Development and functions of adipose tissue in Atlantic salmon

Fettvevets utvikling og funksjoner i Atlantisk laks

Philosophiae Doctor (PhD) Thesis

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This thesis is dedicated to my mother Milka,
who has supported and encouraged me to pursue my dreams.

In memory of my father Ljuban.

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1. AIMS OF THE STUDY

The overall aim of the work presented in this thesis was to increase our understanding of the development and functions of adipose tissue in Atlantic salmon.

The specific aims were to:

Further develop a method for *in vitro* proliferation and differentiation of stromal-vascular cells isolated from the visceral adipose tissue of Atlantic salmon (Article I)

Characterise adipose tissue stromal-vascular cell proliferation and lineage determination towards mature adipocytes by transcriptome analyses (Article III)

Characterise the fatty acid β-oxidation capacity and fat deposition capacity in cells at different developmental stages during adipogenesis and determine how these processes are affected by different fatty acids (Article I)

Study how n-3 fatty acids and oleic acid affect fat deposition and the oxidative stress response in adipocytes, both *in vivo* and *in vitro* (Articles I, II, IV)

To examine how redox processes are involved in the regulation of adipogenesis in Atlantic salmon white adipocytes (Articles III, IV)

2. LIST OF ARTICLES

This thesis is based on the following articles and they will be referred to by theirs roman numerals in the text.

I Marijana Todorčević, Anne Vegusdal, Tor Gjøen, Hilde Sundvold, Bente E. Torstensen, Marte A. Kjær, Bente Ruyter
Changes in fatty acids metabolism during differentiation of Atlantic salmon preadipocytes; Effects of n-3 and n-9 fatty acids
Biochimica et Biophysica Acta 1781 (2008) 326–335

II Marijana Todorčević, Marte A. Kjær, Nataša Djaković, Anne Vegusdal, Bente E. Torstensen, Bente Ruyter
N-3 HUFAs affect fat deposition, susceptibility to oxidative stress, and apoptosis in Atlantic salmon visceral adipose tissue
Comparative Biochemistry and Physiology, Part B 152 (2009) 135–143

III Marijana Todorčević, Stanko Škugor, Aleksei Krasnov, Bente Ruyter
Gene expression profiles in Atlantic salmon adipose-derived stromo-vascular fraction during differentiation into adipocytes
BMC Genomics (2010) 11:39

IV Marijana Todorčević, Stanko Škugor, Bente Ruyter
Alterations in oxidative stress status modulate terminal differentiation in Atlantic salmon adipocytes cultivated in media rich in n-3 fatty acids
(Manuscript submitted)

3. ABBREVIATIONS

aSVF	stromal-vascular fraction of adipose tissue
C/EBP	CCAAT/enhancer-binding protein
CPT	carnitine palmitoyl-transferase
DHA	docosahexaenoic acid
EPA	eicosapentaenoic acid
ER	endoplasmic reticulum
FA	fatty acid
FABP	fatty acid binding protein
FFA	free fatty acid
FATP	fatty acid transport protein
HUFA	highly unsaturated fatty acid
MSC	mesenchymal stem cell
OA	oleic acid
PPAR	peroxisome proliferator-activated recepotor
ROS	reactive oxygen species
TAG	triacylglycerol
UPR	unfolded protein response

4. SUMMARY

The trend in salmon aquaculture is to use feed with high lipid content. In current feeds, energy from lipids can comprise as much as 50% of the total energy, compared to 10% approximately 20 years ago. This change was undertaken in order to increase growth and reduce expensive and scarce marine proteins in the feed [1]. However, these lipid-rich diets lead to high lipid deposition in salmonid fishes both in the fillet and around the internal organs [2].

High levels of visceral adipose tissue result in production losses when the salmon is gutted at harvest [3]. The relationship between excess lipid deposition in viscera and health is not well known for fish, but the occurrence of high mortality in slaughter-sized salmonids due to poor heart health and stress has been observed by Tørud and Hillestad [4].

The overall aim of the work described in this thesis was to gain more insight into Atlantic salmon visceral adipose tissue development and functions as well as the effects of n-3 highly unsaturated fatty acids (HUFAs) on adipocytes by combining *in vivo* fish trials with an *in vitro* cell culture model.

Article I: describes an *in vitro* method for studying the differentiation of preadipocytes isolated from Atlantic salmon visceral adipose tissue. Isolated preadipocytes differentiate from an unspecialized fibroblast like cell type to mature adipocytes filled with lipid droplets in culture. After one week in culture, preadipocytes reach confluence. At this stage, the differentiation process is triggered with hormones. Several adipogenic gene markers were measured in order to follow the differentiation process. The expression of the adipogenic gene markers; peroxisome proliferated activated receptor (PPAR) α, lipoprotein lipase, microsomal triglyceride transfer protein, fatty acid transport protein (FATP) 1 and fatty acid binding protein (FABP) 3 increased during the maturation of adipocytes. In this article, we further describe a novel alternatively spliced form of PPARγ (PPARγ short). The expression of PPARγ short increased during differentiation, while the expression of PPARγ long was down regulated. Eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA) are known to lower the triacylglycerol (TAG) accumulation in human adipocytes both *in vivo* and *in vitro*. We show that this is also the situation in fish, both EPA and DHA significantly lower TAG accumulation and increase fatty acid (FA) β-oxidation in salmon adipocytes compared to OA.

Article II: describes how increasing dietary levels of n-3 HUFAs affects lipid storage and mitochondrial β-oxidation in Atlantic salmon white adipose tissue *in vivo*. Further, it investigates how EPA and DHA influence the susceptibility to oxidative stress. Increased dietary levels of n-

3 HUFAs resulted in lower fat percentage in white adipose tissue; in agreement with the *in vitro* observations in Article I. Mitochondrial FA β-oxidation activity was higher in the fish oil group than it was in the rapeseed oil group. The relative levels of EPA and DHA in phospholipids from white adipose tissue and mitochondrial membranes increased with the increasing dietary levels of these HUFAs. Together with reduced cytochrome c oxidase activity and increased superoxide dismutase activity in the high HUFA groups, these data show an increased incidence of oxidative stress resulting in non-functional mitochondria with no detectable mitochondrial FA β-oxidation activity. The increased activity of caspase 3, in the high n-3 HUFA groups, further indicated some degree of apoptosis occurring in white adipose tissue of these groups. Decreased fat cell number due to apoptosis, may be one factor explaining the lower TAGs percentage found in the high HUFA groups.

Article III: describes development-associated changes in gene expression during determination and terminal differentiation of Atlantic salmon adipose-derived stromo-vascular fraction (aSVF). The determination phase was characterised with cellular heterogeneity. After confluence, however, the cellular heterogeneity decreased as evidenced by the down-regulation of markers of osteo/chondrogenic, myogenic, immune and vasculature cell lineages. Genes involved in nucleotide metabolism and DNA replication, essential processes for cellular division, implied attenuation of proliferation after day 9, in agreement with the number of cells staining positive for proliferating cell nuclear antigen. The terminal differentiation phase was characterised by high lipid accumulation and decreased recruitment of new adipocytes. This was accompanied with increased expressions of several genes involved in lipid and glucose metabolism, including markers of the adipocyte lineage. The gene expression of glyceraldehyde 3-phosphate dehydrogenase and transaldolase, suggested interplay between glycolysis and pentose phosphate pathways in order to secure the production of the glycerol backbone for TAG synthesis. The coordinated-expression of several genes in different antioxidant producing pathways, including the glutathione-based system, pointed to the importance of maintaining a reduced intracellular environment in cells after confluence in order to be able to safely store large amounts of lipids. Signs of endoplasmic reticulum (ER) stress and unfolded protein response (UPR) occurred at the later stages of adipocyte differentiation, in parallel with increased lipid droplet formation and increased gene expression of the secretory proteins adipsin and visfatin. The UPR markers, X-box binding protein 1 and activating transcription factor 6, were induced together with genes involved in ubiquitin-proteasome and lysosomal proteolysis. Notably, changes in expression of a panel of genes belonging to different immune pathways were observed throughout adipogenesis.

Article IV: describes how HUFAs may influence oxidative stress responses in salmon adipocytes *in vitro*. Terminally differentiating adipocytes were cultivated on HUFA-rich media and treated with two agents that affected their oxidative status: buthionine sulfoximine depleted stores of the intracellular antioxidant glutathione and exacerbated oxidative stress, while α-tochopherol protected cells from oxidative stress.

Lipid accumulation and the expression of adipogenic gene markers were lower in cells with high HUFA levels and no antioxidant supplemented than in cells added the antioxidant α-tocopherol. Depletion of glutathione with buthionine sulfoximine was associated with the highest activity of superoxide dismutase and the highest levels of reactive oxygen species (ROS) as measured by increased level of thiobarbituric acid reactive substances. α-tocopherol supplementation mediated a reduction in the oxidative stress response in the glutathione-depleted cells, independent of glutathione peroxidases and superoxide dismutase. α-tocopherol seem to have a strong pro-adipogenic effect, while oxidative stress induced by HUFAs and buthionine sulfoximine have anti-adipogenic effects. In addition, α-tocopherol had anti-apoptotic and anti-inflammatory effects and induced the expression of activating transcription factor 6, a marker of ER-stress. The high expression of transcription factor 6 in the α-tocopherol groups may be explained by the higher lipid level found in these groups, since high lipid level is known to induce ER stress.

5. GENERAL INTRODUCTION

The presence, amount and distribution of adipose tissue vary between species. The lipid storage sites of fish are mesenteric fat, muscle and liver [5]. In Atlantic salmon the primary sites of fat deposition are the visceral adipose tissue [6-8] and the adipose tissue located within the connective tissue sheets called myosepta in the muscle [9;10].

The fat depots that are found around inner organs are collectively referred to as visceral fat. Since individual white adipose tissue depots vary widely in size, molecular and physiological properties and show different gene expression profiles, it has been recently suggested that their status should be upgraded to that of "mini-organs" [11]. In this thesis the focus will be on visceral adipose tissue (around the intestine) of Atlantic salmon.

Visceral adipose tissue is special loose connective tissue composed not only of adipocytes but also of other cell types known as "stromal vascular cells", comprising adipose precursor cells, fibroblasts, endothelial cells, pericytes, monocytes/macrophages and blood cells. The major constituent of visceral adipose tissue is the adipocyte (fat cell), and it has been proposed that this cell type shares a common precursor with osteoblasts, chondrocytes and myocytes [12;13]. The fat cell is adapted for its main function, to store energy in the form triacylglycerols (TAGs) in periods of energy excess and to mobilize this energy during times of feed deprivation [14].

5.1. GROWTH AND DEVELOPMENT OF VISCERAL ADIPOSE TISSUE

Growth in adipose tissue is the result of both hypertrophy (increase in cell size) and hyperplasia (increase in cell number). Mature adipocytes are among the largest cells of the body and can increase in size by incorporating more TAGs [14]. Human adipocytes can change about 20-fold in diameter and several thousand-fold in volume [15].

Most that has been learned about the process of adipogenesis comes from *in vitro* studies of mammalian preadipocytes, and there is very little information on species-specific aspects of differentiation. The first immortal cell line of preadipocytes was introduced by Green and Meuth [16] and Green and Kehinde [17]. This and similar cell lines have provided valuable information in the field of adipocyte biology. Most biological experiments have been performed with immortalized cell lines, since these are readily available and can be expanded without limitation. Established cell lines present a homogenous cell population and can be carried in culture indefinitely. However, such cell lines may differ from cells *in vivo* in important aspects.

Cells that are cultured directly from animal tissue are known as “primary cells”. Fully differentiated primary cells can be kept in culture for a short period and thus have a limited life span (days to weeks), while unspecialised primary stem cells can be kept in culture for longer periods. The function and development of primary cells in culture offers a more relevant model system for the *in vivo* situation than immortalised cell lines. However, working with primary cells in culture presents numerous challenges, including the requirement for unique cell supplements and growth conditions.

No immortalized adipogenic cell lines of fish origin are available. The understanding of adipocyte differentiation from fish progenitor cells to mature adipocytes has been obtained primarily from primary preadipocytes. Primary preadipocyte cells are isolated from the stromal-vascular fraction of adipose tissue (aSVF) and differentiate into adipocytes when treated in cell culture with a combination of adipogenic effectors [18] and (Articles I and III).

Two different phases of adipogenesis have been established: adipogenic lineage determination and terminal differentiation. The molecular events that control the lineage determination of early precursor cells are poorly understood and much less studied than the transcriptional events that accompany the late stages of terminal adipocyte differentiation [12;19-21].

5.1.1. Origin of adipocyte precursors

The development of visceral adipose tissue in mammals is a continuous process in which pluripotent mesenchymal stem cells (MSCs) proliferate and differentiate to adipocytes throughout life [22]. However, the number of these cells decreases with age. MSCs can be isolated from different tissues, and this suggests that they originate from resident tissue cells. Recent evidence led to the hypothesis that MSCs originate from multipotent pericytes residing in blood vessel walls (Figure 1) [23;24]. These smooth-muscle-like pericytes are laid over junctions of endothelial cells [25]. Da Silva Meirelles et al. [26] suggested that once liberated from the endothelial cell, a pericyte is activated and should be considered an MSC. This MSC can readily differentiate into any one of a variety of other cell types, including muscle cells, osteoblasts, chondrocytes and adipocytes (Figure 1) [22;27-30]. Recent studies have demonstrated that multipotent MSCs can be obtained from stromal cells isolated from mature adipose tissue [31-33].

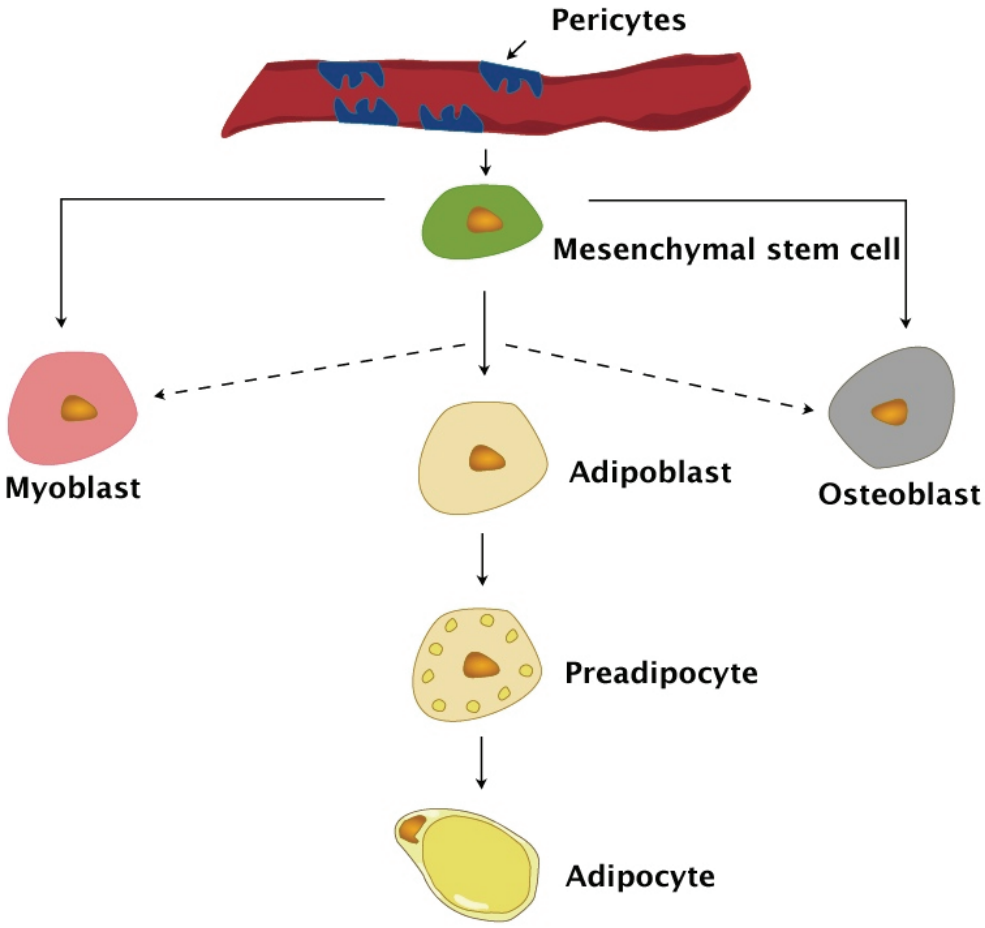

***Figure 1**. The different stages of adipogenesis.*

We show in Article III, for the first time in a fish species, that the pericytic marker transgelin is expressed in SVF cells that are derived from Atlantic salmon adipose tissue, indicating a pericyte origin of salmon preadipocytes. This result parallels the findings of mammalian studies [34]. We further showed high proliferative capacity of these cells.

5.1.2. Adipogenic lineage determination

The first phase, lineage determination, involves the commitment of a pluripotent stem cell to the adipocyte lineage. In this phase, it is not possible to distinguish committed preadipocytes from their precursor cells by their morphology. Committed preadipocytes first undergo growth arrest, which is achieved through contact inhibition at confluence. Subsequently, after hormonal induction, mammalian preadipocytes re-enter the cell cycle and pass through a limited number of cell divisions [12;35-37]. Re-entry into the cell cycle of growth-arrested preadipocytes is known as the "clonal expansion" phase, and this phase is required for the optimal conversion of preadipocytes into matured adipocytes [12;35].

When clonal expansion has been completed, cells become growth-arrested again and initiate the expression of the adipose-specific genes that are involved in the terminal differentiation of adipocytes [12;35].

Growth (proliferation) and differentiation of preadipocytes are controlled by communication between individual cells or between cells and the extracellular environment. Proliferation and differentiation are usually alternative and mutually exclusive pathways, and growth arrest is considered to be a requirement for adipocyte differentiation [38-40]. This suggests that cross-talk takes place between the cell proliferation machinery and the factors controlling cell differentiation [41]. The early phases of adipocyte development have not been studied in fish. Our transcriptome results (Article III) indicate that lineage determination in salmon adipocytes is very similar to the process in mammals.

5.1.3. Terminal differentiation of adipocytes

The second phase of adipogenesis, which is known as "terminal differentiation", has been studied in three fish species: Atlantic salmon [18] (Articles I and III), red sea bream [42] and rainbow trout [43]. In this phase, preadipocytes convert to mature adipocytes that contain the machinery necessary for lipid transport and synthesis, insulin sensitivity and the secretion of adipokines [21]. During mammalian and fish adipocyte differentiation, acquisition of the adipocyte phenotype is characterized by chronological changes in the expression of numerous genes [21], (Articles I and III) and [43;44]. Members of several transcription factor families have been implicated in this process both in mammals and in fish; these members include the CCAAT/enhancer-binding proteins (C/EBPs), C/EBPα, C/EBPβ, C/EBPδ, peroxisome proliferator-activated receptor (PPAR)γ and the adipocyte determination and differentiation-dependent factor-1/sterol regulatory element-binding protein-1c [12;19;45;46].

The adipogenic transcription factors PPARγ and C/EBPs play key roles in the complex transcriptional cascade that occurs during adipogenesis [12;19;45;46]. PPARγ is both necessary and sufficient for adipogenesis [47]. This transcription factor is not only crucial for adipogenesis but is also required for the maintenance of the differentiated state. Several C/EBPs are involved in adipogenesis. C/EBPß and C/EBPδ are induced very early during differentiation and promote adipogenesis by inducing C/EBPα and PPARγ in mammals [19;48;49]. Even though C/EBPs are very important in adipogenesis, they cannot function efficiently if PPARγ is not present [50]. Our gene expression results show that these transcription factors are also important in Atlantic salmon adipogenesis (Articles I and III) and [44]. There are many other transcription factors, such as sterol regulatory element binding protein, Kruppel-like factor 5, the zinc finger

transcription factor and the phosphorylated cAMP response element-binding protein, that play significant roles in this process [21]. This thesis presents the first microarray study performed during adipogenesis of a cold-blooded vertebrate (Article III). Microarray studies in Atlantic salmon allowed us to discover several new transcription factors involved in adipogenesis in fish (Article III).

In addition to the expression of specific transcription factors, the specific lineage commitment of MSCs is largely influenced by the culture conditions, and thus the extracellular matrix environment, cell adhesion molecule expressions and growth factors play crucial roles [51]. Growth factors that have regulatory effects on MSCs, in addition to members of the transforming growth factor beta superfamily and the Wnt superfamily, include the insulin-like growth factors [52], the fibroblast growth factors [53], the epidermal growth factor [54], the platelet-derived growth factor, and the vascular endothelial growth factor [55].

5.1.4. Regulation of intracellular redox state during adipogenesis

Carriere et al. [56;57] and Galinier et al. [58] have recently introduced the idea that free radicals are anti-adipogenic signalling molecules that inhibit preadipocyte proliferation and differentiation. This was further supported by the finding that the formation of free radicals inhibits adipocytes formation by reducing the C/EBP binding capacity [59].

In particular, reactive oxygen species (ROS) produced in mitochondria, are substantially involved in adipogenic processes [60]. Galinier et al. [58] in experiments with Zucker rats showed that the adipose tissues of obese rats have a specific higher content of hydrophilic molecules in a lower redox state than those of lean rats, indicating that obesity is associated with reduced oxidative state. Since ROS inhibits adipogenesis, adipocytes consequently suppress oxidative pathways and ROS formation in order to increase their lipid storing capacities. Over expression of glutathione peroxidases and a high content of hydrophilic antioxidant gluthathione are associated with pro-adipogenic processes and the promotion of obesity [58]. Our microarray study (Article III) also showed that the intracellular redox balance is important during adipocyte differentiation in Atlantic salmon. The down-regulations that occur in ROS-generating pathways were concurrent with the activation of several antioxidant systems (the superoxide dismutase, the catalase and the glutathione peroxidases enzymes). All these endogenous antioxidants have been detected in fish [61;62], but this thesis is the first to present their roles in fish adipose tissue.

Our results therefore indicate that a concerted regulation of several antioxidant systems in developing salmon adipocytes is necessary for safe lipid storage during salmon adipocyte differentiation.

5.1.5. Endoplasmatic reticulum stress in adipogenesis

The results presented in Article III and Article IV support the hypothesis that a significant part of control over adipogenesis is signalled through the endoplasmic reticulum (ER) membranes. This is the case in mammals [63;64]. Under normal conditions, the ER must function in the unique and demanding environment of the adipocyte, adapting to meet the demands of increased protein synthesis and secretion, energy storage in the form of TAG droplet formation, and nutrient sensing. These demands are particular to the differentiated adipocyte. ER stress occurs in various physiological and pathological conditions, including obesity [65]. The ER stress causes cells to activate self-protective mechanisms, which are collectively named the "unfolded protein response" (UPR). The UPR may be considered to be the control of a collection of pathways aimed at restoring/maintaining normal ER function [66;67]. The principal events during the UPR include transcriptional activation of chaperones to aid protein folding, selective attenuation of new protein synthesis, and activation of proteosome-mediated protein degradation. Adipocyte differentiation contributes to weight gain, and we have shown that markers of ER stress/UPR activation, including glucose-regulated protein 78 and activating transcription factor 6, are up-regulated during adipogenesis in salmon (Articles III and IV).

The factors that induce ER stress in obesity are still under debate, but several possibilities have been suggested. The excess of nutrients, as is the situation in adipocytes that store high levels of lipids, may lead to the UPR adaptive response being activated as a feedback mechanism to limit or disable the accumulation of lipid and reduce the stress on the ER, which is engaged in lipid biosynthesis. The UPR activates the expression of numerous genes involved in protein secretion in several secretory cell types [68;69]. Secretory cells, whose ER protein load fluctuates substantially, should depend particularly strongly on the UPR: one example of such cells is the pancreatic β-cell that secretes insulin in response to changes in circulating glucose [70]. Similarly, adipocytes are equipped with an astonishing ability to enlarge their size within a short period in response to the availability of lipid precursors. Furthermore, ER stress and the UPR in adipocytes are linked to major inflammatory and stress-signaling networks via several distinct mechanisms [71].

In conclusion, the UPR in adipocytes may be induced due to an increased demand for protein synthesis under nutrient excess and expansion. In addition, the excess nutrients themselves may serve as signals that induce ER stress. The results presented in Article III also suggest that the UPR is part of the normal maturation of salmon adipocytes specialized in lipid storage. In fact, levels of the two key UPR markers, activating transcription factor 6 and glucose-regulated

protein 78, were elevated in Atlantic salmon adipocytes with high lipid levels. Increased lipid deposition is the probable trigger of the UPR response (Articles III, IV).

5.2. LIPID METABOLISM IN ADIPOCYTES

Adipose tissue is a key organ in the regulation of energy metabolism. Fatty acids (FAs) are stored as TAGs in adipose tissue. All eukaryotic organisms, and some prokaryotes, have the ability to synthesise TAGs, in which the glycerol is esterified with three FAs. TAGs form fatty droplets that exclude water and can be packed very densely. The efficiency of energy storage in fats is probably an important reason why animals store most of their energy as fats, and only a small amount of energy as carbohydrates [14].

Under fasting conditions or during periods of increased energy demand, FAs stored as TAG in adipose tissues are mobilized and released into the circulation as an energy source for other tissues.

5.2.1. Fatty acid uptake

The mechanisms by which FAs are taken up into the adipocytes have been hardly studied in fish. The transport of FAs into adipocytes can occur to some extent via diffusion across the lipid bilayer of the plasma membrane in mammals, but the predominant mechanism is mediated by protein. Proteins that have been associated with the transport of FAs across mammalian adipocyte plasma membranes are: plasma membrane fatty acid binding protein (FABP) [72]; fatty acid transport protein (FATP) [73], fatty acid translocase [74] and caveolin-1 [75]. FAs are bound by accessory proteins such as fatty acid translocase, and then transported into the cell by the FATPs; they are acylated and subsequently bound by FABPs and acyl-CoA binding protein to prevent rapid efflux. We have confirmed the expression of FATP1 in Atlantic salmon adipocytes (Article I). The scavenger receptor class B type I, which it is thought may be involved in the uptake of lipids from the diet, was recently identified in salmon intestine [76]. We have also shown expression of this receptor in adipocytes (Article I).

The free fatty acids (FFAs) inside the adipocyte are bound to FABPs which shuttle FAs between the plasma membrane and intracellular membranes or metabolic compartments. More studies on FA uptake mechanisms in fish are becoming available, and FABP have been identified in several fish tissues. The expression pattern of the adipose tissue type FABP (FABP11) in Atlantic salmon has recently been described [77]. The significant increase in FABP11 expression that

occurs in the mature adipocytes is consistent with the idea that mammalian adipocyte FABP (FABP4/aP2) is a late marker of adipocyte differentiation [14].

5.2.2. Triacylglycerol synthesis and lipid droplet formation

The fate of intracellular FAs depends on the energy status of the organism. In situations of excess energy intake, excess calories in the form of FFAs will be stored in the form of TAG, primarily in adipose tissue [14].

The main source of the glycerol backbone in TAG has long been believed to be glycerol-3-phosphate produced by the catabolism of glucose via glycolysis. However, a significant proportion of the glycerol also seem to be produced by a metabolic pathway known as "glyceroneogenesis" in which pyruvate is an intermediate [78]. This pathway may be important in situations of inadequate glucose supply [79]. The formation of glycerol-3-phosphate from substrates other than glucose may be relevant in salmonid fish, which rely on diets low in carbohydrates. The results presented in Article III support the hypothesis that adipocytes in salmonid fish, at least at some points during development, employ redundant pathways for glycerol-3-phosphate production, possibly in order to increase the TAG. The rapid clearance of FFAs from the bloodstream is an important step to reduce lipotoxicity, and thus the engagement of several pathways seems to be a reasonable evolutionary outcome [14]. Also, such a strategy probably enables better adjustment of exact cellular needs (Article III).

TAGs are stored as lipid droplets in all cell types that have been examined [80]. Lipid droplets are also termed "fat globules", "oil bodies", "lipid particles", "adiposomes". Lipid droplets were long considered to be simple storage sites for neutral lipids. More recent data from mammals, however, support the idea that lipid droplets are highly dynamic organelles with more diverse functions than previously thought [81]. The mature lipid droplet occupies the major portion of the cytoplasm in both mammalian and fish white adipose tissue (Figure 2), and it consists of a neutral lipid core (primarily TAG and/or cholesteryl ester), a phospholipid monolayer, and associated proteins [82-84]. The most abundant lipid droplet-associated proteins found in mammals are members of the PAT family (perilipin, adipose differentiation-related protein, tail-interacting protein of 47 kDa, to which myocardial lipid droplet protein/oxidative tissues–enriched PAT protein and plasma membrane–associated protein also belong) [85-87]. These proteins have not been described in fish.

Lipid droplets are formed in two very different environmental conditions. First, cells accumulate lipid droplets in response to exogenous lipid availability and the cells in this case are highly

directed towards generating energy and forming membranes [88]. Second, many kinds of cellular stress, including inflammation and apoptosis, induce lipid droplet biogenesis [89]. Their metabolic origin and the physiological function are not known in this case.

It has not been determined how these lipid droplets forms, but many studies support the hypothesis that lipid droplets form in the ER. Neutral lipids are synthesized between the two leaflets of the ER membrane. The lipid droplets formed are then thought to bud off into the cytosol together with part of the cytosolic lipid monolayer of the ER [80;82]. Despite recent advances, the lipid droplet is still a poorly understood organelle.

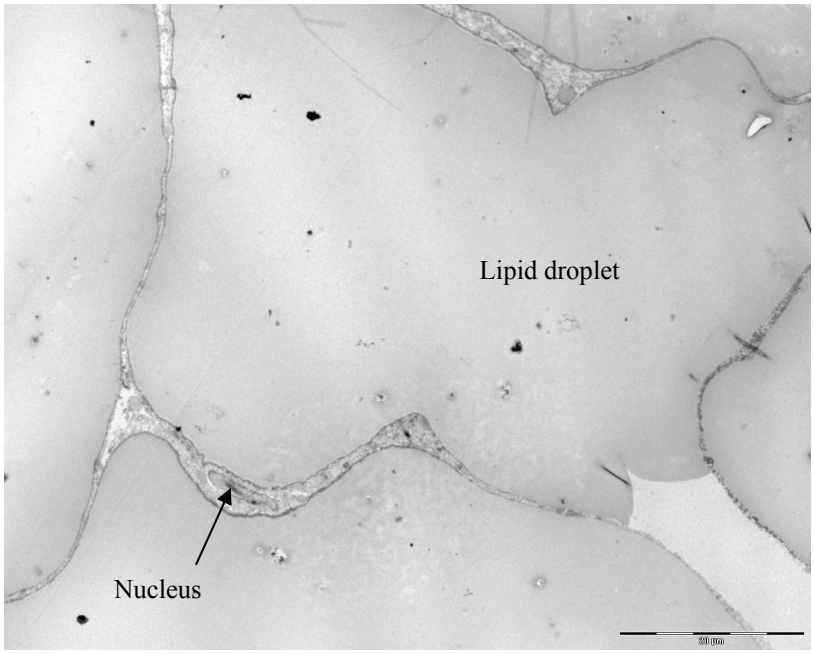

Figure 2. *Electron microscopy of mature Atlantic salmon adipocyte in vivo. The cytoplasm is completely filled with a lipid droplet. The nucleus and cytoplasm are "squeezed" between the lipid droplet and the cell membrane.*

5.2.3. β-oxidation of fatty acids

When energy is needed, TAGs stored in adipocytes are hydrolysed into glycerol and FFAs, and primarily transported to other organs such as the liver, muscles, and the heart for β-oxidation. Some FAs derived from TAG, however, are β-oxidised within the adipocyte.

β-oxidation is the major process by which FAs are catabolised, by the sequential removal of two-carbon units from the acyl chain [90]. FAs are a primary energy source, and thus β-oxidation activity has been studied in several fish species. The capacity for β-oxidation of FAs is particularly high in the liver [91;92] red muscle [93] and the heart [94]. Little is known,

however, about β-oxidation in visceral adipose tissue. We show for the first time (Articles I and III) that Atlantic salmon adipocytes both *in vivo* and *in vitro* have different capacities for β-oxidation, depending on the developmental stage and the diet. Maassen et al. [95] have shown that visceral adipose tissue in mammals is able to oxidize substantial amounts of FAs over a long period by mitochondrial β-oxidation. Visceral adipose tissue represents about 15 to 25% of the body weight of a lean individual, and this percentage can increase to more than 50% in the obese state. This tissue thus can contribute significantly to the total FA oxidation of an organism.

The β-oxidation of FAs occurs in two organelles: the mitochondria and the peroxisomes [96]. Mitochondrial β-oxidation occurs in the inner mitochondrial space, called the "matrix". In fish, as in mammals, short-chain FAs (shorter than C6) and medium-chain FAs (C6-C12) are thought to transverse the mitochondrial membrane through a non-carrier-mediated transport. In contrast, long-chain FAs (C14-C20) must bind to carnitine, a reaction that is catalyzed by the enzyme carnitine palmitoyl-transferase (CPT) I, for mitochondrial entry [96]. Generally, the rate-limiting step in the regulation of mitochondrial β-oxidation is thought to be the step that is catalysed by CPTI, namely the conversion of acyl-CoA to acyl-carnitine. Acyl-carnitine is then transported across the inner mitochondrial membrane in exchange for free carnitine molecules. Inside the mitochondria, the acyl-carnitine esters are converted back to fatty acyl-CoA in a process that is catalyzed by CPTII, and then β-oxidized. Both CPTI and CPTII are present in Atlantic salmon, and we have confirmed the expression of CPTII in adipocytes (Article I). The β-oxidation reaction in fish is thought to be the same as that in mammals, except for a lower production of CO_2 [97]. The fatty acyl-CoA molecules produced in the β-oxidation reaction proceed to the Krebs cycle. The Krebs cycle is a series of enzymatic reactions that catalyzes acetyl CoA molecules to carbon dioxide and water, thereby generating energy for the production of adenosine triphosphate molecules [96]. We show in Article I that the Krebs cycle follows the β-oxidation reaction also in fish adipocytes, since the intermediates acetate and oxalacetate are formed, together with low levels of CO_2.

The β-oxidation in peroxisomes is a four-step reaction similar to that in mitochondria. Peroxisomal β-oxidation is not directly coupled to energy production, as mitochondrial β-oxidation is, but it does generate heat and NADH [98]. It is believed that in fish, as in mammals, the peroxisomal pathway is mainly a chain-shortening mechanism of very long-chain FAs (longer than C20), preparing them for β-oxidation by the mitochondrial system [99]. However it seems that the peroxisomal β-oxidation pathway may be more dominating in fish than in mammals, accounting for up to 50% of total hepatic β-oxidation in some Antarctic species [91;92].

5.3. LIPIDS AS ENERGY SOURCE FOR FISH

Energy in fish can be obtained from three different sources: proteins, carbohydrates and lipids. Lipids and proteins are the most important energy sources for most fish species. Most carnivorous fish species have a limited capacity to utilize carbohydrates, and a common way of reducing feed cost in aquaculture is to replace as much protein as possible with lipids. Lipids are an excellent source of non-protein energy. Protein and lipids should be kept in balance, as an imbalance in the ratio will lead to either the waste of proteins or the use of proteins to synthesize lipids [100].

Increased dietary lipid levels improve palatability and feed conversion ratios in most fish species. However, increased dietary lipid levels may also increase fat deposition in the fish's fat storage organs and thereby reduce harvest yields [101]. Laying down high fat depots in salmonids is probably a strategy that has become adapted during evolution for energy storage in species that may experience periods of starvation. This strategy may become a problem when feed is continuously available. The use of high-lipid feed for cultured fish may also affect fish flesh quality by increasing the percentage of lipids stored in the edible muscle itself [102;103]. Fish in aquaculture, just as the people in the western world, consume high-lipid diets and undertake low levels of exercise, resulting in high lipid stores. In aquaculture, fish are kept in net pens during the grow-out phase, and the degree of exercise depends on the local currents at the fish farm. However, the degree of exercise is low in most cases, which may lead to poor cardio-respiratory fitness and may add to the development of excessive lipid accumulation in adipose tissue. In addition, the feed has been changed from a diet rich in the healthy n-3-rich marine oils to diets rich in plant oils with relatively high levels of the "proinflammatory" n-6 FAs [104]. How the lack of exercise, and high lipid and plant oil diets affect fish health is not known. However, there is growing concern that these factors influence the development of lifestyle-related disorders in fish [105;106].

5.3.1. Dietary fatty acids and adipose tissue

Feed is the major variable cost in aquaculture, and the industry continues to seek improvements in the nutritional-efficiency and cost-effectiveness of diet formulations. The demand for feed in the salmonid aquaculture industry has increased over recent years in parallel with increases in total fish production [107;108]. Traditionally fish feeds have relied mainly on fish oil as the source of lipid. The availability of this oil is limited and the worldwide production of fish oils is already too low to cover the needs of the aquaculture industry. In addition, fish oil is more

expensive than other oils and the future expansion of aquaculture, and in particular salmon production, will depend more and more on alternative oil sources becoming available [109].

Several vegetable oils have been tested in the search for alternatives [104;110-113]. The compositions of vegetable oils differ from those of fish oils, in particular in terms of FAs. VOs are characterised with high levels of 18-carbon FAs such as oleic acid (OA, 18:1n-9) in rapeseed oil and olive oil, linoleic acid (LA, 18:2n-6) in sunflower oil and soybean oil, and alpha-linolenic acid (ALA, 18:3n-3) in linseed oil. The vegetable oils do not contain n-3 highly unsaturated fatty acids (HUFAs) such us eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3). In contrast, fish oils are rich in n-3 HUFAs. The FA composition in feed can modify the FA compositions of different tissues [110;113-119] (Article II), and the changes in composition will further affect many metabolic processes involved in FA uptake, FA catabolism and lipid storage [5;120]. We show in Article II that high dietary levels of n-3 HUFAs reduce the fat level in the visceral adipose tissue of Atlantic salmon in comparison to fish fed vegetable oil diet. Furthermore, we show in Article I that OA leads to a higher lipid accumulation in Atlantic salmon adipocytes in culture than EPA and DHA.

Numerous studies have suggested that fish oil, rich in EPA and DHA, can influence obesity in mammals [121-124]. The precise mechanisms by which the EPA and DHA suppress the excessive accumulation of adipose tissue and exert anti-obesity effects are still largely unknown. However the following mechanisms may be involved: EPA and DHA inhibit key enzymes that are responsible for lipid synthesis, such as fatty acid synthase and stearoyl-CoA desaturase-1; they enhance mitochondrial FA β-oxidation capacity, preventing FFAs from entering adipocytes for lipogenesis; and they induce fat cell death (apoptosis) [125].

We have shown for the first time that high levels of EPA and DHA in the diet induced oxidative stress and apoptosis in Atlantic salmon visceral adipose tissue (Article II). Several other studies have shown that high dietary-HUFA levels increase the susceptibility to FA peroxidation in different fish tissues [126-129]. Lipids in fish are more highly unsaturated than those in mammals [130], which makes fish more prone to the peroxidation of FAs. Indeed, *in vivo* lipid peroxidation caused by oxygen radicals can cause several diseases in fish, such as jaundice [131] and nutritional muscular dystrophy [132]. Clearly, the presence of oxidized lipids can have toxic consequences for fish, whether they arise from dietary input of toxic agents or by deficiencies in essential antioxidant nutrients. However, several authors have suggested that dietary antioxidants, particularly tocopherol, can prevent the production of excessive levels of free-radical-generated toxic compounds and in this way eliminate these pathological compounds [61;130;133;134]. Stephan et al. [126] demonstrated that the HUFA/α-tocopherol ratio is

important in determining the susceptibility of a tissue to FA peroxidation. On the other hand, many studies into the relationship between the HUFA/α-tocopherol ratio and antioxidant defences have failed to demonstrate that such defences are induced in fish fed with high-HUFA diets [129;135-138].

Many different oil qualities are currently used in aquaculture, and oxidative stress and antioxidants have thus again become the focus of research. There is a need to increase our knowledge about suitable antioxidants and their concentrations in fish diets.

Almost no research is being conducted into how alternative oils affect lipid storage in adipose tissue in Atlantic salmon. Our results from Articles I and II imply that more research is needed into the effects of including vegetable oils on adipose tissue development and lipid storage.

5.4. FUNCTIONS OF ADIPOSE TISSUE IN IMMUNITY

In addition to buffering the daily influx of dietary nutrients and maintaining energy homeostasis, adipose tissue is the safest place for the long-term storage of lipids in a manner that is non-toxic to the cell or whole organism [139]. Adipose tissue was previously considered to be simple, static, lipid-storage tissue. Today, adipose tissue is considered to be an active endocrine and secretory organ with multiple functions in metabolic processes (Figure 3). However, some researchers consider adipose tissue to be an ancestral immune organ [140]. The complex relationship that exists between adipose tissue and the immune system is believed to be related to a large extent to the secretion of numerous adipokines. Visceral adipose tissue in mammals produces more than 50 cytokines, termed “adipokines” (Figure 3) [141]. Adipokines are biologically active substances with signaling properties. They are classified according to their functional role: appetite and energy balance, immunity, insulin sensitivity, angiogenesis, blood pressure regulation, lipid metabolism and homeostasis (Figure 3).

An active role for visceral adipose tissue as a contributor to whole-body homeostasis was demonstrated with the discovery of leptin in mammals [142]. Our group was the first to discover leptin expression in fish adipocytes [18]. Recent articles have suggested that leptin has similar functions in fish as it has in mammals. Leptin suppresses food intake in rainbow trout [143]. In addition, Kling et al. [144] found higher levels of leptin in fasted trout than in fed trout. It is now recognized that adipose tissue lies at the heart of a complex autocrine, paracrine and endocrine network and that it participates in the regulation of a variety of diverse biological functions.

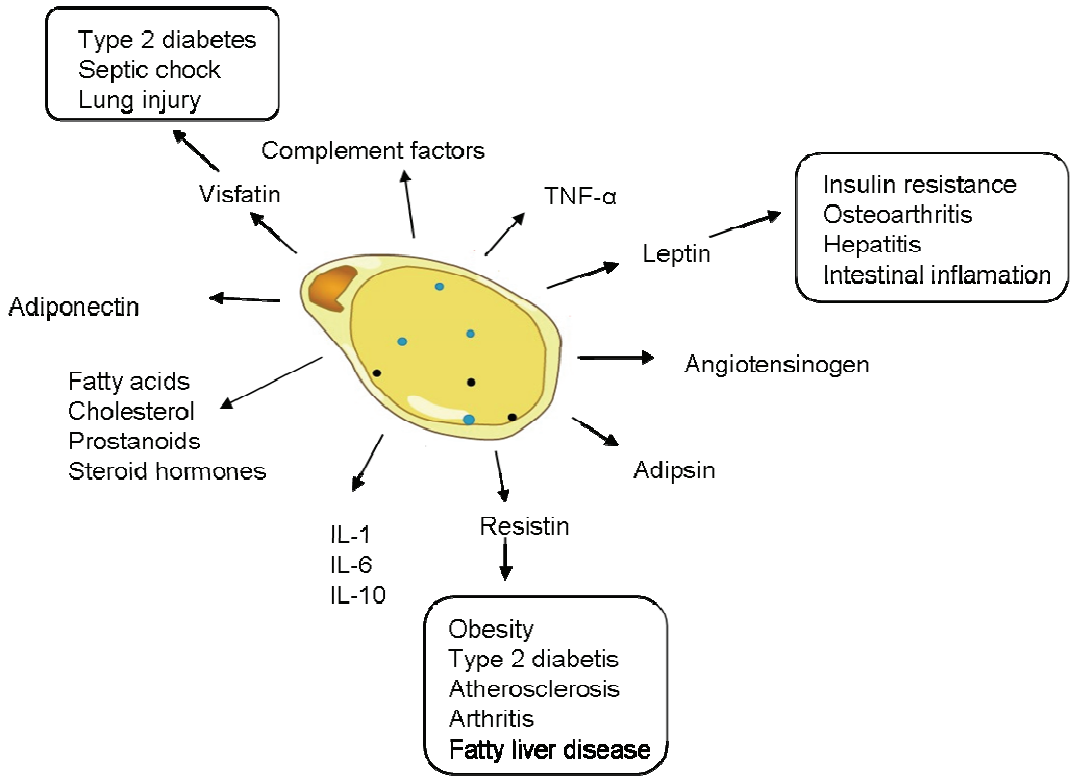

Figure 3. *Adipose tissue as an endocrine organ. The functions of white adipose in mammals include the synthesis and secretion of adipokines, and the uptake, storage and synthesis of lipids. The adipose tissue is also a source of pro-inflammatory factors that modulate the inflammatory response (Figure adapted from [141]).IL –interleukin; TNF- tumor necrosis factor.*

Less is known about the roles of adipose tissue in fish. We have carried out microarray analyses during the differentiation of Atlantic salmon preadipocytes *in vitro* (Article III), and shown that these cells express several of the genes involved in adipokine production.

As has already been mentioned, the cellular composition of visceral adipose tissue is not homogeneous. Indeed, visceral adipose tissue is composed of many cell types. Particularly important is the close interaction between the macrophages and the adipocytes that determine the adipose biology and regulate adipose tissue homeostasis. Accumulated evidence suggests that preadipocytes and macrophages share a great number of common features in mammals [145]. Many functions that were initially thought to be specific for macrophages have now been described in adipocytes and vice versa (Figure 4). Several recent studies have shown that these cells have many similarities: macrophages express many "adipocyte" gene products such as adipocyte fatty acid binding protein and PPARγ, while adipocytes express many "macrophage" gene products such as tumour necrosis factor α, interleukin 6, and matrix metalloproteinases [146-149]. In addition, Charriere et al. [145] compared mouse adipocyte progenitors with macrophages using gene expression profiling, and showed that preadipocytes can be effectively

trans-differentiated into macrophages. Recent results from our lab have also indicated that this is the case in Atlantic salmon preadipocytes [150]. Undoubtedly, adipose precursor cells exhibit a high degree of plasticity. In summary, these observations underline the plasticity of adipose precursor cells and emphasize the relationship between innate immunity and adipose tissue.

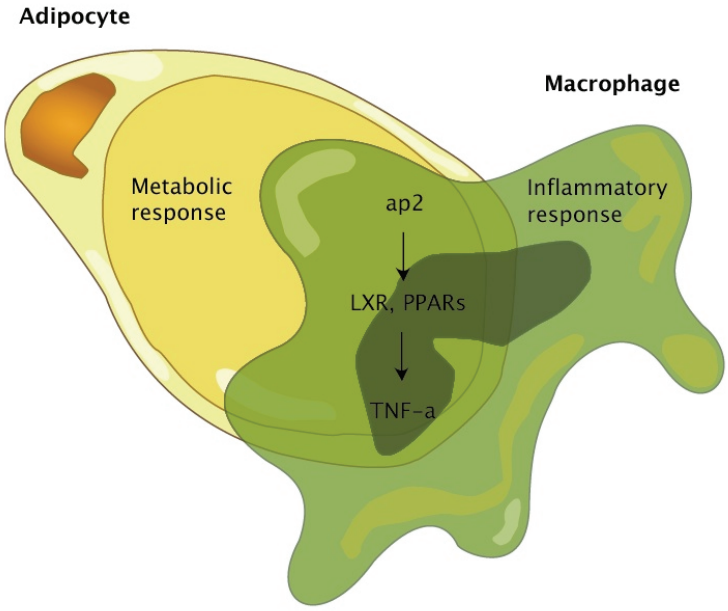

Figure 4. *Integration of metabolic and immune responses in adipocytes and macrophages. Under normal conditions, adipocytes store lipids in the form of triacylglycerol and are responsible for metabolic homeostasis, while macrophages function as pathogen sensors and play an important role in the initiation of inflammatory responses. Each of these cells has the capacity to perform both functions. In obesity, adipose tissue becomes inflamed, both via infiltration of adipose tissue by macrophages and as a result of adipocytes themselves becoming producers of inflammatory cytokines. Adipocytes and macrophages share common features such as the expression of cytokines, fatty acid binding proteins, nuclear hormone receptors, and many other factors (Figure adapted from [151]).aP2-adipocyte fatty acid binding protein; LXR-liver x receptor; PPAR-peroxisome proliferator-activated receptor; TNF-tumor necrosis factor.*

5.5. CONSEQUENCES OF EXCESSIVE ADIPOSE TISSUE GROWTH AND LIPID STORAGE

Although the main function of adipocytes is to store energy and absorb lipids readily, the morphological changes associated with adipose tissue growth are not without consequences for the organism as a whole [152]. When the buffering capacity of mammalian adipose tissue is overwhelmed, lipids are accumulated in other tissues, and other organs become a “sink” for

lipids [153]. This phenomenon, also known as “lipotoxicity”, may lead to dysfunction of the recipient sites, contributing to pathology in, for example, the liver, muscles and heart. Although the factors explaining obesity and their relationship with related diseases have been well-described in mammals, little is known about this relationship in salmonids.

Usually the hepatic lipid content is 5% of fat by weight in mammals. The liver lipid stores exceed this value in mammalian obesity, to give a condition known as “fatty liver” or “liver steatosis” [154;155]. In addition, there is increasing evidence that ectopic fat accumulation inside the heart plays a role in cardiomyopathy, and eventually heart failure in mammals [156]. Very little is known of how obesity affects the development of coronary arteriosclerosis in fish. It was, however, recently shown that atherosclerotic plaques containing foam cells with lipid accumulations appear in the heart of Atlantic salmon [157], indicating that coronary arteriosclerosis may be a factor that causing heart disease also in fish. Farrell [158], however, demonstrated that increased growth and growth rate are associated with coronary arteriosclerosis in Atlantic salmon. Recently, it was shown that approximately 40% of fish from 2,700 farmed salmon (291 families) suffered from inflammation of the heart (epicarditis); 90% had epicardial fat, of which 40% had a considerable amount of fat [159]. It remains to determine whether excess visceral adipose tissue in fish is linked to the “metabolic syndrome” as is the case in mammals [160-162].

The capacity of adipose tissue to expand is critical for accommodating changes in the availability of energy, but this capacity is not unlimited and probably varies between individuals. As already mentioned, it has been proposed that excess lipid accumulation creates in mammals a variety of stresses on adipose tissue [71].

In addition, obesity promotes the apoptosis of metabolically dysregulated adipocytes [163-165]. Adipocyte death promotes macrophage recruitment, accumulation, and persistence in the visceral adipose tissue of obese individuals. The mechanism(s) by which hypertrophy promotes adipocyte death is unclear, but cell stress may be involved [166-169]. It would be interesting to study macrophage recruitment and function in the visceral adipose tissue of fish and to elucidate the underlying causes of obesity-associated visceral adipose tissue inflammation.

The capacity of adipose tissue to store significant amounts of TAGs depends on the creation of new adipocytes or on increasing the size of existing adipocytes. Paracrine factors released by enlarged adipocytes may induce neighbouring preadipocytes to grow and differentiate, bolstering the adipose tissue energy reservoir. The capacity of adipose tissue to expand is critical for accommodating changes in the availability of energy. This capacity, however, is not unlimited

and probably varies between individuals [139;170]. An energy deficit stimulates the release of FFAs from adipocytes via lipolysis, generating smaller adipocytes. Although it was believed until recently that weight loss caused a decrease in cell size only, it is now recognized that the mass of adipose tissue can also shrink due to a loss of cells through apoptosis (Articles II, IV).

Apoptosis, the regulated cell death programme, is a complicated process that occurs during both normal and pathological conditions [171]. The morphological features of apoptosis include chromatin condensation, cell shrinkage and membrane blebbing, while the biochemical features include DNA fragmentation and condensation, the activation of a cascade of proteases, protein cleavage at specific locations, changes in the phospholipid bilayers of cell membranes, and increased mitochondrial membrane permeability [172].

Apoptosis in the cell occurs through two main pathways: internal signals (occurring inside the cells—for example, DNA damage), and external signals (from the cell surface—for example, tumour necrosis factor α). Internal signals are transmitted via intrinsic or mitochondrial pathways that lead to the release of cytochrome c from the mitochondria when stimulated. Once released into the cytosol, cytochrome c interacts with apoptotic protease-activating factor-1, leading to the activation of caspase 9 proenzymes. Active caspase 9 then activates caspase 3, which subsequently activates the rest of the caspase cascade and leads to apoptosis [173].

External signals are transmitted via extrinsic or cytoplasmic pathways that involve the cell surface death receptors (such as the tumour necrosis factor receptor) with their respective ligands. These receptors activate membrane-proximal caspases (caspase 8 and caspase 10), which, in turn, cleave and activate effector caspases such as caspase 3 and caspase 7, leading to apoptosis [173].

Thus both intrinsic and extrinsic pathways converge to a final common pathway involving the activation caspases.

The third pathway is the activation of a mitochondrial intermembrane protein called “apoptosis-inducing factor”. Apoptosis-inducing factor is the first mitochondrial protein shown to induce apoptosis independently of caspases [174-176]. When cells are induced to undergo apoptosis, apoptosis-inducing factor translocates from the mitochondrial intermembrane space to the cytosol, and to the nucleus. Mitochondria probably release death triggers that are specific for both caspase-dependent and caspase-independent apoptotic pathways (cytochrome c and apoptosis-inducing factor respectively) in response to apoptotic stimuli.

5.5.1. Inflammatory stresses

Excess adipose tissue in mammals, which occurs in obesity, is associated with a state of chronic, low-grade inflammation [177;178]. Several lines of evidence have accumulated that show that adipose tissue function is compromised in response to adipocyte hypertrophy during the development of obesity. The dysregulated production of pro-inflammatory and anti-inflammatory adipocytokines that is seen in visceral adipose tissue obesity is associated with the metabolic syndrome [179]. These observations suggest that inflammatory changes within mammalian adipose tissue may contribute critically to the development of many aspects of the metabolic syndrome including the development of heart disease, diabetes, cancer, and premature death [180-183]. It is still unknown if this is also the case in fish.

As mammals grow fatter, the visceral adipose tissue releases more and more inflammatory molecules [163]. Adipose tissue of the obese expresses greater amounts of proinflammatory proteins such as tumour necrosis factor α, interleukin 6, transforming growth factor beta 1, and monocyte chemotactic protein-1 than the adipose tissue of lean individuals [147;184-188]. Adipocytes have been blamed for producing these molecules, but recent results have revealed a strong cross-talk between adipocytes and macrophages.

Macrophages infiltrate the adipose tissue in obese humans and rodents, and their accumulation is a critical component of the development of obesity-induced inflammation. Weisberg et al. [163] showed that the numbers of macrophages present in visceral adipose tissue are directly correlated with the adipocyte size (Figure 5). Furthermore, cytokines from both adipocytes and macrophages may together play a role in activating various inflammatory responses that are considered to be important mediators of cardiovascular disease. Weisberg et al. and Xu et al. [163;189] have shown that the adipose tissue of obese mice and humans contains an increased number of resident macrophages and that macrophages can constitute up to 40% of the cell population within an adipose tissue depot [189]. In addition Weisberg et al. [163] also observed that macrophages surround adipocytes and contain large lipid droplets in obese animals. In contrast, macrophages in adipose tissue from lean mice are rare, and without lipids. The relationship between macrophages and adipocytes in Atlantic salmon adipose tissue has not been studied. Our transcriptome data (Article III) shows that Atlantic salmon adipocytes express many of the same inflammatory genes as macrophages.

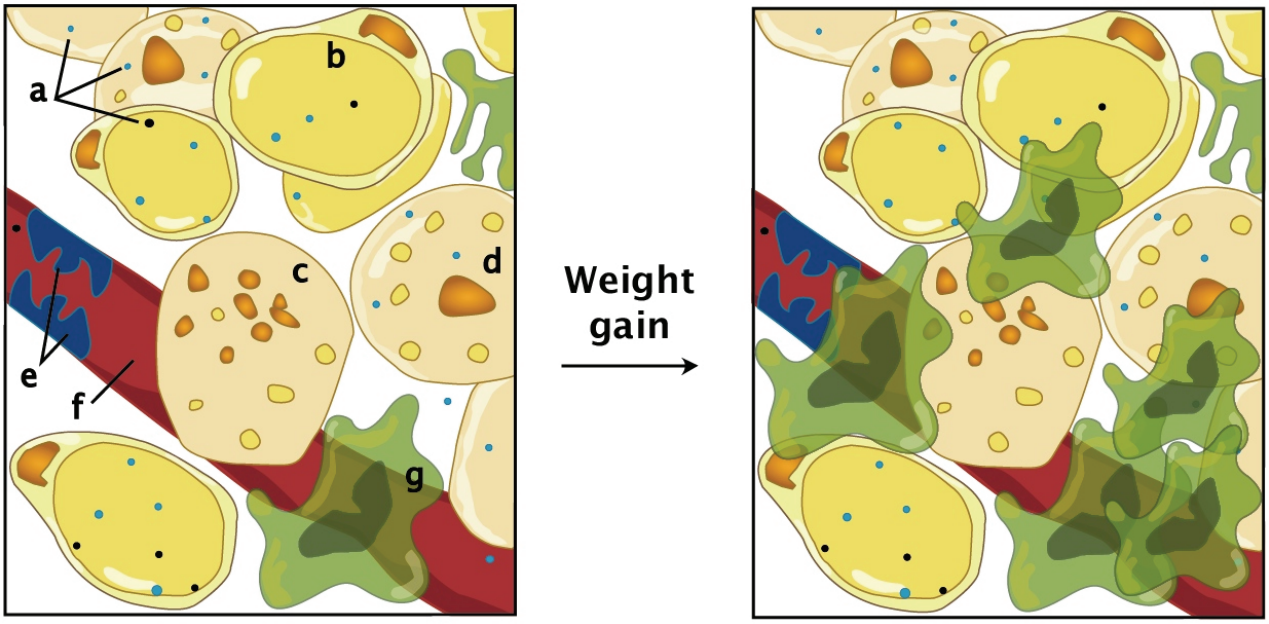

Figure 5. *A) Cross-talk among adipocytes, macrophages, and endothelial cells during adipogenesis. B) Adipose tissue expansion during weight gain leads to the recruitment of macrophages through a variety of signals. These macrophages predominantly localize around dead adipocytes, and they may aggravate the inflammatory state, resulting in increased secretion of proinflammatory cytokines/chemokines, adipokines, and angiogenic factors.*
a-adipokines; b-mature adipocyte; c-apoptotic adipocyte; d-preadipocyte; e-pericyte; f-blood vessel.

6. RESULTS AND DISCUSSION

6.1. ATLANTIC SALMON ADIPOGENESIS

When this PhD work was initiated, knowledge about the functions and the development of white adipose tissue in Atlantic salmon, and in fish in general, was very limited. Microarray (transcriptome analysis) was therefore used as a screening tool in order to gain new ideas about adipose tissue functions and development. Microarray analysis gave us the possibility to measure the expressions of a large number of genes in parallel with high accuracy. Multiple gene expression profiling with the salmonid microarray covered all the key cellular processes and has been applayed in a number of experimental setting [190-192]. The genes studied included genes that are involved in basic cellular and metabolic processes such as transcription, translation and protein folding, and energy storage and release, as well as genes governing cell shape, the structure of the extracellular matrix and immunity. The key to successful interpretation of microarray data necessitates, however, knowledge of the functions of the differentially expressed genes. The crucial step in this process is the annotation of genes. Annotation of genes is currently in progress for the two important salmonid species, rainbow trout and Atlantic salmon, and has reached a fairly satisfactory state. Functional genomics in salmonids still relies heavily on the knowledge of gene functions of their mammalian gene homologues. Hypotheses about adipose tissue development and functions arising from the interpretation of the microarray and qPCR results in this thesis are therefore based primarily on known gene functions from mammalian species. Although the majority of gene functions are well conserved between fish and mammalian species, we cannot exclude the possibility that some of the gene functions discussed in this thesis may have different functions in fish than those described for mammals.

6.1.1. Multipotency of stromal-vascular fraction of adipose tissue

Transcriptome profiling suggested that the Atlantic salmon aSVF contained not only preadipocyte precursors in various stages of differentiation but also several other cell types, including vascular cells, macrophages, leukocytes and pericytes (Article III). This is consistent with the heterogeneity shown by mammalian aSVF [193]. The transcriptome profiling indicated that the diversity of cell types in the Atlantic salmon aSVF fraction quickly decreased with time in culture as implied by the attenuated expression profiles of cell lineage markers for the osteo/chondrogenic, myogenic, immune and vasculature lineages. The expression of adipogenic markers increased with time, showing that the preadipocytes dominated more and more. This is probably also caused by high proliferation capacities of the preadipocyte precursors (Article III).

Not much is known about the first line of unspecialised precursor cells that are committed to the adipogenic lineage in fish. We observed, however, an increase of the pericytic marker transgelin at confluence and two days after hormonal addition, indicating an existence of pericytes of vasculature origin in Atlantic salmon aSVF. Accumulated evidence from mammalian species led to the hypothesis that the origin of MSCs are smooth-muscle-like pericytes that are laid over junctions of endothelial cells in blood vessel walls [25]. Once liberated from the endothelial cell, the pericytes are activated and can be considered to be MSCs [26]. However, the further conversion of a vessel-bound pericyte into a detached and proliferating adipose progenitor cell probably involves substantial changes on the transcriptional level.

The interconnected nature of stem cell differentiation pathways is probably best illustrated by the PPARγ actions. As the adipogenic master regulator, PPARγ simultaneously inhibits myogenesis, osteogenesis and chondrogenesis [194;195]. Before confluence, the expression level of PPARγ peaks, and this may limit the induction of non-adipogenic mesenchymal cell lineages in salmon aSVF (Article III). Non-adipogenic developmental pathways are also expected to be strongly suppressed upon the addition of adipogenic hormones. We observed an increased level of the anti-osteogenic growth arrest specific protein 6 one week after hormonal induction. Interestingly, growth arrest specific protein 6 plays a negative role in osteogenesis in the culture model of vascular pericytes [196].

Growth factor bone morphogenic protein 4 plays a general role in the control of MSC commitment, inhibiting myogenesis and promoting adipose, cartilage and bone lineages, depending on culture conditions [197;198]. The observed expression profile of bone morphogenic protein 4 after hormonal induction confirms its role in pro-adipogenic processes in fish (Article III). The expression of myogenic factor D, an early marker of the myogenic lineage, was somewhat surprising. It was significantly down-regulated from its level at confluence and during early stages of differentiation but its expression increased greatly in terminal differentiated cells. Myogenic factor D plays a role in the regulation of the cell cycle in terminally differentiating myocytes, and this function may be co-opted by mature adipocytes [199]. In this study, myogenic factor D induced the cyclin dependent kinase inhibitor p21 to irreversibly halt the proliferation of muscle cells.

The expression profiles of some of the developmental regulators studied emphasise that lineage determination does not traverse a simple linear pathway to end at a specific cell type; instead, aSVF possesses a multidimensional regulatory network involved in the differentiation of resident stem cells. It is evident from our results that MSCs respond to combinatorial signals during adipose lineage commitment in salmonid fish, and their final fate in the adipocyte lineage is

determined in this way. It appears that adipogenesis in Atlantic salmon involves the active suppression of at least the myogenic and osteogenic/chondrogenic pathways.

6.1.2. Regulation of cell cycle progression

Cell cycle arrest at confluence has not been studied in fish, but it is necessary for optimal adipogenesis in mammalian species [41]. Up-regulation of the gene expression of retinoblastoma-like protein 1 at confluence indicates that growth arrest is important also for Atlantic salmon adipogenesis, since retinoblastoma-like protein 1 plays a role in the stimulation of growth arrest [200;201]. Our results, however, showed relatively high expressions of both mitogenic and anti-mitogenic genes at the confluent stage, which was an unexpected finding. This seemingly contradictory result, may be explained by the fact that not all cells are growth arrested at the same time, giving simultaneous transcriptome signatures of growth arrested cells and mitotic cells. The proportion of cells in mitosis, which stain positive for proliferating cell nuclear antigen, was decreased gradually from the subconfluent stage to the terminal differentiated stage (Article III).

Our microarray results indicated that the cell cycle progression of preadipocytes is regulated by a complex network of transcription factors, as is also the case for mammalian species [202-204]. The transcription factor p53 showed increased gene expression when the cells were growth arrested by the end of terminal differentiation. This agrees with the known function of p53 as an inducer of growth arrest. Further, the expression of a number of genes regulated by p53 strengthens the hypothesis of a major role for this transcription factor in the control of cycle progression in fish adipocytes; p27, a cyclin-dependent kinase inhibitor, regulated by p53, and also growth arrest and DNA-damage inducible 45, which inhibits proliferating cell nuclear antigen, were markedly down-regulated prior to the initiation of terminal differentiation, in a period in which the cells were rapidly proliferating. Another example of the active negative regulation of the pro-mitotic signal at late stages of terminal differentiation was the up-regulation of both the retinoic acid-regulated apoptosis-related protein 3 and the cyclin D1. Yu et al. [205] demonstrated that apoptosis-related protein 3 arrests the cell cycle by suppressing the activity of cyclin D1. Collectively our gene expression results imply that the most extensive cell proliferation occurs around confluence (Article III).

6.1.3. Terminal differentiation

The phenotype of mature adipocytes was determined chemically by oil-red-O staining of lipid accumulation in the cytoplasm (Articles I, II, IV), at the morphological level by light and electron microscopy (Articles I, III, IV), and at the molecular level by observing the gene expression level of specific markers (Articles I, III, IV). The gene expression results showed that the terminal differentiation is orchestrated by a complex network of transcription factors that is known to coordinate the expressions of numerous target genes responsible for establishing the mature adipocyte phenotype [206]. Members of the PPAR and C/EBP families were expressed during the differentiation process in our cell system, which indicates that they play central roles both in cell fate determination and growth inhibition. This is the case for their mammalian homologues [207;208]. In Article I we show for the first time that PPARγ short, an alternatively spliced form of PPARγ, plays an important role in adipogenesis, whereas PPARγ long is induced in the early phase of cultivation and repressed at later stages of differentiation. The PPARγ short form lacks 34 amino acids of the A/B-domain that are present in the previously described PPARγ [209;210]. The length of the PPARγ short A/B-domain is more similar to the mammalian PPARγ1 and PPARγ2 than it is to the salmon PPARγ long. Thus, our results suggest that PPARγ short has important regulatory functions during adipocyte differentiation in Atlantic salmon. The gene expression of PPARα, another member of the PPAR family, was low at confluence, but increased almost linearly towards differentiation. Other authors [211;212] have suggested that PPARα plays a role in the differentiation of adipocytes.

The expressions of PPARγ and C/EBPα in fish seem to be orchestrated by the expressions of C/EBPδ and C/EBPβ, as is the case in mammals [21;50;213;214]. Prior to hormonal induction, cells at confluence expressed retinoblastoma-like protein 1 (a gene in the retinoblastoma family), also known as p107, at high levels (Article III). The retinoblastoma family of proteins promotes the activity of differentiation-associated transcription factors, such as C/EBPs at early stages of differentiation [215]. However, p107 was up-regulated at later stages as well. Our results contrast with other studies that show a loss of p107 in terminally differentiated adipocytes [216;217]. Our cells did not yet reach a stage where the whole cytoplasm is completely filled with lipid droplets, so the high expression of p107 may indicate that the cells have not yet reached the end of the differentiation phase.

The accumulation of lipids during the differentiation of Atlantic salmon preadipocytes was accompanied by increased mRNA expression levels of several genes involved in lipid transport, synthesis and storage. The expression of lipoprotein lipase, a marker for reaching a late stages of

adipogenesis [218;219], increased gradually during maturation into adipocytes (Articles I and III). The expression of microsomal triglyceride transfer protein, which is involved in lipid droplet formation [220], also increased during adipogenesis. Further, our gene expression results suggest that FAs are actively transported over adipocyte cell membranes as a consequence of adipose lipid accumulation. Both the levels of FATP1 (Article I) and FABP3 (Articles I and III) were up-regulated during adipocyte differentiation. Our results are in agreement with mammalian cell line studies [221]. FABP3 may have two functions with opposite effects: transport of FAs into mitochondria or peroxisomes for β-oxidation, and/or transport to the endoplasmic reticulum for TAG synthesis and storage [222;223]. Also, the expressions of genes involved in FA β-oxidation, acyl-coenzyme A dehydrogenase and acyl-coenzyme A binding protein, increased towards the end of adipogenesis (Article III). The up-regulation of the gene coding for the enzymes glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase further implied the importance of the pentose phosphate pathway, which generates the NADPH that is required for the accumulation of lipids. The pentose phosphate pathway can be seen as an alternative to glycolysis, because in addition to generating NADPH and ribose 5-phosphate, it can also provide cells with phosphorylated glycerol backbone for TAG synthesis.

6.2. INTRACELLULAR REDOX STATE AND ADIPOGENESIS

A recently emerged concept introduced by Carriere et al. [56;57] and Galinier et al. [58], is that while ROS are anti-adipogenic signalling molecules that inhibit preadipocyte proliferation and differentiation, high level of antioxidants are pro-adipogenic. These authors show that over expression of glutathione peroxidises and a high content of the hydrophilic antioxidant gluthathione are associated with pro-adipogenic processes and the promotion of obesity [58]. Consequently, adipocytes seem to engage in the suppression of oxidative pathways and the activation of ROS-scavenging pathways in order to maintain their lipid storing capacity. This assumption is supported by the concerted up-regulation of gene expression of ROS scavengers in terminal differentiating salmon adipocytes. This phenomenon can be explained by the need to maintain the highly reduced state of the intracellular environment in adipocytes dedicated to TAG synthesis (Articles III, IV). The up-regulation of glutathione peroxidases was particularly pronounced six days after the administration of lipids. Mammalian studies have shown that adipocytes in the lipid-filling phase of their terminal differentiation need glutathione-based antioxidant systems to ensure that the intracellular redox balance is maintained so that the cells are kept in a pro-adipogenic state e.g.[58]. Glutathione is an electron donor for the glutathione

peroxidase enzymes and, as such, plays a key role in protecting both mammalian [224] and fish cells [225] from oxidative stress.

The work presented in Article IV investigated how compromised ROS scavenging may exacerbate peroxidation in the terminally differentiated salmon adipocytes when high amounts of easily oxidised HUFAs are available as substrates. Buthionine sulfoximine was used to deplete stores of the intracellular antioxidant glutathione in order to exacerbate oxidative stress, while α-tochopherol was used to alleviate the effects of oxidative stress.

Signs of oxidative stress were evident in the groups that were not supplemented with α-tochopherol. Lower percentages of n-3 HUFAs and cardiolipin suggested that the oxidative stress was highest in the buthionine sulfoximine group, in agreement with the previously described negative effects of oxidative stress on the levels of n-3 HUFAs and cardiolipin in membranes [226-228]. Cardiolipin is a mitochondrial phospholipid that is particularly susceptible to oxidative damage due to its high content of HUFAs [227].

The profile of genes that encode a set of well-known apoptotic markers implied a higher incidence of cell death in the cells treated with EPA and DHA, without the addition of antioxidants. This is in agreement with the recent studies that showed increased apoptosis in several tissues of fish fed high HUFA-enriched diets [115;226] (Article II). The mRNA level of Bcl2-associated X protein was highly induced by the buthionine sulfoximine treatment. The Bcl2-associated X protein is involved in the permeabilization of mitochondria and the release of cytochrome c [229;230]. However, the expression of the apoptotic marker caspase 3 was not changed, either at the mRNA or at the protein level in our *in vitro* study (Article IV). This is the opposite of what was found in Article II, where the activity of caspase 3 was higher in fish fed very high levels of EPA and DHA, which induced oxidative stress. In Article IV, on the other hand, highly induced gene expression of the mitochondrial intermembrane space protein apoptosis-inducing factor by the buthionine sulfoximine treatment supported the hypothesis that buthionine sulfoximine stimulates caspase 3-independent apoptosis. Apoptosis-inducing factor-mediated cell death occurs in response to increased levels of lipid peroxidation in mammals [231], while the mitochondrial scavenging of ROS and a role in oxidative phosphorylation are among the suggested cell death-unrelated physiological functions of apoptosis-inducing factor [232]. Cells that had not received additional α-tocopherol contained high numbers of large-sized vesicles that resembled mammalian autophagosomes. Accumulation of autophagosomes and mitochondrial dysfunction caused by oxidative stress can trigger caspase 3-independent autophagic cell death [233;234]. Autophagy involves the highly regulated sequestration of proteins and organelles inside autophagosomes, which are then delivered to lysosomes for

degradation. Interestingly, Singh et al. [235] found that inhibition of autophagy results in an increased accumulation of TAG. High numbers of autophagosome-like structures in the control and buthionine sulfoximine-treated groups suggests the pro-death autophagy scenario and a reciprocal relationship between lipid load and autophagic function in these cells [235].

Preventing the accumulation of ROS is probably a highly prioritised task in TAGs that are loading adipocytes. The level of superoxide dismutase in glutathione-depleted cells was also elevated, which suggests that adipocytes use alternative mechanisms to combat ROS when the glutathione peroxidase system is disabled (Article IV). The activities of superoxide dismutase were also higher in fish fed very high dietary levels of n-3 HUFAs than in fish fed either fish oil or rapeseed oil diets (Article II). The activity of superoxide dismutase correlates well with the production of ROS [137]. It is now recognized that mitochondrial ROS are anti-adipogenic signalling molecules, which explains the need to down-regulate oxidative pathways in lipid-synthesising cells [56-58;60]. Conversely, the up-regulation of the FA degradation and increased production of ROS, assumed to occur at the end of differentiation, may function as a feedback mechanism to limit the deposition of TAG.

6.3. CELLULAR STRESS DURING ADIPOGENESIS

ER stress and UPR are now firmly linked to lipid overload in mammals [65]. The excess of nutrients may lead to the UPR adaptive response being activated as a feedback mechanism to limit or disable the accumulation of lipid and reduce the stress on the ER, which is engaged in lipid biosynthesis. This UPR response has not previously been described in fish, but our transcriptome profiling of Atlantic salmon white adipocytes indicates that the UPR response in fish adipocytes is activated when the cells accumulate lipids. In particular, the marked up-regulation of the gene expressions of X-box binding protein 1 and activating transcription factor 6 in the maturing white adipocytes may be considered as a hallmark of an activated UPR signalling network in fish cells, in agreement with [236]. The UPR activation in salmon adipocytes occurs during terminal differentiation, shortly after the cells become engaged in lipogenic processes. In addition, levels of these two key UPR markers (activating transcription factor 6 and glucose-regulated protein 78) were elevated in cells that had been supplied with additional α-tocopherol, and these cells also had the highest levels of accumulated lipid (Article IV). Increased lipid deposition that is occurring in groups treated with α-tocopherol is probably the trigger of the observed UPR response, in agreement with the mammalian studies discussed above (Article IV).

UPR response is a collection of pathways aimed at restoring ER function. Our results indicate that UPR is highly tailored towards the adipocyte's special needs, as is the case for mammalian species [237]. UPR activation in the fish adipocytes results in the following responses:

1) Reduced efficiency of protein translation: attenuation of general translation is a classical component of the mammalian UPR response [238]. In agreement with this, the expression of genes involved in protein biosynthetic activities was clearly down-regulated at late stages of differentiation. A consequence of the translational arrest of most proteins is the cell cycle growth arrest (growth arrest and DNA damage 45, p53, cyclin D1, cyclin A), and minimizing protein synthesis may thus serve to control the proliferation in terminally differentiating adipocytes. As already mentioned, proliferation activity is highest around confluence and decreases severely when the cells enter terminal differentiation.

2) Increase in proteolysis: the UPR in adipocytes is also thought to be important in helping the ER to recover its functions by activating protein degradation pathways [71]. Misfolded proteins are destined for degradation by the 26S proteasome or by autophagy-lysosomal digestion after their retro-translocation to the cytosol. Components of the membrane protein complexes, derlin 1 and the p97-interacting cofactor p47, which mediate retrograde transport from the ER lumen into the cytosol, are induced at the end of terminal differentiation. Other proteolytic pathways, however, are induced much earlier. UPR-induced proteolysis may be the up-stream regulatory mechanism employed to initiate the breakdown of superfluous proteins and organelles that must be replaced by more highly specialised cellular components during adipocyte development.

3) Ribosomal biogenesis: the biogenesis of ribosomes is closely connected to cell size and growth [239] and is a chief consumer of energy in cells [240], so it is logical to expect that changes in the metabolism and allocation of cellular energy resources will be reflected in the composition of ribosomes. In yeast, the ribosomal subunit ratio that favours protein production is a ratio in which the amount of the 18S component is lower than the amount of the 25S component [241]. Therefore, the down-regulation of 18S in our terminal differentiated cells may signify a switch to a more efficient production of proteins at this stage. This could increase the effectiveness of protein synthesis in fully matured adipocytes that have acquired a secretory phenotype [242]. The expressions of genes coding for the secretory cytokines, the adipokines visfatin and adipsin, increase steadily in differentiating salmon adipocytes, showing that maturing adipocytes can secrete effectively. This shows that the primary role of salmon adipocytes is the storage of TAG, as is the case also for mammalian adipocytes, while the secretion of adipokines is an important second function.

In conclusion, our results examining the expression of genes in salmon adipocytes are consistent with mammalian studies [71], and support a scenario in which the UPR is initiated as part of the normal developmental transformation of lineage-determined adipocyte precursors to mature adipocytes specialized in lipid storing and secretion.

6.4. IMMUNITY IN ADIPOGENESIS

The results presented in Article III imply that the inflammatory response in terminally differentiating adipocytes in salmon can be separated into an early phase and a late phase. The early phase culminates around a week after hormonal induction, and its predominant role may be to act as a first warning sign against the overwhelming TAG deposition. Such a warning sign should confer protection by the UPR-induced changes in gene expression, including the nuclear factor kappa-B-mediated anti-apoptotic responses [71]. Indirect evidence for this is the up-regulation of a number of genes involved in the nuclear factor kappa-B regulatory pathways (tumor necrosis factor receptor-associated factor 3, toll-like receptor 3, receptor (TNFRSF)-interacting serine-threonine kinase 1, Nedd4 family interacting protein 1 etc). Therefore, the likely cause of the induction of "warning" inflammatory genes is that they induce a protective response by the activation of catabolic mechanisms required for lypolysis to relieve the overload of the ER. Terminal differentiation is characterised by a strong induction of complement component C5, Janus kinase-1 and the regulator of eicosanoid immunity 5-lipoxygenase-activating protein, while the expression of most genes related to the immune response diminish when activator protein 1 is established, which coincides with the highest ER-stress and the highest accumulation of TAG. This profile marks the onset of the second phase of the inflammatory response and the radical countermeasures aimed at restoring perturbations of ER functions linked to surplus TAG accumulation. However, it is now also understood that the UPR in higher eukaryotes controls apoptosis in order to eliminate cells in which ER-stress cannot be resolved by the adaptive inflammatory responses [243]. Indeed, we have also observed that genes with pro-apoptotic functions are up-regulated at the latter stages (Article III).

Obesity in mammals induces a state of chronic, low-grade inflammation that is characterised by the accumulation of immune cells in white adipose tissue [163]. Most of the inflammatory mediators possess dual actions: one example is cytokines, which not only have an immunomodulatory function but also act as regulators of energy metabolism [244]. The expression profiles of four immune-related genes are similar and confirm that α-tocopherol possesses anti-inflammatory effects (Article IV). The down-regulation of interleukin 4/13α2

implies that anti-inflammatory interleukin 4 and interleukin 13 signalling is higher in the α-tocopherol groups. The expression of the pro-inflammatory nuclear factor kappa-B transcription factor is compatible with its roles as an anti-adipogenic agent and as a sensor of oxidative stress [245]. Another protein involved in the immune response is JunB, which turned out to be associated both with increased oxidative stress and with increased lipid accumulation in our cells. This was not surprising, given that JunB is a component of the activator protein 1 transcription factor, which has been implicated as a central link between ER stress and TAG overload in murine hepatocytes [65] and adipocytes in fish (Article III).

6.5. EFFECTS OF N-3 FATTY ACIDS ON ADIPOCYTES

Although no research had been performed on the effects of different FAs on fish adipose cell development prior to the work described in this thesis, it was known that n-3 HUFAs, especially EPA and DHA, prevent the development of obesity in mammals by reducing the growth of visceral adipose tissue [123;246]. The exact mechanism behind the adipose tissue lowering effect of n-3 HUFAs is not yet known, but there are likely to be several processes involved in this phenomenon. One mechanism may be that the n-3 HUFAs inhibit the late phase of adipocyte differentiation through the suppression of some genes, such as PPARγ and C/EBPα that regulate terminal differentiation [247]. Other proposed mechanisms are that HUFAs suppress FA synthesis and/or induce mitochondrial FA β-oxidation, leading to the utilisation of stored lipids and a reduction in adipocyte size [124;248]. However, it has recently been suggested that the loss of adipose tissue may also be caused by a decrease in adipocyte number, and that this occurs by, for example, apoptosis [249] induced by oxidative stress. We have examined some of these hypotheses for the effects of EPA and DHA.

First, we performed an *in vitro* trial in which we treated adipocytes at different developmental stages with either EPA, DHA or OA (Article I). Adipocytes cultivated in n-3 HUFA enriched medium had lower TAG levels than cells cultivated in a medium supplemented with OA. Our findings are consistent with those of Kim et al. [249], who observed that HUFAs, including EPA and DHA, suppress TAG accumulation during differentiation in 3T3-L1 cells, and with results from several mammalian studies showing that high-fat diets containing n-3 HUFAs limit the hypertrophy of fat depots [121;122;249;250]. The same results were confirmed in an *in vivo* trial (Article II). Increased dietary levels of EPA and DHA in the diet resulted in lower fat percentages in the adipose tissue of Atlantic salmon (Article II).

It is known that EPA and DHA act as a mitochondrial proliferators and increase mitochondrial β-oxidation activity in fish muscle and liver cells [115;251;252]. Our results from the *in vivo* and *in*

vitro trials of adipocytes are compatible with this, and show that n-3 HUFAs induce mitochondrial FA β-oxidation in salmon adipose tissue and may thus be one factor influencing lipid storage in salmon adipose tissue. The *in vivo* trial showed that Atlantic salmon fed a fish oil diet had higher mitochondrial FA β-oxidation capacity than fish fed a rapeseed oil diet (Article II). We show that the FA β-oxidation capacity in adipocytes cultivated *in vitro* was also higher with EPA and DHA as substrates than with OA as substrate. This was particularly evident at early stages of development around confluence (Article I).

However, in contrast to the expected increase in mitochondrial FA β-oxidation capacities in the fish fed diets highly enriched with EPA and DHA, the opposite was found. There was no detectable mitochondrial β-oxidation activity recovered in adipose tissue of these groups by the method used. We concluded that while moderate levels of EPA and DHA (as were given to fish in the fish oil group) in fish diets reduce lipid accumulation by increasing the FA β- oxidation capacity in adipose tissue, the very high dietary EPA and DHA levels damaged mitochondrial membranes so that they fail to function due to oxidative damage, leading to the induction of apoptosis. This hypothesis was supported by the finding of a 1.5 to 1.6-fold higher degree of membrane disruption (measured as a lack of functional cytochrome c oxidase activity in mitochondria) (Article II). Once in the cytosol, cytochrome c sets off a cascade that results in the activation of a main apoptotic executioner, caspase 3 [253]. In agreement with this, the activity of caspase 3 was higher in fish fed high level of EPA and DHA, strongly suggesting that the high dietary levels of EPA and DHA induced oxidative stress in adipose tissue, leading to mitochondrial membrane damage and a higher incidence of the fat cell death (apoptosis). This may, in turn, be one factor causing the lower fat percentage in our trial. In agreement with our findings in adipose tissue, another study from our group showed reduced cytochrome c oxidase activity, reduced β-oxidation capacity, and increased activity of super oxidase dismutase in livers of fish fed very high dietary n-3 HUFAs levels [115]. The results from this trial strongly indicated that oxidative stress and apoptosis are indeed involved in the lipid-lowering effects of HUFAs in fish. A previous study in mice has shown that n-3 HUFAs limit diet-induced obesity by reducing the hypertrophy and hyperplasia of adipose tissue [123] and, although the authors did not directly measure apoptosis, their results suggest that EPA has an apoptotic effect. Indeed, a rise in apoptosis decreases the number of adipocytes and, consequently, adipose tissue weight in rats [254].

However, in order to understand more about how these oxidative processes are involved in the regulation of adipose tissue development in fish, we followed up these initial findings with studies of how genes that code for intracellular antioxidants and extracellular antioxidants are

involved in regulating redox processes during adipogenesis and lipid storage in Atlantic salmon adipocytes (Articles III and IV).

Both intracellular and endogenous antioxidants protect FAs against peroxidation. In our studies (Article II), very high dietary levels of n-3 HUFAs, however, led to oxidative damage in salmon adipose tissue, even though antioxidants (160 ppm tochopherol and 150 ppm butylated hydrohytoulene) had been added in the diets. Our study indicates that the endogenously added antioxidants were not sufficient to prevent oxidative stress from occurring, further showing the importance of protecting high n-3 HUFA in feed and fish, with both the right level and the proper balance between different antioxidants in order to minimize the peroxidation of FAs. However, there is limited knowledge about the proper balance between antioxidants and other ingredients in fish feed. In agreement with our study (Article II), Stephan et al. [126] showed that a fish oil-enriched diet increases in turbot the susceptibility of cells to FA peroxidation. They found also that increased levels of dietary HUFAs required increased dietary supplementation with antioxidants in order to prevent oxidative damage.

α-tocopherol is one commonly used antioxidant supplement in fish diets, but the mechanisms of its action in fish are not fully understood [137]. We added buthionine sulfoximin to fish adipocytes in order to deplete the cells of the intracellular antioxidant gluthathione and then added α-tocopherol in order to see whether it prevented oxidative stress. α-tocopherol prevented oxidative stress to a certain extent, but the fact that the levels of expression of the genes coding for glutathione peroxidase 2 and glutathione peroxidase 3 did not rise when α-tocopherol was added to the buthionine sulfoximin-treated group, indicates that α-tocopherol has antioxidant effects that are independent of glutathione (Article IV).

The two groups of cells that were treated with α-tocopherol were characterised by the highest deposition of TAG and a marked expression of the master adipogenic regulators PPARγ and C/EBPα. Thus, α-tocopherol seems to be a potent pro-adipogenic vitamin, as it was associated with the up-regulation of the central transcription factors that are essential for the terminal differentiation of adipocytes. A possible mechanism of action involves the direct stimulation of PPARγ [255]. On the other hand, lipid peroxidation in the glutathione-depleted and HUFA-treated cells was associated with increased apoptosis induced by oxidative stress and mediated via caspase 3-independent pathways (Article IV).

Our results illustrate the fact that the capacity to tolerate high HUFA levels depends on a tight balance between the factors that promote the generation of ROS and the factors that inhibit it.

7. CONCLUDING REMARKS AND FUTURE PERSPECTIVES

One of the main objectives of the present work was to gain better understanding of mechanisms regulating adipose tissue growth and development in Atlantic salmon. To the best of our knowledge, no such studies have ever previously been reported.

Transcriptome profiling showed that the mechanisms regulating the processes of new cell recruitment and maturation to lipid filled adipocytes, consists of a multidimensional regulatory network very similar to the complex "machinery" described for development of mammalian adipose tissue. Several studies indicate that nutrition during fetal or neonatal life in mammals may influence the number of adipocytes recruited and thus the risk of developing obesity and metabolic syndrome later in life. The critical time points and nutritional factors affecting fat cell recruitment, excess lipid storage and obesity related disorders in fish is not known and our results points to the need for further research in order to be able to answer these important questions.

There has been a major research focus on utilisation of plant oils in fish diets during the recent years. However, there is a lack of knowledge on how the reduction in the "healthy" n-3 FAs affects adipose tissue development. This is of particular interest since these n-3 FAs are shown to reduce obesity in mammls. Another major objective of this thesis was therefore to study whether the FAs from plant oils affect lipid deposition in visceral adipose tissue. We concluded that OA, a major FA in rapeseed oil which is commonly used in fish feed, led to a higher lipid accumulation in adipocytes than the n-3 FAs, in agreement with what is found in mammalian adipocytes. Obesity is a well-established metabolic and cardiovascular risk factor in humans and it induces adverse local and systemic effects. These effects include adipocyte intracellular lipid accumulation, ER stress and mitochondrial stress, with associated changes in adipokines production, FFAs, and inflammatory mediators. This thesis present the first reports indicating that similar adverse effects may occur in Atlantic salmon. With higher lipid accumulation, salmon adipocytes express higher levels of genes coding for inflammatory factors and endoplasmatic reticulum stress. These results strongly point to the necessity for more research in order to verify whether there is a relationship between dietary FA composition, excess visceral lipid storage and inflammatory diseases in fish.

Cellular and mitochondrial membranes in fish contain relatively high levels of n-3 FAs which are easily susceptible to lipid peroxidation. Another aim of this thesis was therefore to study how dietary n-3 FAs affects adipocytes and mitochondrial membranes. Adipose tissue have relatively low capacity to utilise FAs for energy production, however since this tissue may represent quite a large percentage of total body mass, the mitochondrial FA oxidation may still be important for determining the amount of visceral adipose tissue. We show that while moderate levels of very long chain n-3 FAs (as in fish oil) reduce lipid accumulation in adipose tissue by increasing the FA β-oxidation capacity, very high n-3 FA levels damage mitochondrial membranes so that they fail to function due to oxidative damage with the result of loss of mitochondrial FA β-oxidation capacity. Many different oil qualities, including rancid oils, are currently used in aquaculture, and oxidative stress and antioxidants have thus again become the focus of research. Our results clearly show that there is a need to increase our knowledge about suitable antioxidants in combination with different dietary oils sources in fish diets and their effect on adipose tissue development and fish heath.

This thesis further shows that the salmon body possesses numerous mechanisms to protect itself from adverse effects of excessive lipid storage. However, the evolutionary drive to preserve and store excess calories (which were essential for previously rare periods of abundant food supply), together with the modern aquaculture and high lipid diets have created the perfect environment for occurrence of obesity epidemic and its related health consequences. There is a need to create new ways to reduce excessive fat deposition in Atlantic salmon. Additional research is needed to clarify the intracellular mechanisms of adiposity and the related health consequences and, perhaps more importantly, the most effective mean to implement healthier diets both during early larval feeding as well as later in life.

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Article I

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Changes in fatty acids metabolism during differentiation of Atlantic salmon preadipocytes; Effects of n-3 and n-9 fatty acids

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ABSTRACT

Atlantic salmon (*Salmo salar*) preadipocytes, isolated from visceral adipose tissue, differentiate from an unspecialized fibroblast like cell type to mature adipocytes filled with lipid droplets in culture. The expression of the adipogenic gene markers peroxisome proliferated activated receptor (PPAR) α, lipoprotein lipase (LPL), microsomal triglyceride transfer protein (MTP), fatty acid transport protein (FATP) 1 and fatty acid binding protein (FABP) 3 increased during differentiation. In addition, we describe a novel alternatively spliced form of PPARγ (PPARγ short), the expression of which increased during differentiation. Eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA) lowered the triacylglycerol (TAG) accumulation in mature salmon adipocytes compared to oleic acid (18:1n-9, OA). This finding indicates that a reduced level of highly unsaturated n-3 fatty acids (HUFAs) in fish diets, when the traditional marine oil is exchanged for n-9 fatty acids (FAs) rich vegetable oils (VOs), may influence visceral fat deposition in salmonids. Moreover, major differences in the metabolism of EPA, DHA and OA at different stages during differentiation of adipocytes occur. Most of the EPA and DHA were oxidized in preadipocytes, while they were mainly stored in TAGs in mature adipocytes in contrast to OA which was primarily stored in TAGs at all stages of differentiation.

1. Introduction

The use of high-energy feeds has been of major importance for the development of cost-effective fish farming. By increasing the energy levels in salmonid diets, growth and feed utilisation are improved [1]. However, increased dietary energy also increases fat deposition in the fish's fat storage organs and thereby reduces harvest yields [2]. It is of major importance to develop strategies to prevent excessive fat deposition in cultivated fish in order to strengthen the sustainability of the aquaculture industry. An improved knowledge of the underlying molecular events that regulate the differentiation process of preadipocytes to adipocytes in Atlantic salmon (*Salmo salar*) may open new avenues for the prevention of excessive storage of lipids in this important aquaculture species.

The primary sites for triacylglycerol (TAG) deposition in Atlantic salmon are the visceral adipose tissue [3–5] and myosepta surrounding the muscle [6,7]. Adiposity may arise from both an increased size of individual adipose cells due to lipid accumulation, and from an increased number of adipocytes arising from the proliferation of precursor cells. It has been suggested that in fish, as in mammals, this process occurs not only during early life stages [8] but also throughout life [9]. When energy intake is excessive, both the number and size of fish adipocytes increase [9,10]. The underlying molecular processes that control adipocyte differentiation in fish are poorly known. We have, however, previously shown that primary preadipocytes from Atlantic salmon differentiate to mature adipocytes in vitro and that these cells may be used as a model system for studies of adipose tissue development in fish [11]. In contrast to fish, the differentiation process in several mammalian species has been relatively well described: it is regulated by a complex network of molecular events controlled by signalling from hormones, growth factors and components of the extracellular matrix. The transcription factors peroxisome proliferator activated receptor gamma (PPAR) γ and CCAAT binding proteins (C/EBPs), are key regulators involved in initiating differentiation and inducing the expression of adipose-associated genes during differentiation [12,13].

Nutritional studies in humans and rats have demonstrated that energy balance and body fat content can be affected by changing the dietary long-chain polyunsaturated fatty acid (PUFA) level [14–16]. Diets enriched in n-3 PUFAs decrease adipose tissue mass and

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suppress the development of obesity in rats [17]. De Vos et al. [18] demonstrated that n-3 PUFAs limit the development of visceral adipose tissue by suppressing the late phase of adipocyte differentiation through modifications of PPARγ. Fish oil (FO), being a very rich source of n-3 highly unsaturated fatty acids (HUFAs), has been traditionally used as the dominating lipid component in feed for salmonids. Due to a general shortage of marine feed sources, vegetable oils (VOs) are being included to an ever-increasing degree in Atlantic salmon diets. However, to date it is more or less unknown how changing from dietary FOs to VOs affects visceral fat deposition in Atlantic salmon. In order to avoid an increase in the amount of visceral adipose tissue when replacing a diet rich in n-3 HUFAs with one rich in 18-carbon fatty acids (FAs) from VOs, the different dietary FAs from VOs should preferably be easily β-oxidized rather than being primarily deposited. The role of n-3 HUFAs in promoting FA oxidation and repressing lipid deposition has not been reported in fish so far. This study was therefore conducted in order to investigate firstly, the molecular events regulating adipocyte differentiation in Atlantic salmon; secondly, how oleic acid (18:1n-9, OA), a FA highly present in VOs, affects preadipocyte differentiation, cell morphology, FA deposition and utilisation in comparison to two typical marine FAs, namely eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA); and thirdly, how different FAs are deposited and utilised at different stages of adipocyte differentiation.

2. Materials

Atlantic salmon were obtained from AKVAFORSK (Averøy, Norway). Fetal bovine serum (FBS), essential FA free bovine serum albumin (BSA), antibiotics (mixture of pencillin, streptomycin and amphotericin B), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), L-glutamin, lipid mixture, laminin, Thermanox cover slips, Hank's balanced salt solution (HBSS), oil red O, phosphate buffered saline solution (PBS), Leibowitz-15 (L-15), albumin-solution, diethylether, 2′,7′dichlorofluorescein, petroleum ether, formalin, dexamethasone, biotin, triiodothyronine, panthothenate, isobutylmethylxanthine, non radiolabelled FAs and butylated hydroxytoluene (BHT) were all supplied from Sigma-Aldrich (St. Louis, MO, USA). Insulin (recombinat human Z) was obtained from Invitrogen (Carlsbad, CA, USA). Metacain (MS-222) was obtained from Norsk Medisinaldepot (Oslo, Norway). Collagenase (type I, 220 U/mg) was obtained from Worthington (Lakewood, NJ, USA). Chloroform and phenylethylamine were from Prolabo (Paris, France). Sodium chloride, sodium hydroxide, methanol, ammonium dihydrogenphosphate, perchloric acid, acetonitrile, hexane, acetic acid and thin layer chromatography (TLC) plates (silica gel 60) were all purchased from Merck (Darmstadt, Germany). Whatman filter paper was obtained from Schleicher & Schuell (Dassel, Germany). Nylon filters 250/100 were obtained from Sefar AG (Heiden, Switzerland). RNeasy Mini Kit, QIAshredder columns and RNase-free DNase I were purchased from Qiagen (Valencia, CA, USA). TaqMan® Gold RT-PCR Kit was bought from Applied Biosystems (Foster City, CA, USA).The radiollabeled FAs [1-^{14}C] 18:1n-9, [1-^{14}C] 20:5n-3 and [1-^{14}C] 22:6n-3 (50 mCi/mmol) were obtained from American Radiolabeled Chemicals, Inc. (St.Louis, MO, USA). Cells in culture were observed using a Diaphot inverted light microscope Nikon (Tokyo, Japan). Scinitilant InstaGel II Plus and the dual channel liquid scintillation counter Model 1900 TR TRI-CARB Liquid Scintillation Analyzer were obtained from Packard Instrument (Downers Grove, IL, USA).

Radioactive detector A-100 was obtained from Radiomatic Instrument & Chemicals (Tampa, FL, USA). Tissue culture plastic ware was obtained from NalgeNunc International (Naperville, IL, USA). Glutaraldehyde, osmium tetraoxide, epon resin, copper grids and lead citrate were supplied by Electron Microscopy Sciences (Fort Washington, PA, USA).

3. Methods

3.1. Preadipocyte isolation and culture conditions

Atlantic salmon were reared in sea water (average temperature 12–13 °C) on a commercial diet to an average weight of 2–3 kg. Random fish were sampled and anaesthetized in metacain. After the anaesthesia, arch bows of the gills were cut. After bleeding for a couple of minutes, the fish were killed by a blow to the head and the abdomen was cut open to expose the visceral adipose depot. Visceral fat was carefully excised in order to avoid contamination with intestinal contents. Salmon preadipocytes were mainly isolated as described by Vegusdal et al. [11]. Briefly, the dissected fat tissue was washed with PBS (pH 7.4) to carefully remove blood cells, then minced, and digested in 0.1% collagenase in HBSS (1 g tissue/5 ml HBSS) at 13 °C for 1 h under shaking. Subsequently, the digested tissue suspension was filtered through 250 and 100 μm nylon filters to remove large particulate material. The resulting cell suspension was then centrifuged at 700 ×g for 10 min at 10 °C. The buoyant fat layer with mature adipocytes on the top of the centrifuge tube and the digestion medium was removed by aspiration, while the preadipocytes were pelleted on the bottom. After washing twice, the cells obtained were resuspended in a growth medium containing L-15, 10% FBS, 2 mM L-glutamine, 10 mM HEPES, and antibiotics (mixture of pencillin, streptomycin and amphotericin B) and seeded on laminin coated cell-culture flasks. The adipose tissue was weighed after excision and cells were plated at a density of approximately 10 g tissue/25 cm^2. The cells were kept at 13 °C, a temperature close to the average sea-water temperature where the fish was kept prior to isolation of cells. The medium was changed every three days. The cells reached confluence after approximately 1 week. Confluent preadipocytes were firstly differentiated in an initial differentiation-inducing medium containing growth medium supplemented with 1 μM dexamethasone, 33 μM biotin, 10 nM triiodothyronine, 17 μM panthothenate and 25 μM isobutylmethylxanthine, 20 μg/ml insulin and a lipid mixture (1 μl/ml; corresponding to 45 mg/ml cholesterol, 100 mg/ml cod liver oil FA (methyl esters)). After 48 h the cells were transferred to a maintenance differentiation medium containing growth medium only supplemented with 2 μl/ml of lipid mixture.

The cultured medium was changed every three days until the cells reached the final differentiation step, with morphology of mature adipocytes (day 21). During the cultivating period the differentiation and accumulation of lipids in the mature adipocytes was evaluated by morphological observations. Cells for electron microscopy studies were taken from cultures at three different differentiation stages: at confluence (day 7) and after induction of differentiation (day 9 and day 21). RNA was isolated from the cells at confluence (day 7) and after induction of differentiation (day 9 and day 21).

3.2. Preparation of adipocytes for electron microscopy

Cells were washed in 0.1 M PBS (pH 7.4), then fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at 4 °C for 24 h. The cells were then harvested, rinsed in 0.1 M cacodylate buffer and post-fixed for 60 min in 2% osmium tetroxide containing 1.5% potassium ferrocyanide, followed by en bloc staining with 1.5% uranyl acetate. Cells were dehydrated in a series of ethanol solutions (70%, 90%, 96%, and 100%) and propylene oxide, and then embedded in epon resin, which was polymerized at 60 °C for 24 h. Ultrathin sections (approximately 50 nm) were cut on a Reichert Ultracut E ultramicrotome using a diamond knife. The sections were placed onto formvar/carbon-coated 75-mesh copper grids, post-stained for 2 min with 0.2% lead citrate solution in 0.1 M sodium hydroxide, and examined in a Philips CM 100 transmission electron microscope at an accelerating voltage of 80 kV.

3.3. RNA extraction and cDNA synthesis from salmon adipocytes

Adipocytes for gene expression studies were thoroughly washed in PBS. Adipocytes from six cell flasks were pooled prior to RNA purification. Total RNA was extracted by using RNeasy® Mini Kit, according to the manufacturer's instruction. RNA was treated with RNase-free DNase I to remove any contaminating DNA. All RNA samples used in our experiments had A260/280 ratios between 1.80 and 2.30. The total RNA concentration was determined at 260 nm using spectrophotometry.

Approximately 2 μg of total RNA was reverse-transcribed into cDNA, using TaqMan® Gold real time quantitative polymerase chain reaction (qPCR) Kit, a 100 μl reaction system. All experiments were done in accordance with the protocol: 1 μg of total RNA was used in a 50 μl reaction with a final concentration of 1× TaqMan RT buffer, 5.5 mM magnesium chloride, 500 μM of each dNTP, 2.5 μM oligo d(t)$_{16}$, 0.4 U/μl RNase inhibitor, and 1.25 U/μl Reverse Transcriptase. Then the cDNA synthesis was performed with 10 min primer incubation at 25 °C, 60 min RT step at 48 °C,

and 5 min RT inactivation at 95 °C. The reverse transcription products (cDNA) were stored at −20 °C for qPCR of the target genes.

Analyses were duplicated in the RT step, as it previously has been demonstrated that experimental accuracy is improved by running samples in (at least) duplicate starting with the reverse transcription reaction [19].

3.4. Sequence information and primer design

Primers (Table l) for the qPCR analysis were designed using the Primer Express® Software v2.0 (ABI) and ordered from Invitrogen (Carlsbad, CA, USA), ProOligo (Paris, France) and Applied Biosystems (Foster City, CA, USA). They were designed based on either available salmonid sequences in GenBank®, or by Blast searches with genes from related species in the GRASP EST database [20].

3.5. PPARγ short and PPARγ long

PPARγ short represents an alternatively spliced form of PPARγ that lacks the first 102 nucleotides of exon 3 (nt. 3255–3356) compared to the previously reported variant (acc. no. AJ416952). PPARγ short was amplified by reverse transcription polymerase chain reaction (RT-PCR) using cDNA from adipose tissue as a template, with primers in exon 2 (forward) and exon 4 (reverse) of PPARγ. The two resulting PCR-fragments were further identified by DNA-sequencing (data not shown). The full length cDNA of both PPARγ transcripts were separately cloned and served as positive controls in qPCR analysis. Primers for qPCR were designed in order to specificly amplify each PPARγ-variant. The forward primer of PPARγ short consists of the last 11 nucleotides of exon 2 and the first 9 nucleotides after the deleted region of exon 3 (nt. 3357–3367).

3.6. qPCR, TaqMan

qPCR for the genes encoding fatty acid transport protein (FATP) 1, carnitin palmitoyl transferase (CPT) II, acyl-CoA dehydrogenase (ACD), fatty acid binding protein (FABP) 3 was performed using a modified TaqMan reverse transcription protocol Applied Biosystems (Foster City, CA, USA), before subjecting the sample to qPCR (TaqMan universal PCR master mix, Applied Biosystems, Foster City, CA, USA). qPCR efficiency was monitored using two fold dilution curves of RNA. The qPCR primers and TaqManMGB probes were designed based on published sequences from Atlantic salmon or related species. The forward and reverse primers and probes were as listed in Table 1. Four concentrations were used (from 250 ng total RNA) for the two fold dilution curve. Three parallels (separate 96 well plates) were used at a total RNA concentration of 125 ng (±5%) for analysis of gene expression. The following conditions were used for amplification of cDNA: 2 min at 50 °C followed by denaturation for 10 min at 95 °C followed by 50 cycles of 15 s at 95 °C and 1 min at the annealing temperature listed in Table 1. Thermal cycling and fluorescence detection was done using ABI Prism 7000 Sequence Detection System Applied Biosystems, (Foster City, CA, USA).

3.7. qPCR, SYBR® Green

qPCR for the genes encoding PPARα, PPARγ short, PPARγ long, microsomal triglyceride transfer protein (MTP), scavenger receptor class B type I (SR-BI) type I and lipoprotein lipase (LPL) was performed with the ABI Prism® 7000 system and gene-specific primers. A 2× SYBR® Green PCR Mastermix (ABI), 0.4 μM of each primer, and the cDNA template were mixed in 25 μl volumes. Standard curves for each primer pair were made from serial dilutions of either cDNA or plasmids, and PCR efficiencies (E) were calculated from these according to the formula $E = 10^{(-1/\text{slope})}$ [21]. A two step PCR was run for 40 cycles (15 s at 95 °C, 1 min at 60 °C) with an initial incubation of 2 min at 50 °C and 10 min at 95 °C to activate the hot start AmpliTaq Gold® DNA Polymerase. All amplicons were initially separated by agarose gel electrophoresis to ensure that they were of correct size. A dissociation curve was included in the PCR program to make sure that specific products were obtained in each run.

3.8. Data analyses of gene expression

Relative quantities of gene transcription levels in the cell samples for MTP, LPL, SR-BI, PPARγ short, PPARγ long and PPARα were calculated by the $2^{-\Delta\Delta Ct}$ method adjusted for PCR efficiency. Relative expression was determined using the Relative Expression Software Tool

Table 1

Target gene	Forward primer (5′-3′)	Reverse primer (5′-3′)	Amplicon size (bp)	GenBank accession no	Primer efficiency	Annealing temperature	MGB probe
MTP	CAAAGACCAGCGTCAACAACAA	CGCCTCTGTCTCAAAGCTCACT	107	CA042356.1	2.07		
PPARα	TCCTGGTGGCCTACGGATC	CGTTGAATTTCATGGCGAACT	111	DQ294237	1.99		
PPARγ long*	CATTGTCAGCCTGTCCAGAC	TTGCAGCCCTCACAGACATG	144	AJ416951	2.04		
PPARγ short**	CGTGTATCAAGACGCCAGCT	TTGCAGCCCTCACAGACATG	101	EU655708	2.0		
SR-BI	AACTCAGAGAAGAGGCCAAACTTG	TGCGGCGGTGATGATG	204	DQ266043	1.99		
LPL	TGCTGGTAGCGGAGAAAGACAT	CTGACCACCAGGAAGACACCAT	114	BI468076	2.0		
EF1A (ref gene)	CACCACCGGCCATCTGATCTACAA	TCAGCAGCCTCCTTCTCGAACTTC	77	AF321836	1.97		
18S (ref gene)	TGTGCCGCTAGAGGTGAAATT	GCAAATGCTTTCGCTTTCG	61	AJ427629	2.0		
FATP1	TGGGAGCTTGTGGGTTCAA	ACTTTCATGAGGCGGATTGG	64	CA373015/ AF023258		58	ATCCTGCCCAATGTGT
CPT-II	TGCTCAGCTAGCGTTCCATATG	AGTGCTGCAGGACTCGTATGTG	75	BG934647		54	AGGCAGTATGGGCAGACA
ACD	GCCAAGTACTGGGCGTCTGA	TGGGCTGGATACGGGAATC	127	CB511813		54	AGTACCCCATCGCCAAG
FABP3	CACCGCTGACGACAGGAAA	TGCACGTGAACCATCTTACCA	66	AY509548		60	TCAAGTCCCTAATAACG
β-actin (ref gene)	CCAAAGCCAACAGGGAGAAG	AGGGACAACACTGCCTGGAT	91	BG933897		60	TGACCCAGATCATGTTT
EF1AB (ref gene)	TGCCCCTCCAGGATGTCTAC	CACGGCCCACAGGTACTG	59	BG933853		60	CCAATACCGCCGATTTT

Microsomal triglyceride transfer protein (MTP), peroxisome proliferator-activated receptor gamma (PPARγ) long and short, peroxisome proliferator-activated receptor alpha (PPARα), scavenger receptor class B type I (SR-BI),lipoprotein lipase (LPL),elongation factor 1A (EF1A).

* refers to the previously reported PPARγ (AJ416951).

** refers to an alternatively spliced PPARγ EU655708.

Fatty acid transport protein 1 (FATP1), carnitin palmitoyl transferase II (CPT-II), acyl-CoA dehydrogenase (ACD), fatty acid binding protein 3 (FABP3), elongation factor $1A_{\beta}$ ($EF1A_B$).

*TaqMan probe, not TaqMan MGB probe.

(REST-384© — version 1) [22]. A comparison of gene expression in adipocytes at confluente stage versus adipocytes treated with the differentiation coctail was performed and normalized to the two reference genes 18S rRNA and elongation factor 1 (EF1)A [23]. This analysis sets expression level (ratio between target genes and reference genes) in the confluent cells to 1, and the changes in the test group relative to this. This method calculates a normalization factor based on the geometric mean of multiple housekeeping genes (in this case 2) and thereby reduce the effect of control gene variations [24]. Significant difference from expression in confluent adipocytes was calculated by the Pair Wise Fixed Reallocation Randomization Test© (2000 randomizations) in the software. All samples were reverse transcribed and amplified in parallel, and mean values were used in REST, n=6. Probability values (P) of <0.05 were considered significant.

Validation of assays and data handling of the genes encoding FATP1, FABP3, CPT-II and ACD were according to the ABI Prism 7000 Sequence Detection System User Manual and baseline and cycle threshold (C_T) were set manually for each assay. Each assay was tested on different samples in the same plate to ensure optimal reproducibility. QGene was used for normalisation, and calculation of relative expression data [25]. QGene takes into account the PCR efficacy, calculated on the basis of dilution curves. The gene expression levels were normalised towards a reference gene. Two different reference genes were measured for adipocyte samples; β-actin, and $EF1A_B$. The reference gene stability was tested by geNorm [26], and both reference genes were found to have stability within acceptable limits, however results presented are based on normalisation against $EF1A_B$.

3.9. Incubations with radiolabelled FAs

Preadipocytes (day 7) and adipocytes (day 9 and 21) were washed with serum-free growth medium. 0.5 ml of the substrate stock solution of either [1-^{14}C] OA, [1-^{14}C] EPA or [1-^{14}C] DHA was added to 4.5 ml of incubation medium (incubation medium=growth medium without serum) in each cell flask. This gave a final FA-concentration of 600 µM (4.5 µCi per cell flask). The radiolabelled FAs were added to the medium in the form of their potassium salts bound to BSA (the molar ratio of FA to BSA was 2.7 to 1).

Before incubation, aliquots of 10 µl, 20 µl, and 30 µl of the incubation medium with radioactive FA added were transferred into three different vials for counting of total radioactivity, and the specific radioactivity (cpm/nmol FA) was calculated for each FA substrate.

The cells were incubated for 48 h at 13 °C. At the end of the incubation, the medium from each culture flask was used to analyse radiolabelled lipid classes, acid soluble oxidation products (ASPs) and amount of $^{14}CO_2$ produced. The incubation medium (100 µl) from each cell flask was used for counting of total radioactivity.

The cells in each flask were washed twice in PBS and harvested in 2 ml PBS and stored at −40 °C prior to the analysis of radiolabelled lipid classes.

3.10. Measurement of $^{14}CO_2$ from [1-^{14}C] FAs oxidation

The capacity of preadipocytes on day 7, and adipocytes on day 9 and day 21 to oxidize the different FA substrates was measured by determination of oxidation products (counting ^{14}C-labeled ASPs and the $^{14}CO_2$ formed) after incubation with radiolabelled FAs for 48 h.

FA oxidation was measured essentially as described by Christiansen et al. [27] with minor modifications. Gaseous $^{14}CO_2$ produced during the incubation was determined by transferring 1.5 ml medium to a sealed glass vial with a center well containing a Whatman filter (pore size, Æ 125 mm) paper moistened by adding 0.3 ml of phenylethylamine/methanol (1:1, v/v) (freshly made) with a 1 ml syringe end. The medium was acidified with 0.3 ml 1 M perchloric acid by injecting the acid with a syringe to cell medium. After incubation for 1 h at room temperature, the wells containing the filter papers were placed into vials and dissolved with 8 ml of liquid scintillation fluid for scintillation counting.

3.11. Analyses of ASPs

At the end of the incubation, 1 ml of the incubation medium was added to 0.5 ml ice-cold 2 M perchloric acid. After 60 min at 4 °C, the samples were centrifuged at 17 950 ×g for 10 min at 10 °C and an aliquot

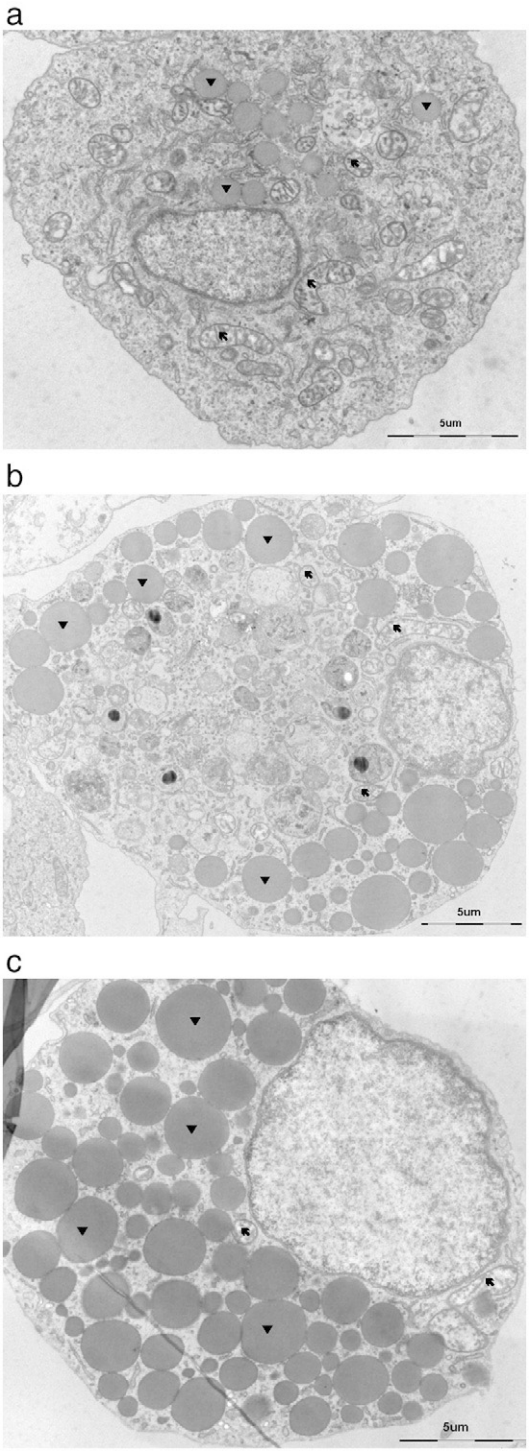
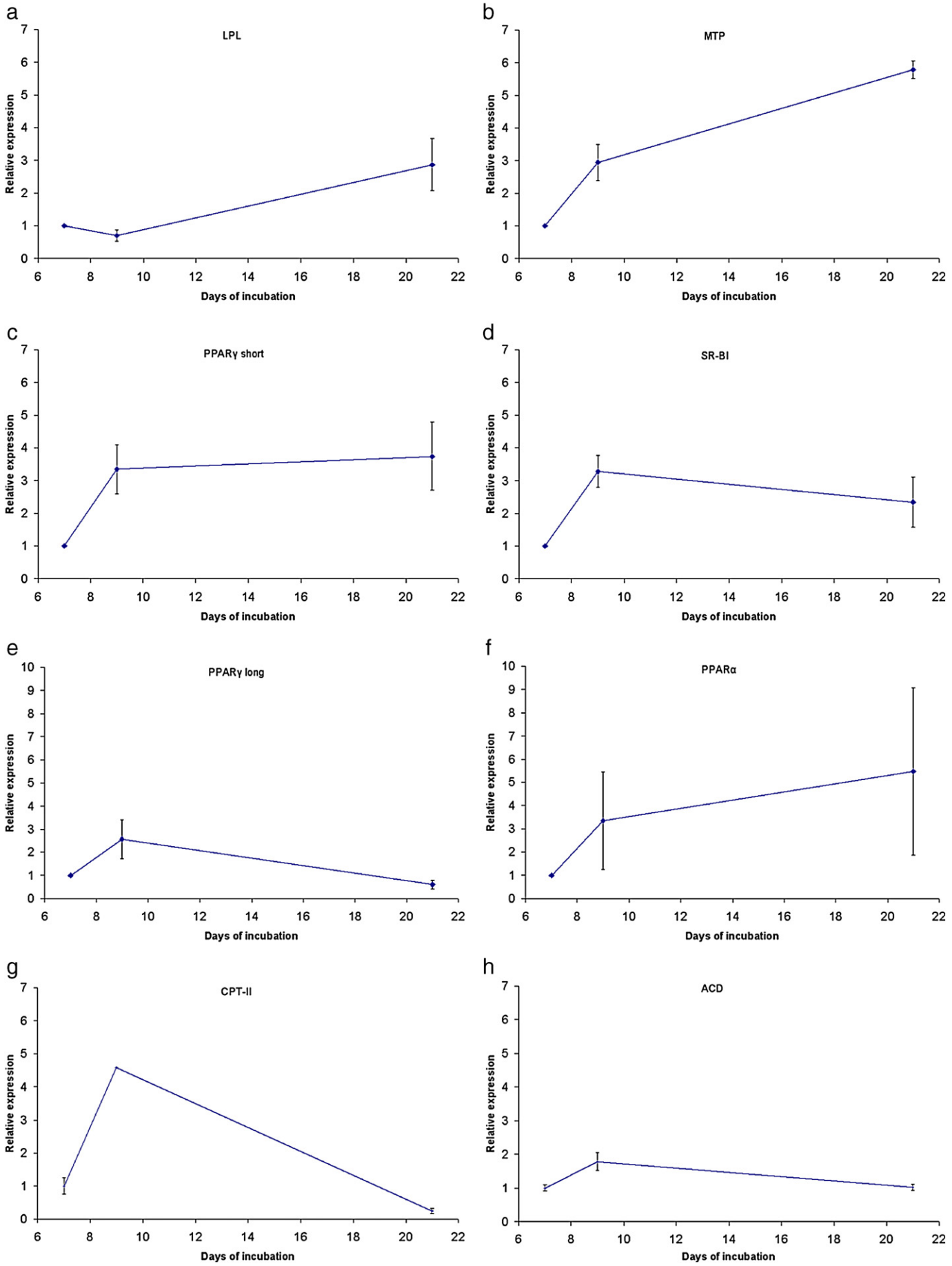


Fig. 1. Electron micrographs of salmon preadipocytes isolated from salmon visceral fat and differentiated in culture. The micrographs show representative cells for three different stages; confluence (day 7) (a); two days after induction of differentiation (day 9) (b); and two weeks after induction of differentiation (day 21) (c). Bars: A=5 µm, B and C=5 µm. Arrows points to mitochondria and arrowheads to lipid droplets.


a
LPL
b
MTP
c
PPARγ short
d
SR-BI
e
PPARγ long
f
PPARα
g
CPT-II
h
ACD
Relative expression
Days of incubation

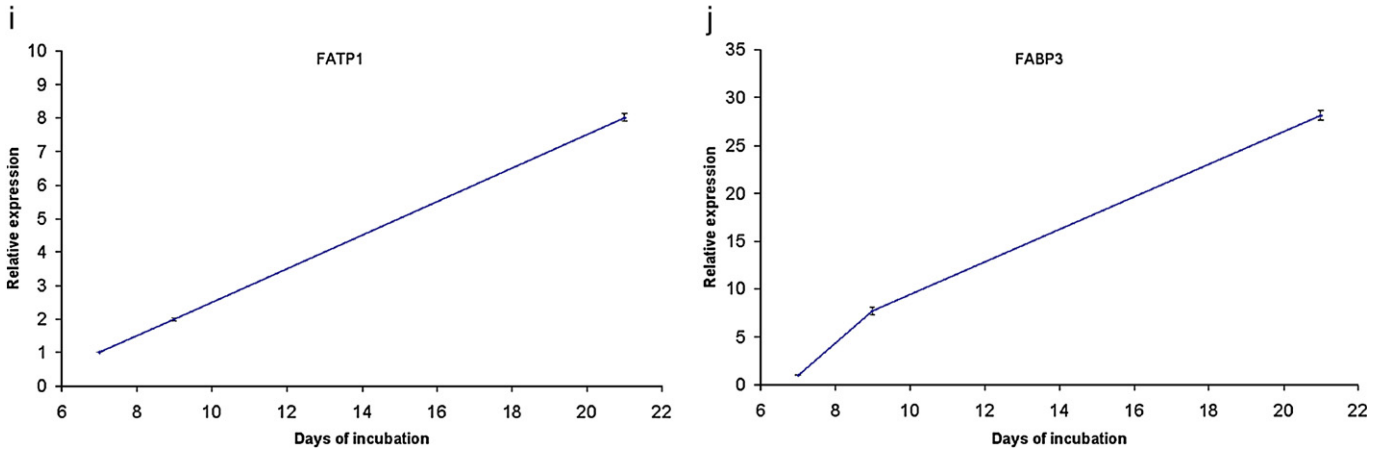


Fig. 2. Gene expression profiles of Atlantic salmon preadipocytes (day 7) and adipocytes (days 9 and 21) cultured in differentiation medium. The expression level of each gene was determined by qPCRs described in the methods section. Each data point represents the mean gene expression value from three qPCR replicates. RNA from the six cell flasks was pooled prior to analyses of gene expression, and SEM represents the variation of the measured RT level. SR-BI = scavenger receptor class B type I; PPARα = peroxisome proliferator-activated receptor alpha; LPL = lipoprotein lipase; MTP = microsomal triglyceride transfer protein; PPARγ short and long = peroxisome proliferator-activated receptor gamma; FATP1 = fatty acid transport protein 1; ACD = acyl-CoA dehydrogenase; CPT-II = carnitine palmitoyl transferase II; FABP3 = fatty acid binding protein 3.

(200 μl) of the supernatant was withdrawn for measurement of the amount of radioactivity in the ASPs. The remaining ASPs were neutralized with sodium hydroxide and the different ASPs were determined by high pressure liquid chromatography (HPLC) by using a ChromSep (250 mm × 4.6 mm stainless steel) Inertsil C8-3 column. The sample was eluted at a flow rate of 1 ml/min with 0.1 M ammonium dihydrogenphosphate adjusted with phosphoric acid to pH 2.5. Eluted components were detected by a UV-detector at 210 nm and a radioactive detector A-100 coupled in series to the UV-detector Radiomatic Instrument & Chemicals (Tampa, FL, USA). The components in the ASPs were identified by co-chromatography with standards.

3.12. Lipid extraction and analyses of lipid classes

Total lipids from preadipocytes on day 7, and adipocytes on day 9 and day 21 were extracted as described by Folch et al. [28]. The chloroform phase (2 ml) with BHT (final concentration 0.7 mg/l) was dried under nitrogen gas and the residual lipid extract was redissolved in 200 μl of hexane. The hexane phase was transferred to the TLC-plate, after which the tubes were rinsed with 100 more μl of hexane. Hexane was applied in a thin bend on the TLC-plate and dried with a hair dryer. Free fatty acid (FFA), phospholipid (PL), mono and diacylglycerol (MDG) and TAG were separated by TLC using a mixture of petroleum ether, diethyl ether and acetic acid (113:20:2 v/v/v) as the mobile phase. The plates were kept in the mixture-solution until the liquid reached 1 cm from the upper edge of the plates. The lipids were visualized by spraying the TLC-plates with 0.2% (wt/v) 2′, 7′-dichlorofluorescein in methanol and they were identified by comparison with known standards under UV-light. The spots corresponding to FFAs, PLs, MDGs and TAGs were scraped off into vials and dissolved with 8 ml of liquid scintillation fluid.

3.13. HPLC separation of FAs in total Folch extract

The compositions of the radioactive FAs of the total lipid fraction (3.8 ml) in preadipocytes on day 7, and adipocytes on day 9 and day 21 were determined by reversed phase, HPLC as described by Narce et al. [29]. The mobile phase was acetonitrile/H_2O (85:15 v/v, isocratic elution) at a flow rate of 1 ml/min at 30 °C. A reversed phase symmetry 3.5 μl C-18 HPLC column from Waters was used. The levels of radioactivity from FAs were measured in a radioactive flow detector A 100 (Packard 1900TR Tri-Carb), Radiomatic Instruments & Chemicals (Tampa, FL, USA). The absorbances of non-radioactive FAs were measured in a diode array detector (Waters 996, Photodiode Array Detector) at 215 nm. The FAs were identified by comparing the sample retention times with the retention times of FA standards. HPLC separation of radiolabeled FAs from the total lipid fraction of cells at all three stages revealed that all three FA substrates were esterified in cellular lipids as OA, EPA and DHA, respectively. Since no other peaks than the added FAs were found, these results are not shown in the result section.

3.14. Incubation with non radiolabelled FAs

Confluent preadipocytes (day 7) were differentiated for 48 h (day 9) as already described and were transferred to a maintenance differentiation medium containing growth medium supplemented with either non radiolabelled OA, EPA or DHA (FA-concentration = 600 μM) and 2 μl/ml of lipid mixture. The cultured medium was changed every three days.

The quantity of lipids in cytoplasm were estimated by oil-red O staining according to Ramirez-Zacarias et al. [30] on day 28. Briefly, after aspirating the medium gently, cells were washed twice with PBS and then fixed with 10% cold formalin for 30 min. The cells were rinsed in water and stained for 2 h with filtered oil red O in isopropanol at room temperature. For relative quantitative measurements of TAGs accumulation, bottles were washed with PBS to remove excess of stain solution, dried, the colour was dissolved in 100% isopropanol and the absorbance was measured spectrophotometrically in a Victor 3 microplate reader PerkinElmer (Wellesley, MA, USA) at 500 nm.

3.15. Statistical analyses

All data, except for gene expression data, for which REST was used, were subjected to two-way analysis of variance (ANOVA), and the differences were ranked by Duncan's multiple range test. We used software package UNISTAT (London, England). The significance level was set at 0.05.

4. Results

4.1. Morphology of preadipocytes at different stages during the differentiation process

After seeding, the isolated preadipocytes were small with a cytoplasm devoid of lipid droplets and morphologically very similar to fibroblasts. Electron microscopy revealed that preadipocytes at confluence (day 7) showed an extensive cytoplasm with nucleus located in a

Table 2
Relative distribution of radioactivity from [1-^{14}C] OA, [1-^{14}C] EPA and [1-^{14}C] DHA recovered in cellular lipids and oxidised products (ASPs and CO_2) in Atlantic salmon preadipocytes on day 7, and adipocytes on day 9 and day 21 (cellular lipids + oxidised lipids = 100%)

	Day 7			Day 9			Day 21		
%	OA	EPA	DHA	OA	EPA	DHA	OA	EPA	DHA
Cellular lipids	68.6±2.23^{c}	13.6±2.24^{a}	9.5±0.91^{a}	95.6±0.68^{d}	63.5±7.38^{b}	56.8±2.89^{c}	88.3±2.48^{d}	88.9±1.69^{d}	86.9±2.43^{d}
ASP	24.5±1.48^{b}	85.3±2.30^{d}	88.0±1.10^{d}	3.1±0.37^{a}	33.3±7.22^{c}	41.8±2.65^{b}	8.2±0.74^{a}	4.4±2.14^{a}	5.2±2.89^{a}
CO_2	6.9±0.81^{c}	1.1±0.07^{a}	2.5±0.20^{ab}	1.3±0.35^{ab}	3.2±0.30^{ab}	1.4±0.28^{ab}	3.5±1.78^{b}	6.7±0.54^{c}	7.9±0.45^{c}

Data are shown as mean±SEM (n=3). Different letters indicate significant differences between different diets and different stages (P≤0.05). OA=oleic acid, 18:1n-9; EPA=eicosapentaenoic acid, 20:5n-3; DHA=docosahexaenoic acid, 22:6n-3; ASPs=acid soluble oxidation products.

central region of the cell. The cells at this stage were relatively rich in mitochondria and were almost devoid of lipid droplets (Fig. 1a).

At confluence, the growth medium was replaced by a differentiation-inducing medium containing a mixture of hormones and lipids. The cells responded rapidly, becoming more rounded in shape, and many lipid droplets of different sizes appeared in the cytoplasm already after 48 h (Fig. 1b). A reduced number of mitochondria were observed at this stage compared to cells at confluence.

Two days after induction of differentiation, the culture medium was changed once again to a maintenance differentiation medium. The cells continued to accumulate lipid droplets of different sizes during the subsequent days. Two weeks after induction of differentiation (21 days in culture), the cytoplasm was almost completely filled with lipid droplets (Fig. 1c). We also observed smaller lipid droplets fusing with larger ones. The cells contained much less mitochondria than cells at earlier stages and the nucleus was "squeezed" between lipid droplets and the cell membrane (Fig. 1c). In comparison, control cells, incubated in growth medium depleted of differentiation-inducing components during the three week cultivating period, exhibited morphology more like cells at confluence, with almost no lipid droplets visible in the cytoplasm (pictures not shown).

4.2. Gene expression during differentiation

The mRNA samples from the Atlantic salmon preadipocytes on day 7, and adipocytes on day 9 and day 21 were analysed by qPCR with well-known markers of adipogenesis (Fig. 2). These data demonstrated that PPARγ short, PPARα, MTP, LPL, FATP1 and FABP3 mRNA expression markedly increased during the differentiation process. On the other hand PPARγ long, CPT-II, SR-BI and ACD were induced during the early phase of differentiation and decreased at later stages of differentiation.

4.3. FA metabolism during differentiation

Preadipocytes from three differentiation stages were incubated with either [1-^{14}C] OA, [1-^{14}C] EPA or [1-^{14}C] DHA for 48 h at 13 °C in order to study their respective capacities for FAs uptake, deposition and β-oxidation.

Table 2 shows the relative distribution of radioactivity recovered in cellular lipids and oxidation products (ASPs+CO_2) in Atlantic salmon preadipocytes on day 7, and adipocytes on day 9 and day 21. Radioactivities from the n-3 substrates, EPA and DHA, were primarily found in ASPs in preadipocytes on day 7 (86–88%), compared to approximately 25% from OA. While in adipocytes on day 9, five times more radioactivity from EPA and DHA substrates and 1.5 times more from OA were found in cellular lipids compared to day 7. In adipocytes at day 21, however, almost 90% of the radioactivity from all three substrates was recovered in cellular lipids and very little of the radioactivity was found in oxidation products (less than 15%). Our results show that the capacity to store FAs in cellular lipids increased from day 7 to day 21.

4.4. FA deposition

Fig. 3 shows the total nmol of [1-14C] OA, [1-^{14}C] EPA and [1-^{14}C] DHA substrates recovered in cellular lipids (total lipid fraction) in Atlantic salmon preadipocytes on day 7, and adipocytes on day 9 and day 21. Our results show that approximately ten times more of all three FA substrates were deposited in mature adipocytes than in preadipocytes at confluence during the 48 h incubation time. A significantly higher level of OA was found in cellular lipids than of EPA and DHA (with the lowest level) in the cells on day 9 and day 21. Oil red O results are in agreement with the metabolic results, showing that EPA and DHA led to lower TAG accumulation in mature adipocytes than compared to cells incubated with a media enriched with OA (Fig. 4).

Figs. 5a and b show the total nmol of [1-^{14}C] OA, [1-^{14}C] EPA and [1-^{14}C] DHA substrates recovered in polar lipids (PLs) and neutral lipids (MGs+DGs+TAGs) at the three stages. At confluence, the majority of the cellular EPA and DHA were found in polar lipids, with only very little recovered in neutral lipids. On the contrary, OA was primarily found in neutral lipids. At day 9, significantly more OA was incorporated in polar lipids than EPA and DHA, while equal amounts of all three FAs were found in neutral lipids. In mature adipocytes the greatest proportion of all three FAs was incorporated into neutral lipids varying from 330 nmol of [1-^{14}C] OA, 260 nmol of [1-^{14}C] EPA to around 180 nmol of [1-^{14}C] DHA. Less than 150 nmol of all three FAs were found in the polar lipid fraction at this late stage.

4.5. FA β-oxidation

The ^{14}C-labelled ASP fractions were further separated by HPLC in order to determine the metabolic fates of products from β-oxidation of FAs. Acetate was the main oxidation product from DHA, accounting for 76% of the total radioactivity in ASPs while in EPA the same product accounted for (50%). Oxaloacetate and malate were the major ASPs from OA (65%) in confluent preadipocytes (day 7). In adipocytes on day 9 and day 21, however, oxaloacetate and malate was the main oxidation product found for all three substrates. Acetate was

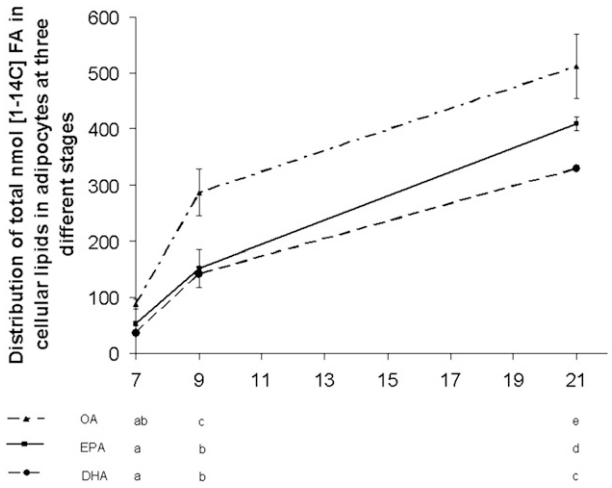


Fig. 3. Total nmol of [1-^{14}C] OA, [1-^{14}C] EPA and [1-^{14}C] DHA recovered in cellular lipids in Atlantic salmon preadipocytes on day 7, and adipocytes on day 9 and day 21. Data are shown as mean±SEM (n=3). Different letters indicate significant differences (P≤0.05). OA=oleic acid, 18:1n-9; EPA=eicosapentaenoic acid, 20:5n-3; DHA=docosahexaenoic acid, 22:6n-3.

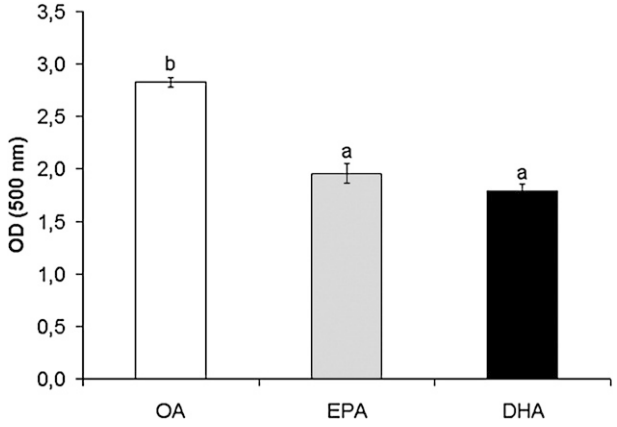


Fig. 4. Adipocytes treated with non-radiolabelled OA, EPA or DHA were fixed and stained with oil red O at day 28. The dye was extracted and absorbance measured at 500 nm. Data are shown as mean±SEM (n=3). Different letters indicate significant differences (P≤0.05). OA=oleic acid, 18:1n-9; EPA=eicosapentaenoic acid, 20:5n-3; DHA=docosahexaenoic acid, 22:6n-3.

recovered with EPA and DHA as substrates but nothing was recovered with OA as substrate (Table 3).

5. Discussion

Atlantic salmon primary preadipocytes differentiate to mature adipocytes when a growth medium containing lipid mixture as the only factor inducing differentiation is added [11]. In this study, however, we show that the hormones insulin, dexamethasone, triiodothyronine and isobutylmethylxanthine also improve the differentiation capacity of Atlantic salmon preadipocytes towards mature adipocytes. Our results agree with results from human preadipocytes [31–33].

The transcription factor PPARγ is a key regulator involved in initiating differentiation and inducing the expression of adipose-associated genes during differentiation in mammals [12,13]. We first discovered this receptor in fish liver [34], but it is not known whether it functions as a key regulator of adipocyte differentiation in fish. However, we show here that an alternatively spliced form of PPARγ exists in Atlantic salmon adipocytes (PPARγ short). This form lacks 34 amino acids of the A/B-domain that are present in the previoulsy described PPARγ [34,35]. The lenght of PPARγ short A/B-domain is more similar to the mammalian PPARγ1 and PPARγ2 than it is to the salmon PPARγ long. Our gene expression results show that PPARγ short is induced during adipocyte differentiation, whereas PPARγ long is induced in the early phase of cultivation and repressed at later stages of differentiation. Thus, our results suggest that the alternatively spliced PPARγ (PPARγ short) has important regulatory functions during adipocyte differentiation in Atlantic salmon. Further studies, however, are needed in order to verify the functions of PPARγ short in fish. The gene expression of PPARα was low in preadipocytes prior to confluence (day 7), but increased almost linearly towards differentiation (from day 9 to day 21). Previous results have suggested that PPARα plays a role in the differentiation of adipocytes [36,37].

The accumulation of lipids during the differentiation of Atlantic salmon preadipocytes was accompanied by increased mRNA expression levels of several genes involved in lipid transport, synthesis and storage. The level of LPL mRNA increased gradually during maturation into adipocytes, indicating that this receptor plays a role in controlling lipid accumulation in Atlantic salmon. This is the case in other species [38,39]. The expression of the MTP gene increased significantly during differentiation. Swift et al. [40] were the first to report MTP expression in mammalian adipose tissue, suggesting a role for MTP in lipid droplet formation. We have also confirmed that SR-BI is expressed during differentiation. The role of this receptor in adipocytes is still unclear, but a recent report shows that SR-BI displays an insulin and angiotensin II-dependent translocation to the plasma membrane in adipocytes and a concomitant decrease in circulating high-density lipoprotein [41]. Cholesterol may be important for signal transduction and TAG accumulation in adipocytes, and the role of this receptor in salmon adipocytes needs to be further elucidated.

There were major differences in the metabolism of OA, EPA and DHA between cells at each differentiation stage. In mature adipocytes, the FA substrates were mainly deposited as TAGs in cellular lipids, with only a small amount oxidized to ASP — approximately ten times more radiolabelled FAs were deposited in cellular lipids in the mature stage than at confluence. We conclude that the cells become more specialized towards lipid deposition as they mature, a conclusion that is compatible with previous mammalian studies showing that lipid accumulation in adipocytes is a marker of adipocyte maturation and the final stage of differentiation [42–46]. This conclusion is further supported by the fact that the gene expressions of typical adipogenic proteins such as LPL, FATP1, MTP and SR-BI are up-regulated. FATP1

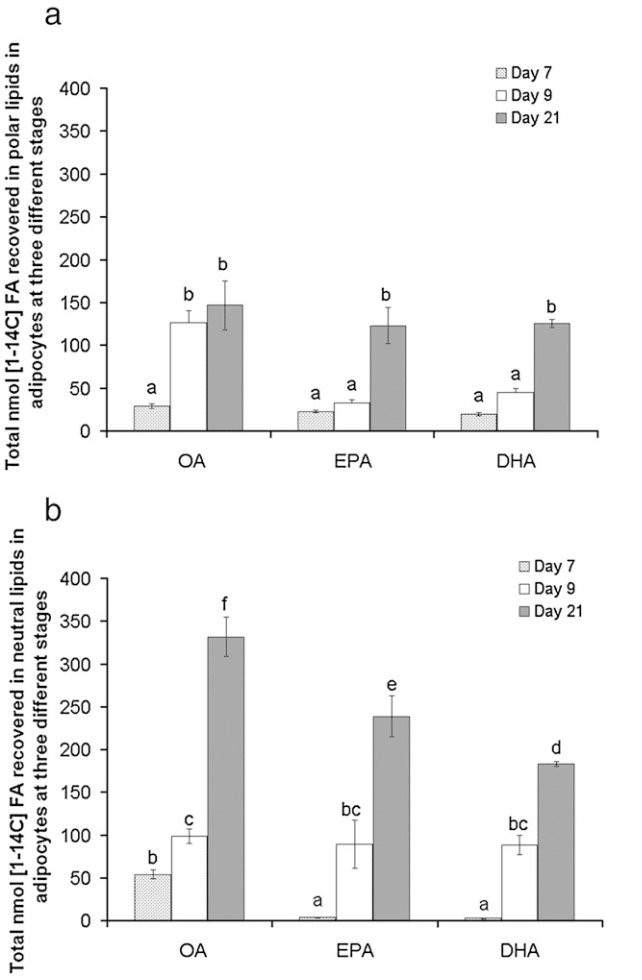


Fig. 5. a: Total nmol of [1-^{14}C] OA, [1-^{14}C] EPA and [1-^{14}C] DHA in polar lipids (PLs) in Atlantic salmon preadipocytes on day 7, and adipocytes on day 9 and day 21. Data are shown as mean±SEM (n=3). Different letters indicate significant differences (P≤0.05). OA=oleic acid, 18:1n-9; EPA=eicosapentaenoic acid, 20:5n-3; DHA=docosahexaenoic acid, 22:6n-3; PLs=phospholipids. b: Total nmol of [1-^{14}C] OA, [1-^{14}C] EPA and [1-^{14}C] DHA in neutral lipids (MDGs+TAGs) in Atlantic salmon preadipocytes on day 7, and adipocytes on day 9 and day 21. Data are shown as mean±SEM (n=3). Different letters indicate significant differences (P≤0.05). OA=oleic acid, 18:1n-9; EPA=eicosapentaenoic acid, 20:5n-3; DHA=docosahexaenoic acid, 22:6n-3; MDGs=mono and diacylglycerols; TAGs=triacylglycerols.

Table 3
Percentage distribution between oxaloacetate, malate and acetate produced from [1-^{14}C] OA, [1-^{14}C] EPA and [1-^{14}C] DHA in Atlantic salmon preadipocytes on day 7, and adipocytes on day 9 and day 21

	Day 7			Day 9			Day 21		
%	OA	EPA	DHA	OA	EPA	DHA	OA	EPA	DHA
Oxaloacetate + malate	65.3±2.98^{c}	50.7±6.20^{a}	24.1±10.60^{b}	100±0.00^{e}	89.2±4.21^{d}	70.5±8.00^{a}	100±0.00^{e}	88.8±0.48^{e}	74.3±2.89^{c}
Acetate	34.7±2.98^{cd}	49.3±6.20^{e}	75.9±10.60^{cd}	0±0.00^{a}	10.8±4.21^{bc}	29.5±8.00^{de}	0±0.00^{a}	11.2±0.48^{ab}	25.7±2.89^{cd}

Total radioactivity recovered as ASPs for each FA = 100% (oxaloacetate + malate + acetate = 100%). Different letters indicate significant differences between different diets and different stages ($P \leq 0.05$). OA = oleic acid, 18:1n-9; EPA = eicosapentaenoic acid, 20:5n-3; DHA = docosahexaenoic acid, 22:6n-3; FA = fatty acid; ASPs = acid soluble oxidation products.

actively transports long chain FAs over adipocyte cell membranes in mammals [47]. The FATP1 mRNA level was also up-regulated as a consequence of adipose conversion in the preadipocyte mammalian cell line 3T3-L1 [48], which agrees with our results. FABP3, which is abundantly expressed in Atlantic salmon red and white muscle [49], was up-regulated during adipocyte differentiation. The protein FABP3 may have two functions with opposite effects: transport of FAs into mitochondria or peroxisomes for β-oxidation, and/or transport to the endoplasmic reticulum for TAG synthesis and storage [50,51]. Furthermore, Li et al. [52] showed that FABP3 mRNA is induced during adipogenic differentiation of stromal vascular cells derived from both adipose tissue and from skeletal muscle.

Significantly more radiolabelled OA, EPA and DHA ($P \leq 0.05$ for all three FAs) were β-oxidised to ASP at confluence than at later differentiated stages, which may be explained by the higher amount of mitochondria at confluence. These results are supported by the higher mRNA expression of CPT-II and ACD, genes coding for important proteins in the mitochondrial β-oxidation pathway. Relatively more EPA and DHA than OA were oxidized to ASP at confluence — we do not know why. However, the high percentage of the peroxisomal product acetate in cells incubated with DHA, indicates a high peroxisomal β-oxidation capacity at the early stage. This agrees with the generally accepted idea that HUFAs are poor substrates for mitochondrial β-oxidation and that these FAs depend on peroxisomes for efficient chain-shortening [53]. The major oxidation products from the OA substrate in cells at confluence (day 7) were the mitochondrial products oxaloacetate and malate ($P \leq 0.05$), indicating that this FA is primarily oxidized in mitochondria.

We have also studied how the n-3 HUFAs (EPA and DHA) affect lipid accumulation in Atlantic salmon adipocytes compared to OA. Relatively more radiolabelled OA than EPA and DHA substrates were deposited in cellular TAGs in cells at all three stages, suggesting that OA leads to a higher degree of lipid filling and fat cell differentiation than EPA and DHA. Supplementation of cell medium with non-labeled OA, EPA or DHA for prolonged periods gave the same result, the oil red O staining of cellular lipid droplets showed that EPA and DHA led to lower TAG accumulation in mature adipocytes than OA. We have previously shown that diets rich in 18-carbon FAs give a higher lipid accumulation in the liver of Atlantic salmon than FO diets at low water temperatures [54]. Our findings are consistent with those of Kim et al. [55], who observed that PUFAs, including EPA and DHA, suppress TAGs accumulation during differentiation in 3T3-L1 cells. Our results agree also with several mammalian studies showing that high fat diets containing n-3 PUFAs limits the hypertrophy of fat depots compared to high-fat diets containing saturated FAs [56,57]. Similar results have been obtained with several animal models, such as obese Zucker rats [58], mice [59], obese ob/ob mice [60], hamsters [61], and with human subjects [14]. Okuno et al. [62] demonstrated that perila oil, which is rich in n-3 PUFAs, prevents the growth of visceral adipose tissue in rats by down-regulating the late phase of adipocyte differentiation.

In conclusion, the differentiation of salmon preadipocytes to mature adipocytes is regulated by a complex network of genes and hormones, more or less in the same way as the process in mammalian cells. We present here a novel alternatively spliced form of PPARγ (PPARγ short), the expression of which increased during differentiation. We suggest that this form plays an important regulatory function during adipogenesis in salmon preadipocytes.

During the differentiation process, the cells change their morphology from an unspecialised fibroblast-like cell type to a mature adipocyte filled with lipid droplets and with a significant increase in the expression of the adipogenic gene markers, LPL, MTP, FATP1 and FABP3.

Preadipocytes have a high capacity for FA β-oxidation, while the mature cells are more specialised towards lipid storage. Preadipocytes in culture given the marine FAs, EPA and DHA had lower lipid levels and appeared as a less mature adipocyte phenotype than cells given OA, indicating that it is necessary to consider the influence on visceral FA deposition when marine FAs are replaced by plant oils in salmon diets.

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N-3 HUFAs affect fat deposition, susceptibility to oxidative stress, and apoptosis in Atlantic salmon visceral adipose tissue

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ABSTRACT

We have investigated how n-3 highly unsaturated fatty acids (HUFAs) in the diet affect fatty acid (FA) utilization, fat storage and oxidative stress (OS) in Atlantic salmon (*Salmo salar*) white adipose tissue (WAT). Four groups of Atlantic salmon were fed for 21 weeks on one of the four diets supplemented with 23% (of dry matter) lipid. Docosahexaenoic acid (DHA; 22:6n-3) and eicosapentaenoic acid (EPA; 20:5n-3) levels increased from 10% of total FAs in the rapeseed oil (RO) diet, to 20% in the fish oil (FO) diet, and to 50% and 55% in the DHA-enriched and EPA-enriched diets, respectively. Increased dietary levels of n-3 HUFAs resulted in lower fat percentage in WAT. Furthermore, mitochondrial FA β-oxidation activity was higher in the FO group than it was in the RO group. The relative levels of DHA and EPA in phospholipids (PLs) from WAT and mitochondrial membranes increased with the increasing dietary levels of these HUFAs. In general, the mitochondrial membrane PLs were characterised by lower relative levels of n-3 HUFAs and higher relative levels of linoleic acid (LA; 18:2 n-6) than WAT membrane PLs. The predominance of LA relative to n-3 HUFAs in mitochondrial membrane PLs may help to protect these PLs from peroxidation. Cytochrome c oxidase measurements revealed higher incidence of disrupted mitochondrial membranes in the DHA and EPA dietary groups than in the FO and RO dietary groups. This disruption further affected the mitochondrial function, resulting in a marked reduction in FA β-oxidation capacities. The reduction in mitochondrial function and the increase in the activity of superoxide dismutase (SOD) in the DHA and EPA groups showed that high dietary dose of DHA and EPA resulted in oxidative stress (OS). The increased activity of caspase 3 in the high n-3 HUFA groups suggested the induction of apoptosis and increased incidence of cell death in WAT, which may be one of the factors explaining the lower fat percentage found in these groups.

1. Introduction

Dietary lipid levels have increased and protein concentrations have decreased in salmonid feeds in recent decades in order to provide cost-efficient growth. High-fat diets lead to increased fat deposition in fish white adipose tissue (WAT) (Stowell et al., 1992; Arzel et al., 1994; Lee et al., 2002) and this causes production losses when the fish are gutted at harvest. Fish oil (FO) rich in n-3 highly unsaturated fatty acids (HUFAs) has traditionally been used as the main lipid source in salmonid diets. The uncertain supply and variable price of FO have led to increased use of vegetable oils (VOs) in fish diets. Little is, however, known about how the reduction in dietary FOs in salmonid diets affects the WAT deposition. We have recently shown that n-3 HUFAs reduce fat cell development and lipid accumulation in salmon preadipocytes *in vitro* (Todorčević et al., 2008). Although not known for fish, n-3 HUFAs are known to suppress fatty acid (FA) synthesis, increase FA-β oxidation, and reduce triacylglycerol (TAG) synthesis in mammals (Al-Hasani et al., 2005). Madsen et al. (2005) showed that diets enriched in n-3 HUFAs decrease WAT mass and suppress the development of obesity in rats. These findings make it important to study whether FOs rich in n-3 HUFAs reduce fat deposition in fish, and if a reduction in the level of these FAs in the diet increases the amount of WAT.

The primary function of WAT is to store lipids. The lipid storage in WAT increases in times of abundant food supply and maintains in this way the healthy levels of circulating lipids. It also provides a source of energy in times of need. Constant energy excess in mammals may, however, lead to obesity and obesity related disorders (Keijer et al., 2008). Lipid overload of WAT in mammals may cause oxidative stress (OS) and changes in mitochondrial health (Keijer et al., 2008). WAT contains few mitochondria, and little is known about their role. Several observations in mammals, however, indicate that WAT mitochondria

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play an important role in the complications associated with obesity. Firstly, obese people have fewer mitochondria in their WAT (Semple et al., 2004; Dahlman et al., 2006). Similarly, in our recent study, the number of mitochondria in Atlantic salmon preadipocytes *in vitro* decreased with the increased lipid accumulation (Todorčević et al., 2008). Secondly, mammals in which the mitochondrial oxidative capacity is mildly impaired have a predisposition to obesity (Pomplun et al., 2007). Little is known for salmonids, but high levels of n-3 HUFAs in their diets, may increase susceptibility to oxidative damage of WAT mitochondria. Mammalian studies have shown that the unsaturation of cell membrane FAs increases rapidly when n-3 FAs are introduced into the diet (Barzanti et al., 1995). The high degree of unsaturation of docosahexaenoic acid (DHA; 22:6n-3) and eicosapentaenoic acid (EPA; 20:5n-3) makes these lipids highly susceptible to peroxidation (Gardner, 1989; Sugihara et al., 1994). Sugihara et al. (1994) showed that EPA is relatively easily peroxidized, whereas oleic acid (OA; 18:1n-9), a mono-unsaturated FA, much less so. Interestingly, OS may cause apoptosis in human adipocytes (Prins et al., 1994) and it has further been suggested that OS and apoptosis contribute to the body fat loss (Kim et al., 2006).

Therefore, the major aims of this study were to examine whether changing the level of n-3 HUFAs in the diet affects lipid storage, mitochondrial function, FA β-oxidation, OS and apoptosis in Atlantic salmon WAT.

2. Materials and methods

2.1. Fish and experimental design

The feeding trial was conducted at Nofima's research station, Sunndalsøra, Norway. Four extruded diets containing approximately 54% (by weight) crude protein and approximately 23% (by weight) crude lipid were manufactured with either 13.5% (by weight) FO, rapeseed oil (RO), DHA-enriched oil, or EPA-enriched oil (Table 1). Four groups of Atlantic salmon (*Salmo salar*) with a mean initial mass of 90 g were distributed into 3 cylinder-conical tanks (of diameter 0.85 m) per diet and fed one of the 4 diets for 21 weeks. The fish were not fasted prior to the final sampling. The average fish mass was 344 g at the end of the growth period and there were no significant difference in weight of the sampled fish between each dietary group. Table 2 shows the FA compositions of the diets.

Table 1
Formulation and chemical compositions of the diets (% total fatty acids)

	Diet			
	RO	FO	DHA	EPA
Formulation (g/kg)				
Fish meal, LT	679	679	679	679
Rapeseed oil	135			
Fish oil		135		
DHAa			135	
EPAb				135
Wheat	171	171	171	171
Vitamin premixc	10	10	10	10
Mineral premixc	4	4	4	4
Yttrium oxided	0.1	0.1	0.1	0.1
Carophyll Pinke (8%)	0.64	0.64	0.64	0.64
Composition				
Dry matter (%)	92.2	92.3	92.2	93.0
% of dry matter				
Crude protein	54.6	55.4	54.4	55.1
Crude fat	23.0	22.5	23.3	22.7
Ash	9.9	9.5	9.7	9.5
Gross energy (MJ/kg)	23.9	23.8	23.8	23.6

[a]Incromega DHA 500TG SR, Croda Chemicals Europe Ltd., Goole, England.
[b]Incromega EPA 500TG SR, Croda Chemicals Europe Ltd., Goole, England.
[c]As described by Mundheim et al. (2004).
[d]Inert marker, Y_2O_3, Sigma.
[e]Hoffman-LaRoche, Basel, Switzerland.
The oils used in the diets had 150 ppm butylated hydroxytoluene (BHT) added to them. RO = rapeseed oil; FO = fish oil; DHA = DHA-enriched oil and EPA = EPA-enriched oil; LT = low-temperature.

Table 2
Fatty acid compositions of diets (% total fatty acids)

	Diet			
Fatty acids	RO	FO	DHA	EPA
16:0	7.6	12.9	5.4	4.9
18:0	1.5	1.5	1.5	0.6
20:0	0.4	3.7	0.4	4.6
22:0	0.3	0.1	n.d.	0.1
24:0	0.1	n.d.	0.5	0.1
Σ Saturated	9.9	18.2	7.8	10.3
16:1 n-7	1.2	4.3	1.4	1.3
18:1 n-7	2.8	1.7	1.1	0.9
18:1 n-9	40.2	7.8	5.3	3.7
20:1 n-9	4.3	10.0	5.2	3.7
22:1 n-7	0.2	0.9	0.8	2.2
22:1 n-9	n.d.	n.d.	0.9	0.3
22:1 n-11	0.2	15.8	6.6	5.3
24:1 n-9	0.5	0.9	1.9	0.3
Σ Monounsaturated	49.4	41.4	23.2	17.7
18:2 n-6	14.1	2.7	1.6	2.2
18:3 n-4	0.1	0.2	0.4	0.3
18:3 n-6	n.d.	0.1	0.5	0.3
18:4 n-3	n.d.	0.2	0.2	0.4
20:2 n-6	0.2	0.3	0.5	0.1
20:3 n-3	n.d.	0.2	0.3	n.d.
20:3 n-6	0.2	0.4	1.3	2.4
20:4 n-3	0.5	1.0	0.7	0.7
20:4 n-6	0.1	0.0	0.3	0.1
20:5 n-3	5.0	7.1	8.7	42.9
21:5 n-3	0.7	0.1	0.1	0.5
22:2 n-6	n.d.	n.d.	n.d.	n.d.
22:5 n-3	0.3	0.8	3.3	1.6
22:5 n-6	2.6	0.4	0.5	1.3
22:6 n-3	5.0	12.2	41.7	12.4
Σ Polyunsaturated	28.8	25.7	60.1	65.2
Σ others[1]	10.4	12.2	6.4	4.7
Σ n-3	18.8	23.3	56.0	59.4
Σ n-6	17.2	4.2	4.8	6.5
20:5n-5+22:6n-3	10.0	19.3	50.4	55.3

RO = rapeseed oil diet; FO = fish oil diet; DHA = DHA enriched oil diet; EPA = EPA-enriched oil diet; n.d. = not detected.
[1]Includes: 14:0; 15:0; 17:0; 19:0; 14:1 n-5; 16:1 n-9; 17:1 n-7; 18:1 n-11; 20:1 n-7; 16:2 n-3; 16:2 n-6; 16:3 n-4; 22:4 n-6.

2.2. Final sampling

Three fish from each dietary group were anaesthetized with metacain (Norsk Medicinal Depot, Oslo, Norway) and weighed and lengths were measured. These fish were killed with a blow to the head and their abdomen was cut open. Approximately 7 g of WAT was taken from each of the 12 fish (3 from each dietary group). Two grams of this was directly frozen in liquid nitrogen, transferred to −80 °C and stored until analysis of lipid composition. The remaining 5 g were transferred to a homogenization buffer (pH 7.4) containing 15 mM Hepes (all chemicals were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA), unless otherwise stated), 0.25 mM sucrose, 1 mM ethylenediaminetetra-acetic acid (Merck, Darmstadt, Germany) and 1 mM ethyleneglycoltetraacetic acid (AppliChem, Darmstadt, Germany), prior to subcellular fractionation.

2.3. Subcellular fractionation

Subcellular fractions were separated by differential centrifugation as first described by de Duve et al. (1955). The homogenate (H) was centrifuged at 1000 g for 10 min to separate out the nuclear fraction (N). Pelletted nuclei were re-homogenized in 1 mL of buffer and centrifuged once more. The two supernatants were pooled and

transferred to a new tube and centrifuged at 2000 g for 10 min to pellet the mitochondria. The crude mitochondrial fraction was diluted in 1.5 mL of homogenization buffer. The supernatant was transferred into a new tube and centrifuged at 25 000 g for 10 min to pellet the peroxisomes. The crude peroxisomal fraction was diluted in 1.5 mL of homogenization buffer. The different fractions were frozen at −80 °C and stored until analysis of different enzyme activities, except for the mitochondrial fraction which was analyzed fresh.

Marker enzymes are discussed below in Section 2.5. Analysis of the marker enzyme activities showed that mitochondria and peroxisomes appeared in the same fraction after differential centrifugation. This fraction (which we here denote as the "M" fraction) contained peroxisomes and mitochondria, but no nuclei, lysosomes or microsomes. It is possible that the difficulties with separating mitochondria from peroxisomes were due to the high amounts of lipid in both organelles in WAT. Peroxisomes from mammalian tissues may be highly heterogeneous with respect to size, shape, lipid and protein composition (Angermuller et al., 1988; Schrader et al., 1994), and if this is also true for fish WAT, it will contribute to the difficulty of separating peroxisomes from mitochondria.

2.4. Protein measurements

Protein concentration was determined based on the method of Lowry et al. (1951) and modified by Peterson (1977), with bovine serum albumin as standard.

2.5. Marker enzymes

The separation of different organelles was followed by measuring the activities of several subcellular marker enzymes.

2.5.1. Catalase

The presence of peroxisomes was assessed by the activity of catalase, using a method based on that of Baudhuin et al. (1964). The substrate hydrogen peroxide (Riedel-de Haen, Seelze, Germany), which is produced in peroxisomes, is broken down by catalase to oxygen and water. This reaction is stopped by the addition of a saturated solution (0.45%) of titanium oxysulphate (Riedel-de Haen) in 2 N sulphuric acid (Merck). Titanium oxysulphate reacts with the remaining hydrogen peroxide to give a yellow solution of peroxy titaniumsulphate. The amount of this product was measured spectrophotometrically at 405 nm in a Victor 3 1420 Multilabel counter spectrophotometer (Perkin Elmer, Norwalk, CT, USA).

2.5.2. Cytochrome c oxidase

The distribution of mitochondrial enzymes in the different fractions was determined by measuring the activity of cytochrome c oxidase (Storrie et al., 1990). This method is based on observation of the decrease in absorbance at 550 nm of Fe^{2+}-cytochrome c caused by its oxidation to Fe^{3+}-cytochrome c by cytochrome c oxidase in mitochondria (measured at room temperature for 3 min). Further, the cytochrome c oxidase assay allowed us to measure the integrity of the outer mitochondrial membrane, by measuring the cytochrome c oxidase activity in the presence and in the absence of the detergent n-dodecyl β-D-maltoside. This detergent allows the maintenance of the cytochrome C oxidase dimer in solution. Absorbance was measured in a GBC UV/VIS 918 spectrophotometer (GBC Scientific Equipment, Victoria, Australia). Control group (FO) was set to 1 and the changes in the test groups (RO, DHA, EPA) were shown relative to this.

2.5.3. Acid phosphatase

Acid phosphatase was measured as described by Bergmeyer et al. (1974). Acid phosphatase is one of the acid hydrolases that normally reside in lysosomes, and it is used as a marker for lysosomes. The measurement is based on the hydrolysis of 4-nitrophenyl phosphate by acid phosphatase. The samples were mixed with 4-nitrophenyl phosphate and citrate buffer solution (0.09 M, pH 4.8), and incubated for 20 min at room temperature. The reaction was stopped by sodium hydroxide (0.5 N). The colour formed was measured spectrophotometrically at 405 nm in a Victor 3 1420 Multilabel counter spectrophotometer (Perkin Elmer).

2.5.4. Esterase

Esterase was first assayed by Beaufay et al. (1974). The enzyme is a marker for microsomes, and catalyzes the production of o-nitrophenol from o-nitrophenyl acetate (MP Biomedicals, Solon,

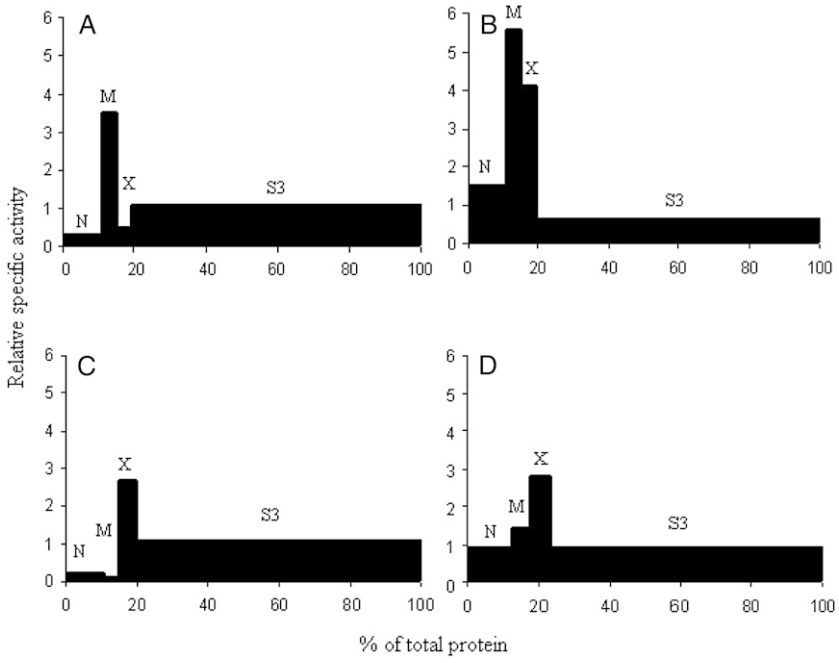

Fig. 1. (A) The relative specific activity of catalase, a marker enzyme for peroxisomes; (B) cytochrome C oxidase, a marker enzyme for mitochondria; (C) esterase, a marker enzyme for microsomes; and (D) acid phosphatase, a marker enzyme for lysosomes in the nucleus pellet (N), the mitochondrial pellet (M), the lysosomal pellet (X) and the final supernatant fraction (S3). Results are expressed as De Duve plots (De Duve et al., 1955) with bars representing the mean of three separate subcellular fraction preparations.

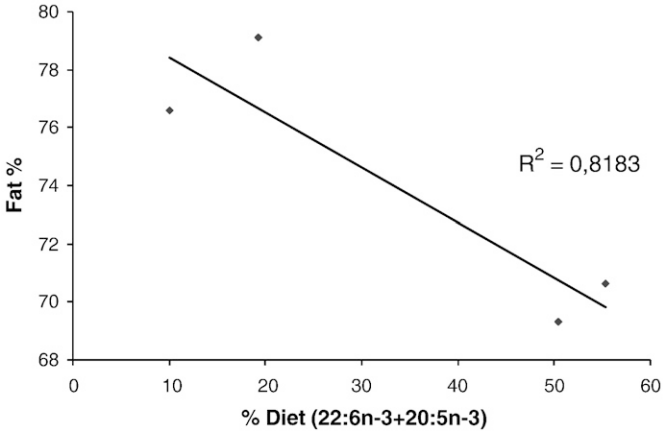

Fig. 2. The plot shows that the relationship between the percentage of 22:6n-3 and 20:5n-3 of the diet and the fat percentage of white adipose tissue, $n=3$.

OH, USA). To 2.7 mL of medium (containing 20 mM potassium phosphate buffer (pH 7.4), 1 mM ethylenediaminetetraacetic acid and 0.1% Triton X-100), 0.25 mL of sample was added. The changes in absorbance were measured at 420 nm for 3 min in a GBC UV/VIS 918 spectrophotometer (GBC Scientific Equipment, Victoria, Australia).

2.6. Fatty acid composition

FA composition in the diets, WAT and crude mitochondrial and peroxisomal fractions were analysed. Total lipids were extracted using the method described by Folch et al. (1957). The chloroform (VWR International, West Chester, PA, USA) phase was dried under nitrogen gas and residual lipid extract was redissolved in chloroform (Merck). Phospholipids (PLs) and TAGs were separated by thin-layer chromatography (TLC) using a mixture of petroleum ether (Riedel-de Haen), diethyl ether (Riedel-de Haen) and acetic acid (VWR) (130:20:2 v/v/v) as the mobile phase. The lipids were visualized by spraying the TLC-plates with 0.2% (w/v) 2′,7′-dichlorofluorescein in methanol (Merck), and the lipids were identified by comparing the positions of the spots with the positions of known standards under UV-light as described by Ruyter et al. (2000). The spots corresponding to PLs and TAGs were scraped into glass tubes and transmethylated overnight with 2.2-dimethoxypropane, methanolic hydrochloric acid and benzene (Merck) at room temperature, as described by Mason and Waller (1964) and by Hoshi et al. (1973). The methyl esters of FAs were separated in a gas chromatograph (Hewlett Packard 6890) with a split injector, using a SGE BPX70 capillary column (length 60 m, internal diameter 0.25 mm and thickness of the film 0.25 μm), flame ionisation detector and HP Chem Station software. The carrier gas was helium, and the injector and detector temperatures were both 280 °C. The oven temperature was raised from 50 to 180 °C at the rate of 10 °C/min, and then raised to 240 °C at a rate of 0.7 °C/min. The relative quantity of each FA present was determined by measuring the area under the peak corresponding to that FA.

2.7. Lipid class composition

Neutral lipid classes composition in the M fraction were quantified using high-performance thin-layer chromatography (HP-TLC) as described by Bell et al. (1993) and Jordal et al. (2007). Total lipid (10 μg) was applied by using an automatic sample applicator (ATS4, CAMAG, Muttenz, Switzerland) onto a 10×20 cm HP-TLC plate that had been pre-run in hexane:diethyl ether (1:1 v/v) and activated at 110 °C for 30 min. The plates were developed fully in hexane:diethyl ether:acetic acid (80:20:2 v/v/v) using an automatic developing chamber (AMD2, CAMAG). Neutral lipid classes were visualised by charring at 160 °C for 15 min after dipping the plate in a glass tank with 3% copper acetate (w/v) in 8% (v/v) phosphoric acid. The lipid classes were identified by comparison with commercially available standards and quantified by scanning densitometry using a CAMAG TLC Scanner 3, the amounts present being calculated using an integrator (WinCATS-Planar

Table 3
Fatty acid compositions of the total lipids, phospholipids and triacylglycerols in white adipose tissue

Fatty acids (% of total)	Total				PL				TAG			
	RO	FO	DHA	EPA	RO	FO	DHA	EPA	RO	FO	DHA	EPA
14:0	2.9±0.15^{a}	5.9±0.10^{b}	3.0±0.08^{a}	3.0±0.12^{a}	2.0±0.07^{a}	3.5±0.09^{c}	2.4±0.1^{bc}	2.8±0.37^{ab}	3.0±0.14^{a}	5.9±0.13^{b}	3.2±0.06^{a}	3.1±0.13^{a}
16:0	9.4±0.30^{b}	13.0±0.13^{c}	8.4±0.11^{a}	7.9±0.43^{a}	11.6±0.78^{a}	14.8±0.07^{a}	12.6±0.90^{a}	13.4±1.83^{a}	9.6±0.26^{b}	13.3±0.12^{c}	9.2±0.10^{b}	8.3±0.48^{a}
18:0	2.5±0.01^{b}	2.3±0.05^{b}	2.4±0.06^{b}	1.7±0.17^{a}	4.3±0.39^{a}	3.9±0.49^{a}	5.1±1.05^{a}	4.7±0.53^{a}	2.5±0.01^{b}	2.3±0.02^{b}	2.6±0.03^{b}	1.8±0.17^{a}
∑ Saturated[1]	15.3±0.37^{a}	21.9±0.15^{b}	15.3±0.17^{a}	13.6±1.13^{a}	18.2±1.12^{a}	22.6±0.25^{a}	21.5±1.33^{a}	21.6±2.73^{a}	15.6±0.40^{b}	22.3±0.23^{c}	16.4±0.11^{b}	13.8±0.79^{a}
16:1 n-7	2.9±0.21^{a}	5.3±0.06^{b}	3.0±0.15^{a}	2.8±0.24^{a}	2.1±0.22^{ab}	2.9±0.22^{b}	2.0±0.05^{a}	2.6±0.25^{ab}	3.0±0.20^{b}	5.4±0.06^{a}	3.2±0.13^{b}	2.9±0.25^{b}
18:1 n-7	3.0±0.07^{d}	2.5±0.01^{c}	1.9±0.05^{b}	1.6±0.11^{a}	2.2±0.02^{a}	2.1±0.12^{a}	1.8±0.07^{a}	2.0±0.26^{a}	3.1±0.02^{b}	2.5±0.01^{a}	2.0±0.04^{d}	1.7±0.10^{c}
18:1 n-9	35.6±0.45^{c}	14.8±0.02^{b}	11.2±0.75^{a}	9.6±1.02^{a}	22.1±0.43^{b}	12.6±0.03^{a}	12.3±1.32^{a}	10.6±0.85^{a}	35.3±0.43^{b}	14.5±0.06^{a}	11.9±0.52^{c}	10.0±0.96^{c}
20:1 n-9	5.3±0.02^{b}	8.8±0.46^{c}	5.3±0.05^{b}	4.0±0.07^{a}	3.6±0.09^{a}	4.8±0.24^{a}	3.8±0.12^{a}	4.3±0.90^{a}	5.3±0.08^{b}	8.6±0.20^{a}	5.7±0.05^{d}	4.2±0.03^{c}
20:1 n-11	1.2±0.11^{a}	2.6±0.14^{b}	1.1±0.02^{a}	3.6±0.23^{c}	1.2±0.22^{a}	1.5±0.17^{a}	1.0±0.05^{a}	2.4±0.09^{b}	1.2±0.10^{b}	2.5±0.11^{a}	1.0±0.03^{b}	3.5±0.24^{c}
22:1 n-7	0.8±0.05^{a}	1.6±0.06^{b}	1.0±0.02^{a}	2.4±0.12^{c}	1.0±0.05^{a}	1.2±0.12^{a}	0.9±0.02^{a}	1.6±0.15^{b}	0.8±0.02^{b}	1.6±0.05^{a}	n.d.	n.d.
22:1 n-11	4.4±0.02^{a}	9.4±0.30^{c}	5.5±0.14^{b}	4.7±0.10^{a}	3.9±0.26^{a}	5.6±0.40^{b}	3.6±0.29^{a}	5.0±0.47^{ab}	4.4±0.05^{b}	9.5±0.25^{a}	1.0±0.01^{d}	2.4±0.14^{c}
∑ Monounsaturated[2]	55.6±0.11^{c}	48.2±0.88^{b}	32.6±0.78^{a}	30.6±0.99^{a}	48.4±1.29^{c}	40.1±0.01^{b}	33.8±0.35^{a}	36.6±2.67^{ab}	55.3±0.26^{b}	48.1±0.22^{a}	33.9±0.50^{d}	31.7±0.92^{c}
18:2 n-6	10.1±0.02^{d}	3.8±0.07^{c}	2.9±0.06^{a}	3.2±0.01^{b}	8.4±0.02^{c}	3.6±0.00^{a}	3.4±0.02^{a}	3.2±0.10^{b}	10.1±0.12^{b}	3.9±0.01^{a}	3.0±0.05^{d}	3.3±0.01^{c}
18:3 n-3	4.1±0.10^{d}	1.7±0.04^{c}	0.9±0.02^{a}	1.2±0.02^{b}	2.6±0.10^{c}	1.3±0.08^{b}	0.9±0.10^{a}	1.0±0.05^{ab}	4.1±0.13^{b}	1.7±0.07^{a}	0.8±0.02^{d}	1.2±0.03^{c}
20:4 n-6	0.3±0.01^{a}	0.4±0.02^{a}	0.9±0.02^{b}	1.6±0.10^{c}	1.7±0.10^{a}	2.3±0.28^{ab}	3.2±0.15^{b}	2.8±0.61^{ab}	0.3±0.01^{a}	0.4±0.02^{a}	0.9±0.02^{c}	1.6±0.12^{b}
20:5 n-3	2.3±0.06^{a}	4.5±0.02^{ab}	5.8±0.12^{b}	25.8±2.20^{c}	8.1±0.38^{a}	10.5±0.29^{a}	8.0±0.24^{a}	17.8±2.31^{b}	2.4±0.16^{a}	4.3±0.02^{a}	5.4±0.15^{a}	24.7±2.23^{b}
22:5 n-3	1.3±0.03^{b}	2.2±0.04^{a}	3.6±0.02^{c}	5.7±0.15^{d}	1.9±0.12^{a}	2.1±0.04^{a}	2.7±0.32^{ab}	3.5±0.46^{b}	1.3±0.02^{b}	2.0±0.05^{a}	3.4±0.02^{d}	5.6±0.21^{c}
22:6 n-3	7.9±0.01^{a}	14.0±0.50^{b}	34.5±0.79^{c}	15.3±0.66^{b}	16.8±0.71^{a}	20.1±0.58^{a}	28.5±0.91^{b}	15.3±2.20^{a}	7.7±0.05^{b}	13.4±0.43^{a}	32.7±0.58^{c}	14.7±0.47^{a}
∑ Polyunsaturated[3]	29.1±0.08^{a}	29.9±0.87^{a}	52.2±0,79^{b}	55.8±1.82^{c}	33.4±0.17^{a}	37.3±0.01^{ab}	44.7±0.98^{b}	41.8±5.40^{ab}	29.1±0.14^{a}	29.6±0.44^{a}	49.7±0.61^{c}	54.5±1.71^{b}
n-3	17.4±0.53^{a}	23.6±0.80^{a}	51.3±3.88^{b}	51.6±3.73^{b}	29.4±0.12^{a}	34.0±0.41^{ab}	40.1±1.09^{b}	37.7±5.01^{ab}	16.3±0.04^{b}	22.9±0.41^{a}	43.4±0.65^{d}	46.8±1.51^{c}
n-6	23.0±0.70^{b}	7.6±1.37^{a}	8.9±1.79^{a}	12.8±0.25^{a}	13.1±0.13^{b}	6.2±0.25^{a}	7.0±0.18^{a}	6.7±0.56^{a}	10.6±0.10^{b}	4.8±0.02^{a}	4.4±0.04^{d}	6.0±0.17^{c}
20:5n-3+22:6n-3	10.2±0.05^{a}	18.4±0.52^{b}	40.3±0.89^{c}	41.1±1.54^{c}	24.9±0.33^{a}	30.6±0.29^{ab}	36.5±0.67^{b}	33.1±4.51^{ab}	10.1±0.11^{b}	17.7±0.41^{a}	38.2±0.69^{c}	39.4±1.76^{c}

n.d. = not detected.
1 Includes: 15:0, 22:0, 24:0.
2 Includes: 14:1 n-5, 16:1 n-5, 16:1 n-9, 17:1 n-7, 18:1 n-11, 20:1 n-7, 22:1 n-9, 24:1 n-9.
3 Includes: 16:2 n-3, 16:2 n-6, 16:3 n-4, 18:3 n-4, 18:3 n-6, 20:2 n-6, 20:3 n-3, 20:3 n-6, 20:4 n-3, 22:2 n-6, 2:4 n-6.
The fatty acid composition of total lipids, polar and neutral lipids of white adipose tissue from fish fed four different diets. The quantity of each fatty acid is given as the percentage of total fatty acids. The values given are means ± SEM ($n=3$). Different letters indicate significant differences ($p \leq 0.05$) between the different dietary treatments within each lipid class. FO = fish oil diet; RO = rapeseed oil diet; DHA = DHA enriched oil diet; EPA = EPA enriched oil diet; n.d. = not detected; PL = phospholipid; TAG = triacylglycerol.

Chromatography, Version 1.3.3). Further, the quantities of lipid classes present were determined by establishing standard equations for each lipid class within a linear area. Furthermore, a standard mix of all the lipid classes was included on each HP-TLC plate to enable the corrections of between-plate variations.

2.8. Mitochondrial β-oxidation

Mitochondrial β-oxidation was measured by the method of Lazarow (1981). This assay measures the amounts of acid-soluble products in mitochondrial fraction. Acid insoluble ^{14}C-palmitoyl-CoA (American Radiolabel Chemicals Inc, St. Louis, MO, USA) is converted to acid-soluble ^{14}C-acetyl-CoA. The buffer for mitochondrial β-oxidation contained 50 mM Tris–HCl (pH 8.0), 20 mM NAD, 330 mM dithiothreitol, 1.5% bovine serum albumin, 10 mM CoA, 1 mM flavin adenine dinucleotide, 0.25 M sucrose, 1 mM L-carnitine, 5 mM unlabeled palmitoyl CoA, and ^{14}C-palmitoyl-CoA (60 Ci/mol, 20 μCi/mL).

The reaction was stopped after 30 min of incubation at room temperature by adding ice-cold perchloric acid (6%) (Merck). The tubes were kept on ice for 1 h. After incubation, the tubes were centrifuged for 2 min, at 13 000 g in a 4 °C cold centrifuge. The supernatant was siphoned off and 500 μL was placed into the scintillation vial, to which 7 mL of the scintillation liquid Insta gel II plus (Packard Instruments, Downers Grove, IL, USA) was added. The samples were counted in a scintillation counter (Packard Model 1900 A).

2.9. Superoxide dismutase assay

Superoxide dismutase (SOD) catalyzes the reduction of superoxide into oxygen and hydrogen peroxide. A commercially available kit was used to assay this enzyme (Cayman Chemicals, Ann Arbor, MI, USA). The kit utilizes a tetrazolium salt to detect superoxide radicals generated by xanthine oxidase and hypoxanthine. One unit of SOD was defined as the amount of enzyme needed to achieve 50% dismutation of the superoxide radical. Colour was measured at 405 nm in a Titertek Multiskan PLUS MKII (Labsystems, Helsinki, Finland) plate reader.

2.10. Caspase 3 assay

Caspase 3 is an intracellular cysteine protease that exists as a proenzyme, becoming activated during the cascade of events associated with apoptosis. We used a caspase 3 colorimetric assay kit (R&D Systems, Inc, Minneapolis, MN, USA) to determine the increase in enzyme activity of the caspase 3 class of proteases in apoptotic cells. The H fraction from each dietary group was first lysed to collect its intracellular contents. The lysate was then tested for protease activity by adding a caspase-specific peptide that conjugates to a color reporter molecule, p-nitroaniline. The cleavage of the peptide by the caspase releases the chromophore p-nitroaniline, which was quantified spectrophotometrically. Colour was measured at 405 nm in a Titertek Multiskan PLUS MKII plate reader.

2.11. Statistical analyses

All data were subjected to one-way ANOVA for the factor “diet”, and the differences were ranked by Duncan's multiple range test. We used the software package UNISTAT (London, England). The significance level was set at 0.05.

3. Results

3.1. Isolation of a crude mitochondrial fraction

Fig. 1A and B show that mitochondria and peroxisomes from WAT were enriched for the same fraction after differential centrifugation. However, the relative specific activity of the mitochondrial enzyme cytochrome C oxidase was substantially higher than the relative

Table 4
Fatty acid composition of the total lipids, phospholipids and triacylglycerols of mitochondrial fraction

Fatty acids (% of total)	TOTAL				PL				TAG			
	RO	FO	DHA	EPA	RO	FO	DHA	EPA	RO	FO	DHA	EPA
14:0	2.0±0.11^{a}	3.7±0.28^{b}	2.6±0.12^{a}	2.3±0.08^{a}	3.6±0.46^{a}	2.8±0.11^{a}	2.6±0.18^{a}	3.2±0.69^{a}	2.7±0.18^{a}	5.6±0.49^{b}	2.8±0.03^{a}	2.8±0.12^{a}
16:0	9.8±0.37^{a}	19.0±1.52^{b}	9.3±0.83^{a}	8.3±0.00^{a}	18.0±1.74^{a}	19.4±0.16^{a}	16.9±1.09^{a}	18.7±0.47^{a}	12.3±1.67^{a}	22.2±0.97^{b}	10.1±1.14^{a}	9.3±0.20^{a}
18:0	3.1±0.23^{a}	7.5±1.65^{b}	3.1±0.46^{a}	2.3±0.09^{a}	4.9±1.45^{a}	10.5±0.39^{a}	9.4±1.68^{a}	9.3±1.20^{a}	4.1±0.75^{ab}	5.6±0.70^{b}	3.0±0.27^{a}	2.5±0.03^{a}
∑ Saturated[1]	15.4±0.67^{a}	31.3±3.08^{b}	16.8±1.03^{a}	13.3±0.27^{a}	27.9±4.89^{a}	36.3±0.65^{a}	32.3±3.14^{a}	33.3±1.99^{a}	19.5±2.48^{a}	34.2±2.16^{b}	18.0±1.40^{a}	15.2±0.35^{a}
16:1 n-7	2.0±0.10^{a}	4.1±0.31^{b}	2.2±0.06^{a}	2.1±0.11^{a}	2.6±0.63^{a}	1.6±0.13^{a}	1.4±0.08^{a}	2.1±0.57^{a}	2.0±0.14^{a}	3.9±0.59^{b}	2.3±0.09^{a}	2.3±0.11^{a}
18:1 n-7	3.1±0.11^{c}	2.6±0.07^{b}	1.8±0.12^{a}	1.5±0.05^{a}	2.2±0.20^{a}	2.0±0.19^{a}	1.6±0.11^{a}	1.8±0.22^{a}	2.9±0.10^{b}	2.4±0.19^{b}	1.6±0.03^{a}	1.5±0.04^{a}
18:1 n-9	39.0±0.77^{c}	12.8±0.29^{b}	9.2±0.18^{a}	8.0±0.54^{a}	22.2±2.48^{b}	7.8±0.28^{a}	7.7±0.25^{a}	9.1±2.55^{a}	36.5±2.02^{c}	17.3±2.29^{a}	9.3±0.15^{c}	8.3±0.52^{c}
18:1 n-11	0.9±0.05^{a}	1.8±0.09^{b}	0.9±0.01^{a}	0.9±0.04^{a}	5.6±2.41^{b}	0.7±0.01^{a}	0.5±0.23^{a}	0.8±0.17^{a}	0.7±0.22^{a}	1.6±0.13^{b}	0.7±0.16^{a}	0.9±0.03^{a}
20:1 n-9	5.2±0.02^{b}	7.3±0.46^{c}	5.0±0.13^{b}	3.7±0.04^{a}	n.d.	2.3±0.15^{a}	2.3±0.34^{a}	2.4±0.42^{a}	4.9±0.17^{b}	6.7±0.59^{c}	5.2±0.15^{b}	3.8±0.07^{a}
22:1 n-7	0.5±0.26^{a}	0.5±0.05^{a}	1.0±0.04^{a}	2.5±0.05^{b}	0.7±0.13^{a}	n.d.	0.3±0.07^{a}	1.3±0.10^{b}	0.7±0.04^{a}	0.9±0.14^{a}	0.9±0.06^{a}	2.5±0.03^{b}
22:1 n-11	1.0±0.04^{a}	1.6±0.54^{b}	1.0±0.10^{ab}	3.6±0.06^{c}	1.8±0.21^{a}	1.9±0.05^{a}	1.3±0.67^{a}	2.1±0.43^{a}	3.7±0.16^{a}	6.6±0.91^{c}	5.6±0.17^{ab}	4.6±0.11b^{a}
∑ Monounsaturated[2]	55.8±1.33^{c}	39.8±1.71^{b}	28.8±0.26^{a}	27.8±0.67^{a}	36.3±3.43^{b}	16.8±0.46^{a}	16.6±1.38^{a}	21.3±4.06^{a}	53.8±2.45^{c}	42.7±0.10^{b}	29.5±0.55^{a}	28.8±0.67^{a}
18:2 n-6	12.6±0.71^{c}	7.1±0.89^{b}	3.8±0.94^{a}	3.3±0.08^{a}	12.6±0.23^{ab}	15.9±1.07^{a}	11.4±4.16^{ab}	7.7±0.56^{a}	10.7±0.49^{c}	4.3±0.81^{b}	2.4±0.10^{a}	2.8±0.03^{a}
18:3 n-3	4.8±0.16^{b}	1.1±0.34^{a}	0.5±0.24^{a}	1.1±0.01^{a}	2.5±0.62^{b}	0.6±0.03^{a}	0.1±0.05^{a}	0.7±0.05^{a}	4.2±0.24^{c}	1.6±0.34^{b}	0.7±0.04^{a}	1.1±0.01^{a}
20:4 n-6	0.6±0.03^{a}	1.3±0.51^{c}	0.9±0.08^{b}	1.0±0.02^{b}	0.9±0.06^{a}	1.6±0.14^{a}	3.1±0.63^{b}	3.0±0.58^{b}	0.2±0.04^{a}	0.2±0.12^{a}	1.0±0.04^{b}	1.7±0.02^{c}
20:5 n-3	1.7±0.02^{a}	4.5±0.56^{b}	6.5±0.32^{c}	26.9±0.84^{d}	2.2±0.68^{a}	4.8±0.31^{ab}	5.6±0.95^{b}	13.0±1.27^{c}	1.6±0.18^{a}	2.2±0.23^{a}	6.1±0.50^{b}	25.4±0.43^{c}
22:5 n-3	0.8±0.09^{a}	0.6±0.06^{a}	3.5±0.16^{b}	6.5±0.19^{c}	0.8±0.05^{a}	n.d.	1.2±0.04^{a}	2.9±0.15^{b}	0.8±0.06^{a}	1.1±0.14^{a}	3.4±0.17^{b}	6.1±0.29^{c}
22:6 n-3	6.2±0.15^{a}	14.2±0.71^{b}	36.2±0.54^{d}	16.1±0.29^{c}	10.1±0.78^{a}	15.4±1.29^{b}	23.2±4.38^{c}	13.6±1.33^{a}	5.7±0.27^{a}	7.5±1.36^{a}	35.3±0.81^{c}	15.0±0.31^{b}
∑ Polyunsaturated[3]	28.8±0.69^{a}	28.9±1.82^{a}	54.4±0.97^{b}	58.9±0.94^{c}	35.8±2.12^{a}	46.9±0.44^{b}	51.1±1.86^{b}	45.4±3.11^{b}	26.7±0.45^{a}	23.1±2.11^{a}	52.5±1.16^{b}	56.0±0.68^{b}
n-3	18.5±3.26^{a}	18.5±1.58^{a}	47.9±1.44^{b}	51.7±0.81^{b}	17.5±2.17^{a}	21.5±1.54^{ab}	30.2±6.14^{b}	30.9±2.72^{b}	14.0±0.29^{a}	14.3±1.84^{a}	47.3±1.44^{b}	49.0±0.51^{b}
n-6	10.7±2.10^{a}	10.0±2.38^{a}	6.3±0.55^{a}	7.1±0.19^{a}	17.8±0.70^{ab}	25.1±1.40^{b}	18.5±4.86^{ab}	13.3±0.67^{a}	12.6±0.23^{c}	8.8±1.15^{b}	5.1±0.46^{a}	6.8±0.20^{ab}
20:5n-3+22:6n-3	7.9±0.17^{a}	18.7±0.93^{b}	42.7±0.86^{c}	42.9±1.03^{c}	12.3±0.99^{a}	20.2±1.59^{ab}	28.8±5.32^{c}	26.6±2.59^{c}	7.3±0.44^{a}	9.6±1.48^{a}	41.4±1.23^{b}	40.4±0.36^{b}

n.d. = not detected.
1 Includes: 15:0, 17:0, 20:0, 24:0.
2 Includes: 14:1 n-5, 16:1 n-5, 16:1 n-9, 17:1 n-7, 20:1 n-7, 22:1 n-9, 24:1 n-9.
3 Includes: 16:2 n-3, 16:2 n-6, 16:3 n-4, 18:3 n-4, 18:3 n-6, 18:4 n-3, 20:2 n-6, 20:3 n-3, 20:4 n-3, 22:2 n-6, 22:4 n-6.
The fatty acid composition of total lipids, polar and neutral lipids of mitochondrial fraction from fish fed four different diets. The quantity of each fatty acid is given as the percentage of total fatty acids. The values given are means ± SEM (n = 3). Different letters indicate significant differences ($p \leq 0.05$) between the different dietary treatments within each lipid class. FO = fish oil diet; RO = rapeseed oil diet; DHA = DHA-enriched oil diet; EPA = EPA-enriched oil diet; n.d. = not detected; PL = phospholipid; TAG = triacylglycerol.

specific activity of the peroxisomal enzyme catalase, showing that the fraction denoted "M" was most highly enriched in mitochondria. Analyses of marker enzymes show that this M fraction contained relatively low levels of nuclei, lysosomes and microsomes (Fig. 1C and D).

3.2. Total fatty acid compositions of white adipose tissue and mitochondrial fraction

The plot in Fig. 2 shows that the relationship between the n-3 HUFA composition of the diet and the fat percentage of WAT was linear. Fish fed high dietary DHA and EPA levels had lower fat percentage of WAT than fish fed diets with low levels of these FAs. Further, the FA compositions of the diets affected the total FA compositions of WAT and that of the M fraction (Tables 3 and 4). The percentage of n-3 HUFAs was approximately 2.5 times greater in the DHA and EPA groups, than it was in the RO group. This was the case both for whole WAT and for the M fraction. The main contributor to n-3 FAs in the FO and DHA dietary groups was 22:6n-3, while the main contributor in the EPA dietary group was 20:5n-3. On the other hand, the percentage of n-6 FAs in WAT was approximately twice as high in the RO dietary group as those of the DHA and EPA dietary groups. The main contributor here was 18:2n-6.

3.3. Fatty acid compositions of neutral and polar lipid fractions of white adipose tissue and the mitochondrial fraction

The total lipid fractions of WAT and of M were separated into polar and neutral lipid fractions by TLC prior to analyses of FA composition.

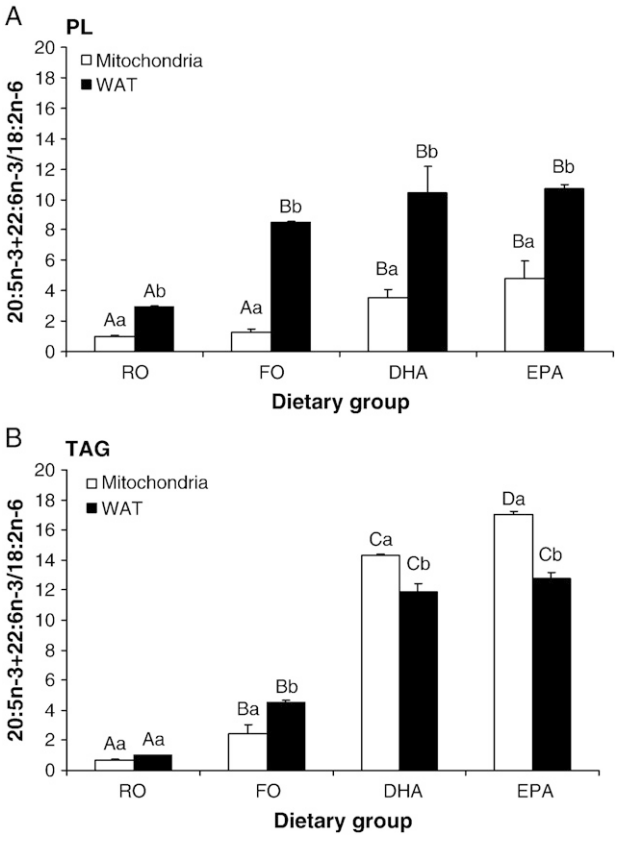

Fig. 3. The ratio of the percentage of 20:5n-3+22:6n-3 to the percentage of 18:2n-6 in the phospholipid fraction (A) and triacylglycerol fraction (B) of whole white adipose tissue and in the mitochondrial fraction. Different capital letters indicate significant differences between different dietary groups; different small letters indicate significant differences within dietary group ($p \leq 0.05$). Data shown are means ± SEM ($n = 3$). FO = fish oil diet; RO = rapeseed oil diet; DHA = DHA-enriched oil diet; EPA = EPA-enriched oil diet. (A) PL = phospholipid; (B) TAG = triacylglycerol.

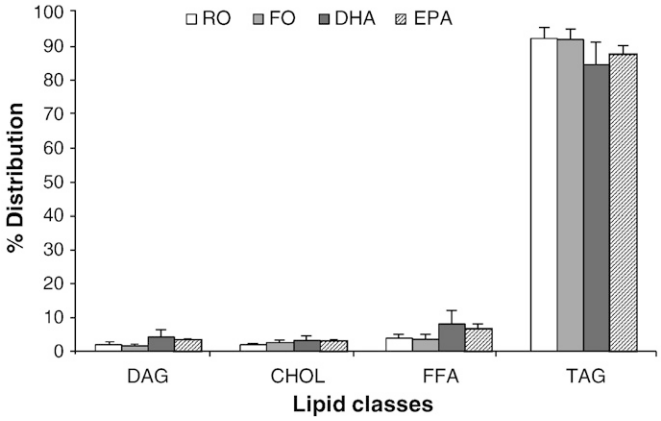

Fig. 4. Relative distribution between neutral lipid classes in white adipose tissue. Different letters indicate significant differences ($p \leq 0.05$). Data shown are means ± SEM ($n = 3$). FO = fish oil diet; RO = rapeseed oil diet; DHA = DHA-enriched oil diet; EPA = EPA-enriched oil diet; CHOL = cholesterol; DAG = diacylglycerol; FFA = free fatty acids; TAG = triacylglycerol.

Tables 3 and 4 show that the percentages of 20:5n-3 and 22:6n-3 were significantly higher in PLs and TAGs in the DHA and EPA dietary groups than in the FO and RO dietary groups. With the low and moderate levels of 20:5n-3 and 22:6n-3 available in the RO and FO diets, the majority of these FAs was found in the PLs and with lower percentages found in TAGs. High dietary levels of 20:5n-3 and 22:6n-3 on the other hand, led to higher relative levels of these FAs in the TAG fractions than in the PLs. Furthermore, the mitochondrial membrane PLs contained lower relative levels of 20:5n-3 and 22:6n-3 and higher relative levels of 18:2n-6 than the membrane lipids of WAT. The ratio of 20:5n-3+22:6n-3 to 18:2n-6 in the PLs of WAT was always more than 2-fold higher than in the PLs of the M fraction; irrespective of dietary group (Fig. 3A). In the TAG fraction, however, the ratio of 20:5n-3+22:6n-3 to 18:2n-6 in WAT relative to M was close to equal in the RO and FO dietary groups and 1.3 fold lower in DHA and EPA groups (Fig. 3B).

3.4. Neutral lipids in the white adipose tissue and the mitochondrial fraction

We measured the neutral lipids in the WAT fraction, and determined the relative distribution between cholesterol (CHOL), free fatty acid (FFA), diacylglycerol (DAG) and TAG present (Fig. 4). Although there were no significant differences in the distribution

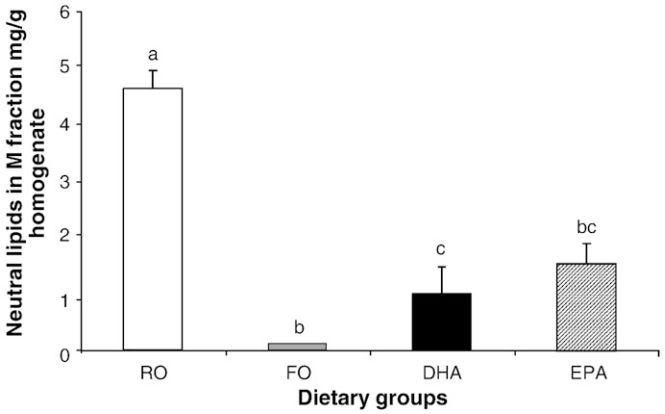

Fig. 5. Total neutral lipid levels in the mitochondrial fraction. Different letters indicate significant differences ($p \leq 0.05$). Data shown are means ± SEM ($n = 3$). FO = fish oil diet; RO = rapeseed oil diet; DHA = DHA-enriched oil diet; EPA = EPA-enriched oil diet; M = mitochondrial fraction.

between the neutral lipid classes between the dietary groups, there was a clear trend towards reduced TAG percentage with increasing dietary levels of n-3 HUFAs.

The M fraction from fish in the RO dietary group contained the highest levels of total neutral lipids compared to any other groups, while the lipid levels in the DHA and EPA dietary groups were significantly higher than in the FO dietary group (Fig. 5).

3.5. Mitochondrial β-oxidation

Mitochondrial FA β-oxidation activity was significantly higher in the FO dietary group than in the RO dietary group. No FA β-oxidation activities were recovered in the DHA and EPA dietary groups (Fig. 6).

3.6. Oxidative stress markers

Cytochrome c oxidase activities were measured in all dietary groups. The activity in the FO group was set to 1, and the changes in the test groups (RO, DHA, EPA) were calculated relative to the FO group. Decrease in the level of functional cytochrome c oxidase was further used to interpret for eventual differences in membrane integrity. The cytochrome C oxidase measurements showed that there were significantly more disrupted mitochondrial membranes in the DHA (1.5±0.03), EPA (1.6±0.02) and RO (1.3±0.06) dietary groups than in the FO (1±0.00) dietary group ($p \leq 0.05$ for all comparisons).

The activity of caspase 3, an apoptotic marker, differed between the dietary groups. The activity was lower in FO and RO-fed fish, and significantly higher in the DHA and EPA dietary groups (Fig. 7).

The activity of SOD, as well, was significantly different between the dietary groups. The activities were lowest in FO and RO-fed fish, and significantly higher in fish fed DHA and EPA-enriched diets (Fig. 8).

4. Discussion

Increased dietary levels of the very long-chain FAs, DHA and EPA, resulted in lower fat percentage in WAT in Atlantic salmon fed 23% dietary lipid. This is consistent with results from several mammalian studies reporting that the intake of high-fat diets containing n-3 HUFAs leads to less hypertrophy of fat depots than the intake of high-fat diets containing saturated FAs (Belzung et al., 1993; Hainault et al., 1993; Couet et al., 1997). Similar results have been obtained in several animal models, such as obese Zucker rats (Carlotti et al., 1993), mice (Ikemoto et al., 1996), obese *ob*/*ob* mice (Cunnane et al., 1986), and hamsters (Jones, 1989). A recent *in vitro* study of our group showed that Atlantic salmon preadipocytes, to which DHA and EPA have been

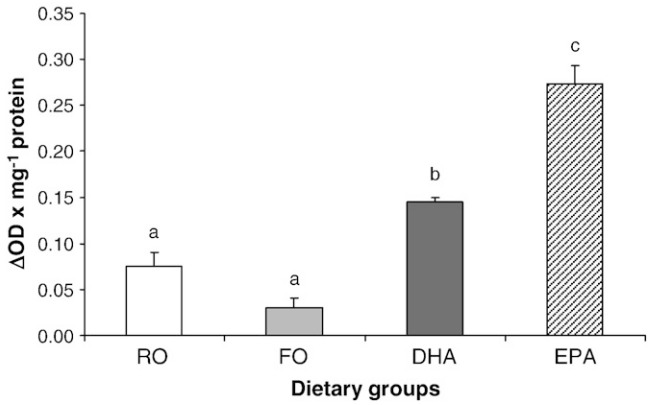

Fig. 7. Caspase 3 activity. Different letters indicate significant differences ($p \leq 0.05$). Data shown are means±SEM ($n=3$). FO = fish oil diet; RO = rapeseed oil diet; DHA = DHA-enriched oil diet EPA = EPA-enriched oil diet.

added, were filled with lipid to a lower extent, and were less differentiated, than cells to which OA have been added (Todorčević et al., 2008). The high dietary levels of DHA and EPA used in the present study also led to increased percentages of these HUFAs in WAT, where they amounted to approximately 40% of the total FAs. A previous study in mice have shown that n-3 HUFAs limit diet induced obesity by reducing hypertrophy and hyperplasia of WAT (Ruzickova et al., 2004) and, although the authors did not directly measure apoptosis, the presented results suggested that EPA may have an apoptotic effect. The caspase 3 activity was significantly higher in WAT of fish fed the high DHA and EPA diets, indicating that a higher incidence of the fat cell death may be one factor causing the lower fat percentage in our trial. Indeed, a rise in apoptosis decreases the number of adipocytes and, consequently, adipose tissue weight in rats (Perez-Matute et al., 2007).

The percentages of n-3 HUFAs increased from 12% to 29% in mitochondrial membrane PLs and from 10% to 41% in mitochondrial TAGs with the increasing dietary doses. Since HUFAs are very susceptible to peroxidation, one aim of this work has also been to study how the high levels of DHA and EPA in membrane lipids affect oxidative stress of mitochondria in WAT. The percentages of n-3 HUFAs were, however, always lower in mitochondrial PLs than in the PLs of WAT. Furthermore, the mitochondrial PLs had higher percentages of LA than WAT PLs. Mitochondria are rich in the cardiolipin (CL), a PL that is particularly susceptible to oxidative damage and that needs to be protected. Tsalouhidou et al. (2006) have suggested that the

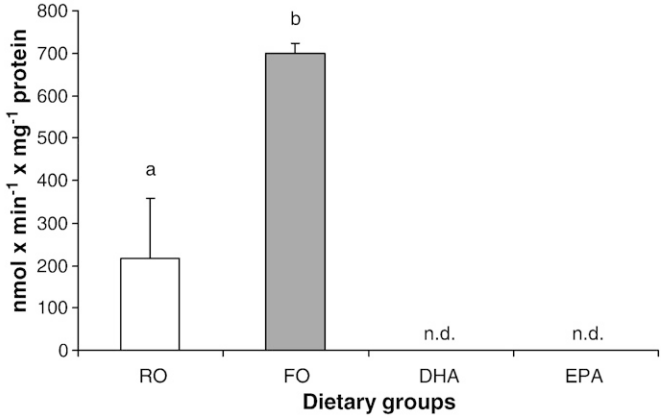

Fig. 6. Mitochondrial fatty acid β-oxidation activity in the mitochondrial fraction. Different letters indicate significant differences ($p \leq 0.05$). Data shown are means±SEM ($n=3$). FO = fish oil diet; RO = rapeseed oil diet; DHA = DHA-enriched oil diet; EPA = EPA-enriched oil diet.

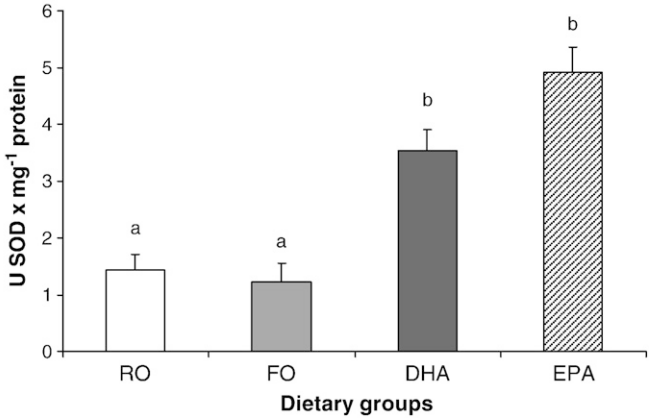

Fig. 8. Superoxide dismutase activity. Different letters indicate significant differences ($p \leq 0.05$). Data shown are means±SEM ($n=3$). FO = fish oil diet; RO = rapeseed oil diet; DHA = DHA-enriched oil diet; EPA = EPA-enriched oil diet.

predominance of LA in the CL in the gastrocnemius muscle mitochondria of rats, and the relatively low percentages of HUFAs, may protect this PL from peroxidation. We also show that EPA is elongated to 22:5n-3 (docosapentaenoic acid, DPA) to a substantial extent and stored in WAT. Ackman (1992) postulated that DPA is a temporary storage form for surplus EPA. We propose that the lower degree of n-3 HUFAs found in PLs of mitochondria than that found in WAT PLs, lead to mitochondrial membranes that are less susceptible to oxidative stress. The mitochondrial FA β-oxidation is a sensitive marker for mitochondrial function. The FA β-oxidation activity was three times higher in the FO group than it was in the RO group. Increased β-oxidation may partly explain the lower degree of lipid deposition in WAT in the fish fed DHA and EPA enriched diets. It is known that EPA, which is present in FO, acts as a mitochondrial proliferator and increases mitochondrial β-oxidation activity (Froyland et al., 1997; Vegusdal et al., 2005). The neutral lipid content of mitochondria was 10 fold higher in the RO group than in the FO group, indicating a more rapid turnover rate of lipids in the latter group. There was however also a 1.3 fold reduction in the functional cytochrome c oxidase in the mitochondrial membranes in the RO group compared to the FO group, which may indicate a moderate reduction in membrane integrity in this group. It is also possible that the relatively high lipid accumulation inside mitochondria in the RO group leads to less functional mitochondria and this itself may also be one factor causing the lower mitochondrial FA β-oxidation capacity in the RO group than in the FO group. Even mildly impaired mitochondrial function lead to a predisposition to obesity (Pomplun et al., 2007).

Adipocytes in mammals actively utilize FAs for energy (Milstein et al., 1959), but mechanisms that control the mitochondrial machinery for FAs oxidation in fish are poorly understood. It was long believed that WAT served only as a fat depot, and that energy was not produced in the tissue by fat burning. WAT may constitute a significant percentage of salmon body mass, and thus even a moderate up- or down regulation of FA β-oxidation capacity in this tissue may significantly affect the total body energy balance.

In contrast to the expected increase in mitochondrial FA β-oxidation in the DHA and EPA groups, the opposite was found; there was no mitochondrial β-oxidation activity recovered in WAT of these groups. We conclude that while moderate levels of n-3 HUFAs in fish diets reduce lipid accumulation in WAT by increasing the FA β-oxidation capacity, the high dietary HUFA levels, damage mitochondrial membranes so that they fail to function. This is possibly caused by OS, resulting in increased fat cell apoptosis, which as a consequence led to the reduced lipid accumulation in WAT. This hypothesis is supported by the finding of 1.5 to 1.6 fold higher degree of membrane disruption (measured as lack of functional cytochrome c oxidase activity in mitochondria). Uncontrolled OS may lead to the creation of pore-like openings in the mitochondrial outer membrane, which is linked to cytochrome c release. Once in the cytosol, cytochrome c sets off a cascade that results in the activation of a main apoptotic executioner, caspase 3 (Gogvadze et al., 2006). The activity of SOD correlates well with the production of reactive oxygen species (ROS) (Mourente et al., 2007). The activities of SOD were higher in both the DHA group and the EPA group than in the FO and RO groups. The levels of caspase 3 were also higher in these groups, strongly suggesting that the high dietary levels of DHA and EPA induced OS in WAT, leading to mitochondrial membrane damage.

Our study shows that n-3 HUFAs in Atlantic salmon diets decrease the fat percentage of WAT and increase the FA β-oxidation capacity. However, very high levels of n-3 HUFAs lead to oxidative stress, loss of mitochondrial functions, induction of caspase 3, indicating an onset of apoptosis of WAT. Further studies with the total amount of WAT quantified in a larger number of fish of production size are needed in order to evaluate whether n-3 HUFAs affect the total WAT lipid deposition.

Acknowledgments

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Article III

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RESEARCH ARTICLE Open Access

Gene expression profiles in Atlantic salmon adipose-derived stromo-vascular fraction during differentiation into adipocytes

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Abstract

Background: Excessive fat deposition is one of the largest problems faced by salmon aquaculture industries, leading to production losses due to high volume of adipose tissue offal. In addition, increased lipid accumulation may impose considerable stress on adipocytes leading to adipocyte activation and production and secretion of inflammatory mediators, as observed in mammals.

Results: Microarray and qPCR analyses were performed to follow transcriptome changes during adipogenesis in the primary culture of adipose stromo-vascular fraction (aSVF) of Atlantic salmon. Cellular heterogeneity decreased by confluence as evidenced by the down-regulation of markers of osteo/chondrogenic, myogenic, immune and vasculature lineages. Transgelin (TAGLN), a marker of the multipotent pericyte, was prominently expressed around confluence while adipogenic PPARγ was up-regulated already in subconfluent cells. Proliferative activity and subsequent cell cycle arrest were reflected in the fluctuations of pro- and anti-mitotic regulators. Marked regulation of genes involved in lipid and glucose metabolism and pathways producing NADPH and glycerol-3-phosphate (G3P) was seen during the terminal differentiation, also characterised by diverse stress responses. Activation of the glutathione and thioredoxin antioxidant systems and changes in the iron metabolism suggested the need for protection against oxidative stress. Signs of endoplasmic reticulum (ER) stress and unfolded protein response (UPR) occured in parallel with the increased lipid droplet (LD) formation and production of secretory proteins (adipsin, visfatin). The UPR markers XBP1 and ATF6 were induced together with genes involved in ubiquitin-proteasome and lysosomal proteolysis. Concurrently, translation was suppressed as evidenced by the down-regulation of genes encoding elongation factors and components of the ribosomal machinery. Notably, expression changes of a panel of genes that belong to different immune pathways were seen throughout adipogenesis. The induction of AP1 (Jun, Fos), which is a master regulator of stress responses, culminated by the end of adipogenesis, concurrent with the maximal observed lipid deposition.

Conclusions: Our data point to an intimate relationship between metabolic regulation and immune responses in white adipocytes of a cold-blooded vertebrate. Stress imposed on adipocytes by LD formation and expansion is prominently reflected in the ER compartment and the activated UPR response could have an important role at visceral obesity in fish.

Background

Feeds used in modern Atlantic salmon aquaculture contain large amounts of lipids, which provide rapid growth, reduce environmental load from farms, but increase visceral fat deposition [1]. Very little is, however, known about the factors regulating development and functions of adipose tissue in fish, and whether increased fat deposition may lead to health problems. Previously regarded principally as energy storage, white adipose tissue (WAT) in mammals is now recognized as a highly active endocrine tissue producing numerous secretory proteins, including adipokines, a suite of small signalling proteins specifically produced in WAT [2,3]. Under normal conditions, adipocytes are

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involved in the regulation of a broad range of physiological processes but at obesity increased production of cytokines and adipokines lead to the chronic low-grade inflammatory state. The extent of conservation of endocrine WAT functions in the cold-blooded vertebrate, including its capacity to influence systemic inflammatory responses and development of life style related disorders has not been studied until now.

In salmonid fish, precursor cells differentiate into adipocytes continuously [4], however, the exact origin of preadipocytes is not known. In mammals, development of white adipocytes is thought to begin with progenitor cells from the perivascular compartment (pericytes) in WAT, characterised by expression of smooth muscle actin 22α, also known as transgelin (TAGLN), and other vascular markers. Isolated pericytes differentiate into mesenchymal stem cells (MSCs), which in turn, give rise to various cell types including osteoblasts, chondrocytes, smooth muscle cells, fibroblasts, macrophages and adipocytes [5-9]. Mammalian adipose stromal-vascular fraction (aSVF) harbours a population of progenitor cells that is also capable to differentiate *ex vivo* into cells and tissues of mesodermal origin, thus suggesting their perivascular origin. Atlantic salmon WAT contains a large number of fibroblast-like precursor cells that can differentiate to mature adipocytes *in vitro* [10,11], however, their stem cell features have not been characterized yet.

In mammals, adipogenesis includes three distinct stages. Following active proliferation and the phase of determination, cells reach confluence, followed by hormonal induction and terminal differentiation. Re-entry into the cell cycle of growth-arrested cells at confluence involves several rounds of proliferation, referred to as mitotic clonal expansion. Secondary growth arrest and induction of the transcription factors CCAAT-enhancer-binding protein (C/EBP) α and peroxisome proliferator-activated receptor (PPAR) γ mark the end of the mitotic clonal expansion phase and entry into terminal differentiation with transcriptional activation of genes defining the mature adipocyte phenotype.

The few performed studies indicate that adipogenesis in fish bears many resemblances to that of terrestrial vertebrates [10-12], though there certainly exist as yet undiscovered species-specific differences. Atlantic salmon precursor cells take longer than mammalian to acquire the mature adipocyte phenotype in culture, due to low incubating temperature, typical of salmonid fish habitat. Further, mammalian cells are able to produce lipid droplets (LDs) from glucose alone, while salmon preadipocytes require lipids in order to achieve the mature phenotype [10]. Hence it is to be expected that white adipocytes and their precursors in a cold-blooded vertebrate may have both evolutionarily conserved and specific features.

The aSVF primary culture enabled investigation of early events involved in the adipogenic determination as well as processes characteristic of the later terminal differentiation phase. Particular emphasis was placed on genes governing nutrient metabolism and stress and inflammatory responses.

Results

Cell culture characterization

A short summary of adipocyte development and cultivation conditions is presented in Fig. 1. In the subconfluent stage during the first seven days of culture, aSVF cells had the highest proliferative activity (Fig. 2A). Approximately at day 7, cells reached the confluent stage. Two-day adipogenic hormonal induction was applied at that time point. This was followed by the terminal differentiation stage during which cells acquired a more rounded shape. Morphological changes observed by electron microscopy images at days 15 and 30 were characteristic of the terminally differentiating phenotype of adipocytes, including a relatively low mitochondrial number, large size of LD and the nucleus located between LDs and cell membrane (Fig. 3A, B). Oil red O staining also showed a high degree of lipid accumulation in mature adipocytes at day 30 (Fig. 4A). A steady decrease in the extracellular superoxide dismutase (SOD) activity was observed from day 1 to day 30 (Fig. 4B).

Subconfluence (days 1 and 4)

Microarray analyses of aSVF in the first days of culture, revealed concurrent expression of genes characteristic of MSCs, immune cells and cells of perivascular origin (Fig. 5). Platelet-derived growth factor A (PDGFA) is a major regulator of proliferation and migration of mammalian adipose-derived MSCs [13]. Glomulin is essential for normal development of the vasculature while the lymphatic vessel endothelial hyaluronan receptor 1 (LYVE1) is a marker of lymph vessels and endothelial cells in mammals. Chemokine receptor 4 (CXCR4) and other chemokine/cytokine receptors are involved in cell mobilisation and retention in several populations of MSCs and in immune cells [14-16]. We have selected eight co-expressed genes encoding proteins involved in nucleotide metabolism, DNA replication and regulation of cell cycle and their averaged profile, due to their involvement in the similar cellular functions, was shown in Fig. 2B. The lowest expression of genes from this category was at day 1 (also see Fig. 5). The subsequent increase was in concordance with the results of proliferating cell nuclear antigen (PCNA) assay that revealed the highest proliferation rate at day 4 (Fig. 2A). In parallel, microarray analyses showed a decreased expression of non-adipogenic cell markers. These genes had high levels at day 1 (Fig. 5). Tumour necrosis factor (TNF) α,

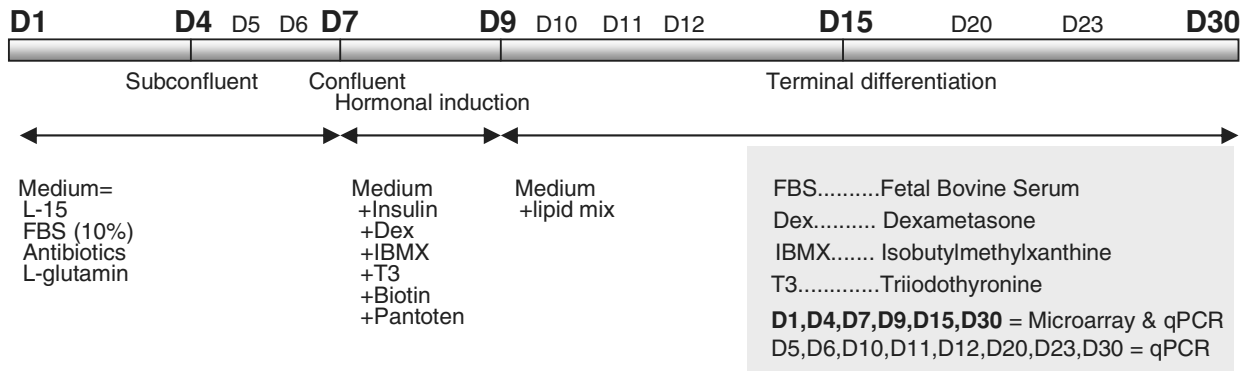

Figure 1 Summary of procedures and the key stages.

a proinflammatory cytokine and a potent negative regulator of adipocyte differentiation in terrestrial vertebrates, was up-regulated before confluence as well as a panel of TNF-related genes and receptors (Fig. 5 and 6). We observed up-regulation of PPARγ already at day 4 (Fig. 7). This is the master regulator that co-ordinately activates transcription of adipocyte-specific genes [17,18].

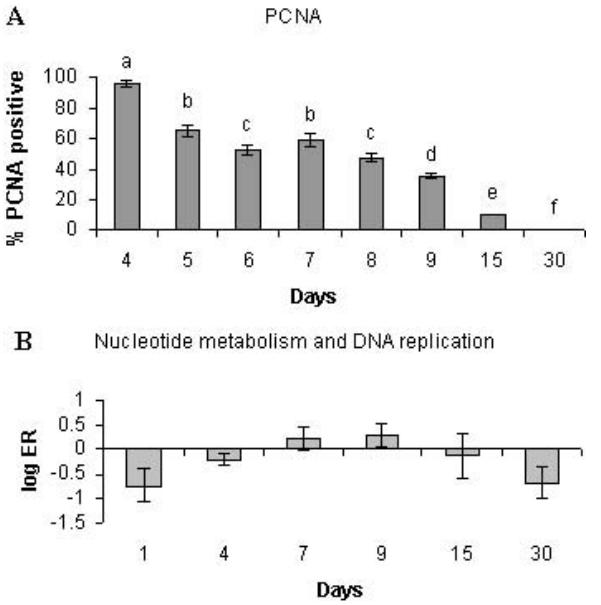

Figure 2 Cell division, DNA replication and nucleotide metabolism. **A**: cell proliferation was assessed by immunocytochemical detection of proliferating cell nuclear antigen (PCNA). Data (labeling index) are shown as mean ± SEM (n = 5). Different letters indicate significant differences ($p \leq 0.05$). **B**: expression changes (mean log-ER ± SE) of eight genes (Cdc45, cell division control protein 42 homolog, anti-silencing function 1B, ribonucleoside-diphosphate reductase large subunit, cold inducible RNA binding protein-1 and 2, chromosome segregation 1-like protein, chromosome-associated kinesin KIF4A) analyzed with microarrays.

Confluence (day 7) and hormonal induction (day 9)

Cells reached confluence at day 7. Up-regulation of retinoblastoma-like protein 1 (RBL1) and p53-like protein (Fig. 5) was in concordance with the temporary cell cycle arrest at G_0/G_1, which is commonly observed at this stage in most mammalian cell lines. After stimulation with hormones, cells re-enter the cell cycle and undergo mitotic clonal expansion before ultimate exit from the cell cycle. At day 9, expression of RBL1 and p53-like protein decreased while cyclins E1 and B2 were stimulated. Two days after addition of the differentiation media, cells changed to a more rounded shape. This coincided with the up-regulation of a suite of motor contractile proteins (Fig. 5). Interestingly, microarray showed elevated expression of TAGLN at day 7 and day 9. TAGLN is a marker of pericytes, smooth muscle-like cells surrounding vasculature, that are closely related to the MSCs and fibroblasts [19]. Positive and negative regulators of myogenic differentiation showed opposite changes: decrease in myogenic factor D (MyoD) and increase in bone morphogenic protein 4 (BMP4) (Fig.7). We also observed up-regulation of several genes for Ca^{2+} binding proteins (Fig. 5). Calcium is involved in the control of the whole adipogenic process, from multipotent stem cells to adipocytes [20,21]. Increases in cytoplasmic calcium during this phase inhibit adipogenesis [21-24]. Days 7 and 9 were marked by the expression changes of C/EBPs. The early adipogenic marker C/EBPβ, was up-regulated until confluence, whereas transient induction of C/EBPδ and consistent increase of C/EBPα were observed after the addition of hormones at day 9 (Fig. 7). Overall, C/EBPβ and C/EBPδ work sequentially and predate the expression of C/EBPα [12]. We observed different expression changes in a number of genes encoding chemokines and cytokines. The leukocyte cell-derived chemotaxin 2 (LECT2) was re-activated at day 9 and remained up-regulated until the end of the study period (Fig. 6). LECT2 is a potent neutrophil chemoattractant, which also affects development of

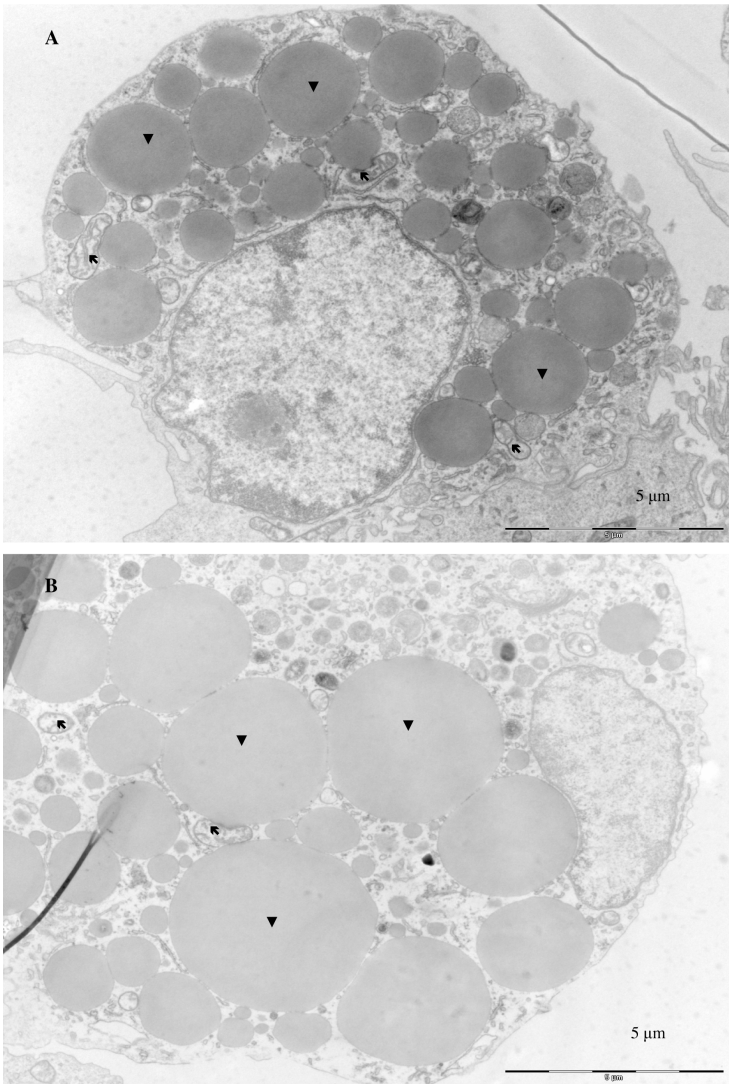

Figure 3 Atlantic salmon aSVF primary cell culture. **A, B**: Electron micrographs of representative salmon adipocytes day 15 (A) and day 30 (B). Three weeks after induction of differentiation (day 30), a large portion of cytoplasmic space was filled with LDs, the number mitochondria was reduced and the nuclei were located between LDs and cell membranes. Bars: 5 μm. Arrows points to mitochondria and arrowheads to lipid droplets

chondrocytes and osteoblasts [25,26]. In contrast, TNFα and related genes were down-regulated (Fig. 5 and 6).

Terminal differentiation and the late phase of white adipocyte maturation (days 12-30)

The increase in intracellular lipid levels measured by Oil Red O (Fig. 4A) was in line with the up-regulation of genes for several proteins of lipid metabolism (Fig. 5), including lipoprotein lipase (LPL) (Fig. 7). The expression of fatty acid synthase (FAS) was highest from day 15 to day 30 (Fig. 7) in agreement with the profile of FAS observed in murine adipocytes [27]. Metabolic alterations were also supported by the regulation of key genes from major carbohydrate pathways (Fig. 8). Glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (PGD) from the oxidative pentose phosphate pathway (PPP) were up-regulated from day 12 to the end of adipogenic differentiation indicating increased need for NADPH that is required for TAG biosynthesis. To synthesise nucleotides, cells need large amounts of ribose-5-phosphate (R5P). The PPP uses glucose to provide R5P and NADPH, however, the output can be modified according to the cells' current needs, towards the production of either one of the products. The increase of phosphofructokinase (PFK) and pyruvate kinase (PK) expression from day 15 could be an evidence for the importance of glycolysis in TAG-accumulating adipocytes. In addition to glycolysis and contrary to what was earlier believed,

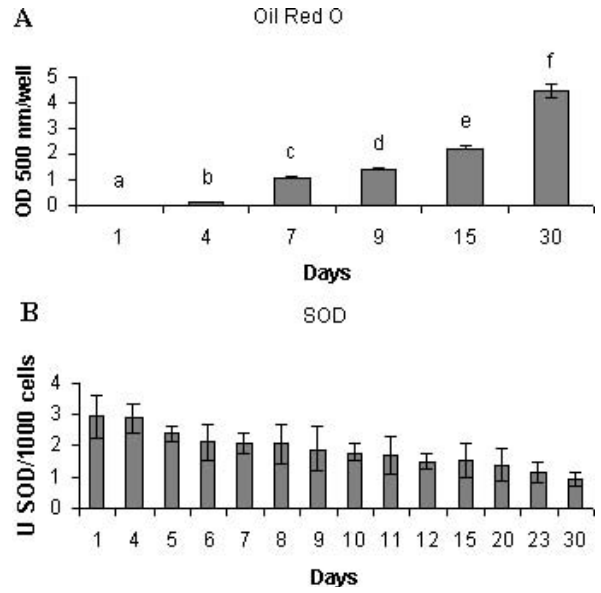

Figure 4 A: lipid content in adipocytes was quantified with Oil Red O. Absorbance of extracted dye was measured at 500 nm. Data are shown as mean ± SEM (n = 5). Different letters indicate significant differences ($p \leq 0.05$). **B**: superoxide dismutase (SOD) activity in culture media. Different letters indicate significant differences ($p \leq 0.05$). Data shown are mean ± SEM (n = 5).

adipocytes seem to be able to use alternative means (glyceroneogenesis) to produce glycerol-3-phosphate (G3P) needed for TAG synthesis [28,29]. Two key glyceroneogenic enzymes, cytoplasmic malate dehydrogenase (MDHC) and cytosolic phosphoenolpyruvate carboxykinase (PEPCKC) were also up-regulated during terminal differentiation. These changes did not necessarily mean a switch to anaerobic metabolism. Microarray analyses revealed a decrease of hypoxia inducible factor (HIF1α) while the negative regulator of HIF1α (HIF prolyl hydroxylase) was induced (Fig. 5).

Day 15 was marked with the expression changes of genes involved in differentiation and cell cycle. Increase was observed in the anti-osteogenic [30] growth arrest specific protein 6 (GAS6) and adipogenic markers adipsin and visfatin (Fig. 5). The up-regulations of RBL1, p53-like protein and growth arrest and DNA-damage-inducible (GADD45) γ were in concordance with the marked attenuation of cell proliferation. Interestingly, many immune-related genes exhibited sustained up-regulations, which began shortly before or at the onset of terminal differentiation (Fig. 6). Programmed death ligand 1 (PD-L1) is involved in the blockage of cell cycle in T-cells [31]. The up-regulation of decoy receptor interleukin (IL) 13 receptor alpha 2 (IL13Rα2) implies suppression of the anti-inflammatory IL4/IL13 axis [32]. Janus kinase 1 (JAK1) and 5-lipoxygenase activating protein (FLAP) play pivotal roles in respectively, cytokine receptor signalling and biosynthesis of eicosanoids (lipid mediators of inflammation). Galectins (Fig. 5) are carbohydrate binding proteins involved in various immune processes [33]. Notably, expression of a panel of immune genes decreased by day 30.

The most noticeable aspect of the last stage of adipogenesis was co-ordinated activation of genes involved in various stress responses. Several components of the activator protein complex 1 (AP1) (c-Fos, c-Jun, JunB1, JunB2, and JunD), which co-ordinates responses to pathogens and stressors, showed highest expression levels at day 30 (Fig. 5). The observed gene expression changes clearly revealed the endoplasmic reticulum (ER) stress in our culture at this stage. The up-regulated oxidant stress-activated serine/threonine kinase 25 (YSK1, Fig. 5) is a Golgi complex-associated regulator of transport of proteins and lipids to plasma membrane [34]. The A-kinase anchor protein 9 (AKAP9) is an essential mediator in lipolytic pathways and is necessary for the maintenance of Golgi structure through interactions with signalling proteins, including the protein kinase N (PKN1); both AKAP9 and PKN1 showed strong down-regulation at day 15 (Fig. 5). The ER stress response involves a set of mechanisms referred to as unfolded protein response (UPR) [35]. The classical UPR markers X-box-binding protein 1 (XBP1) and activating transcription factor 6 (ATF6) were up-regulated during the lipid-loading phase of adipogenesis (Fig. 9). Our findings point to the increased responses to oxidative stress with time. The glutathione peroxidases (GPXs) (Fig. 5 and 9), which are sensitive to lipid mediated peroxidation [36], were similarly induced and a suite of genes coding for proteins of glutathione metabolism also had high levels, at the time when cells engaged in increased LD formation and expansion. The thioredoxin (TXN) antioxidant system is also involved in the regulation of intracellular ROS [37]. The production of ROS is catalyzed with iron and hem, therefore, the up-regulation of ferritin (the intracellular iron storage protein), and two key enzymes in the metabolism of hem, aminolevulinate δ synthetase and heme oxygenase 2 (HMOX2) suggest the induction of mechanisms that protect against oxidative stress at day 30.

The massive down-regulation of components of the translational machinery and translation initiation factors suggested reduction of ribosomal biogenesis and attenuation of protein translation while the highest expression level of 18S at day 15 suggested profound changes in the composition of ribosomes during terminal differentiation (Fig. 5, 9 and 10). Averaged expression profiles of thirty five highly co-expressed ribosomal proteins, components of the 40S and 60S ribosomal subunits, were shown in Fig. 10. Interestingly, eukaryotic initiation translation factor 5 (eIF5) involved in the

Cellular markers	1	4	7	9	15	30
MSC						
Transgelin	-1.61	-0.74	0.91	0.76	NS	NS
Platelet-derived growth factor, A chain	0.65	0.36	NS	NS	ND	-3.44
Notch1	1.23	0.37	NS	-1.6	-4.81	-3.91
Bone/chondrogenic						
Growth arrest specific 6	-0.49	-0.64	-0.58	-0.35	1.61	0.64
Vasculature						
Glomulin	1	NS	-0.44	NS	NS	-1.09
Vascular adhesion protein 1	-0.4	-0.47	-0.28	NS	1.28	NS
Endothelial adhesion molecule 1	NS	NS	1.51	NS	2.65	2.51
Lymphatic hyaluronan receptor 1	0.81	NS	-1.11	NS	ND	-1.78
Immune						
Chemokine receptor 2	1.36	NS	NS	-0.78	ND	ND
Chemokine receptor-like 1	0.65	NS	NS	NS	NS	-1.36
CXC chemokine receptor	0.46	NS	NS	NS	-0.95	NS
CXC chemokine receptor 4	1.22	0.59	-1.54	-1.1	-3.61	-1.9
C-C chemokine receptor type 6	1.15	0.94	1.04	-1.84	NS	NS
C-C chemokine receptor type 7	0.74	NS	-1.6	-0.74	NS	NS
Adipogenic						
Adipsin	-0.78	-0.97	-3.22	-1.13	0.81	2.21
Visfatin	-0.33	NS	-0.43	-1.2	1.16	1.35
Cell cycle						
Cyclin D1	-0.74	NS	0.93	NS	1.95	2.19
Cyclin A2	-0.76	NS	NS	NS	0.62	NS
Cyclin E1	-2.32	NS	NS	0.77	NS	-1.04
Cyclin B2	-1.69	-0.61	NS	1.05	NS	-2.44
Retinoblastoma-like protein 1	NS	NS	1.19	NS	2.69	2.2
p53 related protein	0.49	0.39	0.96	NS	1.23	1.02
Growth arrest and DNA damage 45 γ1	-0.94	NS	-1.05	-0.47	0.94	0.93
Calcium regulated						
Calpain regulatory subunit	NS	NS	0.5	0.66	NS	NS
Calpactin I light chain	NS	-0.87	0.57	1.39	NS	-0.9
Calponin 2	-0.29	-0.58	NS	0.77	-2.25	-0.97
Calmodulin 3	-1.12	NS	0.69	0.97	1.16	NS
Contractility						
Troponin C-1, skeletal muscle	NS	NS	1.34	NS	2.21	2.05
Tropomyosin alpha 3 chain	-3.85	-1.03	0.62	0.88	NS	-2.36
Troponin I, slow skeletal muscle	-0.79	-0.51	-1.25	0.72	ND	ND
Troponin T-1, fast skeletal muscle	-0.74	NS	NS	0.6	-1.13	ND
Myosin regulatory light chain	-2.58	-1.03	0.97	0.92	1.36	NS
Immune related						
TNF decoy receptor	1.86	0.46	-1.28	-3.09	-2.72	-3.6
TNF receptor 2	0.7	NS	-1.45	NS	-2.39	-1.54
TNF receptor associated protein 1	0.64	0.48	NS	NS	-1.49	-2.26
TNF inducible protein TSG-6 precursor	1.37	0.57	NS	-2.53	-4.3	-3.08
TNF receptor associated factor 3	NS	NS	NS	-0.64	1.69	NS
TNF receptor superfamily member 9	NS	NS	-1.22	-1.46	-1.17	1.61
TNF receptor superfamily member 19	NS	NS	0.8	NS	1.57	1.57
IkappaB kinase complex-associated protein 1	0.61	NS	-0.41	NS	-1.99	-1.45
IkappaB kinase complex-associated protein 2	NS	NS	NS	NS	1.21	NS
Galectin-1	-1.56	NS	-0.77	-0.81	1.23	1.27
Galectin 2	-0.28	NS	NS	NS	0.96	NS
Galectin-3	1.33	NS	NS	-0.74	2.29	1.55
Galectin-9	-1.99	NS	-1.25	-1.47	1.24	1.58
Toll-like receptor 3-2	-1.13	-0.49	-0.53	-0.46	1.34	0.83
Interleukin-1 receptor-associated kinase 1-2	NS	NS	0.84	NS	2.07	1.36
Nedd4 family interacting protein 1	NS	NS	NS	NS	NS	0.97
MAPK/ERK kinase kinase kinase 3	-0.54	NS	NS	NS	1.27	1.09
AP1 complex						
Transcription factor jun-B1	NS	NS	-0.97	-1.51	NS	0.87
JunDLa	0.41	NS	-1	-0.38	-1.04	1.14
C-Fos protein	0.53	NS	NS	NS	NS	0.35
Transcription factor jun-B2	0.63	NS	-0.49	-1.78	-0.49	0.9
Proto-oncogene protein c-fos	NS	NS	0.26	NS	1.25	0.97
C-Jun protein	0.68	NS	-1.68	-1.65	-3.92	1
26S proteasome non-ATPase regulatory subunit 14	NS	NS	-0.26	NS	-1.57	0.79

Lipid metabolism	1	4	7	9	15	30
Apolipoprotein E-1	-0.68	NS	NS	-1.19	0.8	1.17
CREB-binding protein	NS	NS	NS	NS	0.89	0.72
Fatty acid-binding protein-3	-0.98	NS	0.84	0.55	1.05	1.01
Cytochrome P450 2J2	NS	NS	NS	NS	2.83	2.91
Cytochrome P450 27	NS	NS	NS	NS	1.03	1.29
Cytochrome P450 3A27	NS	NS	NS	-0.78	1.79	1.74
Cytochrome P450 2K4-2	NS	NS	0.64	NS	NS	0.91
Acyl-CoA-binding protein	NS	NS	1.17	NS	1.82	1.68
Acyl-Coenzyme A dehydrogenase	NS	NS	0.99	NS	1.77	1.44
Acyl-coenzyme A thioester hydrolase	-1.25	NS	NS	NS	2.38	1.26
Carbohydrate metabolism						
Fructose-bisphosphate aldolase A	-0.75	NS	0.78	NS	1.55	0.76
Pyruvate dehydrogenase [lipoamide] kinase	NS	NS	NS	0.6	NS	1.46
Transaldolase	-0.79	NS	NS	NS	0.77	0.46
D-3-phosphoglycerate dehydrogenase	NS	-0.22	NS	0.67	0.99	-0.72
Malate dehydrogenase, cytoplasmic	-0.46	NS	NS	0.87	NS	2.1
ER stress						
X-box binding protein 1	NS	-0.64	0.35	NS	1.45	-1.02
Serine/threonine-protein kinase 25	-0.47	NS	1.12	NS	1.58	0.99
A-kinase anchor protein 9	0.63	NS	-0.73	NS	-2.84	NS
Protein kinase N1	1	0.29	NS	-0.38	-2.7	NS
Calcium homeostasis endoplasmic reticulum protein	0.39	0.31	-0.47	NS	NS	NS
Schwannomin interacting protein 1	-0.74	-0.45	1	NS	1.52	1.47
Derlin-1	NS	NS	NS	NS	NS	0.71
p97-interacting cofactor p47		NS	NS	NS	NS	0.98
p62	NS	-0.57	-2.18	-2.39	-1.12	2.02
Transport-associated protein	NS	0.33	NS	-1	1.36	1.17
Protein translation						
Eukaryotic translation initiation factor 4A	NS	NS	NS	-0.39	-1	-1.27
Eukaryotic translation initiation factor 2	NS	NS	NS	NS	-1.27	NS
60S ribosomal protein L32-3	0.72	NS	0.19	0.34	-0.88	-1.38
60S ribosomal protein L37a	1.04	NS	0.41	NS	-0.95	-1
40S ribosomal protein S21	0.82	NS	NS	NS	-1.27	-2.12
40S ribosomal protein S29	1	-0.37	0.36	0.64	-0.92	-1.62
18 S ribosomal RNA	NS	NS	0.56	NS	1.92	0.675
Eukaryotic translation initiation factor 5	NS	NS	-0.47	-0.87	NS	1.26
Protein folding						
Heat shock factor 2-1	NS	NS	NS	NS	1.51	NS
Heat shock protein 75 kDa-2	0.72	NS	0.63	NS	1.07	NS
Heat shock protein cognate 40	-3.15	-2.88	-2.01	NS	1.47	0.89
Protein degradation						
Proteasome subunit alpha type 3	0.51	0.53	1.83	NS	3.15	2.76
26S protease regulatory subunit 6B 2	-0.44	NS	NS	NS	NS	1.19
26S proteasome non-ATPase regulatory subunit 13	-0.19	NS	NS	NS	1.18	1.55
Ubiquitin	-1.15	-0.75	-1.27	-1.4	NS	2.16
Ubiquitin ligase SIAH1	NS	NS	0.69	NS	2.12	1.84
Ubiquitin-conjugating enzyme E2-18 kDa	NS	NS	0.91	NS	1.75	1.4
Ubiquitin-like protein SMT3A-2	-0.48	-0.63	-0.59	-0.61	NS	1.32
Phosphotyrosine independent ligand for the Lck SH2	NS	-0.57	-2.18	-2.39	-1.12	2.02
Cathepsin D	0.4	-0.45	-0.35	-0.73	0.56	1.52
Cathepsin K	-0.67	-0.58	NS	NS	0.97	1.16
The antioxidant system						
Superoxide dismutase	NS	NS	0.52	NS	1.4	NS
Selenoprotein W	NS	0.72	0.63	-0.66	1.9	1.68
Uroporphyrinogen decarboxylase	NS	NS	0.56	NS	1.65	0.75
Aminolevulinate, delta-, synthetase 1-2	-0.42	NS	NS	NS	0.58	0.75
Heme oxygenase 2	NS	-0.36	-0.37	0.64	-1.36	1.58
Thioredoxin	-0.62	-0.38	-0.95	NS	NS	2.04
Thioredoxin-like protein 1	-0.28	NS	NS	NS	-0.96	1.06
Thioredoxin-dependent peroxide reductase 1	NS	NS	NS	NS	NS	0.89
Thioredoxin domain containing protein 4	NS	NS	0.72	NS	1.7	1.2
Glutathione S-transferase theta 1	-0.82	NS	NS	NS	0.63	NS
Glutathione S-transferase, mitochondrial	NS	0.27	NS	0.39	0.91	NS
Glutathione peroxidase-gastrointestinal	-0.71	NS	-0.91	-0.38	1	1.24
Glutathione reductase, mitochondrial-2	-0.53	-1.01	-1.55	-0.33	1.05	1.87
Glutathione S-transferase P-1	-0.77	-0.84	NS	NS	2.39	1.55
Gastrointestinal glutathione peroxidase 2	-0.46	NS	NS	NS	1.84	1.97
Hypoxia-inducible factor prolyl hydroxylase 1	NS	NS	NS	NS	1.27	0.64
Hypoxia-inducible factor 1 alpha 2	NS	NS	NS	NS	-1.96	NS
Ferritin H-3	-1.01	-1.65	-2.81	-1.87	-0.74	2.47

Figure 5 Results of microarray analyses, selected genes with expression changes. Samples from days 1, 4, 7, 9, 15 and 30 (5 pooled flasks per day) were compared to an equalised mixture from all time-points. Data are log-ER (expression ratios). Significantly up- and down-regulated genes ($p < 0.01$, t-test, 12 spot replicates per gene) are highlighted with red and green scales. NS - not significant, ND - not detected.

joining of ribosomal subunits resulting in the formation of a functional 80S initiation complex was up-regulated at the last time point (Fig. 5).

The increase of several protein folding heat shock proteins and genes involved in protein degradation was evident during this period (Fig. 5 and 10). The components of the 26S proteasome, ubiquitin and several enzymes involved in the ubiquitin conjugation to target proteins were up-regulated at day 15 and even more markedly at the end of the study period. High levels of 26S proteasome non-ATPase regulatory subunit 14 (POH1), were observed at day 30 while up-regulation of lysosomal proteases (cathepsins) was seen from day 15 (Fig. 5).

Discussion

Our transcriptomic analyses suggest, as expected, that early salmon aSVF culture contains a number of cell types, including vascular cells, macrophages and lymphocytes, in addition to preadipocytes. Similar cell composition of aSVF was reported in mammals [38]. The gene expression profiles at days 1 and 4 implied retention of multipotency of aSVF cells during the early

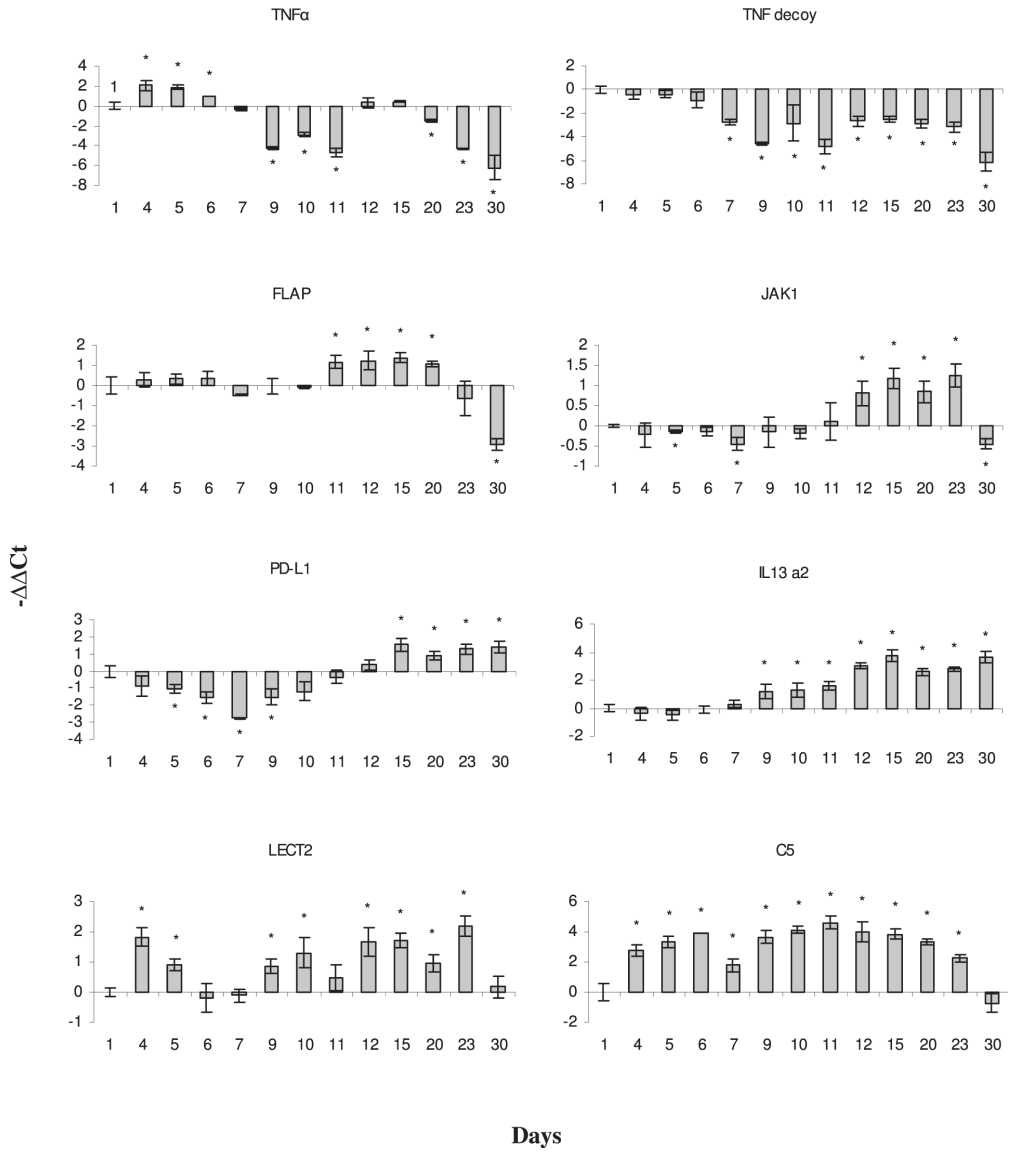

Figure 6 Immune genes analyzed with real-time qPCR. In this and other panels data are shown as -ΔΔCt ± SE (n = 5). Significant difference from day 1 (t-test, $p < 0.05$) is indicated with *. TNFα - tumour necrosis factor alpha, TNF decoy - tumour necrosis factor decoy receptor, FLAP - 5-lipoxygenase-activating protein, JAK1 - janus kinase 1, PD-L1 - programmed cell death ligand 1, IL-13 a2 - interleukin 13, LECT2 - leukocyte cell-derived chemotaxin a2, C5 - complement component 5.

stages of the culture. Decrease in gene expression of markers of osteo/chondrogenic, myogenic, immune and vasculature cell lineages (Fig. 5) and increase in adipogenic markers indicate that the preadipocyte precursors, probably due to their active proliferation, become the preponderant cell type at confluence.

Recent studies in mammals showed that tissue-resident MSCs originate from the smooth-muscle-like pericytes [39,40], which are laid over junctions of endothelial cells in blood vessel walls [41]. Once liberated from the endothelial cell, pericyte is activated and can be considered a stem cell [42]. Characterisics of the WAT population of stem cells and early events in the determination phase are much less studied than the terminal differentiation phase of adipogenesis. The breakthrough in the understanding of elusive origins of the adipogenic MSCs was made only recently in a study of Tang et al. [9], which demonstrated that pericytes surrounding blood vessel walls in WAT are precursor cells of preadipocytes. Gradual attenuation of the vasculature specific genes glomulin and LIVE1 and increase of the pericytic marker TAGLN at days 7 and 9 support perivascular origin of salmon preadipocytes as well. Early PPARγ expression could represent an additional

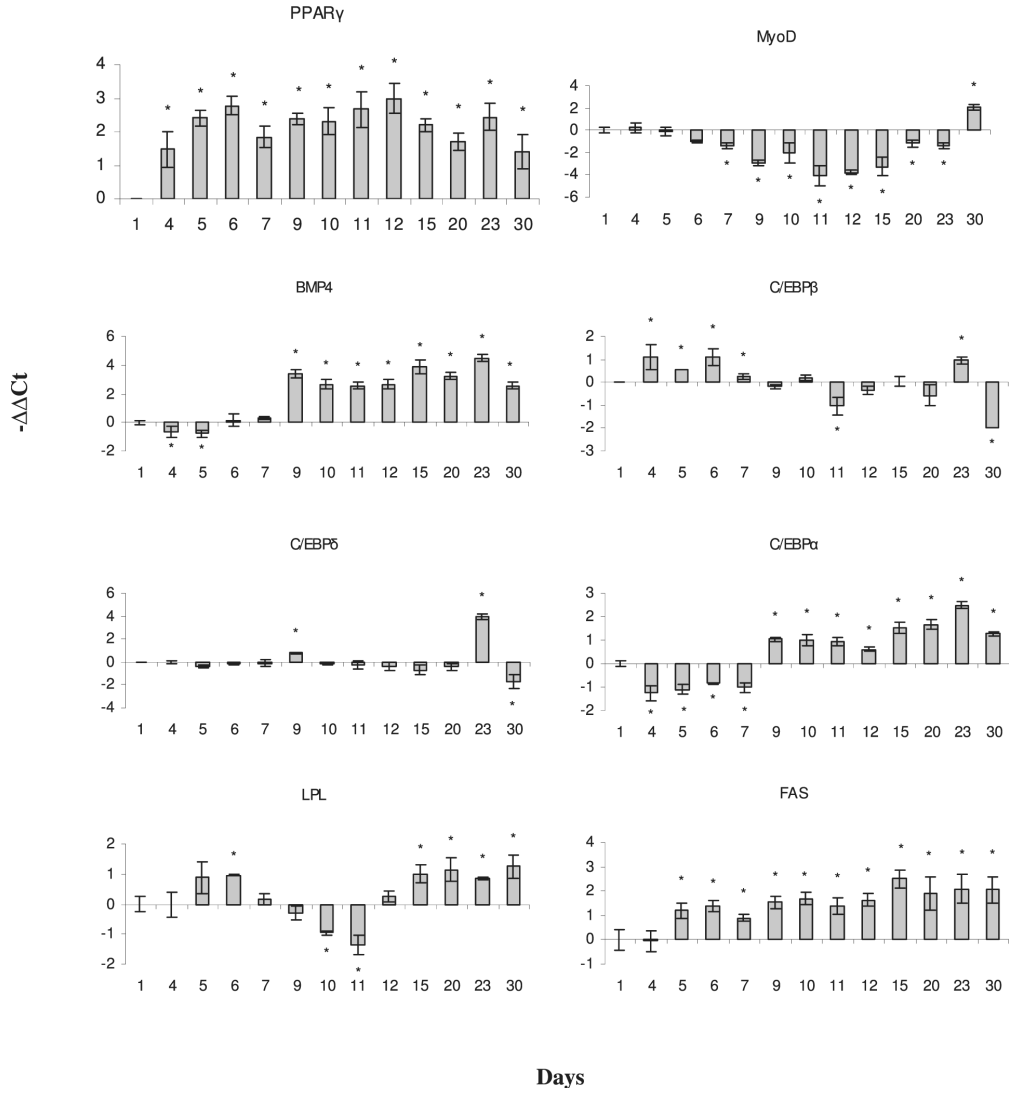

Figure 7 Cell lineage markers analyzed with real-time qPCR. PPARγ - peroxisome proliferator-activated receptor γ, MyoD - myogenic factor D, BMP4 - bone morphogenic protein 4, 1C. C/EBP - CCAAT/enhancer-binding proteins α, β and δ, LPL - lipoprotein lipase, FAS - fatty acid synthase.

evidence of the pericytic identity of adipocytes' precursors in our model since Tang et al. confirmed PPARγ as a specific marker of the perivascular fraction in subconfluent aSVF [9]. Indeed, our finding of high levels of PPARγ mRNA well before confluence contradicts the cell culture studies reviewed in [18] as the expression of C/EBPβ and C/EBPδ predate the expression of PPARγ and C/EBPα [17,18], which then activate adipocyte-specific genes during terminal differentiation. This illustrates the advantage of primary cell cultures, which offer possibilities to gain valuable insight into the early molecular events of adipogenic differentiation.

Commitment of multipotent MSCs to the adipogenic lineage involves simultaneous inhibition of other mesenchymal lineages. Starting from day 6 we observed consistently decreased expression of MyoD, an early marker of the myogenic lineage. MyoD regulates the cell cycle in terminally differentiated myocytes by inducing CDK inhibitor p21, which irreversibly arrests proliferation [43]. MyoD may play a similar role in mature adipocytes and in theory this could account for the increased expression at day 30. Activation of BMP4 from day 9 was an additional evidence for the inhibition of myogenesis. This growth factor equally promotes differentiation of the adipose, cartilage or bone lineages depending on the culture conditions [44,45]. Therefore, activation of BMP4 is necessary but not sufficient for the commitment of preadipocytes. PPARγ is a master

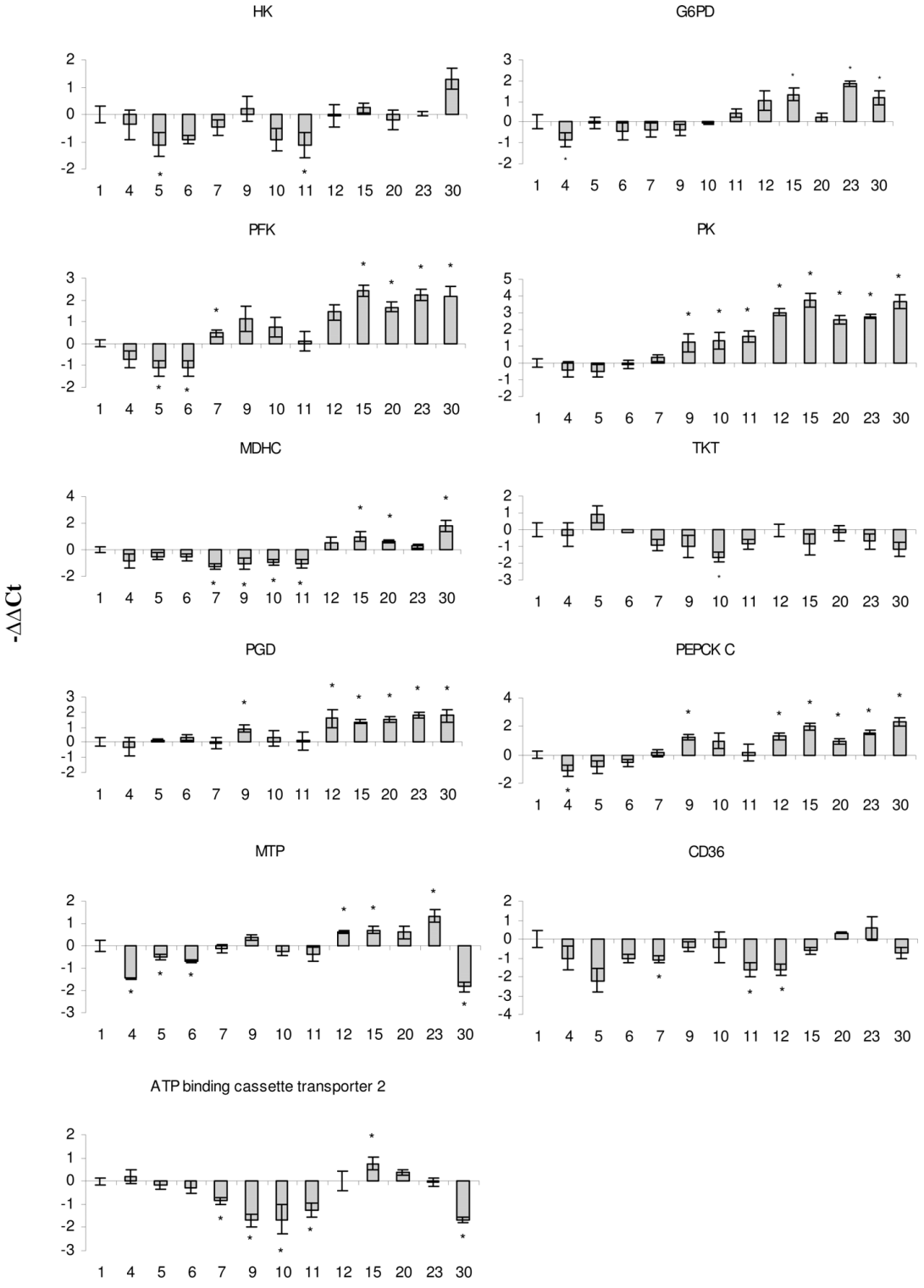

Figure 8 Genes for enzymes from different pathways of carbohydrate and lipid metabolism analyzed with real-time qPCR. HK - hexokinase, G6PD - glucose-6-phosphate dehydrogenase, PFK - phosphofructokinase, PK - pyruvate kinase, MDHC - malate dehydrogenase, PEPCKC - phosphoenolpyruvate carboxykinase C, PGD - 6- phosphogluconate dehydrogenase, TKT - transketolase, MTP - microsomal triglyceride transfer protein, CD36 - cluster of differentiation 36, ATP binding - ATP-binding cassette transporter 2.

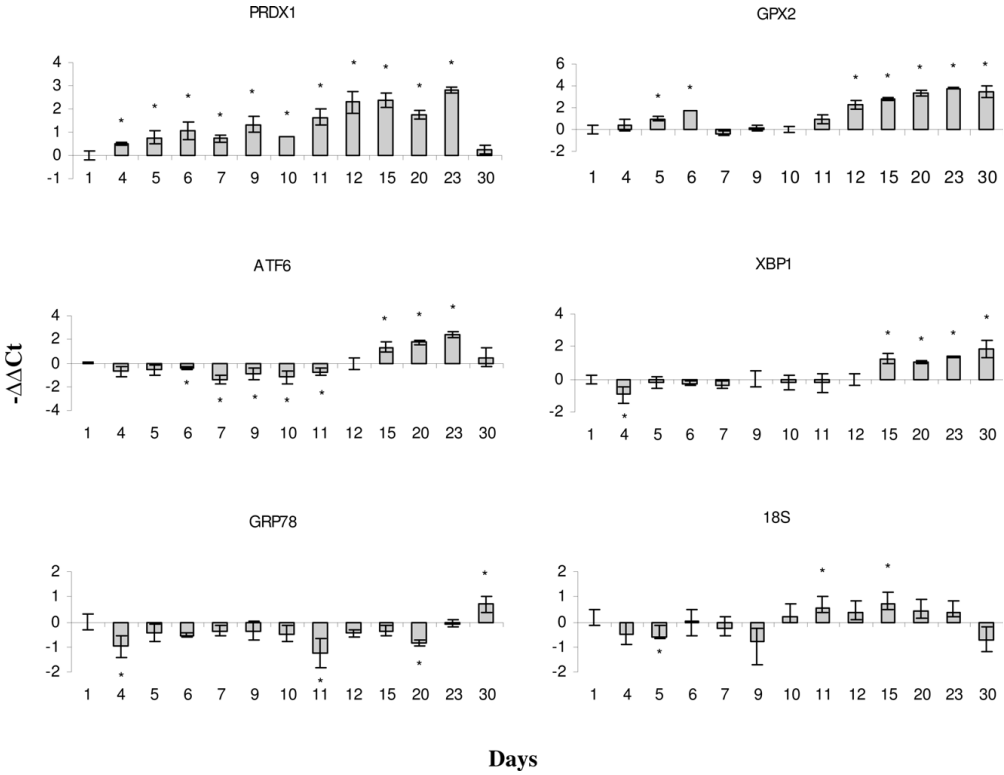

Figure 9 Genes for antioxidants and proteins involved in unfolded protein responses analyzed with real-time qPCR. PRDX1 - peroxiredoxin 1, GPx2 - glutathione peroxidase 2, ATF6 - activating transcription factor 6, XBP1 - x-box binding protein 1, GRP78 - glucose-regulated protein 78, 18S.

adipogenic regulator, which simultaneously inhibits myogenesis, osteogenesis and chondrogenesis [44,46,47]. Thus, high expression of PPARγ observed long before confluence with the peak at day 6 probably posed a barrier to the induction of non-adipogenic mesenchymal cell lineages. Non-adipogenic developmental pathways are expected to be strongly suppressed by the addition of hormones. Yet, we did not find any noticeable changes in the markers of different cell types after day 9 and earliest increased level of the anti-osteogenic GAS6 was observed at day 15.

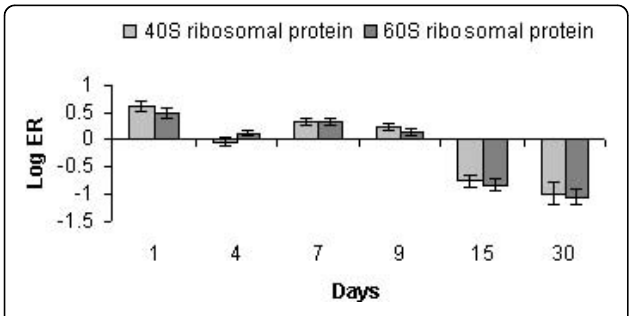

Figure 10 Ribosomal proteins analyzed with microarrays. Data are mean log-(ER) ± SEM of twenty and ten genes involved in 60S and 40S subunit assembly respectively.

Our PCNA results suggested the highest proliferative activity during the first days of culture followed by a moderate reduction of mitotic activity at day 6 and much greater decrease by day 30. Overall, the expression patterns of genes involved in cell cycle were in concordance with these changes. Highest levels of the key negative cell cycle regulators (e.g. RBL1, p53-like, GADD45γ) were observed during days 15-30. An additional evidence for the anti-proliferative status was the up-regulation of several pro-apoptotic genes, including retinoic acid-regulated apoptosis-related protein 3 (APR3). APR3 arrests cell cycle by suppressing the activity of cyclin D1 [48], which was also induced at the latest stage. Differentiation of adipocytes in mammals is associated with a reduction in proliferation at confluence followed by subsequent activation or clonal expansion and eventually with cell cycle arrest at terminal differentiation [49]. Our results revealed the diversity of mechanisms potentially employed in the execution of the similar stage of events that occur during Atlantic salmon adipogenesis. Given that WAT is a highly specialized organ that functions to regulate

metabolic homeostasis and energy balance, nutrient sensing mechanisms could be opted for a role in the control of proliferation. Growth cycle arrest of salmon adipocytes was in parallel with the up-regulation of the fatty acid binding protein 3 (FABP3) that participates in the uptake, intracellular metabolism and transport of long chain fatty acids. FABP3 seems like a good candidate that could be involved in this type of regulation since its growth arrest activity was previously demonstrated in mammalian epithelial cells [50]. Further, minimizing protein synthesis may also control proliferation as cell cycle arrest is a well established consequence of the general translational arrest, implied from day 15 onwards.

Microarray analyses suggested changes in nutrient metabolism in the late phase of adipogenesis (day 30). As a consequence of the up-regulation of pyruvate dehydrogenase kinase that inactivates pyruvate dehydrogenase, the Krebbs cycle most likely relied on the preferential use of acetyl-CoA from FA oxidation rather than pyruvate to cover the cellular energy requirements. Also, the expression of genes involved in FA β-oxidation, acyl-Coenzyme A dehydrogenase and acyl-Coenzyme A binding protein, steadily increased towards the end of adipogenesis. The up-regulation of G6PD and PGD pointed to the importance of the PPP, which generates NADPH required for accumulation of lipids. The PPP can be seen as an alternative to glycolysis, because in addition to generating NADPH and R5P, it can also provide cells with G3P, the phosphorylated glycerol backbone suitable for TAG synthesis. Induction of glycolytic PFK was also in line with the increased production of G3P and TAG biosynthesis. Biosynthesis of carbohydrates in carnivourous fish species could be important due to the very low carbohydrate dietary level in their natural diets [51]. Indeed, we have seen regulations in all major glucose metabolic pathways in our model (Fig. 8); however, differentiation of adipocytes did not involve induction of HK, one of the key enzymes of glucose metabolism that only showed slight up-regulation at day 30. This indirectly suggested that the transformation of gluconeogenic amino acids into glucose is important in adipocytes of Atlantic salmon, at least *in vitro*. In addition, late activity of two key glyceroneogenic genes, MDHC and PEPCKC implied that glyceroneogenesis, which can be fed with mitochondrial intermediates derived from lactate and gluconeogenic amino acids, in addition to pyruvate, was active in developing fish adipocytes. To conclude, these findings illuminate potentially novel aspects of nutrient metabolism in adipocytes, which might take advantage of the coupling of glycolysis, the PPP and glyceroneogenesis, in order to fulfil shifting demands for the three major products of these pathways: G3P, R5P and NADPH.

NADPH produced by PPP is also crucially important for the cellular antioxidant defence since it is required for the regeneration of oxidized glutathione by glutathione reductase (GSR). Up-regulation of GSR and a suite of genes involved in metabolism of glutathione, thioredoxin and iron was a prominent feature of late salmon adipocyte differentiation (days 15-30). Indeed, a recently introduced concept [52-54,4] clearly demonstrated ROS as anti-adipogenic molecules that inhibit preadipocyte proliferation and differentiation. A reduced redox state in WAT is now recognised as a distinct characteristic of visceral obesity in mammals, with overexpression of GPX peroxidases and high content of hydrophilic antioxidant gluthathione associated with pro-adipogenic processes. Induction of the number of GPXs, known to be sensitive to lipid peroxidation, implied ROS-provoked response in our culture and the need to maintain highly reduced state of the intracellular environment. However, contrary to what could be expected in situations of high ROS production, neither gene expression nor activity of SOD increased. In fact, the activity of this important ROS scavenger progressively declined during adipocyte differentiation. Thus, our data corroborated the indespensible role of glutathione-based antioxidant system in the maintenance of the reduced intracellular state in white adipocytes of fish.

Accumulation of lipids in adipocytes coincided with the activation of genes coding for secreted proteins, such as adipokines adipsin and visfatin. Together with the previous finding of leptin secretion by Atlantic salmon adipocytes [10], this supports the notion of WAT as an active endocrine organ in fish. Enhanced secretion may impose an additional load on the ER actively engaged in lipid droplet synthesis [55]. The ER compartment is suspected to exert a great deal of control over adipogenesis and association of obesity with ER-stress is firmly established [56]. Up-regulation of XBP1 and ATF6 at day 15 in salmon adipocytes implied activation of UPR in response to perturbations in the ER homeostasis [57]. Moreover, endoplasmic GRP78, which is located downstream from ATF6 in the UPR signalling cascade was down-regulated throughout the whole studied period but increased in expression at day 30. A number of changes that are typical of UPR [35] was a hallmark of the latest stages of adipogenesis in our model. UPR as a collection of pathways aimed at restoring ER function may serve different adaptive roles and is observed both under pathological and normal physiological situations [58]; for example, it is part of the developmental program in highly specialized secretory B and T cells [59] and ß-pancreatic cells [60]. The revealed UPR in developing fish adipocytes appears as a highly tailored homeostatic mechanism that is activated when

increased number and size of LDs meat the limited ER capacity. Although ER is mainly considered to be a protein-folding factory, all proposed models of LD formation emphasize the engagement of the ER compartment in the process [61].

UPR utilises two broad strategies to relieve stress in the ER: increased clearance of misfolded proteins from the ER and reduction of new protein influx into the ER. We found ample evidence for the activation of the 26S proteasome and autophagy-lysosomal proteolytic pathways, which degrade misfolded proteins. Components of the membrane protein complex, derlin 1 and p97-interacting cofactor p47, which mediate transport from the ER lumen into the cytosol were induced at day 30, while other proteolytic pathways were up-regulated even earlier. UPR-induced proteolysis may be employed to initiate the breakdown of superfluous proteins and organelles that must be replaced by more highly specialised cellular components during the adipocyte development. Without a doubt, considerable remodelling of the cellular architecture occurs during terminal differentiation of Atlantic salmon adipocytes.

UPR also activates genes involved in protein folding. Hsp40s and other J-domain-containing proteins act as co-chaperones for Hsp70s helping to restore homeostasis in the ER and in addition may have pro-degradation roles. Down-regulation of translational machinery is commonly observed during UPR [35], and in our study, it involved a dramatic reduction in expression of ribosomal proteins.

Alteration of the ribosomal composition is most certainly involved in the multiple regulation of the ER-stress response. The curious up-regulation of the 40S subunit 18S rRNA through most of the terminal differentiation against the majority of down-regulated ribosomal proteins is bound to have important consequences given the high level of 18S constitutive expression. This could signify a switch to a less efficient protein translation as increase in 18S negatively affects the ribosomal subunit ratio that favours protein production [62]. On the other side, reduced expression of 18S at the very end of differentiation could improve the effectiveness of protein synthesis in adipocytes that acquire secretory phenotype. In conclusion, fully matured adipocytes seem to reprogram the pattern of gene expression to sustain a certain level of protein production, congruent with the now recognised endocrine functions of WAT.

Secreted adipsin and visfatin have multiple immune functions as most other adipokines [63]. Overall, expression changes of a large number of immune genes were characteristic of Atlantic salmon aSVF culture. Dissimilar temporary profiles suggest different regulatory and physiological roles of these genes. Additionaly, high initial levels and subsequent decrease of the chemokine receptors, TNFα and a panel of TNF-related genes could be explained by the changes in relative abundance of non-adipogenic cells that possess pronounced immune properties. In mammals, TNFα has high anti-adipogenic activity [64], which was recently shown to be conserved in fish [65]. Therefore, down-regulation of the TNF axis at days 7-9 was probably important for the onset of adipocyte differentiation. However, a number of immune genes showed either stable increase since day 4 (complement component C5) or biphasic regulation with the second activation after hormone induction (LECT2). Highest expression levels of many genes with diverse roles in different inflammatory pathways were observed during terminal adipocyte differentiation. Numerous independent studies have provided evidence that a set of inflammatory and stress-response genes is activated in obesity in mammals (reviewed in [66]). Some of these genes likely have important roles in the coordination of homeostasis in WAT, similar to the established roles of TNFα in modulating proliferative abilities in preadipocytes or metabolic activities in mature adipocytes.

An intriguing example is a group of virus responsive genes including TLR3, a receptor of double stranded RNA and a group of galectins, previously not reported to have roles in any aspect of adipocyte biology. The latter includes galectin 9, which showed highly specific responses to viruses in previous studies from our group [67,68]. However, a large portion of immune genes, including complement component C5, JAK1, the key actor of interferon signaling and FLAP, the regulator of eicosanoid metabolism, decreased expression by the time of AP1 establishment. Finally, our results do not permit for major conclusions as to whether immune genes are important for adipogenesis or change expression as a consequence of differentiation and/or stress responses. However, it is clear that Atlantic salmon WAT possesses high potential for immune activity.

It is tempting to explain the activation of immune genes in differentiating adipocytes as a side effect of adaptation to the oxidative and ER stress, since stress and immune responses share common regulatory pathways. Ozcan et al. [56] showed that ER-stress-induced JNK-AP1 axis is the central link between TAG overload in liver and diabetes. Most interestingly, this is in agreement with our study, which shows that the same players are activated in fish adipocytes in response to increased lipid deposition. Continuous administration of lipids led to the maximal observed TAG loading at the end of the studied period, when the collective and highest expression of the AP1 complex components, including all c-Jun and JunB members was also observed. This additionally coincided with the induction of POH1, a regulatory subunit of the 26S proteasome that has a specific

de-ubiquitinase activity towards c-Jun leading to its accumulation and subsequent increase in AP1-mediated gene expression [66]. A suite of highly expressed immune genes at this time supported an inflammatory type reaction in adipocytes. Hence, an unrelieved ER-stress could likely be the major cause of chronic inflammatory responses in lipid overloaded white adipocytes in fish.

Conclusions

Our study revealed concordance between the gene expression profiles and the key events during adipogenic development of the primary culture of aSVF cells, including fluctuations of proliferative activity, induction of adipogenic differentiation and suppression of other mesenchymal cell lineages and final tuning of metabolism towards production and accumulation of lipids. For the first time in a fish species we show that the establishment of mature adipocyte phenotype is associated with high activity of immune genes, activated UPR and responses to oxidative stress. These changes are likely to be part of the normal adipocyte development but may be accentuated when cells are overloaded with lipids. High expression of pro-inflammatory mediators imply that excessive growth of WAT in fish may cause disturbed endocrine function with possible negative effects on health, as seen in mammals.

Methods

Preadipocyte isolation and culture conditions

Atlantic salmon was reared at Nofima Marin station at Averøy, Norway on a commercial diet to weight of 2-3 kg. Fish were anaesthetized with metacain (MS-222), bled by cut of arch bows of the gills and killed by a blow to the head. The experiment was conducted according to the National Guidelines for Animal Care and Welfare of the Norwegian Ministry of Research. Visceral adipose tissue was excised and salmon preadipocytes were isolated as described in Vegusdal et al. [10]. Briefly, the dissected fat tissue was washed with phosphate buffered saline (PBS, pH 7.4) (unless otherwise stated, all chemicals were obtained from Sigma-Aldrich Chemical Co., St. Louis, MO, USA), minced and digested in 0.1% collagenase (type I) in HBSS (1 g tissue/5 mL HBSS) at 13°C for 1 h under shaking and filtered through 250 and 100 μm nylon. The resulting cell suspension was then centrifuged at 700 × *g* for 10 min at 10°C. The buoyant fat layer with mature adipocytes was removed by aspiration, while the preadipocytes were pelleted on the bottom. After washing twice, the cells were resuspended in growth medium containing L-15, 10% fetal bovine serum (FBS), 2 mM L glutamine, 10 mM HEPES, and antibiotics (mixture of penicillin, streptomycin and amphotericin B) and seeded on laminin coated cell-culture flasks at a density of approximately 10 g tissue/25 cm^2. The cells were kept at 13°C and media were changed every 3 days. Cells reached confluence after approximately 1 week. Confluent cells were cultivated for 48 h in an differentiation inducing medium, i.e. growth medium supplemented with 1 μM dexamethasone, 33 μM biotin, 10 nM triiodothyronine, 17 μM panthothenate and 25 μM isobutylmethylxanthine, 20 μg/ml insulin and a lipid mixture (1 μl/ml; corresponding to 45 mg/ml cholesterol, 100 mg/ml cod liver oil FA (methyl esters). After that cells were transferred to a maintenance differentiation media containing growth media only supplemented with 2 μl/ml of lipid mixture. Media was changed every 3 days until the cells reached the final differentiation stage with morphology of mature adipocytes (day 30).

Electron microscopy

Cells for electron microscopy studies were taken from cultures at days 15 and 30. Cells were washed in 0.1 M PBS (pH 7.4), then fixed in 2% glutar aldehyde in 0.1 M cacodylate buffer (pH 7.4) at 4°C for 24 h. The cells were the harvested, rinsed in 0.1 M cacodylate buffer and post-fixed for 60 min in 2% OsO_4 containing 1.5% potassium ferrocyanide, followed by en bloc staining with 1.5% uranyl acetate. Cells were dehydrated in a series of ethanol solutions (70%, 90%, 96%, and 100%) and propylene oxide, and then embedded in epon resin, which was polymerized at 60°C for 24 h. Ultrathin sections (approximately 50 nm) were cut on a Reichert Ultracut E ultramicrotome using a diamond knife. The sections were placed onto formvar/carbon-coated 75-mesh copper grids, post-stained for 2 min with 0.2% lead citrate solution in 0.1 M NaOH, and examined in a Philips CM 100 transmission electron microscope at an accelerating voltage of 80 kV.

Microarray analyses

The samples for microarrays analyses were taken at days 1, 4, 7, 9, 12 and 30. The salmonid fish microarray (SFA2, immunochip) includes 1800 unique clones printed each in six spot replicates. Total RNA was extracted by using RNeasy® Mini Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instruction. RNA was treated with RNase-free DNase I (Qiagen, Valencia, CA, USA), to remove any contaminating DNA. All RNA samples used in our experiments had A260/280 ratios between 1.80 and 2.30. The total RNA concentration was determined at 260 nm using spectrophotometry. Equal inputs from 5 cell flask replicates were pooled for each time point. Equalised control was made by mixing RNA from each time point. The test and control samples (15 μg RNA in each) were labelled with respectively Cy5-dUTP and Cy3-dUTP (Amersham Pharmacia, Little Chalfont, UK). The fluorescent dyes were incorporated in cDNA using the SuperScript™

Table 1 Primers used for real-time qPCR analyses

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Accession number
Tumor necrosis factor alpha (TNFα)	AGGTTGGCTATGGAGGCTGT	TCTGCTTCAATGTATGGTGGG	AF321836
Tumor necrosis factor (TNF) decoy	TCTCCTGGTATTTGCGCTCTGTGGT	TATAAGTCGGTGTGTGAGCGCCTGA	CA351440
Arachidonate 5-lipoxygenase-activating protein (FLAP)	TCTGAGTCATGCTGTCCGTAGTGGT	CCTCCCTCTCTACCTTCGTTGCAAA	CA369467
Tyrosine-protein kinase 1 (Jak1)	GAGGAGTTTGTCCAGTTCGGTCCGT	CATGCACCAGCTTCTTATCCTCCAG	CA368994
Programmed death ligand 1 (PD-L1)	TCAACGACTCTGGGGTGTACCGATG	TCCACCTCATCTCCACCACGTCTC	CA366631
Interleukin 13 receptor alpha 2 (IL13Rα2)	TCTCTGAGCCGCTCAACCTGTCAT	CGTTCCACGACAGCTTTATACGGA	CA348044
Leukocyte cell-derived chemotaxin 2 (LECT2)	CTGTGTTGTCAGAGTGCGAGATGGT	TACACACAATGTCCAGGCCCTGA	EXOB2
Complement component 5 (C5)	AGAACTCTTCCGAGTTGGCATGGT	AGTGATGCTGGGATCCATCTCTGA	CA364804
Proliferator-activated receptor gamma (PPARγ)	CGTGTATCAAGACGCCAGCT	TTGCAGCCCTCACAGACATG	EU655708
Bone morphogenic protein 4 (BMP4)	TCAAGTTGCCCATAGTCAGT	CACCTGAACTCTACCAACCA	FJ195610
Myogenic factor D (MyoD)	CCGCAACACGAAGCAACTATTACAGC	GGAACCCTCCTGGCCTGATAACAC	AJ557150
CCAAT-enhancer-binding protein beta (C/EBPβ)	CAAACTACATTACCAGGC	GTTATGTGTTGCCAGTTG	EU668996
CCAAT-enhancer-binding protein delta (C/EBPδ)	TTGGGCGGTGGAGCCTAT	TTTCCTCGCCCGTGTCAT	EU668997
CCAAT-enhancer-binding protein alpha (C/EBPα)	AGACCTCGGCGAGATTTGT	TGTGGAATAGATCAGCCAGGAA	EU668995
Hexokinase (HK)	GCTGAAGACCAGAGGCATCTTTGA	GCTGCATACCTCCTTGACGATGAT	AY864082
Glucose-6-phosphate dehydrogenase (G6PD)	TGGTGCAGAACCTCATGGTCCTCA	ATCCCGGATGATTCCAAAGTCGTC	BT044902
Phosphofructokinase (PFK)	AATCCATCGGCGTTCTGACAAGC	GCCCGTACAGCAGCATTCATACCTT	BT059256
Pyruvate kinase (PK)	TGCCTTCATTCAGACGCAGCA	CAGATGATTCCGGTGTTGCGA	BT043851
Malate dehydrogenase cytoplasmic (MDHC)	AGACGTCCACCACTGCAAGGTCAA	TTAACAGCGTCGAAGCAGGCCA	BT043497
Cytosolic phosphoenolpyruvate carboxykinase (PEPCKC)	AGGGCATGGACCAGGAACTCC	GGGCTCTCCATCCTGGGATGT	BT072418
6-phosphogluconate dehydrogenase (PGD)	CCAATGAGGCTAAAGGCACCAAGA	CCAGCTTGTCGATGAAGTCATCCA	BT050391
Transketolase (TKT)	TGCCATCTCCGAGAGCAACATC	CCGTGGGAATGGCTCTGAACAT	BT059642
Peroxiredoxin 1 (PRDX1)	CACTGCTGTGGTGGATGGACAGTT	CCAGCGGGTAGAAGAAGAACACCA	est02b08
Glutathione peroxidase 2 (GPX2)	TGTACCTCAAGGAGAAGCTGCCGT	ATTAAGGCCATGGGATCGTCGC	est04e05
Activating transcription factor 6 (ATF6)	CTCACACCATCAAAGCTACAGCGA	GTGTCGCCTCGTCGATTTAACTCA	CA367172
X-box binding protein 1 (XBP1)	CGACTCAAATTCGACAACTGGGC	TTTCTGTCTCTGGCTGTCTGGGCT	CA385697
Microsomal triglyceride transfer protein (MTP)	CAAAGACCAGCGTCAACAACAA	CGCCTCTGTCTCAAAGCTCACT	CA042356
78 kDa glucose-regulated protein (GRP78)	GTGCAGCATGACATCAAGTA	CTCTTCTTCTCGATAACCTT	CA368961
Cluster of differentiation 36 (CD36)	GGATGAACTCCCTGCATGTGA	TGAGGCCAAAGTACTCGTCGA	AY606034
ATP-binding cassette transporter 2	AGCGGGGGAAACAGTAGCAGGA	GCCTGGTCTTGAGATTGTGGGTGT	CA343913
Lipoprotein lipase (LPL)	TGCTGGTAGCGGAGAAAGACAT	CTGACCACCAGGAAGACACCAT	BI468076
Fatty acid(FAS)	TGCCTCAGCACCCTACTCTG	GCTTTACAACCTCAGGATTGGC	BT048827
18S	TGTGCCGCTAGAGGTGAAATT	GCAAATGCTTTCGCTTTCG	AJ427629
Elongation factor 1A (EF1A)	TGCCCCTCCAGGATGTCTAC	CACGGCCCACAGGTACTG	BG933853
Eukaryotic translation initiation factor 3 subunit 6	GTCGCCGTACCAGCAGGTGATT	CGTGGGCCATCTTCTTCTCGA	CX040383

Indirect cDNA Labelling System (Invitrogen, Carlsbad, CA, USA). The cDNA synthesis was performed at 46°C for 3 h in a 20 μl reaction volume, following RNA degradation with 0.2 M NaOH at 37°C for 15 min and alkaline neutralization with 0.6 M Hepes. Labelled cDNA was purified with Microcon YM30 (Millipore, Bedford, MA, USA). The slides were pretreated with 1% BSA fraction V, 5 × SSC, 0.1% SDS (30 min at 50°C) and washed with 2 × SSC (3 min) and 0.2 × SSC (3 min) and hybridized overnight at 60°C in a cocktail containing 1.3 × Denhardt's, 3 × SSC 0.3% SDS, 0.67 μg/μl polyadenylate and 1.4 μg/μl yeast tRNA. After hybridization slides were washed at room temperature in 0.5 × SSC and 0.1% SDS (15 min), 0.5 × SSC and 0.01% SDS (15 min), and twice in 0.06 × SSC (2 and 1 min, respectively). Scanning was performed with Axon GenePix 4100A and images were processed with GenePix 6.0 (Molecular Devices, Sunnyvale, CA, USA). The spots were filtered by criterion (I-B)/(SI+SB) ≥ 0.6, where I and B are the mean signal and background intensities and SI, SB are the standard deviations. Low quality spots were excluded from analysis and genes presented with less than three high quality spots on a slide were discarded. After subtraction of median

background from median signal intensities, the expression ratios were calculated. Lowess normalization was performed first for the whole slide and next for twelve rows and four columns per slide. The differential expression was assessed by difference of the mean log-expression ratios between the slides with reverse labelling (6 spot replicates per gene on each slide, Student's t-test, $p < 0.01$). Complete microarray results are submitted to NCBI GEO Omnibus (GSE18389).

Quantitative real-time RT-PCR

RNA isolated at days 1, 4, 5, 6, 7, 9, 10, 11, 12, 15, 20, 23 and 30 was used for qPCR. Approximately 200 ng of total RNA was reverse-transcribed into cDNA using TaqMan® Gold RT-PCR Kit (Applied Biosystems, Foster City, CA, USA), a 25 μl reaction system according to the manufacturer's protocol. The PCR primers (Table 1) were designed using the Vector NTI (Invitrogen, Carlsbad, CA, USA) and synthesized by Invitrogen. Efficiency was checked from tenfold serial dilutions of cDNA for each primer pair. A 2 × SYBR® Green PCR Mastermix (Roche Diagnostics, Mannheim, Germany), 0.4 μM of each primer, and the cDNA template were mixed in 12 μl volumes. PCR was performed in duplicates in 96-well optical plates on Light Cycler 480 (Roche Diagnostics, Mannheim, Germany). Different controls were used for microarray and qPCR. All time points were compared relative to day 1 in qPCR analyses. Relative expression of mRNA was calculated using the ΔΔCt method. Three commonly used genes (18S, elongation factor 1A and eukaryotic translation initiation factor 3, subunit 6) were tested for stability using the GeNorm and NormFinder. Finally, elongation factor 1A (EF1A) met criteria of stability in the analyzed material. Differences between control and cells at different developmental stages were assessed with Student's t-test ($p < 0.05$).

Cell proliferation

Cell proliferation was assessed by the immunocytochemical detection of PCNA)(Zymed Laboratories, South San Francisco, CA, USA). Cells were washed in PBS and fixed in 70% ethanol for 30 minutes at 4°C. Endogenous peroxidise activity was blocked with with 3% hydrogen peroxide in methanol for 10 min. The cells were washed three times with in PBS, then incubated with a mouse anti PCNA monoclonal antibody (clone PC10) using a PCNA immuno detection kit, following the manufacturer's instructions. The cells were counterstained with Mayer's haematoxylin for 2 min, washed in water, dehydrated in a graded series of alcohol solutions, cleared with xylene, and mounted with Histomount. PCNA-containing nuclei were stained dark brown. Two hundred cells were observed and the percentage of proliferating cell was calculated.

Lipid accumulation in adipocytes

The quantity of lipids in cytoplasm were estimated by oil-red O staining according to Ramirez-Zacarias et al. [70] on days 1, 4, 7, 9, 15 and 30. Briefly, cells were washed twice with PBS, fixed with 10% cold formalin for 30 min, rinsed in water and stained for 2 h with filtered oil red O in isopropanol at room temperature. For relative quantitative measurements of TAGs accumulation, bottles were washed with PBS to remove excess of stain solution, dried, the colour was dissolved in 100% isopropanol and the absorbance was measured spectrophotometrically in a Victor 3 microplate reader PerkinElmer (Wellesley, MA, USA) at 500 nm.

Superoxide dismutase assay

SOD activity was assayed with a kit (Cayman Chemicals, Ann Arbor, MI, USA), which utilizes a tetrazolium salt to detect superoxide radicals generated by xanthine oxidase and hypoxanthine. Colour was measured at 405 nm in a Titertek Multiskan PLUS MKII (Labsystems, Helsinki, Finland) plate reader. One unit of SOD was defined as the amount of enzyme needed to achieve 50% dismutation of the superoxide radical.

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Authors' contributions

MT and BR contributed to the overall experimental design. SS and AK carried out microarray analyses. MT carried out cell culture work, and assay analyses. MT and SS carried out qPCR analyses and produced the first manuscript draft. All authors read, contributed to, and approved the final manuscript.

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Article IV

Alterations in oxidative stress status modulate terminal differentiation in Atlantic salmon adipocytes cultivated in media rich in n-3 fatty acids

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ABSTRACT

BACKGROUND: Regulation of oxidative stress (OS) in adipocytes is an important mediator of their development and dysfunction. n-3 highly unsaturated fatty acids (HUFAs) play essential roles in marine fish, where they have anti-lipogenic effects, but they are prone to peroxidation. The aim of this study was to investigate how the effects of HUFAs in fish adipocytes are modulated by changes in their intracellular redox status.

METHODS: Adipocytes from Atlantic salmon were cultivated on HUFA-rich media and treated with buthionine sulfoximine (BSO) that depleted stores of the antioxidant glutathione (GSH) and exacerbated OS and α-tochopherol (α-TOCH) that protected cells from OS. Gene expression was assessed by qPCR. In addition, phospholipid composition, total fatty acid (FA) composition, TBARS and superoxide dismutase (SOD) activity were determined.

RESULTS: Lipid accumulation and the profile of adipogenic markers were lower in GSH-depleted cells than in α-TOCH-supplemented cells. Large intracellular vesicles were prominent in the BSO and control groups. α-TOCH had anti-apoptotic and anti-inflammatory effects and induced the expression of activating transcription factor 6, a marker of ER-stress. Depletion of GSH was associated with the highest activity of SOD and the highest levels of TBARS. α-TOCH mediated reduction of the OS in the GSH-depleted cells, independent of glutathione peroxidases and the antioxidant SOD.

CONCLUSIONS: α-TOCH is strongly pro-adipogenic while OS induced by HUFAs and BSO have anti-adipogenic effects. Transcriptional modulation of the caspase 3 (CASP3)-independent apoptosis in salmon adipocytes is sensitive to OS.

GENERAL SIGNIFICANCE: Redox metabolism is important in the regulation of piscine adipogenesis and homeostasis.

Key words: adipocytes, HUFA, oxidative stress, gene expression, apoptosis

1. INTRODUCTION

Fatty acids (FAs) play multiple roles in the adipocyte. FAs are major constituents of membrane phospholipids (PL), while FAs in triacylglycerols (TAGs) are a main energy reserve that can be mobilised when required and used as a substrate for β-oxidation. The role of FAs in energy metabolism has stimulated a huge interest in the role that dietary FAs, and particularly long-chain highly unsaturated FAs (HUFAs), play in preventing the development of adiposity in mammals [1] and TAG accumulation in adipose tissue of Atlantic salmon [2]. Fish may be susceptible to oxidative damage in the adipose tissue due to the high levels of n-3 HUFAs in their natural diets. We showed that the unsaturation of FAs of adipocyte membranes increased rapidly by feeding n-3 HUFAs rich diet leading to increased OS and reduced visceral fat level [2]. The mechanisms by which FAs and their derivatives regulate lipid metabolism in adipocytes are complex, and they involve to a large extent the modulation by FAs of the activities of members of the transcription factor PPAR family [3-5]. PPARγ, which is well-conserved between fish and mammals [6-8], regulates many cellular pathways that are necessary for the differentiation of preadipocytes and the establishment of the mature phenotype characterised by an increased ability for lipid storage [9; 10]. HUFAs modulate the activity of both PPARα and PPARγ nuclear receptors in mammals [5; 11; 12] and in fish [13]. HUFAs may exert their anti-lipogenic effects by increasing the production of ROS, accompanied by FA peroxidation, which in turn induce apoptosis. Studies have shown that the number of mitochondria and FA β-oxidation capacity in adipocytes are positively affected by dietary HUFAs in both mammals [14] and fish [2]. HUFA-induced apoptosis has been studied in mammalian tumor cells [15-18] and preadipocytes [5] and adipose tissue in fish [2]. A recently discovered mechanism responsible for the induction of cell death by lipid peroxidiation involves the DNase apoptosis-inducing factor (AIF) [19]. This mechanism does not require caspase 3 (CASP3). Almost all mammalian genes encoding components of the apoptotic machinery have their homologues in Atlantic salmon, including AIF [20].

ROS signalling in adipocytes normally initiates the production of intracellular anti-oxidants that maintain cells in a pro-adipogenic state, while the lack of ROS scavengers inhibits adipogenesis [21]. Several lines of evidence show that protection against increased levels of oxidative stress (OS) in adipocytes relies heavily on the glutathione (GSH) based antioxidant system [22; 23]. Although not studied in adipocytes of fish, deficiency of GSH in mammals increases the risk of intracellular lipid peroxidation and cell death [19]. GSH acts as the reducing agent for glutathione peroxidases (GPXs), which are selenoproteins whose

overexpression promotes obesity in mice [24]. Our recent transcriptome profiling of complete adipogenesis in Atlantic salmon however showed that several GPXs were induced, as were genes involved in GSH metabolism and selenium metabolism in the late phase of terminal differentiation, pointing to the importance of GSH during fish adipogenesis [25]. Further, levels of expression of genes associated with responses to endoplasmic reticulum (ER) stress, and the c-jun N-terminal kinase (JNK) and NFkB pro-inflammatory pathways, were also higher [25]. This agrees with accumulating evidence that shows that the ER becomes overwhelmed when nutrients are present in excess and activates the unfolded protein response (UPR) both in hepatocytes [26] and in adipocytes [25]. The UPR is comprised of several different mechanisms that restore altered homeostasis in the ER [27], and increased lipid deposition in terminally differentiating adipocytes has been characterised by the expression of several classical UPR markers [28]. Inflammatory pathways, including the JNK and nuclear factor kB (NFkB) signalling cascades, are activated together with the UPR in obese mammalian adipose tissue. It has been well-established that transcription factors, activator protein 1 (AP1) from the JNK axis and NFkB are regulated by the intracellular redox state in several tissues [29], and excessive TAG deposition in adipocytes may stimulate their activation through increased lipid peroxidation.

The purpose of the study presented here was to examine the potencies of eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA) to mediate OS in Atlantic salmon white adipocytes. Buthionine sulfoximine (BSO) was used to deplete stores of the intracellular antioxidant GSH in order to exacerbate OS, while α-tochopherol (α-TOCH) was used to alleviate the effects of OS.

We measured by qPCR the expressions of genes coding for proteins in the antioxidant and apoptotic pathways, and the expressions of genes for UPR and inflammatory markers in the presence and absence of α-TOCH and BSO. We measured also the enzymatic activities of the antioxidant superoxide dismutase (SOD) and of the pro-apoptotic CASP3. We estimated the level of lipid peroxidation from the TBARS, the PL composition and the total FA composition.

2. MATERIALS AND METHODS

2.1. Preadipocyte isolation and culture conditions

Atlantic salmon (from Nofima's sea station at Averøy, Norway) were reared on a commercial diet to an average weight of 3 kg. Random fish were sampled and anaesthetized in metacain (MS-222). The arch bows of the gills were cut after the fish had been anaesthetised. The fish were bled for a couple of minutes, and then killed by a blow to the head. The abdomen was cut open to expose the visceral adipose depot. Visceral adipose tissue was carefully excised, avoiding contamination with the intestinal contents. Salmon preadipocytes were isolated essentially as described by Vegusdal et al. [30]. Briefly, the dissected fat tissue was washed with phosphate buffered saline (PBS) (at pH 7.4) (all chemicals were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA), unless otherwise stated) to carefully remove blood cells, then minced, and digested in 0.1% collagenase (type I) in HBSS (1 g tissue/5 mL HBSS) at 13 °C for 1 h under shaking.

The digested tissue suspension was subsequently filtered through 250 and 100 µm nylon filters to remove large particulate material. The resulting cell suspension was centrifuged at 700 x *g* for 10 min at 10 °C. The buoyant fat layer with mature adipocytes on the top of the centrifuge tube and the digestion medium were removed by aspiration, while the preadipocytes were pelleted at the bottom. The cells obtained were washed twice, and then resuspended in growth medium containing L-15, 5% fetal bovine serum (FBS) + 5% of fish serum (FS), 2 mM L glutamine, 10 mM HEPES, and antibiotics (a mixture of penicillin, streptomycin and amphotericin B). The adipose tissue was weighed after excision and isolated cells were seeded onto laminin coated cell culture flasks at a density of approximately 10 g tissue/25 cm^2. The cells were kept at 13 °C. The media were changed every 3 days. The cells reached confluence after approximately 1 week. Confluent preadipocytes were differentiated in an initial differentiation-inducing medium that contained growth medium supplemented with 0.5 µM dexamethasone, 5 nM triiodothyronine, 12 µM isobutylmethylxanthine, and 10 µg/ml insulin. The cells were transferred back to the growth media after 48 h and divided in the four treatment groups. All treatment groups were cultivated in the growth medium supplemented with 0.6 mM EPA and 0.6 mM DHA. The medium for the control group was supplemented with 0.6 mM EPA and 0.6 mM DHA only. The medium for the α-TOCH group was supplemented with 0.1 mM α-TOCH in addition to 0.6 mM EPA and 0.6 mM DHA. The

medium for the BSO group was supplemented with 0.05 mM BSO in addition to 0.6 mM EPA and 0.6 mM DHA. The medium for the BSO+α-TOCH group was supplemented with 0.1 mM α-TOCH and 0.05 mM BSO, in addition to 0.6 mM EPA and 0.6 mM DHA.

The media were changed every 3 days until the cells reached the final differentiation step (which occurred on Day 15). The differentiation and accumulation of lipids in the mature adipocytes were evaluated during the period of cultivation by observing the morphology of the cells. Each experiment was performed in triplicate.

2.2. Fatty acid composition

Total lipids were extracted using the method described by Folch et al. [31]. The chloroform phase was dried under nitrogen gas and the residual lipid extract was re-dissolved in chloroform. The methyl esters of FAs were separated in a gas chromatograph (Hewlett Packard 6890) with a split injector, SGE BPX70 capillary column (length 60 m, internal diameter 0.25 mm and thickness of the film 0.25 μm), flame ionisation detector and HP ChemStation software. Helium was used as carrier gas, and the injector and detector temperatures were both 280 °C. The oven temperature was raised from 50 to 180 °C at the rate of 10 °C min^{-1}, and then raised to 240 °C at a rate of 0.7 °C min^{-1}. The relative quantity of each FA present was determined by measuring the area under the peak corresponding to that FA.

2.3. Phospholipid composition

The phospholipid (PL) composition in adipocytes was quantified using high-performance thin-layer chromatography (HP-TLC). Total lipids were applied using an automatic sample applicator (Linomat 5, CAMAG, Muttenz, Switzerland) onto a 10 x 20 cm HP-TLC plate that had been pre-run in methanol and activated at 120 °C for 20 minutes. The plates were developed fully in methanol:chloroform:hexane using an automatic developing chamber (AMD2, CAMAG, Muttenz, Switzerland). PL classes were visualised with 10% w/v copper sulphate, 4% v/v phosphoric acid (85%) in methanol. The plate was dried (at 140 °C for 5 minutes) on a TLC plate heater (CAMAG, Muttenz, Switzerland). The PL classes were identified by comparison with commercially available standards and the relative distribution between different PL classes was determined by scanning densitometry using a CAMAG TLC Scanner 3, the relative levels present being calculated using an integrator (WinCATS-Planar

Chromatography, Version 1.4.3). Furthermore, a standard mix of all the PL classes was included on each HP-TLC plate to enable the correction of between-plate variations.

2.4. Protein measurements

Protein concentration was determined according to a micro Lowry method [32], with bovine serum albumin as standard.

2.5. Lipid accumulation in adipocytes

The quantity of lipids in the cell cytoplasm was estimated by Oil Red O staining according to Ramirez-Zacarias et al. Briefly, the culture medium was gently aspirated, the cells were washed twice with PBS and then fixed with 10% cold formalin for 30 min. The cells were rinsed in water and stained for 2 h with filtered Oil Red O in isopropanol at room temperature. Relative quantitative measurements of TAG accumulation were obtained from cells washed with PBS to remove excess stain solution and dried. The Oil Red O was dissolved in 100% isopropanol and the absorbance (as a measure of intracellular lipid level) was measured spectrophotometrically in a Victor 3 microplate reader from PerkinElmer (Wellesley, MA, USA) at 500 nm.

2.6. Superoxide dismutase assay

SOD catalyzes the reduction of superoxide into oxygen and hydrogen peroxide. A commercially available kit was used to assay this enzyme activity (Cayman Chemicals, Ann Arbor, MI, USA). The kit utilizes a tetrazolium salt to detect superoxide radicals generated by xanthine oxidase and hypoxanthine. One unit of SOD was defined as the amount of enzyme needed to achieve 50% dismutation of the superoxide radical. Colour was measured at 405 nm in a Titertek Multiskan PLUS MKII (Labsystems, Helsinki, Finland) plate reader.

2.7. Measurements of thiobarbituric acid reactive substances (TBARS)

We used a TBARS colorimetric assay kit (Cayman Chemical, Ann Arbor, MI, USA) to measure lipid peroxidation products. Adipocytes from each treatment group were collected in 100 µl PBS and sonicated three times for 5 seconds on ice. The samples were transferred to 5 ml vials and 100 µl of sodium dodecyl sulfate and 4 ml of colour reagent were added. The vials were sealed and the contents boiled for 1 hour. The vials were then placed on ice for 10 minutes to stop the reaction. The samples were centrifuged for 10 minutes at 1600 x *g* at 4 °C.

150 µl from each vial was loaded onto 96-well plates and the absorbance was measured spectrophotometrically in a Victor 3 microplate reader from PerkinElmer (Wellesley, MA, USA) at 530 nm.

2.8. Caspase 3 activity

Caspase 3 is an intracellular cysteine protease that exists as a proenzyme, becoming activated during the cascade of events associated with apoptosis. A CASP3 colorimetric assay kit (R&D Systems, Inc, Minneapolis, MN, USA) was used to determine whether the different treatments affected the enzyme activity. The cells from each experimental group were lysed to release the intracellular contents. The lysate was then tested for protease activity by adding a caspase-specific peptide that conjugates to a colour reporter molecule, p-nitroaniline. The cleavage of the peptide by the caspase releases the chromophore p-nitroaniline, which was quantified spectrophotometrically. Colour was measured at 405 nm in a Victor 3 microplate reader from PerkinElmer (Wellesley, MA, USA).

2.9. Quantitative real-time PCR

Approximately 200 ng of total RNA was reverse-transcribed into cDNA using a TaqMan® Gold RT-PCR Kit (Applied Biosystems, Foster City, CA, USA) in a reaction system of volume 25 µl. All procedures were carried out using the following protocol: 1 µg of total RNA was used in a 50 µl reaction with a final concentration of 1x TaqMan RT buffer, 5.5 mM $MgCl_2$, 500 µM of each dNTP, 2.5 µM oligo $d(t)_{16}$, 0.4 U/µl RNase inhibitor, and 1.25 U/µl reverse transcriptase. cDNA synthesis was then performed during an incubation of the primer at 25 °C for 10 min, a 60 min RT step at 48 °C, and 5 minutes of RT inactivation at 95 °C. The PCR primers (Table x) were designed using the Vector NTI software (Invitrogen, Carlsbad, CA, USA) and synthesized by Invitrogen. The efficiency of the PCR procedure was checked using tenfold serial dilutions of cDNA for each primer pair. A 2 x SYBR® Green PCR Mastermix (Roche Diagnostics, Mannheim, Germany), 0.4 µM of each primer, and the cDNA template were mixed in 12 µl volumes. PCR was performed in duplicate in 96-well optical plates on a Light Cycler 480 (Roche Diagnostics, Mannheim, Germany). The relative gene expressions represent ΔΔCt, using elongation factor 1A (EF1A) as the reference gene for cell stages. The expression of EF1A did not change during the whole adipogenesis [25]. Elongation factor 1A also met the criteria for stability in this experiment. Differences between

control cells and cells in the different treatment groups were assessed with Student's t-test ($p < 0.05$).

3. RESULTS

3.1. Fatty acid composition of adipocytes

There were no major differences in the FA compositions of adipocytes between the different treatment groups (Table 2). This is expected, since the FA compositions of their respective cultivation media were identical. However, the percentages of HUFAs were significantly lower in the BSO groups than in the control and α-TOCH groups. The HUFAs affected were mainly 20:5n-3 and 22:5n-3. The percentages of saturated FAs in the BSO groups were significantly higher than those of the control and α-TOCH groups.

3.2. Phospholipid composition of adipocytes

Table 3 shows the distributions between different PL classes. Phosphatidylcholine (PC) was the dominating PL class in adipocytes, constituting approximately 60% of the total PLs. Phosphatidylethanolamine (PE) constituted around 15% of the PLs, cardiolipin (CL) around 12%, and sphingomyeline (SM) around 9%. The percentages of CL were significantly lower in the BSO and control groups than in the groups with added α-TOCH. PC was more stable against peroxidation, and this probably meant that it constituted a higher percentage of total PLs when CL was reduced by BSO, as was also the trend for phosphatidylinositol (PI).

3.3. Characterisation of adipocytes

Cells in all treatment groups had a phenotype that was characteristic of adipocytes, with relatively high numbers of accumulated lipid droplets in the cytosol. The lipid levels of adipocytes were quantified by Oil Red O staining (Figure 1). The α-TOCH supplemented cells had significantly higher lipid contents than cells in the control group and BSO group.

Phase contrast microscopy of adipocytes showed that the treatments affected the morphology of the cells. Large intracellular vesicles were more numerous in cells in the control and BSO groups than they were in cells in the α-TOCH group (Figure 2).

The levels of gene expression of two major regulating factors of adipogenesis, namely PPARγ and C/EBPα, were significantly higher in the groups with the highest lipid contents, which

were those to which α-TOCH had been added (Figure 3). However, there were no significant differences in the gene expression of PPARα between the different treatment groups.

3.4. Oxidative stress and apoptosis markers

There were no significant differences in the enzyme activity of CASP3 between the different groups. The activities were 0.039 ± 0.006 ΔOD/mg protein in the control group; 0.038 ± 0.0007 ΔOD/mg protein in the α-TOCH group, 0.044 ± 0.008 ΔOD/mg protein in the BSO group; and 0.024 ± 0.015 ΔOD/mg protein in the BSO+α-TOCH group.

The levels of expression of the apoptosis-related genes, apoptosis inducible factor (AIF), Bcl-2–associated X protein (Bax), and Diablo were, in contrast, significantly higher in the BSO group than in the control group. The level of gene expression of CASP3 was not significantly affected by BSO (Figure 4). Gene coding for AIF significantly decreased in expression in the α-TOCH in comparison to the control group.

The levels of expression of secondary oxidation products, TBARS, were significantly higher in the control group and the BSO group than they were in the cells treated with α-TOCH (Figure 5).

The expressions of gene markers related to intracellular antioxidant protection mechanisms were affected by the treatments (Figure 6a). The gene expressions of GPX3 and GPX2 were significantly lower in cells to which BSO had been added in the culture media than they were in the groups without BSO. Further, the gene expression of GPX3 was higher in the α-TOCH group than in the control group (Figure 6a). The expression of catalase, which reduces hydrogen peroxide, was lower in all treated groups than it was in the control group, with the highest level of reduction in the cells treated with BSO (Figure 6b).

SOD also catalyses the reduction of hydrogen peroxide and this enzyme is an important anti-oxidant. The activities of SOD in the groups to which α-TOCH had been added were less than half of the levels in the control group and BSO group (Figure 7). The activity of SOD was significantly higher in cells in the BSO group than it was in cells in the control group.

3.5 ER stress and genes related to immune responses

The level of expression of activating transcription factor 6 (ATF6), an ER stress-regulated transmembrane transcription factor, was higher in cells in the groups supplemented with α-TOCH than in cells in the other groups (Figure 8). The expression of another UPR marker, GRP78, was highest in the group with combined treatment by BSO and α-TOCH. The expression of the X-box protein 1 (XBP1), a UPR transducer that regulates a subset of ER-resident chaperone genes important in protein folding, did not change with treatment (Figure 8).

mRNA levels of ribosomal protein S15 (RPS15), a component of the 40S ribosomal subunit, and (SIAH1), a key component of the ubiquitin-proteasomal proteloytic system, were reduced in cells treated with α-TOCH and increased in cells treated with BSO (Figure 9b).

Expression of all genes related to the immune response – JunB, IL4/13α2 decoy receptor, and Janus kinase 1 (JAK-1) – was induced by BSO, and it was suppressed by α-TOCH, administered either on its own or in combination with BSO (Figure 9a).

4. DISCUSSION

Mammalian studies have shown that adipocytes in the lipid-filling phase of their terminal differentiation need GSH based antioxidant systems to ensure that the intracellular redox balance is maintained so that the cells are kept in a pro-adipogenic state e.g. [23]. GSH is an electron donor for the GPX enzymes and as such plays a key role in protecting both mammalian [33] and fish cells [34] from OS. Our recently conducted microarray study also showed that the intracellular redox balance is important during the adipocyte differentiation in a cold-blooded vertebrate [25]. The down regulations in ROS generating pathways were concurrent with the activation of several antioxidant systems. In the present study, we wanted to further investigate how compromised ROS scavenging may exacerbate peroxidation in the primary culture of fish adipocytes when high amounts of easily oxidised HUFAs are available as substrates. The *in vitro* system enabled manipulation of the selected pro-oxidant BSO, which depletes GSH, and the exogenous anti-oxidant α-TOCH, against a background of exposure to high levels of HUFAs. As expected, the levels of expression of genes coding for GPX2 and GPX3 were low in cells cultivated in media supplemented with BSO, while the mRNA levels of the classical OS marker NFkB1 were high. We measured the enzymatic activity of the ROS scavenger SOD, which does not require GSH and is highly responsive to OS in fish [35]. The level of SOD in GSH-depleted cells was elevated, which suggests that adipocytes use alternative mechanisms to combat ROS when the GPX system is disabled. This is compatible with the hypothesis that preventing the accumulation of ROS is a highly prioritised task in adipocytes that are loading TAGs. Signs of oxidative stress were nevertheless evident in the groups that were not supplemented with α-TOCH. Lower percentages of HUFAs and CL suggested that the OS was highest in the BSO group, in agreement with the previously described negative effects of OS on the levels of HUFAs and CL in membranes [36-38]. CL is a mitochondrial PL that is particularly susceptible to oxidative damage due to its high content of HUFAs [36]. The total amount of cellular HUFAs was only moderately affected by the treatments, indicating that the degree of OS was moderate. This could be partly due to the increased antioxidant activity of SOD.

The pro-oxidant intracellular state in two groups not supplemented with α-TOCH was further evidenced with the higher levels of the secondary peroxidation products TBARS in these cells. The addition of the external antioxidant clearly prevented a reduction in the levels of

CL. The reduction of the mitochondrial membrane CL content is known to be pro-apoptotic [39].

The mechanisms of α-TOCH action in fish are not fully understood [40]. They may involve the reduction of peroxides from unsaturated lipids by one of the members of the GPX family [41]. The fact that the levels of expression of the genes coding for GPX2 and GPX3 did not rise when α-TOCH was added to the BSO-treated group indicate that α-TOCH has antioxidant effects that are independent of GSH.

The two groups of cells that were treated with α-TOCH were characterised by the highest deposition of TAG and a marked expression of the master adipogenic regulators PPARγ and C/EBPα. Thus, α-TOCH is a potent pro-adipogenic vitamin, as it was associated with the up-regulation of the central transcription factors that are essential for terminal differentiation of adipocytes. A possible mechanism of action involves the direct stimulation of PPARγ [42].

The profile of genes encoding a set of well-known apoptotic markers implied that the incidence of cell death in the groups with no added α-TOCH was higher. This is in agreement with the recent studies that showed increased apoptosis in several tissues of fish fed high HUFA-enriched diets [2; 13; 38]. mRNA level of Bax was highly induced by the BSO treatment. The protein Bax is known to be involved in the permeabilization of mitochondria and release of cytochrome C [43; 44]. The expression of the apoptotic marker CASP3 was however, not changed, either at the mRNA or at the protein level. On the other side, highly induced gene expression of the mitochondrial intermembrane space protein AIF by the BSO treatment supported the assumption that BSO stimulated CASP3 independent apoptosis. AIF-mediated cell death was previously shown to occur in response to increased levels of lipid peroxidation in mammals [19] while mitochondrial scavenging of ROS and the role in oxidative phosphorylation are among the suggested cell death-unrelated physiological functions of AIF [45]. In summary, our results support the hypothesis that GSH depletion has pro-apoptotic effects and that α-TOCH plays a role in promoting cell survival.

Cells that had not received additional α-TOCH contained high numbers of large-sized vesicles that resembled mammalian autophagosomes. Accumulation of autophagosomes and mitochondrial dysfunction caused by OS can trigger CASP3 independent autophagic cell death [46; 47]. Autophagy involves highly regulated sequestration of proteins and organelles inside autophagosomes, which are then delivered to lysosomes for degradation. Interestingly, Singh et al. [48] found that inhibition of autophagy resulted in increased accumulation of TAG. High numbers of autophagosome-like structures in the control and BSO treated groups

suggests the pro-death autophagy scenario in these cells and confirms the reciprocal relation between lipid load and autophagic function [48].

ER stress and UPR are now firmly linked to TAG overload [26]. The excess of nutrients may lead to the UPR adaptive response being activated as a feedback mechanism to limit or unable the accumulation of lipid and reduce the stress on the ER, which is engaged in lipid biosynthesis. In fact, levels of the two key UPR markers, ATF6 and GRP78, were elevated in cells that had been supplied with additional α-TOCH, and these cells also had the highest levels of accumulated lipid. Increased lipid deposition that is occurring in α-TOCH treated groups is the likely trigger of the observed UPR response.

Obesity in mammals induces a state of chronic, low-grade inflammation that contributes to insulin resistance and that is characterised by the accumulation of immune cells in white adipose tissue [49]. Most of the inflammatory mediators were shown to possess dual actions: one example is cytokines, which not only have an immunomodulatory function but also act as regulators of energy metabolism [50]. The expression profiles of four immune-related genes were similar and generally confirmed the anti-inflammatory effects of α-TOCH. The down-regulation of IL4/13α2 implied that anti-inflammatory IL4 and IL13 signalling was higher in the α-TOCH groups. The expression of the pro-inflammatory NFkB transcription factor is compatible with its roles as an anti-adipogenic agent and a sensor of OS [51]. Another protein involved in the immune response is JunB, which turned out to be associated both with increased OS and with increased lipid accumulation in our cells. This was not surprising, given that JunB is a component of the AP1 transcription factor, which has been implied as a central link between ER stress and TAG overload in murine hepatocytes [26] and adipocytes in fish [25].

α-TOCH treatment was associated with the up-regulations of master pro-adipogenic transcription factors and increased TAG biosynthesis. Our data illustrates the fact that the capacity to tolerate high HUFA levels depends on a tight balance between the factors that promote the generation of ROS and the factors that inhibit it. The induction of adipocytes' cell death by HUFAs likely plays an important part in the adipose tissue homeostasis in animals exposed to high dietary HUFA levels.

We suggest that lipid accumulation promoted by α-TOCH is a stronger disturbance to ER homeostasis than the increased OS in GSH-depleted Atlantic salmon adipocytes. On the other

hand, lipid peroxidation in the GSH-depleted and HUFA-treated cells is associated with an increased probability of apoptosis mediated via CASP3-independent pathways that probably involve AIF-dependent pathways and autophagosome-like intracellular vesicles.

5. FIGURE LEGEND

Figure 1. Oil Red O staining. Adipocytes from the four treatment groups were fixed and the intracellular lipid droplets were stained with Oil Red O. The dye was extracted and the absorbance measured at 500 nm. The absorbances correspond with the intracellular lipid level. Data are shown are mean ± SEM (n = 3). Different letters indicate significant differences ($p \leq 0.05$). Control = adipocytes cultivated in growth media supplemented with eicosapentanoic and docosahexanoic acids. All treatment groups were cultivated in the same media as the control cells, with the addition of either α-tocopherol (α-TOCH), BSO, or BSO + α-TOCH.

Figure 2. Adipocyte morphology. A) Control = adipocytes cultivated in growth media supplemented with eicosapentanoic and docosahexanoic acids. All treatment groups were cultivated in the same media as the control cells, with the addition of either B) α-tocopherol (α-TOCH), C) BSO, or D) BSO + α-TOCH. Images were all taken with 40x magnification.

Figure 3. Adipogenic transcription factors analysed with real-time qPCR in mature adipocytes in the four treatment groups. Data are presented as $-\Delta\Delta Ct \pm SE$ (n = 4). The significance level has been set at $p \leq 0.05$. Different letters indicate significant differences between dietary groups within each gene. Control = adipocytes cultivated in growth media supplemented with eicosapentanoic and docosahexanoic acids. All treatment groups were cultivated in the same media as the control cells, with the addition of either α-tocopherol (α-TOCH), BSO, or BSO + α-TOCH. PPARα, γ – peroxisome proliferator-activated receptor α, γ; C/EBP – CCAAT/enhancer-binding proteins α.

Figure 4. Genes involved in apoptosis analysed with real-time qPCR in mature adipocytes in the four treatment groups. Data are presented as $-\Delta\Delta Ct \pm SE$ (n = 4). The significance level has been set at $p \leq 0.05$. Different letters indicate significant differences between dietary groups within each gene. Control = adipocytes cultivated in growth media supplemented with eicosapentanoic and docosahexanoic acids. All treatment groups were cultivated in the same media as the control cells, with the addition of either α-tocopherol (α-TOCH), BSO, or BSO + α-TOCH. CASP3 – caspase 3; AIF – apoptosis inducible factor; Bax – Bcl-2–associated X protein. Diablo.

Figure 5. Thiobarbituric acid reactive substances (TBARS) measured in mature adipocytes in the four treatment groups. Different letters indicate significant differences ($p \leq 0.05$). Data shown are means ± SEM (n = 3). Control = adipocytes cultivated in growth media supplemented with eicosapentanoic and docosahexanoic acids. All treatment groups were cultivated in the same media as the control cells, with the addition of either α-tocopherol (α-TOCH), BSO, or BSO + α-TOCH.

Figures 6a, 6b. Genes for antioxidants analysed with real-time qPCR in mature adipocytes in the four treatment groups. Data are presented as $-\Delta\Delta Ct \pm SE$ (n = 4). The significance level has been set at $p \leq 0.05$. Different letters indicate significant differences between dietary groups within each gene. Control = adipocytes cultivated in growth media supplemented with eicosapentanoic and docosahexanoic acids. All treatment groups were cultivated in the same media as the control cells, with the addition of either α-tocopherol (α-TOCH), BSO, or BSO + α-TOCH. GPX3 – glutathione peroxidase 3; GPX2 – glutathione peroxidase 2; CAT – catalase; PRDX – peroxiredoxin; HMOX2 – heme oxygenase 2.

Figure 7. Intracellular superoxide dismutase activity of mature adipocytes in the four treatment groups. Different letters indicate significant differences ($p \leq 0.05$). Data shown are means ± SEM (n = 3). Control = adipocytes cultivated in growth media supplemented with eicosapentanoic and docosahexanoic acids. All treatment groups were cultivated in the same media as the control cells, with the addition of either α-tocopherol (α-TOCH), BSO, or BSO + α-TOCH.

Figure 8. Genes involved in ER stress analysed with real-time qPCR in mature adipocytes in the four treatment groups. Data are presented as $-\Delta\Delta Ct \pm SE$ (n = 4). The significance level has been set at $p \leq 0.05$. Different letters indicate significant differences between dietary groups within each gene. Control = adipocytes cultivated in growth media supplemented with eicosapentanoic and docosahexanoic acids. All treatment groups were cultivated in the same media as the control cells, with the addition of either α-tocopherol (α-TOCH), BSO, or BSO + α-TOCH.ATF6 – activating transcription factor 6; XBP1 – X box binding protein-1; GRP78 – glucose-regulated protein 78.

Figure 9ab. Genes involved in immune the response analysed with real-time qPCR in mature adipocytes in the four treatment groups. Data are presented as $-\Delta\Delta Ct \pm SE$ (n = 4). The significance level has been set at $p \leq 0.05$. Different letters indicate significant differences between treatment groups within each gene. Control = adipocytes cultivated in growth media supplemented with eicosapentanoic and docosahexanoic acids. All treatment groups were cultivated in the same media as the control cells, with the addition of either α-tocopherol (α-TOCH), BSO, or BSO + α-TOCH. Jun B – Jun B proto-oncogene ; IL4/13a – interleukin 4/13A2 ; NFκB1 – nuclear factor kappa B1; JAK-1 – Janus kinase 1; Siah 1 – ubiquitin ligase SIAH1; RPS15 – ribosomal protein S15.

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Table 1. Primer list

Gene	Forward primer (5'–3')	Reverse primer (5'–3')	Accession number
Proliferator-activated receptor gamma (PPARα)	TCCTGGTGGCCTACGGATC	CGTTGAATTTCATGGCGAACT	DQ294237
Proliferator-activated receptor gamma (PPARγ)	CGTGTATCAAGACGCCAGCT	TTGCAGCCCTCACAGACATG	EU655708
CCAAT-enhancer-binding protein alpha (C/EBPα)	AGACCTCGGCGAGATTTGT	TGTGGAATAGATCAGCCAGGAA	EU668995
Caspase 3 (CASP-3)	ACAGCAAAGAGCTAGAGGTCCAACAC	AAAGCCAGGAGAGTTTGACGCAG	DQ008070
Apoptosis Inducible Factor (AIF)	AGGTGGAGTCCCAAGGAATCTGC	CCCCAAGAAACCTCCTCCAATG	TC37490 tigr est
Bcl-2 associated X protein (BAX)	TGACAGATTTCATCTACGAGCGGG	GCCATCCAGCTCATCTCCAATCT	DY722290
Diablo	GGATGTTACCAGAACGAGCTTGGC	GGGAGAGGTAAGTGTTTGCACTGTCTG	TC55946 tigr est
Glutathione peroxidase 3 (GPX3)	CCTTCCAGTACCTGGAGTTGAATGC	CTCATGATTGTCTCCTGGCTCCTGT	CA345853
Glutathione peroxidase 2 (GPX2)	TGTACCTCAAGGAGAAGCTGCCGT	ATTAAGGCCATGGGATCGTCGC	est04e05
Catalase (CAT)	CCAGATGTGGGCCGCTACAA	TCTGGCGCTCCTCCTCATTC	est04a09
Peroxiredoxin (PRDX)	CACTGCTGTGGTGGATGGACAGTT	CCAGCGGGTAGAAGAAGAACACCA	est02b08
Heme oxygenase 2 (HMOX2)	CA363120_Heme	CA363120_Heme	CA363120
Activating transcription factor 6 (ATF6)	CTCACACCATCAAAGCTACAGCGA	GTGTCGCCTCGTCGATTTAACTCA	CA367172
X-box binding protein 1 (XBP1)	CGACTCAAATTCGACAACTGGGC	TTTCTGTCTCTGGCTGTCTGGGCT	CA385697
Glucose regulated protein 78 (GRP78)	GTGCAGCATGACATCAAGTA	CTCTTCTTCTCGATAACCTT	CA368961
Jun B proto-oncogene (Jun B)	CATCAGAAGTCGGCTCGCTGAA	GGTGTCGGTGTGGTAGTGATGACA	EST1-3A_H06
Interleukin 4/13a2 (IL4/13a2)	TCTCTGAGCCGCTCAACCTGTCAT	CGTTCCACGACAGCTTTATACGGA	CA348044
Nuclear factor kappa B1 (NFκB1)	CAGCGTCCTACCAGGCTAAAGAGAT	GCTGTTCGATCCATCCGCACTAT	CA341859
Janus kinase 1 (JAK-1)	GAGGAGTTTGTCCAGTTCGGTCCGT	CATGCACCAGCTTCTTATCCTCCAG	CA368994
Ubiquitin ligase SIAH1 (SIAH1)	CTGCCCACAGGAACCTCCAAGT	CAGACAGGGCACTCAAACAGGC	CA378361
Ribosomal protein S15 (RPS15)	CAAGCAGCAGTCCCTCCTGA	GACCATGTCCCTGAGGTGAGTCT	EXOB3
Elongation factor 1A (EF1A)	TGCCCCTCCAGGATGTCTAC	CACGGCCCACAGGTACTG	BG933853

Fatty acids (% of total)	Control	α-TOCH	BSO	BSO + α-TOCH
14:0	0.6±0.12[a]	0.5±0.09[a]	0.6±0.09[a]	0.9±0.17[b]
15:0	0.2±0.05[c]	0.2±0.04[cb]	0.3±0.04[b]	0.3±0.02[b]
16:0	6.0±0.06[a]	6.9±0.04[b]	7.5±0.04[c]	7.2±0.02[bc]
17:0	0.3±0.07[a]	0.2±0.05[a]	0.3±0.08[a]	0.4±0.07[b]
18:0	3.7±0.00	3.4±0.06	3.6±0.03	3.7±0.03
20:0	n.d[a].	n.d.[a]	0.2±0.02[b]	0.3±0.06[c]
24:0	0.5±0.06	0.5±0.04	0.6±0.02	0.3±0.05
Σ Saturated	11.3±0.21[ab]	11.8±0.56[b]	13.0±0.54[c]	13.1±0.24[c]
14:1 n-5	0.2±0.09[b]	n.d.[a]	n.d.[a]	n.d.[a]
15:1	0.2±0.01	0.2±0.05	0.2±0.05	0.2±0.00
16:1 n-5	0.2±0.01[b]	0.1±0.01[ab]	n.d.[a]	0.2±0.01[b]
16:1 n-7	1.1±0.11[bc]	0.9±0.08[b]	1.1±0.08[c]	1.1±0.11[bc]
16:1 n-9	0.3±0.06[ab]	0.2±0.05[a]	0.3±0.02[ab]	0.4±0.02[b]
17:1 n-7	0.1±0.04[a]	n.d.[b]	0.2±0.07[a]	n.d.[b]
18:1 n-11	0.4±0.05	0.3±0.04	0.3±0.02	0.3±0.02
18:1 n-7	1.2±0.15	1.2±0.05	1.3±0.10	1.2±0.18
18:1 n-9	7.8±0.55[ab]	7.7±0.43[ab]	7.4±0.01[a]	8.2±0.20[b]
20:1 n-9	0.9±0.02	0.8±0.09	0.8±0.09	0.8±0.07
20:1 n-11	0.2±0.02	0.2±0.03	0.3±0.05	0.2±0.05
22:1 n-11	0.6±0.08	0.6±0.02	0.6±0.03	0.6±0.06
22:1 n-7	0.5±0.03	0.7±0.09	0.5±0.05	0.5±0.08
22:1 n-9	0.2±0.02[b]	n.d.[a]	n.d.[a]	n.d.[a]
24:1 n-9	0.6±0.12	0.5±0.09	0.6±0.16	0.5±0.10
Σ Monounsaturated	14.5±0.41	13.6±0.64	13.6±0.38	14.2±0.70
16:2 n-3	n.d.[a]	n.d.[a]	0.1±0.02[b]	n.d.[a]
18:2 n-6	2.4±0.11[ab]	2.7±0.16[b]	2.2±0.07[a]	2.6±0.30[b]
20:2 n-6	0.5±0.07	0.6±0.17	0.5±0.06	0.5±0.06
22:2 n-6	0.2±0.09[b]	0.4±0.06[c]	0.5±0.08[d]	n.d.[a]
16:3 n-4	0.5±0.09	0.6±0.06	0.5±0.10	0.5±0.09
18:3 n-3	0.4±0.01	0.4±0.05	0.5±0.07	0.4±0.07
20:3 n-3	0.2±0.03[b]	n.d.[a]	n.d.[a]	n.d.[a]
20:3 n-6	0.5±0.04	0.6±0.07	0.5±0.04	0.4±0.0[a]
20:4 n-3	0.2±0.01[c]	0.1±0.01[b]	0.2±0.01[c]	n.d.[a]
20:4 n-6	1.3±0.09[b]	1.3±0.15[b]	1.2±0.02[b]	1.0±0.08[a]
22:4 n-6	0.5±0.05[c]	0.2±0.04[a]	0.4±0.03b[c]	0.3±0.05[ab]
20:5 n-3	29.1±0.40[bc]	29.6±0.68[c]	27.0±0.21[a]	28.5±0.17[b]
22:5 n-3	4.2±0.11[c]	4.3±0.09[c]	3.9±0.09[b]	2.8±0.19[a]
22:6 n-3	30.8±0.95	30.9±1.21	31.3±0.16	30.2±0.63
Σ Highly unsaturated	70.8±0.91[b]	71.5±1.74[b]	68.7±0.53[a]	67.2±0.68[a]
Σ n-3	64.9±1.11[c]	65.3±1.98[bc]	62.9±0.40[ab]	62.0±0.64[a]
Σ n-6	5.4±0.28[b]	5.7±0.22[b]	5.3±1.13[b]	4.8±0.54[a]
EPA+DHA	59.9±1.20[ab]	60.5±1.84[b]	58.2±0.28[a]	58.7±0.70[ab]

Table 2. Fatty acid compositions of the total lipids in mature adipocytes in the four treatment groups. The quantity of each fatty acid is given as the percentage of total fatty acids. The values given are means ± SEM (n = 3). Different letters indicate significant differences ($p \leq 0.05$). Control = adipocytes cultivated in growth media supplemented with eicosapentanoic and docosahexanoic acids. All treatment groups were cultivated in the same media as the control cells, with the addition of either α-tocopherol (α-TOCH), BSO, or BSO + α-TOCH. n.d. = not detected.

Phospholipid class (% of total lipids)	Control	α-TOCH	BSO	BSO + α-TOCH
LPC	2.0±0.53	2.0±0.61	1.6±0.18	2.3±0.28
SM	8.9±0.43	7.9±0.14	7.9±0.19	9.7±0.87
PC	60.7±2.70^{b}	57.2±1.67^{ab}	56.9±1.54^{ab}	53.7±0.55^{a}
PS	1.3±0.0.39	2.9±0.58	2.3±0.55	2.2±0.24
PI	0.9±0.060^{a}	1.14±0.34^{a}	2.2±0.143^{b}	1.8±0.39^{ab}
PE	16.4±1.56	15.7±0.81	15.6±0.43	15.9±0.53
CL	10.1±0.48^{a}	13.3±0.25^{b}	10.7±0.06^{a}	13.2±0.50^{b}

Table 2. Phospholipid compositions of mature adipocytes in the four treatment groups. The relative distributions between different lipid classes are presented. The values given are means ± SEM (n = 3). Different letters indicate significant differences ($p \leq 0.05$). LPC – lysophosphatidyl choline; SM – sphingomyelin; PC – phosphatidylcholine; PS –phosphatidylserine;PI – phosphatidylinositol;CL – cardiolipin;PE – phosphatidylethanolamine. Control = adipocytes cultivated in growth media supplemented with eicosapentanoic and docosahexanoic acids. All treatment groups were cultivated in the same media as the control cells, with the addition of either α-tocopherol (α-TOCH), BSO, or BSO + α-TOCH.

Figure 1

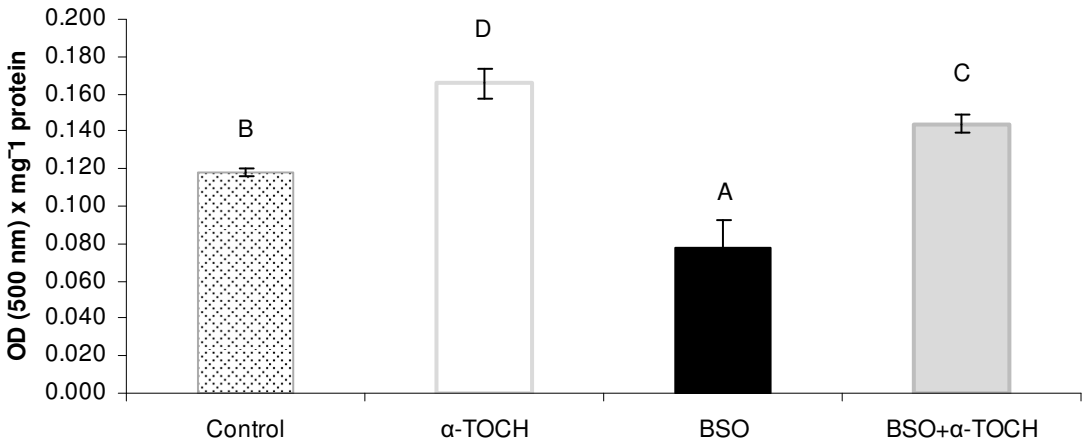

Figure 2

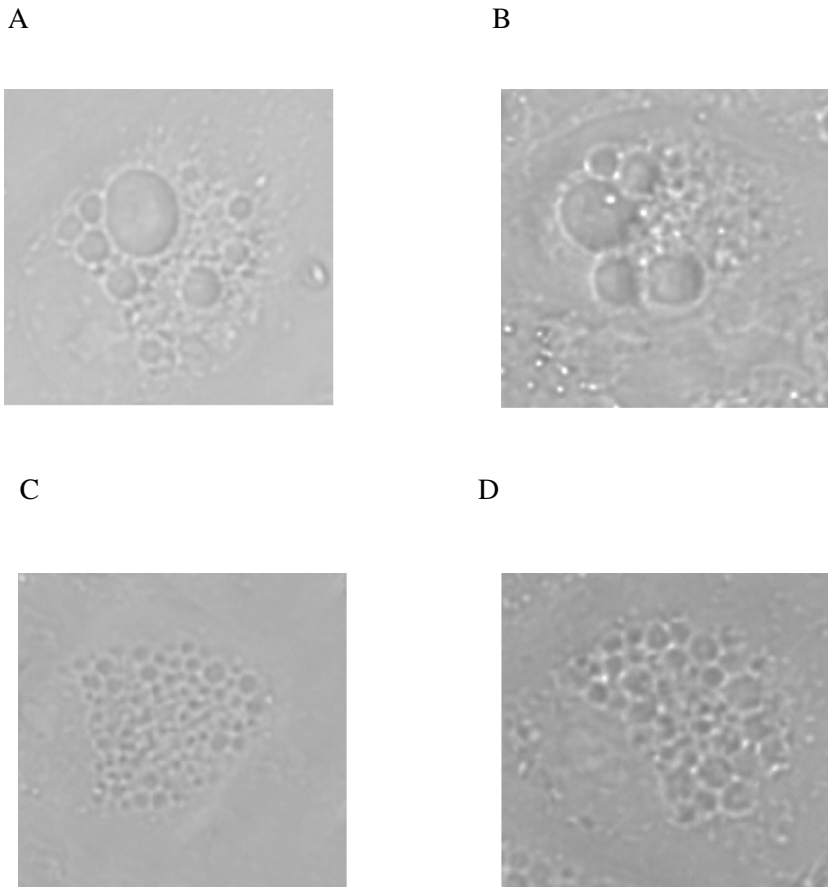

Figure 3

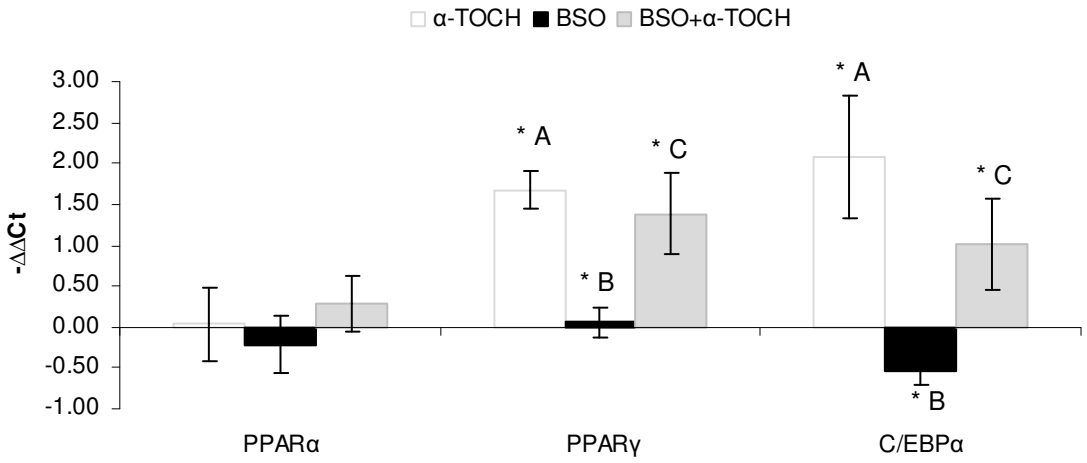

Figure 4

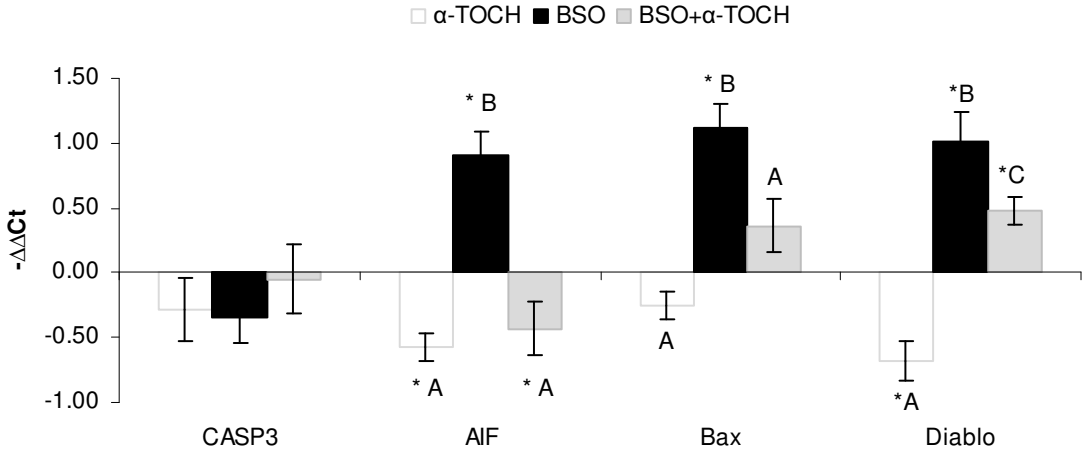

Figure 5

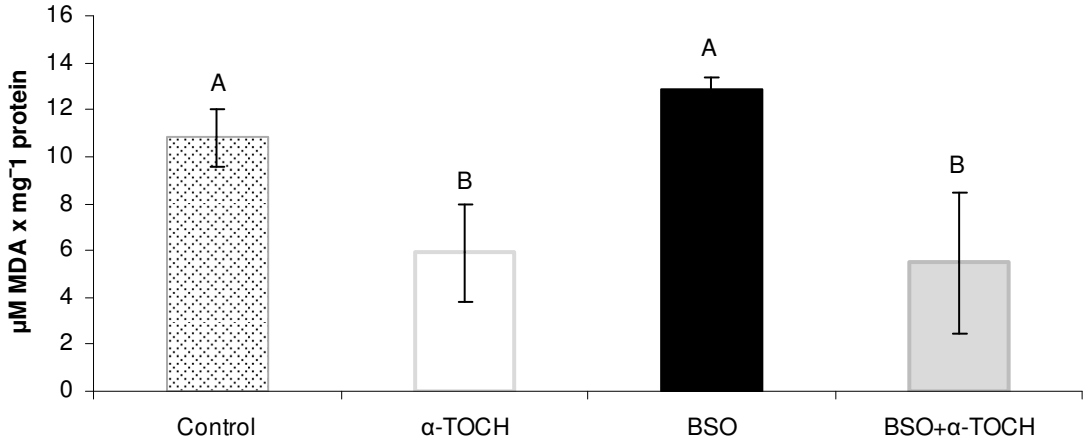

Figure 6a

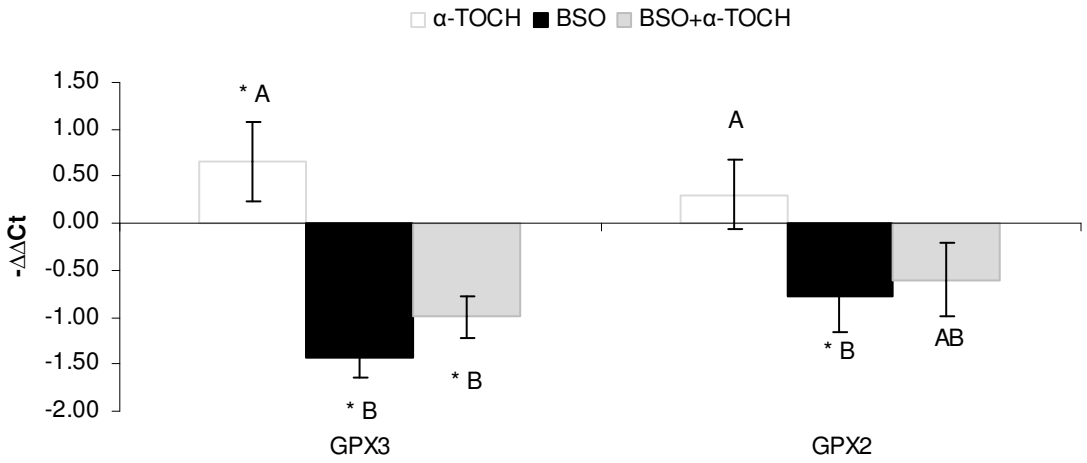

Figure 6b

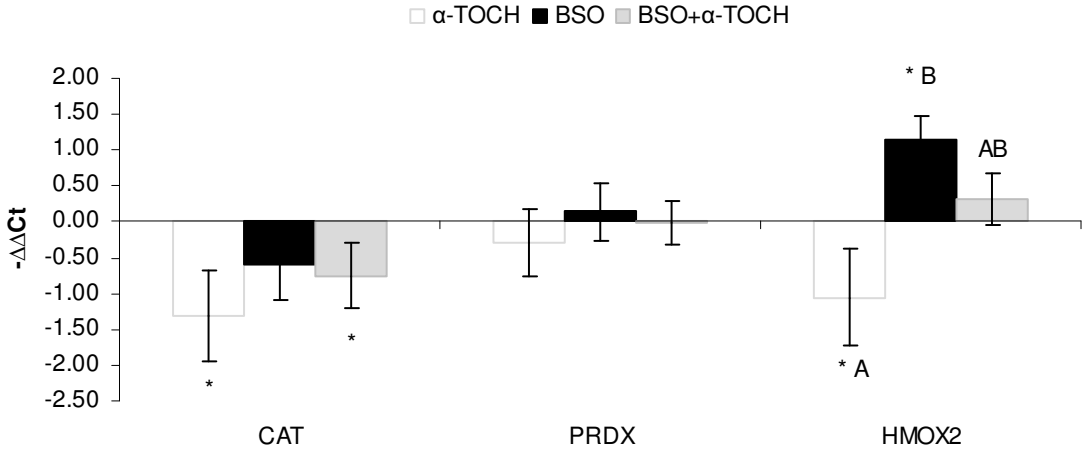

Figure 7

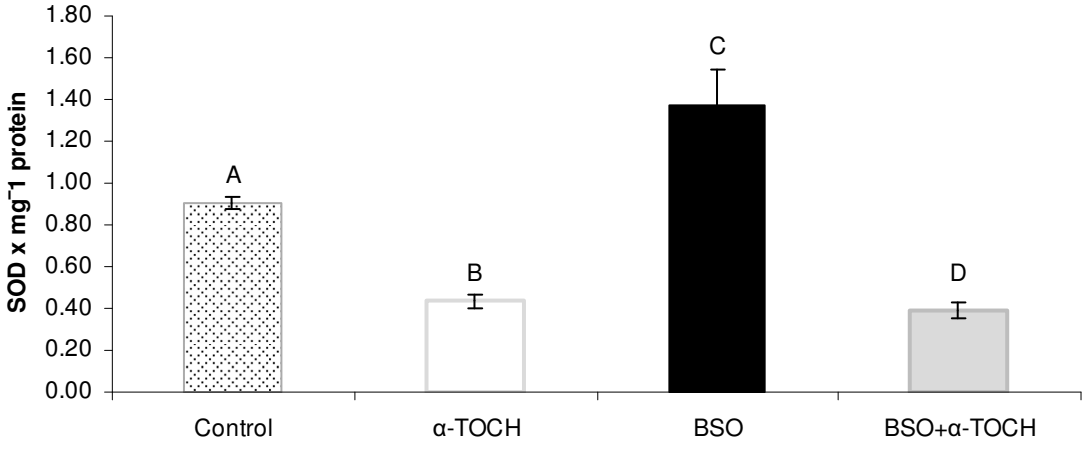

Figure 8

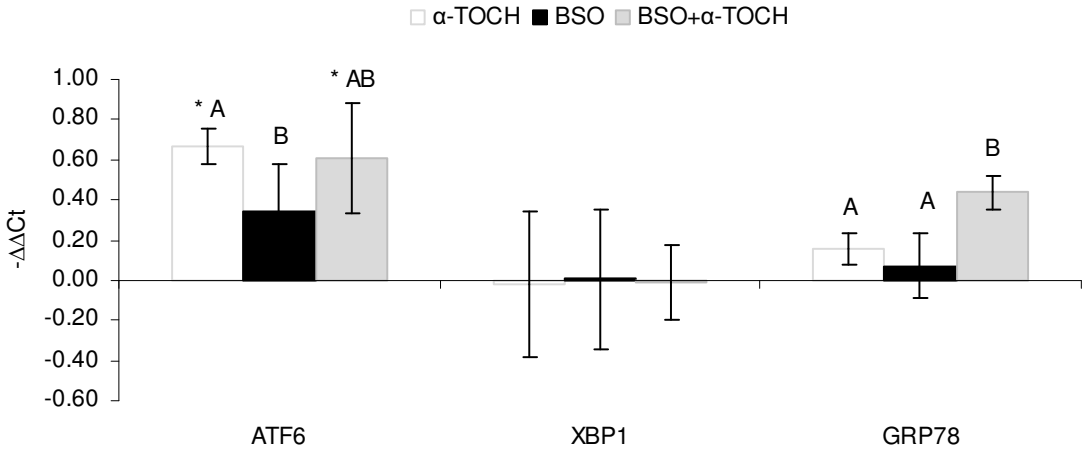

Figure 9a

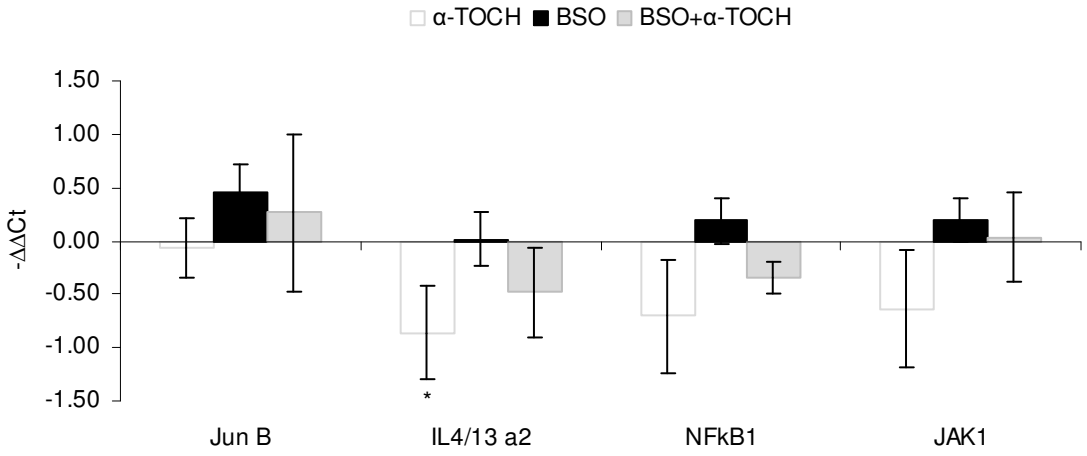

Figure 9b