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A study of the gene expression profiles in muscle, liver and intestine of Atlantic salmon fed different omega- 3 fatty acid levels and with red and pale fillet color.

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Abbreviations

ABC: ATP-binding cassette

ASK: Atlantic salmon kidney cell line

BCMO1: Beta-Carotene 15,15'-Monooxygenase 1

BCMO2: Beta-Carotene 9',10' Oxygenase

Bp: base pair

cDNA: complementary DNA

CCE: carotenoid cleavage enzyme

CCO: carotenoid cleavage oxygenase

CD36: Cluster of differentiation 36

CRISPR/Cas9: Clustered regularly interspaced short palindromic repeats/associated protein 9

Ct: cycle threshold

DNA: Deoxyribonucleic acid

DSB: double strand break

FBS: fetal bovine serum

FM: fish meal

FO: fish oil

mRNA: messenger RNA

NHEJ: non homologous end joining

PAM: protospacer adjacent motif

PCR: Polymerase chain reaction

qPCR: quantitative polymerase chain reaction

RNP: Ribonucleoprotein

sgRNA: single guide RNA

Srb1: Scavenger receptor class B, member 1

Acknowledgment

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Sina Velzi

Abstract

The economic value of Norwegian salmon production is closely associated with various quality factors of salmon meat like red fillet. Carotenoids are the main pigment in the fish fillet which need to be added to the feed. Astaxanthin is the main carotenoid added to salmon feed but its transport from the intestine to the liver and muscle is not completely understood. However, studies have shown not only transportation of this pigment is dependent on the diet but also, genome-wide association studies (GWAS) showed genomic regions in the salmon genome connected with the variation in fillet redness.

In this experiment, two different diets including a standard feed with a composition like commercial feed and a high Omega-3 fatty acid diet, were fed to two groups of salmon previously selected based on high/low fillet pigment. Further, samples from groups were selected for fillet color analysis by VIS/NIR spectroscopy and astaxanthin measurements. Moreover, genes involved in the metabolism of astaxanthin were studied by microarray and qPCR analysis. *In vitro* and *in vivo* trials using Atlantic salmon kidney cell line (ASK) and newly fertilized salmon eggs (1-2 cell stage), were initiated to establish methods to study the function of selected genes using gene editing by the CRISPR/cas9 techniques, respectively. Atlantic

Results showed a significant difference in the expression of *bcmo2-like*, *bcmo2c* genes between dietary groups and *bcmo1* gene between high and low fillet pigment groups of the intestine. Moreover, a significant difference in expression of the *bcmo1-like* gene was observed in the liver between the two dietary groups. Finally, a significant difference between *bcmo1* and *cd36* genes was observed in muscle tissue between dietary groups and *bcmo1* between high and low fillet pigment. This study has identified that both the omega-3 fatty acid levels in the feed and differences in muscle pigmentation significantly influenced the expression of several genes related to carotenoid and/or lipid metabolism. The second major finding based on gene expression analyses was that the liver was identified as an important organ in astaxanthin metabolism.

Taking all together, astaxanthin metabolism might be organ dependent in Atlantic Salmon. Moreover, further research should focus on the establishment of efficient CRISPR/cas9 techniques in ASK cell and salmon embryos to further confirm current results.

1. Introduction and literature review

1.1. Salmon production industry

Aquaculture is one of the fastest-growing industries for food production globally, and its products compose one of the main sources of protein for human consumption. The aquatic food industry has grown steadily and demonstrated substantial growth in GDP contribution in recent years (Richardsen, Myhre & Tyholt, 2019). Moreover, according to a published report from the Norwegian seafood council (2020), the revenue from the aquatic food industry has reached double the amount from 2009. Norwegian salmon farms are the main salmon producers on a global scale and among species exported, the Atlantic salmon (Salmo salar) is standing in the first place.

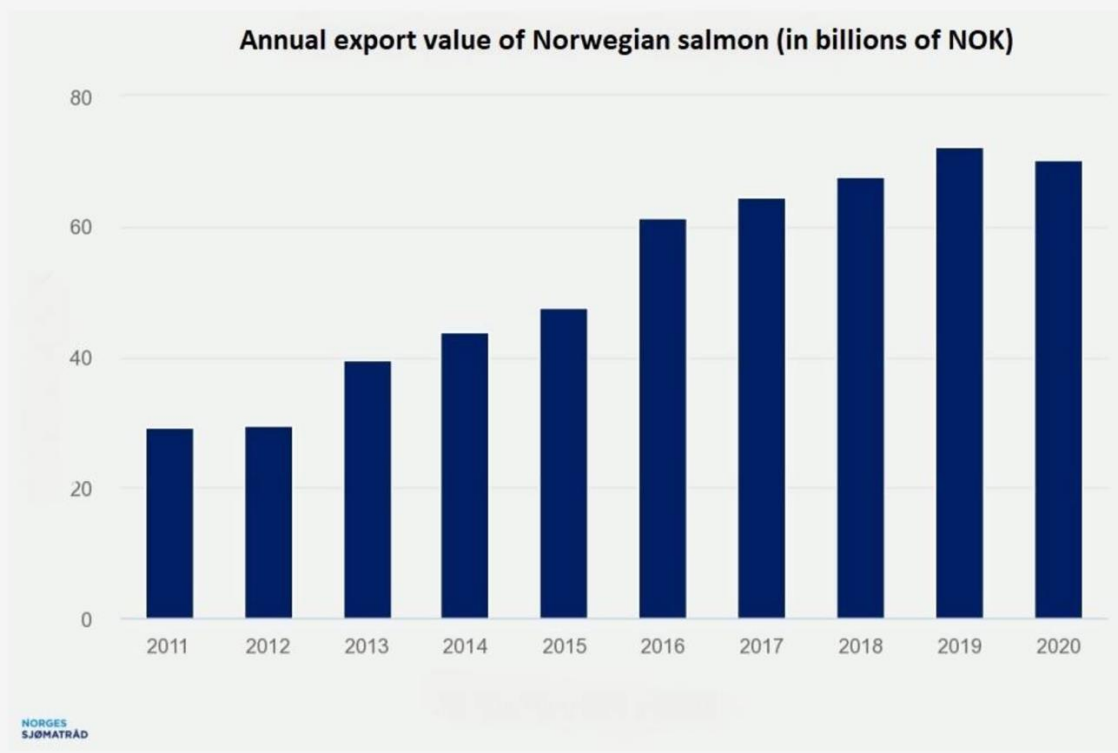


Figure 1.1. Annual export of Norwegian salmon in billions of NOK, the figure is retrieved from https://www.fishfarmingexpert.com/article/norwegian-salmon-export-value-second-highest-ever-in-2020/.

1.2. The red pigment of salmon fillet

All species belonging to the Oncorhynchus, Salvelinus, Salmo, and Parahucho genera of the Salmonidae family have red flesh color which is unique compared to other fish species. The economic value of Norwegian salmon production is closely associated with various quality factors of salmon meat. For instance, texture, smell, and characteristic fillet color (Espe,2008) substantially affect consumers' interest when buying raw (Anderson, 2000) or smoked salmonid products (Gormley, 1992; Rørå et al., 2004b). In fact, consumers prefer to pay higher prices for fillet with red color than pale color (Alfnes, Guttormsen, Steine & Kolstad, 2006). Salmon fish acquires red fillet color by feeding on crustaceans that contain carotenoid pigments in their flesh and shell. Crustaceans are not able to synthesize these pigments. In fact, they obtain them through other food resources like marine phytoplankton and microalgae (Lorenz & Cysewski, 2000). Carotenoids which are consisting of polyene hydrocarbon chains considered a large group of pigments and are present in different diets. These pigments are lipid-soluble compounds and believed to be as the main pigment in the fish which cannot be synthesized by them. Canthaxanthin and astaxanthin both are subcategory of Xanthophylls (a group of carotenoids) are involve in salmon fillet pigmentation (Storebakken & No, 1992; Torrissen, 1989). Mentioned dietary pigments are metabolized and deposited in salmon fillets (Schiedt, Leuenberger, Vecchi & Glinz, 1985). Since carotenoids and their metabolites are involved in more than 100 cellular processes like reproduction, growth, vision, and lipid metabolism they are biologically so essential (Eroglu and Harrison, 2013). Moreover, carotenoids have other benefits like being a strong antioxidant (McNulty et al., 2007) which inhibit lipid peroxidation (Dose et al., 2016). Astaxanthin comprises approximately 90% of salmon flesh's total carotenoid. There are three main natural sources of astaxanthin including microalgae *Haematococcus pluvialis*, yeast *Phaffia rhodozyma*, and products made from crustaceous. However, mentioned sources of astaxanthin are pricey and less effective than the synthetic source of astaxanthin used in diets of farmed salmon. Since pigment deposition in different fish species is not at the same level, the flesh colour varies from pink to red colour. There are different factors influencing the redness of fish flesh. For example, differences in absorption capacities for carotenoids in the digestive system in salmonids most likely play a crucial role in determining the differences in flesh colour between fish species. Atlantic salmon muscle deposits only approximately 10% of the astaxanthin from the feed (Bjerkeng, Hatlen and Wathne, 1999). It is shown that a low absorption rate of dietary astaxanthin in the intestine is one important factor explaining the low deposition of dietary astaxanthin in fillets (Ytrestøyl

et al., 2006) (Bjerkeng et al., 1997). Also, studies have shown there is a correlation between intraperitoneal injection of astaxanthin and increased level of astaxanthin in plasma of Atlantic salmon and rainbow trout. For instance, the level of astaxanthin in muscle and plasma has increased up to 15 times and 20 times, respectively (Ytrestøyl and Bjerkeng, 2007). Hence, since the level of astaxanthin in muscle and plasma increased due to the injection, it indicates uptake and binding capacity of astaxanthin in muscle cannot be the main limiting factor in astaxanthin retention in the muscle. In fact, lower retention of astaxanthin in muscle can also be due to metabolic conversion of astaxanthin to other metabolic products in intestine and liver in addition to lower absorption of astaxanthin in the intestine. In another survey, astaxanthin was injected into white flesh colour fish. By the injection, the astaxanthin level increased in plasma but, it did not change in muscle. In fact, it showed astaxanthin binding receptors in white-fleshed fish are not as high as in red-fleshed fish (Ytrestøyl and Bjerkeng, 2007).

Figure 1.2. Fillet color difference between wild salmon on the left, farmed salmon on the right, the figure is retrieved from: https://laurensworldofwellness.wordpress.com/tag/wild-salmon/

1.3.The physiological function of astaxanthin

Astaxanthin is deposited in the skin, gonads (female), and flesh of Atlantic salmon in different life stages. When salmon is in the young life phase, astaxanthin is deposited in the skin while, at the mature stage, astaxanthin is deposited mainly in muscle (Bjerkeng, Storebakken, and Liaaen-Jensen, 1992). At the next life stage where they get sexually maturated, astaxanthin is

transferred from muscle to skin probably for attracting mating partners (March et al., 1990). Moreover, astaxanthin transfers to the eggs and protects them from lipid peroxidation. During the time astaxanthin is stored in different tissues of the body, it is either bound in protein conjugates or esterified to fatty acids (Hussein et al., 2006). Also, in one study after salmon were subjected to some stress conditions, fillet color ended in a paler muscle which indicates muscle cells act as temporary storage for astaxanthin, and astaxanthin is released from the cells when needed as an antioxidant under stressful situations (Trine Ytrestøyl et al., 2019). Moreover, since astaxanthin has a powerful antioxidizing capacity by free radical scavenging, it is assumed to have positive health effects on humans (Dose et al., 2016; Higuera-Ciapara, Felix-Valenzuela L Fau - Goycoolea & Goycoolea, 2006). However, different sources of astaxanthin are costly and cover almost 15% of total feed cost (Prendergast, 1994). Different fish like freshwater fish (Gross and Budowski, 1966) and marine fish (Yamashita, Arai, and Matsuno, 1996) have enzyme systems that enable them to convert astaxanthin to provitamin A. The provitamin A feature of astaxanthin is not only seen in Atlantic salmon; but, also has been seen in other fish species which shows this metabolic conversion is present in more fish species than it was believed previously (Guillou et al., 1989). The metabolism regarding conversion of astaxanthin to vitamin A is not clear; but, studies have shown idoxanthin (3,3',4'-trihydroxy- β,β-carotene-4-one) is a dominant metabolite in plasma after astaxanthin consumption in Atlantic salmon (Aas, Bjerkeng, Storebakken and Ruyter, 1999).

1.4. Factors affecting salmon fillet pigmentation

1.4.1. Diet formulation

The huge rise in salmon production resulted in higher plant-based feed production which comprised only 25% of marine-based material in 2016 (Aas, Ytrestøyl, and Åsgård, 2019). By feeding a diet supplemented with astaxanthin, the amount of the pigment and redness rate of the fillet increases. Nonetheless, although the astaxanthin supplementation level has increased in the feed recently, flesh redness has decreased. For instance, in 2009 when 30-40 mg of astaxanthin per kg feed was added to feed, it could be ended up in 7 mg/kg salmon muscle. However, in 2019, almost 50-70 mg/kg of astaxanthin per kg feed is required for the resulting 6 mg/kg in salmon muscle (Trine Ytrestøyl et al., 2019). One explanation could be replacing feed mainly from marine-based feed with plant-based feed. Indeed, altering feed to plant-based feed has led to a lower amount of cholesterol, phospholipid, vitamin A and of long-chain

polyunsaturated fatty acids (n-3 LC PUFA) Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) and a higher level of dietary fiber in the feed. All these mentioned variations can affect how much astaxanthin is stored from the feed in the muscle (Desmarchelier and Borel, 2017; Lutfi et al., 2022). Hence, proper diet formulation is critically important to achieving redder salmon muscle. Atlantic salmon is known for its high content of long-chain polyunsaturated fatty acids (n-3 LC PUFA) Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in edible muscle. These fatty acids are mainly found in marine ingredients like fish oil (Colombo et al., 2017) (Qian, Hart, and Colombo, 2020). The rapid growth in aquaculture has led to a decrease in EPA and DHA in Atlantic salmon diets (Sprague, Dick and Tocher, 2016). Furthermore, recent studies suggested reduced EPA and DHA level has a negative impact on flesh quality including the red color (Lutfi et al., 2022) (Kousoulaki et al., 2020).

1.4.2. Other related factors

Many other factors affect salmon flesh colour such as pigment type (Buttle et al. 2001; Skrede & Storebakken 1986; Storebakken et al. 1987), genetic background (Torrissen and Naevdal, 1988), seasonal changes (Mørkøre and Rørvik, 2001), and stressors before slaughtering like starvation (Einen and Thomason, 1998; Erikson and Misimi, 2008; Mørkøre et al., 20). Previous research has established that the color of salmon flesh diminishes while the spawning period (Bjerkeng et al., 1992). Moreover, the lower temperature has adverse impacts on astaxanthin digestibility. In fact, digestibility decreases up to 10% at low temperatures (Ytrestøyl et al., 2005).

1.5.Astaxanthin metabolism

The biochemical and physiological mechanisms engaged in the absorption, tissue deposition, and metabolism of astaxanthin are not completely understood (Schmeisser et al., 2021). Approximately 55% of astaxanthin in the salmon diet, emitted from the digestive system. Also, from the absorbed amount of astaxanthin, 50% of it is metabolized; consequently, lower than 10 % of carotenoids remain in fillet (Torrissen et al., 1989; Storebakken and No, 1992; Bjerkeng and Berge, 2000). In addition, an investigation has shown that according to feeding rate, astaxanthin digestibility varies from 14.5% to 38% (Rørvik et. al. 2010). In order to

understand the mechanism behind salmon flesh coloration, it's crucial to increase the knowledge regarding both absorptions in the gut, enzymatic cleavage, and muscle deposition of astaxanthin. Atlantic salmon pyloric caeca and midgut are important parts of the intestine responsible for astaxanthin absorbance (White, Ørnsrud & Davies, 2003). By consumption of carotenoid-containing foods, carotenoids are released from their food matrix and incorporated into mixed micelles consisting of lipids and bile components (Parker, 1996). Astaxanthin absorption was earlier thought to happen mainly by passive diffusion (Hollander and Ruble, 1978). However, if carotenoid uptake from the digestive system is similar to the mammalian pathway, it suggested several apical membrane proteins are involved in active transport at enterocytes, including scavenger-receptor class B-type I (*srb1*) (Sun, 2012), and Cluster of differentiation 36 (cd36) (Borel et al., 2013). From the provitamin-A carotenoids arrived on the basolateral side of the enterocyte, some portion undergoes centric cleavage by bcmo1, producing retinal. At the following stage, lecithin: retinol acyltransferase (LRAT) esterifies retinol produced at the previous stage (O'Byrne et al., 2005). Then, retinyl esters were produced combined with non-cleaved carotenoids (intact carotenoids) and fatty acids. As a result, the accumulation of fatty acids, non-cleaved carotenoids, and retinyl esters are packed in chylomicrons which is directly secreted to the fish portal vein (D'Ambrosio, Clugston and Blaner, 2011). Also, in mammalian less apolar β-carotene metabolites, which are produced by eccentric cleavage by BCO2, are secreted in the portal blood therefore directly reach the liver (Harrison, 2012). Astaxanthin metabolism in the liver is to a large degree unknown in fish but can probably be repacked in VLDL and released to blood circulation. However, there are some studies indicating that astaxanthin can also be transported in blood circulation through binding to circulating albumin or high-density lipoprotein (HDL) before being taken up into the muscle (Trigatti and Gerber, 1995) (Bjerkeng et al., 1997) (Aas, Ytrestøyl and Åsgård, 2019). Thus, chylomicrons, serum albumin, and high-density lipoproteins (HDL) most likely play important roles in astaxanthin transportation in the bloodstream, although their exact roles are not yet determined in salmon (Fielding and Fielding, 2002). Also, the mechanism of how astaxanthin enters the muscle is not known yet. Some studies indicate by having a high level of astaxanthin in the blood of the white-fleshed fish, the fillet remains white. Thus, carotenoid metabolism regarding entrance to the muscle cells is assumed to be carried out through a salmonid-specific transport protein pathway (Ytrestøyl and Bjerkeng, 2007). Following astaxanthin entrance into the muscle, f-actin filaments help pigment deposit in the muscle cell. Carotenoids that are produced by hydrolysis of esterified astaxanthin in lipoproteins will bind to the actomyosin

complex of muscle fibers and result in the red color of flesh (Aas, Bjerkeng, Storebakken and Ruyter, 1999) and (Saha et al., 2005).

1.6.Genomic markers relevant to fillet pigmentation

It is shown that by keeping a salmon breeding population in the same environment and feeding them the same diet composition, there will be large individual differences in red fillet color (Norris and Cunningham, 2004). One logical explanation for individual differences in fillet color, can be the presence and or expression of different genes. Indeed, genome-wide association studies (GWAS) showed there are individual variations in genomic regions of the salmon connected with variation in fillet redness. This “red fillet phenotypic trait” is assumed to be controlled by specific gene loci (Rajasingh, Gjuvsland, Våge and Omholt, 2008). One of these gene loci regions is positioned on chromosome 26 and contains two genes coding for the enzymes b-carotene oxygenase including b-carotene-15,15'-monooxygenase 1 (*bcmo1*) and its paralog β-carotene-15,15'-monooxygenase 1 like (*bcmo1-like*) (Baranski, Moen and Våge, 2010). The enzymes, *Bcmo1,* and *bcmo1-like,* are considered carotenoid cleavage oxygenases (CCO). These are two major enzymes that have a crucial role in carotenoid metabolism. As a result, mentioned enzymes play an important role in fillet color variation at salmon. Mentioned enzymes are responsible for metabolic conversion of astaxanthin into two vitamin A molecules, and thereby less astaxanthin are available for deposition in salmon muscle (Shete and Quadro, 2013). The exact mechanism of *bcmo1* in salmon is not clear however, by comparing *bcmo1* and *bcmo1-like* protein models they are assumed to have the same functions with differing substrate specificity. Another study demonstrated that the *bcmo1* level in the intestine of pale fleshed salmon is higher than in the intestine of red-fleshed salmon (Zoric N, Torgersen J, Grammes F, von Lintig J, Våge DI., 2017). Furthermore, the expression of the *bcmo1-like* gene showed to be more in red salmon than pale fleshed salmon (Helgeland et al., 2019). Thus, both paralogues are suggested to be closely associated with astaxanthin conversion which negatively affects salmon flesh coloration.

1.7.Receptors and tissue uptake of astaxanthin

In addition to passive diffusion, Atlantic salmon have some proteins that enable them to absorb β-carotene, α-carotene, lutein, and β-cryptoxanthin. The most important protein is scavenger receptor class B, member 1 (*srb1*) and its paralog *srb1-2* which is highly expressed in the intestine and helps carotenoid absorption and carotenoid transport (Kleveland et al., 2006) (Sundvold et al., 2011). Animal studies showed that *srb1* has a crucial role in the intestinal absorption of dietary lipids (Hauser et al., 1998). Similarly, the expression of *srb1* mRNA in the mid gut of Atlantic salmon has been reported at a considerable level (Thuahnai, Lund-Katz, Williams, and Phillips, 2001). *Srb1* which is classified at the ATP-binding cassette (ABC) transporter superfamily, recognizes and binds a varied set of ligands with low substrate specificity; as a result, mediating the transport of many lipophilic substances (Urban et al., 2000). One study in DrosophiLa showed gene encoding *srb1* homologous protein is crucial for the cellular uptake of carotenoids and led to the Importance of *srb1* in carotenoid transport (Kiefer, Sumser, Wernet, and von Lintig, 2002).

1.8.Genome editing as a tool for study gene function

Genome editing is a method where a DNA sequence is inserted, deleted, modified, or replaced in the genome of a living organism. There are different methods for genome editing, including zinc finger nucleases (ZNFs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats CRISPR/CRISPR associated protein 9 (khalil, 2020). Among mentioned methods, the CRISPR method is more precise and cheaper than others (Gratacap, Wargelius, Edvardsen, and Houston, 2019). CRISPR gene editing method has been tested in several fish species (Cleveland, Yamaguchi, Radler, and Shimizu, 2018), particularly in aquaculture freshwater fish like Cyprinus carpio (Zhong et al., 2016). CRISPR and its various elements are constituting a bacterial genome editing system which utilized to modify genes present in cells and different tissues. The CRISPR system is containing two main components including guide RNA and cas-9 nuclease, and together these constitute a ribonucleoprotein (RNP) complex. The guide RNA can be designed with desired base pairs that perfectly match the target DNA site and are located close to a protospacer adjacent motif (PAM) (Hwang et al., 2013). The Cas-9 nuclease works as a pair of scissors and makes double-strand breaks on desired DNA sequence. Following cas9 protein attaching to the target strand

of DNA, conformational modifications occur leading to targeted DSB breaks 3-4 nucleotides upstream of the PAM (Sternberg, LaFrance, Kaplan, and Doudna, 2015). After DSB occurred, broken chromosomal DNA can be repaired by different mechanisms like non-homologous end joining (NHEJ) or homology-directed repair (HDR) pathways. Consequently, NHEJ and HDR can introduce mutations as a result of error occurrence in the mechanism of cellular DNA repair (Parsaeimehr, Ebirim, and Ozbay, 2022).

1.9. The aim of the study

The main aim of this thesis was to characterize differences in gene expression profile in muscle, liver, and intestine of Atlantic salmon with red and pale fillet fed high and low levels of omega-3 fatty acids.

The main goal of this thesis was achieved using the following subgoals:

- Select gene candidates of importance to flesh color based on gene expression profiles.
- Select gene candidates of importance to the omega-3 fatty acid effects on flesh color.
- Optimize the method for gene editing by CRISPR/cas9 using *in vitro* model of the Atlantic salmon kidney (ASK) cell line.
- Optimize the method for gene editing by CRISPR/cas9 in Atlantic salmon embryos to investigate the function of genes involved in salmon fillet pigmentation.

2. Materials and methods

2.1. Dietary treatment and feeding scheme

In June 2020, approximately 900 Atlantic salmon with known pedigree and high and low pigment genotypes from the Mowi breeding nucleus in Norway were distributed across six 5x5m^2 sea pens located in Averøy research station, Norway as part of the EU project AquaImpact. Atlantic salmon from high and low pigment genotypes were fed two different diets including a control diet (CT) containing a standard industry-relevant level of the Omega-3 fatty acids EPA and DHA and a diet containing a higher level of EPA and DHA from a start weight of approximately 200 g in June 2020 to a slaughter weight of approximately 5 Kg in July 2021. All animal handling procedures complied with the National Guidelines for Animal Care and Welfare published by the Norwegian Ministry of Education and Research [Norwegian Food Safety Authority (FOTS), approval 16059].

The two diets were provided by Skretting ARC. During this experiment, the feeds were produced in several batches to fit the different sizes of fish. However, since the feed batch produced for fish from 2.5 kg to 5 kg is more important for final fillet composition than the earlier feed batches, Table 2.1 shows a detailed ingredient composition of the experimental diets for the last growth period. An overview of experimental design is presented in Figure 2.1.

Table 2.1. Two different diets used in the experiment. CT diet supplemented with the standard level of Omega-3 fatty acids and omega diet supplemented with higher level of Omega-3 fatty acids.

Diet formulation	CT diet	Omega diet
Fish meal Scandinavian	5.00	5.00
Wheat gluten	22.00	22.00
Faba bean	6.00	6.00
Soybean concentrate	10.34	10.51
Sunflower meal	7.00	7.00
Wheat	8.35	7.50
Fish oil S.American	7.62	
Fish oil Scandinavian		7.30
Rapeseed oil	22.94	20.53
Veramaris microalgae oil		3.44
Linseed oil	3.00	3.00
Premixes	6.49	6.51
Water/Moisture change	1.27	1.21
Targets		
EPA+DHA, % of FA	6.00	10.00
n6/n3	0.95	0.76

2.2. Experimental design

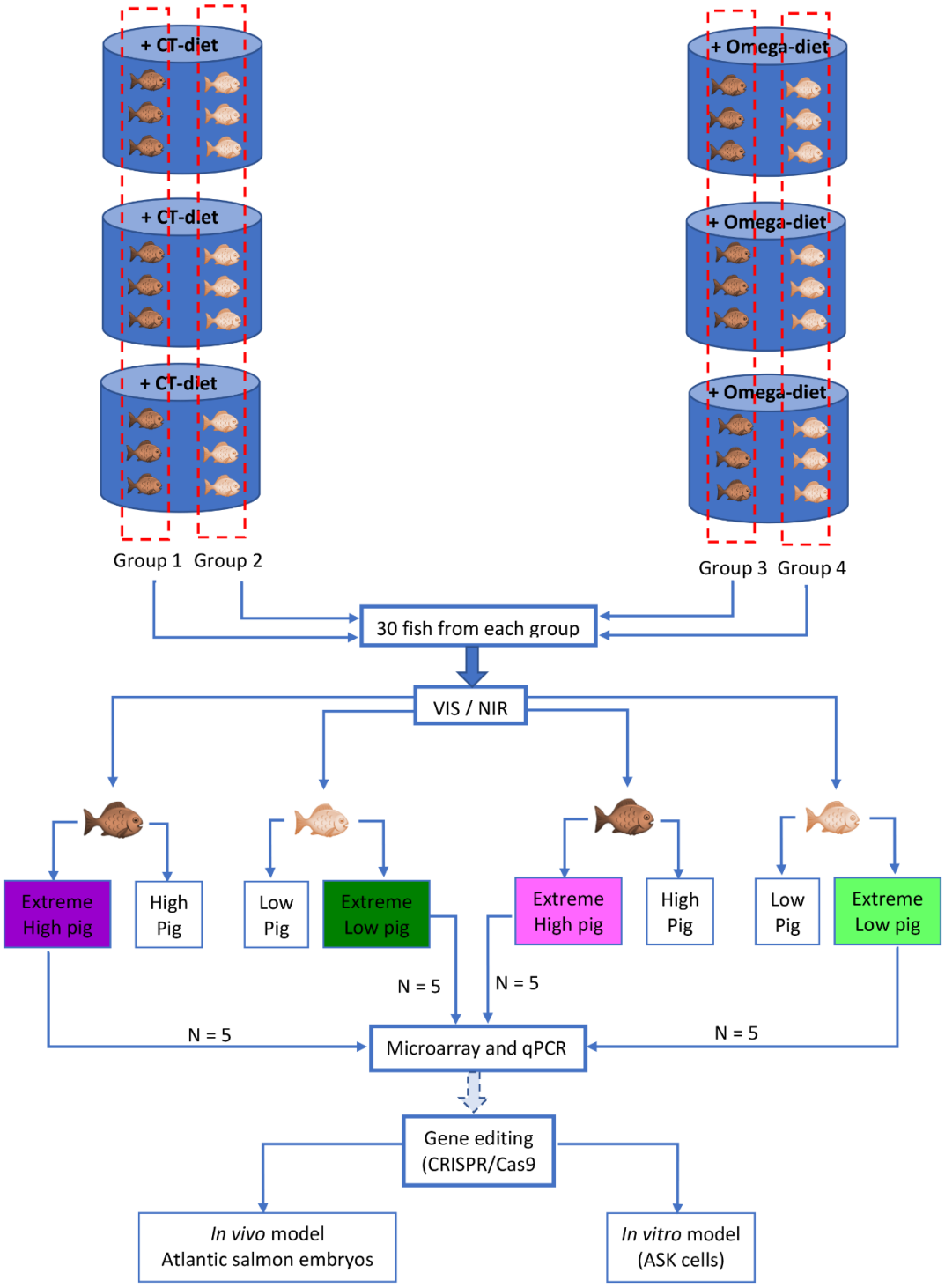


Figure 2.1. The overview and experimental design of the study. Fish with two different pigment phenotypes (high and low, representing by dark and light brown, respectively) were reared at six sea pens. Each sea pen contained both high and low pigment phenotype fish. Three of the sea pens fed Omega diet (+Omega-diet: diet supplemented with high level of omega-3 fatty acid including EPA and DHA) and the other three sea pens fed Control diet (+CT-diet: commercial feed with a standard level of omega-3 fatty acid including EPA and DHA). Following slaughtering, 30 fish from each group were sampled for VIS/NIR spectroscopy. Later, five samples with extreme pigment values from each group were selected for gene expression analysis using microarray and qPCR. Finally, gene editing with the CRISPR technique was conducted using *in vivo* and *in vitro* models.

2.3. Sampling

Prior to sampling, all fish were anesthetized on-site with MS-222 (Metacaine 0.1g/L, Alpharma, UK) and sacrificed by a blow to the head, gill arched cut, and bled out in big containers filled with seawater (3~4 fish per box at the maximum). Salmon's body length and body weight were recorded individually before being gutted. Norwegian quality cut (NQC) of muscle were taken and frozen at -20°C for later analyses of astaxanthin and fatty acid compositions. Small tissue samples of muscle, mid intestine, and liver were sampled on RNA later for later analyses of gene expression. Moreover, gutted salmon were filleted and the pigment content in the fish fillet was measured by scanning with an online VIS/NIR system.

2.3.1. Sample selection for VIS/NIR analysis

All 900 experimental fish were analyzed by on-site VIS/NIR determination of fillet color (see description paragraph below) and based on the results from these analyses, 30 fish from each group were selected for presentation of VIS/NIR results in this thesis (Table 2.2).

Table 2.2. An overview of the naming of the 4 experimental groups. High and Low are salmon group with high and low flesh color, respectively. The CT and omega are two different diets explained in Table 2.1.

Group	Diet
High-Ct	30 fish (originally selected for higher fillet pigmentation), whose fed with standard level of Omega-3 fatty acid (CT)
Low-Ct	30 fish (originally selected for lower fillet pigmentation), whose fed with standard level of Omega-3 fatty acid (CT)
High-Omega	30 fish (originally selected for higher fillet pigmentation), whose fed with higher level of Omega-3 fatty acid (Omega)
Low-Omega	30 fish (originally selected for lower fillet pigmentation), whose fed with higher level of Omega-3 fatty acid (Omega)

2.3.2. Sample selection for microarray and qPCR analysis

In order to create high contrast in color between the experimental groups used for gene expression analyses; five fish from the high VIS/NIR pigment phenotype in the high pigment genotype and five fish of low VIS/NIR pigment phenotype in the low pigment genotype within each of the two diet groups were selected (Table 2.3) and labeled as shown in the Table 2.4.

Table 2.3. The Overview and number of fish selected for microarray analyses from two phenotype and dietary groups.

	Control diet	High Omega-3 fatty acid diet
High pigment genotype	5 fish with high VIS/NIR pigment phenotype	5 fish with high VIS/NIR pigment phenotype
Low pigment genotype	5 fish with low VIS/NIR pigment phenotype	5 fish with low VIS/NIR pigment phenotype

Table 2.4. The labeling of selected fish from different groups. Here high and low represent high and low pigment genotype and ex subscript indicates the highest fillet pigment within each dietary group and CT and omega indicate control and Omega-3 diet respectively.

$High_{ex}$-CT	Phenotypically selected fish (with extreme high fillet color, evaluated by VIS/NIR) within the fish group (originally selected for high fillet pigmentation), and fed standard level of Omega-3 fatty acid (CT)
$High_{ex}$-Omega	Phenotypically selected fish (with extreme high fillet color, evaluated by VIS/NIR) within the fish group (originally selected for high fillet pigmentation), and fed with high level of Omega-3 fatty acid (Omega)
Low_{ex}-CT	Phenotypically selected fish (with extreme low fillet color, evaluated by VIS/NIR) within the fish group (originally selected for low fillet pigmentation), whose fed with standard level of Omega-3 fatty acid (CT)
Low_{ex}-Omega	Phenotypically selected fish (with extreme low fillet color, evaluated by VIS/NIR) within the fish group (originally selected for low fillet pigmentation), whose fed with high level of Omega-3 fatty acid (Omega)

2.4. Color measurement by visible and near infrared (VIS/NIR) spectroscopy

The left fillet was gently wiped clean for liquids and scales and the pigment (mg kg− 1 astaxanthin) and fat (%) content in the fish fillet were measured by scanning the fillet with an on-line VIS/NIR system (QVision 500, TOMRA Sorting Solutions, Belgium) which is an industrial hyperspectral imaging scanner. This scanner records spectral images in the visible and near-infrared range of 460−1040 nm with a spectral resolution of approximately 20 nm. The VIS/NIR instrument was based on interacting measurements where the light was transmitted into the sample and then backscattered to the surface. The optical sampling depth in the salmon fillet was approximately 10-15 mm. The scanner was placed 30 cm above the conveyor belt so there was no physical contact between samples and the instrument. Each VIS/ measurement took less than 1 sec. The image obtained represents the mean spectrum from each fillet that was used to calculate the average fat and pigment content of the whole fillet (Folkestad et al., 2008) (only the pigment values are included in this thesis). Following scanning 30 randomly selected fish salmon with astaxanthin concentrations higher than 8 mg kg− 1 within each diet group were considered high and 30 randomly selected fish within each diet group with less than 6 mg kg^{-1} were considered low pigment (120 experimental fish in total) (Table 2.5). Overall, 30 samples were selected as reference analysis to check whether the VIS/NIR measurements were correct.

Table 2.5. The VIS/NIR results of 30 randomly selected fish within each experimental group (120 experimental fish in total). However, for microarray, qPCR, astaxanthin and fatty acid analyses, 5 salmon within each group with astaxanthin, concentrations higher than 8 mg kg− 1 were selected as high and 5 fish with less than 6 mg kg− 1 within each group were considered low pigment (see Table 3.1 in result section).

Group	Mean (mg kg− 1)	SD	Min	Max
High-CT	6,8	0.8	4.4	9.7
Low-CT	6,4	0.7	5.2	9.1
High-Omega	7,2	0.8	5.5	9.4
Low-Omega	6,9	0.8	4.9	8.8

2.5. Method for analyses of astaxanthin, idoxanthin, and fatty acid composition of muscle

Astaxanthin and idoxanthin levels were analyzed in the selected 20 samples (5 per experimental group) of Norwegian Quality Cut cutlets by Nofima laboratory according to the method described by Bjerkeng et al., 1997. In summary, a mixture of distilled water, methanol, and chloroform was homogenized using the ratio of 1:1:3. following mixing, centrifuged for 10 minutes at 300 rpm. Next, 5 ml of the chloroform phase was removed and re-dissolved in acetone/n-hexane/methanol (20:80:0·1), filtered through a 0·45-μm filter, and analyzed isocratically by an HPLC (Spherisorb S5-CN nitrile column; PhaseSep). After repeating this procedure, the extraction procedure was repeated twice. Standard samples were prepared from crystalline all-E-astaxanthin (Hoffmann-La Roche Ltd.). At the next step, their concentrations were calculated by spectrophotometer (UV-260). For this measurement a molar absorptivity E1 %, 1 cm = 2100 (λmax = 470 nm) in n-hexane containing 4·5 % chloroform was used. Finally, the percentages of idoxanthin and astaxanthin were measured from chromatogram areas and corrected for differences in extinction coefficients (E1 %, 1 cm). The total fat of muscle samples was extracted according to the method described by Folch et al., 1957. Fatty acid methylation (Mason and Waller 1964) and GC analyses of fatty acid composition were analyzed according to the method described by Bou et al (2017).

2.6. RNA extraction from the tissue sample

2.6.1. Tissue sample homogenization

To homogenize tissue samples, 400 microliter lysis buffer was added to tubes containing two magnetic beads. Later, 10 mg of tissue sample were cut and added to the solution at room temperature. Then, 20 microliter proteinase k was added to each tube. Following this step, samples were mixed for 2 minutes at 1800 rpm using the FastPrep-96™ instrument. Next, samples were immediately transferred to a centrifuge machine and spun at 1600 rpm in one minute. later, samples were placed in a 37°C incubator for 45 minutes. Finally, samples were transferred to -80 °C for the next step.

2.6.2. RNA extraction on biomek 4000 using agencourt RNAdvance tissue kit

Homogenized samples of the liver, intestine, and muscle were taken out of the freeze and transferred to 37°C for 30 minutes. RNA extraction was carried out using Agencourt RNAdvanceTM Tissue and Total RNA Purification Kit (Beckman Coulter Inc., CA, USA) following the manufacturer´s. In summary, before launching the program, different reagents were prepared for the robot and transferred to the reservoir including 70% ethanol, wash buffer, isopropanol, binding solution, DNase enzyme, DNase buffer, and elution buffer. RNA extraction from the homogenized tissue was performed in 4 hours.

2.6.3. Quantification of RNA

Following RNA extraction, RNA concentration of samples was determined using nanodrop 8000 spectrophotometer (thermofisger scientific). To start working with the nanodrop, it was washed (blancked) with RNase-free water (1.1 µl) twice. After the blanking step, 1.5 µl of each sample was transferred to the nanodrop to measure the RNA concentration. In this regard, the sample quality was evaluated using Agilent® 2100 BioanalyzerTM RNA 6000 Nano Kit (Agilent Technology Inc., Santa Clara, CA, USA). This step ensures sufficient concentrations for normalization and microarray analysis.

2.7. cDNA synthesis

cDNA synthesis was done using TaqMan Reverse Transcription Kit. This protocol describes the procedure for generating DNA (double-stranded) from an RNA template via reverse transcription, resulting in complementary DNA (cDNA), using the TaqMan Reverse Transcription Kit. After thawing RNA samples, the PCR plate mixed and spun briefly. 250-1000 ng RNA depending on the type of tissue added to the PCR plate and diluted with RNase-free water to the same concentration. Next, 1 µl of DNAse buffer and 1 µl DNase enzyme were added to each well and after briefly spinning the PCR plate, kept at room temperature for 15 minutes. Following incubation, 1 µl of EDTA per well was added to all the samples and transferred to a PCR machine adjusted to 10 minutes at 65°C. Then, for making the Master mix, RT buffer(10x), Mgcl2 (25mM), dNTP(10mM), RNAse inhibitor (20 U/µl), and

MultiScribe™ RT (50 U/µl) were added to each well with the volume of 2 µl, 1.4 µl, 4 µl, 1µl, and 1µl, respectively. Later, 1 µl of random hexamer (50 uM) was added to each well plate separately. Moreover, during the cDNA synthesis, two groups of controls were used including no enzyme control (NEC) (for controlling genomic DNA contamination in the sample) and no template control (NTC) (for controlling genomic DNA contamination of the mix). Finally, the PCR plate was sealed and centrifuged at 2000 rpm for 1 min and transferred to the PCR machine which was programmed to run 25 °C, 37°C, 95°C, and 4°C for 10 min, 30 min, 5min, and infinitely, respectively.

2.8. Evaluation of primer specificity by electrophoresis

Evaluation of primer specificity was tested by running a PCR including the gene-specific primers and pooled cDNA from the 40 samples. Thereafter an agarose gel electrophoresis was run to evaluate if the amplified fragment had the correct size. Primers preparation started with adding 9.5 µl of RNase free water, F and R primers (1 µl), cDNA pool mix (1 µl), and 12.5 µl LongAmp Taq 2X Master Mix, Thermo fisher scientific) to the new PCR well plate as shown in (Table 2.6).

Table 2.6. Master mix reagents for primer preparation at the electrophoresis step

Component	25 µl reaction	50 µl reaction	Final concentration
10 µM Forward Primer	1 µl	2 µl	0.4 µM (0.05_1 µM)
10 µM Reverse Primer	1 µl	2 µl	0.4 µM (0.05_1 µM)
Template DNA	variable	variable	<1.000 ng
LongAmp Taq 2× Master Mix	12.5 µl	25 µl	1×
Nuclease-free water	to 25 µl	to 50 µl	

Following making the master mix, the PCR plate was transferred to the 96 well thermal cyclers which were programmed with the following amplification conditions and cycle steps shown in (Table 2.7).

Table 2.7. Amplification conditions and cycle steps at the electrophoresis step

Step	Temperature	Time
Initial denaturation	94 °C	30 seconds
30 cycles	94 °C 45-65°C 65 °C	10-30 seconds 15-60 seconds 50 seconds per kb
Final extension	65°C	10 minutes
Hold	4-10 °C	

Electrophoresis buffer made by adding 20 ml 50x TAE buffer (Tris/Acetic Acid/EDTA, pH 8.0) to 980 ml distilled water. Then, the solution was poured into the electrophoresis chamber. Preparing electrophoresis gel starts with adding 1.5% agarose to the electrophoresis buffer. Next, the solution boiled up for one minute. Following cooling down of the solution to 60 °C, SYBR® Safe Stain was added to the solution with a 1:10.000 ratio. Later, the comb was placed into the gel casting tray and gel solution was poured into the tray and polymerization happened after 45 minutes. After polymerization, the electrophoresis gel was transferred to the horizontal electrophoresis chamber (Bio-RAD) which was previously filled with 1x TAE buffer. Then, 1 µl and 5 µl of loading dye and PCR product were loaded to the gel, respectively. Next, the agarose gel electrophoresis was run at 80 V for 45 minutes. Later, the gels were photographed under UV light (Bio-Rad).

2.9. Evaluation of primer efficiency by standard curve

Primer efficiency was determined by putting up a standard curve. All samples were diluted to 10 times by mixing 19 µl cDNA with 171 µl of RNase-free water. After diluting all the samples, the pool mix of all the samples was made simply by adding 7 µl of each sample to a 1x dilution tube. Later, the 1x dilution was diluted with RNase-free water to make 2x dilution. Similarly, the cDNA samples were diluted from 1x to 2x, 4x, 8x, 16x, and 32x dilutions. Then, 4 µl of each dilution per well was added to the qPCR well plate. Two replicates of each sample were included to minimize errors during the process. Various genes are tested for the standard curve as shown in (Table 2.8). Following adding all the dilutions to the wells, a master mix was prepared by adding 0.5µl, 0.5µl, and 5 µl of F primer, R primer, and SYBER green respectively and was added to each well plate. After adding the master mix to all the wells from 1x dilution to 32x dilution, the qPCR plate vortexed and was spun at 2000 rpm for 2 minutes. Finally, it

was transferred to QuantStudio real-time PCR system, and a qPCR reaction was performed as shown in Figure 2.2.

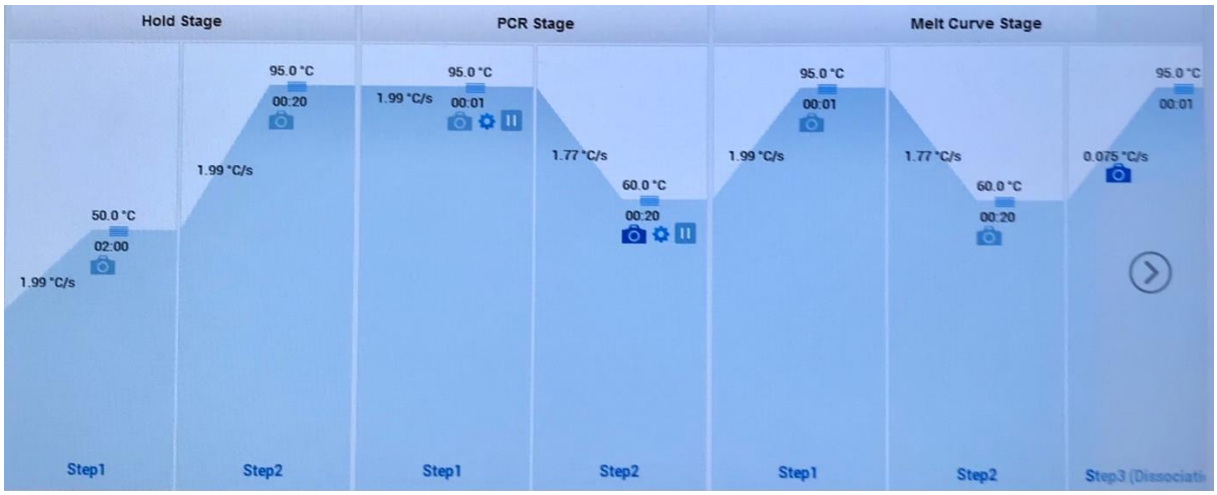


Figure 2.2. Thermal cycling conditions utilized for amplification by qPCR at standard curve run. The program is started with the hold stage and follows by the PCR stage and melt curve stage. Hold stage, PCR stage, and melt curve stage performed at two, two, and three steps respectively. The PCR stage is repeated for 40 cycles before continuing to the melt curve stage.

2.10. Real time qPCR

According to results from the standard curve for analyzing the expression of pigment-related genes, different genes presented in (Table 2.8) were tested in muscle, liver, and intestine. For this purpose, four µl of the previously synthesized cDNA were transferred to 384-Well PCR Plate. Two replicates of each sample ran to minimize errors during the process. Following adding cDNA to the each well plate, 0.5 µl of F primer, 0.5 µl of R primer, and 5 µl of SYBR green were mixed to make master mix and 6 µl of the mix was added to each well (final reaction volume was adjusted to 20 µl using DNases free water. Then, the plate was spun in 2000 rpm for 2 minutes and directly transferred to QuantStudio real-time PCR system and qPCR reaction was performed as shown in Figure 2.2.

Table 2.8. The list of primers and their sequences used for qPCR of all the tissues. L, M, and I are indicating liver, muscle, and intestine, respectively.

Gene symbol	Tissue	Gene name	Sequence (5'-3')	Primer name
bco2-like	L, M, I	Beta-carotene 15, 15-dioxygenase 2, like	GAAACTTAAGGCGTGGCGTC	bco2-like_F
			CCTTCTCTGGCTGTACCGAAA	bco2-like_R
bcmo1-like	L, M, I	beta-carotene oxygenase 1, like	ATGGCTCAAGAGTGGAGTGG	bcmo1-like_F
			GGCTCAGAGGGGTAGCAGTC	bcmo1-like_R
bcmo1	L, M, I	beta-carotene oxygenase 1	TGTTGCAATGTCAGCAGTGG	bcmo1_F
			AATGAACACGGGCTCTGATG	bcmo1_R
cd36	L, M, I	CD36 antigen	GGATGAACTCCCTGCATGTGA	cd36_F
			TGAGGCCAAAGTACTCGTCGA	d36_R
srb1	L, M, I	scavenger receptor BI	AACTCAGTGAAGAGGCCAAACTTG	srb1_F
			TGCGGCGGTGATGATG	srb1_R
bco2a	L, M, I	beta-carotene oxygenase 2a	AGAATAAGGATCATCATTACACAGACG	bcmo2a_F
			CCAGAGTGGATTAGTGGCAATTT	bcmo2a_F
bco2b	L, M, I	beta-carotene oxygenase 2b	TCAGACACCTGGTGGGAGAC	bcmo2b_R
			AGGGAACGAACACAGGCTCT	bcmo2b_F
bco2-like/bcmo2c	L, M, I	beta-carotene 15, 15-dioxygenase 2, like	GACACCTGTTCAGCGACTCC	bcmo2c/bco2l_F
			CAGGCTCAGATGGGAACAGA	bcmo2c/bco2l_R
arf4-like	L, I	ADP-ribosylation factor 4-like	TGTTCAAAGTTATCATCCAGGGTCT	arf4-like_F
			GAACTACATCTCTCAGCTGGTCC	arf4-like_R
apoa1	L, I	apolipoprotein A-I	CCCAGTCCCATAACGACCAA	apoa1_F1
			AGCAGGATGGTTAGTGCGAG	apoa1_R1
arf1-like	I	ADP-ribosylation factor 1-like	CGATGTCACTCACACGAAGAAAC	arf1-like_F1
			GATAAAGCCGATGTCACACGAAG	Arf1-like_R1
eif-3	L, M, I	Eukaryotic translation initiation factor 3	CAGGATGTTGTTGCTGGATGGG	eif-3_F1
			ACCCAACTGGGCAGGTCAAGA	eif-3_R1
rpol2	L, M, I	RNA polymerase 2	TAACGCCTGCCTCTTCACGTTGA	rpol2_F
			ATGAGGGACCTTGTAGCCAGCAA	rpol2_R
ef1a	L, M, I	scavenger receptor BI	CACCACCGGCCATCTGATCTACAA	ef1a_F
			TCAGCAGCCTCCTTCTCGAACTTC	ef1a_R

2.11. Microarray analysis

Extreme samples from liver, intestine, and muscle were subjected to microarray analysis using Nofima's Atlantic salmon DNA oligonucleotide microarray SIQ-6 (custom design, GPL16555, Sacramento, CA, USA), which contains 44 K probes for protein-coding genes Total RNA was isolated from the RNAlater®-preserved extreme samples of liver, intestine and muscle tissue using the Agencourt RNAdvance™ Tissue Total RNA Purification Kit (Beckman Coulter Inc., Brea, CA, USA). The extraction was carried out by the use of a Biomek 4000 pipetting robot (Beckman Coulter). Reagents and equipment were purchased from the same supplier. By adding 110 ng of total RNA template per reaction, RNA amplification and Cy3 labeling were carried out using a One-Color Quick Amp Labelling Kit (write supplier here). Next, for fragmentation of labeled RNA a gene Expression Hybridization kit (supplier here) was used. The arrays were hybridized by placing them in an oven for 17 h at 65 °C with a constant rotation speed of 0.01*g*. Finally, Gene expression Wash Buffers 1 and 2 (supplier) was used for washing arrays and then scanned using an Agilent SureScan microarray scanner. Nofima's bioinformatics package STARS (Salmon and Trout Annotated Reference Sequences) (Krasnov, Timmerhaus, Afanasyev and Jørgensen, 2011) was used for data pre-processing.

2.12. Transfection of ASK cells by electroporation

In vivo experiments were carried out using ASK [Atlantic Salmon Kidney] CRL-2747™. The culture medium was based on L15 supplemented with a 10% volume of fetal bovine serum (Sigma-Aldrich), 1% volume of antibiotic antimycotic solution (Sigma-Aldrich) containing 10,000 units penicillin, 10 mg streptomycin, and 25 µg amphotericin B per mL, 1% volume of HEPES (Sigma-Aldrich) with pH 7.0-7.6. The cells were sub-cultured every 10-17 days in a subculture ratio of 1:2 and 1:3 using trypsin-EDTA (Sigma-Aldrich) solution to detachment. The cells were kept at 20 °C without CO_2 in a humidified atmosphere. A constant room temperature was assumed in the model during the electrical pulse administration. In the *in vitro* study, we aimed to edit two genes including *srb1* and *perilipin1* with different guide RNAs. For the *srb1* gene, sgRNA-srb1, and for targeting the *perilipin1* gene three gRNA including plinB1-sgRNA6-P27, plinB2-sgRNA6-P27 and plinB3-sgRNA6-P27 were used. The guides were designed using CHOPCHOP and ordered from IDT (https://eu.idtdna.com/pages). Electroporation transfection protocols were performed using the Neon Transfection System (ThermoFisher, 2014) and the Neon Transfection System Kit. Firstly, cells were seeded into

96-wells plates according to procedures used in *in vitro* studies. Briefly, the culture medium was removed from the culture bottle, then cells were rinsed with 1 mL Phosphate Buffer Saline (Gibco™ DPBS, no calcium, no magnesium). Afterward, 1 mL of trypsin was used for cell detachment. Cells suspension was centrifuged for 600 × g for 4 minutes and re-suspended in a culture medium at a density of 1 x 10^7 cells/ml and seeded into a 96-wells plate (100 µl per well). After 24 h culture medium was removed from each well and then, solutions of dye (propidium iodide) were added to appropriate samples. Electroporation was performed using selected parameters: 1500 V, 1 electric pulse in 20 ms of duration. Electroporation was performed according to the manufacturer's protocol (Invitrogen, 2014) for the 24-well culture plate format, designed for adherent cells using the 10 µl Neon tips. Cells were suspended in an electroporation buffer to give 1 x 10^5 cells per 10 µl Neon tip. Directly after electroporation, cells were carefully distributed in wells with 0.5 ml culture medium without antibiotics (L-15 Gibco™) + 10% fetal bovine serum (Sigma Aldrich). Thereafter, cells were incubated for 7 days at 20 °C without CO_2 in a humidified atmosphere. Subsequently, microscopic observation was carried out and after reaching to high concentration, DNA was extracted from transfected cells to assess gene editing efficiency.

2.13. DNA extraction from the ASK cells by spin column protocol

After the electroporation procedure, cells were maintained for 5 days and washed twice by PBS as described below:

Old media was removed from the plates and one ml PBS was added. Later, one ml of new media was added to the cells. After getting to the desired concentration, cells were detached by taping the edges of the plate and scraping adherent cells with a scraper. Finally, detached cells were added to the microcentrifuge tubes for DNA extraction. DNA extraction was performed using DNeasy blood and tissue kit (QIAGEN, GERMANY) following the manufacturer´s protocol with minor modification. Firstly, after centrifuging cells with a concentration of 5 × 10^6 in 8000 × g for 5 min, cells were resuspended in 200 µl PBS and 20 µl proteinase k was added later. At the next step, 200 µl buffer AL (without added ethanol) was added and after vortexing incubated at 56 °C for 10 minutes. Subsequently, 200 µl ethanol (96-100%) was added to the sample and mixed by a vortex. Thereafter, the mixture pipetted in the DNeasy Mini spin column was placed in a 2 ml collection tube and centrifuged at 6000 × g for one minute. After centrifuge, the collection tube and flowthrough were discarded and the DNeasy

mini-column was placed in a new 2 ml collection tube. Next, 500 µl buffer AW1 was added to the mixture and centrifuged at 6000 × g for 1 minute. The collection tube and flowthrough were discarded the same as in the previous step and 500 µl buffer AW2 was added to the DNeasy Mini spin column which was placed in a new 2 ml collection tube. At this step, the DNeasy mini spin column was centrifuged in 20000 × g to dry the DNeasy membrane. After the membrane dried, the old collection tube and flow-through were discarded and the DNeasy mini spin column was placed in a new 2 ml microcentrifuge tube for elution. Hence, 50 µl buffer AE was pipetted directly to the DNeasy membrane and after one-minute incubation at room temperature, centrifuged for one minute at 6000 × g to elute.

2.14. Gene sequencing

To evaluate if the genes were edited, the amplicon containing the target sequence was amplified using PCR. The PCR mix was prepared by adding 8.5 µl, 1 µl, 1 µl, 12.5 µl, and 2 µl of nuclease-free water, R primer, F primer, LongAmp Taq 2x master mix, and genomic DNA, respectively. The cycling parameters were 2 min pre-incubation at 94 °C, followed by 35 cycles of amplification at 94 °C for 20 s, 60 °C for 50 s and 65 °C for 50 s, followed by a melting curve at 65 °C for 10 min. At the end, 15 µl of the PCR product with 20 µl of Reverse and Former primer were sent to Eurofins genomics for sequencing by the supreme Sanger sequencing method.

2.15. Salmon egg microinjection

For the *in vivo* study, a guide RNA (sgRNA-slc45a2) targeting *slc45a2* involved in pigmentation (as described in Edvardsen et al., 2014) was ordered from IDT (https://eu.idtdna.com/pages). Knocking out the *slc45a2* gene will result in an albino phenotype. Salmon eggs and milt were purchased from Benchmark genetics. The eggs were fertilized by adding milt to the eggs and incubation for 2 minutes. Thereafter the eggs were washed carefully with 0.5 mM glutathione. The fertilized eggs were kept in the glutathione solution (to soften the chorion) at three degrees until the eggs entered the one-cell stage. Three different CRISPR/cas9 injection mixtures were prepared as described in the (Table 2.9). sgRNA targeting *slc45a2* was used as a positive control. The microinjection glass needles were pulled and ground by the use of Narishige PC-100 Pipette Puller and Narishige's EG-401. The puller can produce glass microneedle by applying heat and pulling apart glass capillaries.

Consequently, different shapes and sizes of microneedle are designed for injection. Finally, the tip of the needles was inspected and ground. The injection solution was added to the capillary through the back end of the glass capillary with the aid of MicroloaderTM pipette tip (Eppendorf, Hamburg, Germany). The filled microneedle was attached to Eppendorf FemtoJet Microinjector. The injection mixture was injected into the germinal disc of the fertilized egg. After injection and throughout the gene editing process, eggs were kept in 4-8 °C H_2O water with and pH of 8. After injection, the eggs were regularly inspected, and dead eggs were removed every two days.

Table 2.9. CRISPR/cas9 injection mixtures used for egg microinjection

	Cas9 protein	**sgRNA**
Mix 1	150 ng/µl	50 ng/µl
Mix 2	225 ng/µl	75 ng/µl
Mix 3	300 ng/µl	100 ng/µl

2.16. Statistical Analysis

GraphPad Prism version 9.1.1 (La Jolla, USA, www.graphpad.com) was used for statistical analyses of the gene expression in qPCR and microarray. For the microarray analysis, the average microarray intensity levels were log2-transformed and normalized against controls. Differentially expressed genes were identified relative to control using Student's t-test ($p < 0.05$). Statistical significance for qPCR results was evaluated by a two-way analysis of variance followed by multiple comparisons (Tukey honest post-hoc test). Differences with p-values < 0.05 were considered statistically significant. Venn diagrams were generated by the venny tool (https://bioinfogp.cnb.csic.es/tools/venny/). Principal component analysis (PCA) was performed for the microarray results from three different tissue. Score plots from (PCA) were used to explore the main trends and clustering in the data.

3. Results

3.1.VIS/NIR spectroscopy analysis of pigment in the fillet

The muscle color data from VIS/NIR spectroscopy of the five fish selected from each experimental group are presented in Table 3.1. The able shows that we succeeded in selecting experimental fish with a relatively large difference in fillet pigmentation. Pigment values of fillets, based on spectroscopy analyses, were approximately 9 mg/kg in the High pigment group compared to 5.4 mg/kg in the Low pigment group, with an average pigment value of both groups combined of 7.2 mg/kg.

Table 3.1. Color results from VIS/NIR spectroscopy from extreme groups. Pigment level from VIS/NIR spectroscopy showed to be higher at $High_{ex}$-CT and $High_{ex}$-Omega groups compared to Low_{ex}-CT and Low_{ex}-Omega. The groups in the Table indicate the four different experimental groups defined in Table 2.4. Data are shown as mean ± SD where SD equals standard deviation. (Five fish are behind the analysis in each experimental group. Significant differences are followed by Tukey's post hoc test and determined by Tukey´s post hoc multiple comparison test. Different subscript letters within each row denote significant differences among groups.

Group	**Pigment NIR (mg kg− 1)**
	Mean (SD)
$High_{ex}$ -CT	$9.0\pm 0.4_{a}$
Low_{ex}-CT	$5.4 \pm 0.1_{b}$
$High_{ex}$-Omega	$8.8 \pm 0.4_{a}$
Low_{ex}-Omega	$5.4 \pm 0.3_{b}$

3.2. Chemical composition analysis of astaxanthin and idoxanthin levels in the muscle

The result from chemical analysis of astaxanthin (Figure 3.1 A) and idoxanthin (Figure 3.1 B) at muscle of the five fish selected from each experimental group are presented in Figure 3.1. Results show an approximately 2-fold higher astaxanthin level in the $High_{ex}$-Omega and $High_{ex}$-CT groups compared to Low_{ex}-CT and Low_{ex}-Omega groups.

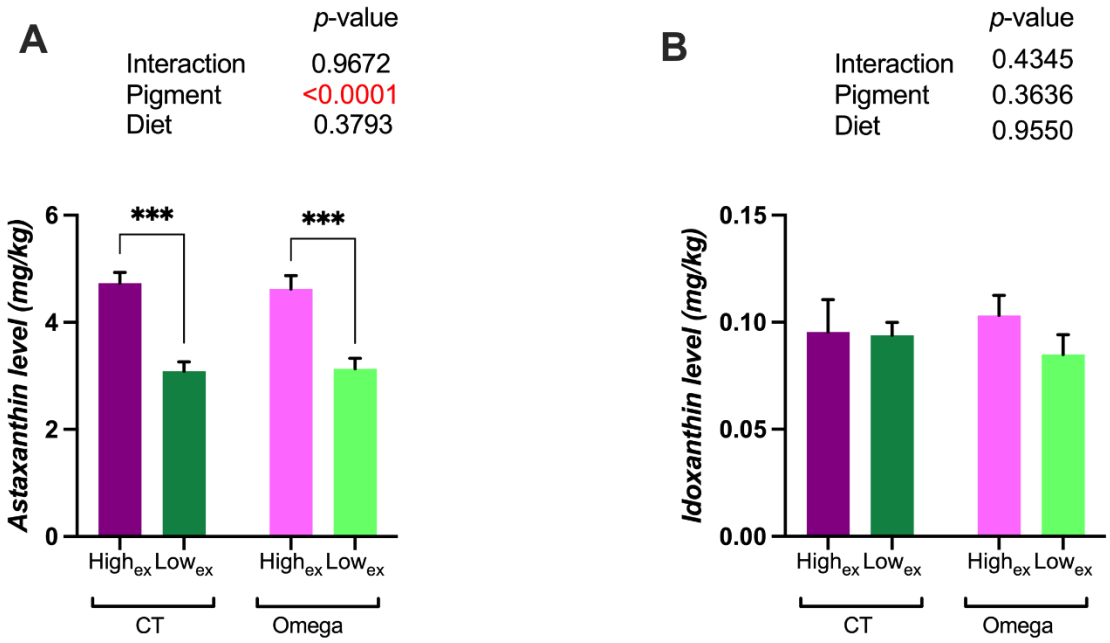


Figure 3.1. Astaxanthin (A) and Idoxanthin (B) concentration (mg/kg) in the salmon muscle of four experimental groups (Highex-Omega, $High_{ex}$-CT, Low_{ex}-Omega and Low_{ex}-CT). CT and Omega are indicating two different diet fed as defined in Table 2.1. Statistical differences in the variability between measurements are indicated in three components: interaction, pigment, and diet using two-way ANOVA. Data are shown as mean± SEM. (n = 5). Significant differences are followed by Tukey's post hoc test and indicated by red values and were determined by Tukey´s post hoc multiple comparison test. Different letters indicate significant differences among groups.

3.3. Fatty acid composition

The result from chemical analysis of EPA (Figure 3.2 A), DHA (Figure 3.2 B) and EPA+DHA (Figure 3.2 C) at muscle of the five fish selected from each experimental group are presented in figure below:

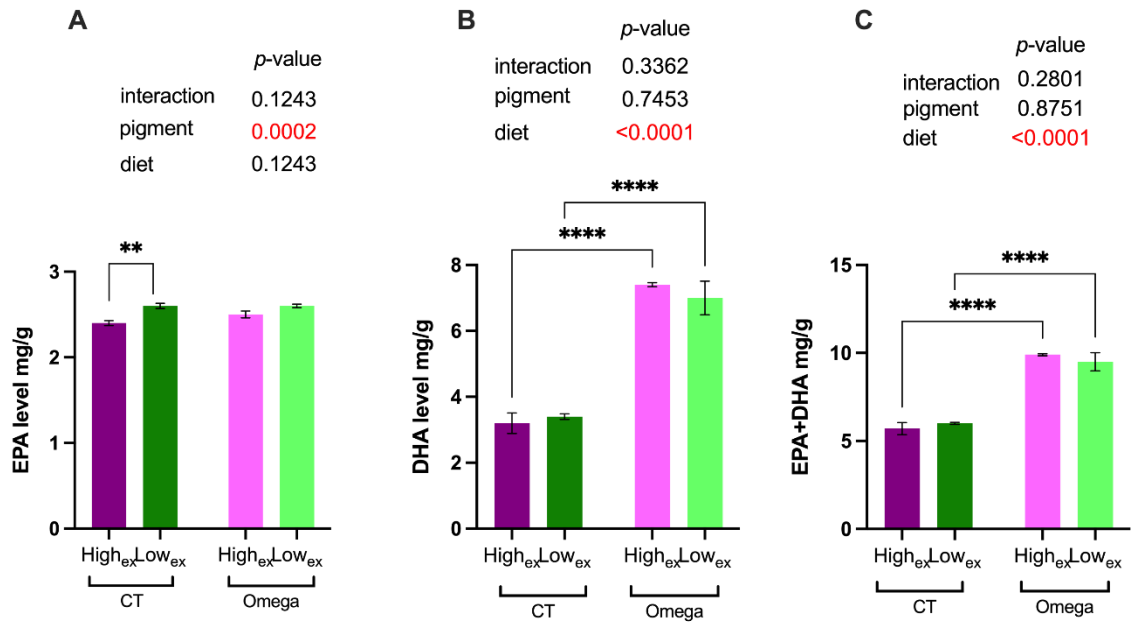


Figure 3.2. EPA (A), DHA(B), and EPA+DHA (C) concentration (mg/kg) in the salmon muscle of four experimental groups ($High_{ex}$-Omega, $High_{ex}$-CT, Low_{ex}-Omega and Low_{ex}-CT). CT and Omega are indicating two different diets fed as defined in Table 2.1. Statistical differences in the variability between measurements are indicated in three components: interaction, pigment, and diet using two-way ANOVA. Data are shown as mean± SD. (n = 5). Significant differences are followed by Tukey's post hoc test and indicated by red values and were determined by Tukey´s post hoc multiple comparison test. Significant differences **($p < 0.01$), ***($p<0.001$) and ****($p<0.0001$) highlighted in red color.

Transcription analyses using microarray in liver, intestine, and muscle

3.4. Multivariate analysis of microarray data

Following analyzing samples by microarray, PCA analyses of microarray data from the intestine, liver, and muscle were conducted to evaluate the specific effects of different experimental diets and pigment groups on various gene expressions in mentioned organs. As shown in Figures 3.3, 3.4, and 3.5 the PCA plots display a clustering of samples with similar gene expression profiles in the same area. Scores of samples belonging to the same pigment group and the same dietary group were clustered together, respectively.

3.4.1. PCA of gene expression from microarray analyses of the liver

As shown in Figure 3.3, scores show the connection between the four experimental groups and the various gene expression in the liver. The scores of genes belonging to each of the $High_{ex}$ and Low_{ex} groups were clustered together. Moreover, the scores of genes belonging to the same dietary group were clustered together. However, gene expressions were less clearly affected by dietary treatment and showed no evident separation between groups. Considering this, the plots show the first principal component (PC1, 28.17%) vs. the second principal component (PC2, 17.90%), summarising 46.07% of the variation. Here, 28.17% of the variation was explained by the first principal component (x-axis), which distributed the samples along the axis from $High_{ex}$ in the left quadrants, to Low_{ex} in the right quadrants. Moreover, individual gene samples from Low_{ex}-CT and $High_{ex}$-CT groups clustered together at the top right and top left quadrant, respectively. However, one biological replicate of Low_{ex}-CT and $High_{ex}$-Omega did not cluster with the others.

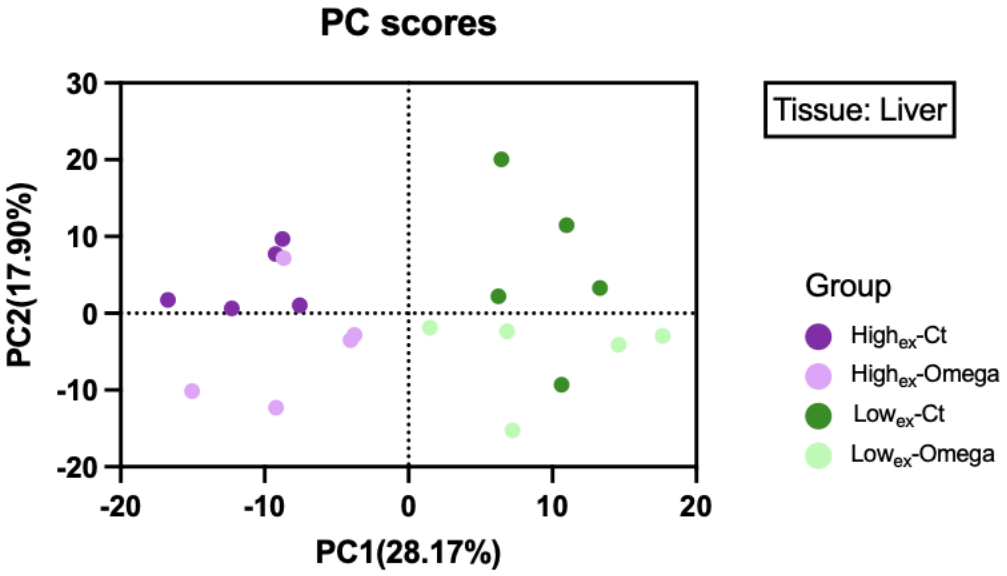


Figure 3.3. PCA score plot of liver. Scores showing the relationship between the effect of different treatments and differently expressed genes at the liver. The scores in the plot indicate the four different experimental groups ($High_{ex}$-Omega, $High_{ex}$-CT, Low_{ex}-CT, and Low_{ex}-Omega) represented in different colors. Each treatment group is defined in Table 2.4.

3.4.2. PCA of gene expression from microarray analyses of the muscle

As displayed in the scoring plot (Figure 3.4), the first two principal components (PC1 and PC2) explained 44.27% of the variability among the data. The $High_{ex}$-CT and Low_{ex}-Omega samples fell in the negative and positive direction of the PC1 axis, respectively. Two biological replicates of $High_{ex}$-CT did not cluster with the others likely due to individual variability between the animals. Although both $High_{ex}$-Omega and Low_{ex}-CT fell in the positive and negative directions of the PC2 axis, respectively individual variability was observed among replicates. This led to no obvious clustering of samples. Therefore, the Low_{ex}-Omega group was the only group that demonstrated clear clustering compared to the three other groups.

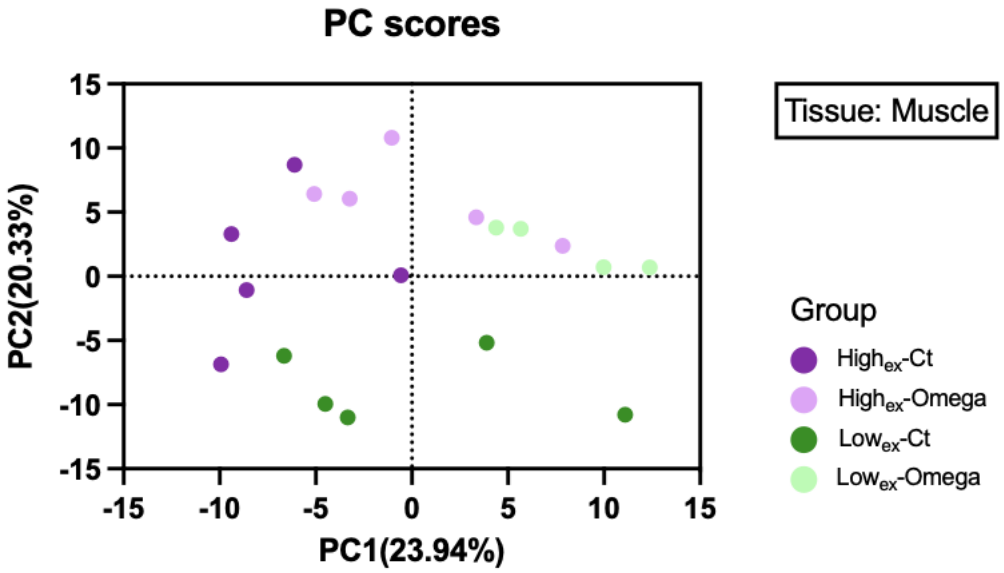


Figure 3.4. PCA score plot of muscle. PCA Scores showing the relationship between the effect of different treatments and differently expressed genes in the muscle. The scores in the plot indicate the four different experimental groups ($High_{ex}$-Omega, $High_{ex}$-CT, Low_{ex}-CT, and Low_{ex}-Omega) represented in different colors. Each treatment group is defined in Table 2.4.

3.4.3. PCA of gene expression from microarray analyses of the intestine

As displayed in the scoring plot (Figure 3.5), the first two principal components (PC1 and PC2) explained 40.08% of the variability among the data. The $High_{ex}$-CT and Low_{ex}-Omega samples fell in the negative and positive direction of the PC1 axis, respectively. Moreover, one biological replicate of $High_{ex}$-CT did not cluster with the others. Further, $High_{ex}$-Omega and Low_{ex}-CT samples fell in the positive and negative directions of the PC2 axis. Nonetheless, one replicate of $High_{ex}$-Omega and two replicates of Low_{ex}-CT did not cluster with others. Therefore, the Low_{ex}-Omega group was the only group that demonstrated evident clustering compared to the three other groups.

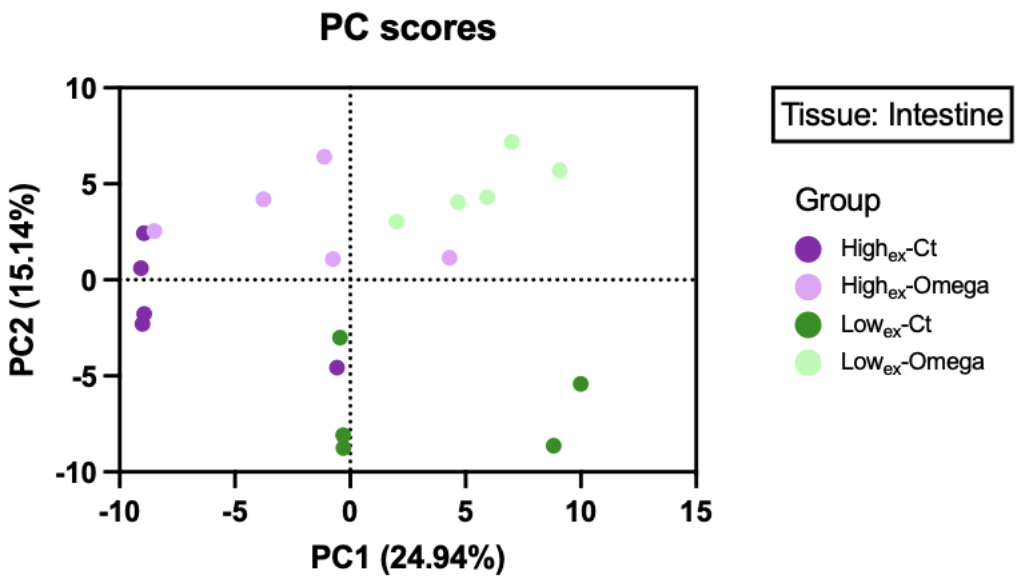


Figure 3.5. PCA score plot of intestine. Scores showing the relationship between the effect of different treatments and differently expressed genes at the intestine. The scores in the plot indicate the four different experimental groups ($High_{ex}$-Omega, $High_{ex}$-CT, Low_{ex}-CT, and Low_{ex}-Omega) represented in different colors. Each treatment group is defined in Table 2.4

3.5. Microarray analysis

Based on microarray results 187, 371, and 170 genes were identified in muscle, liver, and intestine, respectively. There were different criteria behind choosing identified genes for heat map construction. Some of the genes were chosen due to being significantly up and downregulated in different comparisons. Moreover, based on the previous studies there were some candidate genes related to the astaxanthin metabolism pathway. Results are shown as fold change values. To determine the number of differentially expressed genes (DEG), two different comparisons were made. The comparison between the dietary group would reflect the effect of high Omega-3 fatty acid (EPA and DHA) on fillet pigment and a comparison between $High_{ex}$ group vs Low_{ex} group would reflect the effect of high and low pigment flesh on DEG and different genes expression. Results showed a clear effect of the fillet pigment selection on the transcriptional regulation of all tissues and particularly in the liver. Furthermore, diets also had a significant effect within the high and especially the low pigment group.

3.5.1. Tissue-specific gene expression

Venn diagrams were prepared using the Venny online (version 2.2.0) platform to plot differences in the genes differentially expressed in different tissues. (Figure 3.6). The liver showed the largest number of unique differentially expressed genes (315) compared to the intestine (138) and muscle (144). Numbers in the overlap area indicate the mutual gene names.

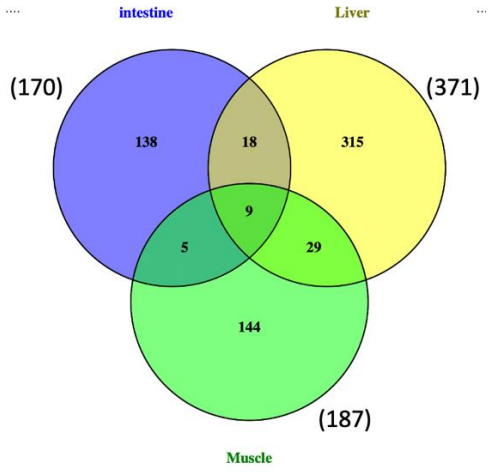


Figure 3.6. Venn diagram illustrating the numbers of common and differentially expressed genes (DEGs) of liver, intestine, and muscle in all treatment groups. It shows that of the 728 genes differentially expressed, only nine are common to the three tissues. The total amount of DEGs in the individual tissues is written in brackets.

3.5.2. Common genes found in liver, intestine, and muscle tissues

The venny diagram showed that nine genes were mutual between three tested organs. As shown in Figure 3.7, the heatmap from tissues shows significant differential expression of H3 histone, family 3A, ATP-dependent RNA helicase DDX42, and von Willebrand factor A domain-containing protein in the intestine, H3 histone, family 3A, ATP-dependent RNA helicase DDX42, Epidermal growth factor receptor kinase substrate, Pecanex homolog, RUN, and SH3 domain-containing protein 1 and von Willebrand factor A domain-containing protein at liver and H3 histone, family 3A, Epidermal growth factor receptor kinase substrate, Pecanex homolog and von Willebrand factor A domain-containing protein in the muscle. Among those with significant different expression, H3 histone, family 3A and von Willebrand factor A domain-containing protein (at all the tissues), the Pecanex homolog gene (at liver and muscle), and Epidermal growth factor receptor kinase substrate in muscle were significantly downregulated at $High_{ex}$ group compared to Low_{ex} group. Additionally, ATP-dependent RNA helicase DDX42 (at intestine and liver), Epidermal growth factor receptor kinase substrate, and RUN and SH3 domain-containing protein 1 (at liver) significantly upregulated in the $High_{ex}$ group compared to Low_{ex} group. Furthermore, the heatmap from the dietary group shows significant differential expression of H3 histone, family 3A, Epidermal growth factor receptor kinase substrate, Lipoprotein lipase, mitochondrial ribosomal protein S34, Pecanex homolog, and von Willebrand factor A domain-containing protein in the intestine mitochondrial ribosomal protein S34, RasGEF domain family, member 1Ba at liver Lipoprotein lipase, mitochondrial ribosomal protein S34 and RUN and SH3 domain-containing protein 1 in the muscle. Among those with significant differential expresion, H3 histone, family 3A, Epidermal growth factor receptor kinase substrate, Pecanex homolog, von Willebrand factor A domain-containing protein in the intestine and RUN and SH3 domain-containing protein 1 in the muscle were significantly upregulated at group fed Omega diet compared to the group fed CT diet. Besides, Lipoprotein lipase (in intestine and muscle), mitochondrial ribosomal protein S34 (in all the tissues), and RasGEF domain family, member 1Ba (in the liver) were significantly downregulated at group fed Omega diet compared to the group fed CT diet.

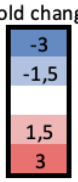
Intestine

Gene	$High_{ex}$ vs Low_{ex}	*p*-value	Omega vs CT	*p*-value
H3 histone, family 3A	-1,3	0,001	0,0	0,001
ATP-dependent RNA helicase DDX42	0,5	0,048	-0,6	0,054
Epidermal growth factor receptor kinase substrate	-0,4	0,075	0,6	0,004
Lipoprotein lipase	0,7	0,090	-1,2	0,005
mitochondrial ribosomal protein S34	0,7	0,350	-1,5	0,030
Pecanex homolog	-0,5	0,101	0,6	0,042
RasGEF domain family, member 1Ba	0,3	0,263	-0,3	0,352
RUN and SH3 domain-containing protein 1	0,5	0,182	-0,4	0,348
von Willebrand factor A domain-containing protein	-0,9	0,001	0,2	0,001

Liver

Gene	$High_{ex}$ vs Low_{ex}	*p*-value	Omega vs CT	*p*-value
H3 histone, family 3A	-1,3	0,000	0,4	0,288
ATP-dependent RNA helicase DDX42	1,1	0,000	0,1	0,788
Epidermal growth factor receptor kinase substrate	1,0	0,038	0,7	0,130
Lipoprotein lipase	0,5	0,216	0,0	0,980
mitochondrial ribosomal protein S34	-0,7	0,286	-1,4	0,018
Pecanex homolog	-0,8	0,004	0,2	0,438
RasGEF domain family, member 1Ba	0,4	0,367	-1,1	0,019
RUN and SH3 domain-containing protein 1	1,1	0,005	-0,1	0,892
von Willebrand factor A domain-containing protein	-1,1	0,003	-0,2	0,554

Fold change



Muscle

Gene	$High_{ex}$ vs Low_{ex}	*p*-value	Omega vs CT	*p*-value
H3 histone, family 3A	-0,9	0,008	0,6	0,263
ATP-dependent RNA helicase DDX42	0,1	0,767	0,5	0,078
Epidermal growth factor receptor kinase substrate	-0,9	0,030	0,3	0,513
Lipoprotein lipase	-0,4	0,306	-0,8	0,017
mitochondrial ribosomal protein S34	-1,2	0,116	-1,6	0,023
Pecanex homolog	-0,8	0,001	0,0	0,982
RasGEF domain family, member 1Ba	0,3	0,202	-0,5	0,066
RUN and SH3 domain-containing protein 1	0,3	0,456	0,8	0,046
von Willebrand factor A domain-containing protein	-1,1	0,005	0,0	0,997

Figure 3.7. Different regulation of nine common genes in three different tissues. The Figure shows common gene expression profiles involved in different metabolism between dietary groups (Omega and CT) and $High_{ex}$ group (Phenotypically selected fish with high fillet color) vs Low_{ex} group (phenotypically selected fish with low fillet color). Red and blue cells indicate upregulated and downregulated values, respectively. Two comparisons with the corresponding *p*-value were done by t-test between the pigment and dietary groups and are included in the Figure. Significant differences ($p < 0.05$) are indicated by red values.

3.5.3. Microarray analysis of selected genes at muscle

As shown in Figure 3.8, a number of genes were found to be significantly expressed between different experimental groups. Those genes contributed to five distinct biological pathways including Cell Folding, protein modification, cell stress, Cell chromosome, and Lipid metabolism. The heatmap from muscle tissue shows a significantly different expression of the following genes between the $High_{ex}$ group vs Low_{ex} group: heat shock protein 30, heat shock protein 30-like, Jun B-1, CCAAT/enhancer binding protein (C/EBP) _ beta, jun proto-oncogene (jun), Jun C, AP1-1, Butyrate response factor 1, JunD-2, H3 histone, family 3A, Acyl-CoA synthetase family member 2. From the genes differentially expressed, following genes significantly up-regulated: heat shock protein 30, heat shock protein 30-like, heat shock cognate 70, Jun B-1, CCAAT/enhancer binding protein (C/EBP) _ beta, jun proto-oncogene (jun), Jun C, AP1-1, Butyrate response factor 1, JunD-2. Additionally, H3 histone, family 3A and Acyl-CoA synthetase family member 2 were significantly downregulated. Moreover, the transcription analyses showed significant differential expression at JunD-2 and Acyl-CoA synthetase family member 2 genes being upregulated at JunD-2 and downregulated at Acyl-CoA synthetase family member 2 between the group fed Omega diet compared to the group fed CT diet. On the basis of these results, the gene with the highest response in the muscle of $High_{ex}$ compared to Low_{ex} seems to be heat shock protein 30 and heat shock protein 30 like.

Gene	$High_{ex}$-Ct	$High_{ex}$-Omega	Low_{ex}-Ct	Low_{ex}-Omega	$High_{ex}$ vs Low_{ex}	*p*-value	Omega vs Ct	*p*-value
Cell Folding, protein modification								
Heat shock protein 30					2,3	0,029	1,1	0,315
heat shock protein 30-like					2,2	0,032	1,1	0,323
Heat shock cognate 70					1,0	0,033	-0,2	0,782
cell stress and Cell chromosome								
Jun B-1					1,6	0,017	-0,2	0,694
CCAAT/enhancer binding protein (C/EBP)_ beta					1,4	0,009	-0,6	0,277
jun proto-oncogene (jun)					1,3	0,020	-1,0	0,063
Jun C					1,1	0,011	-0,7	0,117
AP1-1					1,0	0,006	-0,8	0,069
Butyrate response factor 1					1,0	0,014	-0,4	0,260
JunD-2					1,0	0,038	-0,9	0,011
H3 histone, family 3A					-0,9	0,008	0,6	0,263
Lipid metabolism								
Acyl-CoA synthetase family member 2					-1,8	0,000	0,5	0,013
lipocalin-like					0,7	0,348	2,1	0,806

Fold change: -3, -1,5, 1,5, 3

Figure 3.8. Expressions levels of different genes involved in cell folding, protein modification, cell stress, Cell chromosome, and lipid metabolism in skeletal muscle between dietary groups (Omega vs CT) and phenotypic groups ($High_{ex}$ vs Low_{ex} group). Red and blue cells indicate upregulated and downregulated values, respectively. Two comparisons with the corresponding *p*-value were done by t-test between pigment and dietary groups and are included in the figure. Significant differences ($p < 0.05$) are indicated by red values.

3.5.4. Microarray analysis of selected genes at intestine

As shown in Figure 3.9, a number of genes were found to be significantly expressed between different experimental groups. Those genes contributed to four distinct biological pathways including lipid metabolism, retinoid metabolism, mitochondria metabolism, and cell chromosome. The heatmap from intestine tissue shows a significantly different expression of the following genes between $High_{ex}$ group vs Low_{ex} group: polyunsaturated fatty acid elongase elov12, Diacylglycerol O-acyltransferase 2, Proto-oncogene c-Fos, acyl-CoA synthetase short-chain family member 1 (acss1), low-density lipoprotein receptor class A domain-containing protein 4-like, Beta-carotene dioxygenase 1, electron transfer flavoprotein subunit alpha, mitochondrial-like and H3 histone, family 3A. From the genes differentially expressed, the following genes significantly upregulated: polyunsaturated fatty acid elongase elov12, Diacylglycerol O-acyltransferase 2, Proto-oncogene c-Fos, acyl-CoA synthetase short-chain family member 1 (acss1) and Beta-carotene dioxygenase 1. Besides, low-density lipoprotein receptor class A domain-containing protein 4-like, electron transfer flavoprotein subunit alpha, mitochondrial-like and H3 histone, family 3A genes were significantly downregulated. Furthermore, the transcription analyses showed significantly different expression of following genes at dietary groups (Omega vs CT) at Plastin 3 (T isoform), Probable imidazolonepropionase, Phospholipase C, delta 1b, Potassium channel tetramerisation domain containing 16a genes being up-regulated, and lipid phosphate phosphohydrolase 1-like gene being downregulated.

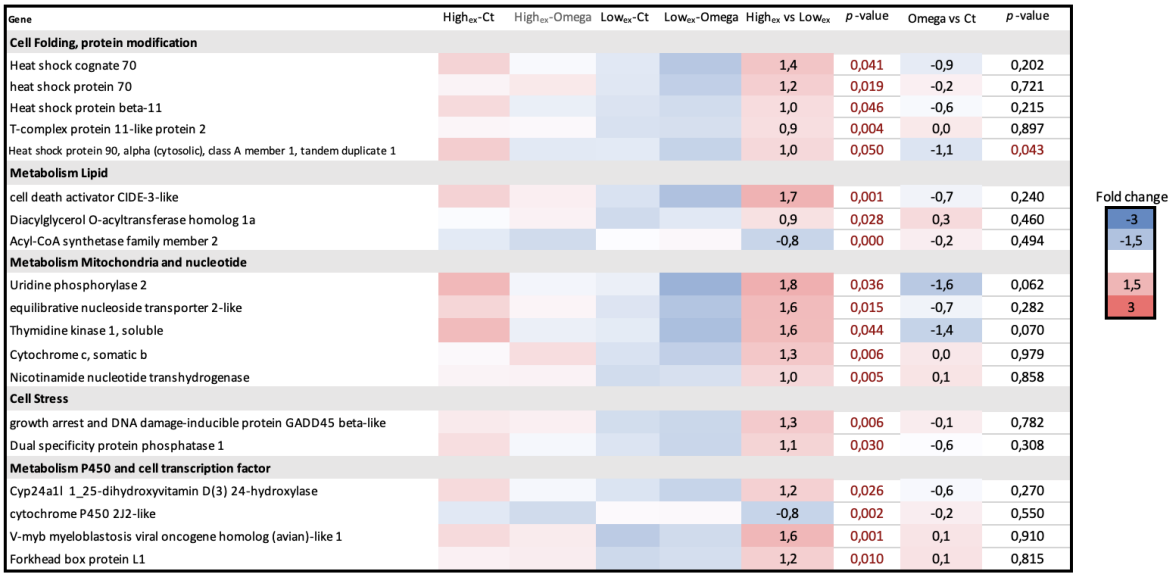
Gene	$High_{ex}$-Ct	$High_{ex}$-Omega	Low_{ex}-Ct	Low_{ex}-Omega	$High_{ex}$ vs Low_{ex}	*p*-value	Omega vs Ct	*P*-value
Lipid metabolism								
polyunsaturated fatty acid elongase elov12					2,2	0,011	-0,5	0,573
Diacylglycerol O-acyltransferase 2					1,4	0,020	-0,5	0,431
Proto-oncogene c-Fos					1,3	0,004	-0,8	0,100
acyl-CoA synthetase short-chain family member 1 (acss1)					0,8	0,013	-0,2	0,515
low-density lipoprotein receptor class A domain-containing protein 4-like					-0,9	0,019	-0,3	0,534
Plastin 3 (T isoform)					-0,4	0,220	0,6	0,040
Probable imidazolonepropionase					0,1	0,717	1,0	0,008
Phospholipase C, delta 1b					-0,3	0,307	0,9	0,004
alpha-(1,3)-fucosyltransferase 9-like					0,4	0,073	-0,4	0,091
Potassium channel tetramerisation domain containing 16a					-0,3	0,212	0,5	0,033
Retinoid metabolism								
Beta-carotene dioxygenase 1					1,0	0,002	0,5	0,189
Lanosterol 14-alpha demethylase					-0,3	0,473	-0,5	0,280
Cytochrome P450 7A1					-1,2	0,345	-1,4	0,186
Mitochondria metabolism and Cell chromosome								
lipid phosphate phosphohydrolase 1-like					-0,2	0,501	-0,5	0,049
electron transfer flavoprotein subunit alpha, mitochondrial-like					-1,4	0,028	0,7	0,287
H3 histone, family 3A					-1,3	0,001	0,0	0,967

Fold change: -3, -1,5, 1,5, 3

Figure 3.9. Expressions of levels of different genes involved in lipid metabolism, retinoid metabolism, mitochondria metabolism, and cell in the intestine between dietary groups (Omega vs CT) and phenotypic groups ($High_{ex}$ vs Low_{ex} group). Red and blue cells indicate upregulated and downregulated values, respectively. Two comparisons with the corresponding *p*-value were done by t-test between pigment and dietary groups and are included in the Figure. Significant differences ($p < 0.05$) are indicated by red values.

3.5.5. Microarray analysis of selected genes at liver

As shown in Figure 3.10, a number of genes were found to differently expressed between different experimental groups. Those genes contributed to several distinct biological pathways including cell Folding, protein modification, lipid metabolism, mitochondria metabolism and nucleotide, cell Stress, metabolism P450, and cell transcription factor. The heatmap from intestine tissue shows a significant different expression of the following genes between $High_{ex}$ group vs low_{ex} group: Heat shock cognate 70, heat shock protein 70, Heat shock protein beta-11, T-complex protein 11-like protein 2 , Heat shock protein 90, alpha (cytosolic), class A member 1, tandem duplicate 1, cell death activator CIDE-3-like, Diacylglycerol O-acyltransferase homolog 1a, Acyl-CoA synthetase family member 2 , Uridine phosphorylase 2, equilibrative nucleoside transporter 2-like , Thymidine kinase 1, soluble, Cytochrome c, somatic b, Nicotinamide nucleotide transhydrogenase, growth arrest and DNA damage-inducible protein GADD45 beta-like, Dual specificity protein phosphatase 1, Cyp24a1l 1_25-dihydroxyvitamin D(3) 24-hydroxylase, Cyp24a1l 1_25-dihydroxyvitamin D(3) 24-hydroxylase, cytochrome P450 2J2-like, V-myb myeloblastosis viral oncogene homolog (avian)-like 1 and Forkhead box protein L1. Among those genes significantly different expressed following genes were significantly upregulated: Heat shock cognate 70, heat shock protein 70, Heat shock protein beta-11, T-complex protein 11-like protein 2 , Heat shock protein 90, alpha (cytosolic), class A member 1, tandem duplicate 1, cell death activator CIDE-3-like, Diacylglycerol O-acyltransferase homolog 1a , Uridine phosphorylase 2, equilibrative nucleoside transporter 2-like , Thymidine kinase 1, soluble, Cytochrome c, somatic b, Nicotinamide nucleotide transhydrogenase, growth arrest and DNA damage-inducible protein GADD45 beta-like, Dual specificity protein phosphatase 1, Cyp24a1l 1_25-dihydroxyvitamin D(3) 24-hydroxylase, Cyp24a1l 1_25-dihydroxyvitamin D(3) 24-hydroxylase, V-myb myeloblastosis viral oncogene homolog (avian)-like 1 and Forkhead box protein L1. Additionally, Acyl-CoA synthetase family member 2 and cytochrome P450 2J2-like were significantly downregulated. Further, the transcription analyses showed Heat shock protein 90, alpha (cytosolic), class A member 1, tandem duplicate 1 significantly downregulated between dietary groups (Omega vs CT).



Gene	$High_{ex}$-Ct	$High_{ex}$-Omega	Low_{ex}-Ct	Low_{ex}-Omega	$High_{ex}$ vs Low_{ex}	*p*-value	Omega vs Ct	*p*-value
Cell Folding, protein modification								
Heat shock cognate 70					1,4	0,041	-0,9	0,202
heat shock protein 70					1,2	0,019	-0,2	0,721
Heat shock protein beta-11					1,0	0,046	-0,6	0,215
T-complex protein 11-like protein 2					0,9	0,004	0,0	0,897
Heat shock protein 90, alpha (cytosolic), class A member 1, tandem duplicate 1					1,0	0,050	-1,1	0,043
Metabolism Lipid								
cell death activator CIDE-3-like					1,7	0,001	-0,7	0,240
Diacylglycerol O-acyltransferase homolog 1a					0,9	0,028	0,3	0,460
Acyl-CoA synthetase family member 2					-0,8	0,000	-0,2	0,494
Metabolism Mitochondria and nucleotide								
Uridine phosphorylase 2					1,8	0,036	-1,6	0,062
equilibrative nucleoside transporter 2-like					1,6	0,015	-0,7	0,282
Thymidine kinase 1, soluble					1,6	0,044	-1,4	0,070
Cytochrome c, somatic b					1,3	0,006	0,0	0,979
Nicotinamide nucleotide transhydrogenase					1,0	0,005	0,1	0,858
Cell Stress								
growth arrest and DNA damage-inducible protein GADD45 beta-like					1,3	0,006	-0,1	0,782
Dual specificity protein phosphatase 1					1,1	0,030	-0,6	0,308
Metabolism P450 and cell transcription factor								
Cyp24a1l 1_25-dihydroxyvitamin D(3) 24-hydroxylase					1,2	0,026	-0,6	0,270
cytochrome P450 2J2-like					-0,8	0,002	-0,2	0,550
V-myb myeloblastosis viral oncogene homolog (avian)-like 1					1,6	0,001	0,1	0,910
Forkhead box protein L1					1,2	0,010	0,1	0,815

Figure 3.10. Expressions levels of different genes involved in cell folding, protein modification, lipid metabolism, mitochondria, nucleotide, cell stress, p450 metabolism, and cell transcription factor in liver between dietary groups (Omega vs CT) and phenotypic groups ($High_{ex}$ vs Low_{ex} group). Red and blue cells indicate upregulated and downregulated values, respectively. Two comparisons with corresponding *p*-value done by t-test between pigment groups and dietary groups are included in the Figure. Significant differences ($p < 0.05$) are indicated by red values.

3.6. Gene expression study using qPCR

The next step was to determine whether four different treatments differentially modified the expression of several candidate genes in the intestine, liver, and muscle. The expression of *bcmo1*, *bcmo1-like*, *bcmo2-like*, *bcmo2c*, *bcmo2b*, *arf1-like*, *arf4-like*, *cd36*, *apoa1*, and *srb1* genes were analyzed by quantitative PCR according to their relevance in carotenoid metabolism. The genes were expressed differently in different organs from groups with different fillet pigmentation that received different diets.

3.6.1. Gene expression analyses of pigmentation related genes in the intestine

Gene expression results for selected genes from the intestine are shown in Figure 3.11. In the current study, the expression of eight different genes was tested including *bcmo1*, *bcmo1-like*, *bcmo2-like*, *bcmo2c*, *arf1-like*, *arf4-like*, *apoa1*, and *cd36*. Results show that the expression level of the *bcmo1* gene was significantly increased (*p*-value= 0.0154) in fish taken from the $High_{ex}$ omega group compared with Low_{ex} group (Figure 3.11 A). Moreover, the results demonstrated a significant increase in mRNA levels for *bcmo2-like* (*p*-value= 0.0113) and *bcmo2c* (*p*-value= 0.0019) genes in fish fed Omega diet compared with fish fed CT diet. Finally, no significant changes were observed in *bcmo1-like*, *arf1-like*, *arf4-like*, *apoa1*, and *cd36* mRNA levels upon feeding with different diets (Figure 3.11. B, E, F, G, and H)

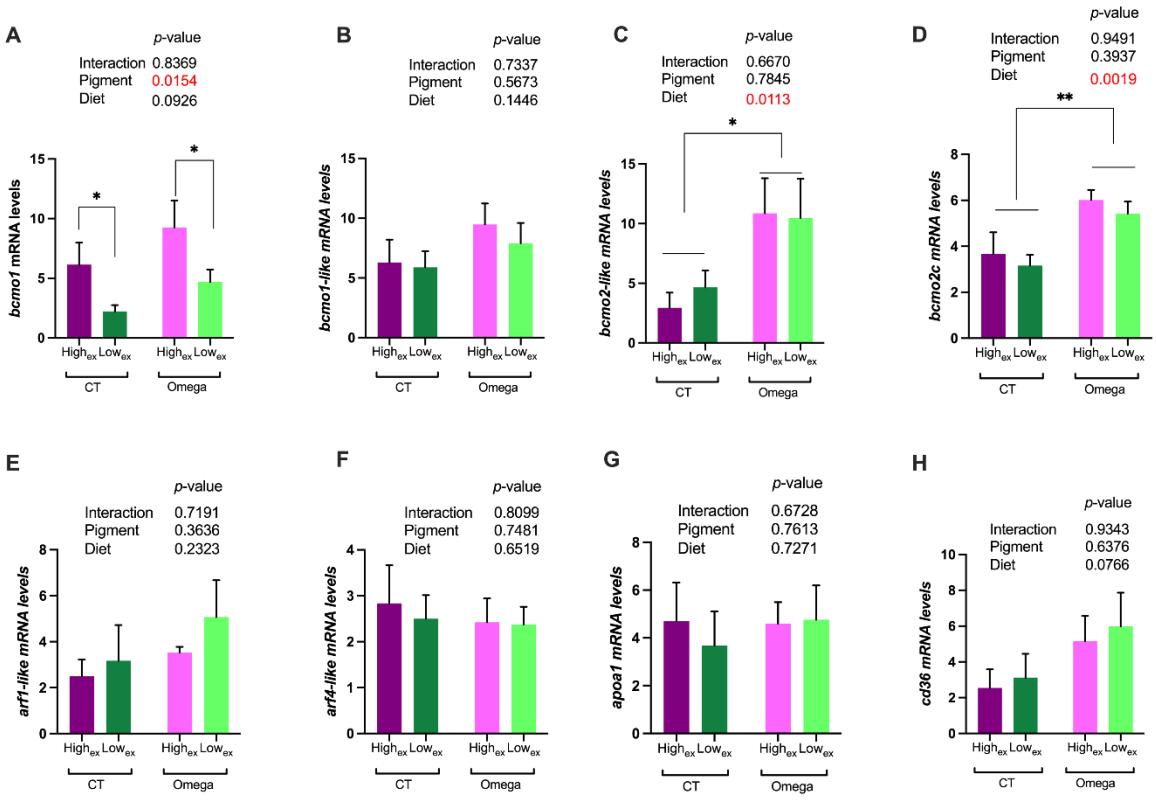


Figure 3.11. Gene expression levels of pigmentation-related genes in four different experimental groups ($High_{ex}$-Omega, $High_{ex}$-CT, Low_{ex}-Omega, and Low_{ex}-CT) in the intestine. CT and Omega are indicating two different diets fed as defined in Table 2.1. mRNA levels of *bcmo1* (A), *bcmo1-like* (B), *bcmo2-like* (C), *bcmo2c* (D), *arf1-like* (E), *arf4-like* (F), *apoa1* (G) and *cd36* (H) are presented. Relative expression levels of all samples were normalized to the geometric mean of three reference genes, *EF1a*, *rpol2*, and *eif-3*. Statistical differences in the variability between measurements are indicated in three components: interaction, pigment, and diet using two-way ANOVA followed by Tukey's post hoc test. Data are shown as mean± SEM. (n = 5). Significant differences * ($p < 0.05$) and ** ($p < 0.01$) highlighted in red color. Abbreviations. beta-carotene oxygenase 1, *bcmo1*; beta-carotene oxygenase 1, like, *bcmo1-like*; Beta-carotene 15, 15-dioxygenase 2, like, *bcmo2-like*; beta-carotene 15, 15-dioxygenase 2, like; *bcmo2c*; ADP-ribosylation factor 1-like, *arf1-like*; ADP-ribosylation factor 4-like, *arf4-like*; apolipoprotein A-I, *apoa1*; cluster of differentiation 36, *cd36*; Eukaryotic initiation factor 3, *eif-3*; elongation factor 1 alpha, *eif1a*; RNA polymerase 2, *rpol2*.

3.6.2. Gene expression analyses of pigmentation related genes in the liver

Gene expression results of selected genes from the liver are displayed in Figure 3.12. For Liver tissue, the expression of seven different genes was tested including *bcmo1*, *bcmo1-like*, *bcmo2-like*, *bcmo2c*, *srb1*, *apoa1*, and *cd36*. Results showed fish fed with the CT diet significantly displayed an increase in mRNA levels of the *bcmo1-like* gene with a *p*-value= 0.05 (Figure 3.12. B) compared with the fish group fed the Omega diet. Finally, no significant changes were observed in *bcmo1*, *bcmo2-like*, *bcmo2c*, *srb1*, *apoa1*, and *cd36* mRNA levels upon treatment (Figure 3.12. A, C, D, E, F, and G).

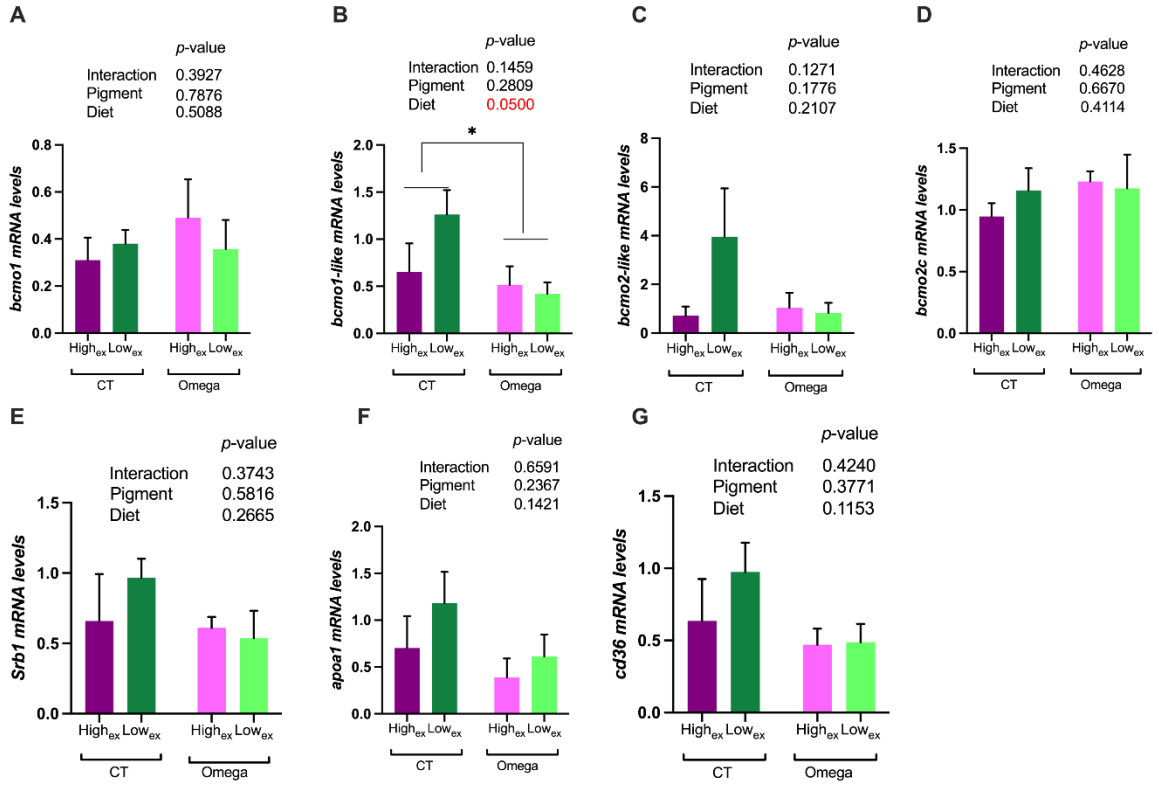


Figure 3.12. Gene expression levels of pigmentation-related genes in four different experimental groups ($High_{ex}$-Omega, $High_{ex}$-CT, Low_{ex}-Omega, and Low_{ex}-CT) in the liver. CT and Omega are indicating two different diets fed as defined in Table 2.1. mRNA levels of *bcmo1* (A), *bcmo1-like* (B), *bcmo2-like* (C), *bcmo2c* (D), *srb1* (E), *apoa1* (F) and *cd36* (G) are presented. Relative expression levels of all samples were normalized to the geometric mean of two reference genes, *eif2b2*, and *rpl12*. Statistical differences in the variability between measurements are indicated in three components: interaction, pigment, and diet using two-way ANOVA followed by Tukey's post hoc test. Data are shown as mean± SEM. (n = 5). Significant differences * ($p < 0.05$) and ** ($p < 0.01$) highlighted in red color. Abbreviations. beta-carotene oxygenase 1, *bcmo1*; beta-carotene oxygenase 1, like, *bcmo1-like*; Beta-carotene 15, 15-dioxygenase 2, like, *bcmo2-like*; beta-carotene 15, 15-dioxygenase 2, like; *bcmo2c*; scavenger receptor BI; *srb1*, apolipoprotein A-I, *apoa1*; cluster of differentiation 36, *cd36;* Eukaryotic initiation factor 3, *eif-3*; elongation factor 1 alpha, *eif1a.*

3.6.3. Gene expression analyses of pigmentation related genes in the muscle

Gene expression results of selected genes from muscle are displayed in Figure 3.13. For this tissue, the expression of the following genes was examined: *bcmo1, bcmo1-like, cd36, bcmo2b, srb1,* and *bcmo2c.* Results showed fish fed Omega diet significantly displayed a decrease in mRNA levels of *bcmo1* (Figure 3.13 A) (*p*-value= 00.368) and *cd36* (Figure 3.13 C) (*p*-value= 0.0370) genes compared with the fish group fed CT diet. Additionally, results showed fish from the Low_{ex}-Ct group significantly demonstrated an increase in mRNA levels of the *bcmo1* gene with *p*-value= 0.0230 compared with fish from the $High_{ex}$-Ct group (Figure 3.13. A). Finally, no significant changes were observed in *bcmo1-like, cd36, bcmo2b, srb1,* and *bcmo2c* mRNA levels upon treatments (Figure 3.13. B, C, D, E, and F).

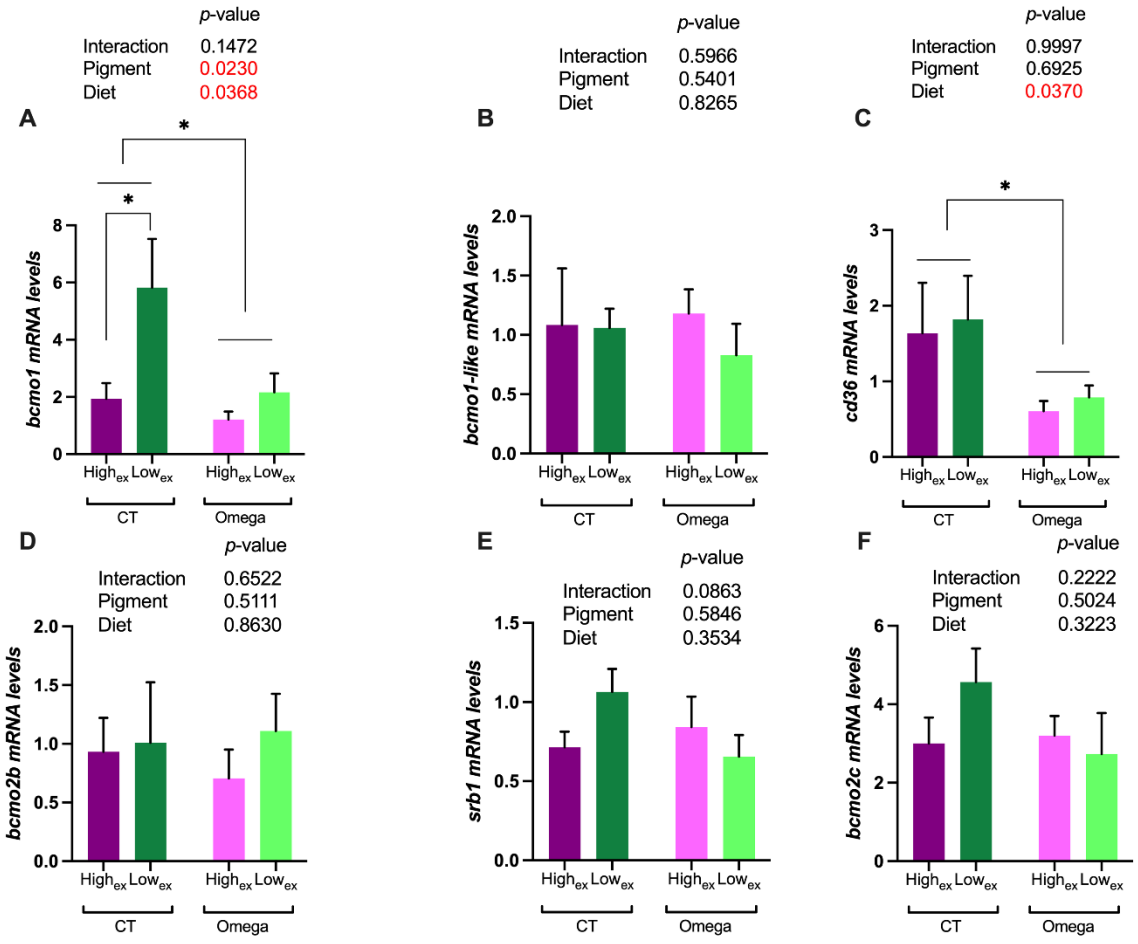


Figure 3.13. Gene expression levels of pigmentation-related genes in four different experimental groups ($High_{ex}$-Omega, $High_{ex}$-Ct, Low_{ex}-Omega, and Low_{ex}-Ct) in the muscle. CT and Omega are indicating two different diets fed as defined in Table 2.1. mRNA levels of *bcmo1* (A), *bcmo1-like* (B), *cd36* (C), *bco2b* (D), *srb1* (E), bcmo2c (F) are presented. Relative expression levels of all samples were normalized to the geometric mean of two reference genes, *eif2b2, rpl12*. Statistical differences in the variability between measurements are indicated in three components: interaction, pigment, and diet using two-way ANOVA followed by Tukey's post hoc test. Data are shown as mean± SEM. (n = 5). Significant differences * ($p < 0.05$) and **($p < 0.01$) highlighted in red color. Abbreviations. beta-carotene oxygenase 1, *bcmo1*; beta-carotene oxygenase 1, like, *bcmo1-like;* cluster of differentiation 36, *cd36*; beta-carotene oxygenase 2b, *bcmo2b*;, scavenger receptor

BI; srb1, beta-carotene 15, 15-dioxygenase 2, like; *bcmo2c*, Eukaryotic initiation factor 3, *eif-3*; elongation factor 1 alpha, *eif1a*.

3.7. qPCR gene expression pattern of *bcmo* genes and *cd36* across all the tissue

The overall gene expression pattern between dietary groups (Table 3.2) and phenotypic groups (Table 3.3) from all the tissue are shown below. Gene expression analysis showed fish from $High_{ex}$-CT group displayed lower mRNA levels of the *bcmo1* gene compared to Low_{ex}-CT in the muscle. However, fish from $High_{ex}$-CT and $High_{ex}$-Omega displayed high mRNA levels of the *bcmo1* gene in the intestine compared to Low_{ex}-CT and Low_{ex}-Omega, respectively. Moreover, results showed fish fed the Omega diet significantly displayed a decrease in mRNA levels of *bcmo1* and *cd36* genes compared to fish fed CT diet in muscle. Moreover, the results demonstrated a significant increase in mRNA levels for *bcmo2c* and *bcmo2-like* genes in the intestine of fish fed Omega diet compared with the fish group fed CT diet. Finally, the *bcmo1-like* gene showed a significantly low mRNA level in fish liver fed Omega diet compared with fish fed CT diet.

Table 3.2. The overview of different genes expression between two fillet pigment at all the tissue. ↓ and ↑ indicate significantly down and up expression of the genes, respectively. The ↑ indicates a high, but close to significant gene expression.

	Pigment H/L (liver)	**Pigment H/L (intestine)**	**Pigment H/L (muscle)**
Bcmo 1		↑	↓
Bcmo 1-like	↑		
Bcmo 2-like			
Bcmo2c			
Cd36			

Table 3.3: The overview of different genes expression between two dietary groups at all the tissue. ↓ and ↑ indicate significantly down and up expression of the genes, respectively. The ↑ indicates a high, but close to significant gene expression.

	Omega H/L (liver)	**Omega H/L (intestine)**	**Omega H/L (muscle)**
Bcmo 1			↓
Bcmo 1-like	↓		
Bcmo 2-like		↑	
Bcmo2c		↑	
Cd36		↑	↓

3.8. *In vitro* gene editing

In the *in vitro* study, we aimed to knock out the two genes *srb1* and *perilipin1*. Multiple sequence alignment of retrieved *srb1* sequence from GenBank, guide RNA and two ASK cell samples sequenced by sanger sequencing using ClustalW2 did not show any indels at the Cas cut site (Figure 3.14). Therefore, transfection of ASK cells with CRISPR/Cas9 construct targeting *srb1* did not edit the gene sequence. This was confirmed by analyzing the Sanger sequences using TIDE.

```
XM_014127943.1          TCATTTCCCAACCCCACTTCTACAATGCTGACCCTGTGCTGCTGGACTACGTACAGGGA    1375
Srb1B_815               TCATTTCCCAACCCCACTTCTAcAaTGCTgacccTGTGCTGCTGGACTACGTACAGGGa    190
Srb1A_815               TCATTTCCCAACCCCACTTCTACAATGCTGACCCTGTGCTGCTGGACTACGTACAGGGA    190
gRNAsrb                 ----------------CTTCTACAATGCTGACCCTG----------------------    20
                                         ********************
```

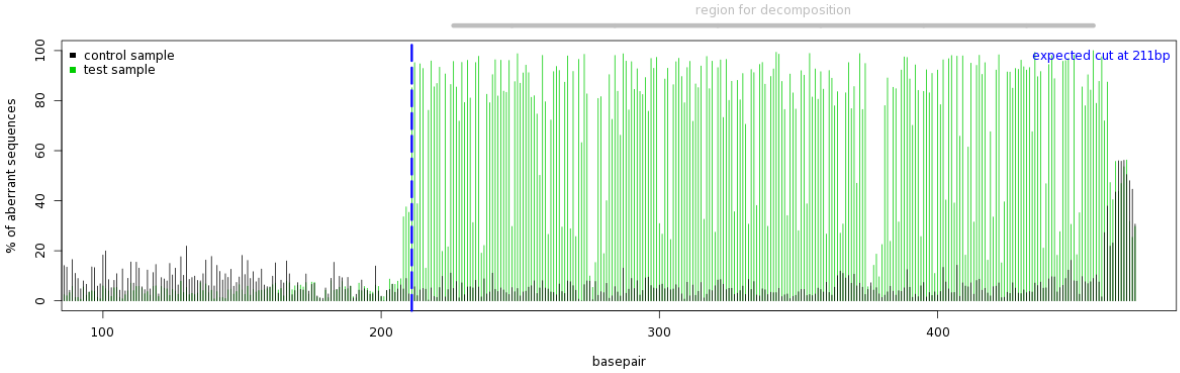
Figure 3.14. Alignment of *srb1* GenBank sequence (XM_014127943.1), two ASK cell samples (srb1A_815 and srb1B_815) sequenced by sanger sequencing and the srb1 guide RNA sequence. The results from srb1 A and srb1 B showed no indel at the Cas 9 cut cite.

For editing the *perilipin1* gene in the ASK cells, three different guide RNAs were tested. The sequencing results of the target sequences for the guide RNAs demonstrated that from three tested guide RNAs, two of the guide RNAs (including sgRNA6 shown in the Figure below) induced changes in the *perilipin1* gene, whereas one guide RNA did not work. As shown in Figure 3.15, alignment of the sequences using ClustalW2 revealed a deletion of one nucleotide at the cas9 cut site. Additionally, an assessment of the gene-editing using TIDE proved editing efficiency of 96.7% around the cut cite (Figure 3.16)

```
11-Ctrl3_p27            TGCTCAGCATGGCATCCAGTTGGCTCCAGTTGACCAGGAGTGCCTTCAGTGATGGCTTTA    236
1-Ctrl1_p27             TGCTCAGCATGGCATCCAGTTGGCTCCAGTTGACCAGGAGTGCCTTCAGTGATGGCTTTA    239
sgRNA6_plin1-KT6        ---------------CCAGTTGGCTCCAGTTGACC------------------------    20
plinB2-sgRNA6_p27       TGctcagCATGGCATCCAgttGgctccaGTT-ACCAGGAgTGCCTTCAGTGATGgcTTTA    235
plinB1-sgRNA6_p27       TGCTCAGCATGGCATCCAGTTGGCTCCAGTT-ACCAgGAGTGCCTTCAgTGATGGCTTTA    233
plinB3-sgRNA6_p27       TGCTCAGCATGGCATCCAgttGgcTCCAGTT-ACCAgGAgTGCCTTCAgTGATGGcTTTA    231
                                        *************** ***
```

Figure 3.15. Alignment of sequences from two control samples (11-Ctrl3_p27, 11-Ctrl1_p27), three ASK cell samples transfected with guide RNAs targeting *perilipin1* (plinB1-sgRNA6_p27, plinB2-sgRNA6_p27 plinB3-sgRNA6_p27) and the guide sequence targeting *perilipin1* (sgRNA6_plin1-KT6). The result from plinB1-sgRNA6_p27, plinB2-sgRNA6_p27 plinB3-sgRNA6_p27 shows a deletion of a G nucleotide at Cas 9 cut site. Yellow marking is indicating deleted nucleotide gene sequence.

A



B

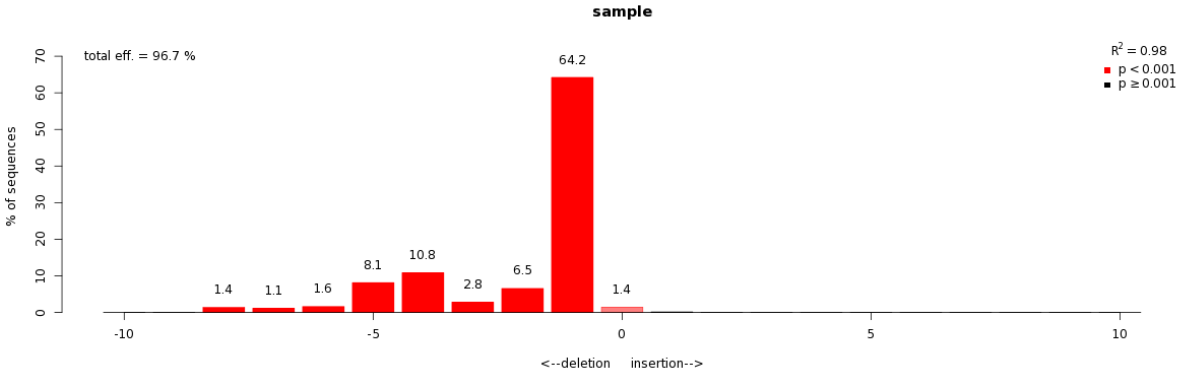


Figure 3.16. TIDE output for gene editing assessment of sequences from ASK cells transfected with guide RNA targeting *perilipin 1*. A) The abnormal signal in non-edited (black) and edited cells (green) and the expected cleavage site at 211 bp (blue line). B) The indel spectrum within ±10 bp from predicted cleavage site. The majority of single nucleotide showed deletion. The assessment is showing 96.7% editing efficiency at Cas 9 cut cite.

3.9.*In vivo* gene editing

A guide targeting a gene involved in pigmentation was used to establish the method for gene editing in salmon eggs as displayed in Figure 3.17. Microinjection of CRISPR/cas9 constructs targeting *slc45a2* in fertilized salmon eggs at a 1-2 cell stage was not successful. The mortality of eggs was up to 50-60% after the injection. Consequently, it was not possible to investigate the gene-editing efficiency of eggs after injection.

Figure 3.17. Microinjection of the eggs from observing under the microscope for confirming one-cell stage of the egg (A) to injection (B) and incubation of the egg in distilled water (C).

4. Discussion

The two main objectives of this thesis were: (i) to characterize differences in gene expression profile between muscle, liver, and intestine of Atlantic salmon with red and pale fillet color fed two different diets, and (ii) to optimize the method for gene editing by CRISPR/Cas9 using *in vitro* cultured salmon cells and embryos to investigate the function of genes involved in salmon fillet pigmentation. The current study compared muscle, liver, and intestine transcription profiles of two Atlantic salmon phenotypes including red fillet color and pale fillet color. Both phenotypes were reared under the same experimental conditions and fed two different diets including a CT diet containing a standard level of Omega-3 fatty acids (EPA and DHA) and an Omega diet with higher levels of Omega-3 fatty acids (EPA and DHA).

4.1. A similar trend of carotenoid was observed following using different measurement methods

The pigment levels of slaughter-sized salmon today normally lie in the area of 6-7 mg pigment per kg fillet (Hamre et al., 2022), showing that the average experimental fish in our trial are within the expected range (6.9 mg pigment per kg fillet). Worth mentioning that a similar trend of astaxanthin was observed in comparable groups using both VIS/NIR and HPLC method. However, when conducting chemical analyses of astaxanthin in the same groups (all four possible combinations), the astaxanthin levels were much lower than those from VIS/NIR spectroscopy analyses. The reason for the difference in pigment values is probably due to the different methods used. The VIS/NIR spectroscopy method analyses the outer surface of the fillet, which can be influenced by texture, fat content, and spectral changes due to for instance oxidation of lipids and proteins on the fillet surface (Kimiya, Sivertsen and Heia, 2013). The chemical analysis of astaxanthin, on the other hand, is a direct HPLC analytical method, which gives more exact values of the actual astaxanthin quantity in the fillets. The chemically analyzed astaxanthin values of fillets for this size of fish found in our trial is however in agreement with astaxanthin values of Atlantic salmon fillets found in a recently published study by Lutfi et al (2022). Although the astaxanthin values obtained using the chemical analyses method show less difference between groups than the VIS/NIR results, the selected High groups have higher pigment values than the low groups, showing that we reached the aim with selected experimental groups with differences in fillet astaxanthin values.

4.2. Omega-3 fatty acids did not affect fillet pigmentation and astaxanthin content

In the current study, Increased Omega-3 fatty acids in diet had no significant effect on the fillet pigmentation in the selected samples when measured both by VIS/NIR spectroscopy and HPLC analyses of astaxanthin. This finding is contrary to previous studies which have suggested that increased dietary levels of EPA and DHA were significantly associated with the fillet pigmentation in Atlantic salmon (Lutfi et al., 2022) (Kousoulaki et al., 2020). These observations may partly be explained by the relatively low number of fish per group.

When looking into the VIS/NIR spectroscopy analyses of all 900 fish in the original data material, there was a significantly increased fillet pigmentation in salmon fed with increased dietary levels of EPA and DHA in both the High pigment and Low pigment (Table 2.5). Similarly, results from the idoxanthin level in the muscle showed no significant difference in the idoxanthin level (Figure 3.1 B). However, the main purpose was to select tissue with large spread in astaxanthin and Omega-3 values in muscle within each dietary group. The idea behind this selection was to find potential gene expression difference at the liver, intestine, and muscle of contrast groups.

4.3. Liver as an essential organ involved in fillet pigment metabolism

PCA is commonly applied for the analysis of multivariate data to decrease the dimensionality of the data and simplify them for further analysis (Wang and Gehan, 2005). In our study, the PCA plot of microarray gene expression data from the liver, intestine, and muscle shows clustering of samples belonging to either High pigment or Low pigment groups, although the separation was most clear for the liver. This means samples that are found in different and separated clusters have each specific gene expression pattern. Furthermore, we have shown that not only phenotypic selection but also Omega-3 fatty acid levels affect several important metabolic pathways including lipid metabolism in the liver. Our results seem to be consistent with those previously reported that different levels of Omega-3 fatty acids (EPA and DHA) in the Atlantic salmon muscle are associated with the expression of genes related to different metabolic processes including lipid catabolism and insulin regulation in the liver (Horn et al., 2019). Our results further highlight the important role of the liver as an essential metabolic tissue in regulating the astaxanthin deposition and fillet color.

4.4.Transcription analysis of selected genes by microarray

4.4.1. Lipid metabolism-related genes identified to have a role in fillet pigment

Gene expression profile from the intestine showed a significant upregulation for several genes coding for different enzymes involved in lipid metabolism in the salmon with High fillet pigment compared to the Low fillet pigment. One of these enzymes is acyl-CoA synthetase short-chain family member 1 (*acss1*), which is hypothesized to be involved in the esterification and transport of fatty acids in plasma (Young et al., 2018). Consequently, it can be assumed that this enzyme indirectly might impact Atlantic salmon fillet pigmentation through lipid metabolism. In accordance with the present results, previous studies have demonstrated that higher expression of lipid metabolism-related genes in the intestine and liver of chinook salmon is associated with high fillet pigmentation (Madaro et al., 2020). Worth mentioning that Madaro in their experiment, used different phenotypes of chinook with red and white fillet color fed the same diet, which poses a diet difference compared with the current study. This study supports evidence from previous observations, where a significant enrichment of lipid metabolism-related genes was observed in the intestine of the Atlantic salmon with red fillet compared to the pale fillet group (Helgeland et al., 2019). Furthermore, current results are in agreement with previous study where different aspects of lipid metabolism were regulated by both β -carotene and astaxanthin (Amengual et al., 2011). However, some other lipid related genes like Acyl-CoA synthetase family member 2 is downregulated in fish group with high pigment fillet compared to low pigment fillet in both liver and muscle. Hence, the data reported here appear to support the assumption that astaxanthin regulation role on lipid metabolism is tissue dependent.

4.4.2. Higher *bcmo1* mRNA level was observed in the intestine of group with higher pigment fillet

The heatmap result from our study showed significant up-regulation of *bcmo1* in the intestine of fish with red fillet pigment compared to pale fillet. Beta carotene dioxygenase 1 (*bcmo1*) is a major enzyme in vitamin A synthesis by symmetrically cleaving carotenoids into 2 molecules of all-trans-retinal. Our study is in accordance with results from (Helgeland et al., 2014) who found higher expression of *bcmo1* in the intestine of red fillet compared to pale fillet. It is somewhat surprising that we documented upregulation of *bcmo1* gene in the intestine while

this gene normally decreases the astaxanthin bioavailability, indirectly contributing to a pale fillet. Considerably more work will need to be done to determine the role of *bcmo1* gene in astaxanthin metabolism in different tissues.

4.4.3. The carotenoid level may affect the stress-related gene expression

In this study, the expression level of genes associated with stress including heat shock protein 30 (*hsp30*) and jun proto-oncogene (*jun*) was downregulated in the liver and muscle of the low pigment group. Heat-shock proteins (Hsps) and their cognates are considered to be the main moderators of cell stress (Chen, Feder, and Kang, 2018). Generally, in the cells that are experiencing stress conditions, the level of Hsps will increase (Chen, Feder and Kang, 2018). Moreover, Ytrestøyl et al (2021), demonstrated up-regulation of stress-related genes like heat shock proteins and cognates and jun in Atlantic salmon fed an astaxanthin-free diet. A possible explanation for this might be that in our study, a low amount (but not zero) of astaxanthin was added to the diet of the Low group. However, it seems that even a low amount of astaxanthin in diet might have a protective effect against stress. This hypothesis is further supported by the previous investigation that showed an antioxidant effect of astaxanthin (Kim and Kim, 2018).

4.4.4. Cytochrome p450 212 like seems to be involved in carotenoid metabolism in Atlantic salmon

In the heat map result of the current study, cytochrome p450 2J2-like in liver is downregulated in the salmon group with red color fillet compared to the salmon group with pale fillet. Opposite to our findings, it has previously been suggested that astaxanthin stimulates the expression of cytochromes p450 (CYPs) in human hepatocytes. (Kiefer, Sumser, Wernet and von Lintig, 2002). Additionally, Madaro et al (2020) reported significantly activated cytochrome p450 in the red chinook group compared to the pale chinook group where they aimed to compare the different transcriptomes of chinook with red fillet compared to pale fillet color. On the contrary, Page and Davies (2002) showed that cytochrome p450 is not involved in astaxanthin metabolism in rainbow trout. Hence, further studies are needed in order to determine the role of Cyt p450 involvement in the carotenoid metabolism in salmonids.

4.4.5. Two carotenoid related genes found in common between liver, intestine, and muscle

From all identified genes using microarray, only nine genes were in common between the three tissues. None of the nine common genes found in this study previously known to have specific roles in carotenoid metabolism in Atlantic salmon. However, one can speculate that at least two of the common genes found in the current study could play a role in fillet pigmentation. Those two genes are coding for H3 histone family, and von Willebrand factor A domain-containing protein (VWA), which are both significantly downregulated in all tissues. A variant of von Willebrand factor domain-containing protein (VWD) is known to be a major part of the lipoprotein vitellogenin which transports carotenoids from muscle to oocytes during oogenesis (Zheng et al., 2012) (Baker, 1988), and our finding might be extendable also for VWA, but this requires further studies to verify. Previous studies showed that epigenetics may alter the expression of genes involved in carotenoid metabolism by histone modification or DNA methylation (Arango et al., 2016). Considering the involvement of H3 histone family in epigenetic mechanisms, this observation may support the hypothesis that epigenetic mechanisms might be involved in carotenoid metabolism in Atlantic salmon with High pigment fillet. Up to now, far too little attention has been paid to the role of H3 histone family in salmon fillet pigmentation. This would be a fruitful area for further work.

4.5.Gene expression analyses by qPCR

4.5.1. *Bcmo1* downregulated in muscle tissue of fish having fillet with high pigmentation level

The expression of genes related to fillet pigmentation were analyzed by quantitative PCR. As shown in (Figure 3.13. A) the *bcmo1* gene was significantly downregulated in the muscle of $High_{ex}$ compared to Low_{ex} pigment group receiving CT diet. The same results were observed for fish group receiving Omega diet. This result points *bcmo1* gene as an important gene differentially expressed in both phenotypically selected fish and those that received Omega-3 fatty acid. However, these results need to be interpreted with caution as a high degree of variation was observed between individuals in Low_{ex} in CT group, leading to a significant level. The *bcmo1* gene is coding one of the main enzymes involved in making vitamin A from carotenoids. Therefore, higher *bcmo1* gene will metabolize the pro-vitamin A carotenoids like

astaxanthin, make them less available for pigmentation. Considering lower expression of *bcmo1* gene in high pigment group receiving CT diet, it can hypothesize that higher expression of *bcmo1* in muscle leads to higher cleavage of carotenoids. Then, higher cleavage of carotenoids results in lower astaxanthin bioavailability and that leads to pale fillet pigment. The knowledge about *bcmo1* function in the muscle is limited. A recent study by Jlali et al., (2012) on the chicken has identified a negative correlation between *bcmo1* expression and the yellow color of chicken pectoral muscle. Interestingly, *bcmo1* is significantly upregulated in the intestine of high pigment muscle compared with low pigment muscle. Therefore, it seems likely that *bcmo1* activity is tissue dependent, hence the mechanism behind carotenoids metabolism might be different from tissue to tissue. However further research needs to be done to explore the role of *bcmo1* in carotenoids metabolism and fillet pigmentation in Atlantic salmon.

4.5.2. Omega-3 fatty acid impacts the *Cd36* expression level at muscle of Atlantic Salmon

In current study, the gene expression pattern of *Cd36* is interesting as this gene significantly expressed (downregulated) only in muscle and not in the intestine or liver (Table 3.2 and Table 3.3). The *cd36* expression level was only affected by the diet type (downregulated in fish group fed Omega diet) and was not different between groups having different level of fillet pigmentation. It has been shown that *cd36* plays an important role in the uptake and transport of long-chain fatty acids in adipocytes (Coburn et al., 2000). Studies by Tsai et al (2020) showed astaxanthin significantly downregulated mRNA levels of fatty acid transport-related genes like *cd36* in 3T3-L1 adipocytes. In our study, downregulation of *cd36* observed mainly due to diet having same amount of astaxanthin but different in Omega level. Current results neither in agreement nor in contrast with Tsai result, as we did not have any group receiving diet with zero amount of astaxanthin. However, it seems likely that different levels of Omega-3 fatty acid impact the expression of *cd36* in muscle of Atlantic salmon. However, the interaction of astaxanthin and lipid accumulation as well as *cd36* in salmon is not clear and requires further studies.

4.6. *In vitro* gene edi*ting*

This study set out to optimize an efficient method to deliver the guide RNAs in ASK cells. The second aim of this trial was to employ the efficient and optimized method *in vivo* for fertilized salmon embryos. For *in vitro,* ASK cells that are fast-growing, easy to maintain, and more adaptable to standard cell culture routines (Rolland et al., 2005) were used. The aim was to knock out two genes including *srb1* and *perilipin 1* in ASK cell lines. Since perilipins are the proteins linked with the lipid droplets (Londos et al., 1995), the idea behind knocking down of *perilipin* gene was to see its impact on salmon fillet pigmentation. From the two genes aimed to edit, we could manage to edit *perilipin 1* with 96% editing efficiency. However, the results for *srb1* gene were not very encouraging. Possible explanations for why the guide RNA targeting the s*rb1* gene did not work are many, including transfection efficiency, chromatin accessibility (Jensen et al., 2017), selection of target site, short guide RNA design, different delivery methods, and off-target effects (Peng, Lin, and Li, 2015). To improve the success rate, several guide RNAs targeting the same gene should be evaluated to select the most efficient guide. Further protocol optimization was not possible due to a lack of time for the master project.

4.7. *In vivo* gene editing

The major limitation of this study is that we were not able to establish the gene-editing by CRISPR/cas9 in the Atlantic salmon embryo model. The main reason was the high mortality rate (up to 60%) after injection. It seems possible that these results are due to egg or water quality. There are, however, other possible explanations for instance the size and shape of the glass microneedles and the volume of the mixture injected into cells. In this study, the injected volume was determined by the pressure supplied by the compressor. Injection of a too high volume might also result in the mortality of eggs. As an attempt to overcome mentioned possibility, different approaches were taken. However, due to lack of time, the method was not established for salmon embryos.

5. Conclusion

To characterize the difference in the gene expression profile of red and pale fillet colors in Atlantic salmon, genes involved in the metabolism of astaxanthin were studied by microarray and qPCR analysis. In addition, we attempted to optimize the method for gene editing by CRISPR/cas9 of *in vitro* cultured salmon cells and salmon embryos.

Results from carotenoids analysis showed that increased dietary inclusion of Omega-3 fatty acids had no significant effect on the muscle astaxanthin content. In addition, this study showed a clear effect of the fillet pigment selection on the transcription profile of important genes in all tissues and particularly in the liver which indicates the potential involvement of this tissue in regulating astaxanthin deposition and fillet color.

Additionally, transcription data from different tissue demonstrated differentially expressed genes involved in regulatory pathways like lipid metabolism that are likely linked with the absorption, metabolism, and transport of astaxanthin in the body. Therefore it can be concluded that astaxanthin metabolism could be different and tissue dependent.

Likewise, our results demonstrated that both the omega-3 fatty acid levels in the feed and differences in muscle pigmentation significantly influenced the expression of several genes related to carotenoid and/or lipid metabolism including *bcmo1, bcmo1-like, bco2-like, bcmo2c,* and *cd36* in the liver, intestine, and muscle.

Furthermore, we aimed to knock down some candidate genes using both *in vitro* and *in vivo* trials. However due to lacking time during the master thesis, method optimization will be further carried out by Nofima. On the basis of these results, it appears that salmon fillet color is less responsive to the content of omega level in the diet. However, it is more likely to be affected by some candidate genes, worth to be investigated further using CRISPR/Cas method.

6. References

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