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New insights into lipid and carbohydrate metabolism in teleost fish: transcriptional and functional characterization of adipocytes

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Doctoral Thesis

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ABBREVIATIONS

List of the main abbreviations used throughout this work. The rest will be described in the text as they appear.

ACC: acetyl-CoA carboxylase

ACO: Acyl-CoA oxidase

ACSL: acyl-coenzyme A synthetase

AdipoR: adiponectin receptor

ADRP: adipose differentiation-related protein

ATGL: adipose triglyceride lipase

BAT: brown adipose tissue

CPT: carnitine palmitoyl transferase

CREB: cyclic AMP response element-binding protein

C/EBP: CCAAT/enhancer binding protein factor

DNL: *de novo* lipogenesis

ECM: extracellular matrix

FA: fatty acid

FABP: fatty acid binding protein

FAS: fatty acid synthase

FAT: fatty acid translocase

FATP: fatty acid transport protein

FFA: free fatty acid

gAD: globular adiponectin

GH: growth hormone

GLUT: glucose transport protein

G6PDH: glucose 6-phosphate dehydrogenase

HSL: hormone-sensitive lipase

KLF: Krüppel-like factor

KROX20: early growth response protein-2

LPL: lipoprotein lipase

LC n-3 PUFA: long-chain omega-3 fatty acids

LXR: liver X receptor

ME: malic enzyme

miRNAs: microRNAs

MSC: mesenchymal stem cells

PAT: perilipin/ADRP/TIP47

PI3K: phosphatidylinositol-3-kinase

PKA: protein kinase A

PLIN: Perilipin

PPAR: peroxisome proliferator activated receptor

PPP: pentose phosphate pathway

PUFA: polyunsaturated fatty acid

SCD: stearoyl-CoA desaturase

SREBP: sterol regulatory element binding protein

SVF: stromal vascular fraction

TAG: triglyceride

Tip-47: tail-interacting protein of 47 kDa

TNFα: tumour necrosis factor-α

TOR: target of rapamycin

UCP-1: uncoupling protein-1

UFA: unsaturated fatty acid

VLDL: very-low-density lipoproteins

WAT: white adipose tissue

Chapter 1. GENERAL INTRODUCTION

1.1 LIPID METABOLISM IN FISH

Lipids constitute the major macronutrient classes required to cover all nutritional and energetic demands in fish, along with proteins and to a lesser extent carbohydrates. Dietary lipids serve a diverse array of purposes in the organism. They are responsible of constituting the cellular building blocks being thus essential for cell viability. Lipids are also in charge of maintaining energy homeostasis by providing energy during different life stages and circumstances (growth, reproduction, migration, starvation…) and storing it in times of excess. On the other hand, lipids play a key role ensuring the right environment and function in the organism. They are known to determine protein structure and function (Menon, 2008), they act as precursors of relevant metabolites, like eicosanoids (Smith and Murphy, 2008), and also as important signalling molecules able to regulate a wide diversity of processes, including the transcription of genes involved in lipid metabolism (Miyazaki and Ntambi, 2008).

Lipids are the main source of energy in aquaculture nutrition, and therefore they are included to a high degree in fish feeds (Bell and Wolfgang, 2010). The development of high-energy diets has allowed the aquaculture industry to reduce the production time while increasing the growth rates and sparing in dietary protein content, which decreases feed costs (Leaver et al., 2008). However, this increase in dietary lipids has some negative issues associated such as an increase in fish fat deposition. The major sites of lipid storage in fish are perivisceral fat, muscle and liver, being the importance of each one species-specific (Sheridan and Kao, 1998, Weil et al., 2013). In the species studied in the present thesis, rainbow trout (*Oncorhynchus mykiss*), Atlantic salmon (*Salmo salar*) and gilthead sea bream (*Sparus aurata*), adipose tissue and muscle represent the main reservoir compartments while liver accumulate lipids to a less degree (Takama et al., 1994, Jobling et al., 1998, Santinha, 1999, Jobling and Johansen, 2003).

Not only the quantity, but also the quality and distribution of the deposited lipids affect to a big extend the aquaculture industry. Visceral fat, which is located in the abdominal cavity around the digestive tract, and subcutaneous fat depots (below the skin) influence carcass and fillet yield, negatively affecting the sector productivity. Intramuscular fat depots affect the quality and organoleptic characteristics of the flesh, and thereby consumer acceptance. Fish are the major dietary source of the long-chain omega-3 fatty acids (LC n-3 PUFA), which are known to have beneficial effects on human health (Lorente-Cebrian et al., 2013, Crupi et al., 2013, Kimmig and Karalis, 2013). Farmed fish are mainly intended for human consumption and

therefore fish nutritional value is an important factor to maintain for the sake of the sector growth and development.

In addition, and despite the fact that an excessive accumulation of lipids in the different organs can trigger adverse effects on fish health, little attention has been paid to this issue. In mammals is well documented that an excess in fat deposition is linked to the development of many aspects of the metabolic disease (Wahba and Mak, 2007). In humans, this disease has been defined as a cluster of the most dangerous heart attack risk factors: diabetes, abdominal obesity, high cholesterol and high blood pressure (Alberti et al., 2005). Special attention must then be paid to understand the different mechanisms regulating the lipid deposition and mobilization in order to ensure the growth of aquaculture without compromising fish welfare and maintaining the nutritional benefits for the consumer.

Fish adiposity is modulated by a different array of extrinsic or environmental and intrinsic factors. The natural life cycle of many fish species includes a fasting period during reproductive phases or periods of low food intake in winter season (Navarro and Gutiérrez, 1995). Fasting leads to the mobilization of different fat depots in a specie-specific manner. All the species studied in this thesis have been reported to present different patterns of energy utilization. Gilthead sea bream preferentially mobilises fat deposited in the liver and in the muscle while visceral adipose tissue needs longer time to be mobilised (Grigorakis and Alexis, 2005). Atlantic salmon mobilises visceral adipose tissue and muscle fat before liver fat depots and rainbow trout shows a preference to use liver and visceral adipose tissue in the first place, leaving the muscle reservoirs for later (Navarro and Gutiérrez, 1995). Other factors, such as fish age (Kiessling et al., 1991), the use of diets with different levels of substitution of marine ingredients by vegetable ingredients (Torstensen and Tocher, 2010) or the breeding of fish selected based on specific traits (Quillet et al., 2005) exert profound effects in the regulation of lipid homeostasis.

In addition, we should keep in mind the high complexity of studying lipid metabolism. The great molecular and structural diversity, the fact that lipids do not present inherent catalytic activity nor obvious functions after being isolated (Dowhan et al., 2008) complicates the task to define the precise property of a given lipid in molecular terms that supports a particular function *in vivo*. Moreover, genes do not encode lipids and therefore enzymes along biosynthetic pathways need to be targeted to make mutants. All this issues represent some of the challenges that need to be overcome in this intricate research field.

1.1.1 Lipid metabolism in adipose tissue

A renewed interest about the biology of the adipose tissue has emerged since it has been demonstrated that adipocytes are not passive storage depots. On the contrary, adipocytes are dynamic cells that play a key role in the energy balance and overall body homeostasis. In humans, dysregulations of lipid metabolism in adipose tissue have been involved in the pathogenesis of several diseases (Berg and Scherer, 2005, Lazar, 2005). Physiological or anatomic abnormalities in this tissue, such as an excessive accumulation of perivisceral fat and/or a hypertrophy of the adipocytes, are considered markers of metabolic failure (Bays et al., 2008). In fish, partial replacement of fish oil by vegetable oil triggers different responses in lipid accumulation and hypertrophy of visceral adipocytes. In Atlantic salmon a high inclusion of plant ingredients in the diet increases the whole body adiposity (Torstensen and Tocher, 2010) and in gilthead sea bream an enlargement of the adipocytes is observed when 66% of the fish oil is replaced by a mixture of vegetable oils (Cruz-Garcia et al., 2011), impairing the storage capacity of the tissue. Therefore, the distribution and architecture of adipose tissue are key aspects that characterise a healthy and functional tissue.

Adipose tissue is a key organ in the regulation of energy metabolism in all vertebrates including fish (Sheridan, 1988). It provides the storage place of excess of energy that comes from the diet. This energy is stored in form of triglycerides (TAGs), which can be utilised in order to fulfil the metabolic requirements of peripheral organs and tissues. Lipids, in particular fatty acids (FA), are efficient fuel storage and therefore this is one of the reasons for the animals to store the majority of their energy in fats and only a minor part as carbohydrates (Hertzel et al., 2008). When they are oxidised they produce large quantities of NADH and $FADH_2$ and subsequently ATP that supply the needs of other organs and tissues. The balance between fat deposition and mobilization responds to different signals (Sheridan, 1988, Leaver et al., 2008) as will be presented below, and is mediated by the different players depicted in Figure 1.

1.1.1.1 Fatty acid uptake and transport

In the vascular endothelium, FAs are liberated from triacylglycerol rich lipoproteins (chylomicrons and very-low-density lipoproteins (VLDL)) through the action of lipoprotein lipase (LPL). This enzyme has been identified in several fish species, including the ones studied in the present thesis; Atlantic salmon (Kleveland et al., 2006), gilthead sea bream (Saera-Vila

et al., 2005) and rainbow trout (Arnault et al., 1996). The activity and expression of this enzyme has been assessed in response to different nutritional challenges and hormonal regulation (Albalat et al., 2006, Albalat et al., 2007, Bouraoui et al., 2012).

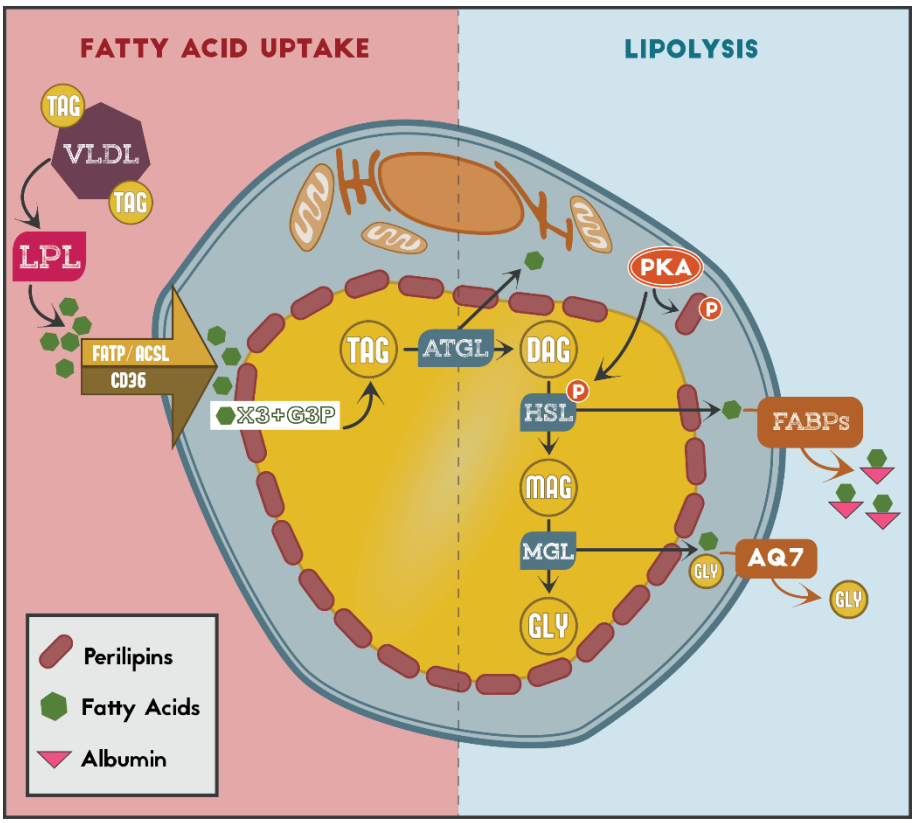

Figure 1. Adipocyte representing the fat deposition process on the left-hand side and the fat mobilization process on the right-hand side. Adapted from Girousse and Langin (2012).

LPL-mediated released FAs are bound by albumin and can be up-taken by tissues. Information on the mechanisms by which FAs enter into fish adipocytes is still limited. In mammals, the transport of FAs, especially long chain ones, is predominantly mediated by carrier proteins, even though FA transport can also occur to some extend via diffusion across the cellular membrane. A large number of proteins participating in the influx of FAs have been identified in mammalian models. However, the mechanisms of action of these transporters are controversially discussed. Proteins implicated in FA uptake are: fatty acid translocase (FAT or CD36), acyl-CoA synthetases, which include fatty acid transport protein (FATP) and acyl-coenzyme A synthetase (ACSL) family members, plasma membrane fatty acid binding protein (FABP), and caveolin-1 (Doege and Stahl, 2006). CD36 might serve as a FA receptor, accepting lipids transported by albumin, which can then be transported into the cell by the FATPs. Once

the FAs are on the inner membrane, a variety of acyl-CoA synthetases catalyse the ATP- and CoA-dependent esterification (Hertzel et al., 2008).

During the last years, more studies on FA uptake mechanisms in fish are becoming available. The mRNA expression of CD36, FABP3, FABP11 and FATP1 has been described in Atlantic salmon tissues, including white adipose tissue (Torstensen et al., 2009, Sánchez-Gurmaches et al., 2011), and in salmon adipocyte culture (Todorcevic et al., 2008, Todorcevic et al., 2010). CD36 presented a tendency to be reduced with maturation of adipocyte (Todorcevic et al., 2010) and be up-regulated by a vegetable oil-based diet in white adipose tissue (Torstensen et al., 2009). On the other hand, FATP1 and FABP3 transcript abundance showed an up-regulation during adipocyte differentiation (Todorcevic et al., 2008) while the replacement of fish oil by vegetable oil decreased the expression of FATP1 and exerted no effect on the regulation of the expression of FABP3 in salmon adipose tissue. A deep *in vivo* and *in vitro* study of the transporters CD36 and FATP1 and their endocrine and nutritional regulation was recently published in Atlantic salmon (Sánchez-Gurmaches et al., 2011) and in rainbow trout (Sánchez-Gurmaches et al., 2012b).

1.1.1.2 Triacylglycerol biosynthesis and lipid droplet formation

Depending on the energetic status of the organism, the intracellular FAs will be redirected and utilized for phospholipid synthesis, oxidation, or TAG synthesis (Figure 1). In a situation of excess of energy intake, TAG synthesis is activated in the adipose tissue, which is thought to occur on or around the plasma membrane of adipocytes (Ost et al., 2005). Fat accumulation in the adipocytes will serve two purposes; it will provide an energy reserve for periods of nutrient deprivation and it will clear out free FAs (FFAs) from the bloodstream that will be otherwise harmful compounds for the organism.

The backbone precursor for TAG formation is primarily glycerol-3-phosphate, derived from glycolysis or glyceroneogenesis within adipocytes. The relevance of these two pathways in Atlantic salmon adipocytes has been reported, suggesting that the parallel activation of pathways with redundant functions might be an efficient strategy to cover all the metabolic cellular needs (Todorcevic et al., 2010). Mammalian fat cells express specific glucose transporters on the plasma membrane to ensure a ready supply of glycolytic intermediates for TAG synthesis, mainly glycerol or acetyl-CoA; being the later a precursor of FAs by the process

of "*de novo*" lipogenesis (DNL) (see section 1.2.2). There are two types of glucose transport proteins in adipose tissue: GLUT1 and GLUT4. GLUT1 is responsible of facilitating transport of glucose down a concentration gradient, while GLUT4 is responsible of the majority of insulin-stimulated glucose transport (Hertzel et al., 2008). A GLUT4 homolog has been cloned and functionally characterized in brown trout (*Salmo trutta*) (Planas et al., 2000a), salmon (*Oncorhynchus Kisutch*) (Capilla et al., 2004a), Atlantic cod (*Gadus morhua*) (Hall et al., 2006, Hall et al., 2014) and Fugu (*Takifugu rubripes*) (Marín-Juez et al., 2013).

Insulin is also the most important physiological stimulus for synthesis of TAG as fat storage in mammals (Hertzel et al., 2008). The implication of this hormone in the regulation of lipid metabolism in fish adipose tissue has also been evidenced (Planas et al., 2000b). Besides, insulin has been recently shown to stimulate the lipogenic potential of rainbow trout adipose tissue (Polakof et al., 2011a, Capilla et al., 2013). Since this increase was observed on fish fed a high carbohydrate diet, the authors hinted at the implication of adipose tissue in glucose metabolism.

The synthesised TAGs are stored in lipid droplets together with cholesteryl esters (forming the neutral core of the droplet), phospholipids, and unesterified cholesterol. The concentration of each lipid class in the lipid droplet changes in a tissue-specific manner and their size can vary in response to cellular signals. Most cells are able to generate lipid droplets, however, the majority of fat is found in adipocytes of white adipose tissue. Until recently, they were considered as an inert storage compartment, but now it has become clear that they represent remarkably flexible, dynamic organelles that participate not only in lipid metabolism, but also in protein degradation, response to endoplasmic reticulum stress, protein glycosylation, and pathogen infection (Wilfling et al., 2014). The lipid droplets are coated by proteins from the PAT family, which is termed after the first three members identified: perilipin, ADRP (also called adipophilin) and Tip-47 (tail-interacting protein of 47 kDa). Two other proteins, S3-12 and OXPAT (also termed MLDP or LSDP5) round out the family. Recently, a unified nomenclature has been proposed for these proteins, being respectively named PLIN1, PLIN2, PLIN3, PLIN4 and PLIN5 (Kimmel et al., 2010). These lipid-droplet associated proteins have not been described in fish so far.

1.1.1.3 Lipolysis and fatty acid oxidation

Lipolysis (Figure 1) refers to the process by which TAG molecules are hydrolysed to FAs and glycerol. In mammals, a family of lipases including adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL), and monoacylglycerol lipase catalyse this reaction sequecially, even though other molecules are involved in this complex process (Hertzel et al., 2008). For instance, lipid droplets-coating proteins described previously are regulators of the lipolytic machinery, protecting or exposing the TAG core of the lipid droplet (Zechner et al., 2009). The phosphorylation of the perilipins and HSL induced by protein kinase A (PKA) initiates the release of the TAG reservoir. The hydrolysed FFAs in adipose tissue are primarily transported to other organs where they will be β-oxidise for energy production. A small part, however, can remain in the adipocyte where they can be either β-oxidise for energy purposes or re-esterified for further storage. The liberated glycerol is shuttled to the liver to undergo oxidation or gluconeogenesis, since adipocytes are unable to reuse glycerol due to the lack of expression of the enzyme glycerol kinase in normal conditions (Hertzel et al., 2008).

Very little is known about the lipid oxidation capacity of fish adipose tissue. In Atlantic salmon this process is regulated by different FAs *in vivo* (Todorcevic et al., 2009) and *in vitro* (Todorcevic et al., 2008). The lipid oxidation potential of rainbow trout adipose tissue has been suggested, possibly for a local use. (Polakof et al., 2011a). On the other hand, and in agreement with the anti-lipolytic effects of insulin, the infusion of this hormone was able to down-regulate FA β-oxidation in rainbow trout adipose tissue (Polakof et al., 2011a).

Numerous stimuli are capable of eliciting the lipolytic response in adipocytes, converging the vast majority on cAMP dependent PKA. In fish adipose tissue, as in mammals, the lipolytic cascade is regulated by hormones, cytokines and nutritional factors. Information on the regulation of lipolysis in gilthead sea bream and rainbow trout has come to light using isolated adipocytes. These studies have confirmed the catabolic action of the growth hormone (GH) and glucagon, as well as the anabolic effects of insulin, by the corresponding increase and decrease respectively in the levels of lipolysis in response to hormone incubation in rainbow trout (Albalat et al., 2005b, Albalat et al., 2005a). On the other hand, fasting (Albalat et al., 2005b) and feeding with experimental diets containing plant protein (Albalat et al., 2005a) or vegetable oils (Cruz-Garcia et al., 2011) also showed and induction of lipolysis in this cell type. Additionally, the pro-lipolytic effects of the cytokine tumour necrosis factor-α (TNFα) were

also confirmed in isolated adipocytes from both species (Albalat et al., 2005c, Saera-Vila et al., 2007).

HSL is the enzyme that hydrolyses intracellular TAG and diacylglycerol, and is one of the key molecules controlling lipolysis (Lafontan and Langin, 2009, Lampidonis et al., 2011). Despite of its relevance governing this process, sparse information concerning the regulation of this enzyme in fish is available (Hazel and Sidell, 2004). The expression of HSL was measured for the first time in perivisceral adipose tissue of gilthead sea bream, where a lack of regulation in response to the level of fish adiposity was found (Cruz-Garcia et al., 2009b). Nevertheless, the activity and expression of the enzyme was regulated in the same species by the replacement of fish oil in the diets by vegetable origin oils (Cruz-Garcia et al., 2011). Mechanisms that control HSL are not well known in fish. In rainbow trout isolated adipocytes an increase in lipolysis after incubation with liver X receptor (LXR) agonists was observed, but was not correlated with an increase in HSL expression indicating that HSL is not an LXR target gene (Cruz-Garcia et al., 2012). The presence of two HSL-encoding mRNAs that are differentially express among and within tissues and independently regulated by the nutritional state has been recently reported in rainbow trout (Kittilson et al., 2011).

In mammalian models, an imbalance in fat deposition/mobilization can lead to the development of metabolic disorders such diabetes, non-alcoholic fatty liver disease and dyslipidemia (Girousse and Langin, 2012). An increase in the understanding of the regulation of these processes in fish will provide important information to improve and secure the development of sustainable aquaculture practices (i.e., implementation of favourable feeding regimes, optimise diet formulation, maintenance of fish welfare…).

1.1.2 Lipid metabolism in liver

The basic biological mechanisms described above for FA uptake and transport, TAG biosynthesis and TAG mobilization are maintained in the liver with some differences characteristic of this tissue. The direction of FA metabolism in the liver depends on the energetic state of the animal. In the fed state, the animal converts carbohydrates to FAs through DNL (see later on and on section 1.2.2), while in the fasted state the most active processes are FA oxidation, ketogenesis and gluconeogenesis (Schulz, 2008). In mammals, an enhanced hepatic lipogenesis and the accumulation of fat in the liver appear to play an important role in

the pathogenesis of the metabolic syndrome (Sato et al., 2010). Even though an increase in fat deposition in fish liver has been extensively reported, its effects on health and welfare have been little explore.

The transport of the dietary lipids to the different organs is mediated by lipoproteins. The basic molecular organization and role in lipid metabolism of these lipoproteins in fish are similar to those of mammals (Tocher, 2003). The liver has a key role regulating TAGs and cholesterol homeostasis through the control of the lipoprotein trafficking (Fielding and Fielding, 2008). VLDL are synthesised in the liver for the export of endogenous TAGs. The synthesis of VLDL seems to be coordinated with TAG synthesis, and both take place in the endoplasmic reticulum of the hepatocytes (Ost et al., 2005).

As described above, the lipid uptake into the tissue is mediated by the action of LPL. In contrast to mammalian models, LPL is expressed in adult fish liver (Lindberg and Olivecrona, 1995) and is seasonally and nutritionally regulated in gilthead sea bream (Saera-Vila et al., 2005), where an increase in plant protein in the diet triggered an increase in LPL expression. In rainbow trout LPL is also nutritionally regulated in the liver (Richard et al., 2006). However, in this species the replacement of fish oil by vegetable oil decreased the activity of the enzyme.

Liver has been described as the main organ where *de novo* lipogenesis (Figure 2) takes place in both, mammals and fish, (Polakof et al., 2011a) and the process is considered to be conserved among them (Sheridan, 1994). Acetyl-CoA produced in the mitochondria is activated to malonyl-CoA by acetyl-CoA carboxylase (ACC). Then in the cytosol the multifunctional fatty acid synthase (FAS) through multiple condensations of malonyl-CoA with acetyl-CoA (or the elongating lipid) and reducing power in the form of NADPH, generates saturated FAs of 16 and 18 carbons. The pentose phosphate pathway (PPP) provides the reducing power needed in this process, principally by the glucose 6-phosphate dehydrogenase (G6PDH) and the malic enzyme (ME). In fish, the contribution of these two enzymes is species-specific and is nutritionally and hormonally regulated (Leaver et al., 2008). The saturated FAs produced can go through desaturation and elongation pathways to be further transformed into polyunsaturated FAs (PUFA).

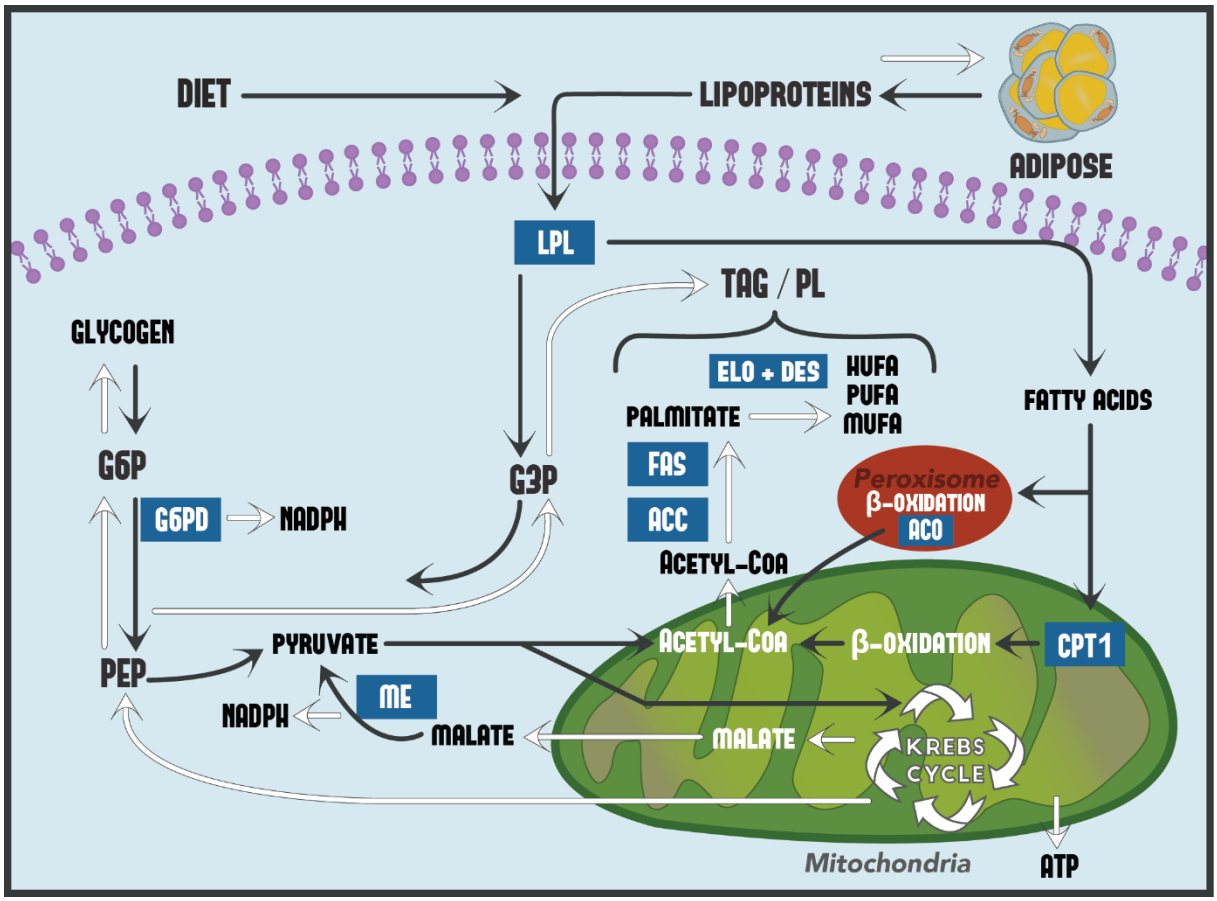

Figure 2. Main metabolic pathways of fatty acids in fish liver. White arrows indicate anabolic pathways, black arrows catabolic routes and enzymes are represented in blue boxes. Diet and adipose tissue are sources of fatty acids that can enter into the liver for energy production though *β*-oxidation in peroxisomes or mitochondria. Abbreviations: ACC, Acyl-CoA-carboxylase; ACO, Acetyl CoA-oxidase; CPT1, Carnitine palmitoyltranferase I; DES, Desaturases; ELO Elogases; FAS, Fatty acid synthase; G3P, glycerol-3-phosphate; G6P, Glucose-6-phosphate; G6PD, Glucose-6-phosphate dehydrogenase; HUFA, Highly unsaturated fatty acids; LPL, Lipoprotein lipase; ME, Malic enzyme; MUFA, Monounsaturated fatty acids; PEP, Phosphoenolpyruvate; PUFA, Polyunsaturated fatty acids. Modified from Leaver et al. (2008).

Regarding nutritional regulation of hepatic lipogenesis in fish, it has been reported that the amount of n-3 PUFA present in the fish oil is the responsible for inhibiting the pathway in salmonids (Alvarez et al., 2000, Menoyo et al., 2003). Therefore, increasing levels of replacement of fish oil by vegetable oils can promote liver lipogenesis (Leaver et al., 2008) but the effects are variable depending on the species and inhibitory effects have been also described in sea bream and sea bass (Menoyo et al., 2004, Dias et al., 2005). Lipogenesis is also regulated by hormones and, as is the case in mammals, insulin stimulates the pathway promoting energy storage (Polakof et al., 2011b, Plagnes-Juan et al., 2008).

In eukaryotic cells, the degradation of FAs takes place in two different organelles, mitochondria and peroxisomes. Fish capacity to catabolize FAs depends on several factors such as fish size, life stage, and season (Leaver et al., 2008). Mitochondrial β-oxidation is mediated by a carnitine-dependent transfer of acyl groups across the inner mitochondrial membrane. Carnitine palmitoyltransferase-1 (CPT1) in the outer mitochondria membrane converts acyl-CoA to acylcarnitine, which is then transported over the inner mitochondrial membrane in exchange with a free carnitine molecule. In the mitochondrial matrix, carnitine palmitoyltransferase-2 (CPT2) catalyses the reversible transfer of acyl residues between carnitine and CoA to form acyl-CoA thioesters that are then β-oxidised (Schulz, 2008). CPT1 is considered as the regulation point of mitochondrial β-oxidation and in fish, as in mammals, this enzyme is inhibited by malonyl-CoA (Froyland et al., 1998, Gutieres et al., 2003). Peroxisomal β-oxidation resembles mitochondrial β-oxidation but with a different enzymatic machinery and representing a less efficient process from an energetic point of view since half of the energy produced is lost in form of heat. In fish, the contribution to the total β-oxidation capacity of both organelles depends on the tissue, being the peroxisomal β-oxidation a relevant pathway of FA degradation in fish liver (Nanton et al., 2003, Stubhaug et al., 2005). Acyl-CoA oxidase (ACO) catalyses the rate-limiting step in the peroxisomal β-oxidation and the nutritional modulation of this enzyme in fish has been analysed (Diez et al., 2007, Morais et al., 2007, Stubhaug et al., 2007).

1.2 CARBOHYDRATE METABOLISM IN FISH

In contrast to terrestrial vertebrates, fish seem to utilise carbohydrate in a less effective way. Therefore, is generally accepted that fish do not have a specific requirement for dietary carbohydrates (NRC, 2011). It has been suggested that they might fulfil their requirements of glycogen through the catabolism of amino acids to a high degree (Leaver et al., 2008). However, proteins are normally the most expensive ingredient in the formulated aquafeed and usually rely on fish meal. In an attempt to reduce production costs and the dependence on overexploited marine fisheries, the development of experimental diets with different inclusion levels of carbohydrate have been tested in different farmed species, showing that the level of acceptance is species-specific.

Gilthead sea bream has been reported to present excellent starch digestibility coefficients (Couto et al., 2012) and elevated activity of the main enzymes implicated in the glycolytic

pathway (Enes et al., 2008, Panserat et al., 2000a, Couto et al., 2008), suggesting an efficient utilization of dietary starch for energy purposes in this species. In salmonids, digestible carbohydrate contents of more than 20-30% of the diet result in prolonged postprandial hyperglycaemia (Kirchner et al., 2003) and impaired growth (Geurden et al., 2007). Despite of an induction in the glycolytic capacity, neither insulin nor dietary carbohydrates trigger the inhibition of the gluconeogenic potential in the liver (Polakof et al., 2011c, Panserat et al., 2001a), which could explain in part the limited ability to regulate glycaemia in this species. However, recent studies have demonstrated that the interaction between dietary components might be one of the key factors affecting glucose homeostasis. In mammals, high levels of FAs disrupt carbohydrate metabolism, eventually causing impaired glucose tolerance (Randle, 1998). A similar interaction has been described in rainbow trout, where fish developed high fat-induced persistent hyperglycaemia and reduced insulin sensitivity (Figueiredo-Silva et al., 2012).

The presence of most of the key enzymes involved in carbohydrate metabolic pathways in fish has been reported, indicating that the poor utilisation may be due to an aberrant hormonal and nutritional regulation caused by evolutionary adaptation (Enes et al., 2009, Polakof et al., 2011c). Most of the studies in farmed fish species regarding high dietary carbohydrate content have taken a hepato-centric approach, despite of the recognised relevance of other peripheral tissues in the control of energy balance, such as muscle or adipose tissue.

1.2.1 Glucose intolerance in fish

In mammals, glucose is the fuel substance used by most tissues. Yet, to what extent can teleost fish use glucose and what is its contribution to the overall energy metabolism is still not well known. Glucose has been described as the main substrate in several tissues, such as red cells in the sea raven (*Hemitripterus americanus*) (Sephton et al., 1991) or brain in rainbow trout (DiAngelo and Heath, 1987). However, carnivorous fish species are traditionally considered as glucose intolerant (Moon, 2001, Wilson, 1994). This is mainly due to the persistent hyperglycaemia exhibited following an ingestion of a high carbohydrates meal or a glucose injection (Polakof et al., 2012).

Glucose homeostasis results from a balance between glucose utilization (glycolysis) and glucose production (gluconeogenesis and glycogenolysis) (Figure 3). In mammals, the intake

of dietary carbohydrates significantly induces the glycolytic pathway and concurrently inhibits gluconeogenesis (Iynedjian, 2009). The impaired postprandial inhibition of hepatic endogenous production of glucose is a metabolic characteristic of rainbow trout (Panserat et al., 2000b, Panserat et al., 2001a, Panserat et al., 2001b). This lack of gluconeogenesis regulation was also observed in gilthead sea bream (Caseras et al., 2002). A deep review concerning this imbalance between glucose uptake and glucose production in fish and its regulation by nutritional factors is available (Enes et al., 2009).

Some studies have pointed to the poor regulation of glucose utilization by the peripheral tissues of carnivorous fish (Moon, 2001, Kamalam et al., 2012, Jin et al., 2014). However, insulin has been shown to improve glucose distribution and uptake by peripheral tissues enhancing the fish ability to regulate plasma glucose (Polakof et al., 2010b, Polakof et al., 2010a). Other reported factors explaining the poor utilization of glucose in fish in comparison with mammals, are the relatively low number of insulin receptors and the lower capacity of glucose to stimulate insulin secretion compared to that from amino acids (Navarro et al., 1999, Navarro et al., 2002). Additionally, the persistent hyperglycaemia observed in carnivorous fish after a carbohydrate load despite the existence of insulin secretion and insulin receptors in major insulin-responsive tissues resembles the insulin resistance state from humans suffering from type II diabetes. Insulin exerts its anabolic effects through the heterotetrameric receptor tyrosine kinase, which activity in fish is lower than that in mammalian models, according to their lower capacity for glucose utilization (Gutiérrez et al., 1995). Along a cascade of protein phosphorylation progresses protein kinase B is activated (AKT/PKB) via phosphatidylinositol-3-kinase (PI3K). This pathway is known to promote cell growth, protein synthesis, lipogenesis and glucose uptake while inhibiting apoptotic processes and lipolysis (Berggreen et al., 2009). The identification and activation of AKT by insulin and IGF-1 has been observed in fish myocytes and adipocytes (Montserrat et al., 2007, Sánchez-Gurmaches et al., 2010, Bouraoui et al., 2010). One of the members of the downstream signalling pathway of PI3K/AKT is the target of rapamycin (TOR). In fish, as in mammals, this protein is considered a nutrient sensor especially involved in protein metabolism (Sengupta et al., 2010, Seiliez et al., 2008).

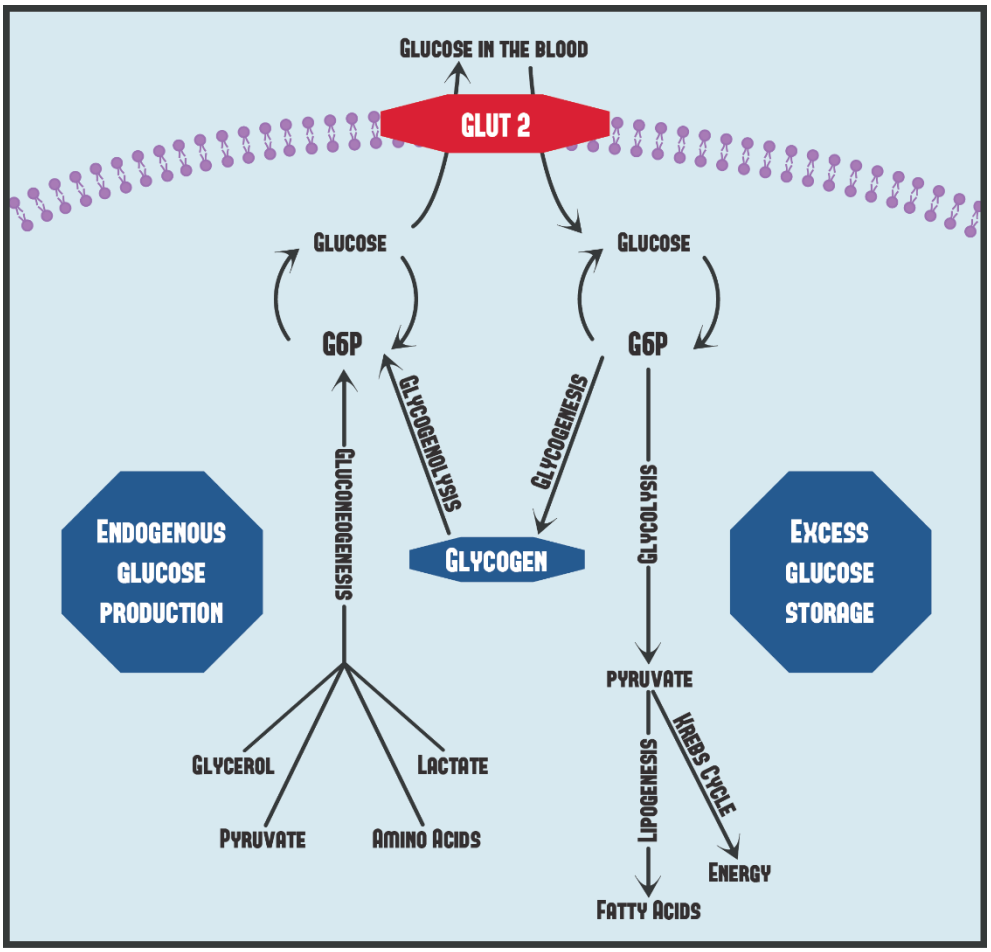

Figure 3. Main metabolic pathways responsible for maintaining glucose homeostasis. Adapted from Enes et al. (2009).

Another factor studied regarding the low capacity of glucose utilization in fish is the glucose transporter GLUT4. Salmon GLUT4 has lower affinity for glucose than mammals, contributing to the glucose intolerance described in salmonids (Capilla et al., 2004a). The sensitivity of this transporter to insulin has been reported in fish. For instance, insulin is able to up-regulate the expression of GLUT4 in rainbow trout skeletal muscle (Polakof et al., 2010b, Diaz et al., 2009). The translocation of the transporter after insulin stimulation has also been demonstrated in trout skeletal muscle cells (Diaz et al., 2007), and in mammalian 3T3-L1 adipocytes transfected with salmon okGLUT4 (Capilla et al., 2004a). In addition, an increase in glucose uptake upon insulin stimulation in trout adipocytes has been reported and suggested to be mediated by GLUT4 (Bouraoui et al., 2010). However, in an *in vivo* approach insulin infusion failed to regulate the expression of GLUT4 in adipose tissue of rainbow trout fed a high-carbohydrate diet (Polakof et al., 2010a).

The low capacity of carbohydrate use in fish, reflected in a variable degree of glucose intolerance, seems to be explained, at least in part, by various factors already mentioned related to glucose metabolism and its control. Nevertheless, other possible causes of this low ability to metabolise carbohydrates remain less explored, such as the possible role of lipogenic pathways.

1.2.2 Metabolic use of glucose: *de novo* lipogenesis (DNL)

DNL provides a physiological pathway for the synthesis of lipids from carbohydrates when a mammal has excess carbohydrate in the diet (Figure 4). In humans, this pathway occurs in essentially all cells, being primarily active in the liver and in adipose tissue. It has been reported that hepatic lipogenesis from glucose is quantitatively more efficient than lipogenesis in the adipose tissue (Lodhi et al., 2011). However, the biological relevance of DNL in both tissues, as well as its regulation, is known to be of extreme importance for the organism. The deregulation of this pathway has been associated with diverse metabolic anomalies, such as insulin resistance, obesity and non-alcoholic fatty liver disease (Ameer et al., 2014). An elevation of DNL in liver cells, which also occurs in cancer cells and during viral infections, has been related with the development of metabolic disease. On the contrary, an increase in DNL in adipose tissue is associated with metabolic health, indicating that this pathway might be linked to health in a tissue-specific manner (Eissing et al., 2013). Therefore, mammalian DNL is a complex and highly regulated metabolic pathway (Ameer et al., 2014).

The products of DNL are saturated fatty acids from 12:0 to 18:0 and unsaturated fatty acids (UFAs), particularly 16:1n-7 and 18:1n-9. The adverse effects of saturated fatty acids accumulation on cell function are well known (Borradaile et al., 2006). However, this potential toxicity is minimized by the coordinate up-regulation of the elongation of FAs and their desaturation by the enzyme stearoyl-CoA desaturase (SCD or delta-9 desaturase) (Collins et al., 2010). Apart from the traditional role as efficient storage of energy, the relevance of this pathway has been highlighted since the FAs produced by DNL and their derivatives present different bioactivities than those provided by the diet, exerting a key role as important signalling molecules implicated in many physiological processes. For instance, 16:1n-7 has been categorised as a lipokine able to strongly stimulate muscle insulin action and suppress hepatosteatosis (Cao et al., 2008). Whether this is the case in fish is an issue that remains to be further elucidated.

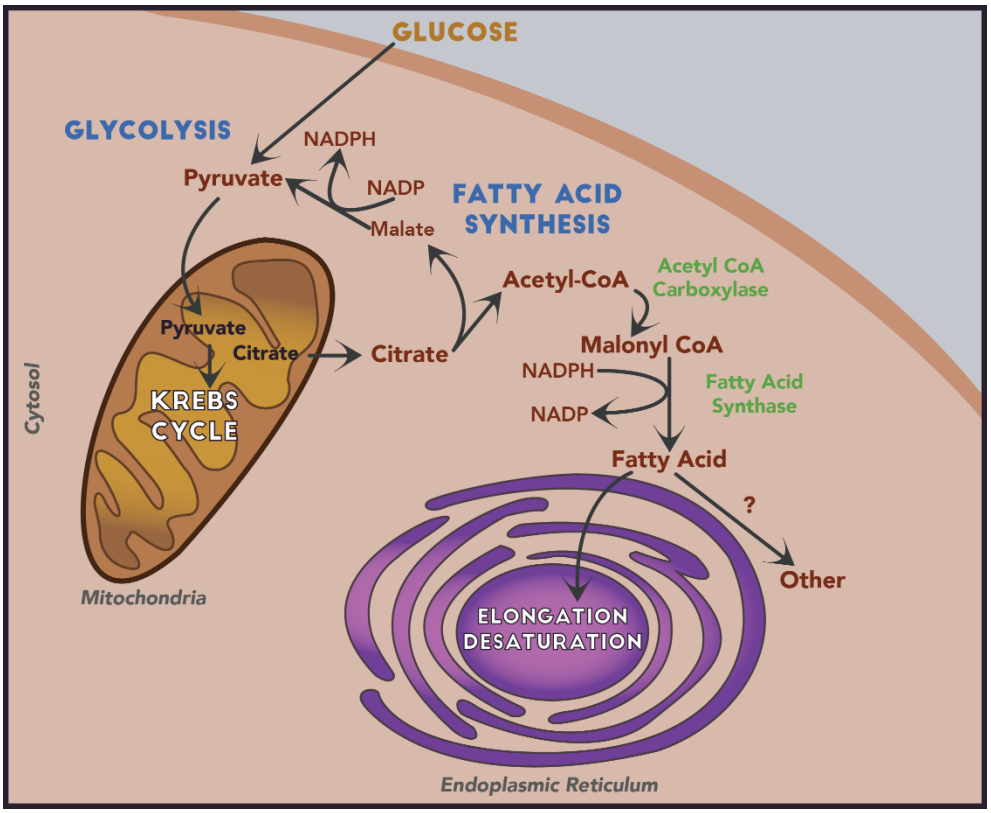

Figure 4. Illustration of the pathway for *de novo* lipogenesis. Modified from Herman and Kahn (2012)

The presence of the main enzymes responsible for *de novo* lipid synthesis have been identified at a biochemical level in fish adipose tissue (Polakof et al., 2011a) and in liver (Polakof et al., 2011b). In salmonids, hepatic DNL is regulated by environmental, physiological and pharmacological factors (Skiba-Cassy et al., 2009, Panserat et al., 2009, Polakof et al., 2011d, Sheridan et al., 1985, Higgs et al., 2009). The nutritional regulation of hepatic DNL has been already mentioned in 1.1.2 section. In a recent work, insulin stimulates *de novo* hepatic lipogenesis in rainbow trout fed a high carbohydrate diet (Polakof et al., 2011b), suggesting the involvement of this pathway as a sink for excess of glucose.

Different approaches have been used to study fish DNL in relation to fish glucose homeostasis during the last years. In rainbow trout two experimental lines have been developed through divergent selection for low (Lean line) or high (Fat line) muscle fat content (Quillet et al., 2005). These two trout lines have been subjected to diverse experimental challenges providing valuable information that sheds light on the mechanisms regulating glucose homeostasis. This study demonstrated an increase in the lipogenic hepatic capacity of the Fat line, suggesting that *de novo* synthesis of lipids from glucose might have a pivotal role in the control of glucose homeostasis (Kamalam et al., 2012, Skiba-Cassy et al., 2009). Besides, further research with

these two lines showed that the Fat line presents an enhance glucose utilization in liver and muscle coupled with reduce hepatic FA oxidation (Kolditz et al., 2008).

An enhanced hepatic lipogenesis was also observed with the use of an anti-diabetic drug and it was linked to a better ability to control glucose homeostasis (Polakof et al., 2011d, Panserat et al., 2009). Metformin exerted a hypoglycaemic effect, however and paradoxically, the use of this drug increased the level of hepatic gluconeogenesis. In addition, in fasted rainbow trout, metformin exerted an unexpected negative effect on insulin action (Polakof et al., 2009), being the nutritional state of the animal a key factor modulating the effect of the drug. Metformin also increased the glucose uptake capacity in the peripheral tissues (muscle and adipose tissue) (Polakof et al., 2011d).

The use of a diet depleted in methionine has been also addressed in rainbow trout, showing that the restriction in this single amino acid in the diet abolishes the hyperglycaemia present after a carbohydrate load (Craig and Moon, 2013). However, the hypoglycaemic phenotype was not linked to an increase in the hepatic lipogenic capacity as previous works did. Instead, an increase in fat oxidation in the liver and a decrease in the glycolytic activity was found.

The implication of fish adipose tissue in the control of glucose metabolism and homeostasis has also been proposed (Polakof et al., 2011a). In gilthead sea bream, the lipogenic activity of adipose tissue decreased with the inclusion of vegetable ingredients in the diet (Bouraoui et al., 2011). However, and despite its importance in controlling fish energy balance, adipose tissue remains largely unexplored. The present thesis will provide new insights into the lipogenic potential of Atlantic salmon adipocytes and their ability to synthesize lipids via DNL from glucose.

1.3 ADIPOSE TISSUE

In mammals, adipose tissue has been classified as white adipose tissue (WAT) or brown adipose tissue (BAT). White adipocytes, which make up the bulk of fat tissue in most animals, contain a large unilocular lipid droplet that comprises the majority of cell volume, while the cytoplasm and nucleus are found at the cell periphery. On the other hand, brown adipocytes contain multiple smaller lipid droplets and dissipate energy in form of heat through the actions of uncoupling protein-1 (UCP-1). Recently, the existence of some adipocytes present in white adipose depots that can be induced to display a brown fat-like phenotype, has been identified

and called beige or brite adipocytes (Park et al., 2014). So far, only one study has tried to characterise the presence of BAT in fish (Chung-Davidson et al., 2013). However, and despite the thermogenic capacity of the adipose tissue analysed by Chung-Davidson and collaborators, no other evidence supporting that this tissue could be considered as BAT was found.

Traditionally, adipose tissue was considered to be simple, static and a mere storage compartment of TAGs. However, it is now clear that adipose tissue is a remarkably complex organ with profound effects on physiology (Rosen and Spiegelman, 2014). Several discoveries linking adipose tissue with the control of energy balance and overall body homeostasis, as well as the obesity epidemic and the increasing rates of type 2 diabetes, have triggered a renew interest on the developmental, functional, and pathophysiological aspects of this organ.

Adipose tissue is a special loose connective tissue. Adipocytes make up around 60-70% of adipose mass in adults. The remaining components of adipose tissue are blood and endothelial cells, adipose precursor cells of varying degrees of differentiation, macrophages, and fibroblasts (collectively referred as the stromal vascular fraction or SVF). The amount and distribution of adipose tissue varies among and within species. In humans, the excessive visceral fat accumulation is associated with metabolic disease (i.e. insulin resistance, type 2 diabetes, dyslipidaemia, hypertension, atherosclerosis, hepatic steatosis, and cancer) while the preferential accumulation of subcutaneous fat is associated with improved insulin sensitivity and low risk for developing type 2 diabetes (Sánchez-Gurmaches and Guertin, 2014a). In rainbow trout regional differences were also detected between visceral and subcutaneous fat depots, pointing out to a higher metabolic activity present in the subcutaneous population (Weil et al., 2009).

In order to understand the development of adiposity, it is crucial to identify the factors that regulate the formation of adipocytes from precursor cells in the adipose tissue. In fish, like in mammals, the development of adipose tissue is a continuous process that takes place throughout all animal life that depends on genetic, hormonal and dietary factors. Adipose tissue has a considerable capacity to expand. Its growth is the result of the recruitment of new adipocytes (hyperplasia) and the expansion of the existent ones (hypertrophy).

The developmental origins of adipose tissue remain poorly defined. Research efforts are being made to trace the origins of mature adipocytes back to their adult precursor and embryonic ancestors. While one hypothesis is that adipose tissue develops from a single lineage (Gesta et al., 2007, Berry and Rodeheffer, 2013), other research groups provided evidences to support

the idea that adipocytes arise from diverse lineages and that this influences fat distribution and function (Sánchez-Gurmaches and Guertin, 2014b).

1.3.1 Adipogenesis

Adipogenesis (Figure 5) is a complex developmental process controlled by a coordinated transcriptional cascade and cell-cycle proteins that regulate gene expression and lead to adipocyte differentiation. Adipocytes derive from multipotent mesenchymal stem cells (MSC) and its transition to mature adipocytes has been characterized as a two-phase process comprising proliferation and determination as a first phase and posteriorly terminal differentiation (Rosen and MacDougald, 2006). During the determination phase, multi-potent stem cells commit to the adipocyte lineage and become preadipocytes. Preadipocytes can then enter the terminal differentiation phase, where they will acquired all the characteristics of mature adipocytes.

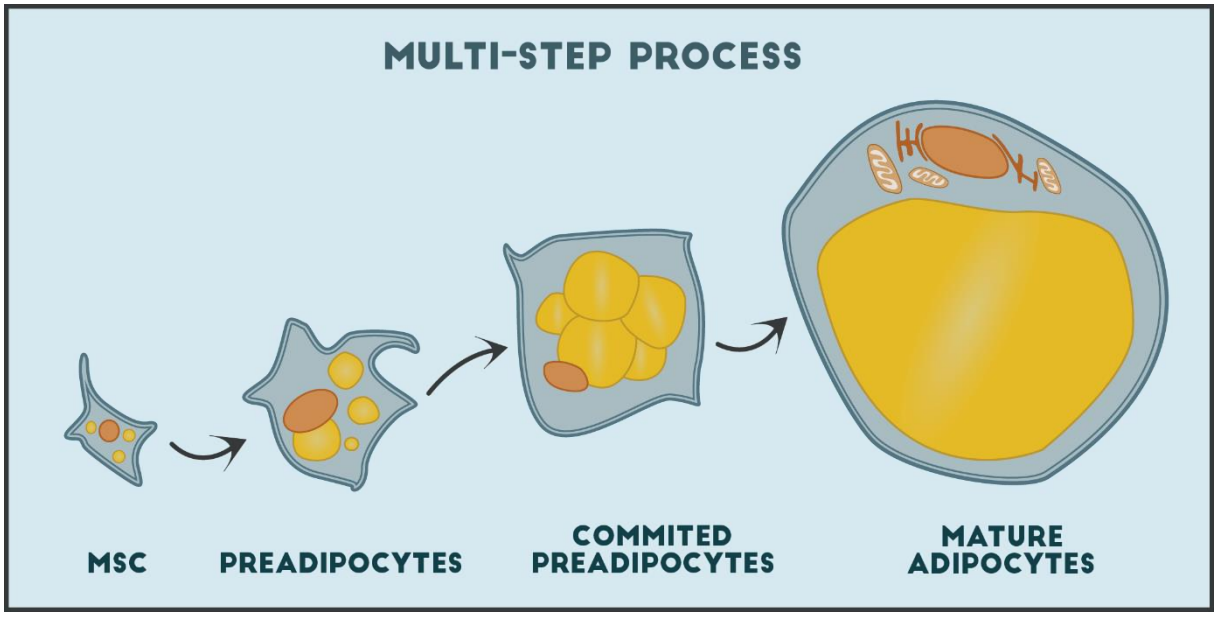

Figure 5. Schematic representation of adipogenesis.

Most of the information available concerning the process of adipogenesis comes from *in vitro* studies using cellular models that are already committed to the adipose lineage (i.e. 3T3-L1, 3T3-F442A, Ob17). That is why terminal differentiation has been more characterized than the

process of determination (Rosen and Spiegelman, 2014). However, multipotent stem cell lines, able to commit to different lineages including adipose, bone and muscle lineage have also been used. In fish, scarce information regarding the early phases of adipocyte development is available. However, a transcriptomic analysis of Atlantic salmon adipocyte development indicates that lineage determination in this species is very similar to that in mammals (Todorcevic et al., 2010).

On the other hand, primary cultures derived from SVF isolated from adipose tissue have been also used to study the processes governing adipogenesis in different mammalian species (Louveau and Gondret, 2004, Boone et al., 2000). Despite of some technical difficulties in the isolation and growth condition requirements when compared to the immortalised cell lines, the use of primary cultures represents a more relevant *in vitro* system to study the *in vivo* context (Boone et al., 2000). In fish, no adipogenic cell lines are available so far. However, primary cultures derived from the SVF of adipose tissue from different fish species have been developed, including Atlantic salmon (Vegusdal et al., 2003), red sea bream (Oku et al., 2006), rainbow trout (Bouraoui et al., 2008), large yellow croaker (Wang et al., 2012) and gilthead sea bream (Salmerón et al., 2013).

Studies with cell lines and primary cell culture shows that during the first phase the committed cells proliferate (exponential growth phase) until reaching confluence, when they undergo growth arrest. Then, and upon hormonal induction, most cell lines undergo one or two rounds of DNA replication and cell doubling, known as mitotic clonal expansion. However, other models, such as C3H10T1/2 and human pre-adipocytes differentiate without post-confluence mitosis. Therefore, whether the mitotic clonal expansion is a fundamental requirement for terminal adipocyte differentiation remains controversial (Otto and Lane, 2005). However, it is clear that some of the checkpoint proteins for mitosis also regulate aspects of adipogenesis (Rosen and MacDougald, 2006). After clonal expansion, the cells are called early or immature adipocytes and they start to express adipocyte-specific genes.

The differentiation of preadipocytes to mature adipocytes is accompanied by marked morphological and biochemical changes. One of the first hallmark of adipogenesis is the dramatic alteration in cell shape since the cells convert from fibroblastic to spherical. These morphological modifications are paralleled by changes in the level and type of extracellular matrix (ECM) components and the level of cytoskeletal components with the involvement of a

proteolytic plasminogen cascade (Selvarajan et al., 2001). Failure to acquire the right shape will compromise the ability of the adipocytes to perform their physiological function (Cao, 2010).

Many different events contribute to the commitment of MSCs into the adipocyte lineage, including the coordination of a complex network of transcription factors, cofactors and signalling intermediates from numerous pathways. For instance, the IGF/insulin signalling pathway, which is strongly adipogenic in mammals (Rosen and Spiegelman, 2014), has also been studied in fish and its implication in the regulation of adipocyte development and its pro-adipogenic function has also been reported (Bouraoui et al., 2008, Bouraoui et al., 2010). Conversely, the Wnt and hedgehog pathways are known to promote osteogenesis and inhibit adipogenesis in mammalian uncommitted and committed cells (Rosen and MacDougald, 2006). Many other signalling pathways have been related to the regulation of adipogenesis; however, the results are still misleading since they strongly vary depending on cell type, stage of differentiation and other experimental conditions. Such is the case for the TGFβ/BMP superfamily. These pathways remain to be explore in fish, at least in the adipocyte context.

Few information is available in fish concerning the main transcription factors involved in the regulation of adipocyte differentiation. Yet, the expression of peroxisome proliferator activated receptor γ (PPARγ) and CCAAT/enhancer binding protein factor (C/EBP) α, β and δ have been characterized during adipogenesis in salmonids (Bouraoui et al., 2008, Todorcevic et al., 2008, Todorcevic et al., 2010, Huang et al., 2010). These transcription factors play a key role in the complex transcriptional cascade that takes place during adipogenesis. PPARγ is considered as the "master regulator" of fat cell formation, since it is both, necessary and sufficient for adipogenesis (Rosen and Spiegelman, 2014). On the other hand, C/EBPβ and δ are early induced during differentiation and promote adipogenesis by inducing C/EBPα and PPARγ in mammals (Rosen and MacDougald, 2006). There are many other transcription factors that are known to be involved in adipose determination, like members from the Krüppel-like factor family (KLFs), the early growth response protein-2 (KROX20), sterol regulatory element binding protein (SREBP) and cyclic AMP response element-binding protein (CREB) among many others (Rosen and MacDougald, 2006, Rosen and Spiegelman, 2014, Ali et al., 2013, White and Stephens, 2010, Moreno-Navarrete and Fernández-Real, 2012). Nevertheless, as far as we know their role in adipogenesis has not been characterized in fish.

The identification of novel factors that contribute to the regulation of adipogenesis in mammals has expanded the research field. Such is the case for epigenetic signals and chromatin-

modifying proteins, which are now known to orchestrate the transcriptional cascade of adipocyte development (Moreno-Navarrete and Fernández-Real, 2012, Musri et al., 2010). Other players recently identified are microRNAs (miRNAs), which are small non-coding RNAs that bind to regulatory sites of target mRNA and modify their expression, either by translational repression or target mRNA degradation, resulting in decreased protein production. A wide number of miRNAs involved in the control of adipogenesis, either accelerating or inhibiting the process, have been reported (Chen et al., 2013). Studies on these areas are lacking in fish. A deeper understanding of the epigenetic mechanisms could provide valuable information regarding not only adipogenesis, but also the control of adipocyte metabolism.

1.3.2 Adipose tissue as an endocrine organ

As discussed above, adipose tissue plays a crucial role in the regulation of whole body FA homeostasis. However, a much more complex and dynamic role of adipose tissue emerged with the discovery of leptin in 1994 (Zhang et al., 1994). The subsequent discovery of many other factors (Table 1), including cytokines, chemokines, and adipokines, stablished adipose tissue as an endocrine organ critical for regulating metabolism in both health and disease (Galic et al., 2010, Ali et al., 2013, Harwood, 2012). These factors are originated from adipocytes, preadipocytes and other cell types within adipose tissue, such as macrophages.

These secreted products interact with central as well as peripheral organs such as the brain, liver, pancreas, and skeletal muscle to control diverse processes, such as appetite regulation, energy expenditure, carbohydrate and lipid metabolism, insulin sensitivity, blood pressure, blood coagulation, and immunological responses (Harwood, 2012). Therefore, cross-talk and interactions between adipose tissue and other organs are of vital importance to maintain the organism homeostasis. Besides, some of the secreted factors can interact in a paracrine fashion to control adipocyte metabolism. When adipose tissue expands, as occurs in obesity, there is a dysregulation of adipokine secretion and action. This dysregulation is linked to the development of a variety of metabolic disorders (Ali et al., 2013).

Table 1. Factors secreted by adipose tissue and their metabolic effects. Reviewed from Harwood (2012).

Secreted proteins	Metabolic effects
Adiponectin	Increases insulin sensitivity; anti-inflammatory and anti-atherosclerotic actions
Adipsin	Stimulates TAG storage in adipocytes; activates alternate complement pathway
ApoE[b]	Protein component of triglyceride-rich lipoproteins
ASP	Stimulates triglyceride synthesis in white adipose tissue; antilipolytic
CETP[b]	Transfers cholesterol esters between lipoproteins
IGF-1	Stimulates proliferation and differentiation of adipocytes
IL6	Proinflammatory; lipolytic; reduces insulin sensitivity
Leptin	Satiety signal; inhibits lipogenesis; stimulates lipolysis; improves insulin sensitivity
LPL[b]	Hydrolyzes triglycerides in triglyceride-rich lipoproteins allowing cellular uptake
MCP-1	Recruits monocytes to sites of injury and inflammation
MIF	Immunoregulator with paracrine actions in white adipose tissue
Omentin	Believed to enhance the actions of insulin
PAI-1	Inhibits plasminogen activation; blocks fibrinolysis
RBP4	Increases insulin resistance
Resistin	Increases insulin resistance; promotes endothelial dysfunction
TGFβ[b]	Regulates preadipocyte proliferation and differentiation and also adipocyte apoptosis
Tissue factor	Initiates the coagulation cascade
TNFα	Lipolytic; increases energy expenditure; induces insulin resistance
VEGF	Stimulates angiogenesis (vascular proliferation) in white adipose tissue
Visfatin	Insulin mimetic produced primarily by visceral adipose tissue

ApoE, apoliproprotein E; ASP, acylation-stimulating protein; CETP, cholesterol ester transfer protein; IGF-1, insulin like growth factor-1; IL6, interleukin-6; LPL, lipoprotein lipase; MCP-1, monocyte chemo-attractant protein-1; MIF, macrophage migration inhibitory factor; PAI-1, platelet activator inhibitor-1; RBP4, retinol binding protein-4; TGFβ, transforming growth factor-β; TNFα, tumor necrosis factor-α; VEGF, vascular endothelial growth factor. Harwood 2012.

[b] Secreted proteins without hormonal actions.

In fish, the most studied endocrine factors are leptin and ghrelin due to their role as hormones regulating appetite and growth (Kling et al., 2012, Salmerón et al., 2015a). Nevertheless, leptin is more expressed in liver than in adipose tissue in fish (Copeland et al., 2011). As in mammals, leptin is consistently anorexigenic in fish. However, and due to gene duplication events and different strategies concerning energy storage, divergent functions for leptin in fish that only partially resemble those of higher vertebrates might be found (Won and Borski, 2013). On the

other hand, ghrelin, which is present in adipose tissue but highly expressed in stomach as in mammals, is also an orexigenic peptide in fish, rising its levels during fasting and decreasing after feeding. However, variable results have been found, suggesting that the regulation of the ghrelin system among teleost is species-specific (Unniappan and Peter, 2005).

1.3.2.1 Adiponectin

Adiponectin, also known as AdipoQ, apM1, ACRP30 and GBP28, is a 30 kDa protein discovered in 1995 (Scherer et al., 1995). Is one of the main proteins secreted by mammalian adipocytes, making adipose tissue the principal contributor of its circulating levels. Adiponectin exists as full length or as a smaller globular domain (gAd) and it forms a wide range of multimer complexes in plasma, being the 3 major oligomeric forms a low-molecular weight (LMW) trimer, a middle–molecular weight (MMW) hexamer, and high-molecular weight (HMW) 12- to 18-mer adiponectin (Kadowaki et al., 2006). This anti-inflammatory adipokine exert its action through binding two receptors: adiponectin receptor 1 (AdipoR1) and adiponectin receptor 2 (adipoR2). These heptahelix receptors present an internal N-terminus and an external C-terminus, being therefore structurally and functionally different from G-protein coupled receptors (Yamauchi et al., 2003). These receptors are expressed differentially in tissues and are involved in the regulation of glucose and lipid metabolism, as well as inflammation and oxidative stress (Yamauchi et al., 2007). AdipoR1 and AdipoR2 exert similar effects but they seem to present individual signalling preferences. Whereas phosphorylation of ERK1/2 may depend on both receptors, AdipoR1 is more prominent in AMPK phosphorylation, and AdipoR2 is involved in PPARα activation (Yamauchi et al., 2007, Lee et al., 2008).

In 2001, three independent research groups demonstrated for the first time an insulin-sensitizing effect of adiponectin in mice (Berg et al., 2001, Fruebis et al., 2001, Yamauchi et al., 2001). Adiponectin has also been shown to reduce glucose production in the liver by inhibiting the expression of the gluconeogenic enzymes phosphenolpyruvate carboxykinase and glucose-6 phosphate via AMPK (Yamauchi et al., 2002). In skeletal muscle it triggers an increase in glucose uptake by promoting the translocation of GLUT4 to the plasma membrane (Yamauchi et al., 2001). Indirect insulin-sensitizing effects of adiponectin were also revealed by its capacity to reduce plasma levels of FFA (Fruebis et al., 2001) and TAG content in liver and muscle (Yamauchi et al., 2001). Moreover, adiponectin is engaged in the maintenance of optimal adiposity. It controls adipocyte differentiation (Yan et al., 2013) and inhibits lipolysis in

adipocytes (Qiao et al., 2011). Adiponectin has also been reported to supress the release pro-inflammatory cytokines, such as TNFα, from human adipocytes and stromal-vascular cells (Dietze-Schroeder et al., 2005). The interplay between these two cytokines is an issue that will be further discuss in this thesis on the fish adipocytes context.

Although many aspects regarding adiponectin biological functions, regulation and mechanisms of action require further investigations, adiponectin plasma levels have been negatively correlated to human obesity, insulin resistance, metabolic syndrome and cardiovascular diseases (Harwood, 2012). Due to all its properties, adiponectin is seen as a promising candidate for human therapeutic use.

The adiponectin system has been characterized in zebrafish (Nishio et al., 2008), where the relationship between this adipokine and appetite regulation was confirmed. In rainbow trout the presence and tissue distribution of adiponectin was also established (Kondo et al., 2011). According to this work, and contrary to what has been described in mammals, adiponectin transcripts were highly expressed in muscle and weakly detected in perivisceral adipose tissue. These results were confirmed by Sánchez-Gurmaches et al. (2012a) in an extensive work, where also the expression of the adipoRs was assessed. In this work, a more ubiquitous distribution of these receptors compared to that reported in humans and mice was found. Nevertheless, another study has considered that the high levels of adiponectin in fish skeletal muscle might be due to both the adipocyte distribution around myosepta and the intermyocyte distribution of the adipocyte-related cells (Han et al., 2013). The first contributions on the effects of endocrine regulation of the adiponectin system in teleost fish were carried out, confirming the role of insulin, GH and TNFα in the regulation of the adiponectin system (Sánchez-Gurmaches et al., 2012a). Furthermore, the same authors reported that adiponectin activated the PI3K/AKT pathway in culture myocytes suggesting that some of its effects are conserved from fish to mammals. Even though the expression of adiponectin in rainbow trout subjected to different nutritional challenges has been studied (Kondo et al., 2012, Craig and Moon, 2013, Sánchez-Gurmaches et al., 2012a), further research needs to be done in order to fully understand the role and regulation of the adiponectin system in fish.

1.3.2.2 TNFα

The secretion of this pro-inflammatory cytokine by adipose tissue was first reported in 1993 (Hotamisligil et al., 1993). Contrary to the above mentioned functions of adiponectin, TNFα production is enhanced in obese and/or diabetic mammalian models (Hotamisligil et al., 1993, Nieto-Vazquez et al., 2008). The increased infiltration of macrophages in the adipose tissue of obese individuals is another important source of locally-produced TNFα (Weisberg et al., 2003). In mammals, this cytokine is known to induce apoptosis and lipolysis, as well as inhibit adipogenesis and impair insulin signalling in adipose tissue (Tang et al., 2010, Ryden and Arner, 2007). TNFα plays a well-documented role in the development of insulin resistance through the reduction of insulin-induced glucose transport, although it can stimulate at the same time basal glucose uptake (Stephens et al., 1997) and, by inhibiting the function of the transcriptional regulator PPARγ (Ye, 2008). An increase in TNFα promotes the expression of other pro-inflammatory cytokines and reduces the expression of anti-inflammatory cytokines (such as adiponectin), resulting in an overall pro-inflammatory state (Wang et al., 2013).

This cytokine has been cloned and sequenced in the three species studied in the present work; rainbow trout (Laing et al., 2001), Atlantic salmon (Ingerslev et al., 2006) and gilthead sea bream (Garcia-Castillo et al., 2002), as well as in other relevant farmed fish species. Its role as a key modulator of lipid metabolism in fish has been suggested, with some conserved activities from fish to mammals. Therefore, the lipolytic capacity of TNFα has been reported in freshly isolated adipocytes from rainbow trout (Albalat et al., 2005c) and gilthead sea bream (Saera-Vila et al., 2007, Cruz-Garcia et al., 2009b). Besides, the capacity of the cytokine to inhibit preadipocyte differentiation was observed in rainbow trout (Bouraoui et al., 2008), as well as its role limiting mesenteric fat deposition in gilthead sea bream (Saera-Vila et al., 2007). On the other hand, the administration of TNFα injections failed to regulate the expression of the adiponectin system in rainbow trout adipose tissue (Sánchez-Gurmaches et al., 2012a). However, in the same species, the incubation of mature isolated adipocytes with the cytokine triggered a down-regulation of adipoR2. In the present work, we will provide more information regarding the interaction between TNFα and the adiponectin systems, as well as their effects on glucose uptake and activation of metabolic pathways in rainbow trout cultured adipocytes.

Chapter 2. OBJECTIVES

The overall aim of the present work is to improve the knowledge on the complex regulation of lipid and carbohydrate metabolism in fish. This thesis is specially focused in fish adipose tissue, since the identification and characterization of the main markers defining the degree of adiposity and lipid deposition capacity provide a valuable information that can be applied in the future to optimize farming conditions as well as for selective breeding. The species studied are rainbow trout, Atlantic salmon and gilthead sea bream, due to their commercial interest for aquaculture.

The general objective can be subdivided into the following specific aims:

1- To elucidate the mechanisms involved in the activation of lipid and carbohydrate metabolic pathways in adipose tissue and liver in response to dietary macronutrient replacements, as well as the effects of these dietary manipulations on growth performance, plasma metabolites and adipocyte size in gilthead sea bream. (Article I)

2- To determine the lipogenic potential of Atlantic salmon adipocytes and their ability to synthesize lipids via *de novo* lipogenesis by studying the metabolic fate of glucose and palmitic acid, and the cell distribution of their transporters. (Article II)

3- To increase the understanding of adipokines in fish by analysing the interplay of the adiponectin system, TNFα and insulin at a transcriptional level and their effects on the activation of main insulin pathways (AKT and TOR), PPARγ and glucose uptake in primary cultures of rainbow trout adipocytes. (Article III)

4- To characterize the dynamics of conversion of preadipocyte into mature adipocytes through the analysis of the transcriptomic profile along the adipocyte culture at different stages of development and to identify possible novel adipogenic mediators and markers in rainbow trout. (Article IV)

Chapter 3. SUPERVISOR’S REPORT

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Barcelona, noviembre de 2015

La Dra Isabel Navarro Álvarez, como directora de la tesis doctoral presentada por Marta Bou Mira titulada "New insights into lipid and carbohydrate metabolism in teleost fish transcriptional and functional characterization of adipocytes." manifesta la veracidad del factor de impacto y la implicación de la doctoranda en los artículos científicos publicados presentados en esta tesis. La doctora Navarro hace constar que Marta Bou Mira ha participado de forma muy activa en la elaboración de los artículos en todos los aspectos, tal y como queda reflejado en la relación de autores, ya que consta como primer autor en todos ellos. Así, Marta Bou Mira ha contribuido de manera principal tanto en el diseño experimental como en la realización de los experimentos, obtención de datos, análisis de resultados, así como en la redacción y elaboración de todas las publicaciones. El artículo 2 deriva de una estancia realizada por la doctoranda en el laboratorio de la Dra. Bente Ruyter en el Centro de Nofima de Ås, Noruega y el artículo 4 se ha realizado en colaboración con el Laboratorio de Fisiologia y Genómica de peces del INRA, Rennes, Francia.

Artículo 1: Adipose tissue and liver metabolic responses to different levels of dietary carbohydrates in gilthead sea bream (*Sparus aurata*)

Autores: Marta Bou, Marijana Todorčević, Ramón Fontanillas, Encarnación Capilla, Joaquim Gutiérrez, Isabel Navarro

Revista: Comparative Biochemistry and Physiology, Part A

Factor de impacto: 1.966 (Quartil 1)

Estado: Publicado

Artículo 2: De novo lipogenesis in Atlantic salmon adipocytes

Autores: Marta Bou, Marijana Todorčević, Jacob Torgersen, Stanko Škugor, Isabel Navarro, Bente Ruyter

Revista: Biochimica et Biophysica Acta

Factor de impacto: 4.381 (Quartil 1)

Estado: Publicado

Artículo 3: Interplay of adiponectin, TNFα and insulin on gene expression, glucose uptake and PPARγ, AKT and TOR pathways in rainbow trout cultured adipocytes

Autores: Marta Bou, Marijana Todorčević, Julia Rodríguez, Encarnación Capilla, Joaquim Gutiérrez, Isabel Navarro.

Revista: General and Comparative Endocrinology

Factor de impacto: 2.470 (Quartil 3)

Estado: Publicado

Artículo 4: Gene expression profile during proliferation and differentiation in rainbow trout adipocyte precursor cells

Autores: Marta Bou, Jerôme Montfort, Aurélie Le Cam, Cécile Rallière, Jean-Charles Gabillard, Claudine Weil, Joaquim Gutiérrez, Pierre-Yves Rescan, Encarnación Capilla and Isabel Navarro

Revista: BMC Genomics

Factor de impacto: 3.986 (Quartil 1)

Estado: En preparación

Dra Isabel Navarro Alvarez

Directora de la tesis doctoral

Chapter 4. GENERAL DISCUSSION

The aim of the present thesis is directed towards the comprehension of the mechanisms involved in the regulation of lipid and carbohydrate metabolism in relevant species in European aquaculture. The aquaculture product is an efficient source of edible protein and an excellent source of omega-3 LC-PUFA able to fulfil the increasing global demand for food (Tacon and Metian, 2013). However, in order to ensure an efficient and sustainable fish production the industry has to provide a nutritious, healthy and sustainable feed for the fish. Aquafeed still relies on fish meal and fish oil due to its high nutritional quality. However, this practice is not sustainable and the dependence on these ingredients threatens the growth of the sector. The use of plant materials as protein and lipid sources has been recognized as a sustainable alternative to fish products (Bouraoui et al., 2011, Ytrestøyl et al., 2014). Nevertheless, protein continues to be the most expensive nutrient. Thus, carbohydrates are very attractive ingredients as they are considered to supply energy at low cost; however carnivorous fish species are traditionally considered as glucose intolerant (Moon, 2001, Wilson, 1994), limiting the inclusion of dietary starch. On the other hand, the use of feed with high lipid content allows the industry to increase fish growth while reducing expenses (Leaver et al., 2008). However, the use of these lipid-rich diets leads to an excessive deposition of fat in fish fillets and around the internal organs (Weil et al., 2013). (Moon, 2001, Wilson, 1994). By increasing our understanding of how fish metabolise and utilise lipids and carbohydrates, the formulation of modern aquafeeds could be improved.

Therefore, and in order to determine the feasibility of replacing different macronutrients in gilthead sea bream diets, a feeding experiment was performed where we have looked at the nutritional regulation at a transcriptional level of some of the main genes involved in lipid and carbohydrate metabolism (Article I). However, it has been extensively reported that the ability to metabolise dietary carbohydrates varies greatly among and within species at different life stages or subjected to certain feeding challenges (Hemre et al., 2002, Moon, 2001, Polakof et al., 2011c). Hence, in order to unravel new mechanisms that can explain carbohydrate intolerance in fish, we have studied the existence of *de novo* lipogenesis pathway in Atlantic salmon adipocytes (Article II). In addition, we have explored the implication of some relevant adipokines, adiponectin and TNFα, in the regulation of glucose uptake in rainbow trout adipocytes. Moreover, we provide new information on the interplay of the adiponectin system and TNFα and the possible relevance of these adipokines in the adipocyte biology (Article III). Finally, to gain a better understanding of the mechanisms regulating adipose tissue growth and development in fish, we have studied the transcriptional changes throughout the culture of

adipose tissue stromal vascular cells from rainbow trout towards mature adipocytes (Article IV).

Overall, these studies have contributed to better understand the mechanisms that regulate lipid and carbohydrate metabolisms in liver and adipose tissue of teleost fish. This work is, however, specially focused on the regulation and biology of adipose tissue. All this information may be applied in the future to modulate and optimize farming practices and in genetic selection programs.

4.1 LIPID AND CARBOHYDRATE METABOLISM IN FISH:

Fish metabolism is an intricate network that is modulated in the context of different physiological challenges to regulate the energetic balance in the animals. Part of the present work has been focused on the characterization of the lipogenic potential and glucose utilization capacity of adipose tissue (Articles I, II and III) and liver (Article I) of teleost fish. Both, *in vivo* and *in vitro* approaches have been used to this end.

4.1.1 Nutritional regulation of lipid and glucose metabolism in gilthead sea bream adipose tissue and liver

A feeding trial was designed in order to analyse the effects of replacing dietary lipids by carbohydrates and carbohydrates by fibre in gilthead sea bream adipose tissue and liver (Article I). These organs were selected based on their metabolic relevance in fat and starch deposition and turnover. As described in the Introduction section, dietary macronutrients can be endogenously converted into other metabolites and thus serve different purposes depending on the fish energy status. Therefore, the fact that they can be directed to cover different biological needs, limits straightforward conclusions. However, analysing the transcriptional activation of lipid and carbohydrate metabolic pathways to better understand the mechanisms behind fish biological responses towards dietary macronutrient replacements might enable us to fulfil fish nutrient requirements more correctly in the future.

Apart from entering in the glycolytic pathway, dietary carbohydrates can be directed to the glycogen synthesis, endogenous lipid synthesis and protein synthesis. In our study, an increased

hepatosomatic index (HSI) was positively related with the inclusion of carbohydrates in gilthead sea bream, which was interpreted as an increase in glycogen liver content rather than to an increase in hepatic lipogenesis. First, an augment in HSI and glycogen liver contend as a response to high-carbohydrate diets has been widely reported in this species (Couto et al., 2008, Ekmann et al., 2013). Moreover, in our conditions, we observed a lack of transcriptional regulation in response to dietary starch levels in the lipogenic enzymes assessed in the liver, namely FAS and G6PDH. Therefore, we concluded that dietary glucose was preferentially directed towards hepatic glycogen synthesis. However, in rainbow trout subjected to different challenges, an enhanced hepatic lipogenesis was linked to a better ability to control excess of hepatic dietary glucose (Kamalam et al., 2012, Skiba-Cassy et al., 2009, Panserat et al., 2009, Polakof et al., 2011d). This suggests that different species use different strategies to deal with dietary carbohydrates.

The lack of hyperglycaemia regardless of the level of starch ingested suggested that gilthead sea bream is able to adapt to different metabolic situations. In the light of our results, a higher glucose uptake from the circulation into the liver might explain the steady level of plasmatic glucose observed. Correspondingly, liver glucose phosphorylation was efficient as indicated by the induction in transcript abundance of GK in response to carbohydrate inclusion. Therefore, and as it has been reported before not only for gilthead sea bream, but also for other fish species (Panserat et al., 2000a, Capilla et al., 2004b, Leung and Woo, 2012), this enzyme presents mammalian-type responses. It is worth noting, however, that GK induction by starch was somehow overridden by the highest substitution of lipids by carbohydrates. Interferences between GK and dietary lipid levels have been reported before in rats (Girard et al., 1991, Lam et al., 2003) as well as in gilthead sea bream (Metón et al., 2004). Despite of the adaptation of hepatic GK to dietary starch, no transcriptional regulation of G6Pase in response to dietary replacements was found. The lack of gluconeogenesis regulation is consistently observed in fish (Caseras et al., 2002, Polakof et al., 2011c, Panserat et al., 2001a) and has been highlighted as one of the factors contributing to the limited ability to utilize carbohydrates.

On the other hand, a high lipid turnover was found in the liver of the fish receiving the high carbohydrate/low lipid diets. This was evidenced by the higher transcript abundance of LPL and HSL. Replacements of carbohydrates by fibre significantly reduced the transcription of LPL, supporting the modulation of this enzyme in a starch dose dependent manner. Nevertheless, the hepatic transcription of HSL was not affected by these substitutions, suggesting that the modulation of this enzyme is more dependent on the dietary lipid

manipulation. This is in agreement with previous studies that have reported a seasonal and a nutritional regulation of the hepatic LPL in this species (Saera-Vila et al., 2005).

Many of the lipid-sensing transcription factors described in mammals have been reported in fish and their role regulating lipid metabolism has been assessed. This is the case of LXRα, which has been recently described in gilthead sea bream, rainbow trout and Atlantic salmon (Cruz-Garcia et al., 2009a, Cruz-Garcia et al., 2009b) and its pro-lipolytic action has been demonstrated before in rainbow trout adipocytes (Cruz-Garcia et al., 2012). The nutritional regulation of this transcription factor in the liver of gilthead sea bream has been previously reported in an experiment where fish oil was replaced with vegetable oil (Cruz-Garcia et al., 2011). We did not find any nutritional hepatic regulation of this transcription factor under our experimental conditions, but in adipose tissue, the mRNA levels of LXRα significantly decreased with increasing levels of dietary starch, regardless of the other macronutrient used to compensate (lipids or fibre). In 3T3-L1 and human adipocytes, the activation of LXRα is known to be associated with a preferential use of lipids over carbohydrates as oxidation substrate for energy production (Stenson et al., 2009). Therefore, it seems that the nutritional regulation of this transcription factor in adipose tissue is maintained from mammals to fish.

PPARs are critical transcriptional regulators of lipid metabolism and energy homeostasis in mammals (Desvergne et al., 2006). However, and despite their importance, their role in fish is still puzzling due to species and tissue specific responses. In line with the mentioned role of LXRα, PPARβ has also been described to change body's fuel preferences from glucose to lipids in rat (Brunmair et al., 2006). This seems to be also the case for gilthead sea bream, where the transcriptional regulation of PPARβ followed the same pattern as LXRα did, with higher levels of transcripts in adipose tissue associated with diets rich in lipids and low in starch. The same was true for the transcript abundance of PPARα in adipose tissue. Both transcription factors, PPARα and β, are known to be involved in lipid β-oxidation (Kota et al., 2005). Thus, it seems that gilthead sea bream is able to adapt its metabolism to efficiently use the nutrients that are more available. However, under our experimental conditions, no nutritional regulation at a transcriptional level of PPARα or β was found in gilthead sea bream liver. Nevertheless, PPARγ hepatic mRNA levels were significantly decreased with high replacement of lipids by carbohydrates. The same trend was observed in gilthead sea bream adipose tissue, being the transcripts coding for PPARγ significantly increased when carbohydrates were replaced by higher amounts of fibre. Since PPARγ promotes lipid accumulation (Tontonoz and Spiegelman, 2008), it is reasonable to consider that its expression will be lower with reduced dietary lipids.

On the other hand, a constant level of dietary lipids with a concominant decrease in carbohydrates, as occurs with replacement of carbohydrate by fibre, also seems to favor the incorporation of FAs in the adipose tissue.

The balance between fat accumulation and utilization in adipose tissue was maintained in gilthead sea bream subjected to dietary manipulation. This was supported by the fact that the mesenteric fat index, the level of transcripts coding for the main enzymes involved in FA uptake and lipolysis, LPL and HSL respectively, and adipocyte size were not significantly affected by the macronutrient replacement used. Previous studies performed in the same species reported an activation of HSL together with an increase in cell size when 66% of the fish oil from the diet was replaced by vegetable oils (Cruz-Garcia et al., 2011). A correlation between adipocyte hypertrophy and high lipolytic rates in mammals has been linked to a defective lipid deposition and turnover (Gregor and Hotamisligil, 2007). The fact that the diets tested in our work were fish oil based together with the careful and balanced formulation of the diets might explain why we did not observe any of these adverse effects. This suggests that high replacements with carbohydrates could be performed provided that the ingredient used are able to satisfy the nutritional needs without triggering aberrant modulations on fish metabolism.

It is well known that TAG accumulation can be favoured by dietary carbohydrates in mammals (Ameer et al., 2014). Lipogenesis of gilthead sea bream adipose tissue was stimulated by the availability of increasing levels of carbohydrates at expenses of lipids through the activation of the PPP at a transcriptional level as evidenced by the mRNA levels of G6PDH. This pathway, activated for the supply of NAPPH for the biosynthesis of FAs, was, however, not modulated when fibre/carbohydrate replacements were tested, suggesting that the dietary modulation of PPP in adipose tissue is only triggered by specific combinations of macronutrients. In this case, the highest carbohydrate and lowest lipid levels. In other species, such as rainbow trout, a lack of dietary regulation in the lipogenic capacity of adipose tissue has been reported (Barroso et al., 2001, Figueiredo-Silva et al., 2012, Kamalam et al., 2012). However, lipogenesis was enhanced in rainbow trout infused the anti-diabetic drug metformin (Polakof et al., 2011d) or insulin (Polakof et al., 2011a) highlighting the lipogenic potential of white adipose tissue. Nevertheless, the capacity of synthesis of TAG has not been studied in detail in salmonids.

Summarizing, the main results of our feeding experiment showed that dietary lipid substitution by carbohydrate up to levels of 28%, and subsequent changes in carbohydrate content by fibre

had moderate effects in adipose tissue and liver metabolism homeostasis without affecting growth performance or feed utilization, thus supporting their commercial use.

4.1.2 Glucose utilization and lipogenic capacities of fish adipose tissue

We have reported that carbohydrate inclusion levels in our experimental conditions in gilthead sea bream diets did not triggered adverse effects in terms of fish health or performance and was accompanied by an increase of lipogenic markers in adipose tissue, (Article I). Nevertheless, the nutritional regulation of lipogenesis seems to be species-specific. This is in agreement with previous studies that report a more efficient use of carbohydrates as energy fuel in gilthead sea bream compare to that from salmonids (Felip et al., 2013). Indeed, salmonids experience reduced growth and prolonged postprandial hyperglycaemia when carbohydrates exceeds 20% of the diet (Wilson, 1994, Panserat et al., 2014, Refstie et al., 2001, Higgs et al., 2009, Polakof et al., 2012).

Taking into consideration the aforementioned and the fact that much more is known about the hepatic regulation of glucose metabolism in fish (Enes et al., 2009) than what is known in adipose tissue, we decided to study the existence of the *de novo* lipogenesis (DNL) in Atlantic salmon adipocytes. DNL is a pathway responsible to convert excess of carbohydrates into FAs, which are then esterified and safely stored as TAGs. In our work, we have provided the first evidences of the existence of this pathway in fish adipocytes (Article II).

Specifically, a radioactive assay revealed that the majority of the glucose added to the adipocytes ended up in Krebs-cycle intermediates. Radiolabelled glucose was also converted to acid soluble products to a high degree in rainbow trout myotubes (Sánchez-Gurmaches et al., 2010). Only a minor part of the radiolabelled glucose used in our study was incorporated into cellular lipids. Further experiments helped us to determine that 16 times more nmols were converted to glycerol from glucose compare to the amount of FAs produced. The identities of the FAs produced from glucose were 16:0, 16:1 n-7 and 18:1 n-9, which are known to be the main products of DNL. This further confirms the activity of DNL pathway in Atlantic salmon adipocytes. In addition, the fact that approximately 74% of the newly made FAs were in the form of the monounsaturated FAs suggests that, as in humans (Collins et al., 2010, Chong et al., 2008), the elongation and desaturation pathways are closely related and coordinated to DNL in order to prevent any potential adverse effects of the saturated FAs. In light of these results,

we concluded that glucose promotes adipocyte lipid accumulation in Atlantic salmon by providing the backbones on which to synthesize TAGs. However, and despite of being produced to a low extent, we cannot rule out the relevance of DNL products and their implication in fish adipocyte biology.

The glyceroneogenesis pathway is an alternative way of producing glycerol, from precursors other than glucose, for the synthesis of TAGs. However, the lack of regulation of PEPCK, the main enzyme regulating this pathway, suggested that the contribution of glyceroneogenesis is not stimulated by the addition of non-radiolabelled glucose. In mammalian adipose tissue, glucose has been shown to reduce the transcriptional rate of PEPCK (Chakravarty et al., 2005, Cadoudal et al., 2005, Beale et al., 2003). Instead, glucose stimulated the oxidative branch of the pentose phosphate pathway, as evidenced by the increase in transcript abundance of PGD, in order to obtain the reducing power for the lipid synthesis. On the other hand, we observed a decreased in the mRNA levels of PFK, the main glycolytic enzyme, and TKT, the main enzyme from the non-oxidative branch of the PPP that serves as a reversible link between PPP and glycolysis. These transcriptional changes might be directed to finely regulate the output from the PPP and related pathways during increases in lipogenic and adipogenic pathways.

As explained above different approaches allowed us to determine that glucose supplementation promoted TAG deposition and accelerated terminal differentiation of the adipocytes. This last statement was based on the transcriptional regulation of relevant adipogenic markers. Thus, adipocytes supplemented with glucose significantly increased the mRNA levels of the late differentiation marker C/EBPα while the early differentiation markers assessed, C/EBPβ and CREB, were decreased. On the other hand, it has been reported that, as adipocytes mature and specialise towards lipid storage, their capacity for β-oxidation decreases (Todorcevic et al., 2008). Our work showed that when glucose was added to the adipocytes, the transcript levels of enzymes coding for mitochondrial and peroxisomal β-oxidation (CPT-1 and ACO respectively) decreased, supporting the higher maturation capacity of these cells.

In comparison with glucose addition, Atlantic salmon mature adipocytes supplemented with palmitic acid presented a significantly higher capacity to accumulate lipids (measured as cellular lipids produced from C^{14}-palmitic acid) since more than 80% of the substrate was incorporated into cellular lipids; mostly in form of neutral lipids (Article II). Yet, it has been suggested that the lipogenic potential of rainbow trout white adipose tissue is higher than that from other salmonids based on the capacity of this tissue to generate reducing power in form of

NADPH (Polakof et al., 2011a). We also showed that the addition of palmitic acid to Atlantic salmon adipocytes significantly increased the transcript abundance of PEPCK suggesting an activation of glyceroneogenesis for the synthesis of glycerol to form the TAG backbones. Experiments performed in 3T3-F442A reported that unsaturated FAs strongly induce PEPCK (Forest et al., 2003). We observed that most of the palmitic acid (96.5%) was transformed to unsaturated FAs, suggesting that PEPCK in fish adipose tissue is regulated in the same way as in mammals.

Fatty acid uptake, together with lipogenic pathways determine the synthesis of TAGs in the cells. However, the mechanisms by which FAs enter the cells remind poorly understood. Sánchez-Gurmaches and co-workers reported for the first time in 2011 the translocation of FATP1 from an intracellular compartment to the plasma membrane of Atlantic salmon myocytes after insulin stimulation. This would be in agreement with previous studies found in the mammalian literature that suggests that FA uptake is regulated by FATP1 dynamics in a similar manner as glucose uptake is regulated by glucose transporters translocation (Glatz et al., 2010). However, recent findings have questioned this idea and suggested other methods of action (Lobo et al., 2007, Wiczer and Bernlohr, 2009). FATP1 was located in the endoplasmic reticulum, and translocation to the plasma membrane was not needed to enhance the basal or insulin-mediated FA uptake in 3T3-L1 adipocytes (Zhan et al., 2012). This is in agreement with our findings, where immunofluorescence antibody staining revealed that FATP1 was located intracellularly in the endoplasmic area of Atlantic salmon adipocytes, with the highest signal in cells treated with palmitic acid.

In contrast, glucose transporter GLUT4, was expressed at a high level in the membrane of salmon adipocytes (always in the presence of insulin), especially in glucose treated cells. Salmon GLUT4 can maintain its main mammalian functions, even though it has a lower affinity for glucose (Capilla et al., 2004a). The translocation of this transporter after insulin stimulation has been demonstrated in trout skeletal muscle cells (Diaz et al., 2007), and an increase in glucose uptake upon insulin stimulation in trout adipocytes has been reported and suggested to be mediated by GLUT4 (Bouraoui et al., 2010).

In the present work, we have also addressed the regulation of glucose uptake by two relevant adipokines, adiponectin and TNFα, as well as their interactions with insulin or IGF-I in rainbow trout adipocytes (Article III). Barely nothing is known about the biological effects of TNFα and adiponectin on fish glucose metabolism and the very few studies performed were in the context

of fish muscle. Thus, in trout cultured muscle cells, TNFα triggered an enhancement of basal glucose uptake (Vraskou et al., 2011) while a short stimulation with adiponectin did not exert any effect (Sánchez-Gurmaches et al., 2012a). We revealed that both adipokines studied stimulated glucose uptake in trout adipocytes without modifying AKT or TOR phosphorylation. This is in agreement to what has been reported in mammalian adipocytes, where both, TNFα (Medina et al., 1999, Cornelius et al., 1990) and adiponectin (Wu et al., 2003), increased basal glucose uptake. Therefore, we concluded that, as in mammals, these adipokines could be important stimulators of glucose transport in fish adipocytes.

However, when we evaluated the interaction between these factors and insulin in relation to glucose uptake, their responses were different from those reported in mammals. While in mammals TNFα induces an insulin resistant state by impairing insulin-stimulated glucose uptake (Porter et al., 2002, Fernández-Veledo et al., 2009), we observed a stimulatory effect of the cytokine on glucose uptake when combined with insulin. On the other hand, and contrary to what has been described in mammals (Wu et al., 2003), glucose uptake in insulin-treated adipocytes was not enhanced by adiponectin. Based on previous studies showing a higher stimulation of glucose uptake by IGF-I compared to that from insulin (Montserrat et al., 2012, Bouraoui et al., 2010), we investigated the interactions between this growth factor and TNFα and adiponectin regulating glucose uptake. Our results showed that neither adiponectin nor TNFα modify the IGF-I stimulatory effect on glucose entry into trout adipocytes. Although these results might be related to an imprecise regulation of glucose metabolism in piscine systems, further research would be needed to elucidate the biological significance of these responses and their implications in the regulation of fish energy homeostasis.

Little attention has been paid to the role of fish adipose tissue in the context of carbohydrate metabolism. However, this work provides evidences supporting its implication in glucose homeostasis. Apart from the results already discussed, the increase in transcript abundance of many genes involved in major carbohydrate pathways, such as glycolysis and gluconeogenesis, during the terminal differentiation of rainbow trout adipocytes (Article IV), highlights the implication of fish adipocytes in the regulation of carbohydrate metabolism. Likewise, the relevance of glyceroneogenesis, PPP pathway and glycolysis during the last stages of maturation of Atlantic salmon adipocytes has been reported (Todorcevic et al., 2010).

4.2 NEW CONTRIBUTION ON FISH ADIPOCYTE BIOLOGY

Adipocytes have been recognized as highly specialized cells controlling energy homeostasis, playing a key role in insulin sensitivity and presenting central endocrine properties (Moreno-Indias and Tinahones, 2015). We have already mentioned the importance of factors secreted by the adipose tissue in relation to glucose homeostasis in trout adipocytes. The implication of fish adipocytes in the control of the whole body energy homeostasis has been suggested before (Polakof et al., 2011a, Bouraoui et al., 2010) and in the present work, we provide new evidences that help to support this statement. Regarding fish adipose tissue adipokines, most studies have been focused on the identification and characterization of leptin, ghrelin and TNFα (Ronnestad et al., 2010, Jonsson, 2013, Salmerón et al., 2015a, Salmerón et al., 2015b, Cruz-Garcia et al., 2009b, Bouraoui et al., 2008), whereas almost nothing is known about the role of adiponectin and its interactions with other factors in fish adipose tissue. In a similar way, few of the well-known key adipogenic transcription factors described in mammals, such as PPARγ and C/EBPα, have been identified and linked to an adipogenic function in fish (Bouraoui et al., 2008, Oku and Umino, 2008, Vegusdal et al., 2003). Nevertheless, the characterization of key factors and molecular mechanisms that regulate adipocyte differentiation are not fully elucidated.

The use of primary cell culture of fish adipocytes is a potent tool to study different aspects of adipocyte biology. Understanding the mechanisms underlying the development of adipose tissue and orchestrating energy homeostasis (Article IV) as well as gaining new knowledge about adipokines (Article III) can contribute to the control of fat deposition in farmed fish.

4.2.1 Interplay of the adiponectin system and TNFα in adipocytes

In the present work, we have evaluated the interplay of TNFα, adiponectin and insulin at a transcriptional level in rainbow trout adipocytes (Article III). Adiponectin is known by its anti-obesogenic and insulin-sensitizing effects in mammals. At the same time, adiponectin system is modulated by insulin (Hajri et al., 2011, Liu et al., 2008). In trout adipocytes, insulin stimulated the presence of mRNA levels of adiponectin and decreased the levels of adipoR1, and to a lesser extent, of adipoR2. This hormonal regulation is similar to the what has been described in mammalian models regarding to both, adiponectin (Halleux et al., 2001, Hajri et al., 2011, Kim et al., 2010) and the receptors (Fang et al., 2005, Liu et al., 2008). Intraperitoneal

administration of insulin in rainbow trout did not reveal a positive effect of insulin in adiponectin transcript abundance in adipose tissue, but significantly reduced the transcript abundance of both receptors (Sánchez-Gurmaches et al., 2012a). Therefore, the opposite regulation of peptide and receptor by insulin might be a regulatory feedback mechanism directed to maintain a functionally active and stable adiponectin system in the adipocyte.

In mammals, TNFα has pleiotropic actions and can interact with other adipokines. In this sense, the administration of TNFα effectively decrease adipose tissue transcript abundance and plasma levels of adiponectin (Kappes and Loffler, 2000, Bruun et al., 2003, Wang et al., 2013). Likewise, adiponectin has been shown to supress the synthesis and secretion of TNFα in mammalian adipose tissue (Maeda et al., 2002, Dietze-Schroeder et al., 2005). Nevertheless, these reciprocal suppressive effects reported in mammals were not present in our model system. However, we did observe an inhibitory effect of TNFα on the insulin-induce increase in adiponectin mRNA levels. Our results are in agreement with previous *in vivo* studies where injections with TNFα also failed to regulate adiponectin in rainbow trout adipose tissue (Sánchez-Gurmaches et al., 2012a). In addition, our results showed a lack of effect of TNFα on its own regulation, while in mammals this cytokine stimulates its expression together with other pro-inflammatory cytokines through the activation of the NF-kB pathway (Ye, 2010). We have reported an effect of TNFα on the increase in mRNA levels of adipoR1, but in mammals, no clear effects of this cytokine on the regulation of adipoRs has been found (Fasshauer et al., 2004).

The nuclear receptor PPARγ, as we have previously mentioned, is a transcription factor that plays a key role in adipocyte biology through the regulation of fat accumulation and adipose tissue development (Tontonoz and Spiegelman, 2008). Differences in structure and tissue distribution between fish and mammals have been reported (Leaver et al., 2008), and even though its physiological function in teleost remains unclear (Cruz-Garcia et al., 2009b), PPARγ also controls lipid deposition in fish adipose tissue (Todorcevic et al., 2008) and we have found that its transcriptional level is dietary regulated (Article I). In the present work, we have studied the effects of adipokines and insulin in the regulation of this transcription factor at a protein and mRNA level. Consistent with the anti-adipogenic effect of TNFα on rainbow trout adipocytes (Bouraoui et al., 2008), this cytokine exerted an inhibitory effect of PPARγ at a protein level, but failed at regulating mRNA levels, suggesting a preferential post-transcriptional regulation. On the other hand, adiponectin did not exert a regulation on PPARγ by itself but when combined with insulin, PPARγ mRNA abundance was increased. Interestingly, the increase of PPARγ

induced by insulin alone was not significant neither at gene nor at protein level. We attributed this to the fact that this transcription factor is already highly expressed in culture salmonids adipocytes during *in vitro* development (Bouraoui et al., 2008, Todorcevic et al., 2010) and therefore higher stimulus or synergic effects might be needed to trigger an up-regulatory effect.

Overall, we have showed that the piscine adiponectin system is under some regulatory mechanisms that differ from those already described in mammals. As it has been described in a previous section, adiponectin failed to increase glucose uptake in insulin-stimulated adipocytes. However, and based on the results regarding the effects of insulin on the transcriptional regulation of the adiponectin system, we confirmed that some of the mammalian-type responses are conserved. Regarding the effects of TNFα on fish adipocytes, some expected responses were also observed, such as a stimulation of basal glucose uptake and its anti-adipogenic role. Yet, the interactions between TNFα and insulin appear not to be well defined in fish. Finally, the previously reported reciprocal suppressive effect of the two studied adipokines is not present in our piscine model.

4.2.2 Rainbow trout adipogenesis

As we have explained in the introduction section, several are the causes that lead to an undesired excessive accumulation of fat in farmed fish. Regardless of the cause, at the cellular level, an increase in adipose tissue is the result of two processes: hypertrophy (lipid accumulation within existing adipocytes) and hyperplasia (recruitment of vascular stromal cells within the adipose tissue to undergo differentiation). Adipose tissue development is accompanied by the production of a network of numerous endocrine factors. Since one of the main functions of adipocytes is to store lipids and buffer peripheral tissues from FA exposure, an efficient storage capacity of excess of energy is required for a healthy and functional adipocyte. In mammals, not only an excessive accumulation of adipose tissue but also an impaired expandability has been associated to the development of metabolic disorders (Moreno-Indias and Tinahones, 2015). Whether this is the case in fish is still unknown.

In this work, we have studied the transcriptomic profile along adipocyte culture in order to shed light into the development and functions of adipose tissue in rainbow trout, and to identify novel proteins that could serve in the future as potential links between adipocyte growth and whole body energy homeostasis (Article IV). Bioinformatics analysis clearly revealed the presence of

two major clusters that corresponded to the main phases observed during the preadipocyte culture, proliferation and differentiation. As it was expected, proliferation was characterized by a large number of genes involved in basic cellular and metabolic processes such as transcription, ribosome biogenesis, translation and protein folding and cell proliferation, while the terminal differentiation was characterized by the presence of genes involved in energy production, glucose and lipid metabolism. The main biological functions of differentially expressed genes associated with this two phases are presented in Figure 6.

One of the first hallmarks of adipogenesis is the dramatic alteration in cell shape, since the cells shift from a fibroblastic to a spherical shape. Transcriptome profiling suggested that the balance between proteases and anti-proteases might play a relevant role throughout the whole developmental process by regulating cell shape and extracellular matrix remodelling (ECM). In this sense, members of the matrix metalloproteinase family as well as their inhibitors and genes from the ADAMTS family are highly regulated during both, early and late phases of adipocyte development. In mammals, cell shape and ECM remodelling regulate preadipocyte commitment and competency by modulating among others, the RHO-family GTPase signalling cascade (Cristancho and Lazar, 2011). Our results suggest that this could be the case for rainbow trout adipocytes. Adipocyte cytoplasm also undergoes an intense reorganization that includes a dynamic change in mitochondrial mass. The presence of a key autophagy gene Atg5 overexpressed during the proliferative phase of the culture imply the relevance of this process in our model. The development of the angiogenic capacity of our cell system was early revealed, which is in agreement with previous studies describing the tight association between angiogenesis and initial stages of adipogenesis (Lijnen, 2008). Regarding adipokines, among others, TNFα (known as a negative regulator of adipocyte differentiation) as well as a panel of TNF-related genes and receptors, were up-regulated before cell confluence and exhibited a down regulation during the later stages of the culture, which might be an important fact for the onset of adipocyte differentiation.

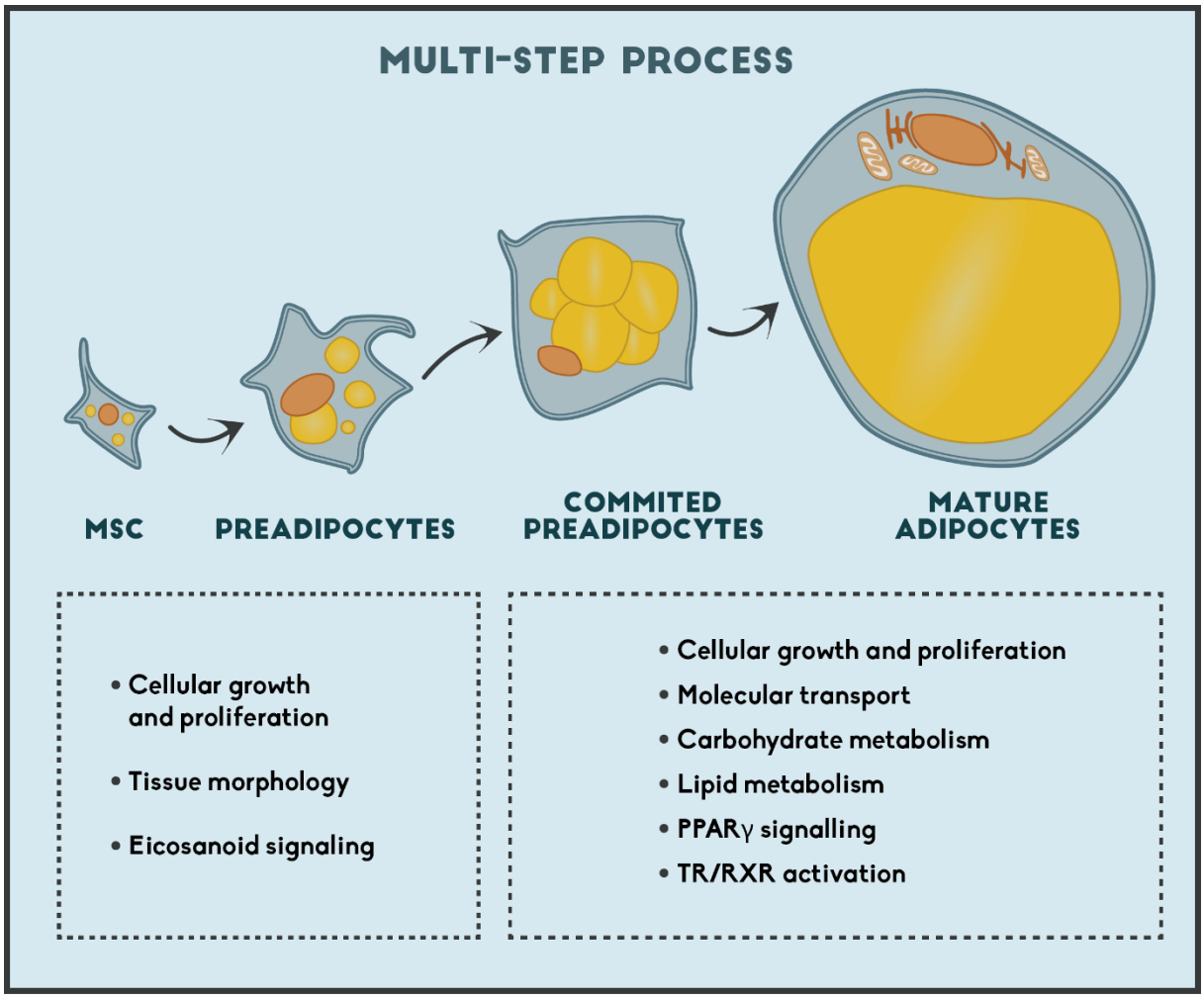

Figure 6. Functional annotation clustering on genes significantly upregulated during the proliferating and differentiating phase.

In mammals, cellular morphological modification are known to regulate adipogenesis by promoting the expression of critical transcription factors and cell-cycle regulators (Gregoire, 2001). The activation of these transcription factors is required in the adipocyte maturation process since they are responsible for the coordinated induction and silencing of a large amount of genes related to the regulation of adipocyte in both, morphology and physiology (Rosen and MacDougald, 2006). We identified a large number of transcription factors known to be implicated in the mammalian adipogenic process. Among many others we observed the up-regulation of members of the activator protein-1 (AP-1) transcription factor complex, the early growth response 2, members from the key family C/EBPs, as well as members from the Krüpperl-like factor (KLF) family, GATA factors and homeobox-containing gene. The different chronological activation of these factors during trout adipocyte development might indicate that, as in mammals, a coordinate cascade is required during fish adipogenesis.

Interestingly, our results suggest that there is an important link between transcriptional regulation and epigenetic modulation, due to the large amount of genes identified encoding epigenetic transcriptional regulators, especially during proliferation phase. The influence of chromatin remodelling on adipogenesis has been reported in other adipocyte cell models (Siersbaek et al., 2012, Musri et al., 2010, Musri and Parrizas, 2012).

Several of the already described intracellular signalling pathways implicated in development of the adipocyte and their regulators in the mammalian models have been found in this piscine model, such as the insulin and IGF system (Garten et al., 2012) and the eicosanoid signalling pathway (Polus et al., 2015). Furthermore, the activation of the thyroid-receptor/retinoic X receptor (TR/RXR) and the PPAR signalling pathway is shown during the differentiation phase. In mammals, PPARs and TRs can crosstalk to regulate different cellular processes, including adipogenesis (Lu and Cheng, 2010). Adipocyte is a specialized cell type responsible for the regulation of the balance between TAG storage and FA release. The activation of the lipid metabolism machinery was highlighted by the enrichment in genes coding for proteins involved in FA transport, FA activation, TAG biosynthesis and FA oxidation during the last stages of the culture, corroborating the maturation degree of the cells. As mentioned before, the implication of adipocytes in carbohydrate metabolism was suggested by the large amount of glucose-related genes induced in the differentiation cluster. The coordinated activation of all major lipid and carbohydrate-related pathways points to their close relation and reinforces the relevance of pathways such as DNL, which acts as a link between them. Moreover, the activation of different pathways with redundant functions might be a strategy for the cells to fulfil all the metabolic needs.

On the other hand, and consistent with the development of the adipocyte, the presence of genes encoding well-known structural proteins involved in the formation and expansion of the lipid droplet was revealed during the differentiation phase. The storage of TAGs within the lipid droplet allows the correct expansion of the adipose tissue while preventing lipotoxicity in other organs (Rutkowski et al., 2015). Therefore, appropriate formation of lipid droplets is considered to be crucial in the regulation of energy homeostasis and in the prevention of insulin resistance (Konige et al., 2014). However lipid droplet development and consequently adipocyte expansion can induce the production of reactive oxygen species (ROS) (Rutkowski et al., 2015). While an excessive production of ROS can trigger adverse effects to the cells, a moderate generation of these products has been described as a factor promoting adipocyte differentiation (Tormos et al., 2011). The lipid-loading phase is paralleled by the activation of the gluthation-

based antioxidant system, demonstrating the importance of controlling the intracellular redox homeostasis.

Overall, our study demonstrates the coordinated expression of functionally related genes during proliferation and differentiation of rainbow trout adipocyte cells, presenting high similarity with the already characterized mammalian adipogenesis. However, functional genomics in fish is significantly based on the knowledge of gene functions of their mammalian gene homologues. Even though the majority of the gene functions are well conserved between fish and mammals, species-specific differences exist. Thus, transcriptome analysis should be further complemented by mechanistic studies on selected genes. Nevertheless, the information generated by microarray analysis provides a solid starting point for the investigation of specific genes involved in different processes of fish adipogenesis.

Chapter 5. CONCLUSIONS

1- Lipid replacement from levels of 23 to 17% by starch raising from 12 to 28%, as well as starch replacement with fibre up to levels of 18%, in diets with a plant-to-animal protein ratio of 2.3, are feasible to be used in gilthead sea bream without impairing growth performance and feed utilization efficiency and triggering only moderate changes in lipid and glucose metabolism.

2- The metabolic adaptation of gilthead sea bream adipose tissue to lipid substitution by carbohydrate in the diet is revealed by an activation of the lipogenic pathway and by the regulation of relevant factors (LXRα, PPARα and PPARβ) directed to modulate the preferential use of lipids or carbohydrates according to availability. Moreover, no differences in terms of adipocyte size or MFI are observed, supporting the integrity and the optimal metabolic function of adipose tissue.

3- The absence of dietary carbohydrate modulation in the transcript abundance of G6Pase and G6PDH points to an apparent lack of regulation of gluconeogenesis and lipogenesis, respectively, in gilthead sea bream. However, an efficient metabolic hepatic adaptation to dietary starch is found through the modulation of enzymes directed to increase glucose metabolization (GK) and to balance FA uptake (LPL) and lipolysis (HSL).

4- It has been demonstrated for the first time that the *de novo* lipogenesis pathway is active in Atlantic salmon adipocytes; however, the capacity of the pathway to convert glucose into cellular lipids is relatively low. Thus, this might be one of the factors contributing to the previously reported glucose intolerance in salmonids.

5- Atlantic salmon adipocytes mainly transform glucose to glycerol and Krebs-cycle intermediates. Therefore, glucose promotes adipocyte lipid accumulation to a certain extend by providing the backbones on which to synthesise TAGs. On the other hand, palmitic acid is mainly incorporated into cellular lipids in Atlantic salmon adipocytes.

6- Coupling of glycolysis, the pentose phosphate pathway and glyceroneogenesis ensure the availability of necessary factors and other substrates for lipid synthesis and storage in Atlantic salmon adipocytes, highlighting the relative relevance of carbohydrate metabolism in this type of cells.

7- GLUT4 immunofluorescence staining indicates that this transporter is expressed at a high level in the membrane of Atlantic salmon adipocytes, especially in presence of glucose. FATP1,

in contrast, is most prominently located intracellularly at a particularly high level after palmitic acid treatment.

8- Insulin regulates the transcription of the adiponectin system in rainbow trout adipocytes enhancing the mRNA levels of the peptide and reducing those of adiponectin receptors, following the mammalian model. However, other aspects of the piscine adiponectin system seem to be governed by different regulatory mechanisms. Thus, adiponectin stimulates glucose uptake without modifying AKT or TOR phosphorylation, but fails to stimulate glucose uptake in insulin-treated adipocytes. In addition, neither adiponectin nor TNFα mRNA levels are affected by each other, showing that the reciprocal suppressive effects reported in mammals are not conserved in fish.

9- TNFα stimulates basal glucose uptake in rainbow trout adipocytes without modifying AKT or TOR phosphorylation and supresses PPARγ at a protein level, corroborating its anti-adipogenic role. On the other hand, TNFα enhances glucose uptake in insulin-stimulated adipocytes, suggesting that interactions between this cytokine and insulin are not so well defined in fish.

10- Rainbow trout adipogenesis is a complex process that involves the coordinated activation or repression of genes associated to the different stages of cell development. It presents high similarities to the mammalian model, being characterized by two main phases: proliferation and differentiation.

11- During rainbow trout adipocyte proliferation, there is an enrichment of genes involved in basic cellular and metabolic processes (transcription, ribosome biogenesis, translation and protein folding), cellular remodelling, autophagy and angiogenesis. In addition, the implication of the eicosanoid signalling pathway is highlighted during the early stages of differentiation. On the other hand, differentiation phase is highly enriched with genes involved in energy production, lipid and carbohydrate metabolism. During this phase, the formation of the lipid droplets is evidenced as well as the activation of the TR/RXR and the PPAR signalling pathways. The whole process is driven by coordinated activation of transcription factors and epigenetic modulators.

Chapter 6. FUTURE PERSPECTIVES

Understanding how fish utilize lipids and carbohydrates to fulfil their biological needs is of utmost importance in order to strengthen the aquaculture industry. As it has been described in this thesis, lipid and carbohydrate metabolism are closely related and play a central role in balancing whole body energy homeostasis. However, the fact that energy homeostasis is a complex physiological system that involves the coordination of multiple tissues and overlapping regulated pathway complicates the task of interpreting biological responses. In addition, fish are a diverse group with many species-specific strategies and adaptive mechanisms to cope with different challenges.

The present work provides new insights into different aspects of lipid and carbohydrate metabolism in three relevant farmed species. However, several questions have arisen during this work, some of which are listed below.

- Although we have shown for the first time that fish adipocytes can synthesise fatty acids *de novo* from the non-lipid precursor glucose, many aspects regarding the relevance of DNL in fish remains unexplored. We observed that the production of DNL-derived fatty acids from glucose was relatively low. Nevertheless, these products could have different bioactivities with profound effects in different physiological processes, as is the case in mammals. On the other hand, the fact that different precursors could be contributing carbon atoms to the pathway should be further explored. Furthermore, determining the relevance of DNL in hepatocytes as well as studying whether there is an association of this pathway with health in a tissue-dependent manner, could provide key information to understand better glucose metabolism in fish.
- The effects of adiponectin in fish are largely unknown. Even though, we have contributed to understand the regulation of this cytokine and its implication in glucose uptake in adipocytes, the effect of adiponectin on fish liver has not been evaluated so far. In mammals, it is known that adiponectin reduces glucose production in the liver by inhibiting the expression of gluconeogenic enzymes. Taking into consideration that neither insulin nor dietary carbohydrates inhibited the gluconeogenic potential in the liver, valuable information concerning the regulation of this pathway might be provided by analysing the interaction with this cytokine.
- We performed a comprehensive examination of the transcriptional regulation along adipocyte differentiation. Further functional analysis on selected candidates should be performed in order to obtain an in depth understanding of the intracellular mechanisms governing fish adipogenesis. Furthermore, the study of fat recruitment and fat

accumulation in different depots would be relevant, since they could be subjected to specific regulatory mechanisms and therefore might present different functions and physiological significance for the fish.

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Chapter 8. ARTICLES

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Adipose tissue and liver metabolic responses to different levels of dietary carbohydrates in gilthead sea bream (*Sparus aurata*)

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ABSTRACT

This study analyzes the effects of replacing dietary lipids by carbohydrates and carbohydrates by fiber on gilthead sea bream growth, as well as lipid and glucose metabolism in adipose tissue and liver over the course of a 15-week feeding trial. Six different diets were formulated and fish were classified into two experimental groups sharing one diet. In the first group (LS), fish were fed four diets where lipids were reduced (23%–17%) by increasing carbohydrates (12%–28%) and, the second group (SF) consisted on three diets where the amount of carbohydrates (28%–11%) was exchanged at expenses of fiber (1%–18%). Differences in growth were not observed; nevertheless, the hepatosomatic index was positively related to dietary starch levels, apparently not due to enhanced hepatic lipogenesis, partly supported by unchanged G6PDH expression. In the LS group, lipogenic activity of adipose tissue was stimulated with low-lipid/high-carbohydrate diets by up-regulating G6PDH expression and a tendency to increase FAS, and promoted carbohydrate utilization *versus* fatty acid oxidation by modulating the transcription factors LXRα, PPARα and PPARβ expression. In the SF group, PPARs and LXRα increased parallel to fiber levels in adipose tissue. Furthermore, an adaptation of hepatic GK to dietary starch inclusion was observed in both groups; however, the lack of effects on G6Pase expression indicated that gluconeogenesis was not nutritionally regulated under the conditions examined. Overall, metabolic adaptations directed to an efficient use of dietary carbohydrates are present in gilthead sea bream, supporting the possibility of increasing carbohydrate or fiber content in diets for aquaculture sustainability.

1. Introduction

Aquaculture production has expanded by almost 12-fold in the last three decades (FAO, 2012). Aquafeed relies on fish meal and fish oil due to its high nutritional quality; however, the sustainability of this practice is not well accepted at present, especially over the long term (Watanabe, 2002). Hence, in order to reduce aquafeed costs and alleviate overexploited marine fisheries pressure, research efforts to find suitable alternatives are currently underway.

The use of raw plant materials as protein and lipid sources has been recognized as a sustainable alternative to fish products (Bouraoui et al., 2011; Nasopoulou and Zabetakis, 2012); nevertheless, protein continues to be the most expensive nutrient and in an attempt to spare protein energy in several fish species, lipid content is rising (Company et al., 1999; Watanabe, 2002; Li et al., 2012). The current trend to use high-lipid diets has been shown to induce undesirable increases in fat depots or even physiological alterations such as induction of oxidative stress (Kjaer et al., 2008). Thus, carbohydrates are attractive ingredients as they are considered to supply energy at a low cost; however, the inclusion of high amounts of dietary carbohydrates remains controversial. Carnivorous fish are considered to present a limited ability to use dietary carbohydrates (Moon, 2001; Hemre et al., 2002), and their effects on growth depend upon many factors such as the source, concentration, level of food intake and digestibility (Brauge et al., 1994). Gilthead sea bream has been reported to present excellent starch digestibility coefficients (Enes et al., 2008; Couto et al., 2012) and elevated activity of the

Abbreviations: eF1α, elongation factor 1α; F, fiber; FAS, fatty acid synthase; FFA, free fatty acids; GK, glucokinase; G6Pase, glucose 6-phosphatase; G6PDH, glucose-6-phosphate dehydrogenase; HSI, hepatosomatic index; HSL, hormone sensitive lipase; L, lipids; LPL, lipoprotein lipase; LXRα, liver X receptor α; MFI, mesenteric fat index; PCR, polymerase chain reaction; PPARs, peroxisome proliferator-activated receptors; RPL27, ribosomal protein 27; S, starch; SGR, specific growth rate.

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main enzymes of the glycolytic pathway (Panserat et al., 2000; Couto et al., 2008; Enes et al., 2008), suggesting a possible efficient utilization of dietary starch for energetic purposes in this species.

Most of the studies in cultured fish species regarding high dietary carbohydrate content have focused on the effects of starch origin and inclusion levels on nutrient digestibility and retention efficiency, as well as growth performance (Capilla et al., 2003; Capilla et al., 2004). The response of glucose, lipid or amino acid metabolism enzymes has been reported mainly in the liver as crucial active metabolic organ (Caseras et al., 2000; Panserat et al., 2000; Caseras et al., 2002; Panserat et al., 2002b; Metón et al., 2004; Enes et al., 2006, 2008; Couto et al., 2008). However, and despite its importance in controlling fish energy balance, scarce information concerning the effects of dietary carbohydrates on adipose tissue is available. It has been reported that the lipogenic activity of adipose tissue is hormonally regulated in rainbow trout and modulated by diet in gilthead sea bream (Bouraoui et al., 2011; Cruz-Garcia et al., 2011; Polakof et al., 2011a), suggesting its possible implication on glucose homeostasis, being glucose one of the main lipogenic precursors. Therefore, the study of both, adipose tissue and liver, as well as their inter-relation may help to better explain how fish use dietary lipids and carbohydrates to grow.

Few studies have analyzed the interactions between dietary lipids and carbohydrates and their effects on glucose metabolism in fish (Polakof et al., 2011b; Figueiredo-Silva et al., 2012). In mammals, it is known that high levels of fatty acids disrupt carbohydrate metabolism, eventually causing impaired glucose tolerance (Randle, 1998). A recent study demonstrated the existence of a similar alteration in rainbow trout, where fish developed high fat-induced persistent hyperglycemia and reduced insulin sensitivity (Figueiredo-Silva et al., 2012). The poor utilization of carbohydrates by rainbow trout was linked, at least to some extent, to the use of high-fat diets (Panserat et al., 2002a). These observations pointed out that a reduction in dietary fat content could improve the glycemic control in carnivorous fish fed high-carbohydrate diets, and highlighted the importance of studying the metabolic interactions between dietary macronutrients.

Although in humans it has been shown that some dietary fibers tend to reduce cholesterolemia and improve glucose tolerance (Johnson, 1990; Kishimoto et al., 1995), the effects of different fiber levels in fish diets are still not clear and some discrepancies between species have been reported. In Atlantic salmon, apparent digestibility of lipids was linearly reduced with the inclusion of cellulose, while starch or protein digestibility was not influenced (Aslaksen et al., 2007). However, no significant effects of cellulose inclusion were found on the digestibility of main nutrients in rainbow trout (Hansen and Storebakken, 2007). Most of the research effort has been focused on the effects of dietary fiber inclusion on growth, digestibility and feces characteristics, whereas little is known about its possible implications on lipid and glucose metabolism. In white sea bream, the use of guar gum in the diet had no effect apparently on glucose utilization but contributed to lower endogenous glucose production (Enes et al., 2013).

In order to characterize the lipogenic potential and glucose utilization capacity of adipose tissue and liver of gilthead sea bream, two experimental groups were established. One group was used to test 4 diets with different lipid-to-carbohydrate ratios, in order to know whether gilthead sea bream is able to efficiently use high levels of starch, and how lowering lipid levels affect the fish energetic status. A second group was used to test 3 diets with different fiber-to-carbohydrate ratios in order to detect specific metabolic changes triggered by modifications in starch levels together with the use of a high content of cellulose as a filler agent. To this end the expression of key enzymes and transcriptional factors involved in glucose and lipid metabolism was evaluated. We chose the enzymes lipoprotein lipase (LPL) and hormone sensitive lipase (HSL) as markers of fatty acid uptake and lipolysis respectively, and enzymes related with lipogenesis, fatty acid synthase (FAS), and one of the main enzymes acting as NADPH donor, glucose-6-phosphate dehydrogenase (G6PDH), in order to assess whether dietary increases in carbohydrate-fiber or carbohydrate-lipid ratios might activate lipogenic processes. The expression of two enzymes, hepatic glucokinase (GK) and glucose 6-phosphatase (G6Pase) involved in glucose uptake and release, respectively, was expected to be regulated by diet composition. The lipid metabolism-related transcription factors determined were: liver X receptor α (LXRα) recently involved in triglyceride breakdown in fish adipose tissue (Cruz-Garcia et al., 2012) and peroxisome proliferator-activated receptors α, β and γ (PPARα, PPARβ, PPARγ), being the α and β isotypes promoters of fatty acids use in mammals (Kota et al., 2005) and PPARγ involved in lipid accumulation and adipogenesis also in fish (Bouraoui et al., 2008). We hypothesize that PPARs transcription levels could be related to the dietary lipid content.

All in all we aimed to elucidate the mechanisms involved in the activation of lipid and carbohydrate metabolic pathways at a transcriptional level, in both, adipose tissue and liver, in response to dietary macronutrient replacements. To generate a complete picture we also studied the effects of these dietary manipulations on growth performance, plasma metabolites and adipocyte size.

2. Materials and methods

2.1. Animals and feeding experiment

All animal handling and experimental procedures were conducted in compliance with the experimental research protocol approved by the Committee of Ethic and Animal Experimentation of the University of Barcelona (CEEA 239/09), and the Departament de Medi Ambient i Habitatge (DMAH permit number 5420, Generalitat de Catalunya, Spain) following regulations and procedures established by the European Union, and by the Spanish and Catalan Governments.

A total of 306 juvenile gilthead sea bream (*Sparus aurata* Linnaeus 1758) with an initial body weight of 115 g were maintained in IRTA-SCR facilities (Tarragona, Spain) in 18 cylindro-conical 400 L indoor tanks connected to a closed recirculation system with feed collectors to measure the food wasted to calculate feed intake, in groups of 17 fish per tank (3 tanks per each dietary condition). Water temperature ranged from 21 to 23 °C under natural photoperiod and fish were fed *ad libitum* twice daily with automatic feeders for 15 weeks. Six different diets were formulated by Skretting (Stavanger, Norway) and fish were classified into two experimental groups which were carried out simultaneously. The first group consisted on four isoproteic diets where lipids (L) were decreased by increasing carbohydrates (S) (LS group, diets $L_{23}S_{12}$, $L_{20}S_{16}$, $L_{19}S_{22}$, $L_{17}S_{28}$), and the second group consisted on three isolipidic and isoproteic diets where the amount of carbohydrates was changed at expenses of fiber (F) (SF group, diets $S_{28}F_1$, $S_{19}F_9$, $S_{11}F_{18}$) starting from $S_{28}F_1$, which was the same diet as $L_{17}S_{28}$ ($L_{17}S_{28}F_1$). Ingredients and proximate composition of the experimental diets are presented in Table 1. Subscripts indicate the percentage of inclusion of each component (L, S or F) in the diet. The ratios of distinct protein sources, calculated from the proportion between plant (corn gluten, wheat gluten and soya concentrate) and animal (fish meal) protein ingredients, were the same in all experimental diets (plant-to-animal ratio of 2.3).

After the 15-week feeding period all fish were fasted 24 h before sampling to avoid regurgitation of food and to obtain basal values of plasma metabolites and killed with a blow to the head under anesthesia (3-aminobenzoic acid ethyl ester, MS-222; 100 μg/ml). We sampled 9 fish for each dietary condition (3 fish from each of 3 tanks). Blood was collected and, mesenteric adipose tissue and liver excised, weighed, frozen in liquid nitrogen and stored at −80 °C until further analyses. Small pieces of adipose tissue were also taken for histological studies. Utmost care was taken to assure that all fish had consumed the feeds, which was checked by visual observation of intestinal tract content. Weight values from all fish from each tank were used to obtain the specific growth rate (SGR) and feed conversion ratio (FCR).

Table 1
Ingredient composition and proximate analysis of the different experimental diets from the LS and SF groups.

	$L_{23}S_{12}$	$L_{20}S_{16}$	$L_{19}S_{22}$	$L_{17}S_{28}F_1$	$S_{19}F_9$	$S_{11}F_{18}$
Diet raw materials (%)						
Cellulose	10.84	6.78	2.43	0.00	7.70	17.07
Fish meal	20.00	20.00	20.00	20.00	20.00	20.00
Corn gluten	13.00	13.00	13.00	13.05	13.10	13.10
Wheat gluten	20.00	20.00	20.00	20.00	20.00	20.00
Fish oil	17.15	15.28	13.28	11.27	11.27	11.27
Soya concentrate	11.52	11.52	11.52	11.53	11.53	11.53
Minerals/vitamins	2.00	2.00	2.00	2.00	2.00	2.00
Yttrium premix	0.10	0.10	0.10	0.10	0.10	0.10
Wheat Starch	5.00	10.93	17.28	21.73	14.18	5.00
Nutrient analysis (%)						
Moisture	6.09	6.70	7.35	7.82	7.04	6.10
Protein	45.47	46.33	47.29	46.49	46.17	45.73
Fat	22.79	20.34	19.05	17.04	17.34	16.44
Ash	5.50	5.53	5.56	5.54	5.34	5.10
Starch	11.50	15.55	22.08	27.83	18.51	10.85
Fiber	11.78	7.76	3.46	1.05	8.68	17.95

Four isoproteic diets (LS group) where lipids (L) were replaced by carbohydrates (S), and three isolipidic and isoproteic diets (SF group) where carbohydrates (S) were replaced by fiber (F) were formulated by Skretting. Subscripts indicate the percentage of inclusion of each component in the diet.

2.2. Biochemical analysis of plasma parameters

Caudal vein blood samples were drawn into propylene tubes containing sodium ethylenediamine tetra-acetic acid (EDTA-Na; 5 mg/ml) and plasma was prepared by centrifugation at 11,000 rpm for 5 min.

Plasma glucose, free fatty acids (FFA), triglycerides and lactate were analyzed in all sampled fish (n = 9) using commercial enzymatic methods (Wako Chemicals GmbH, Neuss, Germany and Spinreact, Sant Esteve d'en Bas, Spain).

2.3. Histology and image analysis

Adipose tissue samples were fixed in paraformaldehyde, embedded in paraffin, cut into 5 μm sections, and stained with hematoxylin and eosin.

Histology sections were observed at 10× magnification and images were obtained with a digital camera coupled to a microscope (Olympus BX40). For measurements of cross-sectional areas of adipocytes the images were converted into a binary format with ImageJ software (Schneider et al., 2012). Threshold and Watershed commands were used for the conversion and binary images were compared with the original ones to ensure an accurate conversion. Minor adjustments, if needed, were made with Erode and Paintbrush commands. Results were directly loaded into a spreadsheet program (Excel, Microsoft Inc., Redmond, WA) for analysis. For measurements of adipocyte cell area, histological preparations from six fish per diet (2 fish from each tank) were studied by processing five images from each individual. Data of each experimental group was divided into four ranges; 0.5–314 μm^2, 324–2827 μm^2, 2827–7854 μm^2 and bigger than 7854 μm^2, and values smaller than 0.50 μm^2 were considered to represent artifacts from the image-conversion process and excluded.

2.4. RNA extraction and cDNA synthesis

Total RNA from adipose tissue and liver was extracted from 6 fish per dietary treatment (2 fish from each tank) using TriReagent (Ambion, Alcobendas, Spain) following the manufacturers' recommendations. The quantity of isolated RNA was determined by spectrophotometry with a ND-2000 NanoDrop (Thermo Fisher Scientific, Alcobendas, Spain). For cDNA synthesis, 1 μg of RNA, 3 μl of a blend 2:1 random hexamers (600 μM)/oligo dT (50 μM), 2 μl dNTP (10 mM), 0.5 μl of reverse transcriptase (20 U/μl), and 0.5 μl of RNAse inhibitor (40 U/μl) were mixed with the kit buffer in a final volume of 20 μl (Transcriptor first strand cDNA synthesis kit, Roche, Sant Cugat del Valles, Spain), and incubated at 55 °C for 30 min, followed by 85 °C for 5 min to inactivate the enzymes.

2.5. Real-time PCR assay

Transcript measurements of lipid and carbohydrate metabolism-related enzymes and transcription factor genes were performed using an iCycler iQ Real-time Detection System (Bio-Rad, El Prat de Llobregat, Spain) as described elsewhere (Calduch-Giner et al., 2003). Briefly, diluted cDNA was used for PCR reactions in 20 μl final volume. Each PCR well contained SYBR Green Master Mix (Bio-Rad, El Prat de Llobregat, Spain) and specific primers at a final concentration of 0.35 μM that were used to obtain amplicons of 77–192 bp in length (Table 2). The reference genes: β-actin, eF1α and RPL27 were tested, being the former the most stable. In the first experimental group (LS) gene expression results are relative to those from diet $L_{23}S_{12}$ in order to analyze the simultaneous increase in carbohydrates and decrease in lipid content, while in the second group (SF) results are expressed relative to those from diet $S_{28}F_{1,}$ where the effects of decreasing the level of carbohydrates at expenses of increasing fiber were tested. PCR efficiency was between 90% and 110%. The specificity of the reactions was verified by analysis of melting curves. Reactions were performed in duplicate and the fluorescence data acquired during the extension phase normalized to β-actin by the delta–delta method (Livak and Schmittgen, 2001).

2.6. Statistical analyses

All data were analyzed using the statistical software SPSS™ (version 17.0, IBM, Chicago, IL). Normality of the data and homogeneity of the variance were tested to ensure that the assumptions of analysis of variance (ANOVA) were satisfied using the Shapiro–Wilk and Levene's tests, respectively. If the variances were normally distributed, the Tukey test for multiple comparisons of means was applied. Differences were considered significant at a level of 95% ($P < 0.05$). If only two means were compared, Student's *t*-test was applied. When variances were not normally distributed a logarithmic transformation was performed and Tukey test applied. When logarithmic transformed variances were not normally distributed, the Kruskall–Wallis non-parametric test was applied to the non-transformed data. Tank was used as the statistical unit (n = 3) with the average values of the tank for body weight, SGR and FCR, while the rest of the data was analyzed using individual values (n = 6 or 9).

3. Results

3.1. Effects of diet on growth performance and feed utilization

Biometric parameters were measured at the end of the feeding experimental trials and values are presented in Table 3. In each group (LS or SF), fish fed with the different diets were comparable and no significant differences due to carbohydrate inclusion levels, changed either by lipids or fiber, were observed after the 15-weeks in terms of growth performance (SGR) or feed utilization (FCR).

In the LS group, dietary effects were reflected in the hepatosomatic index (HSI) of fish, which increased its value in parallel to the percentage of carbohydrates in the diet (Table 3). Therefore, fish fed $L_{17}S_{28}$ diet, with the highest content of starch, displayed a significantly higher HSI compared to fish fed with $L_{23}S_{12}$ and $L_{20}S_{16}$ diets. Moreover, adipose tissue weight and consequently the mesenteric fat index (MFI), showed a tendency to increase in the fish receiving the diet with the higher lipid content ($L_{23}S_{12}$) in comparison to all the other diets (Table 3).

Fish from the SF group fed with the highest amount of fiber and lowest carbohydrate content ($S_{11}F_{18}$) exhibited a significantly lowest HSI compared to the fish receiving the diets with higher levels of

Table 2
Gilthead sea bream primer sequences used for real-time PCR.

Gene	Accession number	Primer sequence (5′–3′) **F**: forward, **R**: reverse	Annealing T (°C)	Position
HSL	EU254478	**F**: GCTTTGCTTCAGTTTACCACCATTTC	65	154–179
		R: GATGTAGCGACCCTTCTGGATGATGTG		275–249
LPL	AY495672	**F**: GAGCACGCAGACAACCAGAA	64	500–520
		R: GGGGTAGATGTCGATGTCGC		691–672
FAS	AM952430	**F**: TGGCAGCATACACACAGACC	60	74–94
		R: CACACAGGGCTTCAGTTTCA		171–151
G6PDH	AY754640	**F**: CAGAATGAAAGATGGGATGGAGTC	60	1029–1053
		R: TTCAGGTAAATGGCTTCGTTCG		1205–1183
GK	AF053330	**F**: GACGCTATCAAGAGACGAGGGGAC	65	692–716
		R: CCACGGTCCTCATCTCCTCCAT		858–836
G6Pase	AF151718	**F**: CTGCTGTGGACGATGGAGAAAG	62	726–748
		R: TGTTGAGGGGCGAGTGAAGAC		868–847
LXRα	FJ502320	**F**: GCACTTCGCCTCCAGGACAAG	62	476–496
		R: CAGTCTTCACACAGCCACATCAGG		582–559
PPARα	AY590299	**F**: TCTCTTCAGCCCACCATCCC	62	106–125
		R: ATCCCAGCGTGTCGTCTCC		221–203
PPARβ	AY590301	**F**: AGGCGAGGGAGAGTGAGGATGAGGAG	69	375–400
		R: CTGTTCTGAAAGCGAGGGTGACGATGTTTG		562–533
PPARγ	AY590304	**F**: CGCCGTGGACCTGTCAGAGC	66	318–337
		R: GGAATGGATGGAGGAGGAGGAGATGG		420–395
β-actin	X89920	**F**: TCCTGCGGAATCCATGAGA	60	811–829
		R: GACGTCGCACTTCATGATGCT		861–841
eF1α	AF184170	**F**: CTTCAACGCTCAGGTCATCAT	60	1024–1047
		R: GCACAGCGAAACGACCAAGGGGA		1286–1264
RPL27	AY188520	**F**: AAGAGGAACACAACTCACTGCCCCAC	68	148–173
		R: GCTTGCCTTTGCCCAGAACTTTGTAG		307–282

carbohydrates ($S_{28}F_1$ and $S_{19}F_9$), and the same tendency with regards to MFI was observed according to the dietary level of lipid (Table 3).

3.2. Effects of diet on plasma metabolites

Plasma glucose and triglycerides levels did not show any differences between diets in any of the experimental groups (Table 3). However, circulating FFA were significantly higher in fish fed the $L_{19}S_{22}$ diet compared to the fish fed the other diets on the LS group (Table 3); and in fish fed $S_{11}F_{18}$ diet among the SF group (Table 3). Lactate analysis also showed differences between fish fed the different experimental diets, presenting the highest values the fish under the $L_{20}S_{16}$ and the $S_{19}F_9$ diets in LS and SF groups, respectively (Table 3).

3.3. Effects of diet on adipocyte size

A representative image of a histological preparation of adipose tissue as well as its conversion into a binary representation are shown in Fig. 1A and B; and the frequency distributions of adipocyte cell area from the LS and SF groups are shown in Fig. 1C and D, respectively. All fish from the LS group showed their maximum relative frequency in the 314–2827 μm^2 range (Fig. 1C). Among this group a slightly increasing

Table 3
Biometric and biochemical plasma parameters of fish fed the different experimental diets from the LS and SF groups.

	$L_{23}S_{12}$	$L_{20}S_{16}$	$L_{19}S_{22}$	$L_{17}S_{28}F_1$	$S_{19}F_9$	$S_{11}F_{18}$
Body mass (g)	366.9 ± 11.6	378.0 ± 0.9	378.4 ± 6.3	361.9 ± 4.6	380.1 ± 12.5	385.5 ± 4.3
Length (cm)	22.82 ± 0.62	22.82 ± 0.38	22.68 ± 0.28	22.78 ± 0.18^{A}	23.16 ± 0.19^{A}	24.01 ± 0.23^{B}
Adipose tissue mass (g)	6.65 ± 1.23	4.69 ± 0.37	4.38 ± 0.30	4.76 ± 0.78	6.01 ± 1.12	4.46 ± 0.52
Liver mass (g)	5.19 ± 0.51	5.21 ± 0.39	6.44 ± 0.35	6.71 ± 0.31	6.98 ± 0.71	5.43 ± 0.27
SGR (%)[(a)]	1.09 ± 0.03	1.12 ± 0.00	1.13 ± 0.02	1.08 ± 0.01	1.13 ± 0.03	1.14 ± 0.01
FCR[(b)]	1.62 ± 0.09	1.59 ± 0.05	1.50 ± 0.04	1.63 ± 0.02	1.38 ± 0.14	1.61 ± 0.07
K (%)[(c)]	2.95 ± 0.06	3.07 ± 0.09	3.12 ± 0.10	3.06 ± 0.10	3.19 ± 0.11	3.03 ± 0.07
HSI (%)[(d)]	1.45 ± 0.10^{a}	1.44 ± 0.11^{a}	1.78 ± 0.10^{ab}	1.87 ± 0.09^{bA}	1.73 ± 0.12^{A}	1.30 ± 0.07^{B}
MFI (%)[(e)]	1.82 ± 0.24	1.29 ± 0.11	1.21 ± 0.08	1.30 ± 0.19	1.49 ± 0.24	1.06 ± 0.12
Adipocyte size (μm^2)	1335.5 ± 144.5	1680.7 ± 239.0	1723.6 ± 241.4	1815.6 ± 181.1	2055.0 ± 251.4	1653.7 ± 240.3
Plasma metabolites						
Glucose (mMol/L)	3.60 ± 0.52	3.74 ± 0.46	3.95 ± 0.37	3.62 ± 0.27	3.98 ± 0.29	3.89 ± 0.32
FFAs (mEq/L)[(f)]	0.22 ± 0.02^{a}	0.27 ± 0.01^{a}	0.31 ± 0.02^{b}	0.23 ± 0.02^{abAB}	0.18 ± 0.01^{A}	0.28 ± 0.02^{B}
Triglycerides (mg/L)	3467 ± 449	2921 ± 231	3945 ± 126	3456 ± 373	2657 ± 189	3293 ± 226
Lactate (mMol/L)	3.40 ± 0.31^{a}	4.30 ± 0.57^{a}	1.94 ± 0.19^{b}	3.14 ± 0.21^{aAB}	3.94 ± 0.29^{A}	2.93 ± 0.31^{B}

Values are the mean ± SEM (n = 3 for body mass, SGR and FCR and n = 9 for the rest of the parameters). Differences between diets were analyzed by one-way ANOVA, followed by Tukey's test. Values that do not share a common letter are significantly different ($P < 0.05$), with lower and upper case letters indicating differences between diets within the LS and SF groups, respectively.
[(a)]Specific growth rate (SGR) = ((ln final wet weight − ln initial wet weight) × 100 / time in days between weights.
[(b)]Feed conversion ratio (FCR) = weight of feed offered / wet weight gain.
[(c)]Condition factor index (K) = (fish weight / (total length)^3) × 100.
[(d)]Hepatosomatic index (HSI) = (liver weight / fish weight) × 100.
[(e)]Mesenteric fat index (MFI) = (mesenteric fat weight / fish weight) × 100.
[(f)]Free fatty acids (FFA).

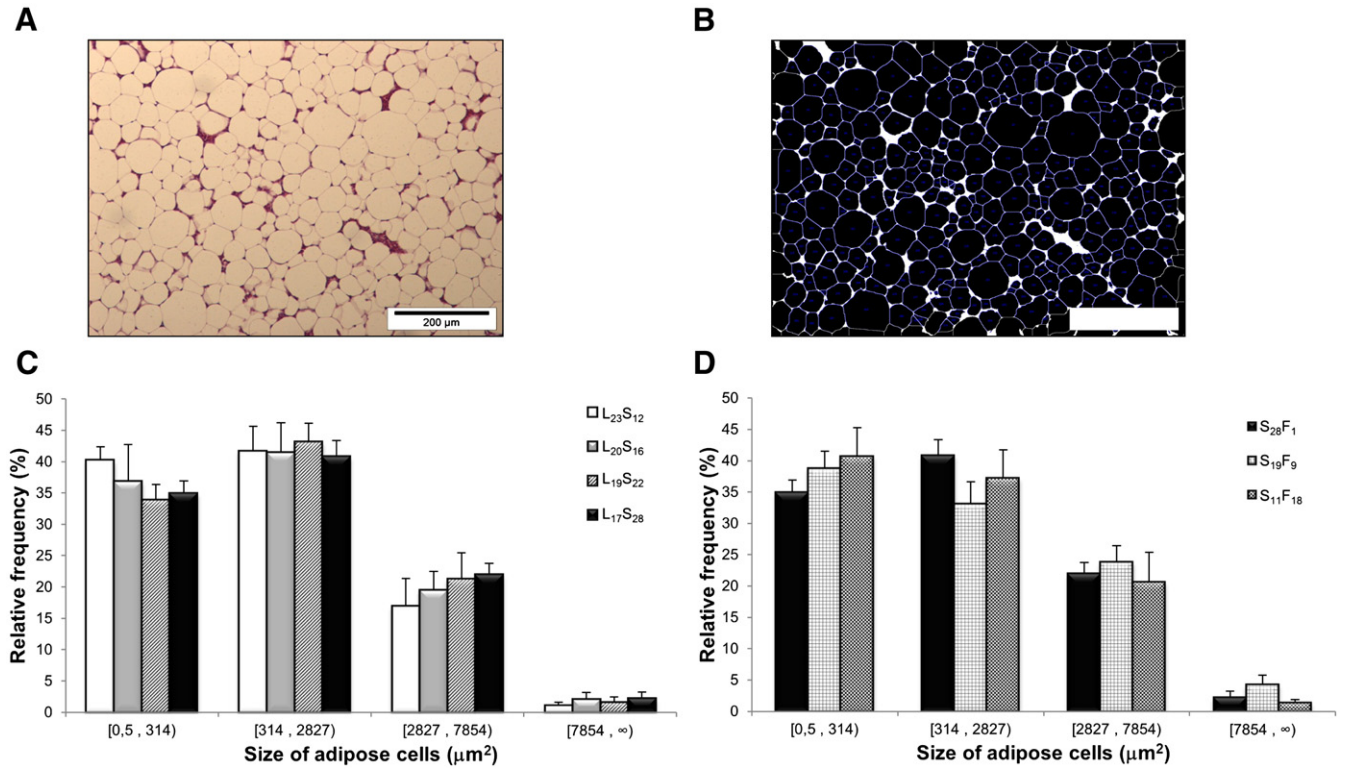

Fig. 1. Adipose tissue histology and adipocyte cell size distribution analysis. Representative hematoxylin and eosin-stained histological sections of adipose tissue (A) and its corresponding computer-converted image into a binary representation (B). Images captured at 10× magnification. Frequency distribution of adipocyte cell area from gilthead sea bream fed the different experimental diets from LS (C) and SF (D) groups. Data are shown as mean ± SEM (n = 6). Results were analyzed by one-way ANOVA, followed by the Tukey's test. No significant differences were found ($P < 0.05$).

pattern was identified, although not significant, in the number of smaller cells in the fish consuming the diet with the highest content of lipids ($L_{23}S_{12}$). The opposite trend was observed when the relative frequency of bigger cells was analyzed, being the experimental diets with higher levels of carbohydrates the ones causing a higher presence of larger adipocytes (Fig. 1C). Gilthead sea bream from the SF group receiving the diet with less carbohydrate content and higher level of fiber ($S_{11}F_{18}$) exhibited a slightly higher proportion of cells with smaller areas (0.5–314 μm^2) than those fed with the other diets (Fig. 1D).

3.4. Effects of diet on gene expression

In the LS group, the substitution of lipids by carbohydrates produced a significant up-regulation of LPL and HSL in the liver, meanwhile the expression of these enzymes was not significantly affected in the adipose tissue additionally, the mRNA content of FAS was not significantly modified in none of the tissues (Fig. 2). Moreover, the dietary carbohydrate level had no effect on the mRNA levels of G6PDH in the liver, whereas a significant induction was observed in the adipose tissue when the carbohydrate included in the diet was at a level of 16% or higher. In the liver, G6Pase was not nutritionally regulated, contrary to GK, which was significantly induced in fish fed the $L_{19}S_{22}$ diet (Fig. 2). Furthermore, hepatic LXRα expression was unaffected in gilthead sea bream, but in the adipose tissue this transcription factor was suppressed as the substitution of lipid by carbohydrates increased (Fig. 3). Besides, PPARα and PPARβ did not show differences in the liver, but PPARγ exhibited a down-regulation in fish fed the two diets with higher content in carbohydrates and lower in lipids, $L_{19}S_{22}$ and $L_{17}S_{28}$. However, in the adipose tissue a different pattern was observed, since PPARγ was not affected by dietary composition but PPARβ showed a significant down-regulation when the lipid level went below 19% (diets $L_{19}S_{22}$ and $L_{17}S_{28}$); and PPARα was significantly induced only by the $L_{20}S_{16}$ diet (Fig. 3).

In the SF group differences in genes involved in fatty acid metabolism such as FAS, LPL and HSL were not found in the adipose tissue, while in the liver LPL was significantly down-regulated in response to the highest inclusion of fiber in the diet ($S_{11}F_{18}$) (Fig. 4). The levels of mRNA of the enzyme G6PDH were significantly higher in the adipose tissue of gilthead sea bream receiving the $S_{11}F_{18}$ diet in comparison with the fish fed the other diets in the SF group, while in the liver its expression was not affected. Regarding the expression of carbohydrate metabolism-related enzymes, in this experimental group no modulation of G6Pase expression in the liver was found. However, lowering the carbohydrate content at the level of 11% significantly inhibited hepatic GK expression (Fig. 4). Furthermore, all PPARs as well as LXRα were significantly induced by fiber inclusion at expenses of carbohydrates in the adipose tissue, especially in the case of PPARγ, and LXRα that were already up-regulated by the intermediate experimental diet $S_{19}F_9$ (Fig. 5). On the other hand, the mRNA levels of these transcription factors remained unchanged in the liver, and so it was the expression of LXRα in this tissue.

4. Discussion

The aim of this study was to gain knowledge on the dietary lipid and carbohydrate use in gilthead sea bream focusing our interest not only in the role of adipose tissue, but also in the liver, both key organs of fat and starch deposition and metabolic turnover. The results demonstrated that in the conditions studied, dietary lipid substitution by carbohydrate up to levels of 28%, and subsequent changes in carbohydrate content by fiber had moderate effects in adipose tissue metabolism homeostasis without affecting growth performance and feed utilization, thus supporting the use of these diets in gilthead sea bream production.

Dietary treatments did not significantly modify the MFI, thus, the balance between fat accumulation and utilization was maintained in visceral adipose tissue. In agreement with this observation, neither

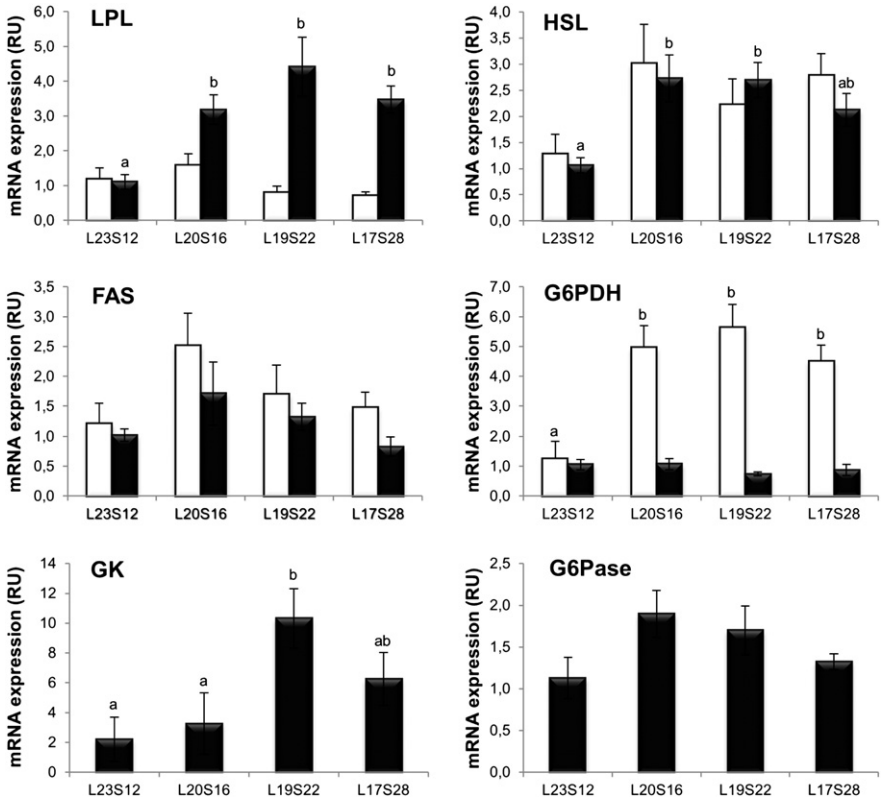

Fig. 2. Gene expression of enzymes involved in different carbohydrate and lipid metabolism pathways of gilthead sea bream fed the different experimental diets from LS group. Adipose tissue (□) and liver (■). Data are presented in relative units (RU) using β-actin as a reference gene (delta–delta method) and shown as mean ± SEM (n = 6). Results were analyzed by one-way ANOVA, followed by Tukey's test. Values that do not share a common letter are significantly different ($P < 0.05$).

adipose cell size nor the transcription pattern of LPL or HSL, was affected. However, it has been previously reported that adipose tissue is able to respond to dietary changes, since high HSL activity and expression, as well as increased adipocyte cell size were observed in gilthead sea bream subjected to a dietary replacement of 66% of fish oil by vegetable oils (Cruz-Garcia et al., 2011). In mammals, a positive correlation between cell size and increased lipolytic rates and HSL activity has been also demonstrated, being indicative of defective lipid deposition and turnover (Berger and Barnard, 1999; Gregor and Hotamisligil, 2007). Besides, hypertrophy of adipocytes is considered a marker of failure in the mechanism of preadipocyte recruitment to develop new and smaller adipocytes (Medina-Gomez and Vidal-Puig, 2005). In the present study, the adipocyte size was not altered pointing out that the use of high quality diets is essential to maintain an adequate lipid metabolic balance.

On the other hand, adipose tissue lipogenesis appeared to be regulated by diet, and in this sense, the activation of the pentose phosphate pathway, at least at a transcriptional level, was observed when lipid

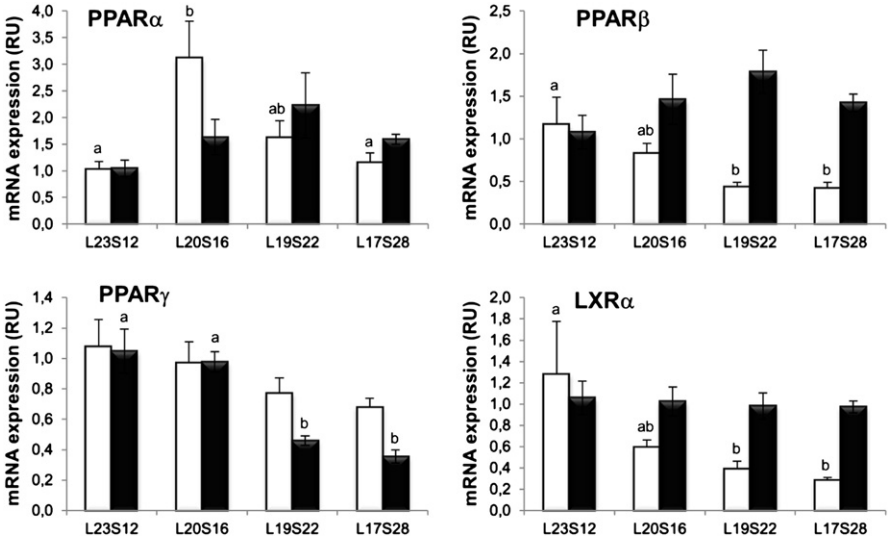

Fig. 3. Gene expression of nuclear receptors of gilthead sea bream fed the different experimental diets from LS group. Adipose tissue (□) and liver (■). Data are presented in relative units (RU) using β-actin as a reference gene (delta–delta method) and shown as mean ± SEM (n = 6). Results were analyzed by one-way ANOVA, followed by Tukey's test. Values that do not share a common letter are significantly different ($P < 0.05$).

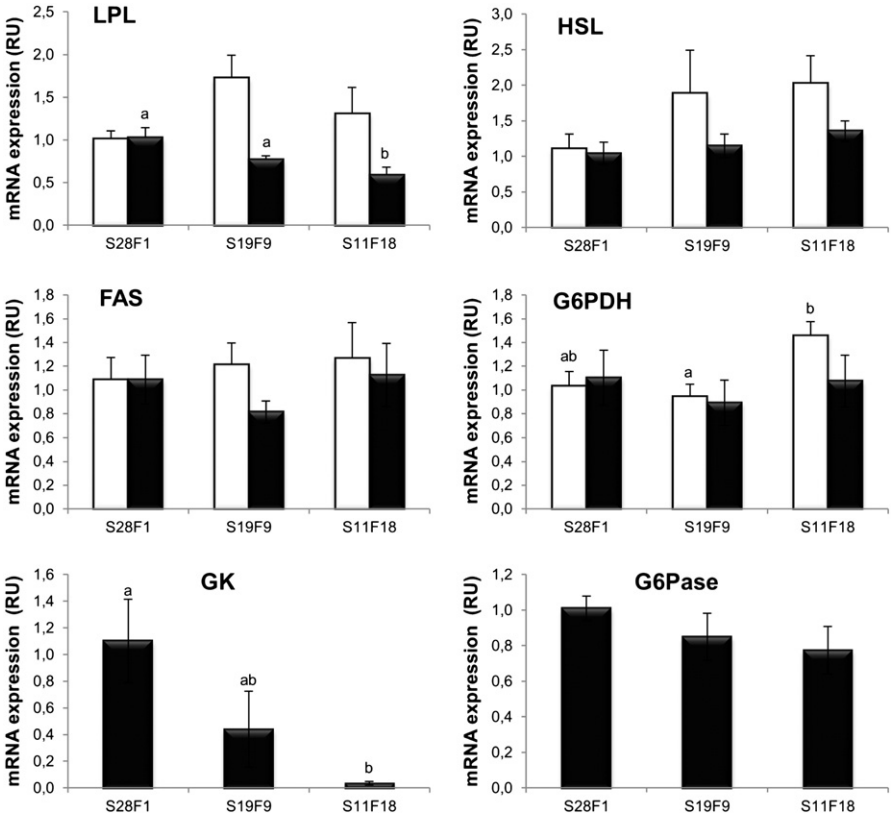

Fig. 4. Gene expression of enzymes involved in different carbohydrate and lipid metabolism pathways of gilthead sea bream fed the different experimental diets from SF group. Adipose tissue (□) and liver (■). Data are presented in relative units (RU) using β-actin as a reference gene (delta–delta method) and shown as mean ± SEM (n = 6). Results were analyzed by one-way ANOVA, followed by Tukey's test. Values that do not share a common letter are significantly different ($P < 0.05$).

content was decreased in exchange of starch in the LS group. This was revealed by a strong up-regulation of G6PDH expression, the main enzyme of this pathway, even from the first level of substitution ($L_{20}S_{16}$ diet), which was accompanied only by a tendency to increase FAS expression. This modulation of the pentose phosphate pathway associated to the level of carbohydrates was not observed in the adipose tissue of animals from the SF group, where the substitution of carbohydrates by cellulose did not induce a parallel decrease in the mRNA levels of G6PDH. Thus, it appears that an increase in starch, together with a reduction in the level of lipid in the diet is necessary to stimulate G6PDH transcription in adipose tissue. It has to be noted that we have studied the regulation at a transcriptional level, and that although gene expression and enzyme activity usually change in parallel, some exceptions can occur. Previous studies regarding the lipogenic potential of adipose tissue are scarce in fish. In rainbow trout, neither carbohydrate deprivation nor the use of rich carbohydrate diets with different fat content modulated the expression of G6PDH and FAS, or G6PDH activity in the adipose tissue, suggesting a lack of dietary regulation on

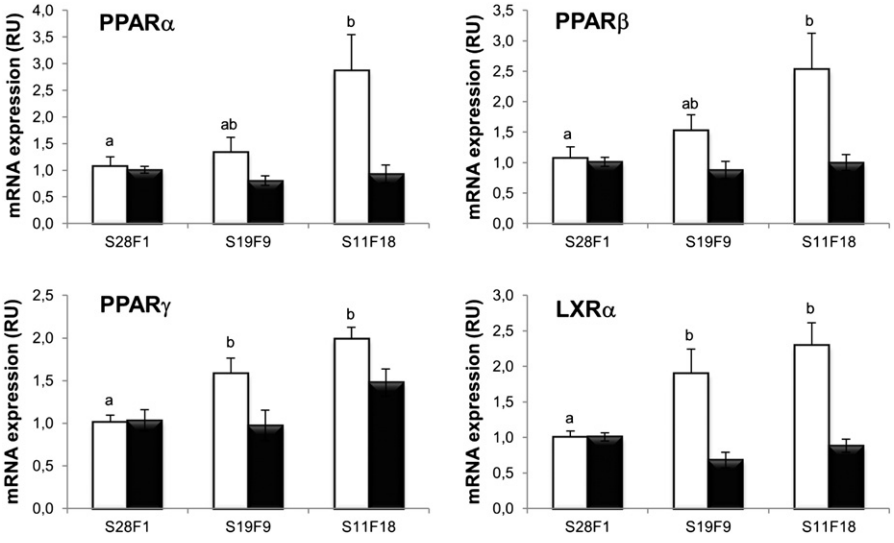

Fig. 5. Gene expression of nuclear receptors of gilthead sea bream fed the different experimental diets from SF group. Adipose tissue (□) and liver (■). Data are presented in relative units (RU) using β-actin as a reference gene (delta–delta method) and shown as mean ± SEM (n = 6). Results were analyzed by one-way ANOVA, followed by Tukey's test. Values that do not share a common letter are significantly different ($P < 0.05$).

lipogenesis in the fat visceral tissue of this species (Barroso et al., 2001; Figueiredo-Silva et al., 2012; Kamalam et al., 2012). On the other hand, gilthead sea bream fed diets with high content of wheat gluten and soybean meal showed a decrease in the activity of G6PDH and malic enzyme in white adipose tissue without affecting hepatic lipogenesis (Bouraoui et al., 2011). All in all, these results suggest that the lipogenic activity of the adipose tissue and its regulation seems to be species-specific, with gilthead sea bream presenting higher dietary modulation than salmonids.

Considering the transcriptional factors studied here, it is worth noting that the nuclear receptor LXRα exhibited a nutritional regulation in the adipose tissue of gilthead sea bream in both experimental groups, being significantly suppressed with the inclusion of carbohydrates in the LS group and activated as the amount of starch decreased in the SF group. This transcription factor has been recently described in gilthead sea bream and salmonids (Cruz-Garcia et al., 2009a, 2009b). In mammals, it is known to be involved in cholesterol, fatty acid and glucose metabolism (Steffensen and Gustafsson, 2004). In 3T3-L1 and human adipocytes, the activation of LXRα has been associated with a switch on substrate oxidation from carbohydrates towards lipids (Stenson et al., 2009), a role that fits well with the changes observed in LXRα expression in the present study. In gilthead sea bream the dietary regulation of LXRα was corroborated by Cruz-Garcia et al. (2011), since the use of vegetable oil in the diet caused down-regulation of this transcription factor in the liver. Moreover, in rainbow trout isolated adipocytes, it has been demonstrated that together with the regulation of cholesterol transport, LXRα has a pro-lipolytic action independent of HSL (Cruz-Garcia et al., 2012). Consequently, the observed changes in LXRα expression in the present dietary groups agree with a pro-lipogenic and anti-lipolytic situation in gilthead sea bream adipose tissue after lipid substitution by starch.

PPARα, β and γ are critical transcriptional regulators of lipid metabolism and energy homeostasis in mammals (Desvergne et al., 2006). The three isotypes have been found in several fish species, among them in gilthead sea bream (Leaver et al., 2005). However, the role of PPARs in lipid metabolism is still puzzling due to both, species and tissue specific responses. Albeit the expression of PPARγ was not significantly modified by dietary treatments in the adipose tissue of the LS group, a decreasing tendency when reducing lipid levels and increasing carbohydrates was observed. In a similar way, in the SF group this PPAR isotype was significantly promoted when diminishing starch availability. Since PPARγ promotes lipid incorporation into adipose tissue (Tontonoz and Spiegelman, 2008), it is reasonable to consider that its expression will be lower with reduced dietary lipids. Accordingly, high-fat feeding was reported to activate PPARγ to enhance adipogenesis in mammals (Davis et al., 2013; Shen et al., 2014). Nevertheless, a constant level of dietary lipids with a concomitant decrease in carbohydrates (SF group) also seems to favor the incorporation of fatty acids in the adipose tissue according to the observed changes in PPARγ expression. Changes in PPAR α and β expression in the adipose tissue followed a similar pattern, decreasing in parallel to the level of lipids and increasing as the content of dietary starch was reduced. Due to the role of PPAR α and β in fatty acid oxidation (Kota et al., 2005), these changes in gilthead sea bream might be related to increased lipid use by β-oxidation when carbohydrates become less available. Besides, PPARβ has been described to change body's fuel preferences from glucose to lipids in rat (Brunmair et al., 2006), as in the case of LXRα (Stenson et al., 2009), in agreement with the fact that PPARβ expression and LXRα expression were maximum with the highest level of dietary lipid and minimum content of carbohydrate ($L_{23}S_{12}$ diet).

Regarding the liver, the inclusion of carbohydrates in both experimental groups significantly increased the HSI, but always inside a normal range of values (Baños et al., 1998) and probably due to an enhancement in glycogen liver deposition. The augmented HSI and glycogen liver content in response to high-carbohydrate diets has been widely reported previously in this species (García de Frutos et al., 1990; Couto et al., 2008; Ekmann et al., 2013) and several other species, such as silver sea bream (Leung and Woo, 2012), sea bass (Peres and Oliva-Teles, 2002; Pérez-Jiménez et al., 2007), flounder (Lee and Lee, 2004), common carp (Capilla et al., 2004) and rainbow trout (Hilton and Atkinson, 1982; Baños et al., 1998; Capilla et al., 2003). However, in other species, such as cod (Hemre et al., 1989), high values of HSI have been associated with fat deposition in the liver, the preferential site of lipid storage in gadoids. Yet, the lack of regulation in the liver of the enzyme G6PDH by our experimental diets suggests that the excess of hepatic dietary glucose is preferentially directed towards glycogen synthesis rather than to enhance hepatic lipogenesis.

Contrariwise to mammals, fish LPL is expressed in the liver, and it is seasonally and nutritionally regulated, being induced in gilthead sea bream with plant protein diet administration (Saera-Vila et al., 2005) and in red sea bream fed low-lipid diets (Liang et al., 2002). Similarly, in the present study, the transcription of this hepatic enzyme was also regulated by diet, being significantly promoted in fish fed by the substitution of lipids by carbohydrates, and significantly suppressed by the highest substitution of starch by fiber in the $S_{11}F_{18}$ diet. Although the pro-lipolytic enzyme HSL was not regulated in the liver of fish subjected to the SF diets, a concomitant up-regulation of HSL with LPL was found in the LS group, reflecting the presence of a dynamic liver with a high lipid turnover.

In regards with hepatic glucose metabolism, the first step consists of the phosphorylation of glucose by GK or other hexokinases. There is molecular and biochemical evidence showing mammalian-type responses of GK to dietary treatments in fish. For instance, the expression and activity of GK have been proved to be induced after a rich-carbohydrate meal in rainbow trout, gilthead sea bream, common carp, perch and silver sea bream (Borrebaek and Christophersen, 2000; Panserat et al., 2000; Capilla et al., 2004; Leung and Woo, 2012). This nutritional regulation was corroborated in the present study, where gilthead sea bream fed the $L_{19}S_{22}$ diet also exhibited an induction of GK expression. However, with the highest inclusion of carbohydrates present in the diet $L_{17}S_{28}$, the increase in GK mRNA levels was not significant, which could be related to interferences with dietary lipid content. In rats (Girard et al., 1991; Jump et al., 1994; Lam et al., 2003) and in gilthead sea bream during refeeding (Metón et al., 2004) an inhibitory effect of FFA on GK expression has been described, leading to partially override the strong induction of this enzyme by dietary carbohydrates. Nevertheless, when we compared the changes on expression of this enzyme in the SF group, a significant repression in fish fed the diets with less starch content was observed, which pointed out the strong dependence on dietary starch for the activation of GK at the transcriptional level in this species. Even though assessing GK activity would be necessary to fully understand the dietary regulation of this enzyme, all these results reinforce the previous described hypothesis that both, starch digestion (Enes et al., 2008; Couto et al., 2012; Ekmann et al., 2013) and liver glucose phosphorylation (Panserat et al., 2000; Caseras et al., 2002), are very efficient in gilthead sea bream fed high-carbohydrate diets.

In contrast to previous studies in mammals, where the hepatic expression of G6Pase is regulated by hormonal and nutritional status (Newgard et al., 1984; Minassian et al., 1995; Massillon et al., 1997; van de Werve et al., 2000), the presence of high carbohydrates or lipids in the diet did not suppress the expression of this enzyme in gilthead sea bream, suggesting an apparent lack of gluconeogenesis regulation by these nutritional factors. A short-term postprandial regulation of G6Pase has been described previously in this species (Caseras et al., 2002; Panserat et al., 2002b), however none of the studies showed long-term effect on hepatic G6Pase expression. Yet, differences between species have been found, since silver sea bream (Woo and Kelly, 1995; Leung and Woo, 2012) and tilapia (Shimeno et al., 1993) showed G6Pase regulation according to diet composition. G6Pase and GK are the two enzymes that regulate the rate and flux through the hepatic glucose/glucose-6-phosphate substrate cycle. Under our experimental diets, it seemed that with higher inclusion of carbohydrates, hepatic

glucose uptake through glucose phosphorylation predominated over glucose-6-phosphate hydrolysis.

In line with previous observations, plasma glucose was not affected by dietary carbohydrates and was maintained at a steady level under all experimental groups. This absence of glucose fluctuation is in agreement with the results observed in other related species such as the silver sea bream, where feeding high-carbohydrate diets did not trigger a hyperglycemic response (Leung and Woo, 2012). However, this was not the case for cod (Hemre et al., 1989) or salmonids (Bergot, 1979; Polakof et al., 2008). The lack of hyperglycemia might be due to a higher glucose uptake from the circulation into the liver, which is in accordance with the hepatic induction of GK mRNA levels by high-carbohydrate diets, and with the moderate increase in the HSI of fish under these diets previously mentioned. This would point out the ability of this carnivorous species to utilize dietary carbohydrates and therefore the possibility of performing a partial substitution of lipids by carbohydrates in commercial diets.

Overall, in the present study we have demonstrated that using diets with relatively high content of vegetable ingredients, neither lipid replacement from levels of 23 to 17% by carbohydrates increasing from 12 to 28%, nor the inclusion of fiber up to 18% at expenses of carbohydrate affects growth performance, and presents only moderate changes in lipid and glucose metabolism in gilthead sea bream. The activation of the lipogenic pathway in response to dietary carbohydrates was showed in adipose tissue and a balance between the expression of transcriptional factors appeared to be directed to modulate the preferential use of lipid or carbohydrate according to their availability. A metabolic adaptation to high levels of carbohydrates was observed by the modulation of hepatic GK. Data suggest that gilthead sea bream liver appears to show efficient regulation, balancing fatty acid uptake and lipolysis regardless of differences in nutrient composition. All these results encourage the formulation of commercial diets including a relatively high level of lipid replacement by carbohydrates or fiber to reduce aquafeed costs, and reliance on fish oil, without compromising growth performance or the physiological state of the fish.

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De novo lipogenesis in Atlantic salmon adipocytes

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ABSTRACT

Background: Carnivorous teleost fish utilize glucose poorly, and the reason for this is not known. It is possible that the capacity of adipocytes to synthesize lipids from carbohydrate precursors through a process known as "*de novo* lipogenesis" (DNL) is one of the factors that contributes to glucose intolerance in Atlantic salmon.
Methods: Primary adipocytes from Atlantic salmon differentiated *in vitro* were incubated with radiolabelled glucose in order to explore the capacity of salmon adipocytes to synthesize and deposit lipids from glucose through DNL. The lipid-storage capacity of adipocytes incubated with glucose was compared with that of cells incubated with the fatty acid palmitic acid. Quantitative PCR and immunohistochemistry were used to assess changes of genes and proteins involved in glucose and lipid transport and metabolism.
Results: Less than 0.1% of the radiolabelled glucose was metabolized to the fatty acids 16:0 and the stearoyl-CoA desaturase products 16:1 and 18:1 by DNL, whereas approximately 40% was converted to glycerol to form the triacylglycerol backbone of lipids. Transcriptional analysis indicated that adipocytes ensure the availability of necessary cofactors and other substrates for lipid synthesis and storage from glycolysis, the pentose phosphate pathway and glyceroneogenesis.
Conclusions: We have shown for the first time that the DNL pathway is active in fish adipocytes. The capacity of the pathway to convert glucose into cellular lipids for storage is relatively low.
General significance: The limited capacity of adipocytes to utilize glucose as a substrate for lipid deposition may contribute to glucose intolerance in salmonids.

1. Introduction

Carnivorous fish species, as well as mammalian dolphin and cat species, are considered to be glucose-intolerant [1–3]. Their natural diets are rich in protein and lipids and very poor in carbohydrates [4–6]. These species utilize dietary carbohydrates poorly, and experience prolonged postprandial hyperglycaemia when fed carbohydrate rich diets [2,3,7]. Also, a low capacity for glycogen synthesis has been shown in mammalian carnivores [3]. It remains, however, unclear why fish have a much poorer ability to utilize dietary carbohydrates than non-carnivorous mammalian species.

The role of dietary factors as modulators of key enzymes of carbohydrate metabolism has been studied, in order to establish the fate of dietary carbohydrates in several carnivorous species [8–13]. The enzymatic machinery responsible for carbohydrate metabolism has been identified in several fish species [14], and basic responses are conserved from fish to mammals (even though some regulatory mechanisms are species-specific).

Glucose and fatty acids are the major oxidative fuels in non-carnivorous mammals like humans and mice, and typically account for 80% of oxidative metabolism. Cells can only dispose of as much ATP as they have a need for, which makes it necessary to have powerful regulatory mechanisms to control the utilization and storage of glucose and fatty acids [15]. Many aspects of the inter-relationships between glucose and fatty acids in mammals have been elucidated after 1963, when the existence of a glucose-fatty acid cycle was proposed for the first time [16], and the process of *de novo* lipogenesis (DNL) has been studied in particular depth [17]. There is a limit to the amounts of glucose and glycogen that can be stored in the body, and thus DNL in the liver and/or adipose tissue provides a physiological pathway for the synthesis of lipids from carbohydrates when a mammal has excess carbohydrate in the diet [18]. DNL is a complex and highly regulated metabolic pathway in mammals [19]. Under normal conditions, DNL converts excess carbohydrate into fatty acids, which are then esterified to become triacylglycerols (TAGs), which are stored. These TAGs can later provide energy through β-oxidation. DNL in humans is active primarily in the liver and adipose tissue but the quantitative contribution of each tissue to this pathway remains controversial [20]. The deregulation of this pathway is associated with several metabolic anomalies, such as insulin resistance, obesity and non-alcoholic fatty liver disease [19]. An increase in DNL in liver cells is related to the development of metabolic disease. An increase in DNL in adipose tissue, in contrast, is associated with metabolic health, which shows that the pathway is related to health in a

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tissue-specific manner [21]. The pathway is not only an efficient way to store energy; the fatty acids and their derivatives produced by DNL have different bioactivities than those provided by the diet, and play a key role as signalling molecules in many physiological processes [22]. It is not known whether salmonids can convert carbohydrates to lipids, nor is it known whether a limited capacity of adipose and liver cells is a factor that explains the carbohydrate intolerance of carnivorous fish.

The role of adipose tissue DNL in regulation of carbohydrate homeostasis in fish is more or less unknown. However, the finding that increased hepatic lipogenic capacity was associated with a better ability to control hyperglycaemia in rainbow trout (*Oncorhynchus mykiss*) suggests an important role of liver DNL in the regulation of glucose homeostasis [23–26]. Early studies have nevertheless suggested a possible role of also adipose tissue in the regulation of glucose metabolism and homeostasis based on its high lipogenic potential [27]. A study showing an increase in transcript levels of genes regulating carbohydrate pathways during the terminal phase of adipocyte differentiation, further indicates the relevance and involvement of adipose tissue in glucose metabolism in Atlantic salmon (*Salmo salar*) [28].

Primary cells in culture are a potent tool to characterize cellular metabolic processes. A method to differentiate unspecialized primary preadipocytes to mature adipocytes in culture has been successfully established for pre-adipocytes from several fish species [29–31]. Unspecialized precursor cells from fish require the addition of lipids to the culture medium in order to differentiate to mature lipid-filled adipocytes, whereas mammalian pre-adipocyte cell lines and primary preadipocytes require only glucose to be added to the medium to differentiate. DNL is essential in this case to convert glucose to lipids, and to allow the cells to differentiate [32–34]. One of the aims of the work presented here, therefore, was to study the ability of Atlantic salmon adipocytes to convert glucose into fatty acids. A further aim was to study how the DNL pathway influences the differentiation of pre-adipocytes.

Human adipocytes can synthesize considerable amounts of fatty acids from non-lipid precursors, and can in this way obtain all of the fatty acids that are necessary for maturation *in vitro* [35]. The main product of DNL is palmitate (16:0), although stearate (18:0) and shorter fatty acids are also generated. The adverse effects of the accumulation of saturated fatty acids (SAFAs) on cell function are well known [36–38]. However, these potentially toxic effects are minimized by the coordinated up-regulation of the elongation of fatty acids and their desaturation by the enzyme stearoyl-CoA desaturase (SCD or delta-9 desaturase) [39]. This enzyme is important, since it is responsible for converting the 16:0 and 18:0 produced by DNL to palmitoleate (16:1 n−7) and oleate (18:1 n−9) respectively. SCD is thus essential for cell function, as it is involved in maintaining an intracellular equilibrium of SAFAs and monounsaturated fatty acids (MUFAs), ensuring cell membrane integrity and optimizing TAG storage.

We have used a radioactive assay to determine the lipogenic potential of Atlantic salmon adipocytes and the ability of the adipocytes to synthesize lipids via *de novo* lipogenesis. We treated the cells with either radiolabelled palmitic acid (PA) or radiolabeled glucose. We used the same method with non-labelled PA and non-labelled glucose, in order to elucidate how these substrates affect some of the main enzymes that are involved in lipid and carbohydrate metabolism at a transcriptional level. Finally, we studied the cellular transport of glucose and fatty acid, using immunofluorescence staining of cells with antibodies against the glucose transporter 4 (GLUT4) and the fatty acid transport protein 1 (FATP1).

2. Materials and methods

2.1. Preadipocyte isolation and culture conditions

Atlantic salmon were reared at the Nofima Research station at Averøy, Norway on a commercial diet from Skretting to an average weight of 2.5 kg. Fish were anaesthetized with metacain (MS-222), bled by cutting the gill arches and killed by a blow to the head. The experiment was conducted according to the National Guidelines for Animal Care and Welfare published by the Norwegian Ministry of Education and Research. Visceral adipose tissue was excised and salmon preadipocytes were isolated as described in Vegusdal et al. [29]. The isolated preadipocytes were resuspended in growth medium containing L-15, 10% foetal bovine serum (FBS), 2 mM L-glutamine, 10 mM HEPES, and 1% antibiotics (a mixture of penicillin, streptomycin and amphotericin B) (unless otherwise stated, all the chemicals were obtained from Sigma Aldrich Chemical Co., St. Louis, MO, USA). The pH of the medium was measured with pH indicator strips to ensure it was around 7. The 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 and the medium was changed every 3 days. Cells reached confluence after approximately 1 week. Confluent cells were cultivated for 48 h in a differentiation-inducing medium that contained growth medium supplemented with 1 μM dexamethasone, 33 μM biotin, 10 nM triiodothyronine, 17 μM panthothenate, 25 μM isobutylmethylxanthine and 20 μg/ml insulin [29]. Cells were subsequently transferred to a maintenance differentiation medium that contained growth medium supplemented with 1 μl/ml of lipid mixture (1 μl/ml; corresponding to 45 mg/ml cholesterol, 100 mg/ml cod liver oil FA (methyl esters)). The medium was changed every 3 days until the cells reached the final differentiation stage with the morphology of mature adipocytes (Day 13).

2.2. Lipid accumulation in adipocytes treated with glucose

The accumulation of lipids in mature adipocytes was estimated by oil red O staining according to Ramirez-Zacarias et al. [40]. The procedure was applied to mature adipocytes cultivated for 13 days in growth medium (control), growth medium supplemented with non-radiolabeled glucose (1 mM or 5.5 mM), and growth medium supplemented with non-radiolabeled PA (40 or 80 μM) for 48 h. All groups were supplemented with 20 μg/ml insulin. Each experiment was performed four times, with each n-value representing a pool of cells coming from at least 10 fish. 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 first rinsed in water and thereafter added filtered oil red O in isopropanol at room temperature to stain the intracellular lipid for 2 h. Relative quantities of cellular oil red O in triacylglycerol (TAG) were measured in cells washed with PBS to remove excess stain solution. The cells were left to dry at room temperature prior to extraction of the oil red O stained lipids in 100% isopropanol. The absorbance of oil red O (as a measure of intracellular lipid level) was measured spectrophotometrically in a Victor 3 microplate reader from PerkinElmer (Wellesley, MA, USA) at 500 nm.

2.3. Incubations with radiolabelled PA and glucose

Adipocytes at Day 13 were washed with serum-free growth medium and treated with an incubation medium that comprised serum-free growth medium plus 20 μg/ml insulin. The incubation medium contained either [1-^{14}C] PA (0.33 μCi per cell well, 20 μM) or [1-^{14}C] glucose (9 μCi per cell well, 1 mM). The radiolabeled FA was added to the medium in the form of its potassium salt bound to FA free bovine serum albumin (BSA) (the molar ratio of FA to BSA was 2.7:1). PA was selected based on the fact that this FA is the main product of DNL and therefore is the output that we expect to be produced from glucose. Each incubation was conducted in six independent parallel experiments ($n = 6$), each representing adipocytes coming from a pool of adipose tissue from an average of 10 fish.

Prior to incubation, aliquots of 10 μl, 20 μl, and 30 μl of the incubation medium with either radioactive PA or radioactive glucose were transferred into different vials together with 5 mL of Ecoscint A scintillation liquid (National Diagnostics Inc., Atlanta, GA, USA) in order to count total radioactivity. The specific radioactivity (cpm/nmol FA; cpm/nmol

glucose) was subsequently calculated for FA and glucose substrates. The samples were counted in a scintillation counter TRI-CARB 1900 TR (Packard Instrument Co., North Chicago, IL, USA).

The cells were incubated for 48 h at 13 °C. After incubation cells containing PA were washed twice in PBS that contained 1% albumin, and once more with regular PBS, while cells containing glucose were washed three times with regular PBS. All cells were harvested in 2 ml of PBS and stored at −40 °C before the radiolabelled lipid classes were analysed. The culture media was used for determination of un-metabolized radiolabelled substrate and oxidation products. Approximately 85% recovery of the added radioactivity was found in each incubation after summing up the total radioactivity recovered in cellular lipids, oxidation products and un-metabolized substrate in the culture media. The total radioactivity recovered in lipids and oxidation products in each incubation was set to 100% for ease of calculation.

2.4. Measurement of $^{14}CO_2$ and ASP production from [1-^{14}C] PA and [1-^{14}C] glucose

The capacity of β-oxidation of PA was measured by determination of oxidation products (counting ^{14}C-labelled ASPs and the $^{14}CO_2$ formed) as described by Christiansen et al. [41]. The amounts of Krebs cycle products from glucose were determined by the same methods. The amount of radioactive CO_2 was measured by transferring 1.5 ml of the medium into 25 ml Erlenmeyer flasks stoppered with a rubber cap and equipped with a suspended central well that contained a piece of Whatman filter paper (pore size ∅ 125 mm) with 0.3 ml phenylethylamine/methanol (1:1 *v*/*v*). The medium was acidified with 0.3 ml 1 M $HClO_4$ and ^{14}C-CO_2 was trapped for 1 h. Each filter paper was placed in a vial and dissolved in 8 ml scintillation fluid for scintillation counting. The amount of ^{14}C-ASP was determined by mixing 1 ml medium from the adipocytes with 0.5 ml ice-cold 2 M $HClO_4$ and incubating the mixture at 4 °C for 1 h. The samples were centrifuged at 17,950 × *g* at 4 °C for 10 min, and 100 μl of the supernatant was collected for scintillation counting. The remaining supernatant was neutralized with NaOH, and the various ASPs were detected by using high-pressure liquid chromatography (HPLC) on a ChromSep (250 mm × 4.6 mm stainless steel) Inertsil C8-3 column (Chrompack Varian, Inc., Palo Alto, CA), a UV-detector at 210 nm (Waters 2996, Photodiode Array Detector), and a radioactivity detector A-100 (Radiomatic Instrument & Chemicals, Tampa, FL, USA) coupled in series to the UV detector. The sample was eluted at a flow rate of 1 ml/min with 0.1 M ammonium dihydrogenphosphate adjusted to a pH of 2.5 with phosphoric acid. The components were identified by comparison with external standards and retention times.

2.5. Lipid extraction and analysis of lipid classes

Total lipids from adipocytes on Day 15 were extracted as described by Folch et al. [42]. The chloroform phase (475 μl) with butylated hydroxytoluene (BHT) (final concentration 0.7 mg/l) was dried under nitrogen gas and the residual lipid extract was redissolved in 25 μl of hexane. Free fatty acids (FFAs), phospholipid (PL), monoacylglycerols and diacylglycerols (MDGs) and TAGs were separated by thin-layer chromatography (TLC) using a mixture of petroleum ether, diethyl ether and acetic acid (113:20:2 *v*/*v*/v) as the mobile phase. The hexane phase was applied onto the TLC-plate and dried. 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 dipping the plates in copper sulphate solution. The spots corresponding to FFAs, PLs, MDGs and TAGs were identified by comparison with known standards by a Bioscan AR-2000 Radio-TLC & Imaging Scanner and quantified with the WinScan Application Version 3.12 (Bioscan Inc., Washington, DC, USA).

2.6. Fatty acid composition

The degrees of unsaturation and elongation were determined by silver-ion TLC essentially as described by Nikolova-Damyanova [43]. The lipid classes were methylated prior to analysis by incubation overnight with 2 ml benzene, 2 ml methanolic-HCl and 200 μl dimethoxypropane. 2 ml of hexane was added and the samples were neutralized with $NaHCO_3$. The benzene/hexane phase, which contained methylated lipids, was collected, dried at 60 °C with nitrogen overflow and redissolved in chloroform. Lipids were separated on silica gel plates impregnated with silver nitrate (4% silver nitrate in methanol/water 9:1, *v*/*v*) in toluene/ethyl acetate (90:10, *v*/*v*) and specific FAs were identified by comparison with known standards by a Bioscan AR-2000 Radio-TLC & Imaging Scanner (Bioscan Inc., Washington, DC, USA). The peaks corresponding to 16:0, 16:1 n−7 and 18:1 n−9 were scraped off into vials and dissolved with 5 ml of scintillation fluid and measured in a TRI-CARB 1900 TR scintillation counter.

2.7. Incubation with non-radiolabelled PA and non-radiolabelled glucose

Adipocytes at Day 13 were washed with serum-free growth medium and treated with the incubation medium, which consisted of growth medium without serum and with insulin. The incubation medium contained either non-radiolabeled 20 μM PA or non-radiolabeled 1 mM glucose, and the incubation continued for 48 h. The cells in each well that contained PA were washed twice in PBS (containing 1% albumin), while the cells in each well that contained glucose were washed with regular PBS. All cells were harvested in RLT buffer containing β-mercaptoethanol, and stored at −80 °C prior to RNA extraction.

2.8. RNA extraction and cDNA synthesis

Total RNA was extracted using an RNeasy® Mini Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. 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.

The amount of 170 ng of total RNA was reverse-transcribed into cDNA using an AffinityScript QPCR cDNA Synthesis Kit (Agilent Technologies, La Jolla, CA, USA) in a 20 μl reaction system. All procedures were carried out according to manufacturer's instructions. The cDNA synthesis was performed with a primer incubation at 25 °C for 5 min, an RT step at 42 °C for 45 min, and an RT inactivation step at 95 °C for 5 min. The reverse-transcription products (cDNA) were stored at −20 °C for qPCR of the target genes.

2.9. Real-time PCR analysis (qPCR)

The relative changes in transcript levels were quantified by real-time qPCR for: genes involved in carbohydrate and lipid metabolism (acetyl-CoA carboxylase (ACC), phosphofructokinase (PFK), transketolase (TKT), 6-phosphogluconate dehydrogenase (PGD), cytosolic phosphoenolpyruvate carboxykinase (PEPCK-C), hexokinase (HK) and pyruvate kinase (PK)), three major adipogenic factors (CCAAT-enhancer-binding protein alpha and beta (C/EBPα, C/EBPβ respectively) and cAMP response element binding protein (CREB)) and genes involved in FA oxidation (acyl-CoA oxidase (ACO) and carnitine palmitotransferase (CPT1)). The PCR primers (Table 1) were designed using the Vector NTI (Invitrogen, Carlsbad, CA, USA) and synthesized by Invitrogen. The efficiency was checked from tenfold serial dilutions of cDNA for each primer pair. A 2× SYBR® Green PCR Mastermix (Roche Diagnostics, Mannheim, Germany), 0.83 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). Two candidate reference genes (eF1α and 18s) were tested using *geNorm* VBA applet for

Table 1
Atlantic salmon primer sequences used for real-time PCR.

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	Accession number
C/EBPα	AGACCTCGGCGAGATTTGT	TGTGGAATAGATCAGCCAGGAA	EU668995
C/EBPβ	CAAACTACATTACCAGGC	GTTATGTGTTGCCAGTTG	EU668996
CREB	CTCCAGCCCAGGCCAACTCC	GGCCAGGCAGGTGAGCTCCT	CA385588
ACO	CCTTCATTGTACCTCTCCGCA	CATTTCAACCTCATCAAAGCCAA	DQ364432
CPT1	GTACCAGCCCCGATGCCTTCAT	TCTCTGTGCGACCCTCTCGGAA	AM230810
ACC	GGATTGCCTGTATCTTGGAC	CTGGACGATACTCTGAGTGTTC	DW573070
PEPCKC	AGGGCATGGACCAGGAACTCC	GGGCTCTCCATCCTGGGATGT	BT072418
PGD	CCAATGAGGCTAAAGGCACCAAGA	CCAGCTTGTCGATGAAGTCATCCA	BT050391
PFK	AATCCATCGGCGTTCTGACAAGC	GCCCGTACAGCAGCATTCATACCTT	BT059256
PK	TGCCTTCATTCAGACGCAGCA	CAGATGATTCCGGTGTTGCGA	BT043851
TKT	TGCCATCTCCGAGAGCAACATC	CCGTGGGAATGGCTCTGAACAT	BT059642
HK	GCTGAAGACCAGAGGCATCTTTGA	GCTGCATACCTCCTTGACGATGAT	AY864082
EF1α	TGCCCCTCCAGGATGTCTAC	CACGGCCCACAGGTACTG	BG933853
18S	TGTGCCGCTAGAGGTGAAATT	GCAAATGCTTTCGCTTTCG	AJ427629

Microsoft Excel [44] and eF1α met the criteria for stability. The specificity of PCR amplification was confirmed by melting curve analysis. Relative quantification of the abundance of transcripts was calculated using the ΔΔCT method and formula $-\Delta\Delta CT = -[(Ct_{\text{target gene}} - Ct_{eF1\alpha})_{\text{treatment}} - (Ct_{\text{target gene}} - Ct_{eF1\alpha})_{\text{control}}$ [45].

2.10. Immunofluorescence

The cellular localizations of GLUT4 and FATP1 were investigated for cells treated with I) growth medium (L-15, 10% FBS, 2 mM L-glutamine, 10 mM HEPES, 20 μg/ml insulin and mixture of antibiotics (penicillin, streptomycin and amphotericin B), II) FBS-free growth medium supplemented with 20 μM PA, and III) FBS-free growth medium supplemented with 1 mM glucose. After 48 h of incubation, cells that contained PA were washed twice with PBS (containing 1% albumin), while cells that contained growth medium or growth medium plus glucose were washed with regular PBS. All groups were subsequently washed for 5 min with PBST (regular PBS containing 0.1% Tween® 20) and fixed with 2% paraformaldehyde in PBS for 10 min at room temperature. After two washing steps of 5 min each with PBST, Cell Mask™ Orange (WGA A594) (Invitrogen) at a dilution of 1:200 was added in PBS for 10 min to stain the cellular membranes. After two PBST washings, each of duration 5 min, the cells were permeabilized by incubating in PBST with 0.2% Triton X-100 for 5 min. The cells were then rinsed with PBST and blocked for 2 h at room temperature with PBST supplemented with 5% dry milk. FATP1 (MAB3304, R&D Systems, Minneapolis, USA) (1:100) and an anti-okGLUT4 (which was a kind gift from Dr. Josep Planas) (1:300) antibody were applied overnight in PBST with 2% dry milk and 0.01% Triton X-100. Cells were extensively washed the next day with PBST and incubated for 2 h with DAPI and secondary antibodies (Alexa Fluor® 647 goat anti-mouse IgG_{2b}, and Alexa Fluor® 488 goat anti-rabbit, Invitrogen, California, USA). Cells were then washed five times with PBST and then cover-slipped using Histomount (Fisher). Microscopy was carried out in a Zeiss Axio Observer Z1 (Carl Zeiss Microimaging GmbH, Göttingen, Germany) equipped for total internal reflection fluorescence (TIRF) microscopy. Negative controls were prepared by omitting the primary antibodies.

2.11. Statistical analyses

All data were analysed using the statistical software package SPSS™ (version 17.0, IBM, Chicago, IL, USA). The normality of the data and homogeneity of the variance were tested to ensure that the assumptions required for analysis of variance (ANOVA) were satisfied. These conditions were tested using the Shapiro–Wilk and Levene tests, respectively. If the variances were normally distributed, the Tukey test for multiple comparisons of means was used. Differences were considered significant at a level of 95% ($p < 0.05$). If only two means were compared, Student's t-test was applied. When variances were not normally distributed, a logarithmically transformation was performed and the Tukey test applied after this. When logarithmic transformed variances were not normally distributed, the Kruskall–Wallis non-parametric test was applied to the non-transformed data.

3. Results

3.1. Influence of glucose on differentiation degree and lipid accumulation in adipocytes

Atlantic salmon adipocytes at confluence were incubated with growth medium alone (control), growth medium supplemented with non-radiolabeled glucose (1 mM or 5.5 mM), and growth medium supplemented with non-radiolabeled PA (40 or 80 μM), to study whether the degrees of differentiation and of lipid deposition are influenced by the different substrates. The intracellular lipid level was determined by oil red O staining of lipid droplets. The lipid level in cells treated with glucose was compared to the lipid level in cells cultivated with either no glucose or with PA (Fig. 1).

The amount of lipids in the cytoplasm was significantly higher in adipocytes cultured with glucose than it was in the control cells. However, the intracellular lipid level in the glucose group was much lower than it was in cells cultured with the PA in the medium (Fig. 1).

The levels of two transport protein markers that increase during the late stages of differentiation were measured by immunofluorescence staining, to further examine how glucose and PA influence the differentiation (maturation degree) of adipocytes [46]. FATP1 and GLUT4 are involved in the uptake and intracellular transport of FAs and glucose in mature Atlantic salmon adipocytes, respectively. FATP1 was located

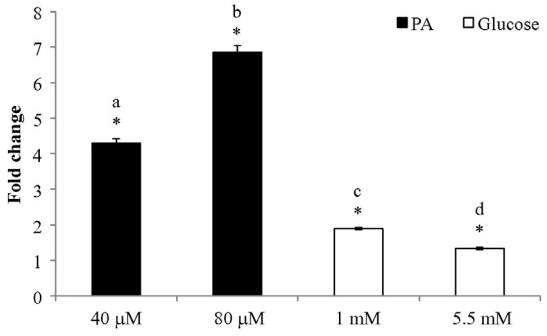

Fig. 1. Lipid content in adipocytes stained with oil red O. Cells treated with non-radiolabeled PA (40 and 80 μM) or non-radiolabeled glucose (1 and 5.5 mM) were fixed and stained on Day 13 after seeding. The absorbance of the extracted dye was measured at 500 nm. Values are expressed as means ± SEM (n = 4). Treatment bars are relative to a control whose value has been set to 1 (not shown). Values that do not share a common letter are significantly different ($p < 0.05$, ANOVA followed by Tukey's test). An asterisk (*) indicates significant differences between control and treated adipocytes ($p < 0.05$, t-test).

throughout the cell in all the conditions studied (Fig. 2). The fluorescence signal of this transporter in the cells supplemented with glucose and PA were, however, slightly larger than in control cells, with the greater rise occurring in cells treated with PA (Fig. 2B). GLUT4 was also expressed throughout the cell in all groups, with higher expression in the PA and glucose groups. GLUT4 tended to accumulate at the plasma membrane in the latter group (Fig. 2C). The high cellular uptake of glucose (Table 2) might be facilitated by the high expression of the GLUT4 protein found in the cells.

The transcript abundance of genes encoding markers of differentiation and fatty acid oxidation was analysed. Cellular mRNA levels of the late differentiation marker C/EBPα increased in cells supplemented with PA or glucose, while cell mRNA transcript levels of the early differentiation markers C/EBPβ and CREB decreased (Fig. 3). The mRNA levels of CPT1 and ACO were lower in the PA and glucose groups than in the control group (Fig. 4).

3.2. Metabolism of radiolabelled glucose and PA

Mature Atlantic salmon adipocytes were incubated with either 20 μM of [1-^{14}C] PA or 1 mM of [1-^{14}C] glucose for 48 h at 13 °C in order to study their capacities for the uptake and metabolism of these two substrates. Table 2 shows the total uptake and the relative distributions of radioactivity from PA and glucose recovered in cellular lipids, water-soluble (ASPs + CO_2) products, and non-metabolized glucose. The total cellular radioactivity recovered for both groups was set to 100%, and the relative contents of different lipid and water-soluble products were calculated. Most of the intracellular radioactivity from the PA was present in cellular lipids (81.31%), while only a minor part from the glucose was (0.079%). Almost 18% of cellular radioactivity from PA and 39% of cellular radioactivity from glucose was present in ASPs, and significantly less CO_2 was produced from glucose (0.03%) than from PA (0.79%). The effect was particularly marked, since glucose is taken up into cells to a higher degree. Approximately 61% of the radioactivity was recovered in un-metabolized glucose.

The ^{14}C-labelled ASP fractions were separated by HPLC in order to determine the amounts of β-oxidation and Krebs-cycle intermediates (Fig. 5). Acetate was the main product from PA, accounting for 35% of the total radioactivity in ASPs. Only 16% of total radioactivity was present in acetate when glucose was used as substrate. Oxaloacetate and malate were the major ASPs formed from glucose, with 55% of the total radioactivity present in these species.

3.3. De novo lipogenesis (DNL)

The results presented above show that more lipids are incorporated in cells incubated with glucose as substrate than in cells incubated with medium alone. This does not, however, prove that glucose is converted into lipids by DNL. Glucose can be converted to either glycerol (to form the TAG backbone of the lipids) or to fatty acids, and further experiments with a radiolabelled tracer are necessary to determine which exact pathway is followed.

Fig. 6A shows the total amounts of radioactivity from [1-^{14}C] PA and from [1-^{14}C] glucose substrates recovered in polar lipids (PLs) and neutral lipids (MAGs + DAGs + TAGs) in mature adipocytes. 3.72 nmol of radioactivity from PA was incorporated in the NLs while a minor part, 0.79 nmol, was present in the PLs. Radioactivity from glucose was present only in neutral lipids. We determined also the composition of the neutral lipids that were formed with the glucose substrate. Fig. 6B shows that most of the radioactivity from glucose was present in the water phase as radiolabelled glycerol after hydrolysis. A minor part in the organic phase was however found as radiolabelled fatty acids, showing that salmon adipocytes are capable of DNL production of fatty acids from glucose.

The main products from DNL in mammals are PA and the desaturated products 16:1 n − 7 and 18:1 n − 9. We determined the identities of the fatty acids formed from glucose by silver-plate TLC separation of the radiolabelled fatty acids. Fig. 7 shows the composition of radiolabelled FAs. Identification of radiolabelled 16:0, 16:1 n − 7 and 18:1 n − 9 is the first proof that *de novo* lipogenesis takes place in fish adipocytes. Most of the radiolabelled PA, not only that produced from glucose but also that added directly to the adipocytes in the form of PA, was converted to the MUFAs 16:1 n − 7 and 18:1 n − 9 presumably by SCD activity.

3.4. Relative transcript abundance of lipogenic genes

Lipogenesis involves fatty acid synthesis followed by triglyceride synthesis. Acetyl-CoA carboxylase (ACC) catalyses the formation of malonyl-CoA, which is an essential substrate for fatty acid synthesis in lipogenic tissues. The abundance of ACC transcripts was higher in the control group and in the glucose group than it was in the PA group. PGD (Fig. 8), an enzyme from the oxidative branch of the pentose phosphate pathway (PPP), was up-regulated by the presence of PA and glucose in the medium compared to the control group. PGD is involved in the production of NADPH that is required for TAG biosynthesis. TKT, an enzyme from the non-oxidative branch of the PPP that provides building blocks for nucleotides and nucleic acids required during cell proliferation, was down-regulated by the two administered substrates.

3.5. Relative transcript abundance of carbohydrate metabolism genes

The transcript abundance of the glycolytic enzymes PFK and PK decreased in the cells to which PA had been added (Fig. 8). Cellular mRNA levels encoding PFK were effectively reduced by glucose, while the abundance of PK transcripts was unaltered. The mRNA level of HK (Fig. 8) was significantly higher in adipocytes that had received PA than it was in adipocytes that had received glucose. PEPCK-C, in contrast, increased its transcript abundance in the PA group (Fig. 8), in which the production of CO_2 was higher (Table 2). This is compatible with the higher amounts of oxaloacetate and malate produced by glucose-treated cells than the amounts produced by cells treated with PA.

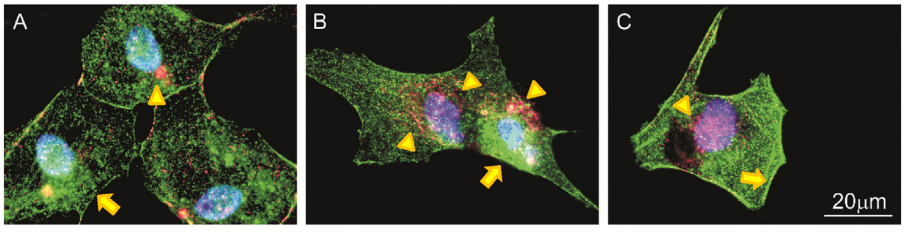

Fig. 2. Immunolocalization of GLUT4 and FATP1 in mature adipocytes. Cells at Day 13 were treated with A) growth medium (L-15, 10% FBS, 2 mM L-glutamine, 10 mM HEPES, 20 μg/ml insulin and mixture of antibiotics (penicillin, streptomycin and amphotericin B), B) FBS-free growth medium supplemented with 20 μM PA, and C) FBS-free growth medium supplemented with 1 mM glucose. After 48 h of incubation, cells were fixed and probed with primary GLUT4 antibody (1:300) shown in green, primary FATP1 antibody (1:100) shown in red, and a secondary antibody, Alexa Fluor 488-conjugated goat anti-rabbit (1:200) and Alexa Fluor 647-conjugated goat anti-rabbit (1:200). Cell nuclei were counterstained with DAPI (blue). Control cells were incubated with blocking buffer alone. Arrows point to GLUT4 and arrowheads to FATP1.

Table 2
Relative distribution of radioactivity from [1-^{14}C 16:0] and [1-^{14}C glucose] recovered in cellular lipids, oxidized products (ASPs and CO_2) and glucose in Atlantic salmon mature adipocytes at Day 13 incubated for 48 h with the radiolabelled substrates. The amount of cellular lipids + oxidized lipids was set to be 100%, which was the total amount (nmol) of radioactive compounds recovered in cells treated with PA. Further, the amount of cellular lipids + oxidized lipids + glucose was set to be 100%, which was the total amount (nmol) of radioactive compounds recovered in cells treated with glucose.

(%)	Cellular lipids	ASP	CO_2	Glucose	Cellular up-take (nmol)
1-^{14}C PA	81.31 ± 1.48 [a]	17.90 ± 1.44 [b]	0.79 ± 0.24 [c]		5.52 ± 0.79
1-^{14}C Glucose	0.079 ± 0.01[a]	39.04 ± 0.62 [b]	0.03 ± 0.004 [c]	60.85 ± 0.63[d]	774.15 ± 9.37

Data are shown as mean ± SEM (n = 6). Different letters indicate significant differences between the products produced from each substrate ($p < 0.05$, Student's t-test). The recovery values were 87.37 ± 9.65% and 85.56 ± 0.77% of the initial radiolabelled PA and radiolabelled glucose respectively.

4. Discussion

We have shown for the first time that mature Atlantic salmon adipocytes can synthesize fatty acids *de novo* from the non-lipid precursor glucose. We have also shown that such synthesis results in an increase in TAG production in adipocytes. The capacity is, however, much lower than that of human adipocytes, in which about 20% of the palmitic acid present in adipocyte TAGs is produced by DNL [47]. Only 5.7% of the neutral lipid fraction derived from radiolabeled glucose was found as radiolabelled fatty acids in the study presented here, and oil red O staining of the cellular lipids showed that the level was much lower than in cells supplemented with the fatty acid PA. However, glucose was able to promote TAG deposition, as shown by a significant increase in oil red O staining compared to the level in control cells. The fact that the activity of this pathway was modest may contribute to the previously reported glucose intolerance in fish. Nevertheless, it must be remembered that other substrates, such as certain amino acids, may contribute carbon atoms to DNL.

The relevance of *de novo* synthesis of lipids from glucose in fish liver has been previously suggested as a mechanism to control glucose homeostasis. Several studies in rainbow trout using fish selected for high fat muscle content have reported a link between a better ability to control hyperglycaemia induced by dietary carbohydrates and an increase in hepatic lipogenesis [23,24]. An enhanced hepatic lipogenesis was also observed in rainbow trout receiving the anti-diabetic drug metformin, which effectively reduced postprandial glycaemia [25,26]. Even though most of the earlier studies of mechanisms regulating glucose homeostasis in fish focus on the liver, the possible implication of adipose tissue in the control of glycaemia has been reported [27] and the lipogenic and glucose uptake potential of this tissue were increased in metformin-infused trout [26]. Our study, irrespective of tissue, is however the first proof that fish are capable of converting glucose to fatty acids by DNL. However, in order to be able to fully understand the role of adipocyte DNL in regulation of whole body glucose homeostasis, future studies are required where the DNL capacities in liver and adipocytes are compared.

The purpose of the DNL pathway in mammals is believed to be to convert carbohydrates into lipids, providing in this way an efficient storage mechanism [18]. The radioactive assay in our study revealed that 16 times more nmols of glycerol were produced from glucose compare to the amount of fatty acids formed. It is thus possible that the mechanism by which glucose promotes adipocyte differentiation in Atlantic salmon is by providing the backbones on which to synthesize TAGs. We cannot, however, rule out the effect of the DNL products on the degree of cell differentiation, even though these products are produced at such a low level. Todorčević et al. [28] reported up-regulation of several genes involved in glyceroneogenesis, PPP pathway and glycolysis during terminal differentiation of Atlantic salmon adipocytes, suggesting the importance of carbohydrate metabolism in fish adipocytes. The glyceroneogenesis pathway is an additional way to provide the cells with the glycerol they need for the synthesis of TAGs. This metabolic pathway regulates the rate of fatty acid re-esterification in adipose tissue, where PEPCK-C is the key enzyme involved in the process [48,49]. The transcription of this gene is regulated by diet and hormones in mammals [50]. Our results showed that the mRNA levels of this gene increased in adipocytes incubated with PA, while they remained equal to the control when the cells were incubated with glucose. We suggest that the contribution of glyceroneogenesis to the synthesis of glycerol

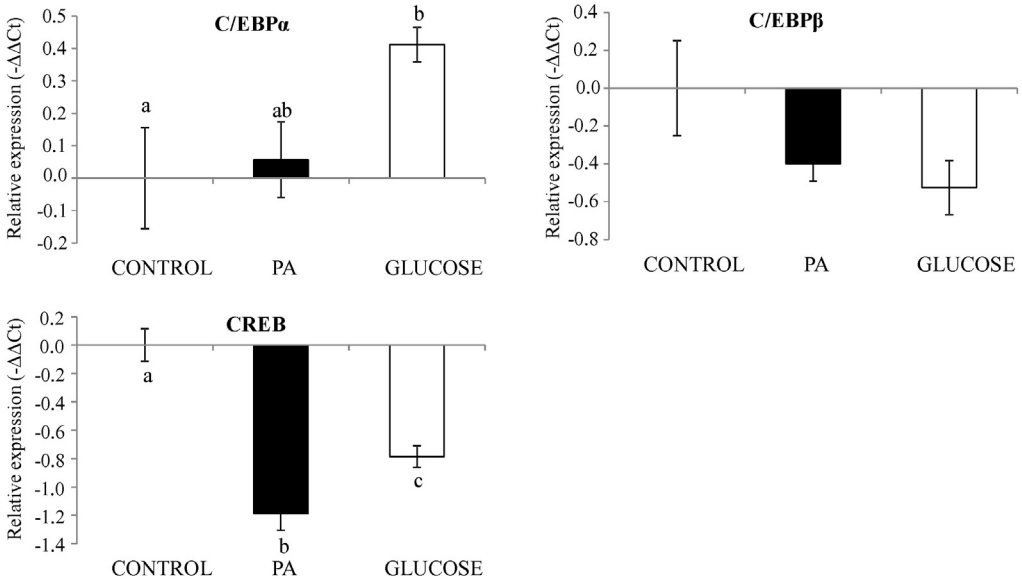

Fig. 3. Relative changes in transcript levels of adipogenic factors. Samples (n = 4–6) are analysed with real-time qPCR; data are presented as –ΔΔCt ± SEM and control was set to zero. Different letters indicate significant differences between treatments ($p < 0.05$, ANOVA followed by Tukey's test). Control = Day 13 adipocytes cultivated in growth medium (L-15, 10% FBS, 2 mM L-glutamine, 10 mM HEPES, 20 μg/ml insulin, 1 μl/ml lipid mixture and mixture of antibiotics (penicillin, streptomycin and amphotericin B)). All treatment groups were cultivated in the same medium as the control cells, with the addition of either PA (20 μM) or glucose (1 mM) for 48 h.

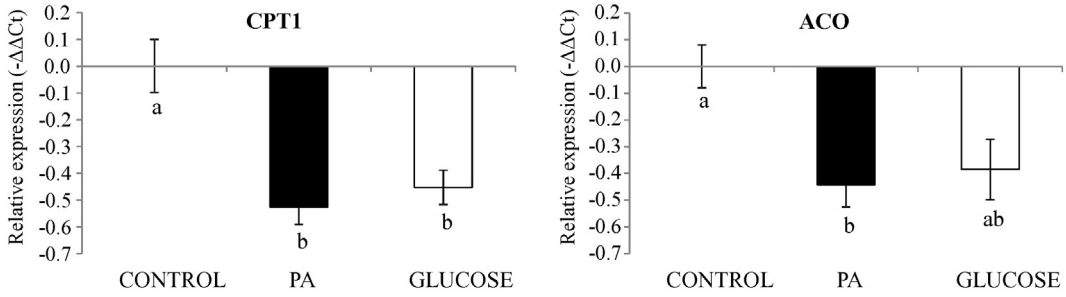

Fig. 4. Relative changes in transcript levels of genes encoding enzymes involved in fatty acid oxidation. Samples (n = 4–6) are analysed with real-time qPCR; data are presented as −ΔΔCt ± SEM and control was set to zero. Different letters indicate significant differences between treatments ($p < 0.05$, ANOVA followed by Tukey's test). Control = Day 13 adipocytes cultivated in growth medium (L-15, 10% FBS, 2 mM L-glutamine, 10 mM HEPES, 20 μg/ml insulin, 1 μl/ml lipid mixture and mixture of antibiotics (penicillin, streptomycin and amphotericin B)). All treatment groups were cultivated in the same medium as the control cells, with the addition of either PA (20 μM) or glucose (1 mM) for 48 h.

in adipocytes of Atlantic salmon is stimulated by the addition of the fatty acid, but not by the addition of glucose. This agrees with reported findings from mammalian adipose tissue, where glucose, together with glucocorticoids, decreases the transcription rate of PEPCK-C [51–53]. Consistent with this, a marked increase in PEPCK-C and glyceroneogenesis occur in adipose tissue when animals are fed a diet devoid of carbohydrates [54,55]. In addition, experiments performed in 3T3-F442A have confirmed that unsaturated fatty acids strongly induce PEPCK-C, although the exact mechanism remains unclear [56]. The high desaturase activity present in the adipocytes treated with PA, and the consequent production of unsaturated fatty acids, indicate that the transcription of PEPCK-C in fish adipose tissue is regulated in the same way as it is in mammals.

TKT, which is the key regulatory enzyme in the non-oxidative branch of the PPP, also serves as a reversible link between this pathway and glycolysis. Decrease in abundance of TKT transcripts by two treatments suggests a need to finely regulate output of the PPP and related pathways during increases in lipogenic and adipogenic pathways, namely the increase in NADPH production by the oxidative PPP branch, and likely a decrease in the amount of glycerol-3-phosphate (G3P) that can enter glycolysis. Furthermore, down-regulation of phosphofructokinase (PFK), which is the most important regulatory glycolytic enzyme, is compatible with the increased activation of PPP at the expense of glycolysis. These results indicate that the mechanism by which glucose stimulates lipid biosynthesis is not primarily DNL to produce fatty acids. The pathway instead delivers glycerol for the carbon backbones and leads to increased availability of cytosolic reducing equivalents. The higher abundance of transcripts encoding PGD, an enzyme from the oxidative branch of the PPP that mediates the production of NADPH to provide reducing power for lipid synthesis, supports this interpretation. The complete PPP pathway can thus be seen as an alternative to glycolysis, since it not only generates the pentose sugar

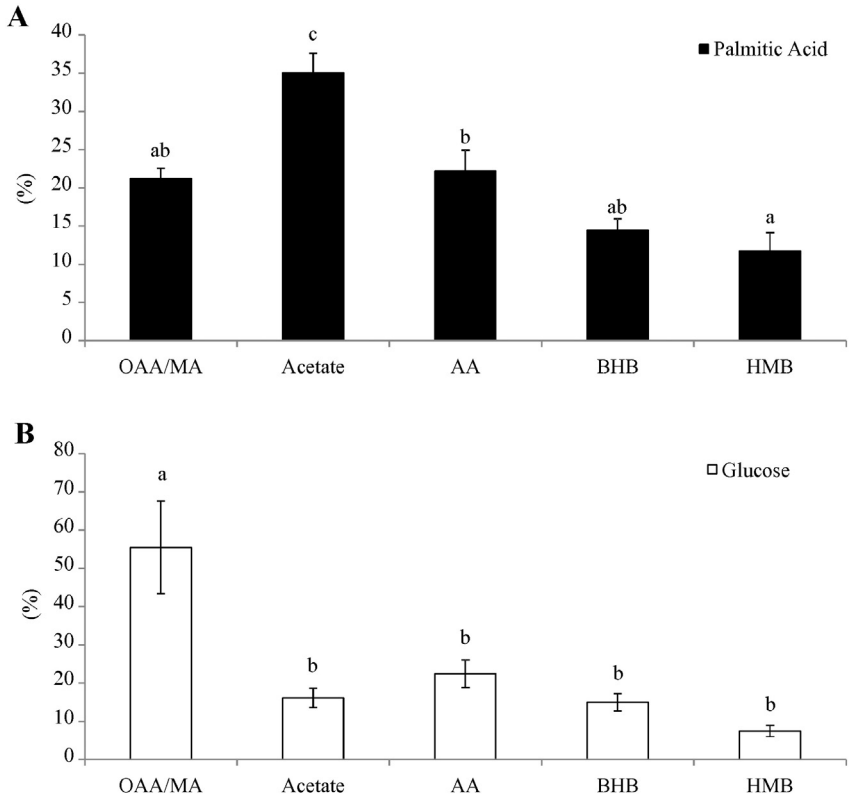

Fig. 5. Percentage distribution between acid-soluble oxidation products produced from [1-^{14}C PA] (A) and [1-^{14}C glucose] (B) in Atlantic salmon mature Day 13 adipocytes incubated for 48 h with the radiolabelled substrates. Total radioactivity recovered as ASPs for each substrate = 100% (oxaloacetate/malate + acetate + acetoacetate + β-hydroxybutyrate + β-hydroxy-β-methylglutarate acid). Data are shown as mean ± SEM (n = 5–6). Different letters indicate significant differences within the treatment group ($p < 0.05$, ANOVA followed by Tukey's test). OAA/MA = oxaloacetate/malate; AA = acetoacetate; BHB = β-hydroxybutyrate; HMB = β-hydroxy-β-methylglutarate acid.

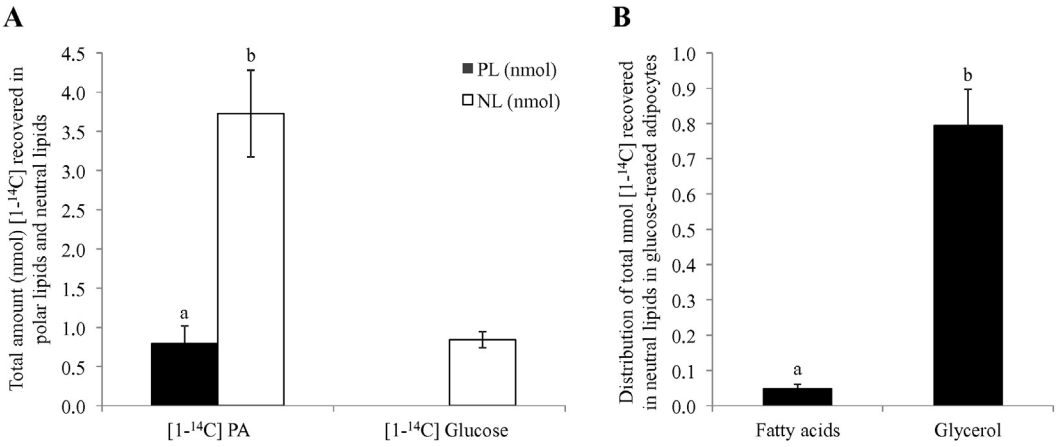

Fig. 6. A) Total amounts (nmol) of [1-^{14}C PA] and [1-^{14}C glucose] in polar lipids (PLs) and in neutral lipids (NLs) in Atlantic salmon adipocytes at Day 13 incubated for 48 h with the radiolabelled substrates. Total nmol radiolabelled products recovered in the cells from each substrate is set to 100% and the % of the total nmol recovered in PL and NL were calculated. Data are shown as mean ± SEM (n = 6 for the PA treatment and n = 3 for the glucose treatment). Different letters indicate significant differences ($p < 0.05$; Student's t-test). B) Total neutral lipid distribution in cells at Day 13 treated with glucose for 48 h (n = 3). Different letters indicate significant differences ($p < 0.05$; Student's t-test).

precursors that are needed for nucleic acid synthesis and the NADPH that is required for the accumulation of lipids, it also provides cells with G3P, the precursor of the glycerol backbone, for TAG synthesis.

SCD activity was higher in the group that received PA, but it was elevated also in the cells that received glucose. As in human adipocytes [39,57], the elongation and desaturation pathways are closely related and coordinated to DNL, in order to avoid any potential toxic properties of the saturated fatty acids. One of the main DNL-derived fatty acids after desaturase activity is 16:1 n−7. This fatty acid makes rodents more sensitive to insulin and has anti-inflammatory effects [22,58]. It has been classified as a lipokine for this reason. Further work should investigate whether this product has similar implications for the metabolic health of fish.

We have incubated differentiated adipocytes with PA and glucose for 48 h in order to study their respective capacities for being metabolized by these cells. As expected, most of the PA substrate was incorporated into cellular lipids, while most of the glucose substrate ended up in Krebs-cycle intermediates, with only a minor part being transformed into cellular lipids. Glucose is converted to ASPs to a high degree in rainbow trout myotubes, where 99.8% of the total radioactivity recovered from glucose was found in this fraction after 48 h of incubation [59]. Oxaloacetate and malate were the major ASPs in adipocytes stimulated with glucose, while in cells incubated with PA acetate was the main oxidation product. This is compatible with a mechanism in which PEPCK-C is induced in the adipocytes cultivated in the presence of PA, since this enzyme is the junction between glycolysis and the Krebs cycle, using oxaloacetate as substrate. Mammalian carnivorous species that

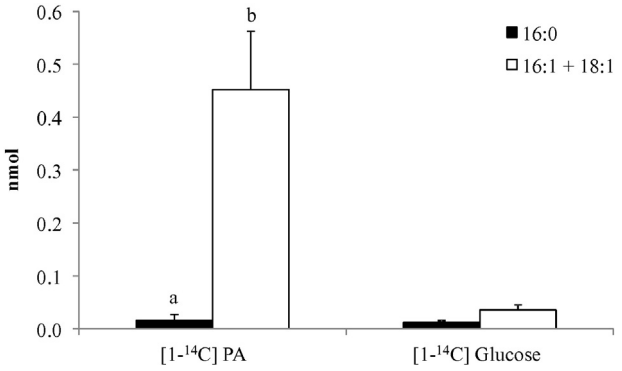

Fig. 7. Total 16:0 and 16:1 + 18:1 produced from [1-^{14}C PA] and [1-^{14}C glucose] in Atlantic salmon mature adipocytes at Day 13 incubated for 48 h with the radiolabelled substrates. Data are shown as mean ± SEM (n = 6 for the PA treatment and n = 3 for the glucose treatment). Different letters indicate significant differences ($p < 0.05$; Student's t-test).

experience difficulty in metabolizing glucose, such as cats, use acetate as a principal precursor of DNL [60]. The contributions of different substrates to fish DNL should be investigated in more depth.

Transcript abundance of genes related to both peroxisomal (ACO) and mitochondrial β-oxidation (CPT-1) decreased regardless of the treatment. Little information regarding lipid oxidation in fish white adipose tissue is available. Studies of CPT-1 regulation in rainbow trout adipose tissue in relation to insulin have shown that the hormone plays an anabolic role in this tissue, and in this way down-regulates the oxidation of FFAs [27]. Our results are compatible with observations by Todorčević et al. [61], in which the capacity for β-oxidation decreased as the cells matured and specialized towards lipid storage. Likewise, and supporting the higher maturation capacity of the adipocytes treated with PA or glucose, terminal differentiation was accelerated by both treatments, as shown by the opposite regulation of the C/EBP transcription factors. The CREB binding protein, which is known to induce key transcription factors, such as C/EBPα and PPARγ [62], was also significantly affected by the treatments. The late adipogenic marker C/EBPα was significantly up-regulated by glucose treatment, while the mRNA abundance of early adipogenic factors C/EBPβ and CREB decreased with both treatments. This is in line with faster maturation occurring in treated cells, and especially by addition of glucose.

The involvement of GLUT4 and FATP1 in the uptake and intracellular transport of glucose and fatty acids respectively, has been previously reported in salmonids [63–65]. Immunofluorescence antibody staining against GLUT4 revealed that this transporter is expressed at a high level in the membrane of insulin-stimulated salmon adipocytes, especially in presence of glucose. FATP1, in contrast, was most prominently expressed intracellularly, and was expressed at a particularly high level after PA treatment. GLUT4 can maintain its main mammalian functions, even though it has a lower affinity for glucose [66]. The translocation of this transporter after insulin stimulation has been demonstrated in trout skeletal muscle cells [65], and an increase in glucose uptake upon insulin stimulation in trout adipocytes has been reported and suggested to be mediated by GLUT4 [67], which is in agreement with our results. On the other hand, many proteins are involved in the cellular uptake of free fatty acids in mammalian models, the most studied and most controversial family being the FATP proteins. Previous studies have suggested that the activity of FATP1 is regulated in a similar manner as glucose uptake is regulated by glucose transporters [68]. However, recent findings have questioned this idea, and suggest other methods of action [69,70]. FATP1 is located in the endoplasmic reticulum, and translocation is not needed to enhance the basal or insulin-mediated fatty acid uptake in 3T3-L1 adipocytes [71]. It is possible that this is the case also for Atlantic salmon adipocytes, since the fatty acid

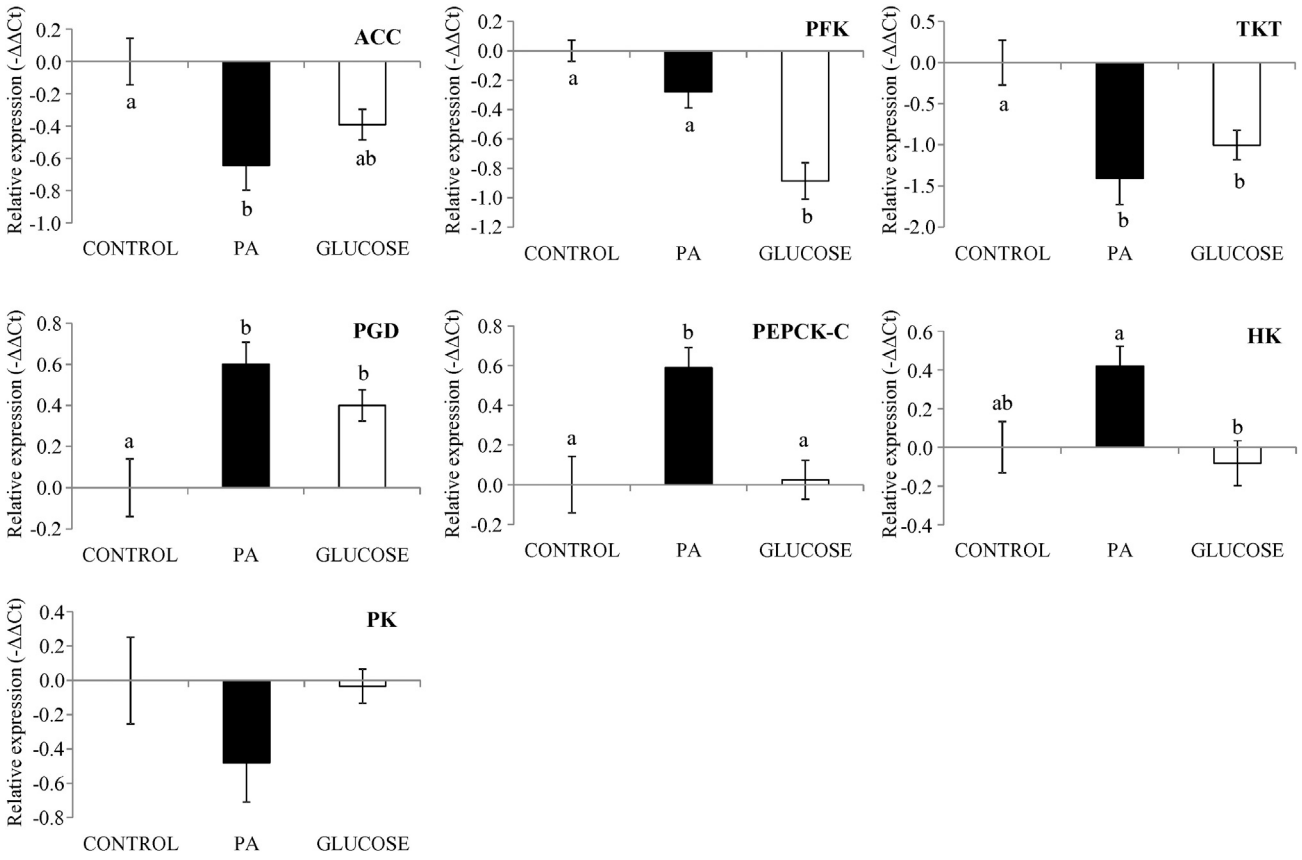

Fig. 8. Relative changes in transcript levels of genes encoding enzymes involved in carbohydrate and lipid metabolism. Samples (n = 4–6) are analysed with real-time qPCR; data are presented as −ΔΔCt ± SEM and control was set to zero. Different letters indicate significant differences between treatments ($p < 0.05$, ANOVA followed by Tukey's test). Control = Day 13 adipocytes cultivated in growth medium (L-15, 10% FBS, 2 mM L-glutamine, 10 mM HEPES, 20 μg/ml insulin, 1 μl/ml lipid mixture and a mixture of antibiotics (penicillin, streptomycin and amphotericin B)). All treatment groups were cultivated in the same medium as the control cells, with addition of either PA (20 μM) or glucose (1 mM) for 48 h.

transporter was located intracellularly in the endoplasmic area, with no translocation, in all treatments studied. However, it should be noted that we investigated expression and localisation of the two transporters 48 h after substrate supplementation. Further time-series experiments are thus necessary to better understand the mechanisms of their action after stimulation in salmon adipocytes.

We have shown that some of the main enzymes responsible for *de novo* lipid synthesis are expressed in salmon adipocytes. We have shown also that DNL can produce cellular lipids from glucose, which suggests that DNL is a functional pathway in this cell type. Salmon adipocytes have only a moderate capacity to convert glucose into fat, but it is possible that substrates other than glucose, such as pyruvate, glutamine or other amino acids, can act as precursors for DNL, as is the case in humans [35]. This relatively low capacity to convert glucose into fat may contribute to the glucose intolerance in carnivorous fish species. However, glucose was found to promote both lipogenic and adipogenic processes to a certain extent, likely acting as a substrate in the generation of molecules required in these processes, as revealed by the transcriptional regulation of key factors with various roles in adipogenic differentiation and carbohydrate and lipid metabolism. The pathways of fatty acid synthesis in fish adipocytes are, however, complex, and the mechanisms involved in lipid and glucose homeostasis and metabolism in fish remain unclear. Further studies of these processes will help to understand the dysregulation of fat balance and the impaired glucose homeostasis that characterize fish with excess visceral fat accumulation.

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Article III

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Interplay of adiponectin, TNFα and insulin on gene expression, glucose uptake and PPARγ, AKT and TOR pathways in rainbow trout cultured adipocytes

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ABSTRACT

Adipose tissue is being increasingly recognized as an important endocrine organ that produces and releases a variety of factors. In the present study we have evaluated in primary cultures of rainbow trout adipocytes, obtained from visceral adipose tissue, the interplay of the adiponectin system, TNFα and insulin at a transcriptional level and, their effects on the adipogenic transcription factor PPARγ, as well as on the activation of main insulin signaling pathways. Likewise, the implication of these adipokines in the regulation of glucose uptake in the adipocyte and their interactions with insulin or IGF-I were also evaluated. Similarly to the mammalian model, insulin enhanced adiponectin gene expression, while it exerted a negative modulation on adiponectin receptors. TNFα increased the mRNA levels of adiponectin receptor 1, but neither adiponectin nor TNFα modulated each other expression. Therefore, the reciprocal suppressive effect of both adipokines previously reported in mammals was not present in this model. Furthermore, the anti-adipogenic effect of TNFα was revealed by the down-regulation of PPARγ at a protein level, meanwhile adiponectin increased PPARγ expression in insulin-stimulated adipocytes, supporting its insulin-sensitizing role. Both adipokines stimulated glucose uptake without modifying AKT or TOR phosphorylation; however, glucose uptake in insulin-treated adipocytes was enhanced by TNFα but not by adiponectin. All in all, these results contribute to gain knowledge on the role of adipokines in rainbow trout adipose tissue and, to better understand the mechanisms that regulate glucose metabolism in this species.

1. Introduction

White adipose tissue is an organ highly specialized in storing and releasing lipids in response to a variety of signals controlling energy balance. Besides, it acts as an endocrine organ (Harwood Jr, 2012) secreting a variety of adipokines that regulate different physiological processes such as appetite, glucose metabolism, immunity, inflammatory responses, angiogenesis, blood pressure and fertility (Ali et al., 2013).

Adiponectin, also known as adipocyte complement-related protein of 30 kDa (ACRP30) (Scherer et al., 1995) or AdipoQ (Hu et al., 1996), is one of the main proteins produced by mammalian adipocytes, making adipose tissue the principal contributor of its circulating levels. This anti-inflammatory adipokine exists as a full-length or as a smaller globular domain in the circulation (Guerre-Millo, 2008). Adiponectin exerts its actions through binding two membrane receptors; adiponectin receptor 1 (adipoR1) and adiponectin receptor 2 (adipoR2). They are known to regulate carbohydrate and lipid metabolism by mediating AMP-activated protein kinase (AMPK) and phosphatidylinositol 3-kinase (PI3K)/AKT pathways and, peroxisome proliferator-activated receptors (PPARs) activity (Yamauchi et al., 2007). Even though the specific biological role of adiponectin has not been fully elucidated, its plasma levels have been negatively correlated to human obesity

Abbreviations: adipoR1, adiponectin receptor 1; adipoR2, adiponectin receptor 2; AMPK, AMP-activated protein kinase; GLUT4, glucose transporter 4; IGF-I, insulin-like growth factor I; PI3K, phosphatidylinositol 3-kinase; PPARγ, peroxisome proliferator-activated receptor γ; p38MAPK, p38 mitogen activated protein kinase; TNFα, tumor necrosis factor α; TOR, target of rapamycin.

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(Arita, 2012), insulin resistance (Yatagai et al., 2003; Yamamoto et al., 2004), metabolic syndrome (Matsushita et al., 2006) and cardiovascular diseases (Im et al., 2006). In 2001, three independent research groups demonstrated for the first time an insulin-sensitizing effect of adiponectin in mice (Berg et al., 2001; Fruebis et al., 2001; Yamauchi et al., 2001). Moreover, some studies have revealed that adiponectin also controls adipocyte differentiation and metabolism (Yan et al., 2013; Lee and Shao, 2013).

Despite the well-known implications of adiponectin in modulating insulin sensitivity, glucose homeostasis and lipid metabolism in mammals, scarce information is available concerning this adipokine in fish. Adiponectin and adipoRs genes were characterized in zebrafish, and the relationship between this adipokine and appetite regulation was confirmed in this species (Nishio et al., 2008). Adiponectin was also identified in rainbow trout (Kondo et al., 2011) and another study in this species concerning the response of the adiponectin and adipoRs system to diverse challenges in several tissues and cell cultures was recently published (Sánchez-Gurmaches et al., 2012). The same authors provided that the adiponectin system is regulated by hormones such as insulin, as well as by feeding restriction. Furthermore, adiponectin activated the PI3K/AKT pathway in rainbow trout myocytes, pointing out to the conservation of some of the signaling pathways of adiponectin among vertebrates.

Adipocytes are also producers of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNFα). Contrary to what has been described for adiponectin, TNFα production is enhanced in obese and/or diabetic mammals (Hotamisligil et al., 1993; Nieto-Vazquez et al., 2008). Moreover, white adipose tissue from obese mice and obese patients has been reported to be highly infiltrated by macrophages, which may be an additional source of locally-produced TNFα (Weisberg et al., 2003). Among the numerous effects of this cytokine in adipose tissue in mammals, the induction of lipolysis and the inhibition of adipogenesis and insulin signaling have been well documented (Gao et al., 2006; Tang et al., 2010). TNFα plays an important role in the development of insulin resistance through the reduction of insulin-induced glucose transport, although it can stimulate at the same time basal glucose uptake (Stephens et al., 1997) and, by inhibiting the function of the transcriptional regulator PPARγ (Ye, 2008). In fish, the involvement of TNFα in the regulation of lipid metabolism has also been addressed. The lipolytic effect of this cytokine has been proved in freshly isolated adipocytes from rainbow trout (Albalat et al., 2005) and gilthead sea bream (Saera-Vila et al., 2007; Cruz-Garcia et al., 2009), as well as the capacity of TNFα to inhibit preadipocyte differentiation in rainbow trout (Bouraoui et al., 2008).

Interactions between TNFα and adiponectin have been reported, since adiponectin is known to suppress the release of TNFα from human adipocytes and stromal-vascular cells (Dietze-Schroeder et al., 2005). On the other hand, TNFα significantly decreased adiponectin mRNA levels in human preadipocytes (Kappes and Löffler, 2000) as well as in 3T3-L1 adipocytes (Fasshauer et al., 2002). In rainbow trout, TNFα injections failed to regulate the expression of the adiponectin system in adipose tissue, meanwhile the stimulation of mature isolated adipocytes with this cytokine provoked a down-regulation of adipoR2 (Sánchez-Gurmaches et al., 2012). However, the effects of TNFα on the adiponectin system remain largely unknown in fish in *in vitro* cultured adipocytes.

The general aim of this study was to gain insight into the interaction between adipokines and insulin using *in vitro* differentiated adipocytes, obtained from visceral adipose tissue of rainbow trout. The first goal of this study was to evaluate how the adiponectin system, TNFα and insulin are mutually regulated at a transcriptional level. Second, the effects of these peptides on 2-deoxyglucose (2-DG) uptake were analyzed. Finally, we studied whether the interplay of these adipokines affects the transcription factor PPARγ and the characteristic metabolic insulin signaling pathways. All in all, these results contribute to increase our understanding of the complex role of adipokines in fish.

2. Materials and methods

2.1. Animals

Rainbow trout (*Oncorhynchus mykiss* Walbaum 1792) were obtained from the "Viveros de los Pirineos" fish farm (Huesca, Spain). Fish were acclimated to a 12 h light:12 h dark photoperiod and to a temperature of 14 ± 1 °C in closed circuit flow systems located in the Faculty of Biology at the University of Barcelona. Fish were fed daily *ad libitum* on a commercial diet based on fishmeal and fish oil (Dibaq Diproteg, Segovia, Spain). All animal handling procedures were approved by the Ethics and Animal Care Committee of the University of Barcelona, following the regulations and procedures established by the EU, and by the Spanish and Catalan governments (permits reference numbers CEEA 237/12 and DAAM 6755).

2.2. Primary culture of preadipocyte cells

All reagents were purchased from Sigma–Aldrich (Tres Cantos, Spain) and all tissue culture plastic ware was obtained from NUNC (Labclinics, Barcelona, Spain) unless stated otherwise.

Fish were fasted 24 h before the experiments. The animals were killed by a blow to the head, which was followed by immersion in 70% ethanol for 30 s to sterilize the external surfaces. Cells for each experiment were isolated from a pool of white adipose tissue of 6–7 fish and cultured as described elsewhere (Bouraoui et al., 2008). Briefly, the dissected visceral fat tissue was washed with Krebs-HEPES buffer (pH 7.4) and was digested for 1 h with type II collagenase 130 UI/mL containing 1% bovine serum albumin (BSA) in Krebs-HEPES buffer at 18 °C. The resulting cell suspension was filtered (100 μm) and centrifuged at 700*g* for 10 min and the pellet was treated with erythrocyte lysing buffer (0.154 M NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM EDTA) for 5 min at room temperature (RT). After washing, the cells were centrifuged again at 700*g* for 10 min. The cell pellet was resuspended in growth medium, consisting on Leibovitz's L-15 medium (L-15) containing 10% fetal bovine serum (FBS), 2 mM L-glutamine and 1% antibiotic/antimycotic solution. Cells were counted, diluted, seeded in pretreated flasks or six-well plates (25 cm^2 or 9.6 cm^2/well respectively), with 1% gelatin at a density of $2{\cdot}10^4$ cells/cm^2 and kept at 18 °C. For each experimental condition either one flask (Western blot), two wells pooled together (gene expression) or three independent wells (glucose uptake) were used. After confluence (day 7), cells were induced to differentiate by means of incubation with a growth medium supplemented with 10 μg/mL insulin, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 0.25 μM dexamethasone and 10 μL/mL lipid mixture. Media were changed every 2 days during the whole procedure.

2.3. Experimental culture treatments

Adipocytes at day 15 were incubated for 44 h with either 2.5 μg/mL human globular adiponectin (BioVision, San Francisco, CA, USA), 100 ng/mL recombinant human TNFα (Sigma–Aldrich, Tres Cantos, Spain) or left untreated (control cells). The concentrations of peptides were chosen according to previous *in vitro* studies performed in rainbow trout (Bouraoui et al., 2008; Sánchez-Gurmaches et al., 2012). Then, all the cells were serum starved in L-15 medium, containing only 0.5% FBS for 4 h.

After this, the cells were incubated with the same L-15 medium with 0.5% FBS in the presence or absence of 1 μM porcine insulin (Sigma–Aldrich, Tres Cantos, Spain) for 30 min, for the analysis of

the activation of signaling pathways and for the glucose uptake assay, in which 100 nM recombinant human insulin-like growth factor-I (IGF-I) (Bachem, Weil am Rhein, Germany) was also tested.

For the gene expression analyses the same initial experimental protocol was followed; later on, cells were incubated with the same L-15 medium with 0.5% FBS with adiponectin or TNFα in the presence or absence of porcine insulin at 1 μM for 6 h.

Thus, the experimental conditions studied in all cases were: Control, Insulin (INS), TNFα (TNF), TNF + INS, Adiponectin (ADIPO) and ADIPO + INS and; in the glucose uptake study, also IGF, TNF + IGF and ADIPO + IGF.

2.4. RNA isolation and cDNA synthesis

Total RNA from adipocyte cells was extracted using the TriReagent method (Ambion, Alcobendas, Spain) following the manufacturer's recommendations. The quantity of isolated RNA was determined by spectrophotometry with a ND-2000 NanoDrop (Thermo Fisher Scientific, Alcobendas, Spain). The amount of 500 ng of total RNA was reverse-transcribed into cDNA using AffinityScript QPCR cDNA Synthesis Kit (Agilent Technologies, La Jolla, CA, USA) in a 20 μL reaction system according to the instructions provided by the manufacturer. The reaction conditions were: 25 °C for 5 min, 42 °C for 45 min and 95 °C for 5 min. The reverse transcribed products (cDNA) were stored at −20 °C for expression of the target genes.

2.5. Real-time PCR analysis (qPCR)

qPCR measurements were performed in triplicate with one fortieth of the cDNA synthesis reaction in an iCycler iQ real-time detection system (Bio-Rad, El Part de Llobregat, Spain) as described in Sánchez-Gurmaches et al. (2012). The qPCR primer sequences for the target genes (TNFα, adiponectin, adipoR1, adipoR2 and PPARγ) and the reference gene, elongation factor 1 alpha (eF1α) are shown in Table 1. The fluorescence data acquired during the extension phase were normalized to eF1α by the delta–delta method (Livak and Schmittgen, 2001). Five independent cultures ($n = 5$) were performed for gene expression analysis.

2.6. Western blot analysis

Protein extraction and Western blot analysis were performed as previously reported (Sánchez-Gurmaches et al., 2012). Twenty micrograms of protein were subjected to SDS–PAGE gel electrophoresis and Western blot analysis performed using the appropriate antibodies for PPARγ (PPARγ; cat. No. 7196; Santa Cruz Biotechnology) and the phosphorylated forms of AKT (AKT-p; antibody cat. #9271) and TOR (TOR-p; antibody cat. #2971), both from Cell Signaling Technology (Beverly, MA). Membranes were stripped and reblotted with the respective antibodies against the total forms of AKT (AKT-t; #9272 Cell Signaling Technology, Beverly, MA) and TOR (TOR-t; T2949, Sigma–Aldrich, Tres Cantos, Spain). Immunoreactive bands were visualized using an enhanced chemiluminescence kit (Pierce ECL Western blotting Substrate; Thermo Scientific, Alcobendas, Spain) and quantified by densitometric scanning using ImageJ software (National Institutes of Health, Bethesda, MD, USA). These antibodies have been shown to successfully cross-react with rainbow trout (Bouraoui et al., 2008; Seiliez et al., 2010). Western blots results were obtained from four independent cultures normalizing the band intensity of the phosphorylated forms against that from the total forms.

2.7. Glucose uptake assay

Determination of 2-DG uptake in rainbow trout adipocytes was performed as previously described (Bouraoui et al., 2010). Adipocytes treated as described in Section 2.3 were rinsed twice with ice-cold PBS and incubated with HEPES-buffered saline containing 2 μCi/mL labeled 2-DG (2-[1,2-^{3}H]-DG; 5–10 Ci/mmol specific activity; Perkin Elmer, Tres Cantos, Spain) and 50 μM cold 2-DG (transport solution) (D6134, Sigma–Aldrich, Tres Cantos, Spain) for 30 min at 18 °C. The transport solution was then aspirated, and cells were rinsed with ice-cold PBS containing 50 mM glucose to stop uptake. Finally, cells were lysed with 0.5 N NaOH, and radioactivity was quantified with a TRICARB 2100 β-counter (Packard Bioscience, Meriden, CT, USA). An aliquot of the lysate was kept for protein determination following the Bradford method (Bradford, 1976).

Cytochalasin B (20 μM), a well-known inhibitor of facilitative glucose transporters (Capilla et al., 2004), was added and incubated simultaneously with the labeled 2-DG to determine non-specific uptake. Glucose uptake was measured in triplicate wells from three independent cultures, normalized to total protein, and expressed as fold change with respect to unstimulated control cells.

2.8. Statistical analysis

Data are reported as mean values ± standard error of mean (S.E.M.). Results were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's test. A significance level of $P < 0.05$ was applied to all statistical tests.

3. Results

3.1. Effects of TNFα, adiponectin, and insulin in adiponectin system and TNFα gene expression in rainbow trout adipocytes

In the present study, we first analyzed adiponectin system and TNFα gene expression after the different experimental treatments:

Table 1
Rainbow trout primer sequences used for real-time PCR.

Gene	Database	Accession number	Amplicon size (bp)	Annealing T(°C)	Direction	Primer sequence (5′-3′)
Adiponectin	DFCI	TC167753	165	62	F	AGCCCGTCATGTTCACCTAC
					R	GAAGGTGGAGTCGTTGGTGT
adipoR1	DFCI	TC165904	199	60	F	TCCACTCCCACCAGATCTTC
					R	CGTGTTCCAGCAGCACTTTA
adipoR2	DFCI	TC140134	196	60	F	CTGATCATGGGCTCCTTTGT
					R	ACACCACTCAGACCCAGACC
PPARγ	DFCI	CA345564	171	60	F	GACGGCGGGTCAGTACTTTA
					R	ATGCTCTTGGCGAACTCTGT
TNFα	GeneBank	NM_001124374	130	60	F	TCTTACCGCTGACACAGTGC
					R	AGAAGCCTGGCTGTAAACGA
eF1α	GeneBank	AF498320	159	58	F	TCCTCTTGGTCGTTTCGCTG
					R	ACCCGAGGGACATCCTGTG

F, forward primer; R, reverse primer; T, temperature.

TNFα, adiponectin, insulin or their combinations (Fig. 1). Insulin incubation significantly increased the expression of adiponectin in rainbow trout adipocytes ($P = 0.04$) (Fig. 1A). However, the mRNA levels of this adipokine were similar in control cells and in cells incubated with adiponectin or TNFα either alone or in combination with insulin, which indicates an inhibition of the insulin-induced increase in adiponectin mRNA levels by TNFα and by adiponectin. On the other hand, insulin exerted a negative regulation in the mRNA levels of adipoR1 ($P = 0.049$) (Fig. 1B). Despite a lack of significance, treatment with adiponectin alone and in combination with insulin, as well as TNFα plus insulin slightly decreased adipoR1 when compared to the control cells. In contrast, TNFα was shown to act as a positive regulator of adipoR1 gene expression ($P = 0.049$) (Fig. 1B). No changes were observed in the mRNA transcript levels of adipoR2 ($P = 0.234$), even though insulin-treated cells showed a decreasing expression tendency of this receptor (Fig. 1C). Furthermore, none of the treatments showed a significant effect on the regulation of TNFα expression ($P = 0.585$) (Fig. 1D).

3.2. Effects of TNFα, adiponectin, insulin and IGF-I on 2-DG uptake in rainbow trout adipocytes

Regarding glucose metabolism regulation, adiponectin and TNFα treated cells, showed an increase in glucose uptake with respect to untreated control cells ($P = 0.011$ and $P = 0.002$, respectively) (Fig. 2A). Cytochalasin B, a glucose transporter inhibitor, effectively exerted a significant inhibition of glucose uptake below the basal levels ($P < 0.001$). Next, the stimulatory effects of insulin on glucose uptake are presented in Fig. 2B ($P < 0.001$). The combination of this hormone with TNFα significantly increased the glucose uptake capacity in rainbow trout adipocytes ($P < 0.001$). Nevertheless, the stimulatory effect was not significant when insulin was combined with adiponectin. On the other hand, IGF-I alone or combined with either TNFα or adiponectin, caused in all cases a significant increase in glucose transport in these adipocytes ($P = 0.037$) (Fig. 2C).

3.3. Effects of TNFα, adiponectin and insulin on AKT and TOR signaling pathways in rainbow trout adipocytes

Next, both cytokines analyzed in this study, adiponectin and TNFα, were ineffective in activating the AKT signaling pathway in cultured rainbow trout adipocytes (Fig. 3A). However, an incubation of the cells with insulin resulted in a significant increase of AKT phosphorylation, up to over a 16-fold ($P < 0.001$). A combination of insulin with adiponectin caused also a significant stimulation of AKT phosphorylation ($P = 0.011$), slightly inferior but not significantly different to the one provoked by insulin alone ($P = 0.258$). On the other hand, TNFα counteracted the insulin effects in the activation of this signaling pathway ($P < 0.001$) (Fig. 3A). Furthermore, insulin triggered a 2.5-fold increase in TOR phosphorylation ($P = 0.019$); meanwhile neither of the cytokines studied showed any effect in the activation of this pathway (Fig. 3B). The promoting effect of insulin on the activation of TOR was observed to a lesser extent when the hormone was combined with either cytokine, since even though the phosphorylation levels of the protein rose they were not significantly different to those from the control cells (Fig. 3B).

3.4. PPARγ regulation by TNFα, adiponectin and insulin in rainbow trout adipocytes

Finally, we analyzed PPARγ expression, both at protein and mRNA levels in response to the different experimental treatments (Fig. 4). TNFα treatment significantly reduced PPARγ expression at protein level ($P = 0.037$), and the addition of insulin did not counteract this effect ($P = 0.037$) (Fig. 4A). Cells treated with adiponectin or insulin alone or in combination did not show significant effects on PPARγ protein levels (Fig. 4A). Regarding the regulation of this transcription factor at a transcriptional level, a significant up-regulation was observed when the cells were treated with adiponectin in combination with insulin ($P = 0.034$) (Fig. 4B). However, despite a 1.87-fold increase in PPARγ gene expression was caused after insulin stimulation, no significant changes were found

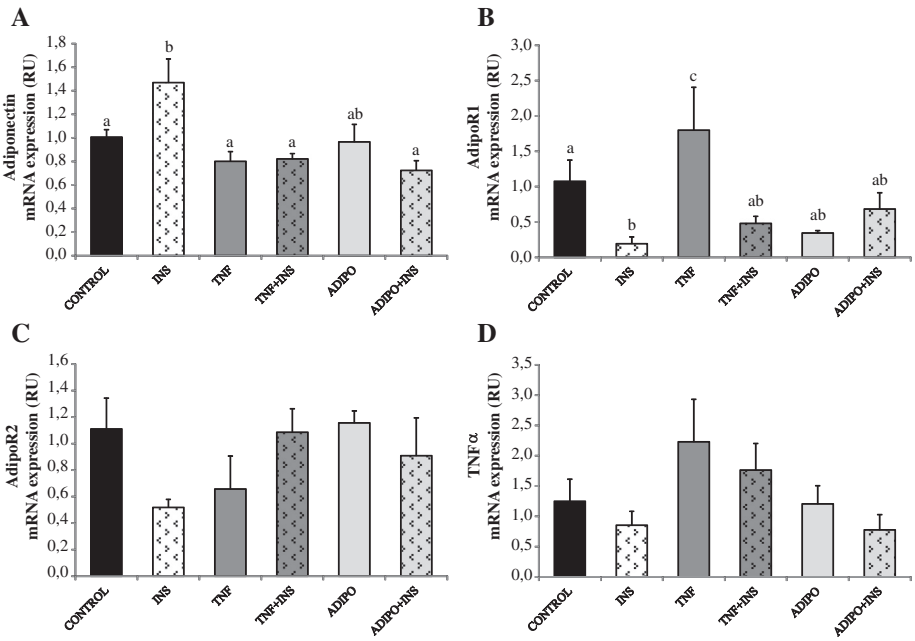

Fig. 1. Adiponectin, TNFα and insulin effects on (A) adiponectin, (B) adipoR1, (C) adipoR2 and (D) TNFα mRNA expression in rainbow trout adipocytes. Cells were incubated with either adiponectin (ADIPO) at 2.5 μg/ml, TNFα (TNF) at 100 ng/ml or were left untreated (CONTROL) for 44 h; then, cells were serum starved for 4 h and incubated in the presence or absence of insulin (INS) at 1 μM for 6 h. Gene expression data are presented as fold change ± S.E.M ($n = 3–5$) using eF1α as a reference gene (delta-delta method). (RU): relative units. Values not sharing letters are significantly different ($P < 0.05$, ANOVA followed by Tukey's test).

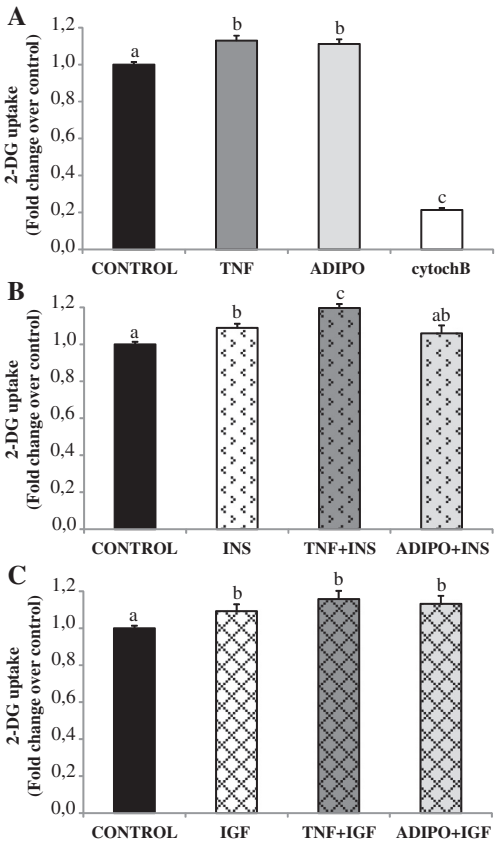

Fig. 2. Adiponectin, TNFα, insulin and IGF-I effects on 2-deoxyglucose (2-DG) uptake in rainbow trout adipocytes. Cells were incubated with either adiponectin (ADIPO) at 2.5 μg/ml, TNFα (TNF) at 100 ng/ml or were left untreated (CONTROL) for 44 h and serum starved for 4 h. Then, the cells were (A) kept with no hormonal stimulation, (B) incubated with insulin (INS) at 1 μM or (C) with IGF-I at 100 nM for 30 min and subsequently incubated with labeled 2-DG for 30 additional min. Cytochalasin B (cytochB) at 20 μM was added during the 30 min of uptake to determine non-specific uptake. Results are expressed as mean ± SEM ($n = 3$). Data was normalized to levels in control cells. Values not sharing letters are significantly different ($P < 0.05$, ANOVA followed by Tukey's test).

($P = 0.289$). The rest of the experimental conditions analyzed had no effect on PPARγ mRNA expression (Fig. 4B).

4. Discussion

In the last years, there has been much effort to define the role of adipokines in mammals (Kwon and Pessin, 2013; Tilg and Moschen, 2006), but not in fish. In the present study, we have evaluated the interplay of TNFα, adiponectin and insulin at a transcriptional level, their effects on glucose uptake and, TOR and AKT signaling pathways activation, including key transcriptional adipogenic factors, in rainbow trout cultured preadipocytes.

The presence of adiponectin in fish has been previously reported only in zebrafish (Nishio et al., 2008) and rainbow trout (Kondo et al., 2011, 2012; Sánchez-Gurmaches et al., 2012), where interestingly, its expression in muscle is higher than in adipose tissue. In mammals, among the factors regulating adiponectin, insulin has been shown to stimulate its expression and secretion in cultured adipocytes (Halleux et al., 2001; Kim et al., 2010; Hajri et al., 2011). In agreement with these reports, in the present study with rainbow trout adipocyte cultures, a stimulatory effect of insulin on adiponectin expression was also observed, together with a decrease in the expression of the receptor adipoR1, and to a lesser extent, of adipoR2. Previous *in vivo* studies using intraperitoneal administration of insulin in trout did not clearly reveal a positive effect of insulin in adiponectin gene expression in adipose tissue, but induced a decrease in adipoR1 and adipoR2 expression (Sánchez-Gurmaches et al., 2012). A down-regulation of adipoR1 and adipoR2 by insulin has been also reported in various mammalian models (Fang et al., 2005; Liu et al., 2008; Sun et al., 2009). This opposite regulation of peptide and receptor by insulin indicated a regulatory feedback mechanism probably directed to maintain a functionally active and stable adiponectin system in the adipocyte.

TNFα, that is considered a biomarker of inflammation, is known to suppress the transcription and secretion of adiponectin in primary human adipocytes (Kappes and Löffler, 2000; Bruun et al., 2003) as well as in 3T3-L1 cells (Fasshauer et al., 2002; Wang et al., 2013). On the other hand, adiponectin has been shown to suppress the synthesis of TNFα in mice adipose tissue (Maeda et al., 2002) and TNFα secretion in human adipocytes (Dietze-Schroeder et al., 2005). However, these reciprocal suppressive effects reported in mammals were not observed in our model, where neither TNFα nor adiponectin mRNA expression was affected by each other in trout adipocytes; although TNFα inhibited the insulin-induced increase in adiponectin mRNA levels. These observations would be in agreement with the lack of effects

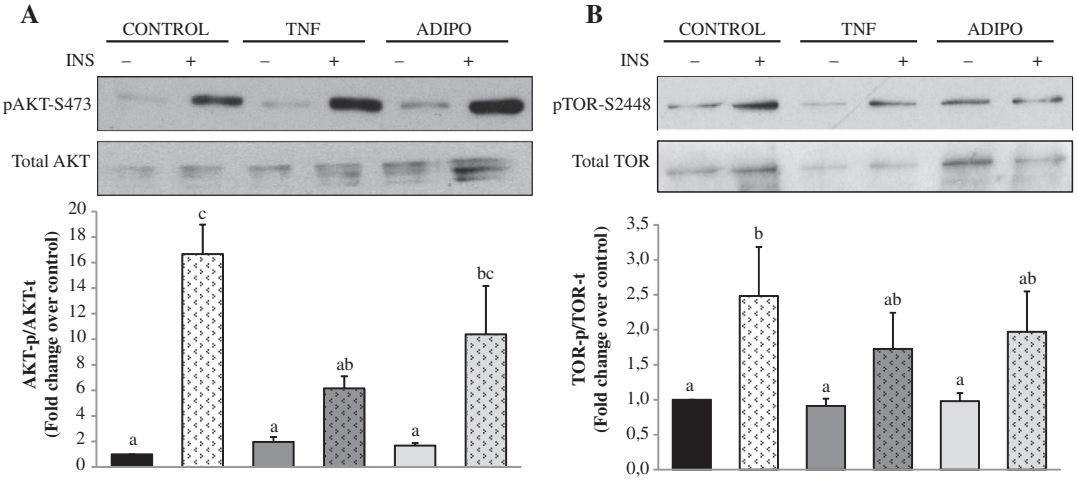

Fig. 3. Adiponectin, TNFα and insulin effects on phosphoinositide-3-kinase (PI3K)/AKT and target of rapamycin (TOR) signaling pathways activation in rainbow trout adipocytes. Cells were incubated with either adiponectin (ADIPO) at 2.5 μg/ml, TNFα (TNF) at 100 ng/ml or were left untreated (CONTROL) for 44 h; then, cells were serum starved for 4 h and incubated in the presence or absence of insulin (INS) at 1 μM for 30 min to assess the effects on the activation of (A) AKT and (B) TOR signaling pathways. A representative blot is shown. Graphs represent the ratio between the phosphorylated protein and the total amount of the targeted protein. Values are expressed as means ± S.E.M. ($n = 4$). Data was normalized to control cells. Values not sharing letters are significantly different ($P < 0.05$, ANOVA followed by Tukey's test).

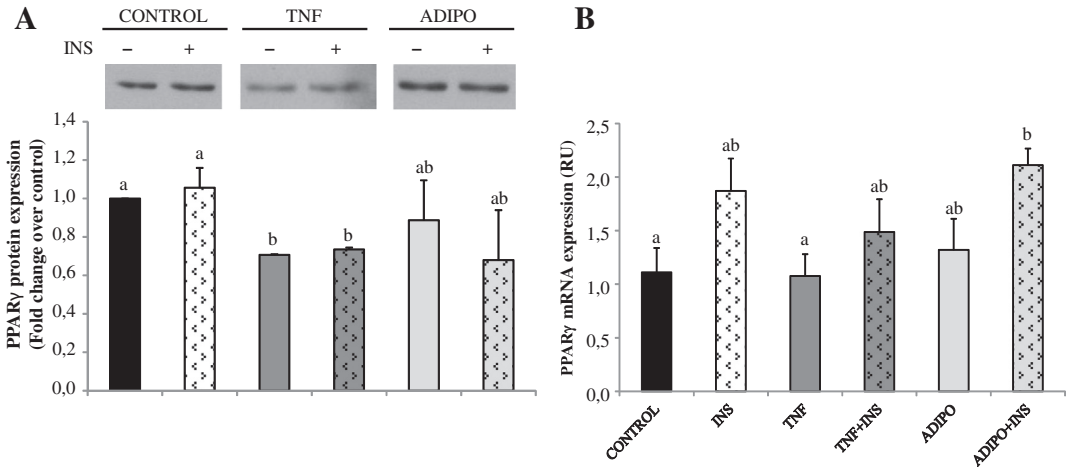

Fig. 4. Adiponectin, TNFα and insulin effects on PPARγ protein and mRNA expression in rainbow trout adipocytes. Cells were incubated with either adiponectin (ADIPO) at 2.5 μg/ml, TNFα (TNF) at 100 ng/ml or were left untreated (CONTROL) for 44 h; then, cells were serum starved for 4 h and incubated in the presence or absence of insulin (INS) at 1 μM. (A) After 30 min of hormonal stimulation PPARγ protein expression was determined. A representative blot is shown. Values are expressed as means ± SEM (n = 4). Data was normalized to control cells. (B) After 6 h of hormonal stimulation PPARγ mRNA expression was quantified. Gene expression data are presented as fold change ± SEM (n = 4–5) using eF1α as a reference gene (delta-delta method). (RU): relative units. Values not sharing letters are significantly different (P < 0.05, ANOVA followed by Tukey's test).

described previously in adiponectin mRNA levels of rainbow trout adipose tissue injected with TNFα (Sánchez-Gurmaches et al., 2012). In a similar way, TNFα only showed a tendency to increase its own expression in trout adipocytes, while in mammals it is well described that TNFα stimulates the NF-kB pathway, which in turn increases the expression of TNFα and other pro-inflammatory adipokines (Ye, 2011). Regarding the effects of TNFα on the regulation of adiponectin receptors we observed that TNFα increased adipoR1 expression, which was also evident when cells were incubated with TNFα and insulin, since the repressing effect caused by insulin alone was counteracted by the addition of TNFα.

In mammalian adipocyte models TNFα has been shown to increase basal glucose uptake (Cornelius et al., 1990; Medina et al., 1999) in agreement with our results, indicating that this cytokine could be an important stimulator of glucose transport in these cells. Nevertheless, in mammals, TNFα induces an insulin-resistant state by impairing insulin-stimulated glucose uptake through the inhibition of insulin signaling at the IRS-1 level (Hotamisligil, 1999; Porter et al., 2002; Fernández-Veledo et al., 2009). This TNFα-insulin interaction appears not to be well defined in fish, according to the observed stimulatory effect of TNFα on glucose uptake when combined with insulin. In fish, the very few studies on the biological effects of TNFα on glucose metabolism have been performed in trout cultured muscle cells, where TNFα also showed an enhancement of basal glucose uptake both, in myoblasts and myotubes (Vraskou et al., 2011). Regarding the signaling pathways, in the present study, TNFα alone modified neither AKT nor TOR; although an inhibitory effect compared to insulin treatment was observed on AKT and TOR phosphorylation when TNFα was added together with insulin, that it was not reflected on glucose uptake.

Regarding adiponectin effects on glucose uptake, it is interesting to note that incubation with adiponectin alone, increased glucose uptake without modifying the phosphorylation of AKT, in accordance with the findings obtained in primary rat adipocytes (Wu et al., 2003). On the other hand, adiponectin did not modify glucose uptake in insulin-treated trout adipocytes, while in rat adipocytes adiponectin enhanced insulin-stimulated glucose uptake (Wu et al., 2003). In mammalian models, the effects and signaling pathways of adiponectin have been studied more extensively in muscle cells than in adipocytes (Fu et al., 2005; Lee and Shao, 2012; Liu and Sweeney, 2014). Regarding adipose tissue it has been reported very recently, associated to the inhibition of chicken preadipocyte differentiation, that adiponectin led to the activation of p38 mitogen activated protein kinase (p38MAPK) and down-regulation of TOR (Yan et al., 2013). However, further studies are needed to elucidate the mechanisms of action of this adipokine in fish adipose tissue.

Moreover, since IGF-I was previously shown to be even more active than insulin stimulating glucose uptake in fish cells (Bouraoui et al., 2010; Montserrat et al., 2012) we found interesting to analyze also the interactions between this growth factor and the adipokines studied in relation to this metabolite uptake. We can conclude from the present results that neither adiponectin nor TNFα modify the IGF-I stimulatory effect on glucose uptake in trout adipocytes.

One of the central regulators of adipocyte biology is the transcriptional factor PPARγ. The observed inhibition of this master regulator of adipogenesis by TNFα in trout adipocytes in the present study is consistent with the anti-adipogenic effects of TNFα on rainbow trout preadipocytes in culture (Bouraoui et al., 2008). This inhibition of PPARγ was observed only at the protein, but not at the mRNA level, suggesting a preferential control by TNFα at a post-transcriptional level in trout adipocytes. Nevertheless, previous studies have shown that TNFα was able to decrease PPARγ gene expression in freshly isolated gilthead sea bream adipocytes (Cruz-Garcia et al., 2009), as well as both at protein and mRNA levels in 3T3-L1 cells (Xing et al., 1997; Wang et al., 2013). The increase of PPARγ induced by insulin was not significant neither at gene nor protein levels, which is in agreement with previous studies in cultured trout preadipocytes (Salmerón et al., unpublished data). This could be due to the fact that, since PPARγ is highly expressed in cultured trout and salmon adipocytes during *in vitro* development (Bouraoui et al., 2008; Todorčević et al., 2010), these cells would probably need a much higher stimulus to increase PPARγ mRNA levels. However, another possible explanation to the lack of insulin effects on PPARγ protein level is that the 30 min incubation with the hormone was not long enough to promote changes in transduction and protein synthesis. In this sense, in freshly isolated adipocytes that appear to be more receptive to hormones than cultured preadipocytes, insulin increased PPARγ gene expression at the same dose, but at longer incubation times than those tested in the present study (Cruz-Garcia et al., unpublished data). On the other hand, adiponectin treatment

appeared not to regulate PPARγ function by itself; however, in insulin-stimulated trout adipocytes this adipokine promoted PPARγ mRNA levels. In mammals, PPARγ has been shown to be a transcriptional activator of adiponectin gene (Iwaki et al., 2003) and in the same direction the use of agonists of this transcription factor such as thiazolidinediones (TZD) have been reported to increase adipose tissue mRNA levels of adiponectin in animal models of obesity and obese humans (Maeda et al., 2001; Phillips et al., 2003). In fact, it has been reported that PPARγ activation counteracts the inhibition of adiponectin expression by TNFα in mice adipocytes (Tsuchida et al., 2005). Therefore, high levels of PPARγ could explain in the present study the lack of an inhibitory effect of TNFα on adiponectin expression; however, further studies would be needed to corroborate this hypothesis.

Overall, from the present study we can conclude that the piscine adiponectin system seems to be under somewhat different regulation from that present in mammals. However, we confirm that insulin enhances adiponectin expression and reduces the expression of adipoR1 and adipoR2 to a lesser extent, as it has been described in some mammalian models. Adiponectin stimulated glucose uptake capacity without modifying AKT or TOR phosphorylation, but failed to increase glucose uptake in insulin-treated rainbow trout adipocytes. Concerning to the studied interplay between TNFα and adiponectin, we can conclude that the previously reported reciprocal suppressive effect of both adipokines in mammals appears to be missing in the present model. On the other hand, the stimulation of basal glucose uptake by TNFα seems to be conserved from fish to mammals, as well as its anti-adipogenic role, as revealed by the inhibition of PPARγ at the protein level, while the opposite effects between insulin and this pro-inflammatory cytokine are not so well defined in fish. Thus, further research is required to elucidate the metabolic effects and mechanisms of action of adipokines in fish, which will provide insights that may lead to control fat deposition in fish.

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Article IV

Gene expression profile during proliferation and differentiation of rainbow trout adipocyte precursor cells

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ABSTRACT

Background: Excessive accumulation of adipose tissue in cultured fish is an outstanding problem in aquaculture. To understand the development of adiposity, it is crucial to identify the factors that regulate the formation of adipocytes from precursor cells. Therefore, the transcriptomic profile at different time points (days 3, 8, 15 and 21) along primary culture development of rainbow trout preadipocytes has been investigated using an Agilent trout oligo microarray.

Results: Our analysis identified 4026 genes differentially expressed (fold-change >3) that were divided into two major clusters corresponding to the main phases observed during the preadipocyte culture: proliferation and differentiation. Proliferation cluster comprised 1028 genes up-regulated from days 3 to 8 of culture meanwhile the differentiation cluster was characterized by 2140 induced genes from days 15 to 21. Proliferation was characterized by the presence of genes involved in basic cellular and metabolic processes (transcription, ribosome biogenesis, translation and protein folding), cellular remodelling, autophagy and angiogenesis. In addition, the implication of the eicosanoid signalling pathway was highlighted during the early stages of differentiation. On the other hand, the terminal differentiation phase was enriched with genes involved in energy production, lipid and carbohydrate metabolism. Moreover, during this phase the formation of the lipid droplets was evidenced as well as the activation of the TR/RXR and the PPARs signalling pathways. The whole adipogenic process was driven by a coordinated activation of transcription factors and epigenetic modulators.

Conclusions: Overall, our study demonstrates the coordinated expression of functionally related genes during proliferation and differentiation of rainbow trout adipocyte cells. Furthermore, the information generated will allow future investigations of specific genes involved in particular stages of fish adipogenesis.

1. BACKGROUND

The ongoing intensification of salmonids aquaculture industry has led to the development of diets with high lipid content, which can induce an increase in subcutaneous, intramuscular and especially in visceral fat depots (Weil et al., 2013). This excessive adiposity may entail a negative impact on the sector productivity. Mature adipocytes are known to play an important role controlling energy balance in mammals by storing fatty acids in the form of triglycerides in periods of excess of energy and by releasing fatty acids when are needed. Despite of the relevance of this issue, very little is known about the factors regulating the development of adipose tissue in fish, and the possible health alterations associated to an increased fat deposition. While excess of adipose tissue in humans, which occurs in obesity, is known to contribute to the development of many aspects of the pathology of metabolic syndrome and other diseases (Alemany, 2013; Rajan et al., 2014), the metabolic consequences of a high adiposity are still not well known in fish.

In order to understand the development of adiposity, it is crucial to identify the factors and mechanisms that regulate the recruitment of mesenchymal stem cells (MSCs) of the vascular stromal fraction of the adipose tissue and its transformation into lipid-filled adipocytes. Adipogenesis has been described as a two-step developmental process consisting on the commitment of undifferentiated MSCs into preadipocytes and the further development of these cells into fully functional mature adipocytes. In fish, like in mammals, adipogenesis takes place as a consequence of normal cell turnover and due to the need of storing energy. Therefore, this tissue growth includes the hypertrophy of already existing adipocytes and the proliferation and differentiation of new ones from MSCs.

Adipogenesis has been extensively studied in mammals (Burton et al., 2004) and several cell and animal models have been successfully used to describe the chronology of the molecular events governing this process. Many are the studies highlighting the importance of the interplay of both, activating and inhibiting signals and thus involving pro-adipogenic and anti-adipogenic factors (Burton et al., 2002; Burton et al., 2004; Guo and Liao, 2000; Soukas et al., 2001). We have previously shown that fish primary preadipocytes differentiate into mature adipocytes *in vitro* and that these cells represent a very helpful model system to study adipose tissue development in fish (Bouraoui et al., 2008; Salmeron et al., 2013). Some of the well-known key adipogenic transcription factors described in mammals, such as peroxisome proliferator activated receptor γ (PPARγ) and CCAAT-enhancer binding protein α (C/EBPα), have been

identified and linked to an adipogenic function in fish as well (Bouraoui et al., 2008; Oku and Umino, 2008; Vegusdal et al., 2003).

Insulin favors differentiation into mature adipocytes in rainbow trout, gilthead sea bream and other fish species of cultured preadipocytes (Bouraoui et al., 2008; Oku et al., 2006; Salmeron et al., 2013). However, a common trait in fish is that lipids, especially high concentrations of fatty acids, play an essential role in adipocyte differentiation; but only in Atlantic salmon, the gene expression profile has been studied during adipogenesis (Todorčević et al., 2010).

Apart from controlling energy storage and mobilization, adipose tissue has an important function as a major endocrine organ secreting diverse adipokines and regulating therefore many physiological aspects (Ali et al., 2013). For instance the cytokine tumor necrosis factor α (TNFα) is expressed in fish adipose tissue and is well known by its anti-adiposity actions (Albalat et al., 2005; Bouraoui et al., 2008; Cruz-Garcia et al., 2009). Leptin and adiponectin are known to be the most abundant proteins secreted by mammalian adipocytes (Tilg and Moschen, 2006), contrary to what has been described so far in teleost species, where leptin is primarily expressed in the liver (Copeland et al., 2011) and adiponectin in muscle (Kondo et al., 2012; Kondo et al., 2011; Sanchez-Gurmaches et al., 2012). These specific features highlight the need to further investigate the physiological and secretory functions of fish adipose tissue.

Here, to characterize the genetic basis of adipogenesis in rainbow trout, a genome-wide expression profiling combined with quantitative PCR (qPCR) and Western blot analysis was performed at 4 different time-points along the adipocyte primary culture. The main goal of this study was to provide deeper insights into the dynamics of adipocyte conversion by defining the cascades of gene expression as well as to identify possible novel adipogenic mediators and markers in this fish species. Likewise, we aimed to unravel yet undiscovered mechanisms and provide a basis for the identification of proteins that could serve in the future as potential links between the adipocyte and the process of whole body energy homeostasis.

2. METHODS

2.1 Animals

Rainbow trout (*Oncorhynchus mykiss* Walbaum 1792) weighting from 200 to 250 g were obtained from the "Truites del Segre" fish farm (Lleida, Spain). Fish were acclimatized to a 12 h light:12 h dark photoperiod and 14 ± 1°C in a closed circuit flow system located in the Faculty of Biology at the University of Barcelona. Fish were fed daily *ad libitum* on a commercial diet based on fishmeal and fish oil (Dibaq Diproteg, Segovia, Spain). All animal handling procedures were approved by the Ethics and Animal Care Committee of the University of Barcelona, following the regulations and procedures established by the EU, and by the Spanish and Catalan governments (permits reference numbers CEEA 237/12 and DAAM 6755).

2.2 Primary culture of preadipocyte cells

All reagents were purchased from Sigma-Aldrich (Tres Cantos, Spain) and all tissue culture plastic ware was obtained from NUNC (Labclinics, Barcelona, Spain) unless stated otherwise.

Fish were fasted 24 h before the experiments. The animals were killed by a blow to the head under anesthesia (3-aminobenzoic acid ethyl ester, MS-222; 100 μg/ml), which was followed by immersion in 70% ethanol for 30 s to sterilize the external surfaces. Cells for each experiment were isolated from a pool of white adipose tissue of 6-7 fish and cultured as described elsewhere (Bouraoui et al., 2008). The use of pooled tissue minimizes fish-specific variation in the experimental measurements. Briefly, the dissected visceral fat tissue was washed with Krebs-HEPES buffer (pH 7.4) and was digested for 1 h with type II collagenase 130 UI/mL containing 1% bovine serum albumin in Krebs-HEPES buffer at 18°C. The resulting cell suspension was filtered (100 μm) and centrifuged at 700 g for 10 min and the pellet was treated with erythrocyte lysing buffer (0.154 M NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM EDTA) for 5 min at room temperature. After washing, the cells were centrifuged again at 700 g for 10 min. The cell pellet was resuspended in growth medium, consisting on Leibovitz's L-15 medium containing 10% fetal bovine serum, 2 mM L-glutamine and 1% antibiotic/antimycotic solution. Cells were counted, diluted, seeded in pretreated flasks or six-well plates (25 cm^2 or 9.6 cm^2/well respectively), with 1% gelatin at a density of $2 \cdot 10^4$ cells/cm^2 and kept at 18°C. For each experimental condition either one flask (RNA extraction) or two wells pooled together (Western blot) were used. After confluence (day 8), cells were induced to differentiate by means of a growth medium supplemented with 10 μg/mL insulin, 0.5 mM 3-isobutyl-1-

methylxanthine (IBMX), 0.25 μM dexamethasone and 10 μL/mL lipid mixture. Media were changed every 2 days during the whole procedure. Samples were collected at day 3 (mesenchymal stem cells), day 8 (proliferated cells), day 15 (committed preadipocytes) and day 21 (mature adipocytes).

2.3 RNA extraction and cDNA synthesis

Total RNA from adipocyte cells was extracted using the TriReagent method (Ambion, Alcobendas, Spain) following the manufacturer's recommendations. The quantity of isolated RNA was determined by spectrophotometry with a ND-2000 NanoDrop (Thermo Fisher Scientific, Alcobendas, Spain) and the quality was assessed using a Bionalyzer (Agilent). The total RNA was used for microarray and qPCR analysis.

For cDNA synthesis 5 μg of total RNA was reverse-transcribed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) in a 25 μl reaction volume according to the instructions provided by the manufacturer. The reaction was set at 200 μl by the addition of nuclease-free water. From each sample two independent reverse transcriptions were performed.

2.4 Quantitative real-time PCR (qPCR) analysis

qPCR measurements were performed in a StepOnePlus Tm system (Applied Biosystems) as described in Weil et al. (2011). Briefly, 5 μl of diluted cDNA was amplified in duplicates containing Fast SYBR Green Master Mix (Applied Biosystems) and specific primers at a final concentration of 600 nM. The qPCR primer sequences for the target genes and the reference gene are shown in Table 1. Primers were designed to span exon-boundaries if possible (Genamics Expression software) using known sequences from the rainbow trout nucleotide databases (INRA-Sigenae) and previously published (Polakof et al., 2012; Weil et al., 2011).

Relative quantity of each gene was determined from a standard curve consisting of serial dilutions of a pool of cDNAs obtained from isolated mature adipocytes and expressed as Arbitrary Units (A.U.). This calculation was performed using the software included in the StepOne plus Tm System which corrects for differences in amplification efficiencies. Primer efficiencies ranged from 90 to 103%. Specificity of the amplification reaction was verified by analysis of melting curves. 18S was used for the normalization of qPCR data since its RNA abundance was stable along the cell culture ($p > 0.05$). The two cDNAs obtained from each sample were run in a single plate for each gene assay together with negative controls (reverse transcriptase-free samples and RNA-free samples).

2.5 Microarray analysis

The adipocyte transcriptome was analyzed using an Agilent-based microarray platform with 8 x 60 K probes per slide previously described (Rescan et al., 2013) and registered in GEO with the platform record GPL15840. Total RNA from cells at different developmental stages (days 3, 8, 15 and 21) from four independent cultures was labelled with Cy3 according to the manufacturer's instructions (Agilent). Briefly, RNA was first reverse transcribed, using a polyDTT7 primer, Cy3 was incorporated by a T7 polymerase mediated transcription and excess dye was removed using an RNeasy kit (Quiagen). The level of dye incorporation was evaluated using a spectrophotometer (Nanodrop ND1000, LabTech). Labelled RNA was then fragmented in the appropriate buffer (Agilent) for 30 min at 60°C before dilution (v/v) in hybridization buffer. Hybridizations were performed in a microarray hybridization oven (Agilent) overnight at 65°C, using two high-density oligonucleotide microarray slides. Following hybridization, the slides were rinsed in gene expression wash buffers 1 and 2 (Agilent) and scanned at a 3 μm resolution using an Agilent G2505 microscanner. Fluorescence intensity was calculated using the standard procedures contained in the Agilent Feature Extraction software version 10.7 and the data were normalized using GeneSpring software. An ANOVA ($p < 0.02$) and an average fold change of >3 were used as the criteria for defining genes as differentially expressed between the different days along the cell culture. For clustering analysis, data were log transformed, median-centered and an average linkage clustering was carried out using CLUSTER software. The results were visualized using TREEVIEW (Eisen et al., 1998). Biological functions and pathways were generated and analyzed using Ingenuity Pathway Analysis software (IPA, Ingenuity Systems, CA). The Database for Annotation, Visualization and Integrated Discovery (DAVID) 6.7 (Jiao et al., 2012) was used as a complementary tool to reinforce the functional studies. The analyses were performed according to the default set of statistical parameters.

2.6 Western blot analysis

Protein extraction and Western blot analysis were performed as previously reported (Bou et al., 2014). Twenty μg of protein were subjected to SDS-PAGE gel electrophoresis and Western blot analysis was performed using the appropriate antibodies for ACSL-1, (cat no 98925), PCNA (cat no 7907), PEPCK (cat no 32879), PLIN2 (cat no 32888), all from Cell Signaling Technology Inc (Beverly, MA) and PPARγ (PPARγ; cat no 7196; Santa Cruz Biotechnology). Immunoreactive bands were visualized using an enhanced chemiluminescence kit (Pierce ECL

Western blotting Substrate; Thermo Scientific, Alcobendas, Spain) and quantified by densitometric scanning using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Some of these antibodies have been previously shown to successfully cross-react with fish, such as PPARγ (Bouraoui et al., 2008) and PCNA (Azizi et al., 2015) . Experiments were performed using homogenates of rat liver as a control to confirm the specificity of immunodetection. Western blots results were obtained from four independent cultures.

Table 1. Rainbow trout primer sequences used for real-time qPCR

Gene	Accession number	Primer sequence (5'-3') **F**: forward, **R**: reverse	Annealing T (°C)
ACSL1	CR363150.p.om.8	**F:** TGCAATCTAGCAAGGTTCCTTTTG	60
		R: TCCAAGCAGAAACCCAGTACAGAA	
GPDH	AF027130.p.om.8	**F:** ATGACCACAGTCCACGCCTACAC	60
		R: GGCAGGTTAGGTCCACCACTGA	
IGFBP5	DQ206713	**F:** ACTTCACGCGCTTCTCCATGGCA	60
		R: CGAGACTCATGATCTATGGGTGGA	
IGFBP7	DQ146965	**F:** GCTCCGATGGAGTGACCTATA	60
		R: ACAATGACAGGTGCTGTTGCG	
PCNA	CA358086.s.om.10	**F:** ATACGGGCAAACTCTCCTGATGGC	60
		R: CAACGCAGACACACTCGCCCTT	
cPEPCK	(Polakof et al., 2012)	**F:** CCCAGTGCCTGTGGGAAAAC	60
		R: CCACACCGAAAAAGCCGTTC	
PLIN2	CB494091.p.om.8	**F:** GATGGCAATGAGGCAGAGAACA	60
		R: AGGCAGAGTGGCTAAGGGACAG	
18S	AF308535	**F:** CGGAGGTTCGAAGACGATCA	60
		R: TCGCTAGTTGGCATCGTTTAT	

3. RESULTS AND DISCUSSION

Gene expression profile during adipogenesis

In order to characterize the process of adipogenesis in rainbow trout, four independent primary cultures of adipocytes were performed and samples were collected at days 3, 8, 15 and 21 and used for microarray experiments. After bioinformatics analysis a total of 4026 genes were found differentially expressed (> 3.0-fold, $p < 0.02$). Hierarchical clustering analysis of these differentially expressed genes was performed and two major clusters were identified (Figure 1) and related to the main phases described during preadipocyte culture; proliferation (cluster 1) and differentiation (cluster 2). Proliferation cluster comprised 1028 genes that were up-regulated from days 3 to 8 of culture meanwhile the cluster characterizing the differentiation phase included 2140 up-regulated genes from days 15 to 21 of culture.

Functional annotation of genes found in cluster 1 (Proliferation) and 2 (Differentiation)

Cluster 1 comprised genes that were up-regulated during days 3 and/or 8 of culture. Significant biological functions associated with the proliferative phase of adipogenesis were determined by using IPA software (Additional file 1) and DAVID (Additional file 2). The early stages of adipocyte development were characterized by a large number of genes involved in basic cellular and metabolic processes such as transcription, ribosome biogenesis, translation and protein folding. During this part of the culture the category of "Cellular Growth and Proliferation" was highly represented, with 254 molecules included in the functional annotation of "proliferation of cells". Among the other significant categories present in the early stages of adipocyte development, the category "Tissue Morphology" that included the functional annotations of "quantity of cells" and "morphology of connective tissue" and the category of "Immune Cell Trafficking" were also found. On the other hand, the "Eicosanoid Signaling" and the "IL-8 Signaling" pathways were found within the top canonical pathways.

On the other hand, cluster 2 consisted on up-regulated genes during days 15 and/or 21 of culture. During this second phase a remarkable number of genes involved in energy production, glucose and lipid metabolism were revealed by IPA and DAVID analyses (Additional files 3 and 4 respectively). IPA analysis highlighted as top categories "Molecular and Cellular Functions" and "Molecular transport", which included the functional annotations "transport of molecules", "concentration of lipid" and "transport of lipid" among others. Other top category represented was "Carbohydrate Metabolism", comprising 128 molecules and including functional annotations such as "glycolysis of cells", "metabolism of carbohydrate" and "gluconeogenesis". The category of "Cellular Growth and Proliferation" was also highly

represented, with 250 molecules included in the functional annotations of "proliferation of cells" and "proliferation of connective tissue cells". "IL-8 Signaling" was present again as one of the top canonical pathways, together with "TR/RXR Activation". Finally, the implication of the PPAR signaling pathway during this phase of the culture was also revealed.

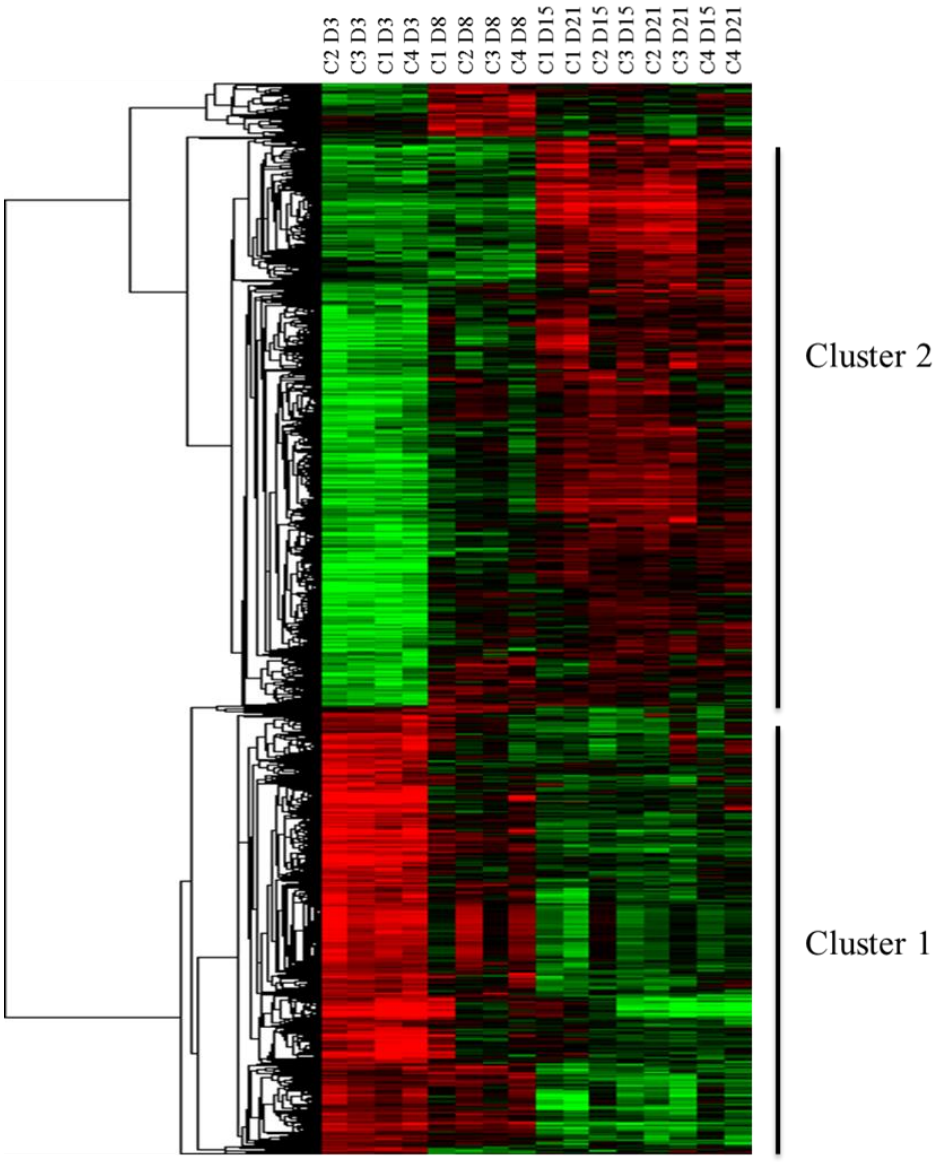

Figure 1. Heat map of the hierarchical clustering of genes differentially expressed (> 3.0-fold, p < 0.02) among different days along rainbow trout adipocyte culture. The horizontal dendrogram represents the correlation distances between gene expression levels. Every column represents one independent culture at one time-point meanwhile the different genes are represented by individual rows. The red color indicates high levels of expression while green represents low levels of expression. The intensity of both colors is related to the transcript expression level.

In order to describe and highlight the different processes that take place during culture development, including the proliferation and differentiation phases, the following results and corresponding discussion will be divided into different sections according to some selected functional groups of molecules. The first section will cover the main processes present during the early stages of adipocyte commitment and development. Next, the coordinated cascade of transcription factors that orchestrate fat cell progression will be presented, followed by the description of the main epigenetic factors identified. Subsequently, the relevance of two regulatory systems, the insulin and insulin-like growth factors (IGFs) and the eicosanoid signaling, will be discussed. Finally, a section concerning the main terminal regulators of adipocyte maturation present in our model system will be provided.

1- Cell commitment and remodelling

Initial phases of adipogenesis are characterized by the presence of pluripotent precursors (i.e. MSCs) of adipocytes. Many genes involved in cell fate (Figure 2A) were differentially induced in the early steps of our cell culture. For instance the presence of Notch1, which is a protein responsible for cell fate decision and implicated in the regulation of adipogenesis in 3T3-L1 cells (Aminova and Wilson, 2007), was revealed during the proliferative phase of the culture. During this phase we found overexpressed also the ribosomal protein S6 kinase delta-1 (Rps6kc1 or S6K1). S6K1 is involved in the commitment of embryonic stem cells to early adipocyte progenitors in mice (Carnevalli et al., 2010) and, is known to promote protein synthesis, cell growth and cell proliferation; processes that are of utmost importance during the early stages of the culture. On the other hand, the expression of the retinoblastoma-like proteins 1 (RBL1/p107) and 2 (RBL2/p130), which control cell cycle progression, was also evident during the first stages of cell development. RBL1 has been reported to be a crucial regulator for determining the adipocyte fate choices of stem cells committing to the white lineage, whereas its suppression is required for commitment to the brown-type fate (De Sousa et al., 2014). In addition, alterations in the expression of both, RBL1 and RBL2 inhibit adipocyte differentiation (Burton et al., 2004). In this sense, their expression in early stages and their down-regulation during days 15 and 21 of our culture will support this observation suggesting a role for these molecules in adipocyte commitment also in rainbow trout.

Various growth factors are known to present an important influence in the commitment of MSCs into different cell types (Ng et al., 2008). In this sense, transcripts encoding some growth

factors are present all along the rainbow trout adipocyte differentiation process (Figure 2B). Some of the adipogenic markers described in human bone marrow-derived MSCs differentiating into the adipogenic lineage (Ng et al., 2008) were present in our transcriptome. Such is the case of dexamethasone-induced Ras-related protein 1 (RASD1), DNA damage-inducible transcript 4-like protein (DDIT4L) and epoxide hydrolase 1 (EPHX1), which are highly expressed during adipocyte development with a peak at day 15 of culture.

The dramatic alteration in cell shape, from a fibroblast to a spherical shape represents one of the first hallmarks of adipogenesis. Our analysis has shown the regulation of a large number of genes involved in this type of processes (Figure 2C). For instance, we found a remarkable presence of members of the matrix metalloproteinases (MMPs) family, as well as transcripts encoding their tissue inhibitors (TIMPs), which are known to regulate MMP activity (Malemud, 2006) all along the culture. MMP9 was up-regulated during the proliferative phase while MMP2 was increased during differentiation. In freshly isolated mature adipocytes from humans both transcripts are present, being the expression of MMP2 higher than MMP9, in agreement with our data. Moreover, in the same line, the expression analysis of MMPs during 3T3F442A preadipocyte differentiation revealed that MMP2 showed a 13-fold increase in mRNA levels after 9 days of differentiation compared with non-differentiated cells, while MMP9 followed a moderate increase during differentiation, reaching a maximal level after 7 days, and decreasing afterwards. Accordingly, the inhibition of MMP2 and MMP9 in the same preadipocyte cell line inhibited the differentiation of adipocytes, highlighting their role during the development of these cells (Bouloumié et al., 2001). Furthermore, we also found MMP14 up-regulated during proliferation. The loss of this protein impairs adipogenesis *in vivo* in mice (Chun et al., 2006). The relevance of the proteolytic activity during rainbow trout adipogenesis was also evidenced by the presence of many transcripts encoding various members of the ADAMTS (a disintegrin and metalloproteinase domain, with thrombospondin motifs) family during the last stages of development (days 15 and 21). Therefore, it seems that is the balance and coordination among these molecules what might have a prominent role during both, early and late phases of adipocyte development.

In addition, cell shape and extracellular matrix remodeling have been found to regulate preadipocyte commitment and competency by modulating among others the RHO-family GTPase signaling cascade in mammals (Cristancho and Lazar, 2011). Our data suggests that this could also be the case for rainbow trout adipocytes, due to the high number of genes of this family especially up-regulated during the early steps of the process. In this sense, cluster 1 was

enriched with many representatives, such as Rho GTPase activating protein 22 (ARHGAP22) and 24 (ARHGAP24), Rho family GTPase 1 (RND1) and 3 (RND3), Rho GDP dissociation inhibitor beta (ARHGDIB), Rap guanine nucleotide exchange factor 1 (RAPGEF1), 2 (RAPGEF2) and 4 (RAPGEF4), Rho guanine nucleotide exchange factor 3 (ARHGEF3) and 4 (ARHGEF4) and T-cell activation RhoGTPase activating protein (TAGAP). Actin and myosin are examples of key molecules involved in cell shape mediated differentiation that are on the contrary up-regulated in late developmental phases (McBeath et al., 2004).

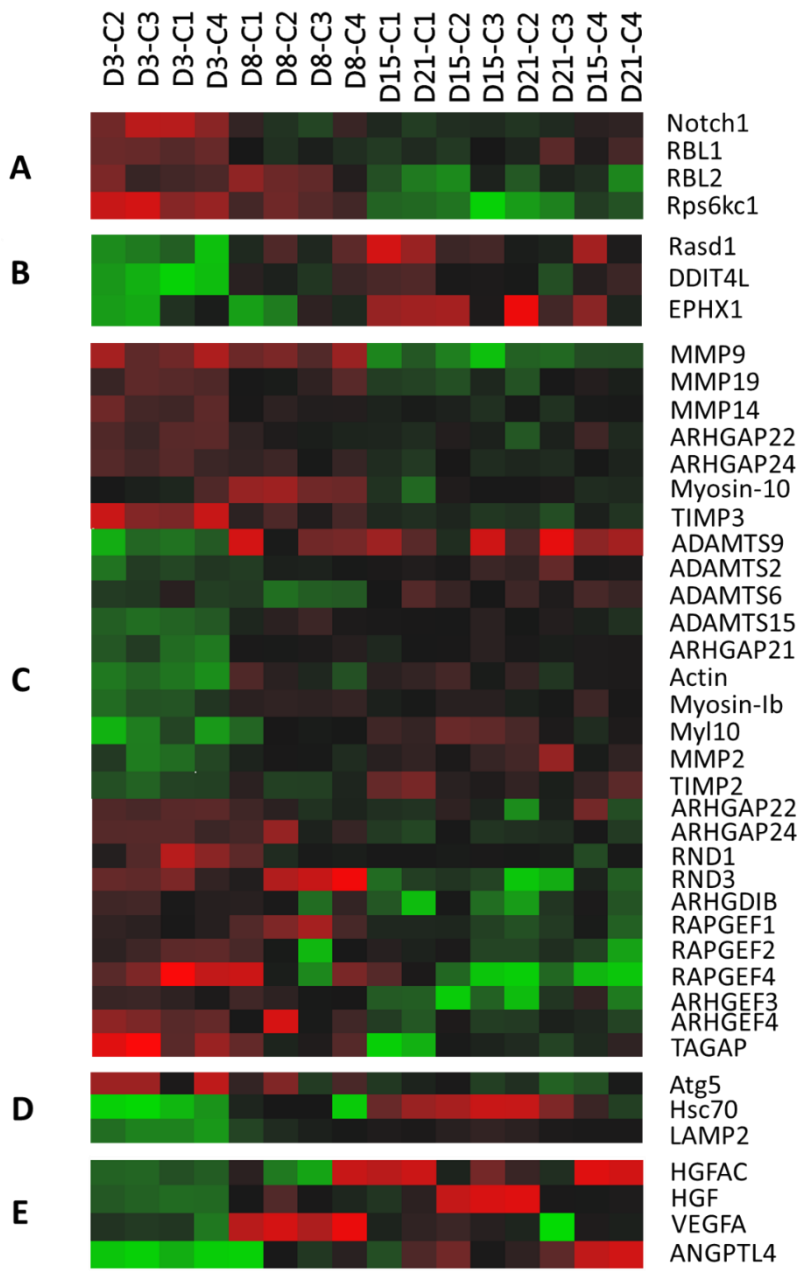

Figure 2. Selected genes involved in cell commitment and remodelling. (**A**) Cell commitment; (**B**) Growth factors; (**C**) Cell remodelling; (**D**) Autophagy; (**E**) Angiogenesis.

In line with the dramatic cytoplasmic reorganization that occurs during adipogenesis, we found in the proliferation cluster the presence of Atg5, a key autophagy gene. Autophagy is a major cytoplasmic degradation pathway that has been linked to the regulation of adipogenesis in mammals (Subramani and Malhotra, 2013). It does not only participate in cellular remodelling, but it also plays an important role controlling the dynamic change in mitochondrial mass that takes place during the maturation process (Zhang et al., 2012). Loss of Atg5 results in impaired white adipose tissue development both, *in vitro* and *in vivo* in mice (Baerga et al., 2009; Singh et al., 2009). In addition, transcripts encoding heat shock cognate protein of 70 kDa (Hsc70) and lysosomal-associated membrane protein type 2A (LAMP2A), relevant proteins involved in chaperone-mediated autophagy (Kon and Cuervo, 2010), were increased in the cluster representing the differentiation phase, indicating that autophagy might be playing an important role during the whole differentiation process (Figure 2D).

In humans, adipose-derived stem cells have been defined as one of the most promising stem cell types for its use in cell-based therapies to treat diverse diseases. This is due to the number of angiogenic and anti-apoptotic growth factors that these cells secrete at bioactive levels. Adipogenesis and angiogenesis are known to be tightly associated in mammals (Lijnen, 2008), being angiogenesis required for adipose tissue expansion (Lafontan, 2014). The angiogenic capacity of our cell system is early displayed (from day 8) (Figure 2E), with the up-regulation of transcripts encoding some of the most relevant genes involved in this process, such as the hepatocyte growth factor (HGF), the vascular endothelial growth factor A (VEGFA) and the angiopoietin-related protein 4 (ANGPTL4). This suggests that, as in mammalian adipose-derived stem cells, which are reported to be involved in the regeneration of the ischemic myocardium (Cai et al., 2007), rainbow trout adipocytes may have also an active role in the regeneration of tissues.

2- Coordinated transcriptional factors during adipocyte differentiation

2.1 AP-1 complex

A large number of transcription factors were up-regulated with different temporal expression patterns over the course of rainbow trout adipocyte differentiation. Experiments using different cellular model systems have described the existence of a tightly regulated cascade of transcription factors that promote the differentiation of fat cells (Rosen and MacDougald,

2006). In white adipocytes this cascade starts with the activation of members of the activator protein-1 (AP-1) transcription factor complex (Sarjeant and Stephens, 2012). AP-1 consists of dimers of proteins belonging to the Fos, Jun and activating transcription factor (ATF) families that have been reported to present different functional properties (Andreucci et al., 2002). AP-1 works as an environmental biosensor of the cell, regulating different aspects of cell physiology in response to stress or growth factors (Wagner, 2001). During the first period of rainbow trout adipocyte development (Figure 3A) we found the presence of c-Fos, which has been described as essential to initiate adipocyte differentiation and identified as part of a nuclear complex that regulates the expression of adipocyte-specific genes (Distel et al., 1987). More recently, the ongoing differentiation process in 3T3-L1 cells was inhibited by the knockdown of this transcription factor (Xiao et al., 2011), and in humans, a mutation in this gene has been associated with the development of congenital generalized lipodystrophy (Knebel et al., 2013). We also found the transcription factor FOS-like antigen 2 (FOSL2, also referred to as Fra2) highly expressed during the first stages of cell development (Cluster 1). Even though this transcription factor is not considered as adipocyte-specific, it has been described to promote the expression of the adipokine leptin in both, human and mouse adipocytes (Wrann et al., 2012). In addition, its implication in adipocyte homeostasis has been evidenced since its deletion triggers a high adipocyte turnover able to regulate body fat mass in mice (Luther et al., 2014). On the other hand, other transcription factor from the AP-1 complex, the protein FosB was also up-regulated during the same period. The overexpression of this gene has been reported to provoke inhibition of adipogenesis in mice and is suggested to exert this action through an interaction with C/EBPβ (Kveiborg et al., 2004). Interestingly, several members of the ATF family (ATF2, ATF3, ATF4, ATF5, and ATF6B) were found overexpressed only during the differentiation phase of the rainbow trout culture. Many of these genes are known to mediate cellular stress response signalling; and for instance ATF3 has been recently involved in adipocyte differentiation although it appears to have an inhibitory action, suggesting that a coordinated equilibrium between members of the different families that conform the AP-1 complex would be necessary (Jang et al., 2012). Overall, the parallelism of gene expression profiles in mammalian models and the present study in AP-1 components suggests a similar role of this transcription factor complex in fish, with some species-specific responses. In this sense, a different temporal activation of this complex was reported in Atlantic salmon adipocytes, where the induction of AP-1 was observed during the last stages of adipogenesis (Todorčević et al., 2010).

2.2 E2F, C/EBP, Krüppel-like factor (KLF) and GATA families

The E2F family of transcription factors is known to regulate cell cycle progression (Burton et al., 2004) as well as adipocyte differentiation (Farmer, 2006). In our study we found E2F4 highly expressed at day 3 of the culture, and then the expression went down (Figure 3B). In mammals, E2F4 forms a complex with RBL2 that is able to repress the transcription of PPARγ, and then is lost after hormonal stimulation to allow adipogenesis to proceed (Fajas et al., 2002). We observed that E2F4 and RBL2 follow the same expression pattern during rainbow trout adipocyte development (Figure 2A and 3B), suggesting that their down-regulation might be required to promote adipogenesis also in this species. Likewise, the recently reported novel regulator of pro-adipogenic genes, zinc finger protein 638 (ZNF638) (Meruvu et al., 2011) was induced early during rainbow trout adipocyte development, presenting its peak of expression at day 3 as well as the early growth response protein 2 (EGR2, also known as KROX20), which has been described as a factor capable of inducing uncommitted fibroblasts to become adipocytes (Chen et al., 2005).

During rainbow trout adipogenesis we also identified a transient induction of several members of the C/EBP family of transcription factors (Figure 3B). C/EBPδ, which is a key early regulator of adipogenesis, presented its peak of expression at day 3 of cell development. C/EBPδ together with C/EBPβ, have been reported to transactivate CEBPα (Cao et al., 1991). In our cell system, CEBPα started to be up-regulated from day 8, with a maximum peak at day 15. This transcription factor, together with PPARγ, is known to coordinate and induce the expression of several genes involved in insulin sensitivity, lipogenesis and lipolysis promoting the adipocyte phenotype (Lowe et al., 2011). Immunofluorescence results showed a high presence of both transcription factors, CEBPα and PPARγ, in rainbow trout differentiating adipocytes at day 11, suggesting their implication in the regulation of these processes (Bouraoui et al., 2008), as supported now by the present results.

Several members of the multigenic Krüppel-like factor (KLF) family have been reported to control adipogenesis, some being considered activators (KLFs 4, 5, 6 and 15) while others are considered repressors of transcription (KLFs 2 and 7) (Lowe et al., 2011; Sarjeant and Stephens, 2012). During the progression of our culture we observed a sequential expression of members of this family (Figure 3B). Thus, during the early stages of cell development we identified transcripts encoding the KLFs 2, 4 and 6, while transcripts encoding the KLFs 11, 15 and 16 were present during adipocyte maturation. Some of them have been extensively studied in the

context of adipocyte progression. For instance, KLF4 has been described as an essential early activator of adipogenesis able to bind to Krox20 to transactivate C/EBPβ (Birsoy et al., 2008). KLF15 is known to play an important role in adipocyte insulin sensitivity through the transactivation of the facilitative glucose transporter (GLUT4) promoter by binding near the myocyte enhancer binding factor-2A (MEF2A) consensus site (Gray et al., 2002). Less is known about the roles of this family of transcription factors in fish. In Fugu, a potential binding site for KLF15 and MEF2 has been identified also in the GLUT4 promoter region, indicating a remarkable conservation among vertebrates (Marín-Juez et al., 2013). In zebrafish, several members have been identified and characterized, and a high degree of functional conservation with the mammalian homologs was reported (Oates et al., 2001) with an special focus on the function of these factors in hematopoiesis (Xue et al., 2015). On the other hand, an important role of this family of proteins in rainbow trout oogenesis has been suggested, being this a conserved feature among vertebrates (Charlier et al., 2012). Therefore, and in light of our transcriptomic data, we could speculate that the KLF family plays a critical function during rainbow trout adipogenesis with some of their actions maintained during evolution from fish to mammals.

GATA factors are generally considered as negative regulators of adipogenesis. We found GATA2 up-regulated during proliferation (Figure 3B), with a significant peak of expression at day 3. This is in agreement with previous findings reported in the mammalian system, where the expression of this transcription factor is restricted to the preadipocyte phase and is down-regulated upon adipocyte differentiation (Tong et al., 2000). Two other members from this family were significantly expressed during rainbow trout adipogenesis with different expression patterns, GATA5 and GATA6 (Figure 3B); however, to the best of our knowledge the implication of these members in adipogenesis has not been reported so far.

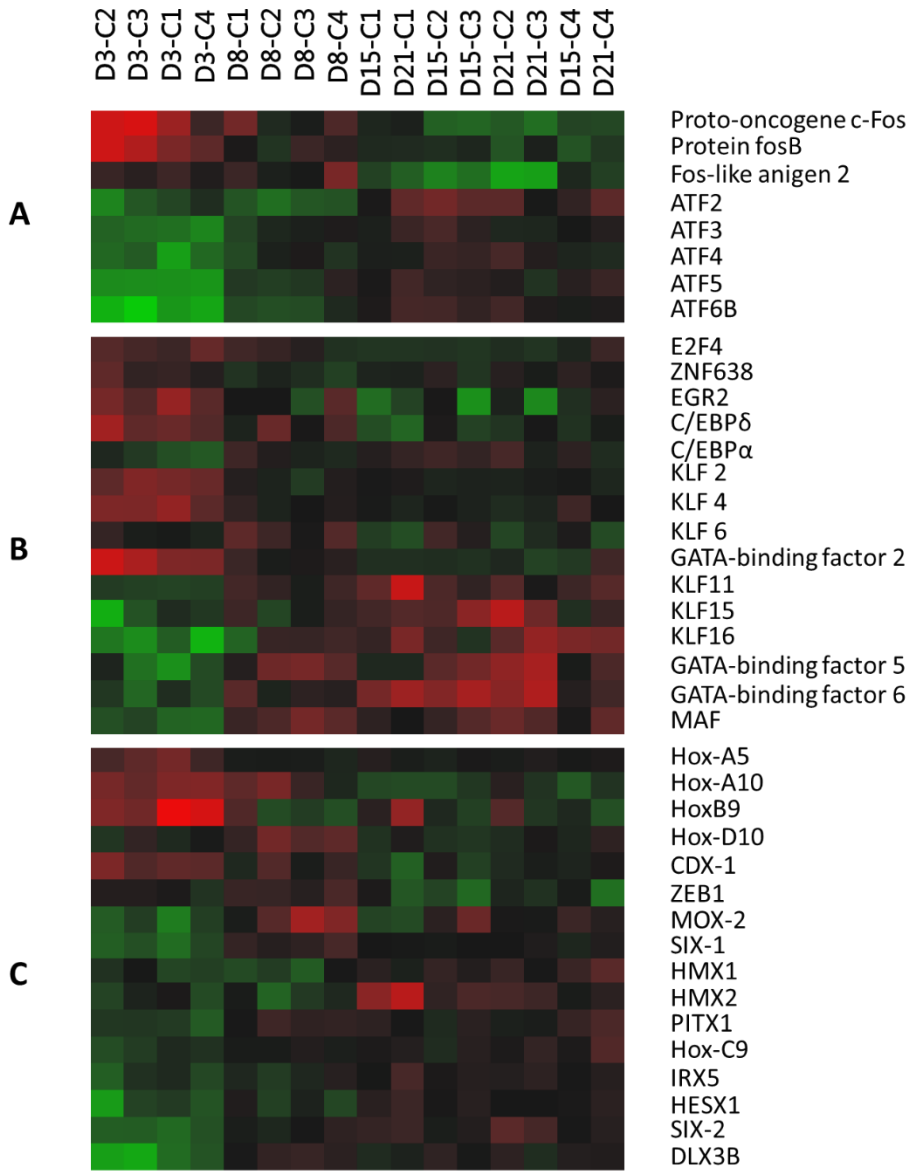

Figure 3. Selected transcriptional regulators overexpressed during rainbow trout adipogenesis. (**A**) AP-1 complex; (**B**) E2F, C/EBP, Krüppel-like factor and GATA families; (**C**) Homeobox containing transcriptional factors.

As previously mentioned, adipocyte development is regulated by a network of multiple transcription factors, and V-maf avian musculoaponeurotic fibrosarcoma oncogene homolog (MAF) is likely to be involved in coordination with other factors (Tsuchiya et al., 2015). The expression of MAF has been reported to increase during human adipogenesis (Bernhard et al., 2013) and this seems to be the case in our culture, since its transcript abundance was increased from day 8 of culture.

2.3 Homeobox genes

Furthermore, with regards to other transcription factors involved in adipogenic differentiation, we also found a large number of transcripts encoding homeobox-containing genes that were differentially regulated (Figure 3C). During the early stages of adipocyte development several Hox family genes, such as HoxA5, HoxA10, HoxB9 and HoxD10 were up-regulated. Hox genes are responsible for embryonic and adult development (Seifert et al., 2015) and even though they have been reported to be involved in the adipogenic process of different model systems (Cantile et al., 2003; Cowherd et al., 1997), their expression pattern differs depending on the localization of fat depots within the human body (Karastergiou et al., 2013). Transcripts from four other homeobox-containing factors were present during the proliferative phase; mesenchyme homeobox 2 (MEOX2), SIX homeobox 1 (SIX1), caudal type homeobox 1 (CDX1) and zinc finger E-box-binding homeobox 1 (ZEB1). Interestingly, ZEB1 has been recently described as a key transcriptional component in the regulation of mouse pre-adipocytes development among other functions (Gubelmann et al., 2014). The second phase of rainbow trout adipogenesis was also characterized by the presence of many transcripts belonging to these groups of proteins. Among them we found up-regulated in the differentiation cluster: H6 family homeobox 1 (HMX1) and 2 (HMX2), paired-like homeodomain 1 (PITX1), homeobox C9 (HoxC9), iroquois homeobox 5 (IRX5), HESX homeobox 1 (HESX1), SIX homeobox 2 (SIX2) and distal-less homeobox 3b (DLX3B). The implication of homeobox transcription factors as relevant players in the regulation of adipose tissue functions have been recently reported (Dankel et al., 2010). The strong enrichment of these kinds of genes during rainbow trout adipogenesis could therefore indicate that they are also relevant molecules regulating adipose tissue development in fish.

All in all, a sequential activation of different transcription factors is present during the transformation of precursor cells into fully differentiated mature adipocytes in rainbow trout. Moreover, the general progression found seems to be very similar to that already described in different mammalian models, although functional studies of some of these factors would reveal possible fish-specific roles during adipogenesis.

3- Epigenetic factors during adipogenesis

The dynamic remodeling of chromatin and its influence on adipogenic gene expression has been previously reported in other cell systems (Mikkelsen et al., 2010; Musri and Parrizas, 2012; Siersbaek et al., 2012). Histone modifications are relevant mechanisms to study since they are known to modulate the transcriptional regulation of factors that govern adipogenesis. However, these kinds of processes have not been explored in fish adipocytes so far.

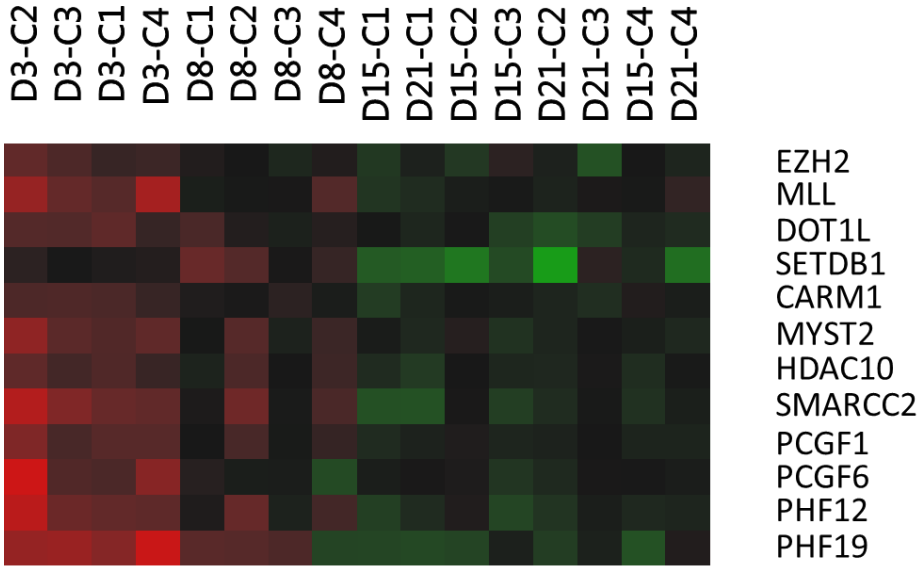

Figure 4. Epigenetic transcriptional regulators overexpressed during the proliferative phase of rainbow trout adipocyte development.

Our results suggest that there is an important link between transcriptional regulation and epigenetic modulation. We have found a large number of up-regulated genes encoding epigenetic transcriptional regulators, especially during the proliferation phase (Figure 4). We detected several histone-lysine N-methyltransferases, such as enhancer of zeste 2 polycomb repressive complex 2 subunit (EZH2), lysine (K)-specific methyltransferase 2A (MLL), DOT1-like histone H3K79 methyltransferase (DOT1L) and SET domain, bifurcated 1 (SETDB1). Surprisingly, SETDB1 is one of the methyltransferases known to inhibit adipogenesis by repressing PPARγ (Takada et al., 2007). On the contrary, EZH2 has been described to present pro-adipogenic activity, since it silences Wnt genes, which are known to be negative regulators of adipogenesis (Wang et al., 2010). Other genes coding for proteins involved in chromatin

remodeling during the first stages of adipocyte development are the histone-arginine methyltransferase CARM1, the histone acetyltransferase MYST2 and the histone deacetylase 10 (HDAC10), SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily c, member 2 (SMARCC2), and members of the Polycomb group (PcG) of proteins such as PCGF1, PCGF6, PHF12 and PHF19.

4- Specific regulatory systems

4.1 Insulin and IGF system

The insulin and IGFs system plays a critical role in mammalian adipocyte models. Insulin, IGF-I and IGF-II expression did not change along the rainbow trout cell culture; however, differential patterns of expression were observed for several IGF binding proteins (IGFBPs). IGF 2 mRNA-binding protein 3 (IGF2BP3), which has been described as a translational activator of IGF-II and further leads to the activation of its immediate downstream effectors promoting proliferation and angiogenesis (Suvasini et al., 2011) was up-regulated during the proliferation phase (Figure 5). IGFBP4 and IGFBP7 were up-regulated during differentiation, while IGFBP5 decreased (Figure 5). IGFBPs can inhibit and/or potentiate IGF actions, depending on the cellular context and experimental conditions (Duan et al., 2010). In vascular smooth muscle cells, when added together with IGF-I, IGFBP4 exerts an inhibitory effect on IGF-I-induced DNA synthesis, while IGFBP5 potentiates the mitogenic effect of IGF-I, which is in concordance with the expression profile of IGFBPs observed in our study. Besides, some IGFBPs such as IGFBP3 and IGFBP5 have been also shown to have intrinsic biological activities that are IGF-independent (Duan and Xu, 2005; Firth and Baxter, 2002). In this sense, IGFBP3 inhibits both 3T3-L1 preadipocyte differentiation initiated by insulin as well as insulin action in differentiated adipocytes. Although no changes in IGFBP3 were observed in our cells, a decline in IGFBP5 was found during the differentiation phase as it has been reported in porcine cells (Baxter and Twigg, 2009).

Moreover, insulin and IGF-I receptors did not show significant variations along the culture but regarding its signaling pathway, insulin receptor substrate 1 (IRS1) was up-regulated during proliferation (Figure 5). IRS-1 and IRS-2 have multiple tyrosine residues, which are used as ‘docking’ sites for downstream signaling molecules and are linked to differential processes (Siddle, 2011). IRS-1 mediates both metabolic and proliferative effects of IGF-I, but its

mitogenic actions are more relevant (Dearth et al., 2006; Siddle, 2011), which agrees with its higher expression during the proliferative phase of our culture compared to the differentiation phase. Several genetically altered mouse models of IGF-I signaling provide clues to understanding the role of this growth factor in adipogenesis. A point mutation in the IRS-1 gene induced growth retardation and reduced the amount of adipose tissue in mice (DeMambro et al., 2010).

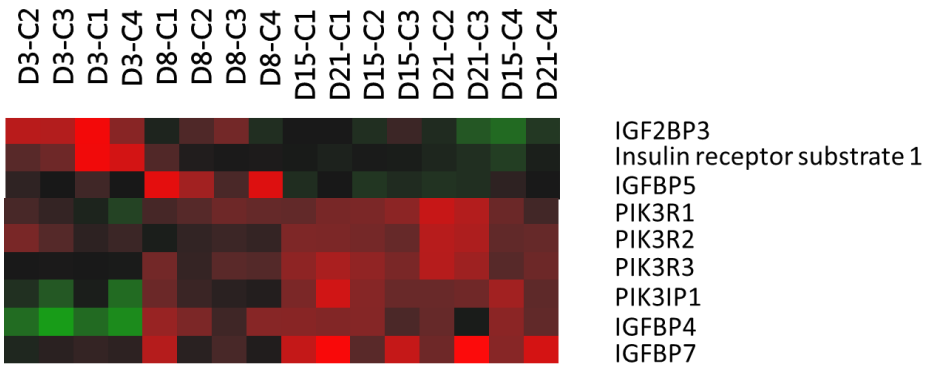

Figure 5. Selected genes involved in the insulin and IGF system overexpressed during rainbow trout adipogenesis.

Key components of the insulin and IGF-I signaling cascade such as phosphatidylinositol 3-kinase regulatory subunits alpha (PIK3R1), beta (PIK3R2), gamma (PIK3R3) and phosphoinositide-3-kinase-interacting protein 1 (PIK3IP1) were up-regulated during differentiation (Figure 5). This regulation agrees with the fact that this pathway activates metabolic processes, abundant during adipocyte maturation in these later phases being for instance key signaling molecules in the stimulation of glucose uptake. Human preadipocytes, as is the case for murine preadipocytes, depend on it to undergo complete differentiation (Aubin et al., 2005).

4.2 Eicosanoid metabolism

Arachidonic acid (AA) is a polyunsaturated omega-6 fatty acid that is released from the membrane phospholipids by the activity of the cytosolic phospholipase A2 (cPLA2). Then, free AA can be metabolized to eicosanoids through three major routes: the lipoxygenase (LOX), the

cyclooxygenase (COX) and the cytochrome P450 (CYP) pathways. These signalling pathways are relevant for adipocyte development, since some of its products have been reported to activate lipid biosynthesis during the acquisition of the adipocyte phenotype (Polus et al., 2015) and are implicated in other functions related to immune response and inflammation. In line with this, the relevance of the activation of different immune pathways during Atlantic salmon adipogenesis has been previously reported (Todorčević et al., 2010). During the early steps of adipocyte culture we found up-regulated different genes encoding members of the PLA2 class of enzymes (Figure 6A), such as PLA2G4A and PLA2G1B, pointing out to the early implication of eicosanoid metabolism in the process of rainbow trout adipocyte development. This is in agreement with the results from the functional analysis performed, where the eicosanoid signaling pathway was highlighted during the proliferative phase of the culture. In mammals, eicosanoids are known to serve as natural ligands to activate PPARs (Grygiel-Górniak, 2014); whether fish adipocytes activate the eicosanoid metabolic pathway to also regulate PPARγ remains unknown.

A critical function of LOX activity in the early steps of mammalian adipocyte development has been reported, since there are enough evidences supporting that LOX-derived endogenous products might be also ligands involved in the transactivation of PPARγ (Madsen et al., 2003). Interestingly, and in agreement with these findings, the search of LOX genes in the microarray data only presented results during the proliferative phase. In the transcriptome data we found an up-regulation of the genes arachidonate lipoxygenases ALOX5, ALOX15b and ALOX12 during days 3 and 8 of the culture (Figure 6A); all of which have been reported to be implicated in mammalian development of the adipocyte (Kuhn et al., 2015).

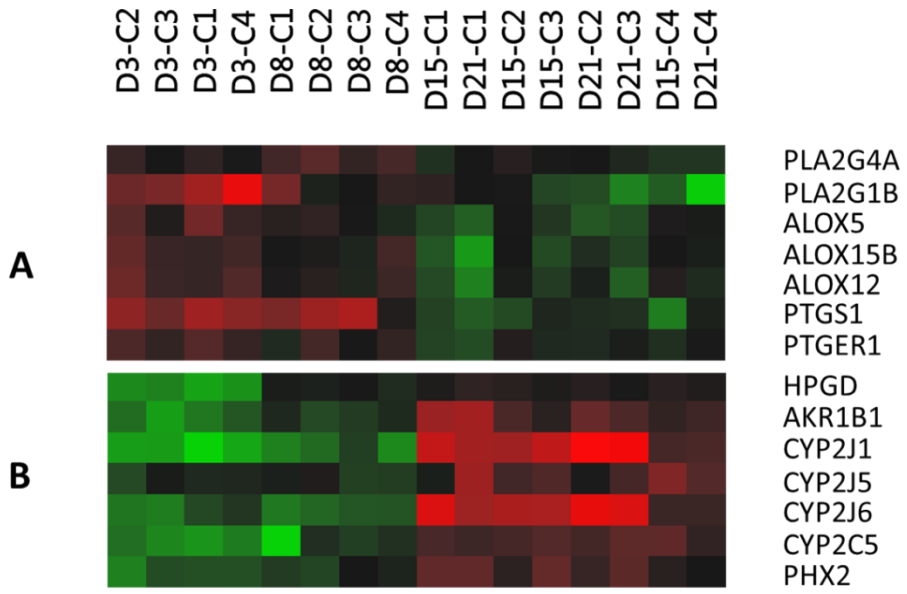

Figure 6. Selected genes involved in eicosanoid metabolism. (**A**) Genes overexpressed during the proliferative phase; (**B**) Genes over expressed during the differentiation phase.

AA metabolites from the COX pathway formed by 3T3-L1 adipocytes have been reported to regulate the progression of adipogenesis through an autocrine control mechanism (Shillabeer et al., 1998; Yan et al., 2003). The identification of several genes involved in the COX pathway exhibiting significant changes in expression during rainbow trout adipogenesis is stressing the possible implication of this pathway in the adipocyte maturation process. Thus, in the cluster associated with proliferation we found up-regulated prostaglandin G/H synthase 1 (PTGS1; also known as COX1) and prostaglandin E2 receptor EP1 subtype (PTGER1) (Figure 6A). During the differentiation phase other genes implicated in the metabolism of prostaglandins were present, such as 15-hydroxyprostaglandin dehydrogenase [NAD+] (HPGD) and the aldose reductase AKR1B1 (Figure 6B), as it was recently shown in human multipotent adipose-derived stem cells (Pastel et al., 2015).

In the CYP pathway, AA is converted to epoxyeicosatrienoic acids (EETs) and 20-HETE by CYP epoxygenases and CYP ω-hydroxylases, respectively (Tacconelli and Patrignani, 2014). EET biosynthesis can be accomplished by different P-450 isozymes, being the members of the CYP2J and CYP2C epoxygenases of especial relevance in this process. Then, EETs are rapidly hydrolyzed by soluble epoxide hydrolase (EPHX2) to form less active dihydroxyeicosatrienoic acids (DHETs). In this sense, the microarray data revealed a remarkable presence of different

members of these genes overexpressed during the differentiation phase such as CYP2J1, CYP2J5, CYP2J6, CYP2C5 and EPHX2 among many others (Figure 6B). A dysregulation of the CYP epoxygenase pathway has been recently reported to be a pathological consequence of obesity (Zha et al., 2014), emphasizing the importance of the bioavailability of EETs for a healthy and functional adipose tissue.

5- Terminal differentiation regulators

As soon as the cells start to present an adipocyte-like phenotype (days 15 and 21), a remarkable number of genes critical for many aspects of carbohydrate and lipid metabolism were overexpressed in comparison with previous stages (days 3 and 8). Regarding the activation of the carbohydrate metabolism, we identified and selected for further validation the gene encoding one of the main enzymes controlling gluconeogenesis; phosphoenolpyruvate carboxykinase 1 (PEPCK). Some other selected genes were also tested by pPCR (see section 6). The development of one of the main functions of adipocytes, which is its high capacity to store energy, was highlighted by the large presence of genes involved in metabolic trapping, glycogen synthesis, the pentose phosphate pathway, glycolysis, oxidative phosphorylation and all aspects of lipogenesis. On the other hand, the differentiation phase was also characterized by up-regulation of genes required for regulating the mobilization of stored energy, corroborating one of the basic roles of adipocyte; to work as a buffer tissue storing and providing energy depending on body demands.

Alongside the development of the machinery involved in lipid and carbohydrate metabolism, the activation of the thyroid-receptor/retinoic X receptor (TR/RXR) and the PPAR signalling pathways was observed during the differentiation phase (Additional file 3). Both, PPARs and TRs exert their activity partly by heterodimerization with RXR and they are known to play key roles in lipid mobilization, lipid degradation, fatty acid oxidation, and glucose metabolism (Moreno et al., 2010). In mammals, PPARs and TRs can crosstalk to regulate different cellular processes including adipogenesis (Lu and Cheng, 2010). The functional annotation analysis in the present study suggests that this might also be the case for rainbow trout adipocytes.

5.1 Fatty acid transport and oxidation during adipocyte maturation

The expression of proteins involved in fatty acid transport were mainly expressed during differentiation. We observed a high representation from the fatty acid binding protein family (FABPs) (Figure 7A). Only FABP2 was up-regulated during proliferation, whilst FABP1, FABP3, FABP4 (which is predominantly expressed in adipose tissue), FABP6 and FABP7 were all up-regulated during differentiation. The increasing levels of FABPs points out to the mobilization of fatty acids to mitochondria and peroxisomes for energy production. In mammals, FABPs are recognized as important signalling molecules with effects not only on the systemic energy metabolism but also on inflammatory processes (Storch and Thumser, 2010). In this sense, FABP4 acts as an adipokine regulating adipocyte and macrophage interactions during inflammation (Thumser et al., 2014). FABP3 transcripts have been predominantly detected in subcutaneous adipocytes (Weil et al., 2009) and intra-peritoneal adipose tissue of rainbow trout (Kondo et al., 2011). The high presence of FABPs suggests an important role for this family of proteins during the maturation process of fish adipocytes. In mammals, it has been reported that some FABPs significantly cooperate with PPARs (Tan et al., 2002). Further studies focusing on the mechanisms of action of these proteins should be performed in fish in order to determine their function in the adipocyte biology.

After transport, fatty acids can be activated to the respective acyl-CoA esters by one of the acyl-CoA synthetases. In this sense, we found a strong enrichment of genes encoding several members of long chain fatty acid acyl-CoA synthetases in the differentiation cluster (Figure 7B), such as long-chain-fatty-acid-CoA ligases ACSL1, ACSL4 and ACSL6, as well as the long-chain fatty acid transport protein 3 (SLC27A3). The presence of different ACSL isoforms might be essential for adipocyte function since they assure the channelling of fatty acids towards degradation or lipid biosynthesis depending on the energetic status of the cell.

A salient feature of the differentiation cluster was the presence of many genes encoding enzymes responsible of catalysing different steps along the triglyceride biosynthetic pathway (Figure 7C). Such is the case for several members of the 1-acylglycerol-3-phosphate O-acyltransferase family (AGPAT4, AGPAT5 and AGPAT9) and the diacylglycerol O-acyltransferase 1 (DGAT1). In addition, genes involved in the production of reducing power for the synthesis of lipids, such as glucose-6 phosphate dehydrogenase (G6PDH) and isocitrate dehydrogenase (ICDH) were also present.

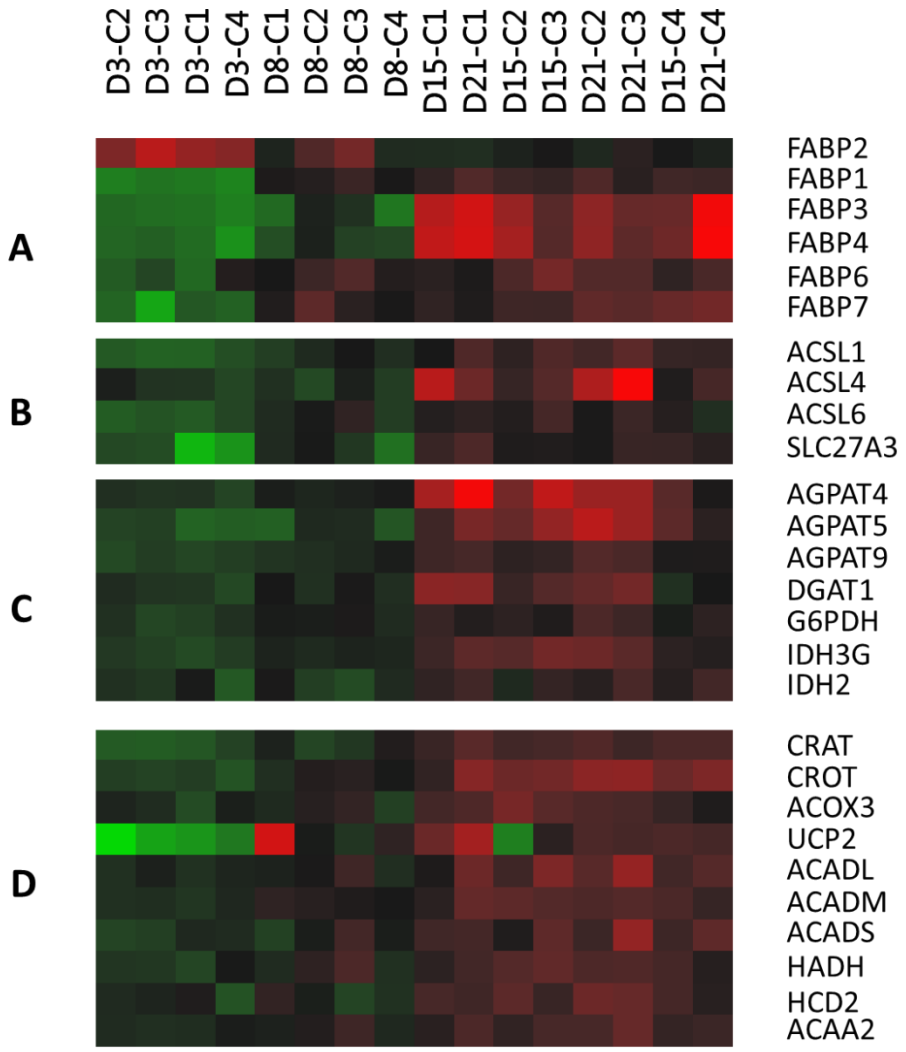

Figure 7. Selected genes involved in lipid metabolism. (**A**) Fatty acid transporters; (**B**) Acyl-CoA synthases; (**C**) Genes coding proteins involved in the synthesis of lipids; (**D**) Genes involved in energy production.

The activation of mechanisms responsible of providing energy to the adipocyte were also evidenced during the last stages of culture development (Figure 7D). In this sense, proteins involved in the oxidation of fatty acids were present in the differentiation cluster, such as carnitine O-acetyltransferase (CRAT) and carnitine O-octanoyltransferase (CROT). These two enzymes convert the end products of the peroxisomal β-oxidation (C8 or C6-CoA) to acylcarnitines so they can be transported out of the peroxisomes and be further oxidized to acetyl-CoA in the mitochondria. We also found up-regulated during differentiation the expression of peroxisomal acyl-coenzyme A oxidase 3 (ACOX3), which is involved in the desaturation of 2-methyl branched fatty acids in peroxisomes. The expression of ACOX3 has been linked to a possible pathway for metabolism of phytanic acid and pristanic acid in

peroxisomes in white adipose tissue (Hunt et al., 2012). In mammals it is known that β-oxidation of fatty acids can take place in both, mitochondria and peroxisomes, serving different functions in the cell (Violante et al., 2013). The transcript abundance of other protein involved in lipid oxidation, like the mitochondrial uncoupling protein 2 (UCP2), was increased from day 8. This protein reduces the ATP yield and may facilitate the oxidation of fatty acids (Cabrero et al., 2001). Many other genes involved in mitochondrial fatty acid beta-oxidation were present, such as acyl-CoA dehydrogenase, long chain (ACADL), acyl-CoA dehydrogenase, C-4 to C-12 straight chain (ACADM), acyl-CoA dehydrogenase, C-2 to C-3 short chain (ACADS), hydroxyacyl-CoA dehydrogenase (HADH), 3-hydroxyacyl-CoA dehydrogenase type-2 (HCD2) and acetyl-CoA acyltransferase 2 (ACAA2) among many others.

Overall, according to the functional characteristics of the mature adipocyte, genes involved in fatty acid transport, lipid synthesis and oxidation were the most relevant in late differentiation phase. The activation of diverse genes with redundant functions might be directed to provide different mechanisms to accomplish and ensure the proper function of mature differentiated adipocytes.

5.2 Lipid droplet formation

Mature white adipocytes are characterized by the presence of a large unilocular lipid droplet that occupies the majority of the cell. A critical aspect of adipocyte development is the formation and expansion of such lipid droplet within the cell. The storage of triglycerides within the lipid droplet allows the correct expansion of the adipose tissue while preventing lipotoxicity in other organs (Rutkowski et al., 2015). Therefore, lipid droplets are considered to be crucial organelles in the regulation of energy homeostasis and in the prevention of insulin resistance (Konige et al., 2014). In the differentiation cluster we found up-regulated some of the well-known structural proteins that constitute the surface of the lipid droplets (Figure 8A). Such is the case of the fat-specific protein 27/Cidec (FSP27) and Cavelin-1. Even though these proteins are not considered to be essential for adipocyte differentiation, their key role in lipid droplet formation has been recognized and thus also, in the acquisition of the adipocyte phenotype (Keller et al., 2008; Le Lay et al., 2009). Apart from their importance as structural proteins they have been described to play an important role in insulin signalling (Konige et al., 2014; Lafontan, 2014). Moreover, we also found highly expressed transcripts of the vesicle-associated membrane protein 4 (VAMP4), a member of the SNARE family known to be involved in the

growth of lipid droplets via fusion (Bostrom et al., 2007). Another protein that has been recently associated to lipid droplets, the microsomal triglyceride transfer protein (MTP), was also present in the differentiation cluster. This protein, which is known to be essential for lipid transport, has been reported to be present in the same droplets as perilipin 2 (PLIN2; ADRP) (Love et al., 2015). Our results showed that PLIN2 exhibited the same pattern of expression as MTP, with significantly higher abundance of transcripts present during the differentiation phase of rainbow trout adipocytes. The study of specific mechanisms concerning the formation and turnover of lipid droplets would be of high value in order to elucidate the function of different associated proteins in lipid droplet biology in fish adipocytes.

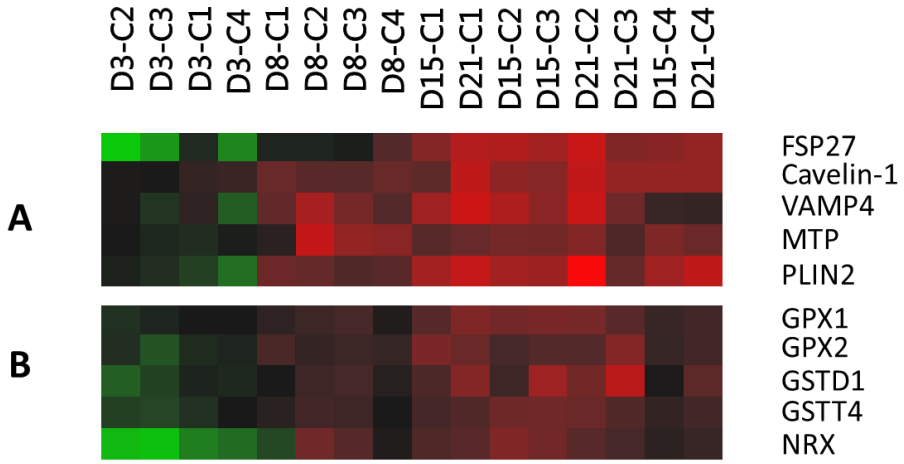

Figure 8. Selected genes highlighted during the terminal phase of adipocyte development. (**A**) Lipid droplet formation; (**B**) Antioxidant system.

5.3 Redox homeostasis

Adipocyte expansion and consequently lipid droplet growth, represents a healthy way of disposing excess of fat in the form of neutral lipids. Even though a continuous or extreme expansion can represent a challenge to adipocytes leading to the production of reactive oxygen species and endoplasmic reticulum stress (Rutkowski et al., 2015), a moderate generation of reactive oxygen species has been described as a factor promoting adipocyte differentiation (Tormos et al., 2011). Therefore, the control of redox homeostasis in the intracellular environment is key for adipogenesis to progress. In line with this, we identified few genes encoding antioxidant enzymes (Figure 8B), such as glutathione peroxidase 1 and 2 (GPX1 and

GPX2), glutathione S-transferase 1, isoform D (GSTD1) and glutathione S-transferase theta-4 (GSTT4), all of them induced during the lipid-loading phase. The modulation of intracellular glutathione has been reported to affect adipogenesis in 3T3-L1 cells (Vigilanza et al., 2011). In addition, the importance of the activation of the gluthation-based antioxidant system in Atlantic salmon adipocytes has been recently highlighted (Todorčević et al., 2010). Likewise, nucleoredoxin (NRX), a member of the thioredoxin family of proteins known to control redox homeostasis in the cell, followed the same regulation in rainbow trout adipocytes. Interestingly, NRX has recently been described as a novel pro-adipogenic factor (Bahn et al., 2015).

6- Temporal profile analysis of mRNA (qPCR) and protein expression

The expression profile along the culture of some selected genes was tested using qPCR (Figure 9). The genes were selected on the basis of their novelty in fish or their relevance and implication in the already described mammalian adipogenic process. The levels of protein of some of them were also tested, in order to assess whether the protein encoded by a specific mRNA and its expression showed a similar profile (Figure 10). Both qPCR and Western blot analysis reinforced the data obtained in the microarray analysis. The high proliferative capacity of the cells during the early stages determined by the gene expression pattern of proliferating cell nuclear antigen (PCNA), which had its highest peak in the transcriptome at day 3 of rainbow trout adipocyte development, was further reinforced by qPCR and Western blot analysis. Likewise, the maturation state of the cells was confirmed by both, microarray and qPCR measures of glycerol-3-phosphate dehydrogenase (GPDH). In addition, the transcript abundance of two IGFBPs aforementioned, IGFBP5 and IGFBP7, were analyzed by qPCR. Despite of the lack of significance, IGFBP5 followed the same pattern as the one observed in the microarray, while the mRNA of IGFBP7 remained fairly static along the culture according to qPCR results. Moreover, two genes representing the development of lipid and carbohydrate metabolism, ACSL1 and PEPCK respectively, were assessed as well by qPCR and Western blot, confirming the specialization of the cells towards mature adipocytes showing higher levels of expression at later days and validating the microarray results. In addition, PLIN2 was also evaluated through all three techniques, exhibiting a higher abundance during the differentiation phase and suggesting thus the involvement of this structural protein in the maturation process of our cell system. It should be noted that, as far as we know, no member from the perilipin family has been characterized in fish until now, therefore the expression and function of this

gene deserves further studies. On the other hand, the protein levels of PPARγ, which is considered the master regulator of adipogenesis, were significantly increased from day 8 and maintained up to day 21. Therefore confirming the relevance of the PPAR signaling pathway during the differentiation phase revealed by the transcriptomic analysis.

Likewise, as we have described in the present paper, many other key molecules known to be involved in the adipogenic process of mammalian preadipocyte cell lines, also changed in the same direction in our culture system. Therefore, these kinds of approaches can be seen as an extra validation of the microarray results.

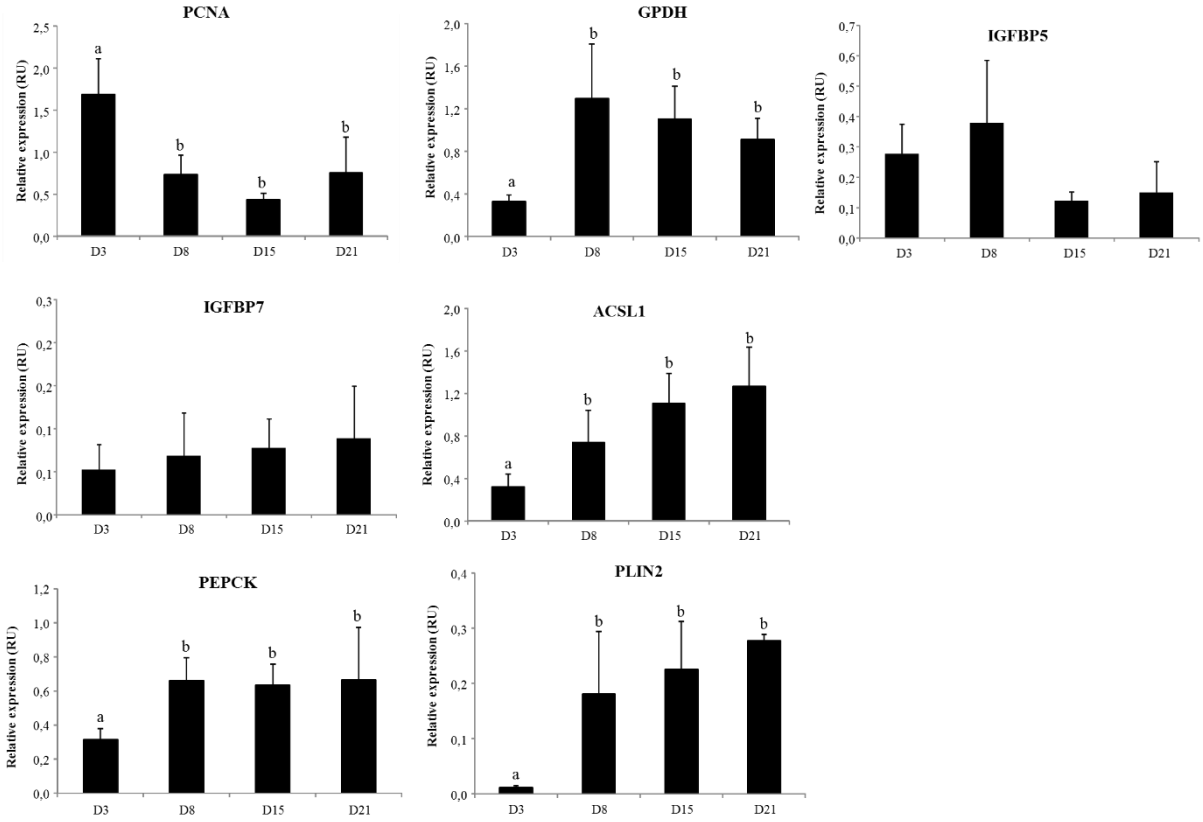

Figure 9. Relative changes in transcript levels of selected genes during trout adipocyte differentiation (days 3, 8, 15 and 21) assessed by real-time qPCR. Transcript abundance is presented as fold change ± S.E.M (n= 4) using 18S as a reference gene (delta-delta method). (RU): relative units. Values not sharing letters are significantly different ($P < 0.05$, ANOVA followed by Tukey's test).

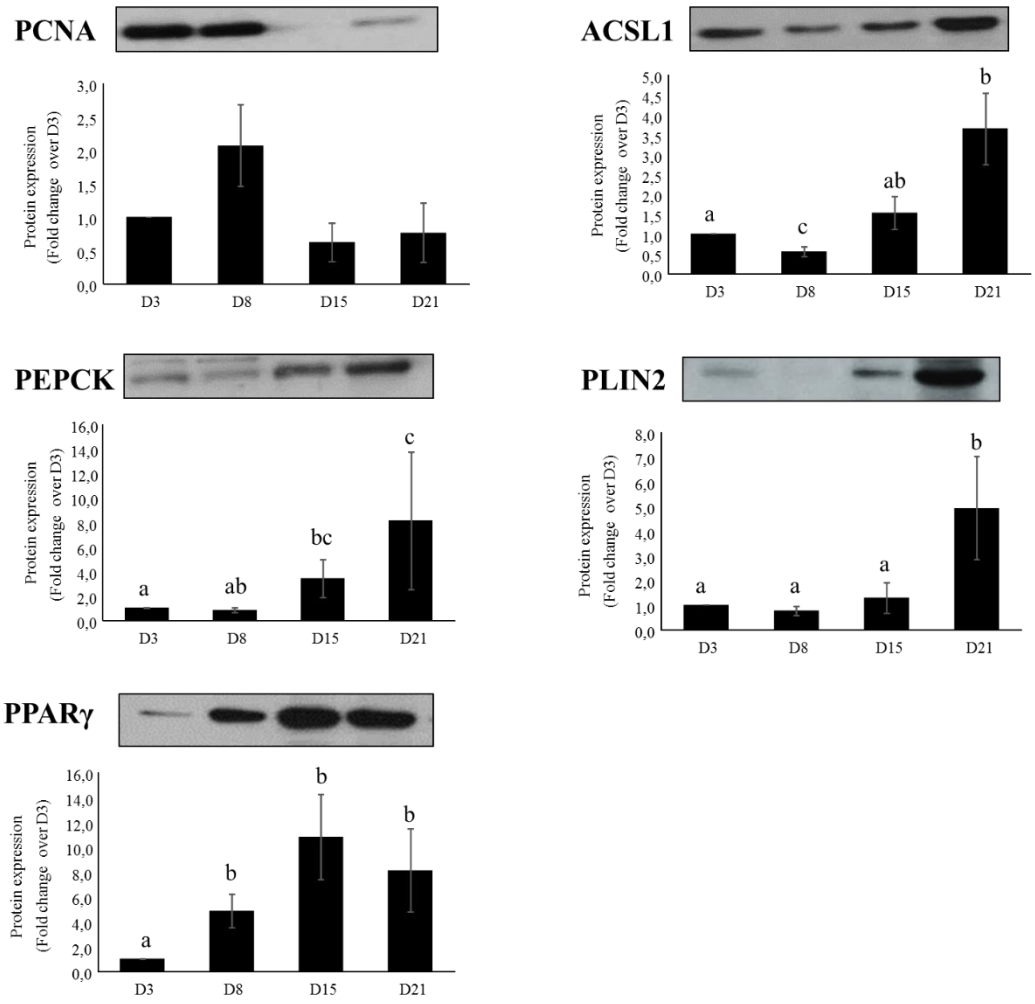

Figure 10. Relative changes in content of selected proteins during rainbow trout adipocyte differentiation (days 3, 8, 15 and 21) assessed by Western blot analysis. Protein abundance (n= 4) was performed as described in the materials and methods section. A representative blot is shown.

4. CONCLUDING REMARKS

This study was performed in order to gain a comprehensive view of the regulatory mechanisms involved in the process of fat cell development in fish. Our cell system presents a high similarity with the already described process of adipocyte differentiation in mammalian models, with abundance of several of the pro-adipogenic transcription factors and many of the mature adipocyte related genes. As it is evidenced by the large number of genes transcriptionally regulated in this study, fish adipogenesis is a complex and tightly coordinated process.

In mammals, adipocyte dysregulation has been linked to the development of metabolic diseases and therefore remarkable efforts are being done to understand the intricacies of adipogenesis.

Many aspects concerning the biology of fish adipose tissue and their contribution to the balance of the overall energy homeostasis remain unexplored. This study provides an important base for further research. However, functional approaches will be needed in order to clarify the role of the genes highlighted in the present work.

ADDITIONAL MATERIAL

Additional File 1: Significant biological functions associated with the proliferative phase of adipogenesis determined by IPA software (data presented in a supplementary CD).

Additional File 2: Significant biological functions associated with the proliferative phase of adipogenesis determined by DAVID software (data presented in a supplementary CD).

Additional File 3: Significant biological functions associated with the differentiation phase of adipogenesis determined by IPA software (data presented in a supplementary CD).

Additional File 4: Significant biological functions associated with the differentiation phase of adipogenesis determined by DAVID software (data presented in a supplementary CD).

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