REPLACING FISH OIL BY VEGETABLE OIL IN A LOW FISH PROTEIN DIET: EFFECTS ON VISCERAL ADIPOSE TISSUE AND INFLAMMATION IN ATLANTIC SALMON

Master in Nutrition of Aquatic Organisms in Aquaculture

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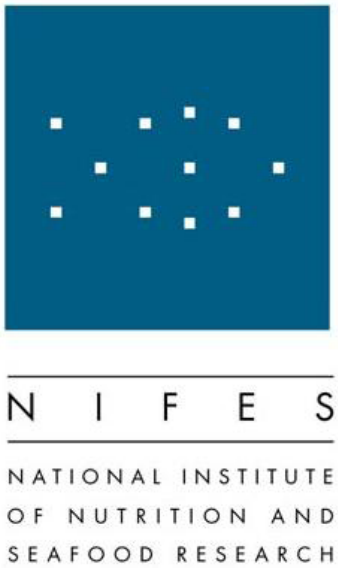

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ABSTRACT

Dietary fish oil (FO) replacement with vegetable oils in combination with high dietary fish meal replacement have previously been shown to increase Atlantic salmon plasma and liver TAG as well as visceral fat deposition. In human subjects increased visceral adiposity may lead to chronic low grade inflammation and may have health implications. Furthermore, decreasing the *n*-3/*n*-6 ratio by increasing dietary *n*-6 fatty acids may influence inflammation status.

We investigated the combined effect of fish oil and fish meal replacement on visceral adipose tissue lipids and inflammation in Atlantic salmon. Fish with an initial weight of ≈ 800 g were fed an experimental diet for 28 weeks where 70% of the fish meal was replaced by a plant protein blend. The control diet contained 100% fish oil, whereas the 80% of the fish oil was replaced by either rapeseed oil (RO), olive oil (OO) or soybean oil (SO) in the three experimental diets. This resulted in high (FO), intermediate (RO and OO) and low (SO) dietary *n*-3/*n*-6 ratio, whereas the marine omega-3 level was stable at 5% in all VO diets.

To this work we analyzed the fatty acid composition of visceral fat, the macrophage infiltration in visceral adipose tissue through histological observation and gene expression of inflammatory markers and macrophage precursors in visceral adipose tissue and head kidney.

There was no statistical significant different in final weight, length, visceral somatic index and in adipocyte size of visceral adipose tissue.

Visceral adipose tissue highly reflected the dietary fatty acid composition. There was no difference in total fatty acids, but there was difference in fatty acid composition between the dietary groups. There was higher eicosapentaenoic acid (20:5 *n*-3, EPA), docosahexaenoic acid (22:6 *n*-3, DHA) and arachidonic acid (20:4 *n*-6, AA) in FO fed fish. On the other hand, 18:2*n*-6, 18:1*n*-9 and 18:3*n*-3 were present in higher levels in fish fed diets with vegetable oils. As a result, the *n*-3/*n*-6 ratio was higher in FO dietary group and fish feed with SO diet had the lowest ratio.

There was macrophage infiltration in all dietary groups but at a very low level, mainly in fish feed with OO and FO. The SO dietary group had the highest macrophage infiltration. Nevertheless the macrophage accumulation was not at a level to be considered as inflammation, being in line with the mRNA levels of cytokines and macrophages genes.

With this we can conclude that the replacement of fish oil by olive oil, rapeseed oil or soybean oil in 80% does not have an influence on adiposity or low-grade inflammation in visceral adipose tissue.

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AA	arachidonic acid (20:4 *n*-6)
DHA	docosahexaenoic acid (22:6 *n*-3)
DPA	docosapentaenoic acid (22:5 *n*-3)
EFA	essential fatty acids
EPA	eicosapentaenoic acid (20:5 *n*-3)
FA	fatty acids
FCR	feed conversion ratio
FM	fishmeal
FO	fish oil
FP	fish protein
H&E	hematoxylin and eosin
HUFA	highly unsaturated fatty acids
IL	interleukine
LNA	linolenic acid (18:3 *n*-3)
LA	linoleic acid (18:2 *n*-6)
OO	olive oil
PUFA	polyunsaturated fatty acids
RO	rapeseed oil
SGR	specific growth ratio
SO	soybean oil
TAG	triacylglycerol
TNF	tumor necrosis factor
VLC *n*-3 PUFA	very long chain *n*-3 polyunsaturated fatty acids
VLDL	very low-density lipoprotein
VO	vegetable oil
VSI	visceral somatic index
WAT	white adipose tissue
WFLL	whole fish lipid level

1. INTRODUCTION

Atlantic salmon (*Salmo salar,* L. 1758) is a member of the family Salmonidae, the only one of Order Salmoniformes. It is present in the cold-water streams, dividing their lives between fresh and salted water in an anadromous form. They are born in fresh water and live there during larval and juvenile life stage. After smoltification – physiologic and morphologic changes – the smolts migrate to sea water, where most feeding, growth and fat storage take place. After maturation, salmon move back to fresh water to reproduction and spawning in the exact home stream. In fresh water the mature fish do not eat and the stored fat is the energy source. The anadromous migration is more related with high latitudes, where the productivity of ocean exceeds in freshwater. The ocean is favourable to adult fish feeding and the river stream is favourable to larval survival (Moyle and Cech, 2004).

Aquaculture is an industry with a enormous growth every year. Norway is the leading aquaculture producer of salmonids 36,4% of world production. Related with export, Norway was the world's second largest exporter, exporting more aquacultured than wild catch fish. Worldwide aquaculture represented 46 percent of total food fish supply, a slightly lower proportion than reported in *The State of World Fisheries and Aquaculture 2008,* but representing a continued increase from 43 percent in 2006. Almost 81 percent (115 million tons) of world fish production in 2008 was destined for human consumption. Since the mid-90's the proportion of fish used for direct human consumption grew. (Tacon *et al.*, 2006).

As a carnivorous fish, Atlantic salmon use amino acids, provided by protein content, mainly to fish growth and lipids as energy source (Schmidt-Nielsen, 1997). In nature, lipids are present in both animal and vegetable sources. They are used as main energy source due the high specific energy value and the almost complete digestibility. In aquaculture fish feed lipids are very important and irreplaceable nutrients (Sargent *et al.*, 2002). Conventionally Atlantic salmon was fed large amounts of fish oil (FO) and fish meal (FM) giving good growth rate and fatty acid composition. Anchoveta, herring and other small pelagics are the main species used for reduction. Production of fishmeal and fish oil is strictly linked to the catches of these species (Tacon *et al.*, 2006). FO provide a high levels of marine fatty acids (FA) with very long chain *n*-3 polyunsaturated fatty acids (VLC *n*-3 PUFA) such as eicosapentaenoic acid (20:5 *n*-3, EPA), docosahexaenoic acid (22:6 *n*-3, DHA) and docosapentaenoic acid (22.5 *n*-3, DPA) (Torstensen *et al.*, 2005, Jordal *et al.*, 2007). When

salmon is fed with FO and fish protein (FP) the amount of *n*-6 fatty acids is low, increasing the *n*-3/*n*-6 fatty acids ratio, which is beneficial to human health (Simopoulos, 2001).

Due the variable availability and environmental issues fish industry has been replacing the FO for other lipid sources to contribute to aquaculture sustainability (Tacon et al., 2006, Turchini et al., 2009). Vegetable oils (VO), such as rapeseed oil (RO), soybean oil (SO), olive oil (OO), palm oil or linseed oil, are promising substitutes, but it is still wanted to study the influences (Torstensen et al., 2011). The concerns with this replacement are the low n-3/n-6 ratio of VO diet, due the increased levels of linoleic acid (18:2 n-6, LA), the presence of monoene FA and the low levels of n-3 PUFAS or with more than 18 carbons in the chain (Torstensen et al., 2008, Nanton et al., 2007, Jordal et al., 2007, Bell et al., 2002).

1.1 Lipids

The lipids are a heterogenic group of molecules insoluble in water but soluble in organic compounds such as benzene, hydrocarbon, chloroform, ether and alcohol. They have long chains of fatty acids esterified to alcohol groups but sterols are the exception. There are two nomenclatures of fatty acids. The first one is, e.g. 22:6*n*-3, which refers that this fatty acid having 22 carbon atoms with six double bounds and the first one is in the third carbon atom from the methyl end. Another one is according the IUPAC nomenclature that the counting begins from the –COOH end and the double bonds are labeled with cis-/trans- rotation.

Lipids are the function of storage energy and also the function of cofactors in enzymes and precursors for essential signal molecules (eicosanoids) and tissue hormones. According with Sargent *et al.* (2002) there are 5 different classes of lipids. Triacylglycerols are lipids formed by three molecules of fatty acids esterified to one glycerol. Their function is storage fat or fat-soluble nutrients. The complete oxidation of fatty acids provides 9kcal/gr. Wax esters are very long single molecule of fatty acid esterified to a single molecule of fatty alcohol. They are very abundant in marine zooplankton. When fish consume wax esters, they are converted to triacylglycerols. It is present mainly in “northern hemisphere fish oil” and in a low percentage in “southern hemisphere fish oil”. It can be also found in body tissue and in the eggs of some fish species. Phospholipids are formed by two molecules of fatty acid saturated or monounsaturated and one alcohol, which specify the phospholipid. They are the main compound lipid bilayer of cells and the compositions of membrane’s fatty acids are according external conditions. Sphingolipids are like phospholipids but constituted from phospholipidic

acid. They are typically saturated or monounsaturated formed by chain with from 16 to 26 carbon atoms by length. Sterols are molecules arranged in four rings of carbon atoms. Cholesterol is major sterol in animal tissue formed by 17 carbon atoms arranged in four rings. It can be present in blood, e.g. C18 - estrogens, C19 - androgens, C21 – progestogens. They are an important component of lipid membrane and a precursor of bile acids.

1.1.1 Adipose Tissue

The adipose tissue belongs to connective tissue. The predominant cells are adipocytes isolated or grouped in considerable amounts, storing energy in form of triglycereol (TAG). TAG are more efficient as energy stores than proteins and carbohydrates due of the high caloric value per gram. They are stimulated by nervous and hormonal stimulus. More than energy function, adipose tissue is also important in shaping the body surface, protect to mechanic injuries, thermal insulation, organ sustaining and secretion of liposoluble nutrients and hormones. The vascularisation in adipose tissue is well supported, if considering the small amount of cytoplasm compared with other cells.

1.1.2 Essential Fatty acids

A fundamental function of adipose tissue is the release of essential fatty acids (EFA) (Sargent *et al.*, 2002). The term “essential” mean that the organism is no able to produce the nutrient *de novo* and it is demanded the present in the diet for normal metabolism, growth and health. *n*-3 and *n*-6 fatty acids are essential fatty acids to fish. *n*-3 fatty acids are characterized by a double bond at position *n*-3 from the methyl end of the carbon chain. They are present in the marine food web, it is the most abundant fatty acid in marine fish and marine finfish and cold water freshwater fish have an essential requirement of them (Turchini *et al.*, 2009, De Silva and Anderson, 1995). *n*-6 fatty acids are the second essential fatty acid family, which is defined by a double bond at position *n*-6 from the methyl end of the carbon chain. This fatty acid is produced by terrestrial plants and by algae in fresh water food web. Freshwater fish from warm waters have been reported to have requirements for both *n*-3 and *n*-6 fatty acids (De Silva and Anderson, 1995).

Theoretically, the only two fatty acids that should be most rigidly termed as essential are linoleic acid (18:2*n*-6, LA) and linolenic acid (18:3*n*-3, LNA), which cannot be

biosynthesized *de novo* by any fish or other vertebrate (Turchini *et al.*, 2009). Almost all vertebrates are potentially able to convert the two basic C_{18} *n*-6 and *n*-3 PUFA into the corresponding C_{20} and C_{22} *n*-6 and *n*-3 highly unsaturated fatty acids (HUFA) by alternating succession of denaturation and elongation (Nakamura and Nara, 2004). With a HUFA-rich environment some species have lost this capability and the consequently these species have a direct requirement of HUFA (Sargent *et al.*, 2002). Thus, it is well accepted that many species have a net requirement for the longer and more unsaturated fatty acids, such as EPA, DHA and arachidonic acid (20:4*n*-6, AA) (Sargent *et al.*, 1999). The same author says that the requirement of the EFA should not be consider in isolation but due their competitive interactions they need to be consider as conjunct and the *n*-3/*n*-6 ratio requires careful considerations (Sargent *et al.*, 1999). When the fish oil is replaced in aquafeeds, mainly with VO rich in *n*-6 PUFA the *n*-3/*n*-6 ratio will be directly modified in fish tissue (Turchini *et al.*, 2009).

Eicosanoids are biological molecules derived by oxidation of pentanoic acids (fatty acids with 20 carbon atoms). Prostaglandins, prostacyclins and lipoxins are examples. These signaling molecules are involved in the reproduction, cardiovascular tone and immune response as pro-inflammatory factors. EPA and AA compete between them in the production of eicosanoids, where the eicosanoids produced by EPA are less active. Thus, the *n*-3/*n*-6 will determine which prostaglandin in cellular membrane will be more common and it will have consequences in immune system (Turchini *et al.*, 2009, Sargent *et al.*, 2002).

Experiments in mice showed that marine *n*-3 PUFA has positive influence in chronic diseases, acting as fuels and structural components of cell and they are very effective in lowering triacylglycerols and very low-density lipoprotein (VLDL) levels (Lombardo *et al.*, 2007). Bell *et al.* (1999) showed that a diet with a lack of EFA decrease specific growth ratio and weight, increase the mortality and a reduction in cellular HUFA, which may predispose to cellular alteration.

1.2 Vegetable oils replacement

With the change dietary fatty acid composition the myosepta, liver and adipose tissue fatty acid composition will be highly affected (Nanton *et al.*, 2007, Torstensen *et al.*, 2004a, Turchini *et al.*, 2009). There will be a lower *n*-3/*n*-6 ratio and lower levels of EPA and DHA in fish fed with VO than fish fed with FO (Torstensen *et al.*, 2004b, Nanton *et al.*, 2007, Bell *et al.*, 2002). Previous studies with FO replacement showed an increase of adipose tissue lipid level in the visceral fat in the fishes fed with VO with high replacement (Nanton et al., 2007). In contrast some studies with the total or partial replacement of FO by VO showed no significant effect in growth, as long as the amino acids requirement is fulfilled (Bell et al., 2002, Torstensen et al., 2005, Espe et al., 2006). Torstensen *et al.* (2005) with the total replacement of the FO by VO did not fnd any different in feed efficiency. The water temperature is also a parameter to concern since that it was reported a negative growth influence due the decrease protein efficiency ratio and utilization in low temperatures (Torstensen *et al.*, 2005). In humans the relationship between inflammation and the normal pattern of bone growth and spinal deformities is known (Hughes *et al.*, 2006, Gratacos *et al.*, 1994). Kvellestad *et al.* (2000) suggest the inflammation as risk factor for spinal deformities in Atlantic salmon. However Martens *et al.* (2010) suggest that VO is not a risk factor for the development of inflammation related with spinal deformities.

1.3 Vegetable Oil and Obesity

A very important association to this present study is the relationship between the obesity and VO diets. Obesity is an excess of visceral adiposity and a chronic pathology with high morbidity rates (Ouchi *et al.*, 2011, Valle *et al.*, 2005). In salmon, visceral adipose tisssue is the major fat storage site, where also occur an excessive fat deposition with obesity (Nanton *et al.*, 2007, Polvi and Ackman, 1992). Madsen *et al.* (2005) demonstrate that a diet enriched with *n*-3 PUFA repress the development of obesity, regulating the transcription of factors involved in adipogenesis and lipid homeostasis in mature adipocytes in rodents. At same time, a diet rich in *n*-3 HUFA had a positive influence in the white adipose tissue (WAT), reducing the fat deposition in fish (Todorcevic *et al.*, 2009). With an excess of energy it lead to obesity and some disorders are related with it, such as oxidative stress and changes in mitochondrial health (Keijer and van Schothorst, 2008, Todorcevic *et al.*, 2009). In Atlantic salmon, fed with *n*-3 HUFAs and increased lipid storage, it was possible to observe more susceptibility to

oxidative stress of mitochondria in WAT and the decrease of mitochondria number (Todorcevic *et al.*, 2008). Furthermore, diets enrich with EPA and DHA decreased lipid accumulation in Atlantic salmon adipocytes (Todorcevic *et al.*, 2008). Dietary EPA and DHA has also been shown to decrease plasma TAG accumulation in mature salmon adipocytes (Huang *et al.*, 2010).

A recent study in mice showed a close relationship between the replacement of FO by VO and the development of obesity (Alvheim *et al.*, 2012c). In this experiment the diets was modulated to increase the dietary level of LA to 8 en% in order to reproduce the increase LA increased uptake in US (Blasbalg *et al.*, 2011). Epidemiological studies linked the increased consumption of LA to the prevalence of the obesity and postulated that AA alter the energy balance towards obesity (Ailhaud *et al.*, 2006). In a diet rich in LA (Alvheim *et al.*, 2012a) the endocannabinoid system is excessively activated. Endocannabinoids are endogenous lipids mediators. AA in phospholipids is the precursor of the two best characterized endocannabinoids 2-arachidonoylglycerol and *N*-arachidonoylethanolamine. The synthesis of endocannabinoids are strongly related with the dietary of *n*-3/*n*-6 ratio, since that *n*-3 and *n*-6 cannot be synthesized *de novo* (Lands *et al.*, 1992). Soybean oil is high in *n*-6 leading to decrease of *n*-3/*n*-6 ratio due the increase of LA in its composition and it is linked with the increase of the obesity in the US during the 20th century (Ailhaud *et al.*, 2006).

Alvheim, *et al.(2012c)* demonstrated for the first time that low fat diets can me made obesogenic by selective inclusion of 8 en% LA subsequently stimulating excessive endocannabinoid activity and inducing both weight gain and adipose tissue inflammation in mice. Even with no effects in body weight or in visceral somatic index in Atlantic salmon, showed that the replacement of FO by SO induces high dietary levels of LA and elevates AA, endocannabinoids activity and TAG accumulation in salmon liver. Thus, lower dietary levels of LA may improve metabolic functions associated with obesity in humans *(*Alvheim *et al.*, 2012b). It is valid also to Atlantic salmon but it is not yet elucidated.

1.4 Obesity and Inflammation

The increased interest in obesity was promoted by the recognition that immune system and inflammation are affected by obesity and at same time due its connection with high morbidity-mortality rate (Valle *et al.*, 2005). Obesity is associated by low-grade systemic inflammation response and it can lead to others chronic diseases, such as the high risk of diabetes and cardiovascular events (Mathis and Shoelson, 2011, Ouchi *et al.*, 2011, Ouchi and Walsh, 2007, Schutte *et al.*, 2010, Wellen and Hotamisligil, 2003) or even cancer and neurodegeneration (Mathis and Shoelson, 2011).

Inflammation and the inflammatory response are part of the innate immune response and system is a fundamental key to the survival and eliminating the invading toxins and/or pathogens (Iwama and Nakanishi, 1996, Goldsby and Kuby, 2002). It constitutes the second line of defense in fish, after protection of integrity of skin, intestinal tract and gill (Magnadóttir, 2006). Inflammation is characterized by redness, swelling, heat and pain. There is an increase in vascular diameter and subsequent increase of blood volume and decreased blood flow (Calder, 2006). Due the increased amount of blood, in that area the temperature and the redness increase. In the end, the permeability of blood capillaries also increases, permitting the leakage of the fluids as well as movement of leukocytes from the bloodstream into the surrounding area, resulting in an edema (Wellen and Hotamisligil, 2005, Calder, 2006). After that there is a migration of leukocytes out of the capillaries into the site of infection, attracted and regulated by adhesion molecules and allowing the diapedisis. Neutrophils are the first cells arriving to the damage tissue. They are phagocytes cells and responsible for releasing mediators, chemokines, to attract monocytes and macrophages to the site of inflammation (Iwama and Nakanishi, 1996, Goldsby and Kuby, 2002).

Macrophages are mononuclear phagocytes belong to connective tissue and at same time to the innate immune system (Junqueira and Carneiro, 2005, Iwama and Nakanishi, 1996, Weisberg *et al.*, 2003). They derive from precursor cells of bone marrow, which split in monocytes and after maturation they become macrophages, increasing the size and the production of proteins. Once the differentiation, the second cells can be divided and produce new macrophages (Junqueira and Carneiro, 2005, Fantuzzi, 2005). Their shape depends with the functional activity and with the tissue where they are. Normally they are between 10 and 30 µm diameter and the nucleus has a kidney shape located eccentrically on cytoplasm. The surface shows an irregular shape due to their phagocytic, pinocytotic and scavenger activity of strange substances. They are the connection of innate immune and adaptive response, due

their capacity to produce pro-inflammation factors and also specific antigens (Calder, 2006, Joerink *et al.*, 2006). The more representative function is the defense by the phagocytisis. They also process antigens and secret cytokines and quimiotatic factors which works in the inflammation process (Iwama and Nakanishi, 1996).

Some decades ago the adipose tissue was considered as a static tissue with the energy storage as the only function. In the past two decades was established that adipose tissue is also an endocrine tissue which is able to produce active molecules, the adipocytokines, which have different functions, such pro- or anti-inflammation function (Ouchi *et al.*, 2011, Valle *et al.*, 2005, Weisberg *et al.*, 2003). Fantuzzi (2005) even says that the adipose tissues is no longer considered an inert tissue and with a considerable importance in regulating physiologic and pathologic processes, like the immunity and inflammation. Mathis (2011) uses the term immunometabolism as "the interplay between immunological and metabolic processes". This integration between them is favorable in normal conditions but it can be a disadvantage under unfavorable metabolic conditions, such as obesity (Wellen and Hotamisligil, 2005).

According with Weisberg *et al.* (2003) the infiltration of macrophages in the adipose tissue is linked with the systemic inflammation and insulin resistance. The change of endocrine and metabolic functions of the adipose tissue is caused by the obesity, leading to an increased release of fatty acids, hormones and pro-inflammatory molecules, the adipokines (Bastard *et al.*, 2006). In lean rodents the macrophages are small and dispersed among the adipose tissue. In a obese rodents the macrophages are present in aggregates and in extremely obese animals some groups of macrophages are surrounding one or a group of adipose cells (Weisberg *et al.*, 2003). Inflammation is the primary event associated with obesity (Wellen and Hotamisligil, 2005, Bastard *et al.*, 2006) but the cause to increased production of pro-inflammatory factors in obesity is not clearly understood in fish (Fantuzzi, 2005, Mathis and Shoelson, 2011).

1.5 Macrophages Accretion

There is known that the expression of the mediators is produced by adipocytes and macrophages to increase the macrophage recruitment until their accumulation. In studies of obesity in mice was observed an increase of adipocytes size and macrophages concentration and their correlation (Ibrahim *et al.*, 2011, Ouchi *et al.*, 2011, Fantuzzi, 2005, Weisberg *et al.*, 2003). The macrophages can express mediator factors alone or with an interaction with adipocytes (Wellen and Hotamisligil, 2005). This integration makes sense because both

participate in the innate immune response – macrophages as killing pathogens and realising cytokines and chemokines to the inflammation and the adipocytes for the modulation of inflammatory state or participate in the pathogens neutralization, through the releasing of fatty acids (Wellen and Hotamisligil, 2005).

The most common referenced cytokines are tumour necrosis factor (TNF)-α, interleukine-6 (IL-6), leptin and resistin (Ouchi *et al.*, 2011). TNF-α is a pro-inflammation cytokine produced by monocytes, macrophages and adipocytes in mammals and also in fishes (Wellen and Hotamisligil, 2005, Weisberg *et al.*, 2003, Ouchi *et al.*, 2011, Iwama and Nakanishi, 1996). The expression of this mediator increases in the adipose tissue and plasma with the increase state of obesity. It is also observed a influence in the insulin receptor, leading to the increase of insulin resistance (Fantuzzi, 2005, Wellen and Hotamisligil, 2003, Bastard *et al.*, 2006, Ouchi *et al.*, 2011, Ouchi and Walsh, 2007). IL-6 is also a pro-inflammatory cytokine produced by adipocytes (Ouchi *et al.*, 2011) and is related when the obesity and insulin resistance (Wellen and Hotamisligil, 2005, Mathis and Shoelson, 2011, Weisberg *et al.*, 2003, Tai and Ding, 2010, Bastard *et al.*, 2006). In other hand, according to Bastard *et al.* (2006) IL-6 is not produced by mature adipocytes but rather by preadipocytes and other cells from stroma vascular fraction. In subjects with weight loss the increased concentration of IL-6 is also observed (Esposito *et al.*, 2003). In mice, leptin is the product of obese gene by adipocytes and it increases in obese individuals. Leptin acts as pro-inflammatory factor because it leads to a major production of TNF-α and IL-6 by monocytes (Ouchi *et al.*, 2011, Valle *et al.*, 2005, Wellen and Hotamisligil, 2005, Bastard *et al.*, 2006). It increases the production of TNF-α and IL-6 but in macrophages (Ouchi *et al.*, 2011). Another complementary cytokines produced by adipocytes and macrophages related with inflammation are IL-1β and IL-10 (Ouchi *et al.*, 2011, Weisberg *et al.*, 2003, Bastard *et al.*, 2006, Valle *et al.*, 2005). In order to find macrophage colonies of macrophages the macrophage colony-stimulating factor receptor (M-CSFR) was tested by Pettersen *et al.* (2008) but without success in Atlantic salmon. Last, denditric cells presence, as precursor of macrophages, were study also as indicative of inflammation in Atlantic salmon (Pettersen *et al.*, 2008, Holen *et al.*, 2011)

In lasts years some theories and hypothesis were formulated to try to explain the inflammation and the macrophage accretion in adipose tissue. Charrièrre (2003) study the relationship between preadipocyte and macrophages and observed that preadipocytes are still present in adipose tissue during the adult stage and they are able to multiply and differentiate into mature adipocytes, according with the energetic request. In an inflammation environment, due the great cellular plasticity, adipose precursor cells also have the ability of convert very rapidly and efficiently into macrophages.

Wellen (2003) said that in obese adipose tissue there are some changes in paracrine functions of adipocyte. There is an increased synthesis of TNF-α, which is the cause for macrophages attraction from the bone marrow to adipose tissue in clusters. There is also an increased production of leptin by adipocytes, which once more lead to macrophage accumulations and a stimulation of more macrophage recruitment. This author says that this could be a vicious cycle of macrophage recruitment, production of inflammatory cytokines and impairment of adipocyte function. Later, the same author (Wellen and Hotamisligil, 2005) speculate two others reasonable explanations to macrophage mobilization. The adipocytes are the first cells affected by the development of obesity or their neighbors hypertrophy. The first speculation is based in the endoplasmic reticulum and its increased request due the increased protein and lipid production and the change in the shape. The endoplasmic reticulum stress leads to release of c-Jun *N*-terminal kinase, a metabolic regulator which is related with macrophage recruitment (Wellen and Hotamisligil, 2005, Ozcan *et al.*, 2004, Nakatani *et al.*, 2005). The second conjecture is based in the oxidative stress. In a hyperglycemic condition, with the increased amount of glucose in the adipose tissue, there is a excessive production of reactive oxygen species in mitochondria, which leads to oxidative damage and production of inflammation factors (Brownlee, 2001).

According with Fantuzzi (2005), the number of the macrophages in the WAT in mice and humans is related with adiposity and adipocyte size. The author demonstrated that the adipocytes present in adipose tissue derived from bone marrow and not from the differentiation of preadipocytes as previous studies (Charrière *et al.*, 2003). With the production of factors as leptin, TNF-α and IL-6 occur the transmigrations of bone marrow-derived monocytes and thus the increased concentration of macrophages in the adipose tissue. In 2003 Weisber *et al.* get the same conclusions about macrophage's origin.

The process of obesity related with inflammation begins with hypertrophy and hyperplasia processes adipose cells (Ouchi et al., 2011). There is increased demand oxygen and nutrients

but the obese adipose tissue could limit the proper flow of vessels and provoke ischemia. At same time they allow the spread of TNF, IL-6, leptin, resistin and others adipokines in body in order to macrophage mobilization. The accumulation of macrophages in obese adipose tissue is proportional to adiposity in both humans and mice. Adipocytes create histological structures composed by macrophages surrounding the dead adipocytes in the inflamed tissue, the crown-like structures (Weisberg *et al.*, 2003, Ouchi *et al.*, 2011). The macrophages stayed there in the way to phagocyte invalid cells, repair the injured tissues and prevent the release of toxic substances (Ouchi *et al.*, 2011). Even though it is significant to remark that, in humans, the adipocyte apoptosis do not increase with the obesity (Spalding *et al.*, 2008).

A diet with considerable concentration of *n*-3 polyunsaturated fatty acids (DHA and EPA) has a suppressive inflammatory action due the inhibition of prostaglandin E2, a suppressor of anti-inflammatory factors TNF-α and IL-6. This prostaglandin also induces the vasodilatation, redness and swelling. In other hand, the prostaglandin E2 for them self are anti-inflammatory in some circumstances, depend which receptors they binding to. (Tai and Ding, 2010, Calder, 2006, Ricciotti and FitzGerald, 2011).

In obese condition there is also production of anti-inflammatory factors, such us adiponectin (Ouchi *et al.*, 2011). Adiponectin is produced exclusively in adipose tissue to control the recruitment of macrophages but it is inhibited by TNF and IL-6, what can lead to a faster inflammatory response. The main function of this adipokine is avoiding the systematic inflammation and cardiovascular disease, in a compensatory response, but at same time it increases the insulin sensitivity (Valle *et al.*, 2005, Ouchi *et al.*, 2011, Beylot *et al.*, 2006, Wellen and Hotamisligil, 2003, Bastard *et al.*, 2006, Ouchi and Walsh, 2007).

The inflammation response occurs during the first 3-4 days, after which the phagocytes return to a resting stage and their number declines, by emigration and also lyses (Iwama and Nakanishi, 1996). When the inflammatory stimuli are not eliminated during an acute inflammation response the immune system starts with chronic inflammation, when the macrophages appear in a large influx and can aggregate together and transform into a multinucleated giant cells (Iwama and Nakanishi, 1996, Goldsby and Kuby, 2002). The uncontrolled or inappropriate inflammatory response, such as hyper-expression of adhesion molecules and mediator factors, can lead to tissue damage.

1.3 Aim of the study

Based on previous data that showing increased adiposity when fish meal and fish oil were replaced by plants ingredients, the aim of this study was to elucidate that the high inclusion level of plant oils together with vegetable protein increase visceral adipose tissue lipid levels in Atlantic salmon followed by low-grade inflammation in visceral adipose tissue; and that the high 18:2*n*-6 (LA) containing in vegetable oil (soybean oil) increase low-garde inflammation in visceral fat irrespective at adipose level.

1.4 Approach

Fatty acids composition, growth and feed intake

With the purpose of identify the influence of the VO diets, body weight, growth ratio and feed intakes were estimated in the present study. The condition factor, visceral fat index and whole fish lipid level were also measured with the aim of find out if the replacement with VO had some differences in lipid storage and fish growth.

Histology

The visceral fat tissue was stained in H&E and exposed to anti-F4/80 mononuclear antibody with the aim of evaluate the macrophage infiltration in the tissue and the influence of the diets in their accumulation. The goal of microscope observation was also measure the mean adipocyte size in the different diets

Gene Expression

Gene expression of inflammation and macrophage precursors was measured to evidence some different concentration in visceral fat and head kidney and thus to be possible evaluate the inflammation stage of the samples, according with the different diets. The intent of analyze the expression of the genes in head kidney was to observe changes in inflammation markers due the diet in comparison with visceral adipose tissue.

2. MATERIAL AND METHODS

2.1 Dietary experiment

This study was a dietary trial thattook place in the period of 15th of April 2010 to 15th of October 2010. This 6 months experiment was conducted at the Lerang Research Station, Skretting ARC, Stavanger, Norway.

Six hundred Atlantic salmon (*Salmo salar*) being 14 months old and a mean weight of 815±28g were equally distributed in 12 tanks (50 fish per tank) with three meters of diameter, a volume of 7000L and a flow between 85-92L/min. The mean water temperature was 9.9 ± 0.6 °C.

During the experiment Atlantic salmon was fed one of 4 diets where up to 70% of the fish meal was replaced by plant protein sources in combination with either 100% fish oil (high *n*-3/*n*-6 ratio) or one of 3 oil mixes replacing up to 80% of the fish oil. The vegetable oils used in the diets were rapeseed, olive oil and soybean oil.

To produce a balanced dietary satured- , monosatured- and polyunsaturated fatty acid level (PUFA), based on RAFOA and Aquamax results (Torstensen *et al.*, 2008, Torstensen *et al.*, 2005), the olive oil and soybean oil feeds contained a mixture of linseed oil and palm oil. The feed with rapeseed oil contain no other vegetable oil than RO. Likewise, plant protein sources was be blended sensibly and crystalline amino acids was be added to reach amino acid requirements (NRC, 1993).

As a result of higher intake than predicted between 15th of April and 31st of August, it was needed to make a second batch of fed, between 1st of September and 15th of October. Due to raw material, there was a minor difference in nutrient composition (table 2.1). The fatty acid content is given in table 3.1.

Table 2.1) Ingredients and proximate composition of experimental feed

Ingredients (g Kg^{-1})	Fish Oil	Olive Oil	Rapeseed Oil	Soybean Oil
Fish meal[1]	180.0	180.0	180.0	180.0
Wheat gluten[2]	162.3	162.3	162.3	162.3
Hi-pro soy[3]	97.3	97.3	97.3	97.3
Soy concentrate[4]	150.0	150.0	150.0	150.0
Wheat[5]	70.0	70.0	70.0	70.0
Fish oil[6]	309.9	62.0	62.0	62.0
Rapeseed oil[7]	-	-	248.0	-
Olive oil[8]	-	208.0	-	-
Soybean oil[9]	-	20.0	-	217.9
Palm oil[10]	-	-	-	30.0
Linseed oil[11]	-	20.0	-	-
Premixes[12]	31.8	31.8	31.8	31.8
Total weight (g)	1001.3	1001.3	1001.3	1001.3
Yttrium oxide[13] premix	1.0	1.0	1.0	1.0

Composition (g 100 g^{-1})	Batch 1	Batch 2	Batch 1	Batch 2	Batch 1	Batch 2	Batch 1	Batch 2
Fat	35.6	34.1	34.6	33.6	34.2	34.5	35.0	33.2
Protein	40.6	41.1	40.5	40.8	41.3	41.3	39.8	40.6
Moisture	6.6	6.8	6.1	6.9	6.3	6.4	6.3	6.6
Ash	5.2	5.3	5.1	5.2	5.2	5.2	5.2	5.2

1 - South Africa fish meal, Skretting, Stavanger, Norway
2 - Cargill Nordic, Charlottenlund, Denmark
3 - Felleskjøpet, Stavanger, Norway
4 - Imcopa, Araucaria, Brazil
5 - Skretting, Stavanger, Norway
6 - Norther hemisphere fish oil. Skretting, Stavanger, Norway
7 - Skretting, Stavanger, Norway
8 - D. Daneilsen AD, Stavanger, Norway
9 - Defona SA, Fredrikstad, Norway
10 - Fritex 24, Aarhus Karishamn, Sweden
11 - Elbe Fetthandel GmbH, Geesthcht, Germany
12 - Include vitamins and minerals; Trouw Nutrition, Boxmeer, the Netherlands, proprietary composition SkrettingARC
13 - Contains 10% Yt_2O_3, Treibacher Auermet, Althofen, Austria

2.2 Sampling

During the experiment three samplings were made: an initial in 09th of April 2010 before the start of fish feed experiment; one intermediate sampling was made in approximately eleven weeks later, 23th and 24th of June 2010; and the final after 28 weeks, 19th and 21st of October 2010.

Samples of all diets were taken and stored at -20 °C.

The samples for this project were collected only during the final sampling, from three fishes per tank. Fishes were unfed during a period of 48 hours before the sampling. They were anesthetized with MS222 in a concentration of 7g/L and killed by a blow to the head. After, the weight and the length were measured one by one. The visceral content (n=5), excluding the liver, were also weighed in order to calculate the visceral somatic index.

Visceral adipose tissue was collected in the most posterior part of visceral adipose tissue. RNA samples was collected (around one gram), placed in eppendorf tubes and immediately frozen on liquid nitrogen to avoid RNA denaturation. To the histology analyses the samples were surrounded with Tissue-Tek O.C.T Compound (Sakura, Torrance, CA, USA), to provide a convenient specimen matrix for cryostat sectioning at low temperatures, and froze in isopropanol. Head kidney samples were taken from the most anterior portion of the organ and immediately frozen in liquid nitrogen. Both visceral fat and head kidney samples were stored at -80 °C until further analyses.

Visceral adipose tissue of three fish was collected and mixed in order to analyze the total lipid composition per tank (n=3). The mixed sample was placed on dry ice and stored at -80 °C until further analyses.

2.3 Dietary and visceral adipose tissue fatty acid composition

Principle: extraction of lipid from samples. Fatty acids were separated and their concentration was determined by Gas Liquid Chromatography (GLC).

Fatty acids composition was analyzed in the diets and visceral adipose tissue. Lipids from the samples were extracted with chloroform/methanol (2:1, v/v) and 19:0 methyl ester was added as internal standard. After extraction of lipids, the samples were filtered, saponified and metylated using 12% of boron trifluoride, according with Lie and Lambertsen (1991). The methyl esters were separated using a Thermo Finnegan Trace 2000 GC (Fison, Elmer, OK, USA) equipped with a fused silica capillary column (Cp-stil 88; 50mx0.32mm internal diameter; Chrompack Ltd, Middelburg, The Netherlands) with temperature programming of 60^{o}C for 1 minute, 160^{o}C for 28 minutes, 190^{o}C for 17 minutes and finally 220^{o}C for 10 minutes with all intervening temperature ramps being at 25^{o}C per minute. The fatty acids were identify by retention time using standard mixtures of methylesters (Nu-chek, Elyian, MN, USA) and the fatty acid composition (weight %) were determined. Data were collected and processed using Totalchrom software (version 6.2; PerkinElmer, Waltham, MA, USA) connected to the gas liquid chromatography.

(This procedure was performed by laboratory technicians at NIFES. In MAR352 - Food Chemistry and Analysis this procedure was tought and experienced by student)

2.4 Molecular Analyses

To collect the RNA molecules from both tissues (visceral fat and head kidney) they were treated with QIAzol lysis reagent (Qiagen, Austin, TX, USA). Both tissues were also treated with DNA-free™ DNAse to remove residual DNA and with NaAc (sodium acetate) treatment to precipitate and remove all phenol and salt residues. To control and evaluate the RNA concentration and its purity, was used the Nanodrop (Saveen Werner, Malmö, Sweden). The RNA integrity (no denaturation) was evaluated by Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). To manage with a DNA molecule it was necessary to convert the RNA into cDNA by the reverse transcriptase reaction and thus it was possible to compare the gene expression through the real time PCR.

Molecular analyses were performed at the molecular biology laboratory at NIFES.

All the chemicals and reagents and listed in appendix II.

2.4.1 RNA extraction

Principle: QIAzol was used to preserve the RNA integrity during the homogenization. Chloroform was added to separate the RNA of DNA and proteins. RNA remains in the water phase which is after precipitated in isopropanol.

To proceed with the homogenization, around 50 mg of head kidney or 120 mg of visceral fat, the samples were placed into 2 ml tube with 4 or 5 zirconium beads and 1 ml of QIAzol. The samples were set in a homogenization instrument with a program of 6000 rpm for 3 times during 15 seconds with a 10 second brake and then incubated at room temperature during at least 5 minutes. In visceral fat, the samples should be centrifuged during 10 minutes at 13000 g in 4 °C and then the supernatant was collected out. To take apart the RNA of DNA and proteins 2 ml of chloroform was added into each tube and then agitated during 15 second. After an incubation of 3 minutes at room temperature the samples was centrifuged during 10 minutes at 13000 g in 4 °C to be able to distinguished two layers, the supernatant was where the RNA was present. The RNA was transferred to fresh tube with 500 µl of isopropanol alcohol to dissolve the target molecule. The tube was centrifuged during 30 minutes at 13000 g in 4 °C to precipitate in a pellet form. Before washing the pellet with 1 ml of cold 75% ethanol it was necessary to take out the liquid part. Later, the sample was mixed, centrifuged during 5 minutes to achieve another RNA precipitation. The ethanol was removed by vacuum suction apparatus (IBS Integra Biosciences, Vacuboy, Switzerland) and the pellet was air dried during 10 minutes to clear the remaining ethanol. Depending on the pellet size, it was dissolved in 30-50 ml of miliQ-water.

Figure 2.1) RNA pellets in an intermediate washing with 75% ethanol

2.4.2 DNA-free™ DNase treatment

Principle: To proceed with the reverse transcription, the samples should be free of DNA molecules. Magnesium and calcium salts were also lead through precipitation. The DNA-free™ kit (Ambion Inc., Applied Biosystems, Foster City, CA, USA) was used.

First, it was added 10% of the sample volume of 10x DNase I buffer, to inactivate the DNase, and 1 µl of rDNase, to react with the remaining DNA molecules. After gently mixed, the solution was incubated during 30 minutes at 37 °C. 0.1 volume of DNase Inactivation reagent was added to the solution, which was incubated during 2 minutes at room temperature and occasionally mixed. The solution was centrifuged at 10000 g during 1.5 minutes. The clean RNA forms a pellet which was dried and, once more, the pellet was dissolved in a miliQ-water volume according with its size and stored at -80 °C until further analyses.

2.4.3 RNA quantity analyses and intermediate quality control

Principle: RNA has its maximum absorption at 260 nm. Through spectrophotometry and UV-absorption was measured the quantity, purity and integrity of RNA. The 260/280 ratio was used to calculate the purity and it should be above 2 to be considered as "pure". If the ratio was lower than 2, it would mean that contamination of protein, phenol or others contaminants which strongly absorb at or near of 280 nm occurs. The 260/230 ratio was used to measure the RNA integrity and the ratio should be over than 2. Samples with a ratio lower than 1 should not be used and it indicates contamination by presence of proteins, chaotropic salts and phenol.

The first step was making a blank with milliQ-water, which was free of RNA. The water should be the same that was used in dilution. A drop of the sample (or milliQ-water to make the blank) with 1.5 µl was placed on the nanodrop ND-1000 petal. After the instrument analysis, the concentration and the ratios were assessed. The concentration of RNA was measured in µg/µl and the ratios indicated the necessity of RNA precipitation or sample contamination. Samples with low ratios and/or contamination make them impossibility to make further analyses (T009-techcical bulletin).

2.4.4 RNA Precipitation

Principle: when occurs contamination of phenol or salts, the sample must be subject to a new precipitation, with NaAc and absolute ethanol. After this treatment the 260/230 ratio and quality of the samples should increase.

2.5 and 0.1 times of the sample volume was added of absolute ethanol and NaAc, respectively. After mixed, the samples were stored one hour at -20 °C and at least 2 hours at -80° C. The samples can be stored at -80° C during the weekend if it was necessary. After being melted, they were centrifuged during 30 minutes at 16000 g in 4 °C. The supernatant was removed and 1 ml of 75% ethanol was added to proceed with a new centrifugation, but now during 5 minutes at 16000 g in 4 °C. Like RNA extraction, the ethanol was removed and the pellet was air dried during 10 minutes to clear the remaining ethanol. Due to the pellet size, it was dissolved in different amounts of milliQ-water, 25 µl to visceral fat and 150 µl to head kidney.

2.4.5 RNA quality analyses

Principle: To measure the quality of the samples (no degradation) the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) was used. 12 samples were chosen randomly and they were diluted to have a final concentration between 100 and 500 µg/µl (if it was applicable). The equipment provided the quality of RNA samples before Real Time PCR analyses. It was used a chip with 16 wells: 12 for samples, 3 for gel dye mix and one for external standard. The RNA fragments were analyzed by capillary electrophoresis. The results were given in form of gel imaging, electropherogram, RNA integrity number (RIM) and the value of rRNA ratio (28S/18S) was calculated

The reagents, RNA Nano Dye Concentrate and Agielent RNA 6000 Nano Marker, were incubated at room temperature during 30 minutes and protected from light. After vortex and spin 0.5 µl of dye was added to a 32.5 µl tube of gel, then properly vortex and centrifuged during 10 minutes at 13000 g in 4°C. The gel was provided in the kit (Aligent RNA 6000 Nano Gel Matrix) but it needed to be filtered. The chip was placed in the chip priming station and 9 µl of gel with the dye were placed to respective wells. In one of them was made some pressure with a plunger help, during exactly 30 seconds. 5 µl of RNA marker was added in the 12 samples wells and in ladder well. The samples and the ladder were incubated during 2 minutes at 70 °C to be denaturated. 1 µl of the ladder was placed in its well and 1µl of each sample was added to the respective wells. The chip was set in the adapter of the IKA vortexer and vortex for 1 minute at 2400 rpm and it needs to be analyzed within 5 minutes. After cleaning the capillaries the Bioanalyzer was prepared to run the chip. After 30 minutes it was possible to analyze the final results. The results of RNA quality are present in appendix IV.

Figure 2.2) RNA nano chip to measure the RNA quality in the Bioanalyzer

2.4.6 Reverse transcription reaction (RT reaction)

Principle: The RNA molecules need to be converted into cDNA for DNA amplification in PCR by the reverse transcriptase enzyme during the RT reaction

A calculated amount of RNA was diluted with ddH_2O in a new tube into a concentration of 50ng/µl ± 5%. Nanodrop ND-1000 (Saveen Werner, Malmö, Sweden) was used to regulate the final concentration.

A standard curve was made with 3µl of each sample together in the same tube and the final concentration was measured. Six tubes of this RNA pool were prepared with the specific RNA concentration: 100ng/µl/well, 50ng/µl/well, 25ng/µl/well, 12.5ng/µl/well, 6.25ng/µl/well and 3.125 ng/µl/well ± 1%.

In the clean room a RT reaction mix was prepared depending with the number of wells per plate. Each well should have 1.3µl of ddH_2O, 3µl of 10x TaqMan RT buffer, 6.6µl of 25mM $MgCl_2$, 6µl of 10mM deoxyNTPS and 1.5µl of Oligo $d(T)_{16}$. This compounds needs to be melted and after that vortexed and spiniged before using. Each well also needs enzymes: 0.6µl of RNAse Inhibitor and 1µl of multiscribe reverse transcriptase. The enzymes should be at -20 °C.

In each well was added 20µl of the RT reaction and 10µl of RNA of the respective samples. As a control two extra wells were made: nac well (19µl of RT reaction without reverse transcriptase, 1µl of ddH_2O and 10µl of RNA) and ntc well (10µl of ddH_2O and 20µl of mix). The aim of these wells was avoid the reverse transcription reaction and show the nonexistence of DNA in the sample. The plate was covered, centrifuged at 100 rpm for 1 minute, placed in the PCR machine (Gene Amp PCR System 9700 PCR machine, Applied Biosystems, Foster City, CA, USA) and the RT reaction was started with specific thermal cycle program for RT reaction (table 2.,2)

Table 2.2) Thermal cycling conditions of RT reaction

Step	**Time (min:sec)**	**Temperature (°C)**
Incubation	10:00	25
Reverse Transcriptase activation	60:00	48
Inactivation of reverse transcriptase	05:00	95

The RT plate was storage at -20°C. In this process we worked with triplicates to ensure correct results.

2.4.7 Real-time Polymerase Chain Reaction (RT-PCR)

Principle: generate a large number of copies of a cDNA sequences and measure their expression with a fluorescence marker. The quantification of stained amplified DNA was relative with a standard curve of known concentration.

The plate was defrosted and centrifuged during 1 minute at 1000 rpm. In the first time the DNA samples were diluted with 30µl of ddH_2O and mixed during 3 minutes at 1300g. The plate was kept on ice.

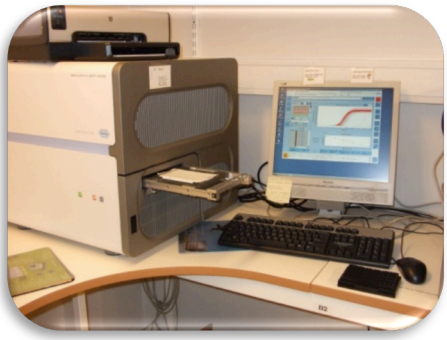

Figure 2.3) Light cycler 480 Real-Time PCR System

In the clean room the primers were defrosted and a mix was prepared to be placed in a 96 well plate with (for each well): 2.8 µl of ddH_2O, 0.1µl of primer forward, 0.1µl of primer reverse and 5µl of SYBR Green, a fluorescent marker which stain DNA double band. 8µl of the mix was added in the new optical plate.

Out of clean room 2µl of the sample was added to the new plate and mixed gently with the pipette. The plate was covered with an optical protection, centrifuged during 2 minutes at 1500rpm and placed on Light cycler 480 Real-Time PCR System (Roche Applied Sciences, Basel, Switzerland) in thermal cycling conditions according with table 2.3.

Table 2.3) Thermal cycling conditions of Real- time quantitative PCR

Step	**Cycles**	**Time (min:sec)**	**Temperature (°C)**
Incubation	1	05:00	25
Amplification	45	00:10 00:20 00:30	95 30 72
Melting curve analysis	1	00:05 01:00 01:00	95 65 72
Cooling	1	00:10	95

The first analyzed genes present in table 2.4 were the reference genes, which were present in all tissues: elongation factor 1AB (EF1Ab), β-actin and ribosomal phosphoprotein (ARP). The target genes (table 2.4) received an improved procedure, using Biomek 3000 Laboratory Automation Workstation (Beckman Coulter, Fullerton, CA USA) where 8µl of the master mix and 2µl of the cDNA from the RT-reaction plate were transferred to a 384-well reaction plate.

Table 2.4) Primer sequences used for gene specific analysis. Each measurement consists of a forward and a reverse sequence (5'-3')

primer	forward	reverse
EF1Ab	TGC CCC TCC AGG ATG TCT AC	CAC GGC CCA CAG GTA CTG
β-actin	CCA AAG CCA ACA GGG AGA AG	AGG GAC AAC ACT GCC TGG AT
ARP	GAA AAT CAT CCA ATT GCT GGA TG	CTT CCC ACG CAA GGA CAG A
IL-10	GGG TGT CAC GCT ATG GAC AG	TGT TTC CGA TGG AGT CGA TG
M-CSFR	GCA GAG GCC CAA ATA CTG C	TAT GTC CAG CGA CCA GGT G
TNF-α	GGC GAG CAT ACC ACT CCT CT	TCG GAC TCA GCA TCA CCG TA
CD-83	CTT GCT GCC CAA TGT AAC G	CCC TCC TGG TTC TGC TCT C
IL-6	ACC AAC AGT TTG TGG AGG AGT	AGC AAA GAG TCT TGG AGA GGT G
IL-1β	ACC AAC AGT TTG TGG AGG AGT T	AGC AAA GAG TCT TGG AGA GGT G

A software program was used in RT-PCR graphs amplification curves to determine cyclic time where fluorescence reaches a threshold level (Ct-value). The Ct-value is inversely proportional of replicated nucleic acid present from the original sample. Samples error and efficiency were also calculated, which determine the possibility to further analyses. An acceptable error should be below 0.04 and the efficiency between 1.8 and 2.2. The standard curve had to be linear. With efficiency above 2.2 meant a inhibition in the transcription from RNA to cDNA and a efficiency below 1.8 meant a inhibition from cDNA to DNA.

The reference gene ARP and the target genes IL-10 and M-CSFR were not used in statistics due qPCR too high efficiency of IL-10 and MCSFR and technical issues in ARP gene.

2.4.8 Primers analysis

Principle: to analyze the specific gene quality and evidence if they were present in the concerning tissues with the One-step RT-PCR kit. In the Agarose gel we can see the different DNA fragments by size. Small fragments will wander further in the gel than larger fragments due to DNA negative charge. With incorporated SyberSafe in the DNA structure it was possible visualize it in UV-light.

Only the new primers (IL-10, CD83 and M-CSFR) were tested in order to control te origin of just one product. In first place the primers were dissolved with TAE buffer to have a final concentration of 50nM.

New tubes were prepared with a final volume of 25µl: 5µ of QIAGEN solution, 5µl of QIAGEN One Step RT-PCR buffer, 1µl of dNTP, 0.3µl of primer, 1µl of Taq (enzymes mix), [1.0 – 1.5nM] of RNA and added water until desired final volume. The tube was placed in the PCR machine with a specific program (table 2.5)

Table 2.5) Thermal cycling conditions of primer test in PCR machine

Step		Cycles	Time (min:sec)	Temperature (°C)
Reverse transcriptase		1	30:00	50
PCR activation		1	15	95
Denaturation	Denaturation	1	00:45	94
	Binding	1	00:45	60
	Extension	1	01:00	72
Final extension		1	10:00	72

The amount of agarose used to make the gel depended with the fragments size. With fragments size between 0.1 and 2kb the percentage of agarose gel was between 0.75 and 1%. The same volume of TAE buffer was added and the solution was heated in the microwave until reach a homogenous state. 5µl of SyberSafe was added and placed in the tray to be solid. In new tubes the sample was mixed with loading dye in the 1:6 fractions. The electrophoresis tray was filled with running buffer and the gel was placed in. 3.5µl of marker was placed in

the first well and the samples in the next wells. The electrode was switched on and ran at 100 watts during one hour. Later than it was possible observe the DNA fragments (figure 2.4).

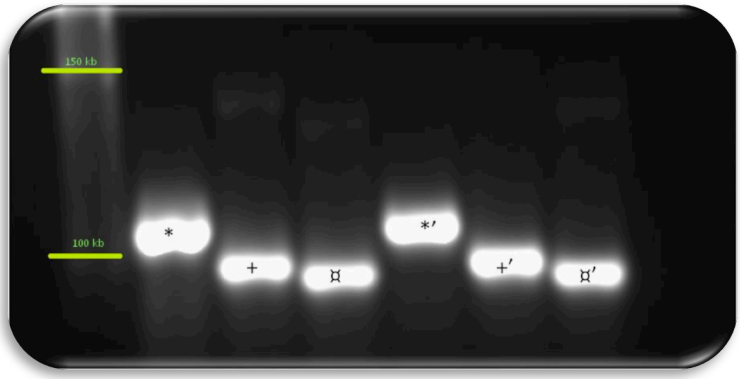

Fig 2.4) Agarose gel. There are represented the target genes: IL-10 (*), CD83 (+) and M-CSFR (¤) in visceral fat and head kidney (‘). In both tissues the gene weight was similar, approximately 110, 100 and 90 kb to IL-10, CD83 and M-CSFR, respectively.

The genes were run in visceral fat and head kidney. Figure 2.4 showed the genes presence in both tissues. The expression of the genes was expected to increase proportionally with the inflammation. Showed results were based in the last sampling.

2.5 Histology analyses

The tissues were fixed to preserve degradation and keep the cell and organelles integrity. The fixative consisted in 0.1M phosphate buffer with 4% paraformaldehyde and it was prepared at the day of sampling. Before use, the solution was filtrated and the samples were immersed in it overnight at 4 °C (fig 2.5A). The samples were washed again with 0.1M phosphate buffer during 30 minutes. Before processing, the samples were dehydrated twice in 95% ethanol during 30 minutes (each immersion), twice in 100% ethanol during 30 minutes, twice in xylene during 30 minutes (to remove the ethanol) and finally in melted paraffin (58°C) during 30 minutes and in again in paraffin (Histowax, Histolab products AB, Göteborg, Sweden) overnight. The samples were placed in paraffin blocks in EC 350 Paraffin embedding center (Microm International GmbH, Walldorf, Germany) (figure 2.5B) and sectioned at 7µm on a microtome instrument (Leica RM2165, Nussloch, Germany) (figure 2.5C).

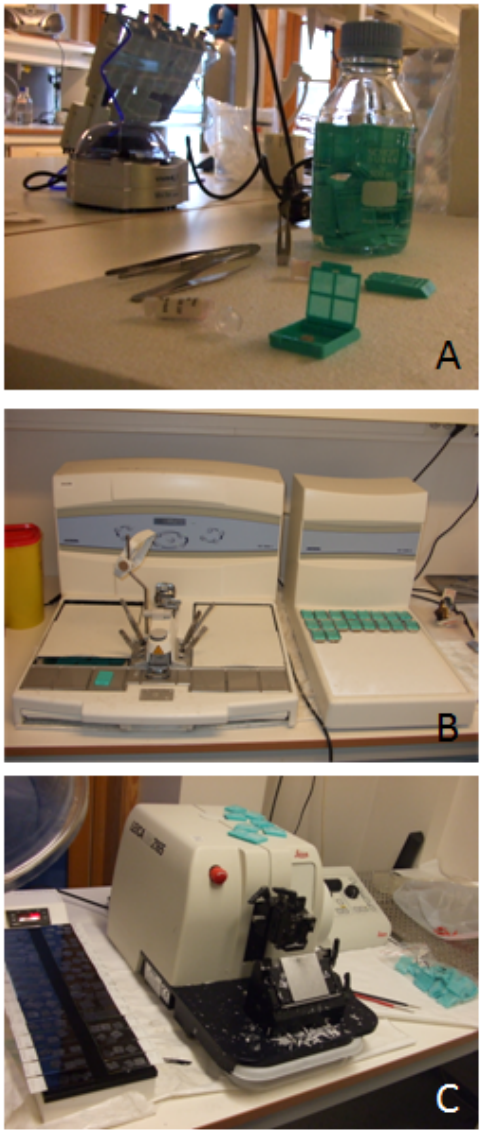

Figure 2.5) Slicing procedure. A:prevention of degradation; B: preparation of paraffin blocks; C: slicing of paraffin blocks

2.5.1 Hematoxylin and eosin staining

Principle: The first staining procedure was hematoxylin and eosin (H&E). Hematoxylin stains the cell nucleus (blue) and eosin stains the cytoplasm (red/pink).

Adipocytes are cells that store fat and have a relatively small nucleus. Through the dehydration steps in ethanol the fat was removed. The staining with H&E makes it possible to observe adipocyte nuclei, cytoplasm and cell membranes. Some connective tissue was also present as well as blood vessels and blood cells.

Before the staining, the sections were rehydrated by immersion in xylene twice (5 min), twice in isopropanol (5 min), 90% ethanol (5 min), 70% ethanol (5 min), 50% ethanol (5 min) and ddH_2O (5 min). Next, the samples were immersed in haematoxylin during 2 minutes, washed in tapwater during 5 minutes, ddH_2O during 1 min, eosine during 30 seconds, dehydrated through the graded series of alcohol and finally xylene as described above. At the end 3 drops of Entellan (Merck, Darmstadt, Germany) were added to the slide and a cover slip was applied. This prevents the tissue from drying out.

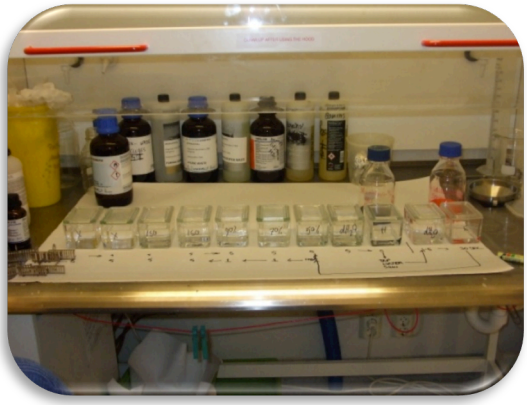

Figure 2.6) Hydratation of histological preparations and staining in H&E

2.5.2 F4/80 mononuclear antibody

Principle: Distinguish macrophages from other cell types in adipose tissue. The presence of F4/80 (a transmembrane protein specific for macrophages) was visualised by immunohistochemistry.

The samples were de-paraffinised and rehydrated as described in 2.5.1 To reduce no*n*-specific staining the sections were incubated in heat-inactivated normal goat serum (10%, 10 min). Sections were then incubated overnight at 4°C with rat anti-mouse F4/80 (1:500; Serotec, Eching, Germany), subsequently washed in tris buffered saline (TBS) during 10 minutes (3 times) and incubated with HRP-conjugated rat anti-goat IgG (1:250; Serotec, Wertheim-Bettingen, Germany) for 2 hours. After washing in TBS during 10 minutes (3 times) specific binding of the antibody to macrophages was visualised using diaminobenzidine. As a positive control samples of mice adipose tissue were stained at the same time (figure 3.10B)

2.5.3 Microscope pictures and cell size measurement

The slides with visceral tissue were visually examined with an Olympus BX 51 binocular microscope (System microscope, Tokyo, Japan) fitted with a Digital Sight DS-Fi1 camera (Nikon, Tokyo, Japan). Adipocyte size was measured using the interactive measurement module of an image analysis system equipped with an Olympus microscope, a Nikon camera and NIS-elements software (Nikon, Tokyo, Japan). In order to calculate the mean size, two hundred adipocytes per tissue were randomly selected and measured by drawing a horizontal line between the cell membranes (figure 2.7).

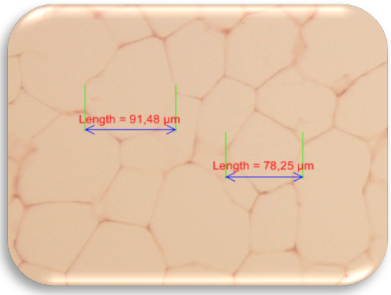

Figure 2.7) Adipocyte cell size measurement

2.5.3 Inflammation evaluation

Principle: with the purpose of evaluate macrophage infiltration and potential inflammation a score table with specific criteria was created in order to make the evaluation as objective as possible.

From each sample, three sections were examined. The considered criteria to evaluate the inflammation were the amount of macrophage clusters and their frequency. The clusters were given the following score (figure 2.8): null, small, medium and severe (0, 1, 2 and 3, respectively). The frequency was scored by the spread of clusters throughout the sample (figure 2.9): null, one spot, some spots, homogeneous (0, 1, 2, and 3).

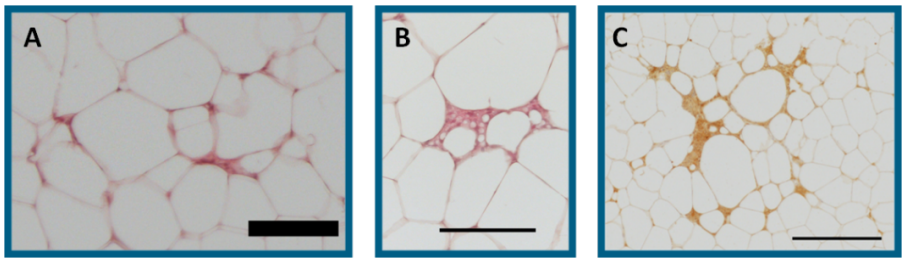

Figure 2.8) Examples of different evaluation of clusters in visceral fat. A: sample 29, score 1, bar = 100µm; B: sample 11, score 2, bar = 250µm; C: mice sample, score 3, bar = 250µm

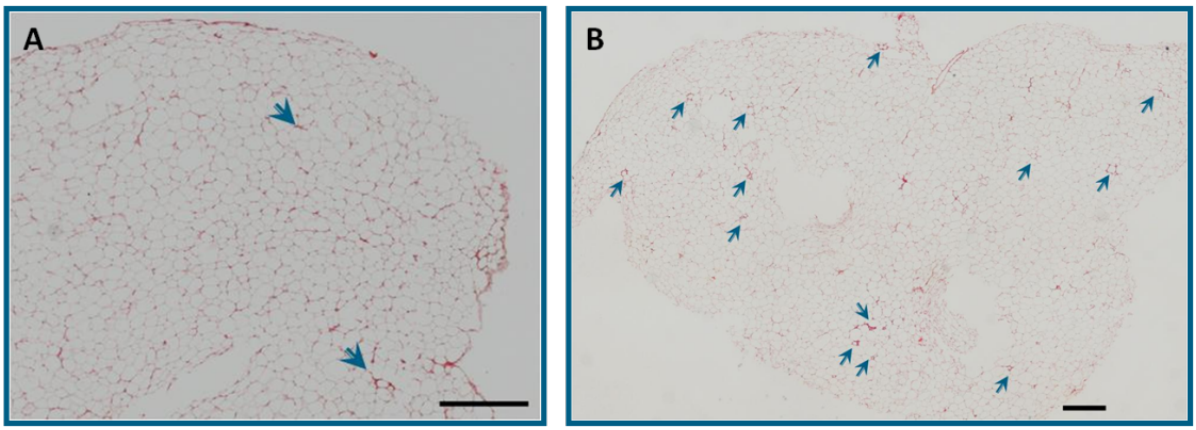

Figure 2.9) Examples of different evaluation of macrophage infiltration frequency . A: sample 29, score 1, bar = 100µm; B: sample 11, score 2, bar = 250µm

Visceral fat of three fish per tank was collected. With three tanks per diet we had a total of nine samples per diet for evaluation of the inflammation by histological observation.

The inflammation evaluation was done on every individual sample using the scoring described above. The highest score was used for the highest degree of macrophage infiltration and/or frequency in the tissues from this experiment. Even that, each case have its particularise and same scores did not mean same stages. A score of zero always means no macrophage infiltration and no clusters. On the other hand, a score of two could mean a relative macrophage infiltration in just one spot or at same time two stops of macrophage but not in the way of clusters.

2.6 Calculations

Specific growth rate

$$SGR = \frac{\ln BW2 - \ln BW1}{t} \times 100$$

Where $BW1$ and $BW2$ represents the initial and final fish body weights (g), respectably; and t represent the time of experiment (days)

Condition factor

$$CF = BW \times FL^{-3} \times 100$$

Where BW represents the body weight (g); and FL represents fork length (cm)

Feed conversion ratio

$$FCR = \frac{DC}{FB} - (IB + SP) + mortalities$$

Where DC represents diet consumed (kg); FB represents final biomass (kg); IB represents the initial biomass (kg); and SP represents the sampled fish (kg)

Visceral somatic index

$$VSI = \frac{VC}{BW} \times 100$$

Where VC represents visceral content without liver (kg); and BW represents body weight (kg)

2.7 Statistical analyzes

2.7.1 Microsoft Excel

Microsoft® Excel 2007 was used to calculate the mean and standard deviation (SD) of data. All the results as presented as mean ± SD.

2.7.2 Statistica

Statistica version 10.0 (Statsoft Inc. USA, 2010) was used to calculate the statistical difference between the dietary groups regarding dietary and visceral adipose tissue fatty acid composition, growth and food intake indexes, adipocytes size and gene expression.

Leven's test (ANOVA) was used in order to evaluate the homogeneity of variance within the groups (normality). When the Leven's test showed significance, the Kruskal-Wallis, a nonparametric test, was used. Without significance the test one-way ANOVA analysis of variance was used, followed by post-hoc Sheffé test to detect significant differences between the groups in question.

A significance level of 0.05 was considered in all statistics, giving a confidence level of 95%.

2.7.1 GeNorm

GeNorm is a visual basic application for Microsoft Excel and a statistical algorithm designed to assess the gene-stability measure (M) based on average multiple housekeeping genes. The genes used as reference genes were elongase factor and β-actine. Both M values were below of 1.5 (0.559 and 0.517), meaning a stability of reference genes. Ct-values from qPCR were used to calculate the gene expressions, which were divided with an estimated normalization factor to achieve the values of relative gene expression level.

2.8 Methodological considerations

We used RT-PCR to measure relative gene expression levels comparing with housekeeping genes (elongation factor 1A – E1Ab, β-actine and ribosomal phosphoprotein - ARP) which should not vary in the tissues under investigation. The method is quick, with automatic controls and sensitive. RNA extraction from fat tissue is a delicate process due the presence of fat layer during the initial steps and this molecule is more labile than DNA (Valasek and Repa, 2005). The RNA integrity number should be above 5 to be considering in good quality or higher than 8 to be considering as perfect to application (Fleige *et al.*, 2006). In this work the RNA quality was measured in Bioanalizer and the samples had very good or perfect quality. The DNA chain should have approximately the same length of all the amplified sequences to reduce the error (Applied Biosystems, 2011). The two extra wells, nac and ntc, were without reverse transcriptase and RNA, respectively, as a control of no transcription.

The normalization through the housekeeping genes should be done to control for experimental error (Pfaffl and Meyer, 2006, Vandesompele *et al.*, 2002). The amount of sample should be similar as like the amount of RNA in the wells to get reliable results (Bustin *et al.*, 2010, Fleige *et al.*, 2006). All the procedures to reduce possible errors were taken in consideration but some primer sequences did not work due inhibition in the transcription. Those primers were not used in normalization and gene expression analyzes. The present molecular biology results followed all the quality requirements and were reliable to static analyzes.

The histological staining quality was good. It was advantageous to identify the macrophage infiltration and also measure the cell size through a horizontal line between the cell membranes. The method used to use measure the average cell size could have some incongruity when used in a small number of cells. The 0.7μm of thickness in the slides used to histological observation just catch a thin part of each cell, what could be the maximum when the cell is sliced in the middle or it could be sliced in an external part, not measuring a representative diameter of cell size. Two hundred cells was the minimum number of cells measured per each slide. This method follow the law of large numbers, a probability theory that says that an independent experiment repeated a large number of times results in a close number to the mean value and tend to become closer as more trials are performed. After that, with more experience in histology and with the samples, a second measurement was performed. The final results of both measurements were similar, thus the total average was used to statistical procedures.

3. RESULTS

3.1 Growth and feed intake

The weight and length were assessed to follow the body development of salmon and detect any influence. This data has been published by Liland, *et al.* (2012). Figure 3.1 presents the finals weight, length and condition factor.

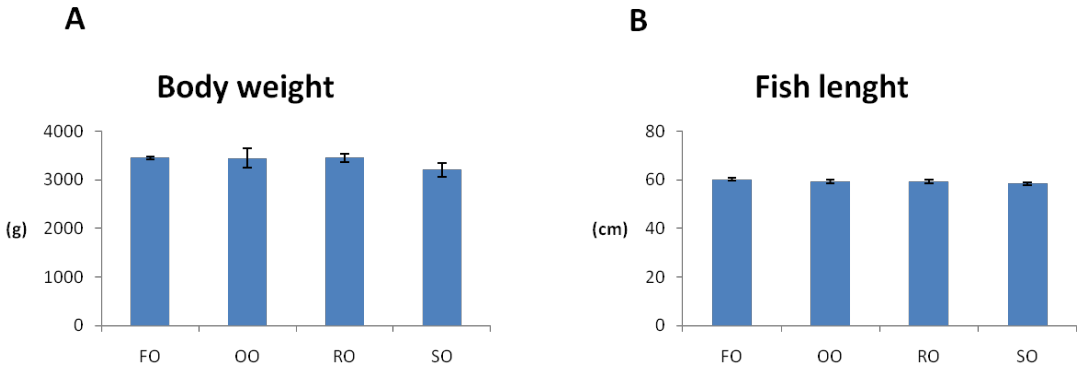

Figure 3.1) A: Final weight (g); B: Final length (cm); FO: fish oil; OO: olive oil; RO: rapeseed oil; SO: soybean oil. (n = 3). Data is given as mean ± STD

With an initial average weight of 815 ± 28g and after 28 weeks of experiment feeding, the final average weight was 3395 ± 534g (fig. 3.1A). No significant difference in final weight was found between the dietary groups ($p = 0.12$).

The final length and condition factor were 59 ± 3 cm and 1.60 ± 0.15, respectively, for all groups together. It is essential to remark that the difference in length between SO and FO diets were almost significant ($p = 0.066$) with a final length of 58.9 and 60.2 cm, respectively. No significant differences in final length and condition factor were found between the dietary groups ($p = 0.07$ and 0.35, respectively).

The average feed intake for all groups was 2765 ± 182g per fish. The major difference was between FO (2558 ± 79g) and SO (2442 ± 99g) dietary trial. The feed intake of OO (2655 ± 164g) and RO (2683 ± 90g) did not show any statistical significant difference ($p > 0.05$).

The feed conversion ratio (FCR) and specific growth ratio (SGR) was calculated for all the dietary groups. Concerning to the FCR, the FO had the highest ratio (1,17 ± 0.03), with a significant difference ($p < 0.001$) between all the VO groups (1.04 ± 0.01). Relatively to SGR, the SO (0.74 ± 0.01) was the dietary trial with lower results and with significant difference between OO (0.78 ± 0.01) and RO (0.79 ± 0.03), $p = 0.02$ and $p = 0.05$, respectively.

The visceral somatic index (VSI) and whole fish lipid level (WFLL) was also calculated for all the dietary groups. Figure 3.2 presents the final VSI and WFLL after 28 week of experiment.

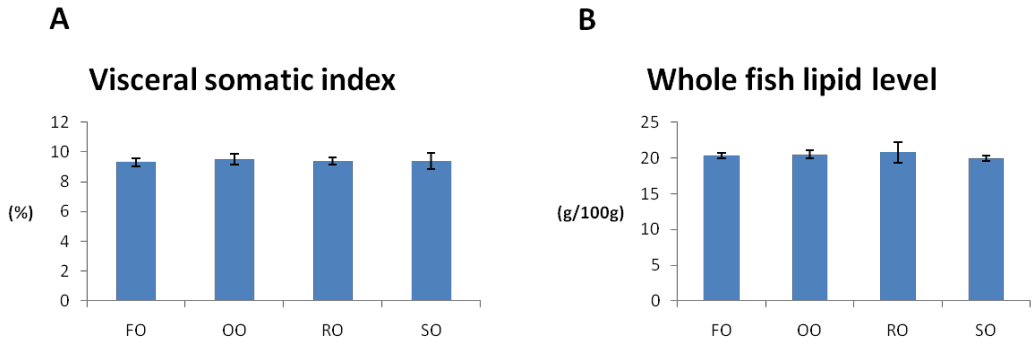

Figure 3.2) A: Visceral somatic index; B: Whole fish lipid level; FO: fish oil; OO: olive oil; RO: rapeseed oil; SO: soybean oil. (n = 3)

The VFI (9.32 ± 0.38) and WFLL (20.31 ± 0.80) were no statistical significant difference ($p = 0.41$ and 0.58, respectively). The OO group was the one with bigger VSI (9.51 ± 0.34) and RO group with higher WFLL (20.80 ± 1.47).

3.2 Dietary fatty acids composition

The FA composition was analyzed in the both diet batches (table 3.1). The fatty acids composition was very similar with only minor differences. The total average of fatty acids in the diets was 34.4 ± 0.8 grams of fat per grams of diet.

Table 3.1) Fatty acid composition (are %) in experimental diets

	Fish Oil		Olive Oil		Rapeseed Oil		Soybean Oil	
	Batch 1	Batch 2	Batch 1	Batch 2	Batch 1	Batch 2	Batch 1	Batch 2
14:0	7.4	6.8	1.4	1.5	1.8	2.0	1.5	1.7
16:0	18.4	15.9	12.3	12.4	7.9	7.8	15.1	14.8
18:0	9.7	2.9	3.4	3.3	2.6	2.5	3.7	3.8
Total saturated FA	31.8	26.8	19.0	18.0	14.4	13.2	22.3	21.2
16:1 *n*-7	6.9	5.9	1.7	1.9	1.8	1.8	1.3	1.5
18:1 *n*-7	2.2	2.0	1.8	2.2	2.9	2.7	1.7	1.6
18:1 *n*-9	9.7	11.9	50.2	50.3	44.2	42.7	22.0	21.5
20:1 *n*-9	3.8	5.6	1.0	1.4	1.8	2.4	0.9	1.4
22:1 *n*-9	0.4	0.1	0.1	0.1	0.2	0	0	0.1
22:1 *n*-11	6.2	10.2	1.1	2.0	1.4	2.8	1.1	2.2
Total monoene FA	30.3	38.5	56.1	58.4	52.4	53.5	26.9	28.6
18:2 *n*-6	2.6	2.7	13.7	12.1	17.3	15.8	40.1	37.5
20:3 *n*-6	0	0.9	0	1.0	0	1.1	0	1.3
20:4 *n*-6	1.1	0.5	0.2	0.1	0.2	0.2	0.2	0.1
18:3 *n*-3	1.1	1.4	4.6	4.1	8.3	7.8	4.9	4.4
18:4 *n*-3	2.6	2.6	0.5	0.5	0.6	0.7	0.5	0.6
20:4 *n*-3	0.8	0.7	0.2	0.2	0.2	0.2	0.2	0.2
20:5 *n*-3	11.0	8.1	2.2	1.9	2.7	2.5	2.1	2.0
22:5 *n*-3	2.0	1.1	0.4	0.3	0.5	0.3	0.4	0.3
22:6 *n*-3	8.9	10.0	1.9	2.3	2.3	3.0	1.7	2.4
Sum *n*-3	29.1	24.9	10.5	9.5	15.2	14.8	10.2	10.1
Sum *n*-6	4.4	4.7	13.9	13.2	17.2	17.2	40.3	39.0
Sum EPA & DHA	20.0	18.1	4.1	4.2	4.9	5.4	2.8	4.5
Sum PUFA	34.3	30.1	2.5	22.8	32.9	32.2	50.6	49.3
n-3/*n*-6	6.7	5.3	0.8	0.7	0.9	0.9	0.3	0.3

Table 3.1 presents fatty acids amount in the four different diets. It is possible see the difference of saturated fatty acids between the FO and VO diets. The FO diet had more saturated FA than the others. Within the VO the SO is the diet with more saturated FA amount and the RO diet with less (half amount than FO).

With regard to monoene FA, VO diets are characterized for the content of oleic acid (18:1*n*-9). The amount of this fatty acid in SO diet was twice than in FO. RO and OO had four and five times more 18:1*n*-9, respectively, than the control group. Even that, SO was the diet with less monoene FA because FO had monoene fatty acids in higher content than VO.

Considering the *n*-6 FA, VO had more than FO due their vegetable origin, even so the essential fatty acid arachidonic acid (20:4 *n*-6) in a concentration in FO four times more than in VO.

On the other hand, the FO had around twice the quantity of *n*-3 PUFA than VO diets. Apart from 18:3*n*-3, that was in lower concentration in FO, all the fatty acids from this group were in an amount four times higher in FO than in VO.

Finally, the *n*-3/*n*-6 ratio was clearly different between FO and VO diets. The FO diet had the higher ratio and in its turn SO had the lower. The difference between them was twenty times. The ratio of *n*-3/*n*-6 in OO and RO were higher than SO but still seven times lower than FO.

3.3 Visceral adipose tissue fatty acids composition

The fatty acids of visceral adipose tissue were analyzed and the more significant fatty acids are present in table 3.2.

Table 3.2) Fatty acids composition (mg FA g^{-1} tissue, w.w.) of visceral adipose tissue from Atlantic salmon fed either fish oil (FO), olive oil (OO), rapeseed oil (RO) and soybean oil (SO) for 28 weeks (October 2010). (n=3)

	FO	OO	RO	SO
14:0	49.6 ± 2.1^{a}	17.1 ± 1.5^{b}	18.4 ± 0.5^{b}	16.1 ± 1.4^{b}
16:0	123.8 ± 5.2^{a}	109.3 ± 6.9^{a}	75.8 ± 1.9^{b}	114.1 ± 11.3^{a}
18:0	26.0 ± 0.8^{ab}	30.4 ± 2.0^{a}	22.5 ± 0.1^{b}	30.8 ± 3.0^{a}
SUM saturated FA	**207.9 ± 7.7**^{a}	**164.2 ± 10.8**^{b}	**124.6 ± 2.3**^{c}	**168.0 ± 16.4**^{b}
16:1*n*-7	58.4 ± 2.7^{a}	21.1 ± 1.8^{bc}	19.0 ± 0.1^{bc}	16.0 ± 1.4^{bd}
18:1*n*-11	6.8 ± 0.5^{a}	1.9 ± 0.1^{b}	2.1 ± 0.3^{b}	1.8 ± 0.2^{b}
18:1*n*-9	163.5 ± 8.4^{a}	503.0 ± 38.4^{b}	393.4 ± 5.7^{c}	232.2 ± 21.8^{d}
20:1*n*-11	4.3 ± 1.0^{a}	1.8 ± 0.5^{b}	1.6 ± 0.4^{b}	1.4 ± 0.3^{b}
20:1*n*-7	1.7 ± 0.2^{a}	0.8 ± 0.1^{bc}	1.0 ± 0.2^{b}	0,6 ± 0.0^{c}
20:1*n*-9	39.3 ± 2.4^{a}	35.9 ± 3.1^{a}	33.4 ± 0.6^{a}	18.4 ± 1.6^{b}
22:1*n*-11	48.5 ± 2.4^{a}	15.3 ± 1.4^{b}	16.9 ± 0.4^{b}	13.9 ± 1.0^{b}
22:1*n*-9	4.4 ± 0.3^{a}	3.4 ± 0.4^{bc}	3.7 ± 0.1^{ac}	2.1 ± 0.2^{d}
SUM Monoene FA	**367.2 ± 18.8**^{a}	**614.3 ± 48.0**^{b}	**506.9 ± 7.5**^{c}	**310.5 ± 27.6**^{a}
18:3*n*-3	18.4 ± 0.9^{a}	34.5 ± 3.8^{b}	53.2 ± 1.3^{c}	33.4 ± 3.0^{b}
20:4*n*-3	16.9 ± 0.4^{a}	7.8 ± 0.5^{b}	10.3 ± 0.2^{c}	6.6 ± 0.6^{b}
20:5*n*-3	65.6 ± 2.9^{a}	16.2 ± 1.9^{b}	18.9 ± 1.2^{b}	15.3 ± 1.4^{b}
22:5*n*-3	36.7 ± 1.1^{a}	8.6 ± 1.1^{bc}	10.6 ± 0.3^{b}	7.9 ± 0.8^{c}
22:6*n*-3	98.6 ± 5.3^{a}	35.1 ± 4.0^{b}	36.0 ± 2.5^{b}	32.0 ± 3.3^{b}
SUM *n*-3	**262.2 ± 11.2**^{a}	**116.9 ± 13.2**^{b}	**145.9 ± 3.1**^{c}	**108.7 ± 9.9**^{b}
18:2*n*-6	57.3 ± 2.1^{a}	129.8 ± 11.2^{b}	145.4 ± 3.1^{b}	306.6 ± 30.0^{c}
20:4*n*-6	6.6 ± 0.6^{a}	1.7 ± 0.2^{b}	1.8 ± 0.3^{b}	2.7 ± 0.3^{b}
SUM *n*-6	**74.4 ± 3.2**^{a}	**147.7 ± 13.1**^{b}	**163.4 ± 2.7**^{b}	**338.7 ± 33.8**^{c}
***n*-3 / *n*-6**	**3.5 ± 0.1**^{a}	**0.8 ± 0.0**^{b}	**0.9 ± 0.0**^{c}	**0.3 ± 0.0**^{d}
rest FA	**5.6 ± 0.3**^{a}	**1.4 ± 0.2**^{b}	**1.5 ± 0.1**^{b}	**1.3 ± 0.1**^{b}
Total FA	**917.3 ± 40.7**	**1044.5 ± 85.1**	**942.3 ± 11.1**	**927.2 ± 86.8**

Data present as mean ± STD (n = 3)
Significant differences between dietary groups are denoted by different letters

Visceral adipose tissue fatty acids composition highly reflected the dietary fatty acid composition. For all fatty acids there was a statistical significant difference in concentration ($p < 0.01$). The main differences were between FO and VO. The EPA, DHA and AA were good examples of this difference, since there was twice, and in some example three times more the concentration of these fatty acids in FO than in VO. On the other hand the amount of oleic acid (18:1*n*-9) was different in all diet groups ($p < 0.01$).

Also within VO diets, fatty acids concentrations were different between each other. The sum of saturated fatty acids of RO diet was statistical smaller than OO and SO diet. On the other hand the sum of *n*-3 fatty acids was statistical higher in RO dietary group than OO and SO, due the statistically higher concentration of linolenic acid (18:3*n*-3) and 20:4*n*-3 in RO diet than in OO and SO dietary groups. In SO diet the total amount of *n*-6 fatty acids are much higher than all samples, due to the high concentration in linoleic acid (18:2*n*-6). The eicosanoic acid (20:1*n*-9) was present in lower concentration in SO than the other diets, which were not statistical significant different between them.

Concerning to *n*-3/*n*-6 ratio there was a statistical significant different between the diet groups ($p = 0.012$). The visceral adipose tissue of FO fed fish had a higher ratio (3.5 ± 0.1) that the VO diets, 0.8, 0.9 and 0.3 (OO, RO and SO, respectively). These differences were explained by the large amount of *n*-3 PUFA in FO and *n*-6 FA in VO.

There was no statistical significant difference in the concentration of total fatty acids ($p = 0.069$).

3.4 Histology

With the successful H&E staining, through microscope observation was possible measure the average adipocyte size of each sample, measuring in minimum 200 adipocytes per each one. The measurement was made twice (at least 400 adipocytes per each sample) and the final presented results are the average of the two.

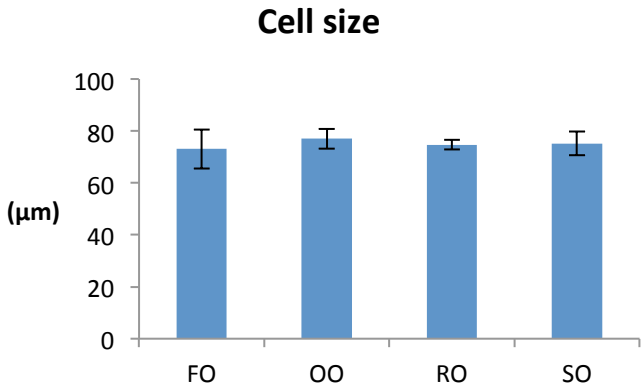

Figure 3.3) Adipocyte size (mean ± SD) in visceral fat. Average based on at least 400 cells per fish. FO: fish oil; OO: olive oil; RO: rapeseed oil; SO: soybean oil. (n = 3)

The mean adipocytes size ranged from 66 nm to 81 nm with an average size of 75 ± 4 µm (n = 3). In the singular samples the adipocytes size ranged from 11.6 µm to 198.3 µm. No significant differences in adipocyte size between groups was seen (p = 0.81).

In order to evaluate macrophage infiltration we created a score table for the presence of macrophages and the frequency in each section.

Table 3.3) Histological evaluation of macrophage infiltration in visceral fat adipocytes. The explanation of this evaluation and the meaning of the criteria are explained more in detail in the section 2.5.3.

diet	sample	clusters null: 0 small: 1 medium: 2 severe: 3	frequency null: 0 one spot: 1 some spots: 2 homogenous: 3	macrophage infiltration score by sample	 score by diet
	4	0	1	1	
	5	0	1	1	
	6	0	1	1	
	13	1	1	2	
FO	14	0	0	0	**1.3 ± 0.6**
	15	2	2	4	
	28	0	0	0	
	29	1	0	1	
	30	1	1	2	
	1	0	0	0	
	2	0	0	0	
	3	0	0	0	
	22	0	0	0	
OO	23	0	1	1	**0.2 ± 0.4**
	24	0	1	1	
	31	0	0	0	
	32	0	0	0	
	33	0	0	0	
	7	1	2	3	
	8	0	2	2	
	9	1	2	3	
	19	1	1	2	
RO	20	0	0	0	**1.9 ± 0.8**
	21	0	1	1	
	34	1	1	2	
	35	1	1	2	
	36	1	1	2	
	10	2	2	4	
	11	2	2	4	
	12	1	1	2	
	16	1	2	3	
SO	17	0	0	0	**2.6 ± 1.1**
	18	0	1	1	
	25	1	1	2	
	26	1	1	2	
	27	2	3	5	

Visceral fat of three fish per tank was collected. With three tanks per diet a total of nine samples per diet were evaluated

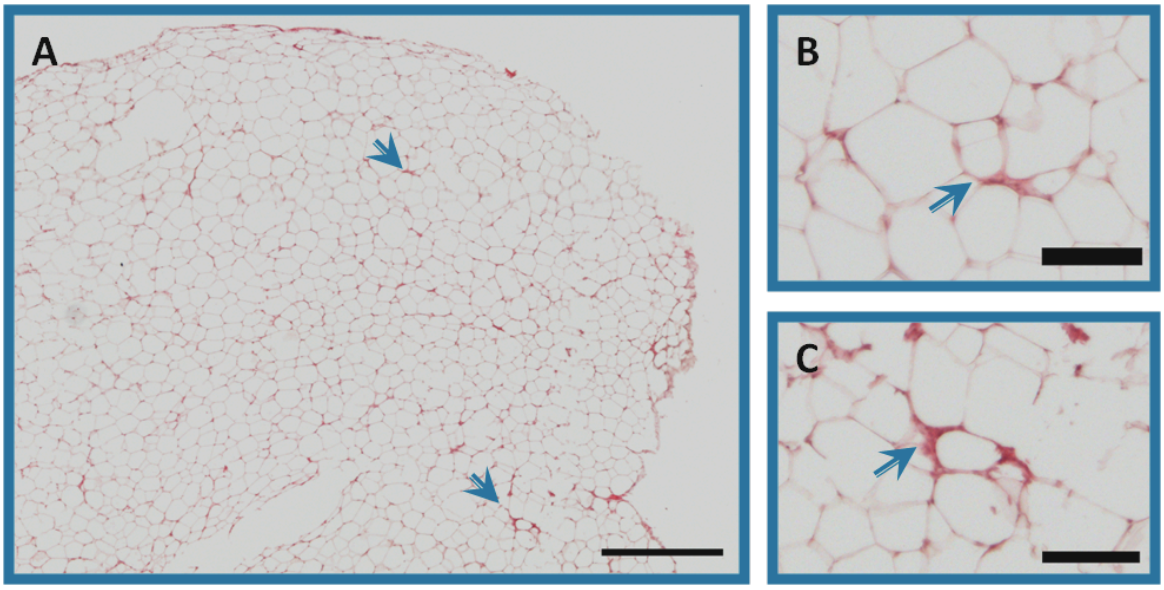

*Figure 3.4) Microscopy pictures of paraffin-embedded sections of visceral fat of Atlantic salmon fed with **FO**. Sample 29. The sections were stained with H&E. Arrows indicate presence of macrophages. A: scale bar = 500µm; B and C: scale bar = 100µm*

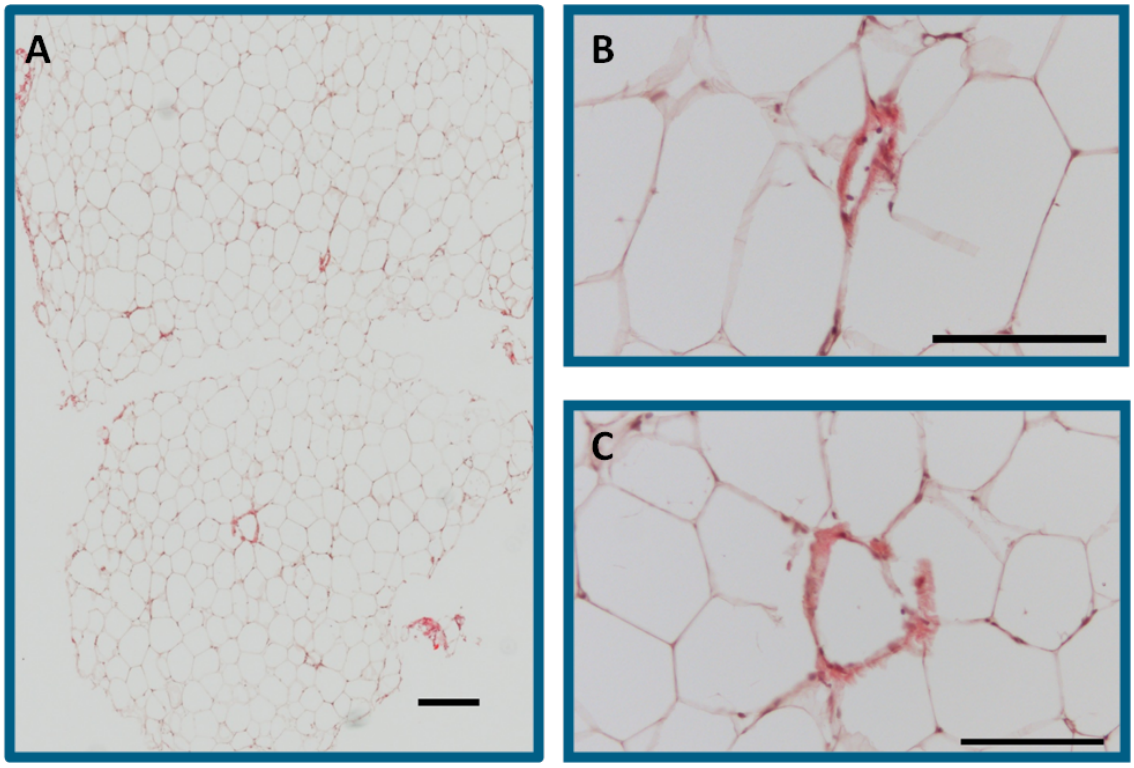

*Figure 3.5) Microscopy pictures of paraffin-embedded sections of visceral fat of Atlantic salmon fed with **OO**. Sample 1. The sections were stained with H&E. Arrows indicate presence of macrophages. Scale bar = 100µm.*

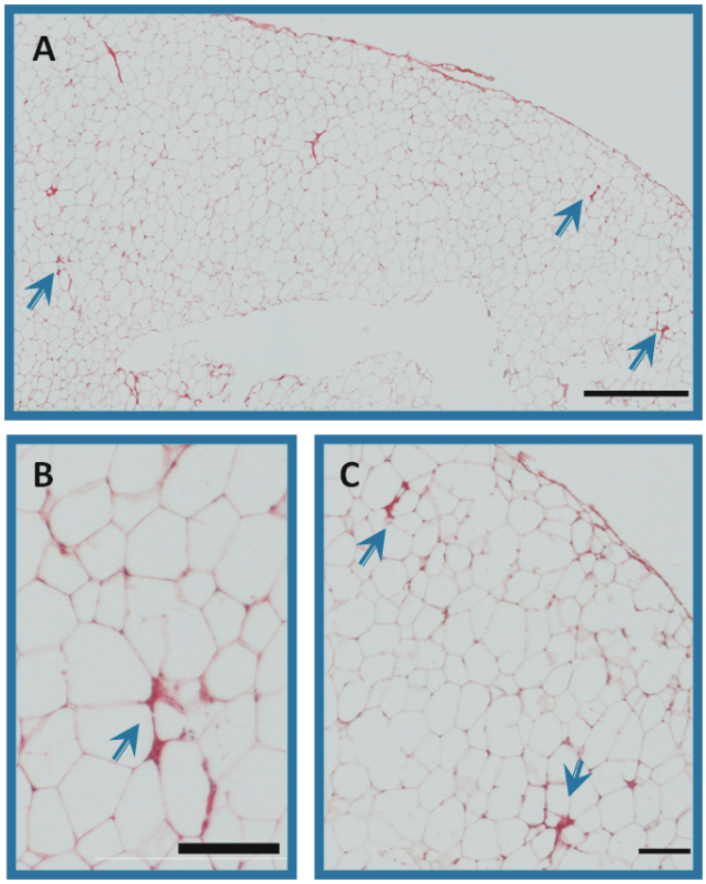

*Figure 3.6) Microscopy pictures of paraffin-embedded sections of visceral fat of Atlantic salmon fed with **RO**. Sample 9. The sections were stained with H&E. Arrows indicate presence of macrophages. A: scale bar = 500µm; B and C: scale bar = 100µm*

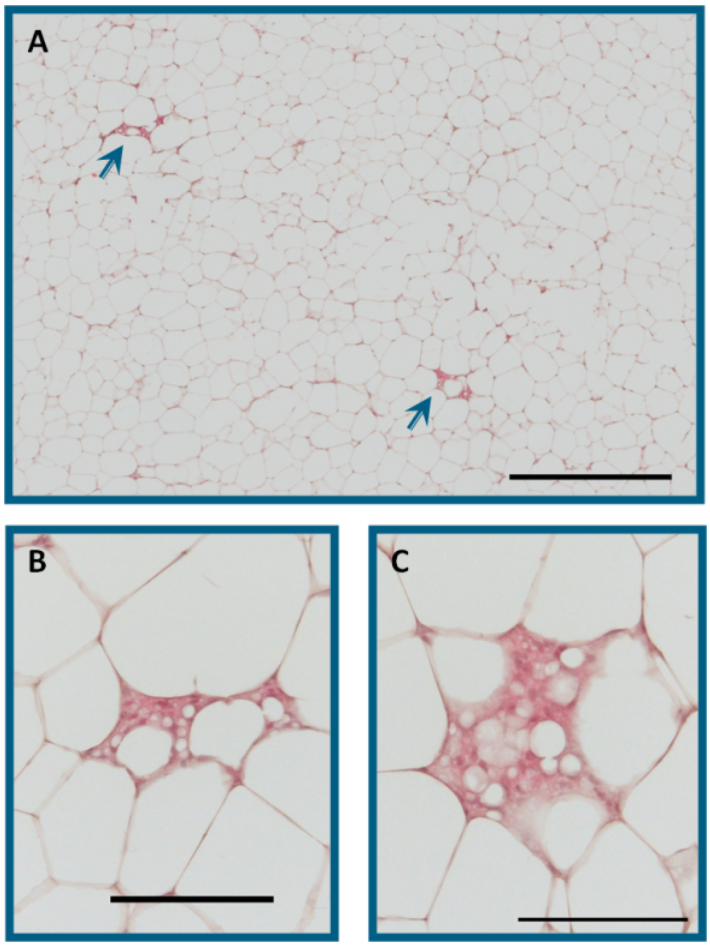

*Figure 3.7) Microscopy pictures of paraffin-embedded sections of visceral fat of Atlantic salmon fed with **SO**. Sample 27. The sections were stained with H&E. Arrows indicate presence of macrophages. A: scale bar = 250µm; B and C: scale bar = 200µm*

The OO diet had the lowest amount of macrophages in white adipose tissue (score = 0.2). Using a low magnification (figure 3.5A) it was possible to see some spots what could be mistaken for macrophages. With a higher magnification (200x) it was possible to see that they were blood vessels and residual tissue (figure 3.5B and C, respectively). This group was considered clean.

The control group (FO dietary group) had also a low grade of inflammation (1.3). In this group there were more samples with an evaluation of zero in the inflammation grade but it was possible to find some macrophages. Sample 15 was the one with highest grade of inflammation of FO group, influencing the FO inflammation score. Figure 3.4A shows that sample 29, as example of FO dietary group, was almost clean with just some small macrophage accumulation.

The RO and SO groups had some considerable inflammation (1.9 and 2.6 respectively). Both diets resulted in increase of macrophage infiltration in the visceral adipose tissue, especially the SO dietary group (figure 3.6B, figure 3.7B and C). The macrophages were more spread in the sample and found easily. The FO dietary group had the highest inflammation score. This group was the only one with scores above 3 and also the only one with a score of 5 (figure 3.8). On the other hand the macrophage infiltration was not so severe and ubiquitous to consider it as inflammation (see figure 3.9B).

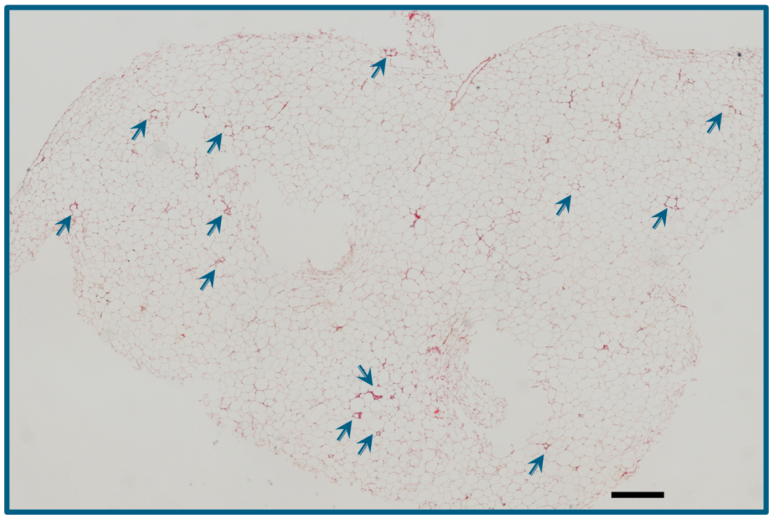

Figure 3.8) Microscopy pictures of paraffin-embedded sections of visceral fat of Atlantic salmon fed with SO. Sample 27. The sections were stained with H&E. Arrows indicate presence of macrophages. Scale bar = 500µm

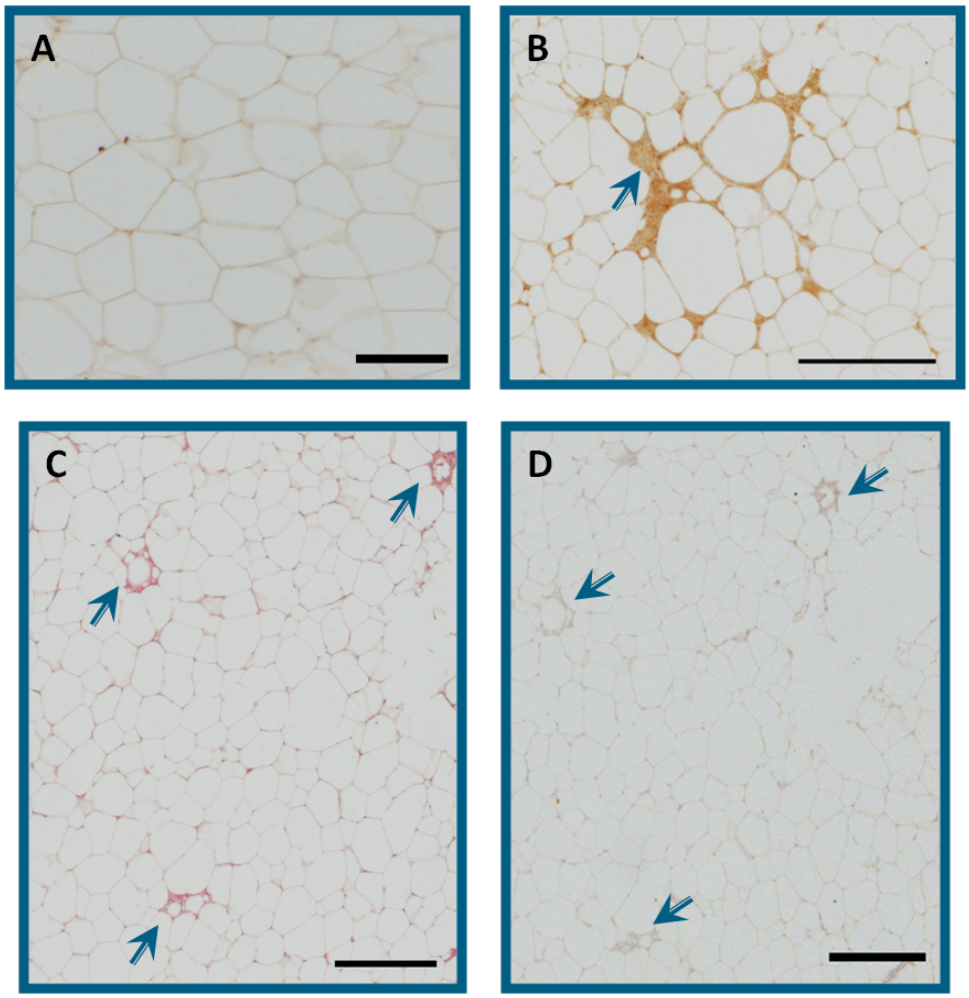

Figure 3.9) Microscopy pictures of paraffin-embedded sections of visceral fat of mice (A and B) and Atlantic salmon fed with FO (C and D). Sample 11. The sections A, B and D were exposed to anti-mouse F4/80 monoclonal antibody and stained with DAB chromogen concentrate. The section C was stained with H&E. Arrows indicate presence of macrophages. Scale bar = 250µm

Figure 3.9A and 3.9B were from mice visceral fat with inflammation that was used as a control for the staining procedure. Figure 3.9A is an example of visceral fat when exposed to anti-mouse F4/80 monoclonal antibody and without macrophages infiltration. Figure 3.9B represent an area with macrophage accumulation seen in severe inflammation. Figure 3.9C is a section from sample 11 (FO) with inflammation score of 4. Figures 3.9C and D are from the same sample (sample 11) and from same place in the tissue, but treated with H&E (figure 3.9C) and antibodies (figure 3.9D). Figure 3.9D shows that the anti-mouse F4/80 monoclonal antibody did not work in Atlantic salmon as judged by the lack of a brownish coloration.

3.5 Gene expression

The expression levels of genes involved in inflammation (TNF-α, IL-6 and IL-1β) and macrophage presence (CD83) were performed in qPCR on RNA extraction of visceral fat and head kidney. The goal of this process was measure the gene expression in the samples, in comparison of reference genes elongation factor and β-actine. Figure 3.10 and 3.11 represents the expression of the target genes in final sampling in head kidney and visceral fat, respectively.

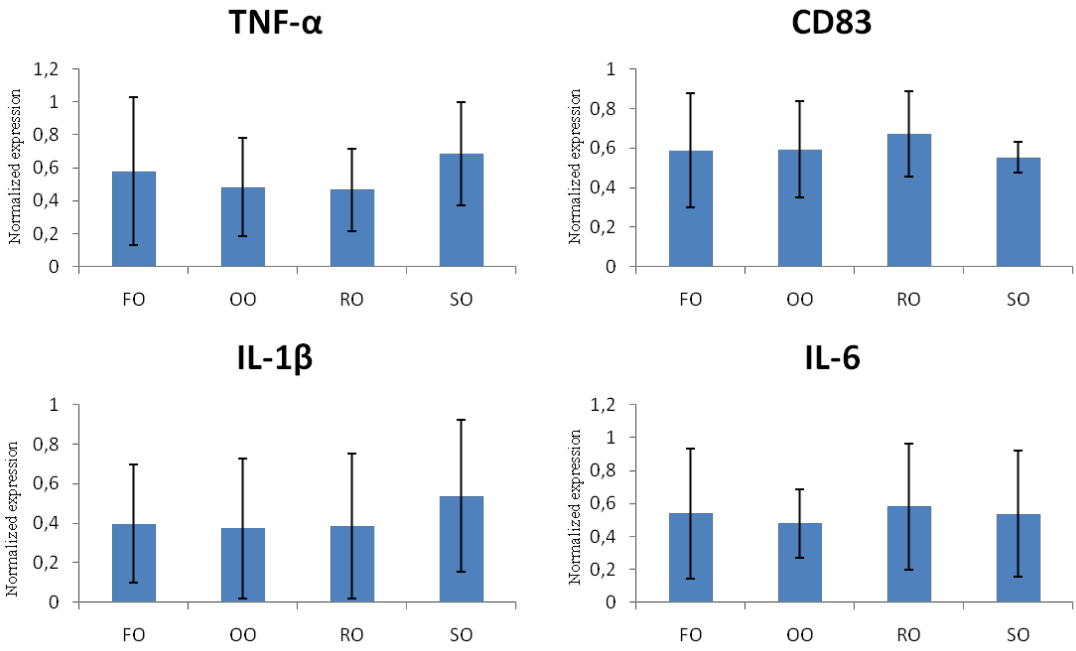

Fig. 3.10) Normalized expression of target genes in head kidney from last sampling. TNF-α: tumour necrose factor-α; CD83: ; IL-1β: interleukin 1β; IL-6: interleukin-6; FO: fish oil; OO: olive oil; RO: rapeseed oil; SO: soybean oil. (n = 3)

In head kidney there was no statistical significant difference relatively to expression of target genes. All the samples have a p-value above 0.05. These results meant no different expression of target genes between the control dietary group and the VO dietary groups. The averages of gene expression were very similar and their standard deviation was too large. Both factors contributed the no statistical differences.

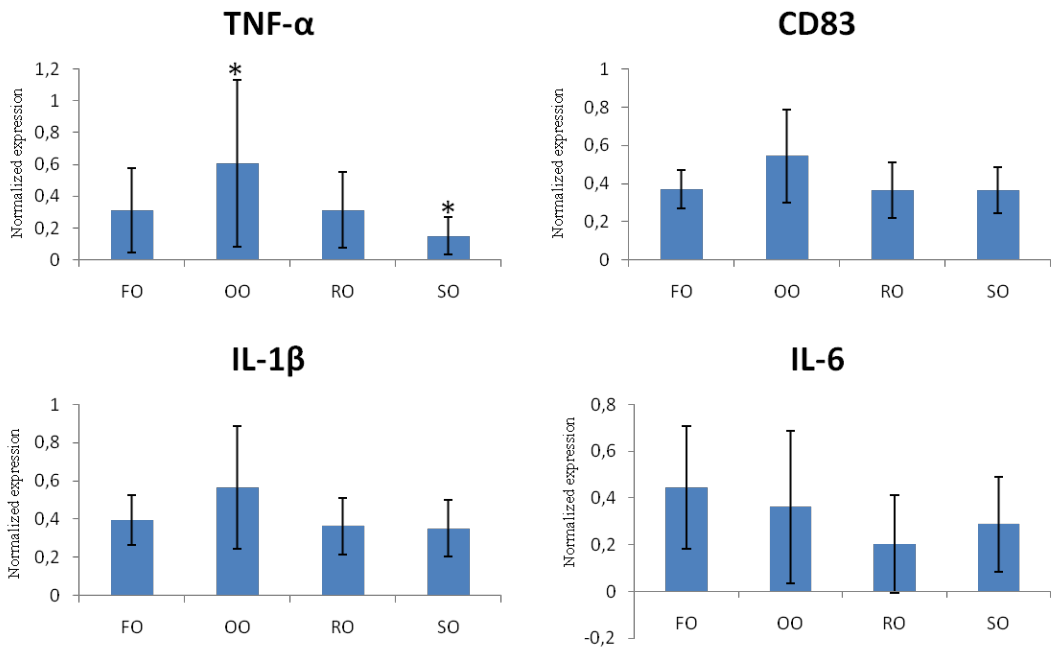

Fig. 3.11) Normalized expression of target genes in visceral adipose tissue from last sampling. TNF-α: tumour necrose factor-α; CD83: ; IL-1β: interleukin 1β; IL-6: interleukin-6; FO: fish oil; OO: olive oil; RO: rapeseed oil; SO: soybean oil. Significant difference is represented with a star (*). (n = 3)

In visceral adipose tissue there was no statistical significant difference in almost all genes and dietary groups either. The standard deviation in this tissue was also high, what difficult find out some statistical significant difference. Contrary of previous gene expressions, there was a statistical significant difference in TNF-α expression ($p = 0.040$) and unexpectedly the difference was between OO and SO diets ($p = 0.046$).

There was no positively related gene expression between both tissues.

4. DISCUSSION

4.1 Fish performance

The replacement of fish meal by 70% of plant protein and 80% of the fish oil by vegetable oils did not cause statistical significant differences in final weight, length and condition factor. Actually the referred replacement provided a good performance in Atlantic salmon growing from 0.8 to 3.4 kg in 28 weeks. Although, the SO dietary group was significantly shorter than fish fed with FO. A significantly reduced feed intake was observed in fish fed with SO and both feed utilization and lipid digestibility were significantly reduce in the FO fed fish (Liland *et al.*, 2012).

In previous studies with FO replacement, it was observed a significantly reduced feed intake and final weight when fish meal and FO were replaced by 80% plant proteins and 70% VO blend (Torstensen *et al.*, 2008, Torstensen *et al.*, 2011). On the other hand, with a partial and complete replacement of FO by VO showed no significant effect in the growth, as long as the amino acids requirement was fulfilled (Torstensen *et al.*, 2005, Bell *et al.*, 2002, Nanton *et al.*, 2007).

The difference in fish growth reported by Torstensen *et al.* (2008) was observed in the dietary group of the highest level of replacement (80% of plant proteins and 70% of vegetable oils blend). The lower feed intake during the first period was the reason of this difference growth. An analogous situation occurred in the fish fed with SO diet in the current experiment. Torstensen *et al.* (2008) did not find differences in nutrient utilization but in the dietary group with the highest level of replacement there was a trend towards lower retention production during the third feeding period. According with previous studies, high inclusion of plant protein did not have influence in protein utilization as long as if the amino acid diet composition was balanced to mimic fish meal diet and if the feed intake was close to that of the control (Espe *et al.*, 2006). Torstensen *et al.* (2005) suggested temperature as a possible explanation to the low growth, where the low temperature (2-6 °C) had a negative impact in fish growth due the lower protein efficiency ratio and protein utilization. At same time low temperature, as one of the most important environmental variables, retard the normal growth in fish (Moyle and Cech, 2004).

In the present experiment the decreased feed efficiency ratio in FO dietary group could be the consequence of lower protein and lipid digestibility, which also negatively influenced the nutrient retention, as discussed by Liland *et al.(Liland et al., 2012)*. Earlier studies showed that high dietary levels of saturated fatty acids reduced the lipid digestibility at 8^{o}C (Torstensen *et al.*, 2000). Ng *et al.* (2004) however did not find any effect in lipid digestibility in palm oil fed salmon at higher temperature, indicating that lipid digestibility is highly dependent on temperature.

The absence of a difference in growth in the present experiment was also found in the experiment of Torstensen *et al.* (2005), where FO was replaced by VO blend in 75 and 100%. A plausible reason to the same growth ratio in both experiments could be the achievement of a similar balance in amino acids content of the dietary group with high replacement. Although the current experiment also demonstrated that the replacement of fish meal by SO dietary group has a negative influence in Atlantic salmon feed intake (Liland *et al.*, 2012).

The reduced feed intake in Torstensen *et al.* (2008) leaded to a reduced fish weight in fish where the FO has replaced in higher level. In the present study the SO dietary groups presented a reduced feed intake but without difference in final weight compared with FO fish fed, which presented reduced feed utilization and reduced lipid digestibility. These data suggest that a prolonged feed period could present similar final weight and no difference between the dietary groups.

4.2 Dietary and visceral adipose tissue fatty acids composition

Visceral adipose tissue fatty acids composition highly reflected the dietary fatty acids composition. The amount of EPA and DHA in diet was clearly higher in FO than in VO due its marine source. At the same time the *n*-3 fatty acids were present in higher concentration in FO diet and in visceral adipose tissue of fish fed with FO diet, whereas the *n*-6 fatty acids were present in highr concentration in SO diet and in visceral adipose tissue of fish fed with SO diet. Our results showed a statistical significant difference in fatty acid composition and also in *n*-3/*n*-6 ratio in visceral adipose tissue between the dietary groups, as expected. There was no statistical significant difference in the concentration of total fatty acids, which is in line with the visceral somatic index and whole fish lipid level.

In the experiment of Torstensen *et al.* (2011) the diet with highest level of replacement (80% of plant proteins and 70% of vegetable oils blend) was characterized by increased percentages of 18:*n*-3, 18:2*n*-6 and 18:1*n*-9 and decrease in highly unsaturated *n*-3 PUFA and long-chain monoenoic fatty acids concentration. With the increased percentage of *n*-6 FA the ratio of *n*-3/*n*-6 FA was much lower than in diet with no replacement. More studies about FO replacement by VO were performed and with similar ratios (Torstensen *et al.*, 2008, Torstensen *et al.*, 2005, Bell *et al.*, 2002, Østbye *et al.*, 2011, Espe *et al.*, 2006, Regost *et al.*, 2003, Peng *et al.*, 2008). On the other hand, Nanton *et al.* (2007) did not experience the same similarity. In this experiment the FA composition of the diets had considerable differences from the diets referred before. However there were no significant differences in total lipid composition and fat content of the tissues for Atlantic salmon fed FO and VO diets.

The RO diet had the highest *n*-3 PUFA amount of the VO diets, but *n*-3 PUFA amount in visceral adipose tissue was less than half of the *n*-3 PUFA concentration in visceral adipose tissue of FO dietary group. The composition of the diet with highest level of replacement in Torstensen *et al.* (2011) was very similar to RO diet in the present study. The SO diet of current study presented a lower *n*-3/*n*-6 ratio than the RO dietary group, due the present lowest *n*-3 FA content and at same time the highest *n*-6 FA amount. Thus, it was expected lower *n*-3/*n*-6 ratio and more dietary influences due the *n*-3/*n*-6 ratio in SO dietary group than the other groups (Turchini *et al.*, 2009).

In current study LNA concentration in visceral adipose tissue was higher in the RO dietary group. The LA level was higher in fish fed with SO, which presented the lowest LNA/LA ratio. In the current experiment the SO dietary group had the highest LA level in whole fish, six times more than fish fed with FO diet (Liland *et al.*, 2012). LA level is correlated with the production of AA as well as the LA/LNA ration (Senadheera *et al.*, 2011). High level of LA and/or low ratio of LA/LNA will induce the production of AA, which is related with the production of pro-inflammatory factors (Harizi *et al.*, 2008).

The EPA/AA is a specific *n*-3/*n*-6 ratio which was statistical significant different ($p < 0.01$) in dietary groups. The fish fed with SO diet presented the lower EPA/AA ratio. EPA and AA are both C_{20} PUFA that compete in the production of eicosanoids. These biological molecules are involved in immune response as pro-inflammatory factors (Tocher *et al.*, 2003, Sargent *et al.*, 2002). The eicosanoids derived by EPA are less active, which means that a lower EPA/AA ratio has more pro-inflammation influence (Tocher *et al.*, 2003, Sargent *et al.*, 1999, Sargent *et al.*, 2002). The higher EPA/AA ratio in the visceral adipose tissue of FO dietary

group is explained by the increased content of EPA in this group and by the reduction of EPA in the SO dietary group, reflecting the EPA content in the diet. The FO contributed with AA, which was more than two fold higher in FO than in SO dietary group. Comparing with RO and OO dietary groups, SO fed fish had 1.5 times more AA in the visceral adipose tissue, due the desaturation and elongation of LA (Liland *et al.*, 2012)

The adipose visceral tissue fatty acid composition followed the diet fatty acid composition in agreement with the initial expectations.

4.3 Visceral adipose tissue lipid stores

The whole fish lipid level and visceral somatic index did not present any statistical significant difference. In addition, no statistical significant difference between the dietary groups was found in the visceral fat adipocyte size.

These findings contradicts Tosrtensen *et al.* (2011) where they reported that FO replacement by 80% of plant proteins and 70% of vegetable oils blend increased whole body lipids level, compared with high inclusion of plant proteins together with low vegetable oil (80% of plant proteins and 35% of vegetable oils blend). It was observed a higher visceral somatic index followed by an increased adiposity. Only few previous studies have been performed with combined high replacement of fish meal and fish oil in Atlantic salmon (Torstensen et al., 2011, Torstensen et al., 2008). The fatty acids are stored as TAG in adipocytes. Diets based on marine source being rich in EPA and DHA and they decrease plasma TAG accumulation in mature Atlantic salmon adipocytes (Huang *et al.*, 2010). In contrast Nanton *et al.* (2007) did not find obvious histological differences between salmon fed with FO and VO diets.

The results in absence of difference in whole fish lipid level, visceral somatic index and adipocyte size suggest that the fish oil replacement in the current proportions had no influence on lipid storage of Atlantic salmon.

In mice and humans the adiposity and consequent obesity are strongly related with atherosclerosis and ischaemic heart (Ouchi *et al.*, 2011). Frøyse A. *et al.* (2012) analyzed the hearts and main coronary arteries from the present experiment through histology, immunohistochemistry, transmission electron microscopy, scanning electron microscopy and PCR. There were no differences between dietary groups. The author suggests that the absence of fat deposition as and immunogenic component serves as an explanation of the remarkable

stability with no sign do denudation of endothelia. They also propose that salmon may be hereditary resistant against developing atherosclerosis.

4.4 Macrophage infiltration, gene expression and inflammation

Macrophage infiltration in all samples was analyzed and a score table was fulfilled according with two criteria (number of cluster and their frequency) and four scores in each one (zero with as minimum and three as maximum). This method was developed in order to make an objective evaluation. FO and OO dietary groups had the lowest score. Both dietary groups showed macrophage infiltration, however OO dietary group was almost absent of macrophage infiltration, and it was consider as clean. Observations in RO and SO dietary groups showed macrophage infiltration in visceral adipose tissue, where the SO dietary group had the highest macrophage infiltration level.

Previous studies showed a relation between the contaminats and the macrophage infiltration (Ibrahim et al., 2011). Still with low level of macrophage infiltration, contamination could be the reason of presence of macrophage as immunity response in the control group. Analysis of the diets (Midtbø *et al. in preparation*) showed that the average sum of PCBs in fish fed with FO diet was 7.50 ng/g, almost three times more than in OO and RO and four times more than in SO dietary group. This organic pollutant is easily stored in fat tissue, accelerated apoptosis and consequently it induces the macrophage infiltration (Ferrante *et al.*, 2011). In contrast, DDTs inhibits the functional macrophages activation in mice (Nunez *et al.*, 2002). The average sum of DDTs was 5.80 ng/g in FO dietary group, around twice the means of both OO and RO and 3.5 times more than SO dietary group mean.

A macrophage antibody specific to macrophage in Atlantic salmon is not available. Therefore to confirm the samples of visceral adipose tissue were exposed to F4/80 mononuclear antibody with expectation of some similarity between the extracellular receptors of both species. With a mice group as control during the staining it was observed a failure of this antibody in Atlantic salmon. Efforts are needed in order to find a specific antibody to macrophages in salmon to facilitate the evaluation of inflammation and with more accuracy.

The reduced level of EPA and DHA and the low ratio of *n*-3/*n*-6 ratio in VO dietary groups supported the hypothesis of a higher macrophage infiltration in VO dietary groups, especially in SO dietary group. However, the macrophage infiltration between the dietary groups was

not so different as to consider that the VO affected negatively Atlantic salmon. In fact, the level of macrophage accumulation and the clusters size in the dietary trial were considered as normal.

In a normal state macrophages can be easily isolated in fish from blood as monocytes, from kidney as a lymphoid organ and in peritoneal cavity (Iwama and Nakanishi, 1996). In early experiments in mice with obesity it was observed an increased adiposity and up-regulation of adipokines by adipocytes to increase the macrophage recruitment (Weisberg *et al.*, 2003, Fantuzzi, 2005, Ouchi *et al.*, 2011). In the experiment of Torstensen *et al.* (2011) fish fed with high level of FO replacement presented higher visceral somatic index and adiposity. Todorcevic *et al.* (2010) showed for the first time that the excessive growth of adipose tissue in fish may cause up-regulation of pro-inflammatory mediators with possible negative effects on health, as seen in mammals. So, with the absence of difference in adipocyte size it was not expected different adipokines production between the dietary groups.

Some genes of cytokines involved in inflammation and related with macrophage presence were selected for performing qPCR on RNA extracted from visceral fat and head kidney of Atlantic salmon. There was no increased expression and no statistical significant difference in the gene expression between the dietary groups in both tissues after 28 weeks.

The inflammation markers TNF-α IL-1β and IL-6 are referred very often in the literature related with the inflammation. There are numerous studies in humans and mice (Wellen and Hotamisligil, 2005, Weisberg *et al.*, 2003, Bastard *et al.*, 2006, Valle *et al.*, 2005, Ouchi *et al.*, 2011) related with obesity and inflammation, whereas the knowledge in fish is still scarce (Magnadóttir, 2006). During the last years some studies about inflammation in fish have been performed (Joerink *et al.*, 2006) and particularly in Atlantic salmon where it was found a relation with the expression of these markers and the inflammation reaction due parasite presence (Fast *et al.*, 2006), virus influence (Wiik-Nielsen *et al.*, 2012) stress conditions (Niklasson *et al.*, 2011) and changes in nutritional regime (Holen *et al.*, 2011, Skugor *et al.*, 2010, Todorcevic *et al.*, 2010). The up-regulation of macrophage precursor CD83 was also referred in salmon as inflammation response (Grammes *et al.*, 2012, Pettersen *et al.*, 2008, Holen *et al.*, 2011)

Todorcevic *et al.* (2010) suggested that adipose tissue possesses high potential for immune activity, however the results hint that the visceral adipose tissue may not be so active endocrine tissue as in mammals. A plausible explanation could be the preference of Atlantic salmon has in which white adipose tissue use first to adipokines production. Mammals have a

preference of visceral adipose tissue to store energy and the subcutaneous adipose tissue as energy source (Junqueira and Carneiro, 2005). Nanton *et al.* (2007) observed a lower proportion of TAG in muscle tissues and a higher proportion of polar lipids in lipid storage tissues, such as myosepta, belly flap and visceral fat. This was reflected in a comparatively lower proportion of *n*-3 PUFA in the muscle and the higher TAG proportion in lipid storage tissues, reflecting the FA composition of the diet more closely.

The results of gene expression are in accordance with histological observation where the macrophage infiltration in the present dietary study was presented in a low level and it was not enough to consider as inflammation.

A possible explanation to this lack of differences and the absence of inflammation would be justified for the non induced inflammation as in some works referred above.

Another possible explanation to the absence of inflammation in adipose visceral tissue could be based in the marine life stage of Atlantic salmon, where it store large amounts of fat in the peritoneal cavity in order to be used as energy source during the fresh water running and spawning (Moyle and Cech, 2004). Make sense that an evolutionary adaptation of the specie does not cause injuries or unnecessary use of energy.

5. CONCLUSION

There was no statistical significant difference in final weight, length and visceral somatic index. The adipocyte size of visceral adipose tissue did not show significant difference.

Visceral adipose tissue highly reflected the dietary fatty acid composition. There were higher levels of *n*-3 fatty acids, such as of eicosapentaenoic acid (20:5 *n*-3, EPA), docosahexaenoic acid (22:6 *n*-3, DHA) and arachidonic acid (20:4 *n*-6, AA) in FO dietary group. On the other hand, *n*-6 fatty acids and C_{18} fatty acids, such as 18:1*n*-9 and 18:3*n*-3, are present in higher amount in fish feed with VO diets. As a result, the *n*-3/*n*-6 ratio was higher in FO dietary group and fish fed with SO diet had the lower ratio.

There was macrophage infiltration but in very low level, mainly in fish feed with OO and FO. The SO dietary group had the higher macrophage infiltration. Nevertheless the macrophage accumulation was not in a level to be considered as inflammation. The lack of statistical difference in gene expression supported the last conjecture.

Together, our results demonstrated that the replacement of fish oil by olive oil, rapeseed oil or soybean oil in 80% does not have negative influence in fish growth, immune system and visceral adipose tissue.

Although visceral adipose tissue *n*-3/*n*-6 ratio decreased more than 10-fold from FO to SO fed salmon, adipose tissue lipid level, adipose size, macrophage infiltration and inflammation markers were not affected.

With the replacement of fish oils by vegetable oils in those proportions the aquaculture becomes more sustainable, the prices of fish food decrease, the demand of fish catch to fishmeal is reduced and the fish quality to the consumer is still good.

7. REFERENCES

AILHAUD, G., MASSIERA, F., WEILL, P., LEGRAND, P., ALESSANDRI, J. M. & GUESNET, P. 2006. Temporal changes in dietary fats: Role of n-6 polyunsaturated fatty acids in excessive adipose tissue development and relationship to obesity. *Progress in Lipid Research,* 45**,** 203-236.

ALVHEIM, A. R., MALDE, M. K., OSEI-HYIAMAN, D., HONG LIN, Y., PAWLOSKY, R. J., MADSEN, L., KRISTIANSEN, K., FROYLAND, L. & HIBBELN, J. R. 2012a. Dietary linoleic acid elevates endogenous 2-AG and anandamide and induces obesity. *Obesity*.

ALVHEIM, A. R., TORSTENSEN, B. E., HONG LIN, Y., LILLEFOSSE, H. H., LOCK, E.-J. J., MADSEN, L., FRØYLAND, L., HIBBELN, J. R. & MALDE, M. K. 2012b. Dietary linoleic acid elevates the endocannabinoids 2-AG and AEA, and induces weight gain and inflammation in mice fed a low fat diet. *Plos One*.

ALVHEIM, A. R., TORSTENSEN, B. E., HONG LIN, Y., LILLEFOSSE, H. H., LOCK, E.-J. J., MADSEN, L., HIBBELN, J. R. & MALDE, M. K. 2012c. Dietary linoleic acid elevates endogenous 2-AG and andandamide in Atlantic salmo (*Salmo salar* L.) and mice, and induces weight gain and inflammation in mice. *Journal of Nutrition*.

BASTARD, J. P., MAACHI, M., LAGATHU, C., KIM, M. J., CARON, M., VIDAL, H., CAPEAU, J. & FEVE, B. 2006. Recent advances in the relationship between obesity, inflammation, and insulin resistance. *European Cytokine Network,* 17**,** 4-12.

BELL, J. G., HENDERSON, R. J., TOCHER, D. R., MCGHEE, F., DICK, J. R., PORTER, A., SMULLEN, R. P. & SARGENT, J. R. 2002. Substituting fish oil with crude palm oil in the diet of Atlantic salmon (*Salmo salar*) affects muscle fatty acid composition and hepatic fatty acid metabolism. *Journal of Nutrition,* 132**,** 222-230.

BELL, J. G., TOCHER, D. R., FARNDALE, B. M., MCVICAR, A. H. & SARGENT, J. R. 1999. Effects of essential fatty acid-deficient diets on growth, mortality, tissue histopathology and fatty acid compositions in juvenile turbot (*Scophthalmus maximus*). *Fish Physiology and Biochemistry,* 20**,** 263-277.

BEYLOT, M., PINTEUR, C. & PERONI, O. 2006. Expression of the adiponectin receptors AdipoR1 and AdipoR2 in lean rats and in obese Zucker rats. *Metabolism-Clinical and Experimental,* 55**,** 396-401.

BLASBALG, T. L., HIBBELN, J. R., RAMSDEN, C. E., MAJCHRZAK, S. F. & RAWLINGS, R. R. 2011. Changes in consumption of omega-3 and omega-6 fatty acids in the United States during the 20th century. *American Journal of Clinical Nutrition,* 93**,** 950-962.

BROWNLEE, M. 2001. Biochemistry and molecular cell biology of diabetic complications. *Nature,* 414**,** 813-820.

BUSTIN, S. A., BEAULIEU, J. F., HUGGETT, J., JAGGI, R., KIBENGE, F. S. B., OLSVIK, P. A., PENNING, L. C. & TOEGEL, S. 2010. MIQE precis: Practical implementation of minimum standard guidelines for fluorescence-based quantitative real-time PCR experiments. *Bmc Molecular Biology,* 11.

CALDER, P. C. 2006. Polyunsaturated fatty acids and inflammation. *Prostaglandins, Leukotrienes and Essential Fatty Acids,* 75**,** 197-202.

CHARRIÈRE, G., COUSIN, B., ARNAUD, E., ANDRÉ, M., BACOU, F., PÉNICAUD, L. & CASTEILLA, L. 2003. Preadipocyte conversion to macrophage - evidence of plasticity. *The Jornal of Biological Chemestry,* 278**,** 6.

DE SILVA, S. S. & ANDERSON, T. A. 1995. *Fish nutrition in aquaculture,* London, Chapman & Hall.

ESPE, M., LEMME, A., PETRI, A. & EL-MOWAFI, A. 2006. Can Atlantic salmon (*Salmo salar*) grow on diets devoid of fish meal? *Aquaculture,* 255**,** 255-262.

ESPOSITO, K., PONTILLO, A., DI PALO, C., GIUGLIANO, G., MASELLA, M., MARFELLA, R. & GIUGLIANO, D. 2003. Effect of weight loss and lifestyle changes on vascular inflammatory markers in obese women - A randomized trial. *Jama-Journal of the American Medical Association,* 289**,** 1799-1804.

FANTUZZI, G. 2005. Adipose tissue, adipokines, and inflammation. *Journal of Allergy and Clinical Immunology,* 115**,** 911-919.

FAST, M. D., ROSS, N. W., MUISE, D. M. & JOHNSON, S. C. 2006. Differential gene expression in Atlantic salmon infected with Lepeophtheirus salmonis. *Journal of Aquatic Animal Health,* 18**,** 116-127.

FERRANTE, M. C., RASO, G. M., ESPOSITO, E., BIANCO, G., IACONO, A., CLAUSI, M. T., AMERO, P., SANTORO, A., SIMEOLI, R., AUTORE, G. & MELI, R. 2011. Effects of non-dioxin-like polychlorinated biphenyl congeners (PCB 101, PCB 153 and PCB 180) alone or mixed on J774A.1 macrophage cell line: modification of apoptotic pathway. *Toxicology Letters,* 202**,** 61-68.

FLEIGE, S., WALF, V., HUCH, S., PRGOMET, C., SEHM, J. & PFAFFL, M. W. 2006. Comparison of relative mRNA quantification models and the impact of RNA integrity in quantitative real-time RT-PCR. *Biotechnology Letters,* 28**,** 1601-1613.

FRØYSE, A., TANGEN, R., ROSENLUND, G., TORSTENSEN, B. E. & KOPPANG, E. O. 2012. Histologic characterisation of coronary changes in the Atlantic salmon.

GOLDSBY, R. A. & KUBY, J. 2002. *Immunology,* New York, Freeman.

GRAMMES, F., RORVIK, K. A. & TAKLE, H. 2012. Tetradecylthioacetic acid modulates cardiac transcription in Atlantic salmon (*Salmo salar* L.) suffering heart and skeletal muscle inflammation. *Journal of Fish Diseases,* 35**,** 109-117.

GRATACOS, J., COLLADO, A., FILELLA, X., SANMARTI, R., CANETE, J., LLENA, J., MOLINA, R., BALLESTA, A. & MUNOZGOMEZ, J. 1994. Serum cytokines (IL-6, TNF-alpha, IL-1-beta and IFN-gamma) in ankylosing-spondylitis - a close correlation between serum IL-6 and disease-activity and severity. *British Journal of Rheumatology,* 33**,** 927-931.

HARIZI, H., CORCUFF, J. B. & GUALDE, N. 2008. Arachidonic-acid-derived eicosanoids: roles in biology and immunopathology. *Trends in Molecular Medicine,* 14**,** 461-469.

HOLEN, E., WINTERTHUN, S., DU, Z. Y. & KROVEL, A. V. 2011. Inhibition of p38 MAPK during cellular activation modulate gene expression of head kidney leukocytes isolated from Atlantic salmon (*Salmo salar*) fed soy bean oil or fish oil based diets. *Fish & Shellfish Immunology,* 30**,** 397-405.

HUANG, T. S., TODORCEVIC, M., RUYTER, B. & TORSTENSEN, B. E. 2010. Altered expression of CCAAT/enhancer binding protein and FABP11 genes during adipogenesis in vitro in Atlantic salmon (*Salmo salar*). *Aquaculture Nutrition,* 16**,** 72-80.

HUGHES, F. J., TURNER, W., BELIBASAKIS, G. & MARTUSCELLI, G. 2006. Effects of growth factors and cytokines on osteoblast differentiation. *Periodontology 2000,* 41**,** 48-72.

IBRAHIM, M. M., FJAERE, E., LOCK, E. J., NAVILLE, D., AMLUND, H., MEUGNIER, E., BATTISTONI, B. L., FROYLAND, L., MADSEN, L., JESSEN, N., LUND, S., VIDAL, H. & RUZZIN, J. 2011. Chronic consumption of farmed salmon containing persistent organic pollutants causes insulin resistance and obesity in mice. *Plos One,* 6.

IWAMA, G. & NAKANISHI, T. 1996. *The fish immune system: organism, pathogen, and environment,* New York, Academic Press.

JOERINK, M., RIBEIRO, C. M. S., STET, R. J. M., HERMSEN, T., SAVELKOUL, H. F. J. & WIEGERTJES, G. F. 2006. Head kidney-derived macrophages of common carp (*Cyprinus carpio* L.) show plasticity and functional polarization upon differential stimulation. *Journal of Immunology,* 177**,** 61-69.

JORDAL, A. E. O., LIE, O. & TORSTENSEN, B. E. 2007. Complete replacement of dietary fish oil with a vegetable oil blend affect liver lipid and plasma lipoprotein levels in Atlantic salmon (Salmo salar L.). *Aquaculture Nutrition,* 13**,** 114-130.

JUNQUEIRA, L. C. & CARNEIRO, J. 2005. *Basic histology: text & atlas,* New York, McGraw-Hill.

KEIJER, J. & VAN SCHOTHORST, E. M. 2008. Adipose tissue failure and mitochondria as a possible target for improvement by bioactive food components. *Current Opinion in Lipidology,* 19**,** 4-10.

KVELLESTAD, A., HØIE, A., THORUD, K., TØRUD, B. & A, L. 2000. Platyspondyly and shortness of vertebral column in farmed Atlantic salmon (*Salmo salar*) in Norway - description and interpretation of pathologic changes. *Diseases Of Aquatic Organisms* 39**,** 97-108.

LANDS, W. E. M., LIBELT, B., MORRIS, A., KRAMER, N. C., PREWITT, T. E., BOWEN, P., SCHMEISSER, D., DAVIDSON, M. H. & BURNS, J. H. 1992. Maintenance of lower proportions of (*n*-6) eicosanoids precursors in phospholipids of human plasma in response to added dietary (*n*-3) fatty acids. *Biochimica Et Biophysica Acta,* 1180**,** 147-162.

LIE, O. & LAMBERTSEN, G. 1991. Fatty acid composition of glycerophospholipids in 7 tissues of cod (*Gadus morhua*), determined by combined high-performance liquid-chromatography and gas-chromatography. *Journal of Chromatography-Biomedical Applications,* 565**,** 119-129.

LILAND, N. S., ROSELUND, G., BERNTSSEN, M. H. G., BRATTELID, T., MADSEN, L. & TORSTENSEN, B. E. 2012. Net production of Atlantic salmon (FIFO, Fish in Fish out < 1) with dietary plant proteins and vegetable oils. *Aquaculture Nutrition*.

LOMBARDO, Y. B., HEIN, G. & CHICCO, A. 2007. Metabolic syndrome: Effects of n-3 PUFAs on a model of dyslipidemia, insulin resistance and adiposity. *Lipids,* 42**,** 427-437.

MADSEN, L., PETERSEN, R. K. & KRISTIANSEN, K. 2005. Regulation of adipocyte differentiation and function by polyunsaturated fatty acids. *Biochimica Et Biophysica Acta-Molecular Basis of Disease,* 1740**,** 266-286.

MAGNADÓTTIR, B. 2006. Innate immunity of fish (overview). *Fish & Shellfish Immunology,* 20**,** 137-151.

MATHIS, D. & SHOELSON, S. E. 2011. Foreword Immunometabolism: an emerging frontier. *Nature Reviews Immunology,* 11**,** 81-83.

MOYLE, P. B. & CECH, J. J. 2004. *Fishes: an introduction to ichthyology,* Upper Saddle River, Prentice Hall.

NAKAMURA, M. T. & NARA, T. Y. 2004. Structure, function, and dietary regulation of Delta 6, Delta 5, and Delta 9 desaturases. *Annual Review of Nutrition,* 24**,** 345-376.

NAKATANI, Y., KANETO, H., KAWAMORI, D., YOSHIUCHI, K., HATAZAKI, M., MATSUOKA, T., OZAWA, K., OGAWA, S., HORI, M., YAMASAKI, Y. & MATSUHISA, M. 2005. Involvement of endoplasmic reticulum stress in insulin resistance and diabetes.(vol 280, pg 847, 2005). *Journal of Biological Chemistry,* 280**,** 30648-30648.

NANTON, D. A., VEGUSDAL, A., RORA, A. M. B., RUYTER, B., BAEVERFJORD, G. & TORSTENSEN, B. E. 2007. Muscle lipid storage pattern, composition, and adipocyte distribution in different parts of Atlantic salmon (*Salmo salar*) fed fish oil and vegetable oil. *Aquaculture,* 265**,** 230-243.

NG, W. K., SIGHOLT, T. & BELL, J. G. 2004. The influence of environmental temperature on the apparent nutrient and fatty acid digestibility in Atlantic salmon (*Salmo salar* L.) fed finishing diets containing different blends of fish oil, rapeseed oil and palm oil. *Aquaculture Research,* 35**,** 1228-1237.

NIKLASSON, L., SUNDH, H., FRIDELL, F., TARANGER, G. L. & SUNDELL, K. 2011. Disturbance of the intestinal mucosal immune system of farmed Atlantic salmon (*Salmo salar*), in response to long-term hypoxic conditions. *Fish & Shellfish Immunology,* 31**,** 1072-1080.

NRC 1993. *National Research Council. Nutrient requirements of fish,* Washington, DC, National Academy Press.

NUNEZ, M. A., ESTRADA, I. & CALDERON-ARANDA, E. S. 2002. DDT inhibits the functional activation of murine macrophages and decreases resistance to infection by Mycobacterium microti. *Toxicology,* 174**,** 201-210.

OUCHI, N., PARKER, J. L., LUGUS, J. J. & WALSH, K. 2011. Adipokines in inflammation and metabolic disease. *Nature Reviews Immunology,* 11**,** 85-97.

OUCHI, N. & WALSH, K. 2007. Adiponectin as an anti-inflammatory factor. *Clinica Chimica Acta,* 380**,** 24-30.

OZCAN, U., CAO, Q., YILMAZ, E., LEE, A. H., IWAKOSHI, N. N., OZDELEN, E., TUNCMAN, G., GORGUN, C., GLIMCHER, L. H. & HOTAMISLIGIL, G. S. 2004. Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes. *Science,* 306**,** 457-461.

PENG, S. M., CHEN, L. Q., QIN, J. G., HOU, J. L., YU, N., LONG, Z. Q., YE, J. Y. & SUN, X. J. 2008. Effects of replacement of dietary fish oil by soybean oil on growth performance and liver biochemical composition in juvenile black seabream, Acanthopagrus schlegeli. *Aquaculture,* 276**,** 154-161.

PETTERSEN, E. F., INGERSLEV, H. C., STAVANG, V., EGENBERG, M. & WERGELAND, H. I. 2008. A highly phagocytic cell line TO from Atlantic salmon is CD83 positive and M-CSFR negative, indicating a dendritic-like cell type. *Fish & Shellfish Immunology,* 25**,** 809-819.

PFAFFL, M. W. & MEYER, H. H. D. 2006. Quantification of candidate gene mRNA expression in food producing animals using qRT-PCR. *Zuchtungskunde,* 78**,** 440-450.

POLVI, S. M. & ACKMAN, R. G. 1992. Atlantic salmon (*Salmo salar*) muscle lipids and theirs response to alternative dietary fatty acids sources. *Journal of Agricultural and Food Chemistry,* 40**,** 1001-1007.

REGOST, C., ARZEL, J., ROBIN, J., ROSENLUND, G. & KAUSHIK, S. J. 2003. Total replacement of fish oil by soybean or linseed oil with a return to fish oil in turbot (*Psetta maxima*) - 1. Growth performance, flesh fatty acid profile, and lipid metabolism. *Aquaculture,* 217**,** 465-482.

RICCIOTTI, E. & FITZGERALD, G. A. 2011. Prostaglandins and inflammation. *Arteriosclerosis Thrombosis and Vascular Biology,* 31**,** 986-1000.

SARGENT, J., BELL, G., MCEVOY, L., TOCHER, D. & ESTEVEZ, A. 1999. Recent developments in the essential fatty acid nutrition of fish. *Aquaculture,* 177**,** 191-199.

SARGENT, J., TOCHER, D. & BELL, G. 2002. The Lipids. *In:* HALVER, J. E. & HARDY, R. W. (eds.) *Fish Nutrition.* California: Elvsevier Science.

SCHMIDT-NIELSEN, K. 1997. *Animal physiology: adaptation and environment,* Cambridge, Cambridge University Press.

SCHUTTE, A. E., HUISMAN, H. W., SCHUTTE, R., VAN ROOYEN, J. M., MALAN, L., FOURIE, C. M. T. & MALAN, N. T. 2010. Adipokines and cardiometabolic function: How are they interlinked? *Regulatory Peptides,* 164**,** 133-138.

SENADHEERA, S. D., TURCHINI, G. M., THANUTHONG, T. & FRANCIS, D. S. 2011. Effects of dietary alpha-linolenic acid (18:3*n*-3)/linoleic acid (18:2*n*-6) ratio on fatty acid metabolism in murray cod (*Maccullochella peelii peelii*). *Journal of Agricultural and Food Chemistry,* 59**,** 1020-1030.

SIMOPOULOS, A. P. 2001. n-3 fatty acids and human health: Defining strategies for public policy. *Lipids,* 36**,** S83-S89.

SKUGOR, S., SKUGOR, A., TODORCEVIC, M., TORGERSEN, J., RUYTER, B. & KRASNOV, A. 2010. Exposure to lipopolysaccharide induces immune genes in cultured preadipocytes of Atlantic salmon. *Fish & Shellfish Immunology,* 29**,** 817-824.

SPALDING, K. L., ARNER, E., WESTERMARK, P. O., BERNARD, S., BUCHHOLZ, B. A., BERGMANN, O., BLOMQVIST, L., HOFFSTEDT, J., NASLUND, E., BRITTON, T., CONCHA, H., HASSAN, M., RYDEN, M., FRISEN, J. & ARNER, P. 2008. Dynamics of fat cell turnover in humans. *Nature,* 453**,** 783-787.

TACON, A. G. J., HASAN, M. R. & SUBASINGHE, R. P. 2006. *Use of fishery resources as feed inputs to aquaculture development : trends and policy implications,* Roma, Food and Agriculture Organization of the United Nations, FAO.

TAI, C. C. & DING, S. T. 2010. N-3 polyunsaturated fatty acids regulate lipid metabolism through several inflammation mediators: mechanisms and implications for obesity prevention. *Journal of Nutritional Biochemistry,* 21**,** 357-363.

TOCHER, D. R., BELL, J. G., MCGHEE, F., DICK, J. R. & FONSECA-MADRIGAL, J. 2003. Effects of dietary lipid level and vegetable oil on fatty acid metabolism in Atlantic salmon (*Salmo salar* L.) over the whole production cycle. *Fish Physiology and Biochemistry,* 29**,** 193-209.

TODORCEVIC, M., KJAER, M. A., DJAKOVIC, N., VEGUSDAL, A., TORSTENSEN, B. E. & RUYTER, B. 2009. N-3 HUFAs affect fat deposition, susceptibility to oxidative stress, and apoptosis in Atlantic salmon visceral adipose tissue. *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology,* 152**,** 135-143.

TODORCEVIC, M., SKUGOR, S., KRASNOV, A. & RUYTER, B. 2010. Gene expression profiles in Atlantic salmon adipose-derived stromo-vascular fraction during differentiation into adipocytes. *Bmc Genomics,* 11.

TODORCEVIC, M., VEGUSDAL, A., GJOEN, T., SUNDVOLD, H., TORSTENSEN, B. E., KJAER, M. A. & RUYTER, B. 2008. Changes in fatty acids metabolism during differentiation of Atlantic salmon preadipocytes; Effects of n-3 and n-9 fatty acids. *Biochimica Et Biophysica Acta-Molecular and Cell Biology of Lipids,* 1781**,** 326-335.

TORSTENSEN, B. E., BELL, J. G., ROSENLUND, G., HENDERSON, R. J., GRAFF, I. E., TOCHER, D. R., LIE, O. & SARGENT, J. R. 2005. Tailoring of Atlantic salmon (*Salmo salar* L.) flesh lipid composition and sensory quality by replacing fish oil with a vegetable oil blend. *Journal of Agricultural and Food Chemistry,* 53**,** 10166-10178.

TORSTENSEN, B. E., ESPE, M., SANDEN, M., STUBHAUG, I., WAAGBO, R., HEMRE, G. I., FONTANILLAS, R., NORDGARDEN, U., HEVROY, E. M., OLSVIK, P. & BERNTSSEN, M. H. G. 2008. Novel production of Atlantic salmon (*Salmo salar*) protein based on combined replacement of fish meal and fish oil with plant meal and vegetable oil blends. *Aquaculture,* 285**,** 193-200.

TORSTENSEN, B. E., ESPE, M., STUBHAUG, I. & LIE, Ø. 2011. Dietary plant proteins and vegetable oil blends increase adiposity and plasma lipids in Atlantic salmon (*Salmo salar* L.). *British Journal of Nutrition,* 106**,** 15.

TORSTENSEN, B. E., FROYLAND, L. & LIE, O. 2004a. Replacing dietary fish oil with increasing levels of rapeseed oil and olive oil - effects on Atlantic salmon (*Salmo salar* L.) tissue and lipoprotein lipid composition and lipogenic enzyme activities. *Aquaculture Nutrition,* 10**,** 175-192.

TORSTENSEN, B. E., FROYLAND, L., ORNSRUD, R. & LIE, O. 2004b. Tailoring of a cardioprotective muscle fatty acid composition of Atlantic salmon (*Salmo salar*) fed vegetable oils. *Food Chemistry,* 87**,** 567-580.

TORSTENSEN, B. E., LIE, O. & FROYLAND, L. 2000. Lipid metabolism and tissue composition in Atlantic salmon (*Salmo salar* L.) - Effects of capelin oil, palm oil, and oleic acid-enriched sunflower oil as dietary lipid sources. *Lipids,* 35**,** 653-664.

TURCHINI, G. M., TORSTENSEN, B. E. & NG, W. K. 2009. Fish oil replacement in finfish nutrition. *Reviews in Aquaculture,* 1**,** 10-57.

VALASEK, M. A. & REPA, J. J. 2005. The power of real-time PCR. *Advances in Physiology Education,* 29**,** 151-159.

VALLE, M., MARTOS, R., GASCON, F., CANETE, R., ZAFRA, M. A. & MORALES, R. 2005. Low-grade systemic inflammation, hypoadiponectinemia and a high concentration of leptin are present in very young obese children, and correlate with metabolic syndrome. *Diabetes & Metabolism,* 31**,** 55-62.

VANDESOMPELE, J., DE PRETER, K., PATTYN, F., POPPE, B., VAN ROY, N., DE PAEPE, A. & SPELEMAN, F. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology,* 3.

WEISBERG, S. P., MCCANN, D., DESAI, M., ROSENBAUM, M., LEIBEL, R. L. & FERRANTE, A. W. 2003. *Obesity is associated with macrophage accumulation in adipose tissue* [Online]. Available: <Go to ISI>://000187348300007 [Accessed 12 112].

WELLEN, K. E. & HOTAMISLIGIL, G. S. 2003. Obesity-induced inflammatory changes in adipose tissue. *Journal of Clinical Investigation,* 112**,** 1785-1788.

WELLEN, K. E. & HOTAMISLIGIL, G. S. 2005. Inflammation, stress, and diabetes. *Journal of Clinical Investigation,* 115**,** 1111-1119.

WIIK-NIELSEN, C. R., LOVOLL, M., SANDLUND, N., FALLER, R., WIIK-NIELSEN, J. & JENSEN, B. B. 2012. First detection of piscine reovirus (PRV) in marine fish species. *Diseases of Aquatic Organisms,* 97**,** 255-258.

ØSTBYE, T. K., KJÆR, M. A., RORA, A. M. B., TORSTENSEN, B. & RUYTER, B. 2011. High n-3 HUFA levels in the diet of Atlantic salmon affect muscle and mitochondrial membrane lipids and their susceptibility to oxidative stress. *Aquaculture Nutrition,* 17**,** 177-190.

8. APPENDIX

Appendix I – Visceral adipose tissue fatty acid composition (mg/g)

	Dietary Groups											
	FO			OO			RO			FO		
	2	5	10	1	8	11	4	6	9	3	7	12
06:0	< 0,01	< 0,01	< 0,01	< 0,01	< 0,01	< 0,01	< 0,01	< 0,01	< 0,01	< 0,01	< 0,01	< 0,01
08:0	< 0,01	< 0,01	< 0,01	< 0,01	< 0,01	< 0,01	< 0,01	< 0,01	< 0,01	< 0,01	< 0,01	< 0,01
10:0	< 0,01	< 0,01	< 0,01	< 0,01	< 0,01	< 0,01	< 0,01	< 0,01	< 0,01	< 0,01	< 0,01	< 0,01
12:0	< 0,01	< 0,01	< 0,01	< 0,01	< 0,01	< 0,01	< 0,01	< 0,01	< 0,01	< 0,01	< 0,01	< 0,01
14:0	52.00	48.80	48.10	18.70	15.80	16.90	14.50	16.80	17.10	18.90	18.40	17.90
14:1*n*-9	0.40	0.40	0.40	0.30	< 0,01	< 0,01	0.30	0.30	< 0,01	0.40	0.40	< 0,01
15:0	3.70	3.30	3.20	1.40	1.20	1.20	1.10	1.30	1.30	1.40	1.40	1.40
16:0	129.40	122.90	119.20	116.80	103.30	107.80	101.10	119.70	121.50	77.50	76.10	73.80
16:1*n*-9	2.30	2.30	2.30	3.20	2.80	3.00	1.60	1.70	1.90	2.30	2.30	2.60
16:1*n*-7	61.52	56.81	57.00	22.87	19.34	21.13	14.37	16.67	17.03	18.84	19.02	18.99
17:0	2.00	2.80	2.80	1.60	1.30	1.50	1.30	1.40	1.40	1.20	1.30	1.30
16:2*n*-4	5.90	5.40	5.50	1.50	1.20	1.40	1.20	1.40	1.40	1.50	1.50	1.40
18:0	26.90	25.70	25.50	32.70	28.90	29.60	27.40	32.10	33.00	22.60	22.40	22.50
16:3*n*-3	< 0,01	0.20	< 0,01	1.50	0.90	1.10	< 0,01	< 0,01	< 0,01	0.30	< 0,01	0.30
18:1*n*-11	7.30	6.40	6.70	2.00	1.80	1.80	1.80	1.90	1.60	2.00	2.40	1.80
18:1*n*-9	172.30	162.70	155.50	543.50	467.10	498.50	211.20	230.60	254.80	387.80	399.20	393.30
18:1*n*-7	29.10	26.80	27.10	26.20	22.30	23.80	16.90	19.40	20.50	28.30	28.50	27.90
16:4*n*-3	4.10	3.70	3.90	2.00	1.10	1.90	1.40	1.70	0.90	1.40	1.30	0.90
18:2*n*-6	59.60	56.90	55.50	142.00	120.00	127.30	272.50	318.00	329.20	142.20	148.30	145.70
18:3*n*-6	0.90	0.90	0.90	3.00	2.90	2.30	8.00	9.10	9.60	2.70	2.20	2.20
20:0	1.80	1.70	1.60	3.00	2.80	2.20	2.00	2.20	2.30	3.20	2.70	3.20
18:3*n*-3	19.30	18.20	17.60	38.60	31.10	33.70	30.00	34.40	35.80	51.90	53.30	54.50
20:1*n*-11	5.40	3.80	3.70	2.20	1.90	1.20	1.20	1.80	1.30	1.30	2.00	1.50
20:1*n*-9	42.10	38.20	37.60	39.50	33.90	34.30	16.50	19.10	19.50	33.00	34.10	33.20
20:1*n*-7	1.80	1.70	1.50	0.90	0.70	0.80	0.60	0.60	0.60	1.10	1.00	0.80
18:4*n*-3	16.70	15.20	15.20	8.30	7.00	6.00	7.90	8.80	9.20	9.40	9.80	8.10
20:2*n*-6	5.60	5.50	5.10	14.20	11.70	12.10	16.80	20.50	21.50	12.90	12.90	13.00
20:3*n*-9	< 0,01	< 0,01	< 0,01	< 0,01	< 0,01	< 0,01	< 0,01	< 0,01	< 0,01	< 0,01	< 0,01	< 0,01
20:3*n*-6	2.60	2.50	2.00	6.10	5.90	5.60	10.50	12.60	13.10	5.40	5.30	4.70
22:0	0.60	0.80	0.60	1.50	1.40	1.40	1.40	1.80	1.80	1.60	1.70	1.70
20:3*n*-3	2.00	2.30	1.80	4.30	3.50	3.60	2.00	2.50	2.60	5.10	5.10	5.80
20:4*n*-6	7.10	6.00	6.70	2.00	1.60	1.60	2.40	2.80	3.00	2.00	1.80	1.50
22:1*n*-11	51.20	47.70	46.70	16.90	14.30	14.70	12.80	14.50	14.50		17.30	16.60
22:1*n*-9	4.60	4.10	4.50	3.80	3.10	3.20	2.00	2.10	2.30	3.70	3.80	3.70
20:4*n*-3	17.30	16.70	16.60	8.40	7.50	7.60	6.00	6.90	7.00	10.60	10.20	10.20
20:5*n*-3	68.30	62.60	65.80	18.00	14.20	16.30	13.90	15.40	16.60	20.00	19.00	17.70
24:0	< 0,01	0.20	0.20	0.50	0.50	0.50	0.40	0.60	0.60	0.60	0.50	0.60
22:4*n*-6	2.40	2.20	2.20	0.70	0.50	0.40	0.40	0.60	0.70	0.70	0.70	0.40
21:5*n*-3	< 0,01	< 0,01	< 0,01	< 0,01	< 0,01	< 0,01	< 0,01	< 0,01	< 0,01	< 0,01	< 0,01	< 0,01
24:1*n*-9	10.40	9.70	9.50	4.50	3.70	3.70	2.90	3.30	3.30	4.90	5.00	4.80
22:5*n*-6	2.30	2.30	1.20	0.50	0.40	< 0,01	0.30	0.30	0.40	0.40	0.30	0.20
22:5*n*-3	37.90	35.70	36.50	9.70	7.60	8.60	7.10	8.00	8.60	10.90	10.40	10.40
22:6*n*-3	104.80	95.80	95.30	39.70	32.90	32.70	28.30	33.10	34.60	37.60	37.30	33.20
24:5*n*-3	4.70	4.30	4.00	1.30	0.80	0.90	1.00	1.10	1.20	0.60	1.30	1.20
24:6*n*-3	2.73	2.54	2.04	1.62	1.38	1.25	1.02	1.48	1.13	1.15	1.46	1.14

Appendix II - Real-time PCR analysis

Table A.1 Chemicals and reagents used in RNA extraction with Quiazol treatment

Product name	Vendor
Qiazol	Qiagen. Austin, TX, USA
Chloroform	Merck, Germany
Isopropanol	Arcus kjemi, Norway
Ethanol	Arcus kjemi, Norway
DEPC (Diethyl pyrocarbonate)	Sigma art. no. F32490, USA
RNase free ddH2O	MilliQ Gradient, Lab-tec, Norway
RNAase Zap	Sigma art. N. R2020, USA

Table A.2 Chemicals and reagents used in RNA precipitation with Ethanol

reagent	Solution components
3 M NaAc pH 5.2	6.15 g NaAc, > 15 ml 0.1 ddH2O
75% EtOH	75 ml absolute ethanol, 25 ml 0.1 ddH2O

Table A.3 Chemicals and reagents used in DNA-free™ DNase treatment

Product name	Vendor
DNA free	Ambion art. Nr. 1906
RNAase Zap	Sigma art. N. R2020, USA

Table A.4 Chemicals and reagents used in RNA integrity on Agilent Bioanalyzer

Product name	Vendor
RNase free ddH2O	MilliQ Gradient, Lab-tec, Norway
RNAase Zap	Sigma art. N. R2020, USA
RNA 6000 Nano LabChip kit	Agilent Technologies art. nr. 5065-4476
RNA ladder RNase	Ambion art.nr 7152

Table A.5 Chemicals and reagents used in RT-reaction

Product name	Vendor
TagMan RT buffer 10x	Biosystems art. nr. N8080234
25 mM magnesium chloride	Biosystems art. nr. N8080234
10mM deoxyNTPs	Biosystems art. nr. N8080234
50µM Oligo $d(T)_{16}$ primer	Biosystems art. nr. N8080234
RNase inhibitor (20 U/µl)	Biosystems art. nr. N8080234
Multiscribe reverse transcriptase (50 U/µl)	Biosystems art. nr. N8080234
RNase free ddH2O	MilliQ Gradient, Lab-tec, Norway

Table A.6 Chemicals and reagents used in reaction mixture for RT-PCR analysis on Lightcycler 480

Product name	Vendor
SYBR GREEN Master	Roche, Norway
Primer forward	Invitrogen Ltd, UK
Primer Reverse	Invitrogen Ltd, UK
RNse free ddH2O	MilliQ Gradient, Lab-tec, Norway

Appendix II - Histology analysis

Table A.7 Chemicals and reagents used in fixation, dehydration, embedding, slicing and dyeing of tissues

Product name	Vendor
37% Formaldehyde	Merck art. no. 1.04003.1000
NaH2PO4 x H2O	Merck art. no. 1.06346.0500
Na2HPO4 x 2H2O	Merck art. no. 1.06580.0500
Ethanol 96%	Arcus kjemi, Norway
Ethanol 100%	Arcus kjemi, Norway
Xylene	Prolabo art. no. 28973.328
Mayer's Hematoxylin	EMS art. no. 26043-05
Eosin Y work solution	Sigma art. no 119830
Methanol	Merck art. no. 1.06018.2500
Microscopy Entellan	Merck art. no. 1.02083.0250
Paraffin	Merck art. no. 1.07337.1000
ddH2O	MilliQ Gradient, Lab-tec, Norway

Appendix IV - RNA integrity on the Bioanalyzer (RNA 6000 Nano)

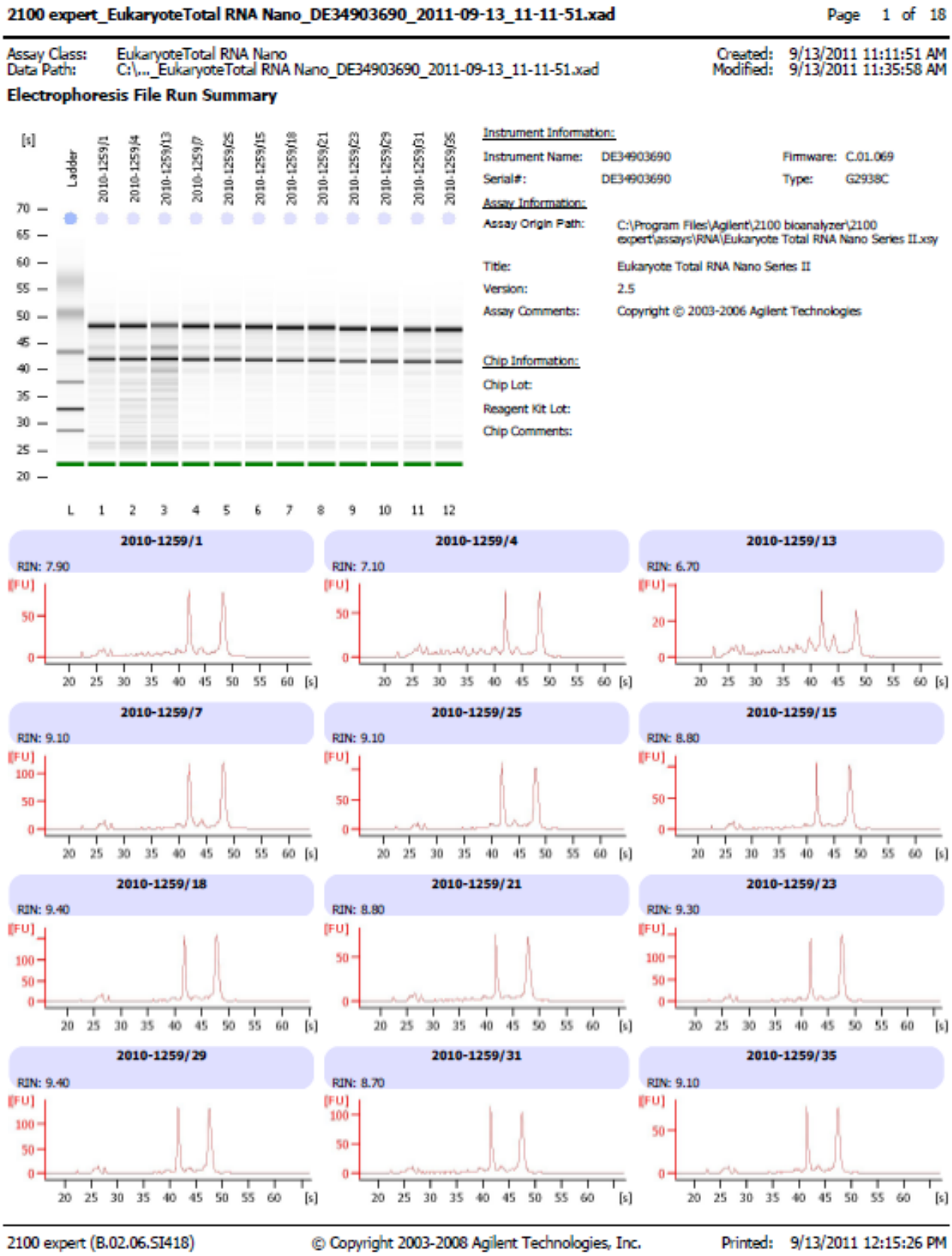

Figure A.1 RNA integrity of visceral fat

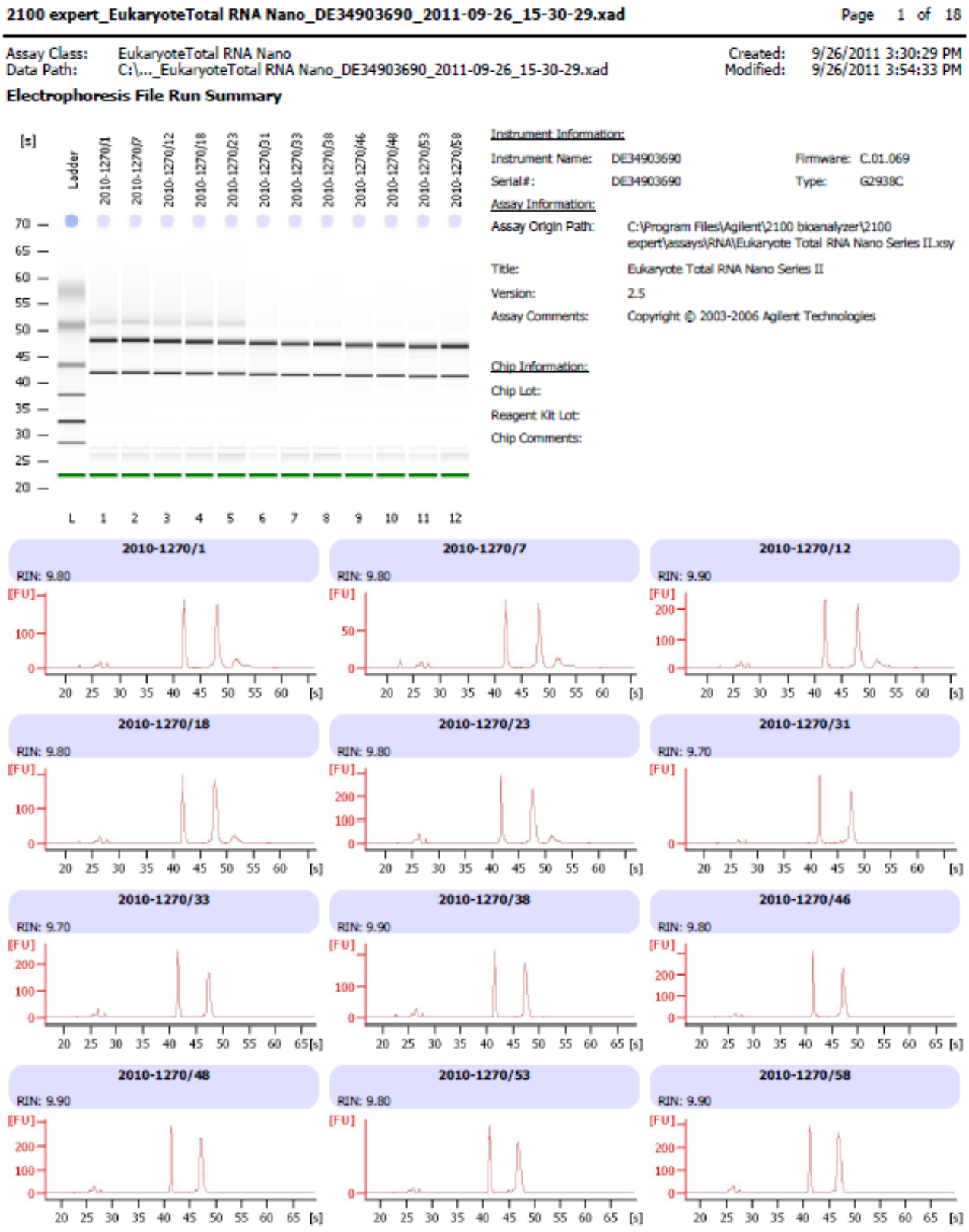

Figure A.2 RNA integrity of head kidney