tissues examined. Cod *elovl4a* transcript showed tissue-specific expression in brain and eye (Fig. 2-4C). Those two tissues are generally thought to have high requirements for VLC-FA and are prominent metabolic sites for the biosynthesis of these fatty acids (Carmona-Antoñanzas et al., 2011). The current cod *elovl4a* transcript expression results do not agree with a previous study on zebrafish *elovl4a*, which was abundantly expressed in a variety of tissues in addition to brain and eye (e.g. intestine, gill, testis, ovary, posterior kidney, heart, spleen, skin, liver, anterior kidney) (Monroig et al., 2010). Unlike the tissue-restricted expression of cod *elovl4a*, transcript expression of cod *elovl4b* was seen in several different tissues (skin, brain, head kidney, posterior kidney, spleen, and eye; Fig. 2-4D). Zebrafish *elovl4b* transcript was shown to be expressed in testis, ovary, eye, and posterior kidney, but not in brain tissue (Monroig et al., 2010), and Atlantic salmon *elovl4* transcript expression was seen in eye, brain and testis (Carmona-Antoñanzas et al., 2011). While the current cod study did not include gonad tissues, a comparison of the current results with those of Monroig et al. (2010) and Carmona-Antoñanzas et al. (2011) suggests that some aspects of *elovl4* transcript expression may be conserved in teleost fishes (e.g. *elovl4b* expression in eye), with other constitutive transcript expression characteristics being different between lineages (e.g. *elovl4a* transcript expression in several tissues in zebrafish where expression was not observed in cod). Functional characterization of zebrafish Elovl4a suggests that this

enzyme is capable of elongating saturated VLC-FA up to C_{36}, with C_{26} as the most preferred substrate (Monroig et al., 2010). In contrast, zebrafish Elovl4b shows preferences toward both C_{20} and C_{22} fatty acids as substrates in the elongation process (Monroig et al., 2010). In a more recent study, *elovl4* has been cloned from marine fish cobia and its expression was detected in a wide range of tissues except red muscle, skin and pyloric caecum; cobia *elovl4* was shown to have a closer evolutionary relationship to zebrafish *elovl4b* than to zebrafish *elovl4a* (Monroig et al., 2011). The cobia Elovl4 is able to synthesize VLC-FA including saturated and polyunsaturated FAs with chain lengths of more than 24 carbons. This enzyme is also involved in the elongation of 20:5ω3 and 22:5ω3, which is a critical step for the production of DHA (Monroig et al., 2011). In the Atlantic cod lineage, the *elovl4c* gene apparently duplicated, giving rise to two paralogues (*elovl4c-1* and *elovl4c-2*) with very different constitutive transcript expression profiles (Fig. 2-3; Fig. 2-4E,F). While cod *elovl4c-1* transcript expression was only seen in two tissues (higher in gill, lower in skin; Fig. 2-4E), cod *elovl4c-2* transcript expression was seen in several tissues including brain, pyloric caecum and liver (Fig. 2-4F). These differences in basal transcript expression profiles suggest that cod *elovl4c-1* and *elovl4c-2* may have diverged (e.g. undergone neofunctionalization or subfunctionalization) after the gene duplication event that gave rise to these paralogues. In addition, my results suggest that Atlantic cod Elovl4 members (especially Elovl4a and Elovl4b) might play roles in the biosynthesis of VLC-FA as well as LC-PUFA in specific tissues that have high constitutive expression (e.g. eye and brain). Whether or not cod Elovl4 members are involved in the production of VLC-FA and LC-PUFA requires further investigation.

The cod *elovl5* transcript was detected in all fifteen tissues tested (Fig. 2-4G). The expression of cod *elovl5* transcript appeared to be higher in some tissues (e.g. brain and gill) than others (e.g. muscle, blood, liver and heart), in agreement with previous work on the tissue distribution of cod *elovl5* transcript (Tocher et al., 2006). In Atlantic salmon, the transcript expression levels of both *elovl5a* and *elovl5b* were highest in intestine (i.e. pyloric caecum), liver and brain (Zheng et al., 2005; Morais et al., 2009). The cloning of the cod *elovl5* gene was first reported in 2005, and the functional characterization study of Elovl5 showed lowest capacity to lengthen ω3 and ω6 PUFA with chain lengths from C_{18} to C_{22}, compared to two other marine species (turbot and sea bream) and other freshwater fish species (Agaba et al., 2005; Monroig et al., 2009). Since cod Elovl5 has limited LC-PUFA biosynthetic capacity (Agaba et al., 2005), the observed relatively high constitutive transcript expression levels of *elovl5*, *elovl4a* and *elovl4b* in cod brain may be needed to maintain sufficient LC-PUFA (e.g. DHA) supply.

Our qualitative RT-PCR analyses of cod *elovl6a* and *elovl6b* transcripts revealed that both paralogues were expressed in skin, eye and brain (Fig. 2-4H,I). Interestingly, *elovl6a* transcript was also detected in liver (Fig. 2-4H), whereas *elovl6b* transcript was expressed in stomach and gill (Fig. 2-4I). This is the first *elovl6* transcript expression study in any teleost species. In rat, *elovl6* transcript was found by RT-PCR to be highly expressed in brain, with lower levels of expression in other tissues including kidney, liver, skin and heart (Wang et al., 2005). In addition, semi-quantitative RT-PCR showed that human *elovl6* transcript was ubiquitously expressed (i.e. in all fifteen tissues examined) (Ohno et al., 2010). The functions of cod Elovl6a and Elovl6b, and the physiological significance of paralogue-specific differences in basal transcript expression profiles in

cod as well as differences in putative orthologous transcript expression between species, remain to be studied.

The transcript expression of *elovl7* was highest in stomach and gill tissues with relatively low levels of expression in skin and posterior kidney (Fig. 2-4J). Prior to the current study, there was no available information on *elovl7* transcript tissue expression distribution in any teleost species. As noted for *elovl6*, there are differences between the observed cod *elovl7* basal transcript expression profile and previously published human *ELOVL7* transcript expression results. For example, cod *elovl7* transcript was detected in only four of the fifteen tissues tested (stomach, gill, skin, and posterior kidney), semi-quantitative RT-PCR showed that human *ELOVL7* expression could be detected in almost all of the fifteen tissues examined (i.e. all except heart and skeletal muscle) (Ohno et al., 2010). Human *ELOVL7* transcript is expressed at high levels in the kidney, pancreas, adrenal gland, and several other tissues based on a Northern blot analysis, in agreement with the RT-PCR results (Tamura et al., 2009). Purified human ELOVL7 enzyme exhibited high activity toward C_{18} acyl-CoAs (Naganuma et al., 2011). This enzyme was confirmed to be involved in the elongation of saturated fatty acids up to C_{24} through the over-expression of ELOVL7 in microsomes (Tamura et al., 2009; Guillou et al., 2010). The physiological importance of the observed high constitutive expression of cod *elovl7* transcript in stomach and gill, and the roles of Elovl7 enzyme in these tissues, remain to be investigated.

2.5.3 Growth performance of cod

The feeding experiment conducted in this study was to evaluate the growth performance of Atlantic cod fed diets containing CO and/or SEFM. Juvenile cod fed either 100CO or 100COSEFM had a significantly lower weight gain than cod fed FO, by 21% and 12% respectively. It is worth noting that fish fed 100CO showed significant lower SGR (1.13% day^{-1}) than fish on FO diet (1.31% day^{-1}), while the SGR of fish fed 100COSEFM (1.21% day^{-1}) was not significantly different from either FO or 100CO. Two previous studies evaluating the replacement of dietary FO with CO, one replacing 80% (Hixson et al. 2013) and the other replacing 100% (Morais et al. 2012), showed no significant differences in growth of cod fed CO-containing diets versus cod fed FO control diets. The growth performance results of Morais et al. (2012) differ from those of the current cod feeding experiment for fish fed diets with 100% of FO replaced by CO. It is possible that the differences in growth results from these studies can be explained by the different amounts of DHA and EPA present in the diets. In the Morais et al. (2012) study, the C100 diet (CO replacing 100% of FO) that was tested had more DHA (6.2% of total fatty acids) and EPA (4.6%), which exceeded essential fatty acid requirements for optimal growth, compared to the DHA (3.8%) and EPA (3.0%) in the 100CO diet in this study (Hixson and Parrish, 2014). In addition, the significant differences in growth observed in the current study could also be due to differences in apparent feed intake between diet groups, as fish fed the FO diet consumed significantly more than fish fed the CO-containing diets (Table 2-3).

2.5.4 Hepatic transcript expression responses to diets containing camelina oil

In order to assess if the elongase and desaturase transcripts in cod liver were inducible by feeding CO-containing diets (i.e. 100CO and 100COSEFM), which contain low amounts of LC-PUFA but are high in C_{18} PUFA, the expression levels of transcripts encoding fatty acyl elongases (Elovl1b, Elovl4c-2, Elovl5 and Elovl6a) and desaturase (Fadsd6) were investigated using QPCR. The selection of these *elovl* family members for the QPCR study was based on their constitutive expression in liver tissue as detected by qualitative RT-PCR (Fig. 2-4). Among *elovl* family members selected for the current QPCR study, at least in mammals including humans, ELOVL1 and ELOVL6 are considered to be preferred for saturated and monounsaturated fatty acids while ELOVL4 and ELOVL5 use PUFA as substrates (Monroig et al., 2010). Hence, the following discussion is divided into two categories: metabolism of non-essential fatty acids, and metabolism of essential PUFA.

The biological significance of non-essential fatty acids, particularly saturated fatty acids, has been linked to some undesirable health effects including obesity, heart failure, and peroxisomal disorders (Guillou et al., 2010). The precise function and preferred substrate of Elovl1 enzymes have not been investigated in any fish species. Since cod *elovl1b* transcript is constitutively expressed in the liver (as well as in several other tissues, Fig. 2-4B), the present study evaluated the hepatic *elovl1b* transcript expression response to diets containing different levels of CO. The transcript expression of *elovl1b* in cod liver was not significantly affected by feeding either of the CO-containing diets (i.e. 100CO and 100COSEFM). A similar result was reported in rats fed olive oil compared to

rats fed FO (Wang et al., 2005); however, in the same study, the transcript expression of *elovl1* in rat liver was induced 2-fold after administration of the peroxisome proliferator-activated receptor alpha (PPARα) agonist Wy14,643. which activated PPARα through binding. Endogenous ligands include fatty acids and some of their eicosanoid metabolites (reviewed in Leaver et al., 2008). This suggests that the transcript expression of rat *elovl1* is under certain nutritional regulation.

It has been shown that in rat, the hepatic *elovl6* transcript expression is regulated by nutritional factors including fasting and refeeding, high-fat diets, and high-PUFA diets (Rodriguez-Cruz et al., 2012). The potential function and preferred substrate of Elovl6 have not been investigated in any fish species. The current study investigated the hepatic gene expression response of *elovl6a* to diets containing different levels of CO, based on the fact that this transcript was constitutively expressed in cod liver (Fig. 2-4H) and therefore may play a role in metabolism. From QPCR analysis, *elovl6a* was 2.13 fold up-regulated (not statistically significant) in cod fed 100COSEFM compared to cod fed FO diet. A previous study in rat showed that *elovl6* transcript was significantly reduced in liver by feeding a diet supplemented with FO (Wang et al., 2005). Additionally, a suppression in the elongation activity of palmitic acid (16:0) was correlated with an decrease in *elovl6* transcript expression in rats fed a FO supplemental diet (Wang et al., 2005). Further studies are needed in regard to *elovl6* gene expression regulation and the function of the Elovl6 enzyme in cod.

The biosynthesis of EPA in vertebrates involves desaturation of 18:3ω3 to 18:4ω3, which is further elongated to 20:4ω3 followed by desaturation to obtain EPA (Zheng et al., 2009; Monroig et al., 2011). The synthesis of DHA from EPA requires two

further elongation processes, an additional desaturation, and a peroxisomal chain shortening step (Sprecher, 2000). In the current QPCR study, the transcript expression of *elovl5* in cod liver did respond to the inclusion of camelina oil in the 100COSEFM fed group by approximately 1.3-fold up-regulation compared to FO. The increase in *elovl5* transcript expression in cod liver (but not pyloric caecum) corresponding to the increased level of C_{18} PUFA in the diet was also shown in a previous feeding trial involving the substitution of vegetable oil blend (i.e. rapeseed, linseed and palm oils) for FO (Tocher et al., 2006). All fish Elovl5 enzymes as functionally characterized by heterologous expression of ORFs in yeast (e.g. *Saccharomyces cerevisiae*), including freshwater fish [zebrafish, African catfish (*Clarius gariepinus*), Nile tilapia (*Oreochromis niloticus*)], salmonids [Atlantic salmon and rainbow trout (*Oncorhynchus mykiss*)], and marine species (cod, turbot, sea bream, and cobia), demonstrated capacity to lengthen ω3 and ω6 PUFA with chain length from C_{18} to C_{22}; Atlantic cod Elovl5 showed the lowest activity of all the species studied (i.e. 7.4% and 0.8% conversion of 18:4ω3 to 20:4ω3 and 20:5ω3 to 22:5ω3, respectively) (Hastings et al., 2004; Meyer et al., 2004; Agaba et al., 2005; Monroig et al., 2009; Morais et al., 2009). Generally speaking, most marine fish species including cod have low LC-PUFA biosynthetic capacity using FA precursors (Agaba et al., 2005; Tocher et al., 2006). The limited ability to produce LC-PUFA in marine fish may be due to both low enzymatic activity of Elovl5 and the absence of *elovl2* genes (as discussed previously). The difference in LC-PUFA biosynthetic capacity between freshwater fish and marine fish may be a reflection of both biological and environmental differences. The high LC-PUFA biosynthetic ability in freshwater fish may be needed for the production of LC-PUFA from shorter chain precursors due to a relative deficiency of

LC-PUFA in their native freshwater habitat, whereas wild marine fish have lower capacity to biosynthesize LC-PUFA potentially due to relatively high levels of LC-PUFA in their diets (Agaba et al., 2005; Tocher et al., 2006; Morais et al., 2009).

As discussed earlier, Atlantic cod Elovl4 members (especially Elovl4a and Elovl4b) might also be involved in the biosynthesis of LC-PUFA in specific tissues that have high basal expression (e.g. eye and brain). However, only *elovl4c-2* was investigated in this study in terms of hepatic transcript expression in response to diets containing camelina oil as this was the only *elovl4* paralogue that showed constitutive expression in liver (Fig. 2-4). QPCR showed that cod *elovl4c-2* transcript was increased by 1.8-fold in 100COSEFM fed fish compared to FO, but this up-regulation was not statistically significant due to high biological variability (Fig. 2-5B). Recently, *elovl4* genes were discovered in zebrafish (Monroig et al., 2010), Atlantic salmon (Carmona-Antoñanzas et al., 2011) and cobia (Monroig et al., 2011); the salmon Elovl4, zebrafish Elovl4b as well as cobia Elovl4 were capable of converting C_{20} and C_{22} LC-PUFA to longer products. Hence these Elovl4s found in fish may participate in the biosynthesis of DHA. This alternative elongase member for the production of DHA is particularly significant in marine fish as it could potentially compensate for the low activity of Elovl5 in the LC-PUFA biosynthetic pathway. The involvement of Atlantic cod Elovl4 members in LC-PUFA synthesis warrants further investigation.

The biosynthesis of LC-PUFA from shorter chain precursors in vertebrates also involves desaturation of appropriate fatty acids by desaturase enzymes, Fadsd5 and Fadsd6. Except for a bifunctional Δ5/Δ6 desaturase reported from rabbitfish (*Siganus canaliculatus*) (Li et al., 2010), only one fatty acyl desaturase, Fadsd6, has been identified

in marine fish including cod (Tocher et al., 2006), cobia (Zheng et al., 2009), gilthead sea bream (Seiliez et al., 2003), Asian sea bass (*Lates calcarifer*) (Mohd-Yusof et al., 2010), European sea bass (*Dicentrarchus labrax*) (González-Rovira et al., 2009), and turbot (Zheng et al., 2004). Interestingly, in the current study *fadsd6* transcript expression was only significantly up-regulated (7.17-fold) in the liver of cod fed 100COSEFM diet compared to cod fed FO diet (Fig. 2-5E). It is worthy to note that the LC-PUFA amounts in 100COSEFM diet were much lower than that of the FO diet (5.1% vs. 21.9%), whereas the amounts of C_{18} PUFA in these diets exhibited opposite trend (36.5% vs. 7.6%) (Hixson and Parrish, 2014). This suggests that the low level of dietary LC-PUFA accompanied with a high level of C_{18} PUFA may have a positive effect on the transcriptional response of *fadsd6*, a critical gene controlling the LC-PUFA biosynthetic pathway. However, two previous studies examining the effect of vegetable oils (i.e. blend of rapeseed, linseed and palm oils or CO) containing diets on cod *fadsd6* gene expression had inconclusive results, as the up-regulation (compared to fish fed FO diet) of *fadsd6* in liver of fish fed vegetable oil diets was not statistically significant, possibly due to high biological variability (Tocher et al., 2006; Morais et al., 2012). In addition, in the 100COSEFM diet included in the current study, the fish meal component was solvent extracted to remove residual FO (~8% in fish meal), resulting in extremely low LC-PUFA in the current experimental diet (100COSEFM) compared to the diets in those two previous cod studies (Tocher et al., 2006; Morais et al., 2012; Hixson and Parrish, 2014); this may have contributed to the different responses in desaturase transcript expression between studies.

2.6. Conclusions

This study shows that Atlantic cod expresses ten members of the *elovl* gene family (*elovl1a*, *elovl1b*, *elovl4a*, *elovl4b*, *elovl4c-1*, *elovl4c-2*, *elovl5*, *elovl6a*, *elovl6b* and *elovl7*) with high sequence similarity to putative orthologues from other fish species such as zebrafish, Atlantic salmon and pufferfish. A study of the constitutive expression of *elovl* family transcripts in fifteen tissues of juvenile cod revealed distinct *elovl* expression profiles in different tissues. For example, *elovl1b* and *elovl5* were ubiquitously expressed in all tissues examined, whereas others such as *elovl4a* and *elovl4c-1* had tissue-specific expression. Four *elovl* members, *elovl1b*, *elovl4c-2*, *elovl5* and *elovl6a*, showed transcript expression in juvenile cod liver, and therefore were selected for the QPCR study on hepatic transcript expression responses to diets containing different levels of CO. Cod fed 100COSEFM showed significantly lower weight gain, with significant up-regulation of *elovl5* and *fadsd6* transcripts, compared with cod on a FO diet. The high ALA and/or low ω3 LC-PUFA levels in the diet may be associated with the up-regulation of *elovl5* and *fadsd6* which may be involved in LC-PUFA biosynthesis in cod. It is important for future studies to define the specific biochemical functions of Atlantic cod Elovl members, especially the newly characterized Elovl4s, in terms of fatty acid biosynthesis.

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CHAPTER 3: Atlantic salmon (*Salmo salar*) liver transcriptome response to diets containing camelina products

3.1 Abstract

Due to increasing demand for fish oil (FO) and fish meal (FM) in aquafeeds, more sustainable alternatives such as plant-derived oils and proteins are needed. *Camelina sativa* products are viable feed ingredients given the high oil and crude protein content in the seed and meal, respectively. Atlantic salmon (initial weight ~240 g) were fed diets with complete or partial replacement of FO and/or FM with camelina oil (CO) and/or camelina meal (CM) in a 16-week feeding trial [Control diet: FO; Test diets: 100% CO replacement of FO (100CO), or 100CO with solvent-extracted FM (100COSEFM), 10% CM (100CO10CM), or SEFM + 10CM (100COSEFM10CM)]. The weight gain and specific growth rate of fish fed the 100CO diet were not significantly reduced compared with FO controls, however these growth parameters were significantly lower in fish fed all other camelina-containing diets. A 44K microarray experiment was conducted to identify transcripts in Atlantic salmon liver that responded to 100COSEFM10CM compared to control diet, and yielded 67 differentially expressed features (FDR < 5%). Ten microarray-identified genes [*cpt1*, *pcb*, *bar*, *igfbp-5b* (2 paralogs), *btg1*, *dnph1*, *lect-2*, *clra*, *klf9*, and *fadsd6a*] and additional genes involved in lipid metabolism [*elovl2*, *elovl5* (2 paralogs), and *fadsd5*] were subjected to QPCR with liver templates from all 5 dietary treatments; of the microarray-identified genes, only *bar* was not validated. Both *igfbp-5b* paralogs were significantly downregulated and *fadsd6a* was significantly upregulated in all 4 camelina-containing diet groups compared with controls. This nutrigenomics approach revealed several salmon genes involved in lipid metabolism, carbohydrate metabolism and immune function that responded to camelina-containing

diets. Genes identified in this work could potentially be used as biomarkers to assist with the development of novel aquafeeds using camelina products.

3.2 Introduction

Fish products are a major source of ω3 long chain polyunsaturated fatty acids (LC-PUFA) in human diets (Tocher et al., 2006). Dietary LC-PUFA such as eicosapentaenoic acid (EPA; 20:5ω3) and docosahexaenoic acid (DHA; 22:6ω3), can benefit human health in several ways including enhancing cardiac health and reducing risk of inflammatory diseases (Calder and Yaqoob, 2009). The worldwide demand for seafood for human consumption, with approximately 50% coming from aquaculture, continues to climb due to flat or decreasing global wild fisheries in the face of rising human population (Agaba et al., 2005; Tocher et al., 2006; FAO, 2009; Bell et al., 2010). Finfish aquaculture, especially that of carnivorous fish such as Atlantic salmon (*Salmo salar*), relies heavily on fish oil (FO) and fish meal (FM) from wild stocks for the production of feeds. Consequently, the increasing demand of FO and FM will exceed the wild fishery supplies, threatening the sustainability of fisheries and aquaculture industries (Tocher et al., 2006).

The need to find alternatives to FO and FM in aquafeeds has been recognized as one of the most important areas of research in aquaculture (Bell et al., 2010). As an oilseed crop, camelina (*Camelina sativa*) has several characteristics that make it desirable for the aquaculture feed industry. Firstly, the oil content of camelina seed is about 40%, and camelina oil (CO) is especially rich in LC-PUFA precursors, α-linolenic acid (ALA, 18:3ω3) and linoleic acid (LNA, 18:2ω6); the levels of these fatty acids in CO are approximately 40% and 15%, respectively (Zubr, 1997; Hixson et al., 2013). Moreover, the ω3/ω6 ratio of CO, which is closely linked to both fish health as well as to the

nutritional value of fish to human consumers, is higher than other plant oils such as soybean oil and palm oil (reviewed in Glencross, 2009). Some by-products of camelina from the oil extraction process, such as the seed meal, may also be used in the aquaculture feed industry. Camelina meal (CM) has a crude protein level of approximately 45%, similar to canola and other rapeseed meal (Acamovic et al., 1999; Frame et al., 2007), and contains several essential amino acids (Zubr, 2003).

Research has previously been performed on FO substitutions with linseed oil (Torstensen et al., 2008), canola oil (Miller et al., 2007) and rapeseed oil (Jordal et al., 2005) for various fish species including Atlantic salmon. Studies have demonstrated that vegetable oil (either singly or as blends) which is low in LC-PUFA and high in C_{18} PUFA such as ALA and LNA, can be used to replace up to 100% of FO without negatively influencing growth in salmonids and marine fish (Bell et al., 2001; Torstensen et al., 2005; Bell et al., 2010; Morais et al., 2012; Hixson et al., 2013). However, the ω3 LC-PUFA content in fish fillets can be reduced significantly if FO is replaced by vegetable oil completely (Bell et al., 2010; Morais et al., 2012). Moreover, genes involved in the LC-PUFA biosynthetic pathway are known to be regulated by vegetable oil diets. Particularly, *delta-5 fatty acyl desaturase* (*fadsd5*) and *fatty acyl elongase* [*elongation of very long chain fatty acids* (*elovl*); e.g. *elovl2* and *elovl5*] genes are often up-regulated in the liver of Atlantic salmon fed diets containing vegetable oil (e.g. rapeseed oil) (reviewed in Leaver et al., 2008a). Compared to marine fish, freshwater fish and salmonids are superior at producing DHA and EPA using ALA (Santigosa et al., 2011). Last but not least, the changes in fatty acid profiles in the diets due to the replacement of FO by vegetable oil may alter fish metabolism, and could potentially affect various

aspects of fish health including susceptibility to infectious diseases (Montero et al., 2003; Mourente et al., 2005).

CO-containing diets have been used in studies involving Atlantic cod (*Gadus morhua*) (Morais et al., 2012; Hixson et al., 2013; Hixson and Parrish, 2014), Atlantic salmon (Bell et al., 2010; Leaver et al., 2011; Morais et al., 2011b; Hixson et al., 2014b), and rainbow trout (*Oncorhynchus mykiss*) (Hixson et al., 2014a). Previously in Atlantic salmon, CO was included in vegetable oil blends (20% CO) with other plant-based oils to study the effect of substituting FO with such oil blends on growth (Bell et al., 2010), ω3 LC-PUFA deposition in the flesh (Leaver et al., 2011), and cholesterol and lipoprotein metabolism (Morais et al., 2011b). In salmon practical diets, both FM and FO have been partially replaced with plant materials simultaneously (Pratoomyot et al., 2010). While high percentage (i.e. ≥ 60%) replacement of FM with plant proteins (especially soybean meal) in the diets of Atlantic salmon has been shown to result in reduced growth performance and altered fish immune responses and gut integrity (Baeverfjord and Krogdahl, 1996; Espe et al., 2006), inclusion of 20% pea protein concentrate in the diet for Atlantic salmon showed no reductions in weight gain and feed intake (Øverland et al., 2009). Recently, an Atlantic salmon feeding trial was conducted to evaluate the growth performance, and the lipid and fatty acid composition in tissues, of fish fed with diets containing full replacement of FO with CO and/or partial inclusion of CM (Hixson et al., 2014b). In the current study, the impact of camelina-containing diets on salmon liver gene expression was investigated in parallel with Hixson et al. (2014b) by analyzing samples from the same individuals using DNA microarray and quantitative reverse transcription – polymerase chain reaction (QPCR). While the diet ingredients and growth performance

data for this feeding trial were previously published (Hixson et al., 2014b); we include them herein as they pertain to the current study as well.

Nutrigenomic approaches [e.g. using DNA microarrays and quantitative reverse transcription – polymerase chain reaction (QPCR)] have been shown to be useful for the identification of genes that are differentially expressed in fish fed altered diet formulations, for example, with FO or FM replaced by plant-based ingredients (Jordal et al., 2005; Leaver et al., 2008b; Panserat et al., 2008a; Morais et al., 2011a). These previous studies focused on hepatic gene expression changes since the liver is the main organ involved in metabolizing carbohydrates, lipids, and proteins into biologically useful materials in vertebrates, and also plays key roles in detoxification and immunity (Vilhelmsson et al., 2004; Panserat et al., 2009). Therefore, the objective of this study was to use a 44,000 feature (44K) salmonid oligonucleotide microarray (Jantzen et al., 2011; Sahlmann et al., 2013) and QPCR to assess the impacts of CO and/or CM containing diets on Atlantic salmon hepatic gene expression in order to identify candidate molecular biomarkers for responses to camelina-containing diets. In addition, the current study included assessment of: 1) salmon performance (growth) and 2) the effect of changes in the fatty acid composition of diets containing different levels of CO on the transcript expression of *fatty acyl elongases* and *desaturases* involved in LC-PUFA biosynthesis. It is anticipated that the molecular biomarkers (i.e. camelina product-responsive genes) identified in this study will be useful in the future development of camelina-containing diets that do not have deleterious effects on fish performance or physiology.

3.3 Materials and methods

3.3.1 Experimental diets and animals

The feeding trial, involving Atlantic salmon post-smolts and test diets containing camelina products (e.g. CO and CM), was conducted in the Dr. Joe Brown Aquatic Research Building (JBARB) at the Ocean Sciences Centre (Memorial University of Newfoundland, Canada) during the period from July 2012 to December 2012. This research was part of a large-scale collaborative project (The Camelina Project; http://www.genomeatlantic.ca/). Experimental diets including the control diet were formulated and produced at the Department of Plant and Animal Science, Faculty of Agriculture, Dalhousie University, Truro, Nova Scotia, Canada, according to the nutritional requirements of Atlantic salmon set by National Research Council (2011) (Table 3-1). All diets were approximately iso-nitrogenous and iso-energetic on a crude protein and gross energy basis. The experimental treatments in this feeding trial were as follows: a control diet with FO and FM (FO); 100% FO replacement with CO (100CO); 100% FO replacement with CO and including solvent extracted FM (100COSEFM); 100% FO replacement with CO and including 10% CM (100CO10CM); 100% FO replacement with CO and including SEFM and 10% CM (100COSEFM10CM). SEFM was employed here to remove FO residue in the FM (about 8%) as much as possible in order to evaluate the full effect of total replacement of fish oil in the diet. Further details on formulation, proximate and fatty acid compositions of the diets are given in Hixson et al. (2014b).

Atlantic salmon post-smolts (242.1 ± 46.0 g) were randomly distributed among

Table 3-1. Compositions of experimental diets used in the Atlantic salmon feeding trial (percentage of total weight as fed)[a].

Ingredient	FO	100CO	100CO SEFM	100CO 10CM	100CO SEFM 10CM
Fish meal	34.9	34.9	-	31.80	-
SEFM[b]	-	-	32.89	-	29.95
Fish oil	14.04	-	-	-	-
Camelina meal	-	-	-	10	10
Camelina oil	-	14.04	17.79	16.08	19.49
Wheat gluten meal	15	15	15	15	15
Empyreal 75®	5	5	5	5	5
D/L Methionine	0.17	0.17	0.17	0.17	0.17
Vitamin/Mineral premix[c]	0.2	0.2	0.2	0.2	0.2
Antioxidant/Pigment premix[d]	0.25	0.25	0.25	0.25	0.25
Choline chloride	0.5	0.5	0.5	0.5	0.5
Whey	5	5	5	5	5
Pregelatinized starch	2.5	2.5	2.5	2.5	2.5
Wheat	22.42	22.42	20.71	13.50	11.94
Total	100	100	100	100	100
Proximate composition analyzed, as-fed basis (n = 3)					
Moisture	8.6 ± 0.2	9.3 ± 0.2	10.9 ± 0.1	8.0 ± 0.04	9.7 ± 0.5
Ash	2.9 ± 0.1	3.2 ± 0.2	2.0 ± 0.1	2.7 ± 0.4	2.9 ± 0.2
Protein	41.3 ± 0.2	42.2 ± 0.5	40.7 ± 0.3	42.2 ± 0.8	41.0 ± 0.7
Lipid	17.6 ± 1.2	18.5 ± 0.2	20.0 ± 0.8	19.3 ± 0.6	22.2 ± 1.2

[a]Note that the diet composition data presented here were previously published (Hixson et al., 2014b); we include them herein as they pertain to the current study as well.
[b]Solvent-extracted fish meal
[c]Vitamin/Mineral Premix contains per kg: Zinc 77.5 mg, Manganese 125 mg, Iron 84 mg, Copper 2.5 mg, Iodine 7.5 mg, Vitamin A 5000IU, Vitamin D 4000IU, Vitamin K 2 mg, Vitamin B12 4 µg, Thiamine 8 mg, Riboflavin 18 mg, Pantothenic acid 40 mg, Niacin 100 mg, Folic acid 4 mg, Biotin 0.6 mg, Pyridoxine 15 mg, Inositol 100 mg, Ethoxyquin 42 mg, Wheat shorts 1372 mg.
[d]Antioxidant/Pigment Premix contains per kg: Selenium 0.220 mg, Vitamin E 250 IU, Vitamin C 200 mg, Astaxanthin 60 mg, Wheat shorts 1988 mg.

fifteen tanks (500 L) with flow-through seawater (~14°C, dissolved oxygen ≥ 10 mg L^{-1}), and all fish were kept on a photoperiod of 12 hours. After the acclimation period (i.e. 1 week in the experimental tanks), all fish were gradually moved from the commercial diet to the control diet (i.e. FO) over 3 days, and were kept on control diet for one week prior to initial sampling. Thereafter, fish were gradually moved onto each assigned experimental or control diet over another 3 days. Triplicate tanks of fish were fed experimental or control diets to apparent satiety, twice each day for a period of 16 weeks. At week 0 (the day before experimental diets were fed), week 1 (3 days of acclimation to the experimental diets plus a full week of feeding experimental diets), week 8 and week 16 of the feeding trial, seven fish from each tank at each time point were euthanized with 400 mg L^{-1} of tricaine-methane-sulfonate (TMS; Syndel Laboratories, Vancouver, BC) after 24 h of fasting. Body weight and fork length of fish were measured and recorded. Liver tissues (50-100 mg $sample^{-1}$) were rapidly dissected from fish, placed in RNase-free 1.5 mL tubes, flash-frozen in liquid nitrogen, and stored at -80°C until RNA extractions were performed. Experimental manipulations and the sampling of the fish tissues were conducted following the Canadian Council on Animal Care guidelines. This study was approved by the Institutional Animal Care Committee of Memorial University of Newfoundland under protocol number 12-50-MR. Further details on fish rearing conditions and sampling for lipid analysis are given in Hixson et al. (2014b).

3.3.2 RNA extraction, DNase treatment, and column purification

Liver samples collected from the feeding experiment were homogenized individually in 2.0 mL tubes containing TRIzol reagent (Invitrogen, Carlsbad, CA) with a

volume of 0.8 mL sample^{-1} and a stainless steel bead (2.5 mm; QIAGEN, Mississauga, ON) using a TissueLyser (QIAGEN) at 25 Hz for 3 min, and further disrupted using QIAshredder spin columns (QIAGEN), and subjected to RNA extraction according to the manufacturers' instructions. After extraction, the quality of total RNA was examined by 1% agarose/ethidium bromide gel electrophoresis, and RNA quantity and purity were measured by NanoDrop spectrophotometry (ThermoFisher, Mississauga, ON). However, due to low 260/230 ratios (i.e. less than 1.2) following TRIzol extraction, all RNA samples were re-extracted using the following phenol-chloroform phase separation method. Thirty µL of each crude RNA sample was diluted 10 times using nuclease-free water (Invitrogen), an equal volume of Acid Phenol:Chloroform:IAA (25:24:1, v/v; pH 6.6; Ambion, Burlington, ON) was added, and after mixing, phases were separated by centrifugation at 15,000 × *g* at 4°C for 20 min. The RNA was then precipitated from the aqueous phase with a mixture of 20 µL 3M sodium acetate (pH 5.2; Ambion) and 400 µL 95% ethanol (Greenfield, Toronto, ON) and incubated at -80°C for one hour, followed by centrifugation at 15,000 × *g* at 4°C for 30 min. The resulting RNA pellet was washed by adding 500 µL of 70% ethanol and centrifuging at 15,000 × *g* at 4°C for 20 min followed by removal of the supernatant, and then air-dried at room temperature for 10 min, and dissolved in 40 µl of nuclease-free water (Invitrogen). Total RNA of each sample was then treated with DNase I (QIAGEN) to degrade residual genomic DNA, and all samples were purified from salts, proteins and nucleotides using the RNeasy Mini Kit (QIAGEN) following the manufacturer's protocols. The quantity and quality of cleaned RNA samples were assessed as previously described. Only high quality (260/280 ratio > 2.0, 260/230 > 1.85, with tight 18S and 28S ribosomal RNA bands) total RNA samples were

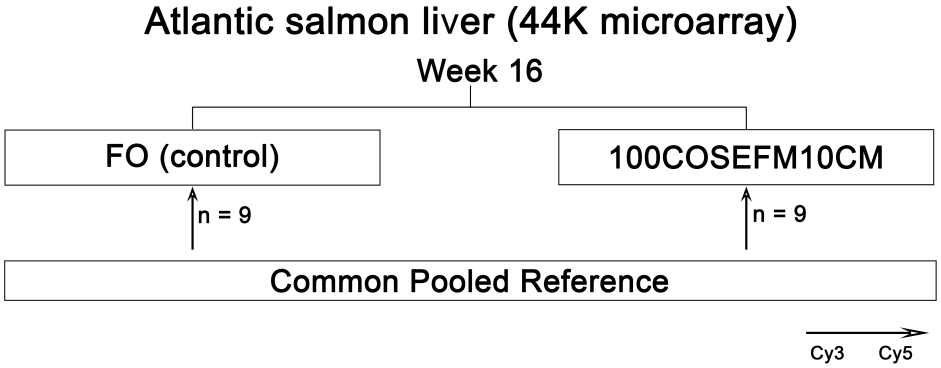


Fig. 3-1. Overview of microarray experimental design (common reference design).

used in RNA amplification and cDNA synthesis reactions for microarray and QPCR experiments, respectively.

3.3.3 Choice of dietary treatment for transcriptomic comparison

The 100COSEFM10CM and control dietary treatments at week 16 were chosen as groups to be compared in this microarray experiment (see Fig. 3-1) since fish performance [assessed by weight gain, final weight and length, and weight-specific growth rate (SGR)] was most different between these groups (Hixson et al., 2014b). Also, this 100COSEFM10CM was the most extreme diet in the feeding trial, with a negligible amount of FO as well as the inclusion of CM. Therefore, it was hypothesized that the 100COSEFM10CM dietary treatment was most likely to lead to the identification of new molecular biomarkers of hepatic transcript expression response to an extreme camelina product-containing diet (associated with significantly reduced growth, see Results section) that could be used in the future development of optimized camelina-based diets for salmon.

3.3.4 Microarray hybridization and data acquisition

Nine individual fish (three from each triplicate tank) each from the 100COSEFM10CM and control diet groups were used in the microarray analysis using a common reference design (Fig. 3-1). Eighteen arrays were used in this study, with one array per individual fish (Fig. 3-1). An equal quantity of each DNase I-treated, column-purified liver total RNA sample involved in the current experiment was pooled to make a common reference for the microarray hybridizations. Anti-sense amplified RNA (aRNA)

was *in vitro* transcribed from 1 µg of each experimental RNA or reference pooled RNA using Ambion's Amino Allyl MessageAmp II aRNA Amplification kit (Life Technologies), following the manufacturer's instructions. The quality and quantity of aRNA were assessed using NanoDrop spectrophotometry and agarose gel electrophoresis. Twenty µg of aRNA was precipitated overnight following standard molecular biology procedures and re-suspended in coupling buffer; the resulting solution was used in a labeling reaction following the manufacturer's protocol. Resulting aRNA was labeled with either Cy3 (for the common reference) or Cy5 (for the experimental individuals) fluor (GE HealthCare, Mississauga, ON) through a dye-coupling reaction, following the manufacturer's instructions. The labeling efficiency was measured using the "microarray" function of the NanoDrop spectrophotometer. Equal quantities (825 ng) of each labeled aRNA from one experimental sample and the common reference were pooled, fragmented following the manufacturer's instructions and co-hybridized to a cGRASP-designed Agilent 44K salmonid oligonucleotide microarray (GEO accession # GPL11299) as per the manufacturer's instructions (Agilent, Mississauga, ON). The arrays were hybridized at 65°C for 17 hours with 10 rpm rotation in an Agilent hybridization oven. The array slides were washed immediately following hybridization as per the manufacturer's instructions.

Each microarray was scanned at 5 µm resolution and 90% of laser power using a ScanArray Gx Plus scanner and ScanArray Express software (v4.0; Perkin Elmer, Woodbridge, ON) with photomultiplier tube (PMT) set to balance fluorescence signal between channels. The resulting TIFF images containing raw microarray data were extracted using Imagene (v9.0; BioDiscovery Inc., El Segundo, CA). Background

correction, data transformation (log_2), print-tip Loess normalization, and removal of low-quality/flagged spots were performed using R and the Bioconductor package mArray using scripts adapted from those described in Booman et al. (2011). After spot quality filtering, features absent in more than 30% of the arrays (i.e. 5 arrays out of 18) were discarded, resulting in a final list of 16,629 probes for statistical analyses. The full microarray data is submitted to Gene Expression Omnibus (GEO) under the accession GSE56784.

3.3.5 Microarray data analysis

The Significance Analysis of Microarrays (SAM) algorithm (Tusher et al., 2001) as implemented in the Bioconductor package siggenes (Schwender et al., 2006) was used to identify genes that were significantly up-regulated or down-regulated in response to the 100COSEFM10CM diet compared with the control diet with a false discovery rate (FDR) cutoff of 5%. Prior to SAM analysis, missing data points for these 16,629 probes were imputed using the EM_array method LSImpute (Bo et al., 2004; Celton et al., 2010). The resulting gene lists were annotated using the contiguous sequences (contigs) from which informative 60mer oligonucleotide probes on the array were designed. BLASTx alignment of these sequences against the NCBI nr database was performed with an E-value threshold of 10^{-5}. BLASTx results for each probe were mapped to Gene Ontology (GO) terms using the Blast2GO program (Conesa et al., 2005).

3.3.6 QPCR analysis

The following microarray-identified genes of interest (GOI) were selected for QPCR assay development and analysis: *delta-6 fatty acyl desaturase a* (*fadsd6a*) (This Atlantic salmon gene represented the putative orthologue of the informative rainbow trout feature C023R134 on the microarray; see Appendix XV for the alignment of *fadsd* paralogues); *carnitine palmitoyltransferase I-like* (*cpt1*); *pyruvate carboxylase* (*pcb*); *bile acid receptor* (*bar*; also known as *farnesoid X receptor*); *insulin-like growth factor binding protein 5* paralogs (*igfbp-5b1* and *igfbp-5b2*) (These Atlantic salmon genes are both likely represented by the informative Atlantic salmon feature C116R063; see Appendix XVI for the alignment of *igfbp-5* paralogues and the associated microarray feature probe sequence); *B-cell translocation gene 1-like* (*btg1*); *2'-deoxynucleoside 5'-phosphate N-hydrolase 1* (*dnph1*); *leukocyte cell-derived chemotaxin 2 precursor* (*lect-2*); *C type lectin receptor A* (*clra*); and *kruppel-like factor 9* (*klf9*). In addition, QPCR assays were developed for four genes (*elovl2*, *elovl5a*, *elovl5b* and *fadsd5*) that play key roles in LC-PUFA biosynthesis. In addition to the 100COSEFM10CM and control diets, the QPCR experiment also included liver samples from fish fed three other diets containing camelina-derived products (100CO, 100COSEFM and 100CO10CM).

QPCR primers were designed using the Primer 3 program [available at http://frodo.wi.mit.edu; (Rozen and Skaletsky, 2000)] either based on contigs/ESTs representing the informative microarray probes identified through BLASTn analyses (for *cpt1*, *pcb*, *bar*, *btg1*, *dnph1*, *lect-2*, *clra*, *klf9*) or sequences from previous publications [for *fadsd5* (Hastings et al., 2004); *fadsd6a* (Zheng et al., 2005a); *elovl2*, *elovl5a* and

elovl5b (Morais et al., 2009); *igfbp-5b1* and *igfbp-5b2* (Macqueen et al., 2013)] (Table 3-2). Paralogue-specific QPCR primers were designed for *fadsd5* and *fadsd6a*, *igfbp-5b1* and *igfbp-5b2*, and *elovl5a* and *elovl5b* (see Appendices XV-XVII). All QPCR primer sets were quality-checked (QC) using a reference cDNA generated by pooling an equal quantity of each individual cDNA involved in this QPCR study. QPCR primer QC procedures, including standard curves and dissociation curves, were conducted as described elsewhere (Rise et al., 2010; Booman et al., 2011). In brief, the amplification efficiency (Pfaffl, 2001) of each primer pair was determined using a 5-point 1:5 dilution series starting with cDNA corresponding to 10 ng of input total RNA, except for *cpt1* and *igfbp-5b1* (5-point 1:3) and *elovl5a* and *btg1* (5-point 1:2). Dissociation curves were carried out to ensure that the primer pairs amplified single products with no detectable primer dimers. Additionally, the expected size of each GOI QPCR amplicon (Table 3-2) was checked by agarose gel electrophoresis.

For each dietary treatment, nine individuals (three from each triplicate tank) were used in the QPCR experiment. All cDNAs were prepared by reverse transcription of 1 μg DNase-I treated, column-cleaned total RNA for each individual sample using random primers (250 ng, Invitrogen) and Moloney murine leukemia virus (M-MLV) reverse transcriptase (RT) (200 U, Invitrogen) at 37°C for 50 min in a 20 μL reaction volume following the manufacturer's instructions. A "no-RT" control with pooled total RNA was performed by omitting reverse transcriptase. The resulting cDNA was further diluted 10 times with nuclease-free water (Invitrogen). QPCR reactions were performed in technical triplicates using Power SYBR Green I dye chemistry in 384-well format on the ViiA™ 7 Real-Time PCR System (Applied Biosystems, Foster City, CA). The QPCR reactions

Table 3-2. QPCR Primers.

Gene		Sequence 5'-3'[a]	Efficiency (%)	r^2	Amplicon size (bp)	Accession number
elovl2	F	GATGCCTGCTCTTCCAGTTC	97.5	0.998	113	FJ237532[b]
	R	GCGACTGGACTTGATGGATT				
elovl5a	F	CAGTGTGGTGGGGACAAAG	101.0	0.980	115	AY170327[b]
	R	TTCCCTCATGGACAAGCA				
elovl5b	F	GGATAGCAGAGGGAGCACAG	90.6	0.995	120	FJ237531[b]
	R	CCTGTTTGGGTCAAGGTTGCT				
fadsd5	F	GTCTGGTTGTCCGTTCGTTT	89.1	0.999	135	AF478472[b]
	R	GAGGCGATCAGCTTGAGAAA				
fadsd6a	F	CCCCAGACGTTTGTGTCAG	88.9	0.997	181	AY458652[b]
	R	CCTGGATTGTTGCTTTGGAT				
btg1	F	GGTCAGCTGCAAGGAAGAAC	94.0	0.995	132	DW555767[c]
	R	TGTGGGGCAGAACTGATACA				
pcb	F	CTCCAGGATGAGGTCGTCTC	92.6	1.000	179	GE787967[c]
	R	CGGGTAAGGTTGTGGAAGTG				
cpt1	F	GCACTGCAAAGGAGACATCA	86.0	0.995	136	EG857609[c]
	R	GCTATCACCTTGGCAACCAT				
bar	F	GCCAAGAGGTAAGCATCTCG	102.1	0.999	120	GO063627[c]
	R	TCAGGAGGTTCTGTGCAATG				
igfbp-5b1	F	GGTGCTTGGGCTCATATGTT	87.0	0.999	209	JX565556[b]
	R	CTTCTCTTCTCCATTTCGCG				
igfbp-5b2	F	GACATTTGTCTTGGGGCTGA	95.2	0.998	127	JX565557[b]
	R	ACAGCCAGGCTCTTTCACG				
lect-2	F	GCCTTCTTCGGGTCTGTGTA	97.5	0.998	150	BT059281[c]
	R	CAGATGGGGACAAGGACACT				
clra	F	ACTGGGAAGTTCATGGCTTG	97.0	0.999	116	EG910992[c]
	R	ATTCGCTGACCTGGTTTGAC				
dnph1	F	TCTGTGGCAGTATTCGTGGA	89.9	0.995	138	DW471353[c]
	R	GCACAGCATCCTCTCCTTTC				
klf9	F	CAAAGAAGACGCATGTGGAA	86.0	0.994	132	EG912132[c]
	R	GTTCCCTAAACGGATGCTGA				
actb	F	CCAAAGCCAACAGGGAGAAG	90.2	0.999	91	BG933897[b]
	R	AGGGACAACACTGCCTGGAT				
rpl32	F	AGGCGGTTTAAGGGTCAGAT	88.9	0.997	119	BT043656[b]
	R	TCGAGCTCCTTGATGTTGTG				

[a]F: forward primer; R: reverse primer.
[b]Nucleotide sequence from GenBank was used for the primer design.
[c]The ESTs representing microarray probes (identified through BLASTn analyses) were used for primer design.

contained 2 μL of 1:10 diluted cDNA (10 ng input total RNA), 50 nM each of forward and reverse primer, and 1× Power SYBR Green PCR Master Mix (Applied Biosystems) in a final volume of 13 μL. The QPCR program consisted of 1 cycle of 50°C for 2 min, 1 cycle of 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min, with the fluorescence signal data collection after each 60°C step. Before performing QPCR expression studies, the absence of genomic DNA contamination in the "no-RT" control sample was confirmed by using each QPCR primer set (i.e. to ensure that any amplification occurring in the QPCR reaction was derived from the reverse transcribed mRNA and not genomic DNA).

The expression levels of each GOI were normalized to the geometric mean of *60S ribosomal protein 32* (*rpl32*) and *β-actin* (*actb*) transcript expression. *Rpl32* and *actb* were chosen as the normalizer genes due to their stable transcript expression in the current study. Six candidate endogenous controls were selected based on the current liver microarray experiment results (for *actin-related protein 2/3 complex subunit 1A* and *NADH dehydrogenase [ubiquinone] iron-sulfur protein 7, mitochondrial precursor*), literature on salmon reference gene evaluation [for *eukaryotic elongation factor 1α-1* (*eef1α-1*; formerly *elongation factor 1α-1*), *eukaryotic elongation factor 1α-2* (*eef1α-2*) and *actb*] (Olsvik et al., 2005), or from previous salmon QPCR studies in the lab (for *rpl32*). Two-thirds of individuals (i.e. 6 from each dietary treatment) were included in the evaluation of the six potential endogenous controls using the *geNorm* algorithm (Vandesompele et al., 2002). *Rpl32* and *actb* were shown to be the most stable (i.e. lowest M-value; a gene-stability measure) across the selected reference genes (data not shown).

In every multi-plate study, a linker control (a pooled cDNA sample from all samples involved in the study) was used to check the inter-plate variability between different plates. The ViiA™ 7 Software v1.2 (Applied Biosystems) was used to determine C_T values over triplicates for each GOI and normalizer. The amplification efficiencies for each GOI and normalizer primer pairs (Table 3-2) were incorporated into the calculation of GOI relative quantity (RQ) using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001), which is implemented in the same software. The individual with the lowest GOI expression was used as the calibrator sample (i.e. RQ = 1) for each GOI study.

3.3.7 Statistical analyses of growth and QPCR data

All statistical analyses of growth-relevant and QPCR data were performed using Prism v5.0 (GraphPad Software Inc, La Jolla. CA) with one-way ANOVA, followed by Tukey post-hoc test for multiple comparisons at the 5% level of significance (i.e. $p < 0.05$), to detect differences between dietary treatments. All data were subjected to normality testing using the Anderson-Darling test. The growth-relevant data (as shown in Table 3-3) were presented as mean ± standard deviation (SD). RQ data were $\log_2$ transformed in Excel in order to meet with statistical assumption (i.e. normality), and were presented as mean ± standard error (SE). For QPCR fold-change calculation, overall fold up-regulation was calculated as 2^{A-B}, where A is the mean of $\log_2$ transformed RQ from an experimental diet (e.g. 100CO or 100COSEFM) group, and B is the mean of $\log_2$ transformed RQ from the control diet (FO) group (Cui and Churchill, 2003).

3.4 Results

3.4.1 Growth performance

The growth performance and fatty acid data for this feeding trial were reported in Hixson et al. (2014b). However, since the growth data are also relevant to the current study, they are briefly described and included in this chapter. Atlantic salmon fed the experimental diets increased in weight from 230-255 g $fish^{-1}$ initially to 529-691 g $fish^{-1}$ after 16 weeks (Table 3-3). The growth performance of salmon, as measured by weight gain and SGR, was significantly reduced in all camelina-containing diet fed groups except fish fed 100CO, compared with the control diet (i.e. FO) group (weight gain, 281-320 g $fish^{-1}$ vs. 471 g $fish^{-1}$; SGR, 0.68-0.76% day^{-1} vs. 0.99% day^{-1}) (Table 3-3). The weight gain in the 100COSEFM10CM group, the lowest among all dietary treatments, was 40% less than that of the FO group. The apparent feed intake (AFI) per fish throughout the feeding trial was affected by diet with fish fed FO diet consuming more than fish fed the CO-containing diets (Table 3-3). However, fish fed all diets had comparable feed conversion ratio (FCR; 1.0-1.25) and condition factor (CF; 1.46-1.53) after 16 weeks of feeding. Additional details on growth and feed efficiency for fish in this feeding trial are reported in Hixson et al. (2014b), which focuses on lipid and fatty acid analyses of diets and tissues.

3.4.2 Liver transcriptome analysis

An experiment involving 18 arrays was conducted to identify transcripts in Atlantic salmon liver that respond to a camelina-based test diet (i.e. 100COSEFM10CM)

Table 3-3. Growth performance of Atlantic salmon after 16 week feeding experiment[1].

	FO	100CO	100CO SEFM	100CO 10CM	100CO SEFM 10CM
Initial weight (g)	230 ± 41	236 ± 56	231 ± 46	255 ± 45	247 ± 11
Final weight (g)	691 ± 153^{a}	613 ± 117^{b}	537 ± 113^{c}	573 ± 136^{bc}	529 ± 121^{c}
Weight gain (g)	471 ± 39^{a}	378 ± 31^{ab}	306 ± 44^{b}	320 ± 57^{b}	281 ± 33^{b}
Initial length (cm)	26.2 ± 2.4	26.8 ± 2.1	26.3 ± 2.3	27.3 ± 1.4	27.6 ± 1.2
Final length (cm)	35.0 ± 4.1^{a}	34.3 ± 2.1^{ab}	33.1 ± 2.4^{bc}	33.3 ± 2.8^{bc}	32.6 ± 2.6^{c}
SGR[2]	0.99 ± 0.1^{a}	0.86 ± 0.1^{ab}	0.76 ± 0.1^{b}	0.73 ± 0.1^{b}	0.68 ± 0.1^{b}
Condition factor[3]	1.53 ± 0.1	1.50 ± 0.1	1.46 ± 0.1	1.53 ± 0.1	1.51 ± 0.1
VSI[4] (%)	9.8 ± 1.1^{a}	10.1 ± 3.6 ^{a}	11.1 ± 1.2^{ab}	11.1 ± 1.5^{ab}	12.0 ± 1.6^{b}
AFI[5]	515 ± 7.6^{a}	436 ± 11^{b}	400 ± 29^{b}	391 ± 15^{b}	381 ± 46^{b}
FCR[6]	1.01 ± 0.1	1.06 ± 0.1	1.21 ± 0.1	1.14 ± 0.2	1.25 ± 0.1

[1]Data are presented as mean ± standard deviation. Means with different letters are significantly different ($p < 0.05$), determined by one-way ANOVA (Minitab 16). Initial measurements, n = 9. n is variable depending on number of fish remaining in tank for final measurements: FO = 48, 100CO = 48, 100COSEFM = 67, 100CO10CM = 66 and 100COSEFM10CM = 66. Weight gain, SGR and FCR were calculated using tank mean, n = 3. Note that all data presented here were previously published (Hixson et al., 2014b); we include them herein as they pertain to the current study as well.
[2]SGR, Specific growth rate (%/day) = 100*[ln(final weight) - ln(initial weight)]/days.
[3]Condition factor = 100*final weight/final length^{3}.
[4]VSI, Viscera somatic index = 100*(viscera weight/body weight).
[5]AFI, Apparent feed intake (g/fish).
[6]FCR, Feed conversion ratio = AFI/weight gain.

compared to a control diet (i.e. FO) after a 16 week feeding trial. The microarray experiment detected 67 significant differentially expressed features (i.e. oligonucleotide probes representing transcripts) with a FDR less than 5% (26 more highly expressed in the salmon fed 100COSEFM10CM diet and 41 more highly expressed in the salmon fed control diet). Of the 67 differentially expressed features, putative identities could be determined for 62 based on sequence similarity using BLASTx searches against protein sequences in the GenBank *nr* database (cutoff E-value = 0.00001; Tables 3-4 and 3-5). Three genes related to lipid metabolism [*long chain fatty acyl-CoA ligase 4* (*facl4)*, *fadsd6* and *aquaporin-8*] were identified as up-regulated in the liver of salmon fed 100COSEFM10CM diet compared to control (Table 3-4). Six genes that are known to be involved in cell differentiation and proliferation (e.g. *dnph1* and *nucleosome assembly protein 1-like 1*) were also represented in the up-regulated gene list (Table 3-4). Other genes identified in the microarray experiment as up-regulated by the camelina-containing test diet play important roles in immune function, DNA synthesis and repair and regulation of transcription (Table 3-4).

Several of the down-regulated genes in the 100COSEFM10CM group compared with the control group were found to be related to carbohydrate, lipid, and/or protein metabolism (Table 3-5). Examples of biological processes associated with genes that were down-regulated by the camelina-containing test diet are gluconeogenesis (*pcb*, *glucose-6-phosphatase*), fatty acid β-oxidation (*cpt1*), and bile acid metabolism (*bar*). Interestingly, 4 genes involved in protein metabolism (*branched-chain-amino-acid aminotransferase*, *syntaxin-16*, *tectonin beta-propeller repeat-containing protein 2*, and *eukaryotic translation initiation factor 4E-binding protein 2*) were identified as

Table 3-4. Genes that were significantly up-regulated in the liver of salmon fed 100COSEFM10CM treatment compared to control (FO).

Probe identifier[2]	BLASTx identification[1]				Fold change
	Gene name (species)[3]	Accession #	E-value	ID (AA %)	
Lipid metabolism					
C104R106	Aquaporin-8 [*Salmo salar*]	NP_001167386	4e-171	259/259 (100%)	2.93
C015R068	Long chain fatty acid-CoA ligase 4 (*facl4*) [*Salmo salar*]	NP_001167160	0	669/669 (100%)	2.90
C023R134[4]	PREDICTED: fatty acid desaturase 2 isoform 3 (Alias: delta-6 fatty acyl desaturase, *fadsd6*) [*Otolemur garnettii*]	XP_003798786	1e-87	85/113 (75%)	**2.04**
Cell differentiation and proliferation					
C014R096	2'-deoxynucleoside 5'-phosphate N-hydrolase 1 (*dnph1*; alias: *rcl*) [*Esox lucius*]	C1BW56	1e-77	129/144 (90%)	**2.29**
C170R067	Baculoviral IAP repeat-containing protein 5 [*Salmo salar*]	ACI66178	6e-89	142/142 (100%)	1.80
C174R002	Ubiquitin-conjugating enzyme E2 C [*Salmo salar*]	ACI69073	1e-111	160/171 (94%)	1.78
C244R160	Nucleosome assembly protein 1-like 1 [*Salmo salar*]	ACM08320	2e-169	394/394 (100%)	1.53
C186R092	Ubiquitin-conjugating enzyme E2 D2 isoform 1 [*Homo sapiens*]	NP_003330	1e-98	147/147 (100%)	1.46
C029R131	FGF2 [*Salmo salar*]	ACJ02099	6e-09	31/45 (69%)	1.44
Immune-relevant					
C263R062	CD200 [*Oncorhynchus mykiss*]	ADV36649	3e-100	148/163 (91%)	2.20
C256R079	SLAM family member 8 precursor [*Salmo salar*]	ACI67051	0	312/312 (100%)	1.82
DNA synthesis and repair					
C040R057	Thymidylate synthase [*Salmo salar*]	NP_001134715	0	333/333 (100%)	2.25
C041R003	Structural maintenance of chromosomes protein 2 [*Cricetulus griseus*]	EGV97289	7e-71	130/155 (84%)	2.13
C187R082	PREDICTED: kinesin-like protein KIF2C-like [*Oreochromis niloticus*]	XP_003437970	2e-41	90/152 (59%)	2.06
C116R127	Adenylosuccinate lyase [*Ctenopharyngodon idella*]	ACB72735	7e-180	249/276 (90%)	1.78
C135R006	DENN domain-containing protein 2D [*Salmo salar*]	ACN11415	0	422/471 (90%)	1.72
C124R122	Deoxyuridine 5'-triphosphate nucleotidohydrolase, mitochondrial-like [*Oryzias latipes*]	XP_004067019	7e-90	133/164 (81%)	1.59
Regulation of transcription					

C234R025	Kruppel-like factor 9 (*klf9*) [*Oplegnathus fasciatus*]	BAM36382	7e-29	49/89 (55%)	**2.06**
C043R148	Chromobox protein homolog 3 [*Salmo salar*]	NP_001134084	2e-98	179/183 (98%)	1.77
Others					
C188R061	Kinesin family member C1/zinc finger protein [*Salmo salar*]	ABO13867	0	625/625 (100%)	2.20
C009R108	PREDICTED: WD repeat-containing protein C2orf44 homolog isoform X1 [*Maylandia zebra*]	XP_004542243	6e-39	74/111 (67%)	1.59
C027R063	PREDICTED: T-complex protein 1 subunit zeta isoform 1 [Oreochromis niloticus]	XP_003447022	1e-113	112/130 (86%)	1.55
C218R117	Eukaryotic translation initiation factor 3 subunit I [*Salmo salar*]	NP_001133273	0	325/325 (100%)	1.41
C230R050	Unknown	N/A	N/A	N/A	2.24
C232R105	Unknown	N/A	N/A	N/A	2.11
C213R142	Unknown	N/A	N/A	N/A	1.60

[1]Each gene was identified by BLASTx of the contig from which the informative microarray probe was designed against the NCBI nr database. The best BLASTx hit with E-value $<10^{-5}$ and an informative gene or protein name is presented in this table with GenBank accession number species affiliation.
[2]Refers to the identity of the probe on the 44K array.
[3]Gene names with bold font are genes of interest for the QPCR analysis.
[4]*Delta-6 fatty acyl desaturase a* (*fadsd6a*) (This Atlantic salmon gene represented the putative orthologue of the informative rainbow trout feature C023R134 on the microarray; see Appendix XV for the alignment of *fadsd* paralogues).

Table 3-5. Genes that were significantly down-regulated in the liver of salmon fed 100COSEFM10CM treatment compared to control (FO).

Probe identifier[2]	BLASTx identification[1]				Fold change
	Gene name (species)[3]	Accession #	E-value	ID (AA %)	
Carbohydrate metabolism					
C110R017	Glucose-6-phosphatase [*Salmo marmoratus*]	ACF75920	1e-86	128/128 (100%)	-1.72
C114R043	Pyruvate carboxylase (*pcb*) [*Danio rerio*]	CAD61259	3e-82	149/167 (89%)	**-1.51**
Lipid metabolism					
C079R110	Adipophilin [*Salmo salar*]	ACN60305	1e-27	62/85 (73%)	-2.15
C052R093	PREDICTED: lysoplasmalogenase-like [Oreochromis niloticus]	XP_003454022	3e-24	52/70 (74%)	-2.10
C118R106	PREDICTED: ATP-binding cassette sub-family A member 1-like [*Oreochromis niloticus*]	XP_003449992	0	233/258 (90%)	-1.96
C089R096	Bile acid receptor (*bar*; Alias: farnesoid X receptor) [*Oncorhynchus mykiss*]	BAN16587	2e-30	72/74 (97%)	**-1.86**
C144R021	Carnitine palmitoyltransferase I-like (*cpt1*) [*Oncorhynchus mykiss*]	NP_001165326	8e-92	147/159 (92%)	**-1.84**
Protein metabolism					
C113R049	Branched-chain-amino-acid aminotransferase, cytosolic [*Salmo salar*]	ACN11196	0	396/396 (100%)	-3.75
C123R018	Syntaxin-16 [*Salmo salar*]	NP_001167314	0	306/306 (100%)	-1.94
C088R082	Tectonin beta-propeller repeat-containing protein 2 [*Danio rerio*]	NP_001038644	0	274/341 (80%)	-1.71
C110R072	Eukaryotic translation initiation factor 4E-binding protein 2 [*Salmo salar*]	ACI33150	4e-63	106/106 (100%)	-1.51
Cell differentiation, proliferation and apoptosis					
C263R103	Lymphocyte G0/G1 switch protein 2 [*Salmo salar*]	ACM08302	1e-60	116/116 (100%)	-2.37
C116R063	IGF binding protein 5 precursor (*igfbp-5*) [*Salmo salar*]	NP_001117121	0	270/270 (100%)	**-1.92**
C135R154	Metalloproteinase inhibitor 3 precursor [*Salmo salar*]	NP_001135315	9e-127	215/215 (100%)	-1.83
C123R147	Steroid receptor RNA activator 1 [*Salmo salar*]	ACM08712	2e-143	238/238 (100%)	-1.62
C089R032	B-cell translocation gene 1-like (*btg1*) [*Salmo salar*]	ACI66378	5e-100	146/146 (100%)	**-1.47**
Immune or stress-relevant					
C134R121	Leukocyte cell-derived chemotaxin 2 precursor (*lect-2*) [*Salmo salar*]	ACI66408	4e-102	156/156 (100%)	**-3.03**

C158R024	Eggshell protein [*Salmo salar*]	CAA04221	0	413/427 (97%)	-2.93
C159R112	Leukocyte cell-derived chemotaxin 2 precursor (*lect-2*) [*Salmo salar*]	ACI66408	4e-102	156/156 (100%)	**-2.92**
C055R131	Chitinase 3 [*Thunnus orientalis*]	BAL14138	7e-18	58/99 (59%)	-2.23
C130R087	PREDICTED: junctional adhesion molecule B-like [*Maylandia zebra*]	XP_004551457	8e-106	165/226 (73%)	-1.85
C108R044	Serum paraoxonase/arylesterase 2 [*Anoplopoma fimbria*]	ACQ58263	4e-163	232/330 (70%)	-1.81
C103R112	CD209 antigen-like protein E (Alias: C type lectin receptor A, *clra*) [*Salmo salar*]	ACI33556	0	255/255 (100%)	**-1.69**
Signal transduction					
C129R088	Small GTPase Ras-dva-2 [*Takifugu rubripes*]	ABB84860	1e-24	68/90 (76%)	-2.36
C128R001	PREDICTED: mitogen-activated protein kinase 12-like [*Takifugu rubripes*]	XP_003967582	2e-173	159/192 (83%)	-1.63
Regulation of transcription					
C035R026	PREDICTED: WW domain-binding protein 4-like [*Maylandia zebra*]	XP_004571287	9e-119	242/430 (56%)	-2.02
C161R038	DnaJ homolog subfamily C member 8 [*Salmo salar*]	NP_001134671	6e-152	256/257 (99%)	-1.81
C078R124	PREDICTED: zinc finger and BTB domain-containing protein 10-like [*Oreochromis niloticus*]	XP_003444217	4e-32	92/124 (74%)	-1.58
C198R123	Cold shock domain-containing protein E1 [*Salmo salar*]	NP_001167093	0	541/563 (96%)	-1.5
Others					
C112R144	PREDICTED: lysoplasmalogenase-like protein TMEM86A-like [*Maylandia zebra*]	XP_004573768	5e-34	65/106 (61%)	-2.08
C110R007	Voltage-dependent calcium channel gamma-like subunit [*Danio rerio*]	NP_998339	3e-22	69/106 (65%)	-1.98
C129R054	TRNA-splicing ligase RtcB homolog [*Danio rerio*]	NP_998268	0	483/505 (96%)	-1.94
C131R073	PREDICTED: protein FAM84A-like [*Maylandia zebra*]	XP_004542015	2e-106	111/142 (78%)	-1.81
C252R049	PREDICTED: lysoplasmalogenase-like [*Oryzias latipes*]	XP_004071563	2e-27	64/99 (65%)	-1.8
C098R023	SLEI family protein [*Leptolyngbya sp. PCC 7376*]	YP_007072112	3e-13	41/124 (33%)	-1.7
C111R024	PREDICTED: solute carrier family 25 member 36-A-like [*Oryzias latipes*]	XP_004079206	6e-119	169/186 (91%)	-1.67
C131R070	RAD21 homolog [*Danio rerio*]	NP_001038585	0	368/423 (87%)	-1.45
C139R027	PREDICTED: zinc finger protein 384-like isoform X3 [*Maylandia zebra*]	XP_004550902	1e-18	43/57 (75%)	-1.42
C060R006	Hemoglobin subunit beta-1 [*Salmo salar*]	ACI66559	2e-42	77/77 (100%)	-1.4

C123R012	Unknown	N/A	N/A	N/A	-1.76
C107R016	Unknown	N/A	N/A	N/A	-1.7

[1]Each gene was identified by BLASTx of the contig from which the informative microarray probe was designed against the NCBI nr database. The best BLASTx hit with E-value $<10^{-5}$ and an informative gene or protein name is presented in this Table with GenBank accession number species affiliation.
[2]Refers to the identity of the probe on the 44K array.
[3]Gene names with bold font are genes of interest for the QPCR analysis.

down-regulated in the liver of salmon fed 100COSEFM10CM diet compared to control (Table 3-5). Transcripts expression of *lymphocyte G0/G1 switch protein 2*, *btg1*, *steroid receptor RNA activator 1*, and *igfbp-5*, which encode protein that are involved in cell differentiation and growth, were more highly expressed in fish fed the control diet. In addition, a number of immune-relevant genes (e.g. *lect-2*, *chitinase 3*, and *clra*) were down-regulated in the liver of salmon fed the experimental diet (i.e. 100COSEFM10CM). Lastly, the microarray experiment identified several genes that play roles in signal transduction (e.g. *small GTPase Ras-dva-2*) and regulation of transcription (e.g. *dnaJ homology subfamily C member 8*) as down-regulated by the camelina-containing test diet (Table 3-5).

3.4.3 QPCR studies

Ten microarray-identified putative camelina-responsive biomarker genes [*cpt1*, *pcb*, *bar*, *igfbp-5b* (2 paralogs), *btg1*, *dnph1*, *lect-2*, *clra*, *klf9*, and *fadsd6a*] were selected for QPCR validation based on their important functional annotations (e.g. carbohydrate and/or lipid metabolism, immune response). In addition, the transcript expression of another *fatty acyl desaturase* gene (*fadsd5*) and three *fatty acyl elongase* genes (*elovl2*, *elovl5a* and *elovl5b*), which are likely to respond to diets containing ω3 PUFA in Atlantic salmon based on Morais et al. (2009), was determined using QPCR. Finally, QPCR assays developed in this study were used to analyze liver templates not only from fish fed the diets used in the microarray experiment but also from fish fed the additional dietary treatments (100CO, 100COSEFM and 100CO10CM) to study the impact of various camelina product-containing diets on biomarker gene expression at week 16 in the

feeding trial. All microarray-identified genes except *bar* were validated by QPCR as being significantly ($p < 0.05$) differentially expressed in the dietary treatment groups that were included in the microarray comparison (i.e. control vs. 100COSEFM10CM) (Figs. 3-2 and 3-3).

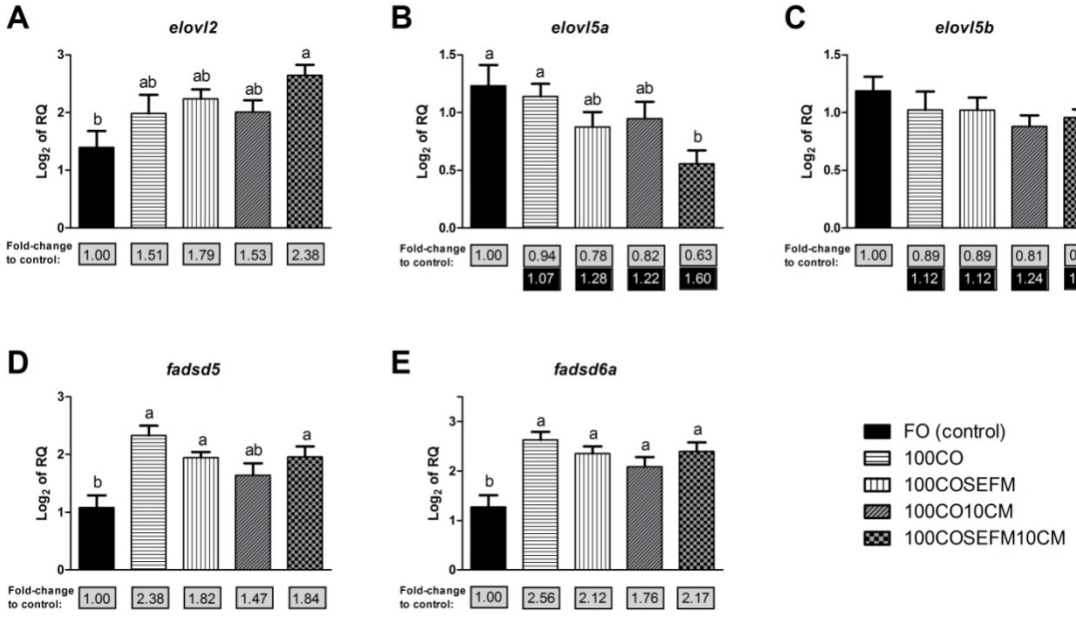
3.4.3.1 Genes involved in fatty acid biosynthesis

Elovl2 was significantly up-regulated (2.38-fold) in salmon fed 100COSEFM10CM compared with control diet fed fish, and showed 1.51 to 1.79-fold up-regulation trends (not significant) in 100CO, 100COSEFM and 100CO10CM groups as shown by QPCR (Fig. 3-2A). Interestingly, *elovl5a* exhibited significant down-regulation in the 100COSEFM10CM group compared with both the control and the 100CO groups (Fig. 3-2B). There was a slight (but not significant) down-regulation, in *elovl5b* mRNA expression in all experimental diet fed groups compared to control (Fig. 3-2C). *Fadsd5* mRNA expression was significantly up-regulated in 100CO, 100COSEFM and 100COSEFM10CM groups (2.38, 1.82 and 1.84-fold, respectively) compared with the control group (Fig. 3-2D). In addition, the QPCR experiment showed that the transcript expression level of *fadsd6a* was increased significantly in all four CO-containing dietary treatments (i.e. 100CO, 100COSEFM, 100CO10CM, and 100COSEFM10CM) compared to the control group by 2.56, 2.12, 1.76 and 2.17-fold, respectively (Fig. 3-2E).

3.4.3.2 Other genes involved in metabolism

The microarray experiment showed that *cpt1*, *pcb*, and *bar* transcript expression

Fig. 3-2. QPCR analysis of transcripts with putative roles in LC-PUFA biosynthesis in liver of salmon fed camelina product-containing test diets [100% camelina oil (CO) replacement of fish oil (100CO); 100CO with solvent-extracted fish meal (100COSEFM); 100CO with 10% camelina meal (100CO10CM); 100CO with SEFM and 10CM inclusion (100COSEFM10CM)] or control diet (FO) at week 16. Transcript relative quantity (RQ) data, presented as mean $\log_2$ transformed data ± standard error. Bars with different letters are significantly different ($p < 0.05$). For each condition, fold up-regulation was calculated as 2^{A-B}, where A is the mean $\log_2$ transformed RQ from an experimental group (e.g. 100CO or 100COSEFM), and B is the mean $\log_2$ transformed RQ from control group (see materials and methods for details); fold down-regulation where appropriate was calculated as the inverse of fold up-regulation, which is shown in a black box.


A
elovl2
Log2 of RQ
b
ab
ab
ab
a
Fold-change to control:
1.00
1.51
1.79
1.53
2.38
B
elovl5a
Log2 of RQ
a
a
ab
ab
b
Fold-change to control:
1.00
0.94
1.07
0.78
1.28
0.82
1.22
0.63
1.60
C
elovl5b
Log2 of RQ
Fold-change to control:
1.00
0.89
1.12
0.89
1.12
0.81
1.24
0.85
1.18
D
fadsd5
Log2 of RQ
b
a
a
ab
a
Fold-change to control:
1.00
2.38
1.82
1.47
1.84
E
fadsd6a
Log2 of RQ
b
a
a
a
a
Fold-change to control:
1.00
2.56
2.12
1.76
2.17
FO (control)
100CO
100COSEFM
100CO10CM
100COSEFM10CM

was significantly down-regulated in 100COSEFM10CM compared with the control group (Table 3-5). QPCR validated the *cpt1* and *pcb* results, and also showed significant down-regulation of *cpt1* in 100CO10CM and of *pcb* in 100COSEFM compared with control fish (Fig. 3-3A,B). QPCR did not validate the *bar* results; while QPCR and microarray showed the same direction of change for bar (i.e. down-regulated in the 100COSEFM10CM compared with control), this was not statistically significant for the QPCR data. Interestingly, *bar* was significantly down-regulated in fish fed 100CO10CM compared with fish fed control, 100CO, and 100COSEFM diets (Fig. 3-3C).

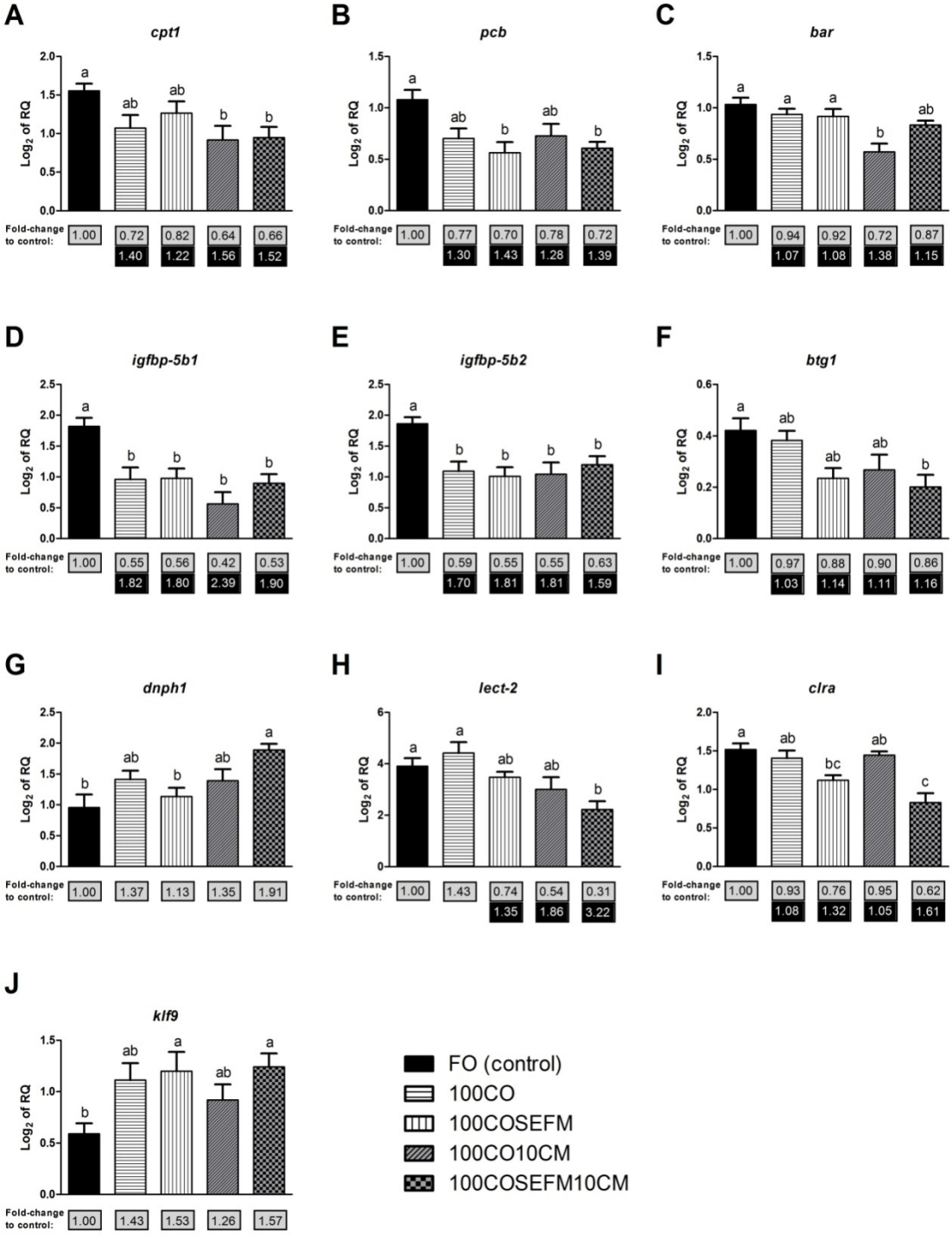
3.4.3.3 Genes involved in cell differentiation and proliferation

Two separate paralogue-specific QPCR assays that distinguish *igfbp-5b1* and *igfbp-5b2* were developed and utilized in the QPCR validation study. Both *igfbp-5b1* and *igfbp-5b2* showed similar expression profiles at the mRNA level. These two genes were significantly down-regulated by all four camelina product-containing diets compared to controls by at least 1.59 fold in the QPCR experiment (Fig. 3-3D,E). *Btg1* was subtle (1.16-fold) but significantly down-regulated in 100COSEFM10CM compared with control fish (Fig. 3-3F). *Dnph1* transcript was significantly higher expressed in 100COSEFM10CM compared to control and 100COSEFM groups (Fig. 3-3G).

3.4.3.4 Genes involved in immune response or regulation of transcription

The QPCR experiment showed that the hepatic expression of *lect-2* transcript was significantly lower in 100COSEFM10CM compared with both control and 100CO groups (Fig. 3-3H). *Lect-2* had the highest fold change of the down-regulated camelina product-

Fig. 3-3. QPCR analysis of transcripts with putative roles in metabolism, cell differentiation and growth, immune function or regulation of transcription in liver of salmon fed camelina product-containing test diets [100% camelina oil (CO) replacement of fish oil (100CO); 100CO with solvent-extracted fish meal (100COSEFM); 100CO with 10% camelina meal (100CO10CM); 100CO with SEFM and 10CM inclusion (100COSEFM10CM)] or control diet (FO) at week 16. Transcript relative quantity (RQ) data, presented as mean $\log_2$ transformed data ± standard error. Bars with different letters are significantly different ($p < 0.05$). For each condition, fold up-regulation was calculated as 2^{A-B}, where A is the mean $\log_2$ transformed RQ from an experimental group (e.g. 100CO or 100COSEFM), and B is the mean $\log_2$ transformed RQ from control group (see materials and methods for details); fold down-regulation where appropriate was calculated as the inverse of fold up-regulation, which is shown in a black box.


A
cpt1
Log2 of RQ
a
ab
ab
b
b
Fold-change to control:
1.00
0.72
0.82
0.64
0.66
1.40
1.22
1.56
1.52
B
pcb
Log2 of RQ
a
ab
b
ab
b
Fold-change to control:
1.00
0.77
0.70
0.78
0.72
1.30
1.43
1.28
1.39
C
bar
Log2 of RQ
a
a
a
b
ab
Fold-change to control:
1.00
0.94
0.92
0.72
0.87
1.07
1.08
1.38
1.15
D
igfbp-5b1
Log2 of RQ
a
b
b
b
b
Fold-change to control:
1.00
0.55
0.56
0.42
0.53
1.82
1.80
2.39
1.90
E
igfbp-5b2
Log2 of RQ
a
b
b
b
b
Fold-change to control:
1.00
0.59
0.55
0.55
0.63
1.70
1.81
1.81
1.59
F
btg1
Log2 of RQ
a
ab
ab
ab
b
Fold-change to control:
1.00
0.97
0.88
0.90
0.86
1.03
1.14
1.11
1.16
G
dnph1
Log2 of RQ
b
ab
b
ab
a
Fold-change to control:
1.00
1.37
1.13
1.35
1.91
H
lect-2
Log2 of RQ
a
a
ab
ab
b
Fold-change to control:
1.00
1.43
0.74
0.54
0.31
1.35
1.86
3.22
I
clra
Log2 of RQ
a
ab
bc
ab
c
Fold-change to control:
1.00
0.93
0.76
0.95
0.62
1.08
1.32
1.05
1.61
J
klf9
Log2 of RQ
b
ab
a
ab
a
Fold-change to control:
1.00
1.43
1.53
1.26
1.57
FO (control)
100CO
100COSEFM
100CO10CM
100COSEFM10CM

responsive biomarker genes in the QPCR study (3.22-fold down-regulated in 100COSEFM10CM compared with the control diet) (Fig. 3-3H). *Clra* was significantly down-regulated in both 100COSEFM and 100COSEFM10CM groups compared to control by 1.32 and 1.61-fold, respectively (Fig. 3-3I). It is important to note that the hepatic transcript expression of both *lect-2* and *clra* was not affected by feeding the 100CO test diet. The mRNA expression of *klf9* was increased in fish fed 100COSEFM and 100COSEFM10CM diets compared to control fed fish by 1.53 and 1.57-fold, respectively (Fig. 3-3J).

3.5 Discussion

3.5.1 Growth performance of salmon

The feeding experiment conducted in this study included evaluation of the growth performance of Atlantic salmon fed diets containing camelina products (CO and/or CM) with either FM or SEFM, versus salmon fed a control diet containing FO and FM (Table 3-1). Atlantic salmon fed a diet with 100% of FO replaced with CO (i.e. 100CO) did not show significantly lower weight gain or SGR compared to FO fed control fish (Table 3-3). Previous studies have also demonstrated that vegetable oil such as linseed oil, canola oil and rapeseed oil (either singly or as blends) can be used to replace up to 100% of FO in the diet without negatively influencing growth in salmonids (Bell et al., 2001; Torstensen et al., 2005; Bell et al., 2010). In contrast, removing lipid residue from FM by solvent extraction and/or adding 10% CM in the experimental diets significantly affected the growth of salmon fed those diets compared to FO fed fish. The growth rate responses

of salmon fed different camelina product-containing diets (e.g. 100CO or 100COSEFM) compared with FO control (Table 3-3) may be related to the amount of essential fatty acids such DHA and EPA provided in the diets. The lipid residue in FM, which is present in the 100CO diet but virtually absent in the 100COSEFM diet (Table 3-1), may provide sufficient amounts of essential fatty acids needed for good growth.

In terms of the impact of plant meal inclusion, a previous study reported that Atlantic salmon fed a diet containing 20% pea protein concentrate showed no reduction in weight gain or feed intake (Øverland et al., 2009). This was not the case in the current study, where the impact of a "double replacement" diet (i.e. 100CO10CM) on Atlantic salmon growth was investigated and showed significantly lower weight gain and SGR compared to the control group (Table 3-3). As mentioned above, 100CO dietary treatment did not significantly reduce weight gain or SGR compared to FO fed control fish. Collectively, these results show that the combination of total replacement of the lipid and relatively low inclusion of camelina meal (10%) resulted in suboptimal growth performance of the salmon. It is important to note that fish fed all five test diets had comparable feed conversion ratios after 16 weeks of feeding (Table 3-3). This suggests that salmon fed camelina-containing diets were able to utilize nutrients as efficiently as fish fed the control diet. The reduction in growth of salmon fed some of the camelina product-containing test diets was deemed to be affected by lower feed intake.

3.5.2 The impact of camelina-containing diets on salmon liver gene expression

A set of 67 informative microarray probes was identified that responded significantly (FDR < 5%) in the liver of Atlantic salmon fed a camelina-containing diet

(100COSEFM10CM) compared to a control diet after a 16-week feeding trial (see Tables 3-4 and 3-5). To confirm the data set and further study camelina-responsive biomarker gene expression, all microarray-identified transcripts, except *bar*, were validated by QPCR. Based on these results, I am confident that the data set is accurate and the genes reported in this chapter represent a trustworthy catalog of camelina-responsive genes in Atlantic salmon liver. The microarray experiment showed that the 100COSEFM10CM diet influenced the expression of genes involved in lipid, energy and protein metabolism, cell differentiation and proliferation, DNA synthesis and repair, immune function, and regulation of transcription. While all of these modulated biological processes are important for a complete understanding of the physiological impact of camelina-containing diets on salmon, the remainder of the discussion is focused on the differentially expressed genes related to metabolism, cell differentiation and proliferation, and immune function, several of which were QPCR validated.

3.5.2.1 Fatty acid biosynthesis

With respect to lipid-related genes, *facl4* encodes an enzyme that is essential for fatty acid metabolism as it converts free fatty acid into fatty acyl-CoA esters, key intermediates for the production of complex lipids (Cao et al., 1998). Previous studies in rainbow trout showed that the removal of dietary fish oil was associated with higher mRNA expression of *facl4* (Panserat et al., 2008a; Panserat et al., 2008b), agreeing with the up-regulation of *facl4* expression from the present microarray study (Table 3-4) and suggesting higher capacity of fatty acid biosynthesis in the liver of salmon fed the 100COSEFM10CM diet. *Fadsd6a*, which is involved in LC-PUFA biosynthesis, was also

shown by microarray to be up-regulated in fish fed the 100COSEFM10CM diet compared with fish fed the control diet (Table 3-4). The transcript expression responses of Atlantic salmon genes involved in LC-PUFA biosynthesis to vegetable oil-containing diets have been studied previously (Zheng et al., 2004; Zheng et al., 2005a; Zheng et al., 2005b; Morais et al., 2009; Monroig et al., 2010; Morais et al., 2011a). Among the Atlantic salmon *fatty acyl desaturase* and *elongase* genes involved in these previously published studies, two desaturases (*fadsd5* and *fadsd6a*) and three elongases (*elovl2*, *elovl5a* and *elovl5b*) were selected for QPCR analyses in the current investigation to determine if these transcripts in Atlantic salmon liver were inducible by feeding CO-containing test diets that were low in LC-PUFA and high in C_{18} PUFA (Hixson et al., 2014b).

QPCR-based transcript analysis showed that, of the three *elovl* genes investigated here, only *elovl2* was significantly up-regulated (2.38-fold) in salmon fed the 100COSEFM10CM diet compared with the control diet fed fish. While the hepatic expression of *elovl2* in salmon fed other camelina-containing diets (100CO, 100COSEFM and 100CO10CM) exhibited 1.51 to 1.79-fold up-regulation compared with controls, these trends were not statistically significant. In fact, the LC-PUFA amounts in diets 100CO, 100COSEFM and 100CO10CM were higher than that of 100COSEFM10CM (3.3%, 2.1% and 2.5% vs. 1.5%, respectively) (Hixson et al., 2014b); it is possible that this may be linked to the different magnitude of hepatic *elovl2* expression responses to the diets. Atlantic salmon Elovl2, functionally characterized in yeast, demonstrated capacity to lengthen ω3 and ω6 PUFA (chain length from C_{20} to C_{24}) with low activity toward C_{18} (Morais et al., 2009). Based on the "Sprecher pathway", Elovl2 is a critical enzyme for producing DHA from fatty acid precursors since it is able to elongate 20:5ω3 to 24:5ω3

following a chain shortening step (Sprecher, 2000). Interestingly, in the current study, *elovl5a* exhibited significant down-regulation (1.6-fold) in the 100COSEFM10CM group compared with the control group. Previous studies evaluating the effect of replacing up to 100% of dietary FO with vegetable oil on Atlantic salmon hepatic *elovl5a* gene expression have yielded inconsistent results (Zheng et al., 2004; Zheng et al., 2005a; Zheng et al., 2005b; Morais et al., 2009). The reason for the varied responses in the *elovl5a* gene expression to different vegetable oil-containing diets is not clear. In the current QPCR study, no significant differences were detected between hepatic transcript expression of *elovl5b* in FO fed fish and any CO-containing diet fed fish. However, a previous study reported that the expression of *elovl5b* was significantly up-regulated in the liver of Atlantic salmon fed rapeseed or soybean oil containing diets, but not linseed oil containing diet, compared to FO fed fish (Morais et al., 2009). Previous functional characterization of Atlantic salmon Elovl5a and Elovl5b suggested that both enzymes are capable of elongating C_{18} to C_{22} with very limited activity towards C_{22} (Morais et al., 2009). The fatty acid analysis of samples from the current feeding experiment, conducted by Hixson et al. (2014b), showed apparent elongated products of 20:3ω3 and 20:4ω3 as they were significantly higher in various tissues (e.g. white muscle) of salmon fed all CO-containing diets compared with FO fed fish. Based on the current QPCR analysis of salmon *elovl5a* and *elovl5b*, it is still not clear whether these two genes have participated in the elongation of C_{18} to C_{20} when salmon were fed CO-containing diets. In order to gain a complete picture of how CO-containing diets influence the fatty acid elongation process in Atlantic salmon, the responses of all fatty acyl elongases at the enzymatic level to CO-containing diets must be investigated.

The biosynthesis of LC-PUFA from C_{18} PUFA in vertebrates also involves Δ5 and Δ6 desaturation of appropriate fatty acids by desaturase enzymes, Fadsd5 and Fadsd6, respectively (Sprecher, 2000). The Atlantic salmon Fadsd5 and Fadsd6a, as functionally characterized in yeast, demonstrated distinct Δ5 and Δ6 desaturation activities, respectively (Hastings et al., 2004; Zheng et al., 2005a). For example, Fadsd5 was more active towards 20:4ω3 for producing 20:5ω3 (Δ5 activity) with very low activity towards 18:3ω3 for producing 18:4ω3 (Δ6 activity). Fadsd6a, on the other hand, showed much higher Δ6 desaturase activity compared to Δ5 activity (Hastings et al., 2004; Zheng et al., 2005a). In the current QPCR study, both *fadsd5* and *fadsd6a* transcripts were significantly up-regulated in the salmon fed all of the CO-containing diets (except for *fadsd5* in 100CO10CM) compared to the control group. The up-regulation of both *fadsd5* and *fadsd6a* agrees with previous studies on the responses of these two genes to vegetable oil replacement diets (Zheng et al., 2005a; Zheng et al., 2005b; Monroig et al., 2010; Morais et al., 2011a). Collectively, these studies show that a low level of dietary LC-PUFA accompanied with a high level of C_{18} PUFA causes the transcriptional induction of Atlantic salmon *fadsd5* and *fadsd6a*, which are critical genes controlling the LC-PUFA biosynthetic pathway.

3.5.2.2 Other metabolism-relevant genes

It is worth noting that in the current microarray experiment, several energy metabolism-relevant transcripts including *adipophilin*, *lysoplasmalogenase-like*, *ATP-binding cassette sub-family A member 1*, *pcb*, *cpt1* and *bar* showed decreased expression in response to the camelina product-containing experimental diet. Replacing FO with CO

or other plant oils may also affect the β-oxidation capacity in response to the changes in fatty acid composition of the diet (Stubhaug et al., 2007; Leaver et al., 2008a). Cpt1 is considered to be the key enzyme in the regulation of mitochondrial fatty acid oxidation since it catalyses the conversion of fatty acyl-CoAs into fatty acyl-carnitines, which are then transported into the mitochondrial matrix followed by oxidation (Leaver et al., 2008a; Morash et al., 2009). In the current QPCR study, *cpt1* transcript was down-regulated in salmon fed all camelina-containing diets (although only significant for 100CO10CM and 100COSEFM10CM) compared to salmon fed the control diet. The pattern of *cpt1* expression reported here is similar to that reported for rainbow trout; fish fed with a high PUFA (especially LC-PUFA) diet significantly increased *cpt1* transcript expression in red muscle, liver and adipose tissue (Morash et al., 2009). Collectively, these results suggest that dietary LC-PUFA are responsible for the modulation of *cpt1* transcript expression in salmonids, possibly through activation of the transcription factor *peroxisome proliferator activated receptor* (*ppar*) as reported in mammals (Price et al., 2000; Morash et al., 2009). Given the above, it was hypothesized that reduced *cpt1* expression in response to CO-containing diets may be needed to prevent the oxidation of newly formed fatty acids through the fatty acid biosynthesis pathway.

Previous microarray studies have indicated that genes involved in the cholesterol biosynthetic pathway (e.g. *squalene epoxidase* and *lathosterol oxidase*) and lipoprotein metabolism (e.g. *apolipoprotein B-100*) were up-regulated following vegetable oil feeding in Atlantic salmon liver, as a result of low cholesterol present in the plant-based diets compared to the FO based diets (Leaver et al., 2008b; Morais et al., 2011b). From the microarray data, a clear influence of the CO-containing diets on transcript expression

of genes linked to the cholesterol biosynthetic pathway and lipoprotein metabolism was not observed (see below). However, the mRNA expression of *ATP-binding cassette sub-family A member 1*, which is involved in intracellular cholesterol transport and reverse cholesterol transport (e.g. from peripheral tissues to liver), was shown to be decreased in fish fed the camelina product-containing diet in the present microarray experiment. This is in agreement with results obtained previously in salmon fed a vegetable oil-containing diet (Morais et al., 2011b). In addition, the current microarray experiment identified *bar* as 1.86-fold down-regulated in salmon fed the 100COSEFM10CM diet, and QPCR showed that *bar* was 1.15-fold down-regulated (not significant) and 1.38-fold down-regulated (significant) in fish fed 100COSEFM10CO and 100CO10CM diets, respectively, compared to fish fed the FO control diet. Mammalian Bar is a nuclear receptor whose role involves maintaining not only bile acid homeostasis, but also lipoprotein and cholesterol metabolism (Lefebvre et al., 2009). The hepatic gene expression of *bar* was increased 2-fold in mice fed a diet supplemented with krill protein hydrolysate, which is high in ω3 LC-PUFA (Ramsvik et al., 2013). Based on the current QPCR analysis of *bar* expression, which showed significant response to only one of the camelina product-containing diets (100CO10CM), it is not possible to conclude whether or not camelina-containing diets influence cholesterol biosynthesis and lipoprotein metabolism in the salmon liver. The response of candidate genes involved in these pathways to camelina-containing diets warrants further investigation using QPCR.

Among the metabolism-relevant genes that were identified by the current microarray analysis, two genes (*glucose 6-phosphatase* and *pcb*, both down-regulated in fish fed the 100COSEFM10CO diet with *pcb* confirmed by QPCR) were related to

carbohydrate metabolism (Suarez and Mommsen, 1987), Both of these genes encode enzymes involved in gluconeogenesis; the responses of these transcripts suggest decreased gluconeogenesis (i.e. glucose production) in the livers of salmon fed the 100COSEFM10CM diet compared with the control diet. This is not surprising given that the pathways of lipogenesis, lipolysis, glycolysis and gluconeogenesis are all interrelated and co-regulate body energy homeostasis (Morais et al., 2011a). Previous studies have shown that salmonids fed plant oil and/or plant protein-containing diets have altered hepatic expression of genes involved in carbohydrate metabolism (Panserat et al., 2009; Morais et al., 2011a). For example, Panserat et al. (2009) reported that the complete replacement of FM and FO by vegetable alternatives in rainbow trout resulted in a decreased hepatic transcript expression of *hexokinase* and *phosphoenolpyruvate carboxykinase*, which are involved in catalyzing the first steps of glycolysis and gluconeogenesis, respectively. Interestingly, the current QPCR experiment showed that *pcb* was significantly ~1.4-fold down-regulated in both 100COSEFM and 100COSEFM10CM fed fish compared with controls. This suggests that *pcb* transcript expression likely responded to the near complete removal of marine lipid and/or the inclusion of camelina oil (i.e. "100COSEFM") rather than to the inclusion of 10% camelina meal in the 100COSEFM10CM diet.

3.5.2.3 Cell differentiation and proliferation

In recent years, the effect of the replacement of FO with plant oil on hepatic gene expression in fish has been studied using microarrays and QPCR (Jordal et al., 2005; Leaver et al., 2008b; Panserat et al., 2008b; Morais et al., 2011a; Morais et al., 2011b).

Most of these studies, like the current functional genomics study, have shown that replacing FO with plant alternatives has a major impact on metabolism-relevant pathways (e.g. lipid, carbohydrate, protein/amino acid metabolism) in the fish liver tissue. In addition to these well-known diet-responsive pathways in liver, the current microarray analysis also revealed that a number of genes related to other pathways (e.g. cell differentiation and proliferation; apoptosis) responded to the 100COSEFM10CM diet. For example, *dnph1* (alias *rcl*) and *nucleosome assembly protein 1-like 1*, both of which play roles in cell proliferation in mammals (Simon et al., 1994; Lewis et al., 1997), were shown by microarray (with *dnph1* confirmed by QPCR) to be significantly up-regulated in the livers of salmon fed the 100COSEFM10CM test diet compared to controls. Furthermore, the down-regulation of *btg1* (shown by both microarray and QPCR), which in humans encodes a protein that exhibits anti-proliferative function (Rouault et al., 1992), appears to be consistent with the higher expression of genes involved in cell proliferation in the livers of salmon fed the camelina product-containing test diet. In addition, the current microarray experiment also revealed that some genes involved in the induction of apoptosis [*lymphocyte G0/G1 switch protein 2* (Welch et al., 2009) and *metalloproteinase inhibitor 3* (Baker et al., 1998)] had lower hepatic transcript expression in salmon fed the 100COSEFM10CM diet compared with the control diet. Collectively, these data support the hypothesis that there was higher cell proliferation and/or lower apoptosis in the livers of salmon fed 100COSEFM10CM, the most extreme camelina product-containing test diet involved in this study.

Higher cell proliferation in the liver does not necessarily correlate with higher somatic growth of the animal. Among various factors that regulate the growth of animals

including mammals and teleost fishes, the insulin-like growth factors (IGFs) and associated signalling pathways play a central role in controlling skeletal muscle growth (Bower and Johnston, 2010). The IGF system includes the following components: the hormones IGF-I, IGF-II, their corresponding receptors, and the IGF binding proteins (IGFBPs) (Picha et al., 2008; Bower and Johnston, 2010). It is well known that IGFBPs can influence the function and activity of IGFs in animals (Picha et al., 2008; Bower and Johnston, 2010). For example, the availability of IGFs is regulated by IGFBPs since proteolytic degradation of IGFBPs by specific proteases can result in release of IGF-I to target tissues (Bower and Johnston, 2010). Based on the current QPCR analysis, transcript expression of both *igfbp-5b1* and *igfbp-5b2* was significantly reduced in the livers of salmon fed all camelina-containing diets compared with the controls. A previous study reported that fast growth in Atlantic salmon muscle was correlated with the up-regulation of several genes within the IGF pathway including *igfbp-5.2* (currently named *igfbp-5a*) (Bower et al., 2008). Furthermore, in gilthead seabream (*Sparus aurata*), the full replacement of FO with vegetable oil resulted in decreased growth and lower plasma IGF-I, indicating an impact on the IGF pathway (reviewed in Picha et al., 2008). Assessed by weight gain and SGR, the growth performance of salmon in the current feeding trial was likewise reduced in all camelina-containing diet groups except 100CO compared with the FO control group. Given the above, I propose that the hepatic expression of *igfbp-5b1* and *igfbp-5b2* may be a potential growth indicator in Atlantic salmon when fed camelina-containing diets.

3.5.2.4 Immune response

The changes in the fatty acid profiles of diets due to the replacement of FO by vegetable oil may alter fish metabolism, which could potentially affect fish health, immune function and pathogen resistance (Montero et al., 2003; Mourente et al., 2005). Although the liver is a primary metabolic organ for metabolizing carbohydrates, lipids, and proteins in animals, it also has other functions such as detoxification and modulation of immune responses, as well as the production of inflammatory mediators (Knolle and Gerken, 2000). Two immune-related genes [*CD200* (also known as *OX-2 membrane glycoprotein*) and *SLAM family member 8 precursor*] were identified by microarray as being up-regulated in liver of salmon fed 100COSEFM10CM diet compared with FO controls. CD200, encoding a membrane glycoprotein belonging to the immunoglobulin superfamily, has been shown to deliver negative signals to T cells and macrophages upon antigen recognition, suggesting a role in immune suppression in animals (Chung et al., 2002). In addition, the microarray-identified and QPCR-validated down-regulation of *clra* (also known as *CD209 antigen-like protein E*) in fish fed 100COSEFM10CM might suggest a decrease in innate immunity in these fish. The transcript expression of this gene was up-regulated in response to infection by *Aeromonas salmonicida* in salmon liver (Soanes et al., 2004), and by *Piscirickettsia salmonis* in salmon macrophage and hematopoietic kidney (Rise et al., 2004).

Additional immune-relevant hepatic transcripts including *lect-2* and chitinase 3 were microarray-identified as down-regulated in response to the 100COSEFM10CM diet. Both *lect-2* and *chitinase 3* genes are involved in inflammation (Kawada et al., 2007; Li

et al., 2008). Lect-2 acts as a chemotactic factor to activate neutrophils, whose transcript expression was shown to be induced in the liver and spleen of *Vibrio alginolyticus*-infected croceine croaker (*Pseudosciaena crocea*) (Li et al., 2008). Mammalian chitinases are involved in positively regulating the inflammatory response (Kawada et al., 2007). For example, mice treated with anti-chitinase 3-like-1 antibody had a significantly lower load of *Salmonella typhimurium* in peripheral tissues (Kawada et al., 2007). The basal expression (i.e. pre-infection) of both of the pro-inflammatory genes (*tumor necrosis factor-α* and *interleukin 1-β*) studied by Montero and colleagues (2010) was reduced in the intestine and head kidney of gilthead seabream fed soybean oil based diets. However, after induced infection, fish fed vegetable oil containing diets showed over-expression of the transcripts encoding both pro-inflammatory cytokines (Montero et al., 2010). Therefore, in order to gain a complete picture of the impact of camelina-containing diets on salmon immune responses, live pathogen challenge experiments after feeding trails are needed. It is also worth noting that the current QPCR experiment showed that the expression of *clra* and *lect-2* was significantly down-regulated in the liver of fish fed 100COSEFM10CM and/or 100COSEFM diets; this result suggests that the near complete removal of marine lipid (as indicated by "100COSEFM") had a suppressive effect on immune-relevant genes and may therefore negatively influence immune function.

3.6. Conclusions

The present study demonstrates the use of functional genomics tools and techniques (DNA microarrays and QPCR) to identify and validate hepatic molecular biomarkers of salmon response to a camelina-containing diet (100COSEFM10CM) that

was associated with reduced growth. I anticipate that these biomarkers will be useful in the future development of camelina-containing diets that do not have deleterious effects on fish growth or physiology. This study revealed several salmon genes involved in lipid metabolism, carbohydrate metabolism, cell differentiation and proliferation, and immune function that responded to camelina-containing diets, particularly in 100COSEFM10CM. Although the total removal of marine lipids is not essential for sustainable aquaculture practice, this study explored the impact of extreme diets with little or no fish oil (i.e. 100COSEFM and 100COSEFM10CM) on Atlantic salmon growth, gene expression, and physiology (based on functional annotations of diet-responsive transcripts). Importantly, most microarray-identified biomarkers of negative effects of an extreme camelina product-containing diet were shown by QPCR to be non-responsive to the more practical 100CO diet; also, weight gain and specific growth rate of fish fed the 100CO diet were not significantly reduced compared with FO controls. Hence, the 100% replacement of FO with CO could be a good option for developing more sustainable diets for Atlantic salmon aquaculture. However, further research is warranted involving the use of candidate biomarkers identified here to evaluate the impact of additional camelina product-containing diets on salmon of different life stages and different genetic backgrounds.

3.7 References

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CHAPTER 4: Summary

4.1 Summary of findings

This thesis research, part of a large-scale collaborative project (The Camelina Project; http://www.genomeatlantic.ca/), aimed to assess the growth performance and hepatic transcript expression responses of Atlantic cod and Atlantic salmon to camelina product-containing diets. Briefly, juvenile cod fed diets with a 100% FO replacement with CO (100CO) or 100CO with solvent extracted fish meal (100COSEFM) had a significantly lower weight gain than cod fed FO, by 21% and 12% respectively, after a 13-week trial. In contrast, the weight gain and SGR of Atlantic salmon were not significantly affected by feeding 100CO after a 16-week trial; however, salmon fed 100COSEFM diet which contains essentially no marine lipid, showed significant reduction in growth. Additionally, the impact of adding 10% CM to 100CO test diets (100CO10CM and 100COSEFM10CM) on Atlantic salmon growth was investigated, and the results showed significantly lower weight gain in both groups, compared to control group. Liver tissues from these cod and salmon feeding trials were used in the subsequent expression analyses.

Only one Atlantic cod *fatty acyl elongase* transcript (i.e. *elovl5*) had been characterized prior to the current study (Agaba et al., 2005). A primary goal of this thesis was to characterize *elovl* family member transcripts in Atlantic cod due to their significance in the LC-PUFA biosynthetic pathway. The publicly available Atlantic cod genomic resources, including a draft genome assembly, allowed the use of bioinformatic techniques to mine sequence databases to identify partial cDNA sequences for ten members of the cod *elovl* gene family; these cod transcripts were further characterized

using RACE and other molecular biology techniques. Additionally, only one *fatty acyl desaturase*, *fadsd6*, which is also involved in the LC-PUFA biosynthetic pathway, has been identified in Atlantic cod (Tocher et al., 2006) (see Chapter 2 for details). In contrast, several *elovl* (Hastings et al., 2004; Morais et al., 2009) and *fadsd* (Hastings et al., 2004; Zheng et al., 2005; Monroig et al., 2010) genes (see Chapter 3 for details) have been characterized previously in Atlantic salmon. All of these gene sequence resources (i.e. publicly available sequences, and cod *elovl* RACE product sequences from the current research) were useful in the present studies of the effect of replacing dietary FO with CO on the hepatic transcript expression of these genes in cod and salmon.

In both Chapters 2 and 3, QPCR was used to investigate how camelina-containing diets affected the hepatic transcript expression of genes related to LC-PUFA biosynthesis in Atlantic cod and Atlantic salmon. This is a very important question for the aquaculture industry, since finding plant oil alternatives to FO is needed to support the sustainability of aquaculture. Briefly, Atlantic cod fed an extreme test diet (100COSEFM), which contains essentially no marine lipid, showed significant up-regulation of both *elovl5* and *fadsd6* transcripts in liver, compared with cod fed control diet after a 13-week trial; however, the transcript expression of other *elovls* (i.e. *elovl1b*, *elovl4c-2*, and *elovl6a*) was not affected. In Atlantic salmon, *elovl2* was significantly up-regulated in salmon fed 100COSEFM10CM compared with the control fish, while *elovl5a* exhibited the opposite direction of change to the 100COSEFM10CM dietary treatment. The transcript expression of Atlantic salmon *fadsd5* and *fadsd6a* was significantly up-regulated in most of the CO-containing diets compared with the control group. Collectively, the high ALA and/or low ω3 LC-PUFA levels in CO-containing diets may associate with the up-

regulation of some *fatty acyl elongase* and *desaturase* genes in Atlantic cod (i.e. *elovl5*, *fadsd6*) and Atlantic salmon (i.e. *elovl2*, *fadsd5*, *fadsd6a*) but not the other members.

Finally, in Chapter 3, the cGRASP-designed Agilent 44K salmonid oligonucleotide microarray was used to study the impact of an extreme camelina-containing diet (100COSEFM10CM; i.e. not a practical camelina-based diet) on liver transcriptome of Atlantic salmon, and to identify biomarkers of hepatic transcript expression response to this extreme test diet that caused a significant decrease in growth. Genes with putative roles in lipid (e.g. *cpt1*) and carbohydrate metabolism (e.g. *pcb*), cell differentiation and proliferation (e.g. *igfbp-5b1*, *btg1*), and immune function (e.g. *lect-2*), were significantly differentially expressed (confirmed by QPCR analysis) in the liver of salmon fed 100COSEFM10CM diet. It is important to note that QPCR showed that 100CO, a more practical test diet, did not significantly alter the expression of most of the biomarkers of response to the more extreme test diet (100COSEFM10CM). It is anticipated that these biomarkers and QPCR assays will be valuable tools for the development of optimal camelina product-containing diets (i.e. diets that do not negatively affect metabolism or immunity). For example, the camelina-responsive biomarkers and associated QPCR assays could be used to assess the impact of graded levels of camelina product inclusion in novel aquafeeds on Atlantic salmon to determine the maximum inclusion level that will not cause deleterious effects on fish growth, physiology and health.

Generally, the 100% replacement of FO with CO (100CO) could be a good option for developing more sustainable diets for Atlantic salmon aquaculture, since this diet did not significantly alter the growth performance nor the hepatic transcript expression of

most microarray-identified camelina-responsive biomarkers. In contrast, Atlantic cod growth was negatively affected by the 100CO diet, indicating that cod in this feeding trial were not able to tolerate full replacement of dietary FO with CO. An earlier experiment in the Camelina Project (not in the current thesis) showed that cod can tolerate up to 80% replacement of FO with CO (80CO) without decreasing growth (Hixson et al., 2013). Since cod can grow optimally with 80CO (Hixson et al., 2013) but not 100CO (current study), then 80CO should potentially be the maximum recommended replacement level of FO with CO for cod aquaculture. In summary, this thesis includes the characterization of cod *elovl* gene family transcripts, the development and application of QPCR assays for genes involved in cod and salmon LC-PUFA biosynthesis, as well as the identification and validation of novel camelina-responsive hepatic molecular biomarkers in Atlantic salmon. The resources and data generated by this research will be valuable in the future development of novel aquafeeds using camelina products.

4.2 Perspectives and future research

I have successfully characterized ten members of the cod *elovl* gene family at the cDNA level; and two out of ten members (*elovl4a*, *elovl4b*) were partially characterized. However, it will be important in future studies to define the specific biochemical functions of each Atlantic cod Elovl member (especially the newly characterized Elovl4 paralogues) in terms of fatty acid biosynthesis. For example, functional characterization of fish *elovl* cDNAs by heterologous expression of ORFs in yeast (e.g. *Saccharomyces cerevisiae*) has previously been used to investigate the efficiency of these enzymes in the LC-PUFA biosynthetic pathway *in vitro* (Hastings et al., 2004; Zheng et al., 2004; Zheng

et al., 2005; Tocher et al., 2006; Monroig et al., 2010). Furthermore, to build on our understanding of the regulation of transcript expression of genes involved in the LC-PUFA biosynthetic pathway in response to CO-containing diets in Atlantic cod and Atlantic salmon, compound-specific stable isotope analysis [which is able to distinguish the source of fatty acids (i.e. marine or terrestrial)], could be utilized to quantify the amount of LC-PUFA (e.g. DHA, EPA) synthesized from ALA provided in the diets from CO.

The use of microarray- and QPCR-based experimentation allowed me to identify and validate several biomarkers of Atlantic salmon hepatic transcript expression response to an extreme test diet (100COSEFM10CM) that caused a significant decrease in growth. Further research is warranted for evaluating the use of these biomarkers in Atlantic salmon by conducting additional feeding trials involving camelina-containing diets for different life stages of salmon (e.g. first-feeding fry, juveniles, adults) and different genetic backgrounds (e.g. selected for superior performance on plant-based diets), to improve the generality of the results. Moreover, the constitutive hepatic transcript expression of several immune-relevant genes was shown by microarray experiment (with *lect-2* and *clra* validated by QPCR) to be significantly reduced in salmon fed the extreme camelina-containing diet (100COSEFM10CM). In order to gain a complete picture of the impact of camelina-containing diets on salmon immune function, future feeding trials can include live pathogen challenge experiments. Finally, to develop a complete understanding of how camelina product-containing diets affect fish metabolism and physiology, it will be important for future studies to investigate how these diets affect

hepatic protein expression and enzyme activity, and to compare the protein data with transcriptome or QPCR-based transcript expression data.

4.3 References

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APPENDICES

Appendix I. Database mining for putative Atlantic cod *elovl* transcript sequences.

Gene	Zebrafish	Protein[1]	Atlantic cod	Genomic sequences[2]
elovl1	Elovl1a	NP_001005989	*elovl1a*	GeneScaffold_4551: 490856-497948
	Elovl1b	NP_998581	*elovl1b*	ENSGMOT00000020099
elovl2	Elovl2	NP_001035452	-	-
elovl4	Elovl4a	NP_957090	*elovl4a*	ENSGMOT00000002680
	Elovl4b	NP_001191453	*elovl4b*	ENSGMOT00000015387
	Elovl4c	AAH60897	*elovl4c-1*	GeneScaffold_1484: 569043-573000
	-	-	*elovl4c-2*	GeneScaffold_1484: 565000-570000
elovl5	Elovl5	NP_956747	*elovl5*	AY660881
elovl6	Elovl6a	NP_955826	*elovl6a*	ENSGMOT00000003128
	Elovl6b	AAH46901	*elovl6b*	GeneScaffold_2788: 6095-10463
elovl7	Elovl7a	NP_956072	*elovl7*	ENSGMOT00000008618
	Elovl7b	AAH45481	-	-

[1]Accession numbers of zebrafish Elovl protein sequences obtained from NCBI.
[2]Putative cod *elovl* transcripts or associated genomic scaffolds obtained through tBLASTn query the Atlantic cod genome (gadMor1 v73.1) or cDNA collection predicted based on their genomic sequences (i.e. GeneScaffolds; available through Ensembl website: http://www.ensembl.org) databases, using zebrafish Elovl protein sequences. These genomic sequences were used in RACE primer design (except *elovl5*, which had available cDNA sequence for primer design).

Appendix II. Primers used for gene cloning and transcript expression studies of Atlantic cod.

Gene	Primer[1]	Primer sequence (5'-3')	Application[2]
elovl1a	ELOVL1a-f1	ACGCCCTCGTTATACAGAGAACTG	3' RACE
	ELOVL1a-r1	AGACGAGAGCCCGTAGTAGAAGTACA	5' RACE
	ELOVL1a-f2	CTTGTTGACCTACGTCTTCCTCTC	3' nested RACE
	ELOVL1a-r2	CTGGCTCTGTTTCTTTCTCAGGAC	5' nested RACE
	ELOVL1a-f3	ACTCCAGATTGGGGGACATATAG	ORF PCR
	ELOVL1a-r3	CTGTCAATCGAGTCTGGGAATAC	ORF PCR
	ELOVL1a-f4	CCTCCATGTCTTCCACCACT	RT-PCR (111 bp)
	ELOVL1a-r4	TGGACCCCTGCATTTATCAT	RT-PCR (111 bp)
	ELOVL1a-f5	CTGCAAGAAATCATGGCAAA	RT-PCR (146 bp)
	ELOVL1a-r5	GCGTACACCGAGAGGAAGAC	RT-PCR (146 bp)
elovl1b	ELOVL1b-f1	GATATCTACGACTACCTCCTGAGTGG	3' RACE
	ELOVL1b-r1	GGTAGTCACAGGTGTCCATGAAGTAG	5' RACE
	ELOVL1b-f2	GTTCTTTGTACTGTACCTCGGACCTC	3' nested RACE
	ELOVL1b-r2	GAGGCCGTAGTAGAAGTACATGACG	5' nested RACE
	ELOVL1b-f3	CTTATTTCGCCTCCTTCTTTCA	ORF PCR
	ELOVL1b-r3	GCTGCACATGTATGTATCCTTCA	ORF PCR
	ELOVL1b-f4	AAACCGCCAAGATGAATGAC	RT-PCR and QPCR (109%, 199 bp)
	ELOVL1b-r4	ACATTGGCAACATCAGTGGA	RT-PCR and QPCR (109%, 199 bp)
	ELOVL1b-f5	TCTGGGTATACGGCACCTTC	RT-PCR (101 bp)
	ELOVL1b-r5	GCTTCGCTTCAAGTCTTGCT	RT-PCR (101 bp)
elovl4a	ELOVL4a-f1	AGCAGCTCGTACCTGCTGTTCCTCTG	3' RACE
	ELOVL4a-r1	CAGGTACTTCTTCCACCACAGGTA	5' RACE
	ELOVL4a-f2	TCCGCAAGACCCTCATCGTCTACAAC	3' nested RACE
	ELOVL4a-r2	GAGGTACTCTACCCCCTTGGAGAC	5' nested RACE
	ELOVL4a-f3	CTTGTGGGCTGCTGATCGTCCTCATTA	Assembly confirmation PCR
	ELOVL4a-r3	CAGGTACTTCTTCCACCACAGGTA	Assembly confirmation PCR
	ELOVL4a-f4	TTTATCCATCGACACGGACA	RT-PCR (184 bp)
	ELOVL4a-r4	AGCCAGAGGAACAGCAGGTA	RT-PCR (184 bp)
	ELOVL4a-f5	ACCTCGACACGGTGTTCTTC	RT-PCR (173 bp)
	ELOVL4a-r5	GTACATCAGCACGTGGATGG	RT-PCR (173 bp)
elovl4b	ELOVL4b-f1	CGTCCTTAGGAAGACCCTCATAGT	3' RACE
	ELOVL4b-r1	GTAGTAGAAGTTGGCGAAGAGGAC	5' RACE
	ELOVL4b-f2	GGTGGTACTACATCTCCAAGGGAGT	3' nested RACE
	ELOVL4b-r2	CAGACCATAGTAGCCGTACATCAG	5' nested RACE
	ELOVL4b-f3	AAAGTTGACTGAACACGGTGACT	Assembly confirmation PCR
	ELOVL4b-r3	TGGTAGTAGAAGTTGGCGAAGAG	Assembly confirmation PCR
	ELOVL4b-f4	CGCCAAAGAGCTCCTGATAG	RT-PCR (150 bp)
	ELOVL4b-r4	ACCGTGTCCAGAAACTCCAC	RT-PCR (150 bp)

	ELOVL4b-f5	GCCTGTACCTGCTCTTCCTG	RT-PCR (107 bp)
	ELOVL4b-r5	GAGGACCACCATGCTGAAGT	RT-PCR (107 bp)
elovl4c-1	ELOVL4c-1-f1	GTACTCTCCGGTCCCAATAGTAGTC	3' RACE
	ELOVL4c-1-r1	CGTGTCACTGAGTTCTATGACCTTG	5' RACE
	ELOVL4c-1-f2	GGTCATAGAACTCAGTGACACGGTA	3' nested RACE
	ELOVL4c-1-r2	GGAGGAGAACTCCTTTCAGATCAAC	5' nested RACE
	ELOVL4c-1-f3	CTTTCAGGGCGACTACAGAGTT	ORF PCR
	ELOVL4c-1-r3	GCCCAACTTTAGGATGGACAGA	ORF PCR
	ELOVL4c-1-f4	TGAAGATGGCCAATGTGTGT	RT-PCR (146 bp)
	ELOVL4c-1-r4	CCAACAGTTGACGATCATGG	RT-PCR (146 bp)
	ELOVL4c-1-f5	GACCCCGATTGATGAAACAC	RT-PCR (197 bp)
	ELOVL4c-1-r5	ACACACATTGGCCATCTTCA	RT-PCR (197 bp)
elovl4c-2	ELOVL4c-2-f1	GGAGAACGGAGATAAGAGGACAGAC	3' RACE
	ELOVL4c-2-r1	CTGTAGTCTACTGGCTGACAGAGGTA	5' RACE
	ELOVL4c-2-f2	GTCTACTCTCCGGTCCCAGTAATAGT	3' nested RACE
	ELOVL4c-2-r2	ACTATTACTGGGACCGGAGAGTAGAC	5' nested RACE
	ELOVL4c-2-f3	TGATTAAGGGGTCTTAAGGGAGA	ORF PCR
	ELOVL4c-2-r3	GGAAAATGAGGCTGACACAATAG	ORF PCR
	ELOVL4c-2-f4	GGTTATCAGGCCAGGTCAAA	RT-PCR (169 bp)
	ELOVL4c-2-r4	GAGTAGACCAGCAGCCAAGG	RT-PCR (169 bp)
	ELOVL4c-2-f5	ACTAGGCCCCACTGGAAGAT	RT-PCR and QPCR (103%, 136 bp)
	ELOVL4c-2-r5	CTGGCCTGATAACCTTTGGA	RT-PCR and QPCR (103%, 136 bp)
elovl5	ELOVL5-f1	AAGACACACACAGCGATGACGAGACC	3' RACE
	ELOVL5-r1	GGAGAGAGAGAGCCATCATACCC	5' RACE
	ELOVL5-f2	TGTACTCCTACTACGGCCTGTCC	3' nested RACE
	ELOVL5-r2	GGACAGGCCGTAGTAGGAGTACA	5' nested RACE
	ELOVL5-f3	AAGCCAAGGTTACACAGCGACT	ORF PCR
	ELOVL5-r3	GAAGCTTGTGTTTAATGGTCTGG	ORF PCR
	ELOVL5-f4	CTCCTGCTCGACAACTACCC	RT-PCR and QPCR (89%, 172 bp)
	ELOVL5-r4	TCGTACCCTCCACTCCTCAC	RT-PCR and QPCR (89%, 172 bp)
elovl6a	ELOVL6a-f1	CTGCTCTACTCCTGGTACTCCTACA	3' RACE
	ELOVL6a-r1	CATCCAGGAGTAGACCAGGTAGTT	5' RACE
	ELOVL6a-f2	GGGCTGTGTGGTCAACTACCTGGTCTACT	3' nested RACE
	ELOVL6a-r2	GTAGACCAGGTAGTTGACCACACAG	5' nested RACE
	ELOVL6a-f3	GAGAGGCAGTTTAACGAAGATGA	ORF PCR
	ELOVL6a-r3	GACTTTAACTTCCCTCCTGCTGT	ORF PCR
	ELOVL6a-f4	TAACGAAGATGAGGCGATCC	RT-PCR and QPCR (95%, 138 bp)
	ELOVL6a-r4	AGCGGTTTCCTCAGTTCAAA	RT-PCR and QPCR (95%, 138 bp)
	ELOVL6a-f5	GGATGCAGGAGAACTGGAAG	RT-PCR (137 bp)
	ELOVL6a-r5	TAGCGTTAGCGACCATAGCA	RT-PCR (137 bp)
elovl6b	ELOVL6b-f1	ATCTGTGACCAGGGCTTCTACTAC	3' RACE
	ELOVL6b-r1	GTCTGGTAGAAGAACTGGGTGAAC	5' RACE
	ELOVL6b-f2	CTACTCCTGGTACTCCTACAAGGACAC	3' nested RACE

	ELOVL6b-r2	GTCCTTGTAGGAGTACCAGGAGTAGAG	5' nested RACE
	ELOVL6b-f3	AGCAGCAGCAGAAACAGAATAAG	ORF PCR
	ELOVL6b-r3	GCCTTCCATTGCTCTTCAATACT	ORF PCR
	ELOVL6b-f4	GATCAGCAGCAGCAGAAACA	RT-PCR (110 bp)
	ELOVL6b-r4	GTCGAAGCTCCTCTCGAATG	RT-PCR (110 bp)
	ELOVL6b-f5	AAAGGAAGAAGCAGCAGCAG	RT-PCR (114 bp)
	ELOVL6b-r5	CAGTGGTTCTGCATCCAGTC	RT-PCR (114 bp)
elovl7	ELOVL7-f1	CCCTCTCGCTATACATGTGCTATG	3' RACE
	ELOVL7-r1	GGTGTAGGCATGGTACCAGAAGTT	5' RACE
	ELOVL7-f2	GTCATGTACACTTACTACGGCCTGAC	3' nested RACE
	ELOVL7-r2	GGTACTTCTTCCACCACAGGTACTTC	5' nested RACE
	ELOVL7-f3	GTTGTCGTTGTAACGGTAGGTTT	ORF PCR
	ELOVL7-r3	GCTCAACACAAAGTGGTTTAAGG	ORF PCR
	ELOVL7-f4	ACAGCATAGTGCACGTCGTC	RT-PCR (176 bp)
	ELOVL7-r4	AAACTGGTAGGGGCAGTCCT	RT-PCR (176 bp)
	ELOVL7-f5	CTGGGGAACAGGCTACACAT	RT-PCR (190 bp)
	ELOVL7-r5	GAAGGGCATGATTGAGTGGT	RT-PCR (190 bp)
eef1α	EEF1α-f1	CCTCAAACTCACCAACACCA	RT-PCR (170 bp)
	EEF1α-r1	GCGTGGTATCACCATTGACA	RT-PCR (170 bp)
fadsd6	FADSD6-f1	ATTGCCATAGGAACGACCAG	QPCR (114%, 178 bp)
	FADSD6-r1	GGCTTCAGGAACTTCTGCAC	QPCR (114%, 178 bp)
rplp1	RPLP1-f1	GGCGACTAGCACCGTTAAAT	QPCR (89%, 118 bp)
	RPLP1-r1	ATCGTCGTGGAGGATCAGAG	QPCR (89%, 118 bp)

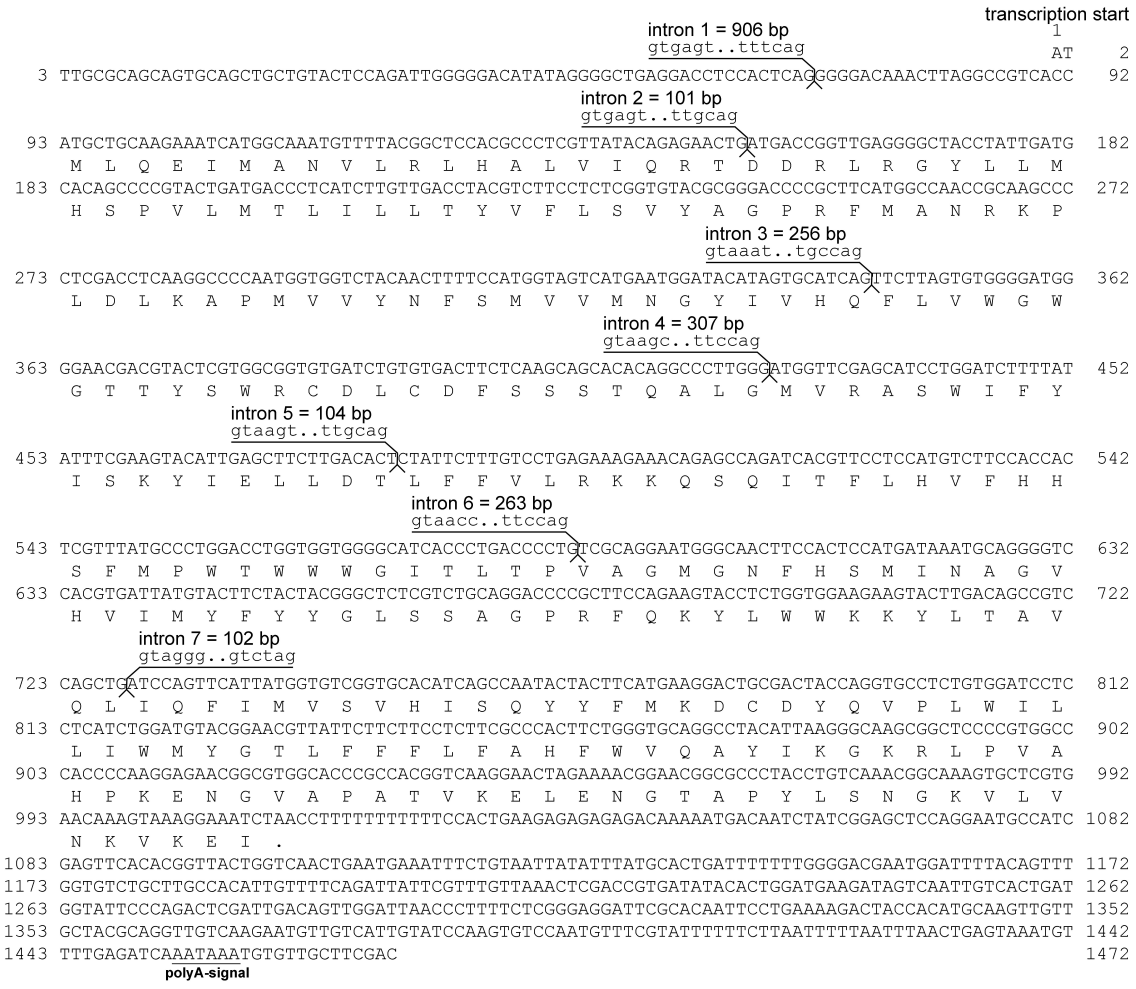
[1]Primer orientation is denoted by "f" or "r" following the gene name for forward or reverse, respectively. *Eef1α* and *rplp1* primer sets were designed based the cod 20K microarray probes: numbers 37006 and 35667, respectively (Booman et al., 2011). The *fadsd6* (*delta-6 fatty acyl desaturase*) primer pair was designed based on the GenBank sequence DQ054840.

[2]For QPCR primers pairs, the amplification efficiency of the primer pair and the size of the amplicon are in parentheses.

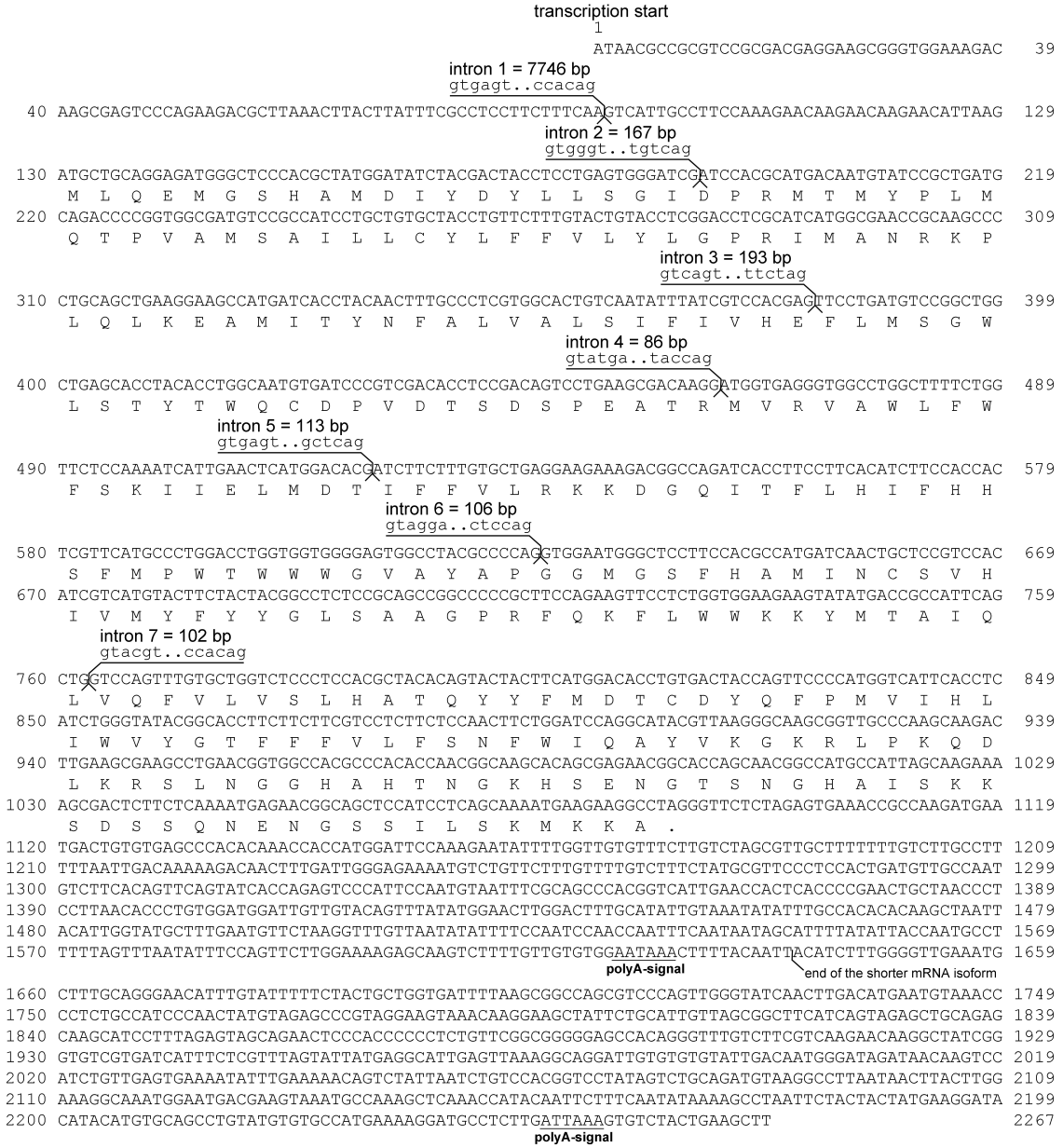
Appendix III. Pairwise percent identity (at the amino acid level) of cod and zebrafish Elovls.

	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*	*18*	*19*	*20*	*21*
1. Cod Elovl1a	***	55	39	40	40	41	36	27	27	52	65	58	37	37	40	42	35	26	27	50	51
2. Cod Elovl1b		***	37	36	40	41	35	22	23	48	61	67	33	39	36	40	35	22	22	50	50
3. Cod Elovl4a			***	70	49	52	45	26	28	43	40	39	50	79	72	52	43	29	29	46	42
4. Cod Elovl4b				***	47	50	47	28	27	45	40	39	45	68	79	49	44	26	26	46	44
5. Cod Elovl4c-1					***	86	45	30	34	48	38	41	53	44	45	80	44	35	35	52	50
6. Cod Elovl4c-2						***	46	31	34	50	40	41	54	46	47	87	45	35	36	54	51
7. Cod Elovl5							***	28	31	42	34	38	56	43	45	46	75	28	29	41	43
8. Cod Elovl6a								***	70	31	23	23	29	23	26	31	29	83	68	33	32
9. Cod Elovl6b									***	32	23	24	34	24	26	34	30	70	76	36	32
10. Cod Elovl7										***	53	53	43	39	42	49	42	31	31	73	72
11. Zebrafish Elovl1a											***	66	33	41	41	39	35	22	23	54	51
12. Zebrafish Elovl1b												***	34	41	40	41	37	22	23	54	51
13. Zebrafish Elovl2													***	44	45	55	58	34	34	46	45
14. Zebrafish Elovl4a														***	75	46	42	23	24	43	38
15. Zebrafish Elovl4b															***	47	44	25	24	44	41
16. Zebrafish Elovl4c																***	47	35	37	53	51
17. Zebrafish Elovl5																	***	28	30	42	43
18. Zebrafish Elovl6a																		***	71	33	31
19. Zebrafish Elovl6b																			***	34.6	32
20. Zebrafish Elovl7a																				***	72
21. Zebrafish Elovl7b																					***

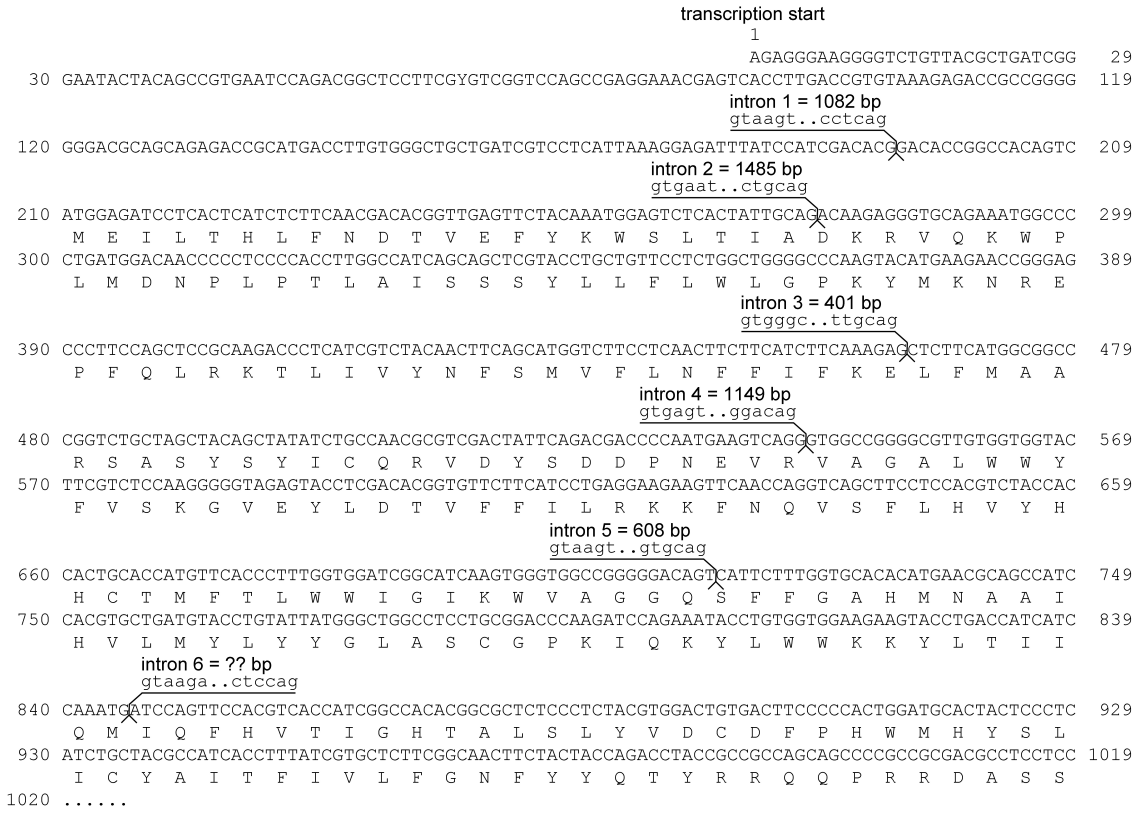
Appendix IV. Nucleotide and hypothetical amino acid sequences of the Atlantic cod *elovl1a* gene (GenBank accession number KF964005). The translation is shown below the putative open reading frame (ORF), with a period indicating the stop codon. The exons are shown in upper case letters, while the introns are shown in lower case letters. The locations and sizes of the introns are indicated. The polyadenylation signal (AATAAA) is underlined in the 3' UTR.


transcription start
intron 1 = 906 bp
gtgagt..tttcag
intron 2 = 101 bp
gtgagt..ttgcag
intron 3 = 256 bp
gtaaat..tgccag
intron 4 = 307 bp
gtaagc..ttccag
intron 5 = 104 bp
gtaagt..ttgcag
intron 6 = 263 bp
gtaacc..ttccag
intron 7 = 102 bp
gtaggg..gtctag
polyA-signal

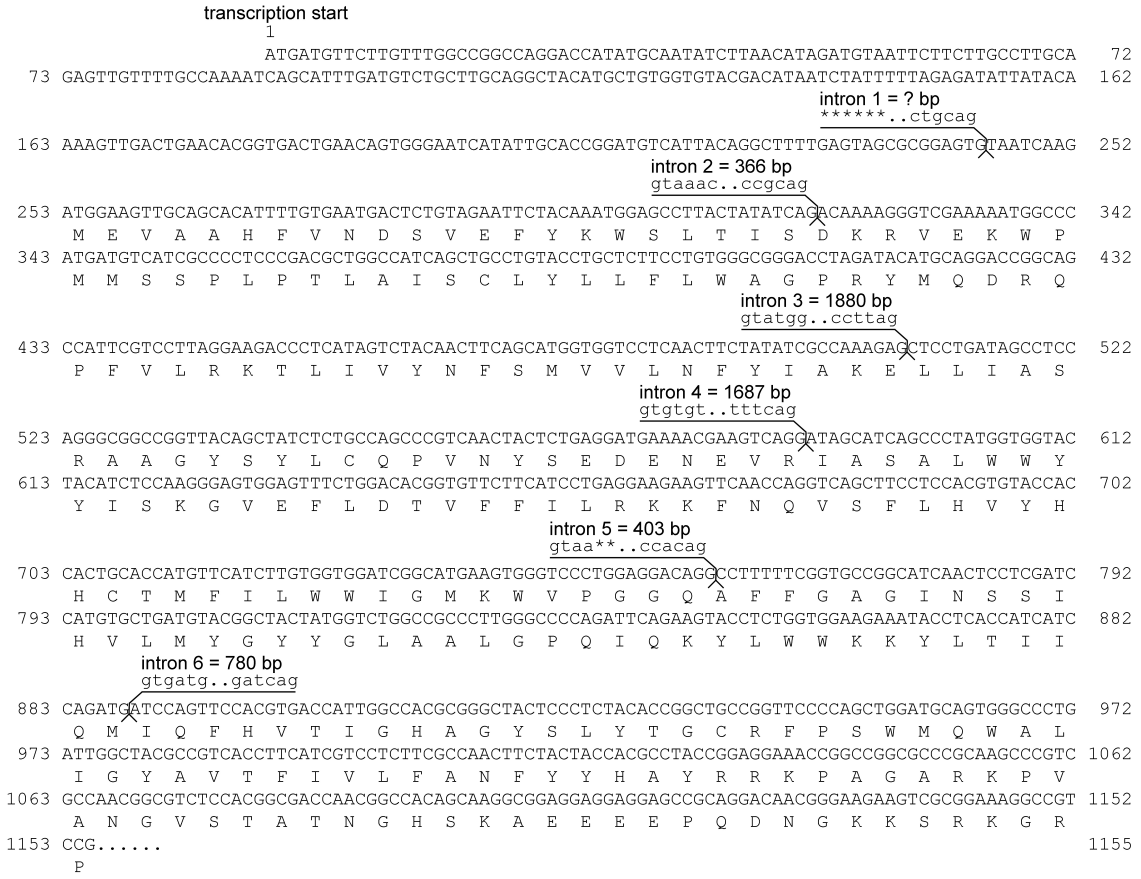
Appendix V. Nucleotide and hypothetical amino acid sequences of the Atlantic cod *elovl1b* gene (GenBank accession numbers KF964006 and KF964007). The translation is shown below the putative open reading frame (ORF), with a period indicating the stop codon. The exons are shown in upper case letters, while the introns are shown in lower case letters. The locations and sizes of the introns are indicated. Two transcript variants that differed at the 3' UTR were identified through the RACE study (short isoform, KF964007; longer isoform, KF964006), and their polyadenylation signals are underlined (i.e. AATAAA for the short isoform and ATTAAA for the longer isoform).


transcription start
1
ATAACGCCGCGTCCGCGACGAGGAAGCGGGTGGAAAGAC 39
intron 1 = 7746 bp
gtgagt..ccacag
40 AAGCGAGTCCCAGAAGACGCTTAAACTTACTTATTTCGCCTCCTTCTTTCAAGTCATTGCCTTCCAAAGAACAAGAACAAGAACATTAAG 129
intron 2 = 167 bp
gtgggt..tgtcag
130 ATGCTGCAGGAGATGGGCTCCCACGCTATGGATATCTACGACTACCTCCTGAGTGGGATCGATCCACGCATGACAATGTATCCGCTGATG 219
M L Q E M G S H A M D I Y D Y L L S G I D P R M T M Y P L M
220 CAGACCCCGGTGGCGATGTCCGCCATCCTGCTGTGCTACCTGTTCTTTGTACTGTACCTCGGACCTCGCATCATGGCGAACCGCAAGCCC 309
Q T P V A M S A I L L C Y L F F V L Y L G P R I M A N R K P
intron 3 = 193 bp
gtcagt..ttctag
310 CTGCAGCTGAAGGAAGCCATGATCACCTACAACTTTGCCCTCGTGGCACTGTCAATATTTATCGTCCACGAGTTCCTGATGTCCGGCTGG 399
L Q L K E A M I T Y N F A L V A L S I F I V H E F L M S G W
intron 4 = 86 bp
gtatga..taccag
400 CTGAGCACCTACACCTGGCAATGTGATCCCGTCGACACCTCCGACAGTCCTGAAGCGACAAGGATGGTGAGGGTGGCCTGGCTTTTCTGG 489
L S T Y T W Q C D P V D T S D S P E A T R M V R V A W L F W
intron 5 = 113 bp
gtgagt..gctcag
490 TTCTCCAAAATCATTGAACTCATGGACACGATCTTCTTTGTGCTGAGGAAGAAAGACGGCCAGATCACCTTCCTTCACATCTTCCACCAC 579
F S K I I E L M D T I F F V L R K K D G Q I T F L H I F H H
intron 6 = 106 bp
gtagga..ctccag
580 TCGTTCATGCCCTGGACCTGGTGGTGGGGAGTGGCCTACGCCCCAGGTGGAATGGGCTCCTTCCACGCCATGATCAACTGCTCCGTCCAC 669
S F M P W T W W W G V A Y A P G G M G S F H A M I N C S V H
670 ATCGTCATGTACTTCTACTACGGCCTCTCCGCAGCCGGCCCCCGCTTCCAGAAGTTCCTCTGGTGGAAGAAGTATATGACCGCCATTCAG 759
I V M Y F Y Y G L S A A G P R F Q K F L W W K K Y M T A I Q
intron 7 = 102 bp
gtacgt..ccacag
760 CTGGTCCAGTTTGTGCTGGTCTCCCTCCACGCTACACAGTACTACTTCATGGACACCTGTGACTACCAGTTCCCCATGGTCATTCACCTC 849
L V Q F V L V S L H A T Q Y Y F M D T C D Y Q F P M V I H L
850 ATCTGGGTATACGGCACCTTCTTCTTCGTCCTCTTCTCCAACTTCTGGATCCAGGCATACGTTAAGGGCAAGCGGTTGCCCAAGCAAGAC 939
I W V Y G T F F F V L F S N F W I Q A Y V K G K R L P K Q D
940 TTGAAGCGAAGCCTGAACGGTGGCCACGCCCACACCAACGGCAAGCACAGCGAGAACGGCACCAGCAACGGCCATGCCATTAGCAAGAAA 1029
L K R S L N G G H A H T N G K H S E N G T S N G H A I S K K
1030 AGCGACTCTTCTCAAAATGAGAACGGCAGCTCCATCCTCAGCAAAATGAAGAAGGCCTAGGGTTCTCTAGAGTGAAACCGCCAAGATGAA 1119
S D S S Q N E N G S S I L S K M K K A .
1120 TGACTGTGTGAGCCCACACAAACCACCATGGATTCCAAAGAATATTTTGGTTGTGTTTCTTGTCTAGCGTTGCTTTTTTTGTCTTGCCTT 1209
1210 TTTAATTGACAAAAAGACAACTTTGATTGGGAGAAAATGTCTGTTCTTTGTTTTGTCTTTCTATGCGTTCCCTCCACTGATGTTGCCAAT 1299
1300 GTCTTCACAGTTCAGTATCACCAGAGTCCCATTCCAATGTAATTTCGCAGCCCACGGTCATTGAACCACTCACCCCGAACTGCTAACCCT 1389
1390 CCTTAACACCCTGTGGATGGATTGTTGTACAGTTTATATGGAACTTGGACTTTGCATATTGTAAATATATTTGCCACACACAAGCTAATT 1479
1480 ACATTGGTATGCTTTGAATGTTCTAAGGTTTGTTAATATATTTTCCAATCCAACCAATTTCAATAATAGCATTTTATATTACCAATGCCT 1569
1570 TTTTAGTTTAATATTTCCAGTTCTTGGAAAAGAGCAAGTCTTTTGTTGTGTGGAATAAACTTTTACAATTACATCTTTGGGGTTGAAATG 1659
polyA-signal
end of the shorter mRNA isoform
1660 CTTTGCAGGGAACATTTGTATTTTTCTACTGCTGGTGATTTTAAGCGGCCAGCGTCCCAGTTGGGTATCAACTTGACATGAATGTAAACC 1749
1750 CCTCTGCCATCCCAACTATGTAGAGCCCGTAGGAAGTAAACAAGGAAGCTATTCTGCATTGTTAGCGGCTTCATCAGTAGAGCTGCAGAG 1839
1840 CAAGCATCCTTTAGAGTAGCAGAACTCCCACCCCCCTCTGTTCGGCGGGGGAGCCACAGGGTTTGTCTTCGTCAAGAACAAGGCTATCGG 1929
1930 GTGTCGTGATCATTTCTCGTTTAGTATTATGAGGCATTGAGTTAAAGGCAGGATTGTGTGTGTATTGACAATGGGATAGATAACAAGTCC 2019
2020 ATCTGTTGAGTGAAAATATTTGAAAAACAGTCTATTAATCTGTCCACGGTCCTATAGTCTGCAGATGTAAGGCCTTAATAACTTACTTGG 2109
2110 AAAGGCAAATGGAATGACGAAGTAAATGCCAAAGCTCAAACCATACAATTCTTTCAATATAAAAGCCTAATTCTACTACTATGAAGGATA 2199
2200 CATACATGTGCAGCCTGTATGTGTGCCATGAAAAGGATGCCTCTTGATTAAAGTGTCTACTGAAGCTT 2267
polyA-signal

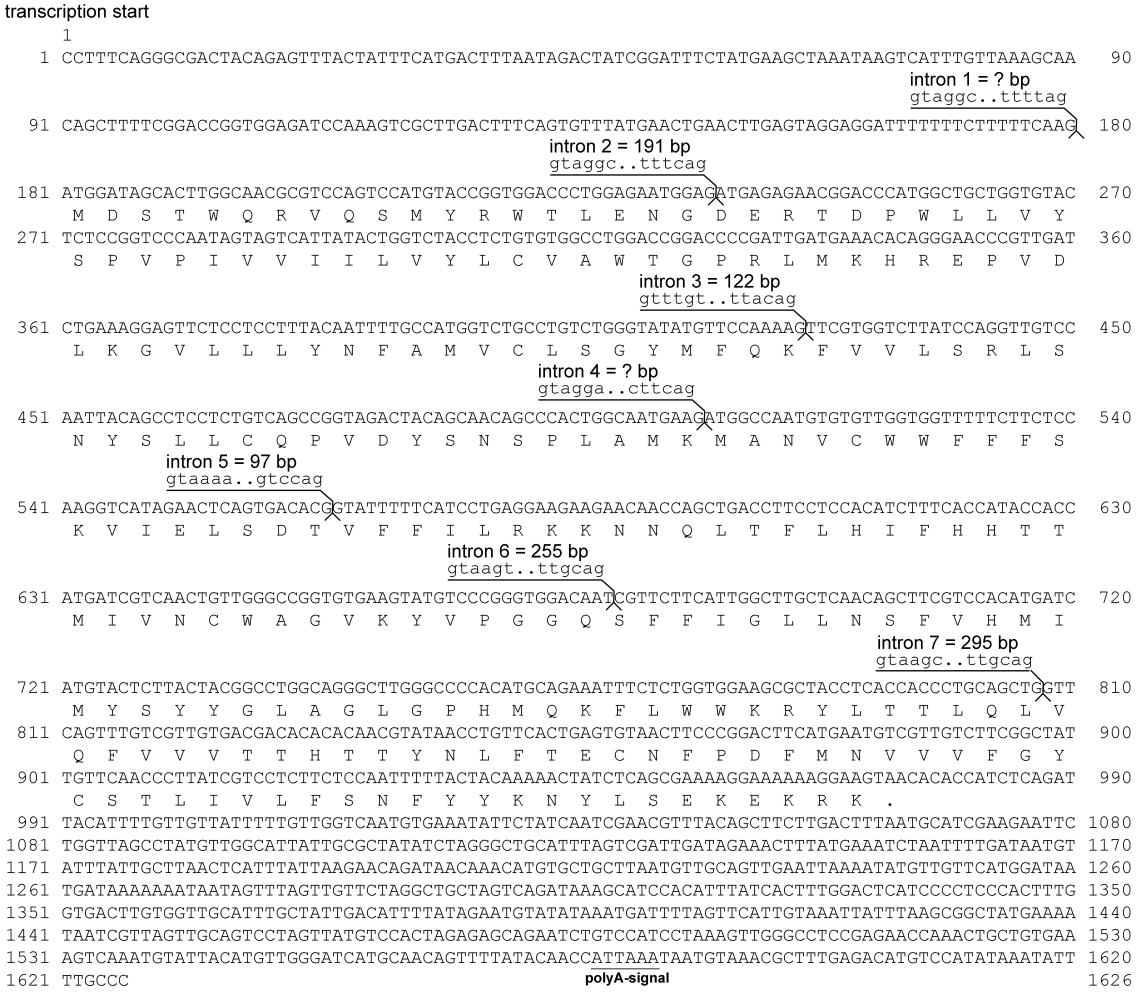
Appendix VI. Nucleotide and hypothetical amino acid sequences of the Atlantic cod *elovl4a* gene (GenBank accession number KF964008). The translation is shown below the putative open reading frame (ORF), with a period indicating the stop codon. The exons are shown in upper case letters, while the introns are shown in lower case letters. The locations and sizes of the introns are indicated. The 3' UTR of *elovl4a* has not yet been fully characterized as indicated by multiple periods.


transcription start
1
AGAGGGAAGGGGTCTGTTACGCTGATCGG 29
30 GAATACTACAGCCGTGAATCCAGACGGCTCCTTCGYGTCGGTCCAGCCGAGGAAACGAGTCACCTTGACCGTGTAAAGAGACCGCCGGGG 119
intron 1 = 1082 bp
gtaagt..cctcag
120 GGGACGCAGCAGAGACCGCATGACCTTGTGGGCTGCTGATCGTCCTCATTAAAGGAGATTTATCCATCGACACGGACACCGGCCACAGTC 209
intron 2 = 1485 bp
gtgaat..ctgcag
210 ATGGAGATCCTCACTCATCTCTTCAACGACACGGTTGAGTTCTACAAATGGAGTCTCACTATTGCAGACAAGAGGGTGCAGAAATGGCCC 299
M E I L T H L F N D T V E F Y K W S L T I A D K R V Q K W P
300 CTGATGGACAACCCCCTCCCCACCTTGGCCATCAGCAGCTCGTACCTGCTGTTCCTCTGGCTGGGGCCCAAGTACATGAAGAACCGGGAG 389
L M D N P L P T L A I S S S Y L L F L W L G P K Y M K N R E
intron 3 = 401 bp
gtgggc..ttgcag
390 CCCTTCCAGCTCCGCAAGACCCTCATCGTCTACAACTTCAGCATGGTCTTCCTCAACTTCTTCATCTTCAAAGAGCTCTTCATGGCGGCC 479
P F Q L R K T L I V Y N F S M V F L N F F I F K E L F M A A
intron 4 = 1149 bp
gtgagt..ggacag
480 CGGTCTGCTAGCTACAGCTATATCTGCCAACGCGTCGACTATTCAGACGACCCCAATGAAGTCAGGGTGGCCGGGGCGTTGTGGTGGTAC 569
R S A S Y S Y I C Q R V D Y S D D P N E V R V A G A L W W Y
570 TTCGTCTCCAAGGGGGTAGAGTACCTCGACACGGTGTTCTTCATCCTGAGGAAGAAGTTCAACCAGGTCAGCTTCCTCCACGTCTACCAC 659
F V S K G V E Y L D T V F F I L R K K F N Q V S F L H V Y H
intron 5 = 608 bp
gtaagt..gtgcag
660 CACTGCACCATGTTCACCCTTTGGTGGATCGGCATCAAGTGGGTGGCCGGGGGACAGTCATTCTTTGGTGCACACATGAACGCAGCCATC 749
H C T M F T L W W I G I K W V A G G Q S F F G A H M N A A I
750 CACGTGCTGATGTACCTGTATTATGGGCTGGCCTCCTGCGGACCCAAGATCCAGAAATACCTGTGGTGGAAGAAGTACCTGACCATCATC 839
H V L M Y L Y Y G L A S C G P K I Q K Y L W W K K Y L T I I
intron 6 = ?? bp
gtaaga..ctccag
840 CAAATGATCCAGTTCCACGTCACCATCGGCCACACGGCGCTCTCCCTCTACGTGGACTGTGACTTCCCCCACTGGATGCACTACTCCCTC 929
Q M I Q F H V T I G H T A L S L Y V D C D F P H W M H Y S L
930 ATCTGCTACGCCATCACCTTTATCGTGCTCTTCGGCAACTTCTACTACCAGACCTACCGCCGCCAGCAGCCCCGCCGCGACGCCTCCTCC 1019
I C Y A I T F I V L F G N F Y Y Q T Y R R Q Q P R R D A S S
1020 ......

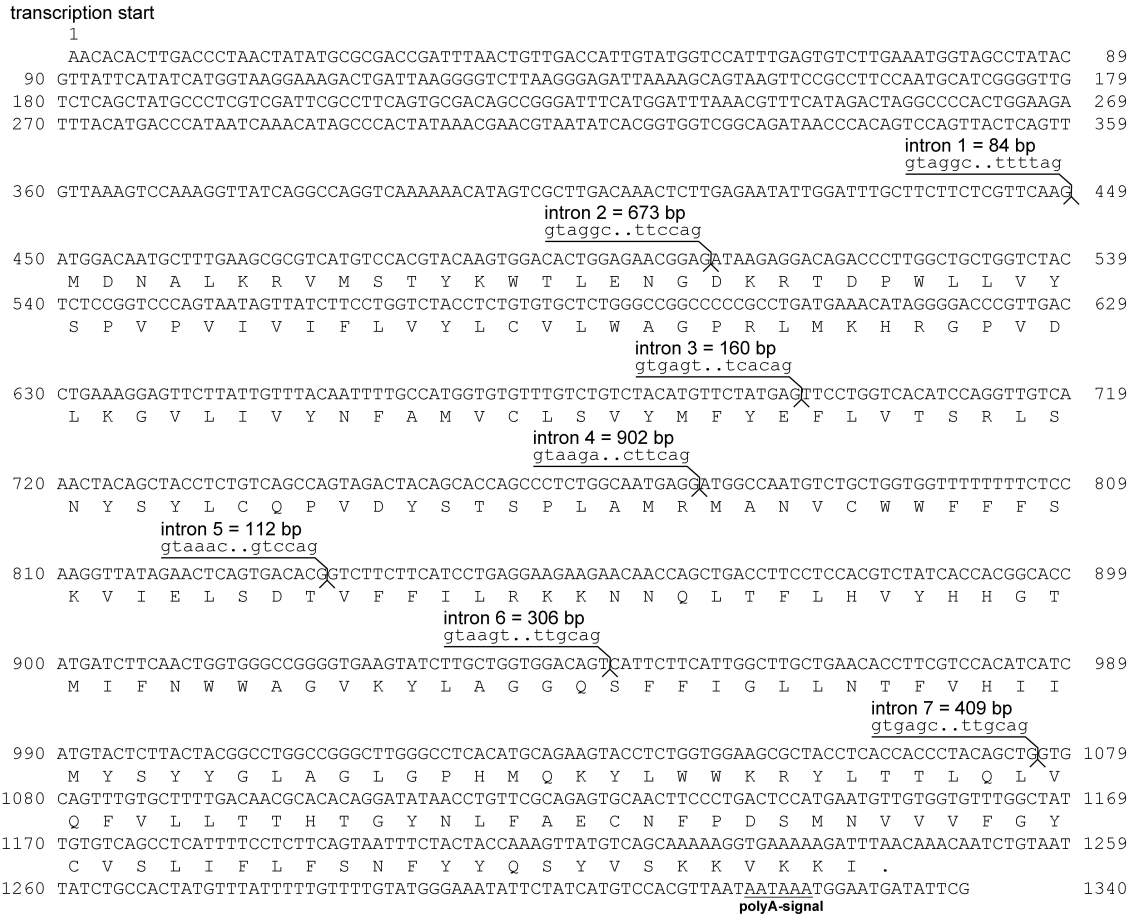
Appendix VII. Nucleotide and hypothetical amino acid sequences of the Atlantic cod *elovl4b* gene (GenBank accession number KF964009). The translation is shown below the putative open reading frame (ORF), with a period indicating the stop codon. The exons are shown in upper case letters, while the introns are shown in lower case letters. The locations and sizes of the introns are indicated. The 3' UTR of *elovl4b* has not yet been fully characterized as indicated by multiple periods.


transcription start
intron 1 = ? bp
******..ctgcag
intron 2 = 366 bp
gtaaac..ccgcag
intron 3 = 1880 bp
gtatgg..ccttag
intron 4 = 1687 bp
gtgtgt..tttcag
intron 5 = 403 bp
gtaa**..ccacag
intron 6 = 780 bp
gtgatg..gatcag

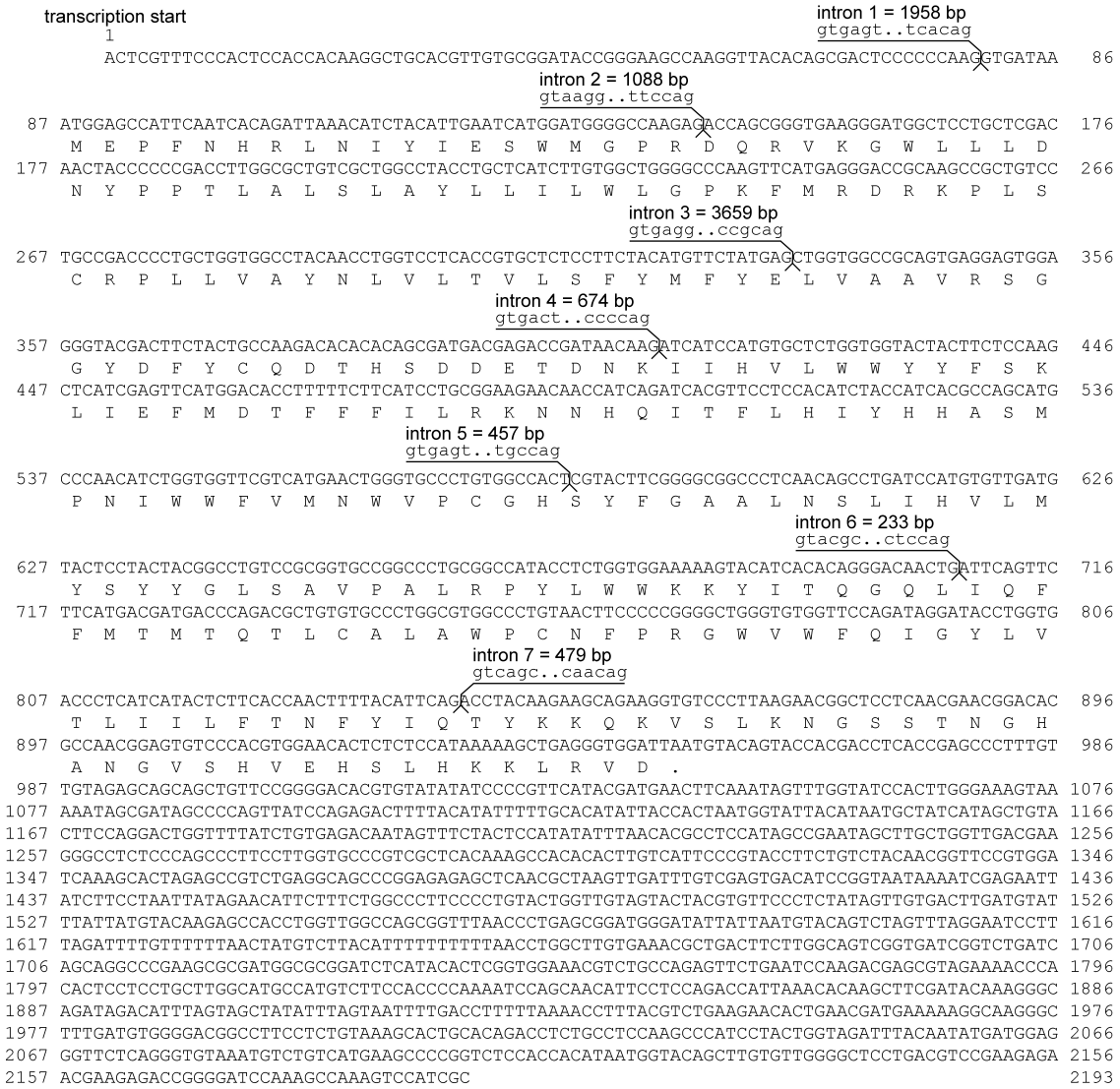
Appendix VIII. Nucleotide and hypothetical amino acid sequences of the Atlantic cod *elovl4c-1* gene (GenBank accession number KF964010). The translation is shown below the putative open reading frame (ORF), with a period indicating the stop codon. The exons are shown in upper case letters, while the introns are shown in lower case letters. The locations and sizes of the introns are indicated. The polyadenylation signal (ATTAAA) is underlined in the 3' UTR.


transcription start
intron 1 = ? bp
gtaggc..ttttag
intron 2 = 191 bp
gtaggc..tttcag
intron 3 = 122 bp
gtttgt..ttacag
intron 4 = ? bp
gtagga..cttcag
intron 5 = 97 bp
gtaaaa..gtccag
intron 6 = 255 bp
gtaagt..ttgcag
intron 7 = 295 bp
gtaagc..ttgcag
polyA-signal

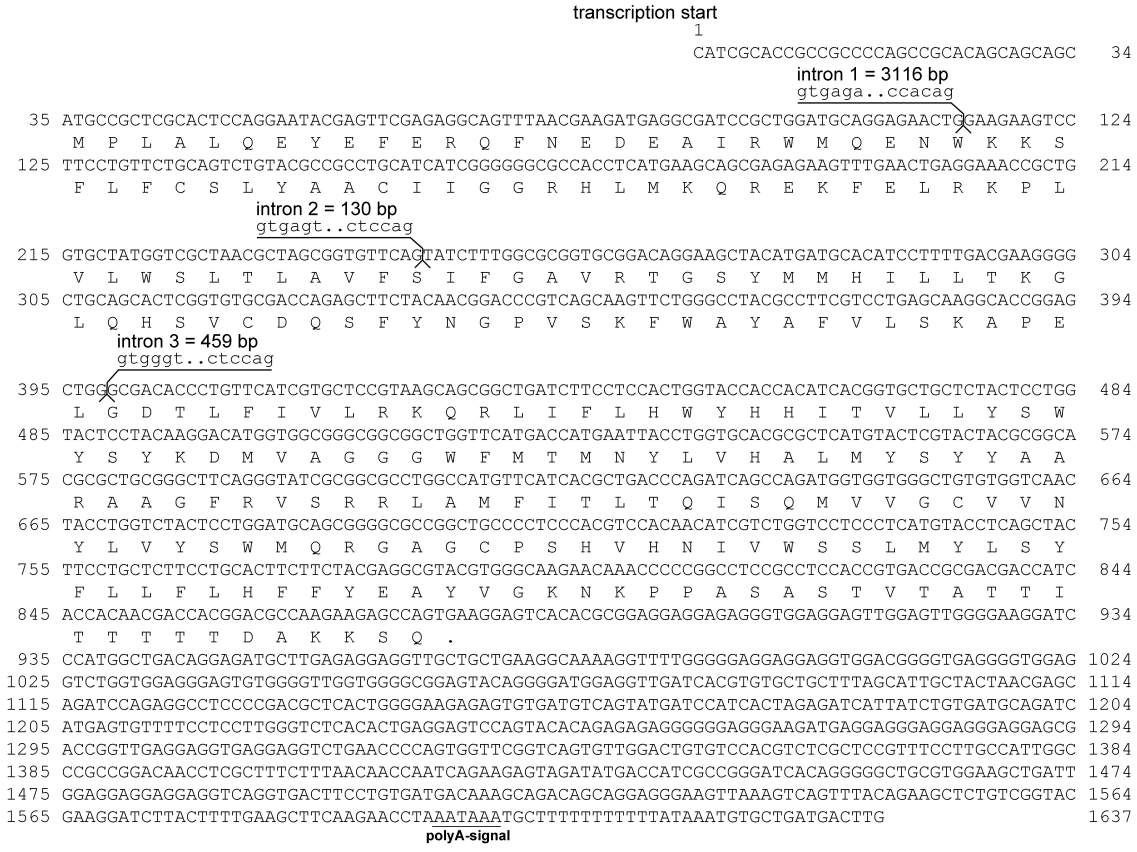
Appendix IX. Nucleotide and hypothetical amino acid sequences of the Atlantic cod *elovl4c-2* gene (GenBank accession number KF964011). The translation is shown below the putative open reading frame (ORF), with a period indicating the stop codon. The exons are shown in upper case letters, while the introns are shown in lower case letters. The locations and sizes of the introns are indicated. The polyadenylation signal (AATAAA) is underlined in the 3' UTR.


transcription start
intron 1 = 84 bp
gtaggc..ttttag
intron 2 = 673 bp
gtaggc..ttccag
intron 3 = 160 bp
gtgagt..tcacag
intron 4 = 902 bp
gtaaga..cttcag
intron 5 = 112 bp
gtaaac..gtccag
intron 6 = 306 bp
gtaagt..ttgcag
intron 7 = 409 bp
gtgagc..ttgcag
polyA-signal

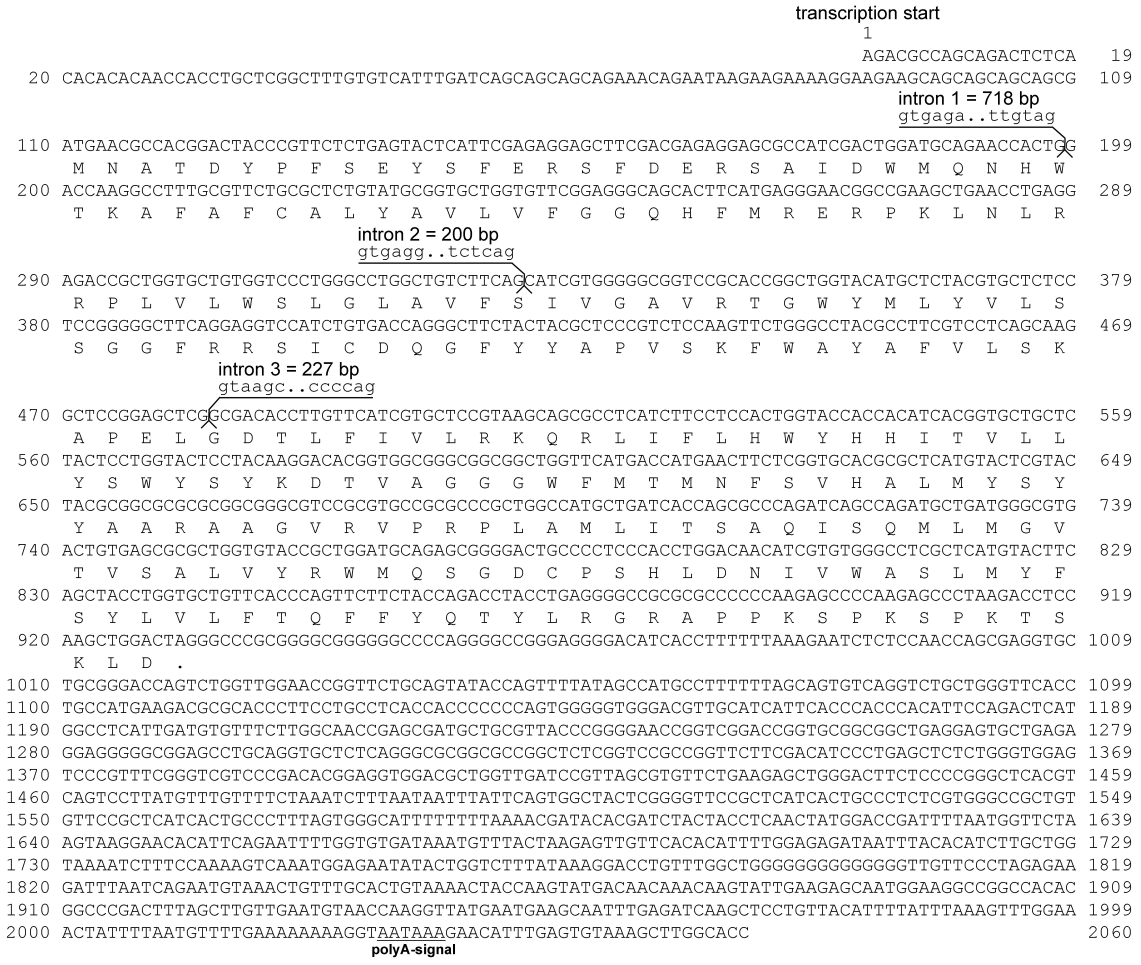
Appendix X. Nucleotide and hypothetical amino acid sequences of the Atlantic cod *elovl5* gene (GenBank accession number KF964012). The translation is shown below the putative open reading frame (ORF), with a period indicating the stop codon. The exons are shown in upper case letters, while the introns are shown in lower case letters. The locations and sizes of the introns are indicated.


transcription start
intron 1 = 1958 bp
gtgagt..tcacag
intron 2 = 1088 bp
gtaagg..ttccag
intron 3 = 3659 bp
gtgagg..ccgcag
intron 4 = 674 bp
gtgact..ccccag
intron 5 = 457 bp
gtgagt..tgccag
intron 6 = 233 bp
gtacgc..ctccag
intron 7 = 479 bp
gtcagc..caacag

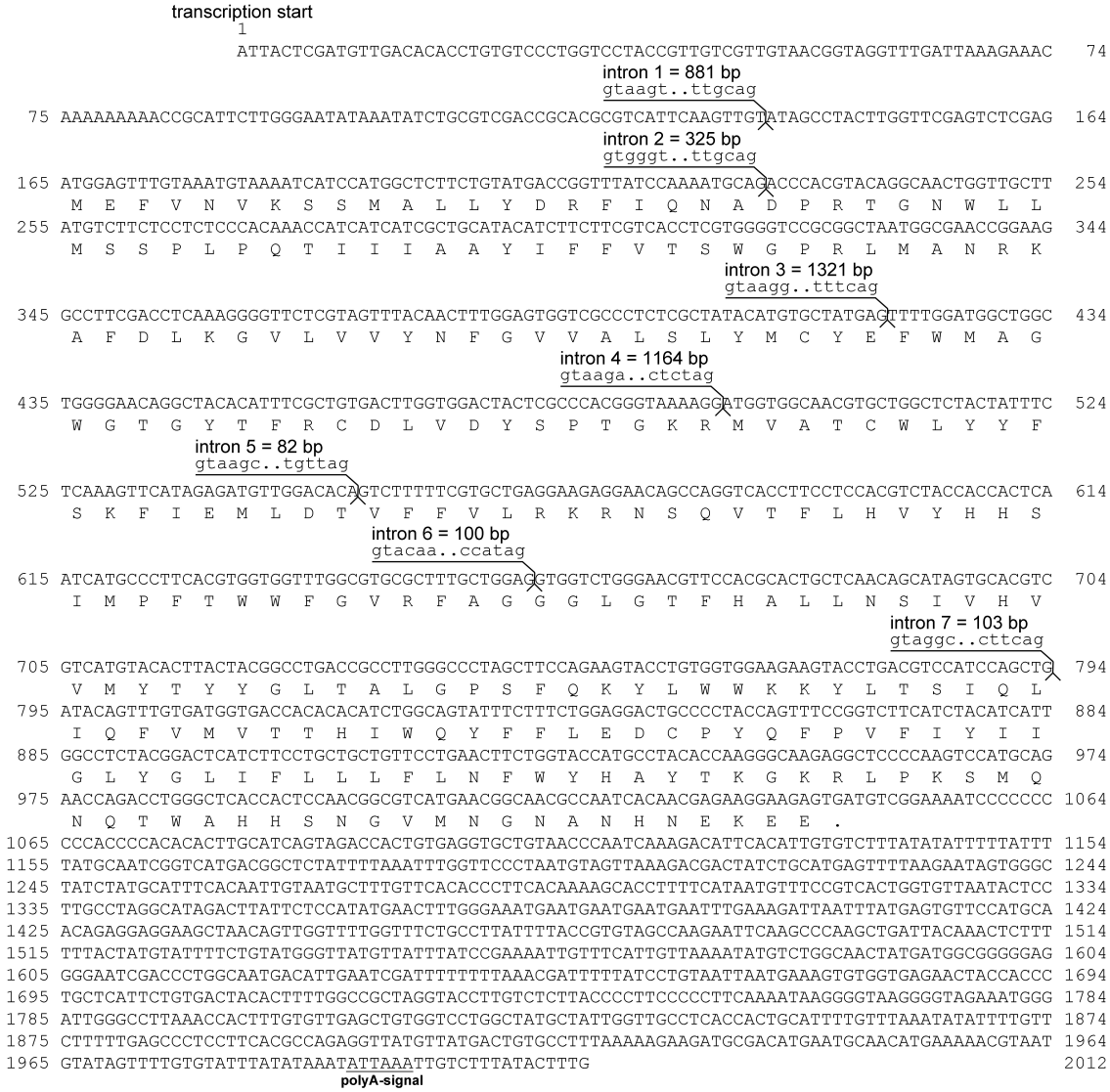
Appendix XI. Nucleotide and hypothetical amino acid sequences of the Atlantic cod *elovl6a* gene (GenBank accession number KF964013). The translation is shown below the putative open reading frame (ORF), with a period indicating the stop codon. The exons are shown in upper case letters, while the introns are shown in lower case letters. The locations and sizes of the introns are indicated. The polyadenylation signal (AATAAA) is underlined in the 3' UTR.


transcription start
1
CATCGCACCGCCGCCCCAGCCGCACAGCAGCAGC 34
intron 1 = 3116 bp
gtgaga..ccacag
35 ATGCCGCTCGCACTCCAGGAATACGAGTTCGAGAGGCAGTTTAACGAAGATGAGGCGATCCGCTGGATGCAGGAGAACTGGAAGAAGTCC 124
M P L A L Q E Y E F E R Q F N E D E A I R W M Q E N W K K S
125 TTCCTGTTCTGCAGTCTGTACGCCGCCTGCATCATCGGGGGGCGCCACCTCATGAAGCAGCGAGAGAAGTTTGAACTGAGGAAACCGCTG 214
F L F C S L Y A A C I I G G R H L M K Q R E K F E L R K P L
intron 2 = 130 bp
gtgagt..ctccag
215 GTGCTATGGTCGCTAACGCTAGCGGTGTTCAGTATCTTTGGCGCGGTGCGGACAGGAAGCTACATGATGCACATCCTTTTGACGAAGGGG 304
V L W S L T L A V F S I F G A V R T G S Y M M H I L L T K G
305 CTGCAGCACTCGGTGTGCGACCAGAGCTTCTACAACGGACCCGTCAGCAAGTTCTGGGCCTACGCCTTCGTCCTGAGCAAGGCACCGGAG 394
L Q H S V C D Q S F Y N G P V S K F W A Y A F V L S K A P E
intron 3 = 459 bp
gtgggt..ctccag
395 CTGGGCGACACCCTGTTCATCGTGCTCCGTAAGCAGCGGCTGATCTTCCTCCACTGGTACCACCACATCACGGTGCTGCTCTACTCCTGG 484
L G D T L F I V L R K Q R L I F L H W Y H H I T V L L Y S W
485 TACTCCTACAAGGACATGGTGGCGGGCGGCGGCTGGTTCATGACCATGAATTACCTGGTGCACGCGCTCATGTACTCGTACTACGCGGCA 574
Y S Y K D M V A G G G W F M T M N Y L V H A L M Y S Y Y A A
575 CGCGCTGCGGGCTTCAGGGTATCGCGGCGCCTGGCCATGTTCATCACGCTGACCCAGATCAGCCAGATGGTGGTGGGCTGTGTGGTCAAC 664
R A A G F R V S R R L A M F I T L T Q I S Q M V V G C V V N
665 TACCTGGTCTACTCCTGGATGCAGCGGGGCGCCGGCTGCCCCTCCCACGTCCACAACATCGTCTGGTCCTCCCTCATGTACCTCAGCTAC 754
Y L V Y S W M Q R G A G C P S H V H N I V W S S L M Y L S Y
755 TTCCTGCTCTTCCTGCACTTCTTCTACGAGGCGTACGTGGGCAAGAACAAACCCCCGGCCTCCGCCTCCACCGTGACCGCGACGACCATC 844
F L L F L H F F Y E A Y V G K N K P P A S A S T V T A T T I
845 ACCACAACGACCACGGACGCCAAGAAGAGCCAGTGAAGGAGTCACACGCGGAGGAGGAGAGGGTGGAGGAGTTGGAGTTGGGGAAGGATC 934
T T T T T D A K K S Q .
935 CCATGGCTGACAGGAGATGCTTGAGAGGAGGTTGCTGCTGAAGGCAAAAGGTTTTGGGGGAGGAGGAGGTGGACGGGGTGAGGGGTGGAG 1024
1025 GTCTGGTGGAGGGAGTGTGGGGTTGGTGGGGCGGAGTACAGGGGATGGAGGTTGATCACGTGTGCTGCTTTAGCATTGCTACTAACGAGC 1114
1115 AGATCCAGAGGCCTCCCCGACGCTCACTGGGGAAGAGAGTGTGATGTCAGTATGATCCATCACTAGAGATCATTATCTGTGATGCAGATC 1204
1205 ATGAGTGTTTTCCTCCTTGGGTCTCACACTGAGGAGTCCAGTACACAGAGAGAGGGGGGAGGGAAGATGAGGAGGGAGGAGGGAGGAGCG 1294
1295 ACCGGTTGAGGAGGTGAGGAGGTCTGAACCCCAGTGGTTCGGTCAGTGTTGGACTGTGTCCACGTCTCGCTCCGTTTCCTTGCCATTGGC 1384
1385 CCGCCGGACAACCTCGCTTTCTTTAACAACCAATCAGAAGAGTAGATATGACCATCGCCGGGATCACAGGGGGCTGCGTGGAAGCTGATT 1474
1475 GGAGGAGGAGGAGGTCAGGTGACTTCCTGTGATGACAAAGCAGACAGCAGGAGGGAAGTTAAAGTCAGTTTACAGAAGCTCTGTCGGTAC 1564
1565 GAAGGATCTTACTTTTGAAGCTTCAAGAACCTAAATAAATGCTTTTTTTTTTTTATAAATGTGCTGATGACTTG 1637
polyA-signal

Appendix XII. Nucleotide and hypothetical amino acid sequences of the Atlantic cod *elovl6b* gene (GenBank accession number KF964014). The translation is shown below the putative open reading frame (ORF), with a period indicating the stop codon. The exons are shown in upper case letters, while the introns are shown in lower case letters. The locations and sizes of the introns are indicated. The polyadenylation signal (AATAAA) is underlined in the 3' UTR.

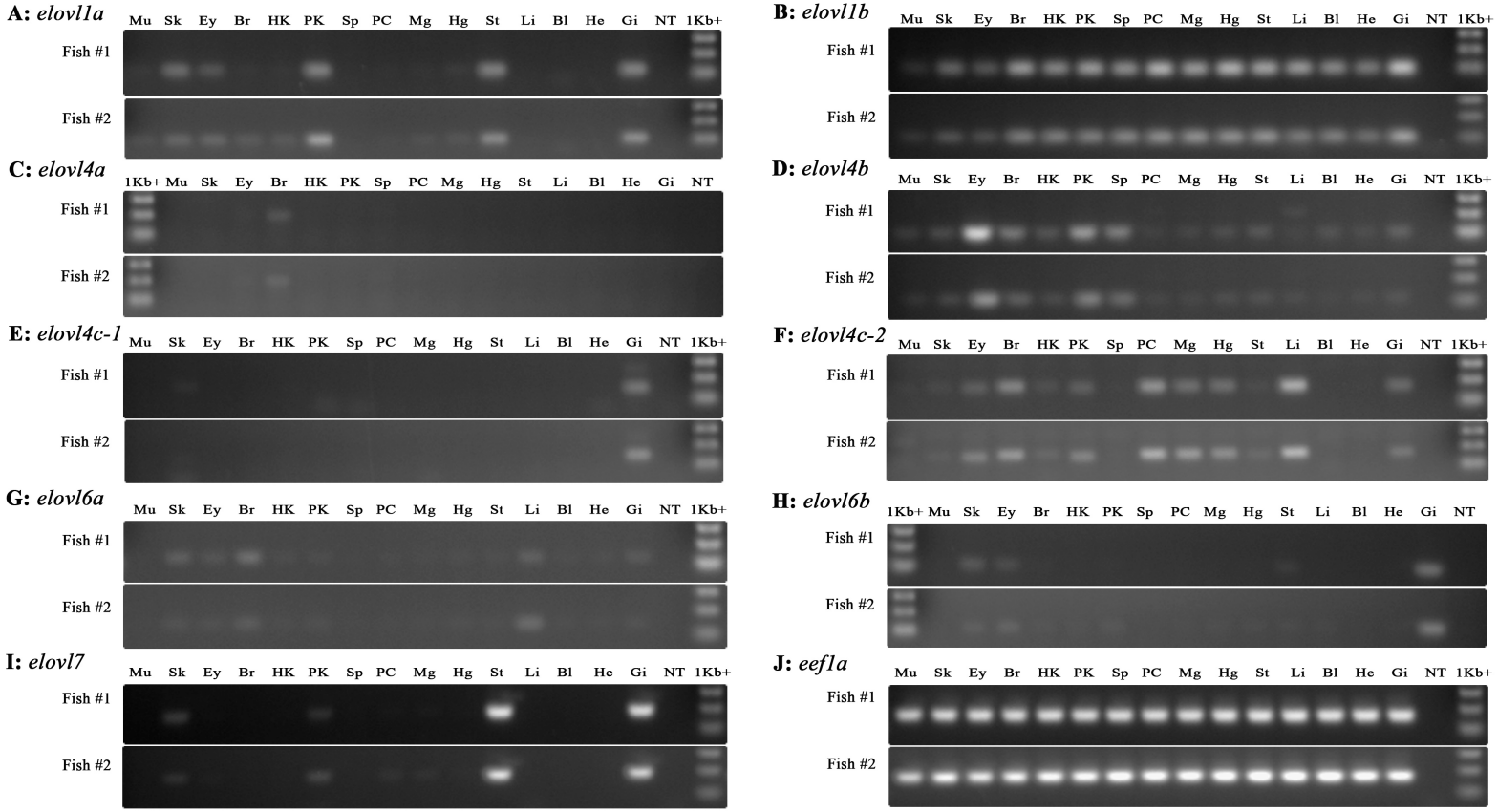

transcription start
1
AGACGCCAGCAGACTCTCA 19
20 CACACACAACCACCTGCTCGGCTTTGTGTCATTTGATCAGCAGCAGCAGAAACAGAATAAGAAGAAAAGGAAGAAGCAGCAGCAGCAGCG 109
intron 1 = 718 bp
gtgaga..ttgtag
110 ATGAACGCCACGGACTACCCGTTCTCTGAGTACTCATTCGAGAGGAGCTTCGACGAGAGGAGCGCCATCGACTGGATGCAGAACCACTGG 199
M N A T D Y P F S E Y S F E R S F D E R S A I D W M Q N H W
200 ACCAAGGCCTTTGCGTTCTGCGCTCTGTATGCGGTGCTGGTGTTCGGAGGGCAGCACTTCATGAGGGAACGGCCGAAGCTGAACCTGAGG 289
T K A F A F C A L Y A V L V F G G Q H F M R E R P K L N L R
intron 2 = 200 bp
gtgagg..tctcag
290 AGACCGCTGGTGCTGTGGTCCCTGGGCCTGGCTGTCTTCAGCATCGTGGGGGCGGTCCGCACCGGCTGGTACATGCTCTACGTGCTCTCC 379
R P L V L W S L G L A V F S I V G A V R T G W Y M L Y V L S
380 TCCGGGGGCTTCAGGAGGTCCATCTGTGACCAGGGCTTCTACTACGCTCCCGTCTCCAAGTTCTGGGCCTACGCCTTCGTCCTCAGCAAG 469
S G G F R R S I C D Q G F Y Y A P V S K F W A Y A F V L S K
intron 3 = 227 bp
gtaagc..ccccag
470 GCTCCGGAGCTCGGCGACACCTTGTTCATCGTGCTCCGTAAGCAGCGCCTCATCTTCCTCCACTGGTACCACCACATCACGGTGCTGCTC 559
A P E L G D T L F I V L R K Q R L I F L H W Y H H I T V L L
560 TACTCCTGGTACTCCTACAAGGACACGGTGGCGGGCGGCGGCTGGTTCATGACCATGAACTTCTCGGTGCACGCGCTCATGTACTCGTAC 649
Y S W Y S Y K D T V A G G G W F M T M N F S V H A L M Y S Y
650 TACGCGGCGCGCGCGGCGGGCGTCCGCGTGCCGCGCCCGCTGGCCATGCTGATCACCAGCGCCCAGATCAGCCAGATGCTGATGGGCGTG 739
Y A A R A A G V R V P R P L A M L I T S A Q I S Q M L M G V
740 ACTGTGAGCGCGCTGGTGTACCGCTGGATGCAGAGCGGGGACTGCCCCTCCCACCTGGACAACATCGTGTGGGCCTCGCTCATGTACTTC 829
T V S A L V Y R W M Q S G D C P S H L D N I V W A S L M Y F
830 AGCTACCTGGTGCTGTTCACCCAGTTCTTCTACCAGACCTACCTGAGGGGCCGCGCGCCCCCCAAGAGCCCCAAGAGCCCTAAGACCTCC 919
S Y L V L F T Q F F Y Q T Y L R G R A P P K S P K S P K T S
920 AAGCTGGACTAGGGCCCGCGGGGCGGGGGGCCCCAGGGGCCGGGAGGGGACATCACCTTTTTTAAAGAATCTCTCCAACCAGCGAGGTGC 1009
K L D .
1010 TGCGGGACCAGTCTGGTTGGAACCGGTTCTGCAGTATACCAGTTTTATAGCCATGCCTTTTTTAGCAGTGTCAGGTCTGCTGGGTTCACC 1099
1100 TGCCATGAAGACGCGCACCCTTCCTGCCTCACCACCCCCCCAGTGGGGGTGGGACGTTGCATCATTCACCCACCCACATTCCAGACTCAT 1189
1190 GGCCTCATTGATGTGTTTCTTGGCAACCGAGCGATGCTGCGTTACCCGGGGAACCGGTCGGACCGGTGCGGCGGCTGAGGAGTGCTGAGA 1279
1280 GGAGGGGGCGGAGCCTGCAGGTGCTCTCAGGGCGCGGCGCCGGCTCTCGGTCCGCCGGTTCTTCGACATCCCTGAGCTCTCTGGGTGGAG 1369
1370 TCCCGTTTCGGGTCGTCCCGACACGGAGGTGGACGCTGGTTGATCCGTTAGCGTGTTCTGAAGAGCTGGGACTTCTCCCCGGGCTCACGT 1459
1460 CAGTCCTTATGTTTGTTTTCTAAATCTTTAATAATTTATTCAGTGGCTACTCGGGGTTCCGCTCATCACTGCCCTCTCGTGGGCCGCTGT 1549
1550 GTTCCGCTCATCACTGCCCTTTAGTGGGCATTTTTTTTTAAAACGATACACGATCTACTACCTCAACTATGGACCGATTTTAATGGTTCTA 1639
1640 AGTAAGGAACACATTCAGAATTTTGGTGTGATAAATGTTTACTAAGAGTTGTTCACACATTTTGGAGAGATAATTTACACATCTTGCTGG 1729
1730 TAAAATCTTTCCAAAAGTCAAATGGAGAATATACTGGTCTTTATAAAGGACCTGTTTGGCTGGGGGGGGGGGGGGGTTGTTCCCTAGAGAA 1819
1820 GATTTAATCAGAATGTAAACTGTTTGCACTGTAAAACTACCAAGTATGACAACAAACAAGTATTGAAGAGCAATGGAAGGCCGGCCACAC 1909
1910 GGCCCGACTTTAGCTTGTTGAATGTAACCAAGGTTATGAATGAAGCAATTTGAGATCAAGCTCCTGTTACATTTTATTTAAAGTTTGGAA 1999
2000 ACTATTTTAATGTTTTGAAAAAAAAGGTAATAAAGAACATTTGAGTGTAAAGCTTGGCACC 2060
polyA-signal

Appendix XIII. Nucleotide and hypothetical amino acid sequences of the Atlantic cod *elovl7* gene (GenBank accession number KF964015). The translation is shown below the putative open reading frame (ORF), with a period indicating the stop codon. The exons are shown in upper case letters, while the introns are shown in lower case letters. The locations and sizes of the introns are indicated. The polyadenylation signal (ATTAAA) is underlined in the 3' UTR.


transcription start
intron 1 = 881 bp
gtaagt..ttgcag
intron 2 = 325 bp
gtgggt..ttgcag
intron 3 = 1321 bp
gtaagg..tttcag
intron 4 = 1164 bp
gtaaga..ctctag
intron 5 = 82 bp
gtaagc..tgttag
intron 6 = 100 bp
gtacaa..ccatag
intron 7 = 103 bp
gtaggc..cttcag
polyA-signal

Appendix XIV. RT-PCR assessment of tissue distribution of *elovl1a*, *elovl1b*, *elovl4a*, *elovl4b*, *elovl4c-1*, *elovl4c-2*, *elovl6a*, *elovl6b* and *elovl7* transcripts in Atlantic cod for various tissues including skeletal muscle (Mu), skin (Sk), eye (Ey), brain (Br), head kidney (HK), posterior kidney (PK), spleen (Sp), pyloric caecum (PC), midgut (Mg), hindgut (Hg), stomach (St), liver (Li), blood (Bl), heart (He), and gill (Gi). These tissues were collected from two juvenile cod. Expression of the housekeeping/reference gene *elongation factor 1α* (*eef1α*) was re-run to ensure the quality of cDNA samples, using the same primer set as shown in Fig. 2-4. NT, no-template control. The primer pairs used in these RT-PCRs were *elovl1a* (f4, r4), *elovl1b* (f5, r5), *elovl4a* (f4, r4), *elovl4b* (f5, r5), *elovl4c-1* (f4, r4), *elovl4c-2* (f4, r4), *elovl6a* (f5, r5), *elovl6b* (f4, r4) and *elovl7* (f5, r5) as listed in Appendix II.

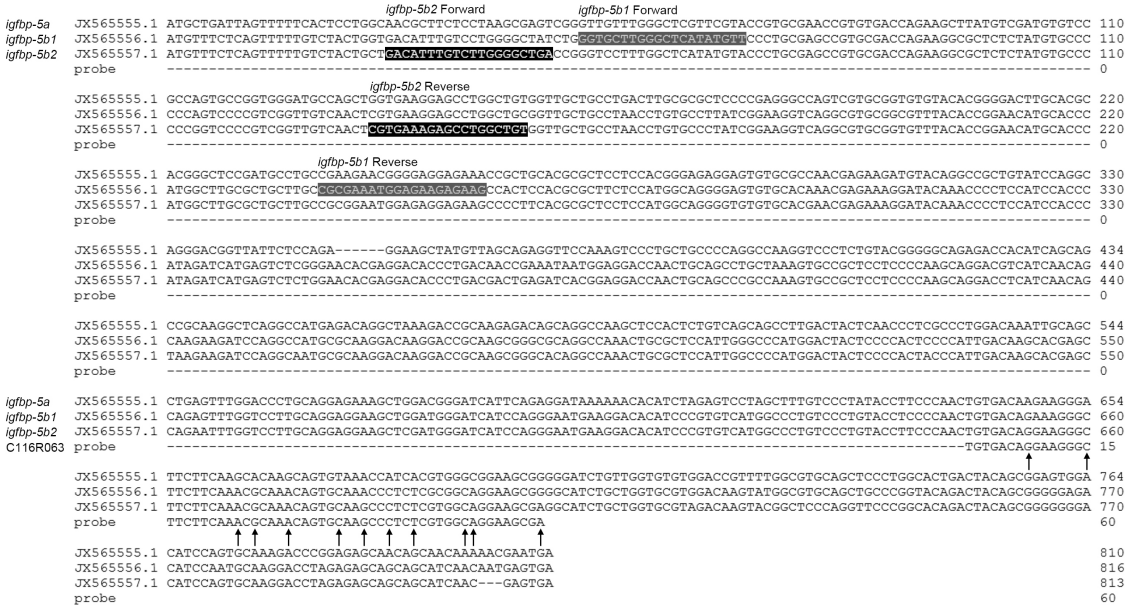
Supplemental figure



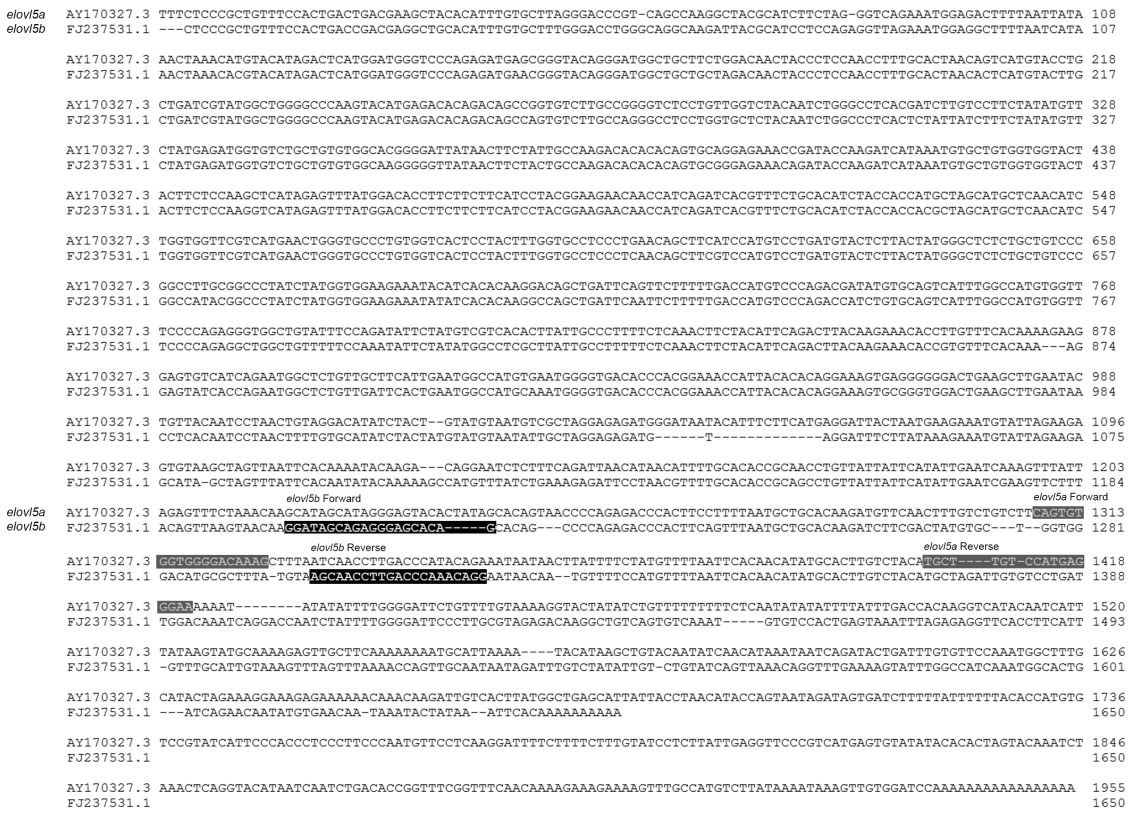
Appendix XV. The design of paralogue-specific primers based on nucleotide sequence alignment (partial) of *fadsd5* (GenBank accession number AF478472), *fadsd6a* (AY458652), *fadsd6b* (GU207400) and *fadsd6c* (GU207401). The locations of primers are marked by shading with each primer pair shaded with the same colour. The alignment was done through the MegAlign function of the Lasergene 7.20 software (DNASTAR, Madison, WI), using the ClustalW method.


fadsd5
fadsd6a
fadsd6b
fadsd6c
fadsd6a Forward
fadsd6a Reverse
fadsd5 Forward
fadsd5 Reverse

Appendix XVI. The design of paralogue-specific primers based on nucleotide sequence alignment of *igfbp-5a* (GenBank accession number JX565555), *igfbp-5b1* (JX565556) and *igfbp-5b2* (JX565557). The locations of primers are marked by shading with each primer pair shaded with the same colour. The alignment was done through the MegAlign function of the Lasergene 7.20 software (DNASTAR, Madison, WI), using the ClustalW method. The microarray probe sequence (i.e. 60mer) representing microarray feature C116R063 was also incorporated in this alignment. Nucleotides that are different between the microarray probe (C116R063) and *igfbp-5a* are indicated with arrows. Based on the aligned region between three transcripts and the microarray probe (C116R063), the microarray probe (C116R063) more likely represents both *igfbp-5b1* and *igfbp-5b2*.


igfbp-5b2 Forward
igfbp-5b1 Forward
igfbp-5a
igfbp-5b1
igfbp-5b2
probe
igfbp-5b2 Reverse
igfbp-5b1 Reverse
C116R063

Appendix XVII. The design of paralogue-specific primers based on nucleotide sequence alignment of *elovl5a* (GenBank accession number AY170327) and *elovl5b* (FJ237531). The locations of primers are marked by shading with each primer pair shaded with the same colour. The alignment was done through the MegAlign function of the Lasergene 7.20 software (DNASTAR, Madison, WI), using the ClustalW method.


elovl5a
elovl5b
AY170327.3 TTTCTCCCGCTGTTTCCACTGACTGACGAAGCTACACATTTGTGCTTAGGGACCCGT-CAGCCAAGGCTACGCATCTTCTAG-GGTCAGAAATGGAGACTTTTAATTATA 108
FJ237531.1 ---CTCCCGCTGTTTCCACTGACCGACGAGGCTGCACATTTGTGCTTTGGGACCTGGGCAGGCAAGATTACGCATCCTCCAGAGGTTAGAAATGGAGGCTTTTAATCATA 107
AY170327.3 AACTAAACATGTACATAGACTCATGGATGGGTCCCAGAGATGAGCGGGTACAGGGATGGCTGCTTCTGGACAACTACCCTCCAACCTTTGCACTAACAGTCATGTACCTG 218
FJ237531.1 AACTAAACACGTACATAGACTCATGGATGGGTCCCAGAGATGAACGGGTACAGGGATGGCTGCTGCTAGACAACTACCCTCCAACCTTTGCACTAACACTCATGTACTTG 217
AY170327.3 CTGATCGTATGGCTGGGGCCCAAGTACATGAGACACAGACAGCCGGTGTCTTGCCGGGGTCTCCTGTTGGTCTACAATCTGGGCCTCACGATCTTGTCCTTCTATATGTT 328
FJ237531.1 CTGATCGTATGGCTGGGGCCCAAGTACATGAGACACAGACAGCCAGTGTCTTGCCAGGGCCTCCTGGTGCTCTACAATCTGGCCCTCACTCTATTATCTTTCTATATGTT 327
AY170327.3 CTATGAGATGGTGTCTGCTGTGTGGCACGGGGATTATAACTTCTATTGCCAAGACACACACAGTGCAGGAGAAACCGATACCAAGATCATAAATGTGCTGTGGTGGTACT 438
FJ237531.1 CTATGAGATGGTGTCTGCTGTGTGGCAAGGGGGTTATAACTTCTACTGCCAAGACACACACAGTGCGGGAGAAACAGATACCAAGATCATAAATGTGCTGTGGTGGTACT 437
AY170327.3 ACTTCTCCAAGCTCATAGAGTTTATGGACACCTTCTTCTTCATCCTACGGAAGAACAACCATCAGATCACGTTTCTGCACATCTACCACCATGCTAGCATGCTCAACATC 548
FJ237531.1 ACTTCTCCAAGGTCATAGAGTTTATGGACACCTTCTTCTTCATCCTACGGAAGAACAACCATCAGATCACGTTTCTGCACATCTACCACCACGCTAGCATGCTCAACATC 547
AY170327.3 TGGTGGTTCGTCATGAACTGGGTGCCCTGTGGTCACTCCTACTTTGGTGCCTCCCTGAACAGCTTCATCCATGTCCTGATGTACTCTTACTATGGGCTCTCTGCTGTCCC 658
FJ237531.1 TGGTGGTTCGTCATGAACTGGGTGCCCTGTGGTCACTCCTACTTTGGTGCCTCCCTCAACAGCTTCGTCCATGTCCTGATGTACTCTTACTATGGGCTCTCTGCTGTCCC 657
AY170327.3 GGCCTTGCGGCCCTATCTATGGTGGAAGAAATACATCACACAAGGACAGCTGATTCAGTTCTTTTTGACCATGTCCCAGACGATATGTGCAGTCATTTGGCCATGTGGTT 768
FJ237531.1 GGCCATACGGCCCTATCTATGGTGGAAGAAATATATCACACAAGGCCAGCTGATTCAATTCTTTTTGACCATGTCCCAGACCATCTGTGCAGTCATTTGGCCATGTGGTT 767
AY170327.3 TCCCCAGAGGGTGGCTGTATTTCCAGATATTCTATGTCGTCACACTTATTGCCCTTTTCTCAAACTTCTACATTCAGACTTACAAGAAACACCTTGTTTCACAAAAGAAG 878
FJ237531.1 TCCCCAGAGGCTGGCTGTTTTTCCAAATATTCTATATGGCCTCGCTTATTGCCTTTTTCTCAAACTTCTACATTCAGACTTACAAGAAACACCGTGTTTCACAAA---AG 874
AY170327.3 GAGTGTCATCAGAATGGCTCTGTTGCTTCATTGAATGGCCATGTGAATGGGGTGACACCCACGGAAACCATTACACACAGGAAAGTGAGGGGGGACTGAAGCTTGAATAC 988
FJ237531.1 GAGTATCACCAGAATGGCTCTGTTGATTCACTGAATGGCCATGCAAATGGGGTGACACCCACGGAAACCATTACACACAGGAAAGTGCGGGTGGACTGAAGCTTGAATAA 984
AY170327.3 TGTTACAATCCTAACTGTAGGACATATCTACT--GTATGTAATGTCGCTAGGAGAGATGGGATAATACATTTCTTCATGAGGATTACTAATGAAGAAATGTATTAGAAGA 1096
FJ237531.1 CCTCACAATCCTAACTTTTGTGCATATCTACTATGTATGTAATATTGCTAGGAGAGATG------T-------------AGGATTTCTTATAAAGAAATGTATTAGAAGA 1075
AY170327.3 GTGTAAGCTAGTTAATTCACAAAATACAAGA---CAGGAATCTCTTTCAGATTAACATAACATTTTGCACACCGCAACCTGTTATTATTCATATTGAATCAAAGTTTATT 1203
FJ237531.1 GCATA-GCTAGTTTATTCACAATATACAAAAAGCCATGTTTATCTGAAAGAGATTCCTAACGTTTTGCACACCGCAGCCTGTTATTATTCATATTGAATCGAAGTTCTTT 1184
elovl5b Forward
elovl5a Forward
elovl5a
elovl5b
AY170327.3 AGAGTTTCTAAACAAGCATAGCATAGGGAGTACACTATAGCACAGTAACCCCAGAGACCCACTTCCTTTTAATGCTGCACAAGATGTTCAACTTTGTCTGTCTTCAGTGT 1313
FJ237531.1 ACAGTTAAGTAACAAGGATAGCAGAGGGAGCACA-----GCACAG---CCCCAGAGACCCACTTCAGTTTAATGCTGCACAAGATCTTCGACTATGTGC---T--GGTGG 1281
elovl5b Reverse
elovl5a Reverse
AY170327.3 GGTGGGGACAAAGCTTTAATCAACCTTGACCCATACAGAAATAATAACTTATTTTCTATGTTTTAATTCACAACATATGCACTTGTCTACAGCGCT----TGT-CCATGAG 1418
FJ237531.1 GACATGCGCTTTA-TGTAAGCAACCTTGACCCAAACAGCAATAACAA--TGTTTTCCATGTTTTAATTCACAACATATGCACTTGTCTACATGCTAGATTGTGTCCTGAT 1388
AY170327.3 GGAAAAAAT--------ATATATTTTGGGGATTCTGTTTTGTAAAAGGTACTATATCTGTTTTTTTTTCTCAATATATATTTTATTTGACCACAAGGTCATACAATCATT 1520
FJ237531.1 TGGACAAATCAGGACCAATCTATTTTGGGGATTCCCTTGCGTAGAGACAAGGCTGTCAGTGTCAAAT-----GTGTCCACTGAGTAAATTTAGAGAGGTTCACCTTCATT 1493
AY170327.3 TATAAGTATGCAAAAGAGTTGCTTCAAAAAAAATGCATTAAAA----TACATAAGCTGTACAATATCAACATAAATAATCAGATACTGATTTGTGTTCCAAATGGCTTTG 1626
FJ237531.1 -GTTTGCATTGTAAAGTTTAGTTTAAAACCAGTTGCAATAATAGATTTGTCTATATTGT-CTGTATCAGTTAAACAGGTTTGAAAAGTATTTGGCCATCAAATGGCACTG 1601
AY170327.3 CATACTAGAAAGGAAAGAGAAAAAACAAACAAGATTGTCACTTATGGCTGAGCATTATTACCTAACATACCAGTAATAGATAGTGATCTTTTTATTTTTTACACCATGTG 1736
FJ237531.1 ---ATCAGAACAATATGTGAACAA-TAAATACTATAA--ATTCACAAAAAAAAAA 1650
AY170327.3 TCCGTATCATTCCCCACCCTCCCTTCCCAATGTTCCTCAAGGATTTTCTTTTCTTTGTATCCTCTTATTGAGGTTCCCGTCATGAGTGTATATACACACTAGTACAAATCT 1846
FJ237531.1 1650
AY170327.3 AAACTCAGGTACATAATCAATCTGACACCGGTTTCGGTTTCAACAAAAGAAAGAAAAGTTTGCCATGTCTTATAAAATAAAGTTGTGGATCCAAAAAAAAAAAAAAAAA 1955
FJ237531.1 1650