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Expression of genes involved in regulation of polyunsaturated fatty acid metabolism in liver of Atlantic salmon (*Salmo salar*) undergoing parr-smolt transformation

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ABBREVIATIONS

ACO	Acyl-CoA oxidase
ARA	Arachidonic acid
Cas9	CRISPR-associated protein 9
cDNA	Complementary DNA
CPP	Cell-penetrating peptide
CRISPR	Clustered regularly interspaced short palindromic repeat
DHA	Docosahexaenoic acid
DMSO	Dimethyl sulfoxide
DPA	Docosapentaenoic acid
DPBS	Dulbecco's phosphate-buffered saline
DSB	DNA double-stranded break
DTT	1,4-Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ELOVL	Very long chain fatty acyl elongase
EMEM	Eagle’s Minimum Essential Medium
EPA	Eicosapentaenoic acid
FA	Fatty acid
FAS	Fatty acid synthetase
FAT	Fatty acid translocase
FATP	Fatty acid transport protein
FBS	Fetal bovine serum
gRNA	Guide RNA

HDR	Homology-directed repair
LA	Linoleic acid
LC-PUFA	Long-chain polyunsaturated fatty acid
L-FABP	Liver cytosolic fatty acid-binding protein
LNA	α-Linoleic acid
miRISC	microRNA-induced silencing complex
ncRNA	Non-coding RNA
NHEJ	Nonhomologous end-joining
NLS	Nuclear localization signal
PAM	Protospacer adjacent motif
PPAR	Peroxisome proliferator-activated receptors
RNP	Ribonucleoprotein
RXRα	Retinoid X receptor alpha
SREBP	Sterol regulatory element binding protein

ABSTRACT

Purposes

The primary aim of this thesis was to study if differences in nutritional and environmental factors influence the regulation of n-3 LC-PUFA synthesis in liver of Atlantic salmon (big smolts) undergoing parr-smolt transformation.

A further aim was to use the cell line RTH-149 to establish a protocol for gene knockout of Δ6 desaturase isoform Δ*6fad_b*.

Methods

The experimental fish were fed a commercial diet until the start of the trial (30 October 2012), and thereafter fed a diet with an oil fraction consisting of only 10% fish oil (FO) and 90% vegetable oil (VO) until the end of the trial. The fish were kept under full light (24 hours per day), except in a period from 20 November 2012 to 16 January 2013, when the light duration was gradually reduced from 24 hours to 12 hours in order to delay the smoltification process in the fish so that a large smolt could be produced in freshwater. Thereafter the fish were subjected to 24 hours light again to prepare it for final smoltification and transfer to seawater in February at approximately 400 gram. The expression of the selected genes (including Δ5 and Δ6 desaturases, transcription factors PPARα and SREBPS, and microRNAs which are related to lipid metabolism) was determined at four time points (30 October 2012, 27 November 2012, 26 February 2013 and 12 March 2013) during this fish trial.

Cell line RTH-149 were transfected at a confluence of approximately 70% by CRISPR-Cas9 system with either sgRNA1, sgRNA2, sgRNA1/2 (a combination of sgRNA1 and sgRNA2, both sgRNA1 and sgRNA2 are targeting gene Δ*6fad_b* but with different cleavage sites) or sgRNA of control random-oligo. The differently transfected cells were further split for time-course study. The expression of the protein coding genes was determined at three selected time points during a 24-hour experiment with an interval of eight hours (i.e. at 8:00, 16:00 and 0:00).

Results

The expression of Δ*5fad* and Δ*6fad_a* increased slightly up to the seawater transfer, and then decreased after seawater transfer. The expressions of these genes in March were almost returned to their starting levels of expression in October. On the contrary, the expression of Δ*6fad_b* and Δ*6fad_c* showed a similar pattern of expression from October to February, but the opposite pattern to Δ*5fad* and Δ*6fad_a* with an increased expression after seawater transfer. The expression of the transcription factors *pparα, srebp-1* and *srebp-2* dropped from October to February and thereafter increased prior to seawater transfer, after which the expression levels of *pparα* and *srebp-1* again significantly decreased after seawater transfer except *srebp-2* which did not change in seawater. The expressions of *mir-122-5p* dropped from October to November; the expression increased prior to seawater transfer and thereafter dropped again in seawater. The gene expression of *mir-30c-3p, mir-8163-3p, mir-27a-3p, mir-15a-5p, mir-429-3p, mir-33a-3p* and *mir-145-3p* showed a similar pattern prior to seawater transfer, but the expression did not change after transfer to seawater.

There were reduced expression levels of Δ*6fad_b* 24 hours after transfection but together with a restrained growth rate and a low viability of the cell cultures. There were relatively moderate and few significant differences in the expression of genes involved in lipid metabolism between the three time points measured during the 24 hour duration of experiment in the RTH cell line. Further, we disclosed a potential positive relationship between Δ*6fad_a*, Δ*6fad_b, elovl2a, elovl2b, elovl5a* and *aco*, and a potential compensatory response of gene Δ*5fad* to the silencing of gene Δ*6fad_b.*

Conclusions

This present study has shown that both nutritional and environmental factors influence expression of genes involved in regulation of LC-PUFA in liver of Atlantic salmon undergoing parr-smolt transformation. This is the first time that transcription factors and microRNAs were involved in a dynamic regulation of the omega-3 metabolic pathway under different environmental conditions at different life stages.

Key words

Smoltification, PUFA, desaturase, peroxisome proliferator activated receptor, sterol regulatory element binding protein, microRNA, cell line, transfection, CRISPR-Cas

1 Introduction

1.1 Parr-smolt transformation (smoltification)

Atlantic salmon (*Salmo salar*) is an anadromous fish, which spend the first 1-2 years of its life cycle in freshwater and thereafter migrate to the sea. They return to freshwater to spawn after spending 1-3 years in sea. Prior to, or accompanying the sea migration, the salmonid juveniles (parr) are shown to undergo a spectrum of simultaneous changes in morphology, behavior and physiology (Table 1.1), which is known as parr-smolt transformation or smoltification (Folmar and Dickhoff, 1980; Wedemeyer et al., 1980).

Table 1.1 Developmental changes occurring during the parr-smolt transformation (Reviewed by Wedemeyer et al., 1980)

Physiological characteristics	Level in smolts compared with parr
Body silvering. fin margin blackening	Increases
Hypoosmotic regulatory capability	Increases
Salinity tolerance and preference	Increases
Weight per unit length (condition factor)	Decreases
Growth rate	Increases
Body total lipid content	Decreases
Oxygen consumption	Increases
Ammonia production	Increases
Liver glycogen	Decreases
Blood glucose	Increases
Endocrine activity Thyroid (T4) Interrenal Pituitary growth hormone	Increases
Gill microsome, Na +, K +-ATPase enzyme activity	Increases
Ability to grow well in full strength (35%) sea-water	Increases
Buoyancy (swim bladder, Atlantic salmon)	Increases
Migratory behavior	Increases

1.1.1 Morphological changes

Among the many changes in morphology, the coloration and body shape are the most dramatic characteristics. Usually, freshwater parr can be readily identified by the parr marks that are darkly pigmented bar-shaped marks caused by the accumulation of melanin on skin. During smoltification, the parr marks fade out

as guanine and hypoxanthine gradually deposit in the skin and scales to produce a characteristic silver appearance of the seawater smolt (Wedemeyer et al., 1980). Another coloration been noted is the caudal and pectoral fin margins become black. Together with the external body silvering and fin margin blackening, the body form and condition index change during the smolting process. The condition index reflect the weight-length relationship. Wild smolt usually weigh less per length than parr do, that is, the smoltification result in a more slender streamlined body form of smolt (Wedemeyer et al., 1980).

1.1.2 Physiological changes

Perhaps the most significant physiological process of smoltification is the increased osmoregulatory ability that preadapts the fish for living in seawater while they are still in freshwater (Folmar and Dickhoff, 1980). This process is accompanied with a shift pattern of water and ion regulation including a decrease in tissue Na+ and chloride, a decrease in glomerular filtration rate, a dramatic increase in gill Na+,K+-ATPase activity and an increase in both number and activity of the Keys-Witlmer (chloride) cells (Wedemeyer et al., 1980). Although there is a phase shift in the distribution of body water upon entry into sea water, total body water remains constant (Folmar and Dickhoff, 1980).

The salinity tolerance increases with fish size until smolting occurs and generally is thought to coincide with migratory behavior. However, if the smolts are prevented from entering seawater, this osmoregulatory capacity then decreases despite of continued growth. This phenomenon is designated as desmoltification or parr-reversion (Wedemeyer et al., 1980).

Body composition also changes during smoltification. Studies with steelhead trout (*Salmo gairdnerii*) have shown that the fatty acid composition of parr was characterized by relatively high proportions of saturated fatty acids and by relatively low proportions of polyunsaturated fatty acids. In contrast, smolts were characterized by relatively low proportions of saturated fatty acids and by relatively high proportions of long-chain polyunsaturated fatty acids (LC-PUFA) (Sheridan et al., 1985). Studies with Atlantic salmon have showed a pre-adaptive increase in hepatocyte fatty acyl desaturation and elongation activities during parr-smolt transformation (Bell et al., 1997; Tocher et al. 2000). Wendt and

Saunders (1973) found both reduced liver glycogen and elevated blood glucose levels in hatchery reared Atlantic salmon during smoltification. At the same time of year, the salmon parr had high liver glycogen levels, low blood glucose (Wedemeyer et al., 1980). During the parr-smolt transformation, a number of endocrine changes have been observed; chiefly, an activation of thyroid, interrenal, and pituitary growth hormone cells (Folmar and Dickhoff, 1980).

1.1.3 Behavioral changes

Juvenile Atlantic salmon (parr) are territorial and live on or near the bottom of the stream, whereas smolts tend to a loosely schooled pattern and swim at the mid of the water column. Saunders (1965) and Pinder and Eales (1969) demonstrated that smolts maintain larger volumes of air in their bladders than do parr, hence, despite the reduced lipid content, smolts are more buoyant than parr. This behavior may play an initiating role in downstream movement (Wedemeyer et al., 1980). Another behavioral change is the preference for increased salinities and downstream migration. It was suggested that both the salinity preference and downstream migration were affected by photoperiod and endocrine activity which is controlled by environmental factors (Wedemeyer et al., 1980).

1.1.4 Regulation of smoltification

The process of smoltification and migration appear to be regulated by both environmental (chiefly photoperiod and temperature) and endogenous factors (critical size). A number of studies have showed that body silvering, fin darkening, salinity tolerance and preference, growth rate, growth hormone cell activity, gill ATPase activity and lipid metabolism can be manipulated by changes of photoperiod (Wedemeyer et al., 1980).

Water temperature chiefly set the threshold and limits of these processes and control the rate of reactions. Elevated water temperatures are usually used to accelerate growth rate and shorten the time needed to produce smolts. However, rearing temperatures also have a strong influence on smoltification as well as desmoltification. Thus, such artificial temperature regimes must be used with caution (Wedemeyer et al., 1980).

The endocrine system is usually thought to be the chemical link between environmental changes and physiological changes in fish. The seasonal growth and migration in parr has corresponding endogenous components which is synchronized with the natural photoperiod cycle (Hoar, 1965). Furthermore, the most important photoperiod cues are the direction and rate of change of the day length, but not the day length per se (Wedemeyer et al., 1980).

The age of parr-smolt transformation in Atlantic salmon is dependent on growth, ranging from 1+ to 7+ in nature (Metcalfe and Thorpe, 1990). In aquaculture, smoltification usually commences in autumn, and is completed in the next spring. Following transfer to seawater, the fish reach harvest size in approximately 18 months in winter (Duston and Saunders, 1995). To stabilize annual production output and shorter production circle, out-of-season smolts have been produced. Underyearling (0+) smolts can be transferred to seawater in autumn and reach harvest size in the next summer (Duston and Saunders, 1995). To produce 0+ smolts in autumn, growth must be accelerated by heating the rearing water, and the fish have to be exposed to appropriate photoperiod regimes to induce the smolting process (Duston and Saunders, 1995).

1.1.5 Big smolts

Atlantic salmon naturally smoltify at a size of 80 to 100 gram, but in this current study, the fish smoltified at approximately 400 gram. The reason why we wished to test large smolt is the fact that the aquaculture industries want to extent the period for the salmon on land in recirculation units or in semi-closed systems and then transfer the fish to sea when they are larger. In this way, they hope to decrease the 10 to 20% mortalities that normally occurs after seawater transfer. The fish will then be larger and hopefully more robust to handle environmental challenges. However, there is very limited knowledge about large smolt production, and also no published articles in this field, which is the reason for this study where the aim is to increase the knowledge about the physiological processes in large smolt production, in particular in relation to the omega-3 metabolic pathway.

1.2 Lipid and fatty acid

Fish lipids are rich in n-3 long chain polyunsaturated fatty acids (n-3 LC-PUFA) that have particularly important roles in animal nutrition, including fish and human nutrition. The predominant saturated fatty acids that occur naturally in animal fats, including fish lipids are 16:0 and 18:0, whereas the main monounsaturated fatty acids are 18:1n-9 and 16:1n-7. In fish, the chief PUFAs to be considered are 20:5n-3 (eicosapentaenoic acid, EPA) and 22:6n-3 (docosahexaenoic acid, DHA) and their metabolic precursor 18:3n-3 (α-linoleic acid, LNA), together with 20:4n-6 (arachidonic acid, ARA) and its metabolic precursor 18:2n-6 (linoleic acid, LA) as the major n-6 PUFAs (Tocher, 2003).

1.2.1 Fatty acid synthesis

The biosynthetic reactions of the new endogenous lipid is termed as lipogenesis. The key pathway in lipogenesis is catalyzed by fatty acid synthetase (FAS) multienzyme complex. The chief products of FAS are the saturated fatty acids 16:0 and 18:0 (Sargent et al., 2002). All known organisms, including fish, are capable to biosynthesize 16:0 and 18:0 de novo, and desaturate them to yield 16:1n-7 and 18:1n-9 respectively (Sargent et al., 2002). The ability of converting the plant-derived C18 PUFAs to health essential C20 and C22 LC-PUFAs varies with species and highly depends on the complement of their fatty acyl desaturases and elongases (Figure 1.1). Δ9 desaturase is found in most animals and plants, whereas Δ12 and Δ15 desaturases generally only present in plants. Therefore, most animals cannot synthesis 18:2n-6 and 18:3n-3 from 18:1n-9, and so it is essential to include 18:2n-6 and 18:3n-3 in their diets (Sargent et al., 2002).

EPA (20:5n-3) is synthesized from 18:3n-3 by desaturation at the Δ6 position, followed by a 2-carbon elongation and another desaturation at Δ5 position. ARA (20:4n-6) is synthesized from 18:2n-6 upon the same enzymatic pattern. The synthesis of DHA (22:6n-3) from EPA has been demonstrated to proceed through two successive chain elongations, another Δ6 desaturation and a peroxisomal β-oxidation chain-shortening reaction. That means the insertion of the last Δ4 ethylene bond in 22:6n-3 does not occur through direct Δ4 desaturation of its immediate precursor 22:5n-3 (Tocher, 2003). Interestingly, some fish including

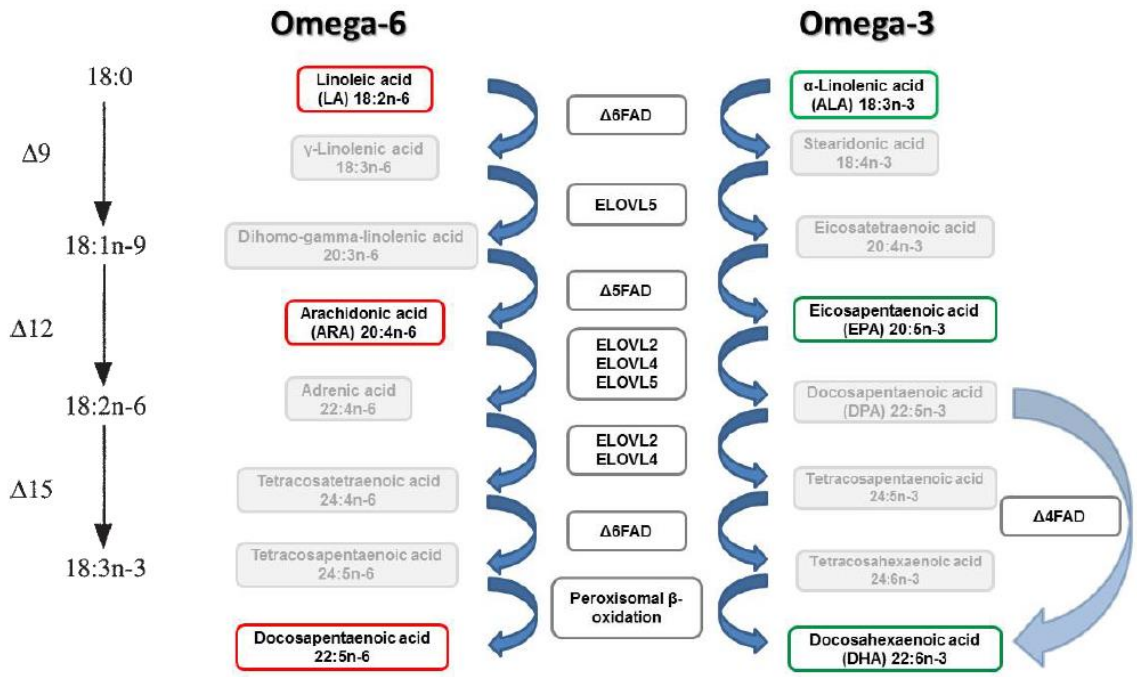


Figure 1.1 Pathways of biosynthesis of C20 and C22 LC-PUFA from n-3 and n-6 C18 precursors (Modified from Vestergeren, 2014)

Siganus canaliculatus (Li et al., 2010) and Solea senegalensis (Morais et al., 2012) recently have been found to synthesize 22:6n-3 via directly Δ4 desaturation from 22:5n-3. The mean end product of 18:3n-3 is DHA (22:6n-3), whereas the mean end product of 18:2n-6 is ARA (20:4n-6) (Tocher, 2003). The enzyme affinity of desaturases is higher for n-3 series than for n-6 series, and the affinity of both n-3 and n-6 series is higher than that of n-9 series (Tocher, 2003).

1.2.2 Defects in marine fish

True marine fish cannot produce DHA at a physiologically significant rate, and this has been attributed to the lack of Δ5 desaturase activity and/or elongase activity. For instance, gilthead sea bream cell line shown active C18 to C20 and C20 to C22 elongase activities but with a very low Δ5 desaturase activity (Mourente and Tocher, 1994; Tocher and Ghioni, 1999). In contrast, study with turbot cell line and Atlantic salmon cell line has shown that the Δ5 desaturase activity of turbot was higher than that of Atlantic salmon, but their C18 to C20 elongase activity was relatively lower (Ghioni et al., 1999). Thus, the limited ability of these LC-PUFAs synthesis in a particular marine fish is likely due to the insufficiently expression of one or more of these required genes, rather than

the complete absence of the required genes (Tocher, 2003), that makes 20:5n-3 and/or 22:6n-3 the dietary essential fatty acids (EFA) for them.

In turn, the activities of these elongases and desaturases are dependent on the extent to the existence of C20 and C22 in their natural diets. The fact is 20:5n-3 and 22:6n-3 are abundant in marine environment, originating mainly in phytoplankton and transmitting to fish via zooplankton. Thus endogenous de novo biosynthesis of these fatty acids in many marine fish is not necessary, as a result, these conversions including chain elongations are likely to be repressed. In contrast, 20:5n-3 and 22:6n-3 are not well supplied in the natural prey of many freshwater fish, therefore, the conversions of 18:3n-3 to 20:5n-3 and 22:6n-3 are necessary (Tocher, 2003).

1.2.3 Roles of EFA and deficiency symptoms

EFA can serve as important sources of cellular energy like all other fatty acids. The extent to which fatty acid is utilized for energy is primarily dependent upon its dietary concentration (Tocher, 2003). However, despite dietary concentration, DHA tends to be conserved chiefly due to its structure which is poor for β-oxidation (Sargent et al. 2002).

PUFA, including EFA, play significant structural and functional roles in cellular membrane as they are essential constituents of phospholipids which are fundamental components of lipid bilayers (Tocher, 2010). Particularly, DHA has important role in neural membranes (Feller, 2008).

Furthermore, PUFA, especially C20 PUFA (ARA and EPA), can be converted to eicosanoids by dioxygenase-catalyzed oxidation. Eicosanoids are highly bioactive autocrine hormones produced in almost all tissues (Tocher, 2010). They have unique metabolic and physiological regulatory roles in immune and inflammatory condition, cardiovascular disease, neurological health and reproduction (Schmitz and Ecker, 2008).

In fish, the deficiency of EFA leads to increased mortality and reduced growth in general. However, a wide range of deficiency pathologies have been noted to the date including myocarditis, pale/swollen (fatty) liver, intestinal steatosis, fin

erosion, bleeding from gills, lordosis, reduced reproductive potential and shock syndrome (reviewed by Tocher, 2010).

Study with Atlantic salmon showed that dietary n-3 PUFAs enhanced the growth and survival rate in fry, while n-6 PUFA seemed to have no such beneficial effects (Ruyter et al., 2000a). Furthermore, the beneficial effects occurred at lower doses with the mixture of 20:5n-3 and 22:6n-3, but the same doses of 18:3n-3 showed no such effects (Ruyter et al., 2000a). In a time-course study, Atlantic salmon with the diet, devoid of n-3 and n-6 fatty acids for 4 months, showed substantial changes in the fatty acid composition of liver and blood, while the fish did not appear to develop a severe deficiency symptom of essential fatty acids during the experimental period (Ruyter et al., 2000b).

1.2.4 Desaturases

In recent years, several fish desaturases have been isolated and functionally characterized. Zebrafish (Hastings et al., 2001), possesses bifunctional desaturase which shows both Δ6 and Δ5 activity, whereas that of common carp, rainbow trout, gilthead seabream, and turbot show predominantly Δ6 activity (Zheng et al., 2005). Atlantic salmon is the first fish species to be shown to possess separate and distinct genes for Δ6 and Δ5 desaturases, as is the case in humans, and possibly in mammals in general (Zheng et al., 2005).

Four genes encoding putative fatty acyl desaturases proteins (Fad) have been cloned and characterized in Atlantic salmon: Δ*5fad* (Hastings et al., 2004), Δ*6fad_a* (Zheng et al., 2005), Δ*6fad_b* and Δ*6fad_c* (Monroig et al., 2010). Both *Δ6fad_a* and *Δ6fad_b* were predominantly expressed in intestine, liver and brain, with *Δ6fad_b* also highly expressed in gill, whereas *Δ6fad_c* had predominant expression levels in brain, with lower distribution in all other tissues. Functional characterization of *Δ6fad_b* and *Δ6fad_c* in yeast shown that both genes had only Δ6 desaturase activity and converted 47 and 12% of 18:3n-3 to 18:4n-3, and 25 and 7% of 18:2n-6 to 18:3n-6, respectively (Monroig et al., 2010). Moreover, in fish fed diets with reduced levels of LC-PUFA, significant increased expression of *Δ6fad_a* and *Δ6fad_b* were observed in liver and intestine respectively, whereas, *Δ6fad_c* showed no response. Interestingly, even though *Δ6fad_a* and

Δ6fad_b were both highly expressed in liver and intestine, *Δ6fad_a* showed no nutritional regulation in intestine whereas *Δ6fad_b* showed low regulation level in liver (Monroig et al., 2010).

1.2.5 Elongases

Very long chain fatty acyl elongases (ELOVL) are membrane-bound proteins responsible for the condensation of activated fatty acids (FAs) with malonyl-CoA in the FA elongation pathway. They belong to a gene family that consists of seven members in mice and humans, which differ in FA substrate specificity and have differing spatial and temporal expression patterns (Jakobsson et al., 2006). Of these seven ELOVL enzymes, ELOVL2 and ELOVL5 have been demonstrated to have a substrate preference for PUFA. In mammals, ELOVL2 has greatest activity in the elongation of C20 and C22 but low or, in the case of human, no activity towards C18 PUFA. In contrast, mammalian ELOVL5 is very active towards C18 PUFA but does not appear to have the capacity to elongate beyond C22 (Leonard et al., 2002).

Fish ELOVL cDNAs have been cloned and functionally characterized from a number of species: the freshwater species zebra fish, common carp, and tilapia; the salmonids, Atlantic salmon and rainbow trout; and the marine species cod, turbot, and sea bream (Agaba et al., 2004, 2005; Meyer et al., 2004; Hastings et al., 2004). Phylogenetic analysis groups all these previously described elovl cDNAs into a cluster with greatest similarity to mammalian ELOVL5. All the fish ELOVL5 cDNAs tested lengthened monounsaturated FA and n-3 and n-6 PUFA with chain lengths from C18 to C22, with residual C22–C24 activity (Agaba et al., 2004, 2005; Hastings et al., 2004). A rainbow trout Elovl5 has also been described with C18 to C22 but no C22–C24 activity (Meyer et al., 2004).

Three elongases have been cloned from Atlantic salmon: *elovl5a*, *elovl5b* and an *elovl2-like* elongase (Hastings et al., 2001, Morais et al., 2009). This is the first *elovl2-like* elongase to be cloned and functionally characterized in fish. In general, *elovl5a, elovl5b,* and *elovl2* are expressed predominately in intestine and liver that are the major sites of lipid synthesis and distribution (Morais et al., 2009). The salmon ELOVL2 protein showed similar low activity towards C18 and high

activity towards C20 and C22 in comparison to mouse ELOVL2 (Morais et al., 2009).

ELOVL4 has recently been shown to be a critical enzyme in biosynthesis of both saturated and polyunsaturated very long-chain fatty acids up to C36 (Carmona-Antoñanzas et al., 2011). ELOVL4 cDNAs have been isolated and characterized from zebrafish, cobia and Atlantic salmon (Carmona-Antoñanzas et al., 2011). Elovl4 are predominately expressed in retina, gonads and brain. ELOVL4 has been shown to elongate C20 and C22 PUFA and to be able to convert EPA and DPA (docosapentaenoic acid; 22:5n-3) to 24:5n-3 the intermediate substrate for DHA biosynthesis (Carmona-Antoñanzas et al., 2011).

Biosynthesis of DHA in vertebrate requires elongation from C22 to a C24 PUFA intermediate, followed by a peroxisomal β-oxidation chain-shortening step (Sprecher et al., 2002). Previous studies had suggested that DHA synthesis in rainbow trout (a closely related salmonid) also proceeds via C24 intermediates (Buzzi et al., 1997). This is consist with the indirectly insertion of the Δ4 double bond. These elongases characterized in Atlantic salmon, together with the previously characterized Δ5 and Δ6 fatty acyl desaturases, indicate the ability to biosynthesize LC-PUFA, including DHA, in Atlantic salmon. Therefore, fish that lack elovl2 would be restricted in their ability to produce these essential fatty acids, particularly DHA.

1.2.6 β-Oxidation

The β-oxidation of fatty acids (FAs) takes place in both mitochondria and peroxisomes. Very long chain FAs, which are too long to be processed directly in the mitochondria, need to undergo initial oxidation in peroxisomes. Peroxisomal acyl-CoA oxidase (ACO) is responsible for peroxisomal chain shortening of very long chain fatty acids in mammal and postulated to catalyze the final step in DHA biosynthesis (Tohcer, 2003). Study with Atlantic salmon has shown that the peroxisomal acyl-CoA oxidase was slightly increased as a response to fibrates treatment (Ruyter et al., 1997).

1.3 Transcriptional regulation

Regulation of lipid metabolism is complex and controlled by several transcription factors and nuclear receptors, including peroxisome proliferator-activated receptors (PPARs) and sterol regulatory element binding proteins (SREBPs).

1.3.1 PPARs

PPARs belong to the nuclear hormone receptor family. They can bind and be activated by a group of small hydrophobic molecules including steroid hormones, retinoids, unsaturated fatty acids and certain eicosanoids. In mammals, three distinct isoforms have been characterized and termed as PPARα, PPARβ (or δ), and PPARγ. PPARα is predominantly expressed in mammalian liver and regulates genes encoding acyl-CoA oxidase, bifunctional enzyme, thiolase and long-chain fatty acid acyl-CoA synthetase. Furthermore, PPARs are involved in fatty acid transportation and uptake by inducing genes such as fatty acid transport protein (FATP), fatty acid translocase (FAT/CD36), and liver cytosolic fatty acid-binding protein (L-FABP) (reviewed by Tocher, 2003).

The first PPAR gene of fish was identified in Atlantic salmon as a homologue of the mammalian PPARγ isoform (Ruyter et al., 1997). Since then, PPARs have been further characterized in plaice (Leaver et al., 1998), Japanese pufferfish (Maglich et al., 2003), sea bass (Boukouvala et al., 2004) and gilthead sea bream (Leaver et al., 2005) which are homologues of the three PPAR isoforms in mammal (Kleveland et al., 2006). In particular, PPARα and PPARβ have similar ligands and functions to their mammalian homologues, while PPARγ present some functional differences (Boukouvala et al., 2004; Leaver et al., 2005).

1.3.2 SREBPs

SREBPs belong to a family of transcription factors that regulate genes involved in lipid homeostasis especially specific to fatty acid and cholesterol synthesis. Three subtypes have been identified in different mammalian species: SREBP-1a, SREBP-1c and SREBP-2. Specifically, SREBP-1a and 1c are produced from a single gene (SREBF-1), but through different transcription start sites and differ in their first exon (exon 1a and exon 1c). SREBP-2 is produced from a separate gene (SREBF-2) (Eberl éet al., 2004). SREBP-1c appear to be implicated in fatty

acid synthesis and glucose metabolism, which is induced by insulin. SREBP-2 is involved specifically in cholesterol synthesis, whereas SREBP-1a has shown to participate in both cholesterol and fatty acid biosynthetic processes (Eberl é et al., 2004).

However, there is no alternatively spliced isoforms of SREBP-1 have been found in Atlantic salmon. The salmon SREBP-1 sequence was more similar to the mammalian SREBP-1a isoform (Minghetti et al., 2011). Studies with Atlantic salmon have shown that the expression of SREBP-1 was increased by cholesterol and decreased by EPA and DHA supplement (Minghetti et al., 2011; Morais et al., 2011). This is consistent with the finding in mammal (Davidson, 2006).

1.4 Posttranscriptional regulation (microRNA/miRNA)

MicroRNAs (miRNAs) are a ubiquitous class of endogenous non-protein coding short RNA molecules which regulate gene expression as part of the miRNA-induced silencing complex (miRISC) at the post-transcriptional level. They bind to the 3'-UTR region of the target mRNAs in a sequence specific manner, resulting in target mRNA degradation or translation inhabitation (He and Hannon, 2004; Bartel, 2004).

1.4.1 MiRNA biogenesis

Briefly, the biogenesis of miRNA begins in the nucleus (Figure 1.2). The miRNA genes are initially transcribed by RNA polymerase II (or polymerase III for some miRNAs) to generate primary miRNAs (pri-miRNAs) which is ranging from hundreds to thousands ribonucleotides (Winter et al., 2009). The pri-miRNAs are further processed by Drosha and other associated proteins to generate shorter precursor miRNAs (pre-miRNAs) approximately 70 nt in length with characteristic hairpin structures (Winter et al., 2009). After transportation from the nucleus to the cytoplasm by Exportin-5, the pre-miRNAs are cleaved by Dicer and other RNA-binding proteins to yield approximately 22-nt-long miRNA duplex. The double-stranded miRNA duplex is then separated and one strand is selected as the mature miRNA and incorporated to form miRISC, whereas most of the other strand is degraded (Winter et al., 2009). Eventually, miRISC binds to 3'-UTR of an mRNA for downregulation of gene expression or it is transported

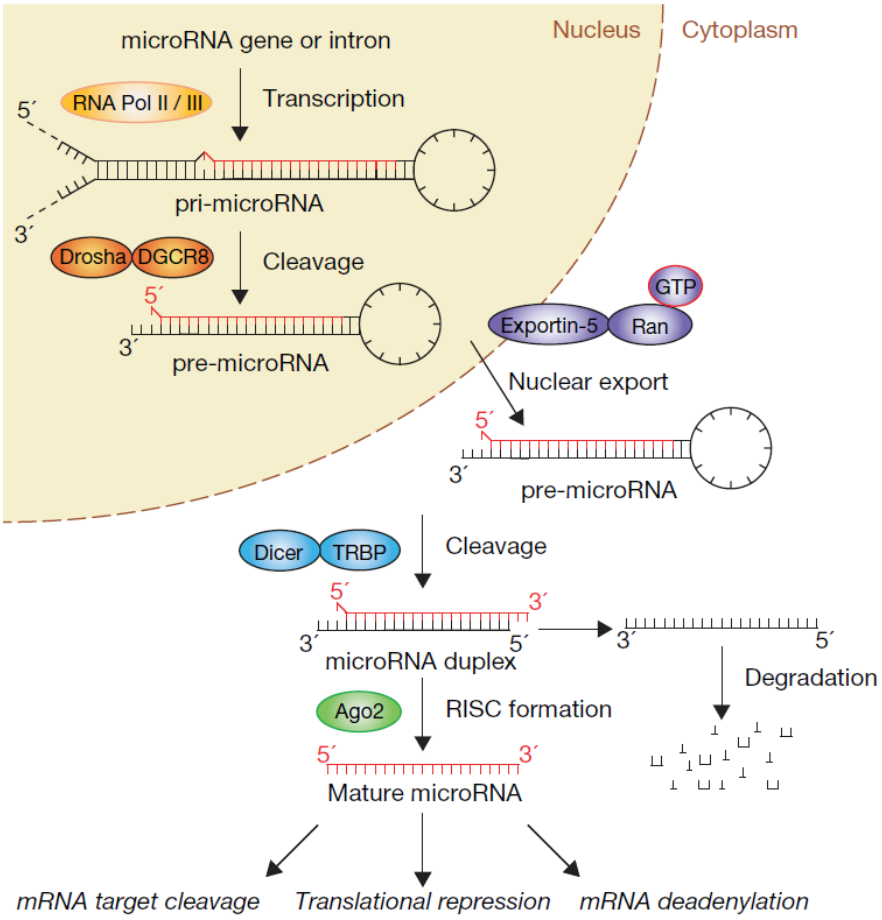


Figure 1.2 The 'linear' canonical pathway of microRNA processing (Winter et al., 2009)

to the nucleus and binds to ncRNAs to repress their processing or interfere with their functions (Winter et al., 2009).

Mature miRNAs are designated by a number in the order of discovery and suffixed with "-5p" or "-3p" to identify their previous location on the pre-miRNA strands (Ambros et al., 2003). The expression of miRNA is often conserved to specific tissues and developmental stages (Bekaert et al., 2013). They are involved in multiple biological processes including growth, developmental timing, stem cell division and apoptosis (He and Hannon, 2004; Bartel, 2004). Study in teleost fish have revealed the important roles of miRNAs in development, organogenesis, tissue differentiation, growth, reproduction, endocrine system and responses to environmental stimuli (Bizuayehu and Babiak, 2014). Recently, a total of 180 distinct mature miRNAs belonging to 106 families of evolutionary

conserved miRNAs, and 13 distincet novel mature miRNAs were discovered and characterized in Atlantic salmon (*Salmo salar*) (Andreassen et al., 2013).

1.4.2 Role of miRNA in lipid metabolism

Emerging evidences have demonstrated that miRNAs are key regulators of lipid synthesis, fatty acid oxidation and lipoprotein formation and secretion, but little has been known in fish.

miR-122 is a liver-enriched miRNA, well-known as the first identified miRNA to regulate lipid metabolism (Yang et al., 2015). Positive relationships between miR-122 and fatty acid synthase (FAS) protein level have been identified in the liver of rainbow trout and mice (Mennigen et al., 2014a; Tsai et al., 2012). Inhibition of miR-122 in mice resulted in increased hepatic fatty acid oxidation and a decrease in hepatic fatty acid and cholesterol synthesis rates (Esau et al., 2006). Inhibition of miR-122 in liver of rainbow trout resulted in decreased postprandial serum triglyceride concentration and conversely cause postprandial hyperglycemia (Mennigen et al., 2014a). In rainbow trout, the abundance of miR-122 was dramatically suppressed by a single high-fat meal (Mennigen et al., 2014b). Furthermore, Laudadio et al. (2012) have showed that miR-122 regulates timing of hepatocyte differentiation in developing zebrafish.

miR-33 is another extensively investigated miRNA regulating genes involved in lipid and cholesterol metabolism. Recent studies have identified miR-33 to be part of the intronic sequences of the sterol regulatory element-binding proteins (SREBPs). Specifically, isoform miR-33a and miR-33b are located within SREBP-2 and SREBP-1 respectively, and is concurrently expressed with its host gene in mammals and teleost fish (Najafi-Shoushtari et al., 2010; Davalos et al., 2011; Mennigen et al., 2014a). At least in mammals, miR-33 act in concert with SREBP to regulate lipid metabolism (Davalos et al., 2011; Mennigen, 2015). Overexpression of miR-33a and -b in human hepatic cell line reduced both fatty acid oxidation and insulin signaling, whereas inhibition of miR-33a and -b increased these two metabolic pathways (Dávalos et al., 2011).

In differentiating human adipocytes, the inhibition of miR-143 significantly inhibited adipocyte differentiation, suggesting the adipocyte differentiation-

promoting role of miR-143 in adipocyte tissue (Easu et al., 2004). In rainbow trout, alevins transitioning from endogenous to exogenous feeding, the expression of miR-143 increases accompanied with the decrease of its predicted target gene, lipolytic gene abhd5, suggesting a role in adipocyte differentiation (Mennigen et al., 2013). This result is consistent with the previous finding in human adipocytes.

miR-17 has been reported to be involved in the regulation of LC-PUFA biosynthesis in rabbitfish (*Siganus canaliculatus*) liver by targeting Δ4 fatty acyl desaturase. However, rabbitfish is among the minority of fish that have been studied to synthesis 22:6n-3 (DHA) via directly Δ4 desaturation from 22:5n-3 (Zhang et al., 2014a).

Subsequent mapping to the partially sequenced genome has revealed that ssa-mir-8163 was located in an intron of a transferrin gene in Atlantic salmon, which suggests a potential unique role for ssa-mir-8163 in iron metabolism in salmon (Andreassen et al., 2013). It was also shown with high expression in liver tissue (Andreassen et al., 2013).

Retinoid X receptor alpha (RXRα) was demonstrated to be the target of both miR-27a and miR-27b and regulate fat metabolism in rat (Ji et al., 2009). Overexpression of miR-27a accelerated adipolysis by releasing significantly more glycerol and free fatty acids in porcine adipocytes (Wang et al., 2011). Furthermore, miR-27a repressed the expression of many lipid metabolism-related genes, including fatty acid synthase (FAS), SREBP1, SREBP2, PPARα, and PPARγ (Shirasaki et al., 2013). Repression of miR-27a increased cellular lipid content in human hepatoma cells (Shirasaki et al., 2013). Study with human liver cells shown that the PPARα protein level were not correlated with PPARα mRNA level, but inversely correlated with miR-21 levels, accompanied with miR-27b levels (Kida et al., 2011). However, the regulatory roles of miR-27 and miR-21 in fish are not known.

Study with blunt snout bream (*Megalobrama amblycephala*) has confirmed the up-regulation of miR-30c and miR-30e and the down-regulation of miR-145 and miR-15a in response to the high-fat diet (Zhang et al., 2014b), indicating the potential roles of miR-30c, miR-30e, miR-145, miR-15a in lipid metabolism. Zaragosi et al. (2011) have demonstrated that the miR-30 family act as a key

regulator of human adipogenesis. Interestingly, in Nile tilapia (*Oreochromis niloticus*), miR-30c and miR-429 are implicated in osmotic stress regulation (Yan et al., 2012a; Yan et al., 2012b).

Recent studies have shown that miR-429, miR-200a/b, miR-30c and miR-10b are implicated in regulation of osmotic stress in fish (Flynt et al., 2009; Yan et al., 2012b). The roles of miRNAs in osmoregulation was disclosed through modulating genes that involved in regulation of membrane dynamics and osmoregulatory signaling such as Na+/H+ exchanger regulatory factor 1 in zebrafish (Flynt et al., 2009) and osmotic stress transcriptional factor 1 in Nile tilapia (Yan et al., 2012b).

Based on the finding in literatures, miR-122, miR-30c, miR-429, miR-8163, miR-27a, miR-33a, miR-145, miR-15a, miR-200a, miR-10b, miR-143, miR-17 and miR-21a were included for analysis in our study. We also included reference genes miR-25 (Johansen and Andreassen, 2014) and miR-92a (Liang et al., 2007) for normalization of real-time qPCR results.

1.5 Gene knockout

1.5.1 CRISPR-Cas9

Targeted genome editing using customized nucleases provides an efficient method for inducing precise deletions, insertions and sequence changes of DNA in a broad range of cell types and organism. These nucleases create DNA double-stranded breaks (DSBs) at a desired location of the genome. Nuclease-induced DSBs can be repaired through nonhomologous end-joining (NHEJ) or homology-directed repair (HDR); these two reparative pathway are operated in nearly all cell types and organism (Sander and Joung, 2014). Recently, a platform based on a bacterial CRISPR-associated protein 9 nuclease (Cas9) from Streptococcus pyogenes has been developed.

The CRISPR (clustered regularly interspaced short palindromic repeat) system is a prokaryotic adaptive immune mechanisms used by many bacteria to protect themselves from foreign nucleic acids, such as viruses or plasmids. In the type II CRISPR system, the bacterial host genome incorporates segments of invading DNA (known as “protospacer”) between CRISPR repeat sequences (Figure 1.3).

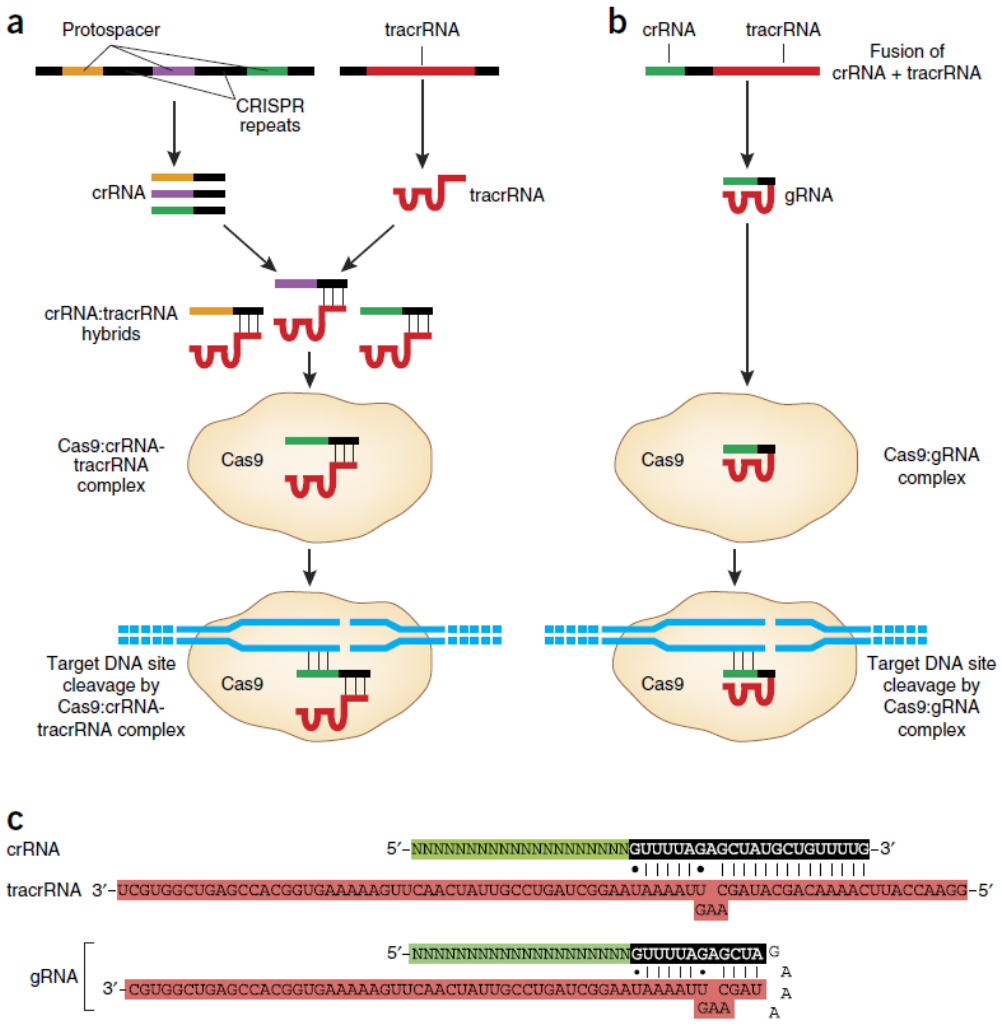


Figure 1.3 Naturally occurring and engineered CRISPR-Cas systems. (a) Naturally occurring CRISPR systems; (b) The most widely used engineered CRISPR-Cas system; (c) Example sequences of a crRNA-tracrRNA hybrid and a gRNA. (Sander and Joung, 2014)

The repeat-spacer array is transcribed as a long precursor and processed into CRISPR RNA (crRNA). Each crRNA hybridizes with an auxiliary transactivating CRISPR RNA (tracrRNA). These two RNA complex with Cas9 nuclease to specifically bind and cleave the target sequence (protospacer). Essential for cleavage is the protospacer sequences incorporated into the CRISPR locus are next to a short sequence known as protospacer adjacent motif (PAM). In this way, CRISPR system is used to recognize and silence exogenous genetic elements at the DNA level (Sander and Joung, 2014).

The type II CRISPR systems has been adapted for targeted genome editing in a variety of cells and organisms. In the most widely used form, the CRISPR system consists of a Cas9 nuclease and a short non-coding guide RNA (gRNA). The gRNA is made up of a fusion of a crRNA and an auxiliary tracrRNA. Twenty nucleotides at the 5' end of the gRNA direct Cas9 nuclease to a specific target DNA locus via standard RNA-DNA complementarity base pairing rule, and cleave the DNA to create a DSB. By altering the first 20 nt of the gRNA to correspond to the target DNA sequence, Cas9 nuclease can be directed to any DNA sequence (Sander and Joung, 2014).

1.5.2 Delivery of CRISPR/Cas9 system

For efficient genome editing via CRISPR/Cas9 system, the successful delivery of gRNA and Cas9 into cell is crucial. The purified recombinant Streptococcus pyogenes Cas9 nuclease protein harbors a nuclear localization signal (NLS) for targeting to the nucleus (Kim et al., 2014). Cell-penetrating peptides (CPPs), such as Lipofectamine, facilitate the delivery of active proteins into cells (Yu et al., 2016). The delivery of purified Cas9 protein and gRNA complexes (Cas9 RNPs) has gained increasing attention due to the high cleavage efficiency and potentially lower off-target effect compared with plasmid DNA transfection (Kim et al. 2014). Although Cas9 RNPs can be delivered into mammalian cells via electroporation with relatively high efficiency, lipid-mediated transfection remains popular due to ease of use, low cost, and adaptation to high throughput system (Yu et al., 2016).

Major aims of the master thesis:

- The primary aim of this thesis was to study if differences in nutritional and environmental factors influence the regulation of n-3 LC-PUFA synthesis in Atlantic salmon (big smolts) undergoing parr-smolt transformation.
- A further aim was to use the cell line, RTH-149, of rainbow trout, a close related species of Atlantic salmon, to establish a protocol for gene knockout of Δ6 desaturase isoform Δ*6fad_b*.

2 Materials and methods

2.1 Chemicals and equipment

Table 2.1 Chemicals and equipment used in this study

Product	Company	Country
accu-jet® pro Pipette Controller	BrandTech Scientific Inc.	CT, USA
Agilent 2100 Bioanalyzer	Agilent Technologies	CA, USA
Agilent RNA 6000 Nano Kit	Agilent Technologies	CA, USA
Avanti™ J-30I Centrifuge	Beckman Coulter, Inc.	CA, USA
Compressor-cooled Incubator ICP 256	Memmert GmbH + Co. KG	Schwabach, Germany
CRISPR/Cas9 Protein	PNA BIO INC	CA, USA
DMSO (Dimethyl Sulfoxide)	Sigma-Aldrich Co. LLC.	MA, USA
DPBS (Dulbecco's Phosphate-Buffered Salines)	Thermo Fisher Scientific Inc.	MA, USA
Dulbecco's Modified Eagle Medium (DMEM)	Thermo Fisher Scientific Inc.	MA, USA
EMEM (Eagle's Minimum Essential Medium)	ATCC	VA, USA
Eppendorf® Microcentrifuge 5415R	Sigma-Aldrich Co. LLC.	MA, USA
Eppendorf® Microcentrifuge 5424	Sigma-Aldrich Co. LLC.	MA, USA
Eppendorf® Multipette® Xstream	Sigma-Aldrich Co. LLC.	MA, USA
Eppendorf® Research® plus, 0.5-10ul	Sigma-Aldrich Co. LLC.	MA, USA
Ethanol 95-97% (v/v) GPR RECTAPUR®	VWR International, LLC.	PA, USA
Ethanol absolute AnalaR NORMAPUR®	VWR International, LLC.	PA, USA
Falcon™ Cell scraper	Thermo Fisher Scientific Inc.	MA, USA
FBS (Fetal Bovine Serum)	Sigma-Aldrich Co. LLC.	MA, USA
Finnpipette Model 4500 Single Channel Pipette	Thermo Fisher Scientific Inc.	MA, USA
Herasafe™ KS12 Biological Safety Cabinets	Thermo Fisher Scientific Inc.	MA, USA
invitrogen™ Cell Counting Chamber Slides	Thermo Fisher Scientific Inc.	MA, USA
invitrogen™ Countess™ Automated Cell Counter	Thermo Fisher Scientific Inc.	MA, USA
Leibovitz's L-15 Medium	Thermo Fisher Scientific Inc.	MA, USA
LightCycler® 480	Roche Diagnostics	Basel, Switzerland
LightCycler® 480 Multiwell Plate 96, white	Roche Diagnostics	Basel, Switzerland
LightCycler® 480 Sealing Foil	Roche Diagnostics	Basel, Switzerland
LightCycler® 480 SYBR Green I Master	Roche Diagnostics	Basel, Switzerland
Lipofectamine™ CRISPRMAX™ Cas9 Transfection Reagent	Thermo Fisher Scientific Inc.	MA, USA

MEM (Minimum Essential Medium), no glutamine	Thermo Fisher Scientific Inc.	MA, USA
mirVana™ miRNA Isolation Kit, with phenol	Thermo Fisher Scientific Inc.	MA, USA
miScript II RT	QIAGEN	Hilden, Germany
miScript PCR System	QIAGEN	Hilden, Germany
MX 3000P qPCR machine	Stratagene	CA, USA
NanoDrop® ND-1000 Spectrophotometer	Thermo Fisher Scientific Inc.	MA, USA
Nikon Diaphot Microscope	Nikon Corporation	Tokyo, Japan
Nunc™ Cell Culture Treated EasYFlasks™	Thermo Fisher Scientific Inc.	MA, USA
Nunc™ Cell Culture Treated EasYFlasks™ Filter	Thermo Fisher Scientific Inc.	MA, USA
Nunc™ Cell-Culture Treated Multidishes 12-well	Thermo Fisher Scientific Inc.	MA, USA
Nunc™ Cell-Culture Treated Multidishes 6-well	Thermo Fisher Scientific Inc.	MA, USA
Pen Strep (Penicillin Streptomycin)	Sigma-Aldrich Co. LLC.	MA, USA
QIAshredder™	QIAGEN	Hilden, Germany
RNA Clean & Concentrator™-5	Zymo Research Corporation	CA, USA
RNase-Free Water	QIAGEN	Hilden, Germany
RNeasy® Plus Mini Kit	QIAGEN	Hilden, Germany
RTH-149 (ATCC® CRL-1710™)	ATCC	VA, USA
Serological Pipette 10ml	SARSTEDT AG & Co	Nümbrecht, Germany
SuperScript® VILO™ cDNA Synthesis Kit	Thermo Fisher Scientific Inc.	MA, USA
Trypsin-EDTA solution (0.25%)	Sigma-Aldrich Co. LLC.	MA, USA
Veriti® 96-Well Thermal Cycler	Thermo Fisher Scientific Inc.	MA, USA
VWR® CryoCooler / -20°C Mini Cooler	VWR International, LLC.	PA, USA

2.2 Fish, experimental conditions and diets

Parent fish (Atlantic salmon) supplied by SalmoBreed AS were selected on the basis of high and low gene expression of the Δ6 desaturase isoform Δ*6fad_b*. Progeny of the high expression families were used in the present study. The fish trial was carried out at Nofima Sunndalsøra Research Station, Norway, from October 2012 to March 2013. The water temperature was 9-12 ℃ until late February, when it dropped to 6-8 ℃ (Figure 2.1). The oxygen levels were adjusted to above 80% saturation.

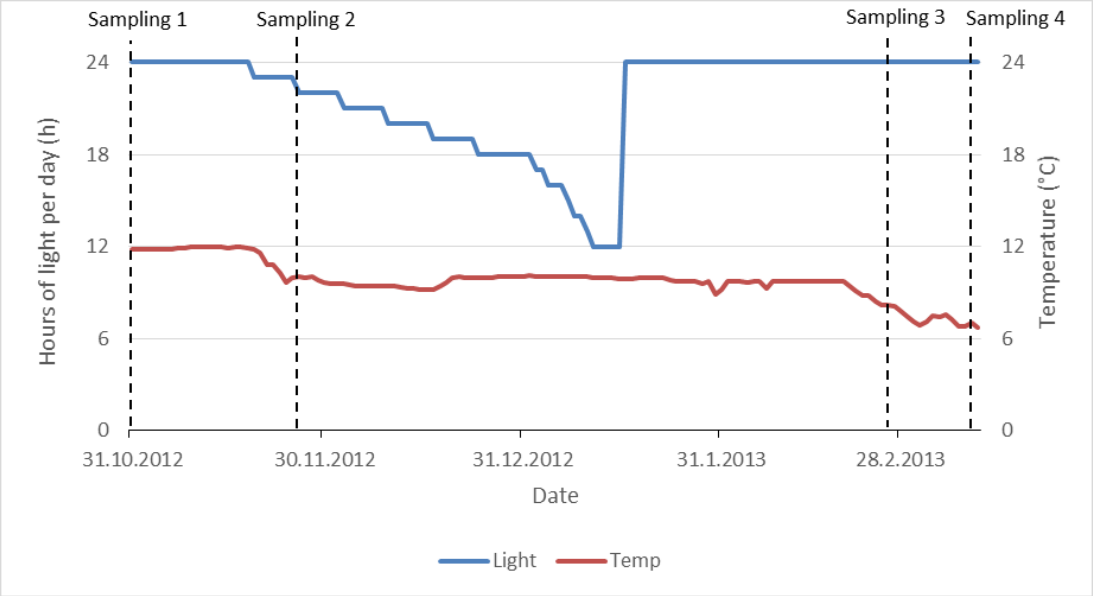


Figure 2.1 Hours of light per day and water temperature throughout the fish trial.
The dotted lines indicate the time of samplings (Sampling 1: 30 October 2012; Sampling 2: 27 November 2012; Sampling 3: 26 February 2013 and Sampling 4: 12 March 2013).

The experimental fish were fed a commercial diet (Table 2.2) until the start of the trial (30 October 2012). On 31 October 2012, group of 100 fish were allocated randomly to triplicate tanks, and thereafter the fish were fed a diet with 10% fish oil (Table 2.2). The initiation of light treatment was started on 19 November 2012. The day light was gradually reduced (approx. 1 hour per week) from 24 hours to 12 hours until 16 January 2013 in order to delay the smoltification process in the fish so that a large smolt could be produced in freshwater. Thereafter the fish were subjected to 24 hours light again to prepare it for final smoltification and transfer to seawater in February at approximately 400 gram.

Sampling were performed at four different time points: 30 October 2012 (Week 0), 27 November 2012 (Week 4), 26 February 2013 (Week 17), and 12 March 2013 (Week 19). At each sampling point, 17-24 fish were taken from the triplicate tanks and anesthetized in MS-222 (Norsk Medisinaldepot, Oslo, Norway). Fish weight and length were recorded at each sampling (Table 2.3). Tissue samples of liver were collected, frozen immediately in liquid nitrogen and stored at -80 ℃ for analysis of gene expression, including the expression of Δ5 and Δ6 desaturases, transcription factors PPARα and SREBPS, and microRNAs which are related to lipid metabolism.

Table 2.2 Fatty acid composition (% of total fatty acids) of the feeds

Fatty acid composition	Commercial diet	10%FO diet
14:0	4.5	1.5
16:0	12.9	7.3
18:0	2.8	2.6
20:0	0.4	0.5
22:0	0.2	0.3
Sum SFA[1]	21.5	12.7
16:1 n-7	5.1	1.8
17:1 n-7	0.2	0.3
18:1 n-9	28.5	45.9
18:1 n-7	2.8	2.3
20:1 n-11	1.4	0.5
20:1 n-9	2.2	1.9
20:1 n-7	0.2	0.1
22:1 n-11	1.9	0.1
24:1 n-9	0.3	0.2
Sum MUFA[2]	44.3	53.4
18:2 n-6	10.3	17.3
18:3 n-6	0.2	0.1
18:3 n-3	4.1	7.5
18:4 n-3	0.1	0.0
20:3 n-6	0.1	0.0
20:4 n-3	0.2	0.0
20:4 n-6	0.3	0.2
20:5 n-3	7.1	4.2
22:5 n-3	1.1	0.4
22:6 n-3	6.7	2.7
Sum PUFA[3]	19.9	14.9
Sum EPA/DHA	13.8	6.9
n-3/n-6 ratio	1.57	0.8

[1]Includes: 15:0, 17:0, 24:0
[2]Includes: 14:1n-5, 15:1, 16:1n-9, 16:1n-5, 16:3n-4, 18:1n-11
[3]Includes: 16:2n-6, 16:2n-3, 18:3n-4

Table 2.3 Weight and length of fish at each sampling point

Time of sampling	Number of fish	Weight (g)	Length (cm)
October 2012	49	136 ± 16	22 ± 2
November 2012	42	159 ± 28	24 ± 1
February 2013	51	426 ± 77	32 ± 2
March 2013	51	419 ± 86	32 ± 2

2.3 Gene expression of fatty acyl desaturases and transcription factors

2.3.1 RNA extraction and reverse transcription

Seventeen to twenty four liver samples per time point were collected, frozen immediately in liquid nitrogen and stored at -80 ℃ prior to RNA extraction. Total RNA was isolated from approximately 20 mg of liver tissue by PureLink™ Pro 96 RNA Purification Kit (Invitrogen Ltd, CA, USA) and recovered in 60 µl molecular biology grade water, following manufacturer's protocol. After purification, 500 ng of total RNA was reverse transcribed into cDNA using AffinityScript™ qPCR cDNA Synthesis Kit (Agilent Technologies, CA, USA) with a reaction volume of 20 µl. The cDNA samples were stored at -20 ℃ prior to qPCR analysis. It is worth mentioning that the RNA isolation and cDNA synthesis of all the liver samples for the fish trial have been performed as part of another master thesis (Arnaud Lefrancois, 2013: The Effect of the Freshwater and the Seawater on the Expression of the Genes Coding for Elongase and Desaturase) prior to the present study.

2.3.2 Real-time qPCR

Table 2.4 Primers used for qPCR analysis

Transcript	Primer sequence (5'-3') forward	Primer sequence (5'-3') reverse	Accession No.
etif3	caggatgttgttgctggatggg	acccaactgggcaggtcaaga	DW542195
Δ5fad	gcttgagcccgatggagg	caagatggaatgcggaaaatg	AF478472
Δ6fad_a	tccccagacgtttgtgtcagatgc	gctttggatcccccattagttcctg	AY458652
Δ6fad_b	tgaccatgtggagagtgaggg	aacttttgtagtacgtgattccagct	GU207400
Δ6fad_c	tgaagaaaggcatcattgatgttg	cacaaacgtctaggaaatgtcc	GU207401
pparα	tcctggtggcctacggatc	cgttgaatttcatggcgaact	NM_001123560
srebp-1	agctgcacggcttccagcag	tcctccgtcttggctccggg	NM_001195818
srebp-2	tgaagggagaggggcggtg	ggcctggctccccattgctg	NM_001195819

The expression of the target genes (Table 2.4) was normalized using *etif3* as a reference gene. The plates containing the cDNA samples were centrifuged at 3000 rpm for 2 min to collect all the samples at the bottom of the wells. All work with the preparation were performed on ice. The reaction mix, consisted of 0.5 µl forward primer (10 µM, with a final concentration of 0.5 µM), 0.5 µl reverse

primer (10 µM, with a final concentration of 0.5 µM) and 5 µl LightCycler® 480 SYBR Green I Master, in a total volume of 10 µl, was added to each well. The plates were sealed with LightCycler® 480 Sealing Foils and centrifuged at 2000 rpm for 2 min to mix the sample with the qPCR reagents and collect the mixture at the bottom. The qPCR was carried out using LightCycler® 480 with the following condition (3-step): denaturation for 5 min at 95 ℃, followed by 45 cycles of 15 s at 95 ℃, 15 s at 60 ℃ and 15 s at 72 ℃, followed by product melting at 95 ℃ for 60 s and 65 ℃ for 60 s to check the purity of the PCR product, followed by cooling at 40 ℃ for 10 s.

2.3.3 Relative gene expression calculation

The relative gene expression levels were calculated according to the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2002).

$$\Delta\Delta C_T = (C_{T\ \text{target gene}} - C_{T\ \text{reference gene}})_{\text{Time x}} - (C_{T\ \text{target gene}} - C_{T\ \text{reference gene}})_{\text{Time 0}}$$

2.4 Gene expression of microRNA (miRNA)

2.4.1 miRNA extraction

Nine liver samples per time point were selected for analysis of gene expression of miRNA during different sampling points. Total RNA was isolated using mirVana™ miRNA Isolation Kit (Thermo Fisher Scientific Inc, MA, USA), following manufacturer's protocol. Tissue samples were immediately placed into a box with dry ice after removed from the -80 ℃ freezer to prevent any possibility of thawing. For each sample, approximately 50 mg of the frozen tissue was cut out and grinded to a powder with liquid nitrogen in a prechilled mortar and pestle sitting in a bed of dry ice. The powdered tissue was scraped into a prechilled tube placed on dry ice by using a prechilled metal spatula. For each sample, 10 volumes (500 µl) of the Lysis/Binding Buffer was added to the tube and mixed rapidly by pipetting and vortexing for homogenization. The mixture was placed on ice until all visible clumps were dispersed. 1/10 volume (50 µl) of miRNA Homogenate Additive was added to the tissue homogenate, and mixed well by vortexing. The homogenate was placed on ice for 10 min. A volume (500 µl) of Acid-Phenol: Chloroform that was equal to the homogenate before addition of the miRNA Homogenate Additive was added. The mixture was vortexed for 30-60 s

to mix. The tube containing the homogenate was centrifuged for 5 min at 100,000 x g at room temperature to separate the aqueous and organic phases.

The aqueous phase was carefully removed without disturbing the lower phase, and transferred to a fresh tube. The volume removed was noted. The RNase-free water was preheated to 95 ℃ for use in eluting the RNA from the filter. 1.25 volumes (350 µl) of room temperature 100% ethanol was added to the aqueous phase. For each sample, a Filter Cartridge was placed into a Collection Tube. The homogenate/ethanol mixture up to 700 µl at a time was then pipetted onto the Filter Cartridge placed into a Collection Tube. The assembly was centrifuged for 15 s at 10,000 x g to pass the mixture through the filter. The flowthrough was discarded. The centrifuge was repeated until all the homogenate/ethanol mixture was through the filter. The Collection Tube was reused for the washing steps. 700 µl miRNA Wash Solution 1 was applied to the Filter Cartridge, and the assembly was centrifuged for 10 s at 10,000 x g. The flowthrough was discarded from the Collection Tube, and the Filter Cartridge was replaced into the same Collection Tube. 500 µl miRNA Wash Solution 2/3 was added to the Filter Cartridge as in the previous step. A second 500 µl miRNA Wash Solution 2/3 was repeated. After discarding the flowthrough from the last wash, the Filter Cartridge was replaced in the same Collection and the assembly was centrifuged for 1 min at 10,000 x g to remove residual fluid from the filter. The Filter Cartridge was then transferred into a new Collection Tube. 100 µl pre-heated (95 ℃) RNase-free water was applied to the center of the filter. The assembly was centrifuged for 30 s at 10,000 x g to recover the RNA. The eluate which contains the total RNA was collected and stored at -80 ℃.

2.4.2 Assessment of RNA quality

The total RNA concentration and purity were determined using NanoDrop® ND-1000 Spectrophotometer. Both 260/280 and 260/230 ratios should be close to 2.0. A low 260/280 ratio indicates the sample is protein contaminated, whereas a low 260/230 ratio indicates the presence of organic contaminants.

The RNA integrity was determined using Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA) and Agilent RNA 6000 Nano Kit (Agilent Technologies,

CA, USA), following manufacturer's instructions. In Agilent 2100 Bioanalyzer, the electrophoretic analysis is based on traditional gel electrophoresis principles that have been transferred to a chip format. A fluorescent dye is used that binds to RNA to determine both the RNA concentration and integrity. The chip format dramatically reduces sample consumption and the separation time. The RNA Integrity Number (RIN) used in the software algorithm allows the classification of total RNA, based on a numbering system from 1 to 10, with 1 being the most degraded and 10 being the most intact. In general, RINs that above 7 indicating a good integrity level.

The RNA samples and RNA ladder was thawed on ice to avoid extensive warming. All reagents was allowed to equilibrate room temperature for 30 min before use. 2.5 µl of each sample was distributed into 200 µl tubes respectively. The tubes were then spun down to collect the samples and RNA ladder at the bottom of each tube. The samples and ladder were degraded for 3 min at 80 ℃ by using a PCR machines. The tubes were immediately cooled on ice. 500 µl of RNA gel matrix was pipette into a spin filter. The assembly was centrifuged for 10 min at 1,500 x g at room temperature. 65 µl filtered gel was distributed into a 0.5 ml RNase-free micro-centrifuge tube. RNA dye concentrate was vortexed for 10 min and spun down. 1 µl of the dye was added into a 65 µl aliquot of filtered gel. The solution was mixed well by vortexing. The tube containing the mixture was centrifuge for 10 min at 13,000 x g at room temperature.

A new RNA chip was put on the chip priming station. 9 µl of gel-dye mix was pipetted in the well marked  . When dispensing the liquid, the pipette tip was always inserted to the bottom of the well. The plunger was positioned at 1 ml and closed. The plunger was then pressed until it was held by the clip. After 30 s, the clip was released. After another 5 s, the plunger was slowly pulled back to 1 ml position. The chip was then taken out from the priming station, and 9 µl of gel-dye mix was pipetted into the two wells marked  respectively. The remaining gel-dye mix was discarded. 5 µl of RNA marker was pipetted into all 12 sample wells and into the well marked  . 1 µl of the prepared ladder was pipetted into the well marked  . 1 µl of sample was pipetted in each of the 12 sample wells. 1 µl of RNA Marker was pipetted in each unused sample wells. The chip was horizontally put in the IKA vortexer and vortex for 1 min at 2400 rpm. The chip

was then run in the Agilent 2100 Bioanalyzer instrument. The chip was removed immediately when the assay was complete, and the electrodes were immediately washed with a cleaning chip.

2.4.3 Reverse transcription for miRNA

Total RNA (100 ng/µl in 20 µl) were reverse transcribed into cDNA by miScript II RT Kit (Qiagen, Hilden, Germany), following manufacturer's instructions. Mature miRNAs were polyadenylated by poly(A) polymerase and reverse transcribed into cDNA using oligo-dT primers (Figure 2.2). The oligo-dT primers have a universal tag sequence on the 5' end, allowing amplification of mature miRNA in the real-time qPCR step.

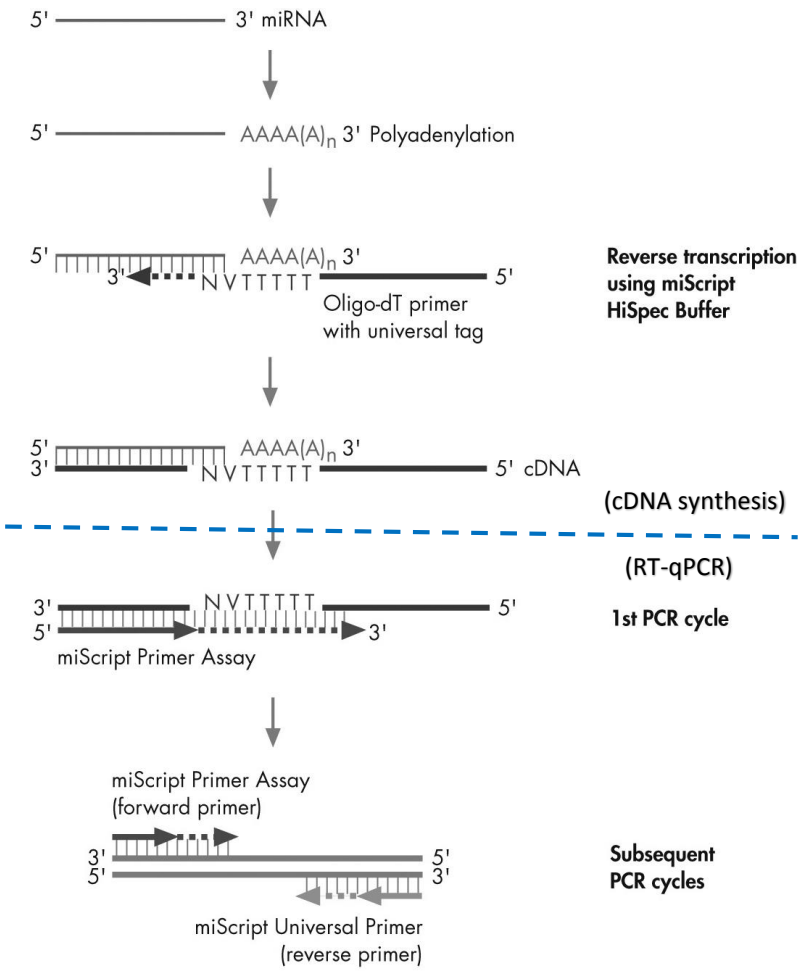


Figure 2.2 Principle of reverse transcription and qPCR for miRNA
Mature miRNAs were polyadenylated by poly(A) polymerase and reverse transcribed into cDNA using oligo-dT primers. The oligo-dT primers have a universal tag sequence on the 5' end, allowing amplification of mature miRNA in the real-time qPCR step.

Total RNA were thawed on ice, 10x miScript Nucleics Mix, RNase-free water, and 5x miScript HiSpec Buffer were thawed at room temperature and then kept on ice. The reaction mixture was consisted of 4 µl 5x miScript HiSpec Buffer, 2 µl 10x miScript Nucleics Mix, 8 µl RNase-free water, 2 µl miScript Reverse Transcriptase Mix and 4 µl template RNA, in a total volume of 20 µl. The reverse transcription was performed using Hybrid express PCR machine with the following condition: 60 min at 37 ℃, followed by 5 min at 95 ℃ to inactivate miScript Reverse Transcriptase Mix. The reaction products were then diluted by adding 200 µl RNase-free water and stored at -20 ℃.

2.4.4 Real-time qPCR for miRNA

Table 2.5 MiRNA specific primers (10 µM, with a final concentration of 1 µM) used for qPCR. All reverse primers were miScript Universal primer (10 µM, with a final concentration of 1 µM) provided in miScript SYBR Green PCR Kit with a confidential sequence.

Primers	Primer sequences (5'-3') forward	Selection criteria	References
ssa-mir-25-3p	cattgcacttgtctcggtctga	Reference gene	Johansen and Andreassen, 2014
ssa-mir-92a-3p	tattgcacttgtcccggcctgt	Reference gene	Liang et al., 2007
ssa-mir-122-5p	tggagtgtgacaatggtgtttg	Lipid metabolism	Yang et al., 2015 Mennigen et al., 2014a, 2014b Tsai et al., 2012
ssa-mir-30c-3p	tgtaaacatccttgactggaagct	Lipid metabolism, osmoregulation	Zhang et al., 2014 Yan et al., 2012
ssa-mir-429-3p	taatactgtctggtaatgccg	Osmoregulation	Flynt et al., 2009
ssa-mir-8163-3p	tcaggtcacatgttcaggata	Iron metabolism, liver enriched	Andreassen et al., 2013
ssa-mir-27a-3p	ttcacagtggctaagttccgct	Lipid metabolism	Ji et al., 2009 Shirasaki et al., 2013 Kida et al., 2011
ssa-mir-33a-3p	caatgtgtctgcagtgcagta	Lipid metabolism	Najafi-Shoushtari et al., 2010 Davalos et al., 2011 Mennigen et al., 2014a Dávalos et al., 2011
ssa-mir-145-3p	attcctggaaatactgttctt	Lipid metabolism	Zhang et al., 2014
ssa-mir-15a-5p	cgtagcagcacgtcatggtttgt	Lipid metabolism	Zhang et al., 2014
ssa-mir-200a-3p	taatactgcctggtaatgatgat	Osmoregulation	Flynt et al., 2009
ssa-mir-10b-5p	taccctgtagaaccgaatttgt	Osmoregulation	Yan et al., 2012
ssa-mir-143-3p	tgagatgaagcactgtagctc	Adipocyte differentiation	Easu et al., 2004 Mennigen et al., 2013a
ssa-mir-17-5p	caaagtgcttacagtgcaggtag	Lipid metabolism	Zhang, X et al., 2014
ssa-mir-21a-5p	tagcttatcagactggtgttgact	Lipid metabolism	Kida et al., 2011

The expression of the miRNAs selected for this study (Table 2.5) was normalized using *ssa-mir-25-3p* and *ssa-mir-92a-3p* as two reference genes. The qPCR for miRNA was performed using miScript SYBR Green PCR Kit (Qiagen, Hilden, Germany). 2x QuantiTect SYBR Green PCR Master Mix, 10x miScript Universal Primer, 10x miScript Primer Assay, template cDNA, and RNase-free water were thawed at room temperature and kept on ice. The individual solutions were mixed gently. The reaction mixture was consisted of 12.5 µl 2x QuantiTect SYBR Green PCR Master Mix, 2.5 µl 10x miScript Universal Primer (10 µM, with a final concentration of 1 µM), 2.5 µl 10x miScript Primer Assay (10 µM, with a final concentration of 1 µM), 5.5 µl RNase-free water and 2 µl template cDNA, in a total volume of 25 µl. Template cDNAs were added into the individual tubes placed on the pre-chilled station. A master mix without template cDNA was made and added to each tube. The tubes were carefully sealed with caps. The real-time qPCR reaction was performed in 96-well plates by MX 3000P qPCR machine (Stratagene, CA, USA) with the following the condition in Table 2.6.

Table 2.6 Cycling condition for real-time PCR

Step	Time	Temperature	Comments
PCR Initial activation step	15 min	95°C	HotStar Taq DNA Polymerase is activated by this heating step.
3-step cycling:			
Denaturation	15 s	94°C	
Annealing	30 s	55°C	
Extension	30 s	70°C	Perform fluorescence data collection.
Cycle number	40 cycles		
Final melting curve analysis			

2.5 Cell line RTH-149 culturing

Molecular studies of fatty acyl desaturases and other lipid homeostatic regulatory system in liver of Atlantic salmon have been constrained by a lack of tractable cellular system. In this study, we used a cell line, designated RTH-149, of rainbow trout, a close related species of Atlantic salmon, to establish a protocol for gene knockout and exploit the regulatory roles of Δ6 desaturase isoform Δ*6fad_b* in omega-3 fatty acid pathway. The cell line RTH-149 was originated from an aflatoxin-induced hepatoma in an adult rainbow trout (Lannan et al., 1984).

2.5.1 Complete growth medium

The base medium for trout hepatoma cell line RTH-149 is ATCC-formulated Eagle's Minimum Essential Medium (EMEM). To make complete growth medium, fetal bovine serum (FBS) was added to the base medium (EMEM) to a final concentration of 10%.

2.5.2 Thawing of frozen cells

The frozen cells were stored in liquid nitrogen vapor phase before thawing. Prior to the addition of the cells, a 75 cm^2 tissue culture flask containing 15 ml complete growth medium was placed in the incubator at 21 ℃ with 5% CO_2 in air atmosphere for at least 15 min to allow the medium to reach its normal pH (7.0 to 7.6).

The vial containing the frozen cells was thawed in a 21 ℃ water bath for approximately 2 min. The O-ring and cap were kept out of the water to reduce the possibility of contamination. The vial with the cells was then removed from the water bath and decontaminated by cleaning with 70% ethanol. The cells were transferred to the prepared culture flask and diluted 1:15 with complete growth medium (1 ml thawed cells were diluted in 15 ml complete growth medium). The cells were incubated at 21 ℃ in the incubator with 5% CO_2 in air atmosphere and washed the following day to remove residuary DMSO (Dimethyl sulfoxide) from the cryopreservation medium.

2.5.3 Washing cells

The cell cultures were washed twice a week to maintain good growth condition. For each culture flask (75 cm^2), the old growth medium was removed, and the cells were rinsed with 10 ml fresh EMEM solution. Then the EMEM was removed and 15 ml fresh complete growth medium was added.

2.5.4 Cell harvesting

Trypsin-EDTA solution (2.5 g porcine trypsin and 0.2 g EDTA per liter of Hanks' Balanced Salt Solution with phenol red) was used to detach the adherent cells from the surface. Trypsin cleaves peptides on the C-terminal side of lysine and

arginine residues. The rate of hydrolysis of this reaction is slowed if an acidic residue is on either side of the cleavage site and hydrolysis is stopped if a proline residue is on the carboxyl side of the cleavage site. The optimal pH for trypsin activity is 7-9. Trypsin can also act to cleave ester and amide linkages of synthetic derivatives of amino acids. EDTA is added to trypsin solutions as a chelating agent that neutralizes calcium and magnesium ions that obscure the peptide bonds on which trypsin acts. Removing these ions increases the enzymatic activity (http://www.sigmaaldrich.com/catalog/product/sigma/t4049).

The old growth medium was removed and the cell culture was rinsed with 10 ml fresh EMEM solution and 2 ml Trypsin-EDTA solution, successively. The rinsing solution was removed. Two ml fresh trypsin solution was added to completely cover the surface of the culture. The culture was incubated at room temperature for approximately 5 min to detach the cells. The cells were continuously checked with microscope to avoid overtrypsinization. The cell suspension was transferred to a fresh tube containing 10 ml complete growth medium to inactive trypsin activity. The procedure was repeated to detach more cells from the culture vessel. The cells were gently pipetted up and down to disrupt cell clumps.

2.5.5 Cell line subculturing

Cells were usually split with a ratio of 1:3 at the confluence of 80% (approximately $1x10^4$-$1x10^5$ per cm^2). The cells were firstly harvested as described in *Section 2.5.4*. The cell suspension was then diluted to 45 ml with complete growth medium and equally distributed into three new culture flasks (15 ml cell suspension for each culture flask). The cells were washed the following day to remove the residuary trypsin from the trypsinization.

2.5.6 Cell line cryopreservation

Cells were cryopreserved for longer-time storage. DMSO was used as a cryoprotectant, added to cell medium to reduce ice formation and thereby prevent cell death during the freezing process.

The cells were firstly harvested as described in *Section 2.5.4*. The final volume of the cell suspension was noted, and the density of the cell suspension was

measured by Countess® Automated Cell Counter (Thermo Fisher Scientific, MA, USA). Ten µl of cell suspension and 10 µl of 0.4% trypan blue solution were mixed in a new 1.5 ml tube by pipetting. Ten µl of the sample mixture was added to the half-moon shaped chamber port on the cell counting chamber slide. The slide was then inserted into the counter. The image was optimized by the "Focus" knob. After running the counting program, result of the cell density and viability were recorded.

The cells were collected by centrifuging at 125 x g for approximately 10 minutes. The supernatant was removed and the cell pellet was resuspended in 1 ml pre-chilled (2 to 8 ℃) cryopreservation medium consisted of complete growth medium with 5% DMSO. The cell suspension was transferred to a pre-chilled (-20 ℃) cryogenic storage vial and stored in the VWR® CryoCooler (-20 ℃). The whole set was stored at -80 ℃ overnight. The cyro-vial containing frozen cells was then transferred to the nitrogen tank and stored in the gas phase above the liquid nitrogen for longer term storage.

2.6 Gene expression analysis on cell line RTH-49

2.6.1 RNA extraction of cell line

Total RNA was isolated using RNeasy® Plus Mini Kit (Qiagen, Hilden, Germany), following manufacturer's instructions. The old growth medium was removed. The cells were washed twice with DPBS solution (10 ml for each time). 20 µl of 2M DTT per ml of Buffer RLT Plus was added to Buffer RLT Plus before using. For every $5x10^6$ cells, 350 µl Buffer RLT Plus (with DTT) was added to the culture vessel to completely cover the surface. The lysate was collected with a rubber policeman and pipetted into a QIAshredder spin column placed in a 2 ml collection tube. The assembly was centrifuged at maximum speed for 2 min. The column was discarded and the flowthrough was saved. The homogenized lysate was transferred to a gDNA Eliminator spin column placed in a 2 ml collection tube. The assembly was centrifuged at 10,000 rpm for 30 s. The column was discarded and the flowthrough was saved. One volume of 70% ethanol was added to the flowthrough and mixed well by pipetting. Up to 700 µl of the sample mixture was transferred to an RNeasy spin column placed in a 2 ml collection

tube. The assembly was centrifuged at 10,000 rpm for 15 s. The flowthrough was discarded and the collection tube was reused. The rest of the sample mixture was transferred to the RNeasy spin column. The assembly was centrifuged at 10,000 rpm for 15 s. The flowthrough was discarded and the collection tube was reused.

For each RNeasy spin column, 700 µl Buffer RW1 was added. The assembly was centrifuged at 10,000 rpm for 15 s to wash the spin column membrane. The flowthrough was discarded and the collection tube was reused. For each RNeasy spin column, 500 µl Buffer RPE was added. The assembly was centrifuged at 10,000 rpm for 15 s to wash the spin column membrane. The flowthrough was discarded and the collection tube was reused. For each RNeasy spin column, another 500 µl Buffer RPE was added. The assembly was centrifuged at 10,000 rpm for 2 min to wash the spin column membrane. The RNeasy Spin Column was placed in a new 2 ml collection tube, and the old collection tube with the flowthrough was discarded. The assembly was centrifuged at full speed for 1 min. The RNeasy Spin Column was placed in a new 1.5 ml collection tube. For each RNeasy Spin Column, 30-50 µl RNeasy-free water was directly added to the spin column membrane and the assembly was centrifuged at 10,000 rpm for 1 min to recover the RNA. The column was discarded and the eluate was kept on ice. RNA quality was measured by NanoDrop® ND-1000 Spectrophotometer. The total RNA samples were stored at -80 ℃ until analysis.

2.6.2 RNA purification

Total RNA was purified using RNA Clean & Concentrator™-5 (Zymo Research Corp., CA, USA), following manufacturer's instructions. Two volumes RNA Binding Buffer was added to the RNA sample and mixed well. An equal volume (after adding RNA Binding Buffer) of ethanol (95-100%) was then added and mixed. The sample was transferred to the Zymo-Spin™ IC Column in a collection tube and centrifuged at 12,000 x g for 30 s. The flowthrough was discarded. 400 µl RNA Prep Buffer was added to the column and centrifuged at 12,000 x g for 30 s. The flowthrough was discarded. 700 µl RNA Wash Buffer was added to the column and centrifuged at 12,000 x g for 30 s. The flowthrough was discarded. Another 400 µl RNA Wash Buffer was added to the column and centrifuged at 12,000 x g for 2 min to ensure complete removal of the wash buffer. The column

was carefully transferred into an RNase-free tube. 25 µl DNase/RNase-Free Water was directly added to the column membrane and incubated at room temperature for 1 min and centrifuged at 10,000 x g for 30 s. The concentration and purity of the RNA sample were assessed by NanoDrop® ND-1000 Spectrophotometer. The purified RNA samples were stored at -80 ℃ until analysis.

2.6.3 cDNA synthesis

Total RNA was reverse transcribed into cDNA by SuperScript® VILO™ cDNA Synthesis Kit (Thermo Fisher Scientific Inc, MA, USA), following manufacturer's instructions. The total RNA sample was thawed on ice. Up to 1 µg of total RNA was diluted to 14 µl with RNase-free water. The reaction mixture, consisted of 4 µl 5X VILO Reaction Mix, 2 µl 10X SuperScript Enzyme Mix and 14 µl prepared total RNA dilution, was added to a 200 µl RNase-free tube on ice with a total volume of 20 µl. Reverse transcription was performed in Veriti® 96-Well Thermal Cycler as following condition: 25 ℃ for 10 minutes, 42 ℃ for 60 minutes and 85 ℃ for 5 minutes. The reaction product was stored at -20 ℃ prior to qPCR analysis.

2.6.4 Real-time qPCR (cell line RTH-149)

The expression of the target genes (Table 2.7) was normalized using *etif3* as a reference gene. All work with the preparation were performed on ice. Four µl of the samples/dilutions was added to each well of the master plate. The master mix, consisted of 0.5 µl forward primer (10 µM, with a final concentration of 0.5 µM), 0.5 µl reverse primer (10 µM, with a final concentration of 0.5 µM) and 5 µl LightCycler® 480 SYBR Green I Master, was added to each well. The plates were sealed with LightCycler® 480 Sealing Foils and centrifuged at 2000 rpm for 2 min to mix the sample with the qPCR reagents and collect the mixture at the bottom. The qPCR was carried out using LightCycler® 480 with the following condition (3-step): denaturation for 5 min at 95 ℃, followed by 45 cycles of 15 s at 95 ℃, 15 s at 60 ℃ and 15 s at 72 ℃, followed by product melting at 95 ℃ for

60 s and 65 ℃ for 60 s to check the purity of the PCR product, followed by cooling at 40 ℃ for 10 s.

Table 2.7 Primers used for qPCR analysis

Transcript	Primer sequence (5'-3') forward	Primer sequence (5'-3') reverse	Accession No.
etif	caggatgttgttgctggatggg	acccaactgggcaggtcaaga	DW542195
Δ5fad	gcttgagcccgatggagg	caagatggaatgcggaaaatg	AF478472
Δ6fad_a	tccccagacgtttgtgtcagatgc	gctttggatcccccattagttcctg	AY458652
Δ6fad_b	tgaccatgtggagagtgaggg	aacttttgtagtacgtgattccagct	GU207400
Δ6fad_c	tgaagaaaggcatcattgatgttg	cacaaacgtctaggaaatgtcc	GU207401
aco	ccttcattgtacctctccgca	catttcaacctcatcaaagccaa	DQ364432
elovl5a	tcccaccctcccttcccaatgt	aaccgaaaccggtgtcagattgatt	NM_001123567
elovl2a	tctacgtccagaaaaagacggc	gtgccattggtggagacaga	NM_001136553*
elovl2b	agacctacagaaaaagacggcc	gtgccattggtggagacaga	NM_001136553*

*We have the sequences of the different isoforms of *elovl2* from an unpublished paper.

Table 2.8 sgRNAs used for transfection

sgRNA	Complete sgRNA sequence	GC%	DNA length
Δ6fad_b sgRNA1	agagaaccgaggaugggggguuuuagagcuagaaaua gcaaguuaaaauaaggcuaguccguuaucaacuugaaa aaguggcaccgagucggugcuuuuuuu	42.7	103
Δ6fad_b sgRNA2	guggcagugcagucuacaccguuuuagagcuagaaaua gcaaguuaaaauaaggcuaguccguuaucaacuugaaa aaguggcaccgagucggugcuuuuuuu		103
sgRNA of control random-oligo	gcauuguggggcaaaaugaaguauuggcaaacauuaag ugucgaacuagaucuaaccuaacgguaagagaguuuca uaauacgucuagccgcaugcgcagg	43	100

2.7 Gene knockout studies

2.7.1 Pilot Trail 1

Cell line RTH-149 were seeded out in two 6-well plates and cultured to 30% confluence. For first time transfection, the vail containing Cas9 nuclease powder (50 µg) was span down to collect all the powder at the bottom. Cas9 nuclease was reconstituted by adding 50 µl nuclease-free water with 20% glycerol (enclosed with the Cas9 nuclease) to the concentration of 1 µg/µl. The vials containing

lyophilized Δ*6fad_b sgRNAs* (50 µg per vial) were span down and reconstituted by adding 50 µl nuclease-free water to the concentration of 1 µg/µl. The vial containing lyophilized control *random-oligo sgRNA* (1168.7 µg) was span down and reconstituted by adding 1168.7 µl nuclease-free water to the concentration of 1 µg/µl. The reconstituted Cas9 nuclease and sgRNAs were aliquoted for single use and stored at -80 ℃.

The Cas9 nuclease/gRNA/Cas9 Plus solution was prepared in [Tube 1] and Lipofectamine™ CRISPRMAX™ Reagent was diluted in [Tube 2] according to Table 2.9. Both solution was incubated for 5 minutes separately at room temperature. Solution in [Tube 1] was then added to [Tube 2], mixed well and incubated for another 10 minutes at room temperature. After the incubation, the transfection reagent complex was added to the cells for different treatments. The cells were transfected at approximately 30% confluence by CRISPR-Cas9 system with either control random-oligo or sgRNA1/2 (a combination of sgRNA1 and sgRNA2, both sgRNA1 and sgRNA2 are targeting the gene Δ6fad_b). In this pilot trials, only one parallel was used for each treatment (i.e. no replicates), because of the limited amounts of cells and reagents available and the high costs of the transfection reagents. The cells were incubated to 70-90% confluence and the RNAs were isolated from the cells for qPCR analysis (according to Section 2.6).

Table 2.9 Overview of component in [Tube 1], [Tube 2] and final volume of transfection reagent complex for Trial 1. Volume in each column are for a single well (in 6-well plate) and account for pipetting variations. Volume was scaled for additional wells.

	Component	Δ6fad_b sgRNA1/2	Control random-oligo
[Tube 1]	EMEM (without serum)	125 µl	125 µl
	Cas9 nuclease	2.5 µl	2.5 µl
	Δ6fad_b sgRNA1	0.5 µl	
	Δ6fad_b sgRNA2	0.5 µl	
	Control random-oligo		1 µl
	Lipofectamine™ Cas9 Plus™ Reagent	5 µl	5 µl
[Tube 2]	EMEM (without serum)	125 µl	125 µl
	Lipofectamine™ CRISPRMAX™ Reagent	7.5 µl	7.5 µl
	Final volume of transfection reagent complex added to each well	250 µl	250 µl

2.7.2 Pilot Trail 2

Several improvements were made in this trial: (1) Cell density was increased to a confluence of 70% prior to the transfection; (2) Single sgRNAs were tested in addition to the combined sgRNAs (i.e. sgRNA1, sgRNA2 or sgRNA1/2).

Cells were seeded out in a 12-well plate and cultured to 70% confluence. The Cas9 nuclease/gRNA/Cas9 Plus solution was prepared in [Tube 1] and Lipofectamine™ CRISPRMAX™ Reagent was diluted in [Tube 2] according to Table 2.10. Both solution was incubated for 5 minutes separately at room temperature. Solution in [Tube 1] was then added to [Tube 2], mixed well and incubated for another 10 minutes at room temperature. After the incubation, the transfection reagent complex was added to the cells for different. In the second trial, cells were transfected by CRISPR-Cas9 system with either sgRNA1, sgRNA2, sgRNA1/2 or sgRNA of control random-oligo. In this pilot trials, only one parallel was used for each treatment (i.e. no replicates), because of the limited amounts of cells and reagents available and the high costs of the transfection reagents. The cells were incubated to 80% confluence and, for each well, cells were split into three new wells and incubated again to 80% confluence. One well of each treatment were harvested for RNA isolation and qPCR analysis (according to Section 2.6).

Table 2.10 Overview of component in [Tube 1], [Tube 2] and final volume of transfection reagent complex for Trial 2. Volume in each column are for a single well (in 12-well plate) and account for pipetting variations. Volume was scaled for additional wells.

	Component	Δ6fad_b sgRNA1	Δ6fad_b sgRNA2	Δ6fad_b sgRNA1/2	Control random-oligo
[Tube 1]	EMEM (without serum)	50 µl	50 µl	50 µl	50 µl
	Cas9 nuclease	1 µl	1 µl	1 µl	1 µl
	Δ6fad_b sgRNA1	0.3 µl		0.15 µl*	
	Δ6fad_b sgRNA2		0.3 µl	0.15 µl*	
	Control random-oligo				0.3 µl
	Lipofectamine™ Cas9 Plus™ Reagent	2 µl	2 µl	2 µl	2 µl
[Tube 2]	EMEM (without serum)	50 µl	50 µl	50 µl	50 µl
	Lipofectamine™ CRISPRMAX™ Reagent	3 µl	3 µl	3 µl	3 µl
	Final volume of transfection reagent complex added to each well	102 µl	102 µl	102 µl	102 µl

*Small volume: 1 µl of each Δ6fad_b sgRNA was mixed and 0.3 µl of the mix was used

2.7.3 Time-course study

The differently transfected cells from the second trial were further split into 9 new wells (in 12-well plates) and incubated to 70-90% confluence. Triplicate wells of each treatment were harvested for RNA isolation every 8 hours during a 24-hour experiment (i.e. the cells were harvested at 8:00, 16:00 and 0:00). The methods for cDNA synthesis and real-time qPCR analysis were according to Section 2.6.

2.8 Statistical analysis

All the data were subjected to a one-way analysis of variance (ANOVA). Significant effects were indicated at a 5% level. The differences were ranked using Duncan's Multiple Range Test. Statistical analyses were conducted using the software package UNISTAT (London, England).

3 Results and discussion

The experimental fish were fed a commercial diet until the start of the trial (30 October 2012), and thereafter fed a diet with an oil fraction consisting of only 10% fish oil (FO) and 90% vegetable oil (VO) until the end of the trial. The fish were kept under full light (24 hours per day), except in a period from 20 November 2012 to 16 January 2013, when the light duration was gradually reduced from 24 hours to 12 hours in order to delay the smoltification process in the fish so that a large smolt could be produced in freshwater. Thereafter the fish were subjected to 24 hours light again to prepare it for final smoltification and transfer to seawater in February at approximately 400 gram. The expression of the selected genes (including Δ5 and Δ6 desaturases, transcription factors PPARα and SREBPS, and microRNAs which are related to lipid metabolism) was determined at four time points (30 October 2012, 27 November 2012, 26 February 2013 and 12 March 2013) during this fish trial.

3.1 Regulation of n-3 LC-PUFA synthesis during smoltification

3.1.1 Effect of diet on gene expression

The experimental fish were transferred from a commercial diet rich in marine ingredients to a new VO-enriched diet right after the first sampling (the start of the trial, 30 October 2012). The second sampling took place after the fish had been fed the new diet for approximately one month (27 November 2012). The change in fatty acid composition of the diet probably explain the change in expression of genes coding for enzymes in the omega-3 pathway as well as transcription factors involved in regulation of the pathway in this first trial period.

All the four fatty acyl desaturase genes Δ*6fad_a,* Δ*6fad_b,* Δ*6fad_c* and Δ*5fad* increased their expression in liver of fish fed with new VO-rich diet for four weeks compared to the start when the fish was fed the commercial marine diet (Figure 3.1). However, the expression of Δ*5fad* showed only a tendency to increased expression and no statistical significant difference. VO are rich in C18 PUFAs but devoid of n-3 LC-PUFAs which are abundant in FO. The C20 and C22 LC-PUFA in FO are thought to suppress the enzyme activities of desaturases in the omega-3 pathway by a feedback inhibition (Sargent et al., 1995; Tocher et

al., 2003b). Our results, showing increased gene expression of desaturases when the VO level in the diet increase, agree with a study by Monroig *et al*. (2010), who showed that the expression of the Δ*6fad_a* gene in liver of Atlantic salmon was significantly up-regulated by VO treatment. The transcript levels of Δ*6fad_b* and Δ*6fad_c* were also higher in liver of fish fed VO than in liver of fish fed a diet rich in FO, although not statistically significant (Monroig *et al*., 2010). In another study, the expression of the Δ5 desaturase gene was also significantly higher in liver of Atlantic salmon fed with a diet rich in VO than that of fed with a diet rich in FO (Zheng *et al.*, 2005). In the present study, we observed that the up-regulation of the gene expression of desaturases coincided with reduced expression of the transcription factors PPARα and SREBPS. Dual regulation of Δ5 and Δ6 desaturase gene expression by SREBP-1 has previously been demonstrated in mouse, but then in the opposite direction to our finding (Matsuzaka et al., 2002). Specifically, the hepatic expression of Δ5 and Δ6 desaturases was highly activated in transgenic mice overexpressing nuclear SREBP-1a, -1c, and -2. Disruption of the SREBP-1 gene significantly reduced the expression of both desaturases in the livers of SREBP-1-deficient mice (Matsuzaka et al., 2002). In addition, administration of fibrate, a pharmacological ligand for PPARα, caused a significant increase in expression of both desaturases (Matsuzaka et al., 2002).

Although the four fatty acyl desaturase genes Δ*6fad_a,* Δ*6fad_b,* Δ*6fad_c* and Δ*5fad* increased their expression in liver of fish fed with new VO-enriched diet, as expected, they may still to some extent be influenced by the expression of the transcription factors. We cannot conclude based on our studies as to whether the increase in expression of these desaturase genes would have been even higher (that the increase to some extent is restrained/controlled) when the transcription factors have not been down-regulated. This is however only a speculation that needs further studies in order to be verified. In addition the diet effects on PPARα is not always consistent with the desaturase gene response. In human and rat Δ6 fad gene promoters, both PUFA and PPARα response regions have been identified to both suppress and induce the Δ6 desaturase expression respectively. Several studies have shown that the molecular mechanisms of transcriptional

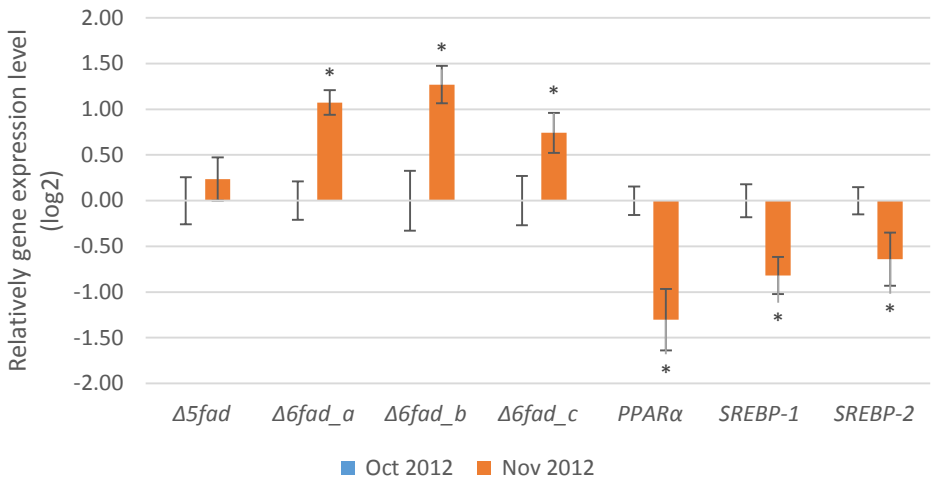


Figure 3.1 Effect of diet on expression of Δ5, Δ6 desaturase and transcription factors Relatively gene expression level of desaturase and transcription factor genes in liver from Atlantic salmon at the first and second sampling points (30 October 2012 and 27 November 2012). Results were normalized to the first sampling point respectively and calculated as the relative expression between the target gene and *etif*, each point represents mean ± S.E. for 17-24 replicate samples. The asterisks (*) are significant difference ($P < 0.05$) between different sampling points.

regulation of the desaturase genes are complex and require further investigation in salmon (Tang et al., 2003; Zheng et al., 2009).

The significantly down-regulation of the transcription factor PPARα by the change from a FO to a VO rich diet (Figure 3.1) is in agreement with a previous study by Morais et al., 2011. This down-regulation may be due to the fact that the FO diet is rich in EPA and DHA fatty acids which are known to act as ligand activators of PPARα and thereby coordinate an up-regulation of lipid oxidation (in both mitochondria and peroxisome) and a down-regulation of lipid synthesis in mammals (Clarke, 2001). Studies have demonstrated that EPA possesses the capacity to induce mitochondrial proliferation and thus stimulate β-oxidation in mitochondria, whereas DHA does not have such effect (Willumsen et al., 1993; Kjær et al., 2008).

The expression of the two transcription factor genes *srebp-1* and *srebp-2* were as mentioned above also significantly down-regulated by the low FO treatment (Figure 3.1), which is the opposite of the expected response to the change in diet. SREBP-1 expression in mammals is increased by depletion of LC-PUFA, while

SREBP-2 is increased by depletion of cholesterol (as is the case when VO is increased in the diet) (Horton *et al.*, 2002). A study with the salmon cell line SHK-1, showed reduced expression levels of SREBP-1 in response to the EPA/DHA treatment that is consistent with the finding in mammals, while the expression levels of SREBP-2 increased by supplementary of EPA/DHA, even though not statistical significant (Minghetti *et al.*, 2011). We do not know the reason why our study shows the opposite of the expected response of SREBPS, but it may be that the physiological smoltification status of the fish influence more than the diet, an assumption supported by the *mir-15a-5p* microRNA results discussed below. Another possible explanation may be that the mRNA transcription and the protein translation processes are unsynchronized. So in order to conclude, the SREBP protein concentration needs to be determined in future studies.

MiR-122, miR-33a, miR-145, miR-27a, miR-15a, miR-143, miR-17 and miR-21a were indicated to be involved in lipid metabolism, while miR-200a, miR-10b, miR-429 and miR-30c were indicated to be involved in osmoregulation. MiR-8163 was suggested to be related to iron metabolism in salmon. Based on the findings in literatures, these genes were included in our study.

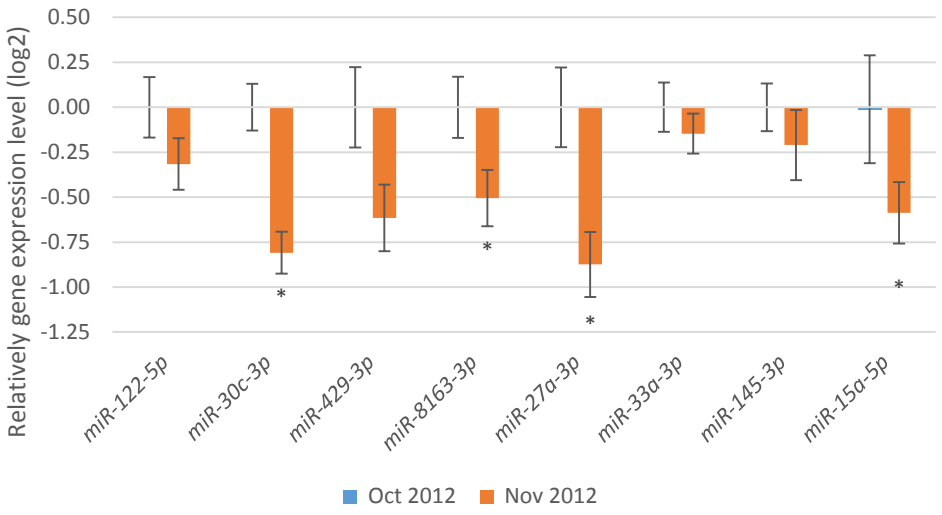


Figure 3.2 Effect of diet on expression of microRNAs
Relatively gene expression level of microRNAs in liver from Atlantic salmon at the first and second sampling points (30 October 2012 and 27 November 2012). Results were normalized to the first sampling point respectively and calculated as the relative expression between the target gene and reference gene geometric mean (miR-25-3p and miR-92a-3p), each point represents mean ± S.E. for nine replicate samples. The asterisks (*) are significant difference ($P < 0.05$) between different sampling points.

The expression of thirteen miRNAs was determined by real-time quantitative PCR at selected time points during the growing cycle. In particular, the gene expression of *mir-200a-3p, mir-10b-5p, mir-143-3p, mir-17-5p* and *mir-21a-5p* showed no significant change throughout the entire fish trial (Appendix Figure 5.1).

The expression of *mir-15a-5p* was significantly down-regulated in November compared to October and, even though not statistically significant, the expression of *mir-122-5p, mir-33a-3p* and *mir-145-3p* showed a trend of decreasing (Figure 3.2). These genes were previously reported to be involved in fatty acid synthesis. Smoltification is an energy demanding period in fish life. In our study, the coordinated down-regulation of these lipogenic miRNAs, together with the SREBPS, in November compared to October pointed in the same direction and may indicate that the salmon goes from a phase where lipid synthesis are prioritized to a phase where it prepares for more lipid utilization and smoltification.

Recent studies have identified miR-33 to be part of the intronic sequences of the sterol regulatory element-binding proteins (SREBPS). Specifically, isoform miR-33a and miR-33b are located within SREBP-2 and SREBP-1 respectively, and are concurrently expressed with its host gene in mammals and teleost fish (Najafi-Shoushtari et al., 2010; Davalos et al., 2011; Mennigen et al., 2014a). The coordinated regulation of *mir-33a-3p* and *srebp-2* in the present study agree with the previous findings.

However, the expression of *mir-27a-3p* and *mir-30c-3p*, which were indicated to be involved in lipid oxidation as also PPARα showed a similar down-regulation (Figure 3.2). The simultaneous regulatory patterns of miRNAs related to both lipid synthesis and oxidation at the same time are seemingly contradictory. However, together with the SREBPS, this may suggest that the influence on these miRNAs and transcription factors are complex and may be more related to environmental factors and smoltification status than the nutritional change in diet.

3.1.2 Effect of light manipulation (day length) on gene expression

It is believed that the smoltification process is primarily controlled by photoperiod (day length), the mechanisms of these environmental triggers regulating omega-3 pathway remains unknown, which is the reason why we wanted to study some possible gene regulators in this study.

In our study, the light duration was gradually reduced from full light (24 hours per day) to 12 hours per day (from 19 November to 16 January), with the aim to first delay the smoltification process in the fish in order to produce a larger smolt of 400 gram than the natural 100 gram smolt, and then returned to full light directly to further stimulate the final preparation of smoltification prior to seawater transfer. The fish were kept in freshwater until the third sampling point (26 February).

Even though not statistically significant, the desaturase genes showed a trend of up-regulation at the sampling in February compared to the sampling in November in agreement with previous studies (Figure 3.3). A study by Zheng et al., (2005) showed that the expression of Δ6 and Δ5 desaturases peaked just prior to seawater transfer. Studies with Atlantic salmon showed a pre-adaptive increase in hepatocyte fatty acyl desaturation/elongation activities prior to seawater transfer (Bell et al., 1997; Tocher et al., 2000). In the present study, the expression of Δ6 and Δ5 desaturase genes was already greatly up-regulated by the change in diet by the second sampling point, the moderate increase after initiation of light treatment could be due to the long-term effect of the VO replacement.

Our results suggest that the light regime initiate a coordinated increase in the hepatic expression of both the transcriptional regulators (i.e. PPARα and SREBPS) as well as the expression of desaturases itself. The three transcription factor genes were significantly up-regulated in this period (Figure 3.3). This is the first report in fish showing regulation of the expression of the transcription factor genes in response to the environmental cue (change of light duration/photoperiod). The up-regulation of PPARα is also in agreement with the fact that the smoltification is an energy demanding process and this transcription factor is involved in regulation of the β-oxidation of fatty acids (Willumsen et al., 1993; Kjær et al., 2008).

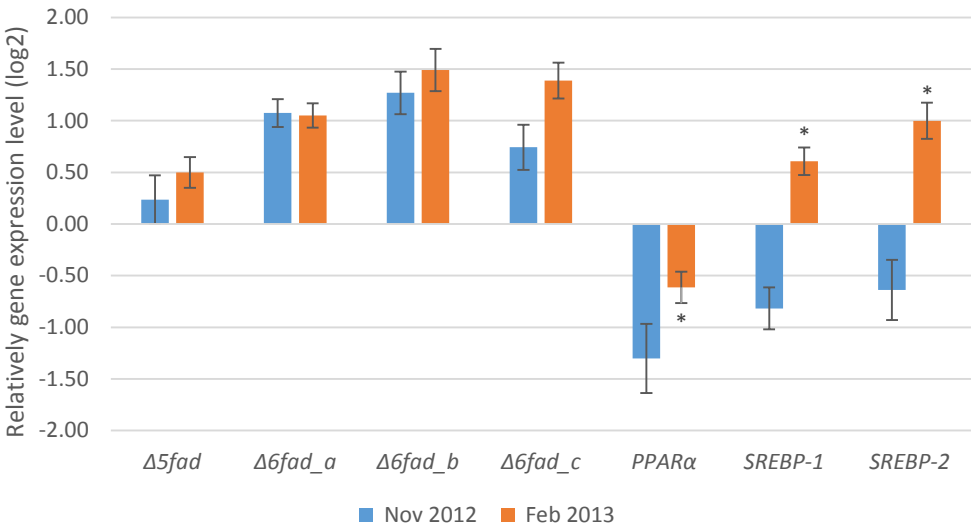


Figure 3.3 Effect of light manipulation on expression of Δ5, Δ6 desaturase and transcription factors Relatively gene expression level of desaturase and transcription factor genes in liver from Atlantic salmon at the second and third sampling points (27 November 2012 and 26 February 2013). Results were normalized to the first sampling point respectively and calculated as the relative expression between the target gene and *Etif*, each point represents mean ± S.E. for 17-24 replicate samples. The asterisks (*) are significant difference ($P < 0.05$) between different sampling points.

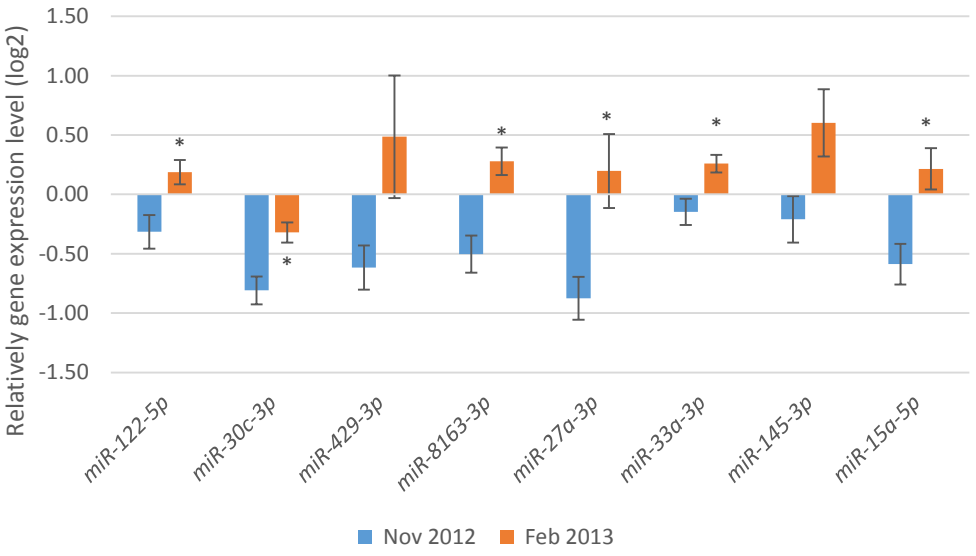


Figure 3.4 Effect of light manipulation on expression of microRNAs
Relatively gene expression level of microRNA in liver from Atlantic salmon at the second and third sampling points (27 November 2012 and 26 February 2013). Results were normalized to the first sampling point respectively and calculated as the relative expression between the target gene and reference gene geometric mean (miR-25-3p and miR-92a-3p), each point represents mean ± S.E. for nine replicate samples. The asterisks (*) are significant difference ($P < 0.05$) between different sampling points.

In this period, there was a clear trend that the gene expression of miRNAs was up-regulated in February compared to that in November and, even though not statistical significant, the expression of *mir-429-3p* and *mir-145-3p* showed a trend of increase (Figure 3.4). We here show for the first time that miRNAs changed the expression levels in response to the change of photoperiod, indicating these miRNAs may be involved in regulation of smoltification. Interestingly, *miR-33a-3p* that is connected to *srebp-2* was once again showing a coordinated regulation with SREBPS.

3.1.3 Effect of seawater transfer on gene expression

The last sampling took place approximately two weeks after the fish had been transferred to the seawater. The expression of the desaturase genes Δ*5fad* and Δ*6fad_a* was significantly down-regulated in response to the seawater transfer in March compared to the sampling in February (Figure 3.5). It is known that the enzyme activities of these desaturases are dependent on the extent to the existence of C20 and C22 in their natural diets. The fact is 20:5n-3 and 22:6n-3 are abundant in marine environment, thus endogenous de novo biosynthesis of these fatty acids in many marine fish is not necessary. As a result, these conversions including chain elongations are likely to be repressed. However, 20:5n-3 and 22:6n-3 are not well supplied in the natural prey of many freshwater fish, therefore, the conversions of 18:3n-3 to 20:5n-3 and 22:6n-3 are necessary (Tocher, 2003). When the fish were transferred from freshwater to the seawater, it is likely that the increased salinity act as an environmental cue to restrain the expression of these desaturases.

Our results agree with Zheng et al. (2005) that the expression of Δ6 and Δ5 desaturase was low after seawater transfer. However, the expression of desaturase genes Δ*6fad_b* and Δ*6fad_c* showed a tendency of up-regulation. This may be due to the design of the fish trial where the parent generation of the experimental fish in the present study was selected for high expression of Δ*6fad_b* in order to maintain a higher capacity for EPA and DHA production. In addition, it is shown by functional characterization of salmon desaturase cDNA that Δ*6fad_a* possess a very low level of Δ5 desaturase activity, whereas Δ*6fad_b* and Δ*6fad_c* had no Δ5 desaturase activity (Zheng et al., 2005; Monroig et al., 2010). This may

explain the relatively coordinated expression pattern between Δ*6fad_b* and Δ*6fad_c*, while Δ6fad_a and Δ5fad were expressed accordantly. Furthermore, the different regulation/gene expression of the Δ6 desaturase isomers indicates that they may possess different functions.

The expression of the transcription factors *pparα* and *srebp-1* was also down-regulated, whereas the expression of *srebp-2* showed no significant change (Figure 3.5). To date, no data of the effect of environmental factors on the expression of transcription factor genes has been reported. The coordinate down-regulation of the *pparα* and *srebp-1*, together with the down-regulation of the desaturases Δ*5fad* and Δ*6fad_a*, suggests the role of environmental cue of seawater to suppress the expression of both desaturases and the transcription factors which regulate the desaturases. Moreover, the different expression pattern of these two SREBPS may indicate different functions between these two isomers.

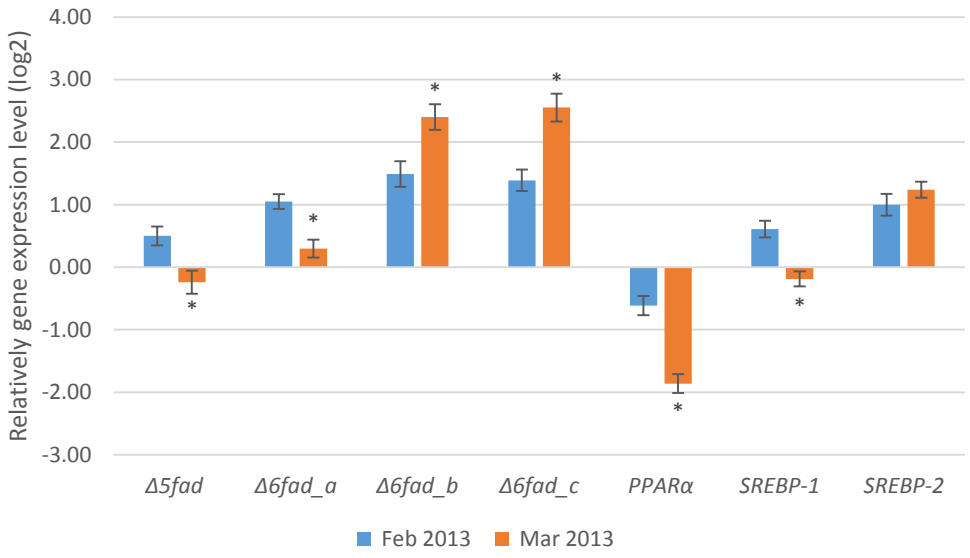


Figure 3.5 Effect of seawater transfer on expression of Δ5, Δ6 desaturase and transcription factors. Relatively gene expression level of desaturase and transcription factor genes in liver from Atlantic salmon at the third and fourth sampling points (26 February 2013 and 12 March 2013). Results were normalized to the first sampling point respectively and calculated as the relative expression between the target gene and *Etif*, each point represents mean ± S.E. for 17-24 replicate samples. The asterisks (*) are significant difference ($P < 0.05$) between different sampling points.

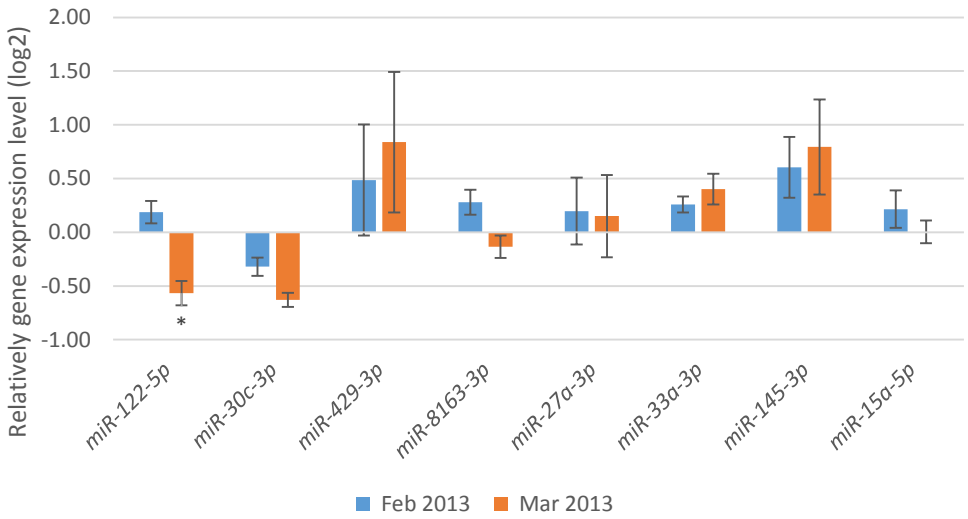


Figure 3.6 Effect of seawater transfer on expression of microRNAs
Relatively gene expression level of microRNA in liver from Atlantic salmon at the third and fourth sampling points (26 February 2013 and 12 March 2013). Results were normalized to the first sampling point respectively and calculated as the relative expression between the target gene and reference gene geometric mean (miR-25-3p and miR-92a-3p), each point represents mean ± S.E. for nine replicate samples. The asterisks (*) are significant difference ($P < 0.05$) between different sampling points.

The gene expression of miRNAs showed no significant change in this period, except *miR-122-5p* which was significantly down-regulated after the seawater transfer (Figure 3.6). The regulation pattern of *mir-122-5p* was once again in line with *srebp-1*. Since *srebp-1* and *mir-122-5p* are both extensively reported to be involved in lipid synthesis, our results indicate they may be regulated by environmental factors similarly.

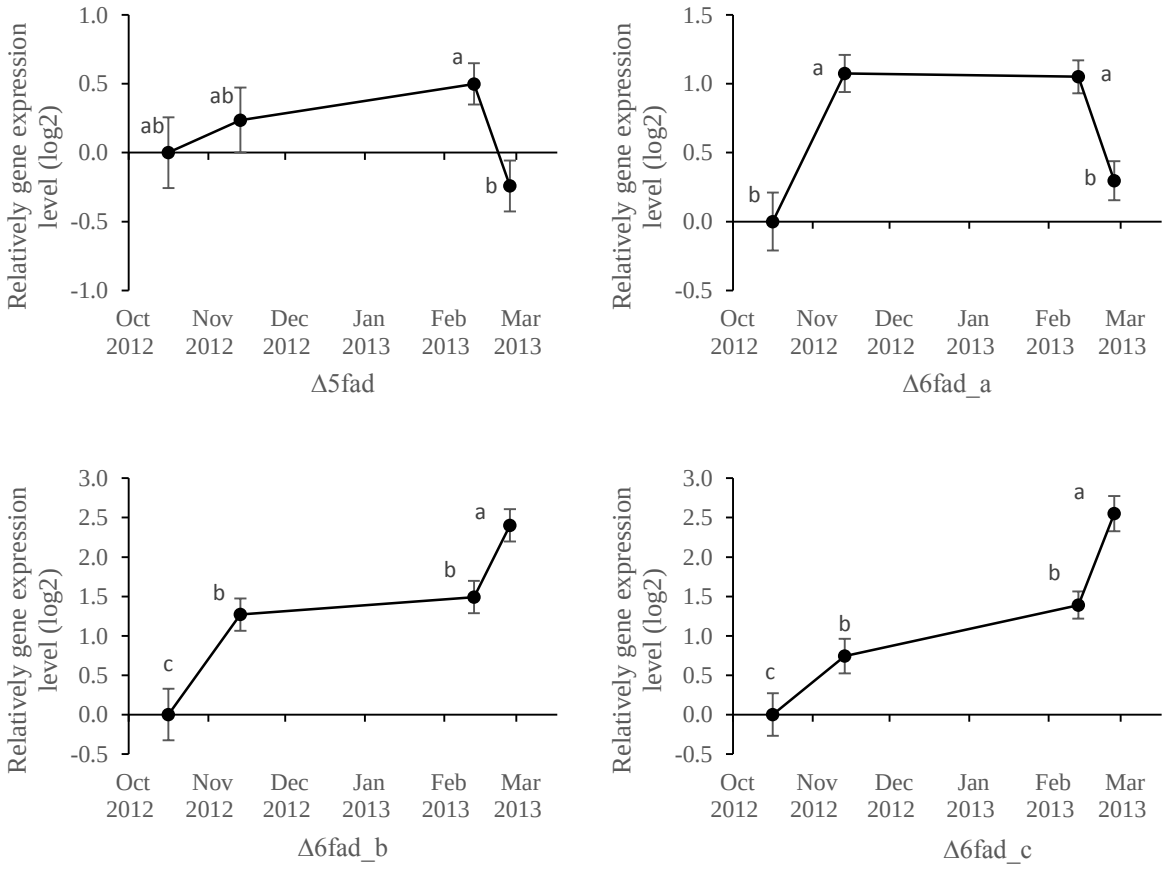
The gene expression of both *srebp-2* and *mir-33a-3p* were not significantly affected by the seawater transfer. In general, the expression of both *srebp-2* and *mir-33a-3p* were highly synchronized during the entire fish trial. Our result is in agreement with the concurrent expression pattern of miR-33a and its host gene SREBP-2 in rainbow trout (Mennigen et al., 2014a) and mammals (Davalos et al., 2011).

3.1.4 A summary of differences in gene expression during the whole time span of the trial

Gene expression of liver fatty acid desaturases and transcription factors was significantly affected by the time points of sampling (Figure 3.7). In particular, the expression of Δ*5fad* and Δ*6fad_a* increased slightly up to the seawater transfer, and then decreased after seawater transfer. The expressions of these genes in March were almost returned to their starting levels of expression in October.

On the contrary, the expression of Δ*6fad_b* and Δ*6fad_c* showed a similar pattern of expression from October to February, but the opposite pattern to Δ*5fad* and Δ*6fad_a* with an increased expression after seawater transfer.

The expression of the transcription factors *pparα, srebp-1* and *srebp-2* dropped from October to February and thereafter increased prior to seawater transfer, after which the expression level *pparα and srebp-1* again significantly decreased after seawater transfer except *srebp-2*, which did not change in seawater.



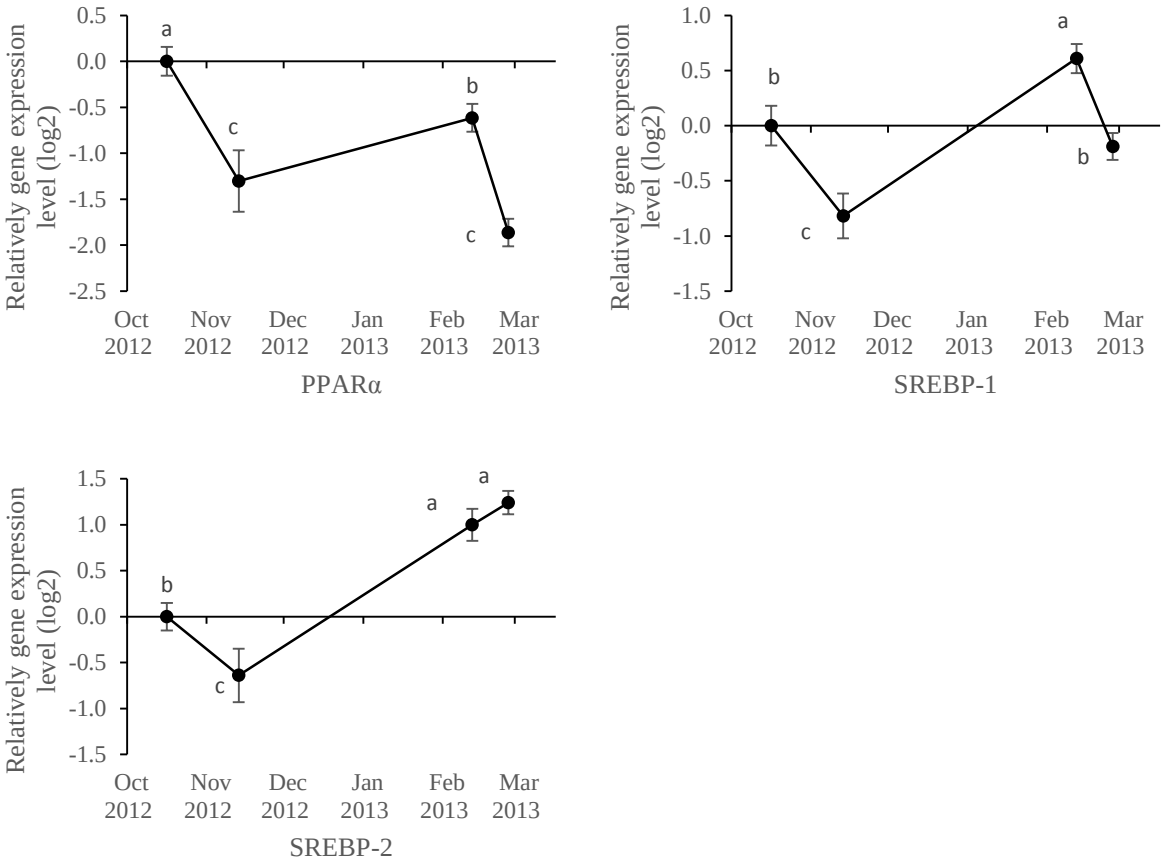
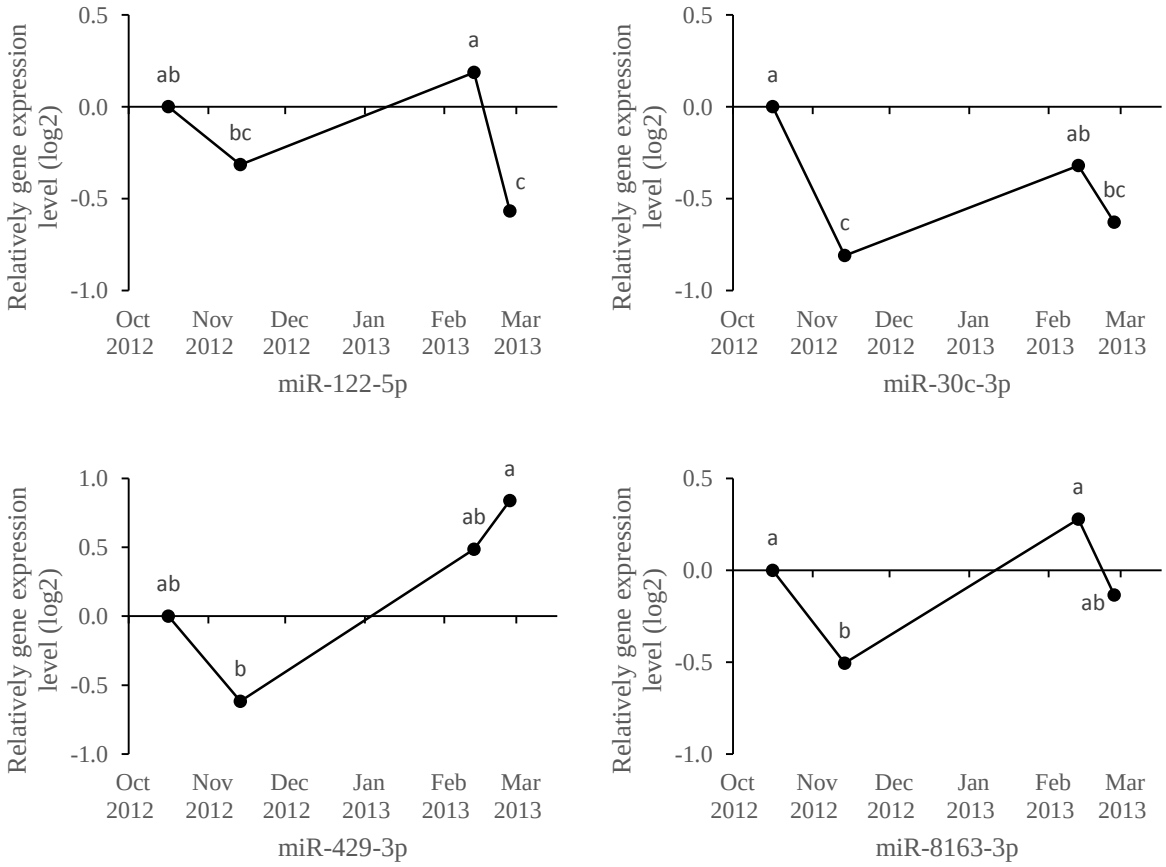


Figure 3.7 Expression of Δ5, Δ6 desaturase and transcription factors at different life stages. Relatively gene expression level of desaturase and transcription factor genes in liver from Atlantic salmon up to seawater transfer. Results were normalized to the first sampling point respectively and calculated as the relative expression between the target gene and *etif*, each point represents mean ± S.E. for 17-24 replicate samples. The different assigned letters are significant difference ($P < 0.05$) between different sampling points (30 October 2012, 27 November 2012, 26 February 2013 and 12 March 2013).

Gene expressions of *mir-200a-3p*, *mir-10b-5p*, *mir-143-3p*, *mir-17-5p* and *mir-21a-5p* showed no significant changes throughout the entire fish trial (Appendix Figure 5.1), whereas the expression of other miRNAs were significantly affected by the time of sampling (Figure 3.8). The expressions of *mir-122-5p* dropped from October to November; the expression increased prior to seawater transfer and thereafter dropped again in seawater. The gene expression of *mir-30c-3p*, *mir-8163-3p*, *mir-27a-3p*, *mir-15a-5p*, *mir-429-3p*, *mir-33a-3p* and *mir-145-3p* showed a similar pattern prior to seawater transfer, but the expression did not change significantly after transfer to seawater (similar to *srebp-2*).

These results indicate that both the transcription factors and miRNA are involved in a dynamic regulation of the omega-3 metabolic pathway under different environmental conditions at different life stages.

The complex mixture of feed effects, environmental effects and different life stages/biological phases (time of sampling) of the fish in the period from October to January complicates the interpretation of the results. More control groups should be integrated in the further studies in order to deviate the feed effects from the environmental and life stage effects.



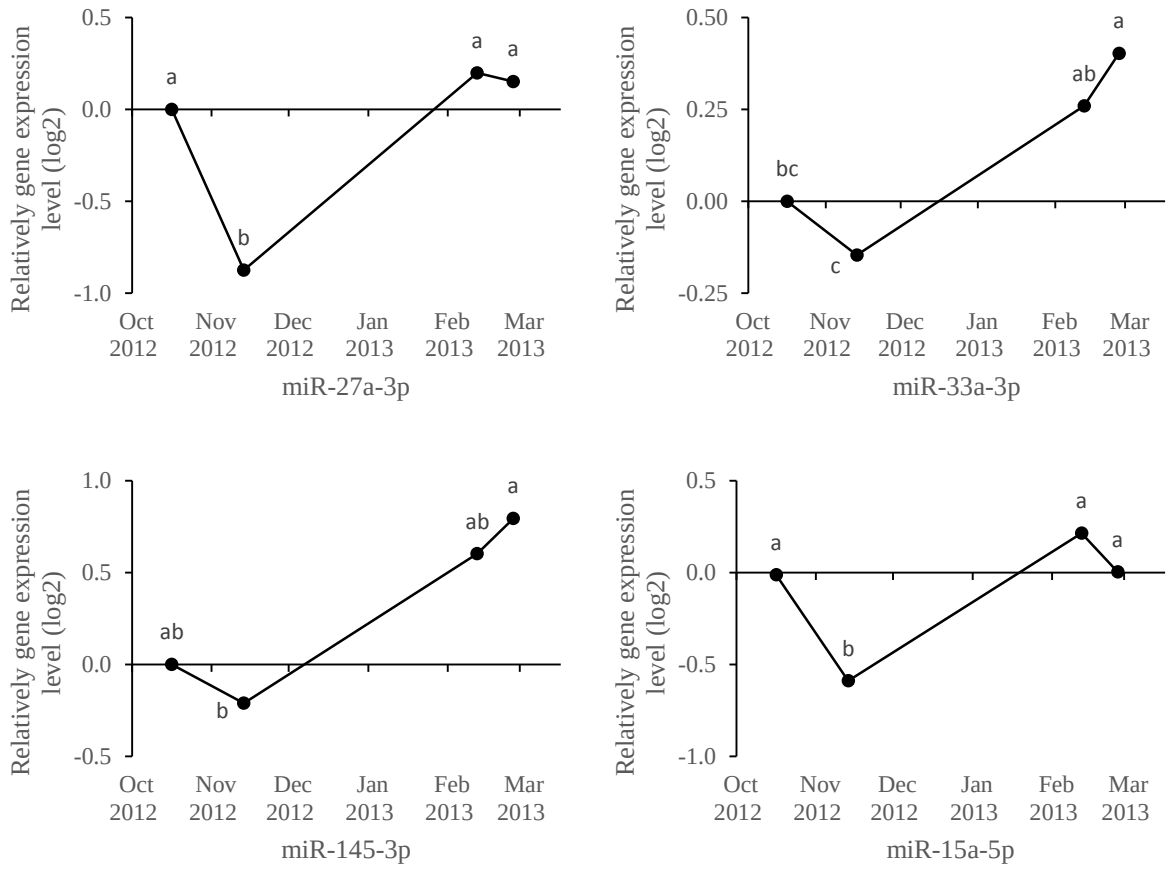


Figure 3.8 Expression of microRNAs at different life stages
Relatively gene expression level of microRNA in liver from Atlantic salmon up to seawater transfer. Results were normalized to the first sampling point respectively and calculated as the relative expression between the target gene and reference gene geometric mean (miR-25-3p and miR-92a-3p), each point represents mean ± S.E. for nine replicate samples. The different assigned letters are significant difference ($P < 0.05$) between different sampling points (30 October 2012, 27 November 2012, 26 February 2013 and 12 March 2013).

3.2 Test the use of CRISPR technique to knock down the gene expression of desaturase *Δ6fad_b*

3.2.1 Method development of transfection

CRISPR technology had not previously been tested in the laboratory in Nofima prior to this trial. We wished to establish this technology in order to knock out the Δ*6fad_b* desaturase gene so that no functional desaturase enzyme will be produced. If this technique succeeds, then it will be a valuable tool in the future to study the function of the different isoforms of desaturases in the omega-3

metabolic pathway. However, the main aim here was only to perform some initial tests in order to establish the technique in the laboratory.

3.2.1.1 Pilot trial 1

The cells were transfected at approximately 30% confluence by CRISPR-Cas9 system with either control random-oligo or sgRNA1/2 (a combination of sgRNA1 and sgRNA2, both sgRNA1 and sgRNA2 are targeting the gene Δ*6fad_b*). In the pilot trials, only one parallel was used for each treatment (i.e. no replicates), because of the limited amounts of cells and reagents available and the high costs of the transfection reagents. All of the cells were incubated under the normal experimental condition for 48 hours. After incubation, the confluence of the transfected cells remained at low levels (around 30%) compared with the non-transfected control group, and dead cells were observed floating in the medium. The morphology of the cells seemed changed and the cells did not look healthy. The reason could be the low density of the cells at the time of transfection, since the degree of confluence of cells is a critical factor for the CRISPR-Cas9 system.

The cells were kept growing until they reached confluence for harvesting (approximately 80%). The growth rate of the cells was dramatically suppressed after transfection (not quantified, just based on observation by microcopy). After approximately three weeks, the total RNA of the cells was harvested. The expression levels of seven protein coding genes (Δ*5fad*, Δ*6fad_a*, Δ*6fad_b*, Δ*6fad_c*, *elovl5a*, *elovl2a*, *elovl2b* and *aco*) were determined by real-time qPCR. The gene expression levels were normalized to the non-transfected group and calculated as the relative expression between the target gene and the reference gene *etif* (Figure 3.9).

The gene expression of Δ*6fad_b* in the sgRNA1/2-treated cells showed slight down-regulation compared to the non-transfected control group. The expression of selected protein coding genes was not expected to be affected by the silencing of control random-oligo. However, the gene expression of Δ*5fad* and Δ*6fad_a* in the control random-oligo-treated cells was remarkably up-regulated after transfection.

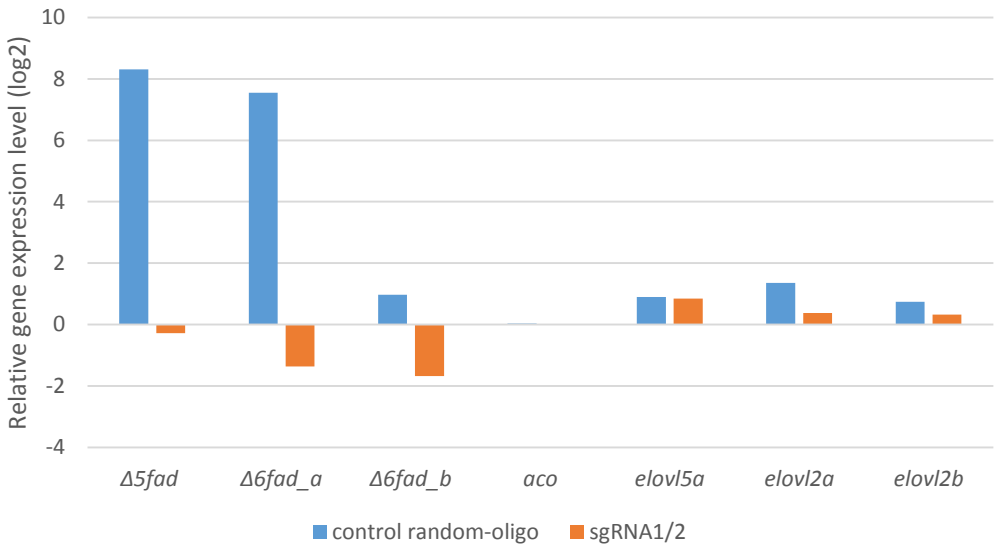


Figure 3.9 Pilot trial 1. Relative gene expression of protein coding genes in trout hepatoma cells transfected by CRISPR-Cas9 system with either control random-oligo or sgRNA1/2 (targeting Δ6fad_b). Results were normalized to the non-transfected control group respectively and calculated as the relative expression between the target gene and *etif*.

According to the observation from the cell cultures, the restrained growth rate and the high mortality could be the results of the low cell density at the time of transfection. The slightly down-regulation of Δ*6fad_b* may indicate that the transfection protocol was effective but needed to be improved. The unexpected up-regulation in the control random-oligo group may imply that the selected oligonucleotide is unqualified as a control. Alternative control random-oligos are not included in the present study due to the time limitation. In the next trial, several improvements were made: (1) Cell density was increased to a confluence of 70% prior to the transfection; (2) Single sgRNAs were tested in addition to the combined sgRNAs (i.e. sgRNA1, sgRNA2 or sgRNA1/2).

3.2.1.2 Pilot trial 2

In the second trial, cells were transfected at a confluence of approximately 70% by CRISPR-Cas9 system with either sgRNA1, sgRNA2, sgRNA1/2 or control random-oligo. After incubation for 48 hours, the density of the transfected cells was reduced compared to the non-transfected control group and many dead cells were observed floating in the medium (Figure 3.10). This was consistent with the observation in the first trial.

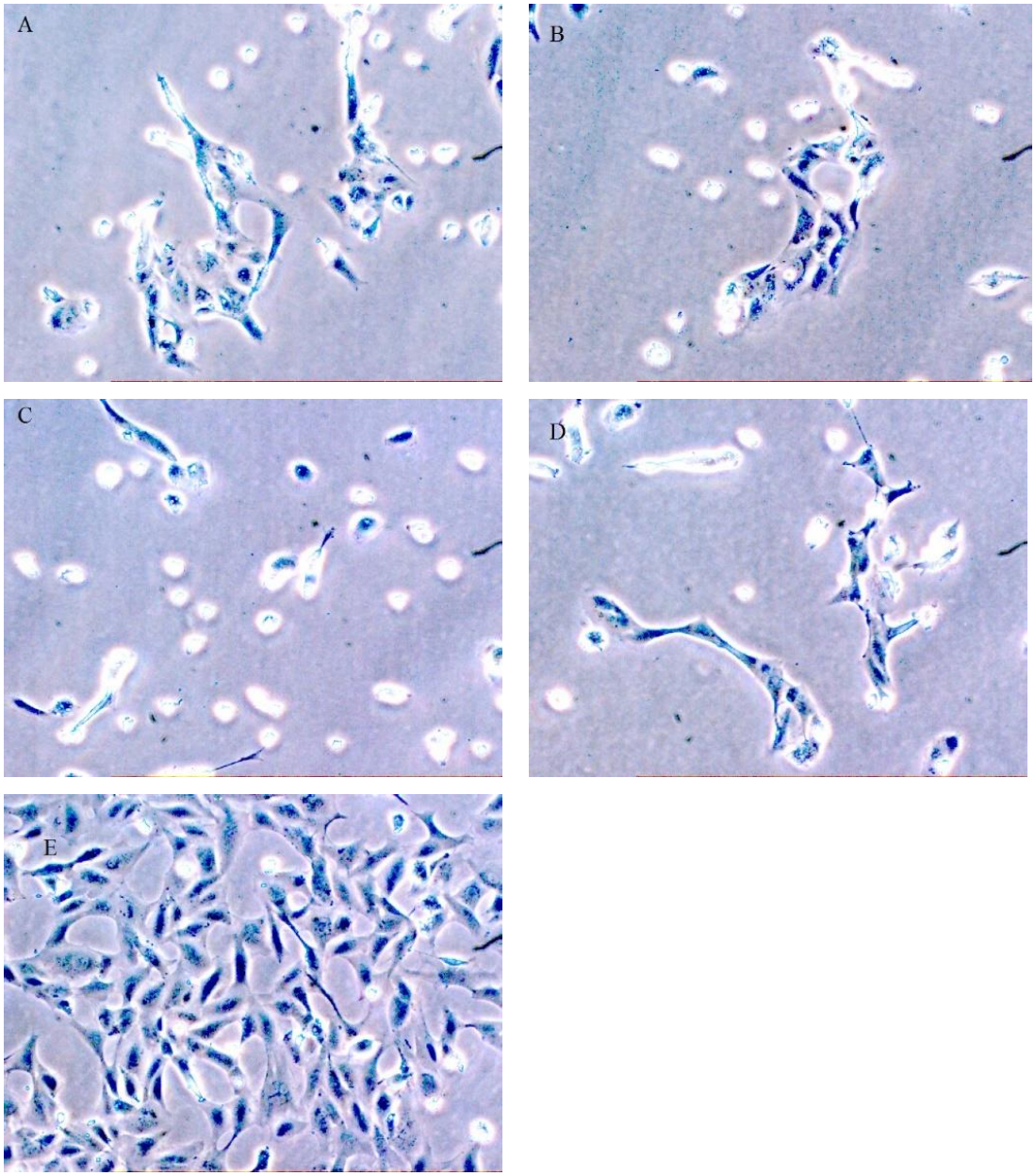


Figure 3.10 Cells from Trial 2, 48 hours after transfection with: A: sgRNA1; B: sgRNA2; C: sgRNA1/2; D: sgRNA of control random-oligo; and E: non-transfected controls.

The cells of different treatments were further split into three new wells. One day after the splitting, more dead cells occurred in the transfected groups, but not in the non-transfected group. The reason could be the intensive impact from the transfection reagent (Lipofectamine™ CRISPRMAX™), which was developed chiefly based on mammalian cells.

The cells were harvested at a confluence of 80% and the total RNA was isolated. Genes, same as in the Trial 1, were tested for expression levels by real-time qPCR

(Figure 3.11). The gene expression of Δ*6fad_b* only showed a slightly down-regulation in the cells treated with either sgRNA2 or sgRNA1/2. In the sgRNA1-treated cells, the expression of Δ*5fad* and Δ*6fad_a* was remarkably up-regulated. The cells treated with control random-oligo also showed a moderate up-regulation in Δ*6fad_a* and a slightly up-regulation in Δ*5fad*.

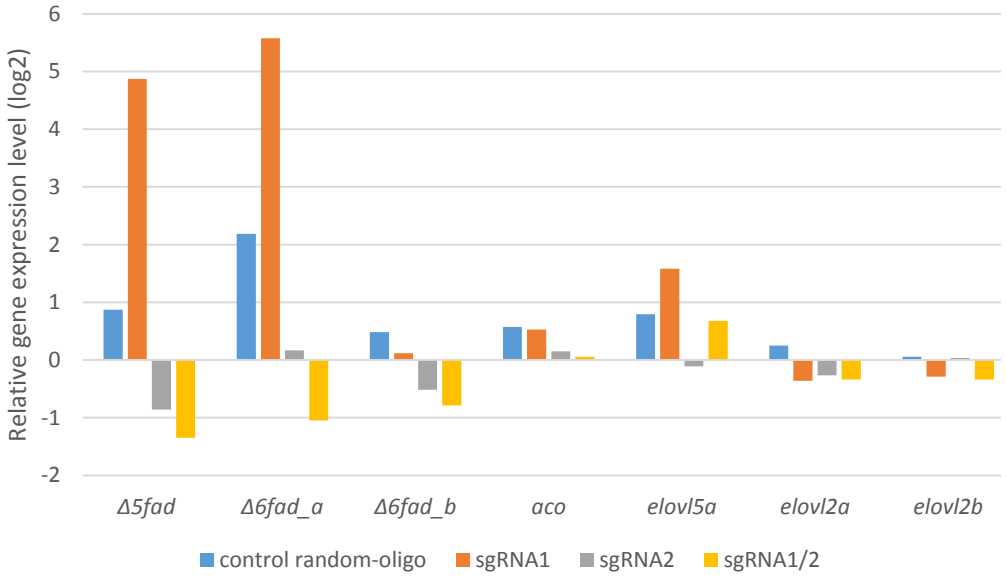


Figure 3.11 Pilot trial 2. Relative gene expression of protein coding genes in trout hepatoma cells transfected by CRISPR-Cas9 system with either control random-oligo, sgRNA1, sgRNA2, sgRNA1/2 (targeting Δ6fad_b) or carried alone. Results were normalized to the non-knockout control respectively and calculated as the relative expression between the target gene and *etif*.

Our results suggest that the protocol of Lipofectamine™ CRISPRMAX™ transfection reagent used in this study may not be the best solution for the gene knockout on trout hepatoma cell line and need further optimization.

The high mortality of the cells in culture after transfection may be primarily in virtue of the formulation of the transfection reagent complex. The volume and proportion of the transfection reagents used in the present study were following manufacture's protocols. However, in the further studies, a gradient experiment could be implemented to explore the best formula of the reagent complex for transfection on the trout cell line.

In addition, increasing sample parallels could give us more significant and robust result. In the next time-course study, we further split the cell culture from the

second trial to study the effect of time on the expression of genes involved in lipid metabolism.

3.2.2 Time-course study

3.2.2.1 Daily variation in expression of genes involved in lipid metabolism

Betancor *et al.*, (2014) have demonstrated significant diurnal variation in expression in the clock gene *bmal1*, transcription factor genes *srebp1, lxr, pparα,* and *pparγ*, and several other lipid metabolism genes in liver of salmon parr. A study with Atlantic salmon hepatocytes also showed that the genes in the omega-3 LC-PUFA pathway had a rhythmic variation in the expression pattern during a 48 hours trial (Kjær et al., 2016, unpublished). In this trial, we therefore wanted to test if the expression of the genes in the omega- 3 pathway changes during 24-hour time span both in non-transfected cell line group and in different transfected cell line groups. To the best of our knowledge, diurnal variation in expression of genes has not previously been documented in the RTH-149 cell line. We further wanted to study if the expression of the genes in the knockout groups was stable or varied over a period of time.

Our results show that there were relatively moderate and few significant differences in the expression of genes involved in lipid metabolism between the three time points measured during the 24 hour duration of experiment in the RTH cell line (Table 3.1). However there was a significant increase in some of the genes at the second time point relative to the first and the third time point and the same trend was shown for most of the genes in the different groups. Although not consistent between the groups, this variation may still indicate that there exists a moderate degree of diurnal variation in gene expression in this cell line. Compared with the primary hepatocytes used in the previous studies, the trout hepatoma cell line in our current study may have some differences at the physiological stand point, since the hepatoma cell line have been subcultured for hundreds of times and always been incubated in dark; this may explain the relatively moderate diurnal variation in the RTH-149 cell line compared to the primary cells isolated from live fish. However, in order to verify with certainty that such diurnal variation exists in the RTH cell line, more time points needs to be included in future studies.

Table 3.1 Daily variation in expression of genes involved in lipid metabolism. Relative gene expression of protein coding genes in trout hepatoma cells transfected differently (non-transfected controls, sgRNA of control random-oligo, sgRNA1, sgRNA2 and sgRNA1/2) at three selected time points (Time point 1: 8:00; Time point 2: 16:00; Time point 3: 0:00). Results were normalized to the non-knockout control at the first time point respectively and calculated as the relative expression between the target gene and *etif*. Data are presented as mean ± S.E. for 3 replicate samples. The different assigned letters are significant difference ($P < 0.05$) between different time points.

Gene	Time point 1 (8:00)				Time point 2 (16:00)				Time point 3 (0:00)			
Cells without transfection												
Δ5fad	0.000	±	0.2324	a	-0.333	±	0.1194	ab	-0.773	±	0.1514	b
Δ6fad_a	0.000	±	0.2679		-0.017	±	0.2912		-0.412	±	0.0852	
Δ6fad_b	0.000	±	0.3325		0.298	±	0.4860		-0.050	±	0.1285	
elovl2a	0.000	±	0.2975		0.140	±	0.3878		-0.231	±	0.0937	
elovl2b	0.000	±	0.2819		0.061	±	0.3392		-0.321	±	0.0851	
elovl5a	0.000	±	0.2895		0.101	±	0.3634		-0.276	±	0.0884	
aco	0.000	±	0.2857		0.081	±	0.3513		-0.299	±	0.0865	
Cells transfected with sgRNA of control random-oligo												
Δ5fad	1.978	±	0.0949	c	3.258	±	0.1602	a	2.660	±	0.0700	b
Δ6fad_a	0.173	±	0.0469	b	0.907	±	0.1100	a	0.384	±	0.1537	b
Δ6fad_b	-1.632	±	0.0073		-1.445	±	0.0608		-1.893	±	0.3775	
elovl2a	-0.729	±	0.0234	b	-0.269	±	0.0852	a	-0.754	±	0.2656	b
elovl2b	-0.278	±	0.0351	b	0.319	±	0.0976	a	-0.185	±	0.2097	b
elovl5a	-0.504	±	0.0292	b	0.025	±	0.0914	a	-0.470	±	0.2377	b
aco	-0.391	±	0.0321	b	0.172	±	0.0945	a	-0.328	±	0.2237	b
Cells transfected with sgRNA1												
Δ5fad	0.247	±	0.1882		0.735	±	0.1952		0.663	±	0.5718	
Δ6fad_a	-0.905	±	0.0813		-0.493	±	0.2258		-0.828	±	0.2958	
Δ6fad_b	-2.057	±	0.3002		-1.722	±	0.2580		-2.320	±	0.0551	
elovl2a	-1.481	±	0.1853		-1.108	±	0.2417		-1.574	±	0.1608	
elovl2b	-1.193	±	0.1302		-0.800	±	0.2337		-1.201	±	0.2277	
elovl5a	-1.337	±	0.1574		-0.954	±	0.2377		-1.388	±	0.1940	
aco	-1.265	±	0.1437		-0.877	±	0.2357		-1.294	±	0.2108	
Cells transfected with sgRNA2												
Δ5fad	1.363	±	0.1482	ab	1.875	±	0.0964	a	0.912	±	0.3683	b
Δ6fad_a	-0.093	±	0.1116		0.146	±	0.0289		-0.380	±	0.3536	
Δ6fad_b	-1.550	±	0.1314		-1.583	±	0.0717		-1.672	±	0.3637	
elovl2a	-0.822	±	0.1143		-0.719	±	0.0373		-1.026	±	0.3556	
elovl2b	-0.458	±	0.1109		-0.286	±	0.0268		-0.703	±	0.3538	
elovl5a	-0.640	±	0.1121		-0.503	±	0.0309		-0.864	±	0.3545	
aco	-0.549	±	0.1114		-0.395	±	0.0285		-0.784	±	0.3541	
Cells transfected with sgRNA1/2												
Δ5fad	0.860	±	0.1862		0.670	±	0.3433		1.052	±	0.2164	
Δ6fad_a	-0.822	±	0.1320		-0.634	±	0.1474		-0.717	±	0.1942	
Δ6fad_b	-2.503	±	0.0838	b	-1.938	±	0.1304	a	-2.485	±	0.1728	b
elovl2a	-1.663	±	0.1067		-1.286	±	0.0890		-1.601	±	0.1834	
elovl2b	-1.242	±	0.1191		-0.960	±	0.1094		-1.159	±	0.1888	
elovl5a	-1.452	±	0.1128		-1.123	±	0.0961		-1.380	±	0.1861	
aco	-1.347	±	0.1160		-1.042	±	0.1021		-1.269	±	0.1874	

3.2.2.2 The effect of silencing of Δ6fad_b on mRNA transcript abundance

The differences in expression of each gene between the different time points in the different groups were very moderate as discussed above. Although not 100% statistically correct, we present here the mean value of the gene expression of the three replicates at the three time-points (even though there were some significant differences between the time points) in order to give an overview illustration on the effects of silencing of desaturase gene Δ*6fad_b* on mRNA transcript abundance (Figure 3.12).

The expression of desaturase genes Δ*6fad_a* and Δ*6fad_b* were significantly suppressed by the CRISPR-Cas9 system targeting gene Δ*6fad_b*. The expression of the elongase genes (*elovl2a, elovl2b* and *elovl5a*) and acyl-CoA oxidase gene (*aco*) was also down-regulated by silencing gene Δ*6fad_b*, indicating the potential positive relation between these protein coding genes. Interestingly, desaturase gene Δ*5fad* was reversely up-regulated; this may suggest a compensatory response of increased gene expression of Δ*5fad* to the Δ*6fad_b* depletion.

Even though both sgRNA1 and sgRNA2 are targeting gene Δ*6fad_b* (with different cleavage sites), the expression patterns shown some variations. In particular, the expression levels of genes in the sgRNA1-treated cells was close to that in the sgRNA1/2-treated (the combined sgRNAs) cells with no statistical significance. The relative expression in the sgRNA2-treated cells was pointing to the same direction, but with significantly lower magnitudes in all of these protein coding genes, except for Δ*5fad* where the sgRNA2-treated cells had greater up-regulation. This finding is in agreement with that choosing sgRNA with different cleavage sites may affect the efficiency of transfection when using CRISPR-Cas systems for genome editing (Sander and Joung, 2014).

Increased expression of Δ*5fad* and Δ*6fad_a*, and decreased expression of Δ*6fad_b* and *elovl2a* were observed in the control random-oilgo-treated cells. This enhance the suspicion in the pilot trials that the random-oligo used in this study may be unqualified as a control oligo and it may target genes involved in lipid metabolism. Multiple control random-oligos rather than single control-oligo could be tested in the future.

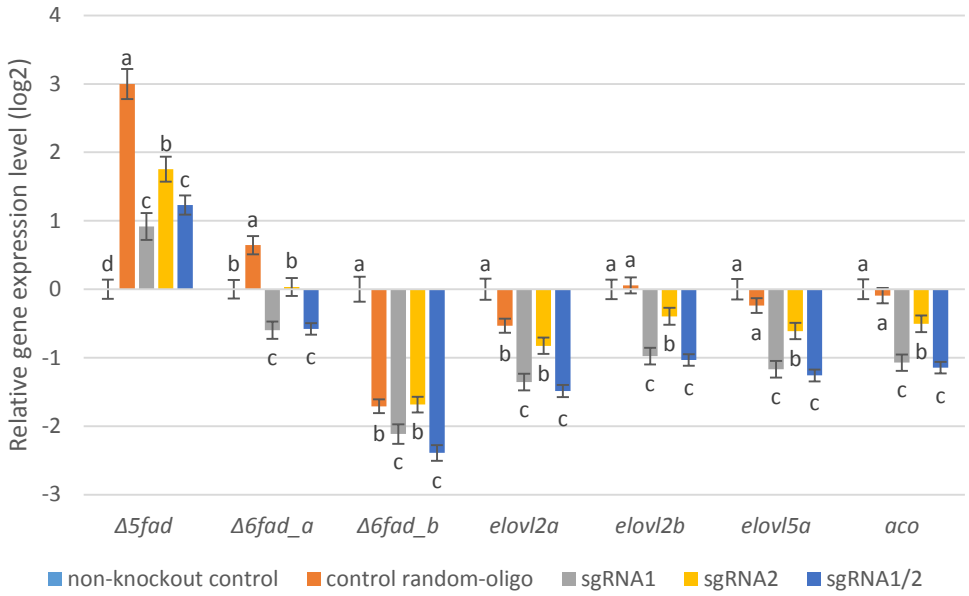


Figure 3.12 Relative gene expression of protein coding genes in trout hepatoma cells transfected by CRISPR-Cas9 system with either control random-oligo, sgRNA1, sgRNA2, sgRNA1/2 (targeting Δ6fad_b). Results were normalized to the non-knockout control respectively and calculated as the relative expression between the target gene and *etif,* each point represents mean ± S.E. for 9 replicate samples. The different assigned letters are significant difference ($P < 0.05$) between different treatments.

Taken together, our result demonstrated reduced expression levels of Δ*6fad_b* 24 hours after transfection but together with a restrained growth rate and a low viability. This may indicate that the knock out protocol used in our study was effective to some extend but perhaps with low transfection efficiencies.

However, to our knowledge, all of the major commercially available transfection reagents are optimized for mammalian cell cultures, including Lipofectamine CRISPRMAX (Thermo Fisher Scientific, USA) that was used in the present study (Yu et al., 2016). Lipofectamine intermediate transfection involves extensive membrane interaction in the form of liposome fusion. Trout cells are cultured at relatively low temperatures (21 ℃) compared to cells of mammal (37 ℃) and lower temperatures may affect the necessary membrane interactions, thus altering liposome fusion and reducing overall transfection performance (Sandbichler et al., 2013). While studying transfected fish cells, it is always advisable to optimize the protocol prior to the experiments. It may increase transfection performance significantly and identify detrimental conditions that affect cell viability in advance.

In the present study, the result may suggest that the manufacture's protocol which is optimized for mammalian cells may not be the best solution for transfection on trout cell line. Since the new Lipofectamine CRISPRMAX is specially developed for delivering Cas9 protein, it is still worthwhile to further optimize the protocol for fish cells. In addition, other transfection reagents could also be taken into consideration. A comparative study with zebrafish cell line has demonstrated that the JetPrime reagent (Polyplus transfection, France) and X-treme HP reagent (Roche) had the relatively better transfection performance than Lipofectamine LTX (Invitrogen) (Sandbichler et al., 2013). Furthermore, electroporation is also a prospective method to be tested.

Our result also disclosed a potential positive relationship between Δ*6fad_a*, Δ*6fad_b*, *elovl2a*, *elovl2b*, *elovl5a* and *aco*, and a potential compensatory response of gene Δ*5fad* to the silencing of gene Δ*6fad_b*.

4 Main findings and conclusions

This present study has shown that both nutritional and environmental factors influence expression of genes involved in regulation of LC-PUFA in liver of Atlantic salmon undergoing parr-smolt transformation.

Our results showed up-regulation of Δ5, Δ6 desaturases and down-regulation of PPARα by introducing VO-enriched diet. In addition, both desaturases and transcription factors that regulate desaturases showed coordinated up-regulation in response to light treatment. Further, we observed that the expression of *pparα* and *srebp-1*, together with the desaturases Δ*5fad* and Δ*6fad_a* was coordinately down-regulated in response to seawater transfer.

This is the first time that microRNAs were observed to be involved in a dynamic regulation of the omega-3 metabolic pathway under different environmental conditions at different life stages of Atlantic salmon. Specifically, we observed a highly coordinated regulatory pattern between *mir-122-5p* and *srebp-1*, and between *mir-33a-3p* and *srebp-2*, indicating they may be regulated by environmental factors similarly.

The complex mixture of feed effects, environmental effects and different life stages/biological phases of the fish in our trial design complicates the interpretation of the results. In the further studies, we could integrate proper control groups to deviate the feed effects from the environmental and ontogenetic effects. We could also increase the frequency of samplings during the fish trial, especially the period when the day length was decreasing, to clarify the changes of fish between the different life stages.

Furthermore, this is for the first time that Lipofectamine CRISPRMAX was tested as transfection reagents on fish cells for gene knockout. Our results showed a potential positive relationship between Δ*6fad_a*, Δ*6fad_b*, *elovl2a*, *elovl2b*, *elovl5a* and *aco*, and a potential compensatory response of gene Δ*5fad* to the gene Δ*6fad_b* silencing. However, due to the restrained growth rate and high mortality during the cell culturing, we suggest that this protocol need further optimization for fish cells.

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APPENDIX

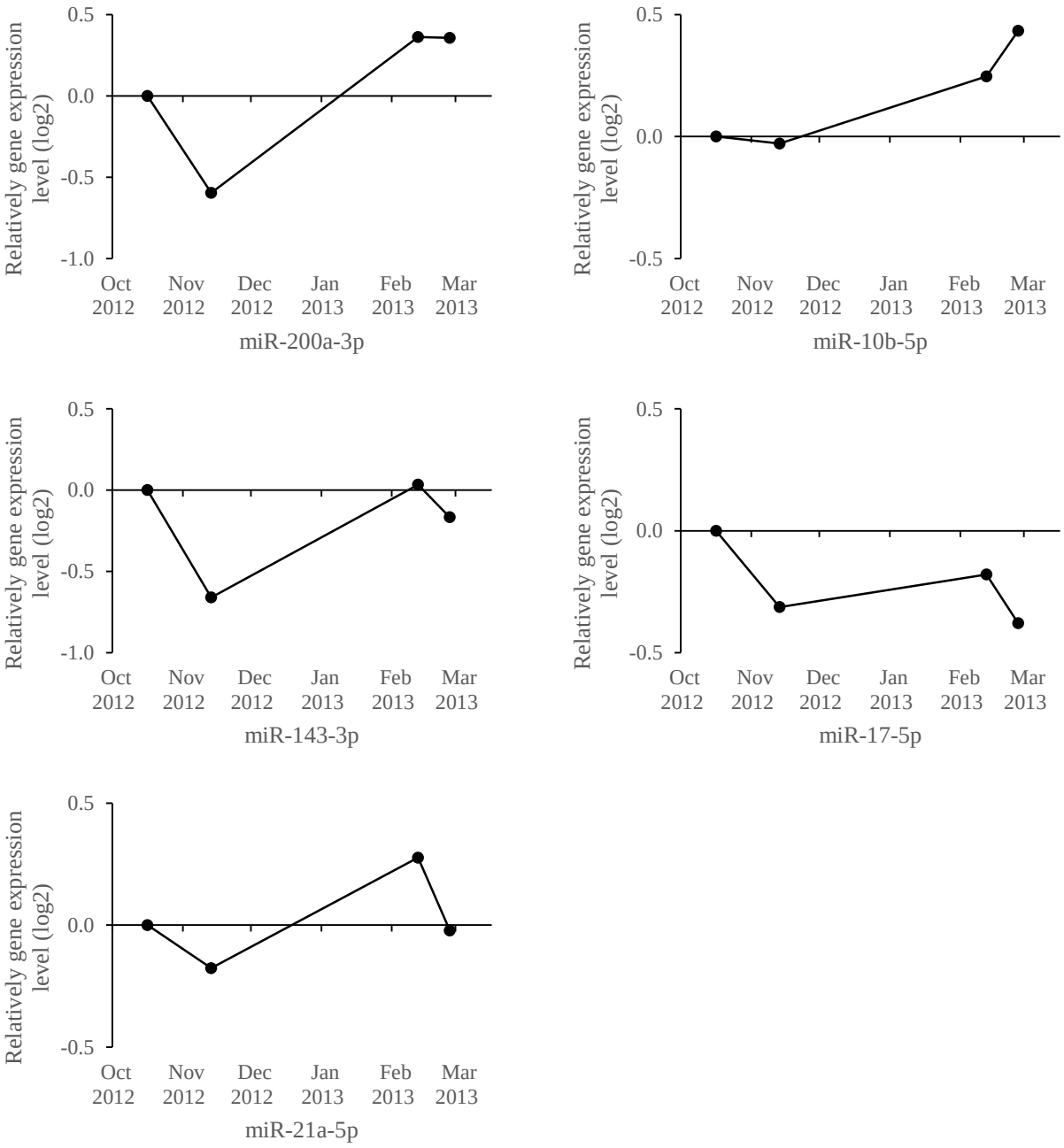

Figure 5.1 Expression of microRNAs that shown no significant change throughout the entire fish trial. Relatively gene expression level of microRNA in liver from Atlantic salmon up to seawater transfer. Results were normalized to the first sampling point respectively and calculated as the relative expression between the target gene and reference gene geometric mean (miR-25-3p and miR-92a-3p), each point represents mean ± S.E. for nine replicate samples. The different assigned letters are significant difference ($P < 0.05$) between different sampling points (30 October 2012, 27 November 2012, 26 February 2013 and 12 March 2013).