UNIVERSITAT DE BARCELONA

Obesity and adipose tissue biology in fish. Influence of nitritional, genetic and environmental factors

Esmail Lutfi Royo

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UNIVERSITAT DE BARCELONA

Department of Cell Biology, Physiology and Immunology

University of Barcelona

Obesity and adipose tissue biology in fish: influence of nutritional, genetic and environmental factors

Supervisors:

Dr. M. Isabel Navarro and Dr. Encarnación Capilla

A thesis submitted by **Esmail Lutfi Royo** for the degree of

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Obesity and adipose tissue biology in fish: influence of nutritional, genetic and environmental factors

Memòria presentada per

Esmail Lutfi Royo

per optar al grau de

Doctor per la Universitat de Barcelona

Doctorand **Directores:**

Esmail Lutfi Royo **Dra. M. Isabel Navarro** **Dra. Encarnación Capilla**

Barcelona, Juny 2017

A la memòria de la "iaia"

Rosina Bieto Malapeira

(1919 – 2014)

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"None but those who have experienced them can conceive of the enticements of science"

Mary Shelley

(Frankenstein or the modern Prometheus)

La ciència és el fruit de la curiositat de l'ésser humà, el resultat de l'esforç en la recerca d'explicacions per entendre millor com funciona el món. Es tracta d'un viatge extraordinari cap al descobriment que ens atrau i ens alliçona a cada pas, que ens fa més forts i ens obliga a aixecar-nos cada cop que fracassem. Per sort, durant la tesi, aquest camí mai el fem sols, i és aquí on tenim l'oportunitat d'agrair tot el suport que hem rebut al llarg de tots aquests anys i sense el qual no hagués estat possible continuar endavant.

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CONTENTS

CONTENTS

ABBREVIATIONS .... **XV**

CHAPTER 1. GENERAL INTRODUCTION **1**

1.1 OBESITY 5

1.1.1 Defining a human epidemic 5

1.1.2 Fat accumulation in farmed fish 5

1.1.3 Fish models for obesity research 8

1.2 ADIPOSE TISSUE **10**

1.2.1 Biological relevance 10

1.2.2 Function and allocation of fat depots 12

1.2.3 Adipogenesis 15

1.2.4 Lipid metabolism 18

1.2.5 Endocrine functions 21

1.3. MULTIFACTORIAL MODULATION OF ADIPOSE TISSUE **24**

1.3.1 Nutritional factors: the influence of diet 24

1.3.2 Genetic factors: inheriting obesity 26

1.3.3 Environmental factors: the “obesogen” theory 28

CHAPTER 2. OBJECTIVES **31**

CHAPTER 3. SUPERVISORS’ REPORT **35**

CHAPTER 4. ARTICLES **39**

ARTICLE I **41**

Caffeic acid and hydroxytyrosol have anti-obesogenic properties in zebrafish and rainbow trout models

ARTICLE II **67**

Eating for two: Consequences of parental methionine nutrition on offspring metabolism in rainbow trout (*Oncorhynchus mykiss*)

ARTICLE III **83**

Breeding selection of rainbow trout for high or low muscle adiposity has differential effects on lipid metabolism, the adiponectin system and oxidative stress

ARTICLE IV **111**

Tributyltin and triphenyltin exposure promotes *in vitro* adipogenic differentiation but alters the adipocyte phenotype in rainbow trout

CHAPTER 5. GENERAL DISCUSSION **125**

5.1 NUTRITIONAL REGULATION OF LIPID HOMEOSTASIS **129**

5.1.1 Anti-obesogenic properties of vegetal antioxidants 129

5.1.2 Dietary methionine deficiency in rainbow trout 133

5.2 GENETIC DETERMINANTS OF FAT DEPOSITION **135**

5.2.1 Metabolic strategies of divergent rainbow trout lines for muscle fat 135

5.3 ENVIRONMENTAL OBESOGENS **139**

5.3.1 Adipogenic role of organotins in rainbow trout adipocytes 139

CHAPTER 6. CONCLUSIONS **143**

REFERENCE LIST **147**

ANNEX **169**

ABBREVIATIONS

List of relevant abbreviations used throughout the present thesis:

ACSL: acyl-coenzyme A synthetase

ADIPOQ: adiponectin

ADIPOR: adiponectin receptor

ATGL: adipose triglyceride lipase

ATX: astaxanthin

BMI: body mass index

CA: caffeic acid

CD36: cluster of differentiation 36

C/EBP: CCAAT/enhancer-binding protein factor

DNL: *de novo* lipogenesis

EDC: endocrine-disrupting chemicals

FA: fatty acid

FABP: fatty acid binding protein

FAS: fatty acid synthase

FATP: fatty acid transport protein

FL: fat line

GLUT: glucose transport protein

HSL: hormone-sensitive lipase

HT: hydroxytyrosol

LL: lean line

LPL: lipoprotein lipase

LXR: liver x receptor

MET: methionine

MGL: monoacylglycerol lipase

MSCs: mesenchymal stem cells

NEFA: non-esterified fatty acid

PPAR: peroxisome proliferator-activated receptor

ROS: reactive oxygen species

RGZ: rosiglitazone

RXR: retinoid x receptor

TAG: triglyceride

TNFα: tumor necrosis factor alpha

TBT: tributyltin

TPT: triphenyltin

WAT: white adipose tissue

ZOT: zebrafish obesogenic test

CHAPTER 1. GENERAL INTRODUCTION

“In nature’s infinite book of secrecy

A little I can read”

William Shakespeare

(Antony and Cleopatra)

1.1 OBESITY

1.1.1 Defining a human epidemic

In the recent years, obesity has become a worldwide epidemic considered one of the most serious public health problems of our time. The prevalence of this disease has more than doubled since 1980, leading to the current situation in which more people worldwide are obese than underweight. Even though this trend appears to be leveling off, the actual rates are alarmingly higher than they were a generation ago, indicating that obesity is still a major public health concern with an important impact in our society (World Health Organisation, 2016). Obesity is traditionally defined as a complex and chronic health condition characterized by an abnormal or excessive fat accumulation in the body that relies upon the homeostatic relationship between energy intake and expenditure. At its core, when intake exceeds expenditure, the body homeostasis is disrupted and leads to storage of energy, primarily as body fat. Nonetheless, despite the simplicity of this equation, the derivation and continuation of this condition stems from the interaction of multiple aspects other than just energy balance (Vliet-Ostaptchouk et al., 2012), suggesting that the conventional view of obesity as a caloric imbalance does not entirely explain its prevalence.

In addition, epidemiological studies have shown that human obesity is related to increased mortality risk and impaired quality life. Apart from the problems inherent in enlarged fat accumulation, pathological overgrowth of fat storage is also associated with a range of related conditions such as type II diabetes, insulin resistance, hypertension and cardiovascular diseases (Blüher, 2013). Several studies in rodents have confirmed the link between a diet-induced obesity and these diseases (Wang and Liao, 2012). In this regard, it is important to notice that many of these complications are the consequence of the metabolic alterations associated with excessive fat deposition and its location in the body. More recently, increasing evidence also links obesity to a growing list of other disorders including cancer and neurodegenerative diseases (Ashrafian et al., 2013; De Pergola and Silvestris, 2013), suggesting that further investigation is needed to better understand this condition and its implications in human health.

1.1.2 Fat accumulation in farmed fish

Aquaculture industry has grown at an impressive rate over the past few decades (8,8% per year) becoming one of the most important sectors of agriculture and animal feed industry (FAO, 2016). With the limited supply of fishery captures, aquaculture has contributed to the advance

in the production of fish for human consumption. In fact, it currently provides half of the fish consumed worldwide, and its share is expected to increase in the next future (Fig. 1). This rapid growth and the subsequent intensification of aquafeeds production, have led to an increase in fish nutrition research, the main focus of which is to improve productivity and to reduce the use of non-sustainable ingredients. In this regard, a dietary supplementation of lipid as a non-protein energy source has been a usual practice in order to satisfy the commercial pressure to increase growth rates and reduce production times (Leaver et al., 2008). Nevertheless, there is a strong relationship between dietary lipid levels and unwanted adiposity (Cowey and Cho, 1993).

Although obesity per se might not be considered a health threat in fish as it is for humans, increased fat deposition is also shown to produce adverse metabolic effects in these species. It has been widely demonstrated that high levels of dietary fat increase adipose tissue abundance and impair lipid and glucose homeostasis in several fish species (Borges et al., 2014; Figueiredo-Silva et al., 2012). Furthermore, excessive fat accumulation also alters flesh quality in terms of fillet yield and organoleptic properties, which directly affect aquaculture productivity. Aside from the level of lipid inclusion, the type of lipid source might also affect physical and organoleptic quality (Thomassen and Røsjø, 1989). In salmonids, visceral adipose tissue and muscle represent the main reservoir compartments. While moderate intramuscular fat depots are appreciated due to improved organoleptic characteristics of the flesh, an excessive fat accumulation in the abdominal cavity has a negative influence on productive performance. Therefore, particular attention must be paid to the dietary lipid composition of fish feeds not only to improve production costs but also to ensure quality of the final product.

Traditionally, animal ingredients, such as fish meal and fish oil, have been the main source of the aquaculture feeds formulation. They are a rich source of highly digestive proteins and long-chain polyunsaturated fatty acids. Nevertheless, due to their limited availability and elevated price, using fish meal and fish oil ingredients is considered an unsustainable practice (Fig. 1). Hence, the contribution of alternative protein sources has aroused great interest for the fish nutrition industry. Many nutrient replacement studies have investigated the effects of different inclusion degrees of plant ingredients mainly focusing in food conversion, digestibility and their potential effects modulating adiposity. Some studies have shown that high content of plant ingredients in the diet increases visceral lipid stores in Atlantic salmon (*Salmo salar,* Torstensen and Tocher 2010; Torstensen et al., 2011) and induces adipocyte hypertrophy and impairs fat accumulation in gilthead sea bream (*Sparus aurata,* Cruz-Garcia et al., 2011), indicating that nutrient composition should be also considered (see section 1.3.1).

On the other hand, fish adiposity is not only modulated by nutritional factors but also, a range of intrinsic and extrinsic factors are thought to have an important implication. As well as in mammals, several factors such as age or gender seem to exert profound effects in the regulation of lipid homeostasis in fish. Age-specific differences in fat metabolism are particularly expected in anadromous fish, which undergo significant physiological changes during adaptation from living in fresh to sea water (Kiessling et al., 1991). Moreover, it has been also demonstrated that the difference in male and female fat accumulation on Atlantic salmon is related to sex-specific reproductive cycles (Dessen et al., 2016). This variation in lipid metabolism strategies can be associated to seasonal changes, suggesting that environmental fluctuations might also influence adiposity dynamics. Indeed, in their natural environment, fish can experience prolonged periods of fasting, either as a result of spawning migration or seasonal variation of low food availability (Byström et al., 2006; McCue, 2010). During these periods, fish can metabolize large quantities of energy reserves affecting the normal regulation of lipid metabolism (Navarro and Gutiérrez, 1995; Wieser et al., 1992).

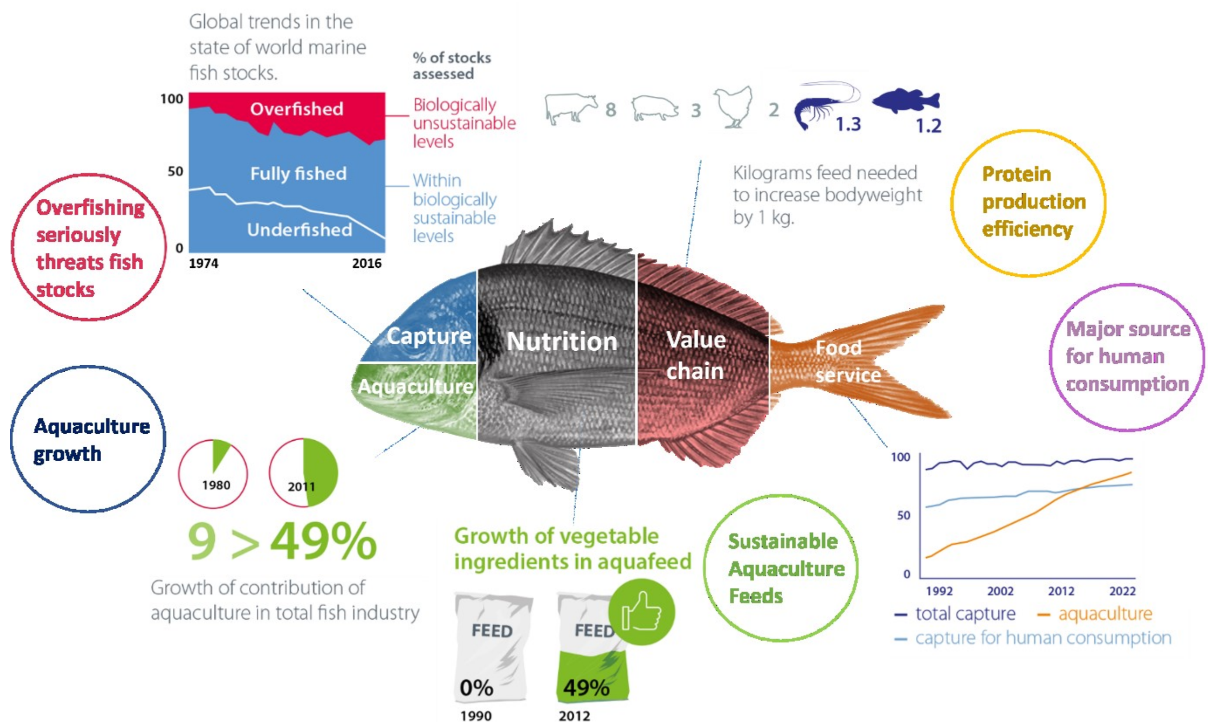


Figure 1. Global trends in fisheries and aquaculture production. Adapted from (FAO, 2016; Rabobank, 2016; World Wildlife Fund, 2012).

1.1.3 Fish models for obesity research

Even though mammalian models (mainly rodents) have been traditionally used in human physiology and disease research due to their anatomical and physiological similarities (Lossi et al., 2016), they could be unsuited for certain types of studies (D'Angelo et al., 2016). In this regard, non-mammalian vertebrates such as fish have been proposed as excellent alternative models for studying human diseases. In the recent years, medaka (*Oryzias latipes*) and specially zebrafish (*Danio rerio*) among other species, have attracted researchers from various fields, including developmental biology, neuroscience and cardiovascular research; however, presently their use is expanding to other areas such as pharmacology, metabolism and drug discovery (Chakraborty et al., 2009; Lin et al., 2016).

Besides their simplicity, fish models are very promising for obesity research, as most of the metabolic pathways linked to the lipid metabolism are conserved between mammals and teleost fish, and they can have similar biological responses (Ichimura et al., 2013; Santoriello and Zon, 2012). For instance, a diet-induced obesity model from both medaka and zebrafish species, has been shown to share common pathophysiological traits with mammals, including increased body mass index (BMI), hypertriglyceridemia and hepatosteatosis (Ichimura et al., 2013; Oka et al., 2010). As vertebrates, they possess many structural similarities with humans that the most well-known non-mammalian obesity models, *Drosophila melanogaster* and *Caenorhabditis elegans*, do not. In this regard, histological studies have revealed evolutionarily conserved morphological structures of zebrafish adipose tissue as well (Minchin et al., 2015). Unlike *C. elegans*, where the major fat storage compartment is the intestine, dietary energy excess in zebrafish is stored almost all around the body, including perivisceral fat, in the form of large unilocular lipid droplets within adipocytes.

The rapid development ex-utero and the optical semi-transparency of the embryonic and early larval stages of zebrafish and medaka, have made teleost fish popular models in applied and basic research. Particularly, it has been shown that semitransparent larvae of these species offer a unique opportunity to study adiposity dynamics *in vivo*, which together with the use of fluorescent transgenic lines and lipophilic dyes (i.e. Oil red O, Nile red or Bodipy) have provided a powerful tool in obesity research (Fig. 2). Moreover, these features afford a suitable platform for high-throughput screening of dietary compounds, drugs and chemicals likely to impair adipocyte fat storage and mobilization (Tingaud-Sequeira et al., 2011). In addition, the uncountable possibilities in developing transgenic fish models represent a significant advance

for the studies of multiple disciplines (Kawakami et al., 2016). They provide a fast, simple and effective *in vivo* approach to investigate gene function and molecular pathways. To date, several zebrafish transgenic lines have been developed to study obesity and its related diseases, showing pathological similarities with mammalian models. As an example, overexpression of endogenous melanocortin antagonist agouti-related protein (AgRP) is regarded to enhance appetite and induce the obese phenotype in zebrafish (Song and Cone, 2007). Moreover, RAC-alpha serine/threonine-protein kinase (Akt1) overexpression show enhanced adipogenesis displaying also an obese phenotype in the same species (Chu et al., 2012). Overall, these results demonstrate that the key adipostat components are conserved between fish and mammals, drawing attention to fish as alternative and effective models for obesity human research.

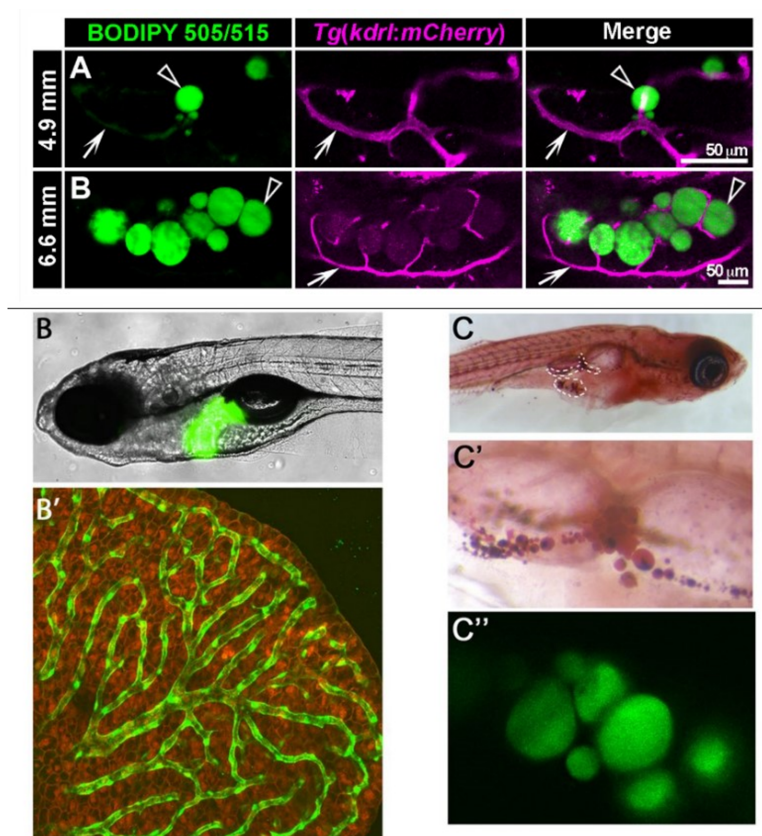


Figure 2. *In vivo* visualization of metabolic tissues in zebrafish. (**A**) Confocal imaging of live zebrafish transgenic line (*Tg*(*kdrl: mCherry*)) stained with Bodipy 505/515 dye showing the close interplay between lipid droplets and the vascular system in visceral adipose tissue. (**B, B'**) Confocal analysis of the liver parenchyma of LiPan/Tg(fli1:EGFP) transgenic zebrafish. (**C, C'**) White adipose tissue of juvenile fish stained with Oil Red O, indicating the location of the adipocytes close to the intestine and pancreas. (**C''**) Confocal imaging of lipid droplets in juvenile fish stained with LipidTox. Adapted from (**A**, Minchin and Rawls 2011) and (**B-C''**, Seth et al., 2013).

This increase in the use of fish models for biomedical research can be also traced back to the relatively recent expansion of fish farming industry. The development in this research field has given great understanding of fish metabolism, providing an invaluable tool for applied comparative studies. Although farmed fish such as Atlantic salmon or rainbow trout (*Oncorhynchus mykiss*) aren't the most conventional models for human disease research, it has been shown that these species can offer an interesting approach to this matter. As well as in mammals, a lipid-rich diet successfully causes an increase in visceral fat content along with changes in the regulation of lipid metabolism and mobilization in salmonids (Libran-Perez et al., 2015; Tocher et al., 2003). Despite the lack of genetically modified obesity models in these species, breeding programs for adiposity traits (Quillet et al., 2005) and the existence of natural variants, such as the "cobalt" rainbow trout, which present high adiposity levels in the abdominal cavity (Yada et al., 2002), provide interesting tools to address metabolic studies. On the other hand, zebrafish, although is not a fish species of interest in aquaculture production, has been used to respond to questions in aquaculture as an experimental model, representing an ideal organism to carry out preliminary evaluation of diets with a particular focus on nutrigenomics (Ulloa et al., 2013).

1.2 ADIPOSE TISSUE

1.2.1 Biological relevance

As a major source of energy storage, adipose tissue is considered a key metabolic organ that plays an important role in the regulation of whole-body energy homeostasis. Until the late 1940s, it was characterized as a form of connective tissue which its primary function was to store excess of energy that comes from the diet, in form of triglycerides (TAG), and to release free fatty acids (FA) to fulfil the metabolic requirements of peripheral tissues. Nonetheless, despite this traditional view as a rather passive storage tissue, it has been recognized as a multi-functional endocrine organ that plays a critical role in modulating several physiological processes, such as appetite, whole-body energy metabolism and homeostasis, as well as tissue inflammation responses (Khan and Joseph, 2014). Henceforth, studies on the functional, developmental and pathophysiological aspects of adipose tissue have expanded noticeably in the last decades (Fig 3).

At the cellular level, hyperplasia (increase in cell number) and hypertrophy (lipid accumulation within existing adipocytes) are two possible growth mechanisms for adipose tissue. Adipocytes

are the major constituent of adipose tissue and, in mammals, can be of three distinct types (white, brown and beige/brite), which exhibit different morphology and functions. Particularly, brown adipose tissue is thermogenic and helps to regulate body temperature, while white adipose tissue is regarded to take part in the classical metabolic and endocrine role of adipose tissue (Giralt and Villarroya, 2013). Moreover, brown-like adipocytes appearing in white adipose tissue are regarded as "inducible, beige, or brite", and are thought to be, at least in part, a sort of adipose cells in a transdifferentiation process of white-to-brown adipocytes. Nevertheless, fish are not considered to possess brown nor beige adipocytes and therefore, they won't be discussed further in this chapter.

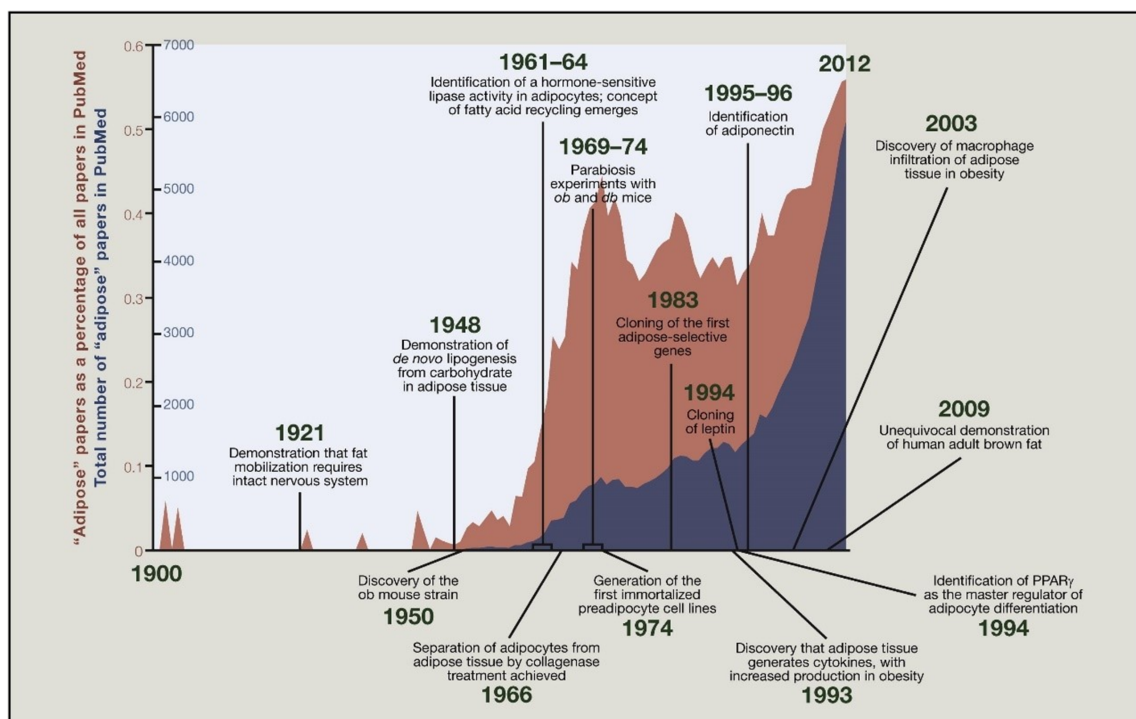


Figure 3. Percentage of publications and important events in adipose biology research. Adipose publications as a percentage of all publications in PubMed are illustrated in red. Total numbers of adipose publications in PubMed are shown in blue. Modified from (Rosen and Spiegelman, 2014).

Although most of the current knowledge about adipose tissue functionality and development comes from mammalian systems, the importance of adipose tissue to aquaculture industry and the increasingly use of teleost models as research tools for biomedical research have provided new insights into adipose tissue regulation in fish. In this regard, a number of studies have utilized fish species as models within the areas of lipid metabolism and adipose tissue biology

(Planas et al., 2000; Todorčević et al., 2010; Zhou et al., 1996). Particularly, development of fish *in vitro* adipocyte cultures has emerged as an easy and useful approach in this type of investigations (see 1.2.3). In fact, using these *in vitro* techniques, it has been well documented that teleost adipocytes express genes related to adipocyte differentiation (Bou et al., 2017; Bouraoui et al., 2012, Bouraoui et al., 2008; Salmerón et al., 2016a; Todorčević and Hodson, 2015), lipolysis (Cruz-Garcia et al., 2015, Cruz- Garcia et al., 2012) as well as adipocyte endocrine function (Sánchez-Gurmaches et al. 2012; Bou et al. 2014; Salmerón et al. 2015), altogether highlighting the particular suitability of fish species to study lipid metabolism and adiposity dynamics.

1.2.2 Function and allocation of fat depots

White adipose tissue is formed at stereotypic times and locations among and within species (Berry et al., 2013). In mammals, the distribution of fat in the body is organized in distinct anatomical depots usually identified as subcutaneous and perivisceral adipose tissues. Each depot has a specific morphology and texture, being the subcutaneous heterogeneously filled with mature unilocular adipocytes intercalated with small multilocular adipocytes, and the perivisceral more uniform with large unilocular adipocytes (Ibrahim, 2010). These deposits differ from each other not only by localization but also by their structural and functional properties. For instance, several features such as adipocyte growth, differentiation and metabolism, developmental gene expression, susceptibility to apoptosis, inflammatory capacity and adipokine secretion, vary among deposits, leading to the provocative conclusion that adipose tissue depots could be considered as mini-organs, serving functions unique to their specific locations (Kirkland et al., 1996).

A number of studies have reported depot-specific differences in the expression of developmental genes during adipocyte differentiation (Gesta et al., 2006; Tchkonia et al., 2006), suggesting a distinct transcriptional regulation of the depots that might contribute to the differences observed in types of obesity and incidence of metabolic disorders (Trujillo and Scherer, 2006). In fact, there is growing evidence that excessive development of some adipose depots, but no others, might produce different physiological outcomes. Increased subcutaneous deposition is shown to have protective effects against certain aspects of metabolic dysfunction (Frayn, 2002), while perivisceral accumulation, is thought to be associated with metabolic complications including diabetes, hyperlipidemia and cardiovascular disease (Wajchenberg,

2000). Unfortunately, despite the clear applicability of this knowledge in specific-target therapies, there are still few data regarding the different functions and regulation of adipose tissue depots.

Similarly, teleost adipose tissue is also depicted in both subcutaneous and perivisceral locations (Fig. 4), suggesting that the developmental programs responsible for adipose tissue formation and localization might have been maintained throughout evolution. Perivisceral adipose tissue is located in the abdominal cavity around the digestive tract and represents a 2–25 % of total body weight, while the subcutaneous fat can be found all around the body with special deposition in dorsal and ventral parts (Weil et al., 2013). One particular aspect of fish, is that they accumulate adipocytes in skeletal muscle, especially within layers of connective tissue, so-called myoseptum (Fig. 4C). Fish muscle can be classified into 2 types: a) red muscle, composed by slow-twitch fibers, which represents a 10 % of the myotomal musculature and is regarded to be used mainly for sustained energy efficient swimming and b) white muscle or fast-twitch fibers, which composes the major part of the skeletal muscle in fish and constitutes more than 70% of the muscle (Kiessling et al., 2006). Salmonid species store significant amounts of lipids in muscle (from 3 to 18%) depending on the diet, and so they are regarded as "fatty" fish (Davidson et al., 2014). In contrast, other species such as turbot (*Psetta maxima*) only have a muscle fat content of < 1% on a fresh wet weight basis (Grigorakis, 2007), suggesting that the regulation of lipid deposition and localization might not be only tissue- but also species-specific in fish. Indeed, lipid distribution within skeletal muscle is shown to be highly diverse among fish species without accounting to their phylogenetic relationship (Kaneko et al., 2016).

The particular features of the zebrafish system are especially well suited to study the developmental origins of different adipose tissue depots and their distribution in the body. Their ex-utero development and optical semi-transparency from fertilization to the onset of adulthood, permit *in vivo* monitoring of adipose tissue dynamics, providing new opportunities to investigate adipose tissue morphogenesis (Seth et al., 2013). In this regard, recent studies have proposed a detailed classification system for zebrafish adipose tissues (Minchin and Rawls, 2017, Minchin and Rawls, 2016), however no specific functions of these depots have been identified yet. Furthermore, adipose tissue development in zebrafish is a step-wise process, with differentiated adipocytes first observed in the visceral region, then the subcutaneous area and finally, the head. This process appears to be regulated by both developmental time and size, being the perivisceral preferentially regulated by age, and the subcutaneous more dependent on

fish size (Imrie and Sadler, 2010). Interestingly, these fat deposits are also mobilized sequentially, in reverse order, in response to starvation, indicating that adipose tissue development is a highly regulated process that follows a well-stablished pattern (Minchin and Rawls, 2011).

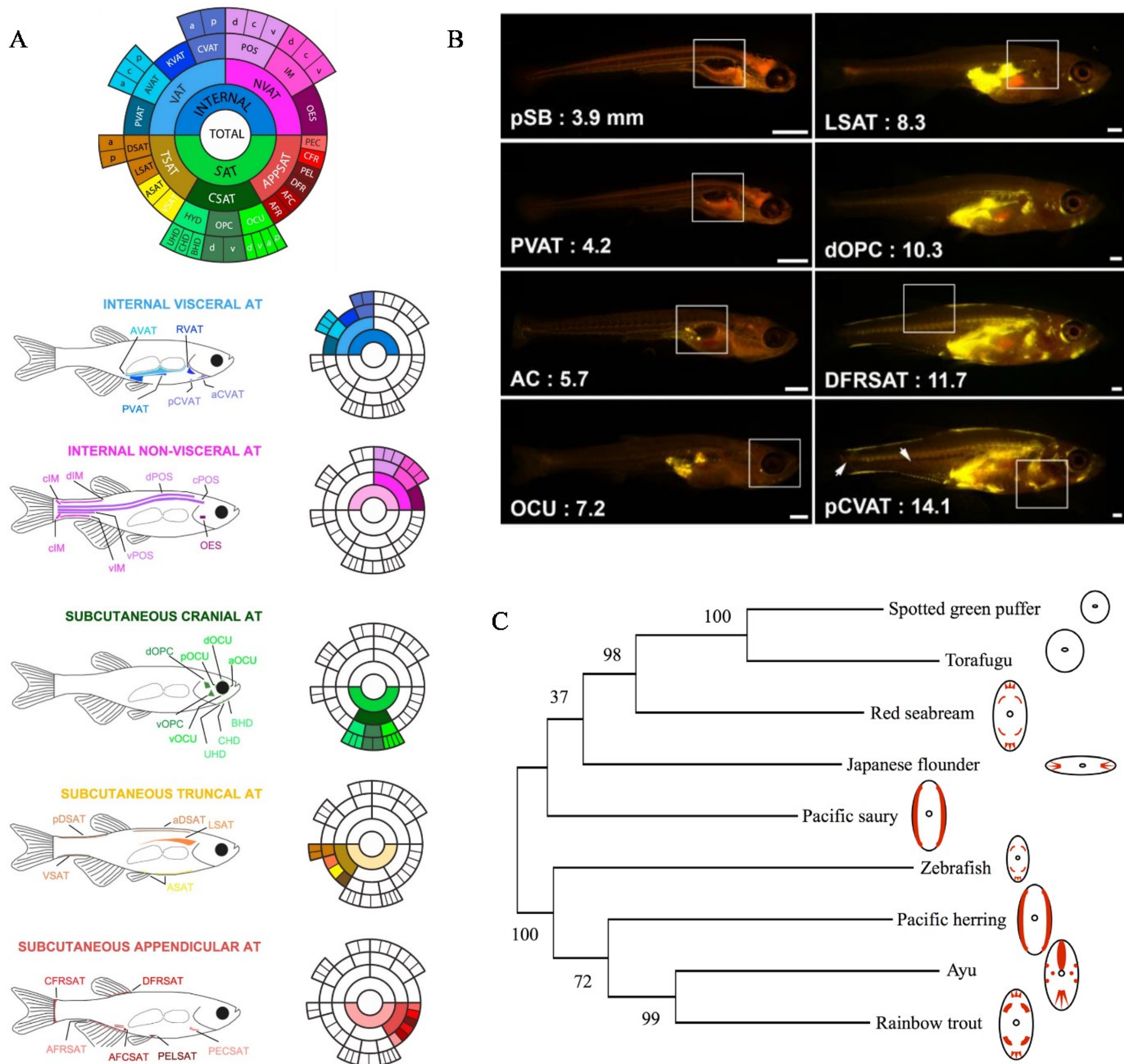


Figure 4. Adipose tissue depots in fish. (**A**) Schematic representation of the anatomical location of zebrafish adipose tissue depots. (**B**) Stereoscopic images of Nile Red-stained postembryonic zebrafish at different stages. White boxes and arrows indicate the appearance of new fat depots in different regions. (**C**) Phylogenetic relationship and schematic representation of muscle lipid distribution in several fish species. Oil red O staining patterns for intramuscular lipids are shown in red. **Abbreviations:** pSB, posterior swim bladder; AC, abdominal cavity; IAT, internal adipose tissue; VAT, visceral adipose tissue; a and p CVAT, anterior and posterior cardiac visceral adipose tissue; PVAT, pancreatic adipose tissue; AVAT abdominal adipose tissue; d, c and v POS, dorsal, central and ventral paraosseal; c, d and v IM, caudal, dorsal and ventral intermuscular; SAT, subcutaneous;

APPSAT, appendicular subcutaneous adipose tissue; CFR, caudal fin ray; DFR, dorsal fin ray; AFR, anal fin ray; AFC, anal fin cluster; PEL, pelvic fin; a, p and l PEC, anterior, posterior and loose pectoral fin; CSAT, cranial adipose tissue; OCU, ocular; d and v OPC, dorsal and ventral opercular; HYD, hyoid; BHD, basihyoid; CHD, ceratohyoid; UHD, urohyoid; TSAT, truncal subcutaneous adipose tissue; LSAT, lateral subcutaneous adipose tissue; a and p DSAT, anterior and posterior dorsal subcutaneous adipose tissue; VSAT, ventral subcutaneous adipose tissue; ASAT, abdominal subcutaneous adipose tissue. Modified from (**A**, Minchin and Rawls, 2016), (**B**, Minchin and Rawls, 2017) and (**C**, Kaneko et al., 2016)**.**

1.2.3 Adipogenesis

Adipogenesis is a multi-step process that determines the differentiation of pre-adipocytes into mature adipocytes, and ultimately controls adipose tissue formation promoting both, lipogenesis and FA uptake (Sethi and Vidal-Puig, 2007). Adipocytes derive from multipotent mesenchymal stem cells (MSCs) that have been described to undergo two phases to become mature adipocytes. First, pluripotent MSCs commit to the adipocyte lineage resulting in a conversion into a pre-adipocyte cell in a process called determination (Fig. 5). At this point, pre-adipocytes lose their ability to differentiate into other cell types and can enter the terminal differentiation phase (Rosen and Spiegelman, 2006).

Most of the current information about the process of adipogenesis comes from mammalian cell lines such as the murine 3T3-L1 (Gregoire, 2001; Rosen et al., 2000), which has been pivotal in advancing the understanding on the role of adipocytes in obesity and its related disorders. Adipocyte cell lines offer numerous advantages, such as they are cost effective, easy to use and provide consistent samples with reproducible results. Nevertheless, even though these *in vitro* models have helped to unravel the mechanistic characterization of adipocyte differentiation, care must be taken when interpreting the results, as cell lines do not always accurately replicate the primary cells (Kaur and Dufour, 2012). In this regard, despite of some technical difficulties inherent to the isolation of stromal vascular cells when compared to the immortalized cell lines, the use of primary cultures represents the situation most closely related to an *in vivo* state (Lerescu et al., 2008). To date, there is not available any adipogenic cell line from fish. Nonetheless, primary cell cultures derived from the stromal vascular fraction of adipose tissue have been developed for several fish species including Atlantic salmon (Vegusdal et al., 2003), red sea bream (*Pargus major,* Oku et al., 2006) rainbow trout (Bouraoui et al., 2012), large yellow croaker (*Pseudosciaena crocea*, Wang et al., 2012) and gilthead sea bream (Salmerón et al., 2013). The use of these *in vitro* approaches has allowed researchers to investigate the

nutritional and hormonal regulation of adipogenesis in these species by focusing in specific factors implicated in lipid metabolism and energy homeostasis (Bou et al., 2017; Bouraoui et al., 2012; Oku et al., 2009; Salmerón et al., 2016b; Todorčević et al., 2010).

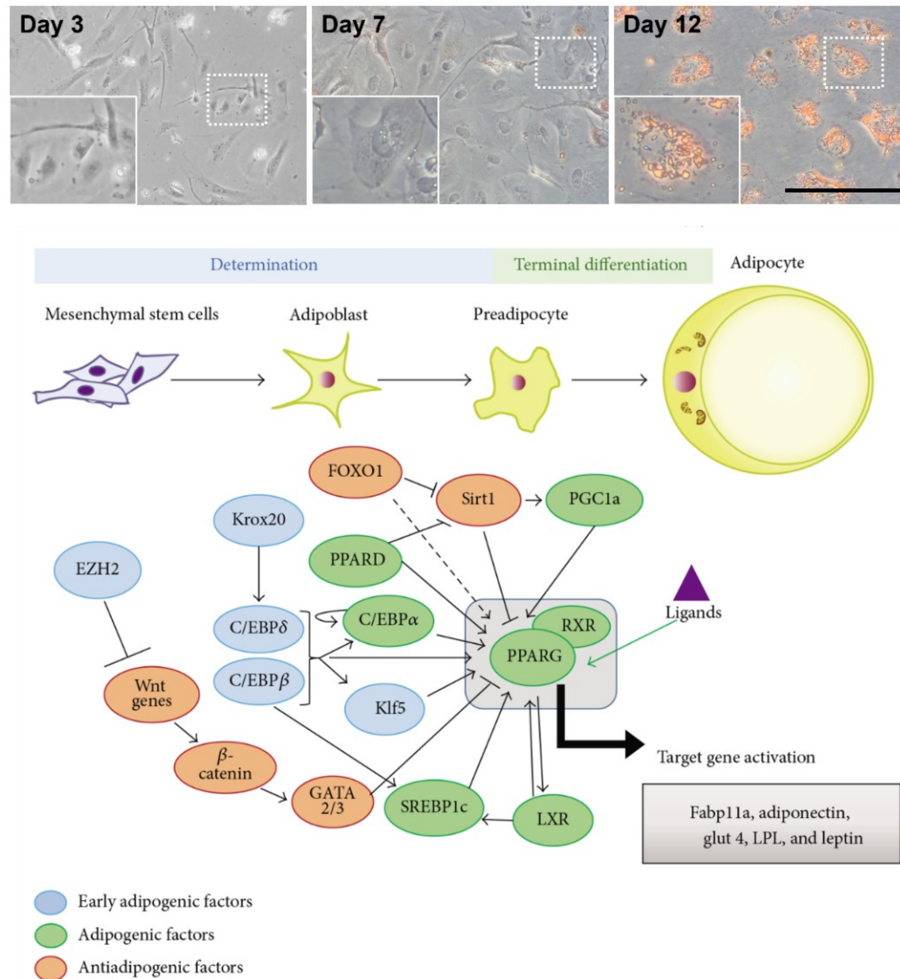


Figure 5. Representative images of rainbow trout Oil Red O-stained adipose cells at different days of culture (top) and transcriptional network of regulatory factors implicated in adipogenesis (bottom). Insets in each image are enlarged views of isolated adipocytes from each panel marked by a white rectangle. Fibroblast-like elongated morphology is observed in left (pre-confluent adipocytes) and middle (confluent adipocytes) panels and the typical mature adipocyte spherical shape in the right panel (differentiated adipocytes). Neutral lipids are stained in red. Magnification, 20 x; scale bar, 100 µm. **Abbreviations:** EZH2, enhancer of zeste homology 2; Krox20, early growth response protein 2; C/EBP (α, β and δ), CCAAT enhancer binding protein (alpha, beta and delta); Kfl5, krüppel-like transcription factor; PPAR (D and G), peroxisome proliferator-activated receptor (delta and gamma); PGC1a, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; RXR, retinoid x receptor; LXR, liver x receptor; SREBP1c, Sterol regulatory element-binding protein 1; β-catenin, beta catenin, GATA 2/3, GATA binding protein 2/3; fabp11a, fatty acid binding protein 11 a; glut 4, glucose transporter 4; LPL, lipoprotein lipase. Modified from (Den Broeder et al., 2015).

The induction of adipocyte differentiation is characterized by sequential changes in the expression of specific genes that determine the profound phenotypical modifications of fibroblast-like pre-adipocytes to become spherical cells filled with lipid droplets (Fig. 5). Although many of the molecular mechanisms of adipogenesis are still unknown, several positive and negative regulators of this network have been elucidated (Lefterova and Lazar, 2009). Among them, the peroxisome proliferator-activated receptor gamma (PPARγ) is considered the master regulator of adipogenesis. This transcription factor, together with CCAAT/enhancer binding protein alpha (C/EBPα), promote adipocyte differentiation by activating adipose-specific genes as well as regulating each other's expression. In mammals, the induction of these two proteins leads to a permanent period of growth arrest followed by manifestation of the fully differentiated phenotype (Rosen, 2005). Therefore, impaired PPARγ signaling, expression and/or activation, directly affect adipose tissue development (Koutnikova et al., 2003) and appear to be implicated in the prevalence of metabolic diseases such as obesity (Sharma and Staels, 2007) and lipodystrophy (Hegele and Leff, 2004). Similar results are found in mice with compromised functionality of C/EBP transcription factors (Linhart et al., 2001; Wang et al., 1995).

Although similar in structure, the C/EBP family members possess specific biological functions (Chen et al., 2000). A transient increase in C/EBPβ and C/EBPδ at the onset of adipogenesis has been reported (Yeh et al., 1995). These early events are followed by the expression of C/EBPα and PPARγ, which remain elevated for the rest of the differentiation process, highlighting their importance in regulating adipogenesis. Further *in vitro* studies have also identified other transcription factors that may be involved in the regulation of adipocyte differentiation and function, including early adipogenic factors such as KLF4, KLF5 and KROX20 (EGR2) (Park et al., 2017); late adipogenic factors, i.e. RXR, SREBP1c and PGC1a (Rosen et al., 2000); and also anti-adipogenic factors including FOXO1, WNT and GATA 2/3 among many others (Cristancho and Lazar, 2011; Den Broeder et al., 2015; Moreno-Navarrete and Fernández-Real, 2012).

Although less intensely investigated, few studies have analyzed the transcriptome profile of adipocyte differentiation in fish, demonstrating that adipogenesis, at least in salmonids, is a complex and tightly coordinated process, as occurs in mammals (Bou et al., 2017; Todorčević et al., 2010). The specific role played by PPARγ and C/EBPα in fish adipocyte development has been characterized in several species (Bouraoui et al., 2008; Den Broeder et al., 2015; Todorčević et al., 2010, Todorčević et al., 2008). A deep review on PPARs in fish in comparison

to mammals is available (Leaver et al., 2008). In contrast to humans and mice, which have three PPARγ isoforms (*pparg1, pparg2* and *pparg3*), only one *pparg* gene has been identified in teleosts (Den Broeder et al., 2015; Wafer et al., 2017). In fish, like in mammals, PPARγ appears coincident with early stages of adipocyte differentiation and increases during adipogenesis (Liu et al., 2015; Salmerón et al., 2016b; Wang et al., 2012). Moreover, *pparg* mRNA is colocalized with Nile Red stained adipocytes within the pancreas and the intestinal epithelium in zebrafish larvae (Imrie and Sadler, 2010). Similarly, *pparg* mRNA levels were shown to be higher in fat than in lean gilthead seabream, altogether suggesting a PPARγ involvement in visceral fat accumulation (Cruz-Garcia et al., 2009).

On the other hand, analysis of the distribution of *cebps* mRNA levels revealed that these isoforms are differentially expressed in different tissues in Atlantic salmon, with highest expression levels in liver and visceral adipose tissue (Huang et al., 2010). Particularly, *cebpd* has been shown to present a peak of expression at day 3 of cell development in rainbow trout adipocytes in primary culture, whereas *cebpa* started to be up-regulated from day 8, with a maximum peak at day 15 (Bou et al., 2017), indicating a similar transcriptional regulation between fish and mammals.

1.2.4 Lipid metabolism

In terms of energy storage and release, adipose tissue is remarkably flexible. Its formation and expansion is dependent on two main processes: enhancement of TAG synthesis (lipogenesis) and fat breakdown (lipolysis) for either FA oxidation or release to be taken by other tissues (Fig. 6). These processes are shown to be orchestrated by the cross-talk of adipose tissue with liver and skeletal muscles (Samdani et al., 2015). The balance between deposition and mobilization of lipids responds to multiple signals and appears to be highly regulated by different mechanisms (Saponaro et al., 2015). Briefly, FA are hydrolyzed from circulating TAG-rich lipoproteins by the action of lipoprotein lipase (LPL) and incorporated into adipocytes through a number of membrane proteins participating in FA influx including acyl-CoA synthetases such as acyl-coenzyme A synthetase (ACSL), fatty acid transport proteins (FATP) and fatty acid translocases (i.e. CD36). Then, depending on the energetic status of the organism, the intracellular FA will be redirected and utilized for TAG synthesis, oxidation or cell membrane formation. Thus, LPL is a key enzyme in lipid metabolism that together with fatty acid synthase (FAS) and diacylglycerol transferase (DGAT), critical enzymes in

lipogenesis and TAG biosynthesis respectively, is considered one of the early markers of adipocyte differentiation (Ranganathan et al., 2006).

The role of LPL has been analyzed in several fish species (Albalat et al., 2007; Arnault et al., 1996) and its regulation appears to be mediated by nutritional and hormonal factors (Albalat et al., 2007; Bouraoui et al., 2012). The presence of LPL in different fish tissues is related to the capacity of these tissues (i.e. adipose tissue and muscle) to hydrolyze TAG of circulating lipoproteins (Babin and Vernier, 1989). Concerning the transport of FA, the mRNA levels of several transporters such as *cd36* and *fatp1*, as well as their nutritional and hormonal regulation (i.e. fasting and insulin administration) have been also reported in salmonids (Sánchez-Gurmaches et al. 2011; Sánchez-Gurmaches et al. 2012). Moreover, *in vitro* studies in Atlantic salmon have shown that *cd36* mRNA levels are slightly reduced in mature adipocytes (Todorcević et al., 2010), while *fatp1*, *fabp3* and *fabp11* mRNA levels are up-regulated during adipocyte differentiation (Huang et al., 2010; Todorčević et al., 2008).

When energy intake exceeds energy expenditure, there is a gain in weight mainly due to TAG stored in adipocytes. This deposition depends on the incorporation of dietary FA, but also on a process called *de novo* lipogenesis (DNL), which synthesizes FA from excess carbohydrates. DNL occurs mainly in the liver; however, it has been demonstrated that mammalian adipocytes are capable to synthetize significant amounts of FA and TAG from non-lipid precursors as well (Collins et al., 2011). In contrast, fish, and especially carnivorous species, utilize dietary carbohydrates poorly and experience prolonged hyperglycaemia after being fed with high-carbohydrate diets (Jin et al., 2014a; Polakof et al., 2012). Indeed, while it has been recently revealed that the DNL pathway is active in fish adipocytes, its capacity to convert glucose into cellular lipids is relatively low compared to mammals (Bou et al., 2016). FAS is an enzyme thought to play an important role in the DNL process, in which catalyzes the synthesis of long chain fatty acids, mainly by using acetyl CoA and malonyl CoA (Smith et al., 2003). An *in vivo* work conducted in rainbow trout fed a high-carbohydrate diet, has shown that insulin increases adipose tissue *fasn* mRNA levels (Polakof et al., 2011). Moreover, it has been shown that *fasn* expression also increases during *in vitro* induced maturation of adipocytes using a differentiation cocktail in Atlantic salmon (Todorcević et al., 2010), indicating altogether the potential action of hormones in FAS activity.

In response to energy demands, stored TAG can be mobilized to be used by peripheral tissues trough the activation of lipolysis releasing FA and glycerol. As shown in fig. 6, lipolysis is

controlled by a complex series of cascades that initiate the activation of TAG hydrolysis, which is induced by a family of lipases including adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL) and monoacylglycerol lipase (MGL) (Ahmadian et al., 2009). In brief, ATGL selectively performs the first step hydrolyzing TAG to form diacylglycerols (DAG) and FA, then HSL hydrolyses DAG to monoacylglycerols (MAG) and these are broken to FA and glycerol by MGL (Lass et al., 2011). ATGL has been recently discovered in mammals by three independent research groups (Jenkins et al., 2004; Villena et al., 2004; Zimmermann et al., 2004), however there is scarce information about its function in fish (Wang et al., 2013). On the other hand, it has been demonstrated that HSL is capable of hydrolyzing different types of acylesters, including TAG, DAG and MAG, indicating the importance of this enzyme in the catabolic mechanism of lipolysis (Lampidonis et al., 2011). As well as LPL, HSL appears to be regulated by both nutritional and hormonal factors in fish species (Bergan et al., 2012; Khieokhajonkhet et al., 2016).

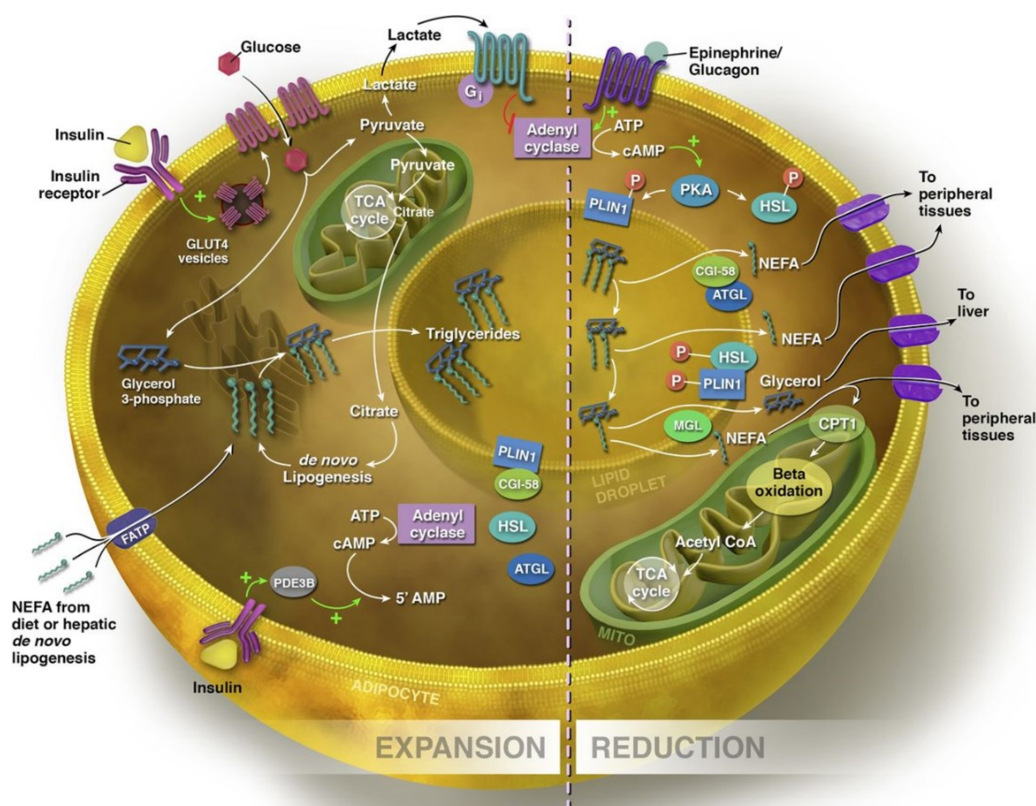


Figure 6. Schematic representation of fat deposition and mobilization in adipocyte cells. The fundamental mechanisms of adipocyte expansion and reduction are depicted on the left-hand side (glucose uptake, *de novo* lipogenesis and fatty acid transport) and right-hand (lipolysis and β-oxidation) respectively. **Abbreviations:** ATGL, adipose triglyceride lipase; CGI-58, comparative gene identification-58; CPT1, Carnitine palmitoyltransferase I; GLUT4, glucose transporter 4; HSL, hormone sensitive lipase; MITO, mitochondrion;

MGL, monoacylglycerol lipase; NEFA, non-esterified fatty acids; PDE3B, phosphodiesterase 3; PKA, protein kinase A; PLIN1, perilipin 1; Adapted from (Rutkowski et al., 2015).

In this regard, it has been demonstrated that fasting and growth hormone administration enhance adipose tissue and hepatic *hsl* mRNA levels in rainbow trout (Bergan et al., 2012) and red sea bream (Khieokhajonkhet et al., 2016). Moreover, dietary soybean lecithin increases *hsl* mRNA levels in gilthead sea bream larvae suggesting a lipolytic effect of this diet (Alves Martins et al., 2010). In addition, gilthead sea bream fed with high vegetable protein an oil diet also presents an increase in gene expression and activity of HSL in adipose tissue (Cruz-Garcia et al., 2011). In any case, the resulting FA from the breakdown are primarily transported to other organs such as liver and skeletal muscle, where they can be oxidized for energy production in the mitochondria and peroxisomes, in a process called β-oxidation. Nevertheless, despite being less important, a small fraction of FA can remain in the adipocyte for further oxidation or re-esterification into newly synthesized TAG (Van Harmelen et al., 1999). β-oxidation pathway and its implication in the regulation of lipid metabolism have been investigated in rainbow trout and Atlantic salmon in different tissues, including liver (Kolditz et al., 2008a), red muscle (Frøyland et al., 1998), white muscle (Torstensen et al., 2009) and more recently, adipose tissue (Polakof et al., 2011).

1.2.5 Endocrine functions

After the identification of leptin as a specific adipocyte cell-derived hormone in 1994, numerous studies have demonstrated that adipose tissue produces a range of factors, named adipokines, with important endocrine functions and whose secretion is affected by metabolic dysregulation (Deng and Scherer, 2010). These factors interact with a range of processes in many different organ systems such as brain, liver, pancreas, skeletal muscle and also, in a paracrine fashion, in adipose tissue (Harwood, 2012); and influence various systemic phenomena including food intake, energy expenditure, inflammation and lipid metabolism. Therefore, the dysregulation of adipokine secretion as it occurs in obesity, results in impaired organ cross-talk communications and metabolic alterations, thereby leading to the development of several diseases such as type II diabetes, insulin resistance and metabolic syndrome (Maury and Brichard, 2010). Imbalanced regulation of these adipokines is observed under conditions of both excessive and lack of adipose tissue.

Most adipokines are regarded to act as pro-inflammatory factors that increase in an obesity context (i.e. leptin, chemerin, resistin and TNFα), with the noted exceptions of several anti-inflammatory factors such as adiponectin, secreted frizzled-related protein 5 (SFRP5), visceral adipose tissue-derived serine protease inhibitor (Vaspin) and omentin-1 (Kwon and Pessin, 2013). Although significant information is available regarding the effects of adipokines in mammals, the metabolic role of these endocrine factors is still not well understood in fish and only few studies are available (Table 1).

Table 1. Depicted pro-inflammatory (top) and anti-inflammatory (bottom) adipokines and their roles in fish metabolism.

Adipokines	Metabolic functions	References
ANGPT2	Activates angiogenesis and increases inflammatory responses	(He et al., 2009)
IL-6	Promotes macrophage growth and inflammatory responses	(Costa et al., 2011; Jørgensen et al., 2000)
Leptin	Satiety signal; inhibits fatty acid uptake and stimulates lipolysis; improves insulin sensitivity	(Salmerón et al., 2015a; Trombley et al., 2014)
TNFα	Lipolytic; increases energy expenditure and induces insulin resistance	(Cruz-Garcia et al., 2009; Wang et al., 2012)
Adiponectin	Increases insulin sensitivity; enhances fatty acid oxidation and glucose uptake	(Sánchez-Gurmaches et al. 2012; Bou et al. 2014)
Apelin	Regulates appetite through anorexigenic effects	(Lin et al., 2014; Volkoff and Wyatt, 2009)
Visfatin	Presents insulin-like effects	(Fujiki et al., 2000)

Abbreviations: ANGPT2, Angiopoietin-Like Protein 2; IL-6, Interleukin 6; TNFα, tumor necrosis factor alpha.

In the recent years, the implication of leptin in regulating appetite and growth in teleost species has been reported (Johnson et al., 2000; Gong et al., 2016). In mammals, leptin reflects fat deposition, regulating food consumption and energy expenditure according to endogenous energy availability. Plasma leptin levels increase postprandially inhibiting appetite and decrease during periods of food deprivation (Weigle et al., 1997). The regulation of appetite through the differential expression of central orexigenic and anorexic neuropeptides is thought to be

conserved between mammals and fish; however, due to gene duplication events, physiological differences in energy storage and diverse life histories, the anorexigenic role of leptin in fish only partially resembles the function found in mammals (Won and Borski, 2013). One difference is that in fish, *leptin* mRNA is more expressed in the liver than in adipose tissue (Kurokawa et al., 2005; Murashita et al., 2008; Gorissen et al., 2009). Moreover, some species exhibit increased plasma or *leptin* mRNA levels during fasting or food restriction (Fuentes et al., 2012; Trombley et al., 2012; Salmerón et al., 2015), whereas other show no leptin modulation by feeding regime (Huising et al., 2006), raising the question whether leptin acts as a lipostatic endocrine signal or might instead drive other functions in fish.

In contrast to the positive correlation of leptin levels with increased adipose tissue mass in mammals, adiponectin is down-regulated in obesity, insulin resistance and metabolic syndrome, positioning itself as a promising candidate for human therapeutic use (Ohashi et al., 2015). Adiponectin, also named Adipo Q, apM1 (adipose most abundant gene transcript 1), GBP28 (gelatin-binding protein) or Acrp30 (adipocyte complement-related protein 30), is a 30 kDa protein secreted by adipose tissue (Hu et al., 1996; Maeda et al., 1996; Nakano et al., 1996; Scherer et al., 1995). Starvation has been shown to produce an increase in adiponectin levels whereas overfeeding and obesity decrease its concentration in rats (Zhou et al., 2005). In addition, adiponectin stimulates FA oxidation in several tissues, decreases plasma TAG levels and improves glucose metabolism by increasing insulin sensitivity (Maeda et al., 2002; Weyer et al., 2001). It binds two different receptors, adiponectin receptor 1 (adipoR1), mainly involved on the regulation of the metabolic functions, and adiponectin receptor 2 (adipoR2), which seems to be more implicated in anti-inflammatory and anti-stress oxidative activities (Miller et al., 2009; Nigro et al., 2013; Yamauchi et al., 2007).

The relationship between this adipokine and lipid homeostasis has been confirmed in zebrafish and rainbow trout (Nishio et al., 2008; Sánchez-Gurmaches et al., 2012). Interestingly, these studies highlighted that while *adipoq* expression is originally identified in adipose tissue in mammals, it is mostly distributed in non-adipose tissues in fish. In fact, Sánchez-Gurmaches and collaborators (2012) confirmed that *adipoq* transcripts are highly expressed in muscle and weakly detected in rainbow trout perivisceral adipose tissue. Nonetheless, this increased *adipoq* expression could be attributed to the adipocyte distribution around and within fish skeletal muscle (Kaneko et al., 2016), altogether indicating that further research is needed in order to fully understand the role and regulation of the adiponectin system in fish.

1.3 MULTIFACTORIAL MODULATION OF ADIPOSE TISSUE

1.3.1 Nutritional factors: the influence of diet

Many dietary factors have been regarded to play a crucial role in the regulation of adipose tissue development and distribution (Melzer et al., 2005). Among them, the macronutrient composition of the diet is the most important. In humans, diets and societal activity patterns have been changing drastically, increasing the reliance upon processed foods as well as adopting sedentary lifestyles. In this regard, much effort has been devoted to investigate the metabolic effects of dietary fat, in order to better understand its potential impact in obesity (Bray and Popkin, 1998; Fava et al., 2013). It is known that high-fat diets promote adipose tissue development showing a direct relationship between the amount of dietary lipid content and the degree of obesity (Golay and Bobbioni, 1997). Furthermore, a direct correlation between high-fat inclusion in diets and the extent of adiposity has been also reported in fish (Grisdale-Helland and Helland, 1997; Landgraf et al., 2017; Panserat et al., 2002). As outlined in section 1.1.2, this pathological increase of fat not only affects key physiological processes in these species, but also has an impact in aquaculture production, as it may alter flesh quality in terms of fillet yield and organoleptic properties.

Besides lipids, the increased reliance on alternative source proteins (i.e. plant-based ingredients) with unbalanced amino acid profiles is showing the need to pay attention to amino acid requirements in fish. Despite the much variability in the literature concerning this, especially depending on the species (Ketola, 1982; Tacon and Cowey, 1985; Wilson and Halver, 1986), excess or deficiency in several essential amino acids has been shown to affect appetite regulation (Nguyen, 2013), growth performance (Choo et al., 1991) and lipid homeostasis (Rathore et al., 2010). For instance, excessive arginine intake reduces growth rate and feed conversion efficiency in Nile tilapia (Santiago and Lovell, 1988) and milkfish (*Chanos chanos,* Borlongan, 1991). Moreover, sub-optimal methionine levels in the diet increase relative liver weight and hepatic FAS activity in Atlantic salmon (Espe et al., 2010), suggesting the direct or indirect implication of this specific amino acid on fish lipid metabolism. In this context, we have investigated the effects of methionine restriction on the response of several metabolism-related genes in rainbow trout fry (Article II).

Furthermore, it is important to remark that plant ingredients contain many natural chemicals. These chemicals, so-called phytonutrients or phytochemicals, are considered to possess a range of properties that might have an impact (positive or negative) on health. On this subject, soy

products are one of the most used ingredients currently incorporated in fish feeds as a non-fish source of essential omega-3 FA and proteins. They have been used as a food additive for human consumption due to their beneficial health effects lowering the risk factors for cardiovascular diseases through the reduction of blood TAG and cholesterol levels (Xiao, 2008), as well as due to their antioxidant (Foti et al., 2005) and anticancer properties (Pavese et al., 2010). Nonetheless, although these studies provide important information about their valuable application in human healthcare, soy foods have become controversial in recent years. Soybean meal products are rich and thus, a primary dietary source of isoflavones, such as genistein and diadzein, which may have a potential impact on growth, hormonal regulation and lipid metabolism in fish. In this regard, it has been shown that genistein depresses the growth performance of Nile tilapia and increases muscle protein degradation in rainbow trout (Chen et al., 2014; Cleveland, 2014).

As well as having an obesogenic effect, dietary composition and feeding strategies may offer practical and efficient solutions for reducing body fat storage deposition. In humans, it has been demonstrated that various plant compounds can improve overweight by inhibiting adipogenic pathways or impairing lipid accumulation (reviewed in González-Castejón & Rodriguez-Casado 2011). Some proposed effects of these plant-derived compounds in mammals include appetite suppression (Tucci, 2010), inhibition of adipocyte differentiation (Andersen et al., 2010) or even apoptosis of fat cells (Rayalam et al., 2008). A number of studies have suggested that several polyphenols such as: phenolic acid derivatives, i.e. chlorogenic acid (Li et al. 2009), flavonols such as quercetin (Pisonero-Vaquero et al., 2015) and stilbenes, i.e. resveratrol (Ahn et al., 2008) can modulate lipid metabolism and the adipocyte lifecycle supporting their potential utilization as dietary anti-obesity agents. Moreover, several functional food ingredients such as components of red wine, olive oil and marine algae are particularly interesting due to their antioxidant properties, which are traditionally linked to longevity and reduced mortality risk of several diseases (González-Molina et al., 2010; Sadowska-Bartosz and Bartosz, 2014; Teixeira et al., 2014). These natural antioxidants have been reported as well to modulate adipose tissue inflammation improving pathological consequences associated with obesity (Huang et al., 2016).

Many recent studies have reported that some phytochemicals and dietary supplements affect the lipid metabolism in fish as well. For instance, adding green tea extract to a regular or a high-fat diet has been shown to exert a potent reduction of body fat in adult rainbow trout and zebrafish (Hasumura et al., 2012; Meguro et al., 2015). Several phytochemicals such as

baicalein, dieckol and kaempferol have been reported to inhibit lipid accumulation in early stages of developing zebrafish (Choi et al., 2015; Lee et al., 2015; Seo et al., 2014). Moreover, the effects of the carotenoid astaxanthin in the modulation of lipid metabolism have been extensively demonstrated in salmonids (Bell et al., 2000; Nakano et al., 1999; Rahman et al., 2016). In this framework, we have evaluated the potential anti-obesogenic effect of three vegetal antioxidants in zebrafish and rainbow trout models (Article I).

1.3.2 Genetic factors: inheriting obesity

There is compelling evidence that inter-individual differences in predisposition to increased fat deposition have strong genetic determinants. Clearly, obesity often tracks in families, even though the family members do not share the same nutritional or exercise habits (Hasselbalch, 2010). On the other hand, it has been previously reported, that some individuals show profound increases of body fat when fed with a high-fat diet, while others fed at the same regime, seem to be more resistant to weight gain suggesting that genetic background is involved. For instance, the experimental A/J and C57BL/KsJ mice strains are relatively resistant to high-fat diet when compared to C57BL/6J (Wang and Liao, 2012). On a bigger scale, some authors explain these genetic variations by differences in the neural encoding for food's preference that predispose some individuals to increase overeating in the presence of high-fat palatable foods (Grimm and Steinle, 2011; Vogel et al., 2017). Others, have postulated different theories supporting the existence of "thrifty genes" that favor some individuals that frequently experienced periods of fasting to increase fat deposition capacity (Neel, 1962). In other words, this theory considers that genetic predisposition to gain weight had a survival advantage. Although it can be a good argument to explain longer survival of "fatty" individuals during times of scarce or no food, this hypothesis has been criticized for several reasons. At first sight, predators would preferentially eat bigger and fatter preys, which would be easier to catch; however, at the same time, fat predators would have more difficulties to catch a prey, suggesting that fatness would be a significant disadvantage. Whatever the case, there is no doubt that these variations in the susceptibility of weight gain have a genetic component. Thus, understanding how genetics plays a role in the development of fat deposition could provide answers not only to treat overweight and obesity but also to highlight molecules and pathways that can be targeted for therapeutic intervention.

On the other hand, these individual differences are also particularly interesting for their potential application in breeding programs for farming industry. In this regard, genetic selection for economically relevant traits has been a common practice for many years in terrestrial animals (Hagedoorn, 1950) and posteriorly in fish (Tave, 1986). This practice, so-called selective breeding or artificial selection, consists in reproducing particular males and females by examining a specific trait (e.g. size, color, disease resistance…) and choosing to breed only those that exhibit better values for that trait (Fig. 7). In fish, growth and flesh quality traits have been widely studied due to their important interest for the industry. In this regard, two experimental rainbow trout lines have been developed through divergent selection for low (Lean line, LL) or high (Fat line, FL) muscle fat content, which in salmonids, has shown to be a highly heritable trait (Quillet et al., 2005). These lines display significant differences in the regulation of glucose and hepatic intermediary metabolism under various dietary regimes (Kolditz et al. 2008; Kamalam et al. 2012). Moreover, recent studies have highlighted the divergent leptin system profiles between the two fish lines, suggesting a different regulation of energy stores and their mobilization (Gong et al. 2016; Johansson et al. 2016). In the present work, we will provide more information regarding the different metabolic regulation of these two rainbow trout genotypes, highlighting their divergent coping mechanisms in response to food deprivation (Article III).

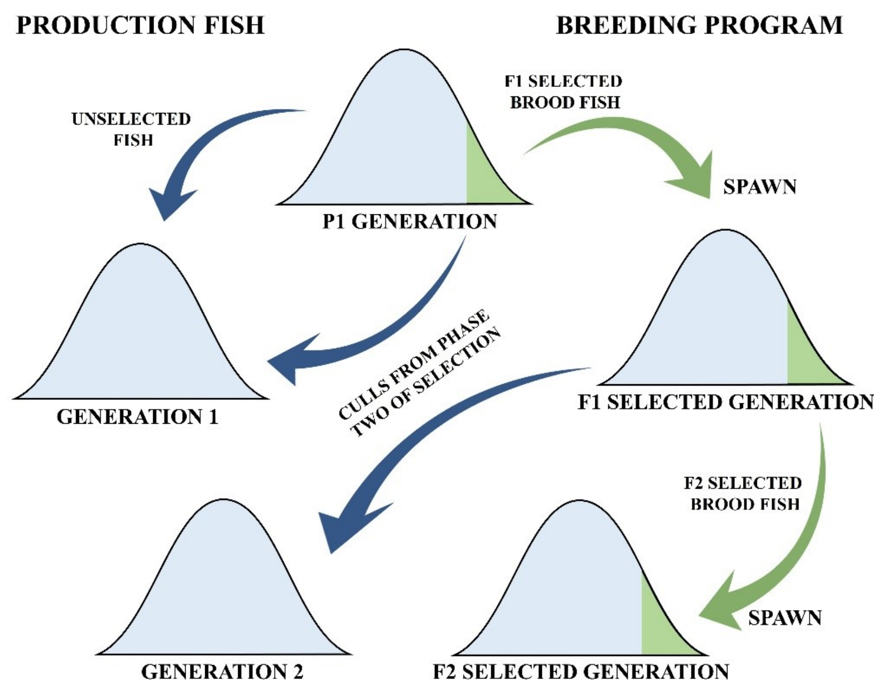


Figure 7. Schematic diagram of breeding selection programs for desirable traits in fish. Adapted from (Tave, 1996).

Recently, particular attention has been drawn on the role of epigenetic changes in determining obesity risk. Several authors have suggested that periconceptional factors, including nutrition and environmental conditions, might influence the risks for many adult health outcomes in the offspring (Feil, 2006; Vickers, 2014). Studies investigating the survivors of the Dutch winter famine of 1944-1945, showed that individuals whose mothers were exposed to famine during early gestation, were more likely to develop obesity and its related diseases. Such phenomenon, so-called nutritional programming, has been largely studied in mammalian models (Burdge and Lillycrop, 2010) and it is supposed to be driven by epigenetic mechanisms. For example, recent studies have shown that mice exposed to soy isoflavones during early neonatal life have improved bone mineral density and higher trabecular inter-connectivity in long bones and lumbar spine at early adulthood (Ward et al., 2016). Although less investigated in fish, studies in rainbow trout have reported that early post-hatch life stimulus with a hyperglucidic diet modulates the expression of specific markers of carbohydrate metabolism and glucose utilization (Geurden et al., 2014, Geurden et al., 2007). Similar results were also found in zebrafish after *in ovo* administrations of glucose (Rocha et al., 2014). Overall, this new approach represents a mechanistic relationship between genetic variation and nutritional factors, and might help to explain the heritable component to obesity. In this regard, we have examined the influence of parental nutrition on offspring metabolism in rainbow trout (Article II).

1.3.3 Environmental factors: the "obesogen" theory

Although less apparent than nutrition and genetics, research into the multifactorial causation of obesity has broaden its scope to potential consequences of environmental factors such as fast food consumption and sedentary lifestyles (Faith and Kral, 2006). In this regard, it has been widely demonstrated that exercise has beneficial effects on body weight, fat storage and endocrine regulation. Indeed, physical activity ameliorates the clinical profile of obese patients delaying at the same time the progression of type II diabetes (Colberg et al., 2010). Moreover, studies in mice have revealed that consumption of a low-fat diet combined with exercise cause significant weight loss along with a decrease in glucose and insulin resistance derived from a high-fat feeding (Carter et al., 2015). On the other hand, sustained exercise in fish is correlated with increases in growth and protein turnover, and several publications have claimed the interest of exercise as a mechanism to improve flesh quality in farmed fish (Palstra and Planas, 2011;

Vélez et al., 2017, Vélez et al., 2016), which is of great interest for aquaculture production. Indeed, it has been shown that moderate and sustained exercise in gilthead seabream can improve not only fish growth and feed conversion but also to induce a reduction of mesenteric fat (Blasco et al., 2015).

Nevertheless, evidence is increasing that exposure to certain environmental compounds, rather than nutrition or life physical activity patterns, could be one of the major determinants of obesity etiology. Thus, it is tempting to assume that the recent increased incidence of obesity and overweight can be associated with the grow in industrialization and the concomitant increase in the use of industrial chemicals over the past decades. This theory proposes the existence of endocrine-disrupting chemicals (EDCs) that can inappropriately regulate particular hormonal signaling pathways and cause adverse metabolic effects, altering fat cell programming and increasing energy storage in adipose tissue (Holtcamp, 2012). These compounds, so-called "obesogens", predispose an exposed individual to subsequent weight gain through different mechanisms of action. Some of them promote fat accumulation through direct or indirect ways, increasing lipid storage in fat cells (hypertrophy) and/or the number of adipocytes (hyperplasia), while others affect hormonal signaling, distressing at the same time appetite regulation and lipid homeostasis (Lustig, 2011).

To date, many obesogens have been identified; supporting the idea that exposure to such chemicals may play an unpredicted role in the obesity epidemic. Among these, estrogenic EDCs such as bisphenol A (BPA) (Rubin et al., 2001), atrazine (Tousignant and Uno, 2015) and dichlorodiphenyltrichloroethane (DDT) (Skinner et al., 2013); organotins like tributyltin (TBT) and triphenyltin (TPT) (Grün and Blumberg, 2009; Kirchner et al., 2010) and phthalates (Hao et al., 2013) induce fat accumulation in animals. For instance, long-term exposure to the herbicide atrazine contributes to the development of insulin resistance and obesity in mice (Lim et al., 2009). On the other hand, mice fed a high-fat diet and exposed to BPA, are shown to consume more food and gain more weight than control animals on the same diet, suggesting a neurological regulation of appetite (MacKay et al., 2013).

Furthermore, in mammals, it has been shown that the metabolic programming of obesity risk may be linked to lifetime contact to obesogenic chemicals but also to perinatal exposure during pregnancy. This early exposure to chemicals in the environment can modify normal cellular and tissue development and function, even at the level of stem cell determination (Heindel et al., 2015). Transgenerational inheritance of obesity resulting from exposure to different

environmental obesogens has been demonstrated (Janesick et al., 2014). For instance, prenatal exposure to TBT is shown to promote obesity in mice generations by permanently altering the development of fat and liver cells through activation of PPARγ and retinoid X receptor (RXR) pathways (Chamorro-García et al., 2013). On the other hand, exposure to phthalates in pregnant mice lead to increased body weight and adipocyte size in male offspring suggesting a sexually dimorphic effect of this compound (Hao et al., 2012).

Some of these chemicals have been utilized as biocides, wood preservatives and antifouling paints for many years, and can be found in significant concentrations in aquatic environments (Boyer, 1989; Fent, 1996). Among them, TBT and TPT are considered the most relevant in aquatic environments, with a proven toxicity risk to humans and wildlife (Antizar-Ladislao, 2008). It has been well documented the endocrine disrupting potential of TBT on shell thickening in oysters (Alzieu et al., 1986), imposex and reduction of growth in mollusks (Birchenough et al., 2002; Salazar and Salazar, 1991) as well as altered sex ratio and suppressed fertility in some fish species (McAllister and Kime, 2003; McGinnis and Crivello, 2011; Santos et al., 2006). However, despite these evidences, there are limited data on the effects of these compounds on fat accumulation and adipogenesis disruption in fish. In this context, we have examined the potential obesogenic effects of these organotins on the regulation of adipogenesis on primary cultured adipocytes in rainbow trout (Article IV).

CHAPTER 2. OBJECTIVES

The overall purpose of the present thesis is to improve the knowledge on the role of adipose tissue in the regulation of lipid homeostasis in fish, specially focusing in adiposity dynamics and the influence of nutritional, genetic and environmental factors. The works presented using zebrafish and rainbow trout models, provide an integrative view of adipose tissue biology related to obesity with potential applications in both, aquaculture and biomedical research.

Specific aims:

1- To investigate the nutritional regulation of lipid metabolism and fat deposition by several vegetal compounds or a dietary essential amino acid deficiency.

   a. To evaluate the anti-obesogenic properties of three antioxidants of plant origin in zebrafish and rainbow trout models and, to determine their specific effects modulating lipid metabolism in adipose tissue through the PPARγ signaling pathway. (Article I)

   b. To assess the effects of different dietary methionine levels on the response of several fat metabolism-related genes in rainbow trout fry. (Article II)

2- To determine the genetic influence on lipid homeostasis with special focus on the effects of nutritional programming and breeding selection.

   a. To evaluate the consequences of feeding rainbow trout broodstock with a diet deficient in methionine on the response of several metabolism-related genes in the offspring. (Article II)

   b. To examine the effects of fasting on the regulation of lipid metabolism, the adiponectin system and oxidative stress status at a transcriptional level, in two experimental rainbow trout lines established through divergent selection for low or high muscle fat content. (Article III)

3- To evaluate the environmental causation of fat accumulation by investigating the obesogenic potential of two organotin compounds on adipocyte development and lipid homeostasis in rainbow trout. (Article IV)

CHAPTER 3. SUPERVISORS' REPORT

UNIVERSITAT DE BARCELONA

Department of Cell Biology, Physiology and Immunology
Faculty of Biology

Edifici Ramon Margalef (plantas 3-A & 4-A)
Av. Diagonal, 643
E-08071 Barcelona
Tel: +34 934021531 Fax: +34 934110358
e-mail: dept-fisiologia@ub.edu

B:KC Barcelona Knowledge Campus

La Dra Encarnación Capilla Campos y la Dra. M. Isabel Navarro Álvarez, como directoras de la tesis doctoral presentada por Esmail Lutfi Royo titulada "Obesity and adipose tissue biology in fish: influence of nutritional, genetic and environmental factors" manifiestan la veracidad del factor de impacto y la implicación del doctorando en los artículos científicos publicados o pendientes de publicación presentados en esta tesis.

Esmail Lutfi Royo participado de forma muy activa en la elaboración de los artículos en todos los aspectos, tal y como queda reflejado en la relación de autores, ya que consta como primer autor en la mayoría de ellos (artículos 1, 3 y 4). Asimismo, Esmail ha contribuido de manera principal en el planteamiento y la realización de los experimentos, obtención de datos, análisis de resultados, así como en la redacción y elaboración de dichos artículos. En el artículo 2, aunque no consta como primer autor, el doctorando ha sido el que ha realizado todos los procesos de análisis y obtención de los resultados relacionados con el metabolismo, así como su interpretación. Por ello, sólo dichos resultados y no los relacionados con el proceso de autofagia son presentados en esta tesis. Los artículos 1 y 2 derivan de dos estancias realizadas por Esmail en los laboratorios del Dr. Patrick J. Babin en la Universidad de Bordeaux y de la Dra. Sandrine Skiba-Cassy del INRA en Saint-Pée-sur-Nivelle, respectivamente. El artículo 3 se realizó en colaboración con el grupo del Dr. Björn Thrandur Björnsson de la Universidad de Göteborg que aportó el diseño experimental. Finalmente, el artículo 4 se realizó contando con la colaboración de la Dra. Cinta Porte del IDAEA-CSIC.

Artículo I: Caffeic acid and hydroxytyrosol have anti-obesogenic properties in zebrafish and rainbow trout models.

Autores: Esmail Lutfi, Patrick Babin, Joaquim Gutiérrez, Encarnación Capilla and Isabel Navarro

Revista: Plos One 12(2017) e0178833

Factor de impacto: **3,06** JCR 2015 (Q1)

Estado: Publicado

Artículo II: Eating for two: Consequences of parental methionine nutrition on offspring metabolism in rainbow trout (*Oncorhynchus mykiss*).

Autores: Iban Seiliez, Emilio J. Vélez, Esmail Lutfi, Karine Dias, Elisabeth Plagnes-Juan, Lucie Marandel, Stéphane Panserat, Inge Geurden and Sandrine Skiba-Cassy.

Revista: Aquaculture 471 (2017) 80–91

Factor de impacto: **1,89** JCR 2015 (Q2)

Estado: Publicado

Artículo III: Breeding selection of rainbow trout for high or low muscle adiposity has differential effects on lipid metabolism and oxidative stress.

Autores: Esmail Lutfi, Ningping Gong, Marcus Johansson, Albert Sánchez-Moya, Björn Thrandur Björnsson, Joaquim Gutiérrez, Isabel Navarro and Encarnación Capilla

Revista: Plos One

Factor de impacto: **3,06** JCR 2015 (Q1)

Estado: Pendiente de enviar

Artículo IV: Tributyltin and triphenyltin exposure promotes *in vitro* adipogenic differentiation but alters the adipocyte phenotype in rainbow trout

Autores: Esmail Lutfi, Natàlia Riera-Heredia, Marlon Córdoba, Cinta Porte, Joaquim Gutiérrez, Encarnación Capilla and Isabel Navarro

Revista: Aquatic Toxicology 188 (2017) 148–158

Factor de impacto: **3,56** JCR 2015 (Q1)

Estado: Publicado

Barcelona, Junio 2017

Dra. M. Isabel Navarro　　　　Dra. Encarnación Capilla

CHAPTER 4. ARTICLES

ARTICLE I

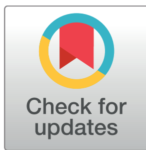
RESEARCH ARTICLE

Caffeic acid and hydroxytyrosol have anti-obesogenic properties in zebrafish and rainbow trout models

Esmail Lutfi[1], Patrick J. Babin[2]*, Joaquim Gutiérrez[1], Encarnación Capilla[1], Isabel Navarro[1]*

1 Department of Cell Biology, Physiology and Immunology, Faculty of Biology, University of Barcelona, Barcelona, Spain, **2** Maladies Rares: Génétique et Métabolisme (MRGM), University of Bordeaux, INSERM, U12211, Pessac, France

* mnavarro@ub.edu (IN); p.babin@gpp.u-bordeaux1.fr (PJB)



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Abstract

Some natural products, known sources of bioactive compounds with a wide range of properties, may have therapeutic values in human health and diseases, as well as agronomic applications. The effect of three compounds of plant origin with well-known dietary antioxidant properties, astaxanthin (ATX), caffeic acid (CA) and hydroxytyrosol (HT), on zebrafish (*Danio rerio*) larval adiposity and rainbow trout (*Onchorynchus mykiss*) adipocytes was assessed. The zebrafish obesogenic test (ZOT) demonstrated the anti-obesogenic activity of CA and HT. These compounds were able to counteract the obesogenic effect produced by the peroxisome proliferator-activated receptor gamma (PPARγ) agonist, rosiglitazone (RGZ). CA and HT suppressed RGZ-increased PPARγ protein expression and lipid accumulation in primary-cultured rainbow trout adipocytes. HT also significantly reduced plasma triacylglycerol concentrations, as well as mRNA levels of the *fasn* adipogenic gene in the adipose tissue of HT-injected rainbow trout. In conclusion, *in vitro* and *in vivo* approaches demonstrated the anti-obesogenic potential of CA and HT on teleost fish models that may be relevant for studying their molecular mode of action. Further studies are required to evaluate the effect of these bioactive components as food supplements for modulating adiposity in farmed fish.

Introduction

Obesity has become a worldwide epidemic and is considered one of the most serious public health problems of our time [1,2]. Overweight and obesity occur when energy intake exceeds energy expenditure, leading to increased storage of triacylglycerols (TAG), mainly in white adipose tissue (WAT). In addition to TAG storage in adipocyte lipid droplets, WAT has been recognized as a multi-functional endocrine organ that plays a critical role in modulating several physiological processes, such as appetite, whole-body energy metabolism and homeostasis, as well as tissue inflammation responses [3]. Consequently, concomitant with enlarged fat

Competing interests: The authors have declared that no competing interests exist.

storage, pathological overgrowth of WAT is associated with a range of related problems, including type II diabetes, insulin resistance, hypertension and cardiovascular diseases [4].

One of the key molecules that modulates WAT activity in response to extrinsic signals is peroxisome proliferator-activated receptor gamma (PPARγ), a master regulator of adipogenesis that activates the transcription of a large number of genes involved in adipocyte differentiation and lipid accumulation [5]. Furthermore, PPARγ controls the expression of many factors secreted by WAT that influence insulin sensitivity, which in turn, modulate the expression of genes involved in glucose homeostasis [6]. Impaired PPARγ signaling, expression and/or activation are thus implicated in the prevalence of metabolic obesogenesis and weight-related diseases, such as diabetes. The most widely studied therapeutic use of PPARγ has been in the treatment of insulin resistance and type II diabetes. Synthetic ligands/agonists of PPARγ, e.g. thiazolidinediones, commonly used as insulin sensitizers for treating hyperglycemia in patients with type II diabetes, are of great clinical significance [7]. Nevertheless, despite their effectiveness in normalizing blood glucose levels, these compounds present detrimental side effects, such as weight gain, edema and cardiovascular complications [8]. Thus, the discovery or development of new compounds that modulate the PPARγ signaling pathway more effectively and safely, while promoting health benefits, is currently a matter of great interest.

Throughout history, natural products have provided a rich source of inspiration for drug discovery. Significant research has recently been undertaken to identify PPARγ modulators, with the aim of formulating a novel treatment to maximize antiobesity effects, in addition to antioxidant and protective properties [9]. Natural antioxidants modulate WAT inflammation produced by the overproduction of reactive oxygen species or pathological processes associated with obesity. While caffeic acid (CA), hydroxytyrosol (HT) and astaxanthin (ATX) are interesting examples of dietary compounds with proven antioxidant properties [10–12], their specific potential for treating obesity has not been fully recognized. Furthermore, the increasing use of plant-based aquafeeds has aroused great interest in the identification of new vegetal ingredients that may respond not only to the demand for sustainable aquaculture, but also, to help develop new diets that may reduce unwanted perivisceral WAT in farmed fish.

In basic research, mammalian models (e.g. mice and rats primarily) have been traditionally used in human physiology and disease research, due to their anatomical and physiological similarities [13]. Nevertheless, they could be unsuited for certain types of studies [14]. In the past decade, teleost species have been regarded as excellent alternative models for studying human diseases [15,16] and now constitute an emerging method for assessing bioactive compounds in food research [17]. A number of *in vitro* and *in vivo* studies have highlighted the applicability of several fish species within the areas of lipid metabolism and adipose tissue biology [18–23]. Besides its simplicity and numerous other advantages, fish research models such as zebrafish (*Danio rerio*) or rainbow trout (*Onchorynchus mykiss*), are very promising for obesity research, as most of the metabolic pathways linked to the lipid metabolism are conserved between mammals and teleost fish [24–26]. Indeed, histological studies have revealed also evolutionarily conserved morphological structures of teleost adipocytes [27–29].

In this study, three selected antioxidant dietary compounds (CA, HT, and ATX) were used *in vitro* and *in vivo*, to assess their potential anti-obesogenic effect on zebrafish and rainbow trout models.

Materials and methods

Animal care and ethics statement

Wild-type zebrafish were produced in our facilities at the University of Bordeaux in accordance with the French Directive (Ministère de l'Agriculture, de l'Agroalimentaire et de la

Forêt), under permit number A33-522-6. All experiments were conducted in conformity with the European Communities Council Directive (2010/63/EU) on the protection of animals used for scientific purposes and local French legislation on the care and use of laboratory animals. Larvae were obtained by natural mating and raised in embryo water (90 μg/ml Instant Ocean [Aquarium Systems, Sarrebourg, France], 0.58 mM $CaSO_4$, $2H_2O$, dissolved in reverse-osmosis purified water) at 28.5˚C with an 11L:13D photoperiod. From 5 days postfertilization until day 15, larvae were fed *ad libitum* on ZF Biolabs formulated diet flakes (Tres Cantos, Spain). They were then nourished with standard diet (SD) for late larvae (TetraMin Baby, Tetra GmbH, Melle, Germany). Animal stages were recorded according to standard length, i.e. the distance from the rostral tip of the larva to the base of the caudal fin.

Juvenile rainbow trout, body weight approximately 80 g for *in vivo* studies and 250 g for extracting WAT to be used in adipocyte primary cultures, were obtained from the "Viveros de los Pirineos" fish farm (El Grado, Huesca, Spain). Animals were maintained according to the Ethics and Animal Care Committee of the University of Barcelona, following the regulations and procedures established by the Spanish and Catalan governments (CEEA 170/14, CEEA 311/15, DAAM 7952).

Reagents

HT (ref. 70604, CAS N˚10597-60-1) and rosiglitazone (RGZ) (ref. 71740, CAS N˚122320-73-4) were purchased from Cayman chemicals (Ann Arbor, MI). CA (ref. C0625, CAS N˚331-39-5), sesame oil (ref. S3547), DMSO (ref. D8418), and ethyl 3-aminobenzoate methanesulfonate (MS-222) (ref. E10521) were provided by Sigma-Aldrich (Tres Cantos, Spain). Certified analytical grade ATX (ref. DRE-CA10307000, CAS N˚472-61-7) was purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Stock solutions were stored at -20˚C and working solutions were diluted in 0.1% DMSO on the day of the experiment.

Zebrafish obesogenic test (ZOT)

The short-term ZOT assay, using Nile red staining, is a non-invasive *in vivo* method for visualizing the effects of the molecules tested on the adiposity dynamics of zebrafish larvae by fluorescence microscopy. The 3-day, *in vivo*, animal treatment protocol was performed as previously described [30]. In replicated experiments, ten larvae ranging from 7 to 9 mm standard length were used per group. Larvae were exposed to the selected compounds or to vehicle alone for one day in a fasting state. All larvae were kept in a fasting state for 24 h prior to exposure and until the end of the trial, to avoid food auto-fluorescence. Treatments were as follows: vehicle control (CT) (0.1% DMSO), CA (0.1% DMSO plus 50 μM CA), HT (0.1% DMSO plus 100 μM HT), and ATX (0.1% DMSO plus 100 μM ATX). Concentrations of each molecule tested were the highest that did not induce any mortality. Quantitative analysis was performed, as previously described [30], by recording the image area of Nile red green fluorescence as a percentage of initial adiposity, using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Rainbow trout adipocyte cell cultures

All cell-culture reagents were purchased from Sigma-Aldrich (Tres Cantos, Spain) and Life Technologies (Alcobendas, Spain). All plastic items and glass cover slips were obtained from Nunc (LabClinics, Barcelona, Spain). Cells were cultured according to the previously established procedure [31], with perivisceral WAT from four to six fish per culture. After counting, cells were seeded at a final density of 2–2.5·10^4 cells/cm^2 in 1% gelatin on pretreated six-well plates (9.6 cm^2/well) for real-time quantitative PCR (qRT-PCR) analyses or twelve-well plates

($2.55\ cm^2$/well), with or without coverslips, for immunofluorescence or Oil red O (ORO) staining, respectively. Plates were kept at 18˚C in Leibovitz's L-15 growth medium, supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic solution (growth medium, GM). When necessary, the standard procedure used for cell differentiation was the following: once confluence was reached (day 7), cells were induced to differentiate by incubating them with a differentiation medium (DM) based on GM and containing 10 μg/mL insulin, 0.5 mM 3-isobutyl-1-methylxanthine and 0.25 μM dexamethasone. The medium was changed every 2 days throughout the procedure.

Immunofluorescence assay

Post-confluent pre-adipocyte cells from day 7 of culture were incubated with vehicle CT (DM ± 0.1% DMSO) or vehicle plus CA (50 μM), HT (100 μM) or RGZ (1 μM), or a combination of CA or HT with RGZ, for 24 h. RGZ was used as a potential rainbow trout peroxisome proliferator-activated receptor gamma (PPARγ) agonist. PPARγ was detected by immunofluorescence, using the protocol described by [32]. The polyclonal rabbit anti-PPARγ (H-100) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary Alexa Fluor® conjugated antibody (A21069, goat anti-rabbit 568) was purchased from Life Technologies (Alcobendas, Spain). Nuclei were counterstained with Hoechst (H1399, Life Technologies, Alcobendas, Spain). Images were obtained at 36x magnification on a Leica TCS-SP5 confocal microscope. Nucleus fluorescence was quantified and normalized to the total number of nuclei in the same field, using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Western blot analysis

Post-confluent pre-adipocyte cells from day 7 of culture were treated as described in the previous immunofluorescence section. Protein extraction and Western blot analysis were performed using the protocol described by [32]. Briefly, the amount of protein from each sample was measured [33] and 20 μg were subjected to electrophoresis (SDS-PAGE) on 10% polyacrylamide gels (125 V for 1 h 30 min). After overnight transfer to a PVDF membrane, a staining with Ponceau S solution (Sigma-Aldrich, Tres Cantos, Spain) was performed, showing similar amounts of transferred proteins on each lane and membranes were scanned for posterior band quantification. Subsequently, membranes were washed and then blocked in non-fat milk 5% and incubated with polyclonal rabbit anti-PPARγ (H-100), an antibody that has been previously shown to successfully cross-react with rainbow trout [31,34]. After washing, membranes were incubated with a peroxidase-conjugated secondary goat anti-rabbit antibody (Cat. No. 31460. Thermo Scientific, Alcobendas, Spain). The immunoreactive band was visualized using an enhanced chemiluminescence kit (Pierce ECL Western blotting Substrate; Thermo Scientific, Alcobendas, Spain) and quantified by densitometric scanning using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Results from the densitometry analysis of each specific band were normalized by the densitometry values of the most abundant band of Ponceau S staining as previously reported [35]. In support of this methodology, it has been shown that reversible Ponceau S staining can be used advantageously over specific proteins detection for quality or control of equal loading in Western blotting [36,37].

Oil red O staining and lipid quantification

After confluence (day 7), cells were incubated with vehicle CT (DM ± 0.1% DMSO) or vehicle plus CA (50 μM), HT (100 μM), LIP (10 μl/ml) or the indicated combination of CA or HT with LIP, for 72 h. Cell differentiation and lipid accumulation were analyzed by ORO staining, as described elsewhere [38], with minor modifications. Briefly, cells were fixed with 10%

formalin for 1 h. Fixed cells were rinsed with PBS, stained with 0.3% ORO prepared in 36% tri-ethyl phosphate for 2 h, and then rinsed three times with distilled water. A 100% 2-propanol solution was used to elute the ORO dye and absorbance was measured at 490 nm. The cells were then stained with Coomassie blue for 1 h and proteins were extracted using 85% propylene glycol at 60˚C for 1 h. Lipid quantification was calculated as the absorbance measured at 490 nm divided by the measurement corresponding to protein at 630 nm.

Cell viability assay

Pre-confluent pre-adipocyte cells (day 5 of culture) were incubated with vehicle CT (GM) or vehicle plus CA (50 μM), HT (100 μM), RGZ (1 μM), or a combination of CA or HT with RGZ, for 24 h. The methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay was performed as previously described elsewhere [38]. Briefly, after 24 h incubation with a final concentration of 0.5 mg/mL MTT, cells were washed with PBS and the blue formazan crystals that formed were resuspended in 250 μL DMSO per well for 2 h. Cell viability values were obtained from the absorbance measured at 570 nm, with 680 nm as the reference wavelength, using a microplate reader (Infinite 200, Tecan).

Measuring cell proliferation

Pre-confluent pre-adipocyte cells (day 5 of culture) were incubated as described in the previous cell viability section. Cell proliferation was evaluated by immunocytochemical detection of proliferating cell nuclear antigen (PCNA), using a commercial staining kit (Cat. No. 93–1143, Life Technologies, Alcobendas, Spain). In brief, after 24 h incubation (see above), cells were washed and fixed in 4% paraformaldehyde (PFA, Sigma-Aldrich, Spain) at room temperature for 15 min. Subsequently, coverslips were post-fixed in 50% and 70% ethanol for 5 min and incubated in PCNA staining reagents, following the manufacturer's suggested protocol. The amount of PCNA-labeled nuclei (positive cells) was evaluated and normalized to the number of nuclei in that field, using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Five to ten images were taken per coverslip with a CC2 camera coupled to a microscope at 40x using analySIS (Soft Imaging System) software.

In vivo experimental treatment in rainbow trout

After 15 days' acclimation in our facilities at 15˚C, juvenile rainbow trout fasted for 24 h were then anesthetized with MS-222 (0.1 g/L) prior to receiving an intraperitoneal injection of 4.64 μL volume per g body weight. Treatments were as follows: vehicle CT containing DMSO diluted in sesame oil (1:3, v/v), vehicle plus CA at 10 μg/g body weight, and vehicle plus HT at 20 μg/g body weight. After 24 h exposure to the compounds in a fasting state, trout were anesthetized, sacrificed by a blow to the head, and blood samples were taken from the caudal aorta. Liver and perivisceral WAT samples were then harvested and preserved at −80˚C pending analysis. None of the molecules tested induced mortality at the concentrations used.

Biochemical analysis of plasma parameters

Plasma samples were analyzed using commercial enzyme kits: glucose (Monlab, Barcelona, Spain), non-esterified fatty acids (NEFAs, Wako Chemicals GmbH, Neuss, Germany), and TAG and glycerol (Sigma-Aldrich, Tres Cantos, Spain).

RNA extraction, cDNA synthesis and qRT-PCR

Total RNAs from WAT (100 mg), liver (50 mg), and primary adipocyte cells (3 wells) were extracted using TriReagent (Ambion, Alcobendas, Spain), according to the manufacturer's recommendations. Treatments for *in vitro* studies were as follows: vehicle CT (DM ± 0.1% DMSO) or vehicle plus CA (50 μM), HT (100 μM), RGZ (1 μM), LIP (10 μl/ml) or the indicated combination of CA or HT with RGZ or LIP, for 24 h. A ND-2000 NanoDrop spectrophotometer (Thermo Fisher Scientific, Alcobendas, Spain) was used to quantify isolated RNAs and 500 ng (cell culture) or 1 μg (tissue) samples of total RNAs were treated with DNase I (Life Technologies, Alcobendas, Spain), following the manufacturer's protocol, to remove all genomic DNA. Afterwards, total RNAs were reverse transcribed with the Transcriptor First Strand cDNA synthesis Kit (Roche, Sant Cugat del Valles, Spain). qRT-PCR was performed as previously described [39]. All the analyses were performed in triplicate using 384-well plates with 2.5 μL itaq SYBR Green Supermix (Bio-Rad, El Prat de Llobregat, Spain), 250 nM forward and reverse primers, and 1 μL cDNA for each sample, in a final volume of 5 μL. The primers were specific for acyl-CoA synthetase-1 (*acsl1*), beta-actin (*actb*), CCAAT/enhancer-binding protein alpha (*cebpa*), elongation factor 1 alpha (*ef1α*), fatty acid synthase (*fasn*), 3-hydroxyacyl-CoA dehydrogenase (*hoad*), hormone sensitive lipase (*lipe*), lipoprotein lipase (*lpl*), adipose triglyceride lipase (*pnpla2*), peroxisome proliferator activated receptor beta (*pparb*) and gamma (*pparg*), and *ubiquitin* (S1 Table).

Statistical analyses

Data were analyzed using IBM SPSS Statistics v.22 (IBM, Armonk, USA) and GraphPad prism 6 (La Jolla, USA, www.graphpad.com) and presented as mean ± SEM, unless stated otherwise. Normal distribution was first analyzed using the Shapiro-Wilk test, followed by Levene's to test homogeneity of variances. Statistical significance was assessed by two-tailed unpaired Student's t-test or one-way analysis of variance (ANOVA), followed by the Tukey *post-hoc* test. When data did not follow a normal distribution, a non-parametric Kruskal-Wallis test was applied, followed by a paired U-Mann Whitney test. Statistical differences were considered significant for all analyses when p-value ≤ 0.05, indicated by asterisks: $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.0001$.

The mRNA level of each gene analyzed was calculated relative to the corresponding reference genes, i.e. geometric means of *ef1a* and *ubiquitin* for the *in vitro* cell culture assays and *ef1a* and *actb* for *in vivo* assays, using the Pfaffl method [40] implemented in the Biorad CFX manager 3.1 (Bio-Rad, El Prat de Llobregat, Spain).

Results

Identifying compounds of plant origin with antioxidant properties able to decrease zebrafish larval adiposity in vivo

To investigate the effect of CA, HT and ATX on the dynamics of zebrafish adiposity, ZOT was used as a non-invasive *in vivo* method for visualizing lipid droplets in living larvae by fluorescence microscopy [30]. The whole-body adiposity dynamics of each larva was expressed as a percentage of variation in Nile red fluorescence signal areas in fish previously fed on standard diet (SD) after one-day's exposure in a fasting state. The results revealed that exposure to 50 μM CA significantly decreased larval adiposity compared to CT-exposed fish (−-22.83 ± 1.02% in CA *versus* −16.99 ± 1.29% with CT, $p \leq 0.01$) (Fig 1A). The same effect was observed with 100 μM HT (−29.72 ± 2.74% in HT *versus* −17.98 ± 1.97% with CT, $p \leq 0.05$)

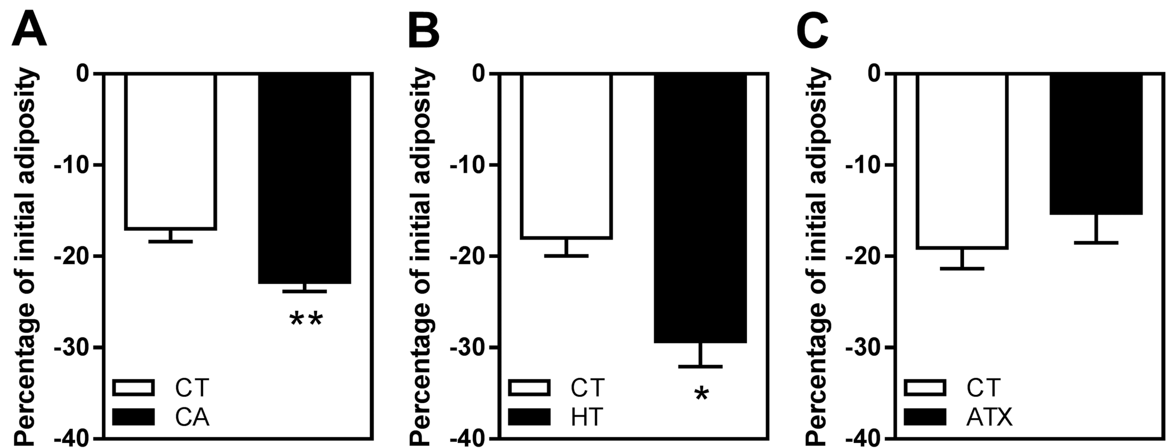

Fig 1. Identification of selected molecules of plant origin able to decrease adiposity *in vivo*. ZOT was conducted on larvae with a standard-length distribution from 7 to 9 mm and initially nourished with SD. Adiposity was quantified in fasting larvae in fish water with 0.1% DMSO as a vehicle control (CT) or 0.1% DMSO plus 50 μM CA (A), 100 μM HT (B), or 100 μM ATX (C). For each larva enrolled, WAT dynamics is expressed as a percentage of initial adiposity. Values are mean ± SEM, n = 4–6 independent experiments (10 animals per group). *$P \leq 0.05$, **$P \leq 0.01$ compared to CT, using two-tailed unpaired Student's t-test.

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(Fig 1B). In contrast, there was no change in adiposity following exposure to 100 μM ATX (Fig 1C).

Differential effect of CA and HT on WAT dynamics in different body parts

ZOT was used to study WAT dynamics in different body parts of zebrafish larvae, during exposure to the selected bioactive compounds. Perivisceral WAT accounted for around 60% of adiposity at these zebrafish larval developmental stages (S1 Fig). The remaining WAT in the head and tail regions represented around 30% and 10%, respectively (S1 Fig). Results showed that exposure to CA mainly affected adiposity in the head region, compared to the same region in CT fish (−10.85 ± 1.95% in CA *versus* −5.53 ± 0.54% with CT, $p \leq 0.05$) (Fig 2A). HT also led to a significant decrease in adiposity in the head (−11.46 ± 1.03% in HT *versus* −7.95 ± 0.24% with CT, $p \leq 0.05$) and viscera (−15.87 ± 2.23% in HT *versus* −7.13 ± 2.46% with CT, $p \leq 0.05$) compared to CT (Fig 2F). In contrast, ATX did not have a significant anti-obesogenic effect in any of the body regions (Fig 2K). Fluorescent images of isolated or grouped adipocytes revealed a marked decrease in oil droplet size following treatment with CA or HT (Fig 2C, 2E, 2H and 2J).

Potential antagonist effect of CA and HT on PPARγ signaling pathway

In view of the significant decrease in zebrafish larvae adiposity observed after treatment with CA and HT, the next step was to elucidate whether these compounds were capable of counter-acting the obesogenic effect of RGZ, a potential PPARγ agonist, *in vivo*. RGZ at 1 μM on an SD background diet was shown to prevent adiposity loss in fasting condition: −10.59 ± 1.35% in RGZ *versus* −18.1 ± 1.52% with CT, $p \leq 0.01$ (Fig 3A) and −14.23 ± 0.98% in RGZ *versus* −20.04 ± 0.88% with CT, $p \leq 0.05$ (Fig 3B). Simultaneous exposure of the larvae to RGZ and CA revealed that the obesogenic effect of RGZ was abolished by this compound (−-19.24 ± 0.40%) (Fig 3A). A similar effect was obtained following co-incubation with RGZ and HT (−23.47 ± 2.17%) (Fig 3B).

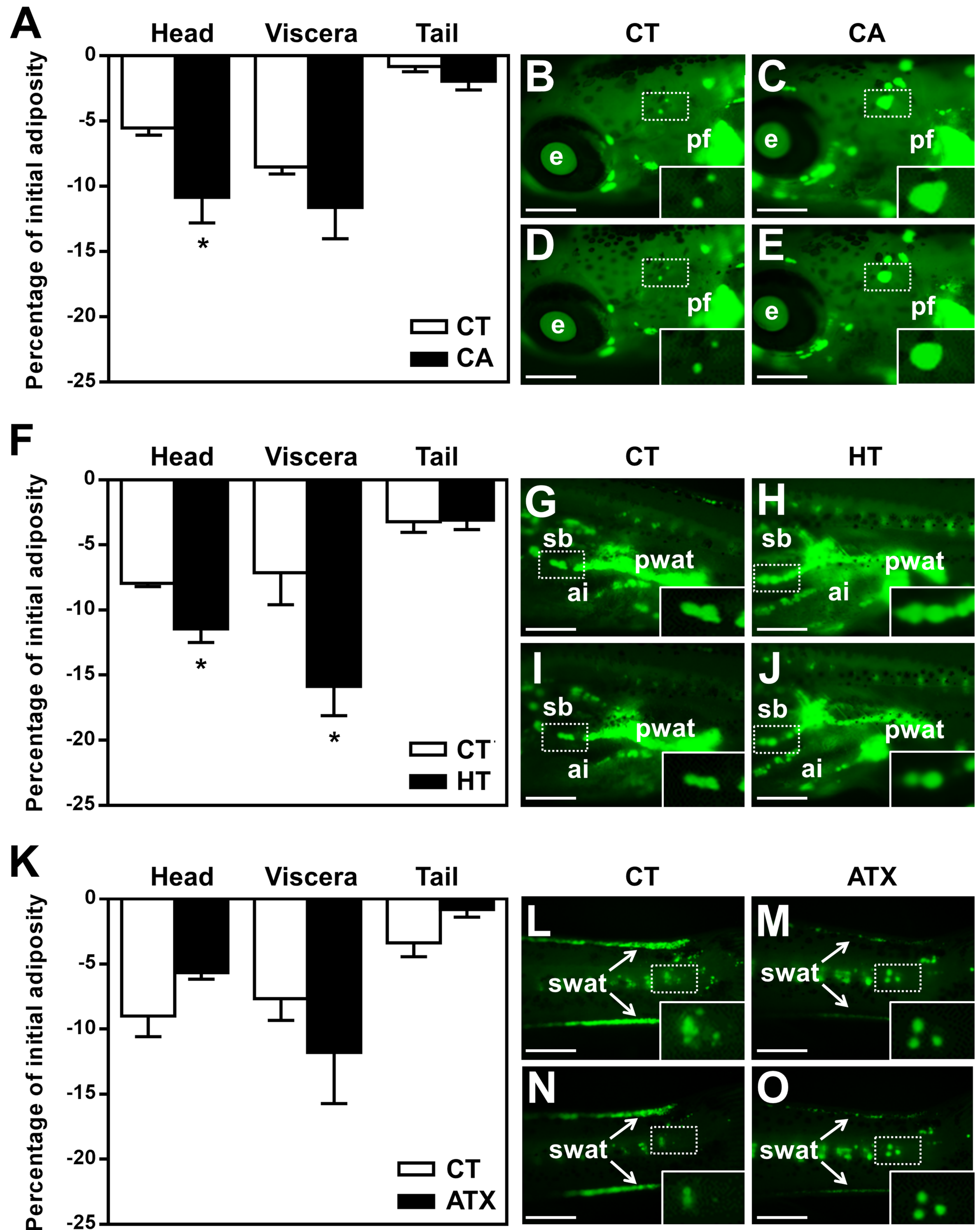

Fig 2. Differential effect of selected vegetal molecules of plant origin on WAT dynamics in different body parts of zebrafish larvae. Quantitative analysis of WAT dynamics was performed according to the ZOT protocol and the results are expressed as a percentage of initial adiposity relative to the amount of WAT fluorescence signal attached to each body region. Fasting larvae were exposed to 50 μM CA in 0.1% DMSO (A, C, E), 100 μM HT in 0.1% DMSO (F, H, J), 100 μM ATX in 0.1% DMSO (K, M, O), or 0.1% DMSO as a vehicle CT (A, B, D, F, G, I, K, L, N). Images of relevant regions in representative larvae are presented: head (B-E), viscera (G-J), and tail (L-O). Lateral views, anterior part on the left and dorsal part at the top, under fluorescence microscope after Nile red staining, recorded before (B, C, G, H, L, M) and after 24 h treatment (D, E, I, J, N, O) with

(CA, HT, ATX) or without (CT) exposure to the compounds. Insets in each image are enlarged views of isolated adipocytes or groups of adipocytes from each panel, marked by a white rectangle. Values are mean + SEM, n = 4–6 independent experiments (10 animals per group). *$p \leq 0.05$ compared to control for each region, using Student's t-test. Scale bar, 500 μm. Abbreviations: e, eye; pf, pectoral fin; pwat, perivisceral white adipose tissue; sb, swim bladder; ai, anterior intestine; swat, subcutaneous white adipose tissue.

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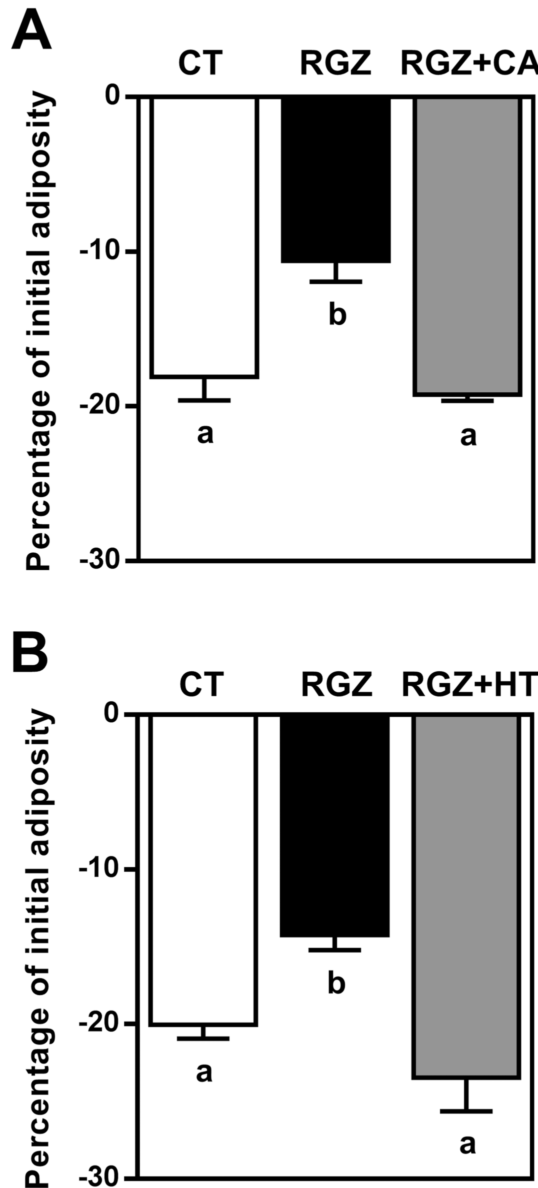

Fig 3. CA and HT abolished the *in vivo* obesogenic effect of RGZ. ZOT was applied to larvae previously nourished with SD. Adiposity was quantified in the presence of 0.1% DMSO as a vehicle CT or 0.1% DMSO plus the indicated combination of compounds. Exposure to 1 μM RGZ induced a significantly smaller decrease in adiposity compared with CT. The effect of RGZ was abolished by CA 50 μM (A) and HT (100 μM) (B). Values are mean ± SEM, n = 4 independent experiments (10 animals per group). Significant differences are shown as different letters ($p \leq 0.05$) using one-way ANOVA test followed by Tukey's *post hoc* test.

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Characterization of CA and HT effects on primary-cultured rainbow trout adipocytes

CA and HT significantly increased adipocyte viability (S2A Fig) without affecting proliferation (S2B Fig), whether the cells were co-treated with RGZ or not. RGZ alone or in combination with treatments did not affect cell viability or the percentage of PCNA-positive cells (S2 Fig). Quantification of the PPARγ immunofluorescence signal revealed that treating adipocytes with CA or HT in combination with RGZ significantly reduced the enhanced PPARγ protein expression signal produced by RGZ alone (Fig 4A and 4B). A very similar pattern although not showing significant differences was observed concerning PPARγ protein expression by Western blot (Fig 4C and 4D). A lower level of lipid storage was observed when these cells were cultured in the presence of lipid mixture (LIP)+CA and especially in the LIP+HT condition, compared to LIP alone (0.93 ± 0.176 and 0.56 ± 0.047 in LIP+CA and LIP+HT, respectively, *versus* 1.34 ± 0.13 with LIP, $p \leq 0.05$) (Fig 4E). Finally, qRT-PCR data revealed that LIP significantly increased the expression of *pparg* and *cebpa* transcripts compared to CT, whereas RGZ did not affect these values (Fig 4F and 4G). In addition, treatments with CA or HT slightly down-regulated *pparg* and *cebpa* mRNA levels when combined with LIP, whereas no changes were observed in combination with RGZ (Fig 4F and 4G).

In vivo effects of CA and HT on rainbow trout lipid metabolism

The next step was to clarify the role of CA and HT in an *in vivo* context by focusing on several key adipogenic, lipolitic and β-oxidation markers, as well as the potential cross talk between liver and WAT, using qRT-PCR and plasma parameters analyses. The data revealed that transcriptional levels of *fasn* in both WAT and liver were significantly decreased by HT treatment (Fig 5A and 5E), indicating a reduction in fatty acid synthesis, possibly resulting in a decrease in TAG formation and fat deposits. Moreover, *pnpla2* expression in WAT but not liver decreased significantly following HT administration (Fig 5D and 5H). On the other hand, *lipe1* mRNA levels in both liver and WAT were unaffected by CA or HT treatment (Fig 5C and 5G). Following CA administration, *lpl* mRNA levels were unaffected in WAT, but increased in liver, suggesting a higher TAG lipase activity in this tissue (Fig 5B and 5F). Regarding β-oxidation markers, *acsl1* mRNA levels presented a reverse regulation between tissues, being significantly increased by HT treatment in WAT and showing an opposite pattern but not significant in liver (Fig 6A and 6D). Aside from that, no changes were found in *hoad* (Fig 6B and 6E) nor *pparb* (Fig 6C and 6F) mRNA levels due to treatment in any tissue.

Since CA and HT were shown to modulate the expression of adipogenic, lipolytic and β-oxidation genes that regulate the lipid metabolism in both WAT and liver, it was useful to obtain further information by measuring blood parameters. Analysis of selected biochemical parameters in plasma revealed a slight decrease in TAG levels in fasting rainbow trout injected with CA and a significantly lower concentration with HT compared to CT (Table 1). However, no significant effects upon treatments were observed in plasma glucose nor NEFAs.

Discussion

While mammalian models have largely contributed to improving our understanding of obesity, in recent years, teleost models have also produced excellent findings on human diseases and offer a number of advantages [41]. Despite obvious differences, teleost fish share a significant amount of genetic identity with humans, and some metabolic pathways and organ systems are also remarkably similar [42,43]. Their rapid development ex-utero and the optical semi-transparency of the embryonic and early larval stages, as well as the advances in fish cell

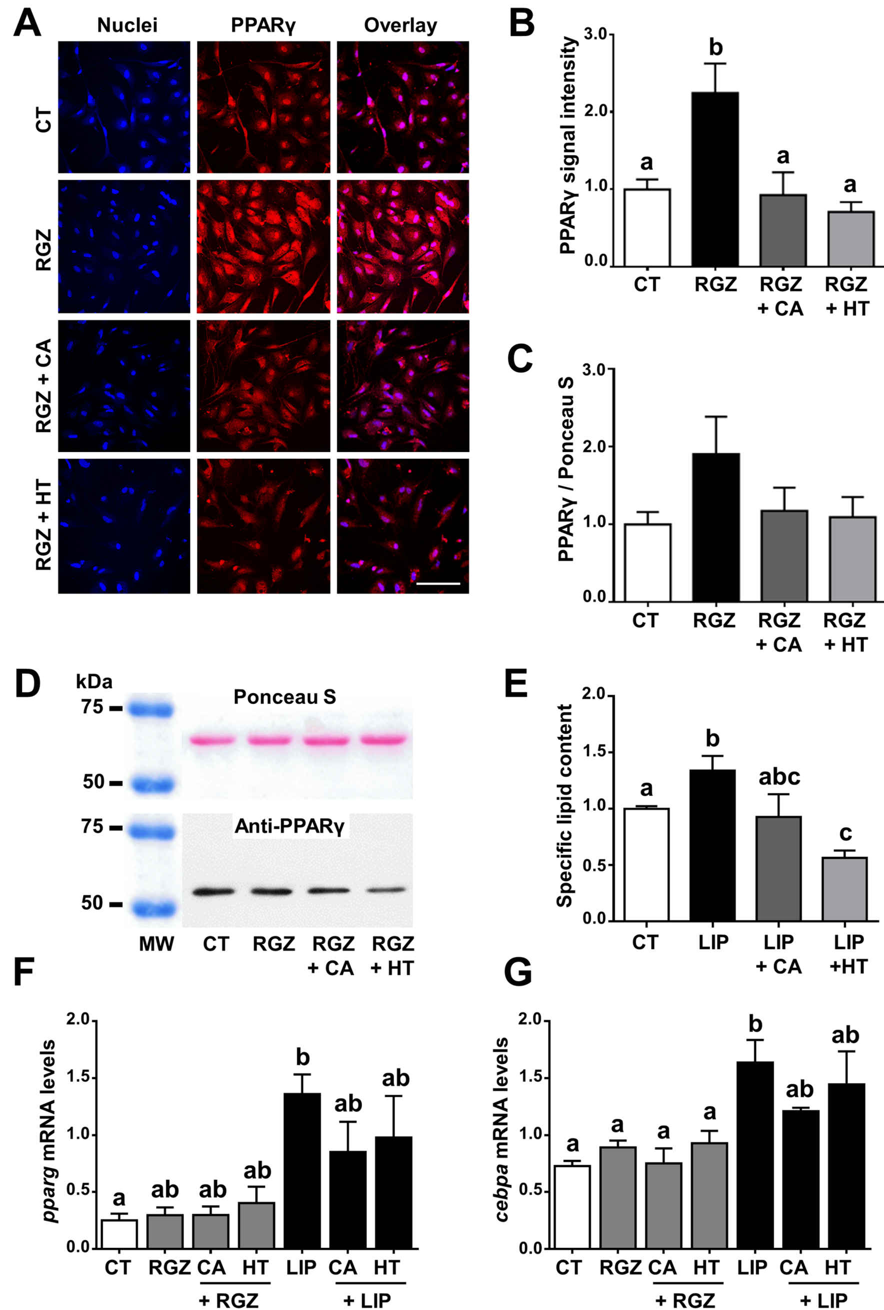

Fig 4. Characterization of potential PPARγ signaling pathway antagonism and inhibition of adipogenesis produced by CA and HT in primary-cultured rainbow trout adipocytes. Representative PPARγ immunofluorescence images (A), quantification of PPARγ immunofluorescence protein signal (B), anti-PPARγ immunoreactive band and quantification of PPARγ protein expression by Western blot (C, D), specific lipid content (E) and mRNA levels of adipogenic genes *pparg* (F) and *cebpa* (G). Immunofluorescence images show Hoechst nuclei staining (left panels), PPARγ (medium panels) and overlay (right panels). Scale bar, 100 μm. For both protein expression analyses (immunofluorescence, A and B; Western blot, C and D) cells were incubated with vehicle (DM) plus CA (50 μM), HT (100 μM), RGZ (1 μM), or the indicated combination of CA or HT with RGZ, or vehicle CT alone, for 24 h (day 7 of culture). RGZ was used as a potential rainbow trout PPARγ agonist. Representative Western blot images of anti-PPARγ immunoreactive band (top) and the same membrane labelled with Ponceau S (bottom) (D). Lipid content expressed spectrophotometrically as the ratio of absorbance value between ORO and Coomassie blue staining (E). For lipid content analysis cells were incubated with vehicle (DM) plus CA (50 μM), HT (100 μM), LIP (10 μl/ml), or the indicated combination of CA or HT with LIP, or vehicle CT alone, for 72 h (day 7 of

culture). mRNA levels of *pparg* (F) and *cebpa* (G) were normalized to the geometric mean of the two reference genes, *ef1a* and *ubiquitin*. For gene expression analyses cells were incubated with vehicle (DM) plus CA (50 μM), HT (100 μM), RGZ (1 μM), LIP (10 μl/ml), or the indicated combination of CA or HT with RGZ or LIP, or vehicle CT alone for 24 h (day 7 of culture). Data are shown as mean ± SEM (n = 3–7 cell cultures). Significant differences ($p \leq 0.05$) are indicated by different letters, using one-way ANOVA followed by Tukey's *post hoc* test (B, C, E, F) or the non-parametric Kruskal-Wallis test followed by paired U-Mann Whitney test (G).

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culture techniques have made teleost fish popular models in applied and basic research [14,21,41,42]. In mammals, fat deposits in specific regions differ from each other not only by localization but also by their structural and functional properties [44]. Similarly, WAT development in zebrafish is a step-wise process, with differentiated adipocytes first observed in the visceral region, then the subcutaneous area and finally, the head [27]. Moreover, zebrafish fat deposits are also mobilized sequentially, in reverse order, in response to starvation [20]; altogether highlighting the particular suitability of this species to study adiposity dynamics in an *in vivo* scenario.

Natural bioactive phytochemicals have recently attracted interest for their potential health benefits in preventing metabolic diseases, including obesity and insulin resistance [45]. Among these, dietary components of olive oil, red wine and marine algae are particularly interesting, due to their antioxidant properties, which are traditionally linked to longevity and reduced mortality from several diseases [46]. Furthermore, as well as obesogenic molecules, which predispose individuals to metabolic changes leading to weight gain [47], there is growing evidence that some plant compounds can improve overweight by inhibiting adipogenic

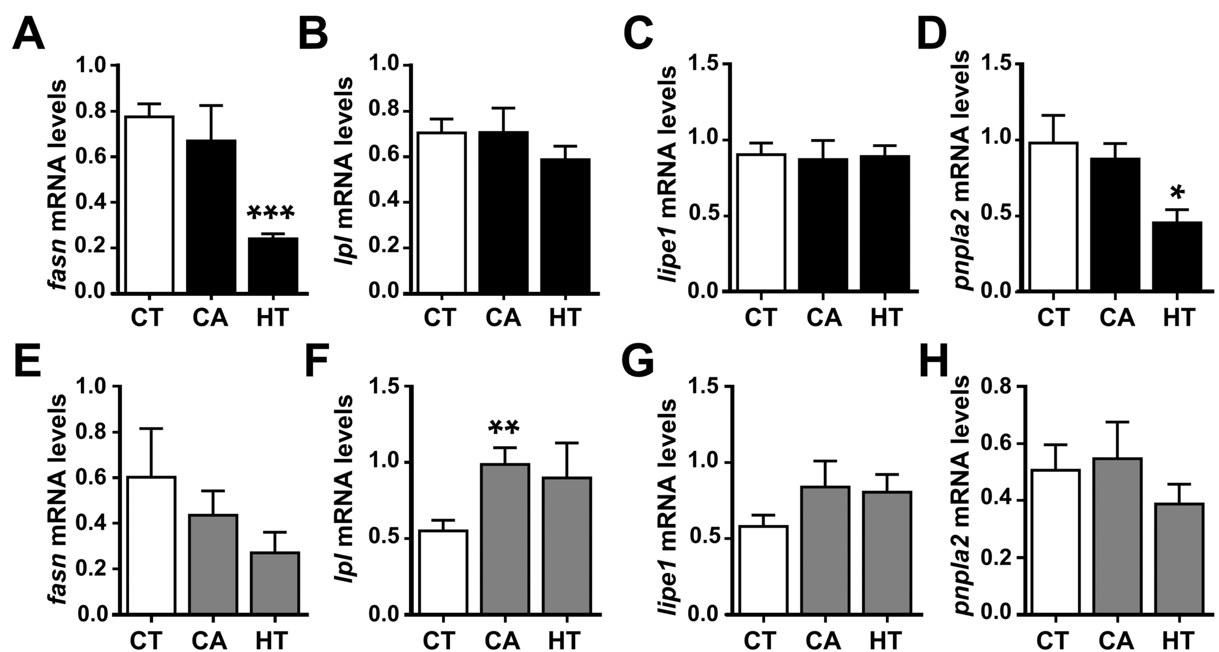

Fig 5. qRT-PCR analysis of selected lipid-metabolism-related gene transcript levels in the dissected perivisceral WAT (A-D) and liver (E-H) of rainbow trout treated with CA and HT or untreated. After one-day fasting, juvenile rainbow trout received intraperitoneal injections of vehicle CT (DMSO in sesame oil 1:3, v/v), vehicle plus CA (10 μg/g body weight), or vehicle plus HT (20 μg/g body weight), and samples were taken after a 24 h exposure period in a fasting state. mRNA levels of *fasn* (A, E), *lpl* (B, F), *lipe1* (C, G) and *pnpla2* (D, H). All mRNA levels were normalized to the geometric mean of the two reference genes, *ef1a* and *actb*. Data are shown as mean ± SEM (n = 7–8 fish per condition). *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.0001$, compared to CT, using Student's t-test.

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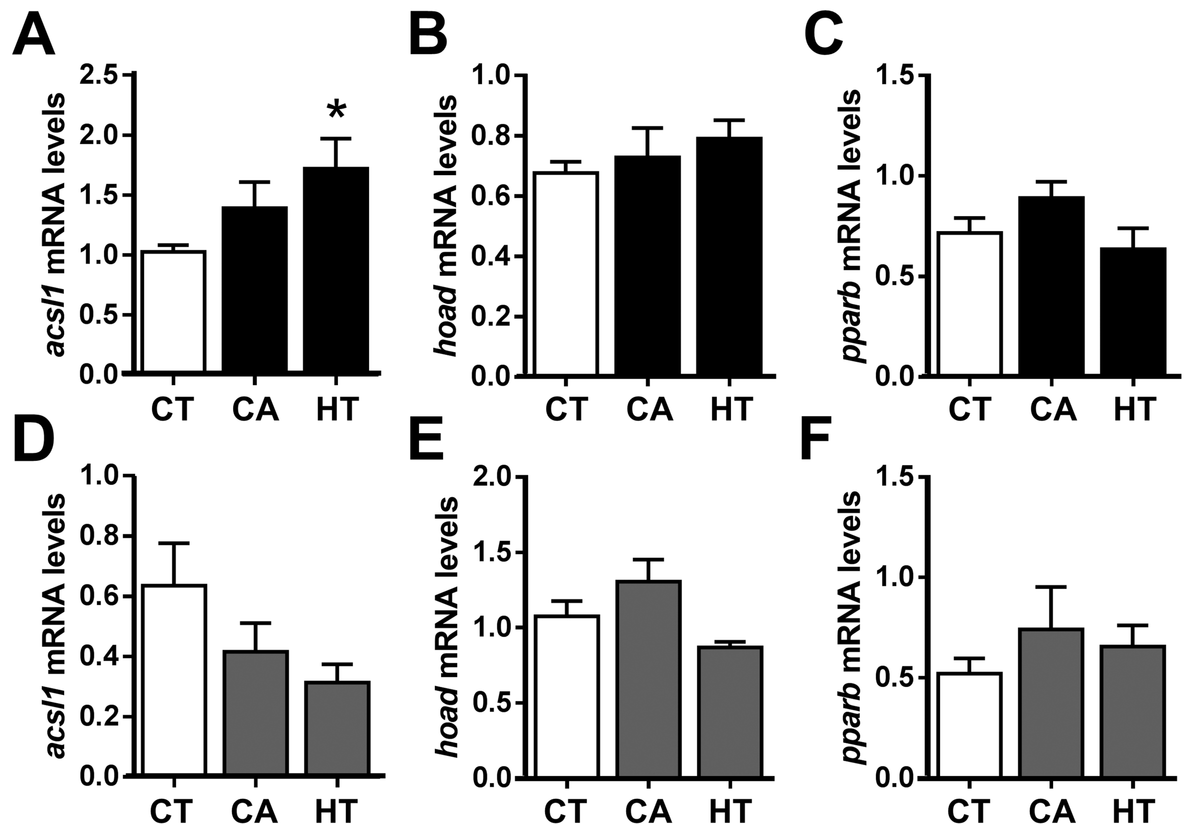

Fig 6. qRT-PCR analysis of selected β-oxidation-related gene transcript levels in the dissected perivisceral WAT (A-C) and liver (D-F) of rainbow trout treated with CA and HT or untreated. After one-day fasting, juvenile rainbow trout received intraperitoneal injections of vehicle CT (DMSO in sesame oil 1:3, v/v), vehicle plus CA (10 μg/g body weight), or vehicle plus HT (20 μg/g body weight), and samples were taken after a 24 h exposure period in a fasting state. mRNA levels of *acsl1* (A, D), *hoad* (B, E) and *pparb* (C, F). All mRNA levels were normalized to the geometric mean of the two reference genes, *ef1a* and *actb*. Data are shown as mean ± SEM (n = 7–8 fish per condition). *$p \leq 0.05$, compared to CT, using Student's t-test.

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pathways or impairing lipid accumulation and may, therefore, be classified as anti-obesogenic. Recent studies have reported that some phytochemicals and dietary supplements affect the lipid metabolism in zebrafish. Adding green tea extract to a high-fat diet significantly reduced body fat storage in adults [48,49]. Other studies of very early-stage larvae reported a decrease in lipids after treatment with compounds originating from plants or algae. The protocol

Table 1. Selected biochemical plasma parameters of rainbow trout intraperitoneally injected with CA or HT.

	CT	CA	HT
TAG (mmol/L)	4.69 ± 0.738	3.53 ± 0.164	2.59 ± 0.478*
Glycerol (DO 540 nm)	0.06 ± 0.0013	0.06 ± 0.0014	0.06 ± 0.0006
Glucose (mmol/L)	8.61 ± 1.077	6.86 ± 0.417	6.83 ± 0.480
NEFAs (mEq/L)	0.96 ± 0.074	1.02 ± 0.086	1.16 ± 0.133

Juvenile rainbow trout kept at 15˚C received intraperitoneal injections after a one-day fast with vehicle CT, vehicle plus CA (10 μg/g), or vehicle plus HT (20 μg/g). After 24 h exposure to the compound, blood samples were harvested for further plasma biochemical analyses. The total fasting period was therefore 48 hours before blood sampling. Values are expressed as means ± SEM (n = 7 to 8 fish per condition). TAG, triacylglycerols; NEFAs, non-esterified fatty acids.

*$p < 0.05$ *versus* control using Student's t-test.

https://doi.org/10.1371/journal.pone.0178833.t001

consisted of a 15-day exposure to the compound on a high-fat diet background, starting at the first feeding period. For example, curcumin [50–53], indole-3-carbinol [54], baicalein [55], dieckol [56], ellagic acid [52], kaempferol [51], quercetin [56], seapolynol [57] and silibinin [58], have all been reported to inhibit lipid accumulation in these early, post-embryonic developmental stages, when the adipocyte lineage is just starting to be established [20,27]. In our work, we used the ZOT protocol at later larval developmental stages, when the animals had a well-established WAT [30]. *In vivo* Nile red staining of adipocyte lipid droplets and quantitative analysis of whole-mount wide-field fluorescence microscopy signals, before and after a one-day exposure to the selected chemical compounds, were used to monitor the dynamics of WAT mass linked to adipocyte droplet size rather than adipocyte cell number. In addition, this methodology discriminated between the lipid signals associated with WAT and liver and identified the potential modulation of different regional WAT fat deposits in zebrafish larvae.

In this context, the aim of the present study was to investigate the anti-obesogenic properties of three antioxidant compounds of plant origin, selected for their capacity to modulate the lipid metabolism in the 3T3-L1 adipocyte cell line and rodent models [59–64]. ATX is a natural red carotenoid pigment found in a wide variety of organisms, CA is one of the most abundant hydroxycinnamic acids in the human diet, and HT is bioavailable in olive leaves and olive oil. In the present work, CA and HT caused a significant, short-term reduction in zebrafish larval adiposity, but CA exhibited stronger anti-obesogenic effects only in the head region, initiating the reversal of the fat mobilization pattern, as described above, while HT affected both the head and visceral regions. These results indicate that these compounds reduce fat mass by decreasing adipocyte size, but they may have different anti-obesogenic capacities according to the WAT location in the body. As mentioned above, it is known that adipose deposits in different parts of the body have different biological functions and biochemical profiles. Several features, such as adipocyte growth and differentiation, developmental gene expression, susceptibility to apoptosis, inflammatory capacity, and adipokine secretion, vary among deposits, as well as fatty-acid processing and WAT enlargement and loss mechanisms [65]. Nevertheless, further investigation is required to determine how CA and HT target the specific needs of the different deposits. On the other hand, ATX did not modify the adiposity level of zebrafish, even when the different regions were analyzed separately. Although several studies support the hypothesis that ATX supplementation reduces body weight and fat accumulation in mice [66], the ATX action mechanism has been demonstrated to be cell-type dependent [62]. In agreement with our data, one study in humans reported that ATX did not boost fat utilization or fat loss [67].

Adipogenesis is a complex process that typically involves the sequential activation of several transcription factors, such as PPARγ, that regulate the differentiation of pre-adipocytes into mature adipocytes, and ultimately control WAT formation promoting both, lipogenesis and fatty acid uptake. In mammals, it has been demonstrated that CA and HT exhibit a significant potential to act as anti-obesity agents by modulating the PPARγ adipogenesis pathway [64,68]. Therefore, the potential antagonism of PPARγ produced by CA and HT may also disrupt fat accumulation in teleost fish and result in a reduction of WAT mass due to lipid storage impairment. Our results revealed that CA and HT abolished the RGZ-induced obesogenic effect in zebrafish. A similar inhibitory effect has been also reported in zebrafish exposed to specific pharmaceutical antagonists of RGZ [30,69], thus supporting the idea that the compounds used in the present study may antagonize PPARγ signaling.

During the last decade, *in vitro* cell culture models from fish species have been widely used for studies of lipid metabolism and adipocyte functionality [31,70,71]. Nevertheless, despite the increasing use of zebrafish as an obesity model, to our knowledge, a primary adipocyte cell culture from this species has not been developed up to date. In this regard, further

investigations using the well-established primary-cultured rainbow trout adipocyte system were performed in order to have a closer look into the potential PPARγ antagonism of CA and HT. The data supported the involvement of PPARγ in the mechanism underlying the anti-obesogenic activity of CA and HT. Concomitant with the counteracting effect downregulating the PPARγ protein expression signal induced by RGZ, CA and especially HT, also reduced the adipocyte lipid content, in agreement with our previous *in vivo* findings in zebrafish. However, qRT-PCR analyses revealed that these two compounds did not modulate the transcript levels of typical key markers of mature adipocytes, i.e. *pparg and cebpa*, when these cells were co-treated with RGZ, and had only a slight effect when they were combined with LIP. These findings suggest that the anti-obesogenic effect of CA and HT may be mediated by post-transcriptional mechanisms.

PPARγ deficiency and/or disruption directly not only affects WAT development and accumulation, but also exerts an impact on the whole body metabolism [72]. The *in vivo* administration experiment in rainbow trout revealed that CA and HT affected the mRNA levels of adipogenic and β-oxidation genes in liver and WAT in different ways. Fat loss is usually caused by the balance of two main processes: enhancement of fat breakdown and inhibition of TAG synthesis and accumulation. This process is orchestrated by the interaction of several tissues, such as WAT, liver and skeletal muscles [73]. The decrease in WAT *fasn* mRNA levels produced by HT treatment was accompanied by a reduction in plasma TAG levels, possibly due to the antagonist effect of this compound on the PPARγ signaling pathway. In agreement with our results, some mammal studies have demonstrated that CA and HT regulate WAT mass by suppressing lipogenic enzyme activity and claimed that PPARγ antagonists can be used to treat hyperlipidemia [74,75]. Interestingly, HT also downregulated *pnpla2* transcript abundance in rainbow trout WAT, which is indicative of decreased lipolysis, leading to a reduction in lipid turnover or compensation for excessive WAT decrease. On the other hand, CA administration increased *lpl* mRNA levels in the liver, also associated with the decreased TAG levels observed in plasma. Concomitant with this decrease, CA and HT also smoothly increased NEFAs levels via TAG hydrolysis. Moreover, a slight decrease was also observed in plasma glucose following treatment, in agreement with mammal studies where CA and HT have been proposed as potential hypoglycemic antidiabetic treatments [76,77]. Within this *in vivo* scenario it is important to notice that CA and HT are antioxidants, with proven effects upon cell viability (S2 Fig), which may have an implication in modulating fatty acid oxidation [78]. In this sense, HT administration upregulated *acsl1* mRNA levels in WAT while slightly decreased its expression in liver, indicating a tissue-specific regulation as previously reported [79]. Even though enhanced *acsl1* expression is traditionally believed to be essential for the synthesis of TAG, several studies in mammals revealed a possible function related to the β-oxidation of fatty acids [80,81], in agreement with our results. Nevertheless, differences were not observed concerning the mRNA levels of *hoad* and *pparb* suggesting that further studies are needed to better understand the potential involvement of CA and HT in WAT β-oxidation.

Conclusions

The findings reported here provide novel insights into the anti-obesogenic role of CA and HT antioxidants, highlighting their potential involvement in negatively modulating the PPARγ signaling pathway. Further studies using specific inhibitors of these nuclear receptors will help to understand the mechanism of action of these anti-obesogenic compounds. The teleost fish models used have been validated for studying the mode of action of these bioactive compounds on WAT, reinforcing their suitability in pharmacological and biomedical research. This study may also help the investigation of new additives to optimize adiposity in farmed animals.

Supporting information

S1 Fig. ZOT as a tool for studying WAT dynamics in different body parts of zebrafish larva. (A) External features of a representative 8 mm SL larva and images of head, viscera, and tail regions under a fluorescence microscope after Nile red staining, using HQ-FITC-BP filter, with adipocytes stained green. Lateral views, anterior part on the left and dorsal part at the top. (B) Quantitative analysis of adipocyte tissue area in each body region, expressed as a percentage of total adiposity. SL distribution of the animals used was from 7 to 9 mm. Boxplot shows median and percentile adiposity values, n = 11 independent experiments (10 animals per group). Scale bar: 0.5 mm. Abbreviations: ai, anterior intestine; cfa, caudal fin adipocytes; e, eye; pf, pectoral fin; pwat, perivisceral white adipose tissue; sb, swim bladder; swat, subcutaneous white adipose tissue.
(TIF)

S2 Fig. CA and HT increased cell viability but did not affect cell proliferation of rainbow trout adipocytes in primary culture. (A) Quantification of cell viability using MTT assay. (B) Cell proliferation determined by immunocytochemistry of PCNA. Cells were incubated with vehicle plus CA (50 μM), HT (100 μM), or RGZ (1 μM), alone or in combination, or vehicle CT alone, for 24 h (day 5 of culture). Data are shown as mean ± SEM (n = 3–4 cell cultures). $^{*}p \leq 0.05$, $^{**}p \leq 0.01$ compared to CT, using one-way ANOVA test followed by Tukey's *post hoc* test.
(TIF)

S1 Table. Nucleotide sequences of the primers used to evaluate mRNA abundance by qRT-PCR in rainbow trout.
(DOC)

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Author Contributions

Conceptualization: EL PJB IN.

Data curation: EL PJB JG EC IN.

Formal analysis: EL PJB.

Funding acquisition: PJB JG EC IN.

Investigation: EL PJB EC.

Methodology: EL PJB EC.

Project administration: PJB IN.

Resources: PJB JG EC IN.

Software: EL PJB.

Supervision: PJB EC IN.

Validation: EL PJB IN.

Visualization: EL PJB.

Writing – original draft: EL PJB IN.

Writing – review & editing: EL PJB JG EC IN.

References

1. Pandita A, Sharma D, Pandita D, Pawar S, Tariq M, Kaul A. Childhood obesity: prevention is better than cure. Diabetes Metab Syndr Obes. 2016; 9: 83–9. https://doi.org/10.2147/DMSO.S90783 PMID: 27042133
2. Mitchell NS, Catenacci VA, Wyatt HR, Hill JO. Obesity: overview of an epidemic. Psychiatr Clin North Am. 2011; 34: 717–32. https://doi.org/10.1016/j.psc.2011.08.005 PMID: 22098799
3. Andrade-Oliveira V, Câmara NOS, Moraes-Vieira PM. Adipokines as drug targets in diabetes and underlying disturbances. J Diabetes Res. 2015; 2015: 681612. https://doi.org/10.1155/2015/681612 PMID: 25918733
4. Blüher M. Adipose tissue dysfunction contributes to obesity related metabolic diseases. Best Pract Res Clin Endocrinol Metab. Elsevier; 2013; 27: 163–77. https://doi.org/10.1016/j.beem.2013.02.005 PMID: 23731879
5. Lee J-E, Ge K. Transcriptional and epigenetic regulation of PPARγ expression during adipogenesis. Cell Biosci. BioMed Central; 2014; 4: 29. https://doi.org/10.1186/2045-3701-4-29 PMID: 24904744
6. Wang L, Waltenberger B, Pferschy-Wenzig E-M, Blunder M, Liu X, Malainer C, et al. Natural product agonists of peroxisome proliferator-activated receptor gamma (PPARγ): a review. Biochem Pharmacol. 2014; 92: 73–89. https://doi.org/10.1016/j.bcp.2014.07.018 PMID: 25083916
7. Grygiel-Górniak B. Peroxisome proliferator-activated receptors and their ligands: nutritional and clinical implications—a review. Nutr J. BioMed Central; 2014; 13: 17. https://doi.org/10.1186/1475-2891-13-17 PMID: 24524207
8. Hussein Z, Wentworth JM, Nankervis AJ, Proietto J, Colman PG. Effectiveness and side effects of thiazolidinediones for type 2 diabetes: real-life experience from a tertiary hospital. Med J Aust. 2004; 181: 536–9. PMID: 15540964
9. Siraj FM, Natarajan S, Huq MA, Kim YJ, Yang DC. Structural investigation of ginsenoside Rf with PPARγ major transcriptional factor of adipogenesis and its impact on adipocyte. J Ginseng Res. 2015; 39: 141–7. https://doi.org/10.1016/j.jgr.2014.10.002 PMID: 26045687
10. Gülçin I. Antioxidant activity of caffeic acid (3,4-dihydroxycinnamic acid). Toxicology. 2006; 217: 213–20. https://doi.org/10.1016/j.tox.2005.09.011 PMID: 16243424
11. Gordon MH, Paiva-Martins F, Almeida M. Antioxidant activity of hydroxytyrosol acetate compared with that of other olive oil polyphenols. J Agric Food Chem. 2001; 49: 2480–5. PMID: 11368623
12. Naguib YM. Antioxidant activities of astaxanthin and related carotenoids. J Agric Food Chem. 2000; 48: 1150–4. PMID: 10775364
13. Lossi L, D'Angelo L, De Girolamo P, Merighi A. Anatomical features for an adequate choice of experimental animal model in biomedicine: II. Small laboratory rodents, rabbit, and pig. Ann Anat—Anat Anzeiger. 2016; 204: 11–28. https://doi.org/10.1016/j.aanat.2015.10.002 PMID: 26527557
14. D'Angelo L, Lossi L, Merighi A, de Girolamo P. Anatomical features for the adequate choice of experimental animal models in biomedicine: I. Fishes. Ann Anat—Anat Anzeiger. 2016; 205: 75–84. https://doi.org/10.1016/j.aanat.2016.02.001 PMID: 26925824
15. Song Y, Cone RD. Creation of a genetic model of obesity in a teleost. FASEB J. Federation of American Societies for Experimental Biology; 2007; 21: 2042–9. https://doi.org/10.1096/fj.06-7503com PMID: 17341684
16. Santoriello C, Zon LI. Hooked! Modeling human disease in zebrafish. J Clin Invest. American Society for Clinical Investigation; 2012; 122: 2337–43. https://doi.org/10.1172/JCI60434 PMID: 22751109
17. Caro M, Iturria I, Martinez-Santos M, Pardo MA, Rainieri S, Tueros I, et al. Zebrafish dives into food research: effectiveness assessment of bioactive compounds. Food Funct. 2016; 7: 2615–23. https://doi.org/10.1039/c6fo00046k PMID: 27109696
18. Yada T, Moriyama S, Suzuki Y, Azuma T, Takahashi A, Hirose S, et al. Relationships between obesity and metabolic hormones in the "cobalt" variant of rainbow trout. Gen Comp Endocrinol. 2002; 128: 36–43. https://doi.org/10.1016/S0016-6480(02)00047-3 PMID: 12270786

19. Albalat A, Saera-Vila A, Capilla E, Gutiérrez J, Pérez-Sánchez J, Navarro I. Insulin regulation of lipoprotein lipase (LPL) activity and expression in gilthead sea bream (*Sparus aurata*). Comp Biochem Physiol Part B Biochem Mol Biol. 2007; 148: 151–159. https://doi.org/10.1016/j.cbpb.2007.05.004 PMID: 17600746
20. Flynn EJ, Trent CM, Rawls JF. Ontogeny and nutritional control of adipogenesis in zebrafish (*Danio rerio*). J Lipid Res. 2009; 50: 1641–52. https://doi.org/10.1194/jlr.M800590-JLR200 PMID: 19366995
21. Bou M, Todorčević M, Torgersen J, Škugor S, Navarro I, Ruyter B. De novo lipogenesis in Atlantic salmon adipocytes. Biochim Biophys Acta—Gen Subj. 2016; 1860: 86–96. https://doi.org/10.1016/j.bbagen.2015.10.022 PMID: 26518346
22. Pignatelli J, Castro R, González Granja A, Abós B, González L, Jensen LB, et al. Immunological characterization of the teleost adipose tissue and its modulation in response to viral infection and fat-content in the diet. Trottein F, editor. PLoS One. 2014; 9: e110920. https://doi.org/10.1371/journal.pone.0110920 PMID: 25333488
23. Johansson M, Morgenroth D, Einarsdottir IE, Gong N, Björnsson BT. Energy stores, lipid mobilization and leptin endocrinology of rainbow trout. J Comp Physiol B. 2016; 186: 759–773. https://doi.org/10.1007/s00360-016-0988-y PMID: 27083432
24. Babin PJ, Vernier JM. Plasma lipoproteins in fish. J Lipid Res. 1989; 30: 467–89. PMID: 2666541
25. Babin PJ, Gibbons GF. The evolution of plasma cholesterol: direct utility or a "spandrel"; of hepatic lipid metabolism? Prog Lipid Res. 2009; 48: 73–91. https://doi.org/10.1016/j.plipres.2008.11.002 PMID: 19049814
26. Den Broeder MJ, Kopylova VA, Kamminga LM, Legler J. Zebrafish as a model to study the role of peroxisome proliferating-activated receptors in adipogenesis and obesity. PPAR Res. 2015; 2015: 358029. https://doi.org/10.1155/2015/358029 PMID: 26697060
27. Imrie D, Sadler KC. White adipose tissue development in zebrafish is regulated by both developmental time and fish size. Dev Dyn. 2010; 239: 3013–23. https://doi.org/10.1002/dvdy.22443 PMID: 20925116
28. McMenamin SK, Minchin JEN, Gordon TN, Rawls JF, Parichy DM. Dwarfism and increased adiposity in the gh1 mutant zebrafish vizzini. Endocrinology. The Endocrine Society; 2013; 154: 1476–87. https://doi.org/10.1210/en.2012-1734 PMID: 23456361
29. Minchin JEN, Dahlman I, Harvey CJ, Mejhert N, Singh MK, Epstein JA, et al. Plexin D1 determines body fat distribution by regulating the type V collagen microenvironment in visceral adipose tissue. Proc Natl Acad Sci U S A. National Academy of Sciences; 2015; 112: 4363–8. https://doi.org/10.1073/pnas.1416412112 PMID: 25831505
30. Tingaud-Sequeira A, Ouadah N, Babin PJ. Zebrafish obesogenic test: a tool for screening molecules that target adiposity. J Lipid Res. 2011; 52: 1765–72. https://doi.org/10.1194/jlr.D017012 PMID: 21724975
31. Bouraoui L, Gutiérrez J, Navarro I. Regulation of proliferation and differentiation of adipocyte precursor cells in rainbow trout (*Oncorhynchus mykiss*). J Endocrinol. 2008; 198: 459–69. https://doi.org/10.1677/JOE-08-0264 PMID: 18579724
32. Vélez EJ, Lutfi E, Jiménez-Amilburu V, Riera-Codina M, Capilla E, Navarro I, et al. IGF-I and amino acids effects through TOR signaling on proliferation and differentiation of gilthead sea bream cultured myocytes. Gen Comp Endocrinol. 2014; 205: 296–304. https://doi.org/10.1016/j.ygcen.2014.05.024 PMID: 24882593
33. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976; 72: 248–254. PMID: 942051
34. Bou M, Todorčević M, Rodríguez J, Capilla E, Gutiérrez J, Navarro I. Interplay of adiponectin, TNFα and insulin on gene expression, glucose uptake and PPARγ, AKT and TOR pathways in rainbow trout cultured adipocytes. Gen Comp Endocrinol. 2014; 205: 218–225. https://doi.org/10.1016/j.ygcen.2014.05.005 PMID: 24846393
35. Olesen KM, Auger AP. Sex differences in fos protein expression in the neonatal rat brain. J Neuroendocrinol. Blackwell Science Ltd; 2005; 17: 255–261. https://doi.org/10.1111/j.1365-2826.2005.01302.x PMID: 15842237
36. Romero-Calvo I, Ocón B, Martínez-Moya P, Suárez MD, Zarzuelo A, Martínez-Augustin O, et al. Reversible Ponceau staining as a loading control alternative to actin in Western blots. Anal Biochem. 2010; 401: 318–320. https://doi.org/10.1016/j.ab.2010.02.036 PMID: 20206115
37. Vélez EJ, Azizi S, Lutfi E, Capilla E, Moya A, Navarro I, et al. Moderate and sustained exercise modulates muscle proteolytic and myogenic markers in gilthead sea bream (*Sparus aurata*). Am J Physiol—Regul Integr Comp Physiol. 2017; ajpregu.00308.2016. https://doi.org/10.1152/ajpregu.00308.2016 PMID: 28228414

38. Capilla E, Teles-García A, Acerete L, Navarro I, Gutiérrez J. Insulin and IGF-I effects on the proliferation of an osteoblast primary culture from sea bream (*Sparus aurata*). Gen Comp Endocrinol. 2011; 172: 107–14. https://doi.org/10.1016/j.ygcen.2011.03.020 PMID: 21447336
39. Salmerón C, García de la serrana D, Jiménez-Amilburu V, Fontanillas R, Navarro I, Johnston IA, et al. Characterisation and expression of calpain family members in relation to nutritional status, diet composition and flesh texture in gilthead sea bream (*Sparus aurata*). PLoS One. 2013; 8: e75349. https://doi.org/10.1371/journal.pone.0075349 PMID: 24086513
40. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 2001; 29: e45. PMID: 11328886
41. Santoro MM. Zebrafish as a model to explore cell metabolism. Trends Endocrinol Metab. Elsevier; 2014; 25: 546–54. https://doi.org/10.1016/j.tem.2014.06.003 PMID: 24997878
42. Seth A, Stemple DL, Barroso I. The emerging use of zebrafish to model metabolic disease. Dis Model Mech. 2013; 6: 1080–8. https://doi.org/10.1242/dmm.011346 PMID: 24046387
43. Oka T, Nishimura Y, Zang L, Hirano M, Shimada Y, Wang Z, et al. Diet-induced obesity in zebrafish shares common pathophysiological pathways with mammalian obesity. BMC Physiol. 2010; 10: 21. https://doi.org/10.1186/1472-6793-10-21 PMID: 20961460
44. Bjørndal B, Burri L, Staalesen V, Skorve J, Berge RK, Berge RK. Different adipose depots: their role in the development of metabolic syndrome and mitochondrial response to hypolipidemic agents. J Obes. Hindawi Publishing Corporation; 2011; 2011: 490650. https://doi.org/10.1155/2011/490650 PMID: 21403826
45. Huang C-C, Tung Y-T, Huang W-C, Chen Y-M, Hsu Y-J, Hsu M-C. Beneficial effects of cocoa, coffee, green tea, and garcinia complex supplement on diet induced obesity in rats. BMC Complement Altern Med. 2016; 16: 100. https://doi.org/10.1186/s12906-016-1077-1 PMID: 26968378
46. Sadowska-Bartosz I, Bartosz G. Effect of antioxidants supplementation on aging and longevity. Biomed Res Int. 2014; 2014: 404680. https://doi.org/10.1155/2014/404680 PMID: 24783202
47. Grün F, Blumberg B. Environmental obesogens: organotins and endocrine disruption via nuclear receptor signaling. Endocrinology. Endocrine Society; 2006; 147: s50–s55. https://doi.org/10.1210/en.2005-1129 PMID: 16690801
48. Hasumura T, Shimada Y, Kuroyanagi J, Nishimura Y, Meguro S, Takema Y, et al. Green tea extract suppresses adiposity and affects the expression of lipid metabolism genes in diet-induced obese zebrafish. Nutr Metab (Lond). 2012; 9: 73. https://doi.org/10.1186/1743-7075-9-73 PMID: 22871059
49. Meguro S, Hasumura T, Hase T. Body fat accumulation in zebrafish is induced by a diet rich in fat and reduced by supplementation with green tea extract. PLoS One. 2015; 10: e0120142. https://doi.org/10.1371/journal.pone.0120142 PMID: 25785691
50. Choi H-S, Jeon H-J, Lee O-H, Lee B-Y. Dieckol, a major phlorotannin in Ecklonia cava, suppresses lipid accumulation in the adipocytes of high-fat diet-fed zebrafish and mice: Inhibition of early adipogenesis via cell-cycle arrest and AMPKα activation. Mol Nutr Food Res. 2015; 59: 1458–71. https://doi.org/10.1002/mnfr.201500021 PMID: 25944759
51. Lee Y-J, Choi H-S, Seo M-J, Jeon H-J, Kim K-J, Lee B-Y. Kaempferol suppresses lipid accumulation by inhibiting early adipogenesis in 3T3-L1 cells and zebrafish. Food Funct. 2015; 6: 2824–33. https://doi.org/10.1039/c5fo00481k PMID: 26174858
52. Woo M-S, Choi H-S, Seo M-J, Jeon H-J, Lee B-Y. Ellagic acid suppresses lipid accumulation by suppressing early adipogenic events and cell cycle arrest. Phytother Res. 2015; 29: 398–406. https://doi.org/10.1002/ptr.5264 PMID: 25462071
53. Kim JH, Kang S, Jung YN, Choi H-S. Cholecalciferol inhibits lipid accumulation by regulating early adipogenesis in cultured adipocytes and zebrafish. Biochem Biophys Res Commun. 2016; 469: 646–53. https://doi.org/10.1016/j.bbrc.2015.12.049 PMID: 26703207
54. Choi H-S, Jeon H-J, Lee O-H, Lee B-Y. Indole-3-carbinol, a vegetable phytochemical, inhibits adipogenesis by regulating cell cycle and AMPKα signaling. Biochimie. 2014; 104: 127–36. https://doi.org/10.1016/j.biochi.2014.06.010 PMID: 24952351
55. Seo M-J, Choi H-S, Jeon H-J, Woo M-S, Lee B-Y. Baicalein inhibits lipid accumulation by regulating early adipogenesis and m-TOR signaling. Food Chem Toxicol. 2014; 67: 57–64. https://doi.org/10.1016/j.fct.2014.02.009 PMID: 24560969
56. Seo M-J, Lee Y-J, Hwang J-H, Kim K-J, Lee B-Y. The inhibitory effects of quercetin on obesity and obesity-induced inflammation by regulation of MAPK signaling. J Nutr Biochem. 2015; 26: 1308–16. https://doi.org/10.1016/j.jnutbio.2015.06.005 PMID: 26277481
57. Jeon H-J, Choi H-S, Lee Y-J, Hwang J-H, Lee O-H, Seo M-J, et al. Seapolynol extracted from ecklonia cava inhibits adipocyte differentiation *in vitro* and decreases fat accumulation *in vivo*. Molecules. 2015; 20: 21715–31. https://doi.org/10.3390/molecules201219796 PMID: 26690099

58. Suh HJ, Cho SY, Kim EY, Choi H-S. Blockade of lipid accumulation by silibinin in adipocytes and zebrafish. Chem Biol Interact. 2015; 227: 53–62. https://doi.org/10.1016/j.cbi.2014.12.027 PMID: 25559859
59. Ikeuchi M, Koyama T, Takahashi J, Yazawa K. Effects of astaxanthin in obese mice fed a high-fat diet. Biosci Biotechnol Biochem. 2007; 71: 893–9. https://doi.org/10.1271/bbb.60521 PMID: 17420580
60. Cho A-S, Jeon S-M, Kim M-J, Yeo J, Seo K-I, Choi M-S, et al. Chlorogenic acid exhibits anti-obesity property and improves lipid metabolism in high-fat diet-induced-obese mice. Food Chem Toxicol. 2010; 48: 937–43. https://doi.org/10.1016/j.fct.2010.01.003 PMID: 20064576
61. Drira R, Chen S, Sakamoto K. Oleuropein and hydroxytyrosol inhibit adipocyte differentiation in 3 T3-L1 cells. Life Sci. 2011; 89: 708–16. https://doi.org/10.1016/j.lfs.2011.08.012 PMID: 21945192
62. Inoue M, Tanabe H, Matsumoto A, Takagi M, Umegaki K, Amagaya S, et al. Astaxanthin functions differently as a selective peroxisome proliferator-activated receptor γ modulator in adipocytes and macrophages. Biochem Pharmacol. Elsevier Inc.; 2012; 84: 692–700. https://doi.org/10.1016/j.bcp.2012.05.021 PMID: 22732454
63. Drira R, Sakamoto K. Modulation of adipogenesis, lipolysis and glucose consumption in 3T3-L1 adipocytes and C2C12 myotubes by hydroxytyrosol acetate: a comparative study. Biochem Biophys Res Commun. 2013; 440: 576–81. https://doi.org/10.1016/j.bbrc.2013.09.106 PMID: 24099771
64. Drira R, Sakamoto K. Hydroxytyrosol stimulates lipolysis via A-kinase and extracellular signal-regulated kinase activation in 3T3-L1 adipocytes. Eur J Nutr. 2014; 53: 743–50. https://doi.org/10.1007/s00394-013-0578-7 PMID: 23995352
65. Tchkonia T, Thomou T, Zhu Y, Karagiannides I, Pothoulakis C, Jensen MD, et al. Mechanisms and metabolic implications of regional differences among fat depots. Cell Metab. 2013; 17: 644–56. https://doi.org/10.1016/j.cmet.2013.03.008 PMID: 23583168
66. Arunkumar E, Bhuvaneswari S, Anuradha CV. An intervention study in obese mice with astaxanthin, a marine carotenoid—effects on insulin signaling and pro-inflammatory cytokines. Food Funct. The Royal Society of Chemistry; 2012; 3: 120–6. https://doi.org/10.1039/c1fo10161g PMID: 22089895
67. Res PT, Cermak NM, Stinkens R, Tollakson TJ, Haenen GR, Bast A, et al. Astaxanthin supplementation does not augment fat use or improve endurance performance. Med Sci Sports Exerc. 2013; 45: 1158–65. https://doi.org/10.1249/MSS.0b013e31827fddc4 PMID: 23274592
68. Liao C, Ou T, Wu C, Wang C. Prevention of diet-induced hyperlipidemia and obesity by caffeic acid in C57BL/6 mice through regulation of hepatic lipogenesis gene expression. J Agric Food Chem. 2013; 61: 11082–11088. https://doi.org/10.1021/jf4026647 PMID: 24156384
69. Ouadah-Boussouf N, Babin PJ. Pharmacological evaluation of the mechanisms involved in increased adiposity in zebrafish triggered by the environmental contaminant tributyltin. Toxicol Appl Pharmacol. 2016; 294: 32–42. https://doi.org/10.1016/j.taap.2016.01.014 PMID: 26812627
70. Vegusdal A, Sundvold H, Gjøen T, Ruyter B. An *in vitro* method for studying the proliferation and differentiation of Atlantic salmon preadipocytes. Lipids. 2003; 38: 289–96. PMID: 12784870
71. Salmerón C, Johansson M, Asaad M, Angotzi AR, Rønnestad I, Stefansson SO, et al. Roles of leptin and ghrelin in adipogenesis and lipid metabolism of rainbow trout adipocytes *in vitro*. Comp Biochem Physiol -Part A Mol Integr Physiol. 2015; 188: 40–48. https://doi.org/10.1016/j.cbpa.2015.06.017 PMID: 26103556
72. Celi FS, Shuldiner AR. The role of peroxisome proliferator-activated receptor gamma in diabetes and obesity. Curr Diab Rep. 2002; 2: 179–185. https://doi.org/10.1007/s11892-002-0078-2 PMID: 12643137
73. Samdani P, Singhal M, Sinha N, Tripathi P, Sharma S, Tikoo K, et al. A Comprehensive inter-tissue crosstalk analysis underlying progression and control of obesity and diabetes. Sci Rep. Nature Publishing Group; 2015; 5: 12340. https://doi.org/10.1038/srep12340 PMID: 26202695
74. Vanella L, Tibullo D, Godos J, Pluchinotta FR, Di Giacomo C, Sorrenti V, et al. Caffeic acid phenethyl ester regulates PPAR's levels in stem cells-derived adipocytes. PPAR Res. 2016; 2016: 7359521. https://doi.org/10.1155/2016/7359521 PMID: 26904104
75. Tutino V, Orlando A, Russo F, Notarnicola M. Hydroxytyrosol inhibits cannabinoid CB1 receptor gene expression in 3T3-L1 preadipocyte cell line. J Cell Physiol. 2016; 231: 483–9. https://doi.org/10.1002/jcp.25094 PMID: 26189725
76. Jung UJ, Lee M-K, Park YB, Jeon S-M, Choi M-S. Antihyperglycemic and antioxidant properties of caffeic acid in db/db mice. J Pharmacol Exp Ther. 2006; 318: 476–83. https://doi.org/10.1124/jpet.106.105163 PMID: 16644902
77. Jemai H, El Feki A, Sayadi S. Antidiabetic and antioxidant effects of hydroxytyrosol and oleuropein from olive leaves in alloxan-diabetic rats. J Agric Food Chem. 2009; 57: 8798–804. https://doi.org/10.1021/jf901280r PMID: 19725535

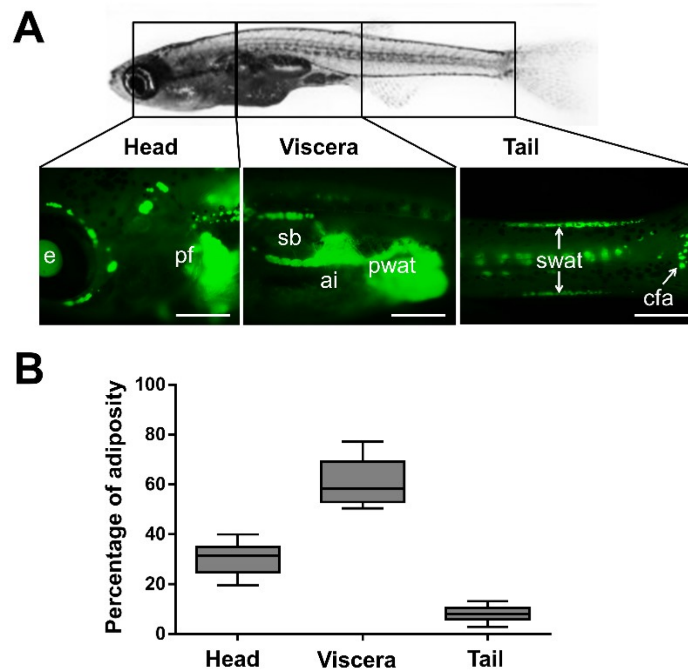
78. Gutierrez VR, de la Puerta R, Catalá A. The effect of tyrosol, hydroxytyrosol and oleuropein on the non-enzymatic lipid peroxidation of rat liver microsomes. Mol Cell Biochem. 2001; 217: 35–41. PMID: 11269663

79. Mashek DG, Li LO, Coleman RA. Long-chain acyl-CoA synthetases and fatty acid channeling. Future Lipidol. NIH Public Access; 2007; 2: 465–476. https://doi.org/10.2217/17460875.2.4.465 PMID: 20354580

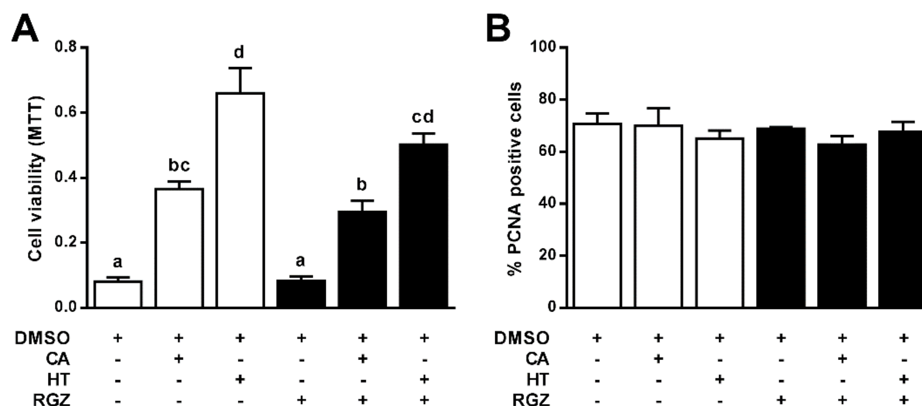
80. Li LO, Ellis JM, Paich HA, Wang S, Gong N, Altshuller G, et al. Liver-specific loss of long chain acyl-CoA synthetase-1 decreases triacylglycerol synthesis and beta-oxidation and alters phospholipid fatty acid composition. J Biol Chem. American Society for Biochemistry and Molecular Biology; 2009; 284: 27816–26. https://doi.org/10.1074/jbc.M109.022467 PMID: 19648649

81. Ellis JM, Li LO, Wu P-C, Koves TR, Ilkayeva O, Stevens RD, et al. Adipose acyl-CoA synthetase-1 directs fatty acids toward beta-oxidation and is required for cold thermogenesis. Cell Metab. NIH Public Access; 2010; 12: 53–64. https://doi.org/10.1016/j.cmet.2010.05.012 PMID: 20620995

Supporting information.



S1 Fig. ZOT as a tool for studying WAT dynamics in different body parts of zebrafish larva. (**A**) External features of a representative 8 mm SL larva and images of head, viscera, and tail regions under a fluorescence microscope after Nile red staining, using HQ-FITC-BP filter, with adipocytes stained green. Lateral views, anterior part on the left and dorsal part at the top. (**B**) Quantitative analysis of adipocyte tissue area in each body region, expressed as a percentage of total adiposity. SL distribution of the animals used was from 7 to 9 mm. Boxplot shows median and percentile adiposity values, n = 11 independent experiments (10 animals per group). Scale bar: 0.5 mm. **Abbreviations:** ai, anterior intestine; cfa, caudal fin adipocytes; e, eye; pf, pectoral fin; pwat, perivisceral white adipose tissue; sb, swim bladder; swat, subcutaneous white adipose tissue.



S2 Fig. CA and HT increased cell viability but did not affect cell proliferation of rainbow trout adipocytes in primary culture. (**A**) Quantification of cell viability using MTT assay. (**B**) Cell proliferation determined by immunocytochemistry of PCNA. Cells were incubated with vehicle plus CA (50 µM), HT (100 µM), or RGZ (1

μM), alone or in combination, or vehicle CT alone, for 24 h (day 5 of culture). Data are shown as mean ± SEM (n = 3–4 cell cultures). $*p \leq 0.05$, $**p \leq 0.01$ compared to CT, using one-way ANOVA test followed by Tukey's post hoc test.

Table S1. Nucleotide sequences of the primers used to evaluate mRNA abundance by qRT-PCR in rainbow trout.

Gene	Primer sequences (5'–3')	Tm ºC	Amplicon size (bp)	Database	Accession number
acsl1	**F**: TGCAATCTAGCAAGGTTCCTTTTG **R**: TCCAAGCAGAAACCCAGTACAGAA	60	137	Sigenae	CR363150.p.om.8
actb	**F**: ATCCTGACGGAGCGCGGTTACAGC **R**: TGCCCATCTCCTGCTCAAAGTCCA	61	112	Genbank	AJ438158
cebpa	**F**: TGTGGCGATAAAGCAAGAGC **R**: CTGGTGGGAATGGTGGTAGG	57	79	Genbank	DQ423469.1
ef1α	**F**: TCCTCTTGGTCGTTTCGCTG **R**: ACCCGAGGGACATCCTGTG	58	159	Genbank	NM_001124339.1
fasn	**F**: GAGACCTAGTGGAGGCTGTC **R**: TCTTGTTGATGGTGAGCTGT	54	186	Sigenae	tcaa0001c.m.06_5.1.om.4
hoad	**F**: GGACAAAGTGGCACCAGCAC **R**: GGGACGGGGTTGAAGAAGTG	59	145	Sigenae	tcad0001a.i.15 3.1.om
lipe1	**F**: AGGGTCATGGTCATCGTCTC **R**: CTTGACGGAGGGACAGCTAC	58	175	Genbank	NM_001197209.1
lpl	**F**: TAATTGGCTGCAGAAAACAC **R**: CGTCAGCAAACTCAAAGGT	59	164	Genbank	AJ224693
pnpla2	**F**: CGTGTCCGAGTTCAAGTC **R**: GGAGAGATGCTGATGGTG	56	174	Genbank	BX318925.2
pparb	**F**: CTGGAGCTGGATGACAGTGA **R**: GTCAGCCATCTTGTTGAGCA	59	195	Genbank	AY356399.1
pparg	**F**: GCCAGTACTGTCGCTTTCAG **R**: TCCATAAACTCAGCCAGCAG	60	171	Genbank	NM_001197212.1
ubiquitin	**F**: ACAACATCCAGAAAGAGTCCA **R**: AGGCGAGCGTAGCACTTG	58	133	Genbank	NM_001124194.1

F: forward; R: reverse; Tm: melting temperature.

ARTICLE II

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Eating for two: Consequences of parental methionine nutrition on offspring metabolism in rainbow trout (*Oncorhynchus mykiss*)

Iban Seiliez [a,*], Emilio J. Vélez [b], Esmail Lutfi [b], Karine Dias [a], Elisabeth Plagnes-Juan [a], Lucie Marandel [a], Stéphane Panserat [a], Inge Geurden [a], Sandrine Skiba-Cassy [a]

[a] INRA-UPPA, UMR1419 Nutrition Métabolisme Aquaculture, F-64310 St-Pée-sur-Nivelle, France
[b] Departament de Biologia Cel·lular, Fisiologia i Immunologia, Facultat de Biologia, Universitat de Barcelona, Spain

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Parental nutrition events may result in permanent changes in the growth potential, health and metabolic status of the next generation. In the present study, we aimed to determine in rainbow trout (*Oncorhynchus mykiss*), the effect of feeding broodstock with a diet deficient in methionine (one of the main methyl-group donor needed for all biological methylation reactions including DNA and histones methylation) on the response of several metabolism- and growth-related genes and proteins in offspring subjected to different dietary methionine levels (deficiency or adequate). Adult males and females were fed for 6 months with either a methionine deficient diet or a control diet. Males and females of the same feeding group were then crossed and the obtained fry were fed with either a methionine deficient or a control diet for 3 weeks from the first exogenous feeding. The obtained results indicated that, whatever the methionine content of the broodstock diet, a 3 weeks dietary methionine deficiency in fry strongly affected the levels of transcripts and/or proteins involved in fatty acid synthesis, cholesterol synthesis and the two main cell quality control mechanisms, namely the ubiquitin-proteasome system and autophagy. In addition, our data also demonstrated that the levels of some of these mRNA and/or proteins are also affected by the dietary methionine deficiency in broodstock. Of interest, some genes (involved in gluconeogenesis and autophagy) were only affected by the parental and not by the fry diet. Together, these results clearly show that the broodstock diet may influence the metabolism of the progeny in rainbow trout and highlight the potential to apply new feeding strategies adapted to plant feedstuffs.

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1. Introduction

Studies in mammals and other vertebrates have shown that dietary influences exerted at critical developmental stages early in life (neonatal nutrition, post-natal (weaning) nutrition) may have long-term consequences on physiological functions in later life (Burdge and Lillycrop, 2010; Lucas, 1998; Patel et al., 2009). This phenomenon, known as nutritional programming, is largely studied in mammalian models for the understanding of diseases such as the metabolic syndrome or diabetes (Burdge and Lillycrop, 2010). Different biological mechanisms have been proposed to be involved in this nutritional programming, including adaptive changes in gene expression (epigenetic phenomenon), preferential clonal selection of adapted cells in programmed tissues and/or programmed differential proliferation of tissue cell types (Jaenisch and Bird, 2003; Lucas, 1998; Symonds et al., 2009; Waterland and Jirtle, 2004).

In fish, the broad concept of nutritional programming is still little studied but seems promising, as reflected by preliminary studies in rainbow trout fry (Geurden et al., 2007; Geurden et al., 2014). Geurden et al. reported that an acute but short (few days) nutritional stimulus (i.e. hyperglucidic diet) applied in early post-hatch life of trout induced a persistent positive effect on the expression of molecular markers involved in carbohydrate digestion (Geurden et al., 2007) and metabolic glucose utilization (Geurden et al., 2014). Similarly, Vagner et al. showed a persistent elevated expression of delta-6 desaturase gene in European sea bass that have been fed during early larval period with a

Abbreviations: *abca1*, ATP-binding cassette transporter A1;; *abcg5*, ATP-binding cassette sub-family G member 5; *abcg8*, ATP-binding cassette sub-family G member 8; *acly*, ATP citrate lyase; *atg4b*, autophagy related 4B; *atg12l*, autophagy related 12-like; *atp6v1a*, ATPase, H+ transporting, lysosomal 70 kDa, V1 subunit A; *cathD*, cathepsin D; *cyp51*, sterol 14α-demethylase; *cyp7a1*, cholesterol 7-alpha-monooxygenase; *dhcr7*, 7-dehydrocholesterol reductase; *eef1a1*, eukaryotic translation elongation factor 1 alpha 1; *fas*, fatty acid synthase; *fbx32*, F-box only protein 32; *gapdh*, Glyceraldehyde 3-phosphate dehydrogenase; *g6pc*, glucose 6-phosphatase, catalytic; *hmgcr*, 3-hydroxy-3-methylglutaryl-CoA reductase; *hmgcs*, 3-hydroxy-3-methylglutaryl-CoA synthase; *lc3/map1lc3*, microtubule-associated protein 1 light chain 3; *lxr*, liver X receptor; *murf*, Muscle Ring-Finger protein; *pck2*, phosphoenolpyruvate carboxykinase 2 (mitochondrial); *sqstm1/p62*, sequestosome 1; *srebp1*, sterol regulatory element-binding protein 1; *ugt1a3*, UDP glucuronosyltransferase family 1 member A3.

* Corresponding author.
E-mail address: iban.seiliez@inra.fr (I. Seiliez).

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diet incorporating low levels of n-3 HUFA, compared to a control group without any early nutritional conditioning (Vagner et al., 2007). More recently, Fang et al. demonstrated the feasibility to permanently modify carbohydrate digestion, transport and metabolism of adult zebrafish through early nutritional programming (Fang et al., 2014). Also in zebrafish, *in ovo* injections of glucose were found to exert long-term effects on molecular markers of glucose metabolism (Rocha et al., 2014). Overall, these studies raise the interesting possibility of directing specific metabolic pathways or functions in juvenile fish, for example to improve the use of substitutes to fish meal and oil, and hence to promote sustainability in aquaculture.

However, studies carried out so far have mainly focused at the onset of exogenous feeding of fish larvae, and it is clearly established that prior to this period, during embryogenesis, an organism has the ability to develop in various ways, depending on the particular environment (Duque-Guimaraes and Ozanne, 2013). Any event that occurs during this period can therefore permanently influence the metabolism and physiology of the organism, and this process has been termed "fetal programming" or more recently "developmental programming" (Reynolds and Caton, 2012). In this regard, in fish, broodstock nutrition that has been shown to influence the yolk-sac reserves (Fernández-Palacios et al., 2011; Izquierdo et al., 2000), as well as larval development and survival (Fernández-Palacios et al., 1995) could also affect profoundly and for long-term the metabolism of progeny. However, except for one recent study in a marine fish species (Izquierdo et al., 2015), the effect of broodstock nutrition on nutritional programming and offspring metabolism during on-growing received very little attention.

Recently, Fontagné-Dicharry et al. demonstrated that lowering the methionine (Met) levels in broodstock diets (for 6 months) below the requirement value for rainbow trout (NRC, 2011) altered the levels of Met and cysteine, as well as those of *S*-adenosyl-methionine (SAM) and *S*-adenosylhomocysteine (SAH) in produced eggs (Fontagné-Dicharry et al., 2017). Furthermore, the size of these eggs as well as survival of the resulting swim-up fry was significantly reduced. Of interest, that study also revealed that parental Met nutritional history affected the growth response of the fry subjected for 21 days to different dietary Met levels, as well as the expression of certain genes involved in Met metabolism, the control of feed intake and of muscle growth in the offspring (Fontagné-Dicharry et al., 2017). However, little attention was paid to the consequences of such nutritional treatments on the expression of intermediary metabolism-related factors. In this context, our objective was to continue the research carried out in this first study and determine the possible effects of parental Met feeding on the offspring's metabolic response, based upon our previously obtained results with rainbow trout fed varying levels of Met (Belghit et al., 2014; Skiba-Cassy et al., 2016). These studies have identified several genes and proteins affected by the Met levels in the diet, being involved in cholesterol, lipid or glucose metabolism (Skiba-Cassy et al., 2016) and also in the two major cell quality control mechanisms, namely the ubiquitin-proteasome system and autophagy (Belghit et al., 2014). These factors have now been analyzed in the present study in fry from Met deficient and Met-adequate (Control) broodstock groups, challenged to feed in turn a Met deficient or Met adequate fry diet with the aim to test the possibility of nutritional programming in fish by means of parental nutrition.

2. Materials andmethods

2.1. Ethics

The experiments were carried out in accordance with the EU legal frameworks, specifically those relating to the protection of animals used for scientific purposes (i.e., Directive 2010/63/EU), and under the French legislation governing the ethical treatment of animals (Decret no. 2001-464, May 29th, 2001). The investigators carrying out the experiment had "level 1" or "level 2" certification, bestowed by the Direction Départementale des Services Vétérinaires (French veterinary services) to carry out animal experiments (INRA 2002-36, April 14th, 2002).

2.2. Experimental design

The experimental design has been detailed in Fontagné-Dicharry et al. (2017). Briefly, rainbow trout female and male broodstock were reared in our experimental fish farm facilities (INRA, Les-Athas, France), in a flow-through rearing system supplied with natural spring water (8 °C) under natural photoperiod (April to October). Fish were randomly divided into three dietary groups (35 fish/group) that were fed for 6 months before spawning one of the three plant-based diet providing three dietary Met levels, set at 0.5, 1 or 2% of the diet in the deficient (BD), adequate (BA) and the excess (BE) diets, respectively (Supplementary Tables 1 and 2). Broodstock growth, relative fecundity, egg size and egg amino acid composition were analyzed and reported in Fontagné-Dicharry et al. (2017). Then, approximately 3000–5000 eggs collected for each female of each nutritional group were fertilized with a pool of sperm (collected from 5 to 8 males fed the corresponding diet). Embryos were reared at INRA experimental facilities at Lées-Athas in 8 °C stream water until the swim-up fry stage at the age of 66 days [8 °C × 66 days = 528 degree days (°D)]; hatching occurred at the age of 44 days (352°D). Following this, the swim-up fry were transferred to the experimental facilities at INRA Donzacq, France, and randomly distributed into 12 circular tanks (50 l; 400 fish/tank) supplied with natural spring water (17 °C) under natural photoperiod. The first-feeding fry were fed for 3 weeks a fry diet containing Met in adequate (FA), deficient (FD) or Excess (FE) amounts ($n = 3$ tank/diet/broodstock group) (Supplementary Tables 1 and 2). At the end of the 21-days feeding trial, survival and growth of fry was significantly affected both by broodstock and fry Met levels as reported in Fontagné-Dicharry et al. (2017) (Supplementary Table 3). At this time point, $n = 9$ fish/tank were killed 8 and 16 h after the last meal by terminal anesthetization by bathing in benzocaine (30 mg/l water) and were then snap-frozen in liquid nitrogen and stored at −80 °C before further mRNA and protein analyses. In the present study, we aimed to precise the parental and dietary effects of a Met deficiency and therefore focused on fry from BD and BA fed broodstock and challenged to fed the FD and FA diets, namely the BD-FD, BD-FA, BA-FD and BA-FA fry groups.

2.3. mRNA levels analysis: quantitative RT-PCR

The extraction of total RNA from whole fry was performed using TRIzol reagent (Invitrogen, 15596018) according to the manufacturer's recommendations. One microgram of the resulting total RNA was reverse transcribed into cDNA, using the SuperScript III RNAseH-reverse transcriptase kit (Invitrogen, 18080085) with random primers (Promega, Charbonniéres, France) according to the manufacturer's instructions. The primer sequences used in the quantitative real-time PCR, as well as the protocol conditions of the assays, have been previously published (Belghit et al., 2014; Marandel et al., 2015; Seiliez et al., 2010; Seiliez et al., 2016; Skiba-Cassy et al., 2016). Primers for genes related to cholesterol metabolism were newly designed using Primer3 software as previously described (Seiliez et al., 2010), and are listed in Table 1. Two set of primers were also designed to amplify together on one hand both *glucose 6-phosphatase b1 (g6pcb1)* ohnologous pair and on the other hand both *g6pcb2* ohnologous pair (Table 1). Indeed these genes were previously described by Marandel et al. (2015) and showed to display the same expression pattern two by two (inside the same ohnologous pair). To confirm specificity of the newly developed RT-PCR assay, the amplicon was purified and sequenced (Beckman-Coulter Genomics, Takeley, UK). For ohnologous pairs (*g6pcb1* and *g6pcb2* pairs), single nucleotide polymorphisms known to

Table 1
Sequences of the primer pairs used for real-time quantitative RT-PCR.

Gene	5′/3′ Forward primer	5′/3′ Reverse primer
abca1	CAGGAAAGACGAGCACCTT	TCTGCCACCTCACACACACTTC
abcg5	CACCGACATGGAGACAGAAA	GACAGATGGAAGGGGATGAA
abcg8	GATACCAGGGTTCCAGAGCA	CCAGAAACAGAGGGACCAGA
acat1	GGCAAGCCTGATGTGGTAGT	ACCGTGCCATTCTCCTTCTG
acat2	TGCTTGTTGTCCCTGGGTTT	GTGTGGCTGTGACGTGTTTC
cyp51	CCCGTTGTCAGCTTTACCA	GCATTGAGATCTTCGTTCTTGC
cyp7a1	ACGTCCGAGTGGCTAAAGAG	GGTCAAAGTGGAGCATCTGG
dhcr7	GTAACCCACCAGACCCAAGA	CCTCTCCTATGCAGCCAAC
hmgcr	GAACGGTGAATGTGCTGTGT	GACCATTTGGGAGCTTGTGT
hmgcs	AGTGGCAAAGAGAGGGTGTG	TTCTGGTTGGAGACGAGGAG
lxrα	TGCAGCAGCCGTATGTGGA	GCGGCGGGAGCTTCTTGTC
srebp2	TAGGCCCCAAAGGGATAAG	TCAGACACGACGAGCACAA
utg1a3	CCACCAGCAAGACAGTCTCA	CAACAGCACAGTGGCTGACT
g6pcb1-glob	AGGGACAGTTCGAAAATGGAG	CCAGAGAGGGAAGAAGATGAAGA
g6pcb2-glob	CCTGCGGAACACCTTCTTTG	TCAATTTGTGGCGCTGATGAG

exist between the two amplified genes were checked to confirm the amplification of both genes by the same primer set. Quantitative RT-PCR was carried out on the Roche LightCycler 480 System (Roche Diagnostics, Neuilly sur Seine, France). The assays were performed using a reaction mix of 6 µl per sample, each of which contained 2 µl of diluted cDNA template, 0.24 µl of each primer (10 µM), 3 µl Light Cycler 480 SY Green Master mix (Roche Diagnostics, 4887352001) and 0.52 µl DNAse/RNAse-free water (5 Prime GmbH, 2500020). The PCR protocol was initiated at 95 °C for 10 min for initial denaturation of the cDNA and hot-start Taq-polymerase activation, followed by 45 cycles of a 3-step amplification program (15 s at 95 °C; 10 s at 60 to 64 °C and 15 s at 72 °C), according to the primer set used (Table 1). Melting curves were systematically monitored (temperature gradient at 1.1 °C/10 s from 65 to 94 °C) at the end of the last amplification cycle to confirm the specificity of the amplification reaction. Each PCR assay included replicate samples (duplicate of reverse transcription and PCR amplification, respectively) and negative controls (reverse transcriptase- and cDNA template-free samples, respectively). For the expression analysis of mRNA, relative quantification of target gene expression was performed using the delta CT method described by Pfaffl et al. (2002). The relative gene expression of *eukaryotic translation elongation factor 1 alpha 1* (*eef1a1*) (or the geometric mean of *eef1α*, *β-actin, gapdh, 18S and R16*) was used for the normalization of measured mRNA as they were both stably expressed in our conditions (data not shown). In all cases, PCR efficiency (E) was measured by the slope of a standard curve using serial dilutions of cDNA. In all cases, PCR efficiency values ranged between 1.8 and 2.2.

2.4. Protein extraction and western blotting

Protein homogenates from whole fry were prepared as previously described (Seiliez et al., 2008). Protein concentrations were determined with the Bradford reagent method (Bradford, 1976). Lysates (10 µg of total protein) were subjected to SDS-PAGE and immunoblotted with an antibody to the microtubule-associated protein 1 light chain 3 (LC3B) (Cell Signaling Technology, 2775), sequestosome 1 (SQSTM1/p62) (Santa-Cruz Biotechnology, sc-10117), β-actin (Santa-Cruz Biotechnology, sc-47778), poly-ubiquitinated proteins (clone FK1 from Upstate/Chemicon Direct, France). These primary antibodies have been shown to cross-react successfully with rainbow trout proteins of interest (Seiliez et al., 2013; Seiliez et al., 2008; Seiliez et al., 2012). After washing, membranes were incubated with an IRDye infrared secondary antibody (LI-COR Inc., 956-32221). Bands were visualized by Infrared Fluorescence using the Odyssey® Imaging System and quantified by Odyssey infrared imaging system software (Application software, version 1.2).

2.5. Statistical analysis

Results are expressed as means ± SD ($N = 6$). Statistical analyses were performed using two-way ANOVA using R software. For all statistical analyses, the level of significance was set at $P < 0.05$.

3. Results

3.1. Effect of Met deficiency in broodstock and/or fry diets on mRNA levels of lipid metabolism-related genes

As illustrated in Fig. 1, analysis of the expression of genes related to lipogenesis indicated that dietary Met deficiency in fry significantly increased the gene expression of *fatty acid synthase* (*fas*) and of the transcription factor *sterol regulatory element-binding protein 1* (*srebp1*) (BD-FD and BA-FD versus BD-FA and BA-FA). No significant effect of the dietary Met content of the broodstock was observed on the expression of these two genes. In contrast, expression of the *ATP citrate lyase* (*acly*) was not affected by the level of Met present in the diet of the fry or the broodstock. We then analyzed the expression of genes related to cholesterol metabolism and found that expression of genes involved in cholesterol synthesis, namely *hmgcs*, *hmgcr*, *cyp51* and *dhcr7*, remained unaffected by the Met content of the broodstock and fry diets (Supplementary Fig. 1). In contrast, expression of the gene coding for the ATP-binding cassette transporter A1 (*abca1*), that mediates the transport of cholesterol and phospholipids from hepatocytes to the apolipoprotein apoA-I in order to generate nascent HDL, significantly increased when fry were fed the Met deficient diet. Dietary fry Met deficiency also increased the expression of *abcg8,* another member of the ATP-binding cassette transporter family, promoting sterol secretion from hepatocytes into bile. We then assessed the expression of genes encoding the cholesterol 7-alpha-monooxygenase also known as cytochrome P450 7A1 (*cyp7a1*), the rate limiting enzyme of bile acid synthesis, and *ugt1a3*, a member of the UDP glucuronosyltransferase (UGT) enzymes family converting hydrophobic bile acids into polar and urinary excretable metabolites. We also found that Met deficiency applied in fry enhanced the expression of *cyp7a1* and *ugt1a3*. Expression of the transcription factor liver X receptor (*lxr*) was found to be upregulated when fry were fed a Met deficient diet. The analysis of the effect of dietary broodstock Met deficiency revealed that *ugt1a3* and *abcg8* were the only two genes affected by the Met content of the broodstock diet with a significant positive effect of Met deficiency.

3.2. Effect of Met deficiency in broodstock and/or fry diets on mRNA levels of gluconeogenic genes

We then monitored the expression of several gluconeogenic genes (*g6pca*, *g6pcb1*, *g6pcb2*, *pck2*) in our samples. As shown in Fig. 2, no significant differences were recorded between fish groups for any of the 3 former analyzed genes. In contrast, the levels of *pck2* transcript exhibited a strong increase in fry from BD fed broodstock (BD-FD and BD-FA) compared to fry from BA fed broodstock (BA-FD and BA-FA).

3.3. Effect of Met deficiency in broodstock and/or fry diets on autophagy

To assess whether methionine deficiency in broodstock and/or fry diets impacts on autophagy in fry, we evaluated the protein levels of LC3-II and SQSTM1/p62, both established markers of autophagy function. We found significantly increased levels of LC3-II and SQSTM1/p62 in fry fed the FD diet (BD-FD and BA-FD) compared to those fed the FA diet (BD-FA and BA-FA) (Fig. 3A). However, no effect of Met deficiency in broodstock diet was evidenced on the levels of these proteins in fry.

We also monitored the expression of several autophagy- (*sqstm1/p62*, *atg12l*, *atg4b*, *lc3b*) and lysosome- (*cathD, atp6v1a*) related genes in our samples. As shown in Fig. 3B, except for *lc3b* and *cathD*, the

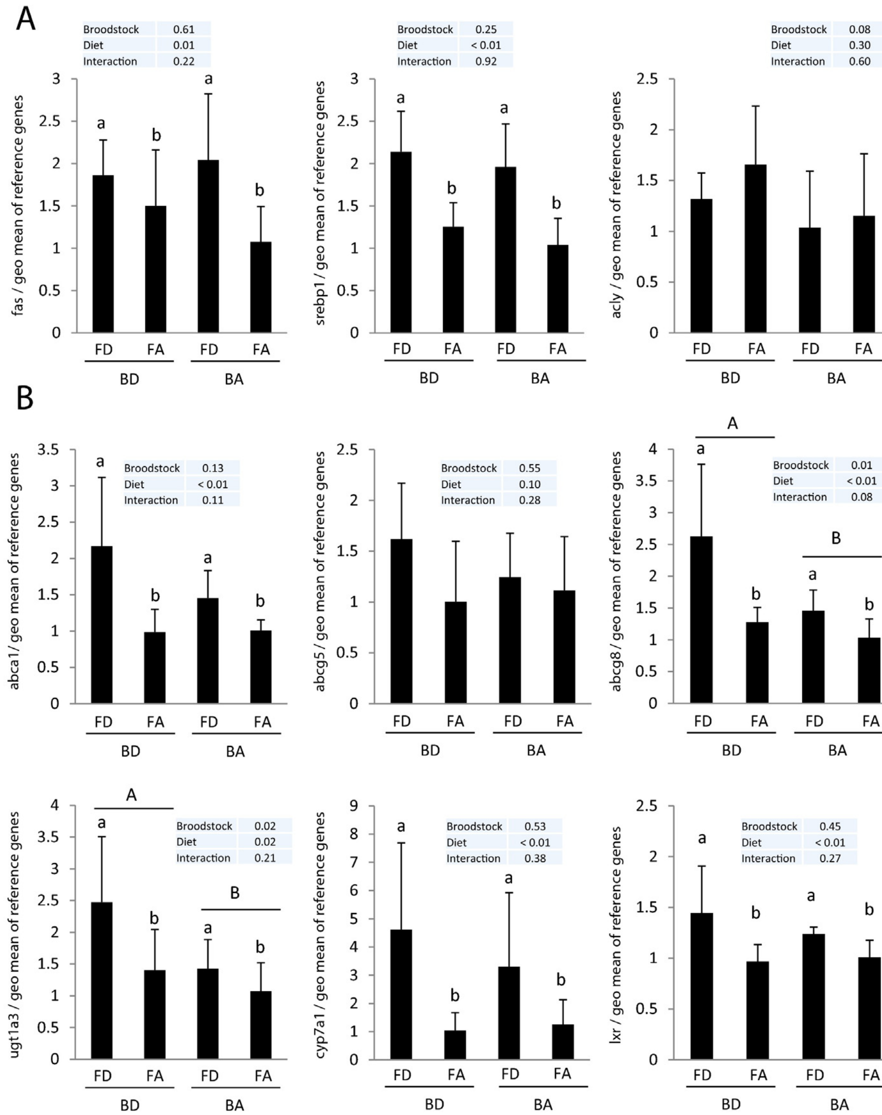

Fig. 1. Effect of a 3 weeks dietary Met deficiency in rainbow trout fry from BD and BA broodstock groups on mRNA levels of lipid and cholesterol metabolism. Analysis by qRT-PCR of mRNA levels of genes involved in (A) fatty acid synthesis (*fasn, srebp1, acly*) and (B) cholesterol synthesis (*abca1, abcg5, abcg8, ugt1a3, cyp7a1, lxr*). Expression values are normalized with the geometric mean of EF1α, β-actin, *gapdh*, 18S and R16. Results are means ± SEM ($n = 6$) and were analyzed using two-way ANOVA. Differences were considered significant at $p < 0.05$. Different (lowercase or uppercase) letters in the graphs indicate difference between groups.

mRNA levels of all other genes displayed a significant increase in fry fed the Met deficient diet (BD-FD and BA-FD) compared to those fed the adequate diet (BD-FA and BA-FA). Interestingly, for *lc3b* and *cathD* (and also *atg4b*), a “broodstock effect” was evidenced, making possible the existence of a parental nutritional ‘imprinting’ event on these genes.

3.4. Effect of Met deficiency in broodstock and/or fry diets on the ubiquitin-proteasome system

We then examined the mRNA levels of muscle-specific E3 ubiquitin ligases coding genes (*fbx32*, *murf1*, *murf2* and *murf3*) thought to target specific proteins for degradation by the Ubiquitin-proteasome system. As shown in Fig. 4A, the mRNA levels of *fbx32* and *murf1* remained the same between the four experimental groups. In contrast, the levels of *murf2* and *murf3* transcripts were higher in fry fed the Met deficient diet (BD-FD and BA-FD) compared to those fed the adequate diet (BD-FA and BA-FA), irrespective of the dietary treatment of broodstocks. In order to assess more directly the function of the ubiquitin-proteasome system, we next analyzed by western blotting the accumulation of ubiquitin-protein conjugates. As shown in Fig. 4B, Met-deficiency in both the fry diet (Diet effect, $p = 0.01$) and broodstock diet (Broodstock effect, $p = 0.04$) enhanced the levels of poly-ubiquitinated proteins.

4. Discussion

Most organisms are constantly faced with environmental changes and stressors. In diverse organisms, there is an anticipatory mechanism during development that can program adult phenotypes. The adult phenotype would be adapted to the predicted environment that occurred during organism maturation. Such an anticipatory mechanism, termed developmental programming, plays likely an important role in fish species which are directly exposed to frequent environmental perturbations such as lowered pH, lowered oxygen levels or rising temperature, as early as the onset of their development. Beside these abiotic factors, parental nutrition which, in oviparous species, can directly affect the composition of produced eggs, may also play an important role in the development of progeny and later on in adult metabolism. In this regard, we recently demonstrated in rainbow trout that lowering the Met levels in broodstock diets below to the requirement value significantly reduced the size of the produced eggs as

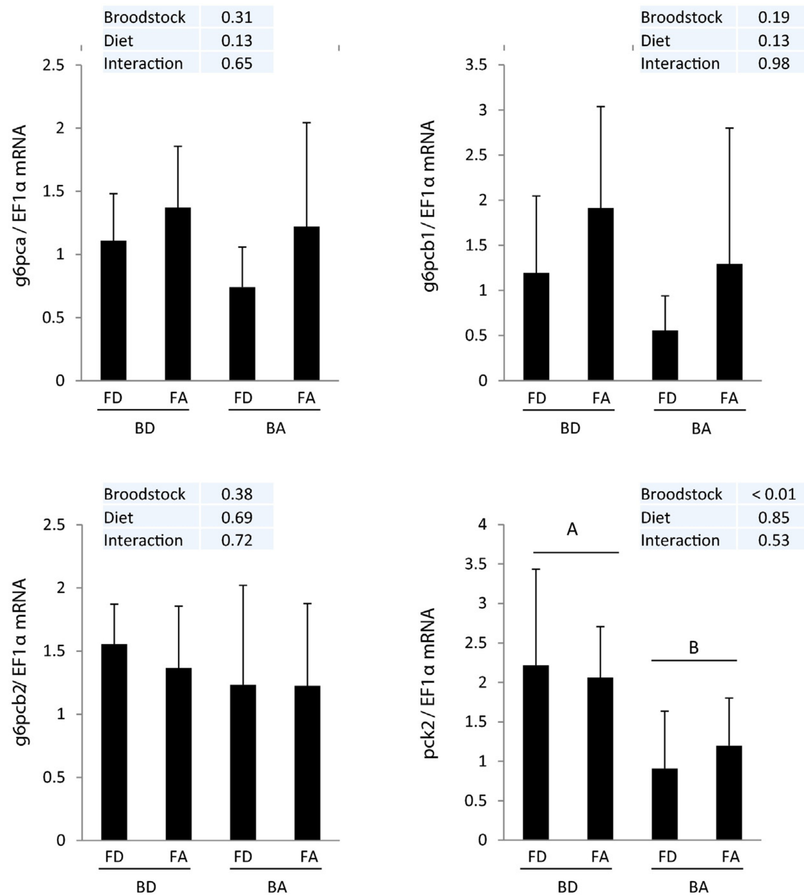

Fig. 2. Effect of a 3 weeks dietary Met deficiency in rainbow trout fry from BD and BA broodstock groups on mRNA levels of gluconeogenic genes. Analysis by qRT-PCR of mRNA levels of gluconeogenic genes (*g6pca*, *G6pcb1*, *G6pcb2*, *pck2*). Expression values are normalized with EF1α-expressed transcripts. Results are means ± SEM ($n = 6$) and were analyzed using two-way ANOVA. Differences were considered significant at $p < 0.05$. Different uppercase letters in the graphs indicate difference between groups.

well as survival of the resulting swim-up fry (Fontagné-Dicharry et al., 2017). The data presented in that study also revealed that parental Met nutritional history affects the growth response of fry to changes in dietary Met levels, as well as the expression of certain genes involved in the regulation of Met metabolism, food intake and muscle growth in offspring (Fontagné-Dicharry et al., 2017). However, little attention was paid to the consequences of such nutritional treatments on the expression of intermediary metabolism-related factors. In this context, the aim of the present study was to precise the metabolic response of fry, from Met deficient and Control broodstock groups, challenged to feed different dietary Met levels (deficiency or adequate), and test the possibility of nutritional programming in fish by the mean of parental nutrition.

Here we show that dietary Met deficiency in male and female broodstock trout during 6 months until the reproduction has very little effect on the expression of genes related to lipogenesis and cholesterol metabolism in the progeny. Among the studied genes, only two (*ugt1a3* and *abcg8*) were affected by the Met content in the broodstock diet with a significant positive effect of Met deficiency. In contrast, Met deficiency applied to the progeny strongly affects the expression of genes involved in lipogenesis and cholesterol metabolism. Met restriction in rat induces a coordinated response between liver and adipose tissue (Perrone et al., 2012). Whereas Met restriction in rat reduces the capacity of the liver to synthesize and export lipids, it enhances the expression of lipogenic and oxidative genes in adipose tissue thereby increasing the capacity of the rat to synthesize and oxidize fatty acids. In the present study, trout fry fed the Met deficient diet for 3 weeks from first feeding displayed enhanced expression of *fas* and *srebp1* which probably contributes to enhance lipogenesis. The gene *lxr* which expression was also increased in trout fry fed the Met deficient diet, might play a key role in the positive regulation of lipogenic gene as it has been found to regulate lipogenesis by directly increasing *srebp1* gene transcription in mice (Schultz et al., 2000). A similar positive effect of Met on lipogenesis has also been demonstrated in Atlantic salmon. In this species, sub-optimal Met level resulted in increased liver weight due to the enhanced hepatic *fas* activity and triacylglycerol accumulation (Espe et al., 2010). However, this positive transcriptional regulation of lipogenesis has not always been observed in rainbow trout. In a previous experiment, we demonstrated that 30% Met deficiency did not alter lipogenic gene expression in liver of juvenile rainbow trout (40 g body weight) (Skiba-Cassy et al., 2016). Similarly, the expression of *fas* and *srebp1* in the liver of male and female broodstock of the present experiment was not affected by feeding the Met deficient diet during 6 months preceding the reproduction (data not shown). In rats, Hasek and coworkers indicated that Met restriction reduced hepatic and muscle triglycerides when initiated after weaning but not when initiated later at 6 months of age (Hasek et al., 2013). Altogether these data suggest that the age or stage of development when dietary Met deficiency is applied may affect the lipogenic response and that very early stages might be more sensitive to dietary Met deficiency.

The early dietary Met deficiency also modified the expression of genes involved in cholesterol metabolism in trout fry. After 3 weeks of deficiency, fry exhibited enhanced expression of genes related to bile acid synthesis (*cyp7a1* and *ugt1A3*), cholesterol export into the bile (*abcg8*) and cholesterol transport to nascent HDL (*abcg1*). The

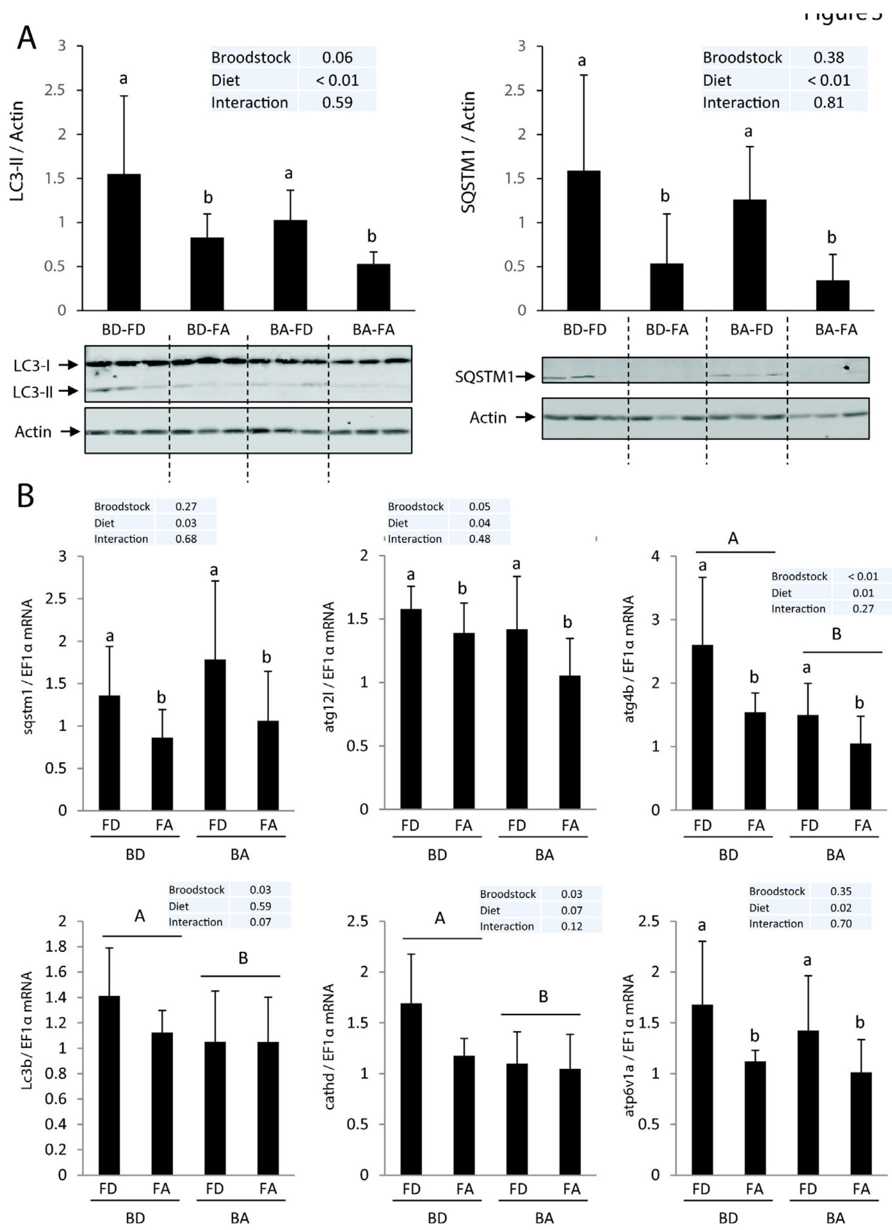

Fig. 3. Effect of a 3 weeks dietary Met deficiency in rainbow trout fry from BD and BA broodstock groups on autophagy. A, Western blot analysis of the two established markers of autophagy function (LC3-II and SQSTM1). Western blots were performed on six individual samples per treatment and a representative blot is shown. Graphs represent the ratio between targeted protein and actin used as loading control. Results are means ± SEM ($n = 6$) and were analyzed using two-way ANOVA. Differences were considered significant at $p < 0.05$. B, mRNA levels of autophagy- (*sqstm1/p62*; *atg12l*; *atg4b*; *lc3b*) and lysosome- (*cathD; atp6v1a*) related genes (qRT-PCR analysis). Expression values are normalized with EF1α-expressed transcripts. Results are means ± SEM ($n = 6$) and were analyzed using two-way ANOVA. Differences were considered significant at $p < 0.05$. Different (lowercase or uppercase) letters in the graphs indicate difference between groups.

expression of *lxr* was also up-regulated in rainbow trout fry fed the Met deficient diet. LXR has been identified as a major regulator of cholesterol metabolism targeting the genes previously cited. Actually, a functional binding site of the oxysterol receptor LXR has been found in the promoter region of the *cyp7A1* gene (Lehmann et al., 1997) which encodes the rate-limiting enzyme converting cholesterol into bile acids as well as in the promoter region of *ugt1A3* which contributes to the detoxification through bile acid glucuronidation (Verreault et al., 2006). The cholesterol transporters of the ATP binding cassette family *abca1* and *abcg5/g8* involved in ApoA1-mediated cholesterol efflux to nascent HDL and sterol excretion into the bile respectively have also been reported as LXR targets (van der Veen et al., 2009; Venkateswaran et al., 2000). Altogether these data support the hypothesis that early dietary Met deficiency promotes hepatic cholesterol elimination and efflux in rainbow trout fry through the activation of the transcription factor LXR. In rats, dietary Met has been shown to induce hypercholesterolaemia by at least enhancing hepatic cholesterol synthesis (Hirche et al., 2006). In the present study, no change in the expression of genes involved in cholesterol synthesis has been observed in trout fry fed Met deficient diet compared to adequate diet. Further investigations are needed to demonstrate if this divergence may be linked to the nutritional carnivorous status of salmonids.

We then analyzed a metabolic pathway essential for the glucose production in carnivorous fish, i.e. gluconeogenesis (for review see (Polakof et al., 2012)). Previous findings in mammals demonstrated that maternal protein restriction could lead to a dysregulation of the carbohydrates metabolism (Fernandez-Twinn and Ozanne, 2010; Jia et al., 2012), through increased expression of two hepatic gluconeogenic genes (*g6pc* and *pck*) in offspring (Vo et al., 2013). The two related enzymes *pck* and *g6pc* catalyze the first and last steps of the gluconeogenesis

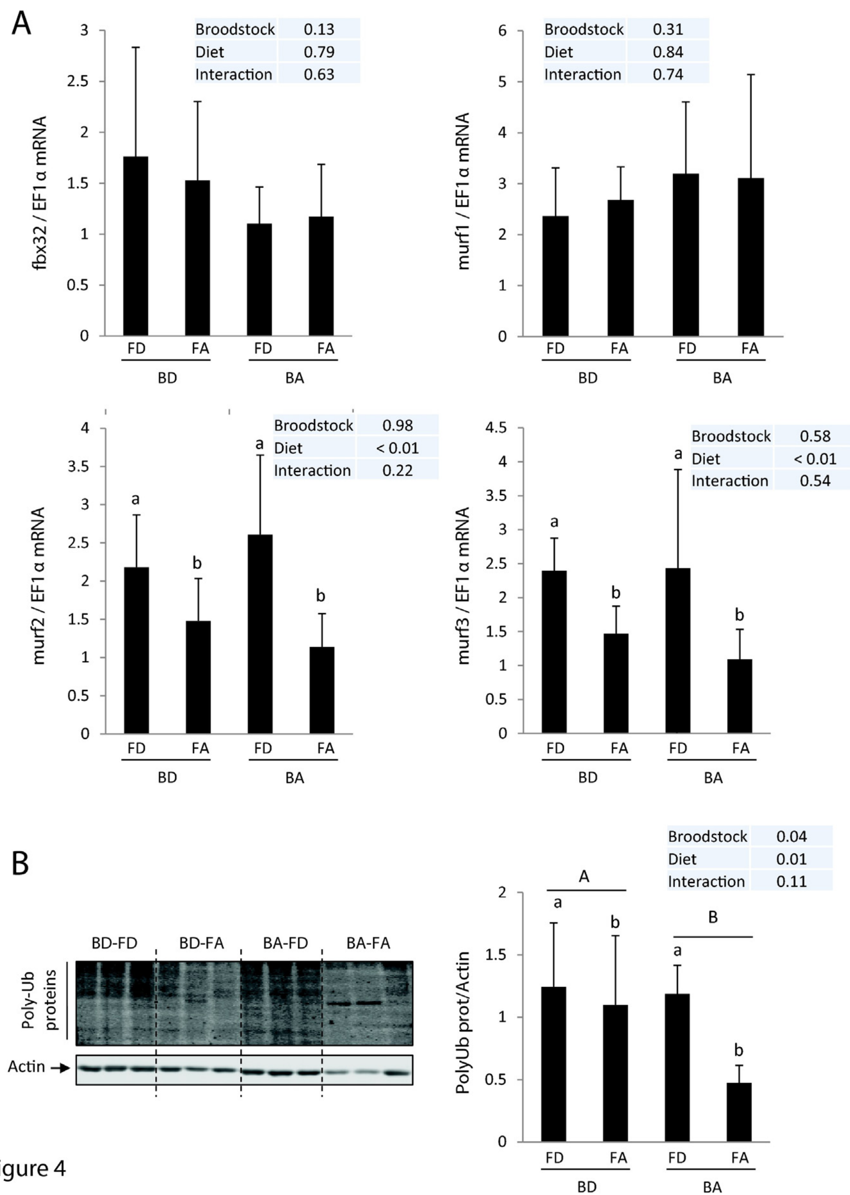

Fig. 4. Effect of a 3 weeks dietary Met deficiency in rainbow trout fry from BD and BA broodstock groups on the ubiquitin-proteasome system. A, mRNA levels of Fbx protein 32 (*fbx32*) and the three muscle-specific RING finger-1 (MuRF-1, 2 and 3) coding for E3 ubiquitin ligases (qRT-PCR analysis). Expression values are normalized with EF1α-expressed transcripts. Results are means ± SEM ($n = 6$) and were analyzed using two-way ANOVA. Differences were considered significant at $p < 0.05$. B, Western blot analysis of poly-ubiquitinated proteins. Western blots were performed on six individual samples per treatment and a representative blot is shown. Graphs represent the ratio between poly-ubiquitinated proteins and actin used as loading control. Results are means ± SEM ($n = 6$) and were analyzed using two-way ANOVA. Differences were considered significant at $p < 0.05$. Different (lowercase or uppercase) letters in the graphs indicate difference between groups.

respectively and have been found to be regulated at a molecular level: *pck2* encodes for the mitochondrial *pck*, which is the major isoform in rainbow trout (Panserat et al., 2001), and duplicated *g6pc* genes (*g6pca*, *g6pcb1* and *g6pcb2*) encode for G6pc enzymes (Marandel et al., 2015; Marandel et al., 2016). In the present study, we observed a strong effect of the parental feeding on *pck2* transcripts, suggesting the possibility in rainbow trout of a developmental programming of glucose metabolism by means of parental nutrition.

We also looked at the effect of dietary Met deficiency in broodstock and/or fry diets on key components of the two main cell quality control mechanisms, namely autophagy and ubiquitin-proteasome system. Autophagy functions as an important catabolic mechanism by mediating the turnover of intracellular organelles and protein complexes through a lysosome dependent degradative pathway (Chen and Klionsky, 2011; Cuervo, 2004). One of the most widely monitored autophagy-related protein is LC3. This ubiquitin-like molecule is present as a nonlipidated form (LC3-I) that is conjugated covalently to phosphatidylethanolamine on the phagophore membrane to form LC3-II, which is a good indicator of autophagosome formation (Klionsky et al., 2016). In the present study, the levels of LC3-II were significantly increased by Met deficiency in the fry diet whatever the parental Met nutritional history, indicating that the autophagosome formation is affected by the profile of amino acids in the diet. These results agree with our previous findings in trout juveniles showing such an effect of dietary Met deficiency on the levels of LC3-II (Belghit et al., 2014). However, they do not allow to conclude on the nature (activating or inhibiting) of the effect of dietary Met deficiency on autophagy. Indeed, while the measure of LC3-II by immunoblot is a reliable indicator of autophagic activity, this method needs to be complemented by assays to estimate overall autophagic flux or rate of flow, since LC3-II is both induced and degraded during autophagy

(Klionsky et al., 2016). Recently, we demonstrated the feasibility of monitoring autophagic flux in vivo in rainbow trout by intraperitoneal injection of colchicine, a pharmacological agent that block fusion of autophagosome with lysosomes and lead to LC3-II accumulation, which can be quantitatively measured (Seiliez et al., 2016). However, fish studied in the present work were too small (mean weight of 0.1–0.3 g) for such an assay. In addition to LC3-II, SQSTM1/p62 can also be used as an autophagy marker. The SQSTM1/p62 protein serves as a link between LC3 and ubiquitinated substrates (Johansen and Lamark, 2011). SQSTM1/p62 and SQSTM1/p62-bound polyubiquitinated proteins become incorporated into the completed autophagosome and are degraded in autolysosomes, thus serving as a readout of autophagic degradation (Klionsky et al., 2016). Here, the levels of SQSTM1/p62 were significantly increased by Met deficiency in the fry diet but no effect of parental Met nutritional history was recorded. It is tempting to conclude that this increase of the levels SQSTM1/p62 in fry fed the Met deficient diet reflects an inhibition of autophagy fuction. However, although analysis of SQSTM1/p62 can assist (at least in certain conditions and/or models) in assessing the autophagy flux, it may have additional functions that need to be considered and particular caution must be taken in interpreting results obtained with this protein (Klionsky et al., 2016). Overall, although doubts remain as to the nature (activating or inhibiting) of the observed effects, our data clearly show that both established markers (LC3-II and SQSTM1/p62) of autophagy function are affected by a Met deficiency in fry diet but not by the parental Met nutritional history.

The induction of autophagy in certain scenarios is also accompanied by an increase in the mRNA levels of certain autophagy genes, such as *atg7* (Bernard and Klionsky, 2015; Bernard et al., 2015), *lc3* (Kirisako et al., 1999), *atg9* (Jin and Klionsky, 2014), *atg12* (Kouroku et al., 2007), *atg14* (Xiong et al., 2012) or *sqstm1/p62* (Sahani et al., 2014). Such induction of the expression of autophagy genes has been thought to allow the replenishment of critical proteins that are destroyed during autophagosome fusion with the lysosome (Sandri, 2010). In the present study, the mRNA levels of several autophagy- and lysosome-related genes was affected not only by Met deficiency in fry diets but also by the parental Met nutritional history. Although, the former dietary effect was already observed previously in trout juveniles (Belghit et al., 2014), to our knowledge, no findings have been published to date on the effect of parental nutrition on the expression of autophagy- and lysosome-related genes in offspring. Interestingly, recent studies have unveiled a epigenetic network that regulates the expression of several autophagy-related genes (Feng et al., 2015; Fullgrabe et al., 2014; Lapierre et al., 2015; Shin et al., 2016). Thus, in the near future, it would be interesting to establish whether or not this observed parental effect on the expression of autophagy- and lysosome-related genes in offspring is accompanied by epigenetic phenomenon, such as DNA and/or histone methylation.

The ubiquitin-proteasomal pathway is another important cell quality control mechanism which has long been considered to be the primary system involved in muscle protein degradation (Attaix et al., 1998; Kumamoto et al., 2000; Lecker et al., 1999; Lecker et al., 2004). Protein breakdown by this pathway relies on selective attachment of ubiquitin molecules to the protein substrate by E3 ubiquitin protein ligases. Following poly-ubiquitination, the targeted proteins are then recognized and degraded by the 26S proteasome. Among genes encoding the E3 ubiquitin ligases, F-box protein 32 (*fbx32*) and Muscle Ring Finger1 (*murf1*), have been studied in depth and shown to play a key role in the control of skeletal muscle mass (Glass, 2010). These genes are both muscle-specific and up-regulated during muscle atrophy (Bonaldo and Sandri, 2013; Schiaffino et al., 2013). In the present study, mRNA levels of two MuRF paralogs (*murf2* and *murf3*) were significantly higher in fry fed the Met deficient diet compared to those fed the adequate diet, irrespective of the dietary treatment of broodstocks. These findings are in agreement with our previous results in trout juveniles showing that dietary Met availability controls the expression of these two paralogs (Belghit et al., 2014). Surprisingly, although we failed to detect any effects of broodstock Met nutritional history on the expression of any of the monitored E3 ligases in fry, the levels of poly-ubiquitinated proteins was altered by the parental dietary Met level ($p = 0.04$). One explanation of this discrepancy is that the levels of poly-ubiquitinated proteins are not solely due to the four E3 ligases studied in the present study but to an extremely complex machinery including hundreds of proteins (e.g., the number of E3 ligases encoded by the mammalian genome is estimated to 600 (Li et al., 2008)). Furthermore, the existence of the newly evidenced crosstalk between the ubiquitin-proteasomal system and autophagy (Wang et al., 2013) indicate that the levels of poly-ubiquitinated proteins not only result from the activity of the former degradative pathway but from the cooperation of both systems. Collectively, these data show that the broodstock dietary history not only affects the expression of certain genes of autophagy but has also a broader effect on the overall cell quality control machinery, as evidenced by the levels of poly-ubiquitinated proteins.

In conclusion, in the present study we show that feeding rainbow trout broodstock for 6 months a diet deficient in Met affected the activation and/or expression of several key metabolic factors in offspring, confirming the possibility of nutritional programming in fish by means of parental nutrition. These results support the decreased growth of offspring from broodstock fed the Met-deficient diet reported in Fontagné-Dicharry et al. (2017) and provide evidence that changes in broodstock Met supply impact on a large variety of metabolism- and growth-related traits in the offspring. Met emerged as a key factor in modulating the cellular availability of the main biological methyl donor SAM needed for all biological methylation reactions (including DNA and histones methylation), and represents a potential critical factor in nutritional programming. In this regard, the reported changes in the levels of SAM and SAH in produced eggs of Met-deficient fed broodstock (Fontagné-Dicharry et al., 2017) could be critical in the observed parental dietary effects on offspring metabolism. In the future, it would be interesting to study whether these effects are accompanied by epigenetic phenomenon, such as DNA and/or histone methylation at the promotor of the concerned genes. Another important issue will be to determine the persistence of the observed "broodstock effects" on offspring metabolism.

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References

Attaix, D., Aurousseau, E., Combaret, L., Kee, A., Larbaud, D., Ralliere, C., Souweine, B., Taillandier, D., Tilignac, T., 1998. Ubiquitin-proteasome-dependent proteolysis in skeletal muscle. Reprod. Nutr. Dev. 38, 153–165.

Belghit, I., Skiba-Cassy, S., Geurden, I., Dias, K., Surget, A., Kaushik, S., Panserat, S., Seiliez, I., 2014. Dietary methionine availability affects the main factors involved in muscle protein turnover in rainbow trout (*Oncorhynchus mykiss*). J Nutr.]–>Br. J. Nutr. 112, 493–503.

Bernard, A., Klionsky, D.J., 2015. Rph1 mediates the nutrient-limitation signaling pathway leading to transcriptional activation of autophagy. Autophagy 11, 718–719.

Bernard, A., Jin, M., Gonzalez-Rodriguez, P., Fullgrabe, J., Delorme-Axford, E., Backues, S.K., Joseph, B., Klionsky, D.J., 2015. Rph1/KDM4 mediates nutrient-limitation signaling that leads to the transcriptional induction of autophagy. Curr. Biol. 25, 546–555.

Bonaldo, P., Sandri, M., 2013. Cellular and molecular mechanisms of muscle atrophy. Dis. Model. Mech. 6, 25–39.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Burdge, G.C., Lillycrop, K.A., 2010. Nutrition, epigenetics, and developmental plasticity: implications for understanding human disease. Annu. Rev. Nutr. 30, 315–339.

Chen, Y., Klionsky, D.J., 2011. The regulation of autophagy - unanswered questions. J. Cell Sci. 124, 161–170.

Cuervo, A.M., 2004. Autophagy: many paths to the same end. Mol. Cell. Biochem. 263, 55–72.

Duque-Guimaraes, D.E., Ozanne, S.E., 2013. Nutritional programming of insulin resistance: causes and consequences. Trends Endocrinol. Metab. 24, 525–535.

Espe, M., Rathore, R.M., Du, Z.Y., Liaset, B., El-Mowafi, A., 2010. Methionine limitation results in increased hepatic FAS activity, higher liver 18:1 to 18:0 fatty acid ratio and hepatic TAG accumulation in Atlantic salmon, *Salmo salar*. Amino Acids 39, 449–460.

Fang, L., Liang, X.F., Zhou, Y., Guo, X.Z., He, Y., Yi, T.L., Liu, L.W., Yuan, X.C., Tao, Y.X., 2014. Programming effects of high-carbohydrate feeding of larvae on adult glucose metabolism in zebrafish, *Danio rerio*. Br. J. Nutr. 111, 808–818.

Feng, Y., Yao, Z., Klionsky, D.J., 2015. How to control self-digestion: transcriptional, post-transcriptional, and post-translational regulation of autophagy. Trends Cell Biol. 25, 354–363.

Fernández-Palacios, H., Izquierdo, M.S., Robaina, L., Valencia, A., Salhi, M., Vergara, J., 1995. Effect of n − 3 HUFA level in broodstock diets on egg quality of gilthead sea bream (*Sparus aurata L.*). Aquaculture 132, 325–337.

Fernández-Palacios, H., Norberg, B., Izquierdo, M., Hamre, K., 2011. Effects of Broodstock Diet on Eggs and Larvae, Larval Fish Nutrition. Wiley-Blackwell, pp. 151–181.

Fernandez-Twinn, D.S., Ozanne, S.E., 2010. Early life nutrition and metabolic programming. N Y Acad Sci.]–>Ann. N. Y. Acad. Sci. 1212, 78–96.

Fontagné-Dicharry, S., Alami-Durante, H., Aragão, C., Kaushik, S., Geurden, I., 2017. Parental and early-feeding effects of dietary methionine in rainbow trout (*Oncorhynchus mykiss*). Aquaculture 469, 16–27.

Fullgrabe, J., Klionsky, D.J., Joseph, B., 2014. The return of the nucleus: transcriptional and epigenetic control of autophagy. Nat. Rev. Mol. Cell Biol. 15, 65–74.

Geurden, I., Aramendi, M., Zambonino-Infante, J., Panserat, S., 2007. Early feeding of carnivorous rainbow trout (*Oncorhynchus mykiss*) with a hyperglucidic diet during a short period: effect on dietary glucose utilization in juveniles. Am. J. Phys. Regul. Integr. Comp. Phys. 292, R2275–R2283.

Geurden, I., Mennigen, J., Plagnes-Juan, E., Veron, V., Cerezo, T., Mazurais, D., Zambonino-Infante, J., Gatesoupe, J., Skiba-Cassy, S., Panserat, S., 2014. High or low dietary carbohydrate:protein ratios during first-feeding affect glucose metabolism and intestinal microbiota in juvenile rainbow trout. J. Exp. Biol. 217, 3396–3406.

Glass, D.J., 2010. Signaling pathways perturbing muscle mass. Curr. Opin. Clin. Nutr. Metab. Care 13, 225–229.

Hasek, B.E., Boudreau, A., Shin, J., Feng, D., Hulver, M., Van, N.T., Laque, A., Stewart, L.K., Stone, K.P., Wanders, D., Ghosh, S., Pessin, J.E., Gettys, T.W., 2013. Remodeling the integration of lipid metabolism between liver and adipose tissue by dietary methionine restriction in rats. Diabetes 62, 3362–3372.

Hirche, F., Schroder, A., Knoth, B., Stangl, G.I., Eder, K., 2006. Effect of dietary methionine on plasma and liver cholesterol concentrations in rats and expression of hepatic genes involved in cholesterol metabolism. Br. J. Nutr. 95, 879–888.

Izquierdo, M.S., Socorro, J., Arantzamendi, L., Hernandez-Cruz, C.M., 2000. Recent advances in lipid nutrition in fish larvae. Fish Physiol. Biochem. 22, 97–107.

Izquierdo, M.S., Turkmen, S., Montero, D., Zamorano, M.J., Afonso, J.M., Karalazos, V., Fernandez-Palacios, H., 2015. Nutritional programming through broodstock diets to improve utilization of very low fishmeal and fish oil diets in gilthead sea bream. Aquaculture 449, 18–26.

Jaenisch, R., Bird, A., 2003. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. Nat. Genet. 33 (Suppl), 245–254.

Jia, Y., Cong, R., Li, R., Yang, X., Sun, Q., Parvizi, N., Zhao, R., 2012. Maternal low-protein diet induces gender-dependent changes in epigenetic regulation of the glucose-6-phosphatase gene in newborn piglet liver. J. Nutr. 142, 1659–1665.

Jin, M., Klionsky, D.J., 2014. Transcriptional regulation of ATG9 by the Pho23-Rpd3 complex modulates the frequency of autophagosome formation. Autophagy 10, 1681–1682.

Johansen, T., Lamark, T., 2011. Selective autophagy mediated by autophagic adapter proteins. Autophagy 7, 279–296.

Kirisako, T., Baba, M., Ishihara, N., Miyazawa, K., Ohsumi, M., Yoshimori, T., Noda, T., Ohsumi, Y., 1999. Formation process of autophagosome is traced with Apg8/Aut7p in yeast. J. Cell Biol. 147, 435–446.

Klionsky, D.J., Abdelmohsen, K., Abe, A., Abedin, M.J., Abeliovich, H., Acevedo Arozena, A., Adachi, H., Adams, C.M., Adams, P.D., Adeli, K., Adhihetty, P.J., Adler, S.G., Agam, G., Agarwal, R., Aghi, M.K., Agnello, M., Agostinis, P., Aguilar, P.V., Aguirre-Ghiso, J., Airoldi, E.M., Ait-Si-Ali, S., Akematsu, T., Akporiaye, E.T., Al-Rubeai, M., Albaiceta, G.M., Albanese, C., Albani, D., Albert, M.L., Aldudo, J., Algul, H., Alirezaei, M., Alloza, I., Almasan, A., Almonte-Beceril, M., Alnemri, E.S., Alonso, C., Altan-Bonnet, N., Altieri, D.C., Alvarez, S., Alvarez-Erviti, L., Alves, S., Amadoro, G., Amano, A., Amantini, C., Ambrosio, S., Amelio, I., Amer, A.O., Amessou, M., Amon, A., An, Z., Anania, F.A., Andersen, S.U., Andley, U.P., Andreadi, C.K., Andrieu-Abadie, N., Anel, A., Ann, D.K., Anoopkumar-Dukie, S., Antonioli, M., Aoki, H., Apostolova, N., Aquila, S., Aquilano, K., Araki, K., Arama, E., Aranda, A., Araya, J., Arcaro, A., Arias, E., Arimoto, H., Ariosa, A.R., Armstrong, J.L., Arnould, T., Arsov, I., Asanuma, K., Askanas, V., Asselin, E., Atarashi, R., Atherton, S.S., Atkin, J.D., Attardi, L.D., Auberger, P., Auburger, G., Aurelian, L., Autelli, R., Avagliano, L., Avantaggiati, M.L., Avrahami, L., Awale, S., Azad, N., Bachetti, T., Backer, J.M., Bae, D.H., Bae, J.S., Bae, O.N., Bae, S.H., Baehrecke, E.H., Baek, S.H., Baghdiguian, S., Bagniewska-Zadworna, A., Bai, H., Bai, J., Bai, X.Y., Bailly, Y., Balaji, K.N., Balduini, W., Ballabio, A., Balzan, R., Banerjee, R., Banhegyi, G., Bao, H., Barbeau, B., Barrachina, M.D., Barreiro, E., Bartel, B., Bartolome, A., Bassham, D.C., Bassi, M.T., Bast Jr., R.C., Basu, A., Batista, M.T., Batoko, H., Battino, M., Bauckman, K., Baumgarner, B.L., Bayer, K.U., Beale, R., Beaulieu, J.F., Beck Jr., G.R., Becker, C., Beckham, J.D., Bedard, P.A., Bednarski, P.J., Begley, T.J., Behl, C., Behrends, C., Behrens, G.M., Behrns, K.E., Bejarano, E., Belaid, A., Belleudi, F., Benard, G., Berchem, G., Bergamaschi, D., Bergami, M., Berkhout, B., Berliocchi, L., Bernard, A., Bernard, M., Bernassola, F., Bertolotti, A., Bess, A.S., Besteiro, S., Bettuzzi, S., Bhalla, S., Bhattacharyya, S., Bhutia, S.K., Biagosch, C., Bianchi, M.W., Biard-Piechaczyk, M., Billes, V., Bincoletto, C., Bingol, B., Bird, S.W., Bitoun, M., Bjedov, I., Blackstone, C., Blanc, L., Blanco, G.A., Blomhoff, H.K., Boada-Romero, E., Bockler, S., Boes, M., Boesze-Battaglia, K., Boise, L.H., Bolino, A., Boman, A., Bonaldo, P., Bordi, M., Bosch, J., Botana, L.M., Botti, J., Bou, G., Bouche, M., Bouchecareilh, M., Boucher, M.J., Boulton, M.E., Bouret, S.G., Boya, P., Boyer-Guittaut, M., Bozhkov, P.V., Brady, N., Braga, V.M., Brancolini, C., Braus, G.H., Bravo-San Pedro, J.M., Brennan, L.A., Bresnick, E.H., Brest, P., Bridges, D., Bringer, M.A., Brini, M., Brito, G.C., Brodin, B., Brookes, P.S., Brown, E.J., Brown, K., Broxmeyer, H.E., Bruhat, A., Brum, P.C., Brumell, J.H., Brunetti-Pierri, N., Bryson-Richardson, R.J., Buch, S., Buchan, A.M., Budak, H., Bulavin, D.V., Bultman, S.J., Bultynck, G., Bumbasirevic, V., Burelle, Y., Burke, R.E., Burmeister, M., Butikofer, P., Caberlotto, L., Cadwell, K., Cahova, M., Cai, D., Cai, J., Cai, Q., Calatayud, S., Camougrand, N., Campanella, M., Campbell, G.R., Campbell, M., Campello, S., Candau, R., Caniggia, I., Cantoni, L., Cao, L., Caplan, A.B., Caraglia, M., Cardinali, C., Cardoso, S.M., Carew, J.S., Carleton, L.A., Carlin, C.R., Carloni, S., Carlsson, S.R., Carmona-Gutierrez, D., Carneiro, L.A., Carnevali, O., Carra, S., Carrier, A., Carroll, B., Casas, C., Casas, J., Cassinelli, G., Castets, P., Castro-Obregon, S., Cavallini, G., Ceccherini, I., Cecconi, F., Cederbaum, A.I., Cena, V., Cenci, S., Cerella, C., Cervia, D., Cetrullo, S., Chaachouay, H., Chae, H.J., Chagin, A.S., Chai, C.Y., Chakrabarti, G., Chamilos, G., Chan, E.Y., Chan, M.T., Chandra, D., Chandra, P., Chang, C.P., Chang, R.C., Chang, T.Y., Chatham, J.C., Chatterjee, S., Chauhan, S., Che, Y., Cheetham, M.E., Cheluvappa, R., Chen, C.J., Chen, G., Chen, G.C., Chen, H., Chen, J.W., Chen, J.K., Chen, M., Chen, P., Chen, Q., Chen, S.D., Chen, S., Chen, S.S., Chen, W., Chen, W.J., Chen, W.Q., Chen, X., Chen, Y.H., Chen, Y.G., Chen, Y., Chen, Y.J., Chen, Y.Q., Chen, Z., Cheng, A., Cheng, C.H., Cheng, H., Cheong, H., Cherry, S., Chesney, J., Cheung, C.H., Chevet, E., Chi, H.C., Chi, S.G., Chiacchiera, F., Chiang, H.L., Chiarelli, R., Chiariello, M., Chieppa, M., Chin, L.S., Chiong, M., Chiu, G.N., Cho, D.H., Cho, S.G., Cho, W.C., Cho, Y.Y., Cho, Y.S., Choi, A.M., Choi, E.J., Choi, E.K., Choi, J., Choi, M.E., Choi, S.I., Chou, T.F., Chouaib, S., Choubey, D., Choubey, V., Chow, K.C., Chowdhury, K., Chu, C.T., Chuang, T.H., Chun, T., Chung, H., Chung, T., Chung, Y.L., Chwae, Y.J., Cianfanelli, V., Ciarcia, R., Ciechomska, I.A., Ciriolo, M.R., Cirone, M., Claerhout, S., Clague, M.J., Claria, J., Clarke, P.G., Clarke, R., Clementi, E., Cleyrat, C., Cnop, M., Coccia, E.M., Cocco, T., Codogno, P., Coers, J., Cohen, E.E., Colecchia, D., Coletto, L., Coll, N.S., Colucci-Guyon, E., Comincini, S., Condello, M., Cook, K.L., Coombs, G.H., Cooper, C.D., Cooper, J.M., Coppens, I., Corasaniti, M.T., Corazzari, M., Corbalan, R., Corcelle-Termeau, E., Cordero, M.D., Corral-Ramos, C., Corti, O., Cossarizza, A., Costelli, P., Costes, S., Cotman, S.L., Coto-Montes, A., Cottet, S., Couve, E., Covey, L.R., Cowart, L.A., Cox, J.S., Coxon, F.P., Coyne, C.B., Cragg, M.S., Craven, R.J., Crepaldi, T., Crespo, J.L., Criollo, A., Crippa, V., Cruz, M.T., Cuervo, A.M., Cuezva, J.M., Cui, T., Cutillas, P.R., Czaja, M.J., Czyzyk-Krzeska, M.F., Dagda, R.K., Dahmen, U., Dai, C., Dai, W., Dai, Y., Dalby, K.N., Dalla Valle, L., Dalmasso, G., D'Amelio, M., Damme, M., Darfeuille-Michaud, A., Dargemont, C., Darley-Usmar, V.M., Dasarathy, S., Dasgupta, B., Dash, S., Dass, C.R., Davey, H.M., Davids, L.M., Davila, D., Davis, R.J., Dawson, T.M., Dawson, V.L., Daza, P., de Belleroche, J., de Figueiredo, P., de Figueiredo, R.C., de la Fuente, J., De Martino, L., De Matteis, A., De Meyer, G.R., De Milito, A., De Santi, M., de Souza, W., De Tata, V., De Zio, D., Debnath, J., Dechant, R., Decuypere, J.P., Deegan, S., Dehay, B., Del Bello, B., Del Re, D.P., Delage-Mourroux, R., Delbridge, L.M., Deldicque, L., Delorme-Axford, E., Deng, Y., Dengjel, J., Denizot, M., Dent, P., Der, C.J., Deretic, V., Derrien, B., Deutsch, E., Devarenne, T.P., Devenish, R.J., Di Bartolomeo, S., Di Daniele, N., Di Domenico, F., Di Nardo, A., Di Paola, S., Di Pietro, A., Di Renzo, L., DiAntonio, A., Diaz-Araya, G., Diaz-Laviada, I., Diaz-Meco, M.T., Diaz-Nido, J., Dickey, C.A., Dickson, R.C., Diederich, M., Digard, P., Dikic, I., Dinesh-Kumar, S.P., Ding, C., Ding, W.X., Ding, Z., Dini, L., Distler, J.H., Diwan, A., Djavaheri-Mergny, M., Dmytruk, K., Dobson, R.C., Doetsch, V., Dokladny, K., Dokudovskaya, S., Donadelli, M., Dong, X.C., Dong, X., Dong, Z., Donohue Jr., T.M., Doran, K.S., D'Orazi, G., Dorn 2nd, G.W., Dosenko, V., Dridi, S., Drucker, L., Du, J., Du, L.L., Du, L., du Toit, A., Dua, P., Duan, L., Duann, P., Dubey, V.K., Duchen, M.R., Duchosal, M.A., Duez, H., Dugail, I., Dumit, V.I., Duncan, M.C., Dunlop, E.A., Dunn Jr., W.A., Dupont, N., Dupuis, L., Duran, R.V., Durcan, T.M., Duvezin-Caubet, S., Duvvuri, U., Eapen, V., Ebrahimi-Fakhari, D., Echard, A., Eckhart, L., Edelstein, C.L., Edinger, A.L., Eichinger, L., Eisenberg, T., Eisenberg-Lerner, A., Eissa, N.T., El-Deiry, W.S., El-Khoury, V., Elazar, Z., Eldar-Finkelman, H., Elliott, C.J., Emanuele, E., Emmenegger, U., Engedal, N., Engelbrecht, A.M., Engelender, S., Enserink, J.M., Erdmann, R., Erenpreisa, J., Eri, R., Eriksen, J.L., Erman, A., Escalante, R., Eskelinen, E.L., Espert, L., Esteban-Martinez, L., Evans, T.J., Fabri, M., Fabrias, G., Fabrizi, C., Facchiano, A., Faergeman, N.J., Faggioni, A., Fairlie, W.D., Fan, C., Fan, D., Fan, J., Fang, S., Fanto, M., Fanzani, A., Farkas, T., Faure, M., Favier, F.B., Fearnhead, H., Federici, M., Fei, E., Felizardo, T.C., Feng, H., Feng, Y., Ferguson, T.A., Fernandez, A.F., Fernandez-Barrena, M.G., Fernandez-Checa, J.C., Fernandez-Lopez, A., Fernandez-Zapico, M.E., Feron, O., Ferraro, E., Ferreira-Halder, C.V., Fesus, L., Feuer, R., Fiesel, F.C., Filippi-Chiela, E.C., Filomeni, G., Fimia, G.M., Fingert, J.H., Finkbeiner, S., Finkel, T., Fiorito, F., Fisher, P.B., Flajolet, M., Flamigni, F., Florey, O., Florio, S., Floto, R.A., Folini, M., Follo, C., Fon, E.A., Fornai, F., Fortunato, F., Fraldi, A., Franco, R., Francois, A., Frankel, L.B., Fraser, I.D., Frey, N., Freyssenet, D.G., Frezza, C., Friedman, S.L., Frigo, D.E., Fu, D., Fuentes, J.M., Fueyo, J., Fujitani, Y., Fujiwara, Y., Fujiya, M., Fukuda, M., Fulda, S., Fusco, C., Gabryel, B., Gaestel, M., Gailly, P., Gajewska, M., Galadari, S., Galili, G., Galindo, I., Galindo, M.F., Galliciotti, G., Galluzzi, L., Galy, V., Gammoh, N., Gandy, S., Ganesan, A.K., Ganesan, S., Ganley, I.G., Gannage, M., Gao, F.B., Gao, F., Gao, J.X., Garcia Nannig, L., Garcia Vescovi, E., Garcia-Macia, M., Garcia-Ruiz, C., Garg, A.D., Garg, P.K., Gargini, R., Gassen, N.C., Gatica, D., Gatti, E.,

Gavard, J., Gavathiotis, E., Ge, L., Ge, P., Ge, S., Gean, P.W., Gelmetti, V., Genazzani, A.A., Geng, J., Genschik, P., Gerner, L., Gestwicki, J.E., Gewirtz, D.A., Ghavami, S., Ghigo, E., Ghosh, D., Giammarioli, A.M., Giampieri, F., Giampietri, C., Giatromanolaki, A., Gibbings, D.J., Gibellini, L., Gibson, S.B., Ginet, V., Giordano, A., Giorgini, F., Giovannetti, E., Girardin, S.E., Gispert, S., Giuliano, S., Gladson, C.L., Glavic, A., Gleave, M., Godefroy, N., Gogal Jr., R.M., Gokulan, K., Goldman, G.H., Goletti, D., Goligorsky, M.S., Gomes, A.V., Gomes, L.C., Gomez, H., Gomez-Manzano, C., Gomez-Sanchez, R., Goncalves, D.A., Goncu, E., Gong, Q., Gongora, C., Gonzalez, C.B., Gonzalez-Alegre, P., Gonzalez-Cabo, P., Gonzalez-Polo, R.A., Goping, I.S., Gorbea, C., Gorbunov, N.V., Goring, D.R., Gorman, A.M., Gorski, S.M., Goruppi, S., Goto-Yamada, S., Gotor, C., Gottlieb, R.A., Gozes, I., Gozuacik, D., Graba, Y., Graef, M., Granato, G.E., Grant, G.D., Grant, S., Gravina, G.L., Green, D.R., Greenhough, A., Greenwood, M.T., Grimaldi, B., Gros, F., Grose, C., Groulx, J.F., Gruber, F., Grumati, P., Grune, T., Guan, J.L., Guan, K.L., Guerra, B., Guillen, C., Gulshan, K., Gunst, J., Guo, C., Guo, L., Guo, M., Guo, W., Guo, X.G., Gust, A.A., Gustafsson, A.B., Gutierrez, E., Gutierrez, M.G., Gwak, H.S., Haas, A., Haber, J.E., Hadano, S., Hagedorn, M., Hahn, D.R., Halayko, A.J., Hamacher-Brady, A., Hamada, K., Hamai, A., Hamann, A., Hamasaki, M., Hamer, I., Hamid, Q., Hammond, E.M., Han, F., Han, W., Handa, J.T., Hanover, J.A., Hansen, M., Harada, M., Harhaji-Trajkovic, L., Harper, J.W., Harrath, A.H., Harris, A.L., Harris, J., Hasler, U., Hasselblatt, P., Hasui, K., Hawley, R.G., Hawley, T.S., He, C., He, C.Y., He, F., He, G., He, R.R., He, X.H., He, Y.W., He, Y.Y., Heath, J.K., Hebert, M.J., Heinzen, R.A., Helgason, G.V., Hensel, M., Henske, E.P., Her, C., Herman, P.K., Hernandez, A., Hernandez, C., Hernandez-Tiedra, S., Hetz, C., Hiesinger, P.R., Higaki, K., Hilfiker, S., Hill, B.G., Hill, J.A., Hill, W.D., Hino, K., Hofius, D., Hofman, P., Hoglinger, G.U., Hohfeld, J., Holz, M.K., Hong, Y., Hood, D.A., Hoozemans, J.J., Hoppe, T., Hsu, C., Hsu, C.Y., Hsu, L.C., Hu, D., Hu, G., Hu, H.M., Hu, H., Hu, M.C., Hu, Y.C., Hu, Z.W., Hua, F., Hua, Y., Huang, C., Huang, H.L., Huang, K.H., Huang, K.Y., Huang, S., Huang, W.P., Huang, Y.R., Huang, Y., Huber, T.B., Huebbe, P., Huh, W.K., Hulmi, J.J., Hur, G.M., Hurley, J.H., Husak, Z., Hussain, S.N., Hussain, S., Hwang, J.J., Hwang, S., Hwang, T.I., Ichihara, A., Imai, Y., Imbriano, C., Inomata, M., Into, T., Iovane, V., Iovanna, J.L., Iozzo, R.V., Ip, N.Y., Irazoqui, J.E., Iribarren, P., Isaka, Y., Isakovic, A.J., Ischiropoulos, H., Isenberg, J.S., Ishaq, M., Ishida, H., Ishii, I., Ishmael, J.E., Isidoro, C., Isobe, K., Isono, E., Issazadeh-Navikas, S., Itahana, K., Itakura, E., Ivanov, A.I., Iyer, A.K., Izquierdo, J.M., Izumi, Y., Izzo, V., Jaattela, M., Jaber, N., Jackson, D.J., Jackson, W.T., Jacob, T.G., Jacques, T.S., Jagannath, C., Jain, A., Jana, N.R., Jang, B.K., Jani, A., Janji, B., Jannig, P.R., Jansson, P.J., Jean, S., Jendrach, M., Jeon, J.H., Jessen, N., Jeung, E.B., Jia, K., Jia, L., Jiang, H., Jiang, L., Jiang, T., Jiang, X., Jiang, Y., Jimenez, A., Jin, C., Jin, H., Jin, L., Jin, M., Jin, S., Jinwal, U.K., Jo, E.K., Johansen, T., Johnson, D.E., Johnson, G.V., Johnson, J.D., Jonasch, E., Jones, C., Joosten, L.A., Jordan, J., Joseph, A.M., Joseph, B., Joubert, A.M., Ju, D., Ju, J., Juan, H.F., Juenemann, K., Juhasz, G., Jung, H.S., Jung, J.U., Jung, Y.K., Jungbluth, H., Justice, M.J., Jutten, B., Kaakoush, N.O., Kaarniranta, K., Kaasik, A., Kabuta, T., Kaeffer, B., Kagedal, K., Kahana, A., Kajimura, S., Kakhlon, O., Kalia, M., Kalvakolanu, D.V., Kamada, Y., Kambas, K., Kaminskyy, V.O., Kampinga, H.H., Kandouz, M., Kang, C., Kang, R., Kang, T.C., Kanki, T., Kanneganti, T.D., Kanno, H., Kanthasamy, A.G., Kantorow, M., Kaparakis-Liaskos, M., Kapuy, O., Karantza, V., Karim, M.R., Karmakar, P., Kaser, A., Kaushik, S., Kawula, T., Kaynar, A.M., Ke, P.Y., Ke, Z.J., Kehrl, J.H., Keller, K.E., Kemper, J.K., Kenworthy, A.K., Kepp, O., Kern, A., Kesari, S., Kessel, D., Ketteler, R., Kettelhut Ido, C., Khambu, B., Khan, M.M., Khandelwal, V.K., Khare, S., Kiang, J.G., Kiger, A.A., Kihara, A., Kim, A.L., Kim, C.H., Kim, D.R., Kim, D.H., Kim, E.K., Kim, H.Y., Kim, H.R., Kim, J.S., Kim, J.H., Kim, J.C., Kim, K.W., Kim, M.D., Kim, M.M., Kim, P.K., Kim, S.W., Kim, S.Y., Kim, Y.S., Kim, Y., Kimchi, A., Kimmelman, A.C., Kimura, T., King, J.S., Kirkegaard, K., Kirkin, V., Kirshenbaum, L.A., Kishi, S., Kitajima, Y., Kitamoto, K., Kitaoka, Y., Kitazato, K., Kley, R.A., Klimecki, W.T., Klinkenberg, M., Klucken, J., Knaevelsrud, H., Knecht, E., Knuppertz, L., Ko, J.L., Kobayashi, S., Koch, J.C., Koechlin-Ramonatxo, C., Koenig, U., Koh, Y.H., Kohler, K., Kohlwein, S.D., Koike, M., Komatsu, M., Kominami, E., Kong, D., Kong, H.J., Konstantakou, E.G., Kopp, B.T., Korcsmaros, T., Korhonen, L., Korolchuk, V.I., Koshkina, N.V., Kou, Y., Koukourakis, M.I., Koumenis, C., Kovacs, A.L., Kovacs, T., Kovacs, W.J., Koya, D., Kraft, C., Krainc, D., Kramer, H., Kravic-Stevovic, T., Krek, W., Kretz-Remy, C., Krick, R., Krishnamurthy, M., Kriston-Vizi, J., Kroemer, G., Kruer, M.C., Kruger, R., Ktistakis, N.T., Kuchitsu, K., Kuhn, C., Kumar, A.P., Kumar, A., Kumar, D., Kumar, R., Kumar, S., Kundu, M., Kung, H.J., Kuno, A., Kuo, S.H., Kuret, J., Kurz, T., Kwok, T., Kwon, T.K., Kwon, Y.T., Kyrmizi, I., La Spada, A.R., Lafont, F., Lahm, T., Lakkaraju, A., Lam, T., Lamark, T., Lancel, S., Landowski, T.H., Lane, D.J., Lane, J.D., Lanzi, C., Lapaquette, P., Lapierre, L.R., Laporte, J., Laukkarinen, J., Laurie, G.W., Lavandero, S., Lavie, L., LaVoie, M.J., Law, B.Y., Law, H.K., Law, K.B., Layfield, R., Lazo, P.A., Le Cam, L., Le Roch, K.G., Le Stunff, H., Leardkamolkarn, V., Lecuit, M., Lee, B.H., Lee, C.H., Lee, E.F., Lee, G.M., Lee, H.J., Lee, H., Lee, J.K., Lee, J., Lee, J.H., Lee, M., Lee, M.S., Lee, P.J., Lee, S.W., Lee, S.J., Lee, S.Y., Lee, S.H., Lee, S.S., Lee, S., Lee, Y.R., Lee, Y.J., Lee, Y.H., Leeuwenburgh, C., Lefort, S., Legouis, R., Lei, J., Lei, Q.Y., Leib, D.A., Leibowitz, G., Lekli, I., Lemaire, S.D., Lemasters, J.J., Lemberg, M.K., Lemoine, A., Leng, S., Lenz, G., Lenzi, P., Lerman, L.O., Lettieri Barbato, D., Leu, J.I., Leung, H.Y., Levine, B., Lewis, P.A., Lezoualc'h, F., Li, C., Li, F., Li, F.J., Li, J., Li, K., Li, L., Li, M., Li, Q., Li, R., Li, S., Li, W., Li, X., Li, Y., Lian, J., Liang, C., Liang, Q., Liao, Y., Liberal, J., Liberski, P.P., Lie, P., Lieberman, A.P., Lim, H.J., Lim, K.L., Lim, K., Lima, R.T., Lin, C.S., Lin, C.F., Lin, F., Lin, F.C., Lin, K., Lin, K.H., Lin, P.H., Lin, T., Lin, W.W., Lin, Y.S., Lin, Y., Linden, R., Lindholm, D., Lindqvist, L.M., Lingor, P., Linkermann, A., Liotta, L.A., Lipinski, M.M., Lira, V.A., Lisanti, M.P., Liton, P.B., Liu, B., Liu, C., Liu, C.F., Liu, F., Liu, H.J., Liu, J., Liu, J.J., Liu, J.L., Liu, K., Liu, L., Liu, Q., Liu, R.Y., Liu, S., Liu, W., Liu, X.D., Liu, X., Liu, X.H., Liu, Y., Liu, Z., Liuzzi, J.P., Lizard, G., Ljujic, M., Lodhi, I.J., Logue, S.E., Lokeshwar, B.L., Long, Y.C., Lonial, S., Loos, B., Lopez-Otin, C., Lopez-Vicario, C., Lorente, M., Lorenzi, P.L., Lorincz, P., Los, M., Lotze, M.T., Lovat, P.E., Lu, B., Lu, J., Lu, Q., Lu, S.M., Lu, S., Lu, Y., Luciano, F., Luckhart, S., Lucocq, J.M., Ludovico, P., Lugea, A., Lukacs, N.W., Lum, J.J., Lund, A.H., Luo, H., Luo, J., Luo, S., Luparello, C., Lyons, T., Ma, J., Ma, Y., Ma, Z., Machado, J., Machado-Santelli, G.M., Macian, F., MacIntosh, G.C., MacKeigan, J.P., Macleod, K.F., MacMicking, J.D., MacMillan-Crow, L.A., Madeo, F., Madesh, M., Madrigal-Matute, J., Maeda, A., Maeda, T., Maegawa, G., Maellaro, E., Maes, H., Magarinos, M., Maiese, K., Maiti, T.K., Maiuri, L., Maiuri, M.C., Maki, C.G., Malli, R., Malorni, W., Maloyan, A., Mami-Chouaib, F., Man, N., Mancias, J.D., Mandelkow, E.M., Mandell, M.A., Manfredi, A.A., Manie, S.N., Manzoni, C., Mao, K., Mao, Z., Mao, Z.W., Marambaud, P., Marconi, A.M., Marelja, Z., Marfe, G., Margeta, M., Margittai, E., Mari, M., Mariani, F.V., Marin, C., Marinelli, S., Marino, G., Markovic, I., Marquez, R., Martelli, A.M., Martens, S., Martin, K.R., Martin, S.J., Martin, S., Martin-Acebes, M.A., Martin-Sanz, P., Martinand-Mari, C., Martinet, W., Martinez, J., Martinez-Lopez, N., Martinez-Outschoorn, U., Martinez-Velazquez, M., Martinez-Vicente, M., Martins, W.K., Mashima, H., Mastrianni, J.A., Matarese, G., Matarrese, P., Mateo, R., Matoba, S., Matsumoto, N., Matsushita, T., Matsuura, A., Matsuzawa, T., Mattson, M.P., Matus, S., Maugeri, N., Mauvezin, C., Mayer, A., Maysinger, D., Mazzolini, G.D., McBrayer, M.K., McCall, K., McCormick, C., McInerney, G.M., McIver, S.C., McKenna, S., McMahon, J.J., McNeish, I.A., Mechta-Grigoriou, F., Medema, J.P., Medina, D.L., Megyeri, K., Mehrpour, M., Mehta, J.L., Mei, Y., Meier, U.C., Meijer, A.J., Melendez, A., Melino, G., Melino, S., de Melo, E.J., Mena, M.A., Meneghini, M.D., Menendez, J.A., Menezes, R., Meng, L., Meng, L.H., Meng, S., Menghini, R., Menko, A.S., Menna-Barreto, R.F., Menon, M.B., Meraz-Rios, M.A., Merla, G., Merlini, L., Merlot, A.M., Meryk, A., Meschini, S., Meyer, J.N., Mi, M.T., Miao, C.Y., Micale, L., Michaeli, S., Michiels, C., Migliaccio, A.R., Mihailidou, A.S., Mijaljica, D., Mikoshiba, K., Milan, E., Miller-Fleming, L., Mills, G.B., Mills, I.G., Minakaki, G., Minassian, B.A., Ming, X.F., Minibayeva, F., Minina, E.A., Mintern, J.D., Minucci, S., Miranda-Vizuete, A., Mitchell, C.H., Miyamoto, S., Miyazawa, K., Mizushima, N., Mnich, K., Mograbi, B., Mohseni, S., Moita, L.F., Molinari, M., Moller, A.B., Mollereau, B., Mollinedo, F., Mongillo, M., Monick, M.M., Montagnaro, S., Montell, C., Moore, D.J., Moore, M.N., Mora-Rodriguez, R., Moreira, P.I., Morel, E., Morelli, M.B., Moreno, S., Morgan, M.J., Moris, A., Moriyasu, Y., Morrison, J.L., Morrison, L.A., Morselli, E., Moscat, J., Moseley, P.L., Mostowy, S., Motori, E., Mottet, D., Mottram, J.C., Moussa, C.E., Mpakou, V.E., Mukhtar, H., Mulcahy Levy, J.M., Muller, S., Munoz-Moreno, R., Munoz-Pinedo, C., Munz, C., Murphy, M.E., Murray, J.T., Murthy, A., Mysorekar, I.U., Nabi, I.R., Nabissi, M., Nader, G.A., Nagahara, Y., Nagai, Y., Nagata, K., Nagelkerke, A., Nagy, P., Naidu, S.R., Nair, S., Nakano, H., Nakatogawa, H., Nanjundan, M., Napolitano, G., Naqvi, N.I., Nardacci, R., Narendra, D.P., Narita, M., Nascimbeni, A.C., Natarajan, R., Navegantes, L.C., Nawrocki, S.T., Nazarko, T.Y., Nazarko, V.Y., Neill, T., Neri, L.M., Netea, M.G., Netea-Maier, R.T., Neves, B.M., Ney, P.A., Nezis, I.P., Nguyen, H.T., Nguyen, H.P., Nicot, A.S., Nilsen, H., Nilsson, P., Nishimura, M., Nishino, I., Niso-Santano, M., Niu, H., Nixon, R.A., Njar, V.C., Noda, T., Noegel, A.A., Nolte, E.M., Norberg, E., Norga, K.K., Noureini, S.K., Notomi, S., Notterpek, L., Nowikovsky, K., Nukina, N., Nurnberger, T., O'Donnell, V.B., O'Donovan, T., O'Dwyer, P.J., Oehme, I., Oeste, C.L., Ogawa, M., Ogretmen, B., Ogura, Y., Oh, Y.J., Ohmuraya, M., Ohshima, T., Ojha, R., Okamoto, K., Okazaki, T., Oliver, F.J., Ollinger, K., Olsson, S., Orban, D.P., Ordonez, P., Orhon, I., Orosz, L., O'Rourke, E.J., Orozco, H., Ortega, A.L., Ortona, E., Osellame, L.D., Oshima, J., Oshima, S., Osiewacz, H.D., Otomo, T., Otsu, K., Ou, J.H., Outeiro, T.F., Ouyang, D.Y., Ouyang, H., Overholtzer, M., Ozbun, M.A., Ozdinler, P.H., Ozpolat, B., Pacelli, C., Paganetti, P., Page, G., Pages, G., Pagnini, U., Pajak, B., Pak, S.C., Pakos-Zebrucka, K., Pakpour, N., Palkova, Z., Palladino, F., Pallauf, K., Pallet, N., Palmieri, M., Paludan, S.R., Palumbo, C., Palumbo, S., Pampliega, O., Pan, H., Pan, W., Panaretakis, T., Pandey, A., Pantazopoulou, A., Papackova, Z., Papademetrio, D.L., Papassideri, I., Papini, A., Parajuli, N., Pardo, J., Parekh, V.V., Parenti, G., Park, J.I., Park, J., Park, O.K., Parker, R., Parlato, R., Parys, J.B., Parzych, K.R., Pasquet, J.M., Pasquier, B., Pasumarthi, K.B., Patschan, D., Patterson, C., Pattingre, S., Pattison, S., Pause, A., Pavenstadt, H., Pavone, F., Pedrozo, Z., Pena, F.J., Penalva, M.A., Pende, M., Peng, J., Penna, F., Penninger, J.M., Pensalfini, A., Pepe, S., Pereira, G.J., Pereira, P.C., Perez-de la Cruz, V., Perez-Perez, M.E., Perez-Rodriguez, D., Perez-Sala, D., Perier, C., Perl, A., Perlmutter, D.H., Perrotta, I., Pervaiz, S., Pesonen, M., Pessin, J.E., Peters, G.J., Petersen, M., Petrache, I., Petrof, B.J., Petrovski, G., Phang, J.M., Piacentini, M., Pierdominici, M., Pierre, P., Pierrefite-Carle, V., Pietrocola, F., Pimentel-Muinos, F.X., Pinar, M., Pineda, B., Pinkas-Kramarski, R., Pinti, M., Pinton, P., Piperdi, B., Piret, J.M., Platanias, L.C., Platta, H.W., Plowey, E.D., Poggeler, S., Poirot, M., Polcic, P., Poletti, A., Poon, A.H., Popelka, H., Popova, B., Poprawa, I., Poulose, S.M., Poulton, J., Powers, S.K., Powers, T., Pozuelo-Rubio, M., Prak, K., Prange, R., Prescott, M., Priault, M., Prince, S., Proia, R.L., Proikas-Cezanne, T., Prokisch, H., Promponas, V.J., Przyklenk, K., Puertollano, R., Pugazhenthi, S., Puglielli, L., Pujol, A., Puyal, J., Pyeon, D., Qi, X., Qian, W.B., Qin, Z.H., Qiu, Y., Qu, Z., Quadrilatero, J., Quinn, F., Raben, N., Rabinowich, H., Radogna, F., Ragusa, M.J., Rahmani, M., Raina, K., Ramanadham, S., Ramesh, R., Rami, A., Randall-Demllo, S., Randow, F., Rao, H., Rao, V.A., Rasmussen, B.B., Rasse, T.M., Ratovitski, E.A., Rautou, P.E., Ray, S.K., Razani, B., Reed, B.H., Reggiori, F., Rehm, M., Reichert, A.S., Rein, T., Reiner, D.J., Reits, E., Ren, J., Ren, X., Renna, M., Reusch, J.E., Revuelta, J.L., Reyes, L., Rezaie, A.R., Richards, R.I., Richardson, D.R., Richetta, C., Riehle, M.A., Rihn, B.H., Rikihisa, Y., Riley, B.E., Rimbach, G., Rippo, M.R., Ritis, K., Rizzi, F., Rizzo, E., Roach, P.J., Robbins, J., Roberge, M., Roca, G., Roccheri, M.C., Rocha, S., Rodrigues, C.M., Rodriguez, C.I., de Cordoba, S.R., Rodriguez-Muela, N., Roelofs, J., Rogov, V.V., Rohn, T.T., Rohrer, B., Romanelli, D., Romani, L., Romano, P.S., Roncero, M.I., Rosa, J.L., Rosello, A., Rosen, K.V., Rosenstiel, P., Rost-Roszkowska, M., Roth, K.A., Roue, G., Rouis, M., Rouschop, K.M., Ruan, D.T., Ruano, D., Rubinsztein, D.C., Rucker 3rd, E.B., Rudich, A., Rudolf, E., Rudolf, R., Ruegg, M.A., Ruiz-Roldan, C., Ruparelia, A.A., Rusmini, P., Russ, D.W., Russo, G.L., Russo, G., Russo, R., Rusten, T.E., Ryabovol, V., Ryan, K.M., Ryter, S.W., Sabatini, D.M., Sacher, M., Sachse, C., Sack, M.N., Sadoshima, J., Saftig, P., Sagi-Eisenberg, R., Sahni, S., Saikumar, P., Saito, T., Saitoh, T., Sakakura, K., Sakoh-Nakatogawa, M., Sakuraba, Y., Salazar-Roa, M., Salomoni, P., Saluja, A.K., Salvaterra, P.M., Salvioli, R., Samali, A., Sanchez, A.M., Sanchez-Alcazar, J.A., Sanchez-Prieto, R., Sandri, M., Sanjuan, M.A., Santaguida, S., Santambrogio, L., Santoni, G., Dos Santos, C.N., Saran, S., Sardiello, M., Sargent, G., Sarkar, P., Sarkar, S., Sarrias, M.R., Sarwal, M.M., Sasakawa, C., Sasaki, M., Sass, M., Sato, K., Sato, M., Satriano, J., Savaraj, N., Saveljeva, S., Schaefer, L., Schaible, U.E., Scharl, M., Schatzl, H.M., Schekman, R., Scheper, W., Schiavi, A., Schipper, H.M., Schmeisser, H., Schmidt, J., Schmitz, I., Schneider, B.E., Schneider, E.M., Schneider, J.L., Schon, E.A., Schonenberger, M.J., Schonthal, A.H., Schorderet, D.F., Schroder, B.,

Schuck, S., Schulze, R.J., Schwarten, M., Schwarz, T.L., Sciarretta, S., Scotto, K., Scovassi, A.I., Screaton, R.A., Screen, M., Seca, H., Sedej, S., Segatori, L., Segev, N., Seglen, P.O., Segui-Simarro, J.M., Segura-Aguilar, J., Seki, E., Sell, C., Seiliez, I., Semenkovich, C.F., Semenza, G.L., Sen, U., Serra, A.L., Serrano-Puebla, A., Sesaki, H., Setoguchi, T., Settembre, C., Shacka, J.J., Shajahan-Haq, A.N., Shapiro, I.M., Sharma, S., She, H., Shen, C.K., Shen, C.C., Shen, H.M., Shen, S., Shen, W., Sheng, R., Sheng, X., Sheng, Z.H., Shepherd, T.G., Shi, J., Shi, Q., Shi, Y., Shibutani, S., Shibuya, K., Shidoji, Y., Shieh, J.J., Shih, C.M., Shimada, Y., Shimizu, S., Shin, D.W., Shinohara, M.L., Shintani, M., Shintani, T., Shioi, T., Shirabe, K., Shiri-Sverdlov, R., Shirihai, O., Shore, G.C., Shu, C.W., Shukla, D., Sibirny, A.A., Sica, V., Sigurdson, C.J., Sigurdsson, E.M., Sijwali, P.S., Sikorska, B., Silveira, W.A., Silvente-Poirot, S., Silverman, G.A., Simak, J., Simmet, T., Simon, A.K., Simon, H.U., Simone, C., Simons, M., Simonsen, A., Singh, R., Singh, S.V., Singh, S.K., Sinha, D., Sinha, S., Sinicrope, F.A., Sirko, A., Sirohi, K., Sishi, B.J., Sittler, A., Siu, P.M., Sivridis, E., Skwarska, A., Slack, R., Slaninova, I., Slavov, N., Smaili, S.S., Smalley, K.S., Smith, D.R., Soenen, S.J., Soleimanpour, S.A., Solhaug, A., Somasundaram, K., Son, J.H., Sonawane, A., Song, C., Song, F., Song, H.K., Song, J.X., Song, W., Soo, K.Y., Sood, A.K., Soong, T.W., Soontornniyomkij, V., Sorice, M., Sotgia, F., Soto-Pantoja, D.R., Sotthibundhu, A., Sousa, M.J., Spaink, H.P., Span, P.N., Spang, A., Sparks, J.D., Speck, P.G., Spector, S.A., Spies, C.D., Springer, W., Clair, D.S., Stacchiotti, A., Staels, B., Stang, M.T., Starczynowski, D.T., Starokadomskyy, P., Steegborn, C., Steele, J.W., Stefanis, L., Steffan, J., Stellrecht, C.M., Stenmark, H., Stepkowski, T.M., Stern, S.T., Stevens, C., Stockwell, B.R., Stoka, V., Storchova, Z., Stork, B., Stratoulias, V., Stravopodis, D.J., Strnad, P., Strohecker, A.M., Strom, A.L., Stromhaug, P., Stulik, J., Su, Y.X., Su, Z., Subauste, C.S., Subramaniam, S., Sue, C.M., Suh, S.W., Sui, X., Sukseree, S., Sulzer, D., Sun, F.L., Sun, J., Sun, S.Y., Sun, Y., Sundaramoorthy, V., Sung, J., Suzuki, H., Suzuki, K., Suzuki, N., Suzuki, T., Suzuki, Y.J., Swanson, M.S., Swanton, C., Sward, K., Swarup, G., Sweeney, S.T., Sylvester, P.W., Szatmari, Z., Szegezdi, E., Szlosarek, P.W., Taegtmeyer, H., Tafani, M., Taillebourg, E., Tait, S.W., Takacs-Vellai, K., Takahashi, Y., Takats, S., Takemura, G., Takigawa, N., Talbot, N.J., Tamagno, E., Tamburini, J., Tan, C.P., Tan, L., Tan, M.L., Tan, M., Tan, Y.J., Tanaka, K., Tanaka, M., Tang, D., Tang, G., Tanida, I., Tanji, K., Tannous, B.A., Tapia, J.A., Tasset-Cuevas, I., Tatar, M., Tavassoly, I., Tavernarakis, N., Taylor, A., Taylor, G.S., Taylor, G.A., Taylor, J.P., Taylor, M.J., Tchetina, E.V., Tee, A.R., Teixeira-Clerc, F., Telang, S., Tencomnao, T., Teng, B.B., Teng, R.J., Terro, F., Tettamanti, G., Theiss, A.L., Theron, A.E., Thomas, K.J., Thome, M.P., Thomes, P.G., Thorburn, A., Thorner, J., Thum, T., Thumm, M., Thurston, T.L., Tian, L., Till, A., Ting, J.P., Titorenko, V.I., Toker, L., Toldo, S., Tooze, S.A., Topisirovic, I., Torgersen, M.L., Torosantucci, L., Torriglia, A., Torrisi, M.R., Tournier, C., Towns, R., Trajkovic, V., Travassos, L.H., Triola, G., Tripathi, D.N., Trisciuoglio, D., Troncoso, R., Trougakos, I.P., Truttmann, A.C., Tsai, K.J., Tschan, M.P., Tseng, Y.H., Tsukuba, T., Tsung, A., Tsvetkov, A.S., Tu, S., Tuan, H.Y., Tucci, M., Tumbarello, D.A., Turk, B., Turk, V., Turner, R.F., Tveita, A.A., Tyagi, S.C., Ubukata, M., Uchiyama, Y., Udelnow, A., Ueno, T., Umekawa, M., Umemiya-Shirafuji, R., Underwood, B.R., Ungermann, C., Ureshino, R.P., Ushioda, R., Uversky, V.N., Uzcategui, N.L., Vaccari, T., Vaccaro, M.I., Vachova, L., Vakifahmetoglu-Norberg, H., Valdor, R., Valente, E.M., Vallette, F., Valverde, A.M., Van den Berghe, G., Van Den Bosch, L., van den Brink, G.R., van der Goot, F.G., van der Klei, I.J., van der Laan, L.J., van Doorn, W.G., van Egmond, M., van Golen, K.L., Van Kaer, L., van Lookeren Campagne, M., Vandenabeele, P., Vandenberghe, W., Vanhorebeek, I., Varela-Nieto, I., Vasconcelos, M.H., Vasko, R., Vavvas, D.G., Vega-Naredo, I., Velasco, G., Velentzas, A.D., Velentzas, P.D., Vellai, T., Vellenga, E., Vendelbo, M.H., Venkatachalam, K., Ventura, N., Ventura, S., Veras, P.S., Verdier, M., Vertessy, B.G., Viale, A., Vidal, M., Vieira, H.L., Vierstra, R.D., Vigneswaran, N., Vij, N., Vila, M., Villar, M., Villar, V.H., Villarroya, J., Vindis, C., Viola, G., Viscomi, M.T., Vitale, G., Vogl, D.T., Voitsekhovskaja, O.V., von Haefen, C., von Schwarzenberg, K., Voth, D.E., Vouret-Craviari, V., Vuori, K., Vyas, J.M., Waeber, C., Walker, C.L., Walker, M.J., Walter, J., Wan, L., Wan, X., Wang, B., Wang, C., Wang, C.Y., Wang, D., Wang, F., Wang, G., Wang, H.J., Wang, H., Wang, H.G., Wang, H.D., Wang, J., Wang, M., Wang, M.Q., Wang, P.Y., Wang, P., Wang, R.C., Wang, S., Wang, T.F., Wang, X., Wang, X.J., Wang, X.W., Wang, Y., Wang, Y.J., Wang, Y.T., Wang, Z.N., Wappner, P., Ward, C., Ward, D.M., Warnes, G., Watada, H., Watanabe, Y., Watase, K., Weaver, T.E., Weekes, C.D., Wei, J., Weide, T., Weihl, C.C., Weindl, G., Weis, S.N., Wen, L., Wen, X., Wen, Y., Westermann, B., Weyand, C.M., White, A.R., White, E., Whitton, J.L., Whitworth, A.J., Wiels, J., Wild, F., Wildenberg, M.E., Wileman, T., Wilkinson, D.S., Wilkinson, S., Willbold, D., Williams, C., Williams, K., Williamson, P.R., Winklhofer, K.F., Witkin, S.S., Wohlgemuth, S.E., Wollert, T., Wolvetang, E.J., Wong, E., Wong, G.W., Wong, R.W., Wong, V.K., Woodcock, E.A., Wright, K.L., Wu, C., Wu, D., Wu, G.S., Wu, J., Wu, M., Wu, S., Wu, W.K., Wu, Y., Wu, Z., Xavier, C.P., Xavier, R.J., Xia, G.X., Xia, T., Xia, W., Xia, Y., Xiao, H., Xiao, J., Xiao, S., Xiao, W., Xie, C.M., Xie, Z., Xilouri, M., Xiong, Y., Xu, C., Xu, F., Xu, H., Xu, J., Xu, L., Xu, X., Xu, Y., Xu, Z.X., Xu, Z., Xue, Y., Yamada, T., Yamamoto, A., Yamanaka, K., Yamashina, S., Yamashiro, S., Yan, B., Yan, X., Yan, Z., Yanagi, Y., Yang, D.S., Yang, J.M., Yang, L., Yang, M., Yang, P.M., Yang, P., Yang, Q., Yang, W., Yang, W.Y., Yang, X., Yang, Y., Yang, Z., Yao, M.C., Yao, P.J., Yao, X., Yao, Z., Yasui, L.S., Ye, M., Yedvobnick, B., Yeganeh, B., Yeh, E.S., Yeyati, P.L., Yi, F., Yi, L., Yin, X.M., Yip, C.K., Yoo, Y.M., Yoo, Y.H., Yoon, S.Y., Yoshida, K., Yoshimori, T., Young, K.H., Yu, H., Yu, J.J., Yu, J.T., Yu, J., Yu, L., Yu, W.H., Yu, X.F., Yu, Z., Yuan, J., Yuan, Z.M., Yue, B.Y., Yue, J., Yue, Z., Zacks, D.N., Zacksenhaus, E., Zaffaroni, N., Zaglia, T., Zakeri, Z., Zecchini, V., Zeng, J., Zeng, M., Zeng, Q., Zervos, A.S., Zhang, D.D., Zhang, F., Zhang, G., Zhang, G.C., Zhang, H., Zhang, J., Zhang, J.P., Zhang, L., Zhang, M.Y., Zhang, X., Zhang, X.D., Zhang, Y., Zhao, M., Zhao, W.L., Zhao, X., Zhao, Y.G., Zhao, Y., Zhao, Y.X., Zhao, Z., Zhao, Z.J., Zheng, D., Zheng, X.L., Zheng, X., Zhivotovsky, B., Zhong, Q., Zhou, G.Z., Zhou, G., Zhou, H., Zhou, S.F., Zhou, X.J., Zhu, H., Zhu, W.G., Zhu, W., Zhu, X.F., Zhu, Y., Zhuang, S.M., Zhuang, X., Ziparo, E., Zois, C.E., Zoladek, T., Zong, W.X., Zorzano, A., Zughaier, S.M., 2016. Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition). Autophagy 12, 1–222.

Kouroku, Y., Fujita, E., Tanida, I., Ueno, T., Isoai, A., Kumagai, H., Ogawa, S., Kaufman, R.J., Kominami, E., Momoi, T., 2007. ER stress (PERK/eIF2alpha phosphorylation) mediates the polyglutamine-induced LC3 conversion, an essential step for autophagy formation. Cell Death Differ. 14, 230–239.

Kumamoto, T., Fujimoto, S., Ito, T., Horinouchi, H., Ueyama, H., Tsuda, T., 2000. Proteasome expression in the skeletal muscles of patients with muscular dystrophy. Acta Neuropathol. 100, 595–602.

Lapierre, L.R., Kumsta, C., Sandri, M., Ballabio, A., Hansen, M., 2015. Transcriptional and epigenetic regulation of autophagy in aging. Autophagy 11, 867–880.

Lecker, S.H., Solomon, V., Mitch, W.E., Goldberg, A.L., 1999. Muscle protein breakdown and the critical role of the ubiquitin-proteasome pathway in normal and disease states. J. Nutr. 129, 227S–237S.

Lecker, S.H., Jagoe, R.T., Gilbert, A., Gomes, M., Baracos, V., Bailey, J., Price, S.R., Mitch, W.E., Goldberg, A.L., 2004. Multiple types of skeletal muscle atrophy involve a common program of changes in gene expression. FASEB J. 18, 39–51.

Lehmann, J.M., Kliewer, S.A., Moore, L.B., Smith-Oliver, T.A., Oliver, B.B., Su, J.L., Sundseth, S.S., Winegar, D.A., Blanchard, D.E., Spencer, T.A., Willson, T.M., 1997. Activation of the nuclear receptor LXR by oxysterols defines a new hormone response pathway. J. Biol. Chem. 272, 3137–3140.

Li, W., Bengtson, M.H., Ulbrich, A., Matsuda, A., Reddy, V.A., Orth, A., Chanda, S.K., Batalov, S., Joazeiro, C.A., 2008. Genome-wide and functional annotation of human E3 ubiquitin ligases identifies MULAN, a mitochondrial E3 that regulates the organelle's dynamics and signaling. PLoS One 3, e1487.

Lucas, A., 1998. Programming by early nutrition: an experimental approach. J. Nutr. 128, 401S–406S.

Marandel, L., Seiliez, I., Veron, V., Skiba-Cassy, S., Panserat, S., 2015. New insights into the nutritional regulation of gluconeogenesis in carnivorous rainbow trout (*Oncorhynchus mykiss*): a gene duplication trail. Physiol. Genomics 47, 253–263.

Marandel, L., Veron, V., Surget, A., Plagnes-Juan, E., Panserat, S., 2016. Glucose metabolism ontogenesis in rainbow trout (*Oncorhynchus mykiss*) in the light of the recently sequenced genome: new tools for intermediary metabolism programming. J. Exp. Biol. 219, 734–743.

NRC, 2011. Nutrient Requirements of Fish and Shrimp. The National Academies Press, Washington, DC.

Panserat, S., Plagnes-Juan, E., Breque, J., Kaushik, S., 2001. Hepatic phosphoenolpyruvate carboxykinase gene expression is not repressed by dietary carbohydrates in rainbow trout (*Oncorhynchus mykiss*). J. Exp. Biol. 204, 359–365.

Patel, M.S., Srinivasan, M., Laychock, S.G., 2009. Metabolic programming: role of nutrition in the immediate postnatal life. J. Inherit. Metab. Dis. 32, 218–228.

Perrone, C.E., Mattocks, D.A., Plummer, J.D., Chittur, S.V., Mohney, R., Vignola, K., Orentreich, D.S., Orentreich, N., 2012. Genomic and metabolic responses to methionine-restricted and methionine-restricted, cysteine-supplemented diets in Fischer 344 rat inguinal adipose tissue, liver and quadriceps muscle. J. Nutrigenet. Nutrigenomics 5, 132–157.

Pfaffl, M.W., Horgan, G.W., Dempfle, L., 2002. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res. 30, e36.

Polakof, S., Panserat, S., Soengas, J.L., Moon, T.W., 2012. Glucose metabolism in fish: a review. J. Comp. Physiol. B. 182, 1015–1045.

Reynolds, L.P., Caton, J.S., 2012. Role of the pre- and post-natal environment in developmental programming of health and productivity. Mol. Cell. Endocrinol. 354, 54–59.

Rocha, F., Dias, J., Engrola, S., Gavaia, P., Geurden, I., Dinis, M.T., Panserat, S., 2014. Glucose overload in yolk has little effect on the long-term modulation of carbohydrate metabolic genes in zebrafish (*Danio rerio*). J. Exp. Biol. 217, 1139–1149.

Sahani, M.H., Itakura, E., Mizushima, N., 2014. Expression of the autophagy substrate SQSTM1/p62 is restored during prolonged starvation depending on transcriptional upregulation and autophagy-derived amino acids. Autophagy 10, 431–441.

Sandri, M., 2010. Autophagy in skeletal muscle. FEBS Lett. 584, 1411–1416.

Schiaffino, S., Dyar, K.A., Ciciliot, S., Blaauw, B., Sandri, M., 2013. Mechanisms regulating skeletal muscle growth and atrophy. FEBS J. 280, 4294–4314.

Schultz, J.R., Tu, H., Luk, A., Repa, J.J., Medina, J.C., Li, L., Schwendner, S., Wang, S., Thoolen, M., Mangelsdorf, D.J., Lustig, K.D., Shan, B., 2000. Role of LXRs in control of lipogenesis. Genes Dev. 14, 2831–2838.

Seiliez, I., Gabillard, J.C., Skiba-Cassy, S., Garcia-Serrana, D., Gutierrez, J., Kaushik, S., Panserat, S., Tesseraud, S., 2008. An in vivo and in vitro assessment of TOR signaling cascade in rainbow trout (*Oncorhynchus mykiss*). Am. J. Phys. Regul. Integr. Comp. Phys. 295, R329–R335.

Seiliez, I., Gutierrez, J., Salmeron, C., Skiba-Cassy, S., Chauvin, C., Dias, K., Kaushik, S., Tesseraud, S., Panserat, S., 2010. An in vivo and in vitro assessment of autophagy-related gene expression in muscle of rainbow trout (*Oncorhynchus mykiss*). Comp. Biochem. Physiol. B Biochem. Mol. Biol. 157, 258–266.

Seiliez, I., Gabillard, J.C., Riflade, M., Sadoul, B., Dias, K., Averous, J., Tesseraud, S., Skiba, S., Panserat, S., 2012. Amino acids downregulate the expression of several autophagy-related genes in rainbow trout myoblasts. Autophagy 8, 364–375.

Seiliez, I., Taty Taty, G.C., Bugeon, J., Dias, K., Sabin, N., Gabillard, J.C., 2013. Myostatin induces atrophy of trout myotubes through inhibiting the TORC1 signaling and promoting Ubiquitin-Proteasome and Autophagy-Lysosome degradative pathways. Gen. Comp. Endocrinol. 186, 9–15.

Seiliez, I., Belghit, I., Gao, Y., Skiba-Cassy, S., Dias, K., Cluzeaud, M., Remond, D., Hafnaoui, N., Salin, B., Camougrand, N., Panserat, S., 2016. Looking at the metabolic consequences of the colchicine-based in vivo autophagic flux assay. Autophagy 12, 343–356.

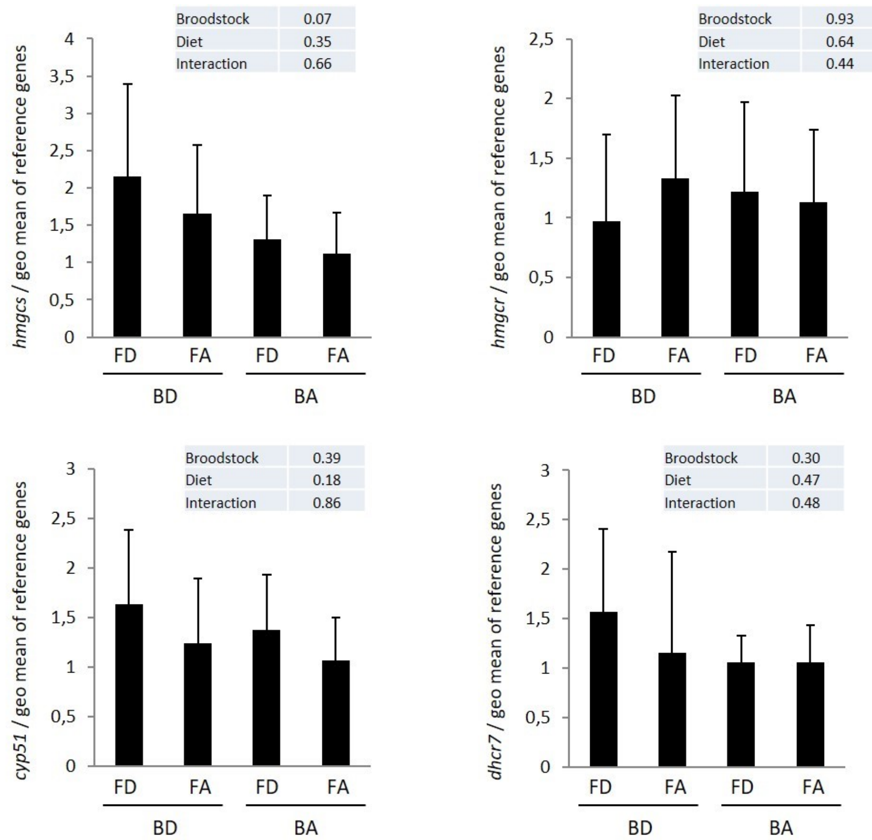
Shin, H.R., Kim, H., Kim, K.I., Baek, S.H., 2016. Epigenetic and transcriptional regulation of autophagy. Autophagy 1–2.

Skiba-Cassy, S., Geurden, I., Panserat, S., Seiliez, I., 2016. Dietary methionine imbalance alters the transcriptional regulation of genes involved in glucose, lipid and amino acid metabolismin the liver of rainbow trout (*Oncorhynchus mykiss*). Aquaculture 454, 56–65.

Symonds, M.E., Sebert, S.P., Hyatt, M.A., Budge, H., 2009. Nutritional programming of the metabolic syndrome. Nat. Rev. Endocrinol. 5, 604–610.

Vagner, M., Infante, U.Z., Robin, J.H., Person-Le Ruyet, J., 2007. Is it possible to influence European sea bass (*Dicentrarchus labrax*) juvenile metabolism by a nutritional conditioning during larval stage? Aquaculture 267, 165–174.
van der Veen, J.N., van Dijk, T.H., Vrins, C.L., van Meer, H., Havinga, R., Bijsterveld, K., Tietge, U.J., Groen, A.K., Kuipers, F., 2009. Activation of the liver X receptor stimulates trans-intestinal excretion of plasma cholesterol. J. Biol. Chem. 284, 19211–19219.
Venkateswaran, A., Laffitte, B.A., Joseph, S.B., Mak, P.A., Wilpitz, D.C., Edwards, P.A., Tontonoz, P., 2000. Control of cellular cholesterol efflux by the nuclear oxysterol receptor LXR alpha. Proc. Natl. Acad. Sci. U. S. A. 97, 12097–12102.
Verreault, M., Senekeo-Effenberger, K., Trottier, J., Bonzo, J.A., Belanger, J., Kaeding, J., Staels, B., Caron, P., Tukey, R.H., Barbier, O., 2006. The liver X-receptor alpha controls hepatic expression of the human bile acid-glucuronidating UGT1A3 enzyme in human cells and transgenic mice. Hepatology 44, 368–378.
Vo, T.X., Revesz, A., Sohi, G., Ma, N., Hardy, D.B., 2013. Maternal protein restriction leads to enhanced hepatic gluconeogenic gene expression in adult male rat offspring due to impaired expression of the liver X receptor. J. Endocrinol. 218, 85–97.
Wang, X.J., Yu, J., Wong, S.H., Cheng, A.S., Chan, F.K., Ng, S.S., Cho, C.H., Sung, J.J., Wu, W.K., 2013. A novel crosstalk between two major protein degradation systems: regulation of proteasomal activity by autophagy. Autophagy 9, 1500–1508.
Waterland, R.A., Jirtle, R.L., 2004. Early nutrition, epigenetic changes at transposons and imprinted genes, and enhanced susceptibility to adult chronic diseases. Nutrition 20, 63–68.
Xiong, X., Tao, R., DePinho, R.A., Dong, X.C., 2012. The autophagy-related gene 14 (Atg14) is regulated by forkhead box O transcription factors and circadian rhythms and plays a critical role in hepatic autophagy and lipid metabolism. J. Biol. Chem. 287, 39107–39114.

Supporting information.



Suppl Fig 1. Effect of a 3 weeks dietary Met deficiency in rainbow trout fry from BD and BA broodstock groups on mRNA levels of genes involved in cholesterol synthesis. Analysis by qRT-PCR of mRNA levels of 3-hydroxy-3-methylglutaryl-CoA synthase (hmgcs), 3-hydroxy-3-methylglutaryl-CoA reductase (hmgcr), sterol 14α-demethylase (cyp51) and 7-dehydrocholesterol reductase (dhcr7). Expression values are normalized with the geometric mean of eef1α, β-actin, gapdh, 18S and R16. Results are means ± SEM (n=6) and were analyzed using two-way ANOVA. Differences were considered significant at $P<0.05$.

ARTICLE III

Breeding selection of rainbow trout for high or low muscle adiposity has differential effects on lipid metabolism and oxidative stress

Esmail Lutfi[1], Ningping Gong[2], Marcus Johansson[2], Albert Sánchez-Moya[1], Björn Thrandur Björnsson[2], Joaquim Gutiérrez[1], Isabel Navarro[1] and Encarnación Capilla[1]*

[1]Department of Cell Biology, Physiology and Immunology, Faculty of Biology, University of Barcelona, Barcelona 08028, Spain

[2]Fish Endocrinology Laboratory, Department of Biological and Environmental Sciences, University of Gothenburg, Box 463, S-40590 Gothenburg, Sweden.

*Corresponding author: Encarnación Capilla

Departament de Biologia Cel·lular, Fisiologia i Immunologia, Facultat de Biologia, Universitat de Barcelona, Av. Diagonal 643, 08028, Barcelona, Spain.

Tel: +34 934039634; Fax: +34 934110358; E-mail: ecapilla@ub.edu

NG, MJ and BThB designed the experimental trial and carried out the sampling. EL and ASM analyzed the samples. EL, JG, IN and EC analyzed and interpreted the data. EL, BThB, JG, IN and EC drafted and critically reviewed the manuscript. All authors read and approved the final paper. The authors have declared no conflict of interest.

ABSTRACT

The present study evaluates the transcriptional regulation of lipid metabolism, adiponectin system and oxidative stress genes in two rainbow trout lines selected for low (lean-line, LL) and high (fat-line, FL) muscle adiposity, subjected to different feeding and fasting regimes. Under feeding conditions, FL fish presented higher muscle lipid content, plasma triglycerides and non-esterified fatty acids than the LL fish, and displayed a different trend in response to fasting. Concerning gene expression analyses, FL fish showed higher fatty acid synthase mRNA levels compared to LL fish in both adipose tissue (AT) and white muscle (WM), and higher lipoprotein lipase mRNA levels only in WM, altogether suggesting an increased lipogenic capacity and fatty acid uptake in the fatty genotype. During fasting, fatty acid synthase mRNA levels presented different expression patterns between lines in both tissues, in agreement with plasma results. Minor differences were observed between genotypes in adiponectin system genes expression, being adiponectin receptor 2 mRNA levels in WM and adiponectin mRNA levels in AT, higher in FL than LL fish after 1 and 4 weeks of fasting respectively. Furthemore, oxidative stress genes mRNA levels in both fish lines showed a different pattern between AT and WM upon fasting, probably indicating a higher protective effect in WM. Overall, the present study reveals a distinct metabolic regulation for each genotype, highlighting their different strategies in response to food deprivation.

1. INTRODUCTION

Energy homeostasis is a critical mechanism by which animals regulate food intake and energy expenditure. When this equilibrium is disrupted such as during fasting or overfeeding, physiological and behavioral compensatory changes take place to restore and maintain energy balance [1,2]. As a major source of energy storage, adipose tissue (AT) is considered to play an important role in the regulation of whole-body energy homeostasis [3]. In addition, AT is an important endocrine tissue secreting hormones such as leptin and adiponectin [4,5]. In mammals, the contribution of these adipokines in regulating energy balance during food restriction is well documented [6,7]. Adiponectin is involved in the modulation of glucose and lipid metabolism [8], and decreased circulating levels are associated with obesity while increased levels are found in fasting patients [9,10]. Furthermore, its anti-inflammatory role has been shown to display protective actions on the development of several metabolic disorders [10,11].

In their natural environment, fish can experience prolonged periods of fasting, either as a result of spawning migration or seasonally limited food availability [12,13]. To survive these periods, fish can metabolize large proportion of their energy reserves without physiological harm [14,15], highlighting their particular suitability as subjects for studies on the effects of long-term fasting. Moreover, for aquaculture production purposes, dietary manipulation in feeding programs has been explored, such as subjecting the animals to periods of fasting, in order to induce compensatory growth during subsequent feeding periods [16]. The molecular mechanisms regulating growth and metabolism have been studied in fish [15,17–19], but there are still many caveats concerning their response to fasting, especially in terms of regulatory functions of relatively recently discovered adipokine hormones such as adiponectin and leptin.

In salmonids, the main energy sources mobilized during fasting are lipids stored in visceral AT, liver and muscle, whereas tissue protein is only mobilized, chiefly from muscle, during long-term starvation periods [20,21]. Salmonids are regarded as "fatty" fish as they store significant amounts of lipids in muscle. Although the muscular fat content can vary greatly depending on intrinsic factors, such as genotype and sexual maturation, as well as extrinsic factors such as environmental or rearing conditions [22], in aquaculture, fat content of rainbow trout (*Oncorhynchus mykiss*) fillet is typically 12-18% [23].

The mobilization of body reserves upon food deprivation in fish is tightly linked to a reduction in metabolic rate as an energy-storing mechanism. However, such metabolic changes can be associated with higher oxidative stress [24], as mitochondria involved in the metabolic responses to fasting also produce damaging reactive oxygen species (ROS). As studies regarding the influence of fasting on antioxidant defenses in liver and flesh lipid oxidation at the molecular level are still scarce [25,26], elucidation of the effects of fasting on rainbow trout lipid metabolism, the adiponectin system regulation and oxidative stress status is of particular interest.

Although controversial, the "thrifty genes" hypothesis postulates that some individuals favor increased fat deposition capacity, in order to explain differential metabolic responses to diverse environmental stressors, such as food deprivation [27,28]. Irrespective of the merit of this hypothesis, there is clearly large individual variability in metabolic strategies depending on several factors such as age, sex, body size or genetic background. These specific contributions to metabolic phenotypes and diseases have been investigated in mammals [29–31], but rarely

in fish [32], even though there is an increased interest within the aquaculture industry to select for specific physiological traits to obtain optimal fish phenotypes.

An important quality trait in farmed salmonids is muscle adiposity, as this may affect organoleptic characteristics as well as yield of the fillets [33]. Breeding selection of rainbow trout for high or low muscle adiposity has shown this parameter to be a highly heritable trait [34]. Through subsequent generations, two rainbow trout lines selected for low (lean line, LL) and high (fat line, FL) muscle adiposity have also diverged in several aspects of glucose and lipid metabolism [35–37]. Thus, the FL fish have higher capability than the LL fish to utilize and store glucose and maintain its homeostasis [35,38]. Further, enhanced lipogenic potential is suggested to be a key mechanism responsible for high muscle adiposity in FL fish [39]. The experiment on which the current study is based was carried out in 2014 on the seventh generation of the breeding selected FL and LL rainbow trout, and both systemic and central effects of leptin endocrinology in relation to energy stores and lipid mobilization have been analyzed [19,40]. However, the role of adipose tissue in the regulation of lipid homeostasis in these lines has not been studied in detail.

The aim of the current study was to elucidate relationships between lipid metabolism, oxidative stress and the adiponectin system by comparing important lipid parameters and expression of key genes in LL and FL rainbow trout during feeding and fasting. As previous studies have reported line-dependent differences in the regulation of metabolic processes such as glucose utilization or some lipid metabolism markers under normal feeding conditions, the hypothesis is that these lines may also follow different strategies to cope with fasting, especially in white muscle (WM) and AT.

2. MATERIALS AND METHODS

2.1 Experimental fish and culture conditions

Adult rainbow trout, approximately 250 g in weight, were maintained at the Institut National de la Recherche Agronomique (INRA) experimental facilities in the Pisciculture Expérimentale des Monts d'Arrée (PEIMA) (Drennec, Sizun, France). These were kept in 1.8 m^3 circular outdoor tanks with water flow of 3 $m^3\ h^{-1}$ and oxygen levels >6.0 mg L^{-1}, under ambient light and temperature conditions (from 10.6 to 13.5°C), and fed five times per day with a commercial diet (Le Gouessant, France). Daily ration was calculated every week based on fish size and

water temperature (from 1.16 to 1.25% body weight day^{-1}). All animal handling procedures complied with the Guidelines of the European Union Council Directive of 24 November 1986 (86/609/EEC), under the official license of L. Laurent (29–036). The PEIMA facility is approved for animal experimentation through license C29-277-02.

2.2 Experimental trial and sampling

The present study was performed using two lines of rainbow trout obtained after seven generations of divergent selection for high or low muscle fat content, designated as fat-line (FL) and lean-line (LL), respectively [34]. After three-week acclimation period, a feeding/fasting experimental protocol was conducted (detailed in Johansson et al. 2016). Briefly, over an experimental period of four weeks (W), FL and LL fish were subjected to four feeding regimes. One feeding regime included no fasting (0W groups), with the fish fed normally throughout. A second feeding regime included feeding for three weeks followed by one week of fasting (1W groups). A third feeding regime included two weeks of feeding followed by two weeks of fasting (2W groups), and a fourth feeding regime consisted of fasting throughout (4W groups). At sampling, ten fish per tank (two tanks per group), thus 20 LL fish and 20 FL fish per feeding regime, were anesthetized with a lethal dose (160 mg l^{-1}) of isoeugenol (ScanAqua, Norway) and sampled.

Body weight was measured and blood taken from the caudal vessels. Blood was immediately placed on ice and centrifuged at 800 × *g* for 5 min at 6 °C and the plasma obtained was frozen at -20 °C. The whole liver and viscera (including intestinal tract and visceral AT) were taken and weighed in order to calculate the corresponding liver and visceral somatic indexes (LSI and VSI, respectively). WM and perivisceral AT samples were taken and snap-frozen in liquid nitrogen. All samples were subsequently transported and stored at −80°C pending analysis.

2.3 Plasma levels of non-esterified fatty acids and total triglycerides

Plasma was analyzed for non-esterified fatty acids (NEFAs, Wako Chemicals GmbH, Neuss, Germany) and total triglycerides (TG) (Sigma-Aldrich, Tres Cantos, Spain), using commercial enzyme kits.

2.4 Muscle lipid content

Lipids were extracted from white muscle samples as described by [41]. Briefly, 70 to 120 mg of tissue were homogenized in a glass tube in 6 ml chloroform:methanol (1:2) for 20 s. Then, chloroform was added to bring the solution to 1:1, homogenized for 15 s and centrifuged at 2095 × g for 10 min. This step was repeated and the supernatants were transferred to a clean glass tube, mixed with 4.1 ml 0.88% KCl solution and stored at 4°C overnight. Next day, the chloroform-lipid phase was removed with a 10 ml Hamilton syringe, placed in a clean pre-weighed 10 ml glass tube and evaporated for 3 h. The tube was re-weighed and lipid content calculated as percentage of the muscle wet weight.

2.5 Lipid peroxidation in muscle

Lipid peroxidation in WM was determined as the end product malondialdehyde (MDA) by thiobarbituric reactive substances (TBARS) assay [42]. Shortly, 100 mg of muscle were homogenized with Precellys Evolution 24 homogenizer coupled to a Cryolis cooler (Bertin Technologies, Hospitalet de Llobregat, Spain) in RIPA buffer (Tris 50 mM pH 7.4, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, NaCl 150 mM, EDTA 2 mM and NaF 50 mM) as previously described [43]. Homogenates were centrifuged twice at 1000 × g for 15 min and once at 16000 × g for 30 min and the pellets were discarded. Then, TBA-MDA reactions were performed by incubating the supernatants at 95°C for 10 min, and measured fluorometrically at an excitation wavelength of 515 nm detecting an emission wavelength of 548 nm. The calibration curve was determined using MDA tetrabutylamonium salt.

2.6 RNA extraction and cDNA synthesis

WM and AT samples (100 mg) were homogenized with Precellys Evolution 24 homogenizer coupled to a Cryolis cooler and total RNAs were extracted using TriReagent (Ambion, Alcobendas, Spain), according to the manufacturer's recommendations. Concentration and RNA purity were determined using a ND-2000 NanoDrop spectrophotometer (Thermo Fisher Scientific, Alcobendas, Spain) and 1000 ng of total RNA were treated with DNase I (Life Technologies, Alcobendas, Spain), following the manufacturer’s protocol, to remove all genomic DNA. Afterwards, the RNA was reverse transcribed with the Transcriptor First Strand

cDNA synthesis Kit (Roche, Sant Cugat del Valles, Spain) and the cDNA obtained was stored at -20 °C for quantitative PCR (qPCR) analyses.

2.7 Real-time quantitative PCR

The mRNA transcript levels of fatty acid synthase, *fasn*; lipoprotein lipase, *lpl*; hormone-sensitive lipase, *lipe1*; glyceraldehyde 3-phosphate dehydrogenase, *gapdh*; liver x receptor, *lxr*; malic enzyme 1, *me1*; peroxisome proliferator-activated receptor alpha, *ppara*; peroxisome proliferator-activated receptor beta, *pparb*; adiponectin, *adipoq*; adiponectin receptor 1, *adipor1*; adiponectin receptor 2, *adipor2*; gamma-glutamyl-cysteine synthetase, *gcs*; superoxide dismutase, *sod*; glutathione peroxidase, *gpx1*; hydroperoxide glutathione peroxidase, *phgpx*; glutathione reductase¸ *gsr* and thioredoxin reductase, *tr;* plus two reference genes (elongation factor 1 alpha, *ef1a* and ubiquitin, *ubq*) were examined with a CFX384™ Real-Time System (Bio-Rad, El Prat de Llobregat, Spain).

All analyses were performed in triplicate wells using 384-well plates with 2.5 µL itaq SYBR Green Supermix (Bio-Rad, El Prat de Llobregat, Spain), 250 nM forward and reverse specific primers (Table 1), and 1 µL diluted cDNA for each sample, in a final volume of 5 µL. The primer sequences as well as the protocol conditions have been published [44–50]. The mRNA levels of each target gene analyzed were calculated relative to the reference genes (geometric mean of *ef1a* and *ubq*) using the Pfaffl method [51] implemented in the BioRad CFX manager 3.1. software.

2.8 Statistical analyses

Data were analyzed using SPSS Statistics v.22 (IBM, Armonk, USA) and GraphPad Prism 6 (La Jolla, USA, www.graphpad.com) and presented as mean ± SEM. Data normality and homoscedasticity were assessed using Shapiro-Wilk and Levene's tests, respectively. Statistical significance was assessed by two-way analysis of variance (two-way ANOVA), followed by Tukey (differences among fasting regimes) and Sidak (differences between lines) *post-hoc* tests. Statistical differences were considered significant for all analyses when p-value ≤ 0.05.

3. RESULTS

3.1 Biometric indexes, plasma parameters and muscle lipids

LSI, VSI, total lipid content and lipid peroxidation products (TBARS) in muscle, and plasma NEFAs and TG levels are presented in Table 1.

Table 1. Liver somatic index (LSI), visceral somatic index (VSI), muscle lipid content and peroxidation products (TBARS) and plasma levels of triglycerides (TG) and non-esterified fatty acids (NEFAs).

Fasting (weeks)		0W	1W	2W	4W
LSI (%)	**LL**	1.16 ± 0.03^{a}	0.88 ± 0.04^{b}	0.81 ± 0.028^{b}	0.89 ± 0.03^{b}
	FL	1.23 ± 0.05^{A}	0.85 ± 0.017^{B}	0.79 ± 0.05^{B}	0.75 ± 0.03^{B*}
VSI (%)	**LL**	9.67 ± 0.23^{a}	8.22 ± 0.24^{b}	7.95 ± 0.22^{bc}	7.09 ± 0.37^{c}
	FL	8.24 ± 0.31^{A**}	7.02 ± 0.40^{B*}	6.69 ± 0.16^{BC**}	5.70 ± 0.26^{C**}
Muscle lipid content (%)	**LL**	3.90 ± 0.54	3.06 ± 0.39	2.99 ± 0.40	3.43 ± 0.62
	FL	7.31 ± 0.72^{A**}	5.10 ± 0.84^{B*}	4.58 ± 0.58^{B}	3.26 ± 0.22^{B}
TBARS (nmol MDA/g)	**LL**	9.83 ± 0.43	9.40 ± 0.57	10.00 ± 0.67	9.69 ± 0.40
	FL	10.72 ± 0.68	12.24 ± 0.68*	9.83 ± 0.70	10.10 ± 0.56
Plasma TG (mmol/L)	**LL**	1.97 ± 0.20^{a}	3.80 ± 0.31^{b}	1.33 ± 0.15^{a}	1.71 ± 0.36^{a}
	FL	4.10 ± 0.33^{A**}	2.33 ± 0.48^{B*}	2.23 ± 0.36^{B}	1.63 ± 0.14^{B}
Plasma NEFAs (mEq/L)	**LL**	0.25 ± 0.03	0.53 ± 0.12	0.35 ± 0.06	0.49 ± 0.10
	FL	0.62 ± 0.16*	0.53 ± 0.09	0.46 ± 0.07	0.39 ± 0.03

Data are shown as mean ± SEM (n = 4-6 fish per feeding regime/treatment). Different letters (lower case for LL and capital for FL) indicate significant differences ($p < 0.05$) among fasting periods within a group (two-way ANOVA followed by Tukey post-hoc test). *$p < 0.05$ and **$p < 0.01$ represent significant differences between LL and FL (two-way ANOVA followed by Sidak post-hoc test).

LSI and VSI decreased significantly already after 1 week of fasting in both fish lines and this trend continued up to 4 weeks, especially for the VSI. Further, LL fish had higher VSI than the FL fish throughout the study, while strain differences were observed in LSI only after 4 weeks of fasting. Muscle lipid content was differentially affected by fasting in the two lines, being significantly decreased with fasting in the FL fish, while no effect was observed in LL fish. In this regard, the significant differences observed between the two fish lines during the fed and 1-week fasted state, disappeared with prolonged fasting (i.e. 2 and 4 weeks). In FL fish, fasting significantly increased TBARS levels after 1 week in comparison with LL fish, returning to

basal levels after 2 weeks. For plasma TG levels, there was a significant interaction between fasting and line (p<0.001). Under feeding conditions (0W groups), FL fish had significantly higher plasma TG levels than LL fish. However, after 1 week of fasting, FL fish had lower plasma TG levels than LL fish. Moreover, in FL fish, plasma TG levels decreased with longer fasting, while in LL fish, the plasma TG levels increased after 1 week of fasting and returned to basal levels after 2 weeks. In fed fish, plasma NEFA levels were significantly higher in FL than LL fish, but during fasting, they decreased to similar levels as in LL fish.

3.2 Gene expression related to lipid metabolism

Relative transcript levels of key lipid metabolism genes in AT are presented in Figure 1. A significant interaction between fasting and genotype was observed in *fasn* and *me1* mRNA levels. Particularly, non-fasted FL fish had higher *fasn* transcripts abundance than LL fish. Besides, *fasn* mRNA levels in FL fish gradually decreased with fasting, while *fasn* mRNA levels in LL fish increased after 1 week, and returned to basal levels at 2 weeks of fasting (Fig. 1A). In both rainbow trout lines, *lpl* and *gapdh* mRNA levels decreased significantly with fasting (Fig. 1B and D), this effect being more pronounced in *gapdh*, where transcript abundance was reduced abruptly already after 1 week. *lipe1* mRNA levels decreased slightly after 2 weeks of fasting in LL fish, while being unchanged in FL fish, indicating significant phenotypic differences after 2 weeks of fasting (Fig. 1C). *pparb* mRNA levels were down-regulated after 1-week fasting, but returned to basal levels in both fish lines after that (Fig. 1H). *me1* mRNA levels remained generally low during fasting, but increased rapidly in FL fish after 4 weeks of fasting in FL (Fig. 1F). *lxr* and *ppara* mRNA levels remained unaltered and similar in both fish lines throughout the experiment (Fig. 1E and G).

Gene expression in WM is shown in Figure 2. For *fasn* and *lpl* mRNA levels, there was a significant interaction between fasting time and genotype, with expression of both genes in fed FL fish significantly higher than in LL fish (Fig. 2A and B). In FL fish, both genes decreased significantly during fasting, while no effect was observed in LL fish (Fig. 2A and B). The *lipe1* mRNA levels progressively increased with fasting in LL fish, but not in FL fish (Fig. 2C), whereas those of *lxr* and *me1* were upregulated after 1 week of fasting and returned to basal levels after 4 weeks of fasting in both fish lines (Fig. 2E and F). A similar profile was observed in FL fish for *pparb* (Fig. 2H). *gapdh* and *ppara* mRNA levels were unaffected by fasting or genotype (Fig. 2D and G).

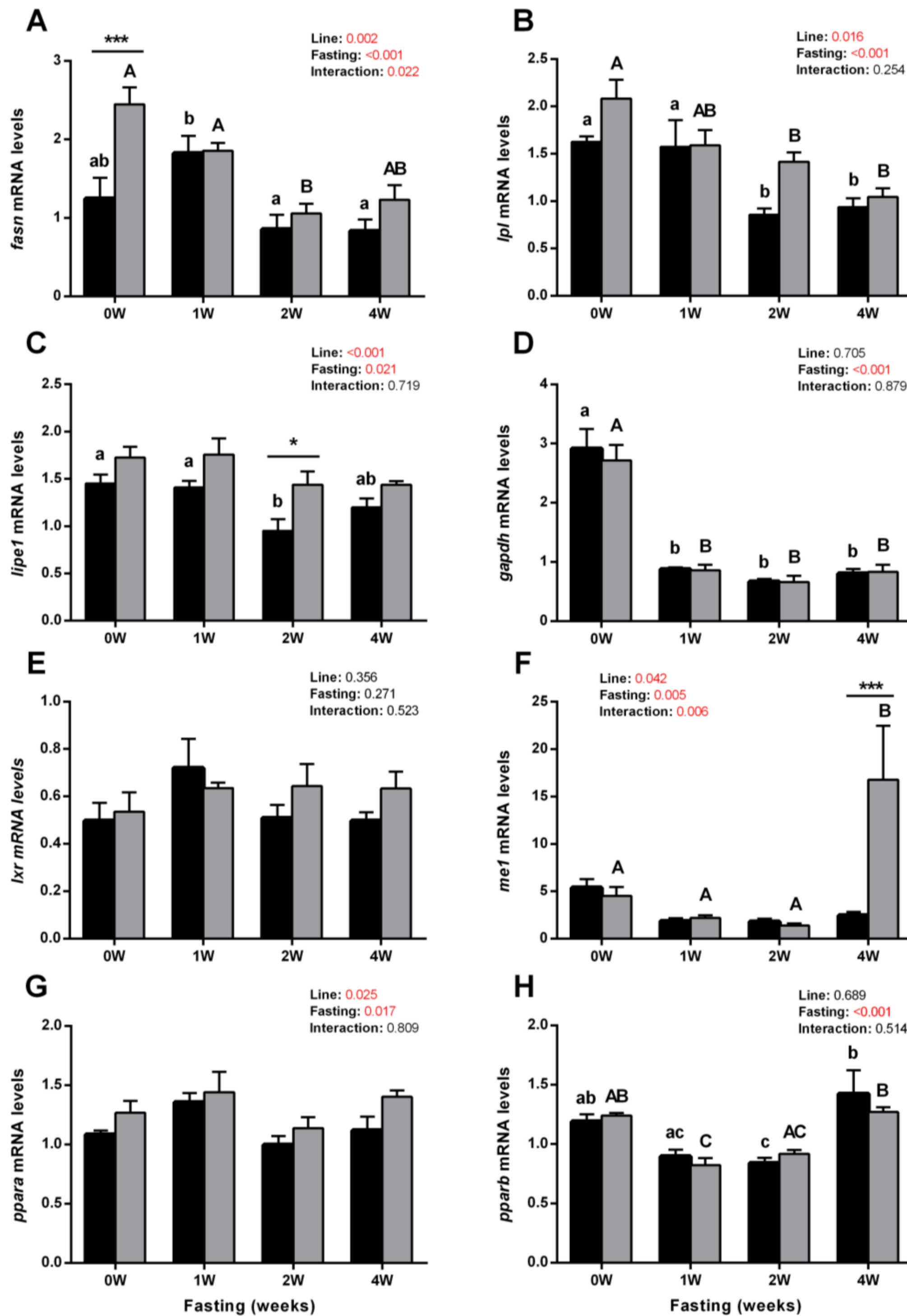


Figure 1. Gene expression analyses of selected lipid-metabolism-related genes in adipose tissue. mRNA levels of *fasn* (A), *lpl* (B), *lipe1* (C), *gapdh* (D), *lxr* (E), *me1* (F), *ppara* (G) and *pparb* (H) from LL (black bars) and FL (grey bars) rainbow trout. Relative expression levels were normalized to the geometric mean of the two reference genes, *ef1a* and *ubq*. Data are shown as mean + SEM (n = 4-6 fish per condition). Statistical differences in the variability between measurements are indicated in 3 components: interaction, line and fasting, using two-way ANOVA. Different letters (lower case for LL and capital for FL) indicate significant differences ($p \leq 0.05$) among fasting periods within a group (two-way ANOVA followed by Tukey *post-hoc* test). *$p \leq 0.05$ and ***$p \leq 0.001$ represent significant differences between LL and FL (two-way ANOVA followed by Sidak *post-hoc* test).

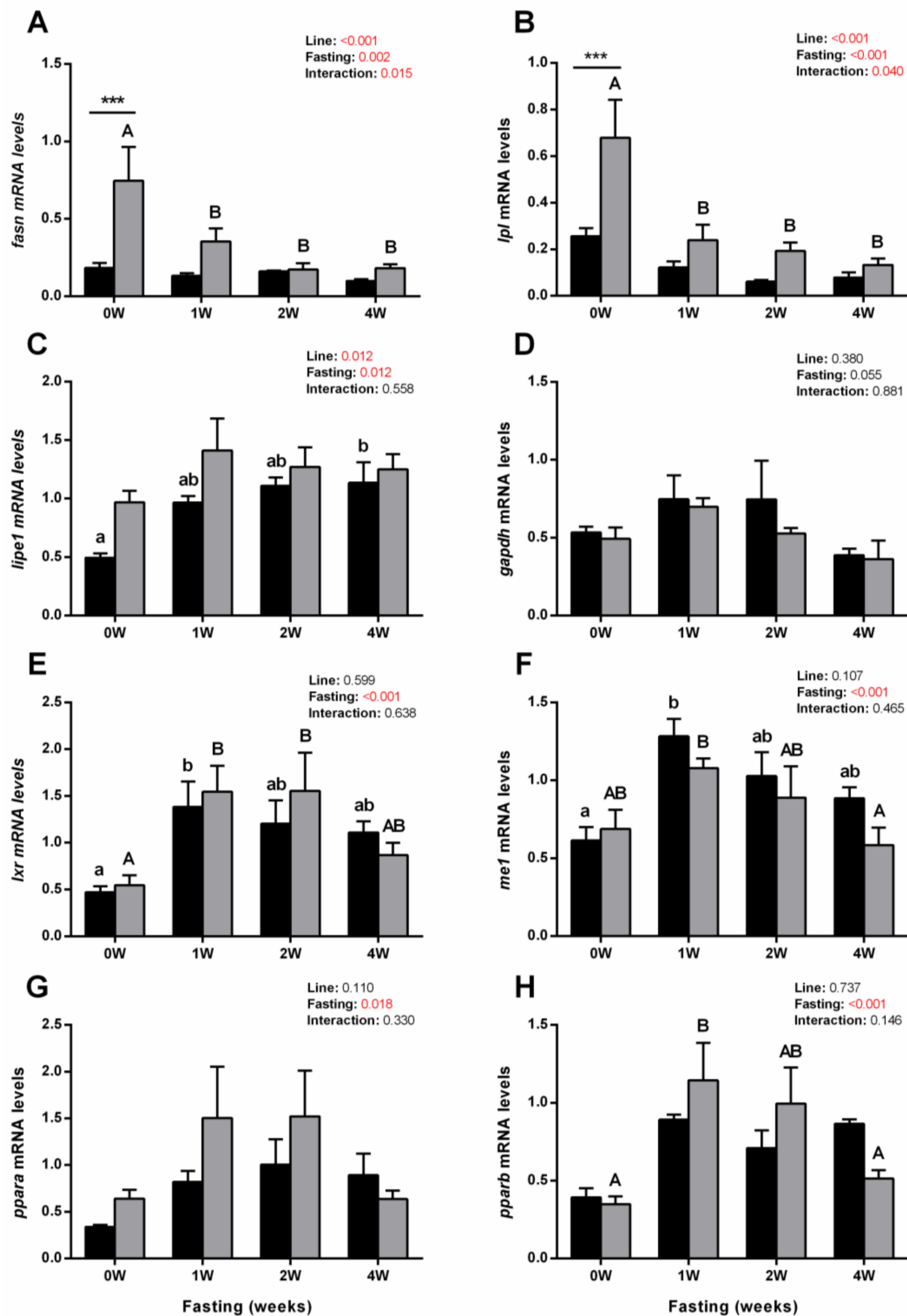


Figure 2. Gene expression analyses of selected lipid-metabolism-related genes in white muscle. mRNA levels of *fasn* (A), *lpl* (B), *lipe1* (C), *gapdh* (D), *lxr* (E), *me1* (F), *ppara* (G) and *pparb* (H) from LL (black bars) and FL (grey bars) rainbow trout. Relative expression levels were normalized to the geometric mean of the two reference genes, *ef1a* and *ubq*. Data are shown as mean + SEM (n = 4-6 fish per condition). Statistical differences in the variability between measurements are indicated in 3 components: interaction, line and fasting, using two-way ANOVA. Different letters (lower case for LL and capital for FL) indicate significant differences ($p \leq 0.05$) among fasting periods within a group (two-way ANOVA followed by Tukey *post-hoc* test). ***$p \leq 0.001$ represent significant differences between LL and FL (two-way ANOVA followed by Sidak *post-hoc* test).

3.3 Expression of adiponectin and adiponectin receptor genes

mRNA levels of adiponectin and adiponectin receptor genes in AT and WM are presented in Figure 3.

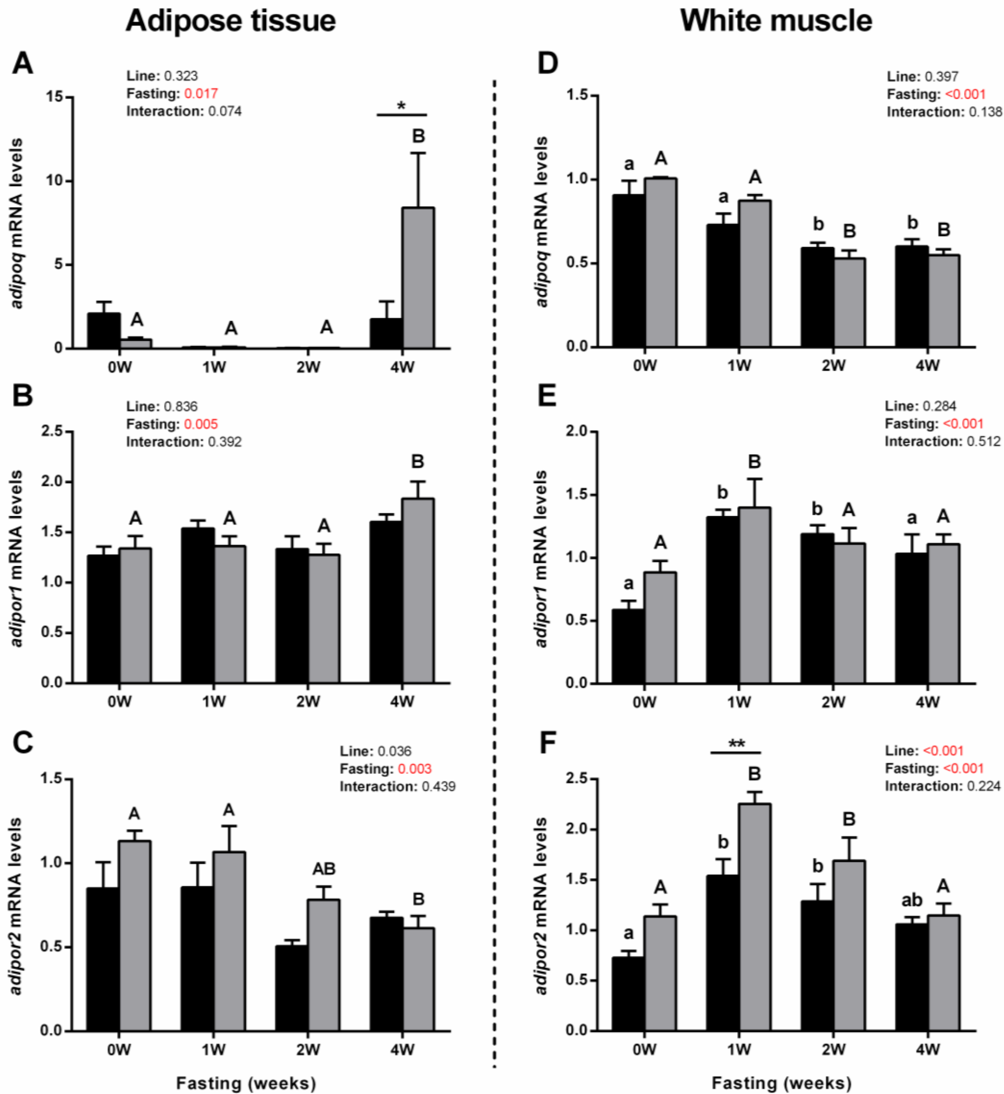


Figure 3. Gene expression analyses of adiponectin system genes in adipose tissue (A, C and E) and white muscle (B, D and F). mRNA levels of *adipoq* (A, B), *adipor1* (C, D) and *adipor2* (E, F) from LL (black bars) and FL (grey bars) rainbow trout. Relative expression levels were normalized to the geometric mean of the two reference genes, *ef1a* and *ubq*. Data are shown as mean + SEM (n = 4-6 fish per condition). Statistical differences in the variability between measurements are indicated in 3 components: interaction, line and fasting, using two-way ANOVA. Different letters (lower case for LL and capital for FL) indicate significant differences ($p \leq 0.05$) among fasting periods within a group (two-way ANOVA followed by Tukey *post-hoc* test). $^{**}p \leq 0.01$ and $^{***}p \leq 0.001$ represent significant differences between LL and FL (two-way ANOVA followed by Sidak *post-hoc* test).

In AT, *adipoq* mRNA levels were very low up to 3 weeks of fasting, to suddenly increase significantly in FL fish at 4 weeks (Fig. 3A). Moreover, *adipor1* mRNA levels were significantly up-regulated after 4 weeks of fasting only in FL fish (Fig. 3B), whereas at the same time *adipor2* expression diminished (Fig. 3C).

In WM, adiponectin system genes expression followed a different pattern. In this tissue, *adipoq* mRNA levels decreased gradually with fasting in both fish lines (Fig. 3D) while *adipor1* and *adipor2* mRNA levels increased after 1 week of fasting and returned to basal levels after 4 weeks (Fig. 3E and F). Further, this increase in *adipor2* mRNA levels after 1 week of fasting was significantly higher in the FL fish (Fig. 3F).

3.4 Gene expression related to oxidative stress

Relative mRNA levels of key oxidative stress markers in AT and WM are presented in Figures 4 and 5. In AT, a significant interaction between fasting and genotype was observed for the *gcs* and *tr* mRNA levels. Expression of these genes was stable for 1 or 2 weeks of fasting in both fish lines, but after 4 weeks of fasting, their expression significantly increased in FL fish only (Fig. 4A and F). *sod* and *phgpx* mRNA levels decreased already after 1 week of fasting in both fish lines. However, in contrast to *phgpx, sod* levels recovered to basal levels after 4 weeks of fasting (Fig. 4B and D). *gpx1* and *gsr* mRNA levels were not affected by fasting or genotype (Fig. 4C and E).

In WM, *gcs* and *tr* mRNA levels increased until week 4 of fasting in LL fish, while in FL fish, the levels increased after 1 week (Fig. 5A and F). *phgpx* mRNA levels increased in FL fish after 1 and 2 weeks of fasting, and then returned to normal levels after 4 weeks of fasting, while the levels remained unaltered in the LL fish throughout the experiment (Fig. 5D). Under feeding conditions, LL fish had higher *gpx1* mRNA levels than FL fish. During fasting, *gpx1* mRNA levels significantly decreased during fasting in LL fish, resulting in similar levels for both genotypes (Fig. 5C). *sod* and *gsr* transcript abundance was not affected by fasting or genotype (Fig. 5B and E).

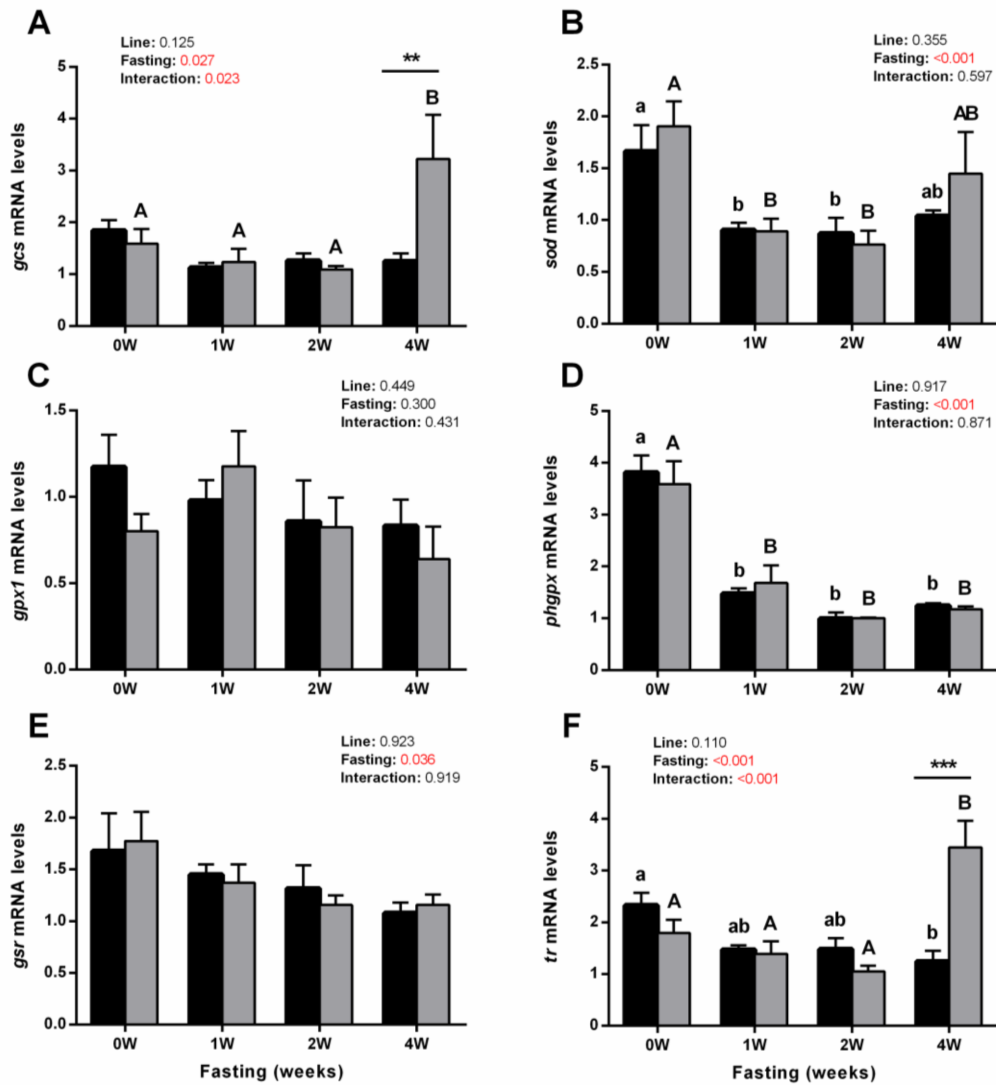


Figure 4. Gene expression analyses of selected oxidative stress genes in adipose tissue. mRNA levels of *gcs* (A), *sod* (B), *gpx1* (C), *phgpx* (D), *gsr* (E) and *tr* (F) from LL (black bars) and FL (grey bars) rainbow trout. Relative expression levels were normalized to the geometric mean of the two reference genes, *ef1a* and *ubq*. Data are shown as mean + SEM (n = 4-6 fish per condition). Statistical differences in the variability between measurements are indicated in 3 components: interaction, line and fasting, using two-way ANOVA. Different letters (lower case for LL and capital for FL) indicate significant differences ($p \leq 0.05$) among fasting periods within a group (two-way ANOVA followed by Tukey *post-hoc* test). *$p \leq 0.05$ and **$p \leq 0.01$ represent significant differences between LL and FL (two-way ANOVA followed by Sidak *post-hoc* test).

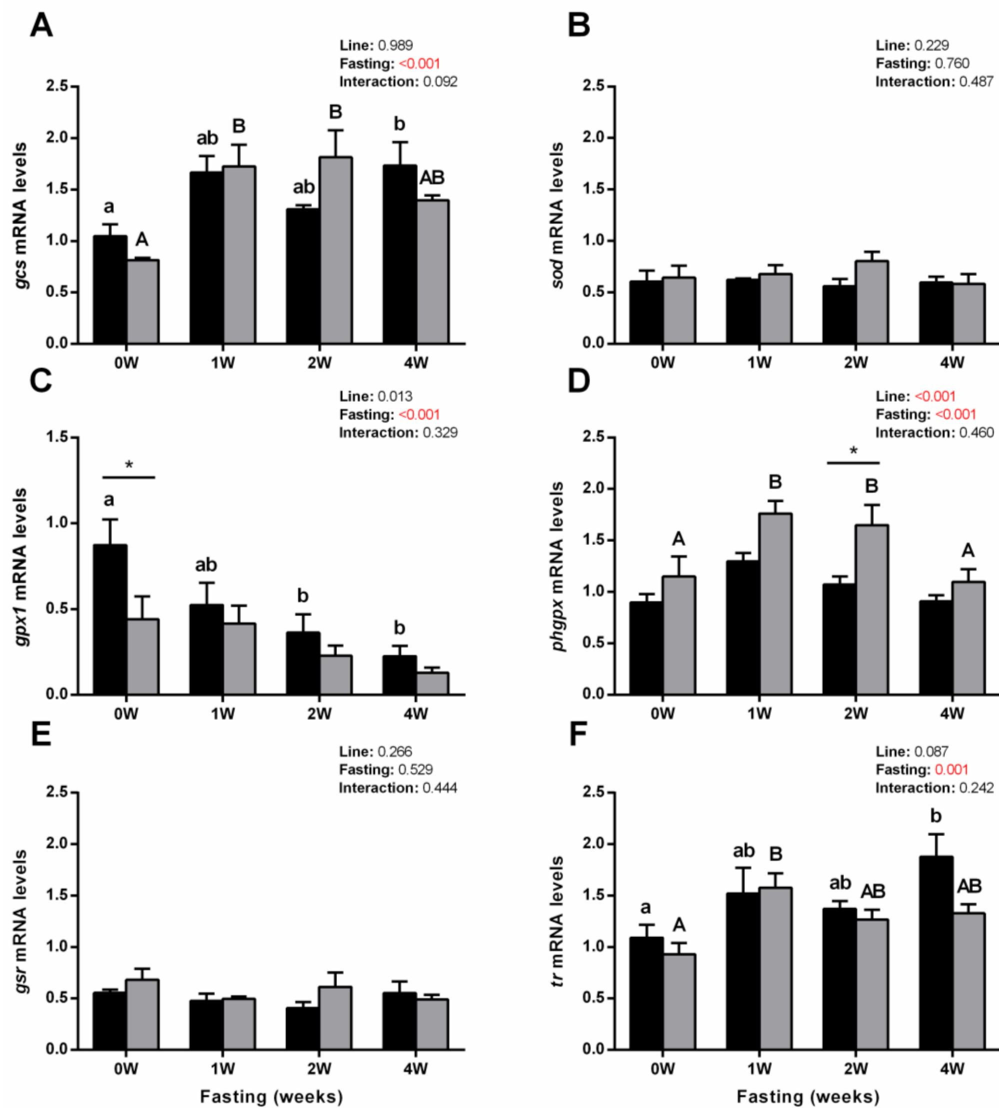


Figure 5. Gene expression analyses of selected oxidative stress genes in white muscle. mRNA levels of *gcs* (A), *sod* (B), *gpx1* (C), *phgpx* (D), *gsr* (E) and *tr* (F) from LL (black bars) and FL (grey bars) rainbow trout are presented. All mRNA levels were normalized to the geometric mean of the two reference genes, *ef1a* and *ubq*. Statistical differences in the variability between measurements are indicated in 3 components: interaction, line and fasting, using two-way ANOVA. Data are shown as mean + SEM (n = 4-6 fish per condition). Different letters (lower case for LL and capital for FL) indicate significant differences ($p \leq 0.05$) among fasting periods within a group (two-way ANOVA followed by Tukey *post-hoc* test). *$p \leq 0.05$ represent significant differences between LL and FL (two-way ANOVA followed by Sidak *post-hoc* test).

4. Discussion

The amount and allocation of fat reserves within the body is of great importance for both human health [52] and quality of animals for food [53]. In production of terrestrial livestock as well as fish species such as salmonids, certain degree of muscle adiposity improves organoleptic

quality of the meat, while large visceral fat deposits are usually wasteful in terms of lower feed conversion efficiency and higher production costs [54]. Further, at least in mammals, different fat depots appear to differ in both physiology and biochemistry, as adipocyte growth and differentiation, as well as fatty-acid processing and adipokine secretion, vary among deposits [55]. The present study describes the differential transcriptional regulation of lipid metabolism, adiponectin system and oxidative stress genes in two divergent rainbow trout lines selected for high (FL) or low (LL) muscle lipid content, both under feeding and fasting conditions. Even though these two rainbow trout lines are designated as fat and lean in terms of muscle adiposity, data on earlier generations indicate that regulatory mechanisms are in play to keep certain level of fat storage, modifying the reserves of different depots [35]. For the seventh-generation fish used in the current study, it appears that the lower muscle fat reserves of the LL fish are partly compensated by greater visceral fat stores [19], supporting the previously suggested divergence of the two fish lines in terms of fat storage depending on the depot. Further, in these fish, the liver does not appear to be affected by the selection process or possible compensatory mechanisms to keep total body lipid reserves constant, as we found that the LSI did not differ between the two genotypes under normal feeding conditions or short-term fasting. In addition, liver and visceral fat reserves were mobilized in a similar manner in both lines in response to fasting, while only the FL fish utilized muscle lipids to fulfill energy requirements. This marked differentiation in energy deposition routes between the two lines, suggests that LL and FL fish also differ in their strategies to maintain energy homeostasis under conditions of energy demand.

Furthermore, the higher TG levels found in non-fasted FL fish suggest increased lipid uptake from diet and supply to muscle in line with its fattier muscle selection compared with the LL fish. Besides, TG levels are found to be very reactive to dietary changes [56]. In agreement with this, after one week of fasting, TG plasma levels drastically decreased in the FL fish while a significant increase was found in the LL fish. Similar results, but less evident, were also found for NEFAs levels. One explanation of these different strategies could be that the FL fish utilize the circulating TG and NEFAs as a source of energy, lowering their levels in comparison with non-fasted animals, while the LL fish first mobilize these products from visceral fat reserves, increasing their plasma levels for delivery to other tissues (i.e. muscle). In agreement with this, several studies in fish have reported that even though fasting usually decreases plasma TG [57], the mobilization of lipids during such periods may differ between species [58], with steady or even increased plasma TG levels at the early stages of fasting [59]. Nevertheless, with

prolonged fasting (2 or 4 weeks), circulating TG decreased in both fish lines confirming that the different strategies in lipid mobilization are mainly observed at the beginning of the fasting period.

Previous studies on the two selected rainbow trout lines (FL and LL) have revealed differential transcriptomic and proteomic regulation in liver and WM between genotypes [60]. Moreover, the two lines differ in glucose and hepatic intermediary metabolism under various dietary regimes. Specifically, the FL fish have a higher capability to metabolize glucose and to synthesize lipids through *de novo* lipogenesis in the liver than the LL fish [38,61]. Nevertheless, in spite of being the major site of energy storage, and subsequently liable to be affected by dietary changes, previous studies have not analyzed the transcriptomic regulation of AT in these trout. Thus, the present study evaluates the expression of several lipid metabolism adipogenic genes in AT and WM in the two lines. From these results, it can be speculated that non-fasted FL fish appear to have higher lipogenic capacity in both AT and WM, as *fasn* mRNA levels were greater in this line compared to the LL fish. This is also supported by the higher plasma levels of TG in the FL fed fish and is in agreement with its previously described higher hepatic lipogenic capacity [36]. Furthermore, *lpl* mRNA levels were found to be higher in WM of non-fasted FL fish, suggesting increased TG incorporation from the circulation under favorable nutritional conditions, leading to fat accumulation. These results agree with earlier findings supporting such augmented fat uptake in terms of *fat/cd36* and *vldl receptor* transcript level up-regulation in the FL muscle [60]. In this context, the decrease in *fasn* and *lpl* with fasting in the WM was more pronounced in the LL fish, while the down-regulation of *lpl* in AT was rather similar in both lines. Remarkably, concomitant with the observed changes in plasma TG levels, *fasn* mRNA levels in the AT decreased progressively with fasting in the FL fish, while in the LL fish, *fasn* mRNA levels increased slightly after 1 week of fasting, thus indicating a differential metabolic regulation under catabolic conditions, also at the transcriptional level.

Furthermore, *lipe1* mRNA levels, only in the LL fish increased gradually with fasting in WM, while they decreased in AT after 2 weeks, indicating significant differences also in lipolytic regulation between both tissues and fish lines. Moreover, *pparb* mRNA levels in adipose tissue decreased at the beginning of fasting in both lines, whereas in white muscle, increased especially in the FL fish, indicating a differential regulation fatty acid oxidation between tissues. Furthermore, the elevated expression of *me1*, which codes for an enzyme that generates NADPH for fatty acid biosynthesis, in the AT of LL trout after a 4-week fasting, could agree with a regulatory mechanism to maintain visceral fat levels, although this was not accompanied

by increases in *fasn.* Nevertheless, in both cases we are assuming that changes in transcript abundance are reflected in protein levels, and we know that this needs to be interpreted with caution.

Following the discovery of several hormones produced by adipose tissues, research has focused on their roles in regulating metabolic and endocrine functions [62]. Among them, adiponectin, which binds two different receptors (adipoR1 and adipoR2), has not been studied extensively in fish [45,63,64]. In mammals, adiponectin expression in adipose tissue inversely correlates with obesity and insulin resistance [65], and is showed to increase with fasting [66]. AdipoR1 appears to be mainly involved in regulating metabolic functions, whereas adipoR2 has rather been linked to anti-inflammatory functions [67,68]. In the present study, an inverse relation was found between *adipoq* and its receptors mRNA levels in WM in response to fasting, as previously reported [64]. Whereas *adipoq* mRNA levels were reduced, *adipor1* and *adipor2* were rapidly up-regulated and progressively returned to basal levels. As considered previously, this response of the adiponectin system could be to maintain the level of muscular fatty acid oxidation. In addition, whereas AT *adipor1* mRNA levels increased after 4 weeks of fasting in the FL fish, only *adipor2* showed an inverse relationship with *adipoq* levels, in agreement with the previous study [64]. Overall only minor differences were observed between genotypes suggesting that the expression of *adipoq* and its receptors is not differentially regulated by fasting in LL and FL fish.

Increased dietary protein and/or lipid intake can increase ROS production [69], but so can long fasting periods [26,70]. The current results show a reverse regulation of several oxidative stress genes in response to fasting between AT and WM. In AT, mRNA levels of the majority of the genes studied, decreased throughout the fasting period while in WM overall displayed a predisposition to increase in both lines. Thus, it is tempting to conclude that the up-regulation of these transcripts in WM might indicate a higher protective effect in this tissue either as an anticipatory mechanism or because of increased oxidative damage. This hypothesis relies on the importance of maintaining structural muscle proteins through primarily avoiding negative ROS effects in this tissue [71]. Thus, the enhanced lipid peroxidation suggested by the increase of MDA production after 1 week of fasting in the WM of the FL fish, might be counteracted by the up-regulated gene expression of *gcs*, *tr* and *phgpx*. Conversely, the lower *gpx1* mRNA levels in non-fasted FL compared with the LL fish, suggest a minor production of ROS derived from fatty acid oxidation, as previously reported [35]. On the other hand, the depletion of AT *sod* and *phgpx* expression in response to fasting should be interpreted carefully. As the production

of ROS in the AT is associated with obesity and inflammatory processes [72], the decreased mRNA expression of these genes may be explained by the reduction of fat mass in response to the fasting. On the other hand, *gcs* and *tr* mRNA levels in this tissue, increased after 4 weeks of fasting in the FL compared to the LL fish, suggesting that the ROS production and/or its oxidative potential in the latter may have stronger effects after prolonged fasting.

5. Conclusions

The current findings reveal differential metabolic regulation between the two genotypes of rainbow trout studied, highlighting their divergent coping mechanisms in response to food deprivation. Changes in plasma TG and NEFA levels, as well as changes at the molecular level, present clear evidence of genotype-specific metabolic and transcriptional strategies. The FL fish had significantly increased *fasn* mRNA levels compared with the LL fish in both AT and WM, and higher *lpl* mRNA levels in WM, altogether suggesting improved lipogenic capacity and enhanced fat uptake in this genotype. Overall, these data improve the understanding of the influence of genetic background on the regulation of lipid metabolism in rainbow trout.

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6. References

1. Wang T, Hung CCY, Randall DJ. The comparative physiology of food deprivation: From Feast to Famine. Annu Rev Physiol. 2006;68: 223–251.

2. Galgani J, Ravussin E. Energy metabolism, fuel selection and body weight regulation. Int J Obes (Lond). NIH Public Access; 2008;32 Suppl 7: S109-19.
3. Viscarra JA, Ortiz RM. Cellular mechanisms regulating fuel metabolism in mammals: Role of adipose tissue and lipids during prolonged food deprivation. Metabolism. 2013;62: 889–897.
4. Kershaw EE, Flier JS. Adipose Tissue as an Endocrine Organ. J Clin Endocrinol Metab. 2004;89: 2548–2556.
5. Khan M, Joseph F. Adipose tissue and adipokines: the association with and application of adipokines in obesity. Scientifica (Cairo). Hindawi Publishing Corporation; 2014;2014: 328592.
6. Gui Y, Silha J V, Mishra S, Murphy LJ. Changes in adipokine expression during food deprivation in the mouse and the relationship to fasting-induced insulin resistance. Can J Physiol Pharmacol. NRC Research Press Ottawa, Canada; 2003;81: 979–985.
7. Pénicaud L. Relationships between adipose tissues and brain: what do we learn from animal studies? Diabetes Metab. 2010;36: S39–S44.
8. Chandran M, Phillips SA, Ciaraldi T, Henry RR. Adiponectin: More Than Just Another Fat Cell Hormone? Diabetes Care. 2003;26.
9. Pannacciulli N, Vettor R, Milan G, Granzotto M, Catucci A, Federspil G, et al. Anorexia Nervosa Is Characterized by Increased Adiponectin Plasma Levels and Reduced Nonoxidative Glucose Metabolism. J Clin Endocrinol Metab. 2003;88: 1748–1752.
10. Fantuzzi G, Wang CC, Goalstone ML, Draznin B, Curat CA, Miranville A, et al. Adipose tissue, adipokines, and inflammation. J Allergy Clin Immunol. Elsevier; 2005;115: 911–9; quiz 920.
11. Bianco A, Mazzarella G, Turchiarelli V, Nigro E, Corbi G, Scudiero O, et al. Adiponectin: an attractive marker for metabolic disorders in Chronic Obstructive Pulmonary Disease (COPD). Nutrients. Multidisciplinary Digital Publishing Institute (MDPI); 2013;5: 4115–25.
12. Byström P, Andersson J, Kiessling A, Eriksson L-O. Size and temperature dependent foraging capacities and metabolism: consequences for winter starvation mortality in fish. Oikos. Blackwell Publishing Ltd; 2006;115: 43–52.
13. McCue MD. Starvation physiology: Reviewing the different strategies animals use to survive a common challenge. Comp Biochem Physiol Part A Mol Integr Physiol. 2010;156: 1–18.
14. Wieser W, Krumschnabel G, Ojwang-Okwor JP. The energetics of starvation and growth after refeeding in juveniles of three cyprinid species. Environ Biol Fishes. Kluwer Academic Publishers; 1992;33: 63–71.
15. Navarro I, Gutiérrez J. Chapter 17 Fasting and starvation. Biochem Mol Biol Fishes. 1995;4: 393–434.
16. Won ET, Borski RJ. Endocrine regulation of compensatory growth in fish. Front Endocrinol (Lausanne). Frontiers Media SA; 2013;4: 74.
17. Ibarz A, Blasco J, Sala-Rabanal M, Gallardo Á, Redondo A, Fernández-Borràs J. Metabolic rate and tissue reserves in gilthead sea bream (*Sparus aurata*) under thermal fluctuations and fasting

and their capacity for recovery. Can J Fish Aquat Sci. NRC Research Press Ottawa, Canada; 2007;64: 1034–1042.

18. López-Luna J, Bermejo-Poza R, Torrent Bravo F, Villarroel M. Effect of degree-days of fasting stress on rainbow trout, *Oncorhynchus mykiss*. Aquaculture. 2016;462: 109–114.
19. Johansson M, Morgenroth D, Einarsdottir IE, Gong N, Björnsson BT. Energy stores, lipid mobilization and leptin endocrinology of rainbow trout. J Comp Physiol B. 2016;186: 759–773.
20. Bower NI, Taylor RG, Johnston IA. Phasing of muscle gene expression with fasting-induced recovery growth in Atlantic salmon. Front Zool. BioMed Central; 2009;6: 18.
21. Bar N, Volkoff H. Adaptation of the Physiological, Endocrine, and Digestive System Functions to Prolonged Food Deprivation in Fish. Comparative Physiology of Fasting, Starvation, and Food Limitation. Berlin, Heidelberg: Springer Berlin Heidelberg; 2012. pp. 69–89.
22. Weil C, Lefèvre F, Bugeon J. Characteristics and metabolism of different adipose tissues in fish. Rev Fish Biol Fish. Springer Netherlands; 2013;23: 157–173.
23. Davidson JW, Kenney PB, Manor M, Good CM, Weber GM, Aussanasuwannakul A, et al. Growth Performance, Fillet Quality, and Reproductive Maturity of Rainbow Trout (*Oncorhynchus mykiss*) Cultured to 5 Kilograms within Freshwater Recirculating Systems. Journal of Aquaculture Research and Development. OMICS Publishing Group; 2014.
24. Feng G, Shi X, Huang X, Zhuang P. Oxidative Stress and Antioxidant Defenses after Long-term Fasting in Blood of Chinese Sturgeon (*Acipenser sinensis*). Procedia Environ Sci. 2011;8: 469–475.
25. Pascual P, Pedrajas JR, Toribio F, López-Barea J, Peinado J. Effect of food deprivation on oxidative stress biomarkers in fish (*Sparus aurata*). Chem Biol Interact. 2003;145: 191–199.
26. Zhang X, Wu T, Cai L, Zhu Y. Influence of fasting on muscle composition and antioxidant defenses of market-size *Sparus macrocephalus*. J Zhejiang Univ Sci B. Zhejiang University Press; 2007;8: 906–11.
27. Neel J V. Diabetes mellitus: a “thrifty” genotype rendered detrimental by “progress”. Am J Hum Genet. 1962;14: 353–62.
28. Chakravarthy M V., Booth FW. Eating, exercise, and “thrifty” genotypes: connecting the dots toward an evolutionary understanding of modern chronic diseases. J Appl Physiol. 2003;96.
29. Masson LF, McNeill G, Avenell A. Genetic variation and the lipid response to dietary intervention: a systematic review. Am J Clin Nutr. American Society for Nutrition; 2003;77: 1098–111.
30. Holmes E, Loo RL, Stamler J, Bictash M, Yap IKS, Chan Q, et al. Human metabolic phenotype diversity and its association with diet and blood pressure. Nature. Nature Publishing Group; 2008;453: 396–400.
31. Li S, Kyvik KO, Duan H, Zhang D, Pang Z, Hjelmborg J, et al. Longitudinal Investigation into Genetics in the Conservation of Metabolic Phenotypes in Danish and Chinese Twins. Caruso C, editor. PLoS One. Public Library of Science; 2016;11: e0162805.

32. Metcalfe NB, Van Leeuwen TE, Killen SS. Does individual variation in metabolic phenotype predict fish behaviour and performance? J Fish Biol. Blackwell Publishing Ltd; 2016;88: 298–321.
33. Bugeon J, Lefevre F, Cardinal M, Uyanik A, Davenel A, Haffray P. Flesh quality in large rainbow trout with high or low fillet yield. J Muscle Foods. 2010;21: 702–721.
34. Quillet E, Le Guillou S, Aubin J, Fauconneau B. Two-way selection for muscle lipid content in pan-size rainbow trout (*Oncorhynchus mykiss*). Aquaculture. 2005;245: 49–61.
35. Kolditz C, Paboeuf G, Borthaire M, Esquerre D, SanCristobal M, Lefevre F, et al. Changes induced by dietary energy intake and divergent selection for muscle fat content in rainbow trout (*Oncorhynchus mykiss*), assessed by transcriptome and proteome analysis of the liver. BMC Genomics. BioMed Central; 2008;9: 16.
36. Skiba-Cassy S, Lansard M, Panserat S, Medale F. Rainbow trout genetically selected for greater muscle fat content display increased activation of liver TOR signaling and lipogenic gene expression. AJP Regul Integr Comp Physiol. 2009;297: R1421–R1429.
37. Kamalam BS, Medale F, Kaushik S, Polakof S, Skiba-Cassy S, Panserat S. Regulation of metabolism by dietary carbohydrates in two lines of rainbow trout divergently selected for muscle fat content. J Exp Biol. 2012;215: 2567–2578.
38. Jin J, Médale F, Kamalam BS, Aguirre P, Véron V, Panserat S. Comparison of Glucose and Lipid Metabolic Gene Expressions between Fat and Lean Lines of Rainbow Trout after a Glucose Load. Planas J V., editor. PLoS One. Public Library of Science; 2014;9: e105548.
39. Jin J, Panserat S, Kamalam BS, Aguirre P, Véron V, Médale F. Insulin regulates lipid and glucose metabolism similarly in two lines of rainbow trout divergently selected for muscle fat content. Gen Comp Endocrinol. 2014;204: 49–59.
40. Gong N, Johansson M, Björnsson BT. Impaired central leptin signaling and sensitivity in rainbow trout with high muscle adiposity. Gen Comp Endocrinol. 2016;235: 48–56.
41. Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem. 1957;226: 497–509.
42. Uchiyama M, Mihara M. Determination of malonaldehyde precursor in tissues by thiobarbituric acid test. Anal Biochem. 1978;86: 271–278.
43. Coimbra-Costa D, Alva N, Duran M, Carbonell T, Rama R. Oxidative stress and apoptosis after acute respiratory hypoxia and reoxygenation in rat brain. Redox Biol. Elsevier; 2017;12: 216–225.
44. Cruz-Garcia L, Sánchez-Gurmaches J, Gutiérrez J, Navarro I. Role of LXR in trout adipocytes: Target genes, hormonal regulation, adipocyte differentiation and relation to lipolysis. Comp Biochem Physiol - A Mol Integr Physiol. Elsevier Inc.; 2012;163: 120–126.
45. Bou M, Todorčević M, Rodríguez J, Capilla E, Gutiérrez J, Navarro I. Interplay of adiponectin, TNFα and insulin on gene expression, glucose uptake and PPARγ, AKT and TOR pathways in rainbow trout cultured adipocytes. Gen Comp Endocrinol. 2014;205: 218–225.

46. Salmerón C, Johansson M, Asaad M, Angotzi AR, Rønnestad I, Stefansson SO, et al. Roles of leptin and ghrelin in adipogenesis and lipid metabolism of rainbow trout adipocytes in vitro. Comp Biochem Physiol -Part A Mol Integr Physiol. 2015;188: 40–48.
47. Seiliez I, Belghit I, Gao Y, Skiba-Cassy S, Dias K, Cluzeaud M, et al. Looking at the metabolic consequences of the colchicine-based in vivo autophagic flux assay. Autophagy. Taylor & Francis; 2016;12: 343–356.
48. Meland S. Ecotoxicological effects of highway and tunnel wash water runoff. 2010.
49. Benedetto A, Brizio P, Squadrone S, Scanzio T, Righetti M, Gasco L, et al. Oxidative stress related to chlorpyrifos exposure in rainbow trout: Acute and medium term effects on genetic biomarkers. Pestic Biochem Physiol. 2016;129: 63–69.
50. Fontagné S, Lataillade E, Brèque J, Kaushik S. Lipid peroxidative stress and antioxidant defence status during ontogeny of rainbow trout (*Oncorhynchus mykiss*). Br J Nutr. 2008;100.
51. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 2001;29: e45.
52. Bjørndal B, Burri L, Staalesen V, Skorve J, Berge RK, Berge RK. Different adipose depots: their role in the development of metabolic syndrome and mitochondrial response to hypolipidemic agents. J Obes. Hindawi Publishing Corporation; 2011;2011: 490650.
53. Wood JD, Enser M, Fisher A V, Nute GR, Sheard PR, Richardson RI, et al. Fat deposition, fatty acid composition and meat quality: A review. Meat Science. 2008. pp. 343–358.
54. Sillence MN. Technologies for the control of fat and lean deposition in livestock. Vet J. 2004;167: 242–257.
55. Tchkonia T, Thomou T, Zhu Y, Karagiannides I, Pothoulakis C, Jensen MD, et al. Mechanisms and metabolic implications of regional differences among fat depots. Cell Metab. 2013;17: 644–56.
56. Kakizawa S, Kaneko T, Hasegawa S, Hirano T. Effects of feeding, fasting, background adaptation, acute stress, and exhaustive exercise on the plasma somatolactin concentrations in rainbow trout. Gen Comp Endocrinol. 1995;98: 137–46.
57. Pérez-Jiménez A, Guedes MJ, Morales AE, Oliva-Teles A. Metabolic responses to short starvation and refeeding in Dicentrarchus labrax. Effect of dietary composition. Aquaculture. 2007;265: 325–335.
58. Furné M, Morales AE, Trenzado CE, García-Gallego M, Carmen Hidalgo M, Domezain A, et al. The metabolic effects of prolonged starvation and refeeding in sturgeon and rainbow trout. J Comp Physiol B. Springer-Verlag; 2012;182: 63–76.
59. Echevarría G, Martínez-Bebiá M, Zamora S. Evolution of Biometric Indices and Plasma Metabolites During Prolonged Starvation in European Sea Bass (*Dicentrarchus labrax, L.*). Comp Biochem Physiol Part A Physiol. 1997;118: 111–123.

60. Kolditz C-I, Plagnes-Juan E, Quillet E, Lefèvre F, Médale F. Changes in white muscle transcriptome induced by dietary energy levels in two lines of rainbow trout (Oncorhynchus mykiss) selected for muscle fat content. Br J Nutr. 2010;103: 629.
61. Kamalam BS, Medale F, Larroquet L, Corraze G, Panserat S. Metabolism and Fatty Acid Profile in Fat and Lean Rainbow Trout Lines Fed with Vegetable Oil: Effect of Carbohydrates. Prunet P, editor. PLoS One. Public Library of Science; 2013;8: e76570.
62. Deng Y, Scherer PE. Adipokines as novel biomarkers and regulators of the metabolic syndrome. Ann N Y Acad Sci. NIH Public Access; 2010;1212: E1–E19.
63. Nishio S-I, Gibert Y, Bernard L, Brunet F, Triqueneaux G, Laudet V. Adiponectin and adiponectin receptor genes are coexpressed during zebrafish embryogenesis and regulated by food deprivation. Dev Dyn. Wiley-Liss, Inc.; 2008;237: 1682–1690.
64. Sánchez-Gurmaches J, Cruz-Garcia L, Gutiérrez J, Navarro I. Adiponectin effects and gene expression in rainbow trout: an in vivo and in vitro approach. J Exp Biol. 2012;215.
65. Kern PA, Di Gregorio GB, Lu T, Rassouli N, Ranganathan G. Adiponectin expression from human adipose tissue: relation to obesity, insulin resistance, and tumor necrosis factor-alpha expression. Diabetes. 2003;52: 1779–85.
66. Liu BH, Wang PH, Wang YC, Cheng WM, Mersmann HJ, Ding ST. Fasting regulates the expression of adiponectin receptors in young growing pigs. J Anim Sci. 2008;86: 3377–3384.
67. Miller M, Cho JY, Pham A, Ramsdell J, Broide DH. Adiponectin and functional adiponectin receptor 1 are expressed by airway epithelial cells in chronic obstructive pulmonary disease. J Immunol. 2009;182: 684–91.
68. Yamauchi T, Nio Y, Maki T, Kobayashi M, Takazawa T, Iwabu M, et al. Targeted disruption of AdipoR1 and AdipoR2 causes abrogation of adiponectin binding and metabolic actions. Nat Med. 2007;13: 332–339.
69. Alvarez MJ, Lopez-Bote CJ, Diez A, Corraze G, Arzel J, Dias J, et al. Dietary fish oil and digestible protein modify susceptibility to lipid peroxidation in the muscle of rainbow trout (Oncorhynchus mykiss) and sea bass (*Dicentrarchus labrax*). Br J Nutr. 1998;80: 281–9.
70. Zhang X-D, Zhu Y-F, Cai L-S, Wu T-X. Effects of fasting on the meat quality and antioxidant defenses of market-size farmed large yellow croaker (*Pseudosciaena crocea*). Aquaculture. 2008;280: 136–139.
71. Barbieri E, Sestili P. Reactive oxygen species in skeletal muscle signaling. J Signal Transduct. Hindawi Publishing Corporation; 2012;2012: 982794.
72. Fernández-Sánchez A, Madrigal-Santillán E, Bautista M, Esquivel-Soto J, Morales-González A, Esquivel-Chirino C, et al. Inflammation, oxidative stress, and obesity. Int J Mol Sci. Multidisciplinary Digital Publishing Institute (MDPI); 2011;12: 3117–32.

Supporting information.

Table 1: Nucleotide sequences of the primers used to evaluate mRNA abundance by quantitative real time PCR (qPCR) in LL and FL rainbow trout adipose tissue and white muscle samples. F: forward; R: reverse; Tm: melting temperature.

Gene	Tm ºC	Primer sequences (5'–3')	
ef1α	58	**F**: TCCTCTTGGTCGTTTCGCTG	**R**: ACCCGAGGGACATCCTGTG
ubq	58	**F**: ACAACATCCAGAAAGAGTCCA	**R**: AGGCGAGCGTAGCACTTG
fasn	54	**F**: GAGACCTAGTGGAGGCTGTC	**R**: TCTTGTTGATGGTGAGCTGT
lpl	59	**F**: TAATTGGCTGCAGAAAACAC	**R**: CGTCAGCAAACTCAAAGGT
lipe1	58	**F**: AGGGTCATGGTCATCGTCTC	**R**: CTTGACGGAGGGACAGCTAC
gapdh	61	**F**: TCTGGAAAGCTGTGGAGGGATGGA	**R**: AACCTTCTTGATGGCATCATAGC
lxr	62	**F**: TGCAGCAGCCGTATGTGGA	**R**: GCGGCGGGAGCTTCTTGTC
me1	60	**F**: TACGTGCGGTGTGTGTGACG	**R**: GTGCCCACATCCAGCATGAC
ppara	54	**F**: CTGGAGCTGGATGACAGTGA	**R**: GGCAAGTTTTTGCAGCAGAT
pparb	60	**F**: CTGGAGCTGGATGACAGTA	**R**: GTCAGCCATCTTGTTGAGCA
adipoq	62	**F**: AGCCCGTCATGTTCACCTAC	**R**: GAAGGTGGAGTCGTTGGTGT
adipor1	60	**F**: TCCACTCCCACCAGATCTTC	**R**: CGTGTTCCAGCAGCACTTTA
adipor2	60	**F**: CTGATCATGGGCTCCTTTGT	**R**: ACACCACTCAGACCCAGACC
gcs	58	**F**: TGATGGACAACACATTCATTAATTGA	**R**: GCGATGCCCGGAACTTATT
sod	54	**F**: CCACGTCCATGCCTTTG	**R**: TCAGCTGCTGCAGTCACGTT
gpx1	60	**F**: CGAGCTCCATGAACGGTACG	**R**: TGCTTCCCGTTCACATCCAC
phgpx	60	**F**: TTGGAGGTCAGGAGCCAGGT	**R**: ACCCTTTCCCTTGGGCTGTT
gsr	54	**F**: CACCAGTGATGGCTTTTTT	**R**: ATATCCGGCCCCCACTATG
tr	58	**F**: ACCGTGCAGCCTAGAATGCT	**R**: GTGATGTCTCTCTTTGCAGTTCCTT

ARTICLE IV

Aquatic Toxicology 188 (2017) 148–158

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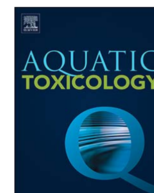

Tributyltin and triphenyltin exposure promotes *in vitro* adipogenic differentiation but alters the adipocyte phenotype in rainbow trout

Esmail Lutfi[a], Natàlia Riera-Heredia[a], Marlon Córdoba[a], Cinta Porte[b], Joaquim Gutiérrez[a], Encarnación Capilla[a], Isabel Navarro[a,*]

[a] Department of Cell Biology, Physiology and Immunology, Faculty of Biology, University of Barcelona, Barcelona 08028, Spain
[b] Environmental Chemistry Department, IDAEA-CSIC, 08034 Barcelona, Spain

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ABSTRACT

Numerous environmental pollutants have been identified as potential obesogenic compounds affecting endocrine signaling and lipid homeostasis. Among them, well-known organotins such as tributyltin (TBT) and triphenyltin (TPT), can be found in significant concentrations in aquatic environments. The aim of the present study was to investigate *in vitro* the effects of TBT and TPT on the development and lipid metabolism of rainbow trout (*Onchorynchus mykiss*) primary cultured adipocytes. Results showed that TBT and TPT induced lipid accumulation and slightly enhanced peroxisome proliferator-activated receptor gamma (PPARγ) and CCAAT enhancer binding protein alpha (C/EBPα) protein expression when compared to a control, both in the presence or absence of lipid mixture. However, the effects were higher when combined with lipid, and in the absence of it, the organotins did not cause complete mature adipocyte morphology. Regarding gene expression analyses, exposure to TBT and TPT caused an increase in *fatty acid synthase* (*fasn*) mRNA levels confirming the pro-adipogenic properties of these compounds. In addition, when added together with lipid, TBT and TPT significantly increased *cebpa, tumor necrosis factor alpha* (*tnfa*) and *ATP-binding cassette transporter 1* (*abca1*) mRNA levels suggesting a synergistic effect. Overall, our data highlighted that TBT and TPT activate adipocyte differentiation in rainbow trout supporting an obesogenic role for these compounds, although by themselves they are not able to induce complete adipocyte development and maturation suggesting that these adipocytes might not be properly functional.

1. Introduction

Obesity is a complex and chronic health condition that develops from the interaction of multiple factors (Chalk, 2004; Mitchell et al., 2011). Among causative factors, poor quality of nutrition and sedentary lifestyle are the most well-known (Egger and Dixon, 2014). Nevertheless, evidence is increasing that the environment could be one of the major determinants of its etiology (Qi and Cho, 2008; Thayer et al., 2012). In this sense, it has been extensively demonstrated that numerous environmental chemicals, such as persistent organic pollutants (POPs) may interfere with complex endocrine signaling pathways and cause adverse metabolic, developmental and reproductive effects in both humans and wildlife (Heindel et al., 2015; Newbold et al., 2008; Tang-Péronard et al., 2011). Significant attention has traditionally focused on the implication of endocrine disruptors (EDs) on cancer (Brophy et al., 2012; Diamanti-Kandarakis et al., 2009) or reproductive health (Jeng, 2014; McLachlan et al., 2006) as well as on obesity in the last years (Grün, 2014; Janesick and Blumberg, 2016; Newbold et al., 2008). Indeed, these chemicals, so-called "obesogens", may predispose an exposed individual to subsequent weight gain; distressing at the same time, hormonal signaling and lipid homeostasis (Janesick and Blumberg, 2016).

Trisubstituted organotins such as tributyltin (TBT) and the related compound triphenyltin (TPT), have been identified as obesogenic compounds, which promote adipogenesis in mouse 3T3-L1 cells (Kanayama et al., 2005) and increase fat deposition *in vivo* in mice and frogs (*Xenopus laevis*) (Grün et al., 2006). A number of studies have shown that TBT and TPT, although different in structure, function in a similar manner as nanomolar agonists of peroxisome proliferator-activated receptor gamma (PPARγ) and retinoid X receptor (RXR) (Harada et al., 2015; Li et al., 2011; Pereira-Fernandes et al., 2013). Nevertheless, differences in the potency of these organotins for PPARγ have been reported (Hiromori et al., 2009), suggesting that they might present different degrees of effectiveness. PPARγ is considered the

* Corresponding author at: Departament de Biologia Cel·lular, Fisiologia i Immunologia, Facultat de Biologia, Universitat de Barcelona, Av. Diagonal 643, 08028 Barcelona, Spain.
E-mail address: mnavarro@ub.edu (I. Navarro).

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master regulator of adipogenesis and together with CCAAT enhancer binding protein alpha (C/EBPα), promote adipocyte differentiation by activating adipose-specific gene expression and regulating each other's expression (Rosen, 2005). Thus, altered activation of these transcription factors could lead to significant changes in adipose tissue metabolism and ultimately contribute to obesity (Grün, 2014; Janesick and Blumberg, 2011). In this regard, several studies have established that these pollutants might also alter cell fate of human and mice adipose-derived stromal stem cells (ADSCs) leading to development of adipocytes at the expense of other cell lineages (Chamorro-García et al., 2013; Kirchner et al., 2010). Nonetheless, the adipocytes generated by such PPARγ activators (*i.e.* TPT), could be non-functional and might display a completely different phenotype (Regnier et al., 2015).

These organotins have been utilized as biocides, wood preservatives and antifouling paints for many years, becoming one of the most common contaminants of marine and fresh water ecosystems (Boyer, 1989; Fent, 1996). Hence, the evaluation of the toxic effects of these compounds to aquatic wildlife is relevant. In this regard, their effects on imposex and reduction of growth in mollusks (Birchenough et al., 2002; Salazar and Salazar, 1991) as well as the shell thickening in oysters (Alzieu et al., 1986) have been well documented. Nevertheless, little is known about them disrupting adipogenesis or lipid metabolism in fish. Recently, in juvenile chinook salmon and zebrafish larvae exposed to TBT an increase in lipid content has been observed (Meador et al., 2011; Ouadah-Boussouf and Babin, 2016; Tingaud-Sequeira et al., 2011). Moreover, it has been shown in RTL-W1 rainbow trout liver cells that TBT, TPT and other EDs alter the expression of lipid metabolism-related genes and the cellular composition in triacylglycerols and membrane lipids; supporting the use of *in vitro* models to study the potential ability of these pollutants to act as obesogenic compounds (Dimastrogiovanni et al., 2015).

Primary culture of fish adipocytes has been proved to be a useful tool to test the effects of hormones on adipogenesis and metabolic markers (Bouraoui et al., 2012, 2010; Salmerón et al., 2015). Thus, in the present study, we aimed to investigate the obesogenic properties of two organotins by comparing the effects of TBT and TPT on adipocyte development and lipid metabolism in rainbow trout (*Onchorynchus mykiss*) by using this specific cell culture system.

2. Materials and methods

2.1. Animal care and ethics statement

Adult rainbow trout (*Oncorhynchus mykiss*) approximately 250 g in weight were obtained from the fishery "AiguaNatura del Ports" (Alfara de Carles, Tarragona, Spain) and were acclimated to the facilities in the Faculty of Biology at the University of Barcelona before use. Fish were kept at a 12L:12D photoperiod in 0.4 m^3 tanks in a temperature-controlled recirculation system (16 ± 1 °C) and were fed *ad libitum* twice daily with a commercial diet (Optiline-sf, Skretting, Burgos, Spain). Fish were fasted 24 h before the experiments in order to avoid contamination from the gastrointestinal tract during the adipose tissue extraction for cell culture. Before sacrifice by cranial concussion, fish were anesthetized with ethyl 3-aminobenzoate methanesulfonate (MS-222) (ref. E10521, Sigma–Aldrich, Tres Cantos, Spain). All animal handling procedures complied with the Guidelines of the European Union Council (86/609/EU) and were approved by Ethics and Animal Care Committee of the University of Barcelona, following the regulations and procedures established by the Spanish and Catalan governments (permit numbers CEEA 311/15, DAAM 7952).

2.2. Primary culture of adipocyte cells

All cell-culture reagents were purchased from Sigma–Aldrich (Tres Cantos, Spain) and Life Technologies (Alcobendas, Spain). All plastic items and glass cover slips were obtained from Nunc (LabClinics, Barcelona, Spain). For the different studies, cells were isolated from rainbow trout adipose tissue and cultured according to the previously established procedure by Bouraoui et al. (2008). After counting, isolated cells were seeded at a final density of 2–2.5 × 10^4 cells/cm^2 on 1% gelatin-pretreated six-well plates (9.6 cm^2/well) for triglyceride determination, Western blot and real-time quantitative PCR (RT-qPCR) or twelve-well plates (2.55 cm^2/well), with or without coverslips, for proliferation analysis, immunofluorescence, Oil red O (ORO) staining and viability assay. Cells were kept at 18 °C in Leibovitz's L-15 medium, supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic solution (growth medium, GM). Medium was changed every 2 days during the whole process. The standard procedure used for cell differentiation was the following: once confluence was reached (day 7), cells were induced to differentiate by incubating them with a differentiation medium (DM) based on GM and containing 10 μg/ml insulin, 0.5 mM 3-isobutyl-1-methylxanthine and 0.25 μM dexamethasone.

2.3. Experimental treatments

Tributyltin chloride 96% (TBT) (ref. T50202), triphenyltin chloride 95% (TPT) (ref. 245712), dimethyl sulfoxide (DMSO) (ref. D8418), lipid mixture (45 mg/ml cholesterol, 100 mg/ml cod liver oil, 250 mg/ml polyoxyethylene sorbitan monooleate and 20 mg/ml D-α-tocopherol acetate) (ref. L5146) were provided by Sigma–Aldrich (Tres Cantos, Spain). Stock solutions of TBT and TPT were stored at −20 °C and working solutions were diluted in 0.1% DMSO on the day of the experiment. For the proliferation and cell viability assays, pre-confluent adipocytes (day 5 of culture) maintained in GM, were incubated with TBT or TPT at different concentrations (1 nM, 10 nM, 100 nM and 1 μM) or left untreated as a control (GM) for 24 h. To evaluate the effect of TBT and TPT during the process of adipogenesis, confluent cells (day 7 of culture) were incubated with only DM as a control (DM), DM plus TBT (100 nM) or DM plus TPT (100 nM), with (+LIP) or without (- LIP) lipid mixture (5 μl/ml) for 24 h (RT-qPCR analyses) or 72 h (ORO, determination of triglycerides and immunofluorescence assays).

2.4. Cell viability assay

The methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay was used to evaluate the cell viability as previously described elsewhere (Capilla et al., 2011). Briefly, cells were incubated with the different treatments with a final concentration of 0.5 mg/ml of MTT for 24 h. Then, cells were washed with PBS and the blue formazan crystals that formed were allowed to resuspend in 250 μl DMSO per well for 2 h. Cell viability values were obtained from the absorbance measured at 570 nm, with 680 nm as the reference wavelength, using a microplate reader (Infinite 200, Tecan).

2.5. Cell proliferation and nuclear morphology analyses

Cell proliferation was evaluated by immunostaining detection of proliferating cell nuclear antigen (PCNA), using a commercial staining kit (P3115-11G, US Biological, Salem, USA). In brief, after 24 h incubation, cells were washed and fixed at room temperature in 4% paraformaldehyde (PFA, Sigma-Aldrich, Spain). Later, coverslips were post-fixed in 50% and 70% ethanol for 5 min, blocked and incubated in PCNA staining reagents, following the manufacturer's suggested protocol. Finally, cells were dehydrated in a graded alcohol series and mounted with histomount. Five to ten images were taken per coverslip with a CC2 camera coupled to a microscope at 40× using analySIS (Soft Imaging System) software and analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The amount of PCNA-labeled nuclei (positive cells) was evaluated and normalized to the number of nuclei in the same field. Immunostaining images were also used to perform a nuclear morphometric analysis. Nuclear area factor (NAF) was used as a cell shape descriptor to evaluate apoptosis

Table 1
Nucleotide sequences of the primers used to evaluate mRNA abundance by quantitative real time PCR (qPCR) in rainbow trout adipocytes in primary culture.

Gene	Primer sequences (5′–3′)	Tm (°C)	Amplicon size (bp)	Database	Accession number
ef1a	**F**: TCCTCTTGGTCGTTTCGCTG **R**: ACCCGAGGGACATCCTGTG	58	159	GenBank	NM_001124339.1
ubq	**F**: ACAACATCCAGAAAGAGTCCA **R**: AGGCGAGCGTAGCACTTG	58	133	GenBank	NM_001124194.1
pparg	**F**: GCCAGTACTGTCGCTTTCAG **R**: TCCATAAACTCAGCCAGCAG	60	112	GenBank	NM_001197212.1
cebpa	**F**: TGTGGCGATAAAGCAAGAGC **R**: CTGGTGGGAATGGTGGTAGG	57	79	GenBank	DQ423469.1
fasn	**F**: GAGACCTAGTGGAGGCTGTC **R**: TCTTGTTGATGGTGAGCTGT	54	186	Sigenae	tcaa0001c.m.06_5.1.om.4
lpl	**F**: TAATTGGCTGCAGAAAACAC **R**: CGTCAGCAAACTCAAAGGT	59	164	GenBank	AJ224693
gapdh	**F**: TCTGGAAAGCTGTGGAGGGATGGA **R**: AACCTTCTTGATGGCATCATAGC	61	210	GenBank	NM_001123561
lxr	**F**: TGCAGCAGCCGTATGTGGA **R**: GCGGCGGGAGCTTCTTGTC	62	171	GenBank	NM_001159338
ppara	**F**: CTGGAGCTGGATGACAGTGA **R**: GGCAAGTTTTTGCAGCAGAT	54	182	GenBank	NM_001197211.1
pparb	**F**: CTGGAGCTGGATGACAGTA **R**: GTCAGCCATCTTGTTGAGCA	60	195	GenBank	AY356399.1
tnfa	**F**: TCTTACCGCTGACACAGTGC **R**: AGAAGCCTGGCTGTAAACGA	60	130	GenBank	NM_001124374
lepa1	**F**: TTGCTCAAACCATGGTGATTAGGA **R**: GTCCATGCCCTCGATCAGGTTA	60	68	GenBank	AB354909
abca1	**F**: CAGGAAAGACGAGCACCTTC **R**: TCTGCCACCTCACACACTTC	58	229	DFCI	TC169876
fatp1	**F**: AGGAGAGAACGTCTCCACCA **R**: CGCATCACAGTCAAATGTCC	60	157	DFCI	CA373015
cd36	**F**: CAAGTCAGCGACAAACCAGA **R**: ACTTCTGAGCCTCCACAGGA	62	106	GenBank	AY606034

F: forward; R: reverse; Tm: melting temperature.

and calculated as the product of nuclear area and roundness, as previously described (Eidet et al., 2014). All images were analyzed by the same researcher.

2.6. Oil red O staining and quantitative determination of triglycerides

Cell differentiation and lipid accumulation were visually analyzed by ORO staining, as previously described (Capilla et al., 2011), with minor modifications. Briefly, cells were fixed with 10% formalin for 1 h, subsequently rinsed with PBS, stained with 0.3% ORO prepared in 36% tri-ethyl phosphate for 2 h, and then rinsed three times with distilled water. The staining effectiveness was evaluated with a Zeiss Axiovert 40C (Carl Zeiss Inc., Germany) inverted research grade microscope equipped with a Canon EOS 1000D digital camera. For triglyceride determination, total lipids were extracted as described by (Folch et al., 1957). In short, cells were washed with PBS and homogenized in a chloroform:methanol mixture (3:1) with 0.1% of 2,6-Di-tert-butyl-4-methylphenol (BHT, Sigma–Aldrich, Germany). Homogenates were vortexed and centrifuged at 2000 × *g* for 30 min, and then the lower layer was extracted and air dried. Next, 2-propanol solution was used to elute the remaining lipid and absorbance was measured at 405 nm (Infinite 200, Tecan) using a triglyceride quantification kit (Sigma–Aldrich, Tres Cantos, Spain).

2.7. Immunofluorescence assay

C/EBPα and PPARγ protein expressions were detected by immunofluorescence using the protocol described by (Vélez et al., 2014). Polyclonal rabbit anti-C/EBPα (14AA) and anti-PPARγ (H-100) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). These antibodies have been shown to successfully cross-react with rainbow trout (Bou et al., 2014; Bouraoui et al., 2012). Secondary antibody Alexa Fluor® 568-conjugated goat anti-rabbit (PPARγ) and Alexa Fluor® 488-conjugated goat anti-rabbit (C/EBPα) were purchased from Life Technologies (Alcobendas, Spain). Nuclei were counterstained with Hoescht (H1399) (Life Technologies, Alcobendas, Spain). Images were obtained with an Olympus Fluoview FV500 confocal microscope.

2.8. Western blot analysis

Protein extraction and Western blot analysis were performed using the protocol described elsewhere (Vélez et al., 2014). Briefly, 20 μg of protein were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 10% polyacrylamide gels (125 V for 1 h 30 min) and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad, El Prat de Llobregat, Spain) (overnight at 4 °C and 100 mA). Subsequently, membranes were washed and blocked in non-fat milk 5% and incubated with anti-C/EBPα and anti-PPARγ antibodies (described in section 2.7) at a dilution of 1:200. After washing, membranes were incubated with a peroxidase-conjugated secondary goat anti-rabbit antibody (Cat. No. 31460, Thermo Scientific, Alcobendas, Spain) at a dilution of 1:15,000. Then, a chemiluminescent detection of the immunoreactive band was performed using an enhanced chemiluminescence kit (Pierce ECL Western blotting Substrate; Thermo Scientific, Alcobendas, Spain) and quantified by densitometric scanning using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Results from the densitometry analysis of each specific band were normalized by the densitometry values of β-Actin (ref. A2066, Sigma–Aldrich, Tres Cantos, Spain), which showed an excellent stability through all the treatments.

2.9. RNA isolation and cDNA synthesis

A pool of cells from three triplicate wells were collected and total RNA extracted using TriReagent (Ambion, Alcobendas, Spain) according to the manufacturer's recommendations. Concentration and RNA purity was determined using a ND-2000 NanoDrop spectrophotometer (Thermo Fisher Scientific, Alcobendas, Spain) and 700 ng of total RNA

were treated with DNase I (Life Technologies, Alcobendas, Spain), following the manufacturer's protocol, to remove all genomic DNA. Afterwards, the RNA was reverse transcribed with the Transcriptor First Strand cDNA synthesis Kit (Roche, Sant Cugat del Valles, Spain) and the cDNA obtained was stored at −20 °C for RT-qPCR analyses.

2.10. Real-time quantitative PCR

The mRNA transcript levels of *cebpa*; *pparg*; *fasn*; *lipoprotein lipase*, *lpl*; *glyceraldehyde 3-phosphate dehydrogenase, gapdh*; *liver × receptor*, *lxr*; *peroxisome proliferator-activated receptor alpha*, *ppara*; *peroxisome proliferator-activated receptor beta*, *pparb*; *tnfa*; *leptin*, *lepa1*; *abca1*; *cluster of differentiation 36*, *cd36 and fatty acid transport protein 1*, *fatp1*; plus two reference genes (*elongation factor 1 alpha*, *ef1a* and *ubiquitin*, *ubq*) were examined with a CFX384™ Real-Time System (Bio-Rad, El Prat de Llobregat, Spain) as previously described elsewhere (Salmerón et al., 2013). All the analyses were performed in triplicate wells using 384-well plates with 2.5 μl itaq SYBR Green Supermix (Bio-Rad, El Prat de Llobregat, Spain), 250 nM forward and reverse primers (Table 1), and 1 μl diluted cDNA for each sample, in a final volume of 5 μl. The primer sequences have been already published (Bou et al., 2014; Carney Almroth et al., 2008; Cruz-Garcia et al., 2012; Lo et al., 2007; Salmerón et al., 2015; Sánchez-Gurmaches et al., 2012) and validated for their specificity. The mRNA level of each target gene analyzed was calculated relative to the reference genes (geometric mean of *ef1a* and *ubq*) using the Pfaffl method (Pfaffl, 2001) implemented in the BioRad CFX manager 3.1. software.

2.11. Statistical analyses

Data were analyzed using SPSS Statistics v.22 (IBM, Armonk, USA) and GraphPad prism 6 (La Jolla, USA, www.graphpad.com) and presented as mean + SEM. Data normality and homoscedasticity were assessed using Shapiro–Wilk and Levene's tests, respectively. Statistical significance was assessed by two-way analysis of variance (two-way ANOVA), followed by the Tukey *post hoc* test. Statistical differences were considered significant for all analyses when p-value ≤ 0.05.

3. Results

3.1. Proliferation, viability and nuclear morphology of adipocyte cells exposed to different concentrations of TBT and TPT

To determine appropriate TBT and TPT concentrations for testing in rainbow trout adipocytes, we first evaluated cell proliferation and viability. Initial experiments first demonstrated that the presence of 0.1% DMSO (*i.e.* the vehicle) in culture medium had no significant effect on cell viability (data not shown). As observed in Fig. 1A, none of the concentrations tested showed significant effects on cell proliferation. Neither compound caused reduced cell viability as the concentration increased up to 100 nM (Fig. 1B). However, at 1 μM, both compounds tended to reduce cell viability compared to the control (GM): the effect was significant in cells treated with TBT. Furthermore, only at the concentration of 1 μM, TBT and TPT clearly affected nuclear morphology (Fig. 1C) showing a consequent reduction in NAF (Fig. 1D), which can be altogether attributed to increased apoptosis. Thus, the highest concentration without any detrimental effect (100 nM) was chosen for the following analyses.

3.2. Changes in adipocyte morphology during differentiation induced by TBT and TPT

Adipocyte morphology and differentiation grade were determined using ORO staining and the quantification of cellular triglyceride content. Results showed that TBT and especially TPT, increased the formation of lipid droplets (Fig. 2A) and the subsequent accumulation of triglycerides in the cells (Fig. 2B), compared to control (DM alone). However, they did not induce the specific adipocyte phenotype as when the cells were treated with lipid mixture (+LIP) (Fig. 2A) (rounded in shape and had high inclusion levels of intracellular lipids). Instead, when treated with TBT or TPT alone, the cells maintained a fibroblastic morphology more similar to undifferentiated cells. Furthermore, the combination of TBT or TPT with lipid mixture (+LIP), showed stronger adipogenic effects, increasing triglyceride content when compared to either TBT, TPT or DM alone (−LIP); although these combinations did not promote further lipid accumulation relative to the condition of DM + LIP (Fig. 2B).

3.3. Induction of C/EBPα and PPARγ protein and gene expression by TBT and TPT

To characterize the contribution of TBT and TPT during adipogenesis we analyzed the expression of C/EBPα and PPARγ, two key transcription factors that regulate the differentiation of pre-adipocytes into mature adipocytes (Rosen, 2005). Immunofluorescence images revealed that both TBT and TPT slightly increased the C/EBPα and PPARγ protein signal compared to control (DM), while cells treated with LIP and its combinations with TBT or TPT produced a higher effect (Fig. 3A). Furthermore, Western blot results also showed an induction tendency, although not significant, in these adipogenic proteins when cells were treated with TBT or TPT alone, but again, stronger effects were observed in the treatments with LIP (Fig. 3B, C and D). Particularly, TBT and TPT together with LIP synergistically enhanced both C/EBPα protein (Fig. 3C) and mRNA levels (*cebpa*, Fig. 3E). In contrast, despite the enhancement of PPARγ protein expression produced by LIP and its combination with TBT (Fig. 3D), no effect was found in *pparg* mRNA levels (Fig. 3F).

3.4. Effects of TBT and TPT on lipid metabolism-related gene expression

The next step was to determine whether TBT and TPT differentially modified the expression of several candidate genes during adipogenesis. Results showed that TBT (slightly) and TPT (significantly) displayed an increase in the mRNA levels of the gene encoding the lipogenic enzyme *fasn*; and similar results were observed when used in combination with LIP (+LIP) (Fig. 4A). Moreover, cells treated with LIP significantly reduced *fasn, lpl, gapdh, lxr and pparb* mRNA levels compared to control (DM) suggesting a negative regulation of these genes when lipids (+LIP) were administered to the medium (Fig. 4A, B, C, D and F). Interestingly, the results on *gapdh* showed an interaction between the treatments and the presence of LIP, suggesting a combined effect, resulting in this case, in a synergistic outcome downregulating *gapdh* mRNA levels (Fig. 4C). Finally, no changes were observed in *ppara* mRNA levels upon treatments (Fig. 4E).

3.5. Effects of TBT and TPT on the expression of fatty acid transport-related genes and genes encoding adipokines

TBT and TPT alone appeared to increase *abca1* mRNA levels as when the LIP was added to the medium (Fig. 5A) while no effect was observed in *cd36* (Fig. 5B). Instead, the treatment with LIP significantly reduced *fatp1* mRNA levels regardless of the treatment combination (Fig. 5C). Moreover, regarding the expression of genes encoding adipokines, TBT and TPT increased *tnfa* mRNA levels when incubated together with LIP (+LIP), especially in the case of TPT, as that combination showed significant differences in comparison to the control (DM alone) (Fig. 6A). On the other hand, *lepa1* mRNA levels were found to rise in the presence of LIP, although differential effects were not observed upon TBT or TPT treatments either with or without LIP (Fig. 6B).

Fig. 1. (A) Quantification of cell proliferation determined by immunocytochemistry of PCNA. Values are shown as a percentage of PCNA-labeled positive cells. (B) Quantification of cell viability using MTT assay. (C) Representative immunostaining images for PCNA showing nuclear morphology. Magnification, 40×; scale bar, 100 µm. (D) Quantitative determination of NAF. Pre-confluent rainbow trout adipocyte cells (day 5 of culture) were incubated with tributyltin (TBT, gray bars) or triphenyltin (TPT, black bars) at different concentrations (1 nM, 10 nM, 100 nM and 1 µM) or left untreated as a control (GM, white bar) for 24 h. Data are shown as mean + SEM (n = 3–4). Significant differences ($p < 0.05$) are indicated by different letters, using two-way ANOVA followed by Tukey's *post hoc* test.

4. Discussion

An emerging view proposes the existence of environmental chemical pollutants that can mimic, antagonize or inappropriately regulate particular metabolic pathways altering fat cell programming and increasing energy storage in adipose tissue (Grün and Blumberg, 2006; Holtcamp, 2012; Lubrano et al., 2013). To date, a number of obesogens have been identified; supporting the idea that exposure to such chemicals may play an unpredicted role in the obesity epidemic. Among them, organotins such as TBT and TPT are considered the most relevant, with proven toxicity risks to humans and non-human organisms (Antizar-Ladislao, 2008). Adipose tissue has been traditionally considered just a rather passive storage organ, although throughout the last decade, it has been recognized as a multi-functional endocrine

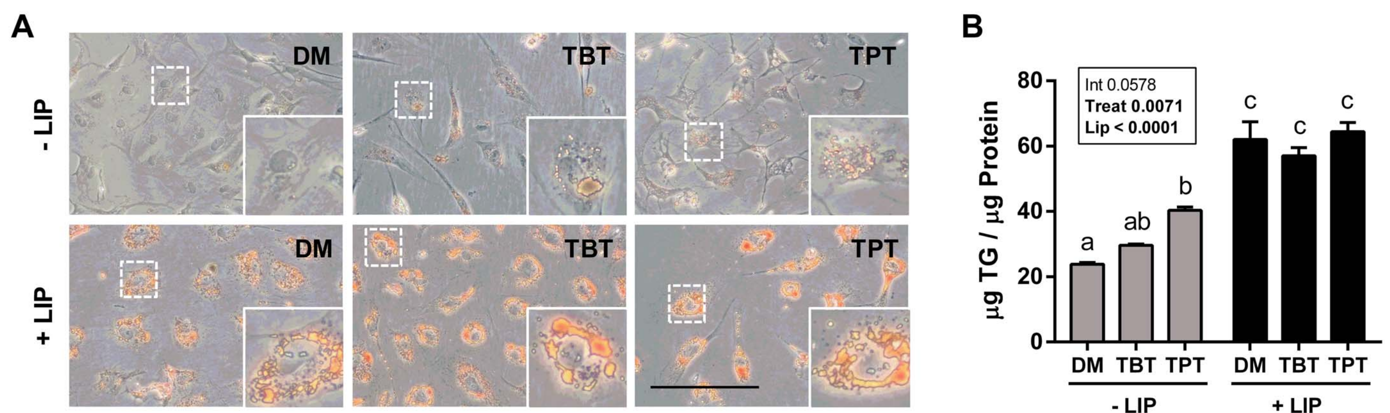

Fig. 2. (A) Representative Oil red O images and (B) quantitative determination of triglycerides of rainbow trout cultured adipocytes. Confluent cells (day 7 of culture) were incubated with differentiation media as a control (DM), DM plus TBT (100 nM) or DM plus TPT (100 nM), with (+LIP) or without (−LIP) lipid mixture (5 µl/ml) for 72 h. Insets in each image are the enlarged views of isolated adipocytes from each panel marked by a white rectangle. Fibroblast-like elongated morphology is observed in the upper panels (−LIP) and the typical mature adipocyte spherical shape in the lower panels (+LIP). Neutral lipids are stained in red. Magnification, 20×; scale bar, 100 µm. Specific triglyceride accumulation is expressed spectrophotometrically as the ratio of absorbance value obtained for triglyceride and protein content. Data are shown as mean values + SEM (n = 3). Statistical differences in the variability between measurements are indicated in three components: interaction (Int), treatment (Treat) and lipid (Lip), using two-way ANOVA. Significant differences ($p < 0.05$), indicated with different letters, were determined using Tukey's *post hoc* multiple comparison test.

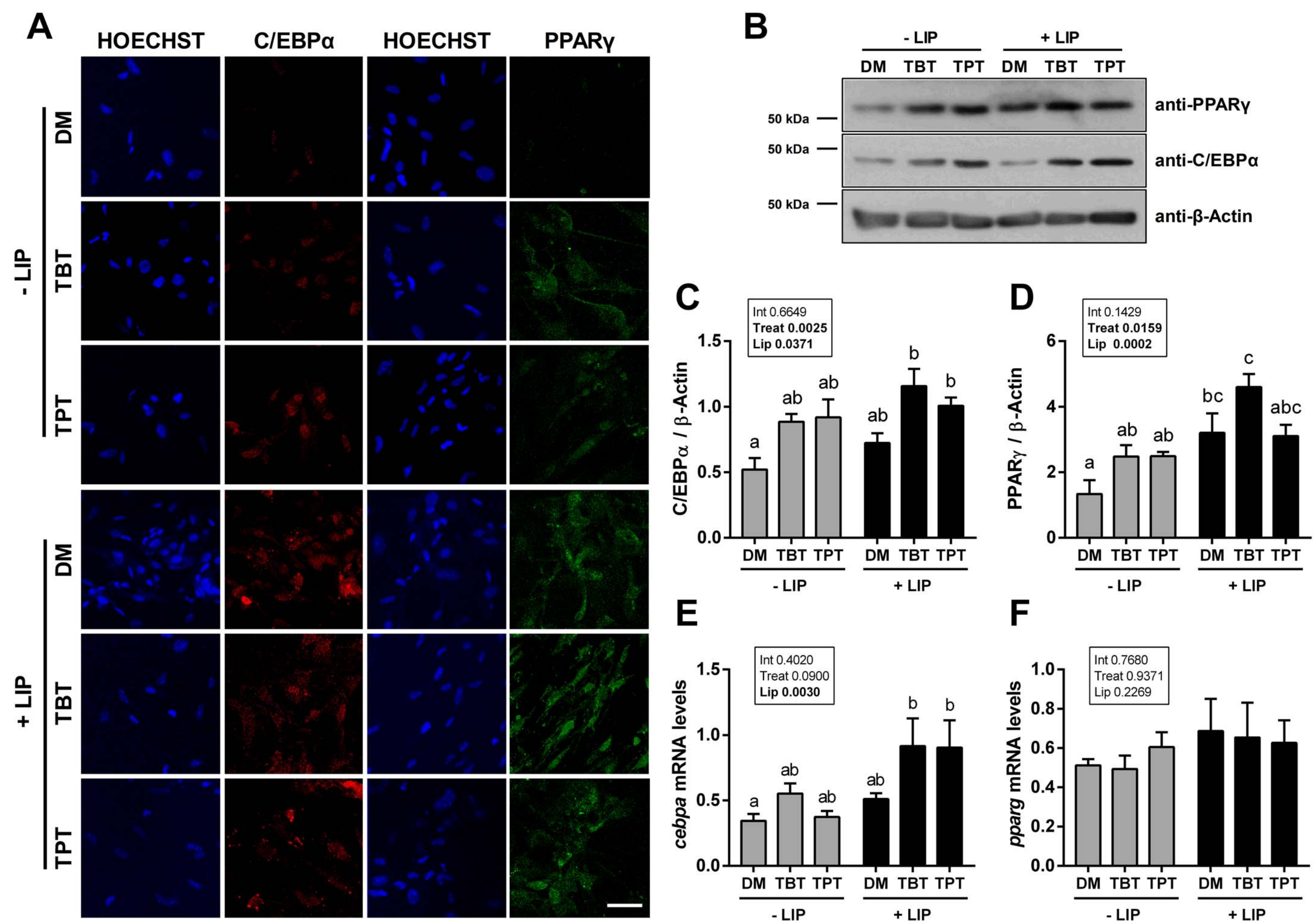

Fig. 3. (A) Representative immunofluorescence images of PPARγ and C/EBPα proteins. Detection was performed using a primary specific antibody and secondary goat anti-rabbit antibodies Alexa Fluor® 488-conjugated (C/EBPα) and Alexa Fluor® 568-conjugated (PPARγ) in combination with Hoechst for nucleus. Magnification, 36×; scale bar, 100 μm. (B) Representative Western blot images and quantification of (C) anti-C/EBPα and (D) anti-PPARγ immunoreactive band normalized by β-Actin. (E) mRNA levels of adipogenic genes *cebpa* and (F) *pparg* normalized to the geometric mean of two reference genes (*ef1a* and *ubq*). Confluent cells (day 7 of culture) were incubated with differentiation media as a control (DM), DM plus TBT (100 nM) or DM plus TPT (100 nM), with (+LIP) or without (−LIP) lipid mixture (5 μl/ml) for 72 h (A, B, C and D) or 24 h (E and F). Data are shown as mean values + SEM (n = 3–6). Statistical differences in the variability between measurements are indicated in three components: interaction (Int), treatment (Treat) and lipid (Lip), using two-way ANOVA. Significant differences ($p < 0.05$) are indicated by different letters, and were determined by Tukey's *post hoc* multiple comparison test. Abbreviations. CCAAT/enhancer-binding protein alpha and its gene, C/EBPα and *cebpa*; peroxisome proliferator-activated receptor gamma and its gene, PPARγ and *pparg*; beta-actin, β-Actin; the gene encoding elongation factor 1 alpha, *ef1a*; the gene encoding ubiquitin, *ubq*.

organ that regulates numerous physiological processes. Actually, its dysfunction can lead to several pathological conditions, such as metabolic syndrome, lipodystrophy, obesity and insulin resistance (Bremer and Jialal, 2013; Kusminski et al., 2016). In overweight and obese conditions, excessive fat is accumulated in adipose tissue through formation of new adipocytes from precursor cells or by hypertrophy of existent ones (Otto and Lane, 2005; Rosen and Spiegelman, 2006). Given the importance of understanding the role of obesogens in adipogenesis and lipid homeostasis, some studies have been performed using adipose tissue-derived cell lines and primary cultures (reviewed in Janesick and Blumberg, 2012). In this regard, it has been demonstrated that TBT and TPT function as pro-adipogenic compounds promoting differentiation of pre-adipocytes into mature adipocytes, thus enabling hyperplastic expansion of adipose tissue in mammalian models. Particularly, these compounds have been found to induce lipid droplet formation along with subsequent triglyceride accumulation in both 3T3-L1 and mouse bone marrow multipotent mesenchymal stem cells (Hiromori et al., 2009; Yanik et al., 2011). Nevertheless, the effects of these contaminants in fish adipocytes are almost unknown.

The regulation of adipocyte development is a key aspect of adipose tissue formation, and works through the reciprocal balance between proliferation and differentiation. In this regard, a decrease in proliferation capacity with organotin exposure has been previously reported (Kirchner et al., 2010) suggesting that the activation of adipogenesis by these compounds might affect proliferation and favor adipogenic differentiation. Similarly, our results showed slightly lower values on cell proliferation as the concentration of TBT and TPT increased, in agreement with the expected stimulation of differentiation. Furthermore, a decrease in cell viability and nuclear area was also observed at 1 μM concentration with both compounds, indicating that exposure to this concentration might induce apoptosis. These results are in agreement with previous studies that reported upregulation of apoptosis-related genes in rat ovaries after TBT administration, leading to cell death in the ovarian follicles (Lee et al., 2012). To avoid these detrimental consequences, the 100 nM concentration was used to determine the adipogenic effects of TBT and TPT in the following experiments.

Induction of adipocyte differentiation triggers profound phenotypical changes of fibroblast-like pre-adipocytes to become spherical cells filled with lipid droplets. These changes appear to be attributed to the activation of a highly orchestrated program of gene expression that requires the sequential activation of numerous transcription factors,

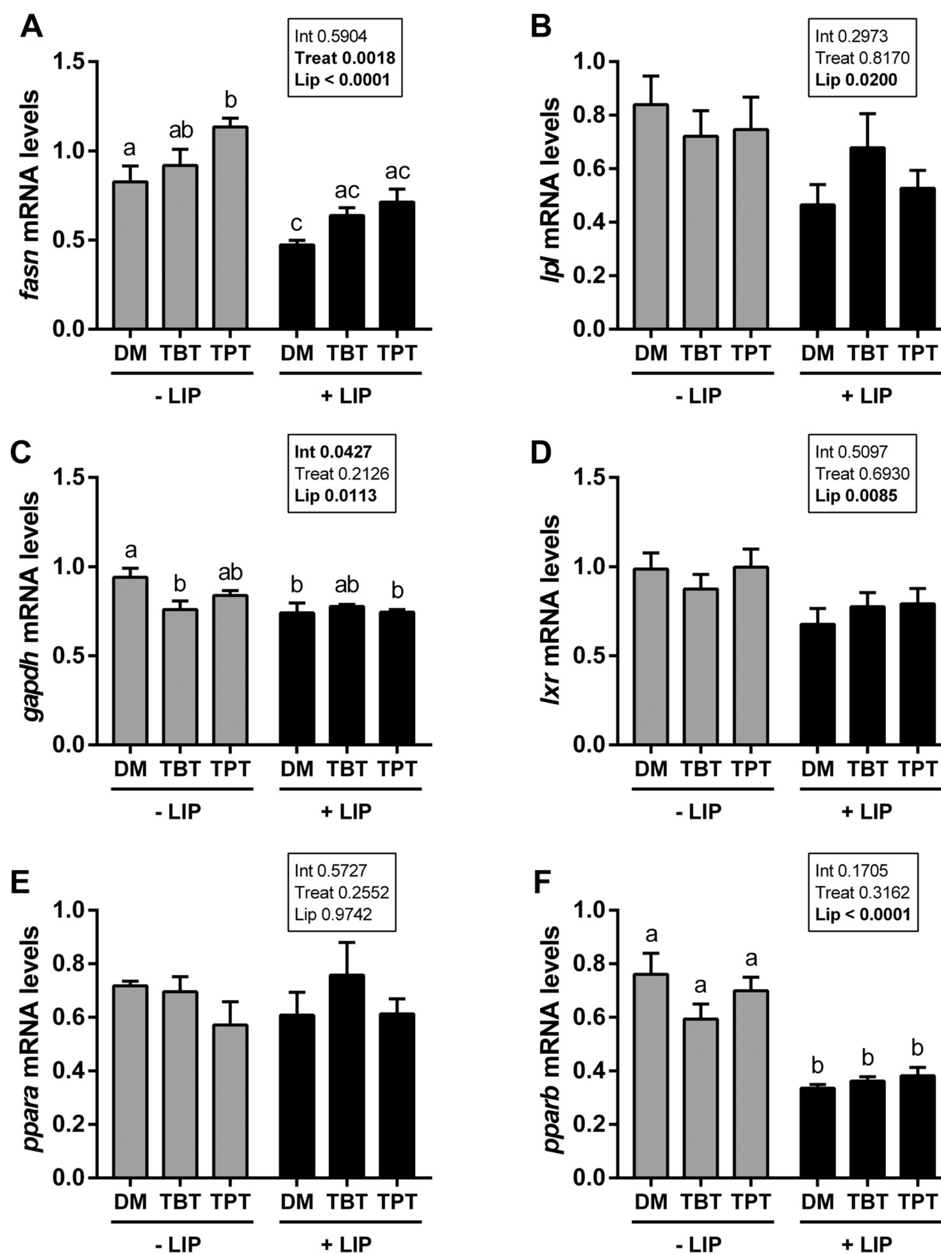

Fig. 4. Relative gene expression of lipid metabolism-related genes in rainbow trout cultured adipocytes. Confluent cells (day 7 of culture) were incubated with differentiation media as a control (DM), DM plus TBT (100 nM) or DM plus TPT (100 nM), with (+LIP) or without (−LIP) lipid mixture (5 μl/ml) for 24 h. mRNA levels of *fasn* (A), *lpl* (B), *gapdh* (C), *lxr* (D), *ppara* (E) and *pparb* (F) are presented. All mRNA levels were normalized to the geometric mean of 2 reference genes (*ef1a* and *ubq*). Statistical differences in the variability between measurements are indicated in three components: interaction (Int), treatment (Treat) and lipid (Lip), using two-way ANOVA. Significant differences ($p < 0.05$) are indicated by different letters, and were determined by Tukey's *post hoc* multiple comparison test. Abbreviations. *fatty acid synthase, fas; lipoprotein lipase, lpl; glyceraldehyde 3-phosphate dehydrogenase, gapdh; liver X receptor, lxr; peroxisome proliferator-activated receptor alpha, ppara; peroxisome proliferator-activated beta, pparb; elongation factor 1 alpha, ef1a; ubiquitin, ubq.*

including C/EBPα and PPARγ (Lefterova and Lazar, 2009; Lowe et al., 2011). In the present work, both TBT and specially TPT induced lipid accumulation in rainbow trout cultured adipocytes, confirming their obesogenic properties in our model; however, did not produce the typical round adipocyte phenotype as when cells were treated with lipid mixture (LIP). Instead, cells retained a fibroblast-like morphology although with evident presence of lipid droplets, indicating that these compounds initiate adipogenesis but might not be capable to induce complete adipocyte differentiation in this species, at least at the concentrations tested. In mammalian adipocytes, specific PPARγ agonists, such as troglitazone and other thiazolidinediones, are commonly used to trigger adipogenesis and initiate the transcriptional cascade that determines the fate of adipocyte precursor cells (Collins et al., 2011). However, recently, it has been reported that 3T3-L1 adipocytes generated by induction with different PPARγ agonists exhibit dissimilar phenotypes affecting adipose tissue functionality and ultimately global energy homeostasis (Regnier et al., 2015). The data presented in that study revealed that whereas both troglitazone and TBT stimulated the expression of *pparg*, the majority of the adipogenic genes tested were responsive to troglitazone but not to TBT. Our results confirmed that TBT and TPT slightly induce C/EBPα and PPARγ protein expression, but an effect on the mRNA levels was not recorded (*cebpa* and *pparg*). Besides, LIP in combination with the organotins produced higher effects, increasing both C/EBPα protein and mRNA levels, suggesting a synergistic effect. Furthermore, LIP and its combination with TBT, significantly upregulated PPARγ protein expression while no effect was observed in mRNA levels, indicating a possible post-transcriptional regulation. Overall, the lack of activation of these two transcription

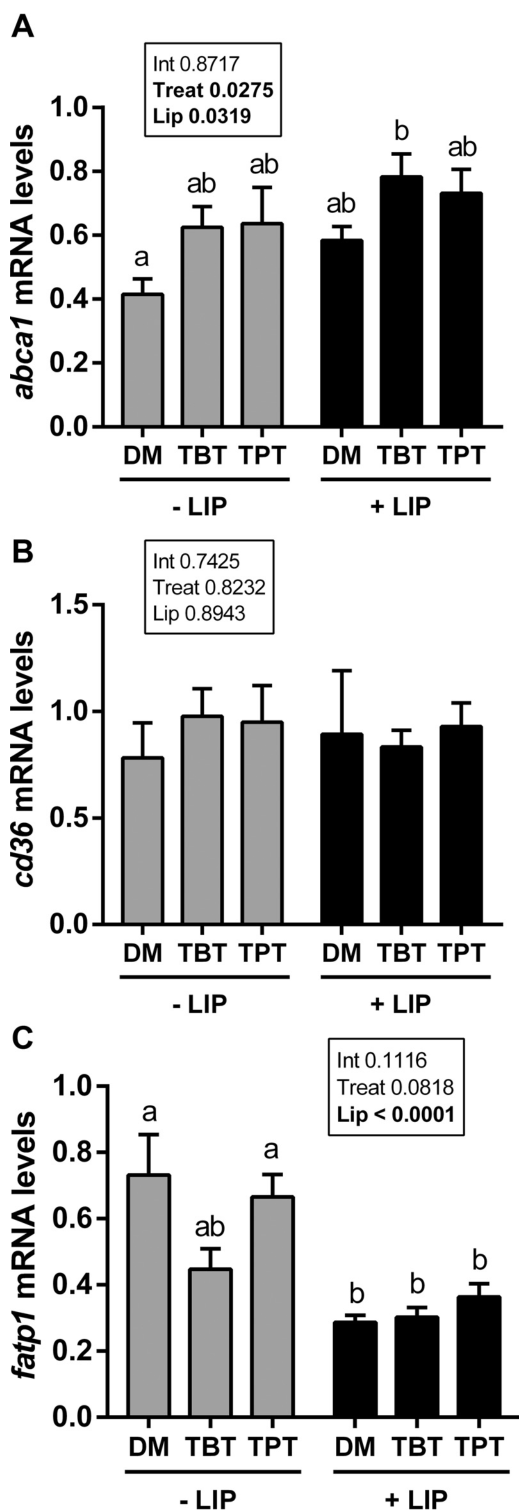

Fig. 5. Relative gene expression of fatty acid transport genes in rainbow trout cultured adipocytes. Confluent cells (day 7 of culture) were incubated with differentiation media as a control (DM), DM plus TBT (100 nM) or DM plus TPT (100 nM), with (+LIP) or without (−LIP) lipid mixture (5 μl/ml) for 24 h. mRNA levels of *abca1* (A), *cd36* (B) and *fatp1* (C) are presented. All mRNA levels were normalized to the geometric mean of 2 reference genes (*ef1a* and *ubq*). Statistical differences in the variability between measurements are indicated in 3 components: interaction (Int), treatment (Treat) and lipid (Lip), using two-way ANOVA. Significant differences ($p < 0.05$) are indicated by different letters, by Tukey's *post hoc* multiple comparison test. Abbreviations: *ATP-binding cassette transporter, abca1; cluster of differentiation 36, cd36; fatty acid transport protein 1, fatp1; elongation factor 1 alpha, ef1a; ubiquitin, ubq.*

factors in the absence of LIP might partly explain the incapability of TBT and TPT to induce by themselves the typical adipocyte morphology.

In this sense, previous publications have highlighted that LIP, as exogenous source of triglycerides, is required to achieve full adipocyte differentiation in both rainbow trout (Bouraoui et al., 2008) and Atlantic salmon (Todorčević et al., 2010). In contrast to fish, mammalian adipocytes have the capacity to mature in response to a differentiation cocktail but without exogenous addition of lipids. This implies the endogenous production of triglycerides in a process known as *de novo* lipogenesis (DNL). DNL is a complex and highly regulated metabolic pathway that converts excess carbohydrate into fatty acids (Ameer et al., 2014). While it has been recently found that the DNL pathway is active in fish adipocytes, its capacity to convert glucose into cellular lipids is relatively low compared to mammals (Bou et al., 2016). Altogether, we can conclude that these particular features of fish adipocytes, may contribute to the difficulty observed under TBT and TPT treatments to induce complete adipocyte development.

Despite the difference in DNL capacity, the fundamental mechanisms that regulate adipogenesis and lipid metabolism pathways appear to be conserved between fish and mammals (Flynn et al., 2009; Seth et al., 2013; Tingaud-Sequeira et al., 2011). During the terminal phase of adipocyte differentiation, the activation of the transcriptional cascade, initiated by C/EBPα and PPARγ, leads to increased activity of enzymes involved in triglyceride synthesis and degradation (Moreno-Navarrete and Fernández-Real, 2012). In the present study, we evaluated the expression of several of these adipogenic markers after TBT and TPT treatments. Both alone and in combination with LIP, these compounds positively upregulated the mRNA levels of *fasn*, the product of which is considered a key enzyme in DNL. Moreover, these organotins also displayed a tendency to increase *abca1*, suggesting that new fatty acid synthesis is enhanced and a positive regulation of cholesterol efflux occurs. Similar results were found in RTL-W1 rainbow trout liver cells, where TBT and TPT, increased both *fasn* and *abca1* mRNA levels (Dimastrogiovanni et al., 2015). Collectively, these findings revealed the adipogenic properties of TBT and TPT and confirmed their implication on the adipocyte differentiation process in rainbow trout. In contrast, LIP significantly downregulated the majority of the genes tested (*fasn*, *lpl*, *gapdh*, *lxr*, *pparb* and *abca1*), which could be secondarily caused by a reduction in lipogenesis due to the availability of fatty acids in the culture medium. Surprisingly, LIP also reduced *fatp1* mRNA levels, which can probably be attributed to a negative feedback regulation after an increased period of fatty acid uptake.

After the identification of leptin as a specific adipocyte cell-derived hormone, numerous studies have clearly established that adipose tissue produces a range of factors, named adipokines, with important endocrine functions and whose secretion is affected by metabolic dysregulation (Deng and Scherer, 2010). Obesogens, such as TBT, have been shown to modulate leptin levels and expression both, in mice under prenatal exposure and in ADSCs treated *in vitro* (Kirchner et al., 2010). In the present study, LIP upregulated *lepa1* mRNA levels as expected in an adipogenic context, however no effect was recorded upon TBT or TPT treatments. One explanation for this lack of effect is that leptin is expressed in late stages of adipogenesis and in this study, adipocytes treated with TBT and TPT seemed to be incapable to reach this point. Furthermore, exposure to organotins together with LIP also caused an increase in *tnfa* mRNA levels, suggesting that in our model TBT and TPT not only induced intracellular lipid accumulation, but also perhaps increased adipocyte inflammation or other TNF*α*-related adipocyte dysfunctions. In a similar way, it has been demonstrated that TNFα production is enhanced in obese and diabetic individuals (Hotamisligil et al., 1993; Nieto-Vazquez et al., 2008). Therefore, increased *tnfa* expression can be explained by the obesogenic properties of these compounds.

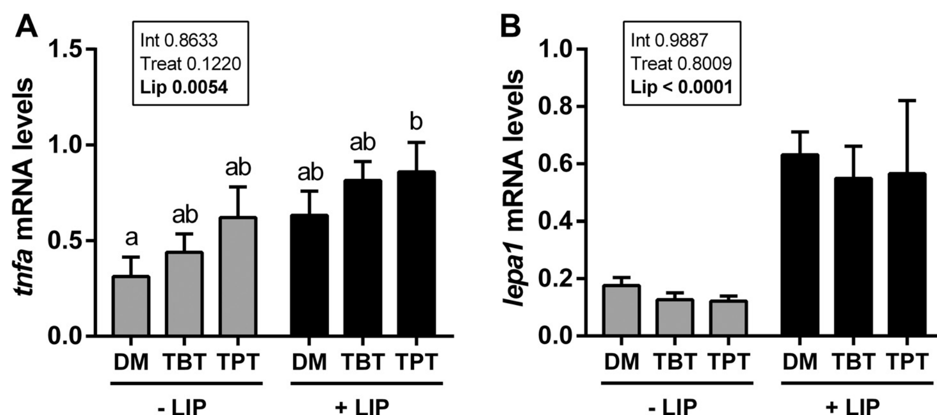

Fig. 6. Relative gene expression of genes encoding adipokines in rainbow trout cultured adipocytes. Confluent cells (day 7 of culture) were incubated with differentiation media as a control (DM), DM plus TBT (100 nM) or DM plus TPT (100 nM), with (+LIP) or without (−LIP) lipid mixture (5 µl/ml) for 24 h. mRNA levels of *tnfa* (A) and *lepa1* (B) are presented. All mRNA levels were normalized to the geometric mean of two reference genes (*ef1a* and *ubq*). Statistical differences in the variability between measurements are indicated in 3 components: interaction (Int), treatment (Treat) and lipid (Lip), using two-way ANOVA. Significant differences ($p < 0.05$) are indicated by different letters, by Tukey's *post hoc* multiple comparison test. Abbreviations: *tumor necrosis factor-alpha, tnfa; leptin, lepa.*

5. Conclusions and future perspectives

From the present study, we can conclude that the environmental pollutants, TBT and TPT, have pro-adipogenic effects in primary cultured adipocytes from rainbow trout. Nevertheless, in contrast to findings reported in mammals, we found for the first time in fish, that these compounds do not induce complete differentiation affecting both adipocyte morphology and most probably functionality. Nonetheless, further characterization using specific agonists and inhibitors is needed to elucidate whether PPARγ, RXRs or other nuclear receptors are involved in the pro-adipogenic effects of TBT and TPT in fish adipocytes. Overall, the present study contributes to improve knowledge on the adipogenic role of obesogens in rainbow trout and suggests that the use of a primary adipocyte cell culture from this species is a valuable *in vitro* tool to estimate the capacity of different compounds and their combinations to interfere with adipocyte differentiation and lipid accumulation.

Conflicts of interest

E.L., N.R-H. and M.C. conducted the research; E.L., E.C. and I.N. designed the experiments, analyzed and interpreted the data. E.L., C.P., J.G., E.C. and I.N. drafted and critically reviewed the manuscript. All authors read and approved the final paper. The authors have declared no conflict of interest.

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References

Alzieu, C.L., Sanjuan, J., Deltreil, J.P., Borel, M., 1986. Tin contamination in Arcachon Bay: effects on oyster shell anomalies. Mar. Pollut. Bull. 17, 494–498.
Ameer, F., Scandiuzzi, L., Hasnain, S., Kalbacher, H., Zaidi, N., 2014. De novo lipogenesis in health and disease. Metabolism 63, 895–902.
Antizar-Ladislao, B., 2008. Environmental levels, toxicity and human exposure to tributyltin (TBT)-contaminated marine environment. A review. Environ. Int. 34, 292–308.
Birchenough, A.C., Barnes, N., Evans, S.M., Hinz, H., Krönke, I., Moss, C., 2002. A review and assessment of tributyltin contamination in the North Sea, based on surveys of butyltin tissue burdens and imposex/intersex in four species of neogastropods. Mar. Pollut. Bull. 44, 534–543.
Bou, M., Todorčević, M., Rodríguez, J., Capilla, E., Gutiérrez, J., Navarro, I., 2014. Interplay of adiponectin, TNFα and insulin on gene expression, glucose uptake and PPARγ, AKT and TOR pathways in rainbow trout cultured adipocytes. Gen. Comp. Endocrinol. 205, 218–225.
Bou, M., Todorčević, M., Torgersen, J., Škugor, S., Navarro, I., Ruyter, B., 2016. De novo lipogenesis in Atlantic salmon adipocytes. Biochim. Biophys. Acta - Gen. Subj. 1860, 86–96.
Bouraoui, L., Capilla, E., Gutiérrez, J., Navarro, I., 2010. Insulin and insulin-like growth factor I signaling pathways in rainbow trout (*Oncorhynchus mykiss*) during adipogenesis and their implication in glucose uptake. Am. J. Physiol. Regul. Integr. Comp. Physiol. 299, R33–R41.
Bouraoui, L., Cruz-Garcia, L., Gutiérrez, J., Capilla, E., Navarro, I., 2012. Regulation of lipoprotein lipase gene expression by insulin and troglitazone in rainbow trout (*Oncorhynchus mykiss*) adipocyte cells in culture. Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 161, 83–88.
Bouraoui, L., Gutiérrez, J., Navarro, I., 2008. Regulation of proliferation and differentiation of adipocyte precursor cells in rainbow trout (*Oncorhynchus mykiss*). J. Endocrinol. 198, 459–469.
Boyer, I.J., 1989. Toxicity of dibutyltin, tributyltin and other organotin compounds to humans and to experimental animals. Toxicology 55, 253–298.
Bremer, A.A., Jialal, I., 2013. Adipose tissue dysfunction in nascent metabolic syndrome. J. Obes. 2013, 1–8.
Brophy, J.T., Keith, M.M., Watterson, A., Park, R., Gilbertson, M., Maticka-Tyndale, E., Beck, M., Abu-Zahra, H., Schneider, K., Reinhartz, A., DeMatteo, R., Luginaah, I., Ferlay, J., Shin, H., Bray, F., Forman, D., Mathers, C., Parkin, D., Sasco, A., Kruk, J., Aboul-Enein, H., Brody, J., Moysich, K., Humblet, O., Attfield, K., Beehler, G., Rudel, R., Brown, P., McCormick, S., Mayer, B., Zavestoski, S., Morello-Frosch, R., Gasior, R., Senier, L., Gray, J., Evans, N., Taylor, B., Rizzo, J., Walker, M., Sieber, S.D.D., Tirona, M., Sehgal, R., Ballester, O., Soto, A., Sonnenschein, C., Birnbaum, L., Fenton, S., Fenton, S., Vandenberg, L., Colborn, T., Hayes, T., Heindel, J., Jacobs, D., Lee, D., Shioda, T., Soto, A., Saal, F.vom, Welshons, W., Zoeller, R., Myers, J., Rudel, R., Attfield, K., Schifano, J., Brody, J., Tomatis, L., Huff, J., Hertz-Picciotto, I., Sandler, D., Bucher, J., Boffetta, P., Axelson, O., Blair, A., Taylor, J., Stayner, L., Barrett, J., Beall, C., Delzell, E., Macaluso, M., Mills, P., Yang, R., Thompson, D., Kriebel, D., Quinn, M., Wegman, D., Eisen, E., Petralia, S., Chow, W., McLaughlin, J., Jin, F., Gao, Y., Dosemeci, M., Tynes, T., Hannevik, M., Andersen, A., Vistnes, A., Haldorsen, T., Band, P., Le, N., Fang, R., Deschamps, M., Gallagher, R., Yang, P., Villeneuve, S., Févotte, J., Anger, A., Truong, T., Lamkarkach, F., Gaye, O., Kerbrat, P., Arveux, P., Miglianico, L., Imbernon, E., Guénel, P., Labrèche, F., Goldberg, M., Valois, M.-F., Nadon, L., Luginaah, I., Gorey, K., Tang, K., Oiamo, T., Holowaty, E., Hamm, C., Wright, F., Gilbertson, M., Brophy, J., Brophy, J., Keith, M., Gorey, K., Laukkanen, E., Hellyer, D., Watterson, A., Reinhartz, A., Gilberston, M., Brophy, J., Keith, M., Gorey, K., Luginaah, I., Laukkanen, E., Hellyer, D., Reinhartz, A., Watterson, A., Abu-Zahra, H., Maticka-Tyndale, E., Schneider, K., Beck, M., Gilbertson, M., Siemiatycki, J., Stewart, P., Stewart, W., Heineman, E., Dosemeci, M., Linet, M., Inskit, P., Teschke, K., Olshan, A., Daniels, J., Roos, A.De, Parks, C., Schulz, M., Vaughan, T., Checkoway, H., Pearce, N., Crawford-Brown, D., Kang, J., Kondo, F., Katayama, Y., Diamanti-Kandarakis, E., Bourguignon, J., Giudice, L., Hauser, R., Prins, G., Soto, A., Zoeller, R., Gore, A., Preston, D., Lubin, J., Pierce, D., Infante, P., Zahm, S., Blair, A., Band, P., Le, N., Fang, R., Deschamps, M., Robert, S., Strombom, I., Trentham-Dietz, A., Hampton, J., McElroy, J., Newcomb, P., Remington, P., Alavanja, M., Sandler, D., McMaster, S., Zahm, S., McDonnell, C., Lynch, C., Pennybacker, M., Rothman, N., Dosemeci, M., Bond, A., Blair, A., Clapp, R., Jacobs, M., Loechler, E., Cohn, B., Wolff, M., Cirillo, P., Sholtz, R., DeMatteo, R., Keith, M., Brophy, F., Wordsworth, A., Watterson, A., Beck, M., Ford, A.R., Gilbertson, M., Phartiyal, J., Rootham, M., Scott, D., Yang, C., Yaniger, S., Jordan, V., Klein, D., Bittner, G., Jm, J.I., Fernández, M., Santa-Marina, L., Olea-Serrano, M., Rivas, A., Aurrekoetxea, J., Expósito, J., Lorenzo, M., Torné, P., Villalobos, M., Pedraza, V., Sasco, A., Olea, N., Lithner, D., Larsson, Å., Dave, G., Gardner, K., Shu, X.O., Jin, F., Dai, Q., Ruan, Z., Thompson, S., Hussey, J., Gao, Y., Zheng, W., Ji, B., Blair, A., Shu, X., Chow, W., Hauptmann, M., Dosemeci, M.,

Yang, G., Lubin, J., Gao, Y., Rothman, N., Zheng, W., Ewertz, M., Holmberg, L., Tretli, S., Pedersen, B., Kristensen, A., Keikotlhaile, B., Spanoghe, P., Steurbaut, W., Geens, T., Aerts, D., Berthot, C., Bourguignon, J., Goeyens, L., Lecomte, P., Maghuin-Rogister, G., Pironnet, A., Pussemier, L., Scippo, M., Loco, J.Van, Covaci, A., Keith, M., Cann, B., Brophy, J., Hellyer, D., Day, M., Egan, S., Mayville, K., Watterson, A., Larsson, M., Boëthius, G., Axelsson, S., Montgomery, S., Park, R., Gammon, M., Sagiv, S., Eng, S., Shantakumar, S., Gaudet, M., Teitelbaum, S., Britton, J., Terry, M., Wang, L., Wang, Q., Stellman, S., Beyea, J., Hatch, M., Kabat, G., Wolff, M., Levin, B., Neugut, A., Santella, R., Petralia, S., Vena, J., Freudenheim, J., Dosemeci, M., Michalek, A., Goldberg, M., Brasure, J., Graham, S., Hansen, J., Høyer, A., Jørgensen, T., Rank, F., Grandjean, P., Gupta, P., Proia, D., Cingoz, O., Weremowicz, J., Naber, S., Weinberg, R., Kuperwasser, C., Blair, A., Linos, A., Stewart, P., Burmeister, L., Gibson, R., Everett, G., Schuman, L., Cantor, K., 2012. Breast cancer risk in relation to occupations with exposure to carcinogens and endocrine disruptors: a Canadian case–control study. Environ. Heal. 11, 87.

Capilla, E., Teles-García, A., Acerete, L., Navarro, I., Gutiérrez, J., 2011. Insulin and IGF-I effects on the proliferation of an osteoblast primary culture from sea bream (*Sparus aurata*). Gen. Comp. Endocrinol. 172, 107–114.

Carney Almroth, B., Albertsson, E., Sturve, J., Förlin, L., 2008. Oxidative stress, evident in antioxidant defences and damage products, in rainbow trout caged outside a sewage treatment plant. Ecotoxicol. Environ. Saf. 70, 370–378.

Chalk, M.B., 2004. Obesity: addressing a multifactorial disease. Case Manager 15, 47–49.

Chamorro-García, R., Sahu, M., Abbey, R.J., Laude, J., Pham, N., Blumberg, B., 2013. Transgenerational inheritance of increased fat depot size, stem cell reprogramming, and hepatic steatosis elicited by prenatal exposure to the obesogen tributyltin in mice. Environ. Health Perspect. 121, 359–366.

Cruz-Garcia, L., Sánchez-Gurmaches, J., Gutiérrez, J., Navarro, I., 2012. Role of LXR in trout adipocytes: Target genes, hormonal regulation, adipocyte differentiation and relation to lipolysis. Comp. Biochem. Physiol. - A Mol. Integr. Physiol. 163, 120–126.

Deng, Y., Scherer, P.E., 2010. Adipokines as novel biomarkers and regulators of the metabolic syndrome. Ann. N. Y. Acad. Sci. 1212, E1–E19.

Diamanti-Kandarakis, E., Bourguignon, J.-P., Giudice, L.C., Hauser, R., Prins, G.S., Soto, A.M., Zoeller, R.T., Gore, A.C., 2009. Endocrine-disrupting chemicals: an Endocrine Society scientific statement. Endocr. Rev. 30, 293–342.

Dimastrogiovanni, G., Córdoba, M., Navarro, I., Jáuregui, O., Porte, C., 2015. Alteration of cellular lipids and lipid metabolism markers in RTL-W1 cells exposed to model endocrine disrupters. Aquat. Toxicol. 165, 277–285.

Egger, G., Dixon, J., 2014. Beyond obesity and lifestyle: a review of 21st century chronic disease determinants. Biomed. Res. Int. 2014, 731685.

Eidet, J.R., Pasovic, L., Maria, R., Jackson, C.J., Utheim, T.P., 2014. Objective assessment of changes in nuclear morphology and cell distribution following induction of apoptosis. Diagn. Pathol. 9, 92.

Fent, K., 1996. Ecotoxicology of organotin compounds. Crit. Rev. Toxicol. 26, 1–117.

Flynn, E.J., Trent, C.M., Rawls, J.F., 2009. Ontogeny and nutritional control of adipogenesis in zebrafish (*Danio rerio*). J. Lipid Res. 50, 1641–1652.

Folch, J., Lees, M., Sloane Stanley, G.H., 1957. A simple method for the isolation and purification of total lipides from animal tissues. J. Biol. Chem. 226, 497–509.

Grün, F., 2014. The obesogen tributyltin. Vitam. Horm. 94, 277–325.

Grün, F., Blumberg, B., 2006. Environmental obesogens: organotins and endocrine disruption via nuclear receptor signaling. Endocrinology 147, s50–s55.

Grün, F., Watanabe, H., Zamanian, Z., Maeda, L., Arima, K., Cubacha, R., Gardiner, D.M., Kanno, J., Iguchi, T., Blumberg, B., 2006. Endocrine-disrupting organotin compounds are potent inducers of adipogenesis in vertebrates. Mol. Endocrinol. 20, 2141–2155.

Harada, S., Hiromori, Y., Nakamura, S., Kawahara, K., Fukakusa, S., Maruno, T., Noda, M., Uchiyama, S., Fukui, K., Nishikawa, J., Nagase, H., Kobayashi, Y., Yoshida, T., Ohkubo, T., Nakanishi, T., 2015. Structural basis for PPARγ transactivation by endocrine-disrupting organotin compounds. Sci. Rep. 5, 8520.

Heindel, J.J., Newbold, R., Schug, T.T., 2015. Endocrine disruptors and obesity. Nat. Rev. Endocrinol. 11, 653–661.

Hiromori, Y., Nishikawa, J., Yoshida, I., Nagase, H., Nakanishi, T., 2009. Structure-dependent activation of peroxisome proliferator-activated receptor (PPAR) γ by organotin compounds. Chem. Biol. Interact. 180, 238–244.

Holtcamp, W., 2012. Obesogens: an environmental link to obesity. Environ. Health Perspect. 120, a62–a68.

Hotamisligil, G.S., Shargill, N.S., Spiegelman, B.M., 1993. Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. Science 259, 87–91.

Janesick, A., Blumberg, B., 2012. Obesogens, stem cells and the developmental programming of obesity. Int. J. Androl. 35, 437–448.

Janesick, A., Blumberg, B., 2011. Endocrine disrupting chemicals and the developmental programming of adipogenesis and obesity. Birth Defects Res. C. Embryo Today 93, 34–50.

Janesick, A.S., Blumberg, B., 2016. Obesogens: an emerging threat to public health. Am. J. Obstet. Gynecol. 214, 559–565.

Jeng, H.A., 2014. Exposure to endocrine disrupting chemicals and male reproductive health. Front. Public Health 2, 55.

Kanayama, T., Kobayashi, N., Mamiya, S., Nakanishi, T., Nishikawa, J.-I., 2005. Organotin compounds promote adipocyte differentiation as agonists of the peroxisome proliferator-activated receptor gamma/retinoid X receptor pathway. Mol. Pharmacol. 67, 766–774.

Kirchner, S., Kieu, T., Chow, C., Casey, S., Blumberg, B., 2010. Prenatal exposure to the environmental obesogen tributyltin predisposes multipotent stem cells to become adipocytes. Mol. Endocrinol. 24, 526–539.

Kusminski, C.M., Bickel, P.E., Scherer, P.E., 2016. Targeting adipose tissue in the treatment of obesity-associated diabetes. Nat. Rev. Drug Discov. 15, 639–660.

Lee, H., Lim, S., Yun, S., Yoon, A., Park, G., Yang, H., 2012. Tributyltin increases the expression of apoptosis- and adipogenesis-related genes in rat ovaries. Clin. Exp. Reprod. Med. 39, 15–21.

Lefterova, M.I., Lazar, M.A., 2009. New developments in adipogenesis. Trends Endocrinol. Metab. 20, 107–114.

Li, X., Ycaza, J., Blumberg, B., 2011. The environmental obesogen tributyltin chloride acts via peroxisome proliferator activated receptor gamma to induce adipogenesis in murine 3T3-L1 preadipocytes. J. Steroid Biochem. Mol. Biol. 127, 9–15.

Lo, J.H., Chiou, P.P., Lin, C.M., Chen, T.T., 2007. Molecular cloning and expression analysis of rainbow trout (*Oncorhynchus mykiss*) CCAAT/enhancer binding protein genes and their responses to induction by GH in vitro and in vivo. J. Endocrinol. 194, 393–406.

Lowe, C.E., O'Rahilly, S., Rochford, J.J., 2011. Adipogenesis at a glance. J. Cell Sci. 124.

Lubrano, C., Genovesi, G., Specchia, P., Costantini, D., Mariani, S., Petrangeli, E., Lenzi, A., Gnessi, L., 2013. Obesity and metabolic comorbidities: environmental diseases? Oxid. Med. Cell. Longev. 2013, 640673.

McLachlan, J.A., Simpson, E., Martin, M., 2006. Endocrine disrupters and female reproductive health. Best Pract. Res. Clin. Endocrinol. Metab. 20, 63–75.

Meador, J.P., Sommers, F.C., Cooper, K.A., Yanagida, G., 2011. Tributyltin and the obesogen metabolic syndrome in a salmonid. Environ. Res. 111, 50–56.

Mitchell, N.S., Catenacci, V.A., Wyatt, H.R., Hill, J.O., 2011. Obesity: overview of an epidemic. Psychiatr. Clin. North Am. 34, 717–732.

Moreno-Navarrete, J.M., Fernández-Real, J.M., 2012. Adipocyte Differentiation. Adipose Tissue Biology. Springer New York, New York, NY, pp. 17–38.

Newbold, R.R., Padilla-Banks, E., Jefferson, W.N., Heindel, J.J., 2008. Effects of endocrine disruptors on obesity. Int. J. Androl. 31, 201–208.

Nieto-Vazquez, I., Fernández-Veledo, S., Krämer, D.K., Vila-Bedmar, R., Garcia-Guerra, L., Lorenzo, M., 2008. Insulin resistance associated to obesity: the link TNF-alpha. Arch. Physiol. Biochem. 114, 183–194.

Otto, T.C., Lane, M.D., 2005. Adipose development: from stem cell to adipocyte. Crit. Rev. Biochem. Mol. Biol. 40, 229–242.

Ouadah-Boussouf, N., Babin, P.J., 2016. Pharmacological evaluation of the mechanisms involved in increased adiposity in zebrafish triggered by the environmental contaminant tributyltin. Toxicol. Appl. Pharmacol. 294, 32–42.

Pereira-Fernandes, A., Vanparys, C., Hectors, T.L.M., Vergauwen, L., Knapen, D., Jorens, P.G., Blust, R., 2013. Unraveling the mode of action of an obesogen: mechanistic analysis of the model obesogen tributyltin in the 3T3-L1 cell line. Mol. Cell. Endocrinol. 370, 52–64.

Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucl. Acids Res. 29, e45.

Qi, L., Cho, Y.A., 2008. Gene-environment interaction and obesity. Nutr. Rev. 66, 684–694.

Regnier, S.M., El-Hashani, E., Kamau, W., Zhang, X., Massad, N.L., Sargis, R.M., 2015. Tributyltin differentially promotes development of a phenotypically distinct adipocyte. Obesity (Silver Spring) 23, 1864–1871.

Rosen, E.D., 2005. The transcriptional basis of adipocyte development. Prostaglandins. Leukot. Essent. Fat. Acids 73, 31–34.

Rosen, E.D., Spiegelman, B.M., 2006. Adipocytes as regulators of energy balance and glucose homeostasis. Nature 444, 847–853.

Salazar, M.H., Salazar, S.M., 1991. Assessing site-specific effects of TBT contamination with mussel growth rates. Mar. Environ. Res. 32, 131–150.

Salmerón, C., García de la serrana, D., Jiménez-Amilburu, V., Fontanillas, R., Navarro, I., Johnston, I.A., Gutiérrez, J., Capilla, E., 2013. Characterisation and expression of calpain family members in relation to nutritional status, diet composition and flesh texture in gilthead sea bream (*Sparus aurata*). PLoS ONE 8, e75349.

Salmerón, C., Johansson, M., Asaad, M., Angotzi, A.R., Rønnestad, I., Stefansson, S.O., Jönsson, E., Björnsson, B.T., Gutiérrez, J., Navarro, I., Capilla, E., 2015. Roles of leptin and ghrelin in adipogenesis and lipid metabolism of rainbow trout adipocytes in vitro. Comp. Biochem. Physiol. A: Mol. Integr. Physiol. 188, 40–48.

Sánchez-Gurmaches, J., Cruz-Garcia, L., Gutiérrez, J., Navarro, I., 2012. Adiponectin effects and gene expression in rainbow trout: an in vivo and in vitro approach. J. Exp. Biol. 215.

Seth, A., Stemple, D.L., Barroso, I., 2013. The emerging use of zebrafish to model metabolic disease. Dis. Model. Mech. 6, 1080–1088.

Tang-Péronard, J.L., Andersen, H.R., Jensen, T.K., Heitmann, B.L., 2011. Endocrine-disrupting chemicals and obesity development in humans: a review. Obes. Rev. 12, 622–636.

Thayer, K.A., Heindel, J.J., Bucher, J.R., Gallo, M.A., 2012. Role of environmental chemicals in diabetes and obesity: a National Toxicology Program workshop review. Environ. Health Perspect. 120, 779–789.

Tingaud-Sequeira, A., Ouadah, N., Babin, P.J., 2011. Zebrafish obesogenic test: a tool for screening molecules that target adiposity. J. Lipid Res. 52, 1765–1772.

Todorčević, M., Škugor, S., Krasnov, A., Ruyter, B., Rora, A., Kvale, A., Morkore, T., Rorvik, K., Steien, S., Thomassen, M., Gregoire, F., Kershaw, E., Flier, J., Fauconneau, B., Andre, S., Chmaitilly, J., Lebail, P., Krieg, F., Kaushik, S., Crisan, M., Yap, S., Casteilla, L., Chen, C., Corselli, M., Park, T., Doherty, M., Ashton, B., Walsh, S., Beresford, J., Grant, M., Canfield, A., Doherty, M., Canfield, A., Farrington-Rock, C., Crofts, N., Doherty, M., Ashton, B., Griffin-Jones, C., Canfield, A., Tang, W., Zeve, D., Suh, J., Bosnakovski, D., Kyba, M., Hammer, R., Vegusdal, A., Sundvold, H., Gjoen, T., Ruyter, B., Todorcevic, M., Vegusdal, A., Gjoen, T., Sundvold, H., Torstensen, B., Kjaer, M., Huang, T., Todorcevic, M., Ruyter, B., Torstensen, B., Kang, Y., Jeon, E., Song, H., Woo, J., Jung, J., Kim, Y., Ratajczak, M., Kucia, M., Reca, R., Majka, M., Janowska-Wieczorek, A., Ratajczak, J., Klein, R., Rubin, J., Rossi, D., Zlotnik, A., Rosen, E., Spiegelman, B., Rosen, E., MacDougald, O., Skalli, O., Pelte, M., Peclet, M., Gabbiani, G., Gugliotta, P., Bussolati, G., Jensen, B., Farach-Carson, M., Kenaley, E., Akanbi, K., Shi, H., Halvorsen, Y., Ellis, P., Wilkison, W., Zemel, M., Miller, C., Casimir, D., Ntambi, J., Neal, J., Clipstone, N., Szabo, E., Qiu, Y., Baksh, S., Michalak,

M., Opas, M., Hiraki, Y., Inoue, H., Kondo, J., Kamizono, A., Yoshitake, Y., Shukunami, C., Mori, Y., Hiraki, Y., Shukunami, C., Kakudo, S., Shiokawa, M., Kagoshima, M., Moldes, M., Boizard, M., Liepvre, X., Fève, B., Dugail, I., Pairault, J., Beale, E., Hammer, R., Antoine, B., Forest, C., Beale, E., Antoine, B., Forest, C., Collett, G., Wood, A., Alexander, M., Varnum, B., Boot-Handford, R., Ohanian, V., Carter, L., Fouser, L., Jussif, J., Fitz, L., Deng, B., Wood, C., Arima, K., Sato, K., Tanaka, G., Kanaji, S., Terada, T., Honjo, E., Rabinovich, G., Riera, C., Landa, C., Sotomayor, C., Preisinger, C., Short, B., Corte, V.De, Bruyneel, E., Haas, A., Kopajtich, R., Malhotra, J., Kaufman, R., Seiler, A., Schneider, M., Forster, H., Roth, S., Wirth, E., Culmsee, C., Tonissen, K., Trapani, G.Di, Caspar-Bauguil, S., Cousin, B., Galinier, A., Segafredo, C., Nibbelink, M., Andrq, M., Traktuev, D., Merfeld-Clauss, S., Li, J., Kolonin, M., Arap, W., Pasqualini, R., Zannettino, A., Paton, S., Arthur, A., Khor, F., Itescu, S., Gimble, J., Gimble, J., Katz, A., Bunnell, B., Meirelles, L., da, S., Caplan, A., Nardi, N., Guo, K., Wang, J., Andres, V., Smith, R., Walsh, K., Bowers, R., Kim, J., Otto, T., Lane, M., Scheideler, M., Elabd, C., Zaragosi, L., Chiellini, C., Hackl, H., Sanchez-Cabo, F., Singh, J., Verma, N., Kansagra, S., Kate, B., Dey, C., Lin, T., Yang, R., Tang, C., Lin, C., Fu, W., Yu, F., Yang, G., Zhao, Z., Ji, L., Cao, Y., Bai, L., Fajas, L., Huynh, H., Larsson, C., Narod, S., Pollak, M., Hemre, G., Mommsen, T., Krogdahl, A., Carriere, A., Fernandez, Y., Rigoulet, M., Penicaud, L., Casteilla, L., Carriere, A., Carmona, M., Fernandez, Y., Rigoulet, M., Wenger, R., Penicaud, L., Galinier, A., Carriere, A., Fernandez, Y., Carpene, C., Andre, M., Caspar-Bauguil, S., Gregor, M., Hotamisligil, G., Ozcan, U., Cao, Q., Yilmaz, E., Lee, A., Iwakoshi, N., Ozdelen, E., Kaufman, R., Kaufman, R., Brunsing, R., Omori, S., Weber, F., Bicknell, A., Friend, L., Rickert, R., Kitiphongspattana, K., Mathews, C., Leiter, E., Gaskins, H., Goodman, J., Bonander, N., Darby, R., Grgic, L., Bora, N., Wen, J., Brogna, S., Fantuzzi, G., Cawthorn, W., Heyd, F., Hegyi, K., Sethi, J., Bouraoui, L., Gutierrez, J., Navarro, I., Wellen, K., Hotamisligil, G., Jorgensen, S., Afanasyev, S., Krasnov, A., Schiøtz, B., Jørgensen, S., Rexroad, C., Gjøen, T., Krasnov, A., Nabhan, J., Ribeiro, P., Ramirez-Zacarias, J., Castro-Munozledo, F., Kuri-Harcuch, W., 2010. Gene expression profiles in Atlantic salmon adipose-derived stromo-vascular fraction during differentiation into adipocytes. BMC Genomics 11, 39.

Vélez, E.J., Lutfi, E., Jiménez-Amilburu, V., Riera-Codina, M., Capilla, E., Navarro, I., Gutiérrez, J., 2014. IGF-I and amino acids effects through TOR signaling on proliferation and differentiation of gilthead sea bream cultured myocytes. Gen. Comp. Endocrinol. 205, 296–304.

Yanik, S.C., Baker, A.H., Mann, K.K., Schlezinger, J.J., 2011. Organotins are potent activators of PPARγ and adipocyte differentiation in bone marrow multipotent mesenchymal stromal cells. Toxicol. Sci. 122, 476–488.

CHAPTER 5. GENERAL DISCUSSION

“Seeking means: to have a goal;

but finding means: to be free,

to be receptive, to have no goal”

Hermann Hesse

(Siddhartha)

The importance of adipose tissue in health and disease in both, humans and fish, is beyond question. In this regard, it has been extensively demonstrated that its dysfunction can lead to several pathological situations (Garg, 2006). Thus, perhaps because it is obvious that diet influences its condition, most work has concentrated on the nutritional regulation of adipose tissue. Nevertheless, it is becoming clear that this view might be an oversimplified approach, as more factors are regarded to play an important role. This is particularly evident in the case of obesity, considering that there is no one single cause that completely explains its prevalence. Indeed, even though the common prescription for weight loss is "Eat less, move more", it has been widely proven that genetics and environment are potentially at least as important as nutrition in this matter (Speakman, 2004).

In this context, and in order to evaluate the influence of nutritional, genetic and environmental factors on adipose tissue biology and lipid homeostasis in fish, four different experimental trials have been conducted to provide an integrative view of the modulation of fat depots in these vertebrates. First, we have examined the potential anti-obesogenic effect of three dietary antioxidants (caffeic acid, hydroxytyrosol and astaxanthin) in zebrafish larvae, and rainbow trout *in vivo* and *in vitro* models, with special focus on adiposity dynamics, and their involvement in modulating adipogenesis through the PPARγ signaling pathway (Article I). Next, we have also investigated the metabolic consequences of feeding rainbow trout broodstock with different dietary methionine levels, on several metabolism-related genes expression in the offspring subjected to the same diets (Article II). Moreover, we provide new information on the regulation of lipid metabolism and oxidative stress at a transcriptional level in two experimental rainbow trout lines genetically selected for high or low muscle fat content and subjected to food deprivation (Article III). Finally, in order to better understand how adipocyte differentiation can be affected by external compounds, we have evaluated the obesogenic potential of two environmental pollutants (the trisubstituted organotins TBT and TPT) by studying adipocyte development and lipid homeostasis in primary culture rainbow trout preadipocytes (Article IV).

5.1 NUTRITIONAL REGULATION OF LIPID HOMEOSTASIS

5.1.1 Anti-obesogenic properties of vegetal antioxidants

Throughout history, natural products from plants and their derivatives have been a rich source of bioactive compounds for drug discovery, with important applications in human health and

agronomic purposes. Some of these compounds, so-called phytochemicals, have recently drawn attention for their potential health benefits in preventing fat deposition while improving insulin resistance and obesity-related symptoms (Mukherjee et al., 2015); therefore, are regarded as anti-obesogenic. In this context, we have examined the potential anti-obesogenic properties of three well-known antioxidants mainly present in red wine (caffeic acid, CA), olive oil (hydroxytyrosol, HT) and marine algae (astaxanthin, ATX), selected for their capacity to modulate adiposity dynamics in mammals (Article I). Specifically, CA is widely available in plants, fruits and vegetables (i.e. thyme, grapes and cinnamon) and is considered the major dietary hydroxycinnamic acid in human diet. It has a variety of potential pharmacological effects including antioxidant (Gülçin, 2006), anticancer (Rajendra Prasad et al., 2011) and anti-inflammatory properties (Chao et al., 2010). In addition, positive effects in the prevention of diet-induced hyperlipidemia and obesity in C57BL/6 mice have been reported (Liao et al., 2013). On the other hand, HT, which is present in olive leaves and olive oil, shows a wide spectrum of biological properties due mainly to its strong antioxidant capacity (Fernandez-Bolaños et al., 2012). Furthermore, *in vitro* studies have shown HT reduces adipocyte hyperplasia and hypertrophy, as well as TAG content by down-regulating adipogenesis-related genes in mouse 3T3L-1 and C3H10 T1/2 adipocyte lines (Drira et al., 2011; Warnke et al., 2011). Finally, ATX is a natural red carotenoid pigment found in a wide variety of organisms including krill and algae. Its antioxidant (Dose et al., 2016; Régnier et al., 2015) as well as anti-obesogenic properties (Ikeuchi et al., 2007) have been extensively reported in mammals.

As outlined in the introduction, the zebrafish model possesses many particular features such as external development and optical transparency from fertilization through the onset of adulthood, which permit *in vivo* imaging of dynamic cellular events during growth, including adipose tissue formation (Minchin and Rawls, 2016). In the present work, we used the Zebrafish Obesogenic Test (ZOT) protocol (Tingaud-Sequeira et al., 2011) to first evaluate the ability of CA, HT and ATX to modulate zebrafish larval adiposity *in vivo*. Our results showed that only CA and HT caused a significant reduction in the fat content of zebrafish larvae, in agreement with previous studies that reported positive effects of CA (Liao et al., 2013) and HT (Cao et al., 2014) in the prevention of diet-induced hyperlipidemia and obesity in mice. However, and even several studies have supported the hypothesis that ATX supplementation reduces body weight and fat accumulation in mice (Arunkumar et al., 2012), this phytochemical did not modify the adiposity level in zebrafish. This lack of effect has been also reported in humans (Res et al., 2013), and to some extent could be attributed to the differences of ATX uptake or action

depending on the species (Østerlie et al., 2000), or on tissue and cell-type (Inoue et al., 2012), respectively.

In mammals, subcutaneous and perivisceral fat depots are shown to respond to external stimuli in a different way. Adipocytes in perivisceral adipose tissue are sensitive to lipolytic stimuli and the first to mobilize fat resources upon necessity, whereas those from subcutaneous depots do not release stored lipids easily, as they have instead a more important structural and thermal isolation role (Bjørndal et al., 2011; Fisher et al., 2002). Besides, insulin and estrogens are thought to increase subcutaneous adipose tissue mass, while perivisceral adipose tissue seems to be more responsive to glucocorticoids (Mattsson and Olsson, 2007). In the present work, CA exhibited stronger anti-obesogenic effects in the head region of zebrafish larva, while HT affected both the head and visceral regions. On the light of these results it is tempting to conclude that these compounds may have different capacities reducing fat accumulation depending on the adipose tissue depot; however, further experiments are needed to demonstrate if they have a specific-depot target or these differences can be attributed to their intrinsic different potency.

In the recent years, significant attention has been paid to the role of PPARγ in obesity and related diseases (Kadowaki et al., 2002) due to its referred importance in the regulation of adipogenesis (Spiegelman et al., 1997). Synthetic PPARγ antagonists such as GW9662 have been shown to prevent high-fat diet-induced obesity (Nakano et al., 2006). However, agonists like rosiglitazone (RGZ), which are commonly used as insulin sensitizers for treating type II diabetes and hyperglycemia (Grygiel-Górniak, 2014), can cause detrimental side effects, such as weight gain and adipose tissue inflammation (Hussein et al., 2004). Hence, targeting PPARγ for therapeutic uses must be considered carefully.

The obesogenic role of RGZ has been previously reported in zebrafish (Tingaud-Sequeira et al., 2011). In that study, RGZ prevented adiposity loss in zebrafish larvae fed with a standard diet and even increased adiposity and lipid droplet size on a high-fat diet background. Moreover, this effect was completely abolished by the PPARγ antagonist T0070907, suggesting a clear involvement of this transcriptional factor in adipocyte hypertrophy. In this regard, our results revealed that CA and HT abolished as well, the RGZ-induced obesogenic effect in zebrafish larvae, in agreement with the PPARγ antagonist effect of these compounds reported in mice (Drira and Sakamoto, 2014; Liao et al., 2013).

In order to have a closer look into the potential effect of CA and HT blocking PPARγ signaling, further investigations using primary-cultured rainbow trout adipocytes were performed. In such system, similar results were found, since CA and HT successfully counteracted the PPARγ protein expression signal induced by RGZ. However, this effect was not observed at a transcriptional level suggesting that the anti-obesogenic effect of CA and HT may be mediated by post-transcriptional mechanisms. Overall, our findings indicate that the decrease in zebrafish larval adiposity along with the observed reduction on lipid accumulation in rainbow trout adipocytes caused by CA and HT treatments might be mediated through PPARγ inhibition. Nonetheless, it is important to note that with the present results we cannot assure that CA and HT function as direct PPARγ antagonists in our teleost models, since further characterization using specific inhibitors is needed.

Even though PPARγ actions are mainly related to adipose tissue, its modulation also exerts an impact on whole-body metabolism. In mammals, this transcription factor regulates the expression of numerous genes involved in lipogenesis and fatty acid uptake, including fatty acid synthase (*fasn*), lipoprotein lipase (*lpl*), fatty acid transporters (*fatps*) and the fatty acid translocase also called cluster of differentiation (*cd36*) (Kadegowda et al., 2009; Lim et al., 2006; Mishima et al., 2011). Although less information concerning the regulatory role of PPARγ on lipid metabolism related-genes is available in fish, the anti-obesogenic effect observed in our models by CA and HT may also involve a transcriptional regulation of these markers.

Precisely, the *in vivo* administration of these two antioxidants to rainbow trout affected the mRNA levels of adipogenic and β-oxidation genes in liver and adipose tissue. HT treatment decreased *fasn* mRNA levels in adipose tissue in agreement with reported studies in 3T3-L1 adipocytes (Tutino et al., 2016). However, the adipose triglyceride lipase *pnpla2* was also down-regulated, which is indicative of decreased lipolysis, suggesting that the anti-obesogenic effects of HT in rainbow trout might be linked to reduced lipogenesis instead of increased lipolytic activity. This down-regulation of *pnpla2* gene expression might be also explained as a compensatory mechanism to counteract a hypothetic previous lipolytic action of this compound. Moreover, in line with this assumption, the increased *acsl1* mRNA levels observed, suggest that HT might activate β-oxidation as well. In this context, despite the fact that enhanced *acsl1* expression is traditionally believed to take part in TAG synthesis, several studies in mammals have revealed a possible function related to the β-oxidation of FA (Li et al. 2009; Ellis et al. 2010), which could support our hypothesis. Nevertheless, no differences were

observed concerning the mRNA levels of two well-known indicators of fatty acid β-oxidation (*hoad* and *pparb*). Furthermore, HT (slightly) and CA (significantly) up-regulated *lpl* expression in liver, indicating increased TAG breakdown, supporting the reduced TAG plasma levels in these fish. Nonetheless, it should be noted that some of our conclusions are based on transcriptional results while no protein expression nor enzymatic activity has been measured; therefore, such results must be interpreted carefully.

Overall, the current findings provide new information on the anti-obesogenic role of CA and HT, highlighting their potential application in alternative therapies for the treatment of obesity and as dietary additives to optimize adiposity in farmed animals.

5.1.2 Dietary methionine deficiency in rainbow trout

With the continued growth of aquaculture, significant attention has been focus on fish nutrition, due to its important impact on both productivity and the sustainable development of this industry. Feeding fish with unbalanced diets not only affects growth and food conversion rates, but also increases susceptibility to disease (Oliva-Teles, 2012). Specifically, unbalanced amino acid diets can induce lipid metabolic alterations in adipose tissue (Albalat et al., 2005). As described in the introduction section, intensive fish farming has traditionally relied on the use of fish meal and fish oil for fish feed formulation. However, due to regulatory measures on fishing and the consequent increased price of these ingredients, the use of alternative raw materials has been proposed essential for the sustainable development of aquaculture. Apart from the recent trend in investigating new dietary plant ingredients with desirable properties for aquaculture production (Article I), plant products have been traditionally used as alternative sources of protein in fish feeds (see Boonyaratpalin, 2015). Nevertheless, it has been shown that plant ingredients often present altered amino acid profiles and should be complemented with crystalline amino acids (Espe et al., 2012) in order to reach the nutritional requirements of each species (NRC 2011).

Methionine (Met) is an essential amino acid implicated in protein synthesis and the regulation of lipid metabolism (Lovell, 1989). Recent studies in rainbow trout have shown that Met restriction cause some glucose and lipid metabolism dysregulations (Craig and Moon, 2017; Skiba-Cassy et al., 2016) that can have long-term consequences on physiological functions in the offspring (Fontagné-Dicharry et al., 2017). This phenomenon, known as nutritional programming, has been largely studied in mammalian models (Rando and Simmons, 2015;

Ward et al., 2016) and also several studies have been reported in fish (Balasubramanian et al., 2016; Izquierdo et al., 2015), and is thought to be driven by epigenetic mechanisms. Interestingly, it has been shown that this parental epigenetic programming can be reversible through Met supplementation in the offspring (Weaver et al., 2005), suggesting a possible interaction between parental and early-life nutritional events. In this context, we have performed a trial aiming to evaluate the metabolic effects of parental and offspring dietary Met deficiency on rainbow trout, with special focus on several genes involved on the regulation of glucose, cholesterol and lipid metabolism (Article II). This study is particularly interesting for the present thesis, as it is an example of the metabolic consequences of an interaction between nutritional and genetic factors.

Our results revealed that feeding for 3 weeks from first feeding a Met deficient diet appears to enhance lipogenesis in trout fry, as reflected by *fasn*, sterol regulatory element-binding protein (*srebp1*) and liver x receptor (*lxr*) increased mRNA levels. A similar positive effect of Met deficiency on lipogenesis was also found in Atlantic salmon, where suboptimal dietary Met levels increased liver weight as a result of enhanced hepatic FAS activity and TAG accumulation (Espe et al., 2010). In contrast, previous studies in juvenile trout showed no effects on lipogenic genes when fed with Met deficient diets (Skiba-Cassy et al., 2016), suggesting that very early stages might be more sensitive to dietary amino acid deficiencies, especially Met. In line with this argument, a study performed in rats demonstrated that Met restriction reduces muscle and hepatic TAG when initiated after weaning but not when initiated in six month old rats (Hasek et al., 2013).

Moreover, early dietary Met deficiency also enhanced the expression of genes involved in cholesterol elimination (*cyp7A1*, *ugt1A3* and *abcg8*) and efflux (*abcg1* and *abca1*), while no effect was observed in cholesterol synthesis (*hmgcs*, *hmgcr*, *cyp51* and *dhcr7*) and gluconeogenic genes (*g6pca*, *g6pcb1*, *g6pcb2* and *pck2*). These results are in agreement with the increased mRNA levels of *lxr*, which is identified as a major regulator of cholesterol metabolism targeting the genes previously cited involved in cholesterol turnover (Lehmann et al., 1997; van der Veen et al., 2009; Venkateswaran et al., 2000; Verreault et al., 2006). Altogether, these data suggest that early dietary Met deficiency promotes hepatic cholesterol elimination and efflux in rainbow trout fry through the activation of the transcription factor LXR. Furthermore, increased *ugt1a3*, *abcg8* and *pck2* expression in trout fry from parents fed Met deficient diets supports the hypothesis of a developmental programming of glucose and lipid metabolism by means of broodstock nutrition, as previously suggested (Balasubramanian

et al., 2016). Similarly, in mammals, it has been shown that protein restriction could lead to dysregulated expression of hepatic gluconeogenic genes, such as *g6pc* and *pck* in the offspring, through modulation of LXR (Vo et al., 2013), highlighting the important role of this transcription factor in nutritional programming of hepatic gluconeogenesis.

In summary, the present results confirm the involvement of dietary Met restriction on modulating lipid, cholesterol and glucose metabolism in rainbow trout fry and provide novel insights into the hypothesis of the influence of parental nutrition on the metabolic outcome in the offspring. However, in order to demonstrate whether these effects can be attributed to epigenetic regulation, further studies such as DNA and/or histone methylation at the promotor of the concerned genes, must be performed.

5.2 GENETIC DETERMINANTS OF FAT DEPOSITION

5.2.1 Metabolic strategies of divergent rainbow trout lines for muscle fat

Genetic selection for economically relevant traits has been a common practice for many years in aquaculture industry. In salmonids, whole body and especially muscle adiposity are remarkable attributes, due to their importance in fillet yield and palatability. Thus, understanding the mechanisms of fat deposition and allocation in these farmed species are particularly interesting. As depicted in the introduction, one key model to achieve this purpose is that of the two divergent rainbow trout lines selected for muscle fat content. Previous studies have shown significant differences in the regulation of glucose and lipid metabolism between the two lines (Kolditz et al. 2008; Kamalam et al. 2012). In particular, increased lipogenic potential in the fat line (FL) was suggested as a key mechanism responsible for its high muscle adiposity (Jin et al. 2014). In fact, it was reported that the ability to synthesize lipids through *de novo* lipogenesis in the liver of FL fish was greater than that of the lean line (LL) (Kamalam et al., 2013). In addition, the FL fish presented a higher capability to metabolize glucose. In this regard, we have examined the likely differential metabolic regulation of these trout lines, with special focus on the transcriptional cross-talk between adipose tissue and white muscle in response to food deprivation (Article III).

These fish lines, despite being designated as fat and lean in terms of muscle adiposity, show differences in lipid content among fat depots, indicating that regulatory mechanisms are in play to keep similar levels of total lipid storage. In the present study, lower white muscle fat reserves of the LL fish appear to be compensated by greater visceral fat stores, in agreement with data

on earlier generations (Kolditz et al., 2008). In contrast, liver does not appear to be affected as we found that the liver somatic index (LSI) did not differ between the two genotypes, supporting the hypothesis that adipose tissue and muscle are the main depot-targets of this selection process. Moreover, the higher circulating TAG and non-esterified fatty acid levels found in FL fish suggest an increased dietary lipid uptake and supply to muscle according to its fattier selection compared with the LL fish.

As commented, previous studies have revealed differential transcriptomic and also proteomic regulation in liver and white muscle between genotypes; however, in spite of being the major site of energy storage and shown to be influenced by the breeding selection, no information is available on the transcriptomic regulation of adipose tissue in these trout. In this regard, gene expression results showed enhanced *fasn* mRNA levels in both adipose tissue and white muscle of the FL compared to the LL fish, altogether suggesting an increased lipogenic capacity in this line as previously reported in liver (Jin et al., 2014b; Skiba-Cassy et al., 2009), and highlighting the possible contribution of adipose tissue to the FA synthesis the novo. On the other hand, *lpl* mRNA levels were higher in white muscle of FL than LL fish, suggesting increased TAG incorporation in this tissue from the circulation. Overall, these results refer to earlier findings that showed up-regulated *fat/cd36* and *vldl* expression (Kolditz et al., 2010), in agreement with our hypothesis of increased fat uptake in the FL muscle.

Apart from these differences in metabolism under normal conditions, FL and LL fish have been reported to differ in muscle and hepatic intermediary metabolism upon changes in dietary factors such as carbohydrate or lipid inclusion. Compared to LL, the FL fish presented lower postprandial plasma glucose levels (Skiba-Cassy et al., 2009) and higher lipogenic potential with an increase in dietary carbohydrate intake (Kamalam et al., 2012), indicating a better ability of this line to metabolize and store glucose. Moreover, the FL fish also exhibited reduced hepatic FA oxidation and enhanced glycolysis in liver and muscle when fed with a high-fat diet (Kolditz et al., 2008b), overall indicating the suitability of these lines to study dietary manipulations. Hence, our hypothesis is that they might also follow different strategies to cope with food deprivation, especially concerning adipose tissue and white muscle responses.

Thus, we evaluated the effect of normal feeding conditions compared to 1, 2 or 4 weeks of fasting in the two lines. In contrast to mammals, fish can experience prolonged periods of fasting and metabolize large quantities of energy reserves without physiological damage (Navarro and Gutiérrez, 1995; Wieser et al., 1992). Both lines mobilized liver and visceral fat

reserves in a similar manner in response to fasting, while only the FL fish utilized muscle lipids to fulfill energy requirements, indicating a marked differentiation in energy deposition routes between the two lines. Besides, after one week of fasting, TAG plasma levels drastically decreased in the FL fish while a significant increase was found in the LL fish. These changes suggest that the FL fish might utilize the circulating TAG as a primary source of energy, whereas the LL fish first mobilize these products from fat reserves (in adipose tissue or liver), increasing their plasma levels for delivery to other tissues such as muscle. However, this tendency did not continue with prolonged fasting (2 or 4 weeks), where circulating TAG decreased in both fish lines, confirming that the different strategies in lipid mobilization are mainly observed with short-term fasting. Similarly, several studies have reported that even though fasting usually decreases TAG in plasma (Pérez-Jiménez et al., 2007), the mobilization of lipids during such periods might differ depending on the length of fasting, showing steady or even a significant increase of circulating TAG at the early stages (Echevarría et al., 1997).

Concomitant with the observed changes in plasma TAG levels, *fasn* transcripts abundance in adipose tissue decreased progressively with fasting in the FL, while LL fish showed a slightly increase after 1 week of fasting. On the other hand, in white muscle, *fasn* and *lpl* mRNA levels of the FL fish decreased right after 1 week, while no effect was observed in the LL fish, in agreement with the decreased lipid content of the former fish line in response to fasting. Peroxisome proliferator-activated receptor beta (*pparb*) mRNA levels in adipose tissue decreased at the beginning of fasting in both lines, whereas in white muscle, increased especially in the FL fish, indicating a differential regulation fatty acid oxidation between tissues. In addition, a differential regulation between tissues was also observed in hormone sensitive lipase (*lipe1*) mRNA levels of the LL fish, being this down-regulated with fasting in adipose tissue and progressively increased in white muscle. This up-regulation of white muscle *lipe1* expression might indicate increased lipolytic activity, even though changes in muscle lipid content were not observed. However, in adipose tissue the results might indicate the involvement of another lipolytic enzyme responsible for the fat mobilization in this tissue. Furthermore, the elevated expression of *me1*, which codes for an enzyme that generates NADPH for fatty acid biosynthesis, in the adipose tissue of LL trout after a 4-week fasting, could agree with a regulatory mechanism to maintain visceral fat levels, although this was not accompanied by increases in *fasn* expression. Again, we should bear in mind that these assumptions are based on transcriptional results, which despite giving an interesting overview

of the metabolic status of these lines in response to fasting, might not completely reflect the enzymatic regulation during this process.

Recent studies have demonstrated different leptin system profiles between the two genotypes (Gong et al. 2016; Johansson et al. 2016), however, in our study only minor differences were observed between lines in the expression of adiponectin (*adipoq*) and its receptors (*adipor1* and *adipor2*) suggesting that this system is not differentially regulated at transcriptional level in LL and FL fish, at least at the present conditions. During fasting, an inverse relation was found between *adipoq* and its receptors mRNA levels in white muscle, as previously reported in this species (Sánchez-Gurmaches et al., 2012). Nevertheless, even though in the later study *adipoq* mRNA levels increased along with fasting in adipose tissue, we only found an up-regulation in the FL fish after 4 weeks, suggesting that the genetic selection may have impaired the *adipoq* regulation in this tissue.

Long periods of fasting can increase oxidative stress in terms of reactive oxygen species (ROS) production (Zhang et al., 2007, Zhang et al., 2008). Our results showed a reverse expression pattern of several oxidative stress genes in response to fasting between adipose tissue and white muscle, supporting the tissue-specific regulation suggested in the case of lipid metabolism as well. In adipose tissue, mRNA levels of the majority of the genes studied, decreased throughout the fasting period while in the white muscle displayed a rapid predisposition to increase, indicating a higher protective effect in this latter tissue as a response mechanism to increased oxidative damage. This assumption relies on the importance of maintaining structural proteins through primarily avoiding negative ROS effects in muscle tissue (Barbieri and Sestili, 2012). Thus, the up-regulation in gamma-glutamyl-cysteine synthetase (*gcs*), hydroperoxide glutathione peroxidase (*phgpx*) and thioredoxin reductase (*tr*) expression in the white muscle of the FL fish after 1 week of fasting, might be driven to counteract excessive ROS damage in this tissue. These results are in agreement with the increased MDA production in the white muscle of this line, indicating an increased lipid peroxidation in comparison to the LL. On the other hand, the lower *gpx1* mRNA levels in non-fasted FL compared with the LL fish, suggest a minor production of ROS derived from fatty acid oxidation, as previously reported (Kolditz et al., 2008b). Moreover, in adipose tissue, the decreased mRNA expression of *sod* and *phgpx* in both lines might be explained by the reduction of fat mass in response to fasting. However, *gcs* and *tr* mRNA levels in this tissue, increased after 4 weeks of fasting in the FL compared to the LL fish, indicating that ROS production and/or its oxidative potential in the latter may have stronger effects after prolonged fasting.

The present findings indicate that rainbow trout selected for low (LL) or high (FL) muscle adiposity present genotype-specific metabolic regulation, highlighting their divergent coping mechanisms in response to fasting. In particular, the FL fish showed improved lipogenic capacity both in adipose tissue and white muscle, and mainly mobilized muscle reserves during fasting.

5.3 ENVIRONMENTAL OBESOGENS

5.3.1 Adipogenic role of organotins in rainbow trout

The idea that environmental chemicals could be contributing to obesity through direct actions in adipose tissue development has garnered increased attention in the recent years. These chemicals interfere with complex metabolic pathways impairing hormonal signaling and lipid homeostasis (Heindel et al., 2015; Holtcamp, 2012; Janesick and Blumberg, 2016). As previously mentioned, tributyltin (TBT) and triphenyltin (TPT), have been identified as obesogenic compounds with a proven toxicity risk to humans and non-human organisms. Given its important role in regulating adipogenesis and lipid homeostasis, several studies have been performed using mammalian adipose tissue derived cell lines (i.e. 3T3L-1) and primary cultures (reviewed in Janesick & Blumberg 2012). Indeed, those studies demonstrated that TBT and TPT function as pro-adipogenic compounds promoting differentiation of pre-adipocytes into mature adipocytes, increasing hyperplastic expansion of adipose tissue. However, it has been recently reported that the adipocytes generated by these compounds, could be non-functional and might display an altered phenotype (Regnier et al., 2015). Despite the reported evidences in mammals, no studies have previously investigated the effects of these obesogens in fish adipocyte models, and only little information is available about their ability to modulate whole-body adiposity in fish (Lyssimachou et al., 2015; Meador et al., 2011; Ouadah-Boussouf and Babin, 2016). In this framework, we have evaluated for the first time in an *in vitro* fish adipocyte model, the potential obesogenic effects of TBT and TPT on adipocyte development and lipid metabolism in rainbow trout (Article IV).

In the recent years, *in vitro* cell culture models from fish species have been commonly used to study lipid metabolism and adipogenesis (Bouraoui et al., 2008; Vegusdal et al., 2003; Salmerón et al., 2015). Like in mammals, the fundamental regulatory mechanism for adipose tissue formation in fish, is based on the alternative balance between cell proliferation and differentiation. Proliferation is generally enhanced at early adipogenic stages (pre-adipocyte

cells), while at the differentiation phase (see 1.2.3), it is consequently reduced. In this regard, previous studies revealed that TBT exposure decreases proliferation capacity of adipose-derived stromal stem cells in mice, suggesting a likely compensation of the increase in differentiation (Kirchner et al., 2010). In our study, TBT and TPT treatment caused slightly lower values on cell proliferation as their concentration increased, in agreement with the adipogenic role of these compounds. However, the observed dose-dependent tendency to decrease cell viability, together with nuclei morphology changes as well as the reduction in nuclear area factor, indicate that high doses (1 μM) of these compounds might also induce apoptosis as previously described in rodents (Lee et al., 2012). Therefore, in order to test the potential pro-adipogenic capacity of these compounds we used the highest concentration (100 nM) without any detrimental effect in the adipocytes for the following experiments.

As described in the introduction, adipogenesis is a complex multi-step process that regulates the differentiation of fibroblast-like pre-adipocytes into spherical adipocytes filled with lipid droplets. The induction of these changes is ultimately attributed to the sequential activation of several transcription factors, including C/EBPα and PPARγ (Lefterova and Lazar, 2009; Lowe et al., 2011; Rosen et al., 2002). Thus, stimulation of these transcription factors lead to the consequent differentiation of adipocyte cells. In this regard, many studies have demonstrated that TBT and TPT, function in a similar manner as other PPARγ/RxR agonists, such as RGZ or troglitazone, to promote adipocyte differentiation (Harada et al., 2015; Li et al., 2011; Pereira-Fernandes et al., 2013). In the present study, both TBT and particularly TPT, induced lipid droplet formation, in agreement with their proposed pro-adipogenic role in mammals. However, cells retained a fibroblast-like shape, in comparison to the typical round adipocyte phenotype induced by lipid mixture treatment (LIP). In addition, even though immunofluorescence and Western blot results revealed that TBT and TPT slightly induce C/EBPα and PPARγ protein expression, these effects were higher when using LIP. Overall, the lack of complete activation of these two transcription factors in the absence of LIP might partly explain the incapability of TBT and TPT to induce by themselves the typical mature adipocyte morphology, indicating that these compounds initiate adipogenesis but are not capable to promote complete adipocyte differentiation in our model.

Previous studies using fish adipocyte models have highlighted that addition of an exogenous source of lipids, is required to achieve full adipocyte differentiation (Bouraoui et al., 2012; Todorcević et al., 2010). In contrast to fish, mammalian adipocytes have the capacity to undergo differentiation and initiate the transcriptional cascade that determines the fate of adipocyte

precursor cells without outer addition of lipids, suggesting an endogenous production of TAG through activation of DNL. As outlined in section 1.2.4, the ability of fish to convert glucose into cellular lipids is relatively low compared to mammals (Bou et al., 2016), leading to the conclusion that these particular features of fish adipocytes, may also explain the difficulty observed under TBT and TPT treatments to induce complete adipocyte development. On the other hand, previous studies demonstrated that TBT has obesogenic properties in zebrafish larvae, increasing adiposity through enlarged adipocyte hypertrophy (Ouadah-Boussouf and Babin, 2016; Tingaud-Sequeira et al., 2011). Thus, taking into account our findings, this increased lipid accumulation in zebrafish adipocytes might also imply a hypothetic upregulation of lipogenesis in other tissues in addition to than adipose tissue, such as the liver. Nevertheless, further studies comparing *in vivo* and *in vitro* scenarios are needed in order to completely address this possibility.

Furthermore, most of the studies investigating the obesogenic properties of these organotins have been focused on their role in regulating fat accumulation and their ability to induce adipocyte differentiation through PPARγ signaling without paying attention to the ulterior metabolic and phenotypic consequences of this activation. Nevertheless, it has been reported that 3T3-L1 adipocytes generated by induction with different PPARγ agonists exhibit dissimilar phenotypes and might affect adipose tissue functionality, and global energy homeostasis (Regnier et al., 2015). This study revealed that while both troglitazone and TBT stimulated *pparg* mRNA levels and induced similar lipid accumulation, the adipogenic genes tested were responsive to troglitazone but not to TBT, indicating that adipocytes generated by different PPARγ activators are not equal and therefore, might display different adipogenic expression patterns.

Similarly, in the present study the most relevant effects were produced by LIP while only few alterations were caused by the organotins. LIP treatment significantly downregulated the majority of the genes tested including *fasn*, *gapdh* and *lxr*, which could be secondarily caused by a reduction in lipogenesis due to an incorporation of fatty acids from the culture medium. In contrast, TBT and TPT increased *fasn* mRNA levels in comparison to LIP, and slightly enhanced *abca1* mRNA levels, especially when combined with LIP, altogether suggesting an increased fatty acid synthesis and a positive regulation of cholesterol efflux. In line with these arguments, these organotins significantly upregulated *fasn* and *abca1* mRNA levels in RTL-W1 rainbow trout liver cells (Dimastrogiovanni et al., 2015), supporting the hypothesis that these compounds have pro-adipogenic properties in this species. Nevertheless, as mentioned

above, this possible induction of lipogenesis was not enough to induce a mature adipocyte phenotype in our model. Furthermore, whereas TBT and TPT did not exert any effect on *lepa1* mRNA levels, LIP significantly upregulated its expression as expected in an adipogenic context. In contrast, previous studies in mammals have shown that, obesogens, such as TBT, successfully increased leptin expression in human and mice adipose-derived stromal cells treated *in vitro* (Kirchner et al., 2010), indicating once again marked differences between the actions observed in rainbow trout cells and those observed in mammalian models. One explanation for this lack of effect is that leptin is expressed in late stages of adipogenesis and in our study, adipocytes treated with TBT and TPT seemed to be incapable to reach this point. In addition, when TBT and TPT were incubated together with LIP also increased *tnfa* mRNA levels, suggesting that in our model, TBT and TPT, not only induced intra-cellular lipid accumulation, but also perhaps increased adipocyte inflammation or other TNF-related adipocyte dysfunctions as previously suggested in mammals (Hotamisligil et al., 1993; Nieto-Vazquez et al., 2008).

In conclusion, the present results indicate that the environmental pollutants, TBT and TPT, have pro-adipogenic effects in primary cultured adipocytes from rainbow trout, but do not induce complete differentiation affecting both adipocyte morphology and most probably functionality. These results diverge from the current findings in most mammalian studies where both compounds promote fully differentiated adipocytes, and suggest species-specific differences in response to these organotins. Furthermore, the current study contributes to improve knowledge on the adipogenic role of these obesogens in rainbow trout and indicate that further studies are needed to fully elucidate the biological meaning of such particular regulation in rainbow trout adipocytes.

CHAPTER 5: CONCLUSIONS

1. The natural antioxidants caffeic acid (CA) and hydroxytyrosol (HT) display *in vivo* anti-obesogenic properties in zebrafish, decreasing larval adiposity in a specific fat-depot manner. In addition, both compounds successfully counteract the obesogenic effect of rosiglitazone (RGZ), indicating a potential antagonism of PPARγ signaling pathway. Contrarily, astaxanthin has no effect in modulating fat storage in zebrafish larvae.

2. CA and HT decrease lipid accumulation and PPARγ protein expression in rainbow trout primary cultured adipocytes, supporting our previous findings in zebrafish. Moreover, HT reduces plasma triglycerides and the mRNA levels of the *fasn* adipogenic gene in adipose tissue in rainbow trout *in vivo*. These results validate the use of these fish models for screening dietary compounds with potential ability to modulate adipose tissue dynamics to be applied in aquaculture and human nutrition.

3. Early dietary methionine restriction increases the expression of lipogenic and cholesterol metabolism-related genes in rainbow trout fry, suggesting an effect in hepatic intermediary metabolism and confirming previous information about methionine requirements in this species.

4. Parental methionine deficiency affects the expression of several key metabolic factors in the offspring, indicating that broodstock methionine supply has an impact on progeny performance, supporting the hypothesis of nutritional programming in fish. These results provide new insights into the influence of parental nutrition and may help to design novel dietary approaches for aquaculture industry.

5. Rainbow trout selected for low (LL) or high (FL) muscle adiposity present genotype-specific regulatory mechanisms in muscle and adipose tissue at a transcriptional level concerning lipid metabolism, suggesting increased lipogenic capacity and fatty acid uptake in the FL fish.

6. The genetic background influences the metabolic responses of the two rainbow trout lines, which follow different strategies to cope with food deprivation. Moreover, oxidative stress genes expression in both fish lines show a different pattern between tissues upon fasting, probably indicating a higher protective effect in muscle.

7. Tributyltin (TBT) and triphenyltin (TPT) initiate adipogenic differentiation of primary cultured adipocytes from rainbow trout, inducing lipid accumulation and slightly modulating activation of PPARγ and C/EBPα expression.

8. These organotins however, has been demonstrated for the first time in fish that do not induce complete adipocyte differentiation, since they impair normal adipocyte morphology and most probably functionality.

9. Overall, the present thesis demonstrates that nutritional, genetic and environmental factors influence adipose tissue development and lipid homeostasis in fish, and provide novel insights into the regulation of fat deposition in zebrafish and rainbow trout models, with potential applications in both, aquaculture and biomedical research.

REFERENCE LIST

Ahmadian, M., Duncan, R.E., Sul, H.S., 2009. The skinny on fat: lipolysis and fatty acid utilization in adipocytes. Trends Endocrinol. Metab. 20, 424–8.

Ahn, J., Cho, I., Kim, S., Kwon, D., Ha, T., 2008. Dietary resveratrol alters lipid metabolism-related gene expression of mice on an atherogenic diet. J. Hepatol. 49, 1019–1028.

Albalat, A., Liarte, C., MacKenzie, S., Tort, L., Planas, J. V, Navarro, I., 2005. Control of adipose tissue lipid metabolism by tumor necrosis factor-alpha in rainbow trout (*Oncorhynchus mykiss*). J. Endocrinol. 184, 527–34.

Albalat, A., Saera-Vila, A., Capilla, E., Gutiérrez, J., Pérez-Sánchez, J., Navarro, I., 2007. Insulin regulation of lipoprotein lipase (LPL) activity and expression in gilthead sea bream (*Sparus aurata*). Comp. Biochem. Physiol. Part B Biochem. Mol. Biol. 148, 151–159.

Alves Martins, D., Estévez, A., Stickland, N.C., Simbi, B.H., Yúfera, M., 2010. Dietary lecithin source affects growth potential and gene expression in sparus aurata larvae. Lipids 45, 1011–1023.

Alzieu, C.L., Sanjuan, J., Deltreil, J.P., Borel, M., 1986. Tin contamination in Arcachon Bay: Effects on oyster shell anomalies. Mar. Pollut. Bull. 17, 494–498.

Andersen, C., Rayalam, S., Della-Fera, M.A., Baile, C.A., 2010. Phytochemicals and adipogenesis. BioFactors 36, 415–422.

Antizar-Ladislao, B., 2008. Environmental levels, toxicity and human exposure to tributyltin (TBT) contaminated marine environment. A review. Environ. Int. 34, 292–308.

Arnault, F., Etienne, J., Noé, L., Raisonnier, A., Brault, D., Harney, J.W., Berry, M.J., Tse, C., Fromental-Ramain, C., Hamelin, J., Galibert, F., 1996. Human lipoprotein lipase last exon is not translated, in contrast to lower vertebrates. J. Mol. Evol. 43, 109–15.

Arunkumar, E., Bhuvaneswari, S., Anuradha, C.V., 2012. An intervention study in obese mice with astaxanthin, a marine carotenoid--effects on insulin signaling and pro-inflammatory cytokines. Food Funct. 3, 120–6.

Ashrafian, H., Harling, L., Darzi, A., Athanasiou, T., 2013. Neurodegenerative disease and obesity: what is the role of weight loss and bariatric interventions? Metab. Brain Dis. 28, 341–353.

Babin, P.J., Vernier, J.M., 1989. Plasma lipoproteins in fish. J. Lipid Res. 30, 467–89.

Balasubramanian, M.N., Panserat, S., Dupont-Nivet, M., Quillet, E., Montfort, J., Le Cam, A., Medale, F., Kaushik, S.J., Geurden, I., 2016. Molecular pathways associated with the nutritional programming of plant-based diet acceptance in rainbow trout following an early feeding exposure. BMC Genomics 17, 449.

Barbieri, E., Sestili, P., 2012. Reactive oxygen species in skeletal muscle signaling. J. Signal Transduct. 2012, 982794.

Bell, J.G., McEvoy, J., Tocher, D.R., Sargent, J.R., 2000. Depletion of alpha-tocopherol and astaxanthin in Atlantic salmon (*Salmo salar*) affects autoxidative defense and fatty acid metabolism. J. Nutr. 130, 1800–8.

Bergan, H.E., Kittilson, J.D., Sheridan, M.A., 2012. Nutrition-regulated lipolysis in rainbow trout (*Oncorhynchus mykiss*) is associated with alterations in the ERK, PI3K-Akt, JAK-STAT, and PKC signaling pathways. Gen. Comp. Endocrinol. 176, 367–376.

Berry, D.C., Stenesen, D., Zeve, D., Graff, J.M., 2013. The developmental origins of adipose tissue. Development 140, 3939–49.

Birchenough, A.C., Barnes, N., Evans, S.M., Hinz, H., Krönke, I., Moss, C., 2002. A review and assessment of tributyltin contamination in the North Sea, based on surveys of butyltin tissue burdens and imposex/intersex in four species of neogastropods. Mar. Pollut. Bull. 44, 534–43.

Bjørndal, B., Burri, L., Staalesen, V., Skorve, J., Berge, R.K., Berge, R.K., 2011. Different adipose depots: their role in the development of metabolic syndrome and mitochondrial response to hypolipidemic agents. J. Obes. 2011, 490650.

Blasco, J., Moya, A., Millán-Cubillo, A., Vélez, E.J., Capilla, E., Pérez-Sánchez, J., Gutiérrez, J., Fernández- Borrás, J., 2015. Growth-promoting effects of sustained swimming in fingerlings of gilthead sea bream (*Sparus aurata L.*). J. Comp. Physiol. B 185, 859–868.

Blüher, M., 2013. Adipose tissue dysfunction contributes to obesity related metabolic diseases. Best Pract. Res. Clin. Endocrinol. Metab. 27, 163–77.

Boonyaratpalin, M., 2015. Overview on the use of plant protein in aquaculture feed 105–107.

Borges, P., Valente, L.M.P., Véron, V., Dias, K., Panserat, S., Médale, F., 2014. High dietary lipid level is associated with persistent hyperglycaemia and downregulation of muscle Akt-mTOR pathway in Senegalese sole (*Solea senegalensis*). PLoS One 9, e102196.

Borlongan, I.G., 1991. Arginine and threonine requirements of milkfish (*Chanos chanos Forsskal*) juveniles. Aquaculture 93, 313–322.

Bou, M., Montfort, J., Le Cam, A., Rallière, C., Lebret, V., Gabillard, J.-C., Weil, C., Gutiérrez, J., Rescan, P.-Y., Capilla, E., Navarro, I., 2017. Gene expression profile during proliferation and differentiation of rainbow trout adipocyte precursor cells. BMC Genomics 18, 347.

Bou, M., Todorčević, M., Rodríguez, J., Capilla, E., Gutiérrez, J., Navarro, I., 2014. Interplay of adiponectin, TNFα and insulin on gene expression, glucose uptake and PPARγ, AKT and TOR pathways in rainbow trout cultured adipocytes. Gen. Comp. Endocrinol. 205, 218–225.

Bou, M., Todorčević, M., Torgersen, J., Škugor, S., Navarro, I., Ruyter, B., 2016. De novo lipogenesis in Atlantic salmon adipocytes. Biochim. Biophys. Acta - Gen. Subj. 1860, 86–96.

Bouraoui, L., Cruz-Garcia, L., Gutiérrez, J., Capilla, E., Navarro, I., 2012. Regulation of lipoprotein lipase gene expression by insulin and troglitazone in rainbow trout (*Oncorhynchus mykiss*) adipocyte cells in culture. Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 161, 83–8.

Bouraoui, L., Gutiérrez, J., Navarro, I., 2008. Regulation of proliferation and differentiation of adipocyte precursor cells in rainbow trout (*Oncorhynchus mykiss*). J. Endocrinol. 198, 459–69.

Boyer, I.J., 1989. Toxicity of dibutyltin, tributyltin and other organotin compounds to humans and to experimental animals. Toxicology 55, 253–98.

Bray, G.A., Popkin, B.M., 1998. Dietary fat intake does affect obesity! Am. J. Clin. Nutr. 68, 1157–73.

Burdge, G.C., Lillycrop, K.A., 2010. Nutrition, Epigenetics, and Developmental Plasticity: Implications for Understanding Human Disease. Annu. Rev. Nutr. 30, 315–339.

Byström, P., Andersson, J., Kiessling, A., Eriksson, L.-O., 2006. Size and temperature dependent foraging capacities and metabolism: consequences for winter starvation mortality in fish. Oikos 115, 43–52.

Cao, K., Xu, J., Zou, X., Li, Y., Chen, C., Zheng, A., Li, H., Li, H., Szeto, I.M.-Y., Shi, Y., Long, J., Liu, J., Feng, Z., 2014. Hydroxytyrosol prevents diet-induced metabolic syndrome and attenuates mitochondrial abnormalities in obese mice. Free Radic. Biol. Med. 67, 396–407.

Carter, L.G., Ngo Tenlep, S.Y., Woollett, L.A., Pearson, K.J., 2015. Exercise Improves Glucose Disposal and Insulin Signaling in Pregnant Mice Fed a High Fat Diet. J. Diabetes Metab. 6.

Chakraborty, C., Hsu, C., Wen, Z., Lin, C., Agoramoorthy, G., 2009. Zebrafish: A Complete Animal Model for In Vivo Drug Discovery and Development. Curr. Drug Metab. 10, 116–124.

Chamorro-García, R., Sahu, M., Abbey, R.J., Laude, J., Pham, N., Blumberg, B., 2013. Transgenerational inheritance of increased fat depot size, stem cell reprogramming, and hepatic steatosis elicited by prenatal exposure to the obesogen tributyltin in mice. Environ. Health Perspect. 121, 359–66.

Chao, C., Mong, M., Chan, K., Yin, M., 2010. Anti-glycative and anti-inflammatory effects of caffeic acid and ellagic acid in kidney of diabetic mice. Mol. Nutr. Food Res. 54, 388–395.

Chen, D., Wang, W., Ru, S., 2014. Effect of dietary genistein on growth performance, digestive enzyme activity, and body composition of Nile tilapia *Oreochromis niloticus*. Chinese J. Oceanol. Limnol. 33, 77–83.

Chen, S.S., Chen, J.F., Johnson, P.F., Muppala, V., Lee, Y.H., 2000. C/EBPbeta, when expressed from the C/ebpalpha gene locus, can functionally replace C/EBPalpha in liver but not in adipose tissue. Mol. Cell. Biol. 20, 7292–9.

Choi, H.-S., Jeon, H.-J., Lee, O.-H., Lee, B.-Y., 2015. Dieckol, a major phlorotannin in *Ecklonia cava*, suppresses lipid accumulation in the adipocytes of high-fat diet-fed zebrafish and mice: Inhibition of early adipogenesis via cell-cycle arrest and AMPKα activation. Mol. Nutr. Food Res. 59, 1458–71.

Choo, P.S., Smith, T.K., Cho, C.Y., Ferguson, H.W., 1991. Dietary excesses of leucine influence growth and body composition of rainbow trout. J. Nutr. 121, 1932–9.

Chu, C.-Y., Chen, C.-F., Rajendran, R.S., Shen, C.-N., Chen, T.-H., Yen, C.-C., Chuang, C.-K., Lin, D.-S., Hsiao, C.-D., 2012. Overexpression of Akt1 Enhances Adipogenesis and Leads to Lipoma Formation in Zebrafish. PLoS One 7, e36474.

Cleveland, B.M., 2014. In vitro and in vivo effects of phytoestrogens on protein turnover in rainbow trout (*Oncorhynchus mykiss*) white muscle. Comp. Biochem. Physiol. C. Toxicol. Pharmacol. 165, 9–16.

Colberg, S.R., Sigal, R.J., Fernhall, B., Regensteiner, J.G., Blissmer, B.J., Rubin, R.R., Chasan-Taber, L., Albright, A.L., Braun, B., American College of Sports Medicine, American Diabetes Association, 2010. Exercise and type 2 diabetes: the American College of Sports Medicine and the American Diabetes Association: joint position statement. Diabetes Care 33, e147-67.

Collins, J.M., Neville, M.J., Pinnick, K.E., Hodson, L., Ruyter, B., van Dijk, T.H., Reijngoud, D.J., Fielding, M.D., Frayn, K.N., 2011. De novo lipogenesis in the differentiating human adipocyte can provide all fatty acids necessary for maturation. J. Lipid Res. 52, 1683–92.

Committee on the Nutrient Requirements of Fish and Shrimp, 2011. Nutrient Requirements of Fish and Shrimp, National Academies Press. National Academies Press, Washington, D.C.

Costa, M.M., Maehr, T., Diaz-Rosales, P., Secombes, C.J., Wang, T., 2011. Bioactivity studies of rainbow trout (*Oncorhynchus mykiss*) interleukin-6: Effects on macrophage growth and antimicrobial peptide gene expression. Mol. Immunol. 48, 1903–1916.

Cowey, C.B., Cho, C.Y., 1993. Nutritional requirements of fish. Proc. Nutr. Soc. 52, 417–26.

Craig, P.M., Moon, T.W., 2017. Methionine restriction affects the phenotypic and transcriptional response of rainbow trout (*Oncorhynchus mykiss*) to carbohydrate-enriched diets.

Cristancho, A.G., Lazar, M.A., 2011. Forming functional fat: a growing understanding of adipocyte differentiation. Nat. Rev. Mol. Cell Biol. 12, 722–734.

Cruz-Garcia, L., Sánchez-Gurmaches, J., Gutiérrez, J., Navarro, I., 2012. Role of LXR in trout adipocytes: Target genes, hormonal regulation, adipocyte differentiation and relation to lipolysis. Comp. Biochem. Physiol. - A Mol. Integr. Physiol. 163, 120–126.

Cruz-Garcia, L., Saera-Vila, A., Navarro, I., Calduch-Giner, J., Pérez-Sánchez, J., 2009. Targets for TNFα-induced lipolysis in gilthead sea bream (*Sparus aurata L.*) adipocytes isolated from lean and fat juvenile fish. J. Exp. Biol. 212.

Cruz-Garcia, L., Sánchez-Gurmaches, J., Bouraoui, L., Saera-Vila, A., Pérez-Sánchez, J., Gutiérrez, J., Navarro, I., 2011. Changes in adipocyte cell size, gene expression of lipid metabolism markers, and lipolytic responses induced by dietary fish oil replacement in gilthead sea bream (*Sparus aurata L.*). Comp. Biochem. Physiol. Part A Mol. Integr. Physiol. 158, 391–399.

Cruz-Garcia, L., Sánchez-Gurmaches, J., Monroy, M., Gutiérrez, J., Navarro, I., 2015. Regulation of lipid metabolism and peroxisome proliferator-activated receptors in rainbow trout adipose tissue by lipolytic and antilipolytic endocrine factors. Domest. Anim. Endocrinol. 51, 86–95.

D'Angelo, L., Lossi, L., Merighi, A., de Girolamo, P., 2016. Anatomical features for the adequate choice of experimental animal models in biomedicine: I. Fishes. Ann. Anat. - Anat. Anzeiger 205, 75–84.

Davidson, J.W., Kenney, P.B., Manor, M., Good, C.M., Weber, G.M., Aussanasuwannakul, A., Kenney, P.B., Good, C.M., Summerfelt, S.T., 2014. Growth Performance, Fillet Quality, and Reproductive Maturity of Rainbow Trout (*Oncorhynchus mykiss*) Cultured to 5 Kilograms within Freshwater Recirculating Systems, Journal of Aquaculture Research and Development. OMICS Publishing Group.

De Pergola, G., Silvestris, F., 2013. Obesity as a major risk factor for cancer. J. Obes. 2013, 291546.

Den Broeder, M.J., Kopylova, V.A., Kamminga, L.M., Legler, J., 2015. Zebrafish as a Model to Study the Role of Peroxisome Proliferating-Activated Receptors in Adipogenesis and Obesity. PPAR Res. 2015, 358029.

Deng, Y., Scherer, P.E., 2010. Adipokines as novel biomarkers and regulators of the metabolic syndrome. Ann. N. Y. Acad. Sci. 1212, E1–E19.

Dessen, J.-E., Arge, R., Thomassen, M.S., Rørvik, K.-A., 2016. Differences in fat accumulation between immature male and female Atlantic salmon *Salmo salar* after dietary administration of tetradecylthioacetic acid. J. Fish Biol. 89, 2085–2097.

Dimastrogiovanni, G., Córdoba, M., Navarro, I., Jáuregui, O., Porte, C., 2015. Alteration of cellular lipids and lipid metabolism markers in RTL-W1 cells exposed to model endocrine disrupters. Aquat. Toxicol. 165, 277–85.

Dose, J., Matsugo, S., Yokokawa, H., Koshida, Y., Okazaki, S., Seidel, U., Eggersdorfer, M., Rimbach, G., Esatbeyoglu, T., 2016. Free radical scavenging and cellular antioxidant properties of astaxanthin. Int. J. Mol. Sci. 17.

Drira, R., Chen, S., Sakamoto, K., 2011. Oleuropein and hydroxytyrosol inhibit adipocyte differentiation in 3 T3-L1 cells. Life Sci. 89, 708–16.

Drira, R., Sakamoto, K., 2014. Hydroxytyrosol stimulates lipolysis via A-kinase and extracellular signal-regulated kinase activation in 3T3-L1 adipocytes. Eur. J. Nutr. 53, 743–750.

Echevarría, G., Martínez-Bebiá, M., Zamora, S., 1997. Evolution of Biometric Indices and Plasma Metabolites During Prolonged Starvation in European Sea Bass (*Dicentrarchus labrax, L.*). Comp. Biochem. Physiol. Part A Physiol. 118, 111–123.

Ellis, J.M., Li, L.O., Wu, P.C., Koves, T.R., Ilkayeva, O., Stevens, R.D., Watkins, S.M., Muoio, D.M., Coleman, R.A., 2010. Adipose Acyl-CoA synthetase-1 directs fatty acids toward b-oxidation and is required for cold thermogenesis. Cell Metab. 12, 53–64.

Espe, M., El-mowafi, A., Ruohonen, K., 2012. Replacement of fishmeal with plant protein ingredients in diets to Atlantic salmon (*Salmo salar*) – Effects on weight gain and accretion. Aquaculture 43–58.

Espe, M., Rathore, R.M., Du, Z.-Y., Liaset, B., El-Mowafi, A., 2010. Methionine limitation results in increased hepatic FAS activity, higher liver 18:1 to 18:0 fatty acid ratio and hepatic TAG accumulation in Atlantic salmon, *Salmo salar*. Amino Acids 39, 449–460.

Faith, M.S., Kral, T.V.E., 2006. Social Environmental and Genetic Influences on Obesity and Obesity-Promoting Behaviors: Fostering Research Integration.

FAO, 2016. The State of World Fisheries and Aquaculture 2016. Contributing to food security and nutrition for all. 200.

Fava, F., Gitau, R., Griffin, B.A., Gibson, G.R., Tuohy, K.M., Lovegrove, J.A., 2013. The type and quantity of dietary fat and carbohydrate alter faecal microbiome and short-chain fatty acid excretion in a metabolic syndrome "at-risk" population. Int. J. Obes. 37, 216–223.

Feil, R., 2006. Environmental and nutritional effects on the epigenetic regulation of genes. Mutat. Res. Mol. Mech. Mutagen. 600, 46–57.

Fent, K., 1996. Ecotoxicology of organotin compounds. Crit. Rev. Toxicol. 26, 1–117.

Fernandez-Bolaños, J.G., Lopez, O., Lopez-Garcia, M.A., Marset, A., 2012. Biological properties of hydroxytyrosol and its derivatives. Sect. Title Biomol. Their Synth. Analog. 375–396.

Figueiredo-Silva, A.C., Panserat, S., Kaushik, S., Geurden, I., Polakof, S., 2012. High levels of dietary fat impair glucose homeostasis in rainbow trout. J. Exp. Biol. 215, 169–78.

Fisher, R.M., Thörne, A., Hamsten, A., Arner, P., 2002. Fatty acid binding protein expression in different human adipose tissue depots in relation to rates of lipolysis and insulin concentration in obese individuals. Mol. Cell. Biochem. 239, 95–100.

Fontagné-Dicharry, S., Alami-Durante, H., Aragão, C., Kaushik, S.J., Geurden, I., 2017. Parental and early-feeding effects of dietary methionine in rainbow trout (*Oncorhynchus mykiss*). Aquaculture 469, 16–27.

Foti, P., Erba, D., Riso, P., Spadafranca, A., Criscuoli, F., Testolin, G., 2005. Comparison between daidzein and genistein antioxidant activity in primary and cancer lymphocytes. Arch. Biochem. Biophys. 433, 421–7.

Frayn, K., 2002. Adipose tissue as a buffer for daily lipid flux. Diabetologia 45, 1201–1210.

Frøyland, L., Madsen, L., Eckhoff, K.M., Lie, O., Berge, R.K., 1998. Carnitine palmitoyltransferase I, carnitine palmitoyltransferase II, and acyl-CoA oxidase activities in Atlantic salmon (*Salmo salar*). Lipids 33, 923–30.

Fuentes, E.N., Kling, P., Einarsdottir, I.E., Alvarez, M., Valdés, J.A., Molina, A., Björnsson, B.T., 2012. Plasma leptin and growth hormone levels in the fine flounder (*Paralichthys adspersus*) increase gradually during fasting and decline rapidly after refeeding. Gen. Comp. Endocrinol. 177, 120–127.

Fujiki, K., Shin, D.-H., Nakao, M., Yano, T., 2000. Molecular cloning and expression analysis of the putative carp (*Cyprinus carpio*) pre-B cell enhancing factor. Fish Shellfish Immunol. 10, 383–385.

Garg, A., 2006. Adipose tissue dysfunction in obesity and lipodystrophy. Clin. Cornerstone 8 Suppl 4, S7–S13.

Gesta, S., Blüher, M., Yamamoto, Y., Norris, A.W., Berndt, J., Kralisch, S., Boucher, J., Lewis, C., Kahn, C.R., 2006. Evidence for a role of developmental genes in the origin of obesity and body fat distribution. Proc. Natl. Acad. Sci. U. S. A. 103, 6676–81.

Geurden, I., Aramendi, M., Zambonino-Infante, J., Panserat, S., 2007. Early feeding of carnivorous rainbow trout (*Oncorhynchus mykiss*) with a hyperglucidic diet during a short period: effect on dietary glucose utilization in juveniles. Am. J. Physiol. - Regul. Integr. Comp. Physiol. 292.

Geurden, I., Mennigen, J., Plagnes-Juan, E., Veron, V., Cerezo, T., Mazurais, D., Zambonino-Infante, J., Gatesoupe, J., Skiba-Cassy, S., Panserat, S., 2014. High or low dietary carbohydrate:protein ratios during first-feeding affect glucose metabolism and intestinal microbiota in juvenile rainbow trout. J. Exp. Biol. 217.

Giralt, M., Villarroya, F., 2013. White, Brown, Beige/Brite: Different Adipose Cells for Different Functions? Endocrinology 154, 2992–3000.

Golay, A., Bobbioni, E., 1997. The role of dietary fat in obesity. Int. J. Obes. Relat. Metab. Disord. 21 Suppl 3, S2-11.

Gong, N., Johansson, M., Björnsson, B.T., 2016a. Impaired central leptin signaling and sensitivity in rainbow trout with high muscle adiposity. Gen. Comp. Endocrinol. 235, 48–56.

Gong, N., Jönsson, E., Björnsson, B.T., 2016b. Acute anorexigenic action of leptin in rainbow trout is mediated by the hypothalamic Pi3k pathway. J. Mol. Endocrinol. 56, 227–238.

González-Castejón, M., Rodriguez-Casado, A., 2011. Dietary phytochemicals and their potential effects on obesity: A review. Pharmacol. Res. 64, 438–455.

González-Molina, E., Domínguez-Perles, R., Moreno, D.A., García-Viguera, C., 2010. Natural bioactive compounds of *Citrus limon* for food and health. J. Pharm. Biomed. Anal. 51, 327–345.

Gorissen, M., Bernier, N.J., Nabuurs, S.B., Flik, G., Huising, M.O., 2009. Two divergent leptin paralogues in zebrafish (*Danio rerio*) that originate early in teleostean evolution. J. Endocrinol. 201, 329–39.

Gregoire, F.M., 2001. Adipocyte differentiation: from fibroblast to endocrine cell. Exp. Biol. Med. (Maywood). 226, 997–1002.

Grigorakis, K., 2007. Compositional and organoleptic quality of farmed and wild gilthead sea bream (*Sparus aurata*) and sea bass (*Dicentrarchus labrax*) and factors affecting it: A review. Aquaculture 272, 55–75.

Grimm, E.R., Steinle, N.I., 2011. Genetics of eating behavior: established and emerging concepts. Nutr. Rev. 69, 52–60.

Grisdale-Helland, B., Helland, S.J., 1997. Replacement of protein by fat and carbohydrate in diets for atlantic salmon (*Salmo salar*) at the end of the freshwater stage. Aquaculture 152, 167–180.

Grün, F., Blumberg, B., 2009. Minireview: the case for obesogens. Mol. Endocrinol. 23, 1127–34.

Grygiel-Górniak, B., 2014. Peroxisome proliferator-activated receptors and their ligands: nutritional and clinical implications--a review. Nutr. J. 13, 17.

Gülçin, I., 2006. Antioxidant activity of caffeic acid (3,4-dihydroxycinnamic acid). Toxicology 217, 213–20.

Hagedoorn, A.L., 1950. Animal breeding., Animal breeding, Crosby Lockwood & Son LTD., 39, Thurloe street, S.W.7., London. London: Crosby Lockwood & Son Ltd.

Hao, C., Cheng, X., Guo, J., Xia, H., Ma, X., 2013. Perinatal exposure to diethyl-hexyl-phthalate induces obesity in mice. Front. Biosci. (Elite Ed). 5, 725–33.

Hao, C., Cheng, X., Xia, H., Ma, X., 2012. The endocrine disruptor mono-(2-ethylhexyl) phthalate promotes adipocyte differentiation and induces obesity in mice. Biosci. Rep. 32, 619–29.

Harada, S., Hiromori, Y., Nakamura, S., Kawahara, K., Fukakusa, S., Maruno, T., Noda, M., Uchiyama, S., Fukui, K., Nishikawa, J., Nagase, H., Kobayashi, Y., Yoshida, T., Ohkubo, T., Nakanishi, T., 2015. Structural basis for PPARγ transactivation by endocrine-disrupting organotin compounds. Sci. Rep. 5, 8520.

Harwood, H.J., 2012. The adipocyte as an endocrine organ in the regulation of metabolic homeostasis. Neuropharmacology 63, 57–75.

Hasek, B.E., Boudreau, A., Shin, J., Feng, D., Hulver, M., Van, N.T., Laque, A., Stewart, L.K., Stone, K.P., Wanders, D., Ghosh, S., Pessin, J.E., Gettys, T.W., 2013. Remodeling the integration of lipid metabolism between liver and adipose tissue by dietary methionine restriction in rats. Diabetes 62, 3362–72.

Hasselbalch, A.L., 2010. Genetics of dietary habits and obesity - a twin study. Dan. Med. Bull. 57, B4182.

Hasumura, T., Shimada, Y., Kuroyanagi, J., Nishimura, Y., Meguro, S., Takema, Y., Tanaka, T., 2012. Green tea extract suppresses adiposity and affects the expression of lipid metabolism genes in diet-induced obese zebrafish. Nutr. Metab. (Lond). 9, 73.

He, M.-F., Liu, L., Ge, W., Shaw, P.-C., Jiang, R., Wu, L.-W., But, P.P.-H., 2009. Antiangiogenic activity of *Tripterygium wilfordii* and its terpenoids. J. Ethnopharmacol. 121, 61–68.

Hegele, R.A., Leff, T., 2004. Unbuckling lipodystrophy from insulin resistance and hypertension. J. Clin. Invest. 114, 163–5.

Heindel, J.J., Newbold, R., Schug, T.T., 2015. Endocrine disruptors and obesity. Nat. Rev. Endocrinol. 11, 653–61.

Holtcamp, W., 2012. Obesogens: an environmental link to obesity. Environ. Health Perspect. 120, a62-8.

Hotamisligil, G.S., Shargill, N.S., Spiegelman, B.M., 1993. Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. Science 259, 87–91.

Hu, E., Liang, P., Spiegelman, B.M., 1996. AdipoQ is a novel adipose-specific gene dysregulated in obesity. J. Biol. Chem. 271, 10697–703.

Huang, C.-C., Tung, Y.-T., Huang, W.-C., Chen, Y.-M., Hsu, Y.-J., Hsu, M.-C., 2016. Beneficial effects of cocoa, coffee, green tea, and garcinia complex supplement on diet induced obesity in rats. BMC Complement. Altern. Med. 16, 100.

Huang, T.S., Todorčević, M., Ruyter, B., Torstensen, B.E., 2010. Altered expression of CCAAT/enhancer binding protein and FABP11 genes during adipogenesis in vitro in Atlantic salmon (*Salmo salar*). Aquac. Nutr. 16, 72–80.

Huising, M.O., Geven, E.J.W., Kruiswijk, C.P., Nabuurs, S.B., Stolte, E.H., Spanings, F.A.T., Verburg-van Kemenade, B.M.L., Flik, G., 2006. Increased Leptin Expression in Common Carp (*Cyprinus carpio*) after Food Intake But Not after Fasting or Feeding to Satiation. Endocrinology 147, 5786–5797.

Hussein, Z., Wentworth, J.M., Nankervis, A.J., Proietto, J., Colman, P.G., 2004. Effectiveness and side effects of thiazolidinediones for type 2 diabetes: real-life experience from a tertiary hospital. Med. J. Aust. 181, 536–9.

Ibrahim, M.M., 2010. Subcutaneous and visceral adipose tissue: structural and functional differences. Obes. Rev. 11, 11–18.

Ichimura, K., Kawashima, Y., Nakamura, T., Powell, R., Hidoh, Y., Terai, S., Sakaida, I., Kodera, Y., Tsuji, T., Ma, J.-X., Sakai, T., Matsumoto, H., Obara, T., 2013. Medaka fish, *Oryzias latipes*, as a model for human obesity-related glomerulopathy. Biochem. Biophys. Res. Commun. 431, 712–7.

Ikeuchi, M., Koyama, T., Takahashi, J., Yazawa, K., 2007. Effects of astaxanthin in obese mice fed a high-fat diet. Biosci. Biotechnol. Biochem. 71, 893–9.

Imrie, D., Sadler, K.C., 2010. White adipose tissue development in zebrafish is regulated by both developmental time and fish size. Dev. Dyn. 239, 3013–23.

Inoue, M., Tanabe, H., Matsumoto, A., Takagi, M., Umegaki, K., Amagaya, S., Takahashi, J., 2012. Astaxanthin functions differently as a selective peroxisome proliferator-activated receptor γ modulator in adipocytes and macrophages. Biochem. Pharmacol. 84, 692–700.

Izquierdo, M.S., Turkmen, S., Montero, D., Zamorano, M.J., Afonso, J.M., Karalazos, V., Fernández-Palacios, H., 2015. Nutritional programming through broodstock diets to improve utilization of very low fishmeal and fish oil diets in gilthead sea bream. Aquaculture 449, 18–26.

Janesick, A., Blumberg, B., 2012. Obesogens, stem cells and the developmental programming of obesity. Int. J. Androl. 35, 437–48.

Janesick, A.S., Blumberg, B., 2016. Obesogens: an emerging threat to public health. Am. J. Obstet. Gynecol. 214, 559–565.

Janesick, A.S., Shioda, T., Blumberg, B., 2014. Transgenerational inheritance of prenatal obesogen exposure. Mol. Cell. Endocrinol. 398, 31–5.

Jenkins, C.M., Mancuso, D.J., Yan, W., Sims, H.F., Gibson, B., Gross, R.W., 2004. Identification, Cloning, Expression, and Purification of Three Novel Human Calcium-independent Phospholipase A2 Family Members Possessing Triacylglycerol Lipase and Acylglycerol Transacylase Activities. J. Biol. Chem. 279, 48968–48975.

Jin, J., Médale, F., Kamalam, B.S., Aguirre, P., Véron, V., Panserat, S., 2014a. Comparison of Glucose and Lipid Metabolic Gene Expressions between Fat and Lean Lines of Rainbow Trout after a Glucose Load. PLoS One 9, e105548.

Jin, J., Panserat, S., Kamalam, B.S., Aguirre, P., Véron, V., Médale, F., 2014b. Insulin regulates lipid and glucose metabolism similarly in two lines of rainbow trout divergently selected for muscle fat content. Gen. Comp. Endocrinol. 204, 49–59.

Johansson, M., Morgenroth, D., Einarsdottir, I.E., Gong, N., Björnsson, B.T., 2016. Energy stores, lipid mobilization and leptin endocrinology of rainbow trout. J. Comp. Physiol. B 186, 759–773.

Johnson, R.M., Johnson, T.M., Londraville, R.L., 2000. Evidence for leptin expression in fishes. J. Exp. Zool. 286, 718–24.

Jørgensen, J.B., Lunde, H., Jensen, L., Whitehead, A.S., Robertsen, B., 2000. Serum amyloid A transcription in Atlantic salmon (*Salmo salar L.*) hepatocytes is enhanced by stimulation with macrophage factors, recombinant human IL-1β, IL-6 and TNFα or bacterial lipopolysaccharide. Dev. Comp. Immunol. 24, 553–563.

Kadegowda, A.K.G., Bionaz, M., Piperova, L.S., Erdman, R.A., Loor, J.J., 2009. Peroxisome proliferator-activated receptor-γ activation and long-chain fatty acids alter lipogenic gene networks in bovine mammary epithelial cells to various extents. J. Dairy Sci. 92, 4276–4289.

Kadowaki, T., Hara, K., Kubota, N., Tobe, K., Terauchi, Y., Yamauchi, T., Eto, K., Kadowaki, H., Noda, M., Hagura, R., Akanuma, Y., 2002. The role of PPARγ in high-fat diet-induced obesity and insulin resistance, in: Journal of Diabetes and Its Complications. Elsevier, pp. 41–45.

Kamalam, B.S., MÃ©dale, F., Larroquet, L., Corraze, G., Panserat, S., 2013. Metabolism and Fatty Acid Profile in Fat and Lean Rainbow Trout Lines Fed with Vegetable Oil: Effect of Carbohydrates. PLoS One 8, e76570.

Kamalam, B.S., Medale, F., Kaushik, S., Polakof, S., Skiba-Cassy, S., Panserat, S., 2012. Regulation of metabolism by dietary carbohydrates in two lines of rainbow trout divergently selected for muscle fat content. J. Exp. Biol. 215, 2567–2578.

Kaneko, G., Shirakami, H., Hirano, Y., Oba, M., Yoshinaga, H., Khieokhajonkhet, A., Nagasaka, R., Kondo, H., Hirono, I., Ushio, H., 2016. Diversity of Lipid Distribution in Fish Skeletal Muscle. Zoolog. Sci. 33, 170–178.

Kaur, G., Dufour, J.M., 2012. Cell lines: Valuable tools or useless artifacts. Spermatogenesis 2, 1–5.

Kawakami, K., Asakawa, K., Hibi, M., Itoh, M., Muto, A., Wada, H., 2016. Gal4 Driver Transgenic Zebrafish, in: Advances in Genetics. pp. 65–87.

Ketola, H.G., 1982. Amino acid nutrition of fishes: Requirements and supplementation of diets. Comp. Biochem. Physiol. Part B Comp. Biochem. 73, 17–24.

Khan, M., Joseph, F., 2014. Adipose tissue and adipokines: the association with and application of adipokines in obesity. Scientifica (Cairo). 2014, 328592.

Khieokhajonkhet, A., Kaneko, G., Hirano, Y., Wang, L., Ushio, H., 2016. Different effects of growth hormone and fasting on the induction patterns of two hormone-sensitive lipase genes in red seabream Pagrus major. Gen. Comp. Endocrinol. 236, 121–130.

Kiessling, A., Kiessling, K.-H., Storebakken, T., Åsgård, T., 1991. Changes in the structure and function of the epaxial muscle of rainbow trout (*Oncorhynchus mykiss*) in relation to ration and age. Aquaculture 93, 357–372.

Kiessling, A., Ruohonen, K., Bjørnevik, M., 2006. Muscle fibre growth and quality in fish. Arch. Tierz. Dummerstorf 49, 137–146.

Kirchner, S., Kieu, T., Chow, C., Casey, S., Blumberg, B., 2010. Prenatal Exposure to the Environmental Obesogen Tributyltin Predisposes Multipotent Stem Cells to Become Adipocytes. Mol. Endocrinol. 24, 526–539.

Kirkland, J.L., Hollenberg, C.H., Gillon, W.S., 1996. Effects of fat depot site on differentiation-dependent gene expression in rat preadipocytes. Int. J. Obes. Relat. Metab. Disord. 20 Suppl 3, S102-7.

Kolditz, C.-I., Plagnes-Juan, E., Quillet, E., Lefèvre, F., Médale, F., 2010. Changes in white muscle transcriptome induced by dietary energy levels in two lines of rainbow trout (*Oncorhynchus mykiss*) selected for muscle fat content. Br. J. Nutr. 103, 629.

Kolditz, C., Borthaire, M., Richard, N., Corraze, G., Panserat, S., Vachot, C., Lefèvre, F., Médale, F., 2008a. Liver and muscle metabolic changes induced by dietary energy content and genetic selection in rainbow trout (*Oncorhynchus mykiss*). Am. J. Physiol. Regul. Integr. Comp. Physiol. 294, R1154-64.

Kolditz, C., Paboeuf, G., Borthaire, M., Esquerre, D., SanCristobal, M., Lefevre, F., Medale, F., 2008b. Changes induced by dietary energy intake and divergent selection for muscle fat content in rainbow trout (*Oncorhynchus mykiss*), assessed by transcriptome and proteome analysis of the liver. BMC Genomics 9, 16.

Koutnikova, H., Cock, T.-A., Watanabe, M., Houten, S.M., Champy, M.-F., Dierich, A., Auwerx, J., 2003. Compensation by the muscle limits the metabolic consequences of lipodystrophy in PPAR hypomorphic mice. Proc. Natl. Acad. Sci. 100, 14457–14462.

Kurokawa, T., Uji, S., Suzuki, T., 2005. Identification of cDNA coding for a homologue to mammalian leptin from pufferfish, *Takifugu rubripes*. Peptides 26, 745–750.

Kwon, H., Pessin, J.E., 2013. Adipokines Mediate Inflammation and Insulin Resistance. Front. Endocrinol. (Lausanne). 4, 71.

Lampidonis, A.D., Rogdakis, E., Voutsinas, G.E., Stravopodis, D.J., 2011. The resurgence of Hormone-Sensitive Lipase (HSL) in mammalian lipolysis. Gene 477, 1–11.

Landgraf, K., Schuster, S., Meusel, A., Garten, A., Riemer, T., Schleinitz, D., Kiess, W., Körner, A., 2017. Short-term overfeeding of zebrafish with normal or high-fat diet as a model for the development of metabolically healthy versus unhealthy obesity. BMC Physiol. 17, 4.

Lass, A., Zimmermann, R., Oberer, M., Zechner, R., 2011. Lipolysis - a highly regulated multi-enzyme complex mediates the catabolism of cellular fat stores. Prog. Lipid Res. 50, 14–27.

Leaver, M.J., Bautista, J.M., Björnsson, B.T., Jönsson, E., Krey, G., Tocher, D.R., Torstensen, B.E., 2008. Towards Fish Lipid Nutrigenomics: Current State and Prospects for Fin-Fish Aquaculture. Rev. Fish. Sci. 16, 73–94.

Lee, H., Lim, S., Yun, S., Yoon, A., Park, G., Yang, H., 2012. Tributyltin increases the expression of apoptosis- and adipogenesis-related genes in rat ovaries. Clin. Exp. Reprod. Med. 39, 15–21.

Lee, Y.-J., Choi, H.-S., Seo, M.-J., Jeon, H.-J., Kim, K.-J., Lee, B.-Y., 2015. Kaempferol suppresses lipid accumulation by inhibiting early adipogenesis in 3T3-L1 cells and zebrafish. Food Funct. 6, 2824–33.

Lefterova, M.I., Lazar, M.A., 2009. New developments in adipogenesis. Trends Endocrinol. Metab. 20, 107–114.

Lehmann, J.M., Kliewer, S.A., Moore, L.B., Smith-Oliver, T.A., Oliver, B.B., Su, J.L., Sundseth, S.S., Winegar, D.A., Blanchard, D.E., Spencer, T.A., Willson, T.M., 1997. Activation of the nuclear receptor LXR by oxysterols defines a new hormone response pathway. J. Biol. Chem. 272, 3137–40.

Lerescu, L., Tucureanu, C., Caras, I., Neagu, S., Melinceanu, L., Salageanu, A., 2008. Primary cell culture of human adenocarcinomas--practical considerations. Roum Arch Microbiol Immunol 67, 55–66.

Li, L.O., Ellis, J.M., Paich, H.A., Wang, S., Gong, N., Altshuller, G., Thresher, R.J., Koves, T.R., Watkins, S.M., Muoio, D.M., Cline, G.W., Shulman, G.I., Coleman, R.A., 2009. Liver-specific loss

of long chain Acyl-CoA synthetase-1 decreases triacylglycerol synthesis and β-oxidation and alters phospholipid fatty acid composition. J. Biol. Chem. 284, 27816–27826.

Li, S.-Y., Chang, C.-Q., Ma, F.-Y., Yu, C.-L., 2009. Modulating effects of chlorogenic acid on lipids and glucose metabolism and expression of hepatic peroxisome proliferator-activated receptor-α in golden hamsters fed on high fat diet. Biomed. Environ. Sci. 22, 122–129.

Li, X., Ycaza, J., Blumberg, B., 2011. The environmental obesogen tributyltin chloride acts via peroxisome proliferator activated receptor gamma to induce adipogenesis in murine 3T3-L1 preadipocytes. J. Steroid Biochem. Mol. Biol. 127, 9–15.

Liao, C., Ou, T., Wu, C., Wang, C., 2013. Prevention of Diet-Induced Hyperlipidemia and Obesity by Ca ff eic Acid in C57BL/6 Mice through Regulation of Hepatic Lipogenesis Gene Expression. J. Agric. Food Chem. 61, 11082–11088.

Libran-Perez, M., Geurden, I., Dias, K., Corraze, G., Panserat, S., Soengas, J.L., 2015. Feeding rainbow trout with a lipid-enriched diet: effects on fatty acid sensing, regulation of food intake and cellular signaling pathways. J. Exp. Biol. 218, 2610–2619.

Lim, H.-J., Lee, S., Lee, K.-S., Park, J.-H., Jang, Y., Lee, E.J., Park, H.-Y., 2006. PPARγ activation induces CD36 expression and stimulates foam cell like changes in rVSMCs. Prostaglandins Other Lipid Mediat. 80, 165–174.

Lim, S., Ahn, S.Y., Song, I.C., Chung, M.H., Jang, H.C., Park, K.S., Lee, K.-U., Pak, Y.K., Lee, H.K., 2009. Chronic Exposure to the Herbicide, Atrazine, Causes Mitochondrial Dysfunction and Insulin Resistance. PLoS One 4, e5186.

Lin, C.-Y., Chiang, C.-Y., Tsai, H.-J., 2016. Zebrafish and Medaka: new model organisms for modern biomedical research. J. Biomed. Sci. 23, 19.

Lin, F., Wu, H., Chen, H., Xin, Z., Yuan, D., Wang, T., Liu, J., Gao, Y., Zhang, X., Zhou, C., Wei, R., Chen, D., Yang, S., Wang, Y., Pu, Y., Li, Z., 2014. Molecular and physiological evidences for the role in appetite regulation of apelin and its receptor APJ in Ya-fish (*Schizothorax prenanti*). Mol. Cell. Endocrinol. 396, 46–57.

Linhart, H.G., Ishimura-Oka, K., DeMayo, F., Kibe, T., Repka, D., Poindexter, B., Bick, R.J., Darlington, G.J., 2001. C/EBPalpha is required for differentiation of white, but not brown, adipose tissue. Proc. Natl. Acad. Sci. U. S. A. 98, 12532–7.

Liu, P., Ji, H., Li, C., Tian, J., Wang, Y., Yu, P., 2015. Ontogenetic development of adipose tissue in grass carp (*Ctenopharyngodon idellus*). Fish Physiol. Biochem. 41, 867–878.

Lossi, L., D'Angelo, L., De Girolamo, P., Merighi, A., 2016. Anatomical features for an adequate choice of experimental animal model in biomedicine: II. Small laboratory rodents, rabbit, and pig. Ann. Anat. Anat. Anzeiger 204, 11–28.

Lovell, T., 1989. Nutrition and Feeding of Fish (An AVI Book). Kluwer Academic Publishers.

Lowe, C.E., O'Rahilly, S., Rochford, J.J., 2011. Adipogenesis at a glance. J. Cell Sci. 124.

Lustig, R.H., 2011. Obesity before birth: maternal and prenatal influences on the offspring. Springer Science+Business Media, LLC.

Lyssimachou, A., Santos, J.G., André, A., Soares, J., Lima, D., Guimarães, L., Almeida, C.M.R., Teixeira, C., Castro, L.F.C., Santos, M.M., 2015. The Mammalian "Obesogen"; Tributyltin Targets Hepatic Triglyceride Accumulation and the Transcriptional Regulation of Lipid Metabolism in the Liver and Brain of Zebrafish. PLoS One 10, e0143911.

MacKay, H., Patterson, Z.R., Khazall, R., Patel, S., Tsirlin, D., Abizaid, A., 2013. Organizational Effects of Perinatal Exposure to Bisphenol-A and Diethylstilbestrol on Arcuate Nucleus Circuitry Controlling Food Intake and Energy Expenditure in Male and Female CD-1 Mice. Endocrinology 154, 1465–1475.

Maeda, K., Okubo, K., Shimomura, I., Funahashi, T., Matsuzawa, Y., Matsubara, K., 1996. cDNA Cloning and Expression of a Novel Adipose Specific Collagen-like Factor, apM1 (AdiposeMost Abundant Gene Transcript 1). Biochem. Biophys. Res. Commun. 221, 286–289.

Maeda, N., Shimomura, I., Kishida, K., Nishizawa, H., Matsuda, M., Nagaretani, H., Furuyama, N., Kondo, H., Takahashi, M., Arita, Y., Komuro, R., Ouchi, N., Kihara, S., Tochino, Y., Okutomi, K., Horie, M., Takeda, S., Aoyama, T., Funahashi, T., Matsuzawa, Y., 2002. Diet-induced insulin resistance in mice lacking adiponectin/ACRP30. Nat. Med. 8, 731–737.

Mattsson, C., Olsson, T., 2007. Estrogens and glucocorticoid hormones in adipose tissue metabolism. Curr. Med. Chem. 14, 2918–24.

Maury, E., Brichard, S.M., 2010. Adipokine dysregulation, adipose tissue inflammation and metabolic syndrome. Mol. Cell. Endocrinol. 314, 1–16.

McAllister, B.G., Kime, D.E., 2003. Early life exposure to environmental levels of the aromatase inhibitor tributyltin causes masculinisation and irreversible sperm damage in zebrafish (*Danio rerio*). Aquat. Toxicol. 65, 309–16.

McCue, M.D., 2010. Starvation physiology: Reviewing the different strategies animals use to survive a common challenge. Comp. Biochem. Physiol. Part A Mol. Integr. Physiol. 156, 1–18.

McGinnis, C.L., Crivello, J.F., 2011. Elucidating the mechanism of action of tributyltin (TBT) in zebrafish. Aquat. Toxicol. 103, 25–31.

Meador, J.P., Sommers, F.C., Cooper, K.A., Yanagida, G., 2011. Tributyltin and the obesogen metabolic syndrome in a salmonid. Environ. Res. 111, 50–6.

Meguro, S., Hasumura, T., Hase, T., 2015. Body fat accumulation in zebrafish is induced by a diet rich in fat and reduced by supplementation with green tea extract. PLoS One 10, e0120142.

Melzer, K., Kayser, B., Saris, W.H.M., Pichard, C., 2005. Dietary factors associated with obesity indicators and level of sports participation in Flemish adults: a cross-sectional study. Clin. Nutr.

Miller, M., Cho, J.Y., Pham, A., Ramsdell, J., Broide, D.H., 2009. Adiponectin and functional adiponectin receptor 1 are expressed by airway epithelial cells in chronic obstructive pulmonary disease. J. Immunol. 182, 684–91.

Minchin, J.E.N., Dahlman, I., Harvey, C.J., Mejhert, N., Singh, M.K., Epstein, J.A., Arner, P., Torres-Vázquez, J., Rawls, J.F., 2015. Plexin D1 determines body fat distribution by regulating the type V collagen microenvironment in visceral adipose tissue. Proc. Natl. Acad. Sci. U. S. A. 112, 4363–8.

Minchin, J.E.N., Rawls, J.F., 2017. A classification system for zebrafish adipose tissues. Dis. Model. Mech. dmm.025759.

Minchin, J.E.N., Rawls, J.F., 2016. In vivo imaging and quantification of regional adiposity in zebrafish. Methods Cell Biol. 138, 3–27.

Minchin, J.E.N., Rawls, J.F., 2011. In vivo analysis of white adipose tissue in zebrafish. Methods Cell Biol. 105, 63–86.

Mishima, T., Miner, J.H., Morizane, M., Stahl, A., Sadovsky, Y., 2011. The Expression and Function of Fatty Acid Transport Protein-2 and -4 in the Murine Placenta. PLoS One 6, e25865.

Moreno-Navarrete, J.M., Fernández-Real, J.M., 2012. Adipocyte Differentiation, in: Adipose Tissue Biology. Springer New York, New York, NY, pp. 17–38.

Mukherjee, A., Mukherjee, S., Biswas, J., Roy, M., 2015. Phytochemicals in obesity control. Int.J.Curr.Microbiol.App.Sci 4, 558–567.

Murashita, K., Uji, S., Yamamoto, T., Rønnestad, I., Kurokawa, T., 2008. Production of recombinant leptin and its effects on food intake in rainbow trout (*Oncorhynchus mykiss*). Comp. Biochem. Physiol. Part B Biochem. Mol. Biol. 150, 377–384.

Nakano, R., Kurosaki, E., Yoshida, S., Yokono, M., Shimaya, A., Maruyama, T., Shibasaki, M., 2006. Antagonism of peroxisome proliferator-activated receptor γ prevents high-fat diet-induced obesity in vivo. Biochem. Pharmacol. 72, 42–52.

Nakano, T., Kanmuri, T., Sato, M., Takeuchi, M., 1999. Effect of astaxanthin rich red yeast (*Phaffia rhodozyma*) on oxidative stress in rainbow trout. Biochim. Biophys. Acta - Gen. Subj. 1426, 119–125.

Nakano, Y., Tobe, T., Choi-Miura, N.H., Mazda, T., Tomita, M., 1996. Isolation and characterization of GBP28, a novel gelatin-binding protein purified from human plasma. J. Biochem. 120, 803–12.

Navarro, I., Gutiérrez, J., 1995. Chapter 17 Fasting and starvation. Biochem. Mol. Biol. Fishes 4, 393–434.

Neel, J. V, 1962. Diabetes mellitus: a "thrifty"; genotype rendered detrimental by "progress" Am. J. Hum. Genet. 14, 353–62.

Nguyen, M. Van, 2013. The impact of lysine and arginine ratios in plant-based protein diets on appetite, growth performance and gene expression of brain neuropeptide Y (NPY) and cholecystokinin (CCK) in juvenile cobia (*Rachycentron canadum*).

Nieto-Vazquez, I., Fernández-Veledo, S., Krämer, D.K., Vila-Bedmar, R., Garcia-Guerra, L., Lorenzo, M., 2008. Insulin resistance associated to obesity: the link TNF-alpha. Arch. Physiol. Biochem. 114, 183–194.

Nigro, E., Scudiero, O., Sarnataro, D., Mazzarella, G., Sofia, M., Bianco, A., Daniele, A., 2013. Adiponectin affects lung epithelial A549 cell viability counteracting TNFa and IL-1ß toxicity through AdipoR1. Int. J. Biochem. Cell Biol. 45, 1145–1153.

Nishio, S.-I., Gibert, Y., Bernard, L., Brunet, F., Triqueneaux, G., Laudet, V., 2008. Adiponectin and adiponectin receptor genes are coexpressed during zebrafish embryogenesis and regulated by food deprivation. Dev. Dyn. 237, 1682–1690.

Ohashi, K., Yuasa, D., Shibata, R., Murohara, T., Ouchi, N., 2015. Adiponectin as a Target in Obesity-related Inflammatory State. Endocr. Metab. Immune Disord. Drug Targets 15, 145–50.

Oka, T., Nishimura, Y., Zang, L., Hirano, M., Shimada, Y., Wang, Z., Umemoto, N., Kuroyanagi, J., Nishimura, N., Tanaka, T., 2010. Diet-induced obesity in zebrafish shares common pathophysiological pathways with mammalian obesity. BMC Physiol. 10, 21.

Oku, H., Koizumi, N., Okumura, T., Kobayashi, T., Umino, T., 2006. Molecular characterization of lipoprotein lipase, hepatic lipase and pancreatic lipase genes: Effects of fasting and refeeding on their gene expression in red sea bream *Pagrus major*. Comp. Biochem. Physiol. Part B Biochem. Mol. Biol. 145, 168–178.

Oku, H., Tokuda, M., Umino, T., 2009. The effects of 2-bromopalmitate on the fatty acid composition in differentiating adipocytes of red sea bream (*Pagrus major*). Comp. Biochem. Physiol. Part B Biochem. Mol. Biol. 152, 370–375.

Oliva-Teles, A., 2012. Nutrition and health of aquaculture fish. J. Fish Dis.

Østerlie, M., Bjerkeng, B., Liaaen-Jensen, S., 2000. Plasma appearance and distribution of astaxanthin E/Z and R/S isomers in plasma lipoproteins of men after single dose administration of astaxanthin. J. Nutr. Biochem 11, 482–490.

Ouadah-Boussouf, N., Babin, P.J., 2016. Pharmacological evaluation of the mechanisms involved in increased adiposity in zebrafish triggered by the environmental contaminant tributyltin. Toxicol. Appl. Pharmacol. 294, 32–42.

Palstra, A.P., Planas, J. V, 2011. Fish under exercise. Fish Physiol. Biochem. 37, 259–72.

Panserat, S., Perrin, A., Kaushik, S., 2002. High dietary lipids induce liver glucose-6-phosphatase expression in rainbow trout (*Oncorhynchus mykiss*). J. Nutr. 132, 137–41.

Park, Y.-K., Wang, L., Giampietro, A., Lai, B., Lee, J.-E., Ge, K., 2017. Distinct Roles of Transcription Factors KLF4, Krox20, and Peroxisome Proliferator-Activated Receptor γ in Adipogenesis. Mol. Cell. Biol. 37, MCB.00554-16.

Pavese, J.M., Farmer, R.L., Bergan, R.C., 2010. Inhibition of cancer cell invasion and metastasis by genistein. Cancer Metastasis Rev. 29, 465–82.

Pereira-Fernandes, A., Vanparys, C., Hectors, T.L.M., Vergauwen, L., Knapen, D., Jorens, P.G., Blust, R., 2013. Unraveling the mode of action of an obesogen: mechanistic analysis of the model obesogen tributyltin in the 3T3-L1 cell line. Mol. Cell. Endocrinol. 370, 52–64.

Pérez-Jiménez, A., Guedes, M.J., Morales, A.E., Oliva-Teles, A., 2007. Metabolic responses to short starvation and refeeding in *Dicentrarchus labrax*. Effect of dietary composition. Aquaculture 265, 325–335.

Pisonero-Vaquero, S., Martínez-Ferreras, Á., García-Mediavilla, M.V., Martínez-Flórez, S., Fernández, A., Benet, M., Olcoz, J.L., Jover, R., González-Gallego, J., Sánchez-Campos, S., 2015. Quercetin ameliorates dysregulation of lipid metabolism genes via the PI3K/AKT pathway in a diet-induced mouse model of nonalcoholic fatty liver disease. Mol. Nutr. Food Res. 59, 879–893.

Planas, J. V, Méndez, E., Baños, N., Capilla, E., Navarro, I., Gutiérrez, J., 2000. Insulin and IGF-I receptors in trout adipose tissue are physiologically regulated by circulating hormone levels. J. Exp. Biol. 203, 1153–9.

Polakof, S., Médale, F., Larroquet, L., Vachot, C., Corraze, G., Panserat, S., 2011. Insulin stimulates lipogenesis and attenuates Beta-oxidation in white adipose tissue of fed rainbow trout. Lipids 46, 189–99.

Polakof, S., Panserat, S., Soengas, J.L., Moon, T.W., 2012. Glucose metabolism in fish: a review. J. Comp. Physiol. B 182, 1015–1045.

Quillet, E., Le Guillou, S., Aubin, J., Fauconneau, B., 2005. Two-way selection for muscle lipid content in pan-size rainbow trout (*Oncorhynchus mykiss*). Aquaculture 245, 49–61.

Rabobank, 2016. How to feed the world in 2050? [WWW Document]. URL https://www.rabobank.com/en/about-rabobank/food-agribusiness/sectors/from-animals/sustainable-seafood/index.html (accessed 5.15.17).

Rahman, M.M., Khosravi, S., Chang, K.H., Lee, S.-M., 2016. Effects of Dietary Inclusion of Astaxanthin on Growth, Muscle Pigmentation and Antioxidant Capacity of Juvenile Rainbow Trout (*Oncorhynchus mykiss*). Prev. Nutr. food Sci. 21, 281–288.

Rajendra Prasad, N., Karthikeyan, A., Karthikeyan, S., Venkata Reddy, B., 2011. Inhibitory effect of caffeic acid on cancer cell proliferation by oxidative mechanism in human HT-1080 fibrosarcoma cell line. Mol. Cell. Biochem. 349, 11–19.

Rando, O.J., Simmons, R.A., 2015. I'm eating for two: parental dietary effects on offspring metabolism. Cell 161, 93–105.

Ranganathan, G., Unal, R., Pokrovskaya, I., Yao-Borengasser, A., Phanavanh, B., Lecka-Czernik, B., Rasouli, N., Kern, P.A., 2006. The lipogenic enzymes DGAT1, FAS, and LPL in adipose tissue: effects of obesity, insulin resistance, and TZD treatment. J. Lipid Res. 47, 2444–50.

Rathore, R.M., Liaset, B., Hevrøy, E.M., El-Mowafi, A., Espe, M., 2010. Lysine limitation alters the storage pattern of protein, lipid and glycogen in on-growing Atlantic salmon. Aquac. Res. 41, e751–e759.

Rayalam, S., Della-Fera, M.A., Baile, C.A., 2008. Phytochemicals and regulation of the adipocyte life cycle. J. Nutr. Biochem.

Régnier, P., Bastias, J., Rodriguez-Ruiz, V., Caballero-Casero, N., Caballo, C., Sicilia, D., Fuentes, A., Maire, M., Crepin, M., Letourneur, D., Gueguen, V., Rubio, S., Pavon-Djavid, G., 2015. Astaxanthin

from Haematococcus pluvialis Prevents Oxidative Stress on Human Endothelial Cells without Toxicity. Mar. Drugs 13, 2857–74.

Regnier, S.M., El-Hashani, E., Kamau, W., Zhang, X., Massad, N.L., Sargis, R.M., 2015. Tributyltin differentially promotes development of a phenotypically distinct adipocyte. Obesity (Silver Spring). 23, 1864–71.

Res, P.T., Cermak, N.M., Stinkens, R., Tollakson, T.J., Haenen, G.R., Bast, A., Van Loon, L.J.C., 2013. Astaxanthin supplementation does not augment fat use or improve endurance performance. Med. Sci. Sports Exerc. 45, 1158–65.

Rocha, F., Dias, J., Engrola, S., Gavaia, P., Geurden, I., Dinis, M.T., Panserat, S., 2014. Glucose overload in yolk has little effect on the long-term modulation of carbohydrate metabolic genes in zebrafish (*Danio rerio*). J. Exp. Biol. 217.

Rosen, E.D., 2005. The transcriptional basis of adipocyte development. Prostaglandins, Leukot. Essent. Fat. Acids 73, 31–34.

Rosen, E.D., Hsu, C.-H., Wang, X., Sakai, S., Freeman, M.W., Gonzalez, F.J., Spiegelman, B.M., 2002. C/EBPalpha induces adipogenesis through PPARgamma: a unified pathway. Genes Dev. 16, 22–26.

Rosen, E.D., Spiegelman, B.M., 2014. What We Talk About When We Talk About Fat. Cell 156, 20–44.

Rosen, E.D., Spiegelman, B.M., 2006. Adipocytes as regulators of energy balance and glucose homeostasis. Nature 444, 847–853.

Rosen, E.D., Walkey, C.J., Puigserver, P., Spiegelman, B.M., 2000. Transcriptional regulation of adipogenesis. Genes Dev. 14, 1293–307.

Rubin, B.S., Murray, M.K., Damassa, D.A., King, J.C., Soto, A.M., 2001. Perinatal exposure to low doses of bisphenol A affects body weight, patterns of estrous cyclicity, and plasma LH levels. Environ. Health Perspect. 109, 675–80.

Rutkowski, J.M., Stern, J.H., Scherer, P.E., 2015. The cell biology of fat expansion. J. Cell Biol. 208.

Sadowska-Bartosz, I., Bartosz, G., 2014. Effect of antioxidants supplementation on aging and longevity. Biomed Res. Int. 2014, 404680.

Salazar, M.H., Salazar, S.M., 1991. Assessing site-specific effects of TBT contamination with mussel growth rates. Mar. Environ. Res. 32, 131–150.

Salmerón, C., Acerete, L., Gutiérrez, J., Navarro, I., Capilla, E., 2013. Characterization and endocrine regulation of proliferation and differentiation of primary cultured preadipocytes from gilthead sea bream (*Sparus aurata*). Domest. Anim. Endocrinol. 45, 1–10.

Salmerón, C., Johansson, M., Angotzi, A.R., Rønnestad, I., Jönsson, E., Björnsson, B.T., Gutiérrez, J., Navarro, I., Capilla, E., 2015a. Effects of nutritional status on plasma leptin levels and in vitro regulation of adipocyte leptin expression and secretion in rainbow trout. Gen. Comp. Endocrinol. 210, 114–123.

Salmerón, C., Johansson, M., Asaad, M., Angotzi, A.R., Rønnestad, I., Stefansson, S.O., Jönsson, E., Björnsson, B.T., Gutiérrez, J., Navarro, I., Capilla, E., 2015b. Roles of leptin and ghrelin in adipogenesis and lipid metabolism of rainbow trout adipocytes in vitro. Comp. Biochem. Physiol. - Part A Mol. Integr. Physiol. 188, 40–48.

Salmerón, C., Riera-Heredia, N., Gutiérrez, J., Navarro, I., Capilla, E., 2016a. Adipogenic Gene Expression in Gilthead Sea Bream Mesenchymal Stem Cells from Different Origin. Front. Endocrinol. (Lausanne). 7, 113.

Samdani, P., Singhal, M., Sinha, N., Tripathi, P., Sharma, S., Tikoo, K., Rao, K.V.S., Kumar, D., 2015. A Comprehensive Inter-Tissue Crosstalk Analysis Underlying Progression and Control of Obesity and Diabetes. Sci. Rep. 5, 12340.

Sánchez-Gurmaches, J., Cruz-Garcia, L., Gutiérrez, J., Navarro, I., 2012. Adiponectin effects and gene expression in rainbow trout: an in vivo and in vitro approach. J. Exp. Biol. 215.

Sánchez-Gurmaches, J., Cruz-Garcia, L., Gutiérrez, J., Navarro, I., 2012. mRNA expression of fatty acid transporters in rainbow trout: In vivo and in vitro regulation by insulin, fasting and inflammation and infection mediators. Comp. Biochem. Physiol. - A Mol. Integr. Physiol. 163, 177–188.

Sanchez-Gurmaches, J., Ostbye, T.-K., Navarro, I., Torgersen, J., Hevroy, E.M., Ruyter, B., Torstensen, B.E., 2011. In vivo and in vitro insulin and fasting control of the transmembrane fatty acid transport proteins in Atlantic salmon (*Salmo salar*). AJP Regul. Integr. Comp. Physiol. 301, R947–R957.

Santiago, C.B., Lovell, R.T., 1988. Amino acid requirements for growth of Nile tilapia. J. Nutr. 118, 1540–6.

Santoriello, C., Zon, L.I., 2012. Hooked! Modeling human disease in zebrafish. J. Clin. Invest. 122, 2337–43.

Santos, M.M., Micael, J., Carvalho, A.P., Morabito, R., Booy, P., Massanisso, P., Lamoree, M., Reis-Henriques, M.A., 2006. Estrogens counteract the masculinizing effect of tributyltin in zebrafish. Comp. Biochem. Physiol. C. Toxicol. Pharmacol. 142, 151–5.

Saponaro, C., Gaggini, M., Carli, F., Gastaldelli, A., 2015. The Subtle Balance between Lipolysis and Lipogenesis: A Critical Point in Metabolic Homeostasis. Nutrients 7, 9453–9474.

Scherer, P.E., Williams, S., Fogliano, M., Baldini, G., Lodish, H.F., 1995. A novel serum protein similar to C1q, produced exclusively in adipocytes. J. Biol. Chem. 270, 26746–9.

Seo, M.-J., Choi, H.-S., Jeon, H.-J., Woo, M.-S., Lee, B.-Y., 2014. Baicalein inhibits lipid accumulation by regulating early adipogenesis and m-TOR signaling. Food Chem. Toxicol. 67, 57–64.

Seth, A., Stemple, D.L., Barroso, I., 2013. The emerging use of zebrafish to model metabolic disease. Dis. Model. Mech. 6, 1080–8.

Sethi, J.K., Vidal-Puig, A.J., 2007. Thematic review series: adipocyte biology. Adipose tissue function and plasticity orchestrate nutritional adaptation. J. Lipid Res. 48, 1253–62.

Sharma, A.M., Staels, B., 2007. Peroxisome Proliferator-Activated Receptor γ and Adipose Tissue—Understanding Obesity-Related Changes in Regulation of Lipid and Glucose Metabolism. J. Clin. Endocrinol. Metab. 92, 386–395.

Skiba-Cassy, S., Geurden, I., Panserat, S., Seiliez, I., 2016. Dietary methionine imbalance alters the transcriptional regulation of genes involved in glucose, lipid and amino acid metabolism in the liver of rainbow trout (*Oncorhynchus mykiss*). Aquaculture 454, 56–65.

Skiba-Cassy, S., Lansard, M., Panserat, S., Medale, F., 2009. Rainbow trout genetically selected for greater muscle fat content display increased activation of liver TOR signaling and lipogenic gene expression. AJP Regul. Integr. Comp. Physiol. 297, R1421–R1429.

Skinner, M.K., Manikkam, M., Tracey, R., Guerrero-Bosagna, C., Haque, M., Nilsson, E.E., 2013. Ancestral dichlorodiphenyltrichloroethane (DDT) exposure promotes epigenetic transgenerational inheritance of obesity. BMC Med. 11, 228.

Smith, S., Witkowski, A., Joshi, A.K., 2003. Structural and functional organization of the animal fatty acid synthase. Prog. Lipid Res. 42, 289–317.

Song, Y., Cone, R.D., 2007. Creation of a genetic model of obesity in a teleost. FASEB J. 21, 2042–9.

Speakman, J.R., 2004. Obesity: the integrated roles of environment and genetics. J. Nutr. 134, 2090S–2105S.

Spiegelman, B.M., Hu, E., Kim, J.B., Brun, R., 1997. PPARγ and the control of adipogenesis. Biochimie 79, 111–112.

Tacon, A.G.J., Cowey, C.B., 1985. Protein and Amino Acid Requirements, in: Fish Energetics. Springer Netherlands, Dordrecht, pp. 155–183.

Tave, D., 1996. Selective breeding programmes for medium-sized fish farms - Contents, Chemistry & biodiversity. Food and Agriculture Organization of the United Nations.

Tave, D., 1986. Genetics for fish hatchery managers. Genet. fish Hatch. Manag.

Tchkonia, T., Giorgadze, N., Pirtskhalava, T., Thomou, T., DePonte, M., Koo, A., Forse, R.A., Chinnappan, D., Martin-Ruiz, C., von Zglinicki, T., Kirkland, J.L., 2006. Fat Depot–Specific Characteristics Are Retained in Strains Derived From Single Human Preadipocytes. Diabetes 55, 2571–2578.

Teixeira, A., Baenas, N., Dominguez-Perles, R., Barros, A., Rosa, E., Moreno, D., Garcia-Viguera, C., 2014. Natural Bioactive Compounds from Winery By-Products as Health Promoters: A Review. Int. J. Mol. Sci. 15, 15638–15678.

Thomassen, M.S., Røsjø, C., 1989. Different fats in feed for salmon: Influence on sensory parameters, growth rate and fatty acids in muscle and heart. Aquaculture 79, 129–135.

Tingaud-Sequeira, A., Ouadah, N., Babin, P.J., 2011. Zebrafish obesogenic test: a tool for screening molecules that target adiposity. J. Lipid Res. 52, 1765–72.

Tocher, D.R., Bell, J.G., McGhee, F., Dick, J.R., Fonseca-Madrigal, J., 2003. Effects of dietary lipid level and vegetable oil on fatty acid metabolism in Atlantic salmon (*Salmo salar L.*) over the whole production cycle. Fish Physiol. Biochem. 29, 193–209.

Todorčević, M., Hodson, L., 2015. The Effect of Marine Derived n-3 Fatty Acids on Adipose Tissue Metabolism and Function. J. Clin. Med. 5.

Todorcević, M., Skugor, S., Krasnov, A., Ruyter, B., 2010. Gene expression profiles in Atlantic salmon adipose-derived stromo-vascular fraction during differentiation into adipocytes. BMC Genomics 11, 39.

Todorčević, M., Vegusdal, A., Gjøen, T., Sundvold, H., Torstensen, B.E., Kjær, M.A., Ruyter, B., 2008. Changes in fatty acids metabolism during differentiation of Atlantic salmon preadipocytes; Effects of n-3 and n-9 fatty acids. Biochim. Biophys. Acta - Mol. Cell Biol. Lipids 1781, 326–335.

Torstensen, B., Tocher, D., 2010. The Effects of Fish Oil Replacement on Lipid Metabolism of Fish, in: Fish Oil Replacement and Alternative Lipid Sources in Aquaculture Feeds. CRC Press, pp. 405–437.

Torstensen, B.E., Espe, M., Stubhaug, I., Lie, Ø., 2011. Dietary plant proteins and vegetable oil blends increase adiposity and plasma lipids in Atlantic salmon (*Salmo salar L.*). Br. J. Nutr. 106, 633–647.

Torstensen, B.E., Nanton, D.A., Olsvik, P.A., Sundvold, H., Stubhaug, I., 2009. Gene expression of fatty acid-binding proteins, fatty acid transport proteins (cd36 and FATP) and β-oxidation-related genes in Atlantic salmon (*Salmo salar L.*) fed fish oil or vegetable oil. Aquac. Nutr. 15, 440–451.

Tousignant, K., Uno, J., 2015. The effect of obesogens on the microbiota and systemic health in zebrafish, The FASEB Journal. Federation of American Societies for Experimental Biology.

Trombley, S., Maugars, G., Kling, P., Björnsson, B.T., Schmitz, M., 2012. Effects of long-term restricted feeding on plasma leptin, hepatic leptin expression and leptin receptor expression in juvenile Atlantic salmon (*Salmo salar L.*). Gen. Comp. Endocrinol. 175, 92–99.

Trombley, S., Mustafa, A., Schmitz, M., 2014. Regulation of the seasonal leptin and leptin receptor expression profile during early sexual maturation and feed restriction in male Atlantic salmon, *Salmo salar L.*, parr. Gen. Comp. Endocrinol. 204, 60–70.

Trujillo, M.E., Scherer, P.E., 2006. Adipose Tissue-Derived Factors: Impact on Health and Disease. Endocr. Rev. 27, 762–778.

Tucci, S.A., 2010. Phytochemicals in the Control of Human Appetite and Body Weight. Pharmaceuticals (Basel). 3, 748–763.

Tutino, V., Orlando, A., Russo, F., Notarnicola, M., 2016. Hydroxytyrosol Inhibits Cannabinoid CB1 Receptor Gene Expression in 3T3-L1 Preadipocyte Cell Line. J. Cell. Physiol. 231, 483–9.

Ulloa, P.E., Peña, A.A., Lizama, C.D., Araneda, C., Iturra, P., Neira, R., Medrano, J.F., 2013. Growth Response and Expression of Muscle Growth–Related Candidate Genes in Adult Zebrafish Fed Plant and Fishmeal Protein–Based Diets. Zebrafish 10, 99–109.

van der Veen, J.N., van Dijk, T.H., Vrins, C.L.J., van Meer, H., Havinga, R., Bijsterveld, K., Tietge, U.J.F., Groen, A.K., Kuipers, F., 2009. Activation of the liver X receptor stimulates trans-intestinal excretion of plasma cholesterol. J. Biol. Chem. 284, 19211–9.

Van Harmelen, V., Reynisdottir, S., Cianflone, K., Degerman, E., Hoffstedt, J., Nilsell, K., Sniderman, A., Arner, P., 1999. Mechanisms involved in the regulation of free fatty acid release from isolated human fat cells by acylation-stimulating protein and insulin. J. Biol. Chem. 274, 18243–51.

Vegusdal, A., Sundvold, H., Gjøen, T., Ruyter, B., 2003. An in vitro method for studying the proliferation and differentiation of Atlantic salmon preadipocytes. Lipids 38, 289–96.

Vélez, E.J., Azizi, S., Lutfi, E., Capilla, E., Moya, A., Navarro, I., Fernández-Borràs, J., Blasco, J., Gutiérrez, J., 2017. Moderate and sustained exercise modulates muscle proteolytic and myogenic markers in gilthead sea bream (*Sparus aurata*). Am. J. Physiol. - Regul. Integr. Comp. Physiol. ajpregu.00308.2016.

Vélez, E.J., Azizi, S., Millán-Cubillo, A., Fernández-Borràs, J., Blasco, J., Chan, S.J., Calduch-Giner, J.A., Pérez-Sánchez, J., Navarro, I., Capilla, E., Gutiérrez, J., 2016. Effects of sustained exercise on GH-IGFs axis in gilthead sea bream (*Sparus aurata*). Am. J. Physiol. - Regul. Integr. Comp. Physiol. 310, R313–R322.

Venkateswaran, A., Laffitte, B.A., Joseph, S.B., Mak, P.A., Wilpitz, D.C., Edwards, P.A., Tontonoz, P., 2000. Control of cellular cholesterol efflux by the nuclear oxysterol receptor LXR alpha. Proc. Natl. Acad. Sci. U. S. A. 97, 12097–102.

Verreault, M., Senekeo-Effenberger, K., Trottier, J., Bonzo, J.A., Bélanger, J., Kaeding, J., Staels, B., Caron, P., Tukey, R.H., Barbier, O., 2006. The liver X-receptor alpha controls hepatic expression of the human bile acid-glucuronidating UGT1A3 enzyme in human cells and transgenic mice. Hepatology 44, 368–378.

Vickers, M.H., 2014. Early life nutrition, epigenetics and programming of later life disease. Nutrients 6, 2165–78.

Villena, J.A., Roy, S., Sarkadi-Nagy, E., Kim, K.-H., Sul, H.S., 2004. Desnutrin, an Adipocyte Gene Encoding a Novel Patatin Domain-containing Protein, Is Induced by Fasting and Glucocorticoids: ectopic expression of desnutrin increases triglyceride hydrolysis. J. Biol. Chem. 279, 47066–47075.

Vliet-Ostaptchouk, J. V., Snieder, H., Lagou, V., 2012. Gene–lifestyle interactions in obesity. Curr Nutr Rep 1, 184–196.

Vo, T.X., Revesz, A., Sohi, G., Ma, N., Hardy, D.B., 2013. Maternal protein restriction leads to enhanced hepatic gluconeogenic gene expression in adult male rat offspring due to impaired expression of the liver X receptor. J. Endocrinol. 218, 85–97.

Vogel, H., Kraemer, M., Rabasa, C., Askevik, K., Adan, R.A.H., Dickson, S.L., 2017. Genetic predisposition to obesity affects behavioural traits including food reward and anxiety-like behaviour in rats. Behav. Brain Res. 328, 95–104.

Volkoff, H., Wyatt, J.L., 2009. Apelin in goldfish (*Carassius auratus*): Cloning, distribution and role in appetite regulation. Peptides 30, 1434–1440.

Wafer, R., Tandon, P., Minchin, J., 2017. The role of Peroxisome proliferator-activated gamma (PPARG) in adipogenesis: applying knowledge from the aquaculture industry to biomedical research. Front. Endocrinol. (Lausanne). 8, 102.

Wajchenberg, B.L., 2000. Subcutaneous and Visceral Adipose Tissue: Their Relation to the Metabolic Syndrome. Endocr. Rev. 21, 697–738.

Wang, C.-Y., Liao, J.K., 2012. A mouse model of diet-induced obesity and insulin resistance. Methods Mol. Biol. 821, 421–33.

Wang, N.D., Finegold, M.J., Bradley, A., Ou, C.N., Abdelsayed, S. V, Wilde, M.D., Taylor, L.R., Wilson, D.R., Darlington, G.J., 1995. Impaired energy homeostasis in C/EBP alpha knockout mice. Science 269, 1108–12.

Wang, X., Huang, M., Wang, Y., Pan, X., Zhang, J., 2012. The Effect of Insulin, TNFα and DHA on the Proliferation, Differentiation and Lipolysis of Preadipocytes Isolated from Large Yellow Croaker (*Pseudosciaena Crocea R*.). PLoS One 7, e48069.

Wang, X., Wang, Y., Li, Y., 2013. Adipose Triglyceride Lipase (ATGL) Clone, Expression Pattern, and Regulation by Different Lipid Sources and Lipid Levels in Large Yellow Croaker (*Pseudosciaena crocea R*.). Mar. Biotechnol. 15, 197–205.

Ward, W.E., Kaludjerovic, J., Dinsdale, E.C., 2016. A Mouse Model for Studying Nutritional Programming: Effects of Early Life Exposure to Soy Isoflavones on Bone and Reproductive Health. Int. J. Environ. Res. Public Health 13.

Warnke, I., Goralczyk, R., Fuhrer, E., Schwager, J., 2011. Dietary constituents reduce lipid accumulation in murine C3H10 T1/2 adipocytes: A novel fluorescent method to quantify fat droplets. Nutr. Metab. (Lond). 8, 30.

Weaver, I.C.G., Champagne, F.A., Brown, S.E., Dymov, S., Sharma, S., Meaney, M.J., Szyf, M., 2005. Reversal of Maternal Programming of Stress Responses in Adult Offspring through Methyl Supplementation: Altering Epigenetic Marking Later in Life. J. Neurosci. 25.

Weigle, D.S., Duell, P.B., Connor, W.E., Steiner, R.A., Soules, M.R., Kuijper, J.L., 1997. Effect of Fasting, Refeeding, and Dietary Fat Restriction on Plasma Leptin Levels 1. J. Clin. Endocrinol. Metab. 82, 561–565.

Weil, C., Lefèvre, F., Bugeon, J., 2013. Characteristics and metabolism of different adipose tissues in fish. Rev. Fish Biol. Fish. 23, 157–173.

Weyer, C., Funahashi, T., Tanaka, S., Hotta, K., Matsuzawa, Y., Pratley, R.E., Tataranni, P.A., 2001. Hypoadiponectinemia in Obesity and Type 2 Diabetes: Close Association with Insulin Resistance and Hyperinsulinemia. J. Clin. Endocrinol. Metab. 86, 1930–1935.

Wieser, W., Krumschnabel, G., Ojwang-Okwor, J.P., 1992. The energetics of starvation and growth after refeeding in juveniles of three cyprinid species. Environ. Biol. Fishes 33, 63–71.

Wilson, R.P., Halver, J.E., 1986. Protein and Amino Acid Requirements of Fishes. Annu. Rev. Nutr. 6, 225–244.

Won, E.T., Borski, R.J., 2013. Endocrine regulation of compensatory growth in fish. Front. Endocrinol. (Lausanne). 4, 74.

World Health Organisation, 2016. WHO | Obesity and overweight. WHO.

World Wildlife Fund, 2012. Banking on Sustainability (WWF) [WWW Document]. URL https://www.worldwildlife.org/stories/banking-on-sustainability (accessed 5.15.17).

Xiao, C.W., 2008. Health Effects of Soy Protein and Isoflavones in Humans. J. Nutr. 138, 1244S–1249.

Yada, T., Moriyama, S., Suzuki, Y., Azuma, T., Takahashi, A., Hirose, S., Naito, N., 2002. Relationships between obesity and metabolic hormones in the "cobalt" variant of rainbow trout. Gen. Comp. Endocrinol. 128, 36–43.

Yamauchi, T., Nio, Y., Maki, T., Kobayashi, M., Takazawa, T., Iwabu, M., Okada-Iwabu, M., Kawamoto, S., Kubota, N., Kubota, T., Ito, Y., Kamon, J., Tsuchida, A., Kumagai, K., Kozono, H., Hada, Y., Ogata, H., Tokuyama, K., Tsunoda, M., Ide, T., Murakami, K., Awazawa, M., Takamoto, I., Froguel, P., Hara, K., Tobe, K., Nagai, R., Ueki, K., Kadowaki, T., 2007. Targeted disruption of AdipoR1 and AdipoR2 causes abrogation of adiponectin binding and metabolic actions. Nat. Med. 13, 332–339.

Yeh, W.C., Cao, Z., Classon, M., McKnight, S.L., 1995. Cascade regulation of terminal adipocyte differentiation by three members of the C/EBP family of leucine zipper proteins. Genes Dev. 9, 168–81.

Zhang, X.-D., Zhu, Y.-F., Cai, L.-S., Wu, T.-X., 2008. Effects of fasting on the meat quality and antioxidant defenses of market-size farmed large yellow croaker (*Pseudosciaena crocea*). Aquaculture 280, 136–139.

Zhang, X., Wu, T., Cai, L., Zhu, Y., 2007. Influence of fasting on muscle composition and antioxidant defenses of market-size *Sparus macrocephalus*. J. Zhejiang Univ. Sci. B 8, 906–11.

Zhou, H., Song, X., Briggs, M., Violand, B., Salsgiver, W., Gulve, E.A., Luo, Y., 2005. Adiponectin represses gluconeogenesis independent of insulin in hepatocytes. Biochem. Biophys. Res. Commun. 338, 793–799.

Zhou, S., Ackman, R.G., Morrison, C., 1996. Adipocytes and lipid distribution in the muscle tissue of Atlantic salmon (*Salmo salar*). Can. J. Fish. Aquat. Sci. 53, 326–332.

Zimmermann, R., Strauss, J.G., Haemmerle, G., Schoiswohl, G., Birner-Gruenberger, R., Riederer, M., Lass, A., Neuberger, G., Eisenhaber, F., Hermetter, A., Zechner, R., 2004. Fat Mobilization in Adipose Tissue Is Promoted by Adipose Triglyceride Lipase. Science (80). 306, 1383–1386.

ANNEX

ANNEX CONTENTS

LIST OF ADDITIONAL PUBLICATIONS **173**

DOCTORAL INTERNSHIPS

University of Bordeaux (France) 175

INRA Saint-Pée-sur-Nivelle (France) 177

Università Politecnica della Marche (Italy) 179

List of peer-reviewed publications not included in the present thesis:

Title: IGF-I and amino acids effects through TOR signaling on proliferation and differentiation of gilthead sea bream cultured myocytes

Authors: Vélez, E.J.*, **Lutfi, E.***, Jiménez-Amilburu, V., Capilla, E., Navarro, I., and Gutiérrez, J. (*equal contribution).

Journal: General and Comparative Endocrinology 205 (2014) 296-304

Impact factor: 2.470 Q3 (14)

Title: Lysine and leucine deficiencies affect myocytes development and IGF signaling in gilthead sea bream (*Sparus aurata*)

Authors: Azizi, Sh., Nematollahi, M.A., Amiri, B.M., Vélez, E.J., **Lutfi, E.**, Navarro, I., Capilla, E. and Gutiérrez, J.

Journal: Plos One 11 (2016) e0147618

Impact factor: 3.23 Q1 (14)

Title: Contribution of in vitro myocytes studies to understanding fish muscle physiology

Authors: Vélez, E.J., **Lutfi, E.**, Azizi, Sh., Montserrat, N., Riera-Codina, M., Capilla, E., Navarro, I. and Gutiérrez, J.

Journal: Comparative Biochemistry and Physiology B 199 (2016) 67-73

Impact factor: 1.55 Q2 (15)

Title: Understanding fish muscle growth regulation to optimize aquaculture production

Authors: Vélez, E.J., **Lutfi, E.**, Azizi, Sh., Perelló, M., Salmerón, C., Riera-Codina, M., Ibarz, A., Fernández-Borràs, J., Blasco, J., Capilla, E., Navarro, I. and Gutiérrez, J.

Journal: Aquaculture 20 (2017) 28-40

Impact factor: 1.893 Q2 (15)

Title: Moderate and sustained exercise modulates muscle proteolytic and myogenic markers in gilthead sea bream (*Sparus aurata*)

Authors: Vélez, E.J., Azizi, Sh., **Lutfi, E.**, Capilla, E., Moya, A., Navarro, I., Fernández-Borràs, J., Blasco, J., and Gutiérrez, J.

Journal: AJP, Regulatory Integrative and Comparative Physiology 312 (2017) R643-R653

Impact factor: 2.91 Q2 (15)

Professeur Patrick J. Babin

Laboratoire MRGM, Université de Bordeaux,
Allée Geoffroy St-Hilaire, Bât. B2, CS 50023
33615 Pessac cedex
FRANCE

Email : p.babin@gpp.u-bordeaux1.fr
Tel.: +33 (0)5 40 00 87 76

To whom it may concern,

May 30, 2017

M. Esmail Lutfi Royo was invited to join my laboratory, from March 18 to July 15 and from August 11 to September 10, 2014 through a collaborative research project engaged with Pr. Isabel Navarro from the Departament de Fisiologia i Immunologia, Facultat de Biologia, University of Barcelona, Spain. This project was entitled "Identification of new obesogenic molecules targeting adipocyte by using the zebrafish obesogenic test (ZOT)". This test was previously developed in our laboratory and Esmail used this test to evaluate the anti-obesogenic activity of some bioactive compounds of plant origin. It has been demonstrated the anti-obesogenic activity of caffeic acid (CA) and hydroxytyrosol (HT) *in vivo*. These compounds were able to counteract the obesogenic effect produced by the peroxisome proliferator-activated receptor gamma agonist, rosiglitazone. Further experiments performed in Isabel Navarro' lab demonstrated this anti-obesogenic activity on trout adipocyte cultured cells and some effects were also retrieved in the adipose tissue of HT-injected rainbow trout. *In vitro* and *in vivo* approaches demonstrated the anti-obesogenic potential of CA and HT on teleost fish models that may be relevant for studying their molecular mode of action.

Esmail did a very productive job within our laboratory and in the months that followed, which allowed the formalization of a scientific publication in PLOS ONE that will undoubtedly date in our field of research (Lutfi E, Babin PJ, Joaquim Gutiérrez J, Capilla E, Navarro I. Caffeic acid and hydroxytyrosol have anti-obesogenic properties in zebrafish and rainbow trout models. PLOS ONE, in press, 2017). This work was nicely complemented by a recently published work (Lutfi E et al., Aquat Toxicol. 188:148-158, 2017) also Esmail as a first author. Esmail is now obviously ready to defend his PhD thesis.

Sincerely,

Professor Patrick J. Babin

INRA SCIENCE & IMPACT

REPUBLIQUE FRANÇAISE
INSTITUT NATIONAL DE LA RECHERCHE AGRONOMIQUE
CENTRE DE RECHERCHES DE BORDEAUX-AQUITAINE

Aquapôle
UR 1067 Nutrition, Metabolism, Aquaculture (NuMeA)

Saint Pée-sur-Nivelle, June, 1st 2017

From 21 of August 2015 to 18 of December 2015, I had the pleasure to supervise Esmail Lutfi Royo within the research unit "Nutrition, Metabolism, Aquaculture" from the National Institute for Agronomic Research (INRA) in Saint Pée sur Nivelle, France. Esmail has joined our research unit in order to perform an internship abroad during his PhD.

During his stay in France, Esmail has worked on an experiment performed in the framework of the ARRAINA FP7 European project. The objective of this study was to determine in rainbow trout (Oncorhynchus mykiss), the effect of feeding broodstock with a methionine deficient diet on the response of several metabolism- and growth-related genes and proteins in offspring subjected to different dietary methionine levels (deficient or adequate).

Esmail has obtained original and interesting results that have been valued in a peer reviewed article.

Seiliez, I. ; Vélez, E. J. ; **Lutfi, E.** ; Dias, K. ; Plagnes- Juan, E. ; Marandel, L. ; Panserat, S. ; Geurden, I. ; Skiba-Cassy, S. Eating for two: Consequences of parental methionine nutrition on offspring metabolism in rainbow trout (Oncorhynchus mykiss). Aquaculture, 2017, 471 : 80-91.

During his internship, Esmail has improved his scientific skills in nutrition and metabolism as well as his expertise in molecular biology methods. Esmail was serious and dynamic; he became quickly autonomous in the lab and was highly appreciated by all.

Sandrine Skiba, INRA Research Director, PhD

Aquapôle, INRA, Quartier Ibarron, 64310 Saint-Pée-sur-Nivelle, France
Tel: +33 (0)5 59 51 59 51 - Fax: +33 (0)5 59 54 51 52
Tél. direct : +33 (0) 5 59 51 59 93- mail: sandrine.skiba@inra.fr – http ://www6.bordeaux-aquitaine.inra.fr/st_pee/

Etablissement Public à caractère Scientifique et Technologique placé sous la tutelle conjointe des ministres chargés de la Recherche et de l'Agriculture

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Dipartimento
di Scienze
della Vita
e dell'Ambiente
DISVA

Ancona, 16/05/2017

Support letter for Esmail Lutfi Royo

Esmail did an internship under my supervision in the lab of Biology of Development and Reproduction from 11th of July to 7th of November 2016. During this time, he was involved in two projects.

The aim of the former was to evaluate the duration of the effects of a probiotic *Lactobacillus rhamnosus* administration on growth and glucose metabolism during zebrafish (*Danio rerio*) development. As regard to the second project, the goal was to establish whether different photoperiod (24 h of light, 24 h of dark or 12/12 light/dark) could interfere with the microbial ecology of zebrafish larvae and in turn affects physiological response of the ones administered with probiotic.

To accomplish both goals, he approached using different techniques such real-time PCR and Western blot that permitted to evaluate the expression of a wide network of genes and several proteins involved in appetite, lipid metabolism and growth. In addition, further metagenomic analysis will establish eventual core microbiota changes in response to different photoperiod exposure. The elaboration of results is currently in progress.

Esmail actively participated to all laboratory activities and performed analyses at different levels, ranging from animal treatments, biochemistry and molecular biology to discussion and interpretation of data. The obtained results are particularly interesting due to their originality and will help to better understand the effects of the *Lactobacillus rhamnosus* on zebrafish gut microbial community under different photoperiod regime and their consequences on zebrafish metabolism.

Sincerely,
Oliana Carnevali

Prof. Biologia dello sviluppo
Dipartimento Scienze della vita e dell'ambiente
Università Politecnica delle Marche
Via Brecce Bianche
Ancona, Italy