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Abstract

L'obiettivo di questo studio è quello di valutare gli effetti del silenziamento del recettore dei glucocorticoidi (GR) (*Danio rerio*) sul metabolismo lipidico epatico in zebrafish**.**

I glucocorticoidi sono ormoni steroidei coinvolti in diversi processi fisiologici come la regolazione della risposta infiammatoria (hanno infatti effetti immunosoppressori), la risposta allo stress e il controllo dell'omeostasi energetica, soprattutto nel caso di disfunzioni metaboliche come l'insulino resistenza e il diabete legato all'obesità. Per poter svolgere la loro funzione, i glucocorticoidi, diffondono attraverso la membrana plasmatica e si legano a specifici recettori (GR). Questi ultimi, nella loro forma inattiva, sono legati alle proteine Hsp90 nel citoplasma, ma una volta avvenuto il legame con i glucocorticoidi migrano nel nucleo assieme al proprio ligando, dimerizzano e si legano a specifiche sequenze di DNA, gli elementi di risposta per i glucocorticoidi (GRE), poste nelle regioni promotrici dei geni di cui regoleranno l'espressione. Inoltre, è stato dimostrato che questi ormoni, possono legarsi a volte con un'affinità addirittura maggiore, ai recettori dei mineralcorticoidi (MR). La sintesi basale e il rilascio dei glucocorticoidi, sono influenzati dal ritmo circadiano, infatti la loro concentrazione fluttua

raggiungendo un picco di espressione di mattina negli animali diurni, all'inizio della fase notturna, invece, in quelli notturni. I gluococorticoidi stessi, inoltre, agiscono sui tessuti periferici, tra cui il fegato, dove le proteine clock regolano l'espressione di numerosi e differenti geni coinvolti nella biosintesi dei lipidi e nell'ossidazione degli acidi grassi. In questo studio, sono stati utilizzati degli zebrafish di due linee: wild-type e mutanti $gr^{-/-}$, della stessa età, con lo scopo di valutare gli effetti del silenziamento sul metabolismo lipidico. Inoltre, considerando il cross-talk tra ritmo circadiano e attività dei GC, gli zebrafish sono stati campionati in quattro momenti differenti: alle 5 (ZT 21), alle 11(ZT 3) , alle 17 (ZT 9) e alle 23 (ZT 15). La linea mutante $gr^{-/-}$ utilizzata è stata ottenuta nel laboratorio della Prof Dalla Valle nell'Università di Padova, (Facchinello et al., 2017) utilizzando la tecnologia CRISPR-Cas9. In questo studio, utilizzando un approccio integrato di dati molecolari, istologici e spettroscopici, sono stati analizzati gli effetti della mutazione gr, in relazione al ritmo circadiano, in pesci maschi e femmine.

Sia nella linea wt che in quella $gr^{-/-}$ è stato valutato il profilo di espressione di diversi geni coinvolti nel metabolismo lipidico, tra cui tre importanti geni master coinvolti nella sintesi dei lipidi, *srebp1*, *c/ebp* e *pparγ*. Nei mutanti è stata misurata una significativa up-regolazione, durante la fase diurna, dei

livelli di espressione di *srebp1*, che svolge un ruolo centrale nel controllo dell'espressione di geni coinvolti nella sintesi e nell'assorbimento dei lipidi, e di *c/ebp*, coinvolto nella secrezione di adipochine, nel metabolismo epatico del glucosio e nella sensibilità insulinica. Per quanto riguarda invece *pparγ*, esso è up-regolato nella linea $gr^{-/-}$, in tutti e quattro i tempi in cui è stato eseguito il campionamento ad eccezione di ZT 15:00 h nelle femmine, in cui risulta down-regolato. Passando all'analisi dei geni lipogenici *agpat4*, *fasn* e *hnf4*: nei mutanti di entrambi i sessi, è stata evidenziata un'acrofase diurna con una significativa up-regolazione di *agpat4*; *fasn* mostra un livello di espressione costante durante tutto il giorno; mentre, *hnf4* ha un'acrofase diurna ma è significativamente downregolato proprio in corrispondenza di essa. È stata poi analizzata l'espressione di *pepck2*, enzima chiave della gluconeogenesi nel fegato, i cui livelli, nei pesci $gr^{-/-}$, sono significativamente down-regolati, e, per quanto riguarda il trasporto, sono stati presi in considerazione *apoA4* e *apoBa*, anch'essi down-regolati. Questi risultati supportano l'ipotesi che la mutazione gr abbia degli effetti sul metabolismo lipidico epatico, tanto da modificare il profilo di espressione osservato nel ceppo wild type, scandito dal ritmo circadiano. Queste evidenze, sono supportate dalle analisi istologiche sul fegato che hanno messo in luce un aumento significativo di lipidi nel tessuto dei mutanti, che appare, così,

steatosico, sia nei maschi che nelle femmine. Maggiori informazioni sono state ricavate grazie all'analisi di microspettroscopia FT-IR, che ha messo in luce le differenze nella composizione chimica del fegato causate dalla mutazione. Concentrandosi sulla composizione lipidica, l'analisi FT-IR ha rivelato un aumento dei lipidi totali nei mutanti, in particolare riguardante i fosfolipidi e gli acidi grassi, conferamando i dati ottenuti dall'analisi molecolare. Per quanto riguarda gli zuccheri, sono evidenti differenze significative tra $gr^{-/-}$ maschi e femmine: nei primi è stato riscontrato un aumento generale nel livello di carboidrati e, in particolare, del glicogeno, contrariamente a quanto emerso nelle femmine, dove c'è una diminuzione netta degli stessi, facendo supporre una diversa richiesta energetica fra i due sessi. Inoltre, è stato valutato il profilo proteico: esso è significativamente ridotto in entrambi i sessi, nei fegati della linea mutante. I risultati ottenuti potrebbero essere spiegati con il fatto che, nonostante il recettore dei glucocorticoidi sia stato silenziato, essendo questi ormoni in grado di legarsi anche al recettore dei mineralcorticoidi (MR), potrebbero svolgere lo stesso la loro funzione legandosi all'MR. Quest'ultimo, è in grado di indurre la trascrizione dei geni selezionati, che proprio nei mutanti si osserva durante le fasi di luce, le quali corrispondono ad un aumento dei livelli di glucocorticoidi. L'altra ipotesi è che i glucocorticoidi potrebbero mediare

l'azione svolta dal sistema endocannabinoide, esercitando su di esso un'azione stimolatoria. Il fegato è infatti un importante tessuto target per l'azione svolta dagli endocannabinoidi e questi ultimi, andrebbero a stimolare la sintesi *de novo* di acidi grassi. Tutti questi risultati mostrano gli effetti dovuti al silenziamento del recettore dei glucocorticoidi sul metabolismo lipidico epatico indicando che la mutazione altera il profilo lipidico e che, insieme al corretto funzionamento del ritmo circadiano, risulta fondamentale per un normale metabolismo energetico. È stato inoltre possibile osservare una risposta sesso specifica della mutazione. Alla luce di questi risultati, si suppone di poter utilizzare la linea $gr^{-/-}$ di zebrafish, come modello per lo studio di malattie croniche del metabolismo e quindi valutare l'efficacia di possibili terapie mediche in questa linea mutante avente un profilo fisiologico simile a quello di pesci "obesi".

1. Introduction

1.1 Glucocorticoid hormones

Glucocorticoids (GCs) are steroid hormones secreted by adrenal cortex in mammals and by the interrenal tissue of the head kidney in teleosts. The main endogenous GC in humans and teleosts is cortisol, while corticosterone is the most present GC in rodents, reptiles and amphibians (Facchinello et al., 2017; Buckingham et al., 2016). The production of GCs is under control of a neuroendocrine feedback system, the hypotalamus-pituitary-interrenal (HPI) axis in fish, which corresponds to the hypotalamus-pituitary-adrenal (HPA) axis of mammals (Facchinello et al., 2017; Alsop et al., 2009).

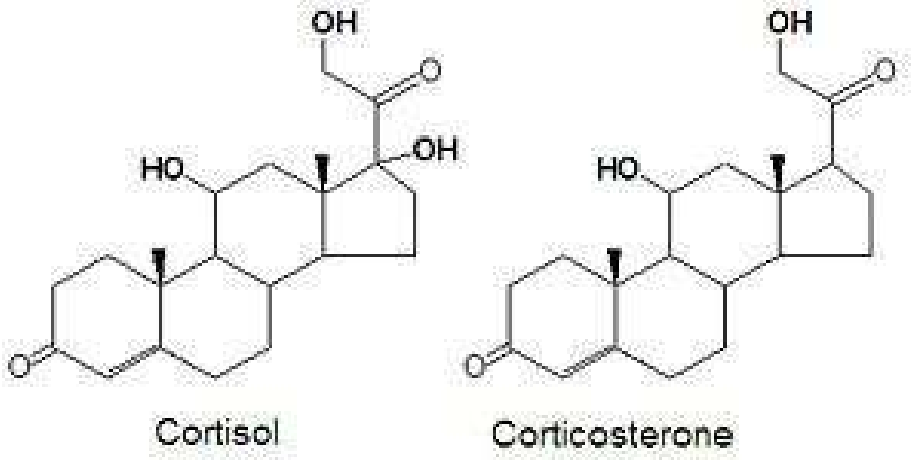


Figure 1. Chemical structure of two of the most common glucocorticoids: cortisol and corticosterone.

Glucocorticoids are involved in the regulation of different physiological processes, including the regulation of inflammation and stress response. Moreover, the role played in the control of energy homeostasis is important, above all in the case of metabolic dysfunction, including insulin resistance, obesity-related diabetes, fasting and starvation (Rose AJ et al., 2013; de Guia et al., 2014; Baschant et al., 2013). Their involvement in embryonic development is also important (K. S. Wilson et al., 2013).

To exert their effects and activate the GC signalling pathway, these hormones bind to their cognate, intracellular receptor, the glucocorticoid receptor (GR), that is ubiquitously expressed in almost all cells. Glucocorticoid receptor was cloned in 1985 by Ron Evans.

GR is a member of a superfamily of ligand-inducible transcription factors that controls a variety of physiological functions and also includes mineralocorticoid receptor (MR), progesterone receptors (PRs), estrogen receptors (ERs) and androgen receptors (ARs). These receptors have a common structural organization consisting of three major domains: a N-terminal domain (NTD), a DNA binding domain (DBD), a C-terminal ligand binding domain (LBD). The amino terminal domain (NTD) is variable among receptors with the exception of a region rich in negatively charged acidic amino acids, that is known as AF-1 (activation function-1) and activates

transcription by a direct interaction with basal transcriptional machinery. In the absence of ligands, the GR resides in the cytosol complexed with some proteins, among which the heat shock protein 90 (HSP90) that interacts with LBD and prevents the receptor from binding DNA. LBD also contains a second activation function (AF-2) domain, which interacts with transcriptional co-activators and co-repressors. After GC binding, GR is released from HSP90 and translocates into the nucleus where it can regulate gene expression via one of two pathways: transactivation, whereby it serves as a DNA sequence-specific transcriptional regulator; transrepression, in which GR takes action through direct protein-protein interactions with other transcriptional regulators (de Guia et al., 2014; Heitzner et al., 2007).

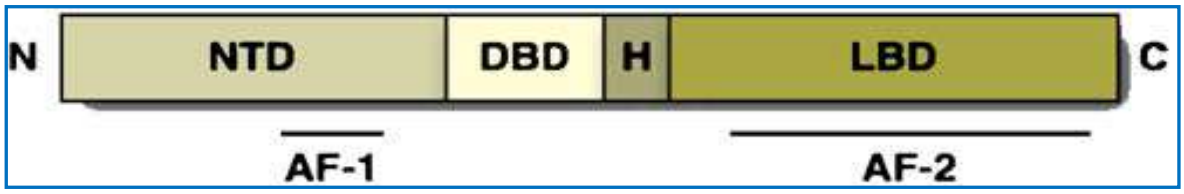


Figure 2. General schematic of steroid hormone receptor domains (Heitzer et al., 2007).

Unlike many teleosts that have two *gr* genes, *gr1* and *gr2,* zebrafish (*Danio rerio*) has only one glucocorticoid receptor gene (*gr, nr3c1*), with a close organization to that of human gene, *hGR.* Both genes consist of 9 exons and two isoforms, Gr-α and Gr-β are present in zebrafish and humans, deriving

from alternative splicing processes. An important difference is the location of the sequence encoding β-isoform-specific amino acids. In the human gene, this sequence is located in exon 9, whereas in the zebrafish gene it is found in exon 8.

1.2 Hepatic lipid metabolism and hepatic functions of GCs

The liver is a central metabolic organ involved in the control of mammalian glucose and lipid homeostasis (van den Berghe, 1991), and is responsible for several functions: detoxifies various metabolites, synthesizes proteins and produces biochemicals necessary for digestion. It plays an important role in lipid metabolism; a complex process including the synthesis of fatty acids, triglycerides and lipoproteins (Nguyen et al., 2007). At the time when energy is required, such as during the fasting, triglycerides are broken down and fatty acids and glycerol are transported to the cells, being this process regulated by stress hormones, catecholamines and glucocorticoids (ie, cortisol in human and corticosterone in rodents) (Peckett et al., 2011).

Glucocorticoids in fact, play an important role on this regard. Genome-wide analysis of GC-regulated target gene networks has shown that the GR controls many aspects of hepatic energy metabolism. More than 50 genes seem to be direct, regulatory targets of GC action. In many cases, the GR functionally

interacts with other transcription factors to control specific genetic networks in the liver (Le Phuc et al., 2005), among which only few have been characterized.

To date, it is known that they induce lipogenic genes, regulate lipolysis, counteract adipose tissue inflammation in obesity and are involved in the differentiation of adipocyte precursors (Lee et al., 2013). Moreover, the orexigenic function of glucocorticoids appear to be under the control of GR action in the nervous system. Specifically, GCs do not directly affect appetite, but interact with neuropeptide Y (NPY) and agouti-related peptide (AgRP). Stimulation of NPY/AgRP neuron in arcuate nucleus promotes food intake: AgRP released from the neurons can induce a delayed and prolonged feeding behavior while NPY works for the rapid response of feeding action (Hirayama et al., 2018). Under normal, basal (fasted) physiological state, GCs promote lipolysis in adipose tissues by inducing activity of major lipases, e.g. pancreatic lipases, and reducing activity the lipoprotein lipase (LPL); these hormones also increase the lipogenic function of insuline. *In vivo* and i*n vitro* studies have shown that GCs regulate the expression of acetyl-CoA carboxylase (ACC) and fatty acid synthase (FASN), a multi-enzyme protein that catalyzes fatty acid synthesis; they are also involved in the regulation of 1-acylglycerol-3-phosphate acyltransferase (AGPAT2), lipin 1 (LPIN1),

strearoyl-CoA desaturase (SCD1/2) and dyacylglyceride acyltransferase (AGPAT2) (de Guia et al., 2014).

GC/GR pro-lipogenic activity in the liver seems to require insulin signaling (Dich et al., 1983; Mangiapane et al., 1986). This process is possibly important in the development of hepatic steatosis prior to the onset of insulin resistance in Metabolic Syndrome (Choi et al., 2011). Hepatic steatosis is, in fact, a prominent negative side-effect of long- term GCs treatment (Vegiopouloset al., 2007). In accordance to this, liver specific GR loss-of function reduces TG levels in normal (Opherk et al., 2004) as well as diabetic (Lemke et al., 2008) mice and reduces accumulation of TG in liver during liver regeneration (Shteyer et al., 2004).

Apart from potential direct effects on the enzymes of de novo lipogenic pathway and TG synthesis, GCs can regulate expression of secreted factors that can likewise affect lipid load in hepatocytes. GCs induce secretion of a series of factor, with well described pro-lipolytic activity able to trigger the release of NEFA in the circulation which can serve as substrate for TG synthesis in the liver. The liver, aside from being at the receiving end for NEFA, can transport lipids to peripheral tissues via very-low density lipoproteins (VLDL). Promotion of VLDL secretion by GCs is possibly due

to increased production and stabilization of *ApoB* together with increased triglyceride synthesis (Wang et al., 1995).

Much attention has focused on the role played by GCs on hepatic lipogenesis, since they result to be positively involved in this process (Peckett et al., 2011). Lipid metabolism can be studied at molecular level considering the expression of those genes involved in the synthesis (lipogenesis) and degradation (lipolysis) of fats within the cells. Among those genes playing a role in lipogenesis, in this contest, we focused on:

- sterol regulatory element-binding protein (*srebp*), is a central player in controlling the expression of signals involved in lipid synthesis and uptake, resulting mainly the isoform 1 (*srebp1*) involved in fatty acid metabolism;
- fatty acid synthase (*fasn*), codifies for a multi-enzyme protein that catalyzes fatty acid synthesis;
- peroxisome proliferator-activated receptor-γ (*pparγ*), is a critical gene in adipogenesis that orchestrates adipocyte function and differentiation as well as lipid storage within adipocytes;
- CCAAT/enhancer binding protein (*c/ebp*), is involved in the secretion of adipokynes, insulin sensitivity and glucose hepatic metabolism;

- 1-acylglycerol-3-phosphate o-acyltransferase (*agpat4*), a coding gene for a protein that catalyzes the second acylation step in the glycerol phosphate pathway;
- hepatocyte nuclear factor 4 (*hnf4*), a major transcriptional regulator of many genes related to lipid homeostasis in the liver and a marker of liver condition;
- phosphoenolpyruvate carboxykinase 2 (*pepck2*), the key enzyme of gluconeogenesis in the liver.

A side to lipid synthesis, transport should also be considered, focusing as key signal on :

- apolipoprotein B (*apoBa*), involved in the assembly of very low density lipoprotein;
- apolipoprotein a-IV (*apoA4*), involved in chylomicrons and VLDL secretion, should be considered as key signal involved in the process (Santangeli et al., 2018; Forner-Piquer et al., 2017; Paredes et al., 2015; Falcinelli et al., 2015).

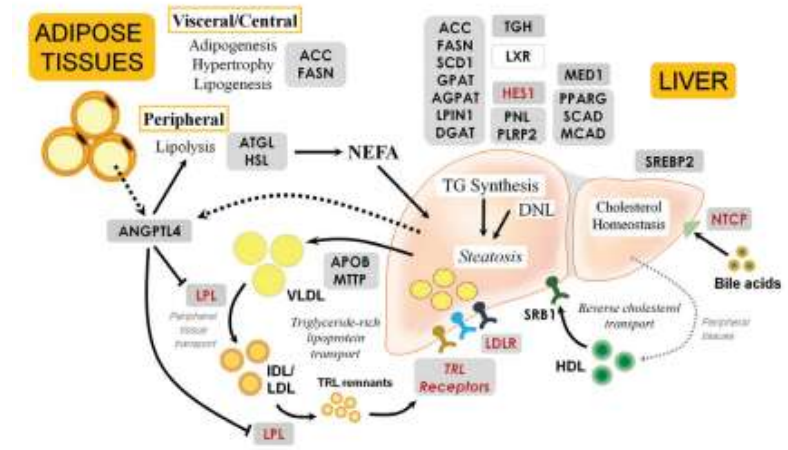


Figure 3. Lipid metabolic processes regulated by GC/GR signalling in adipose tissues and the liver (de Guia et al., 2014).

1.3 Circadian clock

Living organisms live in an environment cyclically marked by events such as the alternation of light between day and night or the seasons. In relation to these variations, a biological timekeeping, by an internal clock, is universal to all living species, from bacteria to mammals. So every organism adapts its behavior and physiology to a 24-hour cycle, the circadian rhythm. The term "circadian" coined by Franz Halberg, comes from the Latin *circa diem* and means "around the day".

Circadian rhythms are important to regulate a wide variety of biological processes that influence cellular metabolic pathways and organ functions,

including sleep, hormone regulation and thermoregulation. Important hallmarks of endogenous circadian oscillators are:

- the capacity to synchronize to external signals, called Zeitgeber (from German Zeit: “Time”; geben: “to give”), which can be natural light or environmental temperature or stimuli of a social nature (time of lunch)
- the temperature compensation, indeed the organism performance to compensate for effects caused by changes in temperature (Weger et al., 2013; Tsuchiya et al., 2003)

The circadian clock consists of three major components:

1. a central biochemical oscillator with a period of about 24 hours that keeps time;
2. a series of input pathways to this central oscillator to allow entrainment of the clock;
3. a series of output pathways that regulate overt rhythms in physiological and behavioral activities.

Vertebrate biological clock keeps the circadian rhythm through a cross-talk between an endogenous pacemaker, that receives external signals from photoreceptors, and several peripheral clocks. The central pacemaker located

in the brain and peripheral clocks, that can be in various cells and tissues, are in contact through nervous and hormonal messages.

At the cellular level, the central pacemaker in mammals has been found in the hypothalamic suprachiasmatic nucleus, which receives information of light and darkness from the photosensitive retinal cells. However, in the hypothalamus, SCN neurons interact also with neighboring nuclei. Instead, in zebrafish, as in other nonmammalian vertebrates, this role belongs to the pineal gland: it is photoreceptive and contains an intrinsic circadian clock that drives rhythmic synthesis of the hormone melatonin. This hormone has a nocturnal expression, in fact its level decreases during the day, regulated by the enzyme AANAT (serotonin-N-acetyl-transferase). Furthermore, unlike mammals, peripheral zebrafish clocks are directly influenced by light, as demonstrated in the study done by Whitmore on zebrafish Pac-2 embryonic cell lines (Idda et al., 2012; Vatine et al., 2009; Whitmore et al., 2000).

At the molecular level the mammalian circadian rhythms consist of a transcriptional autoregulatory feedback loop that involves core clock genes. These genes include transcriptional activators, ie circadian locomotor output cycles kaput (CLOCK), the closely related neuronal PAS domain protein 2 (NPAS2) and brain and muscle ARNT-like protein 1 (BMAL1). The heterodimeric complex CLOCK-BMAL1 is able to bind the E-box regions of

Period (PER1, 2, and 3) and *Cryptochrome* (CRY1 and 2) promoting their transcription. This transciption continues during the night until the nuclear levels of the Per and Cry proteins become high enough to suppress the activation of *Clock/Bmal1*. The decrease of Per/Cry levels in the early hours of the morning then allows a new transcription of genes and the beginning of a new cycle (Hirayama et al., 2018).

This situation is even more complex in fish: the zebrafish genome contains far more copies of different clock genes than the mammalian genome, probably as a consequence of a genome duplication event in teleost evolution. Thus, zebrafish has six *cry* (*cry1a, 1b, 2a, 2b, 3 and 4*) and four *period* genes (*period 1a, 1b, 2, 3*). Unlike mammals, the *Clock* gene expression exhibits daily fluctuations (Idda et al., 2012; Whitmore et al.,1998).

1.3.1 Circadian clock and glucocorticoids

The concentration of glucocorticoid hormones fluctuates in the organism with a circadian rhythm. In diurnal animals, including humans, the levels of glucocorticoids reach a peak in the morning, while in nocturnal animals the peak is evident at the beginning of the night. In turn, glucocorticoids act on

the circadian rhythm, in particular on the peripheral clocks of some organs and tissues, influencing their gene expression.

The *Per1* gene promoter contents tandem GREs (glucocorticoid response element): its transcription is dependent on GCs. The *Clock/Bmal1* heterodimer uses the C-terminal region of the Clock protein to interact with GR, preventing the binding to its cognate DNA sequence GREs. Thus, *Clock/Bmal1* is a negative regulator of GCs action in target tissues. Therefore, glucocorticoids are used by the pacemaker as a signal to control the rhythm of the body's peripheral clocks and are in turn regulated by the central clock (Nader et al., 2010).

Specifically in mammals, it has been shown that glucocorticoids alone are able to synchronize expression of about 60% of the circadian transcriptome. Furthermore, the *HNF4* gene was identified within the liver as a mediator of circadian and glucocorticoid-regulated transcription (Reddy et al., 2007).

1.3.2 Circadian clock and lipid metabolism

In mammals, clock genes function both at the central pacemaker level and in peripheral organs involved in metabolism, such as liver, adipose tissue,

pancreas and skeletal muscle. Many studies conducted on rodents show that they regulate glucose metabolism in the liver, in fact:

- in *Clock∆19* mutant and *Bmal1* knockout mice the gluconeogesis is compromised;
- hepatic glucose export is dysregulated in liver-specific *Bmal1* knockout mice.

CLOCK and BMAL1 regulate mitochondrial biogenesis and respiratory function in skeletal muscle and are also involved in pancreatic insulin secretion. PER2 and BMAL1 act on adipose tissue, regulating adipocyte differentiation, de novo lipogenesis and fatty acid oxidation (Yoshino and Klein, 2013).

Shostak et al. have conducted studies in wild-type mice and genetic mouse models (ClockΔ19 mutant, Bmal1 KO and Per::Luciferase knock-in mice); the experiments demonstrate an important function of clock genes in regulating lipolytic activity in white adipose tissue and in providing a rhythmic release of fatty acids and glycerol from adipocytes. These latter processes are essential for survival during fasting and prolonged physical activity. Indeed, disruption of central or peripheral circadian rhythms can cause an increase in food intake and obesity and consequently alteration of

metabolic pathways (Shostak et al., 2013). Furthermore, as regards gene expression, it has been shown that the regulation of different genes involved in lipid metabolism is mediated by two nuclear receptor superfamilies, the REV-ERBs and the retinoic acid receptor-related orphan receptors (RORs). RORα and REV-ERBα are major regulators of the cyclic expression of BMAL1; and specifically, REV-ERB1α controls the expression of genes involved in cholesterol and lipid homeostasis through sterol regulatory element binding protein (SREBP) pathways and SREBP target genes, such as *Srebp1* (involved in fatty acid metabolism) and *Srebp2* (essential for cholesterol biosynthesis). Other transcription factors under the influence of the circadian rhythm are the peroxisome proliferator-activated receptor-α (PPARα) and PPARγ, which regulate energy homeostasis in several tissues (Solt et al., 2011; Betancor et al., 2014). Thus, clock-controlled systems and metabolic pathways interact in a feedback loop; mammalian liver shows lipolytic and lipogenic rhythms with the acrophase located at the different time points of the daily rhythm, in order to prevent metabolic disorders such as diabetes or obesity (Hernandez-Pérez et al., 2016).

However, unlike mammals, little is known about the circadian regulation of lipid metabolism in teleosts. Daily rhythms in the activity of enzymes related to energy metabolism have been observed in fish liver (Polakof et al., 2007b).

An important study was conducted by Hernandez-Pérez with the aim of investigating in a teleost (rainbow trout) possible daily changes of the activity and mRNA abundance of proteins involved in carbohydrate and lipid metabolism in liver. This study also aimed at investigating whether there was an influence of light-dark cycle or food availability on these rhythms. The results showed that the analyzed genes, including *pepck, glut2, pk* and *fas,* are characterized by circadian rhythms, even in the absence of light or food (Hernandez-Pérez et al., 2015).

In a study on zebrafish, it was firstly described the daily rhythms of expression of lipid metabolic genes in the liver and their synchronization to light/dark and feeding cycles. In particular:

- lipolytic genes (*lpl, pparα* and *hadh*) displayed an acrophase in the dark phase between ZT 02:17 h (11:17 p.m.) and ZT 18:31 h (3:31 a.m.);
- the genes associated with lipogenesis (*lepa, srebp, lxr, pparγ and fas*) showed an acrophase between ZT 15:25 h (00:30 a.m.) and 20:06 h (5:06 a.m.).

These results demonstrate that gene expression in the zebrafish liver is controlled by both light and feeding (Paredes et al., 2015).

1.4 The gr$^{-/-}$ mutant line

In this study a zebrafish mutant line is used. This line was created with the aim to obtain a zebrafish model completely devoid of transcriptional GC activity; for this purpose, a mutation was made on the *gr* gene (*n3rc1*) using CRISPR/Cas9 technology. The F2 generation was obtained by using a heterozygous F1 offspring with a 5-nt insertion in exon 2. This insertion caused a frameshift mutation leading to the presence of a premature stop codon located upstream of DBD. The corresponding encoded protein contains 331 aa, of which the first 301 belong to the zebrafish Gr and in addition there are other 20 new aa.

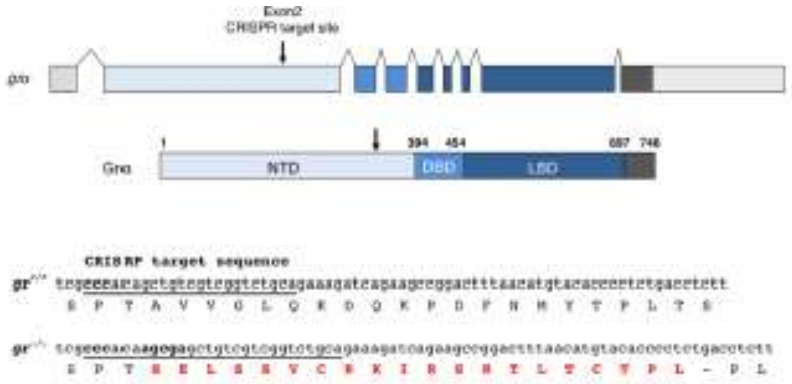


Figure 4. The image shows the zebrafish *gr* gene. Arrow indicates the site in exon 2 that was modified with CRISPR/Cas9 technology. The 20 new aa resulting from the insertion are shown in red. (Facchinello et al., 2017)

Experiments on *gr*$^{-/-}$ mutants revealed that they are fertile, but their reproductive capabilities fall at around 10 months of age, when intestinal and cardiac abnormalities become more pronounced and increased fat deposits are also observed. The HIP axis is dysregulated in the gr mutant lines, which show higher levels of whole-body cortisol. Histology of 8-month-old *gr*$^{-/-}$ zebrafish reveals an intestinal mucosa with sloughing epithelium at the villous tips and reduced height of villi, consistent increase of subcutaneous adipose tissue and reduced extension of pancreas. Futhermore, the immune response is also compromised in the *gr*$^{-/-}$ line (Facchinello et al., 2017).

Experimental model

Zebrafish as model organism: a brief description

Zebrafish (*Danio rerio*) is a freshwater fish, belonging to the Cyprinidae family and native of the Himalayan region. Nowadays, it is an important and widely used vertebrate model organism alongside with the mouse in scientific research for studies of vertebrate development and gene function. The use of zebrafish is advantageous for several reasons such as the small size of both larvae and fish, the high fertility of the species, the transparency of the eggs/embyo during the first phases of development and the short life-cycle reaching the sexual maturity in few months. Moreover, its genome has been

fully sequenced, and this organism exhibits a diurnal sleep cycle with similarities to mammalian sleep behavior. All these hallmarks confer to this model the great interest of the scientific community, increased by the availability of several mutant and transgenic lines (see https://zfin.org) (Hill et al.,2005; Penglase et al.,2012).

Aim of the thesis

Glucocorticoids play an important role on the control of energy homeostasis and in the regulation of many physiological processes through the activation of its cognate receptor (GR) and their concentration fluctuates with a circadian rhythm under the control of an internal clock. Moreover, clock genes exert an action in different peripheral organs involved in the metabolism, including the liver. Starting from these considerations, we used 6-mpf wild-type zebrafish and $gr^{-/-}$ mutants to assess the effects of gr silencing on lipid metabolism. Considering the crosstalk existing between circadian rhythms and Gr activity, fish were sampled at 4 different time points: 5 (ZT 21), alle 11(ZT 3), alle 17 (ZT 9) e alle 23 (ZT 15). Overall this thesis aimed to analyze and compare the hepatic expression profile of several important genes involved in lipid metabolism, in the two zebrafish lines mentioned above: wild-type and $gr^{-/-}$ mutants. Moreover, potential sex

specific effects of the mutation on metabolism regulation were as well evaluated. Thanks to an integrated approach of molecular, histological and spectroscopic data, evidence of the effects of gr mutation, in relation to the circadian clock, were also provided in male and female fish.

2. Materials and Methods

2.1 Animal rearing

Adult female and male zebrafish (*Danio rerio*, wild-type strain and gr $^{-/-}$ mutants) were maintained in 100-L aquaria with oxygenated water under controlled conditions (28.0±0.5°C) in accordance with protocols and procedures approved by the University of Padova Animal Care Committee. Fish were fed two times per day, once with commercial adult zebrafish complete diet (Zeigler Bros., Inc.) and once with *Artemia salina* nauplii. A photoperiod was set at a 12h:12h LD cycle, with lights on (Zeitgeber time 0, ZT0) at 8.00 a.m. They were sampled at ZT3 (11 a.m.), ZT9 (5 p.m.), ZT15 (11 p.m.) and ZT21 (5 a.m.). For histology and FTIRM analysis, fish analyzed were sampled at ZT9.

2.2 RNA extraction and cDNA synthesis

Total RNA was extracted from 10 liver pieces per group, wt and gr $^{-/-}$ using RNAzol solution (Sigma Aldrich, Milan, Italy). RNA concentrations were determined with a NanoDrop™ 1000 Spectrophotometer (NanoPhotometer™ P-Class, IMPLEN, Germany). RNA quality was assessed with 1% agarose gel electrophoresis. Two micrograms of total RNA was used for cDNA synthesis

using iScript cDNA Synthesis Kit (Bio-Rad, Milano, Italy) and stored at 20°C.

2.3 Real-time PCR

qRT-PCRs were performed with SYBR green in a CFX thermal cycler (Bio-Rad) as described in Carnevali et al., (2017). Ribosomal protein L13 (*rpl13*) was used as housekeeping gene in order to standardize the results and eliminate variation in mRNA and cDNA quantity and quality. Primer sequences, GenBank accession numbers and annealing temperature for the genes analyzed are reported in **Table 1**. No amplification products were observed in negative controls and no primer-dimer formations were observed in the control templates Primers used at a final concentration of 10 pmoL/mL. Data were analyzed using iQ5 Optical System version 2.1 (Bio-Rad) including Genex Macro iQ5 Conversion and Genex Macro iQ5 files. Modification of gene expression among the experimental groups are reported as relative mRNA abundance (Arbitrary Units). Primers used at a final concentration of 10 pmoL/mL.

Gene	Abb.	Forward primer 5'-3'	Reverse primer 3'-5'	NCBI Acc Number	Ta (˚C)
Ribosomal protein L13	*rplp13*	TCTGGAGGACTGTAAGAG GTATGC	AGACGCACAATCTTGAGA GCAG	NM_212784.1	59
sterol regulatory element-binding protein 1	*srebp1*	GACACTTCTCTGGACACTC TG	ATCGAACAGCCCAAACTCC	NM_001105129	60
peroxisome proliferator-activated receptors γ	*pparγ*	CTGCCGCATACACAAGAAG A	TCACGTCACTGGAGAACTC G	NM_131467	59
CCAAT/enhancer binding protein	*c/ebp*	AACGGAGCGAGCTTGACTT	AAATCATGCCCATTAGCTG C	NM_131885	57
fatty acid synthase	*fasn*	ATGAAACACACAGGGACTC AGG	TTCTTGAATCTGAACGCGG GTA	XM_001923608	60
phosphoenolpyruvate carboxykinase 2	*pepck2*	CTGTGTGCTCATCCAAACT CC	GATCTCATAGCTGCACCAA CG	NM_213192	60
1-acylglycerol-3-phosphate o-acyltransferase	*agpat4*	TGCTGAAAACTCAGTTGCT G	GACCATAAACGGTGCCAA CT	NM_212992	56
hepatocyte nuclear factor 4α	*hnf4*	ACGGTTCGGCGAGCTGCTT C	TCCTGGACCAGATGGGGG TGT	NM_194368	58
apolipoprotein B	*apoBa*	TGAGAATGGGGCTTTGGG TCTA	ATGTCGTGAGGGACGGGA AA	XM_689735	60
apolipoprotein a-IV	*apoA4*	GGCCTACTGGTGGACTAAG C	TGGTTTGGGCTCATCAGCA T	NM_001079861	60

Table 1. Primer sequences used for qPCR analysis. The table shows the gene target name and acronym, the annealing temperature (Ta), and the accession numbers.

2.4 Liver histology

Livers were collected from 5 wt and 5 $gr^{-/-}$ different fish specimens and fixed by immersion in Bouin's solution (Sigma-Aldrich, Milano, Italy) and stored at 4 °C for 24 h.

The samples were dehydrated through a graded series of ethanol and embedded in Paraplast plus (Leica, 39602004). The samples were serially cut into 7/8-µm sections on a LKB microtome. After rehydration, the sections were stained with haematoxylin and eosin and mounted with Eukitt (BioOptica, 09-00100) for microscopic examination.

2.5 FTIRM analysis and data treatment

Fourier Transform Infrared Microspectroscopy (FTIRM) was used to analyze liver section of male and female specimens of WT and GR zebrafish. Five liver samples were collected from each experimental group (WT_M, WT_F, GR_M and GR_F) and stored at -80°C. Just before IR measurements, samples cut by a cryomicrotome. From each sample, three sections (≈10 µm thickness) were obtained at 200 µm interval, immediately deposited without any fixation process onto CaF_2 optical windows (1 mm thickness, 13 mm diameter) and air-dried for 30 min (Giorgini et al., 2015a, 2015b). FTIRM measurements were performed by using a Perkin Elmer Spectrum GX1 spectrometer (Waltham, Massachusetts, USA), equipped with a Perkin Elmer AutoIMAGE microscope and a photoconductive HgCdTe array detector, operating at liquid nitrogen temperature (Spectrum Image 5.1.0 software package, Perkin Elmer). By means of a microscope television camera, on each section ~ 30 areas

(50x50 μm) were selected, on which the IR spectra were acquired in transmission mode in the 4000-800 cm^{-1} spectral range (spectral resolution 4 cm^{-1}, 64 scans). Before each acquisition, the background spectrum was acquired on a clean portion of the CaF_2 optical window.

Raw IR spectra were preprocessed with OPUS Atmospheric Compensation routine (to correct the atmospheric contributions of carbon dioxide and water vapor), and then vector normalized on the full frequency range (to avoid artifacts due to local thickness variations) (OPUS 7.1 software package).

Pre-processed spectra were converted in second derivative mode (9-points smoothing) and submitted to Principal Component Analysis (PCA) by exploiting the 'Principal Component Analysis for Spectroscopy' tool on OriginPro 2018b (OriginLab Corporation, Northampton, MA). PCA was performed in a pair-wise manner (WT_M *vs* GR_M and WT_F *vs* GR_F). To detect the spectral variation among the experimental groups, PC scores were also displayed by loading spectra, which contained positive and negative peaks. PCA was, then, coupled with linear discriminant analysis (LDA): in first instance, PCA was used to reduce redundant information from the spectral dataset to PC scores, which were then used as variables for LDA (OriginPro 2018b). The number of PCs selected corresponded to the sum of

PC scores explaining a 95% of cumulative variance. A leave-one-out cross-validation was performed.

Based on PCA-LDA analysis, IR spectra were integrated under the following spectral ranges: 3000-2826 cm^{-1} (stretching modes of lipid alkyl chains, representative of lipids, LIP); 1768-1709 cm^{-1} (stretching mode of C=O moiety, representative of fatty acids, FA); 1709-1487 cm^{-1} (vibrational modes of peptide linkage, representative of proteins, PRT); 1284-1203 cm^{-1} (asymmetric stretching mode of phosphate groups, PH1); 1184-1132 cm^{-1} (stretching mode of C-OH moiety, representative of carbohydrates, COH); 1125-1059 cm^{-1} (symmetric stretching mode of C phosphate groups, PH2); 1059-984 cm^{-1} (vibrational modes of glycogen, GLY). The following band area ratios were then calculated: LIP/LIVER (representative of total liver lipids); FA/LIVER (representative of total liver fatty acids); PRT/LIVER (representative of total liver proteins); PH1/LIVER and PH2/LIVER (representative of total liver phosphates); COH/LIVER (representative of total liver carbohydrates), and GLY/LIVER (representative of total liver glycogen). LIVER, calculated as the sum of the integrated area 3000-2826 cm^{-1} and 1768-900 cm^{-1}, was considered representative of the total liver biomass.

2.6 Statistical analysis

Real time PCR results are presented as means ±SD and Two-Way ANOVA followed by the Tukey test as a multiple comparisons test was used for comparison between the two experimental groups at the different ZTs.

FTIRM results are presented as mean ±SD and are analyzed followed by Student's t-test. All statistical analyses were performed using the statistical software package Prism5 (Graphpad Software, Inc. USA) with significance accepted at $P < 0.05$. Asterisks (*) indicate statistical significant differences between wt and gr fish.

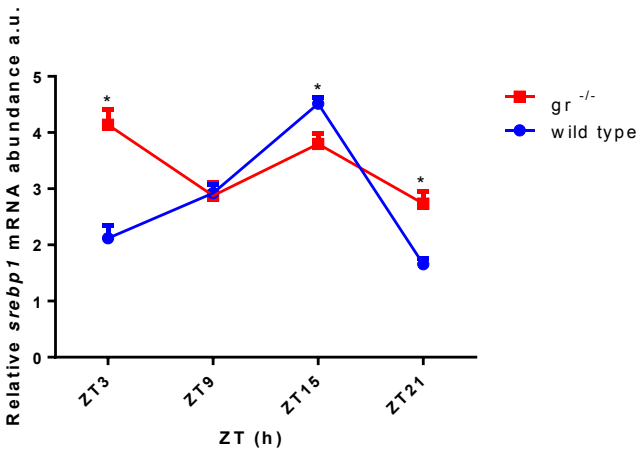
3. Results

3.1 Molecular findings

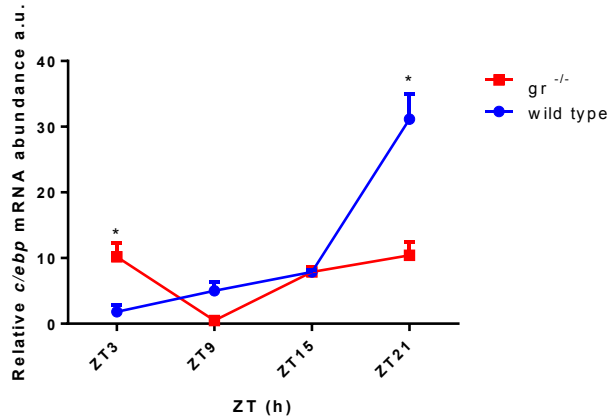
3.1.1 Gene expression analysis in wild-type and gr $^{-/-}$ zebrafish mutants: hepatic mRNA abundance according to their daily rhythms in males

The master genes hepatic lipid metabolism analyzed were *srebp1*, *c/ebp* and *pparγ*. In male, *srebp1* mRNA expression, showed an acrophase in the dark phase (ZT 15:00 h) in the wild-type strain; the acrophase is shifted to ZT 3:00 h in gr$^{-/-}$ mutant line. An up-regulation at ZT 3:00 h and ZT 21:00 h was reported in mutants (Fig. 5a). In the wild-type strain, *c/ebp* acrophase was observed in the dark phase (ZT 21:00 h); while conversely, was diurnal in mutants (ZT 3:00 h). *c/ebp* mRNA expression was significantly increased at ZT 21:00 h in wild-type, and at ZT 3:00 h in *gr*$^{-/-}$ zebrafish (Fig. 5b). Concerning *pparγ,* its expression displayed a diurnal acrophase (ZT 3:00 h) both in wild-type and mutant fishes; an up-regulation of general mRNA abundance was reported in *gr*$^{-/-}$ mutant group (Fig. 5c).

a)


Relative srebp1 mRNA abundance a.u.
0
1
2
3
4
5
*
*
*
ZT3
ZT9
ZT15
ZT21
ZT (h)
gr -/-
wild type

b)


Relative c/ebp mRNA abundance a.u.
0
10
20
30
40
*
*
ZT3
ZT9
ZT15
ZT21
ZT (h)
gr -/-
wild type

c)

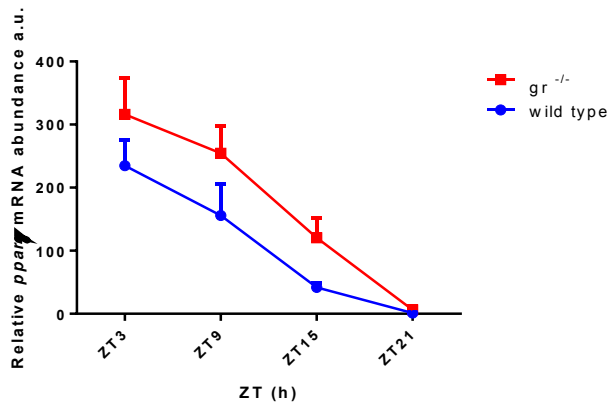

Relative ppar mRNA abundance a.u.
0
100
200
300
400
ZT3
ZT9
ZT15
ZT21
ZT (h)
gr -/-
wild type

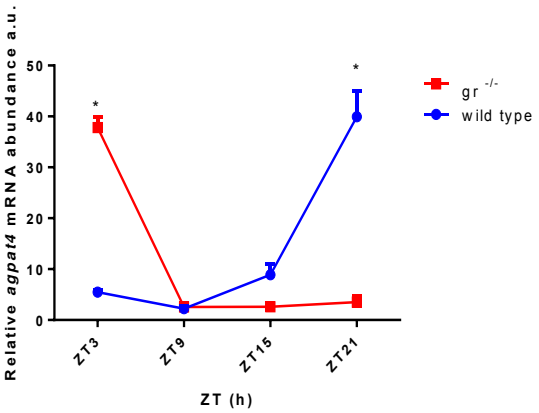
Figure 5. Transcription profile of master genes of hepatic lipid metabolism. *Srebp1* (a); *c/ebp* (b); *pparγ* (c) mRNA expression in the liver of male zebrafish. In wild-type the trend of expression is shown in blue, while mutants are in red. Asterisks denote statistical significant differences between wt and $gr^{-/-}$ at each specific ZT.

Among lipogenic genes, mRNA levels of *agpat4, fasn* and *hnf4* were analyzed.

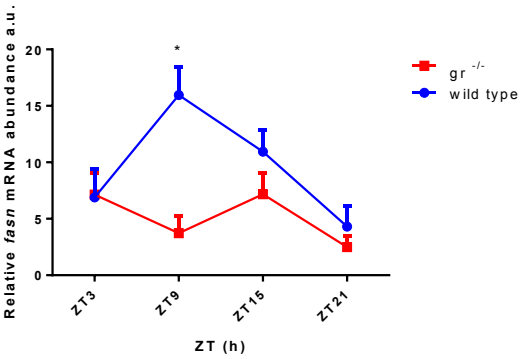
agpat4 mRNA displayed an acrophase at ZT 21:00 h in the wild-type strain, while it was shifted to daylight hours in $gr^{-/-}$ mutant line (ZT 3:00 h). Moreover, *agpat4* expression level was up-regulated at ZT 21:00 h in the wild-type strain and at ZT 3:00 h in $gr^{-/-}$ mutants (Fig 6a). *fasn* mRNA displayed a diurnal acrophase in wild-type zebrafish and at ZT 9, the level was significantly higher than in mutants (ZT 9:00 h) where this gene showed no significant rhythmicity, with the level remaining almost constant during the day (Fig. 6b).

Regarding *hnf4* a diurnal acrophase was shown (ZT 9:00 h) in wild-type zebrafish, with a similar trend, both in wild-type and $gr^{-/-}$ lines from ZT3 to ZT 15. mRNA value at ZT 9:00 was significantly higher in wt, while at ZT21 h, mRNA levels were significantly higher in $gr^{-/-}$ (Fig. 6c).

a)



b)



c)

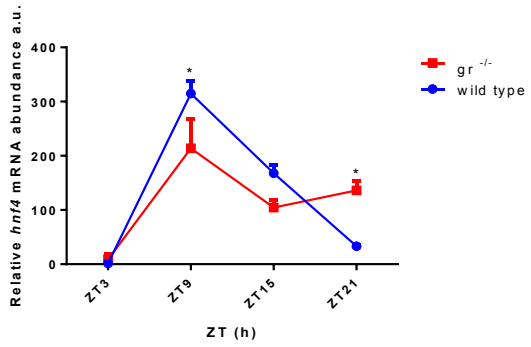


Figure 6. Transcription profile of lipogenic genes. *Agpat4* (a); *fasn* (b); *hnf4* (c) mRNA expression in the liver of male zebrafish. In wild-type the trend of

expression is shown in blue, while mutants are in red. Asterisks denote statistical significant differences between wt and *gr* $^{-/-}$ at each specific ZT.

In wt male zebrafish, *pepck2* showed a nocturnal acrophase (ZT 15:00 h) corrisponding to a significant increase in mRNA level. In gr$^{-/-}$ line, levels resulted significantly lower, without significant variation among the ZTs (Fig. 7).

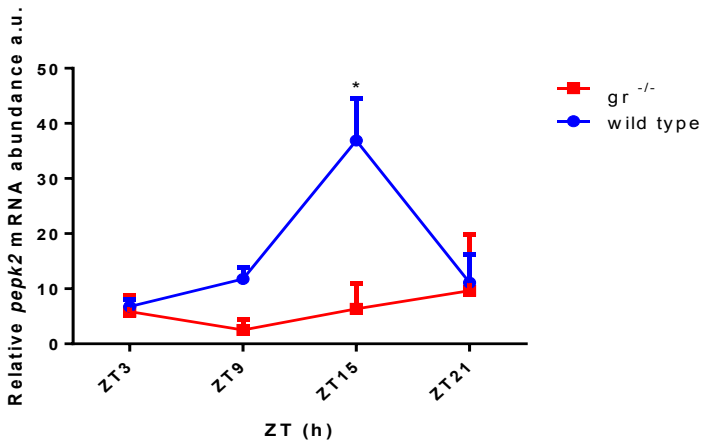
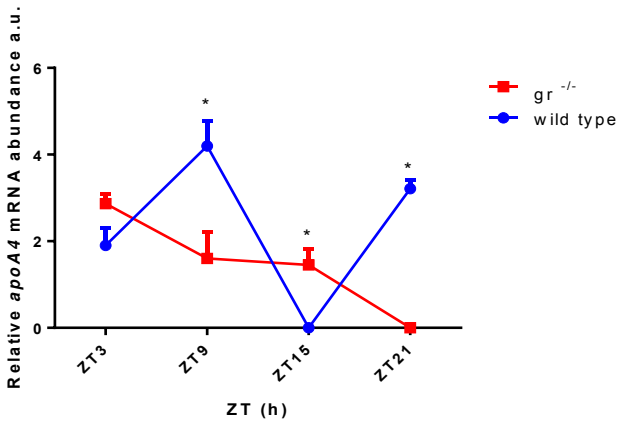


Figure 7. Transcription profile of *pepck2*; mRNA expression in the liver of male zebrafish. In wild-type the trend of expression is shown in blue, while mutants are in red. Asterisks denote statistical significant differences between wt and *gr* $^{-/-}$ at each specific ZT.

Regarding transport, *apoA4* and *apoBa* mRNA levels were analyzed. As showed in the figure 8, *apoA4* showed two different expression peaks in wild-type strain: one diurnal (ZT 9:00 h), the other nocturnal (ZT 21:00 h). For mutants, the expression level was globally reduced with an acrophase at ZT 3:00 h. An up-regulation at ZT 9:00 h and 21:00 h was reported in wild-type

and at ZT 15:00 h in $gr^{-/-}$ line (Fig. 8a). Similarly, *apoBa* mRNA displayed two expression peaks at ZT 3:00 h and 21:00 h in wild-type strain; with a down-regulation of mRNA abundance in $gr^{-/-}$ line (Fig. 8b).

a)



b)

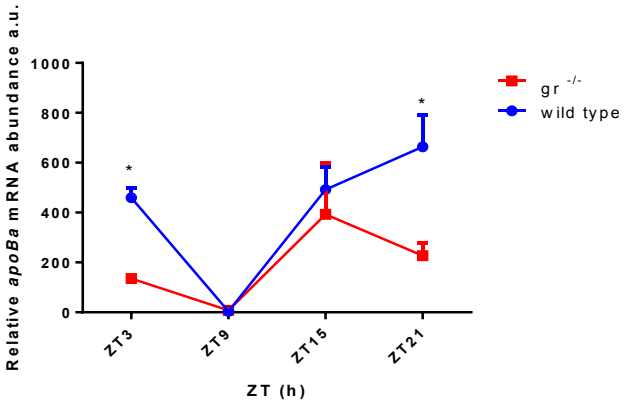
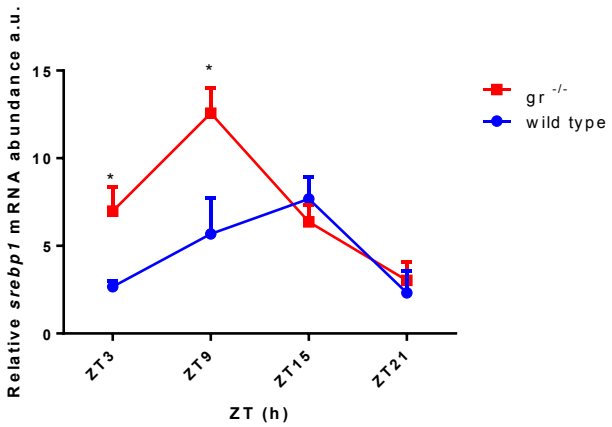


Figure 8. Transcription profile of genes involved in transport. *ApoA4* (a)*; apoBa* (b) mRNA expression in the liver of male zebrafish. In wild-type the trend of expression is shown in blue, while mutants are in red. Asterisks denote statistical significant differences between wt and *gr* $^{-/-}$ at each specific ZT.

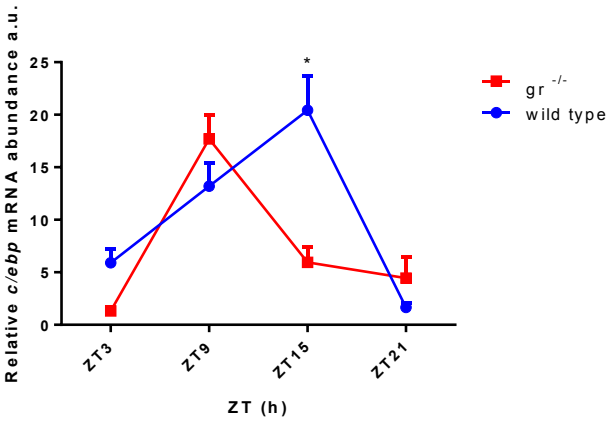
3.1.2 Gene expression analysis in wild-type and gr $^{-/-}$ zebrafish mutants: hepatic mRNA abundance according to their daily rhythms in females.

Regarding *srebp1* (Fig. 9a) a nocturnal acrophase was found (ZT 15:00 h) in wild-type strain; it was shifted to daylight hours in gr$^{-/-}$ mutant line with an expression peak at ZT 9:00 h. An increase of mRNA levels was measured in mutants at ZT 3 and ZT 9. *c/ebp* acrophase in wild-type strain was observed in the dark phase (ZT 15:00 h); shifted to diurnal in mutants (ZT 9:00 h). As shown in the figure, *c/ebp* mRNA expression was significantly increased at ZT 15:00 in wild-type (Fig. 9b). *Pparγ* showed an acrophase at ZT 3:00 h with an up-regulation in *gr$^{-/-}$* line at all ZT points, except for ZT 15:00 h where a down-regulation was found (Fig. 9c).

a)



b)



c)

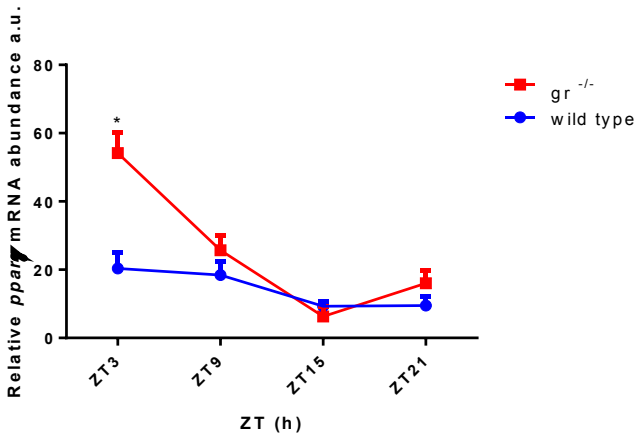
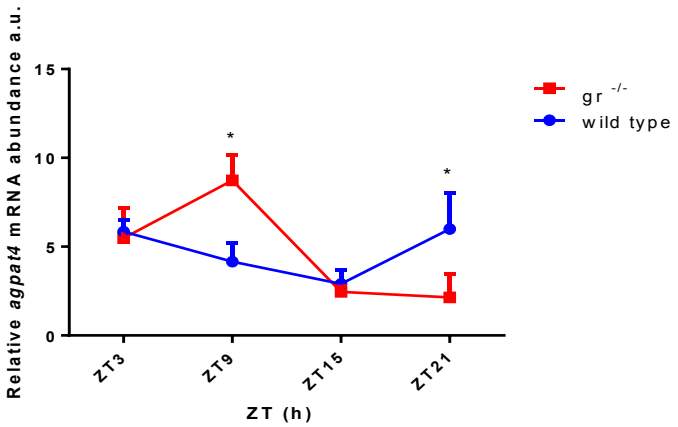


Figure 9. Transcription profile of master genes of hepatic lipid metabolism. *Srebp1* (a); *c/ebp* (b); *pparγ* (c) mRNA expression in the liver of female zebrafish. In wild-type the trend of expression is shown in blue, while mutants are in red. Asterisks denote statistical significant differences between wt and *gr* $^{-/-}$ at each specific ZT.

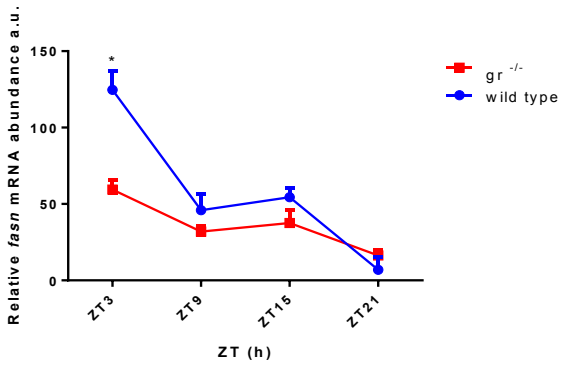
The lipogenesis markers analyzed were *agpat4*, *fasn* and *hnf4*.

The expression of *agpat4* showed an acrophase at ZT 21:00 h in wild-type and at ZT 9:00 h in *gr*$^{-/-}$ zebrafish. These acrophases corresponded to a significant increase of mRNA levels in the two respective groups (Fig. 10a). *fasn* displayed a diurnal acrophase in wild-type zebrafish (ZT 3:00 h), when mRNA levels were significantly higher respect to mutants. In gr $^{-/-}$ no significant rhythmicity was observed and the expression level remained constant throughout the day (Fig. 10b). Also the expression of *hnf4* showed a diurnal acrophase (ZT 9:00 h) with a globally constant trend, both in wild-type and mutant fishes. Moreover, as described in the figure 10, regarding *hnf4* (10c) no significant changes of mRNA levels were measured.

a)



b)



c)

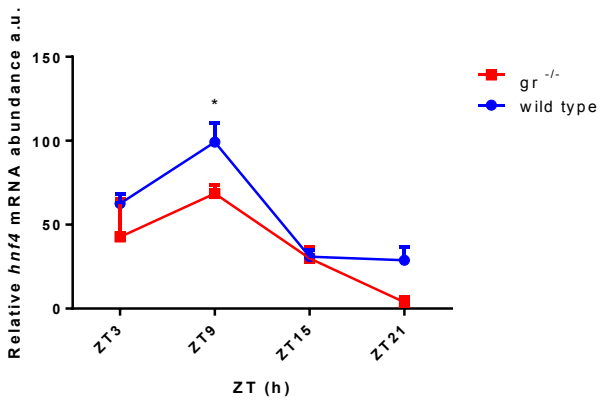


Figure 10. Transcription profile of lipogenic genes. *Agpat4* (a); *fasn* (b); *hnf4* (c) mRNA expression in the liver of female zebrafish. In wild-type the trend of expression is shown in blue, while mutants are in red. Asterisks denote statistical significant differences between wt and *gr* $^{-/-}$ at each specific ZT.

As shown in figure 11, regarding *pepck2* the trend was the same in wild-type and *gr*$^{-/-}$ mutants with an acrophase at ZT 9:00 h. A significant up-regulation was reported at ZT 9:00 h in wild-type strain (Fig. 11).

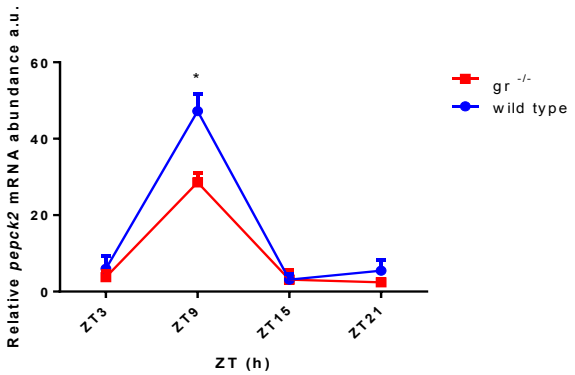
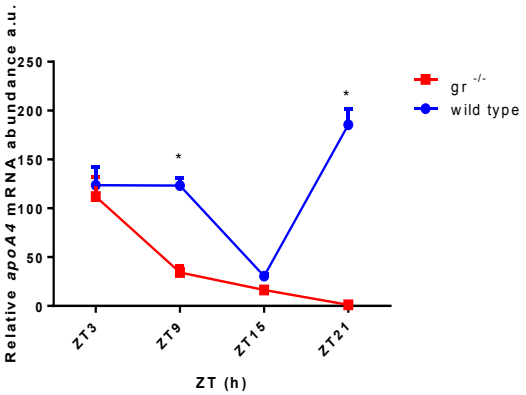


Figure 11. Transcription profile of *pepck2*; mRNA expression in the liver of female zebrafish. In wild-type the trend of expression is shown in blue, while mutants are in red. Asterisks denote statistical significant differences between wt and *gr* $^{-/-}$ at each specific ZT.

Apoa 4 mRNA expression showed a nocturnal expression peak in wild-type strain (ZT 21:00 h). In mutants, the expression level was globally reduced with an acrophase at ZT 3:00 h. A significant up-regulation at ZT 9:00 h and 21:00 h was reported in wild-type (Fig. 12a). Interestingly, *apoAb* showed no changes between wt and *gr*$^{-/-}$, with an acrophase at ZT 15:00 h (Fig. 12b).

a)



b)

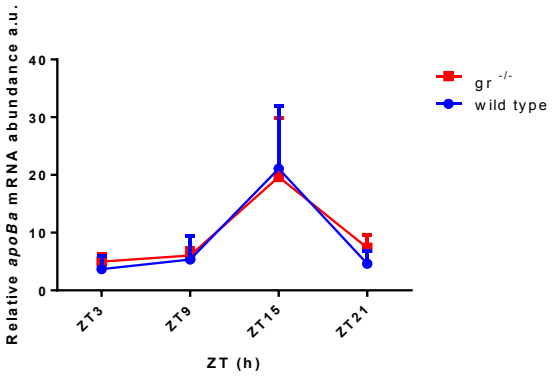


Figure 12. Transcription profile of genes involved in transport. *ApoA4* (a)*; apoBa* (b) mRNA expression in the liver of female zebrafish. In wild-type the trend of expression is shown in blue, while mutants are in red. Asterisks denote statistical significant differences between wt and *gr* $^{-/-}$ at each specific ZT.

3.2 Histological features

Histological analysis on the livers collected from 5 wt and 5 *gr*$^{-/-}$ different fish specimens, sampled at ZT3, revealed a consistent lipid accumulation in the hepatic tissue of the *gr*$^{-/-}$ zebrafish, with an increased hepatocyte size, which loose their classical hexagonal shape to became rounder (Fig. 13). No significant differences were found between males and females.

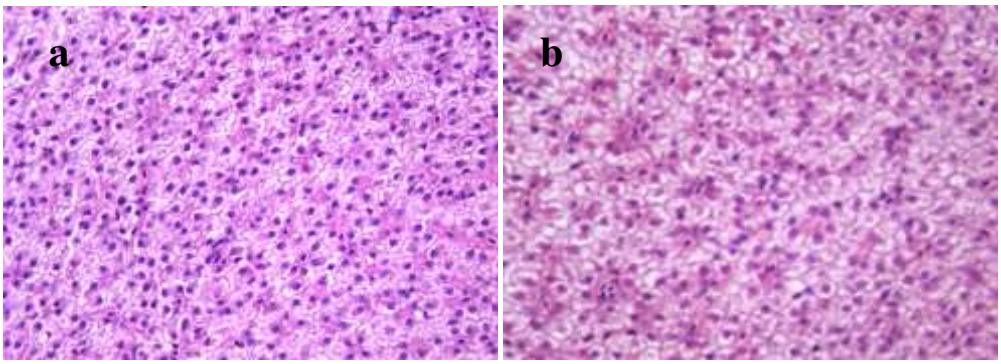


Figure 13. Histological sections of liver. Panel with representative pictures of male zebrafish liver, sampled at ZT3: wt (a) and *gr*$^{-/-}$ (b).

3.3 Fourier Transform Infrared Microspectroscopy analysis

Liver sections of wt and *gr* $^{-/-}$ male and female zebrafish were analyzed by Fourier Transform Infrared Microspectroscopy (FTIRM). To assess the effects of gr silencing on the biochemical composition of zebrafish liver, and to highlight the spectral features discriminating WT_M from GR_M (Fig. 14)

and WT_F from GR_F (Fig. 15), PCA-LDA of pre-processed spectra from each experimental group was performed.

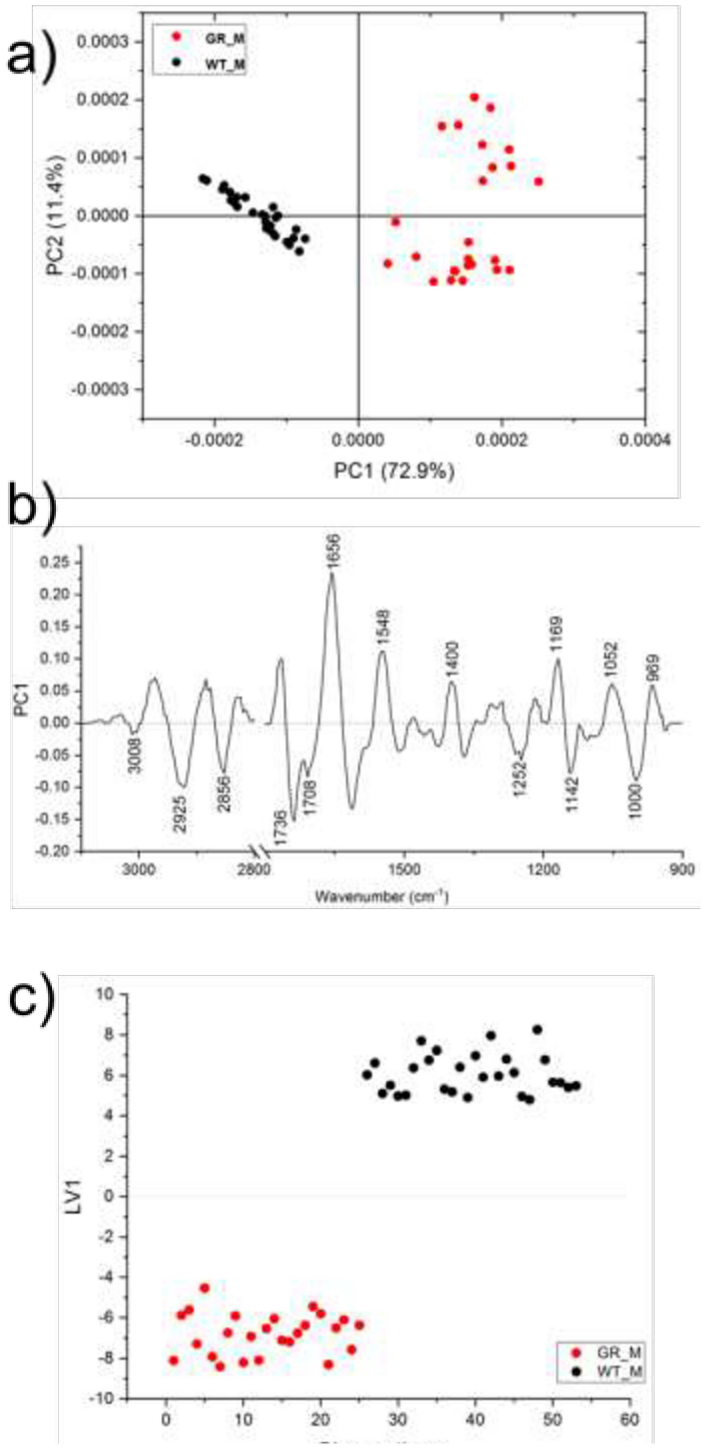


Figure 14. a) PCA score plot of WT_M vs GR_M spectral data; b) PC1 loadings of WT_M vs GR_M populations. Numbers along loading spectra indicate the most discriminant among the groups; c) one-dimensional PCA-LDA score plots WT_M vs GR_M.

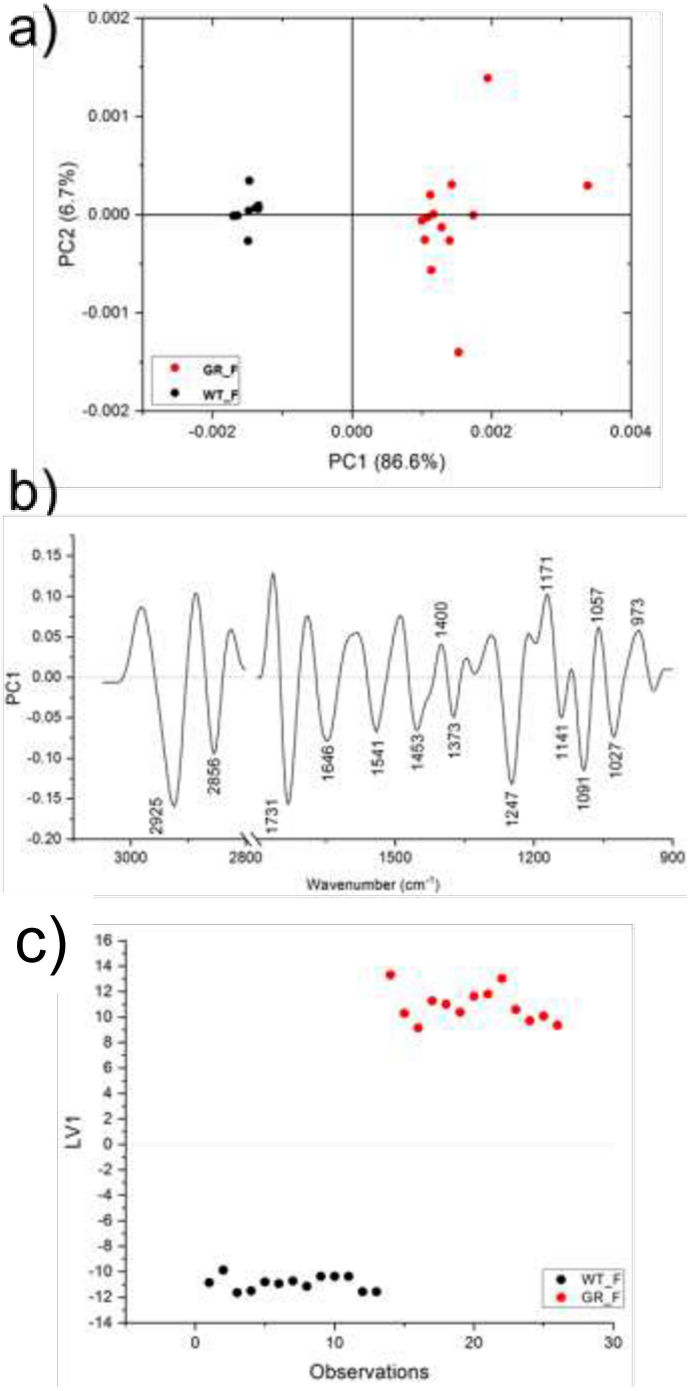


Figure 15. a) PCA score plot of WT_F vs GR_F spectral data; b) PC1 loadings of WT_F vs GR_F populations. Numbers along loading spectra indicate the most discriminant among the groups; c) one-dimensional PCA-LDA score plots WT_F vs GR_F.

To this aim, for each comparison, PC scores obtained from PCA were used as input variables for LDA (WT_M*vs*GR_M, 10 PCs: 95.14% explained variance; WT_F*vs*GR_M, 5 PCs: 95.67% explained variance).

Fig. 14 and Fig. 15 display the results of the pair-wise PCA and PCA-LDA analyses, in terms of WT_M*vs*GR_M and WT_F*vs*GR_F scores plots (Fig. 14 a, Fig. 15a), the corresponding PC1 loadings (Fig. 14 b, Fig. 15b), and the PCA-LDA one-dimensional score plots (Fig. 14 c, Fig. 15c). In both WT_M*vs*GR_M and WT_F*vs*GR_F score plots, a complete segregation between the two groups of spectra was observed along PC1 (72.9% and 86.6% explained variance, respectively). By the analysis of PC1 loadings of WT_MvsGR_M and WT_FvsGR_F populations, spectral modifications were revealed mainly in the following regions: 3050-2800 cm^{-1} (representative of lipids), 1760-1700 cm^{-1} (representative of fatty acids), 1700-1500 cm^{-1} (representative of proteins), and 1150-1000 cm^{-1} (representative of phosphates and carbohydrates). The PC scores of pair-wise PCAs were used as input variables for LDA. PCA-LDA was performed using 10 PCs (95.14% explained variance) for WT_MvsGR_M comparison and 5 PCs (95.67%

explained variance) for WT_FvsGR_F comparison. One dimensional PCA-LDA score plots corresponding to the two pair-wise comparisons are reported in Fig. 14c, Fig. 15c; the plots clearly shows that the two pair-wise PCA-LDA separate spectra collected from WT samples and those of GR samples. The leave-one-out cross-validation procedure evidenced a 0.00% error rate for both WT_MvsGR_M and WT_FvsGR_F.

Based on the results obtained from multivariate statistical procedures, a semi-quantitative analysis was performed on pre-processed spectra, in order to highlight the spectral features discriminating between wild-type and $gr^{-/-}$ populations. The statistical analysis evidenced marked differences among wild-type and $gr^{-/-}$ samples. In particular, regarding male zebrafish (Fig. 16 and 17), some considerations can be drawn. A statistically significant increase of total lipids (LIP/TBM) and of fatty acids (FA/TBM) was found in $gr^{-/-}$ with respect to wt. In this mutant line, a statistically significant higher amount of phosphate groups (PH1/TBM and PH2/TBM) was also observed; moreover, as phosphate groups (PH1 and PH2) and fatty acids (FA) had the same trend, the increase of PH1/TBM and PH2/TBM could be likely attributed to the occurrence of phospholipids. Concerning carbohydrates (COH/TBM) and, in particular glycogen (GLY/TBM), a statistically significant increase was

found. Instead, as expected, a decreasing tendency was detected in the total amount of proteins (PRT/TB) in *gr*$^{-/-}$ livers.

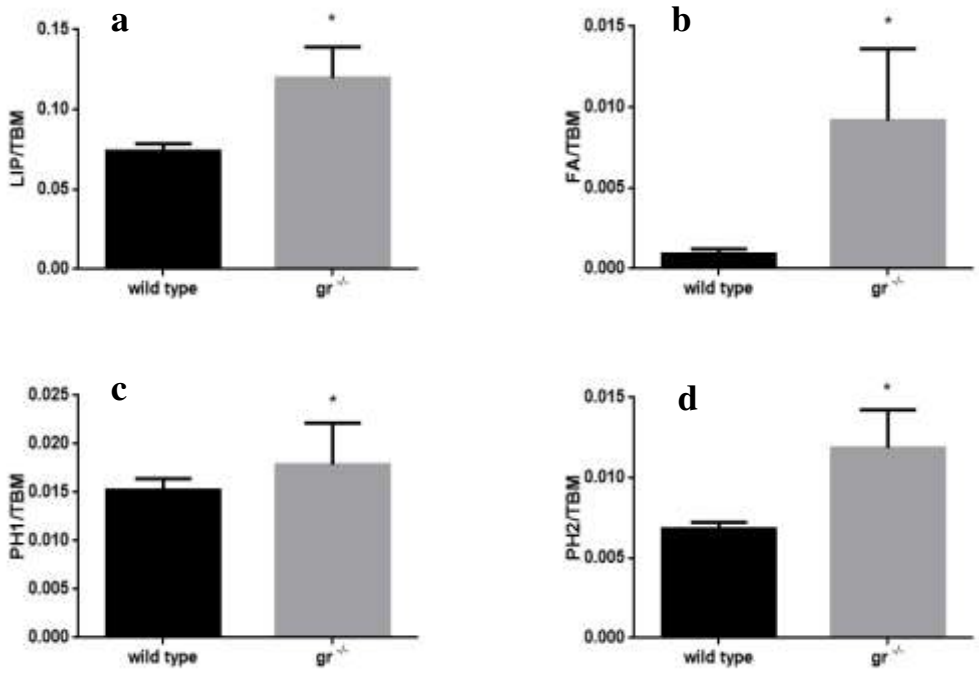
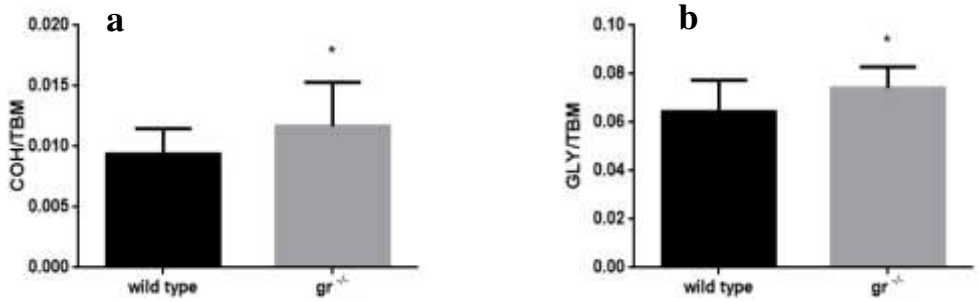


Figure 16 Effects of gr silencing on lipid hepatic composition, in male zebrafish, in terms of: (a) total lipids (LIP/TBM); (b) fatty acids (FA/TBM); (c) phosphate groups 1 (PH1/TBM); (d) phosphate groups 2 (PH2/TBM). Rations are expressed as mean ± SD. Asterisks denote statistically significant differences between wt and *gr* $^{-/-}$, analyzed by Student's t-Test.



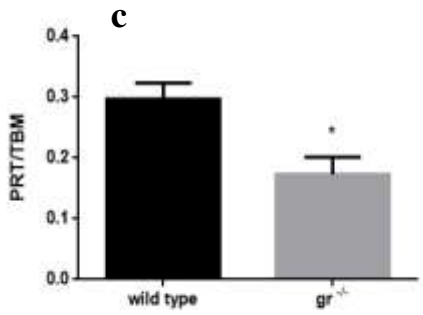
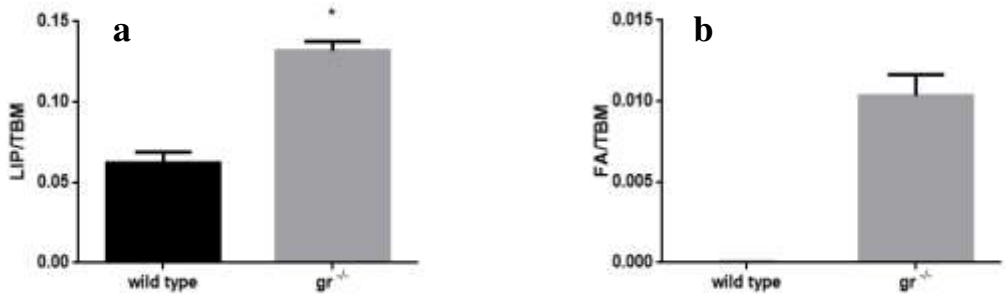


Figure 17. Effects of gr silencing on hepatic composition, in male zebrafish, in terms of: (a) carbohydrates (COH/TBM); (b) glycogen (GLY/TBM); (c) total proteins (PRT/TBM). Asterisks denote statistical significant differences between wt and *gr* $^{-/-}$, analyzed by Student's t-Test.

In female zebrafish (Fig. 18 and 19), with respect to wt samples, *gr*$^{-/-}$ livers had a statistically significant higher amount of total lipids (LIP/TBM) and of phosphate groups (PH1/TBM and PH2/TBM). These phosphate groups referred to the occurrence of phospholipids. Concerning fatty acids (FA/TBM), the band was not detected in wt, resulting markedly higher in *gr*$^{-/-}$ samples.

A decreasing tendency was detected in the total amount of glycogen (GLY/TBM) and of proteins (PRT/TBM) in *gr*$^{-/-}$ livers.



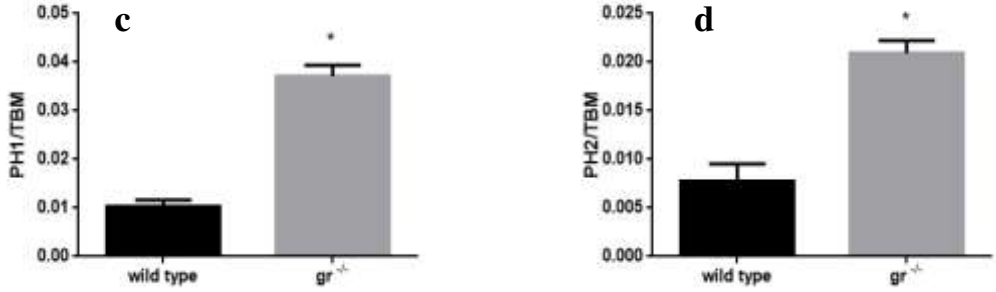


Figure 18. Effects of gr silencing on lipid hepatic composition, in female zebrafish, in terms of: (a) total lipids (LIP/TBM); (b) fatty acids (FA/TBM); (c) phosphate groups 1 (PH1/TBM); (d) phosphate groups 2 (PH2/TBM). Asterisks denote statistical significant differences between wt and *gr* $^{-/-}$, analyzed by Student's t-Test.

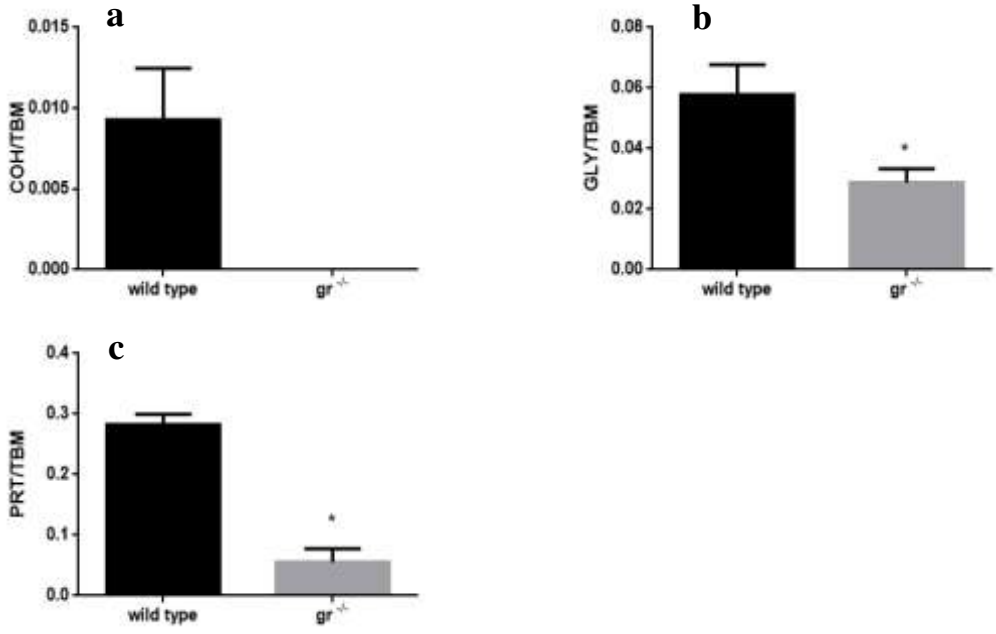


Figure 19. Effects of gr silencing on hepatic composition, in female zebrafish, in terms of: (a) carbohydrates (COH/TBM); (b) glycogen (GLY/TBM); (c) total proteins (PRT/TBM). Asterisks denote statistical significant differences between wt and *gr* $^{-/-}$, analyzed by Student's t-Test.

4. Discussion

At date, several studies have focused on glucocorticoids and the roles played by these hormones (de Guia et al., 2014; Baschant et al., 2013). The release of glucocorticoids from the adrenal cortex can readily activate two types of corticosteroid receptors: the type I mineralocorticoid receptor (MR) and type II glucocorticoid receptor (GR) (Hollenberg et al., 1985; Arriza et al., 1987). MR and GR function as transcription factors that reside within the cytoplasm in the ligand-free state. Once bound by ligands, they dimerize and translocate to the nucleus, allowing for transcriptional control over a variety of target genes. Transcriptional control can happen directly through positive or negative regulation of targeted genes or indirectly through modulation of other transcription factors via protein-protein interactions (Yang-Yen et al., 1990; Stocklin et al., 1996). Additionally, rapid non-genomic effects can occur with bound receptors functioning as cytoplasmic monomers that can interact with a variety of cellular proteins or through interactions with membrane-bound G protein-coupled receptors (Tasker et al., 2006). These rapid non-genomic effects appear crucial for generating the immediate behavioral and physiological responses needed to maintain homeostasis as stress hormones can rise significantly within minutes after exposure to a stressor (Reichardt et al., 2001).To add further understanding to the role

played by GC-GR complex, a stable zebrafish *gr* mutant line completely devoid of transcriptional GC activity was generated by Facchinello et al. in 2017, using CRISPR/Cas9 approach. The line, *nr3c1*$^{ia30/ia30}$, also called *gr*$^{-/-}$ was viable and fertile. However, *gr*$^{-/-}$ mutants had intestinal and cardiac abnormalities, that became more pronounced at around 10 months of age, when increased fat deposits were also observed; the hypothalamus-pituitary-interrenal (HIP) axis was dysregulated in the *gr* mutant line, which showed higher levels of whole-body cortisol (Facchinello et al., 2017). Moreover, many studies described the circadian rhythm of GCs and the existence of a direct crosstalk between the circadian rhythms and GCs activity (Nader et al., 2010, Reddy et al., 2007).

Starting from these considerations, in this thesis, the effects of Gr silencing have been analyzed in 6-months old wild-type zebrafish and *gr*$^{-/-}$ mutants, sampled at 4 different time points (5 and 11 am; 5 and 11 pm), in order to analyze the occurrence of alteration of hepatic lipid metabolism that could be ascribed to the mutation. In addition, possible sex-specific alterations were also analyzed. The histological analyses revealed an increase of lipid accumulation in the livers of mutants animals, characterized by an increased hepatocyte size. Starting from this evidence, the expression profile of *srebp1*, *c/ebp* and *pparγ,* important genes involved in lipid metabolism was

investigated (Santangeli et al., 2018; Carnevali et al., 2017). During the diurnal phase, in *gr*$^{-/-}$ mutants a statistically significant up-regulation of *srebp1* expression, a central player in controlling the expression of genes involved in lipid synthesis and uptake and *c/ebp*, responsible for the secretion of adipokines, hepatic glucose metabolism, insulin sensitivity and inflammation, was observed. Concerning *pparγ*, that orchestrates adipocyte function and differentiation as well as lipid storage within adipocytes, an up-regulation in *gr*$^{-/-}$ line at all ZT points, except for ZT 15:00 h, where a down-regulation was found in females. In accordance with the study done in zebrafish by Paredes et al (2015), in wt line *srebp1* showed a nocturnal acrophase, which was shifted to the diurnal phase in mutants; contrary to what was observed in the same study, where *pparγ* had a nocturnal acrophase, in wt males a diurnal expression peak was observed. Regarding *cebp*, in literature a significant rhythmicty is not documented.

Among lipogenic genes, mRNA levels of *agpat4, fasn* and *hnf4* were analyzed. Regarding *apgat4* expression, a diurnal acrophase (ZT3 in males and ZT9 in females) associated to a significant increase of mRNA was measured in *gr*$^{-/-}$ mutants. Concerning *hnf4*, a gene involved in glucose and cholesterol metabolism and in transport of low density lipoporotein from the liver (Watt et al., 2003 ; Hayhurst et al., 2001), in gr$^{-/-}$ zebrafish, a diurnal

peak expression was found (ZT 9), with a significant downregulation of its mRNA level. Moreover, in *gr*$^{-/-}$ males, an upregulation was found at ZT 21. In gr$^{-/-}$ line, *fasn* had no significant rhythmicty, with the level remaining almost constant during the day. In wt strain, *fasn* had a diurnal acrophase (ZT9 in males and ZT3 in females).A similar trend has been described in Gilthead Sea bream liver (Paredes et al., 2014), different to was observed in Atlantic salmon where absence of significant variation of fasn was measured (Betancor et al., 2014). The lower expression of *fasn* in both male and female gr $^{-/-}$, can be due to the higher amount of fats within the hepatocytes. This gene transcription is in fact negatively regulated by fat concentration.

The expression of *pepck2,* a key enzyme of gluconeogenesis in the liver (Carnevali et al., 2017), resulted down-regulated in mutant males. Our results clearly demonstrate that gr silencing affects the circadian rhythm of the analyzed genes: the acrophase of those genes, whose a nocturnal phase is documented (Paredes et al., 2015), shifted to a diurnal phase. This could be caused to the fact that in diurnal animals, including zebrafish, glucocorticoids reach a peak in the morning (Nader et al., 2010) and could elicit their function by binding the mineralcorticoid receptor (MR) (John et al., 2015). In particular, MR affinity for cortisol (in human) and corticosterone (in rodents) is > 10-fold higher than that of the glucocorticoid receptor (GR) itself (Caprio

et al., 2007); it is possible that many GC-mediated responses attributed to GR action, may be under the direct control of MR (John et al., 2015; Arriza et al., 1987). A recent study, revealed that in either MR or GR knockout zebrafish, a distinct and complementary role for these receptors in stress axis function was observed (Faught and Vijayan, 2018). $GR^{-/-}$ mutants were hypercortisolemic and failed to elicit a cortisol stress response, while $MR^{-/-}$ mutants showed a delayed, but sustained cortisol response post-stressor. We can speculate that, despite gr silencing, most effects found in our results could be due to GC-MR binding. Another possibility could be that glucocorticoids, can activate the endocannabinoid system. In fact, GCs through a non-genomic action acts on corticotropin-releasing hormone (CRH)-neurons thus stimulating the release of endocannabinoids; the latter in turn acting by presynaptic way would reduce the excitatory glutaminergic message, reducing the secretion of CRH, an anorectic hormone, thus giving rise to a final action to stimulate the appetite (Pagotto et al., 2008). Furthermore, the liver is an important target of the endocannabinoid system, whose activation stimulates de novo synthesis of fatty acids (Osei-Hyiaman et al., 2005).

As shown in several studies, there is a bidirectional relation between obesity and stress. This can be partly traced back to a third key player affecting stress and obesity: increased GCs action. The latter is influenced by individual GC

sensitivity and altered levels of GCs, which have both been associated with body composition (van der Valk et al., 2018). Therefore we can speculate about the activation of a feedback loop orchestratd by the diurnal upregulation of glucocorticoids caused by the increase in obesity, which in turn is linked to a stress increase in $gr^{-/-}$ mutant line.

Once synthesized, lipids, in particular triacylglycerol (TAG) and cholesterol esters, are secreted into the circulation as very low density lipoprotein (VLDL) particles, delivering lipids to extrahepatic tissues (Santangeli et al., 2018; Gruffat et al., 1996). Regarding transport, a significant down-regulation of *apoA4* and *apoBa* was reported in $gr^{-/-}$ line; *apoA4* facilitates the packaging of additional lipid into chylomicron particle (Kohan et al., 2012; Otis et al., 2015) and *apoBa* is involved in the assembly of very low density lipoproteins, deputy to cholesterol transport (Haas et al., 2013), thus suggesting that the reduction of these messenger mRNAs, responsible for transport of lipids outside of liver, could be one of the reason of the observed increase of hepatic lipid concentration. A similar trend of expression between *srebp* and *apo4a*, has been described in mouse liver, where *apo4a* expression enhances triglyceride secretion and reduces hepatic lipid content by promoting very low density lipoprotein particle expansion, without significantly affecting *apoB* secretion (VerHague et al., 2013). Also expression confirms these results; a

significant downregulation was shown in $gr^{-/-}$ line during the diurnal phase (ZT 9), suggesting that the transport of low density lipoproteins from the liver was reduced.

In general term, while a similar trend of expression was found for most of genes in both wild type male and female, a marked sex-specificity was found in gr mutants. In males, a lack of acrophase was found for most of the genes analyzed, and levels remained constant across the 4 ZTs, while in females, the peak of expression was shifted to the diurnal phase also for those genes, which display a nocturnal acrophase. As third approach of this study, FTIR microspectroscopy was used to elucidate the effects of gr silencing on the macromolecular composition of the liver. Focusing on lipid composition FTIR analysis revealed an increase of total lipids in $gr^{-/-}$ mutants, in particular of phospholipids and fatty acids, supporting the evidence obtained from the molecular data. In particular, in females peaks of PH1 and PH2 resulted higher than in male, suggesting an higher content of phospholipids. This evidence is also supported by the significantly increase of *agpat4*, codifying for an integral membrane protein that converts lysophosphatidic acid to phosphatidic acid, the second step in de novo phospholipid biosynthesis.

The increase of fats inside the hepatocytes is associated to a decrease of PRT content. Differences have been found in the content of glycogen, with an

increase in males and a decrease in female. On this regard, interestingly, a marked downregulation of *pepck2*, codifying for a key enzyme in glucose and lipid metabolism was found in males. Phisiologically, this gene is activated by the cortisol/GR complex, which binds to the gene GRE element, and since the lack of GR, the lack of gene transcription is justified. Moreover, *pepck2* downregulation has been shown to induce fatty degeneration or hepatic steatosis (Hakimi et al., 2005; She et al., 2003), as also shown by the results herein discussed. At the same time, since the highest GLY levels detected in gr mutant male, we can also speculate a negative feedback loop inhibiting the gene transcription. In female $gr^{-/-}$, *pepck2* trend is similar to that of wild type, and the decrease of GLY could be due to an higher request by the organisms: zebrafish is a daily spawner and daily a number of eggs are ovulated with a great demand of energy. The high levels of GLY found in males are confirmed at the molecular level even with up-regulation of *hnf4* to ZT21, contrary to what is found in females.

All these results obtained by this multidisciplinary approach, strongly demonstrate the effects due to gr silencing on the hepatic lipid metabolism, showing that this gene has an important role in tho organism physiology. The mutation impairs the normal lipid metabolism and together with the correct functioning of the circadian system is essential for normal energy metabolism.

5. Conclusion

In conclusion, these results demonstrate the pivotal role of Gr in the regulation of lipid metabolism. Noteworthy is that while few sex specific differences have been observed in wild type fish, several differences can be detected concerning the regulation of the daily transcription of the signal involved in lipid metabolism between male and female. The integration of molecular, histological data spectroscopic data evidenced in gr mutants an increase of lipid content, potentially caused by a decrease of the levels of lipid transporters. As novelty we also demonstrated also the link existing between gr mutation and the circadian clock. Zebrafish, similarly to mammals is a diurnal species and lipolytic process should occur during the night, while lipogenesis take places during the diurnal phase. We demonstrated a clear diurnal shift of the acrophase of most of the signal analyzed, and this let hypothesized that lipid metabolism in this mutants mainly occurs in the light phase, concomitantly with the peak of glucocorticoids. Since obesity is a major health burden, affecting > 20% of Western populations both in the United States and globally and it is related to metabolic and chronic diseases (Wang et al., 2014; Santangeli et al., 2018), these preliminary results suggest the possibility to use this zebrafish line as a model for human chronic diseases of metabolism such as obesity and other co-morbidities.

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