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In vivo and *in vitro* effects of antioxidant compounds and PPAR γ modulators on rainbow trout oxidative stress and lipid metabolism

Tesi di laurea in:

Adattamenti dei vertebrati all'ambiente marino

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1. Introduction

The amount of fish killed for human feeding and the consumer demand for fish are increasing each year rapidly, as well as the world's human population. The populations of wild fish are decreasing because of overfishing and, consequently, the number of farmed fish has increased greatly during the last decades. From previously answering for 9% of the total of fish killed for human consumption in the middle of the 1980s, aquaculture system produced 46% of the eaten fish in 2008 (Food and Agriculture Organization of the United Nations, 2010), although this percentage is lower in the developing countries. The rising consumer demand for high quality fish meat, as well as the increasing public concern for fish welfare and environmental sustainability, had led to a boom of demand for improvements of fish farm practices (Lambooij et al, 2004). Besides, the increasing public alarm concerning fish wellness in aquaculture (Huntingford et al, 2006), has resulted in a substantial amount of research during the last decade. Aquaculture production has risen by almost 12-fold in the last three decades (FAO, 2012). Aquafeed is based on fish meal and fish oil because of their high nutritional quality; however, the sustainability of this practice is not occurring at present, especially over the long term (Watanabe, 2002). Moreover, in order to reduce aquafeed costs and decrease 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 considered and approved as a sustainable alternative to fish products (Bouraoui et al., 2011; Nasopoulou and Zabetakis, 2012); nevertheless, proteins continue to be the most expensive nutrients 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 increase in fat depots or even physiological alterations such as induction of oxidative stress (Kiaer et al., 2008). Some aquaculture practices, such as the employment of hyperlipidic diets or new raw dietary materials, in synergism with the moderate swimming activity of reared fish, might have negative effects, contributing to reduce fish growth and animal welfare and

increasing visceral adiposity (Benedito-Palos et al., 2008; Montero et al., 2003). Fat increment can induce precocious sexual maturation, alterations in lipid metabolism, as well as a change in the fatty acid profile of the flesh (Cruz-Garcia et al., 2011; Einen and Skrede, 1998). Fat mass increase might be due either to hypertrophy (build up in size of existing adipocytes) or to hyperplasia (rise in cell number by proliferation of precursor cells). Fish oil is the most common source of lipid used in fish feeding, in particular for carnivorous species. Many favourable characteristics contribute to this massive use of fish oil in aquaculture: in fact it is the best source of digestible energy, it is readily palatable by the farmed fish, it contains essential fatty acids, it does not affect flesh composition and taste of the flesh and can spare protein utilization to varying degrees (Sargent et al., 2002; Olivia-Teles, 2012). The falling in global fish oil availability, from 1.5 million tons in 1994 to 1.07 million tons in 2009 (FAO, 2012), due to the depletion of world fisheries resources, environmental concerns related to fish oil production, expansion in global aquaculture production and the development of fish oil-based tablets for human consumption, during the last two decades, concur with the rise in fish oil cost (Klinger et al., 2012; FAO, 2012; NRC, 2011). At present, a rising proportion of fish for human nutrition comes from aquaculture, where formulation of feeds with fish meal and fish oil ensures n-3 LC-PUFA (Long Chain Polyunsaturated Fatty Acid) rich meat production. Together with the expansion of aquaculture, the demand for this marine products also increases, but global supply has stagnated and the reduction of fishery that provides it is considered unsustainable and over-exploitative of natural resources (Naylor et al., 2000). When addressing the imminent deficiency, replacement of fish oil by vegetable oils challenges the maintenance of the recognized health benefits due to n-3 LC-PUFA content. Several studies have shown that complete or partial substitution of fish oil with single vegetable oil such as rapeseed oil, linseed oil, palm oil or with a vegetable oil blend has reduced effect on growth but marked consequences on lipid metabolism, tissue lipid composition and correlated factors such as digestibility, fatty acid catabolism, lipid transport and uptake, lipogenesis, fatty acid desaturation and elongation and eicosanoid synthesis

(Turchini et al., 2009; Leaver et al., 2008; Panserat et al., 2008). Overall, dietary fatty acid composition is reflected in the flesh and lipid stores of the fish. However, the magnitude of the change is dependent on the species and fatness deposition of tissue (Turchini et al., 2009). Aquaculture's dependence on marine oils is considered as one of the major constraints in determining the sector's role in fisheries conservation (Naylor et al., 2009). In this way, in addition with economical problems, it is not strange that fish oil replacement is a major issue of focus in farmed fish nutrition research and development (Turchini et al., 2009). A different multitude of chemical, biochemical and physiological effects of the introduction of alternative dietary lipid sources for farmed fish have been intensively studied over the past two decades (Turchini et al., 2010). Apart from this, there is very little information about the potential effects of different dietary lipid sources on lipid peroxidation and the consequential formation of DNA-reactive compounds, which can significantly affect overall fish health. Many studies in mammals have underlined and revealed the main role of oxidative stress and lipid peroxidation in carcinogenesis and that DNA adducts generated by lipid peroxides are associated with an inflammatory process in their etiopathogenesis (Blair, 2008; Cooke et al., 2003; Hagenlocher et al., 2001; Medeiros, 2009; Nair et al., 1997; Nair et al., 2007). To limit lipid peroxidation, all organisms which practice aerobiosis have got two types of antioxidant defense systems: free radical scavengers, usually of low molecular weight, and antioxidant enzymes (Halliwell and Gutteridge, 2007). These antioxidant enzymes are active during the overall development of *O. mykiss* and are modulated by feeding moderate levels of oxidized lipid (Fontagnè et al., 2008). Early stages of fish's development seem to be particularly sensitive to oxidative stress caused by the overpowering effects of prooxidants on antioxidant defenses of cells that can induce alteration in development (Boglione et al., 2013; Fontagnè et al., 2006; Hata and Kaneda, 1980; Lewis –McCrea and Lall, 2007, 2010). Early life stages of fish also need a dietary phospholipid supply for good development (Boglione et al., 2013; Cahu et al., 2009; Tocher et al., 2008). It has been shown that a diet rich in phospholipids plays an active role in absorption of neutral lipids in fish, even if phospholipids are also known to exert

an antioxidant effect on different oils and fats, due not only to the side-chain amino group but also to the conjunct effect of the hydroxyl group in the side-chain (Tocher et al., 2008). Some studies have demonstrated the efficiency of single and combined treatments with natural antioxidants in the inhibition of lipid oxidation of fish oil and muscle (Fagbenro and Jaucey, 1994; Pazos, Alonso, Fernández-Bolaños, Torres and Medina, 2006; Pérez-Mateos, Lanier and Boyd, 2005; Shahidi and Naczk, 1995; Tang, Sheehan, Buckley, Morrissey and Kerry, 2001). In addition to the ongoing debate about the use of synthetic food additives, such results have lead to the introduction of plant extracts as antioxidants in food that contains fish oil, and this fact became a common practice in the last years. Antioxidants are naturally found in combination with other compounds usually in the form of esters. Similarly to other phenolic compounds, they can directly trap free radicals or scavenge them through a series of coupled reactions with antioxidant enzymes, in this way delaying the starting of lipid oxidation via the decomposition of hydroperoxides (Lewis, 1993). In the past, dietary fish content in fish nutrition was limited by the production technology and pelletized feeds of 1970s-1980s contained dietary lipids ranging between 8% and 15% (Johnsen and Wandsvik, 1991). A major trend for commercial diets for salmonids has been the development of extruded high-nutrient or high-energy diet which have shifted the upper limit of fat content in dry feed to 20-30% (Cho and Cowey, 1991; Green et al., 2002; Karalazos et al., 2007), and even 47% (Hemre and Sandnes, 1999). This kind of diet has got many advantages, which include better growth, feed utilization, nitrogen (protein) retention and reduced nutrient discharges in the environment (Alstead, 1991; Cho, 1992; Hillestad and Johnsen, 1994) and protein sparing effect (Sargent et al., 2002). In every case, there is a strong relationship between high dietary lipid levels and undesirable metabolic consequences beyond limited dietary protein utilization as source of energy. Such consequences include growth delay due to reduced feed consumption (Daniels and Robinson, 1986; Ellis and Reigh, 1991; Page and Andrews, 1973; Watanabe, 1982), increase in body lipid deposition and its effect on carcass quality (Bromley, 1980; Hillestad and Johnsen, 1994).

1.1 Anatomy, biology and habitat of the Rainbow trout (*Oncorhynchus mykiss*)

Considerable attention has been focused on maximixing the production of farmed salmonids at minimal cost even if, in recent years, there has been a rising care on ways in which the food quality attributes of reared fish can be manipulated (Haard, 1992; Shearer, 1994). The rainbow trout, *Oncorhynchus mykiss* (Walbaum, 1792), is a salmonid species that is widely spreaded because of its introduction into many countries for farming and sport fishing purposes. Such is the economic importance of the rainbow trout that entire symposia have been devoted to discussions of the biology and cultivation of the species (e.g. Gall, 1992).

The rainbow trout (**Class**: Actinopterygii; **Order**: Salmoniformes; **Family**: Salmonidae; **Genus**: *Oncorhynchus*; **Species**: *O. mykiss*) (Fig. 1) is a member of the Pacific trout and salmon subgroup of the salmon family which also includes the redband trout, the cutthrout trout and the golden trout. The name of this species means ‘’hooked snout’’ refering to the distinctly hooked upper or lower jaw of mature males.

Fig. 1. Image of the *O. mykiss* (Source: C. Griffiths).

Rainbow trout has a fusiform body shape that is usually olive-green dorsaly with brassy to purple iridescence; back and sides are marked with dark olive to black spots, even if this fish is highly variable in colour. The dorsal, adipose and

caudal fins are light olive to amber marked with dark coloured spots. Lower fins are usually pale shades of amber, orange, red, purple or gray while the anal and pelvic fins are often white tipped. There are radiating rows of black spots on the caudal fin and a pink to red stripe on the side. The upper jaw reaches barely behind the eye in young and female individuals but well behind the eye in large males. The largest rainbow trout ever captured was 1.22 m and 16.3 Kg (GISD, 2012); the average river *O. mykiss* in South Africa is approximately 0.5 m of lenght and 1.5 Kg of weight (Picker and Griffiths, 2011). *O. mykiss* is a fish living in cold water native to North America from Alaska to Mexico (Fig. 2): it is mainly a freshwater species which need of high quality, unpolluted and well-oxygenated water to survive.

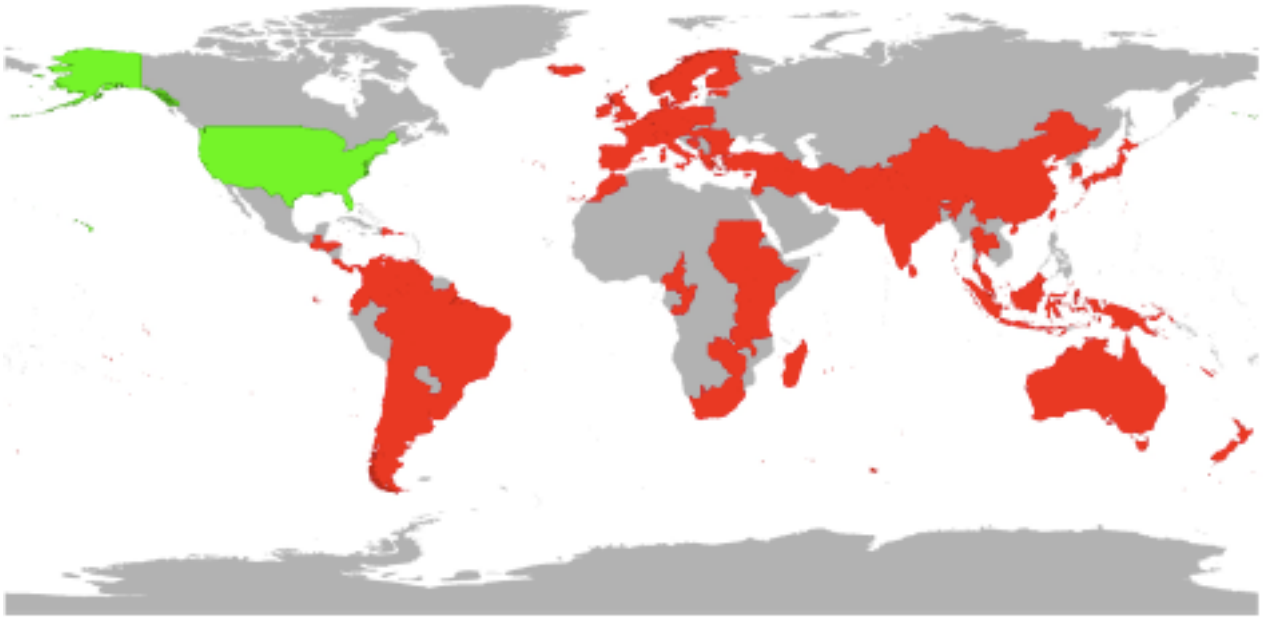

Fig. 2. Native (green) and introduced (red) distribution of *O. mykiss* in the world (Source: GISD, 2012).

Some rainbow trout populations are migratory and spend the majority of their life in seawater, returning to freshwater just to breed; for this cause they have an anadromous life cicle. About the life span of wild rainbow trout, maturation starts before they are one year old with average survival of three to four years; the maximum known longevity is eleven years. Because of its anadromous nature, rainbow trout can survive in a range of salinity from 0 to 35‰ (Molony, 2001)

and dissolved oxygen levels seem to be the major limiting factor for this species, with the minimum O_2 concentration estimated in 2.5 mg/L (Rowe and Chisnall, 1995). Wild individuals of *O. mykiss* typically spawn during the late winter and spring with a peak in March and April when water temperatures are between 10 and 15°C. Spawning actitvity occurs once a year and it is usually induced by increasing day lenght, water flow and water temperature. Females of *O. mykiss* are able to produce 2000 eggs/Kg of body weight. Rainbow trout are opportunistic feeders that prey on invertebrates, small fish, fish eggs and aquatic insects, although, in the wild, *O. mykiss* eat freshwater shrimps, responsible for the fish's pink flesh. Rainbow trout was first farmed in 1879 in California. After, during the 19th and 20th centuries, this species spread across the world and today aquaculture of *O. mykiss* is more popular in its introduced range than in its native one (Vandeputte et al., 2008). In the 1950s, farming of rainbow trout expanded with the diffusion of feed in a pelleted form. At present, the majority of the farming is in the form of extensive monoculture. The main standards on which this kind of aquaculture is based is the availability of good quality and well aerated water, so that trouts can be reared in the following systems: 1) in concrete raceways or ponds with a continuous supply of flowing river water, 2) in tanks connected to recirculating systems or 3) in cages that are usually anchored in dams (FAO, 2012). *O. mykiss* has been introduced to almost all continents, except Antarctica, for the purpose of angling and aquaculture (FAO, 2012) and this introduction has taken place in at least 87 countries (GISD, 2012) (Fig. 2). The incredible spreading of trout aquaculture is helped by easy production and technological advances in culture techniques, allowing trout farming to be possible in almost every environment with a suitable supply of cold clean water. Because of rainbow trout spreading and popularity, it has been classified and listed as one of the World's Worst 100 Invasive Alien Species (GISD, 2012) and this list has been compiled by the Global Invasive Species Database and La Fondation TOTAL d'Enterprise in collaboration with the International Union for Conservation of Nature (IUCN) and the Invasive Species Specialist Group (ISSG).

1.2 Metabolism and functions of adipose tissue in Teleost fish

The term ‘’lipid’’ is used referring to a wide and miscellaneous kind of substances which are grouped together because of their high solubility in non-polar solvents or their similarities to them. Lipids usually contain fatty acids esterified to alcohol groups in the case of glycerides and to amino groups in the case of sphingolipids. Animal lipids, including fish lipids, can be divided into two groups, polar lipids, composed mainly of phospholipids, and neutral lipids composed principally of triacylglycerol (triglycerides). At ambient temperature, triacylglycerols can be either solid, in which case they can be named fats, or liquid, in which case they can be termed oils. Fish triacylglycerols are invariably oils. The most part of lipid compounds in eukaryotes organisms derives from acetyl-CoA and can be part of one of the three main classes of substances: 1) straight chain fatty acids, 2) branched, cyclic and other specialized fatty acids, 3) polyprenoid compounds, which include carotenoids and sterols with all their derivates. Lipids have a crucial role in the structural composition of cell membranes and are indispensable for energy supply and storage. Lipids and fatty acids, together with proteins, are the most important macronutrients for fish (Sargent et al., 2002). Based on previous studies, fish have not evolved efficient systems to employ carbohydrates in their metabolism unlike terrestrial vertebrates, in which carbohydrates represent the major components of their diet. In this way, carbohydrates are quantitatively less important as nutrients for the majority of the fish than the other vertebrate organisms. This fact suggests that fish may obtain structural carbohydrates and storage carbohydrates (glycogen) mainly catabolising amino acids (Cowey and Walton, 1989). Fish exhibit a lot of unique aspects of lipid metabolism. Unlike homeotherms, which store lipid in a single depot site (adipose tissue) mainly as triacylglycerol, fish store a big quantity of lipids among many depots (mesenteric fat, liver and red muscle) as triacylglycerol and other lipid classes. Besides, the constituent fatty acid of fish tend to be more complex (longer chain length and higher degree of unsaturation) comparing with those of homeotherms (Sheridan, 1988). Lipid

accumulation is influenced by new lipid synthesis and even by the deposition from different plasma lipoproteins. For example, lipoprotein lipase (LPL) hydrolyzes triacylglycerols and phospholipids in chylomicrons and very low density lipoproteins, making possible the uptake of the lipid products into the neighbouring tissues (Lindberg and Olivecrona, 2002). In condition of metabolism need and demand, stored lipids are mobilized from depot sites by the action of hormone-sensitive lipase (HSL) (Sheridan, 1988, 1994; Gonzalez-Yanes and Sanchez-Margalet, 2006). White adipose tissue is an organ highly specialized in storing and releasing lipids, in the form of triglycerides, in response to a variety of signals controlling energy balance. In addition, it has the function of an endocrine organ (Harwood, 2012) secreting a multitude of adipokines that regulate different physiological processes such as appetite, glucose metabolism, immunity, inflammatory responses, angiogenesis, blood pressure and fertility (Ali et al., 2013). The study of fat deposition has become a key area of interest in fish farming with the aim of obtaining high quality products, rich in good nutritional elements, and maintaining fish health. The management of high lipid feed can lead to an undesired increase in fat deposition that alters sensory and organoleptic characteristics (Kjaer et al., 2008; Turchini et al., 2009). Moreover, some studies have been conducted about the lipid metabolic disorders in association with the content and type of lipids in the diet (Turchini et al., 2009; Benedito-Palos et al., 2008) and the dietary effects on macrophage function and stress susceptibility (Sitjà-Bobadilla et al., 2005; Gjøen et al., 2004).

Digestion

Lipolytic activity in fish is usually very intense in the proximal part of the intenstine and the pyloric caeca if present, but it might be extended into the lower part of the intestine with the activity decreasing progressively. The pancreas or hepatopancreas is generally considered to be the major source of digestive lipase enzymes in both fish and mammals (Kapoor et al., 1975; Fange and Grove, 1979). Moreover, digestive lipases may also be secreted by the

intestinal mucosa, as many studies have found high lipase activity in mucous layers or intestinal segments of several fish species, although these lipase activities might come from the pancreas, resulting from the absorption of pancreatic enzymes into the intestinal mucosa (Fänge and Grove, 1797; Smith,1989a). There is other evidence, such as that of substrate specificities, that supports the idea that intestinal cells can actively secrete lipolytic enzymes (Borlongan, 1990; Uematsu et al., 1992; Koven et al., 1994a). Lipolytic activity crosschecked in stomach is not probable of pancreatic origin and this fact suggests the hypothesis that this tissue is also a source of lipases, not excluding a bacterial origin for some lipolytic activities in the digestive portion of fish (Olsen and Ringo, 1997).

Triacylglycerol is the most extensive lipid class present in the diet of marine fish and it is usually the main component in the diet of freshwater fish too. In mammalian gut, triacylglycerol hydrolysis is caused by two main lipases, the pancreatic lipase-colipase system (EC 3.1.1.3) and the less specific bile salt-activated lipase (EC 3.1.1.1). Many evidence indicate the presence of a bile salt-activated lipase in teleost fish; stimulation of lipolytic activity has been found in intestinal extracts by bile salts both in marine and freshwater fish (Olsen and Ringo, 1997). It was seen that lipase activity in *O. mykiss* pyloric caecal extracts widely disappeared upon removal of bile salts by dialysis and it was restored by adding exogenous bile salts and also, porcine colipase was not capable of preventing the inhibition of lipolytic activities by cholate (Tocher and Sargent, 1984a).

Moreover, phospholipids, especially phosphoglycerides, constitute an important part of the dietary lipid in fish. It is presumed that both in fish and mammals, dietary phosphoglycerides are digested by pancreatic or intestinal phospholipases with the consequent formation of 1-acyl lyso-glycerophosholipids and free fatty acids which are absorbed by the intestinal mucosal cells (Henderson and Tocher, 1987; Sargent et al., 1989). It is not clear yet if fish possess a specific wax ester hydrolase, since no specific enzymes have been purified or characterised.

Absorption

The main products of lipid digestion in fish are free fatty acids produced by lipolytic activity on all major lipid classes. Moreover, in fish the partial action of acylglycerols, especially of 2-monoacylglycerols, plays an important role, but probably also the activity of diacylglycerol and glycerol from the digestion of triacylglycerols (1-acyl-glycerophospholipids from the digestion of phosphoglicerides) are important and cholesterol and long chain alcohols come from the hydrolysis of cholesteryl and wax esters, respectively. Identically to mammals, lipid absorption in fish happens mostly in the proximal part of the intestine that coincides with the highest lipolytic activity. Anyway, lipid absorption happens along the entire lengh of the intestine, even if in diminishing amount. In the intestinal mucosal cells, the main destiny of the absorbed fatty acids is the re-esterification with glycerol, in part acyl glycerol and lysophospholipids to reform triacylglycerols and phosphoglycerides (Sargent et al., 1989). Sterol and wax esters might be reformed, even though free cholesterol is easily transported from the mucosal cells and the wide majority of fatty alcohols are oxidised to the relative fatty acid in the epithelial cells (Bauermeister and Sargent, 1979). The most part of the phosphoglycerides are absorbed and digested through 1-acyl-phosphoglyceride intermediate by re-esterification, before being exported from the intestinal cells.

Transport

In the extracellular transport mechanism, lipids are exported from the intestine in the form of lipoproteins. The reactions of re-esterification take place above all in the endoplasmic reticulum, leading to the production of chylomicrons, like very low density proteins and particles in the lumen, as it was observed in particular for freshwater species. Chylomicrons are produced exclusively in the intestine but, although some very low density lipoprotein (VLDL) can also be synthesized in the gut, the majority of VLDL which are in the plasma are synthesized in the liver. The main enzymes of lipoprotein metabolism and remodelling,

encountering LPL and hepatic lipase have been found in both marine and freshwater fish. The lipid amount and their degree of unsaturation bears upon lipoprotein production, with high dietary lipid and PUFA, bringing to the production of larger chylomicrons, while high dietary fatty acids result in the production of smaller VLDL particles. In mammals, the transport of intestinal lipoproteins happens from the intestine especially trough the lymphatic system. In a similar way, in fish the majority of the intestinal lipoproteins are transported via the lymphatic system before appearing in the circulatory system and they are delivered to the liver (Sheridan et al., 1985). Anyway, a part of intestinal lipoproteins might also be transported directly through the portal system. Teleost fish have a major plasma protein with almost the same size, solubility and electrophoretic mobility of mammalian serum albumin (De Smet, 1978) and this protein plays an important role in transporting free fatty acids in the blood from adipose tissue depots to peripheral tissues under appropriate physiological conditions, as it happens in mammals (Sheridan, 1988). The intracellular transport of free fatty acids in mammals is helped by specific low molecular weight and highly conserved cytoplasmic proteins that bind either long-chain fatty acids or other hydrophobic ligands. These fatty acid binding proteins (FABPs) are tissue specific and those from liver, intestine, adipose tissue, brain and heart have been widely characterised in mammals (Veerkamp and Maatman, 1995). Many FABPs have been analysed from fish tissues, such as the elasmobranch liver (Bass et al., 1991; Baba et al., 1999; Cordoba et al., 1999). The FABP purified from the liver of the catfish (*Rhamdia sapo*) seemed to result more similar to the chicken liver FABP than to the FABPs of elasmobranchs or mammals (Dipietro et al., 1996). Intracellular FABPs were studied also in different species of Antarctic fishes (Londraville and Sidell, 1995) and two distinct types were isolated from heart tissue, one similar to the mammalian heart-type and the other closed to mammalian adipose tissue-type (Vayada et al., 1998). The deduced protein sequence for the trout heart FABP resulted 75% identical to that of rat heart FABP (Ando et al., 1998). These findings support the general thesis that intracellular transport of fatty acids in fish is essentially the same of higher vertebrates.

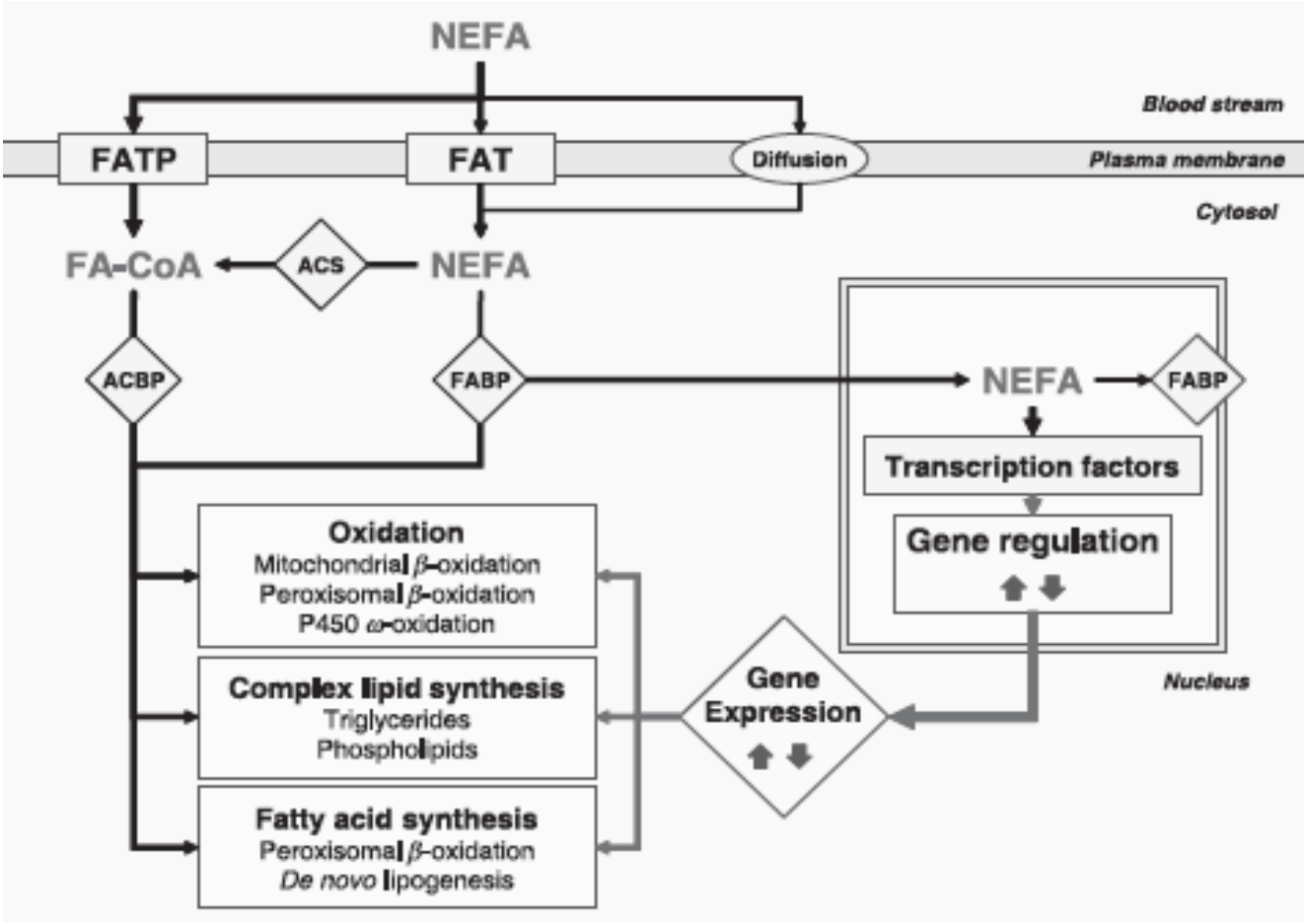

Fig. 3. Trans-membrane fatty acids transport and their intracellular activation, together with the main pathways of activated fatty acids that might explain intracellular triacylglycerol storage. FA= Fatty Acid; FATP= Fatty Acid Transport Protein; FAT= Fatty Acid Translocase; ACS= Acyl-coenzyme A Synthase; NEFA= Non-esterified Fatty Acid; FABP= Fatty Acid Binding Protein; ACBP= Acyl-coenzyme A Binding Protein; MTP= Microsomal Triglyceride Transfer Protein and TAG= Triacylglycerol (Source: Journal of Animal Physiology and Animal Nutrition, 2008).

Lipogenesis

The process of lipogenesis concerns to the biosynthetic reactions that bring to the formation of new endogenous lipid. The ultimate carbon source for the biosynthesis of new lipids is acetyl-CoA, formed in mitochondria from oxidative decarboxylation of pyruvate (carbohydrates source) or the oxidative breaking of some amino acids (proteins source). The central pathway in lipogenesis is

catalysed by the cytosolic fatty acid synthetase (FAS), a multienzyme complex that has been described in fish (Sargent et al., 1989). The most important products of FAS are the saturated fatty acids 16:0 (palmitic acid) and 18:0 (stearic acid) which all known organisms, fish included, are able to biosynthesise ex novo (Sargent et al., 1989). To biosynthesise saturated fatty acids 16:0, eight two-carbon acetyl units are indispensable: one acetyl-CoA unit has the function of a primer while the other seven acetyl units are carboxylated by acetyl-CoA carboxylase to malonyl-CoA, to be combined through FAS in a series of sequential condensation steps requiring nicotinamide adenine dinucleotide phosphate (NADPH) (Henderson and Sargent, 1985). Other key pathways in lipogenesis have the aim of creating the reducing elements, NADPH, encompassing enzymes of the pentose phosphate pathway and malate dehydrogenase, with glucose-6-dehydrogenase probably being the main NADPH generating enzyme in fish (Dias et al., 1999). Despite adipose tissue exerts some lipogenic activity in rainbow trout, liver is quantitatively the main site of lipogenesis in this species (Henderson and Sargent, 1985). In addition, referring to carbon sources, the rate of fatty acid synthesis from alanine was remarkably greater than that from glucose in trout liver, bringing to the conclusion that amino acids are the favourable carbon source for lipogenesis in this fish. The rate of lipogenesis is regulated by a number of dietary/nutritional factors; in particular it was seen that a diet rich in lipids suppresses lipogenesis.

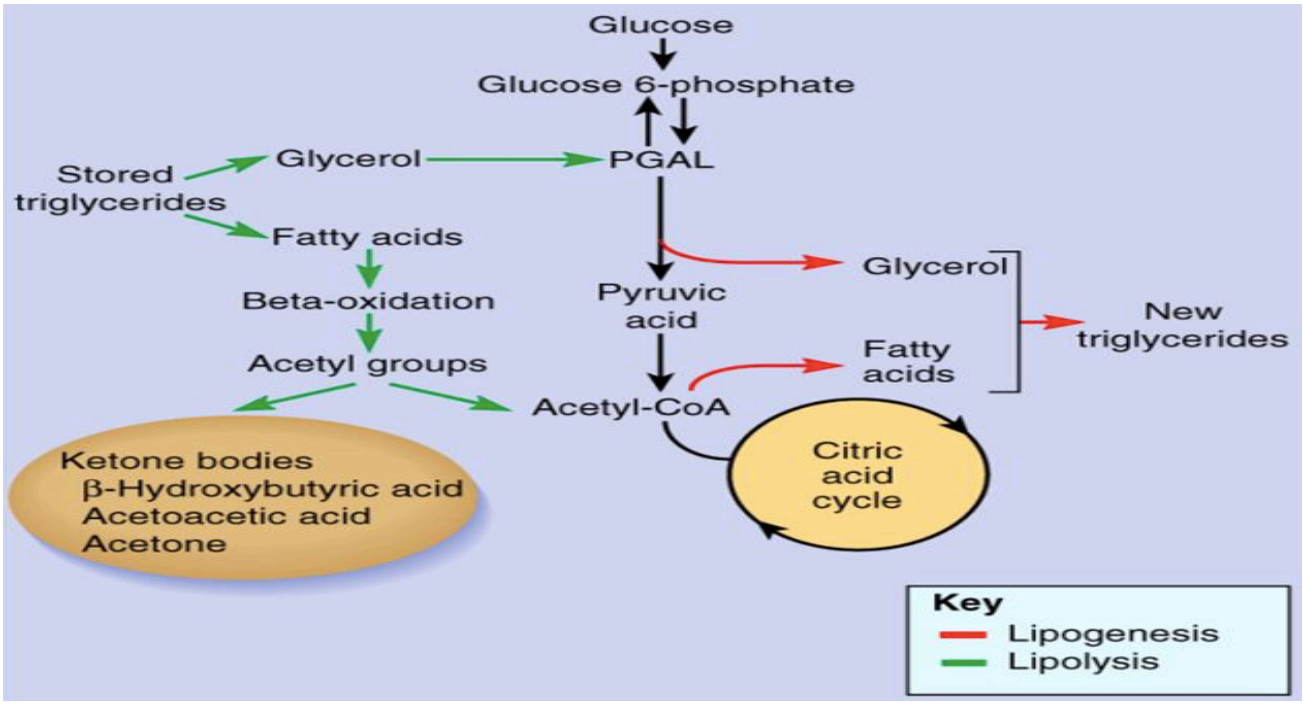

Fig. 4. Lipogenesis and lipolysis pathways (Source: lookfordiagnosis.com).

Triacylglycerol mobilisation

Triacylglycerols are accumulated in adipose tissue mainly as a long-term source of energy useful when the energy requirement of the animals exceeds that available from the diet. An example of this mechanism in fish happens during reproduction when the production of a huge number of gametes is very intensive in terms of energy. Other examples might be starvation and the long distance migrations that happen before the moment of reproduction in many fish species: fish energy requirements are very high because of swimming red muscles consumption, so that energy is greatly provided by fatty acids. Lipids are probably mobilised initially from the main adipose tissue, even though later they will be mobilised from the secondary lipid storage organs (muscle and liver). A necessary enzyme in triacylglycerol mobilisation is HSL, which is regulated by different hormones through reversible phosphorylations by the action of kinases and phosphatases under the influence of many activators and inhibitors. It has been seen that the process of triacylglycerol mobilisation is under hormonal control, at least in the liver, indirectly implying the presence of HSL in fish (Sheridan, 1994). Other studies demonstrated that triglycerides

mobilisation in trout was regulated through the phosphorylation state of the triacylglycerol lipase in both liver and adipose tissue (Sheridan, 1994). In this way, during starvation or periods of sober, lipid mobilisation from liver and adipose tissue, either in fish or in mammals, is under ß-adrenergic control with adrenalin and noradrenalin that stimulate tricylglycerol hydrolysis and the increase of free fatty acids in the plasma in salmonids and in other fish orders, although the influence of the two catecholamines is not the same among different species (Sheridan, 1994; Fabbri et al., 1998). Apart from this, many other hormones (glucagon, cortisol, adrenocorticotropic hormone, growth hormone, somatostatin, thyroxine and prolactin) act to stimulate lipid mobilisation in depot organs, liver in particular, through the activation of HSL, bringing to the breakdown and the secretion of free fatty acids; on the contrary insulin inhibits the activity of HSL (Sheridan, 1994).

Fatty Acid Catabolism

Fatty acid catabolism is the major source of energy in many species of fish. The biosynthesis of fatty acids happens in the cytosol, instead their catabolism occurs in mitochondria and in peroxisomes through a completely different set of enzymes. The whole process is given the name of ß-oxidation and involves the sequential cleavage of two-carbon units, released as acetyl-CoA, through a cyclic series of reactions catalysed by several distinct enzyme activities rather than a multi-enzyme complex. Activated fatty acids are brought to the inner membrane of mitochondria in the form of fatty acylcarnitine esters, formed by the action of carnitine acyltransferase, converted back into fatty acid acyl-CoA derivates and then undergo a round of dehydrogenation, hydration, second hydrogenation and cleavage steps to produce acetyl-CoA and NADH. The acetyl-CoA can be metabolised through the tricarboxylic cycle in order to form more NADH, which can provide metabolic energy in the form of ATP via the process of oxidative phosphorylation. In sober condition or starvation, acetyl-CoA can be exported from the liver as ketone bodies (acetoacetate and 3-

hydroxybutyrate), which are employed by peripheral tissues as a source of energy through re-oxidation to acetyl-CoA.

1.3 Lipid metabolism in liver

The major role of the liver is to maintain a fairly constant supply of circulating nutrients during variations in dietary intake. The maintenance of blood glucose levels in starvation periods is absolutely important because the brain has a great requirement for glucose. The response to hunger is controlled by a rise in the circulating levels of glucagon and glucocorticoids and a fall in the levels of insulin. During short period of fasting, liver glycogen is mobilized to release glucose. As fasting continues, gluconeogenesis from pyruvate, lactate, glycerol and amino acids begins. Gluconeogenesis from dietary proteins will also occur if the diet is poor in carbohydrates. Fatty acids can not act as substrates for gluconeogenesis, so the major energy store in the body can not be used to maintain blood glucose and the animals have to rely on proteins. During the period of fasting the energy requirements of the liver are supplied by the oxidation of fatty acids from the circulation that will have been mobilized from adipose tissue. Fatty acids can also be converted to ketone bodies that are released into the circulation and have the function of alternative fuel. In a situation of well feeding, the direction of metabolism goes towards the storage of glycogen and the synthesis of triacylglycerols, which are synthesized from fatty acids, derived from circulating chylomicrons and from fatty acids synthesized in the liver too. The liver storage of fatty acids is very little but the majority are exported as lipoproteins from the accumulated adipose tissue. Many studies have shown that glucose is a poor substrate for liver at any circulating concentration under the threshold of about 12 mmol/L. It appears that gluconeogenic precursors are better substrates than glucose for both fatty acid and glycogen biosynthesis. As soon as the blood glucose increases, the metabolism of gluconeogenic substrates switches from the production of glucose towards glycogen synthesis, fatty acid synthesis and also oxidation through the citric acid cycle for energy production. The liver is also the most

important organ for the biosynthesis of purines and steroids and the only one that synthesizes urea (Martin, 1987).

Fatty acid oxidation and biosynthesis

After the entry of fatty acids into the cell, they are esterified with coenzyme A; the pyrophosphate produced is rapidly hydrolysed to inorganic phosphorus, increasing the overall negative free energy of the reaction, making more favourable the production of acyl CoA. The mitochondrial membrane is impermeable to fatty acyl CoA, so fatty acyl units are put inside the mitochondria, where ß-oxidation takes place, through the carnitine shuttle. This event involves the synthesis of acyl carnitine from acyl CoA and carnitine in the cytoplasm and the regeneration of acyl CoA in the mitochondria by the reverse reaction. Acyl carnitine enters the mitochondria on a carrier in exchange for free carnitine. The cytoplasmic transferase is inhibited by malonyl CoA, one of the intermediates in the biosynthesis of fatty acids, formed in the cytoplasm by acetyl CoA carboxylase, which is the first enzyme exclusively involved in fatty acid biosynthesis. This enzyme is activated by insulin and inhibited by adrenaline. The main carbon sources for fatty acid biosynthesis are fructose and lactate, in contrast with fat cells where the main carbon source is glucose.
The level of circulating free fatty acids is determined mainly by the rate of lipolysis in adipose tissue. Lipolysis is activated by glucagon during starvation and by adrenaline during stress or exercise and both these processes work through cyclic AMP. Insulin inhibits lipolysis and the downfall in the circulation of this hormone is the main factor in rising rates of lipolysis during hunger. In this way, the rate of fatty acid oxidation is under hormonal control but its effect is indirect because it is a consequence of the hormonal effects on the different tissues. An increase in fatty acid oxidation in the liver brings to a marked rising in the level of acetyl CoA in the mitochondria and this fact is seen as an important signal for the activation of gluconeogenesis and ketone bodies biosynthesis (Martin, 1987).

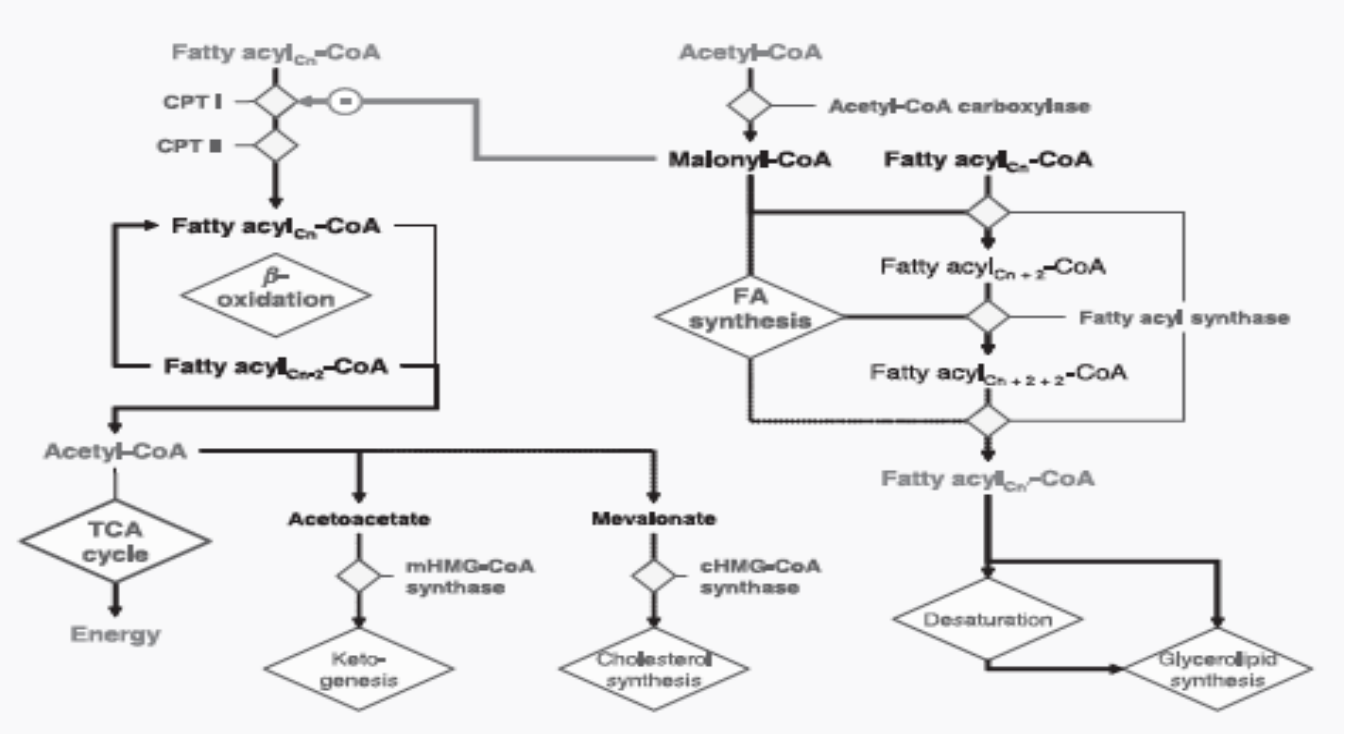

Fig. 5. Main steps of fatty acid oxidation and synthesis. CPT I, CPT II= Carnitine Palmitoyltransferase I and II); mHMG-CoA-synthase= Mitochondrial Hydroxymethylglutaryl-coenzyme A synthase; cHMG-CoA synthase= Cytosolic HMG-CoA synthase) and TCA= Tricarboxylic Acid Cycle; FA= Fatty Acids (Source: Journal of Animal Physiology and Animal Nutrition, 2008).

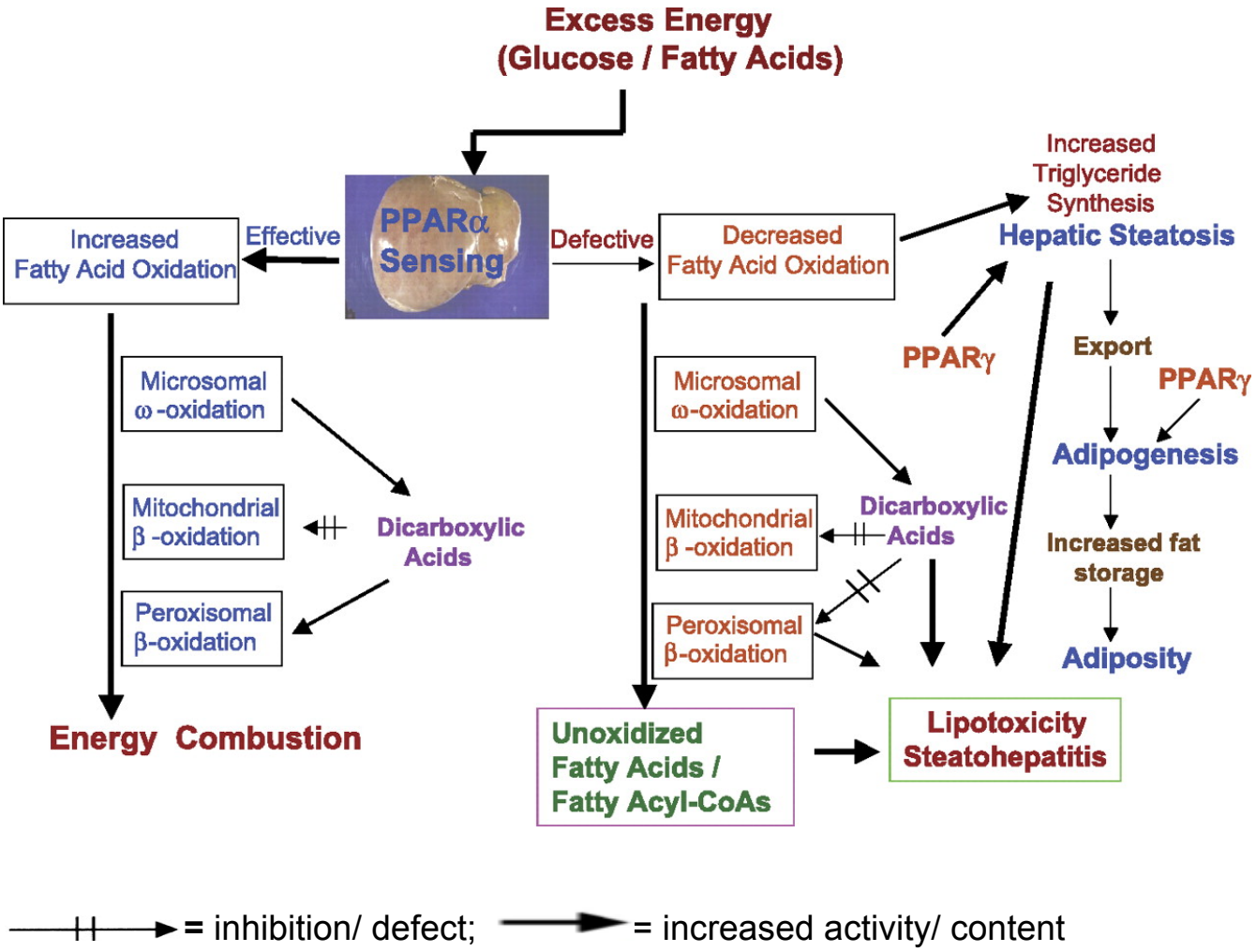

Fig. 6. Fatty acid oxidation path in liver (Source: ajpgi.physiology.org).

Ketone bodies synthesis

In a period of starvation, mitochondrial acetyl CoA levels grow because of increased fatty acid oxidation and the main factor involved in the regulation of ketone bodies synthesis is the resulting substrate stimulation of ß-hydroxymethyl glutaryl CoA synthetase (HMG-CoA synthetase), an enzyme inhibited by succinyl CoA. In every case, glucagon or dibutyryl cyclic AMP stimulate ketone bodies synthesis through a cyclic AMP-mediated mechanism independent from variations in circulating free fatty acids. As said before, HGM-CoA synthetase is inhibited by succinyl CoA, which occupies a site normally filled by acetate. The reverse reaction of the inhibition is quite slow and favoured by acetyl CoA. This process provide an additional mechanism whereby an increase in acetyl CoA might activate ketone bodies synthesis and it can also

give an explanation for the direct glucagon effect, since this hormone reduces the concentration of succinyl CoA in the liver. It is largely accepted that ketone bodies synthesis can be activated by a rise in mitochondrial acetyl CoA levels, which is definitively a consequence of hormonal stimulation of lipolysis in adipose tissue and a fall in succinyl CoA, that results from a direct effect of glucagon on the liver cells (Nguyen et al, 2007).

1.4 Oxidative stress in fish

Many stresses that are induced by the environment frequently activate the endogenous production of reactive oxygen species (ROS), the majority of which are generated as side products of tissue respiration. As a consequence, a continuous exposure to stressors may enhance ROS-mediated oxidative damage. Oxidative stress can be defined as a situation in which steady-state ROS concentration is transiently or chronically enhanced, disturbing cellular metabolism and its regulation, damaging cellular constituents (Lushchak, 2011). The activation of oxidative manifestations leads to the response of antioxidants, activation of expression of genes which encode antioxidant enzymes and elevation of the concentration of ROS scavengers. Oxidative stress occurs when the ROS generation rate exceeds that of their removal (Sies, 1986). Its deleterious effects include oxidation of proteins, DNA and steroid components, as well as peroxidation of unsaturated lipids in the cell membrane. All that produces unstable lipid hydroperoxides, the products of which, decomposing, are highly reactive. These products can also break down into free radicals that can continue the destructive cycle of lipid peroxidation by chain reactions. Biological oxidation is a primitive process and, in the face of the inevitable consequences of O_2 toxicity, evolution has provided appropriate defensive strategies. The first line of defense is the employment of antioxidant substances, such as vitamin C, vitamin E, uric acid, glutathione and carotenoids. In addition, different antioxidant enzymes prevent the cascade of oxidant reactions, intercepting and inactivating the reactive intermediates of oxygen, concluding the lipid peroxidation cycle. Antioxidant enzymes are crucial in the effort to

counteract oxygen toxicity when the supply of other antioxidant compounds is scarce or depleted (Ahmad, 1995). Like all aerobic organisms, fish are susceptible to be attacked by ROS and have developed antioxidant defenses. Specially adapted enzymes, such as catalase, superoxide dismutase and enzymes dependent on glutathione (glutathione peroxidase and glutathione reductase) have been found in most fish species (Rudneva, 1997). Together with these enzymes, lower-molecular-weight antioxidants, such as carotenoids, vitamins E, K and C, amino acids and peptides (glutathione) have been detected as antioxidant defenses in fish. Differently from the model of oxidative stress that is usually caused by singular substances acting under controlled laboratory conditions (concentration and period) (Bagnyukova et al., 2005, 2006, 2007; Kubrak et al., 2010; Lushchak et al., 2007, 2008, 2009 a, b, c; Yi et al., 2007; Sun et al., 2008), environmental impact is usually developed according to multiple stressor effects. In fact, ecosystems are under the pressure of complex mixtures of contaminants released to the environment, due to different human activities. They can originate from miscellaneous sources, which might be chemical and drug manufactures, domestic sewage, polymer and petrochemical-based industries, oil refineries, mining, glass blowing, battery manufacture and many others. Hydrological changes, hydromorphological degradation and invasive species can also contribute to the set of stressing factors (Amado et al., 2006; Sureda et al., 2006; dos Anjos et al., 2011). As a consequence of all these factors, aquatic environment is a sink for many environmental contaminants that can be absorbed by aquatic organisms leading to the disturbance of antioxidant/prooxidant balance in fish (Lackner, 1998; Livingstone, 2001; Lushchak, 2011). Synergistic or antagonistic effects of mixtures of pollutants are hardly interpreted and predicted exclusively from the chemical analyses. Some contaminants are substantially stored in specific tissues without recorded toxic effects (Viarengo and Nott, 1993), while others produce high toxicity even at low levels. For this cause, oxidative stress response of fish cannot be predicted using data on the level of certain pollutants in their tissues. Basing on the intensity and duration of toxicant effect and resistance of many studied organisms, different manifestations of oxidative

stress can be expected. The long-term effects of pollutants, typical for chronically and heavily polluted areas, the increase of ROS levels and perturbation of antioxidant efficiency often prelude the starting of significant alterations like proteins and DNA damage, lipid peroxidation and enzyme inhibition (Winston and Di Giulio, 1991). Fish are particularly threatened by aquatic pollution and the environmental stress they have to face may help to constitute their ecology, evolution or biological systems (Padmini, 2010). The expression of specific damage known to be caused specifically by oxidative stress (e.g. lipid peroxidation and membrane lesions, oxidized bases in DNA and accumulation of lipofuscin pigments) were found in many aquatic animals exposed to contaminants (Winston, 1991). The end-products of lipid peroxidation/oxidation (LPO) can be accumulated in lysosomes as insoluble granules containing autofluorescent pigments and are usually referred as lipofuscin. The presence of these pigments in the lysosome vacuolar system of fish hepatocytes can be also used for the assessment of the level of membrane LPO (Viarengo et al., 2007). In any case, whilst in the laboratory a wide spectrum of the previously mentioned effects can be measured, only a single parameter is often analyzed in Environmental Risk Assessment. Hence, the rate of oxidative damage is the control point of the effective adaptation to oxidative stress.

LPO usually measured as level of thiobarbituric acid reactive substances (TBARS), has been used very often to analyse the effects of pollutants (Livingstone, 2001; Lushchak et al., 2007, 2008, 2009 a, b, c, 2011). The big amount of LPO in fish from heavily polluted field sites was observed (Ferreira et al., 2005; Farombi et al., 2007; Sanchez et al., 2007) and an example of this can be referred to the African catfish (*Clarias gariepinus*) from the Ogun River (Nigeria), in which TBARS levels resulted significantly higher in the liver, kidney, gills and heart compared to those from fish farmed of the same species, which were considered as a reference site (Farombi et al., 2007). The formed free radicals cause various kinds of genotoxicity, especially modifications to DNA bases. The majority of the analytical assays have been focused on measuring products of guanosine hydroxylation, namely 8-OHdG or 8-oxodG, and its free

base 8-hydroxyguanine in urine as an indirect method for oxidative damage by free radicals (Shigenaga and Ames, 1991). A study with the fish gilthead sea bream found that 8-oxodG determination in chromosomal DNA was a potentially useful biomarker of oxidative stress caused by urban and industrial environmental pollution (Rodriguez-Ariza et al., 1999).

Proteins are considered to be important targets of free radicals attack in cells (Eustace and Jay, 2005; Almroth et al., 2008 b; Lushchak, 2011) and thus, compromise antioxidant defense, cellular function and survival (Padmini, 2010). Therefore, protein oxidation, often under investigation in proteomic studies, has been recently proposed as a biomarker of oxidative stress (Sheehan, 2006; Lushchak, 2011).

1.5 Antioxidant compounds and PPARs receptors

In recent years, the addition of natural substances with antioxidant properties is an emerging strategy for protecting biological systems and foodstuffs from oxidative damage. Tea catechins (He and Shahidi, 1997), grape proanthocyanidins (Pazos et al., 2006), rosemary extracts (Medina et al., 2003) and hydroxytyrosol (HT) extracted from olive oil (Pazos et al., 2008) have demonstrated elevated potential to inhibit lipid oxidation in fish muscle-based food products. Among the natural substances, hydroxycinnamic acids, including caffeic (Caf-OH), ferulic, o-coumaric or chlorogenic acids have also attracted considerable attention as food antioxidant additives due to their potential biological and antioxidant activities (Nardini et al., 1995). In particular, chlorogenic acid inhibits carcinogenesis in the colon, liver and tongue and also protects against oxidative stress *in vivo* (Mori et al., 1986; Tanaka et al., 1993; Tsuchiya et al., 1996). Chlorogenic acid has been claimed to modulate the glucose-6-phosphatase involved in glucose metabolism (Hemmerle et al., 1997) and to reduce the risk of cardiovascular disease by decreasing oxidation of low-density lipoproteins (LDL), cholesterol and total cholesterol (Nardini et al., 1995). More recently, it was reported that chlorogenic acid inhibites

preadipocyte population growth, which may provide a proposed mechanism for reducing obesity (Hsu et al., 2006).

Caf-OH and in general o-dihydroxyphenolics, can protect lipids from oxidation by at least two well-described mechanisms: 1) scavenging free radicals as a primary antioxidant method and 2) chelating active transition metals to form inactive metallic complexes (Frankel, 1998).

Many experiments with the polyphenols demonstrated substantial modulation of physiological and molecular pathways involved in energy metabolism and adiposity (Meydani and Hasan, 2010). Catechins from green tea (Lin et al., 2005), resveratrol from red grapes (Floyd et al., 2008) and curcumin from curcuma (Ejaz et al., 2009) suppressed adipogenesis on the 3T3-L1 cell line through several mechanisms including attenuation of peroxisome proliferator-activated receptor γ (PPARγ) regulatory pathway (Manach et al., 2004). Also the olive tree (*Olea europaea*) and its products are a source of at least 30 phenolic compounds and, particularly, extra-virgin olive oil contains considerable amounts of phenolic compounds, such as oleuropein (OL) and the product of its hydrolysis, HT. *In vitro*, OL reduced the expression of PPARγ, inhibithed adipogenesis and enhanced osteoblastogenesis in stem cells derived from human bone marrow (Santiago-Mora et al., 2011). It was seen that OL, in concentration higher than 100 μM, acted on the 3T3-L1 cells to reduce preadipocyte differentiation and lipid storage regulating the size of fat cells. OL between 10 and 400 μM concentrations did not affect activity of PPARα or PPARβ/γ. Contrary, PPARγ activity, either basal or rosiglitazone activated, was inhibited by OL. Some datas suggest that OL exerts anti-adipogenic effect through direct inhibition of PPARγ transcriptional activity. The expression of key transcriptional modulators of adipocyte differentiation genes PPARγ, CCAAT-/enhancer binding protein a (C/EBP1a), the sterol regulatory element binding transcription factor 1c (SREBP-1c) and their downstream target genes were suppressed by OL in a dose dependent way during the process of differentiation (Drira et al., 2011).

It has been shown that HT is highly efficient to prevent oxidation in fish oils, fish oil in water emulsions and fish fillets (Pazos et al., 2008). This is particularly interesting for stabilizing functional products containing ω-3 PUFA. In addition, HT has exhibited very interesting biological properties, such as inhibition of human LDL oxidation, a critical step in atherosclerosis, (Visioli et al., 1995), inhibition of platelet aggregation (Petroni et al., 1995), anti-inflammatory (de la Puerta et al., 1999) and also anticancer properties (Owen et al., 2000). HT is strongly hydrophilic and this property makes difficult its incorporation into fats and oils. It has been reported that both OL and HT are able to exert apoptotic effects in human MCF-7 BC cell line (Han et al., 2009). In addition, it has been recently demonstrated the ability of HT and OL to inhibit MCF-7 BC cells proliferation through a mechanism that does not involve a classic estrogen receptor-mediated gene regulation but an ERK 1/2 (Extracellular Regulated Kinase 1 and 2) activation (Sirianni et al., 2010). This last observation lets hypothesize that the inhibitory effect exerted by the two phenols might be attributable to their ability to interfere with a non-genomic action of estrogen.

Since their initial discovery as transcription factors, which were activated by a variety of chemical and pharmacological compounds known to induce peroxisome proliferator factors in rodents (Issemann and Green, 1990), PPARs have been intensively studied in humans and rodents and have been the subjects of many reviews (Desvergne et al., 2004; Hihi et al., 2002). PPARs are ligand-activated nuclear hormone receptors acting as heterodimers with retinoid X receptors (RXR). PPAR/RXR heterodimers bind to DNA sequences that consist of the nucleotide repetition AGGTCA separated by one base pair (DR1 sequence).

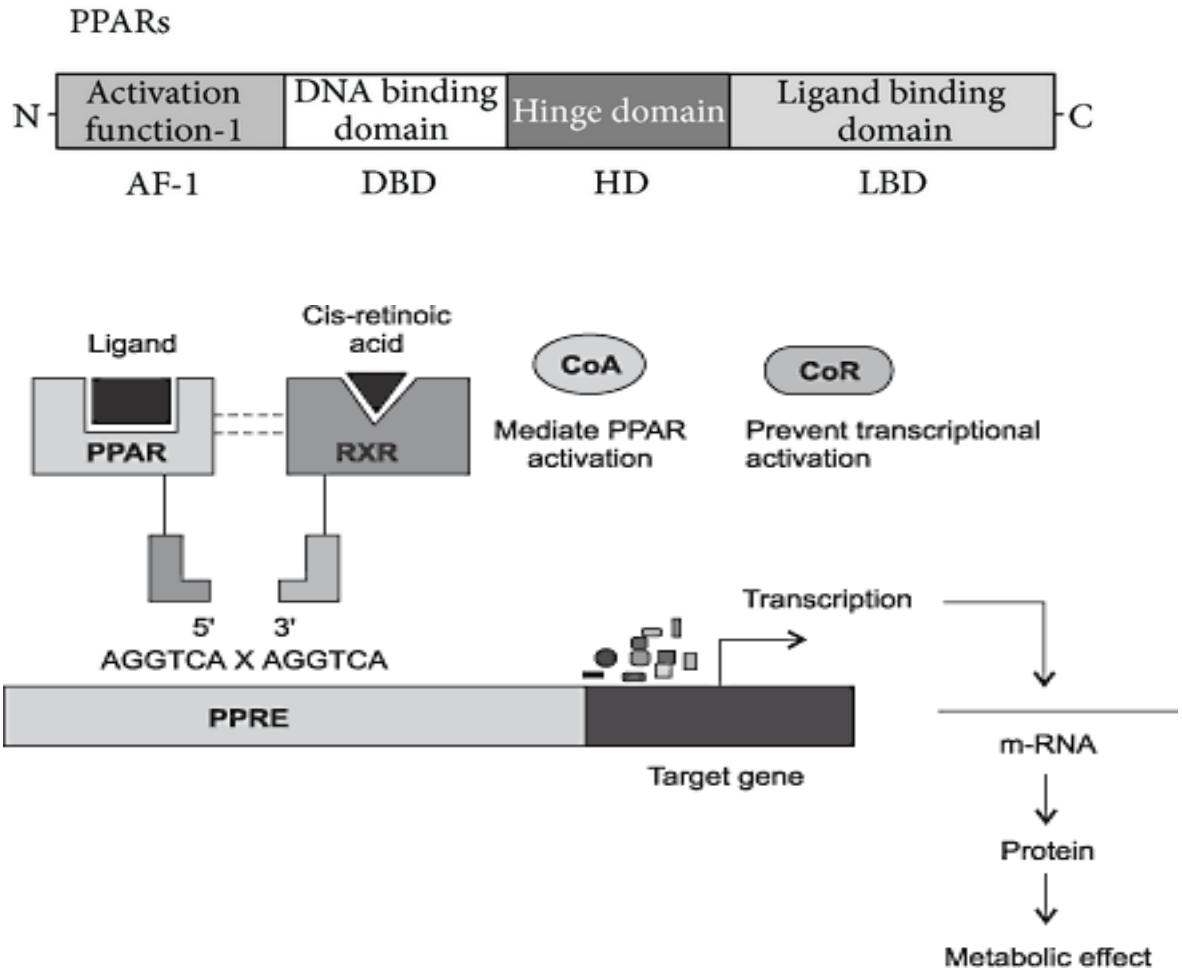

Fig. 7. PPARs structure and pathway. PPRE= Peroxisome Proliferator Response Element (Source: hindawi.com; journals.prous.com).

The PPAR/RXR heterodimers are considered ‘’permissive’’ complexes because they can be activated either by PPAR ligands or by RXR ligands. In mammals, were found three genes encoding PPARs: PPARα, PPARβ (or δ) and PPARγ. These genes have different tissue expression profiles that, along with the tissue specific expression of co-repressor and co-activator proteins and diversities in ligand selectivity, determine their action in regulating genes involved in fatty acid homeostasis (Desvergne et. al., 2006). All mammalian PPARs bind and are activated by unsaturated fatty acids, even though a variety of multitude of oxidized derivatives of fatty acids, such as eicosanoids, have higher affinity and are also more subtype specific. This fact, has led to the thought that PPARs are general fatty acid sensors that respond to increased cellular fatty acid levels or

their metabolites, which result in changes in nutritional status and energy metabolism (Michalik et al., 2006). Because of their important functions in fatty acid metabolism, PPARs have also been the subject of many studies in fish, including Atlantic salmon and sea bream. PPARγ is encoded by a single gene in mammals that, through alternative splicing, gives rise to two proteins, PPARγ 1 e PPARγ 2, with distinct N-terminal sequence (Tontonoz et al., 1994). The second one is highly expressed in adipose tissue, whereas PPARγ 1 is expressed to a high level in gut and at a lower level in a variety of other tissues too (Escher et al., 2001). Mammalian PPARγ is essential for fat storage, especially in adipocytes, but also in lipid accumulating macrophages (Rosen et al., 1999; Fajas et al., 2001). PPARγ was the first subtype to be identified in fish, with cDNAs characterized from Atlantic salmon and plaice (Ruyter et al., 1997; Leaver et al., 1998). After that, the availability of genome information for zebrafish, stickleback, medaka and pufferfish has shown that, similarly as in mammals, these fish possess a single PPARγ gene, such as other studies on sea bream, sea bass and plaice also indicate the presence of a single gene (Maglich et al., 2003; Boukouvala et al., 2004; Leaver et al., 2005). There are no available indications yet of alternatively spliced products and thus alternative PPARγ proteins in fish. Either fatty acid or mammalian PPARγ-specific ligand fail to activate sea bream or plaice PPARγ (Leaver et al., 2005) and, at the same time, Atlantic salmon PPARγ is unresponsive to typical mammalian PPARγ agonists (Leaver et al., unpublished). Even though fish PPARγ is an evident homologue of mammalian PPARγ, all PPARγ proteins of fish identified up to now have a critical amino acid substitution, in which a tyrosine residue in all mammalian PPARs and essential for binding the carboxylic head group of fatty acids and synthetic ligands is substituted by a methonine. This fact suggests the idea that fatty acids and synthetic ligands are not able to activate PPARγ of fish (Leaver et al., 2005; Maglich et al., 2003). In this case, is likely that piscine PPARγ is activated by unidentified compounds, even if, because of its similarity with the entire structure of mammalian PPARγ, these unidentified compounds are probably related to fatty acids. Many differences between

mammalian and PPARγ of fish are also evident from tissue expression profiles. It was seen that PPARγ is expressed at a high level relative to other PPARs and, differently to mammalian PPARγ, in a wide range of sea bream and plaice tissues (Leaver et al., 2005). In addition, the expression of sea bream hepatic PPARγ mRNA levels increase upon feeding, whereas in adipose tissue they remain constant. It was also observed in rainbow trout that the gene transcription levels of PPARs changed differentially during fasting and refeeding and that tumoral necrosis factor α (TNFα) and/or lipopolysaccharide administration suggested the idea that the regulation of PPARs helps maintain metabolic adipose tissue homeostasis. In mammals and, as it would appear, in fish, PPARγ is highly expressed in adipose tissue and acts as a promoter of fat storage and adipocyte differentiation (Bouraoui et al., 2008; Cruz-Garcia et al., 2009; Yin et al., 1998). It was found that insulin administration upregulated PPARγ isotype highly significative in trout adipose tissue and *in vitro* cell preparations, in line with the prolipogenic actions of this hormone. Similarly, the presence of insulin during trout adipogenesis induced preadipocyte differentiation and lipid accumulation, which occurred together with an increase in PPARγ protein levels (Bouraoui et al., 2008). At this point, the identification of agonists for piscine PPARγ is a necessary step for further studies.

A variety of environmental contaminants are agonists of the PPARs. In sensitive rodent models, it was observed that chronic exposure to PPAR-active substances causes cellular peroxisome proliferation, elevates enzymatic markers of oxidative stress and it is linked to the development of hepatic tumors (Gervois et al., 2000; Suga et al., 2004). Peroxisome proliferation is now recommended as a biomarker of exposure to PPAR-active compounds in environmental pollution assessment (Cajaraville et al., 2003). Many compounds that are known PPAR agonists are contaminants in the aquatic environment, including phthalate plasticizers (McDowell et al., 2001) and fibrate drugs (Metcalfe et al., 2003). These compounds could affect the health of aquatic organisms, including fish, and a number of studies report that fish respond at the cellular level to PPAR agonists (Donohue et al., 1993; Scarano et al., 1994). Detection of PPAR-active substances using analytical techniques, such as liquid

chromatography-mass spectrometry, is expensive and time consuming, so that cell-based reporter gene assays represent alternative methods to detect PPAR-active substances. Reporter gene assays with fish cells have been also developed to determine the presence of estrogenic substances in the environment (Petit et al., 1997; Ackermann et al., 2002). Thiazolidinediones (TZDs) are a group of PPARγ agonists, a class of anti-diabetic drugs, which include rosiglitazone (RGZ) and pioglitazone. The activation of PPARγ by TZDs leads to the redistribution of fat from visceral to subcutaneous adipose tissue, increased trapping of fatty acids in adipose tissue and a modified secretion of hormones from adipose tissue; all factors known to improve insulin sensitivity (Semple et al., 2006). Anyway, administration of TZDs has been associated with unexpected side effects such as weight gain, edema, and hepatotoxicity (Guan et al., 2005; Pan et al., 2006).

Recently, identification of new PPARγ partial agonists that do not present the adverse side effects caused by PPARγ full agonists have received considerable attention for developing powerful drugs against diabetes. The ligands acting as partial agonists induce submaximal receptor activation and have been demonstrated to retain beneficial anti-diabetic properties with reduced side effects. Despite the lower activation of the receptor, they are still able to induce PPARγ target genes responsible for anti-hyperglycemia and insulin sensitivity, but not for the unwanted PPARγ actions (Bhalla et al., 2011; Agrawal et al., 2012). The reason for this is not entirely understood.

1.6 Objectives

Fat loss may be normally caused by two main processes: enhancement of lipolysis and inhibition of triglycerides accumulation. In mammals, it has been demonstrated that CA and HT inhibit adipocytes differentiation and thus lipid cell content, reducing the activation of PPARγ (Juman et al., 2010; Warnke et al., 2011). However the potential lipolytic effect of these compounds remains unclear. It is commonly known that PPARγ regulates adipocytes differentiation and the subsequent accumulation of cytoplasmic lipid droplets. Moreover, RGZ is a PPARγ agonist that regulates genes involved in lipid metabolism and homeostasis. It has been confirmed that RGZ has a clear effect in stimulating adipocytes hypertrophy in zebrafish (Tingaud-Sequeira et al., 2011).

In this frame work, the major aims of the present study are using rainbow trout as model:

1) to determine the effect of an intraperitoneal injection of CA and HT on lipid metabolism and oxidative stress related genes in liver and adipose tissue;

2) to define the effect of an *in vitro* incubation of CA and HT on lipid metabolism in primary cultured adipocytes;

3) to describe the potential anti-adipogenic effect of CA and HT, analyzing the effects of these compounds on viability, proliferation and differentiation in rainbow trout primary cultured adipocytes;

4) to describe how CA and HT can interfere with PPARγ gene expression in primary cultured adipocytes, by immunofluorescence analysis for PPARγ.

2. Material and methods

2.1 Animals

Juvenile (intraperitoneal experiments) and adult (cell cultures) rainbow trout (*O. mykiss*) weighing between 80 and 250 g respectively were maintained either in 0.2 m^3 and 0.4 m^3 tanks with a temperature-controlled freshwater recirculation system (18 ± 1°C) and 12 hours light/12 hours dark photoperiod. 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). The trouts were fed daily at will with a commercial diet (Skretting).

2.2 Intraperitoneal injections

After 15 days of acclimation, animals used in intraperitoneal injection were fasted from 2 h before the injection to 24 h post-injection (26 h) when sampling was carried out. Juvenile fish were anesthetized with ethyl 3-aminobenzoate methanesulfonate (MS-222 0.1 g/L) and received intraperitoneal injections of 4.64 μL of volume per g of body mass of the different treatments. Treatments were diluted 1:3 in sesame oil as follows: 1) control containing dimethyl sulfoxide (DMSO) diluted in sesame oil; 2) CA at 10 μg/g and 3) HT at 20 μg/g (n = 9). After 24 h, fish were first anesthetized and then sacrificed by a sharp blow to the head; after that, their masses were recorded and blood samples were taken from the caudal aorta. Samples of liver and perivisceral adipose tissue were harvested and stored at -80°C until analysis.

2.3 Cell culture

To avoid contamination from the gastrointestinal tract, adult fish were fasted 24 hours before the experiments. The cell culture procedure was performed as described in Bouraoui et al., 2008. After counting, cells were seeded (800.000 cells in 2mL or 400.000 cells in 1 mL) in gelatin pre-treated six-wells or twelve-wells plates (9.6 cm^2/well or 2.55 cm^2/well respectively) and kept at 18°C with growth medium. After confluence (day 7), cells were induced to differentiate by means of incubation with a growth medium supplemented with 10 μg/mL of insulin, 0.5 mM of 3-isobutyl-1-methylxanthine (IBMX), 0.25 μM of dexamethasone and 10 μL/mL of lipid mixture. Media were changed every two days during the whole procedure.

2.3.1 Reagents preparation

- **Krebs:**

Prepare 500 mL of stock Krebs (x10) solution the day before starting the culture, aliquot it in the same solution in identical parts of 50 mL and, finally, freeze it. The following amounts are for trout culture:

Reagents	Concentration (mM)	x 500 mL (g) (x10)
NaCl	117.5	34.33
KCl	5.6	2.09
$CaCl_2$ (dehydrated)	2.52	1.85
$MgSO_4$	1.18	1.45
NaH_2PO_4 (monohydrated)	1.28	0.88

The day of cultivation prepare 500 mL of working solution (Krebs x1) and agitate it with a magnet inside.

Reagents	Amounts
Stock Krebs (x 10)	50 mL
Distilled water or Milli Q	445 mL
$NaHCO_3$ (25 mM)	1.05 g
D-glucose	0.495 g
HEPES pH 7.4	5 mL

- **HEPES (1M, pH=7.4)**

- **Erythrocyte buffer:**

Prepare 100 mL of solution at pH 7.3 and putting it into the autoclave/steamer.

Reagents	x 100 mL (g)
NH_4Cl	0.822
$KHCO_3$	0.101
EDTA	0.0292

- **Collagenase:**

Prepare collagenase Type II sigma C6885 (130 U/mL) according to the formula below. Filter collagenase with a 0.22 μm filter (Millipore CatN° SVGP01050).

(130 U/mL) x (mg/475 U of collagenase) x (mL of Krebs buffer to add)=
X mg collagenase in *X* mL of Krebs bovine serum albumin (KBSA) 1%.

475= collagenase units written on the bottle.
mL of Krebs buffer= volume of Buffer Krebs to add, depending on the amount of tissue (1g/5 mL).

- **Medium of growth:**

These amounts are to prepare 500 mL of medium:

Reagents	Amounts
Feral bovine serum (FBS, Sigma F7524-500 mL)	50 mL
Antibiotic (1%) (mix Sigma A5955-100 mL)	5 mL
L-15 (Leibovitz's Ref. 11415)	Add to complete the volume

- **Medium of differentiation (x 50 mL):**

- Insulin (I5523, Sigma; PM 5807 g)⇒ 171.87 µL (to keep into the freezer)

- Dexametasone (4°C, D2915, Sigma 1mM into water; PM 392.5 g)⇒ 12,5 µL

- IBMX (-20°C, 17018, Sigma stock 1M in DMSO; PM 222,25 g)⇒ 50 µL

- Lipids (L5146, Sigma 4°C)⇒ 250 µL (to keep into the freezer)

- **Gelatin 1%:**

Gelatine dust must be sterile, so that it must be used inside the hood. A definite amount of gelatin (1.5 g of gelatin + 150 mL of distilled water) can be used outside the hood but, after the preparation, it has to be autoclaved.

- **Feral Bovine Serum (FBS):**

FBS is at the liquid state and it is stored (in aliquots of 30 mL) into the freezer at -20° C.

2.3.2 Procedure

The day before culture

Keep out of the freezer the FBS and the stock Krebs (x 10). Keep the plates inside the aspirant hood with gelatin 1% (1 mL per well). Put the plates into the incubator at 18°C for three h; then wash them twice (500 μL/well) with antiseptic phosphate Buffered Saline(PBS). At the end, put the PBS in the each well again and store the plates into the incubator at 18°C.

The day of culture

Prepare 500 mL of Krebs-HEPES solution and mix it with CO_2 5% for 7 min. Weight 0.5 g of the anaesthetic compound MS-222 and filter about 370 mL of Krebs-HEPES solution. Put 5 mL for each of Krebs-HEPES filtered solution plus 50 μL of antibiotic into 4 falcons.

Anesthetize 3-5 trout with MS-222 and kill them by a blow to the head. After that, weigh the fishes and extract 20 g of adipose tissue from them (5 g of tissue for each falcon). Add 28,13 mg of collagenase to the Krebs-HEPES solution plus 1% of BSA. Then filter the collagenase inside the Krebs-HEPES solution.

Add the content of each falcon into a Petri plate and mince the adipose tissue with two scalpels. Put the minced tissue in two new falcons, add 5 mL of collagenase in Krebs-HEPES solution in each falcon and put them inside the incubator for 1 h at 18°C. After that, integrate the volume of the two falcons to 50 mL with Krebs-HEPES solution and centrifuge at 1500 rpm and 4°C for 10 min. After the use of centrifuge, collect just the precipitated part inside the falcons (the pre-adipocytes + the erythrocytes) and add 5 mL of erythrocyte lysis buffer to each falcon for no more than 10 min, so that the precipitated part will dilute into the buffer solution. Add the content of two falcons into a new one with the help of a suitable filter.

Wash the empty falcons with 5 mL of Krebs-HEPES solution, from the first filtration procedure, and transfer the content to the washed falcon with a specific filter, avoiding the floating part made of adipocytes. Add to the previous falcons Krebs-HEPES solution until reaching the volume of 35 mL. Put again the falcon into the centrifuge at the same condition, before mentioned. After this last step, remove the floating part of the centrifuged falcon and add 1 mL of growth medium.

The following step is the cell count; to do this collect and mix: 20 μL of pre-adipocytes, 10 μL of growth medium and 10 μL of trypan blue, a colorant. Get 20 μL of the previous solution and put it into a Neubauer chamber, made of 4 squares. Count the cells inside the 4 squares and calculate an arithmetic mean of the numbers expected for each square.

Day following to culture

Put 500 μL of L-15 inside every well and observe the plates by the microscope. We are interested in gray cells with a fibroblastic shape and not to round, glittering cells.

Remove L-15 from the plates and put 500 μL of growth medium in each well. Then, close the plates.
Replace the growth medium every 2 days approxymately.

Differentiation

When the cells flow together and become confluent, add the differentiation medium.

2.4 MTT proliferation assay

MTT is a colorimetric assay which allow us to determine an enzymatic activity because of a change in colour. This assay is based on metabolic reduction of Bromide 3-(4,5-dimetiltiazol-2-ilo)-2,5-difeniltetrazol (MTT) (a water solvable salt of yellow colour), performed by the mitochondrial enzyme succinate-dehydrogenase in a light blue compound (fomazan), allowing to determine mitochondrial functionality of treated cells. MTT method is very useful to analyze survival and proliferation of cells. The amount of living cells is proportional to abundance of formazan produced. Metabolized MTT in formazan forms water insoluble crystals which remained trapped into the cells and they have to be solved into organic dissolvent, such as dimethylsulfoxide (DMSO), before doing colorimetry. This method was realized by Mosmann in 1983 and it was modified in 1986 by François Denizot and Rita Lang.

MTT Stock solution

Add 5 mg of MTT (Ref. M5655 500 mg, Sigma) per mL of PBS (Phosphate Buffered Saline). MTT stock solution must be stored covered in aluminum paper at 4°C because it is sensitive to light, so a possible degradation of this might cause false positive results. MTT stock solution, when it is stored, lasts approximately one month.

MTT treatments

At day 5 of cell culture (cellular proliferation), the following treatments were tested:

1. Control (Growth medium+DMSO)
2. CA 50 μM
3. HT 100 μM
4. RGZ 1 μM
5. CA 50 μM + RGZ 1 μM
6. HT 100 μM + RGZ 1μM

The initial concentration of the compounds used in the treatments were the same for all *in vitro* experiments and were the following: CA=10 mM, HT=10mM and RGZ=100 μM. After 24 h of incubation, the MTT protocol can be applied.

MTT protocol

1. Put 50 μL of MTT stock solution into every single well + 450 μL of differentiation medium (for a 12 wells plate) or 100 μL + 900 μL (for a 6 wells plate), in the way of covering the complete surface of each well. Besides, a single well is used as control, so that it is added PBS instead of MTT.

2. Keep the plate with MTT inside the incubator over night, in the case of adipocites. At the moment of keeping out of the incubator the MTT solution, we should observe many light blue crystals by the microscope.

3. Wash once every well of the plate with 1 mL of PBS solution.

4. Add 150 μL (for a 12 wells plate) or 250 μL (for a 6 wells plate) of DMSO (Dimethylsulfoxide) to dissolve the pre-formed crystals. After that, store the MTT plate into the incubator for two h.

5. Put 100 μL of the solution from every plate (for a 12 wells plate) or 200 μL (for a 12 wells plate) inside an ELISA plate to mesure the absorbance at 570 nm and at 650 nm by a TECAN plate spectrophotometer. The formazan solution absorbs the light at 570 nm but not at 650 nm; for this reason the final optical density (OD) obtained from the formazan can be calculated as the difference between 570 and 650 nm absorbances.

6. To the obtained OD values, the OD value from the ‘’control’’ well containing PBS solution has to be subtracted. At the end, the data can be normalized compared with one of the treatment (‘’control’’ situation).

2.5 ORO coloration and triglycerides (TG) quantification

Oil Red O (ORO) is a technique useful to demonstrate the presence of fat or lipids in fresh and/or frozen tissue sections. ORO was introduced by French in 1926 and it is a fat-soluble diazo dye, classified as one of the Sudan dyes which have been in use since the late 1800s. ORO is not a true special stain, since it can not form bonds with lipid components but it is actually a pigment that functions as an oil-soluble colorant; so this technique represents a physical method of staining. The basis for staining lipids with an oil-soluble dye is due to its increased solubility in fatty substances, differently from the dye solvents which are used in routine tissue processing, and propylene glycol is the

preferred solvent for this technique. The end result is that fat and lipids in tissue sections stain bright red (Fig. 8).

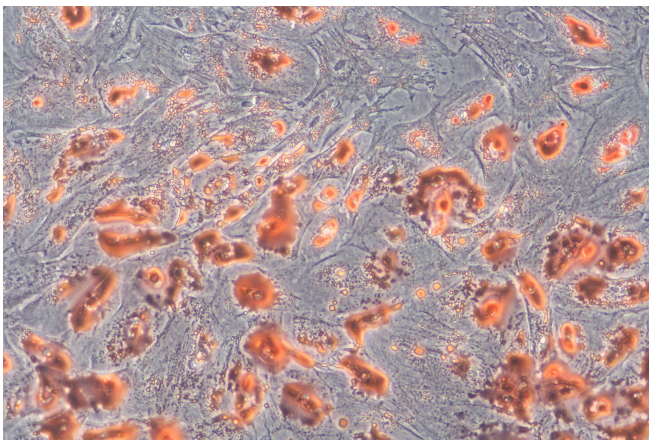

Fig. 8. Picture of adipocytes treated with Oil Red O coloration at day 14 taken by an Olympus optical microscope.

ORO treatments

At day 7 of the cell culture (starting of the cellular differentiation), the following treatments were applied:

1. Control (Differentiation medium + DMSO)
2. RGZ 1 μM
3. RGZ 1 μM + CA 50 μM
4. RGZ 1 μM + HT 100 μM
5. Lipid
6. Lipid + HT 100 μM

7. Lipid + CA 100 μM

After 72 h the ORO protocol can be applied.

Coloration of TG

Stock Solution ORO

- 500 mg ORO/100 ml of 60% TEP (Triethyl phosphate).
- Dilute 12 ml of stock solution with 8 ml of water.
- Filter with paper Whatmann (n. 4).

Working Solution ORO (in 36% TEP)

1. Wash the cells with PBS.
2. Fix the cells with 3.7% formaldehyde for 1 h.
3. Wash three times with wáter for 30 s.
4. Color the cells with Working solution ORO for two h.
5. Wash three times again with water for 30 s.

TG extraction

- Dry cells completely.
- Add isopropanol: 250 µl for plate of 6 wells or 150 µl for plates of 12 wells for 30 min.
- Put in 100 µl x 2 for plates of 6 wells or 100 µl for plates of 12 wells in an ELISA plate at 490 nm (TECAN).
- Add water and let the plate rest overnight.

Coloration and proteins extraction

- Wash twice with distilled water.
- Add 1 ml of Comassie blue solution for 1 h at room temperature.
- Wash twice again with distilled water.
- Add propylene glicol: 1 ml for plates of 6 wells or 500 µl for plates of 12 wells and for 1 h incubate at 60°C.
- Put in 100 µl x 2 for plate of 6 wells or 100 µl for plates of 12 wells and analyse by an ELISA plate at 630 nm (TECAN).

Calculation TG/proteins: simply divide the OD of 490/630 nm.

2.6 Immunofluorescence

Immunofluorescence is a technique primarily used on microbiological samples. It is based on the specificity of antibodies to their antigen to identify a specific target biomolecule with a fluorescent dye, called fluorophore or fluorochrome, allowing the visualization of the distribution of the target molecule through the sample. Immunofluorescence can be used in tissue sections, cultured cell lines or individual cells and may be used to analyze the distribution of proteins, glycans and small biological and not biological molecules. Indirect immunofluorescence relies on two antibodies: the primary antibody and the secondary antibody. The first one detects and joints to a target molecule while the second one, marked with a molecule of fluore, identifies the primary antibody and binds to it. The fluorophore allows visualization of the target distribution in the sample under an epifluorescent or a confocal microscope.

Immunofluorescence treatments

The following treatments were put at day 7 of cell culture:

1. Control (Differentiation medium+DMSO)
2. RGZ 1 μM
3. Lipid
4. RGZ 1 μM + HT 100 μM
5. RGZ 1 μM + CA 50 μM
6. Lipid+HT 100 μM

7. Lipid+CA 50 μM

Immunofluorescence protocol was applied after 72 hours.

Reagents

- PBS (Phosphate Buffered Saline).

- PFA (Paraformaldehylde 4% solution, pH 7.4).

- Triton X 100 (humectante to permeabilize).

- BSA (Albumine Bovine Serum) at 4°C.

- Tween 20 (Polyoxythylenesorbitan monolaurate detergent).

- Prolong (fixation of coverslip on the glass slide).

- HOECHST or DAPI (nucleus colouring).

Solutions attainment

PBS solution 1M at pH 7.4 (x 250 mL of solution):

- 31.15 g of Na_2HPO_4 $2H_2O$.
- 11.70 g of NaH_2PO_4 $2H_2O$.

Dilute in milliQ sterile water, adjust the pH at 7.4 and complete the volume at 250 mL.

PFA 4% solution/PBS (x 250 mL of solution):

- 10 g of PFA.
- 225 mL of milliQ water.
- 25 µL of NaOH.

PFA solution must be heated up to 65°C, until it becomes transparent, under a specific hood for gases extraction because of the toxicity of the solution. As soon as the solution becomes colder, just add 100 mL of PBS at pH 7.4.

PBS solution/Triton x 100 0.2%:

In 100 mL of PBS add 200 µL of Triton x 100 and store the solution at 4°C.

PBS solution/Tween 20 0.1%:

In 100 mL of PBS add 100 µL of Tween 20 and store the solution at 4°C.

BSA solution 3%/Tween 20 0.1%:

In 100 mL of PBS-Tween 20 0.1% add 3 g and store the solution at 4°C.

Procedure

Fixing

1. Take out the plate from the incubator and do two washes with PBS solution (remove the medium from the wells and add 0.5 mL of PBS to each well until its total coverage).

2. Add 0.5 mL of PFA 4%/PBS to each well, or another amount until covering each well totally, for 30 minutes at room temperature.

3. After 30 min, wash three times with PBS for 5 min. At the last wash with PBS, leave the solution inside the wells and store the plate at 4°C for a week.

Permeabilization

1. Remove PBS from the wells and add 0.5 mL of BSA solution 3%/Tween 20 at 0.1% during 1 h.

2. Wash once with PBS for 5 min (add 0.5 mL into each wells for a 12 wells plate).

Primary antibody incubation

1. Dilute the primary antibody with the blocking solution (1:100), add 50 µL of the previous solution in each well and incubate for ¾ h at room temperature (or overnight at 4°C).

At this point, can be added water in the empty space near the wells to simulate the effect of a wet chamber.

2. Do two washes with PBS/Tween 20 solution 0.1% during 5 min.

3. Do another wash with PBS during 5 min.

In a negative control condition primary antibody was not added, just blocking solution. In this way, it can be observed the specificity of secondary antiboby and it should not be seen any fluorescence.

Incubation of secondary antibody and nucleus marking by HOECHST

1. Dilute the secondary antibody with PBS/Tween 20 0.1% solution (1:1000) and add the same amount of nuclear stainer (HOECHST 1000x) and secondary antibody. Add 50 µL of the previous solution in each well and incubate during 1 h at room temperature and in darkness condition.

2. Wash 5/6 times with PBS/Tween 20 0.1% during 5 min.

Assembling

1. After the last wash with PBS, collect the coverslips, previously put inside every well with the help of a croocked blunt bit and a clamp. Bathe the coverslips into distilled water and put them on the slides, previously prepared with a drop (3 μL) of PROLONG.

2. Leave the slides become dry and store them until the following analisys by the microscope.

The microscope analysis was done by a ‘’Leica 63x/1.40-0.6 oil zoom’’ microscope. A series of 10 photos were taken for each treatment and the photos were analysed afterwards with the programme ‘’ImageJ’’.

2.7 PCNA immunohistochemistry

The proliferating cell nuclear antigen (PCNA) is a 36 kDa molecular weight protein also known as cyclin. It was originally identified as an auxiliary factor for DNA polymerase δ and has been described as a DNA sliding clamp that acts as a polymerase processivity factor. PCNA aids in processivity through association with various DNA replication associated proteins. In addition to DNA replication, PCNA function is associated with chromatin remodeling, proliferation, sister-chromatid cohesion, cell cycle control and DNA repair. In addition, PCNA is often used as a marker for cell proliferation. In cells fixed with organic solvents, PCNA is seen to be strongly associated in the nuclear regions where DNA synthesis is occurring, whereas in cells fixed with aldehydes the staining is more diffuse but intense and occurs throughout the cell cycle. This is due to the presence of two basic forms of the PCNA protein, a soluble form sensitive to organic fixation not involved in replication, and a second form that is insoluble and is associated with ongoing DNA synthesis.

Materials

- Coverslips.
- PBS.
- Eppendorfs 1.5 mL.
- Ethanol 70%.
- Paraformaldehyde 4%.
- Clamps.
- Distilled water.
- Kit PCNA.
- Wet chamber to incubate the coverslips.
- Different and gradational series of ethanol (70%, 80%, 90% and absolut).

PCNA treatments

At day 5 of cell culture the following treatments were tested:

1. Control (Growth medium+DMSO)
2. CA 50 μM
3. HT 100 μM
4. RGZ 1μM
5. CA 50 μM + RGZ 1μM
6. HT 100μM + RGZ 1 μM

Procedure

Pre-treatment in culture

1. Aspirate the medium from the wells with the coverslips inside.

2. Wash the wells three times with PBS.

3. Add paraformaldehyde 4% in PBS and let rest during 15 min at room temperature to fix the cells.

4. Aspirate the paraformaldehyde and add 70% ethanol for 5 min at room temperature (repeat twice). The plates can be stored at -20°C until the following step.

5. Remove the coverslips from the wells by the clapms, previously bathed in ethanol, and let dry the coverslips totally.

Staining protocol

1. Put the coverslip in a wet chamber to incubate.

2. Add 100 µL of blocking solution (reactive 1) to each sample and let incubate during 10 min.

3. Dry the solution by absorbant paper.

4. Add 100 µL of primary antibody anti-mouse (reactive 2) to each sample and let incubate in a wet chamder during 60 min.

5. Wash the coverslips three times with PBS during two minutes to remove primary antibody.

6. Add 100 µl of STREPTAVIDINA-PEROXIDASE (reactive 3) to each sample and incubate during 10 minutes at room temperature.

7. Do three washes with PBS for 2 min.

8. Add two drops of reactives 4A, two of reactive 4B and two of reactive 4C of the PCNA kit (Invitrogen) in 1 mL of distilled water and mix the solution (CROMOGEN DAB). Protect the coverslips from the light during the following hour.

9. Add 60 µL of CROMOGEN DAB (reactive 4) to each sample and let incubate for 5 min in a wet chamber.

10. Add 100 µL of HEMATOXILINA (reactive 5) to each sample and incubate for 2 min.

11. Wash the coverslips with current water and bathe them inside PBS, until the colour blue appears (about 30 min). Blue cells are not proliferative.

12. Dehydrate the coverslips doing two washes by a series of gradational alcohols (70%, 80%, 90% and absolut) and a final wash with xilol (50 µL for each sample.

13. Once the coverslips are well dried, add 100 µL of HISTOMOUNT to each sample.

14. Fix the coverslip on a slide and store them into a box for histology.

The microscope analisys was done by an ‘’Olympus BX40 10x/0.25 optical’’ microscope and a series of 5 photos for each treatment were taken. The photos were analysed afterwards with the programme ‘’ImageJ’’.

2.8 RNA extraction, cDNA synthesis and qPCR

Total RNA from adipose (100 mg), liver (50 mg) tissue and primary adipocyte cell cultures (3 wells) were extracted using TriReagent (Ambion) following the manufacturers' recommendations. The quantity of isolated RNA was determined by spectrophotometry with a ND-2000 NanoDrop (Thermo Fisher Scientific). Afterwards, 500-1000 ng of total RNA were treated with Dnase I (Life technologies) following the manifacturers' recommendations to remove all genomic DNA and further the RNA was reverse transcribed with the Transcriptor First Strand cDNA synthesis kit (Roche).

Tissue homogenization

Materials

- Falcons.
- Eppendorfs 1.5 mL.
- TRI Reagent Solution (RNA reagent isolation).
- Polytron.
- MQ water (0.5-1 M).
- NaOH (0.5-1 M).

Procedure

1. Add 1 mL of TriReagent solution to each falcon.

2. Weigh samples (depending on tissue).

3. Put samples inside every falcon together with TriReagent.

4. Homogenize the samples by polytron: 2-3 times for 30 s at 12000 rpm.

5. Put homogenized samples into new eppendorfs.

6. Centrifuge samples at 4°C for 10 min at 12000 rpm.

7. Get only the upper part of the homogenated samples and reject pellet and lipids.

8. Store the samples at -80°C until their use.

Notes: TriReagent must be maintained always into ice; it is a reagent based on water and phenol which extracts nucleic acids and protects from RNAses degradation.

RNA extraction from tissues

Materials

- Chloroform
- Isopropanol
- Ethanol 75%
- Eppendorfs 1.5 mL
- MQ water

Procedure

1. Put 200 µL of chlorophorm into each eppendorf with homogenated tissue and vortex for 20 s.

2. Let rest for 5 min at room temperature so that we can have different phases.

3. Centrifuge at 4°C for 15 minutes at 13000 rpm; then there will be three well differentiated phases: a lower with chlorophorm, an intermediate with protein and a higher one which contains RNA plus water.

4. Get only the upper phase and put it into new eppendorfs.

5. Add 500 µL of isopropanol, that helps RNA to precipitate, and mix 4-5 times by inversion.

6. Let rest the samples for 10 min at room temperature.

7. Centrifuge at 4°C for 10 minutes at 13000 rpm to obtain a higher aqueous phase and a white pellet, which contains RNA.

8. Reject the aqueous part without touching the pellet.

9. Add 1 mL of ethanol 75%.

10. Centrifuge at 4°C for 5 minutes at 7500 rpm.

11. Repeat the last three steps, so that we do two washes with ethanol 75%.

12. Reject aqueous phase and dry pellet so that ethanol evaporates.

13. Re-suspend pellet by adding 30 µL or less of RNAse Free water.

14. Finally, quantify RNA samples by Nanodrop.

cDNA synthesis and retrotranscription

DNAse treatment Kit

Steps:

1. Label 200 µL RNAse free eppendorfs.

2. Calculate the amount of DEPC water in µL to use with Excel, based on the amount of RNA (µg).

3. Prepare mix: DNAse (1µL) + Buffer (1µL) + 2-3 pipetting error. Keep on ice.

4. Add the DEPC water corresponding to each sample (see Excel sheet).

5. Add the amount of RNA from each sample except CNTC (control without RNA) (see Excel sheet).

6. Add mix (2 µL/tube) and put the timer 15 min from the first sample (room temperature).

7. After 15 min, add 1 µL of EDTA to stop reaction.

8. Place tubes in PCR machine and select ‘’DNAse protocol’’ (10 min at 65°C).

Retrotranscription Kit cDNA

Steps:

1. Label the final eppendorfs with sample code.

2. Make a mix with oligodT's (1µL) + Random hexamer (2 µL) + 2-3 pipetting error. Keep on ice.

3. Add 10 µL from the samples to new eppendorfs.

4. Add 3 µL of the mix per tube.

5. Place tubes in PCR machine and select ''Denaturalization protocol'' (10' at 65°C).

6. Prepare mix with buffer (4 µL + Protector (0.5 µL) + dNTPs (2 µL) + RT enzyme (0.5 µL) + 2-3 pipetting error. Keep on ice.

7. Add 7 µL of mix per tube to all samples, except for CRTC (add water instead of RT enzyme).

8. Place the eppendorfs in PCR machine and select ''RT protocol'' (approximately 90 min).

***In vivo* and *in vitro* RT-qPCR real time and gene expression analysis**

The analysis were performed using 2.5 µL of IQ SYBR Green Supermix (Bio-Rad), 250 nM of forward and reverse primers and 1 µL of cDNA for each samples in a final volume of 5 µL. Reactions were performed in triplicate in 384-well plates using an iCycler iQ Real-Time Detection System (Biorad), in the

same conditions previously described by Salmerón et al., 2013. Before the analyses, a dilution curve with a pool of samples was run to confirm primer efficiency and to determine the appropriate cDNA dilution. SYBR Green fluorescence was recorded during the annealing-extending phase of cycling. Negative controls (NTC: No Template Control; RTC: no Reverse Transcriptase Control and PCR: water) were included and ran in duplicate.

2.9 Statistical analysis

All data were analyzed using the statistical software IBM SPSS Statistics 19. Data were reported as mean values ± SEM (Standard Error of the Mean). For *in vitro* experiments, n=4 independent experiments were used. For *in vivo* experiments, n=9 fish were analyzed. To perform the statistical analyses, first the data were $\log_{10}$-transformed. Then, it was confirmed that the data were normally distributed according to the Shapiro-Wilk test and that presented homogeneity in the variance based on Levene test. Finally, statistical differences were analyzed by one-way ANOVA followed by Tukey *post hoc* test (Levene test not significant) or Dunnett test post hoc (Levene test significant). When the data did not follow the ANOVA presumptions, the non-parametric Kruskal-Wallis followed by Mann-Whitney tests were performed. Differences were considered statistically significant at $P<0.05$.

3. Caffeic acid/ Hydroxytyrosol *in vitro* results

MTT results

The effects of CA and HT on cell viability are reported in Fig. 9. CA and HT alone increased cell viability significantly. RGZ alone, instead, had no significant effect on cell viability and when combined with CA or HT reduced their positive effects slightly.

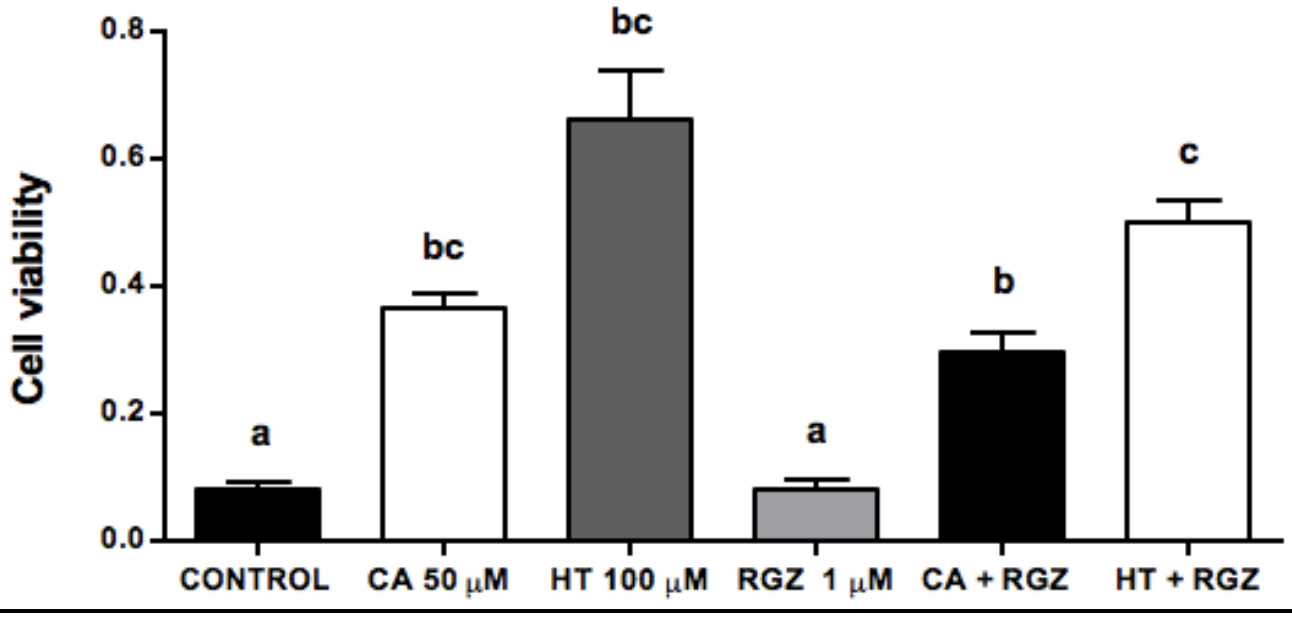

Fig. 9. Effects of CA, HT and RGZ on rainbow trout adipocytes cell viability. Cell viability was identified by the addition of MTT reagent at day 5 of cell culture during 3 h. Data is shown as mean ± SEM (n=4). Different letters indicate significant differences (P<0.05). CA= caffeic acid, HT= hydroxytyrosol, RGZ= rosiglitazone.

PCNA results

Regarding cell proliferation, measured as percentage of PCNA-positive cells, (Fig. 10) statistical significant differences among the treatments were not observed.

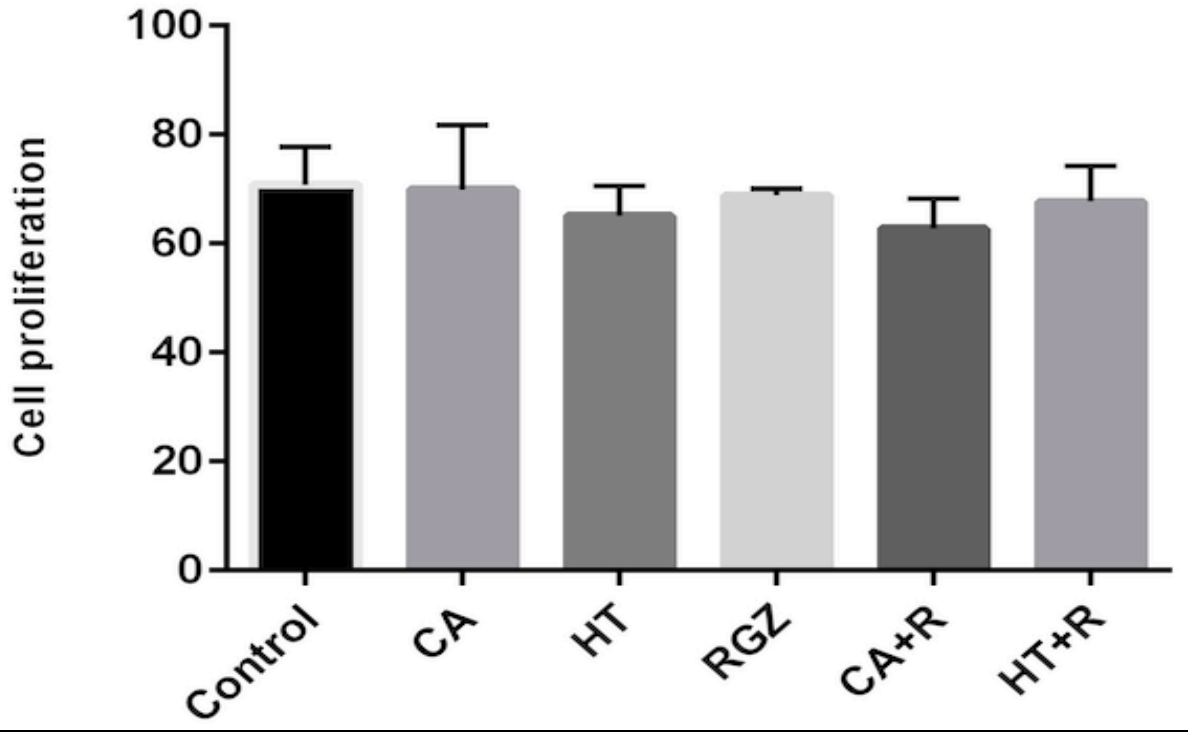

Fig. 10. Effects of CA and HT on proliferation in rainbow trout adipocytes at day 5 of cell culture. Data is shown as mean ± SEM (n=4). Values were considered significant at $P<0.05$. CA= caffeic acid, HT= hydroxytyrosol, RGZ= rosiglitazone.

ORO results

In the case of ORO assay (Fig. 11), it was observed a clear anti-obesogenic effect of HT. In fact, in the treatment in which it was placed in combination with a lipid mixture, this substance showed a significant decrease of lipid specific content, compared to the control or lipid alone. Concerning CA, it had a tendency to decrease the specific lipid content, combined with lipid, but not significantly. Lipid alone increased the specific lipid content slightly.

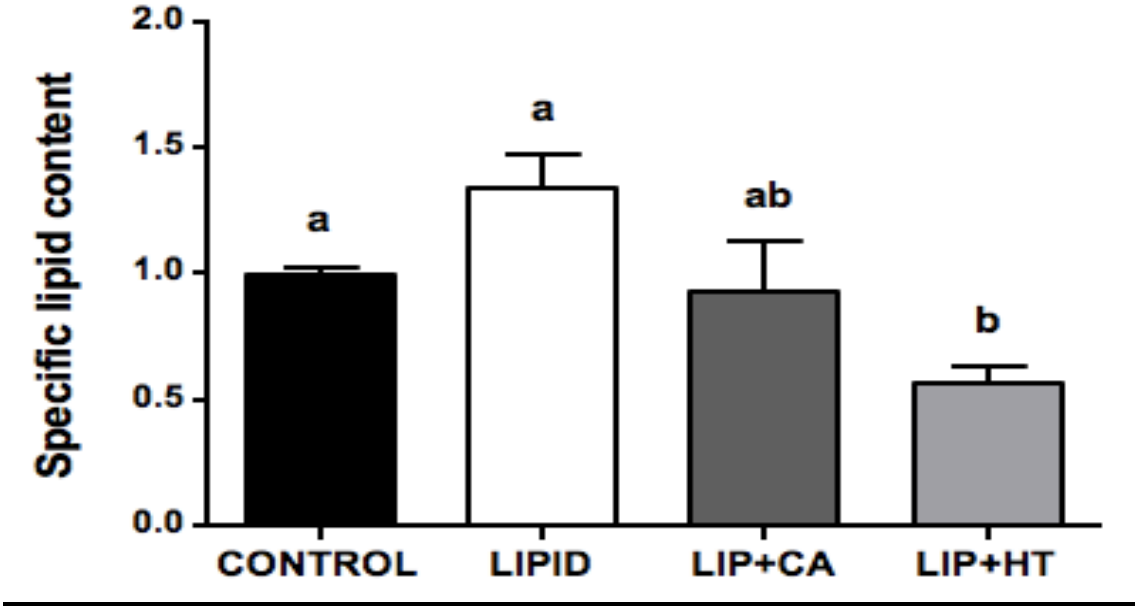

Fig. 11. Effects of CA and HT on the specific lipid content in raibow trout adipocytes at day 7 of cell culture during 2 h. Error bars represent a standard error ± SEM of 4 independent experiments. Different letters indicate significant differences (P<0.05). CA= caffeic acid, HT= hydroxytyrosol, LIP= lipid mixture.

Immunofluorescence results

As regards to the immunofluorescence assay to detect PPARγ (Fig. 12), both CA and HT, combined with RGZ, reduced significantly the obesogenic effect of RGZ, decreasing the fluorescence at the control level, symptom of a lesser expression of the PPARγ. As it can be observed in fig. 13, RGZ alone promoted signal intensity of PPARγ expression with evidence.

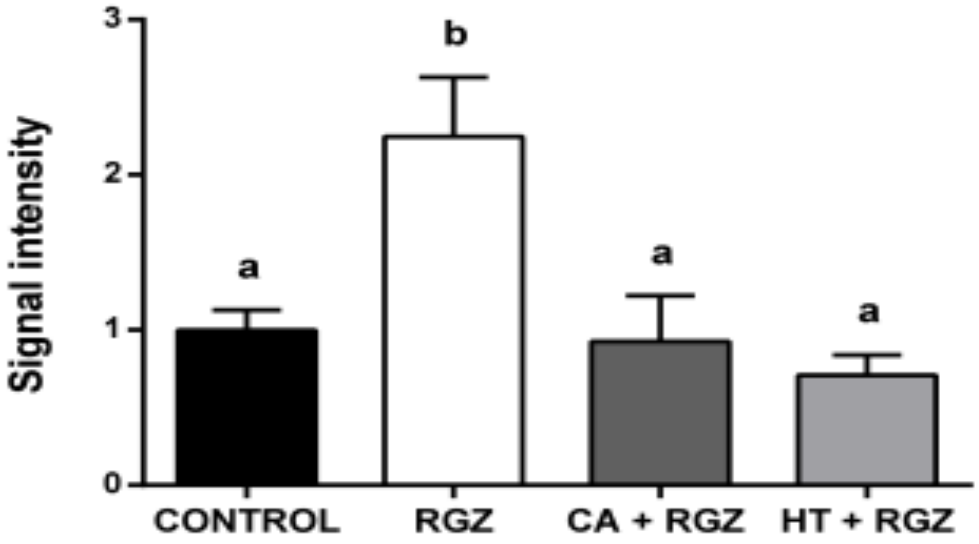

Fig. 12. Effects of CA and HT on signal intensity in rainbow trout adipocytes cell culture at day 7. Data is shown as mean ± SEM (n=4). Signal intensity is relative to the control. Different letters indicate significant differences (P<0.05). CA= caffeic acid, HT= hydroxytyrosol, RGZ= rosiglitazone.

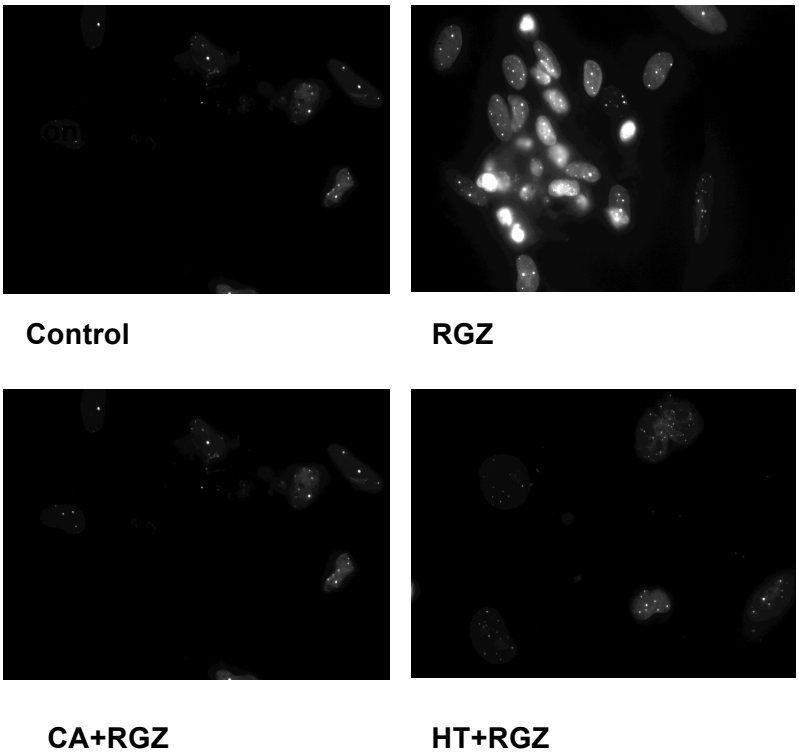

Fig. 13. Immunofluorescence representative of PPARγ images from 4 independent experiments taken by a Leica 63x/1.40-0.6 oil zoom

epifluorescence microscope. CA= caffeic acid, HT= hydroxytyrosol, RGZ= rosiglitazone.

In these images from the same immunofluorescence assay taken by a confocal microscope (Fig. 14) we can see the differences among the treatments respect to the control. As it can be observed either from the graph (Fig. 12) or from the epifluorescent microscope (Fig. 13), RGZ promoted signal intensity, while in combination with CA or HT, this effect was decreased.

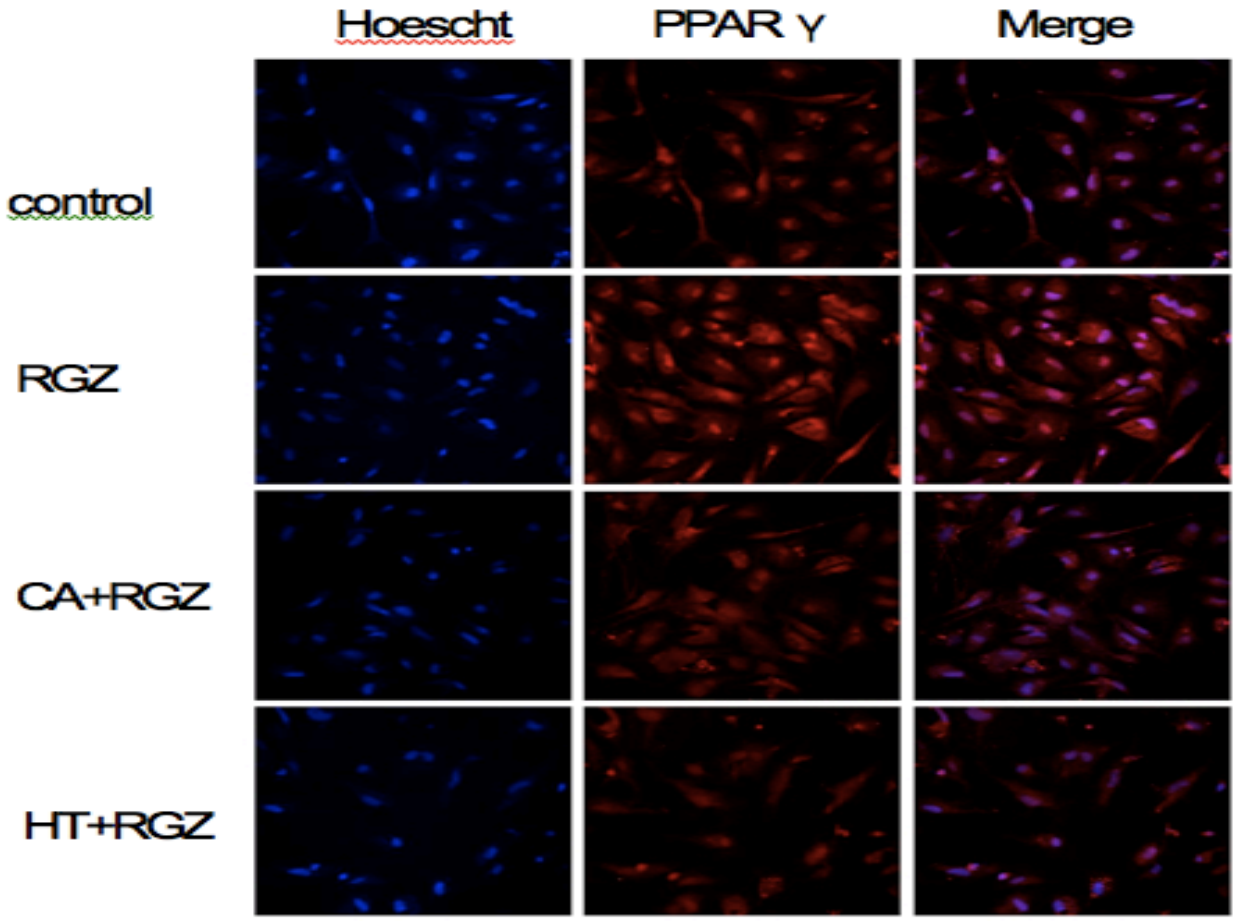

Fig. 14. Immunofluorescence representative images from 4 independent experiments taken by confocal microscope. In cells treated with the stain Hoescht we can observe nuclei coloured in blue; in the red photos luminescence is proportional to PPARγ expression. CA= caffeic acid, HT= hydroxytyrosol, RGZ= rosiglitazone.

qPCR results

The relative expression of different adipogenic genes, in response to the different treatments, is shown in fig. 15. No significant differences among the treatments were observed for FAS or LPL gene expression (Figs. 15A and 15B). The highest mRNA expression levels of HSL (Fig. 15C) were found in the treatments where a lipid mixture was put, whereas lower levels compared to the control were observed when RGZ was added. CA and HT did not cause any variation to HSL expression when combined, neither with RGZ or lipid. PPARγ resulted significantly up-regulated in the presence of a lipid mixture (Fig. 15 D). In combination with lipid, both CA and HT, tended to decrease the lipid effect on PPARγ regulation. On the other hand, RGZ determined only a slight rising in PPARγ expression, but not significantly, even when combined with CA or HT. Concerning C/EBPα (Fig. 15E), only lipids increased the expression of this gene significantly. CA and HT, on the contrary, had a tendency to limit the lipid effect on C/EBPα expression. RGZ raised C/EBPα regulation slightly, even in the presence of CA and HT.

Gene	Sequence 5'-3' (F+R)	Annealing temperature
Ubiquitin	ACAACATCCAGAAAGAGTCCAC AGGCGAGCGTAGCACTTG	58
EF1α	CTTCAACGCTCAGGTCATCAT GCACAGCGAAACGACCAAGGGGA	59
β-actin	ATCCTGACAGAGCGCGGTTACAGT TGCCCATCTCCTGCTCAAAGTCCA	62
FAS	GAGACCTAGTGGAGGCTGTC TCTTGTTGATGGTGAGCTGT	54
LPL	GAGCACGCAGACAACCAGAA GGGGTAGATGTCGATGTCGC	59
PPARγ	GCCAGTACTGTCGCTTTCAG TCCATAAACTCAGCCAGCAG	60
C/EBPα	TGTGGCGATAAAGCAAGAGC CTGGTGGGAATGGTGGTAGG	57
HSL	AGGGTCAATGGTCATCGTCTC CTTGACGGAGGGACAGCTAC	58

Table 1. Rainbow trout primer sequences (Forward and Reverse) used for *in vitro* real-time PCR.

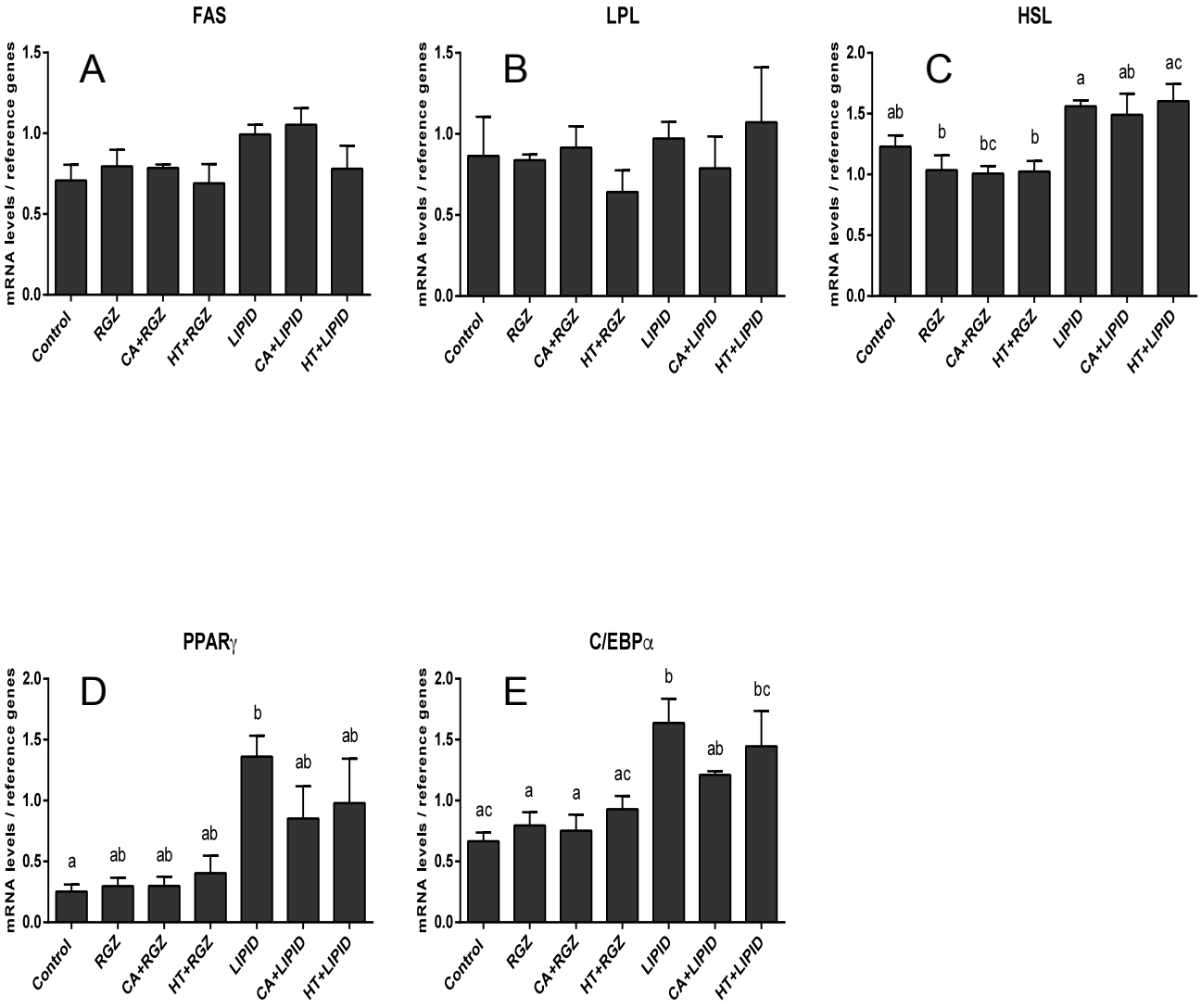

Fig. 15. Relative expression of the genes FAS (**A**), LPL (**B**), HSL (**C**), PPARγ (**D**) and C/EBPα (**E**) in rainbow trout primary cultured adipocytes under different treatments. EF1α, ubiquitin and β-actin were used as reference genes. Values are reported as mean ± SEM of 4 independent cultures. Different letters indicate significant differences between treatments at $P<0.05$. FAS= Fatty acid synthase, LPL= Lipoprotein lipase, HSL= Hormone-sensitive lipase, PPARγ= Peroxisome proliferator-activated receptor gamma, C/EBPα= CCAAT/enhancer binding protein alpha.

3.1 Caffeic acid/ Hydroxytyrosol *in vivo* results

The relative expression of different adipogenic genes and related oxidative stress genes, in response to the treatments used (Control, CA 10μg/g, HT 20μg/g), is shown in fig. 16. Concerning adipose tissue (Fig. 16A), none of the mRNA expression levels of the genes examined showed significant differences among the treatments with the exception of FAS, where the mRNA levels were influenced by the treatments slightly. In particular, CA had a tendency to decrease FAS levels while HT produced a net and significant fall of FAS expression, compared to the control.

In liver (Fig. 16B), HT decreased IGF-II transcript levels significantly but it did not produce any significant effect on the expression of the other genes analyzed. It was just observed a mild but not significant rising in GHR-II and GPX1 levels produced by HT. CA, instead, had a significant effect on GHR-I, GHR-II and GPX1 levels, increasing their mRNA amount visibly.

Table 2. Rainbow trout primer sequences (Forward and Reverse) used for *in vivo* real-time PCR.

Gene	Sequence 5'-3' (F+R)	Annealing temperature
Ubiquitin	ACAACATCCAGAAAGAGTCCAC AGGCGAGCGTAGCACTTG	58
EF1α	CTTCAACGCTCAGGTCATCAT GCACAGCGAAACGACCAAGGGGA	59
β-actin	ATCCTGACAGAGCGCGGTTACAGT TGCCCATCTCCTGCTCAAAGTCCA	62
FAS	GAGACCTAGTGGAGGCTGTC TCTTGTTGATGGTGAGCTGT	54
GHR1	CGTCCTCATCCTTCCAGTTTTA GTTCTGTGAGGTTCTGGAAAAC	55
GHR2	TCTCCTCATCCTAACAGTTGTG GCTCTGTGAGGTTCTGGAATGT	55
IGF1	TGGACACGCTGCAGTTTGTGTGT CACTCGTCCACAATACCACGGTT	64
IGF2	CGGCAGAAACGCTATGTGGA TGCTGGTTGGCCTACTGAAA	60
IGFR1α	AGAGATAGACGACGCCTCCTA CACCAAATAGATCCCTACGT	54
GPX1	CGAGCTCCATGAACGGTACG TGCTTCCCGTTCACATCCAC	60
HPGPX	TTGGAGGTCAGGAGCCAGGT ACCCTTTCCCTTGGGCTGTT	60
SOD	CCATGGTAAGAATCATGGCGG CGTGGATCACCATGGTTCTG	54
TR	ACCGTGCAGCCTAGAATGCT GTGATGTCTCTCTTTGCAGTTCCTT	58

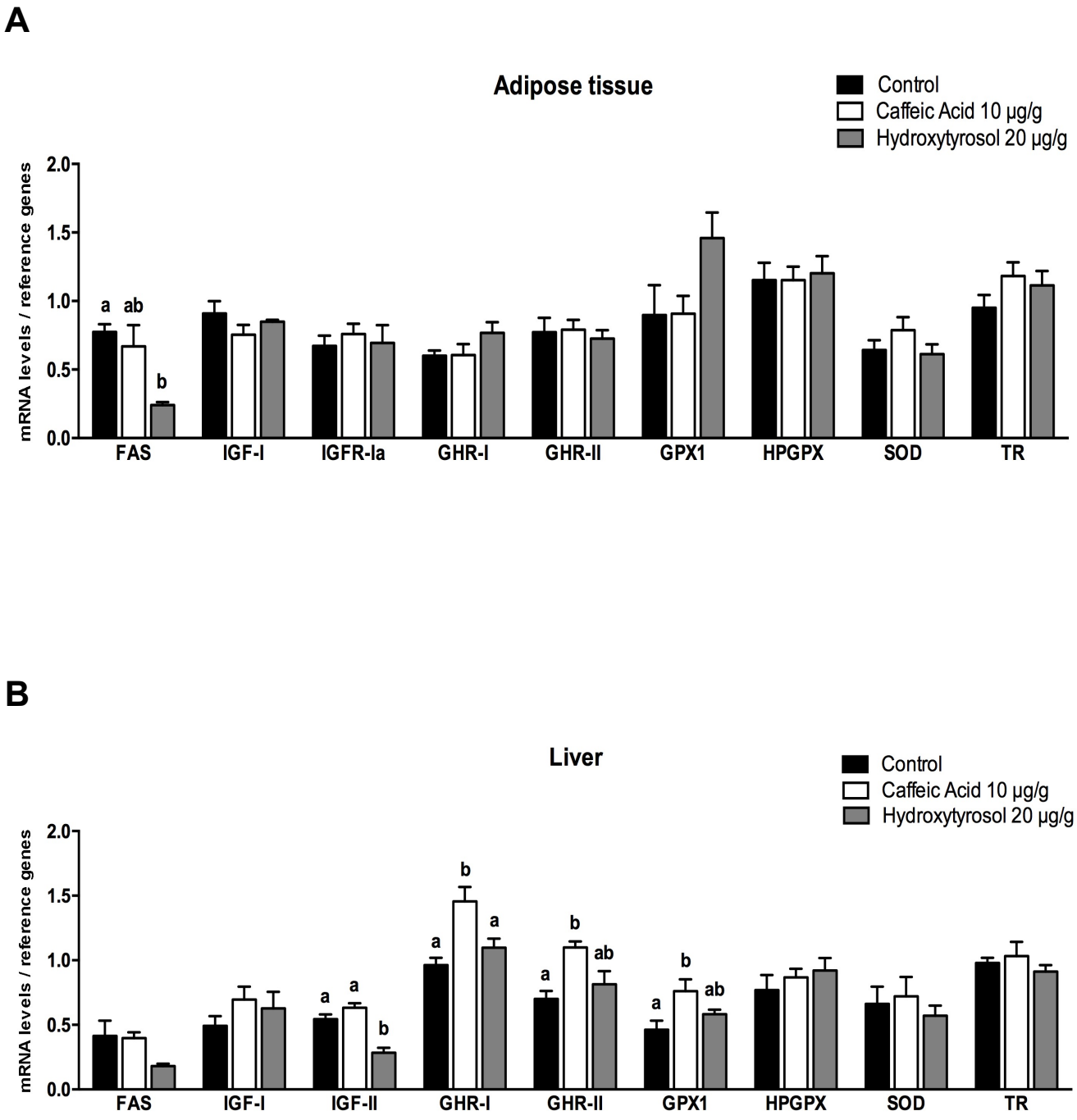

Fig. 16. Relative expression of the genes FAS, IGF-I, IGF-II, IGFR-Iα, GHR-I, GHR-II, GPX1, HPGPX, SOD and TR in adipose tissue and liver of rainbow trout under different treatments (Control, CA 10µg/g, HT 20µg/g). EF1α, ubiquitin and β-actin were used as reference genes. Values are reported as mean ± SEM (n=9). Different letters indicate significant differences between treatments at $P<0.05$. FAS= fatty acid synthase, IGF-I= insulin-like growth factor-1, IGF-II= insulin- like growth factor-2, IGFR-Iα= insulin-like growth factor receptor-Iα, GHR-I= growth hormone receptor-1, GHR-II= growth hormone receptor-2, GPX1= glutathione peroxidase1, HPGPX= phospholipid-

hydroperoxide glutathione peroxidase, SOD= superoxide dismutase, TR= thioredoxin-disulfide reductase.

4. Discussions

In vitro results confirmed our initial assumption: the potential anti-obesogenic effects of the compounds CA and HT on the adipose tissue of the rainbow trout (*O. mykiss*). All *in vitro* tests were carried on to test this hypothesis and resulted in accordance among them; only results from proliferating cell nuclear antigen (PCNA) assay were not significant. In the MTT assay it was observed that CA and HT, used alone in the treatments, increased cell viability appreciably. Cell viability was not affected in presence of RGZ (an agonist of PPARγ). Differently from our MTT results, in a study made on 3T3-L1 cells of rats, it was observed that neither of the phenolic compounds (HT and OL) used in the experiment produced any significant effects on cell viability at the concentrations used (0, 100, 150, 200 μmol/L) in the experiment (Drira et al., 2011). The increase in cell viability observed does not necessarily mean that our result is not in agreement with this last study, in fact we can obtain a higher amount of cells but not differentiated. What does not agree is the fact that we do not see changes in PCNA. Moreover, both HT and OL exhibited anti-adipogenic effects essentially in the early stage of cells differentiation, in fact their effects during the middle and late stages were very low, with no significant differences seen between the control and the treated cells (Drira, et al., 2011). In addition, it was seen by Drira et al. (2014) that HT attenuated triglyceride accumulation and stimulated glycerol release in fully differentiated adipocytes in a dose- and time-dependent manner (Drira, et al., 2014). To test the effects of CA and HT on rainbow trout pre-adipocytes proliferation, PCNA test was carried on, but we did not obtain any significant result from this test. From the ORO assay, emerged that lipids had a mild adipogenic effect, in fact they tended to increase the specific lipid content in rainbow trout adipocytes, which is in agreement with the fact that lipid mixture is important in trout adipocyte differentiation (Bouraoui, et al., 2008). In

Salmon pre-adipocytes, it was observed also that they differentiate only in the presence of lipid mixture (Vegusdal et al. 2003). CA and HT, instead, did not contribute to the amount of adipocytes in rainbow trout adipose tissue, even in association with lipids. A previous study conducted by Shin et al., (2014) showed that 40μM of caffeic acid phenethyl ester (CAPE) completely inhibited adipogenesis of 3T3-L1 preadipocytes. Moreover, the same authors proved that, in rats fed with high fat diet, CAPE showed decreased level of adiposity in a dose-dependent manner. We also observed, by immunofluorescence assay, a reduced expression of PPARγ, which is the main promoter of adipogenesis, caused by the action of CA and HT, even when these two compounds are along with RGZ. Unfortunately, we do not have any specific study on the effects of CA and HT in fish to make a comparison with, only many works done in mammals. For example, it was previously demonstrated in other studies (Juman et al., 2010a; Warnke et al., 2011) that CA and HT were able to inhibit adipocytes differentiation and cell lipid content in mammals through an inhibition of PPARγ expression. In a study published by the same authors (Juman et al, 2010b) it was shown how the addition of CA during adipocytes differentiation of 3T3-L1 mouse fibroblasts cells produced a reduction in intracellular lipid storage. Treatments with CA, through staining with ORO, caused a dose-dependent suppression on oil droplet accumulation, a reduction in the size of the same droplets and a significant decrement in triglycerides deposition (Juman et al., 2010b). These results suggest that CA usage could be negative for the process of adipocytes differentiation.

As regard for HT, it was suggested that short-term control mechanisms of fatty acid and cholesterol synthesis by HT and high phenol extract, such as OL, were effective in liver cells determining a strong inhibition in total synthesis of cholesterol, fatty acids and neutral lipids *in vivo* and *in vitro* treatments (Jemai et al, 2008; Park et al, 2011; Priore et al, 2014). These data is in support of our results.

To have a more complete view about the effects of CA and HT on rainbow trout adipocytes differentiation, *in vitro* qPCR was performed. The results obtained, in agreement with our previous *in vitro* results, showed that the two compounds

had a mild anti-adipogenic effect. In fact, CA and HT tended to decrease PPARγ and C/EBPα expression, even if not in a significant way. HT alone, also up-regulated HSL mRNA levels slightly. Lipids, instead, increased PPARγ and C/EBPα expression significantly, in agreement with Vegusdal et al., (2003). According to these authors, the first evidence of the adipogenic process was the accumulation of lipid droplets: one or more components of the lipid mixture acted as a adipogenic factors, and the mixture also provided cholesterol and lipids for the fat droplets that were increasing in number and size (Vegusdal, et al., 2003). In addition, lipids increased FAS slightly, in concordance with its obesogenic effect. In fact FAS is a key enzyme involved in *de novo* lipogenesis that catalyzes the synthesis of long chain fatty acids, mainly by catalyzing acetyl coenzyme A and malonyl coenzyme A (Smith et al., 2003). However, FAS gene cloning is limited in fish, and data on its gene expression are scarce (Weil et al., 2013). No effect by Lipids, CA and HT was seen on LPL, that is considered a key enzyme in lipid deposition and metabolism in mammals and in fish (Bouraoui, et al., 2011), and it is one of the precocious markers of preadipocyte differentiation into mature adipocytes (Weil, et al., 2013).

Regarding RGZ, it was observed no effect on FAS and LPL, but a slight down-regulation of HSL and a mild up-regulation of PPARγ and C/EBPα. In support of these results, RGZ is an agonist of PPARγ (Semple et al, 2006) and it belongs, along with troglitazone, to the drug class of Thiazolidinediones (TZDs), known as specific anti-diabetic agents that decrease plasma glucose levels and enhance insulin sensitivity in obese mammals (Olefsky and Saltiel, 2000; Lebovitz, 2002; Reynolds and Goldberg, 2006). TZDs induce differentiation of preadipocytes in a wide variety of cell culture systems (Tafuri, 1996; Hausman et al., 2008) via activation of PPARγ, which is considered one of the main transcription factors involved in the regulation of adipocyte differentiation (Rosen et al., 1999; Hammarstedt et al., 2005). During this process, the TZDs promote uptake of glucose and fatty acids, storage of TAG and production of adiponectin (Yki-Jarvinen, 2004; Tontonoz and Spiegelman, 2008).

An *in vitro* study done on 3T3-L1 mouse adipocytes by Drira et al. (2014), was

shown that HT down-regulated HSL, in contrast with our result, in which HT up-regulated slightly HSL expression in rainbow trout adipocytes. On the contrary, our results are in concordance with the role HSL plays both in mammals and fish, namely it is the principal enzyme that catalyzes the hydrolysis of TAGs (triglycerides). In particular, it is involved in the first two steps of this process (breakdown of TAG into dyacylglycerol (DAG) and then into monoacylglycerol (MAG), (Weil et al., 2012). In addition, diet composition can modify HSL activity; in fact, it was showed that basal lipolysis increased in visceral mature adipocytes collected from gilthead seabream fed with plant enriched diets (Cruz-Garcia et al., 2011) or without vegetable oils. (Albalat et al., 2005a), corroborating our results.

Adipocyte differentiation is induced by a series of programed changes in gene expression in mammals (Hwang et al. 1997) as in fish. In both of them PPARγ and C/EBPα are key adipogenic transcription factors (Tchkonia et al., 2002), that activate adipocyte-specific genes and are involved in the growth arrest required for adipocyte differentiation (Niemela et al., 2007).

However, while PPARγ is considered the master regulator of adipogenesis, so it can induce adipogenesis in C/EBPα-deficient cells, C/EBPα, on the contrary, is incapable of driving the adipogenic program in the absence of PPARγ (Rosen et al., 2002). This observation suggests that C/EBPα and PPARγ participate in a single pathway of adipose development, in which the second one is the dominant factor (Farmer, 2006). Moreover, it was seen that these two transcription factors are necessary for the transition of pre-adipocytes into adipocytes *in vitro* (Bouraoui, et al., 2008). In this way, as just said, this confirmed our hyphotesis from the *in vitro* results about the role of CA and HT on PPARγ and C/EBPα mRNA levels, and thus inhibiting adipogenesis. Moreover, in support of these results, Drira et al. (2014) demonstrated that HT down-regulated the expression of PPARγ and C/EBPα also in 3T3-L1 mouse adipocytes.

In vivo results showed a anti-obesogenic effect of HT in adipose tissue, as observed by the significant decrease in FAS gene expression. Regarding

oxidative stress, we could hypothesize an eventual anti-oxidant role of CA in liver. In adipose tissue oxidative-stress related genes were not differentially expressed. In liver, CA was seen to increase GHR-I and GHR-II expression, but no effect was observed on IGF-II. A possible explanation could be that the relative contribution of fish GHRs on IGF regulation probably depends on many factors, such as fish lineage, age, nutritional and environmental condition (Saera-Vila et al., 2007). Differently, in non-salmonid fish, as gilthead seabream, it was highlighted the key role of GHR-I in the tissue-specific regulation of IGFs, but not of GHR-II.

We took into account GH hormone (even if not measured in our experiments) and its GHRs receptors because it is not only linked with growth, but also involved in other processes, among which metabolism (Bjornsson et al., 2004; Norrelund, 2005). In particular, in fish as in mammals, GH is an anabolic protein while promoting lipid and carbohydrate breakdown. In fact, the lipolytic effect of GH in fish was first demonstrated in adipose tissue and liver of juvenile coho salmon *in vivo* (Sheridan, 1986) and GH was also shown to directly stimulate lipolysis in liver isolated from rainbow trout (O'Connor et al., 1993) and adipose tissue isolated from seabream (Albalat et al., 2005). GH also exerted an anti-lipogenic effect in the liver of gilthead seabream, in which it regulated acetil-CoA carboxylase (Yin et al., 1998). More recently, Norbeck et al. (2007) demonstrated that, during periods of food deprivation, lipid depletion is accompanied by increases in plasma levels of GH in the face of reduced plasma levels of insuline and IGF-I in mammals and fish (Norbeck et al., 2007). In line with that we said previously, Bergan et al., (2012) showed that fasting-induced lipolysis in trout liver (as well as in adipose tissue and red and white skeletal muscle) was accompanied by the deactivation of Akt, JAK2, STAT5 and by the activation of ERK and PKC. In particular, GH stimulated lipolysis during fasting through the activation of PKC and ERK in conjunction with the deactivation of PI3K/Akt and JAK-STAT, enhancing the expression of HSL mRNA levels, as well as phosphorilating HSL via the activation of PKC and ERK (Bergan et al., 2013); even if the mechanisms of action involved in GH proliferative and metabolic effects in fish are not well known (Bergan et al., 2013).

Furthermore, in a previous study on rainbow trout (Norbeck et al., 2007), it was demonstrated that decreased hepatic sensitiveness to GH was also supported by reduced hepatic levels of GHR-I and GHR-II, so that, in our work, an increase in hepatic levels of GHRs caused by CA could determine the contrary effect as on trout hepatocytes, making them more responsive to GH action in the liver. Moreover, considering the fact that the actions of GH are initiated by binding to the GHR on the cell membrane of target tissues, we can suppose that CA increasing GHRs mRNA levels could be enhancing an eventual lipolytic action of GH in rainbow trout liver (Norbeck et al., 2007).

In adipose tissue, as CA in liver, it was seen that HT had a possible lipolytic effect, decreasing FAS strongly. In fact, FAS, as we said previously, is involved in lipogenesis (Wakil, 1989).

Regarding CA and HT effects on oxidative-stress related genes in liver, only GPX1 was significantly expressed, among the genes analyzed (SOD, TR and HPGPX). In particular, it was observed an up-regulation of GPX1 by CA, having an indirect effect against oxidative stress by modulating gene expression and enzyme activity, which enhances enzymatic antioxidant defenses (Oliveras-Lopez et al., 2008). HT, instead, had only a tendency to up-regulate GPX1.

In concordance with these results, it is known that to decrease reactive oxygen species levels and avoid lipid peroxidation, all aerobic organisms possess two types of antioxidant defense systems. One is represented by enzymes and includes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX). SOD is a metalloenzyme that catalyses the dismutation of the superoxide anion (O_2^-) into oxygen and H_2O_2 (Fridovich, 1997). The Cu/Zn-SOD is located in the cytosol and nucleus, while Mn-SOD is localized within the mitochondrial matrix. Subsequently, H_2O_2 is reduced to water by CAT in the peroxisomes. GPX catalyses the same reaction as CAT in the cytosol and also converts lipid hydroperoxides into lipid hydroxides, which are more stable products (Fontagnè et al., 2008).

In mammals, several studies were done on the effects of polyphenolic compounds derived from extra virgin olive oil, such as HT, on oxidative stress. For example, in a study carried on mice by Oliveras-Lopez et al. (2008), it was

observed that olive oil exerted both a direct and an indirect anti-oxidant activity through an up-regulation of SOD, CAT and GPX expression in mice liver (Olivera-Lopez et al., 2008). In mammals, it was reported that, as HT, also CA can have anti-oxidant effects (Li et al., 2015). In a work on rats it was demonstrated that CAPE had an anti-oxidant role in liver, with a dose-dependent increase of SOD and CAT mRNA levels. In another study, Karthikesan et al. (2007), found similar results to the previous work, with an increase in the level of enzymic antioxidants enzymes (SOD, GPX, CAT) in rat liver produced by a dose of CA (12 mg/kg of body weight).

Unfortunately, we have not specific *in vivo* studies in fish regarding the effects of CA and HT on the oxidative stress, but just a few works on natural products such as propolis and olive oil, containing CA and HT respectively. For example, a study on rainbow trout showed that propolis (10ppm) had *in vivo* anti-oxidant activity, with an increase of malondialdehyde (MDA) levels and catalase (CAT) activities in spleen and heart tissues, showing significant therapeutic effects (Aldemir et al., 2014). Similar *in vivo* effects of propolis were shown in another study on carp (*Cyprinus carpio)* liver, gill and muscle tissues by Talas et al., (2014). A case-study made in cod liver (De Leonardis et al., 2001) reported that elevated doses of CA could preserve the original fish oil composition and limit the loss of its nutritional quality, inhibiting its double-bond conjugation, so reducing the decrement of PUFA-n3. Concerning HT, it was seen how this substance, in nutritionally relevant amounts, is able to positively modulate the glutathione-driven antioxidant enzymatic machinery in the adipose tissue, probably via homeostatic feedback (Giordano et al., 2014).

5. Conclusions

- CA and HT significantly increase cell viability *in vitro*, although they do not affect cell proliferation.
- CA and HT suppress the increase in lipid content caused by lipids and the protein expression of PPARγ by RGZ in rainbow trout adipocytes, demonstrating they are anti-obesogenic compounds.
- CA and HT *in vitro* do not show any effect on lipogenic genes (FAS and LPL) and only slightly affect the induction of the lipolytic gene HSL, caused by the lipid mixture.
- Lipid mixture significantly increase the gene expression of the transcriptional markers (PPARγ and C/EBPα) while CA and HT slightly decrease this effect, supporting their anti-adipogenic role.
- *In vivo* HT has a clear anti-obesogenic role, strongly reducing FAS expression in rainbow trout adipose tissue; CA has a potential anti-obesogenic effect in trout liver, enhancing GHR-I and GHR-II expression.
- A possible antioxidant action of CA is observed in rainbow trout liver *in vivo*.

Further studies would be useful and necessary in the future to better clarify the effects of antioxidant and anti-obesogenic compounds on fish target tissues, responding to the growing demand of aquaculture products and to improve in this way the flesh taste and quality.

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