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In vivo and *in vitro* effects of genistein as obesogenic/anti-obesogenic compound in the lipid metabolism of rainbow trout (*Oncorhynchus mykiss*)

Tesi di laurea in

Adattamenti degli animali all'ambiente marino

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To my family…..

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Abstract

Nowadays, soy is one of the most used ingredients in the formulation of fish feed, due to the ample market supply, lower market price, high protein concentration and favorable amino acid composition. Nevertheless, soybean meal products are rich and primary diet source of phytoestrogens, as genistein, which may have a potential negative impact on growth, hormonal regulation and lipid metabolism in fish. The principal aim of this study was to better understand in vivo and in vitro genistein's effects on lipid metabolism of rainbow trout. In adipose tissue it was showed an unclear role of genistein on lipid metabolism in rainbow trout, and in liver an anti-obesogenic effect, with an up-regulation of autophagy-related genes LC3b (in adipose tissue) and ATG4b (in liver and adipose tissue), a down-regulation of apoptosis-related genes CASP3 (in adipose tissue) and CASP8 (in liver). An increase of VTG mRNA levels in liver was also observed. Genistein partially exerted these effects via estrogen- receptor dependent mechanism. In white muscle, genistein seemed to promote lipid turnover, up-regulating lipogenic (FAS and LXR) and lipolytic (HSL, PPARα and PPARβ) genes. It seemed that genistein could exert its lipolytic role via autophagic way (up-regulation of ATG4b and ATG12l), not through an apoptotic pathway (down-regulation of CASP3). The effects of genistein on lipid-metabolism and apoptosis-related genes in trout muscle were not dose-dependent, only on autophagy-related genes ATG4b and ATG12l. Moreover, a partial estrogenic activity of this phytoestrogen was also seen. Through in vitro analysis (MTT and ORO assay), instead, it was observed an anti-obesogenic effect of genistein on rainbow trout adipocytes, and this effect was not mediated by ERs. Both in vivo and in vitro, genistein exerted its effects in a dose-dependent manner.

1. Introduction

World aquaculture production has increased significantly in the last 50 years , with an average annual growth rate of 6.1% in volume between 2004 and 2006 (FAO, 2009). Fish meal and fish oil are the main raw materials used in the formulation of fish feeds. Due to the expansion of aquaculture, marine fisheries will not be able to sustain the needs of aquaculture in the not too distant future. The global demand for fishmeal for aquafeeds may exceed total available supplies around the year 2020 and for fish oil well before 2010 (New& Wijkstroem, 2002). Thus, alternatives to the use of marine materials in fish feeds must be found. Several studies have investigated the replacement of fish oil by vegetable oils in fish feed. Partial replacement of fish oils by vegetable oils such as rapeseed, soybean, linseed or palm oils in fish feeds has no negative impacts on growth and survival of Atlantic salmon (Rosenlund et al., 2001), brook char (Guillou et al., 1995), gilthead sea bream and European seabass (Izquierdo et al., 2003) and rainbow trout (Greene & Selivonchick,1990; Caballero et al., 2002). Regarding protein supply, soybean meal (SBM) and soy protein concentrate (SPC) are ingredients currently incorporated in aquafeeds to partially replace fish meal without negative effects on growth performance (Kaushik et al., 1995; Refstie et al., 2010).

A class of compounds of high concentration in soy are isoflavones that, for their capacity of binding to and activating estrogen receptors (Latonnelle et al., 2002), were classified as phytoestrogens. The phytoestrogen of greatest abundance in soy products is genistein, which is present in defatted SBM at an average of 114,7 mg/100g SBM (Bhagwat et al., 2008), although concentrations potentially vary between seasons and soybean varieties (Wang and Murphy, 1994). Epidemiological studies have shown that a regular intake of soy foods is associated with a reduced risk of several chronic pathologies, such as coronary heart disease, cancer, (Setchell and Cassidy, 1999) and atherosclerosis,

associated with oxidative damage (Anthony et al., 1998; Hertog et al.,1998) . Different hypotheses have been suggested to explain these health benefits, such as the involvement of the two major isoflavones in soy foods, genistein and daidzein, whose different biological activities have been documented *in vitro* and *in vivo* studies. For example, it has been proposed that the protective effect exerted by genistein against atherosclerosis could be related to its antioxidant properties (Ferretti et al., 2003); in fact, genistein is able to inhibit lipid peroxidation induced *in vitro* by several pro-oxidant agents on model and natural membranes (Jha et al., 1985), on cultured cells (Guo et al., 2002; Ho et al.,2003), and on low density lipoproteins (LDL) (Kerry et al., 1998; Wilson et al., 2002).

On the other hand, it has been reported, for example, that genistein can impact negatively the growth performance. This happen if, considering that a maximum concentration of genistein in soy products is ~ 5900 µg/g, this is added to a concentration of 3000µg/g of genistein, which is more or less equal to genistein levels when there is a total replacement of fish meal by soybean meal (Chen et al., 2014). So, it has been observed that the high dietary levels of genistein (3000 µg/g) can depress the growth performance in fishes, as Nile Tilapia, while lower levels of it (0-300µg/g) haven't effect on growth performance (Chen et al., 2014); similar results have also been reported in rainbow trout (*Oncorhynchus mykiss*) (Catherine et al., 2001) for a dietary genistein supplementation of 500µg/g. Here, this suppressing effect of genistein can be partly due to its capacity of inhibit the activity of major digestive enzymes: stomach protease and intestine amylase. *In vivo* and *in vitro* studies indicate genistein (and dadzein) is capable of binding to and activating estrogen receptors in rainbow trout (Latonnelle et al., 2000; Bennetau-Pelissero et al., 2001; Denny et al., 2005; Cosnefroy et al., 2009; Cleveland, 2014). This ability suggests that they may have negative effects on growth- related mechanisms

that are parallel to those of estradiol. In fact, in salmonids E2 negatively affects physiological and metabolic processes that support anabolic growth, as down-regulating the growth hormone (GH)/insulin-like growth factor (IGF) axis (Holloway and Leatherland, 1998; Norbeck and Sheridan, 2011), and promoting catabolic effects on protein turnover in skeletal muscle (reducing rates of protein synthesis and increasing rates of protein degradation). Cleveland and Weber, in 2011, demonstrated that also phytoestrogens (as genistein) are capable of these effects. For example, phytoestrogens have effects on protein turnover and cell proliferation in rainbow trout white muscle, in particular, high concentrations of these have negative effects on skeletal growth via estrogen receptor-dependent and –independent mechanisms (Cleveland, 2014). Many studies have been done on the effects that phytoestrogens can have on metabolic processes in mammals, as on lipid metabolism and glucose tolerance, through estrogen receptor-dependent and -independent mechanisms, the latter including AMPK and PPAR activation and inhibition of tyrosine kinase activity (Orgaard and Jensen, 2008; Arunkumar and Anuradha, 2012; Palacios-Gonzalez et al., 2014). But few of these studies have been done on the effects of phytoestrogens and their mechanisms of action in fish adipose tissue.

Thus, in this study we want to examine the *in vivo* effects of the phytoestrogen, genistein, on lipid metabolism in adipose tissue primarily, but also in liver and white muscle, and its *in vitro* effects on adipocytes in culture from rainbow trout (*Oncorhynchus mykiss*). In addition, *in vivo* effects of genistein on apoptosis-related genes expression and autophagy-related genes expression in all three tissue of rainbow trout were studied.

1.1 *Oncorhynchus mikyss* (common name “Rainbow trout”)

1.1.1 Taxonomy

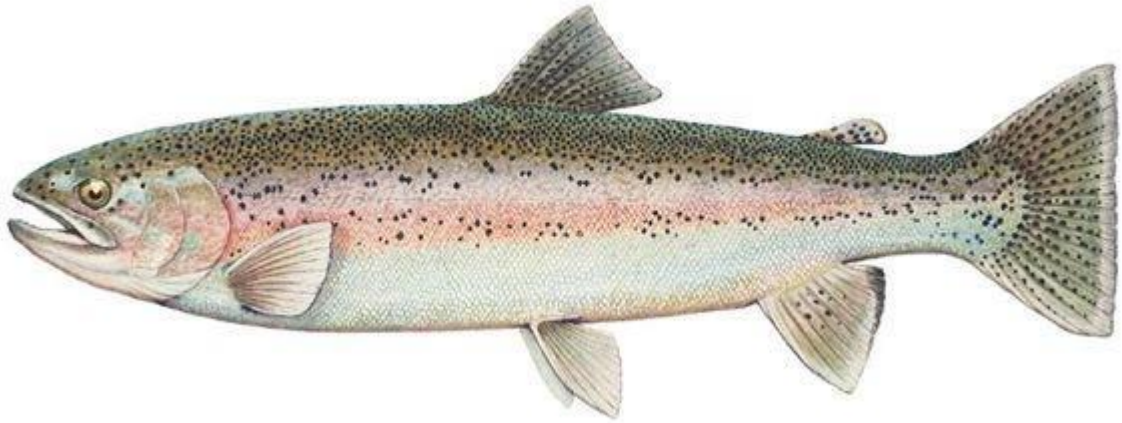
Class: Actinopterygii

Order: Salmoniformes

Family: Salmonidae

Genre: Oncorhynchus

Species: O. mykiss Walbaum, 1792



1.1.2 Morphology

The rainbow trout has an elongate body, the length of which is generally 5 times greater than its height. The head has a conical shape and the mouth is slightly oblique, with the maxillary bone which extends to the posterior edge of the eye. The trout teeth are arranged in 1 or 2 series and are present only on the stem of the ploughshare. The lateral line is nearly horizontal and back, before the tail

fin, is an adipose fin with a black edge. There aren't nuptial tubercles, but changes take place about the head, mouth and colour in spawning males. The body colour of the rainbow trout may vary according to habitat, the size of the specimens and their stage of sexual maturation. Generally the body of this salmonid has a green-blue shade into dorsal part, while the hip area shows a pigmentation which gradually tends to clear and take silvery reflections. On the sides there is a pink band along the lateral line which assumes more intense shades tending to iridescent when arrives the breeding season. The abdomen of the trout has further a colour lighter, almost whitish.

1.1.3 Biology and habitat

Rainbow trout is a species native to north America, that since 1874 it has been introduced in most of the rivers on the planet, for recreational fishery and aquaculture. This species has an anadromous life cycle, in fact, it spends a few years of life at sea, then, returns to freshwater when spawning begins. These animals are characterize by a very rapid growth and, are be able to, in just three years, accumulate from 7 to 10 kg of weigh, while normal rainbow trout, in the same time, increase by a maximum of 4.5kg. The rainbow trout was introduced in Italy about a century ago, and, as a result of its outstanding ability to adapt to various environmental conditions, the presence of this salmonid can be found in most of the rivers and lakes on the national territory, nowadays. It is a species that is easily reproduced in captivity, and, is characterized by very short rates of growth.

Rainbow trout fry have dimensions greater than those of the majority fish species, and, this gives the possibility of being able to administer artificial diet during the early stages of breeding, so to abbreviate the weaning times. This species is very well suited to various temperature conditions and can survive in environments characterized by a temperature range between 0 and 27° C. The

growth of trout takes place in water temperatures that can vary from 6° to 20° C with a thermal optimum between 14° and 18° C , while as regards the reproductive activity , the optimal thermal levels are between 10° and 12°C.

Usually, the maturation of sexual organs takes place on reaching the 3rd-4th year of age, but acting on the diet and thermal conditions of the environment, this can be anticipated. The eggs of the trout have a diameter ranging from 3 to 7 mm, and, the females can also produce up to 2000 per kg of body weight. In breeding, changing some environmental parameters or through hormonal treatments depending on broodstock, it is possible to obtain only females or sterile individuals. These last show development similar to that of females, but more rapid than that of males. Spawning occurs in the natural environment in spring (January-May), but in breeding, this stage can be moved in time, acting on nutrition, on the conditions of photoperiod or through hormonal induction.

In the wild, adult trout feed on aquatic and terrestrial insects, molluscs, crustaceans, fish eggs, minnows, and other small fishes, but the most important food is freshwater shrimp, containing the carotenoid pigments responsible for the orange-pink colour in the flesh. In aquaculture, the inclusion of the synthetic pigments astaxanthin and canthaxanthin in aquafeeds causes this pink colouration to be produced.

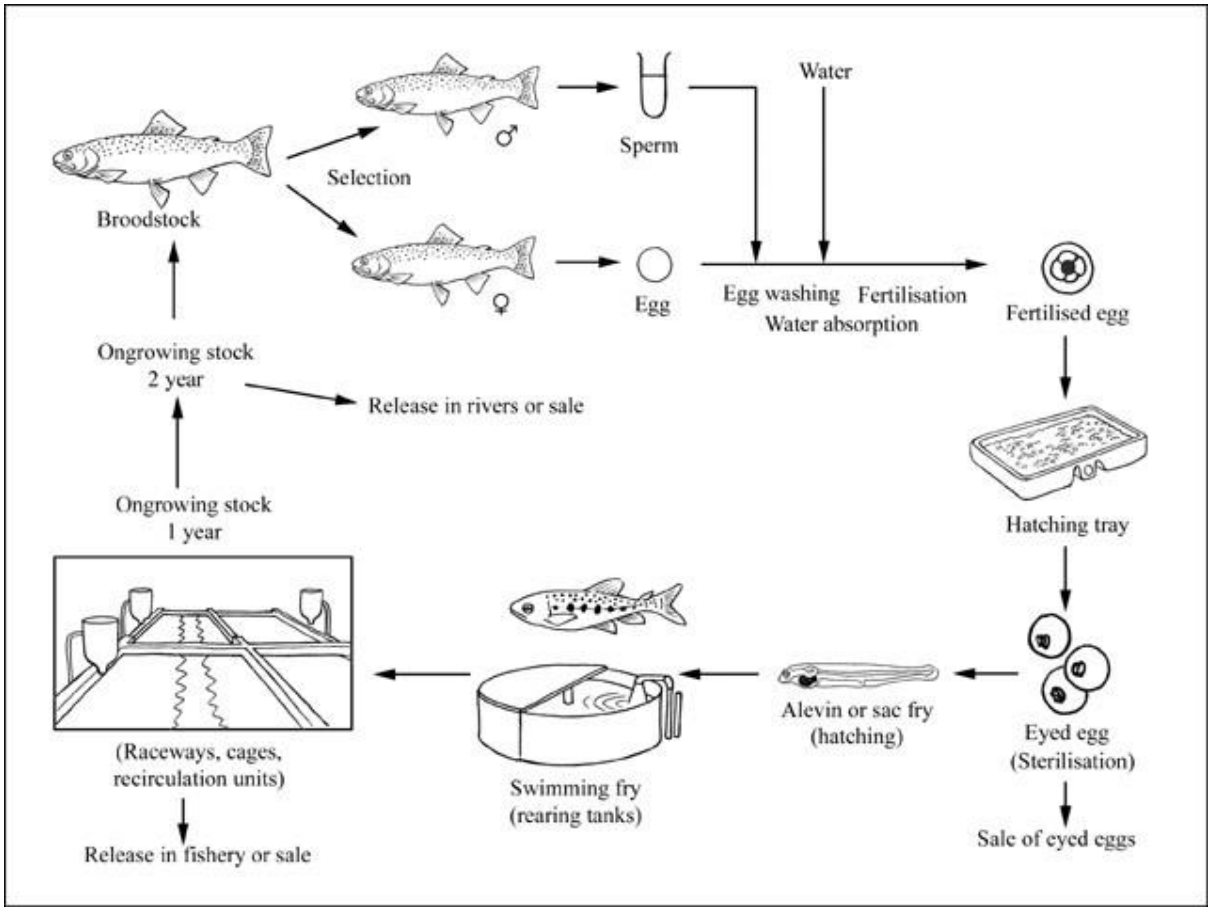


Fig. 1 Production cycle of Oncorhynchus mykiss.

1.1.4 Farm techniques

The breeding of the trout is generally practiced within intensive structure, in which it must constantly be the opportunity to take advantage of waters of excellent quality (without aeration - 1 l/min/kg of trout without aeration or 5 l/sec/tonne of trout with aeration), that meets a number of criteria:

DO_2:	near saturation.
CO_2:	<2.0 ppm.
Temperature:	12-21ºC.
pH:	6.5-8.5.
Alkalinity (as $CaCO_3$):	10-400 mg/litre.
Manganese:	<0.01 mg/litre.
Iron:	<1.0 mg/litre.
Zinc:	<0.05 mg/litre.
Copper:	<0.006 mg/litre in soft water or <0.3 mg/litre in hard water.

Tab.1

Trout is very sensitive to the presence of nitrogenous substances which are derived from the catabolism of proteins, such as , ammonia, nitrates and nitrites. A parameter that must always be kept under control in the plants of trout farming that use water (with dissolved nitrogen) pumped from underground wells , is the oversaturation gaseous water, which must be avoided because it causes the formation of gas bubbles in the blood of the fish. Alternatively, river water can be used, but temperature and flow fluctuations alter production capacity. Trout are animals that can tolerate an higher alkalinity and hardness of water compared to acidity, but in any case, it is good practice to use neutral or medially alkaline water with a pH between 6.5 and 8.5. However, when the above criteria are met, trout are generally on-grown in raceways or ponds supplied with flowing water, but some are produced in cages and recirculating systems.

1.1.5 Fry production

Trout will not spawn naturally in culture systems; thus juveniles must be obtained either by artificial spawning in a hatchery or by collecting eggs from wild stocks. Larvae are well developed at hatching. The breeders, males and females, after being selected, are generally kept separate. The choice of their number depends on the number of fry required, and, the ratio which is most frequently adopted between males and females is 1:3. The females don't spawn naturally in captivity environment, and when are fully mature, to induce the release of the eggs, it is necessary human intervention. Trout are subjected to an anesthetic treatment, and subsequently, eggs are removed manually from females by applying a slight pressure on the abdomen from the pelvic fins to the vent area. To reduce the stress of the animals, it has been ideated another method of extraction, "air spawning" ; it consists of insertion of a hypodermic needle about 10 mm into the body cavity near the pelvic fins and air pressure (2 psi) expels the eggs. The air is removed from the body cavity by massaging the

sides of the fish. Up to 2000 eggs/kg body weight are collected in a dry pan and kept dry, improving fertilization. Males are stripped in the same way as females; the semen is collected into containers by pressing, and then, added to eggs. Water is added to activate the sperm and allow the eggs to increase in size by about 20 percent by filling the perivitelline space between the shell and yoke; a process known as 'water-hardening'.

Fertilized eggs are incubated in hatcheries until the eyed stage is reached; the others (non-viable eggs) are removed. A single water source flows (3-4 L/min) up through the eggs, spills over into the tray below, thus becoming aerated, allowing large numbers of eggs to hatch in a minimal amount of space and water. Time required to hatch varies according to the water temperature. With a temperature of 3.9°C it takes 100 days , while with 14.4°C requires 21 days, for a total of about 370 degrees/day. Fry can remain in trays until swim-up at about 10 to 14 days after hatching. Hatching of the batch of eggs usually takes 2-3 days, during which time all eggshells are regularly removed, as well as dead and deformed fry. Eggs incubated separately from rearing troughs are transferred to rearing troughs after hatching.

After hatching, the trays are removed and trough water depth is kept shallow (8-10 cm) with a reduced flow until fry reach 'swim-up' stage, the yolk sac is absorbed, and active food searching . Larvae are able to absorb the yolk sac in a period ranging from 2 to 6 weeks , depending on the temperature conditions, and, when the yolk sac has been absorbed for about 2/3, begins the artificial administration of food with frequent meals and diets properly formulated. In these rearing troughs, feed pellets, made of fish meal (80 percent), fish oils and grains, provide nutritional balance, encouraging growth and product quality, and are formulated to contain approximately 50 percent protein, 12-15 percent fat, vitamins (A, D and E), minerals (calcium, phosphorus and sodium) and a pigment to achieve pink flesh (where desirable). In this phase, it is very

important the efficiency of the water exchange; in case of poor availability of water, it is appropriate to reduce the loading of the animals in the tanks up to 10 kg / m3, so as to ensure at least 6 changes of water per day.

During this period and also in the subsequent phases, it is extremely important to calibrate fishes to form groups of uniform size, to facilitate the management, and, to reduce losses due to cannibalism. Fry remain in these tanks until the completion of ossification of cartilaginous tissues, which usually occurs around the 12th week of life.

1.1.6 Fattening techniques-intensive farming

When fry reach 8-10 cm in length (250 fish/kg), they are transferred into fattening facilities, as concrete raceways, ponds or cages. The raceways are the most used in the traditional trout farming, which, usually, are rectangular tanks 2-3 m wide, 12-30 m long and 1-1.2 m deep. The amount of food that is given, the amount of water exchange, and, the amount of oxygen to add in the water body, will have to be appropriate to bred biomass and average size of trout. The sustainable load into tanks must be established as a function of environmental conditions and water temperature. The factor that has the greatest impact on growth of trout is the water temperature, which should never deviate too much from the optimal temperature levels. With temperatures between 10°C and 14°C, the trout can reach a weight of 80-100g and a length of 15-20cm in only 6-8 months after hatching, and using diets high in energy, they reach the size of commercialization (250-300 g) in 10-12 months. Alternative on-growing systems for trout include cage culture (6 m by 6 m by 4-5 m deep) production systems where fish (up to 100 000) are held in floating cages in freshwater and marine environments, ensuring good water supply and sufficient dissolved oxygen. This method uses existing water bodies at a lower capital cost than flow-through systems. However, on one hand, stocks are vulnerable to external

water quality problems and fish eating predators (rats and birds), and growth rates depend on ambient temperature; on the other, high stocking densities can be achieved (30-40 kg/m²) and fish transferred to marine cages have faster growth rates, reaching larger market size. Fry of about 70 g weight can attain 3 kg in less than 18 months.

1.1.7 Nutrition of farmed trout

Feeds for rainbow trout have been modified over the years. Rainbow trout feeds have undergone a shift since the 1970s (fig. 2); in the same period, the percentage of digestible protein has increased, making modern trout feeds much more efficient and less polluting.

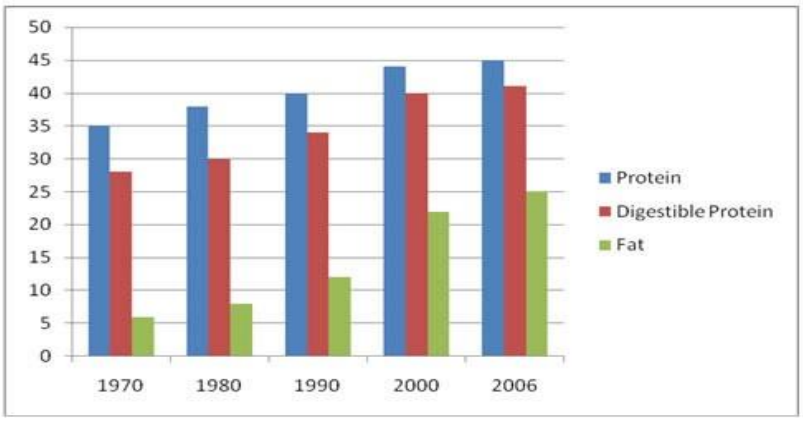


Fig.2 Changes in percent protein, digestible protein and fat in rainbow trout feed. (FAO)

An additional change in trout feeds has been a reduction of the percentage of protein provided by fishmeal and a corresponding increase in the contribution of alternate proteins, mainly plant protein concentrates and animal proteins, such as poultry byproduct meal. Another shift in formulation of rainbow trout feeds is replacement of fish oil with plant oils. In 2006, 88 percent of global production of fish oil was consumed in aquafeeds (Tacon and Metian, 2008); for this, in 2006/2007, there was an increase of fish oil price that leads feed producers to replace portions of fish oil with plant oils such as rapeseed oil and soy bean oil.

About feed ingredients used in rainbow trout feed formulations, these are similar throughout the world; in Tab.2, are shown some examples of rainbow trout feed formulations:

Ingredient composition (%)	**Life stages/ size class**				
	Early fry	**Fry**	**Fingerling**	**Grower**	**Broodstock**
Fish meal	68	68	46	30	34
Corn gluten meal	0	0	2	4	4
Poultry byproduct meal	2	2	5	6	8
Feather meal	0	0	4	6	5
Soybean meal	0	0	5	12	10
Blood meal, avian	1	1	2	4	4
Ground wheat	17	17	20	22	20
Soybean oil	0	0	0	5	0
Fish oil	10	10	12	9	10
Vitamin	1.5	1.5	1.5	1.5	1.5
Mineral	0.5	0.5	0.5	0.5	0.5

Tab.2 Feed formulae (ingredient composition) of commonly used feed for different life stages of rainbow trout in intensive farming structures.

1.1.8 Main producer countries

Main areas where it is practiced aquaculture trout are located in Europe, North America, Chile, Japan and Australia.

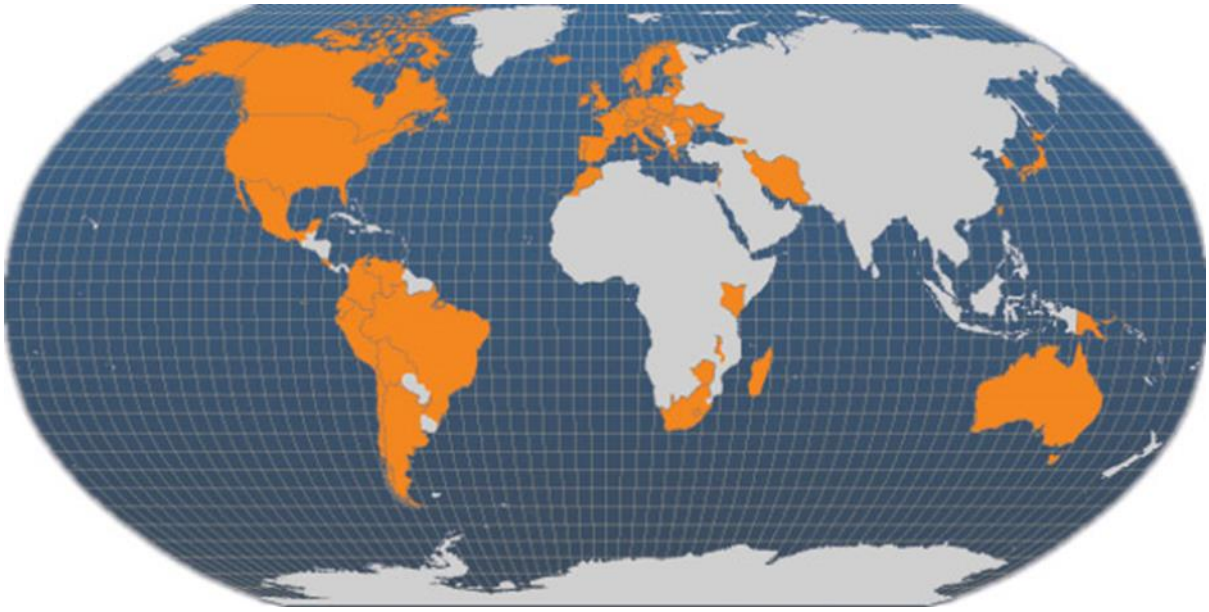


Fig. 3 Main producer countries of Oncorhynchus mykiss (FAO Fishery Statistics, 2006).

1.2 Lipid metabolism in liver and adipose tissue

1.2.1 An introduction to lipids

The noun lipid refers to a large and heterogeneous group of substances classified together on the basis of their high solubility in non-polar solvents or their relatedness to such compounds. Most of the lipids in eukaryotes are derived from acetyl-CoA and belong to three major classes: straight chain fatty acids; branched, cyclic, and other specialized fatty acids; and polyprenoid compounds, including carotenoids and sterols and their derivatives. Lipids have

a number of major roles in all organisms. Perhaps most importantly, they are structural components of cell membranes and are essential for energy provision and storage. Lipids and fatty acids, along with proteins, are the major macronutrients for fish (Sargent et al., 2002). Fish seem not to have evolved efficient carbohydrate utilization systems in contrast to terrestrial vertebrates. So, carbohydrates are less important as nutrients (quantitatively) for most fish, suggesting that fish may satisfy structural carbohydrate and storage carbohydrate (glycogen) requirements principally by catabolizing amino acids (Cowey and Walton, 1989). The types of lipids that are most vital to energy metabolism are fatty acids and triglycerides. Fatty acids are the most commonly stored and circulating forms of energy, and triglycerides are the most common non-toxic form of fatty acids. Fatty acids/triglycerides may originate from four sources (pool input): de novo fatty acids synthesis, cytoplasmic triacylglycerol stores, fatty acids derived from triglycerides of lipoprotein remnants directly taken up by the liver, and plasma non-esterified fatty acids (NEFA) released by adipose tissue. The relative importance of these sources depends on species differences and on short- and long-term nutritional status and energy balance.

Fatty acids and triglycerides may also be used in different ways (pool output). Triglycerides may accumulate in hepatocytes (while NEFA or activated forms of NEFA may not) unless NEFA are oxidized (more or less completely) or triglycerides are exported as constituents of very low density lipoproteins (VLDL). The triacylglycerol content of hepatocytes is regulated by the activity of cellular molecules that facilitates hepatic fatty acid uptake, fatty acid synthesis, and esterification (‘input’) and hepatic fatty acid oxidation and triacylglycerol export (output). Moreover, fatty acids regulate lipid metabolism by binding nuclear receptors that modulate gene transcription. Peroxisome proliferator-activated receptors (PPAR) are ligand binding transcription factors of the nuclear receptor superfamily, which includes receptors for steroids,

thyroids and retinoids (Han et al., 2007; Sertznig et al., 2007). Three types of PPAR have been identified (a, b, ɤ), each encoded by distinct genes and expressed differently in many parts of the body (Sertznig et al., 2007). They form heterodimers with the retinoid X receptor, and these complexes subsequently bind to a specific DNA sequence, the peroxisome proliferating response element (PPRE) that is located in the promoter region of PPAR target genes and modulates their transcription (Tachibana et al., 2008). Gender and stage of life cycle influence expression levels of all the PPARs in brown trout; estrogen appears to play an important role in differential expression of PPARs (Batista-Pinto et al., 2009). In rainbow trout adipose tissue, the gene transcriptional levels of PPARs change differentially with the nutritional status (Cruz-Garcia et al., 2015). As in mammals, it was found in fish that PPARɤ 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; Albalat et al., 2007). In concordance with this, it was seen that insulin administration upregulated this PPAR isotype highly significantly in trout adipose tissue *in vivo* and in *in vitro* in line with the pro-lipogenic actions of this hormone (Cruz-Garcia et al., 2015). PPARɤ gene expression increased during adipocyte differentiation in rainbow trout (Bouraoui et al., 2008), but not with differentiation of trout myocytes, in agreement with the myogenic development program (Rescan, 2008). PPARα and PPARβ are regulated by a transcriptional factor LXR (liver X receptor) in trout myocytes (Cruz-Garcia et al., 2011); PPARα is regulated by insulin in trout adipose tissue, not instead PPARβ (Cruz-Garcia et al.,2015). PPARβ has a controversial role in fish as in mammals; it seems to mediate lipolytic effects in gilthead sea bream (Cruz-Garcia et al., 2009).

In mammals, PPARɤ is expressed strongly in adipose tissue and is a master regulator of adipocyte differentiation (Lehrke et al., 2005). In addition to its role

in adipogenesis, PPARɤ is an important transcriptional regulator of glucose and lipid metabolism, and is implicated in the regulation of insulin sensitivity, atherosclerosis, and inflammation (Lehrke et al., 2005; Semple et al., 2006). PPARγ affects transcription rates of a variety of lipogenic target genes such as FABP4 (fatty acid binding protein 4), CD36 (thrombospondin receptor), LPL (lipoprotein lipase), leptin, ACC (Acetyl-CoA carboxylase), FAS (fatty acid synthase), and SCD1 (Stearoyl-CoA desaturase 1) (Lee and Hossner, 2002). Additionally, PPARα is responsible for regulating fatty acid β-oxidation (Varga et al., 2011). Regarding PPARβ, little is known about it in the context of target tissues, target genes, lipid homeostasis, and functional overlap with PPARα, and PPARɤ. The activation of PPARβ in skeletal muscle cells programs a cascade of gene expression designed to activate catabolism, and energy expenditure (Dressel, et al., 2003). In adipose tissue, instead, even if PPARβ is not directly implicated in the control of adipogenesis, because alone is not able to promote lipogenesis (but only together with PPARɤ), however, it plays a role in the adaptive response of adipose tissue to dietary fatty acid content, (Neels and Grimaldi, 2014).

Definitely, fatty acids can be produced from acetyl CoA into “de novo” Lipogenesis, and can be broken down to acetyl CoA by β-oxidation, a cyclical pathway. Triglycerides, instead, accounted as the main storage form of fatty acids, are produced through the process known as Lipogenesis or synthesis of TG, while their hydrolysis is known as Lipolysis, with release of fatty acids.

1.2.2 Hormonal regulation of lipid metabolism and its dependence on diet.

Adipose tissue is a specialized organ that functions as one of the major storage sites for fat in the form of triglycerides and provides a buffer for energy imbalances. In mammals, the equilibrium between lipolytic and lipogenic pathways in adipose tissue is influenced by nutritional and endocrine factors

and by components of the immune response (Rosen et al., 2006). There is limited knowledge on the hormonal control of lipid turnover in fish adipose tissue (Albalat et al., 2005; Polakof et al., 2011). It has been seen that in mammals, GH reduces body fat, inhibits adipocyte differentiation, decreases lipogenesis, and increases lipolysis (Herrington et al.,2001; Xu et al., 2009). Also in isolated trout adipocytes, GH enhances lipolysis and inhibits, at least in part, lipogenesis. In the first case, through the direct modulation of HSL activity probably, not at a transcriptional level; in the second, through the down-regulation of FAS expression during fasting (Cruz-Garcia et al., 2015). It has been reported that growth hormone (GH), in addition to being a growth promoter, exerts a lipolytic effect in gilthead sea bream adipocytes too (Albalat et al., 2005; Salmeron et al., 2013). GH transgenic coho salmon (Leggatt et al., 2009) are shown to increase their utilization of lipids for synthetic roles to maintain accelerated growth, but the specific effects in adipose tissue of GH overexpression remain unknown.

Insulin acts as a promoter of carbohydrate and lipid deposition by reservoir tissues during the post-feeding period in fish, although its concentration decreases during fasting periods (Navarro et al., 2004), with an increase in circulating GH (Cruz-Garcia et al., 2015). During fasting, insulin down-regulates PPARα expression, suggesting a reduction in fatty acids oxidation associated with the enhanced use of fatty acids for re-esterification and triglyceride synthesis. Studies have demonstrated the anabolic role of insulin in rainbow trout adipocytes and myocytes, where it stimulates glucose and fatty acid uptake (Capilla et al., 2004; Sanchez-Gurmachez et al., 2010). Insulin also stimulates lipoprotein lipase (LPL) activity in rainbow trout adipose tissue (Albalat et al., 2006) and reduces the basal lipolysis level in rainbow trout and gilthead sea bream adipocytes (Albalat et al., 2005; Albalat et al., 2005).

Insulin-like growth factor (IGF)-I is structurally and functionally similar to insulin but is more potent as a growth factor and a metabolic controller in rainbow trout and gilthead sea bream myocytes and adipocytes (Salmeròn et al., 2013; Bouraoui et al., 2010; Codina et al., 2008; Castillo et al., 2004). In vertebrates, many of the growth-promoting actions of GH are known to be mediated indirectly through the stimulation of IGF transcription, mainly by the liver, or locally by extra hepatic tissues (Chia, 2014), but the mechanisms of action involved in GH proliferative and metabolic effects in fish are not well known (Bergan et al., 2013).

In contrast, tumor necrosis factor a (TNFα), secreted by adipose tissue, that a part from acts as a pro-inflammatory cytokine, it also regulates lipid cell uptake and degradation of triglycerides because it is lipolytic in fish and mammalian adipose tissue (Saera-Vila et al., 2007; Albalat et al., 2005; Zhang et al., 2002). TNFα has been described as a limiting factor for adiposity in gilthead sea bream (Bouraoui et al., 2008; Saera-Vila et al., 2007), and it has been shown to promote lipolysis in isolated adipocytes of rainbow trout and gilthead sea bream (Albalat et al., 2005; Cruz-Garcia et al., 2009). The mechanisms underlying the action of TNFa on lipolysis are complex, and modulation of the expression of peroxisome proliferator-activated receptors (PPARs) appears to be significant (Cruz-Garcia et al., 2009). In fact, it has been reported by Cruz-Garcia that TNFα decreases PPARɤ expression in adipocytes in line with its anti-fat storage effects and anti-insulin actions described in relation to the lipid metabolism of mammals and fish (Bou et al., 2015; Nieto-Vazquez et al., 2008). Regarding PPARα expression after TNFα treatment in isolated cells, it remains to be elucidated, but points to a possible pathway for the inhibition of fatty acid oxidation in adipose tissue.

The management of fat deposition has become a key area of interest in fish farming in the quest to obtain a high-quality product with good nutritional value

and to maintain fish health. Regarding the growth in fin-fish aquaculture, it has been made possible by the development of artificial diets or feeds formulated to satisfy essential requirements (amino acids, fatty acids, vitamins and minerals, etc.), and provide macronutrients (protein, lipid, carbohydrate) and energy in balance to optimize growth. Thus, it has been the requirements of aquaculture that have driven research into fish nutrition, including lipid and fatty acid metabolism and its regulation. Dietary lipid and fatty acids can have three primary fates in fish. They can be incorporated into cell membranes and hence the flesh of the fish, they can be oxidized to provide energy, or lipid can be deposited in adipose or other tissues as energy storage (Tocher, 2003). Therefore, from an aquaculture perspective, the lipid (fat) content and the fatty acid composition of the diet must be optimized to enable high growth rates, ensure fish health, and, at the same time, maintain the nutritional benefits of fish for the human consumer (Sargent et al., 2002; Tocher, 2003). The lipid content of pelleted diets has increased greatly in recent years due in part to the technical advancements in the production of feed. This increase is driven by the observation that more energy can be supplied by dietary lipid, less protein will be used for energy, and so more dietary protein can be "spared" for synthesis of new tissue/flesh (Hemre and Sandnes, 1999). However, although protein sparing by dietary lipid is widely accepted, the limits to its effectiveness, or the mechanisms by which it might occur, have not been accurately defined for any fish (see Company et al., 1999). Consequently, dietary formulations have maximized lipid content in order to satisfy commercial pressure to increase growth rates and reduce production times, despite the fact that there is a strong relationship between dietary lipid levels and undesirable levels of lipid in the carcass of fish (Cowey and Cho, 1993). In fact, the administration of high-lipid feed can lead to an unwanted increase in fat deposition that alters sensory and organoleptic characteristics (Kjaer et al., 2008; Turchini et al., 2009). In addition, because of fatty acid composition of fish lipids generally reflects the

fatty acid profile of the diets (Watanabe, 1982), studies have been conducted into the lipid metabolic disorders associated 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;Gjoen et al.,2004). Unfortunately, while in higher vertebrates, many studies have shown that changes in dietary fatty acid composition can induce modification of hepatic lipogenesis (Blake & Clarke,1990; Clarke et al. 1990, Salati & Amir-Ahmady, 2001), lipid transport in blood (Grundy & Denke, 1990; Fernandez & West, 2005) and tissue lipid uptake (Montalto & Bensadoun, 1993; Raclot et al., 1997), a few of them have been done on fishes as on rainbow trout. One of these studies on rainbow trout, conducted by Richard et al., in 2006, has shown that a dietary vegetable oils can have a little effects on hepatic lipogenesis, lipid transport and tissue lipid uptake; in particular, it was seen that hepatic lipogenesis and lipid uptake in perivisceral adipose tissue, white muscle and liver weren't modified; only regarding the lipid composition of plasma, it was seen a decrease in plasma cholesterol and LDL (low density lipoproteins), because of expression of LDL receptor gene in the liver that was down-regulated.

1.2.3 Fatty acids synthesis and uptake

Two major tissues produce fatty acids in the body: the liver and the adipose tissue. Fatty acids synthesized in the liver are exported through lipoprotein production, and thus provide an energy source and structural components for membrane building. In adipose tissue, de novo synthesis of fatty acid directly contributes to in situ fat deposition and long-term energy storage. Fatty acids synthesis occurs in the cytosol and uses acetyl CoA to build a long chain fatty acid. As shown in fig.4, the first step of fatty acids synthesis consists in a conversion of two-carbon acetyl CoA into a three-carbon malonyl CoA (the substrate of the multiprotein enzyme fatty acid synthase), this thanks to an

enzyme, acetyl CoA carboxylase. Fatty acids synthase uses malonyl CoA (and reducing energy of NADPH) to extend the length of the fatty acid, adding two carbons in a series of four reactions: 1) condensation, 2) reduction, 3) dehydration, 4) reduction. At the end of one cycle (5), the fatty acid shifts to the initial position to allow the next malonyl CoA to bind. After seven cycles, when the fatty acid has grown to 16 carbons, palmitate has been produced; this is a common component of fatty acid stores and from it can be produced fatty acids with longer chains. (Fig. 4).

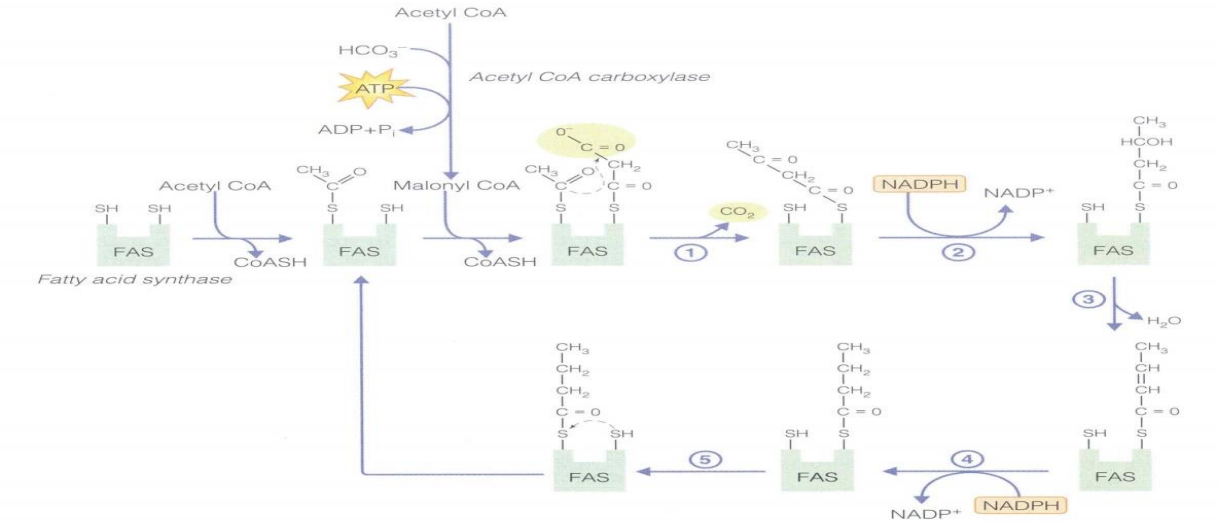


Fig.4 Fatty acid synthesis. (Animal Physiolology, Moyes and Schulte, 2006).

Besides the synthesis capacity of adipose tissue and liver, these tissues take up NEFA from the blood in proportion to their concentration. NEFA enter cells via transporters (fatty acid transport protein (FATP) or fatty acid translocase (FAT)) or diffusion. Into cells, long-chain fatty acids of 14 carbons or more are covalently bound and activated by fatty acid binding protein (FABP) or acyl-CoA synthetases (ACS) found in the microsomes and outer mitochondrial membrane. These transport fatty acids to intracellular compartments (for metabolism) or the cellular nucleus (to interact with transcription factors).

However the cells with these fatty acids, rapidly, assimilate them into neutral and polar lipids, and some are oxidized. The result of these metabolic pathways is to keep intracellular NEFA and fatty acyl-CoA very low.

1.2.4 B-oxidation of fatty acids

The catabolism of fatty acids can occur by β-oxidation in two different organelles in the cells, mitochondria and peroxisomes. Red muscle, liver, and heart are generally regarded as the most important tissues for β-oxidation in fish. The fatty acid oxidation pathway happens primarily into mitochondria and results in the production of acetyl CoA. Depending on conditions, this acetyl-

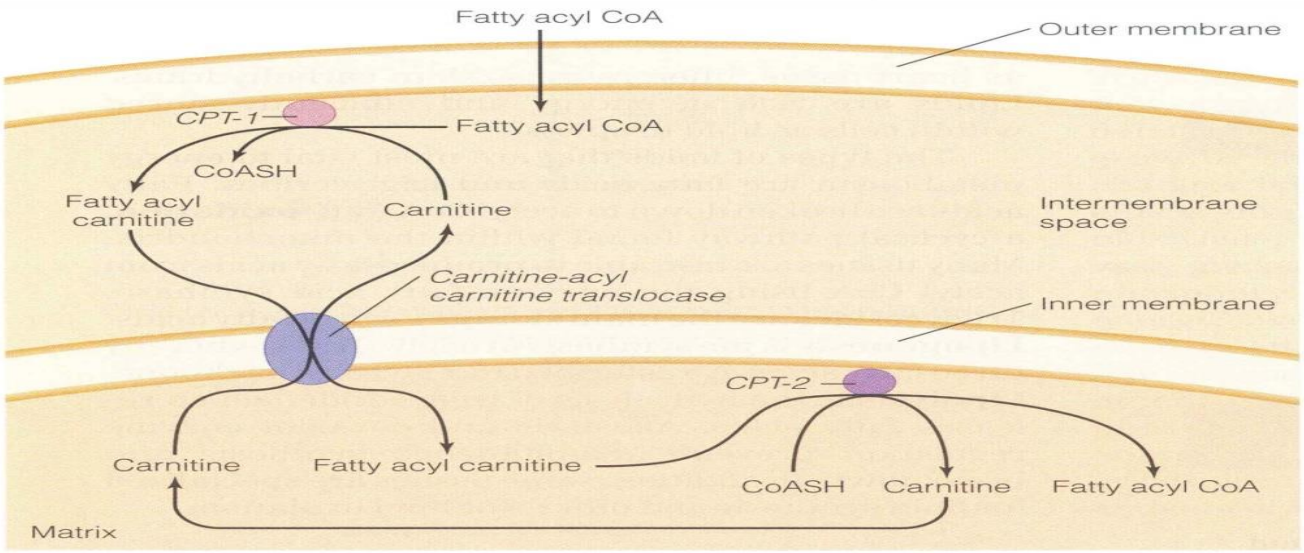


Fig. 5 Fatty acid transport into mitochondria. (Animal Physiolology, Moyes and Schulte, 2006).

CoA can enter the TCA cycle or be destined to other pathways. Because the substrate for β-oxidation is fatty acyl CoA, cells must first convert fatty acids to their CoA esters using a fatty acyl CoA synthase. While short and medium chain fatty acids are able to enter the mitochondria directly (where they are activated by a mitochondrial fatty acyl CoA synthase), Palmitate (and others as it) that cannot cross outer mitochondrial membrane, is activated by an extramitochondrial fatty acyl CoA synthase. Because mitochondria aren't able to import any fatty acyl CoA directly, they use a multienzyme complex (known

as carnitine palmitoyl transferase, CPT) and this transport process is called the "carnitine shuttle" (Fig.5). After that fatty acyl CoA has crossed the outer mitochondrial membrane, is converted to fatty acyl carnitine by the enzyme CPT-1 releasing coenzyme A (CoASH). After that fatty acyl carnitine is transported across the inner mitochondrial membrane by the carnitine-acyl carnitine translocase, the enzyme CPT-2 converts it into fatty acyl CoA. This elaborate transport process provides an additional level of control for the oxidation of long chain fatty acids as Palmitate. Once inside the mitochondria, fatty acids enter the β-oxidation pathway (Fig.6). This is a cyclical pathway that sequentially cuts pairs of carbons of fatty acid to form acetyl CoA. B- oxidation consists of 4 steps:

1. Oxidation
2. Hydration
3. Oxidation
4. Thiolysis

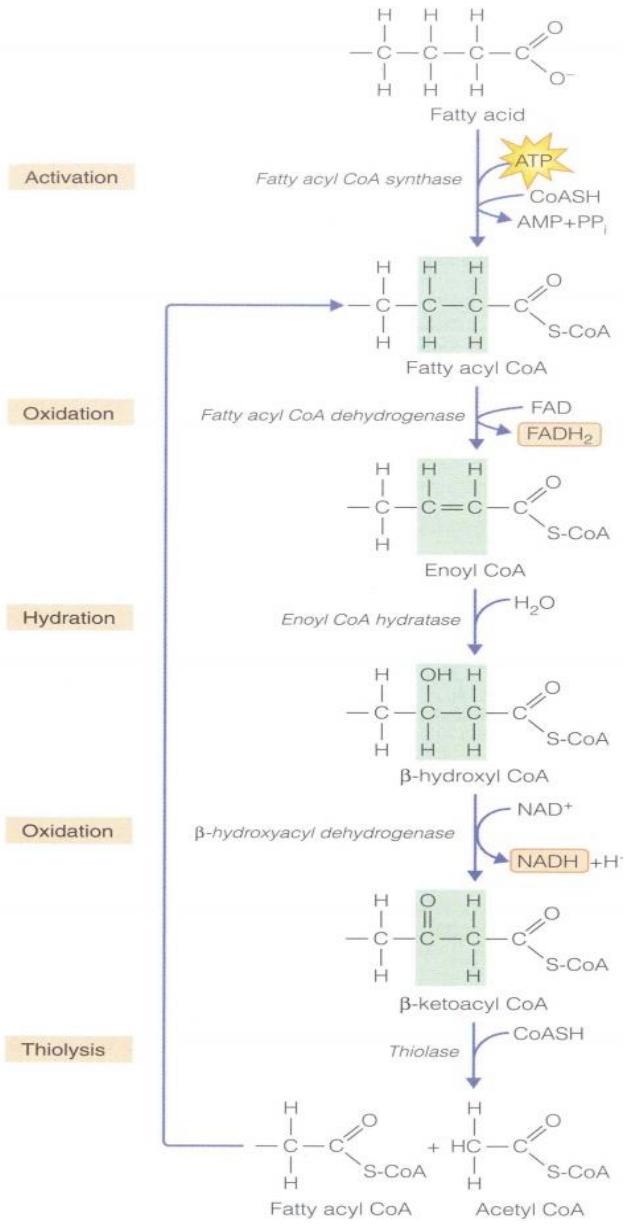


Fig.6 Fatty acid oxidation. (Animal Physiolology, Moyes and Schulte, 2006).

This cycle is repeated until all the acyl CoA of the fatty acid are converted to acetyl CoA.

1. The first step provides a dehydrogenation of the fatty acyl CoA (a loss of hydrogen), forming double bind between carbon 1 and carbon 2 of the molecule; this reaction is catalysed by enzyme acyl CoA dehydrogenase (present on mitochondrial inner membrane) that uses as coenzyme FAD that, in turn, gaining the hydrogen atoms dissociated, become $FADH_2$. The oxidation reaction is the following:

   Fatty acyl CoA $\xrightarrow{\text{acyl CoA dehydrogenase}}$ **Enoyl CoA + $FADH_2$**

2. The second step is a reaction of hydration catalyzed by the enzyme Enoyl CoA hydratase, with the addition of a H_2O molecule to double bind formed to have β-hydroxyl CoA, according to this reaction:

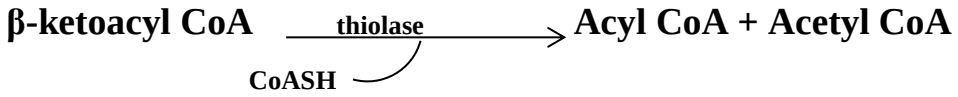
   Enoyl CoA $\xrightarrow{H_2O}$ **β-hydroxyl CoA**

3. The third step is an another oxidation reaction catalyzed by enzyme β-hydroxyacyl dehydrogenase that has as co-factor NAD^+; this reaction of dehydrogenation provides a transformation of the hydroxyl group on the C3 into a carbonyl group, with the loss of two hydrogen atoms, one of which is gained by NAD^+ that become, in turn, NADH according to the following reaction:

   B-hydroxyl CoA $\xrightarrow{\text{β-hydroxyacyl dehydrogenase}}$ **β-ketoacyl CoA**

4. The last step is a thiolysis reaction that provides a separation of the remaining carbonyl group; it is catalyzed by an enzyme thiolase that uses

as co-enzyme CoASH which, in turn, acts as lytic agent to form an acetyl CoA plus a fatty acyl CoA, following the reaction:



About 30% of the energy liberated from fatty acids derives from reducing equivalents (FAD and NAD^+) produced in β-oxidation. The remaining 70% derives, instead, from oxidation of acetyl CoA in the TCA cycle (citric acid cycle, also known as Krebs cycle). Mitochondrial β-oxidation isn't the only pathway trough which it happens fatty acids breakdown, but many cells have other pathways that are complementary to the mitochondrial. In the peroxisomes occurs a β-oxidation that processes very long chain fatty acids (more than 22 carbons), which aren't efficiently oxidized in the mitochondrial pathway; this process occurs through a few cycles, releasing the shortened fatty acids into the cytoplasm where they can be oxidized by mitochondrial β-oxidation. A third pathway of fatty acid oxidation is called ω-oxidation and occurs in the endoplasmic reticulum of liver and kidney. However, this process isn't important for the production of energy but can used in order to synthetize other metabolites.

1.2.5 Lipogenesis

A triglyceride is an ester composed of glycerol and three fatty acids. A lot of tissues can produce triglyceride from fatty acids for a long-term storage of this metabolic fuel. The tissues that act as lipid storage tissues, producing o releasing fatty acids on behalf of other tissues, are adipose tissue and liver in vertebrates. About liver, it has been seen that species with limited hepatic

lipogenesis have less ability to secrete triacylglycerol from the liver compared with species in which the liver is a major or moderate source of lipogenesis (Pullen et al., 1990). In these species, fatty acids could preferentially be esterified into phospholipids that would be incorporated into membranes, then transferred to pre-high-density lipoprotein particles (Yokoyama, 2006). However, in some cases, the liver can also synthesize triglycerides when high concentrations of NEFA are present and phospholipid transfer to membranes is exceeded. Triglyceride synthesis, or lipogenesis, is a multistep pathway through which from glycerol 3-phosphate (produced from glycolysis), fatty acids (activated into their CoA ester by fatty acyl CoA synthase) are added sequentially to carbon 1 then carbon 2 to form phosphatidic acid; after removal of the phosphate group, 1,2-diacylglicerol is formed. At the end with the addition of a third fatty acid, triglyceride is formed (Fig.7). Triacylglycerol synthesis is under the control of transcription factors and nuclear receptors such as sterol regulatory element binding proteins SREBP-1c, carbohydrate regulatory element binding protein (ChREBP) (Dentin et al., 2006), peroxisome proliferator-activated receptor c (PPARɤ), liver X receptors (LXRs) and their ligands. These play an important role together with hormonal and nutritional regulators, such as insulin, carbohydrate, and fatty acids (Coleman and Lee, 2004).

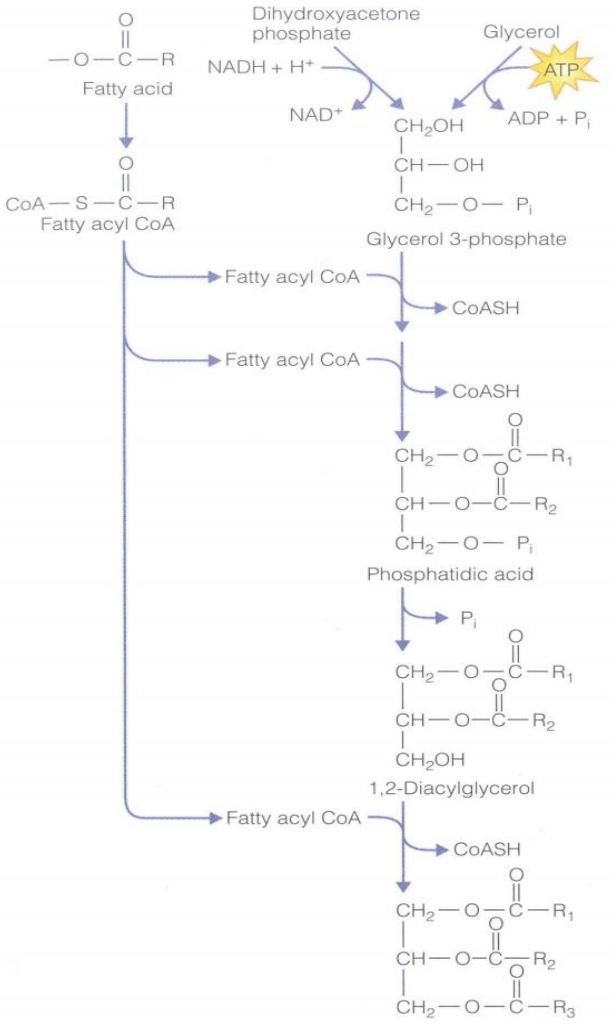


Fig.7 Triglyceride synthesis. (Animal Physiolology, Moyes and Schulte, 2006).

1.2.6 Triglyceride export (VLDL synthesis and secretion)

The liver synthetize VLDL (very low density lipoproteins), enclosing inside them a large amount of triglycerides. VLDL are lipoproteins that, like the other (chylomicrons, IDL, LDL, HDL), are constituted of a central core with lipids inside, and an outer shell constituted of one or more apoproteins, phospholipids with polar groups on the outside and free cholesterol; but, differ from the other lipoproteins in lipid composition and apoproteins, which subserve both the lipid transport (allowing them to move in the aqueous smoothly), and the regulation of lipoproteins metabolism, through interaction with plasma enzymes and with specific cellular receptors. In particular, chylomicrons provide the transport of exogenous lipids from the intestine to the tissues; LDL and HDL are characterized by an high content in cholesterol, carrying it into blood; but, while the first give it to the tissues, the latter, instead, remove superfluous cholesterol from pheripheral tissues and transport it to the liver.

VLDL, synthetized into liver, transport endogenous triglycerides from liver to the tissues. Apoprotein B100 (apoB100; and apoB48 in a few species) is the key component whose rate of synthesis in the rough endoplasmic reticulum controls the overall rate of VLDL production. Lipid components that are synthesized in the smooth endoplasmic reticulum are added by the microsomal triacylglycerol transfer protein to apoprotein B (White et al., 1998). After being carried to the Golgi apparatus in transport vesicles, the apoproteins are glycosylated. Secretory vesicles bud off the Golgi membrane, migrate to the membrane of the hepatocyte, then fuse with the membrane and release the VLDL into blood , through which , the latter come to pheripheral tissues, as muscle or adipose tissue, where release triglycerides transported. After they have given a good part of triglycerides to the tissues, their density and content in cholesterol increase,

so, firstly, become IDL (intermediate density lipoprotein), then, losing triglycerides again, LDL (low density lipoprotein). There are important species differences in the ability to export triglycerides from the liver as VLDL despite similar rates of esterification of fatty acids to triglycerides. It has been suggested that among different species, the rate of export of triglycerides as VLDL from the liver is proportional to its lipogenic capacity. For example, some animals that don't synthetize fatty acids in the liver also have low rates of triglycerides export from the liver, with an increased risk of fat accumulation in this tissue; instead, others, in which lipogenesis occurs predominantly in the liver (chicken and fish), or in adipose tissue and liver (rabbits and rats) produce very high/intermediate rates of VLDL (Pullen et al., 1990). The origin of the fatty acids, that constitute triglycerides, can affect the rate of VLDL export. In obese mice, de novo fatty acids synthesis in the liver does not stimulate VLDL production (Wiegman et al., 2003). Rather, plasma exogenous NEFA, seem to play an important role in enhancing hepatic esterification and stimulating VLDL production (Julius, 2003), as it has been seen in rats.

1.2.7 Lipolysis

Triglyceride breakdown, or lipolysis, needs enzymes called lipases that attack triglyceride molecule, breaking the bond between the fatty acid and glycerol. There are two types of lipases, an hormone-sensitive lipase (HSL) that breaks off two fatty acid from triglyceride molecule to form diacylglyceride, and, monocylglyceride lipase that completes the breakdown of triglyceride, releasing the last fatty acid and separating it from glycerol. The liberated fatty acids are either used directly into the cell or introduced into circulation for uptake by other tissues (such as liver or muscle) that can use them for energy metabolism.

1.2.8 Adipose tissue and "Adipogenesis"

The mesenteric adipose tissue is an organ of fat accumulation in fish which, together with the muscle and liver, controls the lipid homeostasis and energetic balance of the animal (Jobling and Johansen, 2003; Sheridan and Kao, 1998), and, at the same time, it also acts as an endocrine organ secreting adipokines which act as potent messengers to distant organs such as the liver and muscle to maintain the body's energy balance (Gregor and Hotamisligil, 2007).

Adipose tissue stores lipids and provides energy from lipid stores. Triglycerides, which come from the diet, are hydrolyzed by lipoprotein lipase (LPL) and the fatty acids released are taken up by the adipocytes and accumulated in droplet form. In response to energy demands, (HSL), after phosphorylation by protein kinase A, can access the lipid droplet and hydrolyze the triglycerides into glycerol and fatty acids (Lafontan and Langin, 2009).In fish as in mammals, the development of adipose tissue is a continuous process which includes the hypertrophy of existing adipocytes and the proliferation of new ones. The training process of new adipocytes from undifferentiated mesenchymal cells is called adipogenesis (fig.8); this occurs thanks to a transcriptional factor PPARɣ (Peroxisome Proliferator Activated Receptor gamma,), that activates other transcriptional factors, and by which, the precursor cells become lipoblast, and then, pre-adipocytes; the latter, then, proliferate and differentiate into adipocytes, which, in turn, accumulate lipid droplets.

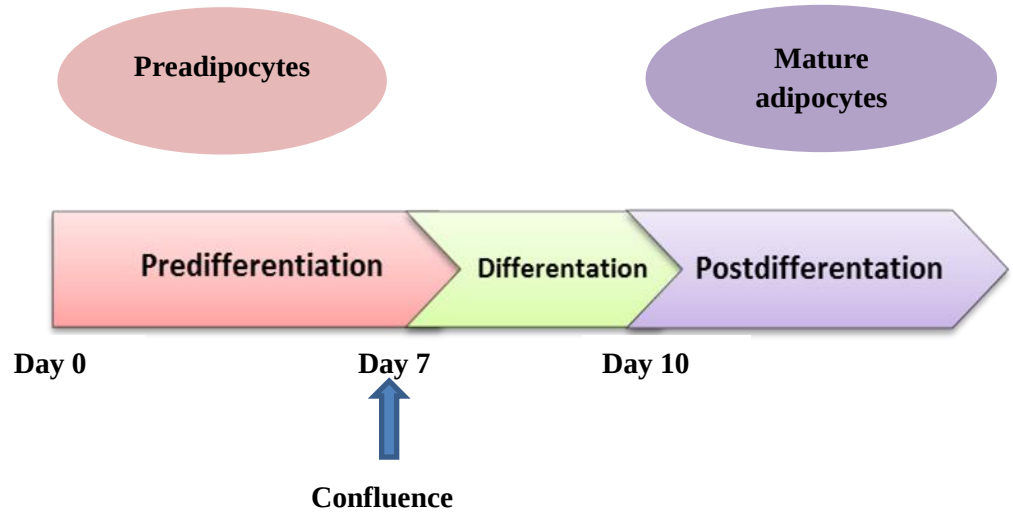


Fig.8 Adipogenesis

These processes are known to be affected by diet in mammals, but how dietary changes affect the capacity for enlargement of adipocytes or the differentiation of new ones is poorly understood in fish. For example, it was seen by Cruz-Garcia et al. in a study conducted in 2011 on gilthead sea bream (*Sparus Aurata L.*) that fish oil substitution by 66% vegetable oils in a diet results in an increase of lipolytic activity and adipocyte cell size. Therefore, dietary vegetable oil, causing changes in tissue fatty acids composition, can affect the metabolism of gilthead sea bream adipocytes and the proliferation of new cells which could potentially affect other organs such as the liver. It has been suggested that excessive lipid accumulation in the liver or steatosis is due in part to the increased hepatic uptake of fatty acids released from adipose tissue with enhanced lipolysis (Benedito-Palos et al., 2008).

1.2.9 The adipocyte life cycle

Adipocytes are derived from mesenchymal stem cells, which have the potential to differentiate into myoblasts, chondroblasts, osteoblasts or adipocytes. The adipocyte life cycle includes alteration of cell shape and growth arrest, clonal storage of lipid and finally cell death (Gregoire, 2001) (fig. 9). During the growth phase, preadipocytes resemble fibroblasts morphologically. Pref-1, a

preadipocyte-secreted factor serves as a marker for preadipocytes and is extinguished during adipocyte differentiation (Wang, et al., 2006). At confluence, preadipocytes enter a resting phase called growth arrest before undergoing the differentiation process. Two transcription factors, CCAAT/enhancer binding protein (C/EBPα), and peroxisome proliferator-activated receptor PPARγ were shown to be involved in the preadipocyte growth arrest that is required for adipocyte differentiation (Umek, et al., 1991). Following growth arrest, preadipocytes must receive an appropriate combination of mitogenic and adipogenic signals to continue through the subsequent differentiation steps. During the process of differentiation, preadipocytes undergo one round of DNA replication leading to clonal amplification of committed cells (Pairault, et al., 1979). The induction of differentiation also results in drastic change in cell shape as the cells convert from fibroblastic to spherical shape. Following induction, a dramatic decrease in Pref-1 expression accompanies a rapid increase in the expression of C/EBPβ, followed by expression of C/EBPα and PPARγ (Rosen, et al., 2002). During the terminal stages of differentiation, the mRNA levels for enzymes involved in triacylglycerol metabolism like glycerol-3-phosphate dehydrogenase, fatty acid synthase and glyceraldehyde-3-phosphate dehydrogenase, increase to a great extent (Paulauskis, et al., 1988; Spiegelman, et al., 1983). Finally, the total number of adipocytes change throughout life, and new adipocytes can be formed or can be removed by the process of apoptosis.

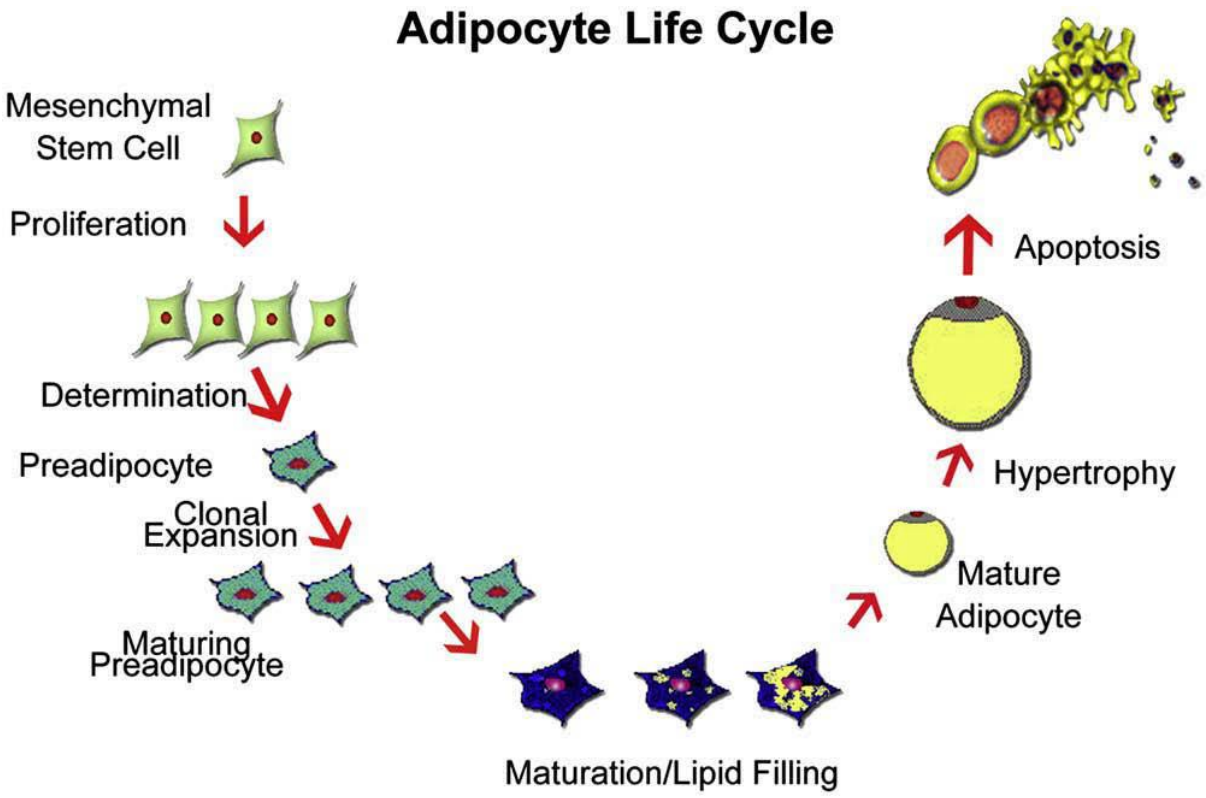


Fig.9 Mesenchymal stem cells are the precursors of several different types of cells, including myoblasts, chondroblasts, osteoblasts and preadipocytes. Once preadipocytes are triggered to mature, they begin to change shape and undergo a round of cell division known as clonal expansion, followed by initiation of the genetic program that allows them to synthesize and store triglycerides. Mature adipocytes can continue storing lipid when energy intake exceeds output, and they can mobilize and oxidize lipid when energy output exceeds input. Mature adipocytes can also undergo apoptotic cell death under certain conditions.

1.3 Apoptosis and autophagy

Cell death has been subdivided into the categories apoptosis (Type I), autophagic cell death (Type II), and necrosis (Type III). The boundary between Type I and II has never been completely clear and perhaps does not exist due to intrinsic factors among different cell types and the crosstalk among organelles within each type. Apoptosis can begin with autophagy, autophagy can end with apoptosis, and blockage of caspase activity can cause a cell to default to Type II cell death from Type I.

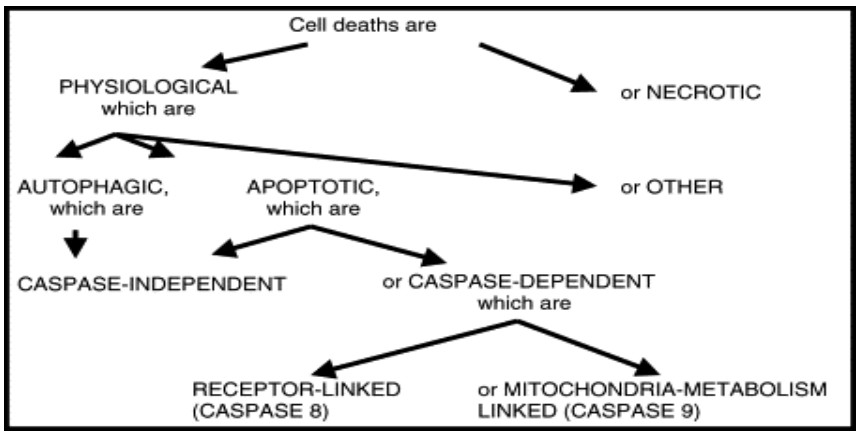


Fig.9 Types of cell deaths. (Richard A. Lockshin and Zahra Zakeri, 2004)

The controlled cells deaths frequently display substantial caspase-independent autophagy or they are predominantly apoptotic. Most apoptotic deaths are caspase-dependent, but there are claims of apoptotic morphology in situations in which caspase activity is equivocal. Caspase activation can occur by means of ligation of a membrane-bound receptor or by means of metabolic changes resulting in depolarization of mitochondria and release of cytochrome *c* and APAF-1. Other metabolic means of activating apoptosis include UPR (unfolded protein response) that is a cellular stress response related to the "ER (endoplasmatic reticulum) stress; this process is usually activated in response to an accumulation of unfolded or misfolded proteins in the lumen of the endoplasmatic reticulum. If it doesn't restore the normal function of the cell, the UPR process can lead to apoptosis (Lockshin and Zakeri, 2004). The "Apoptosis" process was described for the first time by Kerr et al., in 1972, who asserted that apoptosis is a mechanism of controlled cell deletion, which appears to play a complementary but opposite role to mitosis in the regulation of animal cell populations. Its morphological features suggest that it is an active, inherently programmed phenomenon, and it has been shown that it can be initiated or inhibited by a variety of environmental stimuli, both physiological and pathological (Kerry et al., 1972). In particular, they described the morphological changes that take place during the evolution of apoptosis. The latter characteristically affects disseminated single cells, and is manifested

histologically by the formation of small, roughly spherical or ovoid cytoplasmic fragments. Electron microscopy shows that the structural changes in apoptosis take place in two discrete stages (Fig. 10) (Kerr et al., 1972): the first comprises the formation of apoptotic bodies, the second their phagocytosis and degradation by other cells. The condensation is presumably a consequence of the extrusion of water, but its mechanism is still unknown. The formation of apoptotic bodies involves marked condensation of both nucleus and cytoplasm, nuclear fragmentation, and separation of protuberances that form on the cell surface (Fig. 10) (Kerr, 1971) to produce many membrane compact, but otherwise well preserved cell remnants of greatly varying size. The initial morphological events have not been identified: apoptotic cells have already condensed and separated from their neighbours, and the nuclear chromatin is aggregated in dense masses beneath the nuclear envelope (Kerr, 1971). Fully developed apoptotic bodies show closely packed organelles, which may themselves be condensed, but which are apparently intact, both chemically (Kerr, 1965, 1967; Ballard and Holt, 1968) and structurally. Lucent cytoplasmic vacuoles and dense masses of nuclear material are seen in some bodies.

The content of an apoptotic body depends on the cellular constituents that happened to be present in the cytoplasmic protuberance that gave rise to it; small bodies thus occasionally consist almost entirely of condensed nuclear chromatin , whereas others are composed only of cytoplasmic elements. It is difficult to determine precisely the time taken for the sequence of events described above, even when augmented by various stimuli, the process appears to start in individual cells of the same organ or tissue at different times. However, examination of the serial changes that take place in several experimental models (Kerr, 1971; Crawford et al., 1972; Wyllie et al., 1972b) suggests that the process is completed fairly rapidly: bodies may form and disappear within 24 hours.

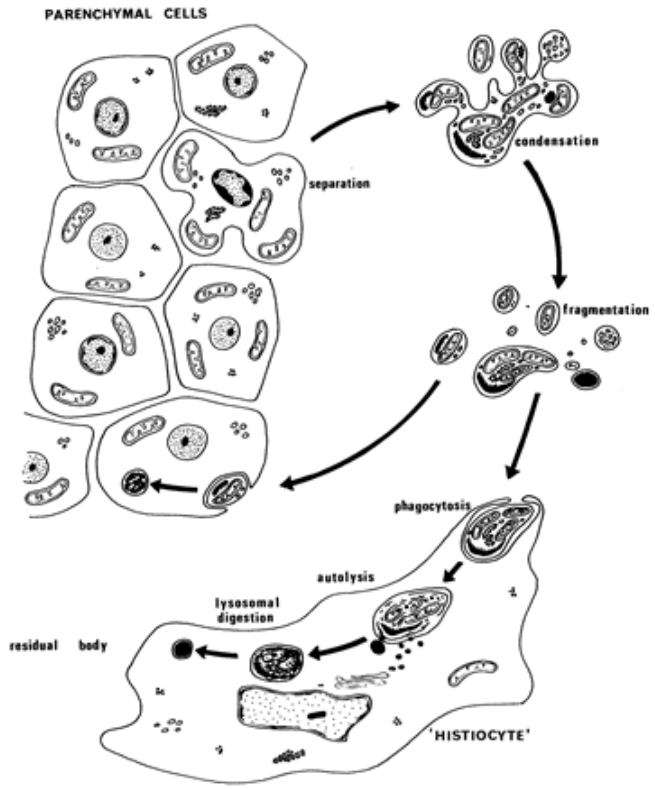


Fig.10 Morphological features of apoptosis. (Kerr et al. 1972).

Currently, the morphology and behaviour of apoptotic cells is largely explained by activation of caspases (cysteinyl aspartate specific proteinase), and apoptosis is considered to be nearly synonymous with caspases activation. Among them, caspase-3 is a frequently activated death protease, catalyzing the specific cleavage of many key cellular proteins. However, the specific requirements of this (or any other) caspase in apoptosis have remained largely unknown until now. Pathways to caspase-3 activation have been identified that are either dependent on or independent of mitochondrial cytochrome c release and caspase-9 function. Caspase-3 is required for some typical hallmarks of apoptosis, and is indispensable for apoptotic chromatin condensation and DNA fragmentation in all cell types examined. Thus, caspase-3 is essential for certain processes associated with the dismantling of the cell and the formation of apoptotic bodies, but it may also function before or at the stage when there is the loss of cell viability (Alan et al., 1998).In most cells the machinery for killing the cell is present but inactive long before the cell is induced to die, and

death appears to be a release from inhibition. So, it is assumed that classical apoptosis is a caspase-dependent form of cell death, whether triggered by extrinsic (cell surface receptor) or intrinsic (mitochondrial depolarization) means. So, the vast majority of maturing or mature cells possess the machinery for self-destruction in the form of inactive proenzymes (pro-caspases) as well as machinery for regulating or adjusting the level at which the proenzymes can be activated. Cells normally hold the machinery in abeyance, and default to its activation when any of numerous conditions define an imperfect situation for the cells. Finally, we can say that as an antagonist of cellular proliferation, apoptosis contributes to maintaining homeostasis between cell production and elimination of superfluous cells produced, that elude mechanisms of control of cell development or that undergo genetic damage.

About autophagy process, this is usually defined as a non-selective vacuolar degradative pathway conserved in eukaryotic cells, starting with the formation in the cytoplasm of a multi-membrane-bound compartment named autophagosome. The discovery of autophagy was contemporary to that of lysosomes by de Duve and Wattiaux (de Duve & Wattiaux, 1966). At the same time, the term autophagic cell death or type II programmed cell death (PCD II) was introduced to describe a cell death different from apoptosis or type I programmed cell death (PCD I) (Schweichel & Merker, 1973) and reviewed by Clarke (1990). From these data it appeared that autophagy is a cell response to stress, which under certain circumstances can lead to cell death. However, the precise role of autophagy as a cell-death mechanism remains to be explored (Lipinski, & Degterev, 2003). Autophagy is an evolutionarily conserved lysosomal pathway involved in the turnover of long-lived proteins and organelles (Dunn, 1994; Klionsky & Ohsumi, 1999; Seglen & Bohley, 1992). Autophagy starts with the formation of a multilayer-membrane bound vacuole (autophagosoma), in this step, cytoplasmic constituents, including organelles,

are sequestered by a unique membrane called the phagophore or isolation membrane, which is a very flat organelle like a Golgi cisterna. Complete sequestration by the elongating phagophore results in formation of the autophagosome, which is typically a double-membraned organelle. This step is a simple sequestration, and no degradation occurs. The autophagosomal membrane is derived from a pre-autophagosomal structure of uncertain origin (Mizushima et al., 2001; Suzuki et al., 2001). A first step towards the formation of the autophagosome is the expansion of the pre-autophagosomal membrane. This step is dependent upon signaling molecules that modulate the activity and the expression of some "autophagy genes". In the next step, autophagosomes fuse with lysosomes (in metazoan cells) or vacuoles (in yeast and plant cells). The inner membrane of the autophagosome and the cytoplasm-derived materials contained in the autophagosome are then degraded by lysosomal/vacuolar hydrolases. These degrading structures are often called "autolysosomes" or "autophagolysosomes". In mammalian cells, most autophagosomes receive input from endocytic compartments (Stromhaug & Seglen, 1993; Liou, Geuze, Geelen, & Slot, 1997) to form a hybrid organelle called the amphisome (Stromhaug & Seglen, 1993). Studies in rat hepatocytes showed that all lysosomes are able to fuse with autophagic vacuoles to degrade the sequestered material (Fengsrud et al., 1995). In some cases direct fusion of autophagosomes with lysosomes was observed (Lawrence & Brown, 1992). However, once macromolecules have been degraded in the lysosome/vacuole, monomeric units (e.g., amino acids) are exported to the cytosol for reuse; for example, amino acids can be used as an energy source through the tricarboxylic acid (TCA) cycle, or they can be used to synthetize proteins, which are important for adaptation to starvation environments; however, little is known about this step. So about roles of autophagy , it is constitutive in all cell types containing a lysosomal compartment. This function is involved in cytoplasmic homeostasis because it controls the turnover of long-lived proteins and probably also the

remodeling of the cytoplasm. But autophagy can be also stimulated in response to different situations of stress, such as starvation, change in cell volume, oxidative stress, accumulation of misfolded protein, hormonal signaling, irradiation and xenobiotic treatment. About role of autophagy in adaptation to starvation, it was seen that the breakdown of proteins by autophagy produces amino acids and other elements needed for intermediary metabolism and for biosynthetic pathways. For this, it's clear that amino acids are regulators of autophagy. Although autophagy is considered to be a non-selective process, selective sequestration of organelles can be observed in various pathophysiological and/or stress situations. Definitively, in addition to diseases as myopathies, neurodegenerative disorders and cancer, where alterations in the autophagic pathway and/or deficiency in autophagy genes are involved, the role of autophagy during physiological processes falls into three categories: (1) autophagy is involved in remodeling during development and differentiation; (2) autophagy is involved in the production of amino acids when nutrients fall short; (3) autophagy is involved in the elimination of unwanted and damaged organelles and molecules, which is likely to be an important function during the adult life span.

Free fatty acids (FFAs) are taken up by hepatocytes and converted into triglycerides (TGs) for storage with cholesterol in lipid droplets (LDs). LD-sequestered TGs continually undergo hydrolysis, generating FFAs that are predominantly re-esterified back into TGs for storage. Nutrient deprivation upregulates TG hydrolysis to supply FFAs for oxidation to meet cellular energy demands. An alternative energy source in times of nutrient scarcity is provided by the breakdown of cellular components by autophagy. In 2009, this new role of autophagy was discovered by a group of researchers (Singh, et al., 2009); they saw that lipid droplets and autophagic components associated during nutrient deprivation, and inhibition of autophagy in cultured hepatocytes and

mouse liver increased triglyceride storage in lipid droplets. So, the regulatory and functional similarities between autophagy and lipolysis, along with the capability of lysosomes to degrade lipids, indicated that autophagy may contribute to LD and TG breakdown.

1.4 The phytochemicals

Phytochemicals are a large group of plant-derived compounds that are commonly found in fruits, vegetables, beans, cereals and plant-based beverages such as tea and wine (Arts & Hollman, 2005). Based on their chemical structure, phytochemicals can principally be categorized into alkaloids, flavonoids, pigments, phenolics, terpenoids, steroids and essential oils.

1.4.1 Flavonoids

Flavonoids are diphenylpropanes that constitute one of the most characteristic classes of secondary metabolites in plants (Cao et al., 1997). It has been shown that flavonoids are potent antioxidants, capable of removing hydroxyl radicals, superoxide anions and lipid peroxy radicals, and have been reported as having antibacterial, anti-inflammatory, antiallergic, antimutagenic, antiviral, antineoplastic, anti-thrombotic and vasodilatory actions (Yao et al., 2004; Chakraborty & Hancz, 2011). The structural components common to these molecules include two benzene rings on either side of a 3-carbon ring, and multiple combinations of hydroxyl groups, sugars, oxygens and methyl groups attached to these structures create the various classes of flavonoids: flavanols, flavanones, flavones, flavan-3-ols (catechins), anthocyanins and isoflavones.

The use of medicinal plants as fertility enhancers and sex reversal agents in fish has been receiving some attention. Moreover, as the components of fish diet and/or compounds present in the aquatic environment, phytochemicals may induce biological responses in fish including estrogenic effects and delayed reproduction, and for this are considered as endocrine disrupting chemicals

(EDCs) (Ng et al., 2006; Cheshenko et al., 2008). Among Phytochemicals, flavonoids are an important group of endocrine disrupting chemicals. Among them there are genistein and dadzein (isoflavones), often called phytoestrogens, because of their structural resemblance with 17b-oestradiol and estrogenic and/or anti-estrogenic properties. In particular, there are numerous reports of genistein exerting estrogenic effects in fish. For example, Bennetau-Pelissero et al. (2001) reported increased plasma vitellogenin concentrations in male and female fish fed diets containing either 500 or 1000 ppm genistein until spawning. Male fish fed a diet with 500 ppm genistein showed a slight decrease in plasma levels of FSH and LH at the end of spermatogenesis, testicular development was accelerated, and sperm motility and concentration were decreased in a dose-dependent way at spawning. Female fed a diet containing 50ppm of genistein showed decreased FSH and LH levels, delayed spawning and impaired gamete quality. Contrarily, in yellow perch, Perca flavescens, Koet al. (1999) reported that genistein (0.75 and 7.5 mg g 1 diet) haven't apparent estrogenic effects on reproductive function. This happens because of dual role of genistein as not only acts as an estrogenic agonist, but also as an antagonist blocking estrogen's action. About mechanism of action of phytoestrogens as endocrine disruptors, it has been seen that phytoestrogens, including isoflavones such as genistein and daidzein, bind weakly to estrogen receptors so that they can produce or inhibit estrogenic effects.

1.5 Genistein: sources, structure and metabolism

Epidemiological data strongly suggested that consumption of plant-based foods rich in isoflavones provides beneficial effects for human health (Ososki and Kennelly, 2003). Thus, much attention has been given to investigating the effects of these compounds, especially genistein, in human physiological and pathological states. Soybean is the main source of isoflavones in the human diet, it contains between 0.6 and 3.8 g isoflavones/kg fresh weight (Cassidy et al.,

2000). The three principal isoflavones found in soy are genistein, daidzein and glycetein, generally in a concentration ratio of 1:1:0.2 (Manach et al., 2004).

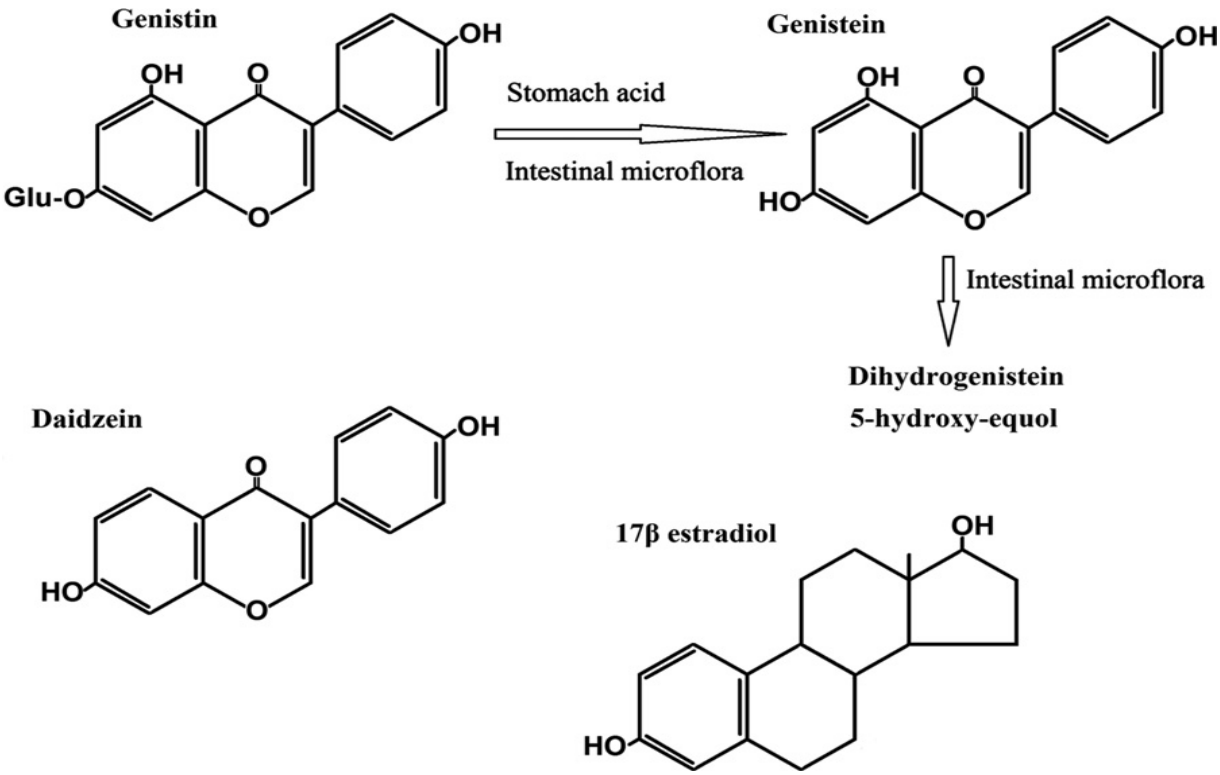


Fig.11 Chemical structure of genistein, genistin, daidzein and 17b estradiol and are presentation of genistein metabolism. (Behloul et al., 2013).

The particular similitude of isoflavones to 17β-estradiol, enables them to bind to estrogen receptors and thus can at least partly explain their effects. After ingestion, genistein is released from the glucoside genistin by acid hydrolysis in the stomach or by microflora hydrolysis in the intestine. The resulting aglycone can either be absorbed or further metabolized to specific metabolites (Dihydrogenistein,5-hydroxy-equol) (Matthies et al., 2012). Genistein (4,5,7-trihydroxyisoflavone) was originally isolated by Perkin and Newbury in 1899 from Dyer's Broom (Genista tinctoria) (Perkin and Newbury,1899).This naturally derived compound is a member of the isoflavone group of the flavonoid family of small molecules, which includes over 5,000 compounds (Andersen and Markham, 2006; Veitch and Grayer, 2008). The isoflavones are structurally characterized by their 3-phenylchromen-4- one core, that is formed

by two benzene rings linked by a heterocyclic pyran ring. In addition to this heterocyclic core, genistein and other members of isoflavone family are polyphenols, because of they contain several hydroxyl groups attached to core phenyl rings. These phenols confer an antioxidant activity to this class of compounds which possess a significant activity against free radicals in tissue (Andersen and Markham, 2006). As previously mentioned, genistein's structural characteristics are similar to those of 17β-estradiol; in particular, shares both a near identical molecular weight as well as a similar hydroxylation pattern, with two key phenolic groups at C7 and C4′ (Fig.12) (Dixon and Ferreira, 2002). Importantly, the C7 hydroxyl group is needed for genistein to bind to the estrogen receptor (ER). Furthermore, the distance (~11.5 Å) between the C7 and C4′ phenolic groups (highlighted in green, see Fig. 12, below) allows for optimal binding of genistein to the ER, as they are in very similar positions to the key hydroxyl groups on the estradiol core (Andersen and Markham, 2006). It has been shown that both the C4′ and C7 phenolic group form key contacts with ERβ, with the C4′ phenol binding to Glu305 and Arg306 and the C7 phenol to His475 (Pike et al., 1999). Because of these structural characteristics, genistein can bind to both α and β isoforms of the ER (Kuiper et al., 1998; Mueller et al., 2004), although it binds to ERβ with 20-fold higher affinity than ERα (Kuiper et al., 1997).

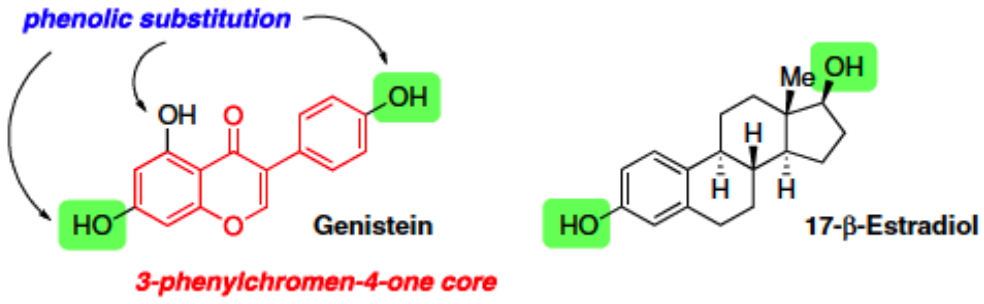


Fig.12 Structures of genistein (left) and 17-β-estradiol (right). Genistein's structure is characterized by a 3-phenylchromen-4-one core (highlighted in red) and phenolic substitution at the C4′, C5, and C7 positions. The C4′ and C7 phenols of genistein are in very similar positions to key hydroxyl groups on estradiol (highlighted in green),allowing genistein to bind to the ER. (Pavese et al., 2010).

1.6 Genistein: a compound with pleiotropic effects

Genistein, a soy derived isoflavone, has been reported as a potential therapeutic agent with anti-cancer, anti-osteoporosis, anti-oxidant and anti-inflammatory effects. Regarding cancer, genistein has been shown to induce apoptosis and differentiation in cancer cells, inhibit cell proliferation, modulate cell cycling, exert anti-oxidant effects, inhibit angiogenesis and suppress osteoclast and lymphocyte functions; these make genistein a promising agent into treatment of cancer. In relation to osteoporosis, it has been seen that genistein exerts a significant estrogenic activity and may be an efficient agent in retaining bone

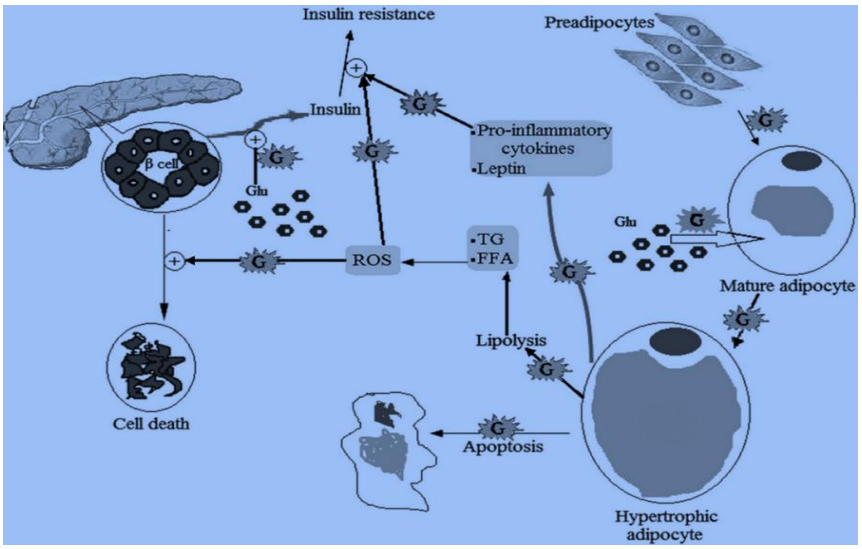


Fig. 13 Pleitropic effects of genistein.Genistein(G) inhibits preadipocytes differentiation,lipidaccumulationandglucose(Glu)uptake in mature adipocyte.It enhances lipolysis and adipocyte apoptosis and reduces the secretion of pro-inflammatory cytokines and leptin.It also enhances glucose-stimulated insulin secretion, reduces reactive oxygenspecies-induced b cell damage and ameliorates the insulin resistance state by counteracting reactive oxygen species,pro-inflammatory cytokines and leptin actions. ROS: reactiv eoxygen species; TG: triglyceride; FFA: free fatty acids. (Behloul et al., 2013).

mass. Studies with ovariectomized rats (lacking in endogenous estrogens) have demonstrate that genistein is active like estrogens in maintaining bone health (Anderson et al., 1998). In tissue cultures *in vitro* genistein, like estradiol, exerts a significant protective activity against experimentally induced bone resorption (Yamaguchi et al., 1998) ,and moreover, it stimulates osteoblast-mediated bone formation. It has been proved that genistein, not only as agonist of estradiol acts on bone health, but also blocking osteoclastic function *in vitro* through tyrosine kinase mechanism. Genistein has been proposed as a promising compound for

the treatment of metabolic disorders and for ameliorating diabetes and obesity states (Fig. 13).

The pleiotropic effects of genistein are due to its multiple mechanisms of action and involvement of several cellular signaling pathways as estrogen-like activity, tyrosine kinase inhibitory effect and 5' AMP- activated protein kinase (AMPK) pathway. Studies conducted on obese diabetic mice and non- obese diabetic mice (Choi et al., 2008) have shown that genistein ameliorated glucose and lipid metabolism, elevated insulin levels and preserved pancreatic β-cells. Moreover, at physiologically achievable concentrations, genistein potentiated glucose-stimulated insulin secretion in both insulin-secreting cell lines and mouse pancreatic islets (Liu et al., 2006). Genistein also protected pancreatic β-cells against cytokine-induced toxicity through inhibition of iNOS (inducible nitric oxide synthase) gene expression, nitric oxide production, and also, the suppression of Erk-1/2 and JAK/signal transducer and activator of transcription (STAT) pathways (Kim et al., 2007). Recently, it has been seen that genistein protects human pancreas cells, that express estrogen receptor β, against high glucose-induced cell apoptosis and the inhibition of their proliferation, through estrogen receptor and Bcl-2 pathways (Zhong et al., 2011).

It has been repeatedly highlighted the bond between oxidative stress and metabolic disorders as diabetes, obesity and cardiovascular diseases (Fatehi-Hassanabad et al., 2010; Grattagliano et al., 2008; Rains and Jain, 2011; Schonfeld and Wojtczak, 2008). Chronic exposure to reactive oxygen species (ROS) activates stress pathways that affect negatively insulin signaling and are involved into insulin resistance, impaired glucose intolerance, β-cell and mitochondrial dysfunctions and finally into diabetes state. The main cellular producers of ROS are mitochondria and plasma membrane. Free fatty acids levels, which are elevated during obesity and diabetes, have been shown to influence the production of ROS by a partial inhibition of complexes 1 and 3 of

the respiratory chain into mitochondria and my modulating the reduced NADPH (nicotinamide adenine dinucleotide phosphate) activity in plasma membrane.

Regarding the anti-oxidant activity of genistein, it has been emphasize genistein as a compound capable of counteract ROS detrimental effects. Following which, its effect on activation of the transcription factors – Nrf1 and Nrf2, which have been implicated in the regulation of genes involved in response to oxidative stress, was investigated (Hernandez-Montes et al., 2006). These transcription factors are involved in the regulation of c-GCS and other detoxification proteins. Genistein was found to induce the cytosolic accumulation and nuclear translocation of Nrf1 and Nrf2. Ultimately, genistein is an antioxidant thanks to its inhibition of the activation of NF-kB stimulated by oxidant stress (Davis et al., 2001).

Beside the oxidative stress, metabolic disorders are also linked to inflammation (Garcia et al., 2010), and genistein seems to ameliorate this inflammatory state. For example, it reduce the expression levels of TNFα and pro-inflammatory citokines in cerebral endothelial cells (Lu et al., 2009), decreases the plasma levels of TNFα and interleukin 6 (IL6) in fructose-fed rats (Palanisamy et al., 2011), and also inhibits the inflammatory process and the progression of non-alcoholic steatohepatitis induced by high fat diet in rats by decreasing TNFα and IL6 levels in serum and liver, by activation of JNK and inhibition of NFkB p65 nuclear translocation and IkBα phosphorylation.

1.6.1 Effects of genistein on adipocytes

Many studies have reported that genistein has an anti-obesogenic effect in ovariectomized mice; in particular, it seems that genistein decreases food intake, body weight, fat pad weight and increases apoptosis of adipose tissue (Kim et al., 2006; Naaz et al., 2003). Many researchers supposed that these effects of genistein may be due to decreased leptin secretion which is the most important

adipose derived hormone, implicated in the regulation of energy homeostasis, obesity, reproduction, bone formation, wound healing and immunity among other biological functions (Peelman et al., 2006). Leptin in mammals is produced and secreted primarily by mature white adipocytes (Matson et al., 1996; Zhang et al.,1994); when fat mass decreases, circulating leptin is reduced, leading to stimulated appetite and suppressed energy expenditure (Ahima et al., 1996; Coppari and Bjørbæk, 2012). In salmonids as in other teleost species studied, the liver appears to be the main production site of leptin, as evidenced by high hepatic Lep gene expression (Gong et al., 2013; Gorissen et al., 2009; Huising et al., 2006; Kling et al., 2012; Kurokawa et al., 2005; Kurokawa and Murashita, 2009; Murashita et al., 2008; Pfundt et al., 2009; Rønnestad et al., 2010), even if leptin has been immunohistochemically detected in primary cultured mature adipocytes of Atlantic salmon (Vegusdal et al., 2003) and in rainbow trout adipose tissue (Pfundt et al., 2009) using mammalian antibodies. So, it is clear the role of leptin as a satiety signal, not only in mammalian, but also in teleost species as rainbow trout (*Oncorhynchus mykiss*), goldfish (*Carassius auratus*), Atlantic salmon. Many of the leptin effects are linked to its actions in the central nervous system, in particular, in the basomedial hypothalamus where leptin receptor β mRNA is highly expressed (Schwartz et al., 2000).

Nowadays, the idea of the leptin resistance state is associated with common forms of human obesity, and, two hypothesis have been proposed to explain it: the failure of circulating leptin to reach its targets into brain and/or the inhibition of the intracellular leptin receptor β; in this case, two inhibitory molecules have been identified: SOCS3 (suppressor of cytokine signaling 3), and, PTP1B (protein tyrosine phosphatase) (Munzberg et al., 2005). It has been demonstrated that genistein disrupts leptin synthesis in 3T3-L1 preadipocytes (Phrakonkham et al., 2008), inhibits its secretion not via estrogen receptor in

murine adipocytes (Niwa et al., 2010), but it has been supposed that genistein might reduce this leptin resistance also by ameliorating the inflammatory state of obesity. Especially, this amelioration could restore the normal leptin influx to its targets in brain and/or down regulate the expression of molecules (SOCS3, PTP1B), that inhibit leptin receptor β signaling. So, genistein could contribute to the recovery of a negative feedback that would decrease the synthesis and the secretion of leptin.

Nowadays, is well known that the process of adipogenesis is controlled by a myriad of adipocyte-specific genes expressions such as expression of peroxisome proliferator-activated receptor ɤ (PPARɤ), CCAAT/enhancer binding protein(C/EBP a) and adipocyte determination and differentiation-dependent factor1/sterol regulatory element binding protein1c (ADD1/SREBP1c). Genistein alone or in combination with other compounds suppresses differentiation, adipogenesis and lipid accumulation and increases lipolysis (Harmon and Harp, 2001) in 3T3-L1adipocytes. Regarding the mechanisms (Fig. 14),

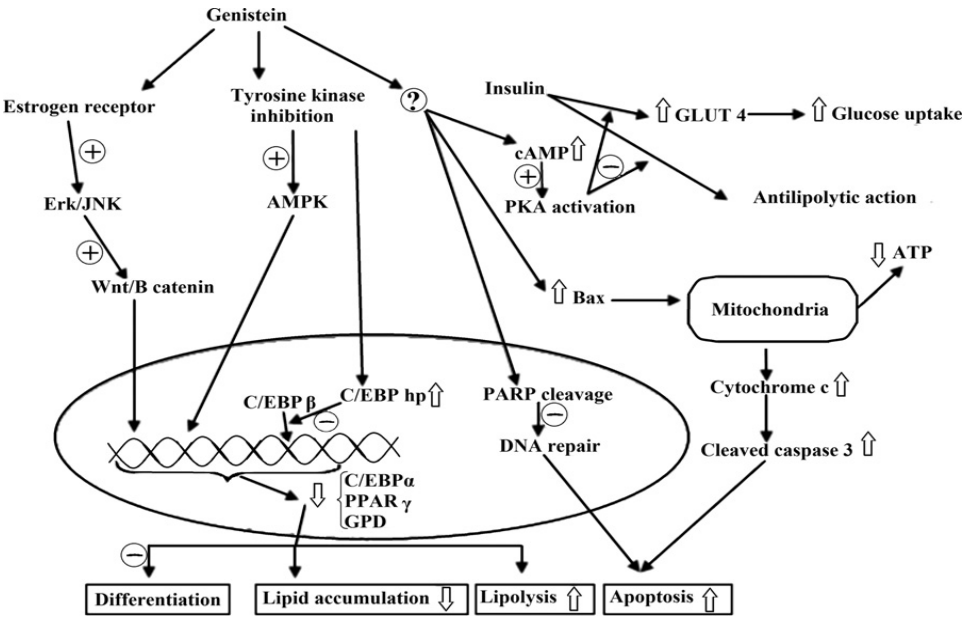


Fig.14 Simplified schema of genistein effects on adipocytes and the different suggested mechanisms. Erk/JNK: extracellular signal-regulated kinase/c-Jun N-terminal kinase; Wnt/b-catenin: wingless and Int/ b-catenin pathway;AMPK:50 AMP-activated protein kinase; C/EBPb: CCAAT/Enhancer binding protein b; C/EBPhp: CCAAT/Enhancer binding protein homologous protein; C/EBPa: CCAAT/Enhancer binding protein a; PPARɤ : peroxisome proliferator activated receptor ɤ; GPD:glycerol-3-phosphate dehydrogenase ;PARP: poly(ADP-ribose)polymerase; Bax:Bcl-2-associated X protein; PKA: protein kinase A; GLUT4:glucose transporter4;(+):activation;(-):inhibition; ⬆: increased level; ⬇: decreased level;(?): the mechanism of action is not elucidated yet. (Behloul et al., 2013).

it has been reported that genistein inhibits adipogenic differentiation of human adipose tissue-derived mesenchymal stem cell via Wingless and Int/β catenin (Wnt/β-catenin) signaling pathway, such as Extracellular signal-regulated kinase/c-Jun-N-terminal kinase (Erk/JNK) signaling, in an estrogen-receptor-dependent way (Kim et al., 2010). Genistein also inhibits lipid accumulation in a dose-dependent manner, via down-regulation of adipocyte specific transcription factors as PPARɤ, C/EBPα and glycerol-3-phosphate dehydrogenase (Park et al., 2009). Previously, it has been shown that genistein also inhibits adipocyte differentiation via activation of 5’ AMP- activated protein kinase (Hwang et al., 2005), and promotes expression of C/EBP homologous protein that blocks the DNA binding and the transcriptional activity of C/EBPβ during differentiation (Harmon et al., 2002).

It has been reported that genistein, because of its tyrosine kinase inhibition activity, inhibits glucose uptake in MC3T3-G2/PA6 adipocytes and directly, through the glucose transporter 4 (GLUT4), counteracts glucose uptake in 3T3-L1 adipose cell (Bazuine et al., 2005; Nomura et al., 2008). Recently, it has been seen that genistein also inhibits insulin-stimulated glucose uptake and reduce ATP in adipocytes and the mechanism proposed seems to involve a mitochondrial dysfunction (Szkudelska et al., 2011). Moreover, genistein counteracts the anti-lipolytic action of insulin (Abler et al., 1992), and also, inhibits, in an estrogen receptor-independent manner, the inhibitory action of insulin on epinephrine-induced lipolysis in isolated rat adipocytes; this action seems to be due to protein kinase A (PKA) activation and elevation of cAMP levels (Szkudelska et al., 2008). Genistein has been shown to induce cell apoptosis via mitochondrial damage; although on adipocytes, low doses of genistein increase cell viability, while high doses decrease it (Park et al., 2009). *In vivo* and *in vitro* studies have shown that genistein has anti-obesogenic effects (Tab.3), unfortunately, not yet it has been done this type of studies on fishes.

Animal/cell line	**Dose of genistein**	**Effects of genistein**	**Mechanism described**	**Ref.**
3T3-L1 adipocytes	100µM/L	Increased lipolysis.		Harmon et Harp (2001).
3T3-L1 adipocytes	200µM/L	Inhibition of adipocyte differentiation.	Activation of AMP-activated protein kinase.	Hwang et al. (2005).
MC3T3-G2/PA6 adipocytes	50µM/L	Inhibition of glucose uptake	Tyrosine kinase inhibition activity.	Bazuine et al. (2005).
Ovariectomized C57/BL6 female mice;	1500µM/L	Reduction of food intake, body weight and fat pad weight.		Kim et al. (2006).

3T3-L1 mouse embryo fibroblasts	400µM/L	Adipose tissue apoptosis.		Kim et al. (2006).
3T3-L1 mouse embryo fibroblasts	100µM/L	Inhibition of leptin synthesis.		Phrakonkham et al. (2008).
3T3-L1 adipocytes	10µM/L	Abrogation of GLUT4-mediated glucose uptake	Tyrosine kinase inhibition activity.	Nomura et al. (2008).
Rat adipocytes	100µM/L	Inhibition of the antilipolyitc action of insulin.	Activation of protein kinase A and elevation of cAMP levels.	Szkudelska et al. (2008).
Synovial fibroblasts		Inhibition of leptin secretion.		Relic et al. (2009).
Primary human pre-adipocytes	50µM/L	Inhibition of lipid accumulation.	Down-regulation of PPARɤ, C/EBPα and glycerol-3-phosphate dehydrogenase.	Park et al. (2009).
Murine adipocytes	40µM/L	Inhibition of leptin secretion.		Niwa et al. (2010).
Human adipose tissue-derived mesenchymal stem cell	100µM/L	Inhibition of adipogenic differentiation	Wnt/b-catenin signaling pathway.	Kim et al. (2010).
Obese postmenopausal women	60.8mg/day	Reduction of serum leptin levels.		Llaneza et al. (2011).
Freshly isolated rat adipocytes	50µM/L	Inhibition of isulin-stimulated glucose uptake and reduction of ATP levels.	Mitochondrial dysfunctions.	Szkudelska et al. (2011).

Tab.3 In vivo and in vitro anti-obesity effects of genistein. (Behloul et al. 2013).

Because of plasma concentrations of genistein, after a regular consumption of it, are much lower than those *in vitro*, combinations with other compounds were investigated (Tab.4).

Combination	**Cell line**	**Effects**	**Mechanisms of action**	**Ref.**
Genistein with guggulsterone	3T3-L1 adipocytes	Apoptosis and suppression of differentiation and adipogenesis.	Production of active caspase. Expression of pro-apoptotic Bax. Release of cytochrome c.	Yang et al. (2007).
Genistein with green tea catechin and capsaicin	3T3_L1 adipocytes	Suppression of differentiation, adipogenesis and lipid accumulation, and induction of apoptosis.	Release of intracellular reactive oxygen species and activation of AMPK pathway.	Hwang et al. (2005).
Genistein with resveratrol	3T3_L1 adipocytes	Inhibition of adipogenesis; induction of apoptosis; stimulation of lipolysis.	Down-regulation of PPARɤ and CCAAT/enhancer binding protein α	Rayalam et al. (2007).
Genistein with vitamin D	3T3_L1 adipocytes	Inhibition of lipid accumulation, and induction of apoptosis.	Suppression of PPARɤ, C/EBPα, and enhancing vitamin D receptor expression.	Rayalam et al. (2008).

Tab.4 Synergistic combinations of genistein with other compounds. (Behloul et al. 2013)

For example, genistein with guggulsterone induces apoptosis and suppression of differentiation and adipogenesis in 3T3-L1 adipocytes, through an increase in production of active caspase 3, and an induction of the expression of pro-apoptotic Bcl-2-associated x protein (Bax) and the release of cytochrome c. Genistein with vitamin D combined promote the inhibition of lipid accumulation and the induction of apoptosis in 3T3-L1 adipocytes, through a suppression of PPARɤ, C/EBPα, and an induction of vitamin D receptor expression (Rayalam et al., 2008).

2. Aims of research

The management of fat deposition has become a key area of interest in fish farming in the quest to obtain a high-quality product with good nutritional value and to maintain fish health. The administration of high-lipid feed usually can lead to an unwanted increase in fat deposition that alters sensory and organoleptic characteristics (Kjær et al., 2008; Turchini et al., 2009). On one hand the potential anti-obesogenic effect of genistein in rainbow trout can be positive as ingredient in fish diets, on the other, it isn't (i.e adipocytes apoptosis). So, the hypothesis of work is that genistein can have *in vivo* and *in vitro* anti-adipogenic and anti-obesogenic effects, as shown previously in mammals. Moreover, we want to analyze the main pathways through which it exercises this role on *Oncorhynchus mykiss* lipid metabolism. Following, are reported the purposes of the research in detail:

1. To determine the effect of an intraperitoneal injection of genistein on lipid metabolism related genes expression; to evaluate the effect of genistein on apoptosis-related genes expression, and on autophagy-related genes expression. All of this, in adipose tissue, liver and white muscle of rainbow trout.
2. To describe the potential anti-obesogenic effect of genistein, in particular, analyzing the effects of genistein on viability and differentiation in rainbow trout adipocytes in primary culture.

3. Materials and methods

3.1 Animals

Juvenile (*in vivo* experiment) and adult (*in vitro* experiment) rainbow trout, approximatively 80g and 250g in weight respectively, were obtained from Spanish commercial fish farm and maintained either in 0,2m^3 and 0,4m^3 tanks with a temperature controlled freshwater recirculation system (18±1°C), and 12L:12D photoperiod in the facilities of the Faculty of Biology at the University of Barcelona. Fish were fed with a commercial diet (Skretting, Burgos, Spain) twice daily. Juvenile animals were fasted 2 hours before the injection to 24 hours post-injection; adult animals for *in vitro* experiment were fasted 24 hours.

3.2 Experimental design *in vivo*: intraperitoneal injection

After 15 days of acclimation, juvenile rainbow trout were anesthetized (MS-222, 0.1g/L), and received intraperitoneal injections of 4.64µL volume per g body mass containing genistein at different dose (see above). Both for the experiment *in vivo* than that *in vitro*, genistein, 17β-estradiol (E2), and fulvestrant (estrogen receptor antagonist) were initially resuspended in DMSO (Dimethyl sulfoxide). Only for *in vivo* experiments, genistein and E2 were diluted then 1:3 in sesame oil. Treatments were :

1) Control containing DMSO diluted 1:3 in sesame oil;
2) Genistein at 5µg/g of body mass;
3) Genistein at 50µg/g of body mass;
4) 17β-estradiol at 5µg/g of body mass.

After 24 hours, fish were first anesthetized, and then sacrificed by a blow to the head; blood samples are taken from the caudal aorta, samples of liver, perivisceral adipose tissue and white muscle were harvested and stored at -80°C until analysis.

3.3 Gene expression analysis

3.3.1 RNA isolation

All tissue samples were processed individually, both those of adipose tissue that those of the liver (N=31), and those of white muscle. To isolate total RNA from tissues, approximately 0,1g (adipose tissue), 0,02-0,03g (liver), and 0,1g (white muscle) were homogenized in 1mL TRIzol (Ambion, Alcobendas, Spain) according to the following protocol:

Homogenization of tissues for RNA

Material

-Falcons

-Eppendorfs 1.5 mL

-Tri-Reagent SOLN (RNA reagent isolation)

- Polytron

-Water mQ

-NaOH (0.5-1M)

Procedure

1. Add to every falcon 1mL of "TRIZOL"
2. Weigh samples; they must be more or less 0.1g
3. Put them into every falcon with "TRIZOL"
4. Homogenize samples by a polytron: 2-3 times (30") at 12000 rpm
5. Samples homogenates must be put into new eppendorfs

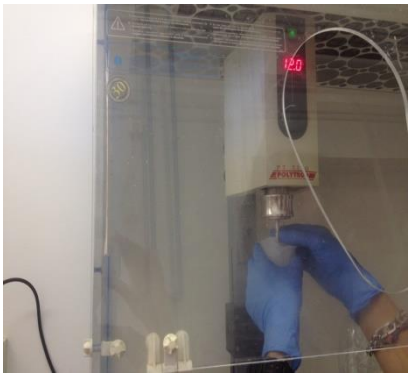


Fig. 15 Samples Homogenization by a polytron.

6. Centrifuge at 4°C for 10 min at 12000 rcf
7. Get only the upper part and put it into new eppendorfs and reject pellet

Fig.16 Centrifuge machine.

Notes

The "TRIZOL" must be always into ice; it is a reaction based on water and phenol which extracts nucleic acids and protects from degradation of RNAses.

3.3.2 Extraction of RNA from tissues

Material

-Chloroform

-Isopropanol

-Ethanol 75%

-Eppendorfs 1.5mL

-Water mQ

Procedure

1. Put 200µL of Chloroform into every eppendorf with homogenate tissue and vortex for 20"

2. Let rest 5 min at room temperature so that the phases begin to separate themselves
3. Centrifuge at 4°C for 15 min at 13000 rcf ; then there will be 3 phases well differentiated: a lower with chloroform, an intermediate with protein and an higher with RNA plus water
4. Get only the upper phase and put it into new eppendorfs
5. Put 500µL of Isopropanol (it helps to precipitate RNA) and mix by inversion (4-5 times)
6. Let rest 10 min at room temperature
7. Centrifuge at 4°C for 10 min at 13000 rcf , so that there will be an higher aqueous phase and a white pellet (it includes RNA)
8. Reject an aqueous phase and don't touch pellet
9. Add 1mL of Ethanol 75%
10. Centrifuge at 4°C for 5 min at 7500 rcf
11. Repeat the last three steps, so that we have two washes by Ethanol 75%
12. Reject aqueous phase and let dry pellet so that Ethanol evaporates
13. Re-suspend pellet by adding 30µL of RNAse Free water
14. Quantify RNA into samples by NANODROP

The quantity of the RNA was determined by measuring the absorbance at 260nm and 280nm with a ND-2000 NanoDrop (Thermo Fisher Scientific, Alcobendas, Spain).

Fig.17 Nanodrop 2000 machine.

3.3.3 Complementary DNA synthesis

For the reverse transcriptase reaction (RT), 500-1000ng of total RNA were treated with DNase I (Life Technologies, Alcobendas, Spain) in order to remove all genomic DNA, and furthermore the RNA was reverse transcribed with the Transcriptor First Strand cDNA synthesis (Roche, Sant Cugat del Valles, Spain), according to the following protocol:

DNase + RT protocol

- <u>cDNA synthesis (DNase + Denaturalization+ RT)</u>

DNase treatment kit (vf= 11µL)

Eppendorfs 200µL RNase-free

Calculate the amount of DEPC H_2O (µL) to use with Excel, based on the amount of RNA (µg).

CNTC= no RNA

CRTC= no RT enzim. Use RNA from higher concentrated sample

STEPS:

1. Label the eppendorfs
2. Prepare mix: DNase (1µL) + Buffer (1µL) (+2-3 pipetting error). Keep on ice.
3. Add the DEPC water corresponding to each sample (see Excel).
4. Add the amount of RNA from each sample except CNTC (see Excel).
5. Add MIX (2µL/eppendorfs) and put the timer 15 min from the first sample (room temperature).
6. After 15min, add 1µL of EDTA to stop reaction.

7. Place eppendorfs in PCR machine and select DNase protocol (10min at 65°C).

Fig.18 PCR machine Bio-Rad.

Denaturalization kit cDNA (vf= 13µL)

STEPS:

1. Label the final eppendorfs with sample code.
2. Make a MIX with oligdT's (1µL) + Random hexamer (2µL) (+2-3 pipetting error). Keep on ice.
3. Add 10µL from the sample (DNase treatment) to new eppendorfs.
4. Add MIX (3µL/Eppendorf).
5. Place eppendorfs into PCR machine and select Denaturalization protocol (10min at 65°C).

RT. Retro- transcription kit cDNA (vf= 20µL)

STEPS:

1. Prepare MIX with Buffer (4µL) + Protector (0.5µL) + dNTPs (2µL) + RT enzim (0.5µL) (+2-3 pipetting error). Keep on ice.

2. Add MIX (7µL/Eppendorf) to all samples except the CRTC (add water instead of RT enzim).
3. Place eppendorfs into PCR machine and select RT protocol (~90min).

3.3.4 Real-time quantitative reverse transcription PCR (Real-time qRT-PCR)

A serial dilution in nuclease-free water of cDNA derived from a RNA pool of experimental samples (liver, adipose tissue and white muscle) was amplified to construct standard curves for both target and reference genes. Standard curves were included in each run to determine amplification efficiency (E) and dilution at which it has the highest gene expression.After then, both cDNA liver samples and adipose tissue samples were diluted 1:10 and 1:50 in nuclease free-water; while white muscle samples were diluted 1:10, 1:50, 1:100, 1:200 in nuclease free water. Real-time qRT-PCR measurements were performed by preparing 4µL PCR mix (2,5µL of Sybr-Green; 0,25µL of primer (F+R), and 1,25 of MQ water) and by applying 1µL of diluted cDNA samples; for some genes, 3µL of PCR mix was prepared (2,5µL of Sybr-Green; 0,125µL of primer (F+R), and 0,375 of MQ water, or 2,5µL of Sybr-Green, 0,25µL of primer (F+R), and 0,25µL of MQ water) with 2µL of diluted cDNA samples. The quantitative polymerase chain reaction primer sequences for the genes analyzed (EF1α LPL, GAPDH, HSL, FAS, EM, LXR, PPARβ, PPARα, β-actin, Ubiquitin, Lep, ER2α, ER2β, VTG, LC3B, SQSTM1, ATG12L, ATG4B,

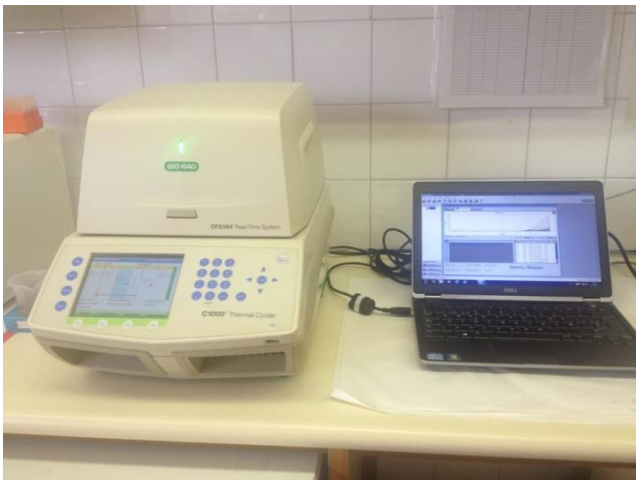


Fig.19 Real-time PCR machine Bio-rad.

CASP3, CASP8, P53, NUPR1), and the reference genes of adipose tissue, liver and white muscle are shown in Tabl. 23.

Tab.5 Rainbow trout sequences used for qPCR.

Gene symbol	T°	Sequence 5'-3'
Reference genes for adipose tissue and liver		
Ubiquitin F	58°	5'- ACAACATCCAGAAAG-3'
Ubiquitin R	-	5'- AGGCGGGCGTAGCACTTG-3'
β-actin F	62°	5'-ATCCTGACAGAGCGCGGTTACAGT-3'
β-actin R	-	5'-TGCCCATCTCCTGCTCAAAGTCCA-3'
EF1αF	59°	5'-TCCTCTTGGTCGTTTCGCTG-3'
EF1αR	-	5'-ACCCGAGGGACATCCTGTG-3'
Reference genes for white muscle		
Ubiquitin F	58°	5'- ACAACATCCAGAAAG-3'
Ubiquitin R	-	5'- AGGCGGGCGTAGCACTTG-3'
EF1αF	59°	5'-TCCTCTTGGTCGTTTCGCTG-3'
EF1αR	-	5'-ACCCGAGGGACATCCTGTG-3'
Lipid metabolism-related genes		
LPLF	59°	5'-TAATTGGCTGCAGAAAACAC-3'
LPLR	-	5'-CGTCAGCAAACTCAAAGGT-3'
GAPDHF	62°	5'-GACACCTAGTGGAGGCTGTC-3'
GAPDHR	-	5'-ATGACCTTGCCCACAGCCTT-3'
HSLF	58°	5'-AGGGTCATGGTCATCGTCTC-3'
HSLR	-	5'-CTTGACGGAGGGACAGCTAC-3'
FASF	54°	5'-GAGACCTAGTGGAGGCTGTC-3'
FASR	-	5'-TCTTGTTGATGCTGAGCTGT-3'
PPARαF	54°	5'-CTGGAGCTGGATGACAGTGA-3'
PPARαR	-	5'-GGCAAGTTTTTGCAGCAGAT-3'
PPARβF	59°	5'-CTGGAGCTGGATGACAGTGA-3'
PPARβR	-	5'-GTCAGCCATCTTGTTGAGCA-3'

LEPF	60°	5’-TTGCTCAAACCATGGTGATTAGCA-3’
LEPR	-	5’-GTCCATGCCCTCGATCAGGTTA-3’
LXRF	62°	5’-TGCAGCCGTATGTGGA-3’
LXRR	-	5’-GCGGCGGGAGCTTCTTGTC-3’
ER2αF	60°	5’-AGACGGTCATCTCGCTGGAAG-3’
ER2αR	-	5’-ACACTTTGTCATGCCCACTTCGTA-3’
ER2βF	60°	5’-AGAGGAAGTGAACTCCTCCTCAGG-3’
ER2βR	-	5’-GATAGTAGCACTGGTTAGTTGCTGGAC-3’
VTGF	58°	5’-GAGCTAAGGTCCGCACAATTG-3’
VTGR	-	5’-GGGAAACAGGGAAAGCTTCAA-3’
Autophagy-related genes		
LC3BF	57°	5’-GAACAGTTTGACCTGCGTGAA-3’
LC3BR	-	5’-TCTCTCAAtGATGACCGGAATCT-3’
SQSTM1F	57°	5’-AGCCCACTGGGTATCGATGT-3’
SQSTM1R	-	5’-GGTCACGTGAGTCCATTCCT-3’
ATG12F	60°	5’-GATGGAGGCCAATGAACAGC-3’
ATG12R	-	5’-GCGTTTGAACTGAAAAGGGCTAA-3’
ATG4BF	60°	5’-TATGCGCTTCCGAAAGTTGTC-3’
ATG4BR	-	5’-CAGGATCGTTGGGGTTCTGC-3’
Apoptosis-related genes		
CASP3F	57°	5’-TTTGGGAGTAGATTGCAGGG-3’
CASP3R	-	5’-TGCACATCCACGATTTGATT-3’
CASP8F	56°	5’-CAGCATAGAGAAGCAAGGGG-3’
CASP8R	-	5’-TGACTGAGGGGAGCTGAGTT-3’
P53F	56°	5’-GTGGAATTTGATCCGAGTCTGT-3’
P53R	-	5’-AGTGTCCAGGGTAGAAATGGAG-3’
NUPR1F	56°	5’-CGAAGAAGCACACTACGATCAA-3’
NUPR1R	-	5’-TCAGTCCGATTTCTCTCTTGGT-3’

Abbreviations: EF1α, elongation factor 1α; LPL, lipoprotein lipase; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase ;HSL, hormone sensitive lipase; FAS, fatty acid synthase; PPAR, peroxisome proliferator-activated receptors; LEP, leptin; LXR, liver X receptor; ER, estrogen receptor; VTG, vitellogenin;LC3B (MAP1LC3B), Microtubule-associated protein 1 light chain 3 beta; SQSTM1, sequestome-1; ATG, autophagy- related; CASP, caspase; P53, phosphoprotein 53; NUPR1, nuclear protein; qPCR, quantitative polymerase chain reaction.

3.4 Experimental design *in vitro*: cell culture

To avoid contamination from the gastrointestinal tract, adult fishes were fasted 24 hours before the experiments. The cell culture was performed according to the following protocol:

Pre-adipocytes protocol

Solutions:

1. **Stock Krebs preparation (x 10)**

   It is necessary to prepare 500mL of Krebs the day before beginning the culture, and, to aliquot it in equal parts of 50mL, and, to freeze it. These amounts are good for cultivation trout.

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

Reagents	Amounts
Stock Krebs (x 10)	50mL
Distilled water (mQ)	445mL
$NaHCO_3$ (25mM)	1.05g
D-Glucose (5.5mM)	0.495g
HEPES pH 7.4	5mL

2. **Stock HEPES (1M) pH 7.4**

3. Erythrocyte buffer preparation

To prepare 100mL of buffer:

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

Notes: Adjust pH at 7.3 and put the solution in the autoclave.

4. Collagenase

Preparing collagenase Type II sigma C6885 (130U/mL). Filtering collagenase by filter 0.22µm (Millipore CatN° SVGP01050).

(130 U/mL) * (mg/475U) * mL of Buffer Krebs = Xmg of collagenase into XmL of KBSA 1%

- 475= units of collagenase that are written on bottle (this value can varies)
- mL of Buffer Krebs= volume of Buffer Krebs to add. It is depends on amount of tissue (per gram of tissue extract, it need to add 5mL (1g/5mL).

5. Medium of growth (for 500mL)

Reagents	Amounts
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

6. Medium of differentiation (for 50mL)

- 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)

7. Gelatine 1%

The bottle (dust) must be preserved sterile, and, it must be opened into cape. It is possible to choose an amount, and, when we go to prepare it outside cape (for example: 150mL), we put 1.5g of gelatin plus 150mL of distilled water, and then, we put the solution in the autoclave. Finally, this solution must be rated.

8. Feral bovine Serum (FBS)

It is liquid, must be rated (~ 30mL) and it must be put into freezer at -20°C.

Procedure

The day before culture

Prepare the plates with gelatine 1% (1mL in every well) into cape, put them 3 hours into incubator at 18°C, and then, wash with PBS (Phosphate Buffered Saline) sterile (500μL/ well) twice, then, put the plates with PBS into incubator until the day of the culture.

The day of culture

- Turn on the cap and select UV.
- Prepare 500mL di Krebs-HEPES solution and mix it with CO_2 5%.
- Weight 0.5g of anesthetic ms222 (Ethyl 3-aminobenzoate methanesulfonate).
- Filter ~ 370mL of Krebs-HEPES solution with help of syringe and filter of 0.22µm (Millipore CatN° SVGP01050), the remaining mL aren't filtered and into them put 1.3g of BSA 1% (Albumin from bovine serum), then put them into incubator.
- Put the dissection material into the cape.
- Put 4 falcons with 5mL of Krebs-HEPES solution plus 50µ of antibiotic and put this solution into ice.

Sacrifice fish

Anaesthetize two fishes at a time, and then, kill them by a blow to the head. After that, weight fishes and extract adipose tissue from them (5g for every falcon).

Digestion and incubation

- Put into the cape a beaker for waste.
- Prepare the amount of collagenase type II (130U/mL) to put into the Krebs-HEPES solution (not filtered) plus 1% BSA. This depends on amount of adipose tissue extract. Prepare 5mL of medium/gram of tissue extract. For example if we extract 16g of tissue (4g for 4 falcons),we will have more or less 130mL:

$$\frac{130U \times 130mL}{693.2} = 24.3mg \text{ of collagenase}$$

- Filter collagenase into the cape with help of syringe and filters (Stevirex GP 0.22µm).
- Put adipose tissue of falcons (one by one) into a petri and, with help of two scalpels, cut tissue and put it into a new falcon, adding 5mL of Krebs filtered.
- Remove lower part of falcons and add to every falcon 5mL/g of medium filtered with collagenase.
- Put falcons into the incubator at 18°C for an hour on the roller.
- Put falcons in ice and filter the content in a new falcon with help of filters for falcons (100µm) (Filters falcon Ref. 352360).
- Complete the volume up to 50ml with Krebs-HEPES and centrifuge at 1500 rpm for 10 minutes at 4°C.
- After centrifuging, take only the precipitate (erythrocytes plus pre-adipocytes), remove the remainder by decantation.
- Put in every falcon 5ml of erythrocytes buffer for 5-10 minutes at room temperature and with help of sterile pipette dilute precipitate in buffer.
- Join the content of two falcons in a new one, filtering it by filters for falcons (100µm) without taking lipids in the upper part.
- Wash falcon (with remaining lipids) by 5ml of Krebs-HEPES, then, take only the Krebs (without lipids) and put it in the new falcon.
- Add to falcon (with the filtered content) Krebs-HEPES until to arrive at 35ml.
- Centrifuge in the same conditions.

- Remove the upper part of the centrifuged from falcons, put 1ml of medium of growth and re-suspend by a pipette; then, add the remaining medium of growth to arrive 2ml.

Cells count

Put into eppendorf:

- 20µl of pre-adipocytes
- 10µl of medium of growth
- 10µl of trypan blue

} Dilution 1:2

Put into Neubauer chamber culture room 20µl after having put a coverslip.

After cells count, it needs to do a media, multiply by 2 (factor of dilution), multiply by the total volume and multiply by 10000 (factor of plate) to obtain total number of cells; while, without total volume to obtain number of cells/ml. After this, cells are seeded in pre-treated six-well or twelve-well plates (9,6cm^2/well or 2,55cm^2/well respectively).

The day after the culture

- Take from the fridge L-15 and medium of growth and put them into cap for about 30 minutes.
- Remove medium from the plates with help of pipette.
- Put 1ml of L-15 in every well (into plates composed of 12 wells) to do the washing of plates.
- Remove L-15 and put 1ml of medium of growth.

- Change medium of growth every 2 days.

Differentiation

When cells are confluent (seventh day of culture), add medium of differentiation.

Treatments were the following:

1. Control containing 0,1% of DMSO;
2. Genistein at 10µM;
3. Genistein at 100µM;
4. E2 at 1µM;
5. Genistein (10µM) + Fulvestrant (2µM);
6. Genistein (100µM) + Fulvestrant (2µM);
7. E2 (1µM) + Fulvestrant (2µM).

The initial concentrations of compounds were:

1. Genistein: 10mM
2. E2: 100µM
3. Fulvestrant: 100µM

3.5 Cytotoxicity assay: MTT assay for the cellular viability

To describe the potential non-viable effect of genistein in rainbow trout adipocytes in primary culture, it was performed a colorimetric assay: bromide of 3-(4,5-dimethylthiazol-2-ilo)-2,5-diphenyltetrazolium (MTT).It is a colorimetric assay (use to examine the cellular survival and proliferation, mainly), through which we can determine a certain enzyme activity, thanks to a change of colour. This assay is based on the metabolic reduction of MTT (a yellow water-soluble

salt) to a blue compound (known as formazan); this reaction happens thanks to mitochondrial succinate-dehydrogenase enzyme that dissociates the tetrazolium ring of MTT molecule. In this way, we can estimate the number of active mitochondria, and so, the number of life cells, because the amount of alive cells is proportional to the amount of formazan produced. The MTT metabolized to formazan forms water-insoluble crystals which are entrapped into cells, so they must be solubilized in an organic solvent before carrying out a colorimetry.

Preparation of the stock solution MTT

Put 5 mg of MTT (ref. M5655 500mg, SIGMA) per millilitre of PBS (Phosphate Buffered Saline). Then, wrap it in an aluminum foil, this because of it is photo-sensible, and so, it could damage itself.

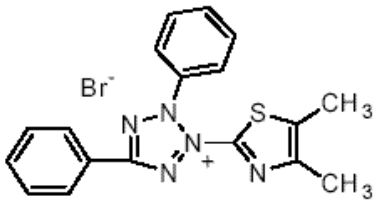


Fig.20 MTT salt.

After the preparation of stock solution MTT, treatments were prepared, and on the fifth day of culture (beginning of cell proliferation) it was performed stimulation of the plates with the following treatments:

1. Control (GM+ DMSO)
2. G10µM
3. G100µM
4. E2 1µM
5. E2 (2µM) + Fulvestrant (2µM)

6. G (10µM) + Fulvestrant (2µM)
7. G (100µM) + Fulvestrant (2µM)

Then, after six hours the solution of MTT was placed, according to the following protocol:

MTT Protocol

1. Put 50µL of MTT plus 450µL of medium in every well of plate (if it is composed of 12 wells), or, 100µL of MTT plus 900µL of medium if it is composed of 6 wells.
2. You leave it in the incubator for 3 hours. When you jab out it from the incubator, it is necessary to observe the blue crystals under the microscope.
3. Wash the wells with PBS once (1mL in every well).
4. Add 150µL (if the plate is composed of 12 wells), or, 250µL (if it is composed of 6 wells) of DMSO (Dimethyl sulfoxide) in every well to dissolve crystals formed. Then, put the plates into incubator for 2 hours (in case of osteoblasts), or, we examine them immediately (in case of adipocytes).
5. Put 100µL (once, if the plate is composed of 12 wells; twice, if it is composed of 6 wells) into a plate ELISA and measure the absorbance at 570nm and at 650nm by a spectrophotometer (TECAN). The formazan solution absorbs light at 570nm, not at 650nm; for this, the final optical density (OD), obtained about formazan produced, can be calculate by subtraction between two absorbance (570nm-650nm).

"DMSO" is an uncolored organic solvent with the capacity of crossing epidermis and cellular membranes, quickly. It is a solvent that in solution

doesn’t release protons to form hydrogen bonds and it is highly polar. It is miscible both water and organic solvents.

3.6 Cytochemical assay: ORO (Oil red oil) assay

The lipophilic dye Oil Red O (ORO) is widely used to characterize the fat cells differentiated, because it is a fat-soluble dye capable of staining neutral triglycerides and lipids, highlighting them in red.

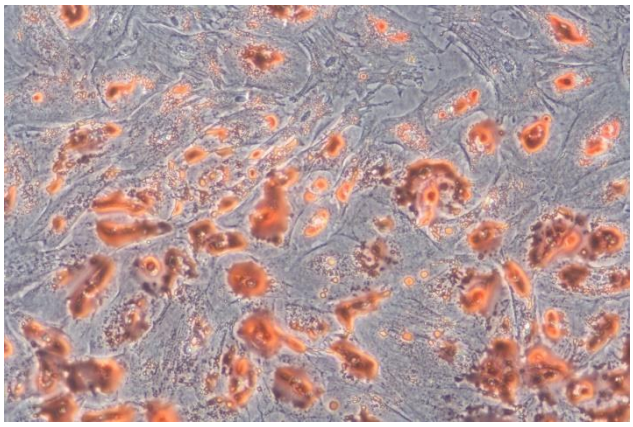


Fig.21 Rainbow trout mature adipocytes highlighted in red with the dye ORO.

In the present work, to investigate the potential anti-differentiation effect of genistein in rainbow trout adipocytes in primary culture, the seventh day of the cell culture, stimulation of the plates was performed with the following treatments:

1. Control (DM + DMSO)
2. G10µM
3. G100µM
4. E2 1µM
5. E2 (2µM) + Fulvestrant (2µM)

6. G (10µM) + Fulvestrant (2µM)
7. G (100µM) + Fulvestrant (2µM)

After preparing the Working solution ORO (in 36% TEP):

Stock solution ORO: 500mg ORO/ 100ml 60% TEP (Triethyl phosphate)

12ml stock solution ORO + 8ml H_2O

Filter with paper Whatmann (n°4)

Working solution ORO (in 36%TEP)

3 days after (72h), it was done the dye of triglycerides with ORO, according to this protocol:

- Wash cells with PBS.
- Fix them for an hour with formalin 10% (or formaldehyde 3,7%).
- Do three washes with distilled water (1ml in every well into plates composed of 12 wells).
- Dye with Working solution ORO (1ml in every well into plates composed of 12 wells) for 2 hours.
- Do three washes with distilled water.

Extraction of triglycerides

- Let dry cells.
- Add isopropanol: 250µL into plates composed of 6 wells (or 150µL for plates composed of 12 wells) for 30 minutes.

- Put 100µL x2 (for plates composed of 6 wells), or 100µL (for plates composed of 12 wells) into wells of ELISA plate and analyze at 490nm with TECAN.

Dye and extraction of proteins

- 2 washes with distilled water
- Add 1ml of Comassie solution for an hour at room temperature.
- Washes with distilled water twice.
- Add propyl glycol: 1ml (for plates composed of 6 wells) or 500µL (for plates composed of 12 wells) for an hour at 60°C.
- Put 100µl x2 (for plates composed of 6 wells) or 100µL (for plates composed of 12 wells) into wells of ELISA plate and analyze at 630nm with TECAN.

Count TG/proteins: divide the reading at 490nm for reading at 630nm.

3.7 Statistical analysis

All data were analyzed using the statistical software IBM SPSS statistics 19. Data are presented as means values ± SEM (standard error of the mean). For *in vitro* experiments, n=4 independent experiments from 4 different cell isolations were used. For *in vivo* experiments, n=9 fish were analyzed. Statistical differences were analyzed in log-transformed data. To verify data normality, it was done Shapiro-Wilk test; to verify the homogeneity of variances, Levene test was done. The results were analyzed by 1-way ANOVA followed by Tukey test

(Levene test not significant) or Dunnett test (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 significant at $P<0.05$.

4. Results

4.1 In *vivo* results: Gene expression analysis in adipose tissue and liver

Expression of estrogen receptors, and lipid metabolism-related genes after genistein or 17β-estradiol administration, in adipose tissue and liver, are presented in fig.22. Expression of VTG was also determined in liver to confirm the effectiveness of the injected compounds.

In adipose tissue, high concentration of genistein (50µg/g) increased FAS, PPARβ, GAPDH and LXR expression. Regarding LPL, HSL and LEP, no significant effect was seen.

In liver, instead, genistein at 50µg/g increased HSL, and GAPDH expression, but no significant effect in the other genes was observed.

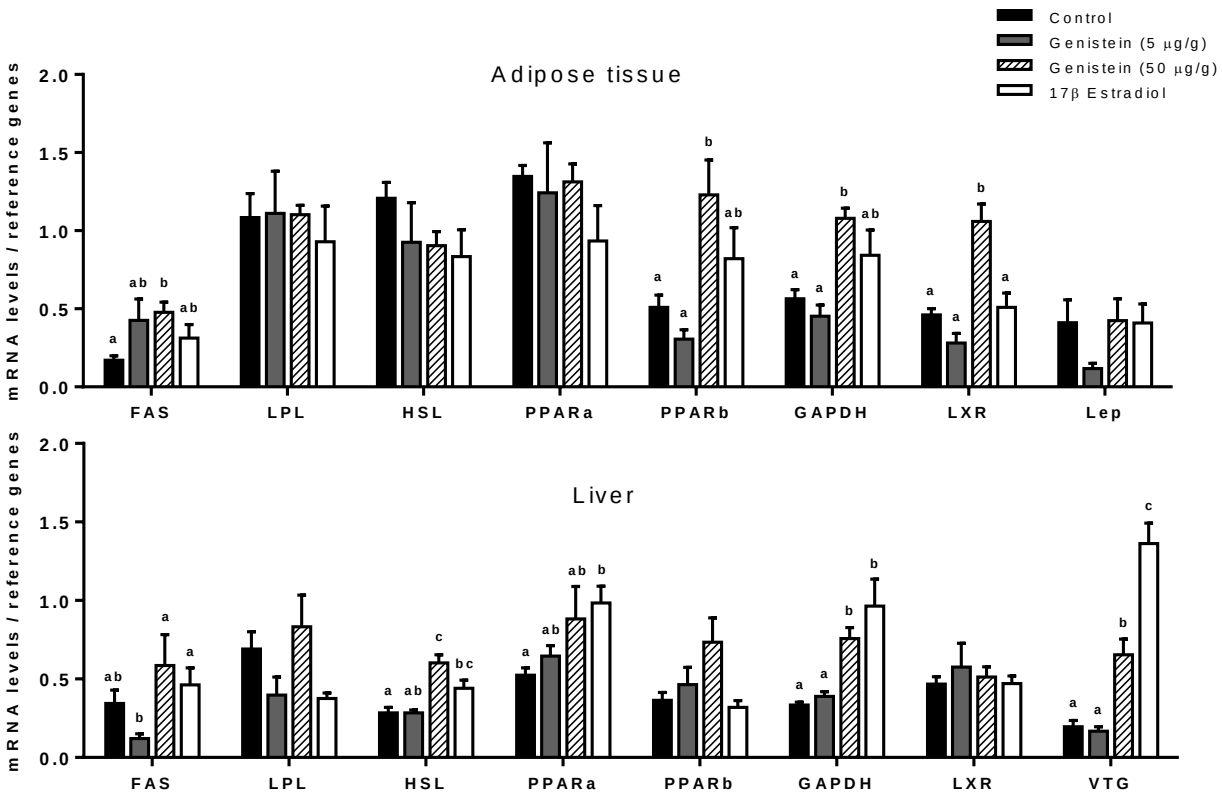
In adipose tissue, E2 induced a tendency to increase FAS, PPARβ, GAPDH and LXR expression, but it isn't a significant effect;

In liver, E2 slightly increased FAS and HSL expression, but not significantly; while, E2 increased PPARα and GAPDH expression strongly.

Regarding VTG in liver, both E2 and the high dose of genistein (50µg/g) increased its expression significantly.

Regarding estrogen receptors ER2α and ER2β, the high dose of genistein (50µg/g) decreased ER2α both in adipose tissue and liver, while in liver it decreased ER2β.

E2, instead, decreased only ER2β in liver.



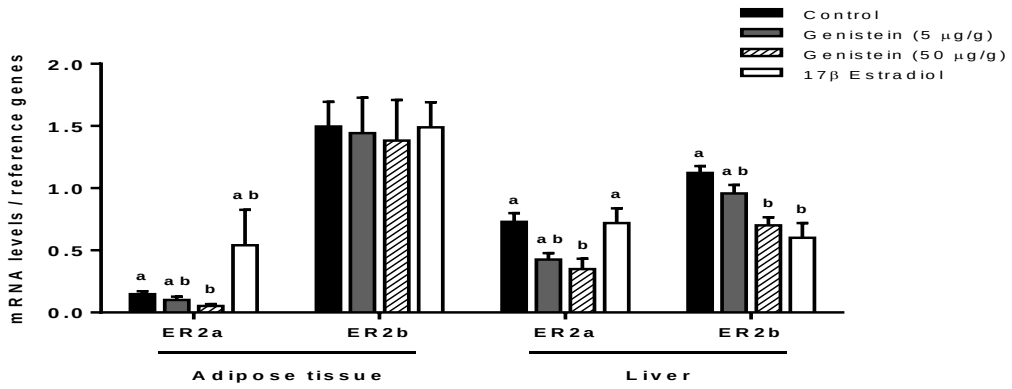


Fig.22 Effects of E2 and genistein (5µg/g and 50µg/g) on expression of estrogen receptors (ERs), vitellogenin (VTG) and lipid metabolism related genes of *Oncorhynchus mykiss* both adipose tissue and liver. Gene expression data are presented as relative units using Ubiquitin, EF1α and β-actin as housekeeping genes. Data are shown as means ± S.E.M. Treatments bars are relative to the control. Different letters indicate significant differences, P< 0,05. FAS, fatty acid synthase; LPL, lipoprotein lipase; HSL, hormone sensitive lipase; PPARs, peroxisome proliferator-activated receptor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; LXR, liver X receptor; LEP, leptin; VTG, vitellogenin.

Expression of apoptosis-related genes and autophagy-related genes after genistein or 17β-estradiol administration, in adipose tissue and liver, are presented in fig. 23. The autophagy-related genes were: CASP3, CASP8, and P53; while, autophagy-related genes were: LC3b, ATG4b, ATG12l.

In adipose tissue, genistein at 50µg/g decreased CASP3, and increased LC3b and ATG4b.

E2, instead, induced a tendency to decrease CASP3, and to increase ATG4b, but these effects weren't significant statistically.

In liver, genistein did not have a significant effect on CASP3 and CASP8, only the lower dose of genistein (5µg/g) decreased CASP8, significantly. On the other hand, the high dose of genistein (50µg/g) increased autophagy-related gene ATG4b, significantly, and it had a tendency to increases ATG12l, but not significantly.

E2, in liver, increased apoptosis-related genes CASP3 and CASP8, appreciably; regarding autophagy-related genes, E2 decreased LC3b, and increased ATG12l, a tendency to increased ATG4b was also observed, but the effect was not significant.

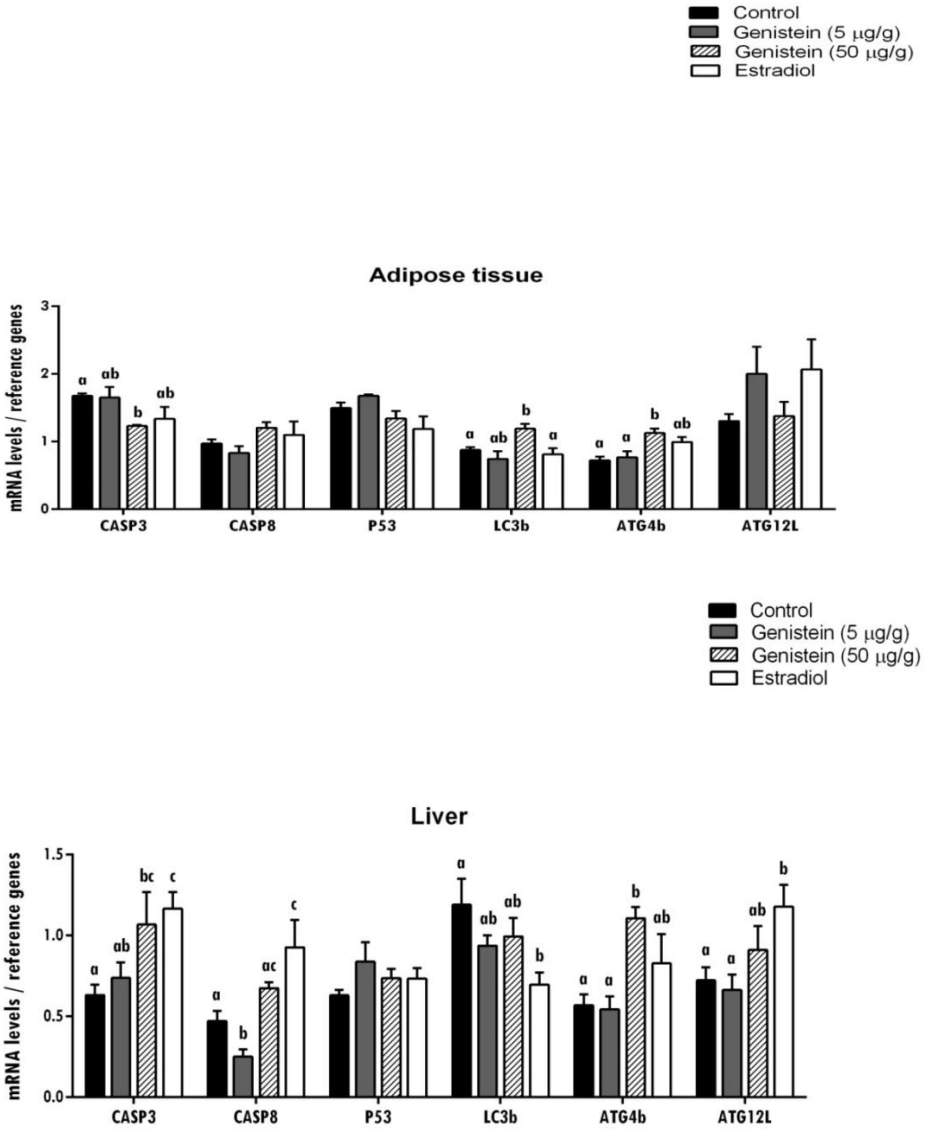


Fig.23 Effects of E2 and genistein (5µg/g and 50µg/g) on apoptosis-related genes and autophagy-related genes of *Oncorhynchus mykiss* both adipose tissue and liver. Gene expression data are presented as relative units using Ubiquitin, EF1α and β-actin as housekeeping genes. Data are shown as means ± S.E.M. Treatments bars are relative to the control. Different letters indicate significant differences, P< 0,05. CASP3, Caspase 3; CASP8, Caspase 8; P53, Tumor protein; LC3b, light chain 3b protein; ATG4b, autophagy related 4, cysteine peptidase; ATG12l, autophagy-related 12.

4.*2* ***In vivo*** **results: Gene expression analysis in white muscle**

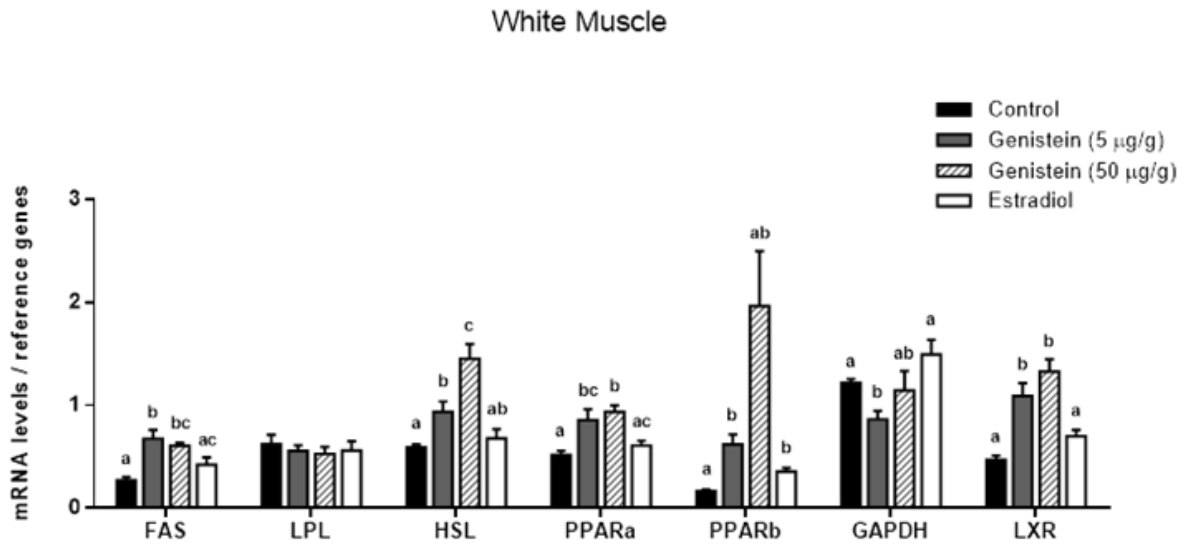
Expression of estrogen receptors, and lipid metabolism-related genes after injection of genistein or 17-β estradiol in white muscle are showed in fig. 24.

The lower dose of genistein (5µg/g) increased FAS, HSL, PPARα, PPARβ and LXR significantly. A significant down-regulation of GAPDH by genistein at 5µg/g was also seen. Genistein at 50µg/g raised FAS, HSL, PPARα, and LXR, with a tendency to increase PPARβ, even if not significantly. GAPDH, instead, was not modified.

E2 increased only PPARβ mRNA levels appreciably, with a tendency to rise the other genes (such as FAS, GAPDH and LXR) but not significantly.

Regarding estrogen receptors ER2α and ER2β, the lower dose of genistein (5µg/g) increased both of them significantly; while the high dose of genistein (50µg/g) increased ER2β strongly, and tended to up-regulate ER2α mRNA levels, but this effect was not significant.

E2 had a tendency to increase ER2β and ER2α, but not significantly.



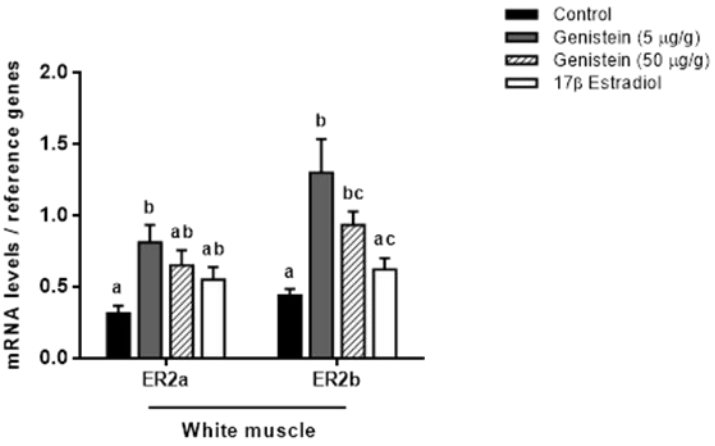


Fig.24 Effects of E2 and genistein (5µg/g and 50µg/g) on expression of lipid metabolism-related genes of *Oncorhynchus mykiss,* and estrogen receptors (ERs) in white muscle. Gene expression data are presented as relative units using Ubiquitin and EF1α as housekeeping genes. Data are shown as means ± S.E.M. Treatments bars are relative to the control. Different letters indicate significant differences, P< 0,05. FAS, fatty acid synthase; LPL, lipoprotein lipase; HSL, hormone sensitive lipase; PPARs, peroxisome proliferator-activated receptor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; LXR, liver X receptor.

Expression of apoptosis-related genes and autophagy-related genes after genistein or E2 administration in trout white muscle are shown in fig. 25.

The lower dose of genistein rised ATG12l mRNA levels in a significant way, but not ATG4b. Regarding apoptosis-related genes, genistein at 5µg/g decreased only CASP3 significantly, while this dose of genistein had a tendency to down-regulate P53 and to up-regulate CASP8.

The high dose of genistein (50µg/g) increased autophagy-related genes ATG4b and ATG12l significantly; the same dose of genistein decreased CASP3 in a significant manner; it tended to decrease P53, although not significantly, and to increase CASP8, even if also this effect was not significant.

E2, instead, increased only CASP8 in a significant way, while a tendency to down-regulate CASP3 was observed.

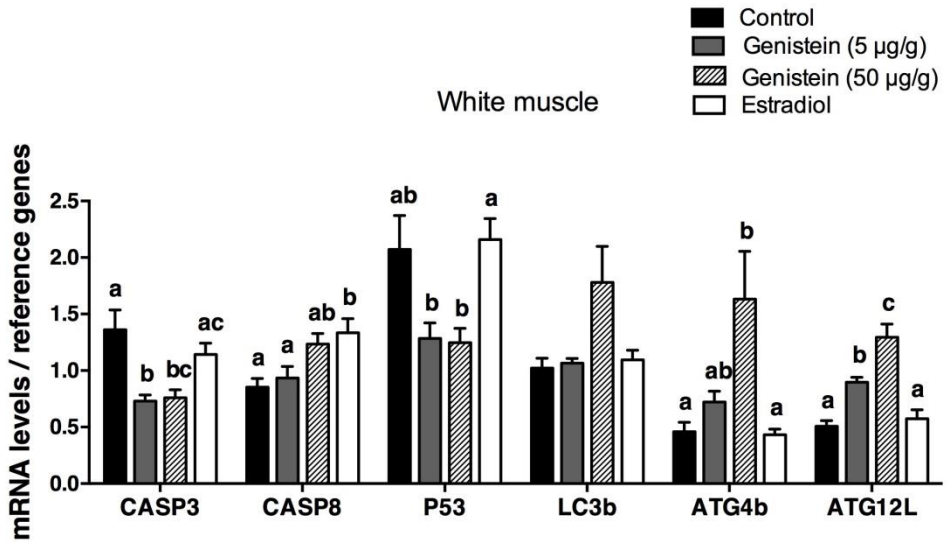


Fig. 25 Effects of E2 and genistein (5µg/g and 50µg/g) on apoptosis-related genes and autophagy-related genes of *Oncorhynchus mykiss* in white muscle. Gene expression data are presented as relative units using Ubiquitin and EF1α as housekeeping genes. Data are shown as means ± S.E.M. Treatments bars are relative to the control. Different letters indicate significant differences, P< 0,05. CASP3, Caspase 3; CASP8, Caspase 8; P53, Tumor protein; LC3b, light chain 3b protein; ATG4b, autophagy related 4, cysteine peptidase; ATG12l, autophagy-related 12.

4.3 In vitro results: MTT assay

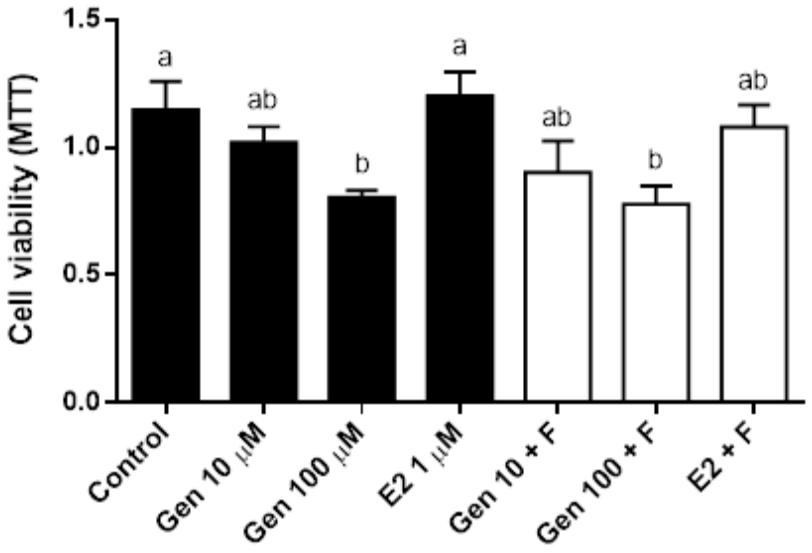


Fig.25 Cell viability profile of rainbow trout adipocytes under different treatments measured using the MTT assay. Data are means ± SEM of 4 independent experiments. Cell viability is relative to the control value. P value <0.05.

To investigate cell proliferation of the adipocytes in culture, MTT assay was performed. The results obtained with the MTT assay performed after 24h of E2 and genistein stimulation at two different doses, are shown in fig.23. After 24h post-stimulation, genistein showed a clear dose-response curve, with significant decrease in cell proliferation at the concentration of 100µM with respect to the control and between two concentrations (Fig.25). Moreover, it was observed that genistein at 100µM+ Fulvestrant (an inhibitor of ERs) had the same effect of genistein at 100µM, namely decreased cell viability in the same proportion, indicating that an high dose of genistein had a significant effect on cell viability and the proliferation cells, but it didn't, either alone or in combination with fulvestrant, exert this effect via estrogen receptor-dependent mechanism. Regarding E2, it had not significant effect on cell viability and the proliferation cells.

4.4 In vitro results: Oil red oil (ORO) assay

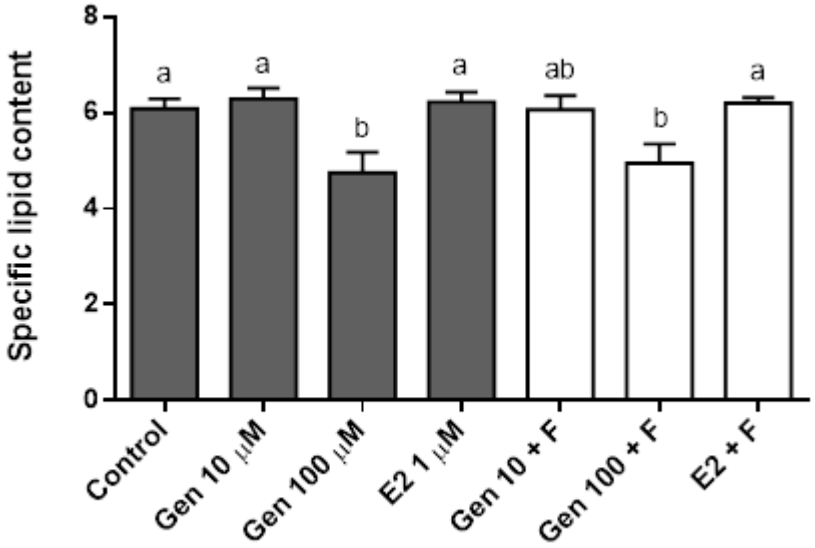


Fig.26 Lipid content profile of rainbow trout adipocytes under different treatments measured using the ORO assay. Data are means ± SEM of 4 independent experiments. Lipid content is relative to the control value. P value <0.05.

To investigate the differentiation of rainbow trout adipocytes in culture and to quantify lipid content of the cells, ORO assay was performed. The results obtained with the ORO assay performed after 72h of E2 and genistein stimulation at two different doses, are shown in fig.26. After 72h post-stimulation, the high dose of genistein (100µM) showed a clear anti-differentiation effect,where genistein at 10µM hadn't significant effect on cell differentiation, as well as E2. Interestingly, genistein at 100µM+Fulvestrant, (an inhibitor of ERs), had the same effect of genistein at 100µM, namely it decreased specific lipid content of adipocytes significantly. Also in this case, as for cell viability and cell proliferation, data suggest that genistein exerts its effect via estrogen-receptor independent mechanism.

5. Discussion

5.1 Effects in adipose tissue and liver

In vivo results from this study showed that genistein had an unclear effect on adipose tissue of *Oncorhynchus mykiss*, increasing FAS, PPARβ, GAPDH and LXR expression. In fact, while FAS and GAPDH are two lipogenic genes, and this would lead us to consider genistein as adipogenic compound, PPARb and LXR have a controversial role in fish and in mammalian adipose tissue. 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 in all vertebrats (Smith et al., 2003); GAPDH is a lipogenic enzyme involved in cell differentiation in mammals and in fish (Bouraoui, et al., 2008). Regarding PPARβ, little is known about it in the context of target tissues, target genes, lipid homeostasis, and functional overlap with PPARα, and PPARɤ (Dressel et al., 2003). In mammalian adipose tissue, this gene is not directly implicated in the control of adipogenesis, because alone is not able to promote lipogenesis (but only together with PPARɤ), however, it plays a role in the adaptive response of adipose tissue to dietary fatty acid content (Neels and Grimaldi, 2014). It has been reported that PPARβ expression in adipose tissue changes with nutritional situation in trout (Cruz-Garcia et al., 2015), and it seems to mediate lipolytic effects in gilthead sea bream (Cruz-Garcia et al., 2009). Regarding LXR, some studies showed that it induces lipogenic genes, adipogenesis and lipid accumulation in adipose tissue (Juvet et al., 2003; Seo et al., 2004), others demonstrated opposite results (Ross et al., 2002; Stulnig et al., 2002; Hummasti et al., 2004; Sekiya et al., 2007; Stenson et al., 2011). Nevertheless, recent studies in humans and mice demonstrate that LXR seems to be more involved in lipid mobilization than lipogenesis in adipose tissue (Korach-Andrè et al., 2011; Stenson et al., 2011). No information is available on the role of LXR in teleost adipose tissue, only in *Oncorhynchus mykiss*, a

group of researchers (Cruz-Garcia et al., 2012) studied its possible roles, showing that it seems to regulate cholesterol transport through ABCA1 (ATP-binding cassette transporter A1), and it is not related to synthetic pathways in trout adipose tissue, but it could be considered an indicator of increased lipolysis in this tissue, a phenomen described already in *Sparus aurata* fed vegetable ingredients (Cruz-Garcia et al., 2011; Albalat et al., 2005a).

Unfortunately, we have not studies on genistein's effects on lipid metabolism in fish in order to do a comparisons, only one done by Cleveland and Manor (2015) on effects of genistein in rainbow trout, however the effects in adipose tissue were not studied. Rather, several studies on mammals have been reported, showing anti-obesogenic effects of genistein in adipose tissue, through up-regulation of β-oxidation-related genes (as PPARα), and down-regulation of LXRs (Mi-Hyun Kim et al., 2010). Furthermore, genistein stimulated a dose-dependent increase in lipolysis, and restricted fatty acid synthesis and/or their esterification in rat adipocytes (Kandulska, et al., 1999; Szkudelska, et al., 2002), and decreased adipose weight and adipocyte circumference at higher doses, and lipoprotein lipase (LPL) mRNA in mice (Naaz, et al., 2003).

In fish, we have only studies that showed the effects of dietary vegetable oil (as soybean oil) on adipose tissue and also in hepatic lipid metabolism–related genes, lipid deposition, growth or tissue fatty acids composition. Therefore, there are previous studies on gilthead seabram (*Sparus aurata*) showed that plant components increase basal lipolysis levels in adipocytes (Albalat et al., 2005a) as well as it was observed in the measurements of the basal lipolysis levels in isolated adipocytes from animals fed vegetable oils (66%VO). This is in agreement with an enhanced HSL activity, and up-regulation of LXR expression in adipocytes by 66%VO. Furthermore, liver weight values were higher in fish fed 66%V0, suggesting that the fatty acids released from adipose tissue could produce lipid accumulation in the liver, as previously suggested

(Benedito-Palos et al., 2008), and as it has been described in humans (Browning and Horton, 2004) (Cruz-Garcia, et al., 2011). Also Caballero et al. (2004) found that a diet with 60% soybean oil as replacement induced fat liver accumulation and steatosis in sea bream. The authors concluded that the reduction of dietary essential fatty acids due to the inclusion of vegetable oil, and the type of non-essential fatty acid included in the diet, affects hepatic morphology and lipid content (Bouraoui, et al., 2011). Similar effects were seen by Li et al., (2015) in blunt snout bream (*Megalobrama amblycephala)*, where an increasing SO (soybean oil) levels in the diet (from 20% to 100%) down-regulated PPARα and PPARβ expression, explaining the increase in lipid accumulation in the liver (Li, et al., 2015). Nevertheless, many other factors beside genistein, can affect lipid metabolism in these vegetable diets studies.

In our study, genistein had a lipolytic effect in liver of rainbow trout, underlined by an increase of HSL expression, and by tendency to increase PPARα. Previously, the same lipolytic effect of genistein was seen in other studies, in which genistein decreased lipogenesis in rat liver, through a reduction in fatty acid synthesis and an increase in β-oxidation (Takahashi, et al., 2009). Genistein decreased fat liver accumulation, reducing levels of hepatic TG and cholesterol, increasing PGC-1 (PPARɤ co-activator involved in mitocondrial fatty acids oxidation) mRNA, mitochondrial medium chain acyl-CoA dehydrogenase (MCAD) (PPARα target gene, involved into fatty acid β-oxidation) mRNA, and uncoupling protein (UCP-2) (a mitochondrial inner-membrane protein that mediates proton leakage by uncoupling adenosine triphosphate synthesis) mRNA levels, up-regulation of which may compromise cellular adenosine triphosphate levels and decrease metabolic efficiency and, thus, decrease fat accumulation (Lee, et al., 2006). Through plasma analysis of *Oncorhynchus mykiss*, with a significant decrease of TAG levels or increase of NEFA levels, a similar lipolytic effect of genistein was seen (Lutfi, et al., 2015 unpublished).

Also a previous study regarding the effects of genistein on growth-related and lipogenic-genes in rainbow trout (Cleveland and Manor, 2015) showed that E2 and genistein promote changes in gene expression that support a regulation of lipid metabolism in liver.

Moreover, the high dose of genistein, along with E2, can have estrogenic effects in liver, evidenced as up-regulation of vitellogenin (VTG). Therefore, up-regulation of this gene in fish exposed to genistein, confirm the ability of this phytoestrogen to produce estrogenic effects *in vivo* (Pelissero et al., 1991; Inudo et al., 2004; Kaush et al., 2008; Schiller et al., 2014). Like in previous studies, in which a similarity between E2 and genistein responses in liver suggested that effects of genistein are partially mediated through activation of estrogen receptors (ERs) (Cleveland, et al., 2015), also in this study, it seemed to be a certain parallelism between the effects of estradiol and those corresponding to the high dose of genistein on ER2β (down-regulation) and VTG (up-regulation) expression in liver. Moreover, as in other studies (Cleveland, et al., 2015), genes expression responses to genistein increased with injected amount, indicating that the estrogenic response in vivo can be dose-dependent, and therefore could vary depending on the amount of phytoestrogens in the diet.

Studies on mammals indicate that genistein may affect lipid metabolism through additional mechanisms, not only through activation of ERs; however for some effect it was not discovered a mechanism of action yet. For example, genistein can have an anti-obesogenic role, decreasing food intake and increasing body weight loss (Kim et al., 2006), or decreasing leptin secretion (Brennan and Mantzoros, 2006) or inhibiting leptin synthesis (Phrakonkham, et al., 2008). *In vitro* genistein can also induce on 3T3-L1 mouse adipocyte apoptosis, which may be contributor to genistein's reducing effect on body weight (Kim, et al., 2006), but the pathway is unknown at the moment. For the other anti-obesogenic effects of genistein, instead, the mechanism of action has been

clarified. In fact, this phytoestrogens is capable of inhibiting lipid accumulation in primary human pre-adipocytes, via down-regulation of PPARɤ, C/EBPα, and glycerol-3-phosphate dehydrogenase (Park et al., 2009), and inhibiting the antilipolytic action of insulin, through an activation of protein kinase A and elevation of cAMP levels (Szkudelska et al., 2008). In *Oncorhynchus mykiss*, we observed as the *in vivo* effects of genistein are partially estrogen receptor-dependent in liver; then, genistein may act through the autophagic way, as shown by an increase of autophagy-related genes LC3b in adipose tissue, and ATG4b both in liver and adipose tissue, while these effects were not observed after estrogen administration. This autophagic pathway could be considered as possible way through which genistein exerts its lipolytic role in liver. Finally, apoptotic pathway was analyzed in order to see if a potential anti-obesogenic effect of genistein happened through an activation of apoptosis in adipose tissue; differently from it was expected and observed in mammals, genistein not seemed to affect apoptosis-related genes significantly, while it not seemed to have an apoptotic effect *in vivo*, rather it was seen a decrease of CASP3 caused by genistein at lower concentration (5µg/g) in adipose tissue; this, in agreement with that reported by Alan et al., (1999) according to them CASP3 is essential for certain processes associated with the dismantling of the cell and the formation of apoptotic bodies, in particular it is is required for some typical hallmarks of apoptosis, and is indispensable for apoptotic chromatin condensation and DNA fragmentation in all cell types examined (Alan et al., 1999).

Regarding autophagy pathway, what we observed in our work is in agreement with the recently reported by Kuma et al., (2010), namely autophagy involvement in lipid metabolism of liver and adipose tissue. Inhibition of autophagy in hepatocytes *in vitro* and *in vivo* leads to an increase in triglyceride storage in lipid droplets (Komatsu et al., 2005; Singh et al., 2009). Moreover, it

was proposed that lipid droplets are sequestered and degraded through autophagy during starvation (Singh et al., 2009), which would lead us to believe that lipolytic role of genistein in liver detected in our study could be positive avoiding excessive lipid accumulation in liver. These findings indicate a new role for autophagy in regulating intracellular lipid stores in the liver. Autophagy also has an impact on adipogenesis. Autophagy is induced during *in vitro* adipogenesis (Baerga et al., 2009), and inhibition of autophagy impairs adipogenesis in cultured mouse embryonic fibroblasts and mouse adipose tissues (Baerga et al., 2009; Zhang et al., 2009; Singh et al., 2009) (Kuma et al., 2010). This could induce us to think that genistein, increasing autophagy-related genes LC3b and ATG4b in adipose tissue, could promote "adipogenesis", even if it is just an idea that need more studies to be clarified.

Regarding *in vitro* studies, phytochemicals, as genistein, are considered in mammals potential agents to inhibit proliferation and differentiation of pre-adipocytes, stimulate lipolysis, and induce apoptosis of existing adipocytes *in vitro*, and reduce body weight and adipose tissues mass in animal models (Andersen et al., 2010). However, we don't know how genistein affects *in vitro* preadipocyte proliferation and differentation in fish, in order to compare the present results, only studies in mammals have been reported. For example, one of these studies done on mammals showed that genistein inhibits the proliferation and differentiation of MCF-7 and 3T3-L1 cells via the regulation of ERα expression and induction of apoptosis in a concentration- dependent manner (Choi et al., 2014); the same results were obtained also in other works, with a difference: genistein may exert these effects through different mechanisms of action, as via Wingless and Int/β catenin (Wnt/β-catenin), such as Extracellular signal-regulated kinase/c-Jun-N-terminal kinase (ERk/JNK) signaling, in an estrogen-receptor independent manner (Behloul et al., 2013). In our work, to determine if the observed effects of genistein on rainbow trout

adipose tissue were connected with its estrogenic activity, adipocytes were also incubated with estradiol and fulvestrant, an inhibitor of ERs. It was observed that, in contrast to genistein, estradiol did not affect preadipocyte cell proliferation and differentiation, and genistein, also in association with fulvestrant, decreased pre-adypocites cell proliferation and their specific lipid content. So, as in mammals, our results *in vitro* from MTT assay and ORO assay not only showed high dose of genistein (50µg/g) decreased in a dose-dependent manner adipose tissue adipogenesis of rainbow trout, but also indicated that the observed effects of genistein were not mediated by estrogen receptor. These *in vitro* data are inconsistent with that obtained *in vivo*, because we didn't have clear results regarding effects of genistein on rainbow trout lipid metabolism in adipose tissue.

5.2 Effects in white muscle

Results from this study showed that genistein affected lipid metabolism-related genes in white muscle of rainbow trout, up-regulating FAS, HSL, PPARα and PPARβ significantly. As previously cited, FAS is a lipogenic gene involved into fatty acids synthesis, while HSL is a lipolytic gene and PPARα and PPARβ arerelated to catabolisms of fatty acids. HSL is the principal enzyme that catalyzes the hydrolysis of triglycerides (Weil et al., 2012), PPARα and PPARβ are transcriptional factors that have been shown to stimulate fatty acid β-oxidation in muscle, liver (PPARα) and adipose tissue (PPARβ) in fish (Cruz-Garcia et al., 2011). This effect of genistein could be seen as a contribute to promote a lipid turnover in white muscle, allowing muscle to respond to the treatments with genistein with an increase of lipogenesis and lipolysis, in agreement with the high capacity of salmonid muscle to accumulate and to oxidize lipids (Cruz-Garcia et al., 2011). Regarding LXR, in our study, it was

observed that both doses of genistein rised it in the same way. However, it's quite difficult to explaine this effect, because few studies have addressed the functional role of LXR in fish (Archer et al., 2008; Cruz-Garcia et al., 2009a; Cruz-Garcia et al., 2009b; Cruz-Garcia et al., 2011), whereas in mammals, it is known that LXRs are involved in the regulation of cholesterol homeostasis (Zhang and Mangelsdorf, 2002; Tontonoz and Mangelsdorf, 2003; Steffensen and Gustafsson, 2004), fatty acid synthesis (Repa et al., 2000; Schultz et al., 2000), carbohydrate metabolism (Laffitte et al., 2003; Mitro et al., 2007) and anti-inflammatory effects (Fowler et al., 2003; Valledor, 2005), as they activate the transcription of genes involved in these processes. Mammalian muscle is a key regulator of lipid, particularly cholesterol, metabolism through the action of LXR (Muscat et al., 2002). In fish, one study was done by a group of researchers (Cruz-Garcia et al., 2011) in muscle trout, *in vitro* showing that LXR is hormonally regulated by insulin and GH, and in turn, it regulates the transcription of key genes (such as FAS, PPARα, LPL, PPARβ, ABCA1) involved in muscle lipid metabolism in trout. In particular, LXR may play a lipogenic role through insulin stimulation and a tendency to promote anabolic effects through GH on trout myocites.

There is scarce information on the regulation of lipid metabolism in teleost muscle, although muscle accounts for a high proportion of the total weight of the animal and the final product in aquaculture. For this reason, this study was done also on lipid metabolism in muscle trout. Moreover, we have not other fish studies done on the effects of genistein in muscle lipid metabolism in order to do a comparisons, only some works in other animals. It was seen that soy isoflavones (which include a mixture of daidzein, genistein and glycitein) lead to improved growth performance, antioxidant capacity, carcass traits and meat quality in several studies (Cook et al., 1998; Payne et al., 2001). Furthermore, isoflavones may have efficacy as a feed supplement to decrease fat deposition in

animals because of its estrogen-like function (Payne et al., 2001). Cook (1998) evaluating the *in vivo* effects of dietary soybean isoflavones on carcass muscle content and body growth rate in pigs, observed that the isoflavones (670 mg genistein, 705 mg daidzein and 210 mg glycitein/kg diet) increased growth rate and carcass muscling, but carcass fat was not affected in pigs from 6 to 32 kg of body weight. Similar results for rats are reported by Cook (1998). Payne et al. (2001) reported that addition of isoflavones to a corn-soy protein concentrate diet (C-SPC), increased carcass leanness and decreased carcass fat and results in carcass traits similar to, or better than, those of barrows fed corn-soybean meal (C-SBM) (Payne et al., 2001).

However, a few of studies on the effects of individual flavonoid compounds (as genistein) were performed. A study done by Kamboh et al. (2013) on the effects of genistein on growth, meat characteristics, oxidative stability of lipids and sensory quality of raw meat in chickens showed that genistein reduced lipid oxidation in muscle, without affecting the sensory quality of breast meat, and in general improved meat quality in a dose-dependent manner. In an other study Kamboh et al., (2013) showed that genistein decreased cholesterol and tryglicerides levels in breast muscle with increasing levels of this phytoestrogen, and also fatty acids composition of chicken meat was improved in a dose-dependent way.

Flesh quality of fish is strongly influenced by diet. Carotenoids, lipid sources, antioxidants, and other dietary components can affect color (Nickell et al., 1998; Coral et al., 1998), fatty acid profile (Waagbo et al., 1993; Skonberg et al., 1994), texture (Rora et al., 2003; Faergemand et al., 1995), and flavor (Johnsen et al., 1991; Skonberg et al., 1993) of farm-raised fish. Similarly, the absorption of feed components such as isoflavones in the fish muscle may offer additional benefits to consumers (D'Souza et al., 2005). Although few of studies was done on the effects of genistein in fish muscle, first

step toward explaining the effects of individual soy components (as genistein) on some of the important quality attributes of fish it was a study done by D'Souza et al., (2005) on rainbow trout white muscle, in which the authors showed that dietary genistein resulted in genistein deposition in fish flesh without adversely affecting the flavor, color, or proximate composition of the fillets; however, the genistein levels found in trout fillets were much lower than that found in commercial soy foods, for example, tofu and tempeh, which contain approximately 20.6 and 24.8 mg of genistein/100 g of sample (U.S. Department of Agriculture, 2002). It is for this reason that it is difficult to draw any conclusions about the potential human health benefits from these low amounts of genistein. More studies are needed to understand the effects of dietary genistein on the oxidative stability of fish fillets. Another study in trout, instead, showed that the high doses of genistein affects muscle protein turnover by increasing rates of protein degradation and proteolysis-related gene expression, via estrogen receptor-dependent and –independent mechanisms, so to have negative effects on skeletal growth (Cleveland et al., 2014).

More studies on the effects of partial or total replacement of fish meal/oil by soybean or, in general, by vegetable meal/oils on growth, muscle fatty acid composition and organoleptic quality of flesh were performed. It was seen that inclusion of vegetable oils in fish diets modifies body fatty acid profiles and may significantly affect fish flesh quality and sensory characteristics (Guillou et al., 1995; Morris et al., 1995). For example, in one of these studies lipid concentrations in the muscle of cobia fed soy-based diets were found to significantly increase with an increase in soybean meal, while muscle ash and protein varied without a definite trend (Chou et al., 2004); in an other work, Izquierdo et al. (2005) observed that it is possible to substitute up to 60% fish oil by vegetable oils in diets for gilthead seabream without affecting growth and feed utilization even for a long feeding period.

In the present study, genistein's possible-mechanisms of action were also studied, in particular the autophagy and apoptotic pathways. Regarding autophagy, in mammals several changes occur during catabolic conditions: proteins are mobilized to sustain gluconeogenesis in the liver and to provide alternative energy substrates for organs, mitochondrial and sarcoplasmic networks are remodeled and myonuclei are lost. In addition, the daily contractions can mechanically and metabolically damage/alter muscle proteins and organelles. Muscle cells therefore require an efficient system for removing and eliminating unfolded and toxic proteins as well as abnormal and dysfunctional organelles. The autophagy system is responsible for this action, generating double membrane vesicles that engulf portion of cytoplasm, organelles, glycogen and protein aggregates (Levine and Kroemer, 2008; Mizushima et al., 2008). Autophagosomes are then delivered to lysosomes for degradation of their contents. Despite this important function, the role of autophagy in the control of muscle mass has only recently begun to be investigated (Sandri, 2010). It was observed that an inhibition/alteration of autophagy can contribute to myofiber degeneration and weakness in muscle disorders characterized by accumulation of abnormal mitochondria and inclusions (Masiero et al., 2009). As in mammals, also in fish the autophagic proteolytic system represents a largely non-selective route of protein degradation which serves to recycle amino acids during periods of amino acid or energy deficiency (Kuma and Mizushima, 2010). This pathway has been implicated as a major contributor to increased muscle proteolysis during sexual maturation in salmon and rainbow trout (Yamashita and Konagaya, 1990; Cleveland et al., 2012).

In mammals different signaling pathways may control autophagosome formation during short (hours) or long (days) periods of induced autophagy. Denervation is able to induce autophagy in skeletal muscles, although at a

slower rate than after fasting (O'Leary and Hood, 2008; O'Leary and Hood, 2009). This effect is mediated by Runx1 (an autophagy suppressor), which is upregulated in denervated muscles and is required to preserve muscle mass (Wang et al., 2005). The mechanisms of Runx1-mediated autophagy suppression is unclear but recent evidence shows that Runx1 can inhibit FoxO3 action (Wildey and Howe, 2009). Another negative regulator of autophagy in muscle cells is the phosphatase Jumpy. Reduction of Jumpy protein by RNAi results in the formation of autophagosomes in C2C12 myoblasts and an increased rate of proteolysis observed both in normal and starvation media (Vergne et al., 2009). However, the regulation of Jumpy in normal and atrophic muscles is still unknown. The most potent autophagy inhibitor in skeletal muscles is the kinase Akt. Acute activation of Akt in adult mice or in muscle cell cultures completely inhibits autophagosome formation and lysosomal-dependent protein degradation during fasting (Mammucari et al., 2007; Zhao et al., 2007; Zhao et al., 2008). Mammalian TOR (mTOR) is a nutrient-sensitive kinase downstream of Akt that is important for cell growth. However, the role of mTOR in autophagy regulation is not so important and therefore, mTOR, at least in skeletal muscles, does not mediate the negative effect of Akt on the autophagy pathway. The upregulation of several autophagy-related genes in atrophying muscles (Lecker et al., 2004; Mammucari et al., 2007; Zhao et al., 2007; O'Leary and Hood, 2008; O'Leary and Hood, 2009) suggests the contribution of one or more transcription factors to autophagy regulation. Recently, it was identified FoxO3 as the critical factor for autophagy control in adult muscles. Expression of FoxO3 is sufficient and required to activate lysosomal-dependent protein breakdown in cell culture and *in vivo*. Moreover several autophagy genes including LC3, Gabarap, Bnip3, VPS34, ATG12 are under FoxO3 regulation. Recently, the p38 αβ MAPK pathway was also described to regulate expression of autophagy-related genes independently of FoxO3 during oxidative stress (McClung et al., 2009). Differently from

mammals, the mechanisms responsible for the regulation of autophagy have not been investigated in teleosts, known to exhibit different muscle growth dynamics. One study was done by Seiliez et al., (2010), in which the authors investigated both *in vivo* and *in vitro* the transcriptional regulation of several major genes involved in autophagy (LC3b, gabarapl1, ATG12l, ATG4b) in the white skeletal muscle of rainbow trout.

In mammals, FoxO transcritional factors are also known as a downstream target of the IGF1/insulin-PI3K-Akt signalling pathway; when the IGF1/insulin-PI3K-Akt axis is active, FoxO proteins are phosphorylated and sequestered in the cytosol, but in catabolic states, the unphosphorylated transcription factors enter the nucleus and induce different proteasome- and autophagy-related genes (Sandri et al., 2004; Mammucari et al., 2007; Zhao et al., 2007). To examine the involvement of the Akt–FoxO signalling pathway in the regulation of the autophagy-related gene expression in trout, Seliez et al. (2010) investigated the specific effect of IGF1 on the Akt–FoxO signalling pathway and the expression of autophagy-related genes in cell culture model. IGF1 induced the phosphorilation of Akt and FoxO3 in primary culture of trout muscle cells, but had a weak or no effect on the expression of autophagy-related genes, suggesting a moderate role for FoxO3 in the regulation of the expression of the autophagy-related genes in trout myocytes. For the first time they showed the existence and the regulation of genes involved into autophagy in fish, and the overexpression of the autophagy-related genes during starvation to indicate that in rainbow trout muscle, these genes are regulated by the feeding status similarly to what is observed in mammals (Seiliez et al., 2010).

In a study of Cleveland (2014) on rainbow trout skeletal muscle, it was seen that high concentrations of genistein, increased autophagy-related genes, among which ATG4b, to support the idea that dietary genistein potentially could be considered as a regulator of autophagy, implicated into protein degradation

process. Also in our study, the high dose of genistein (50µg/g) increased ATG4b and ATG12l. So, we can suppose that, in a similar way than in trout liver, genistein may act through the autophagic pathway in order to exert its lipolytic role into rainbow trout white muscle.

In our study, also a lipogenic role of genistein into trout white muscle was observed, as shown by a significant increase of FAS enzyme involved into fatty acid synthase, and LXR. In mammals, lipotoxicity (excess lipid accumulation resulting from an elevated supply of plasma fatty acids) leads to cellular dysfunction and death in non-adipose tissues including the heart, pancreas and liver. While lipoapoptosis has been shown in cultured skeletal muscle cells (by several specific measures, including caspase-3 activation) (Turpin et al., 2006), *in vivo* instead, it was observed as a lipid overload did not induce skeletal muscle apoptosis (Turpin et al., 2009). In our *in vivo* study, both doses of genistein decreased apoptosis-related gene CASP3 significantly, so we can suppose that genistein, despite its possible obesogenic role, is capable of protect trout muscle cells against an eventual lipid overload-induced cell apoptosis. Or simply, a possible excessive lipid accumulation in trout muscle caused by genistein and following lipid overload-induced cellular apoptosis, can be counteracted by skeletal muscle itself, considering that, as in mammals as in fish, unlike the pancreas and liver, this tissue has a comparatively large capacity to oxidize FAs that may afford the tissue protection from lipoapoptosis. At the moment, these are only speculations that need additional studies to be investigated.

Regarding the supposed anti-obesogenic role of genistein, it is not possible to assert that, because of results obtained, genistein exerts it through apoptotic pathway in trout white muscle.

Differently from the previous studies and unlike we obtained in adipose tissue and liver, the effects of genistein in trout white muscle were not dose-dependent, except in the case of autophagy-related genes ATG4b and ATG12l. Regarding the estrogenic activity of phytoestrogen genistein, even in rainbow trout muscle, it was seen a certain parallelism between genistein and E2; in particular, it was observed a similarity between the effects of the lower dose of genistein and those of E2 on PPARβ (up-regulation), and between the effects of the high dose of genistein and those of E2 on ER2α (mild up-regulation), suggesting that genistein role could be partly mediated by estrogen receptors, as in trout liver. Likewise in a previous study (Cleveland, 2014) it was observed that the high dose of genistein can exert its effects on protein turnover in trout muscle also via estrogen-receptors.

6. Conclusions

From the current study it resulted that genistein can have *in vitro* anti-obesogenic effects on rainbow trout pre-adipocytes via estrogen-receptor independent mechanism. *In vivo* unclear results about role of genistein in *Oncorhynchus mykiss* adipose tissue were obtained.

Regarding liver, genistein had a clear lipolytic effect *in vivo*, with a significant increase of HSL expression, mediated also by a possible autophagic way, enhancing a process that, along with lipolysis, contributes to lipid droplets and triglycerides breackdown. In liver, genistein seems to have a partial estrogenic role.

The *in vivo and in vitro* anti-adipogenic effect of high dose of genistein (50µg/g) in liver and adipose tissue respectively, showed that genistein exerts its role in a dose-dependent way. Globally, data obtained are very interesting both in order to avoid an excess of adiposity of the animal that might affect

sensory and organoleptics characteristics of the fish, which can have a benefit in aquaculture.

In vivo results obtained in trout white muscle, showed that genistein affected lipid metabolism-related genes in this tissue, up-regulating FAS, HSL, PPARα and PPARβ significantly. This double effect of genistein (lipogenic and lipolytic effect) could be positive if interpreted as a contribute to promote a lipid turnover in trout white muscle.

Regarding genistein's mechanisms of action, it was seen that genistein could exert its lipolytic role in trout muscle via autophagic way, increasing ATG4b and ATG12l, not through the apoptotic pathway, because of it decreased CASP3 significantly, with a tendency to decrease P53. Rather, this down-regulation of CASP3 expression involved into apoptosis process, could be seen as a capacity of genistein to counteract an eventual lipid overload-induced muscle cell apoptosis.

The effects of genistein on lipid-metabolism and apoptosis-related genes in trout muscle were not dose-dependent, only on autophagy-related genes ATG4b and ATG12l. Moreover, a partial estrogenic activity of this phytoestrogen was also seen.

Definitely, our data need additional studies both to clarify the *in vivo* effect of genistein on rainbow trout lipid metabolism in adipose tissue, along with its mechanisms of action, and to better understand if the autophagic way in adipose tissue could be a possible pathway through which genistein may exert a possible adipogenic role, directed to the maintenance of the adipose tissue healthy and funcionality.

Regarding rainbow trout white muscle, instead, further studies would be necessary to confirm the double role of genistein in this tissue, and to investigate if genistein can be seen as a possible compound counteracting

cellular apoptotis induced by an eventual lipotoxicity. These data will contribute to know if the inclusion of soybean (that is rich in genistein) into *Oncorhynchus mykiss* diet, as an alternative to fishmeal for aquaculture, could be a benefit or not.

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8. WEB Resources

- www.agraria.org/pesci/trotairidea.htm
- www.docenteunicas.it/lipoproteine_dispense.pdf
- www.fao.org/fishery/culturedspecies/Ocorhynchus_mykiss/en
- www.fao.org/fishery/affris/species-profiles/rainbowt-trout/feed-formulation/en
- www.treccani.it/enciclopedia/adipogenesi