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Modulation of circadian rhythms by glucocorticoids

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Abstract

Behavior is conceived as a stimulus-response dependent relationship between a sensory input and a motor output. While moving from an input to an output, internal homeostasis is continuously shaped to maintain an optimal energies expenditure balance. The ultimate purpose of enabling animals to adjust their homeostasis with the surrounding world is by producing adaptive behaviors in order to increase their fitness in light of natural selection.

The environment can be either predictable or unpredictable. The former condition led to the evolution of the circadian rhythms to promote active behavior at the time you mostly benefit from, while the latter take advantage of glucocorticoids axis to face sudden challenges. Thus, a crosstalk between the circadian and the glucocorticoid systems allows a fine tuning of animal's activity.

My goal is to understand the circadian-glucocorticoids dialogue by monitoring the locomotor daily/circadian behavior and its molecular oscillation counterpart under differentially phased light and feeding cycle.

My model species is the zebrafish, particularly, I utilized a CRISPR/Cas9 mutant lacking the capability to coordinate glucocorticoids transcription because it lacks functional receptors which permit a correct ligand-receptor interaction. As a result, level of circulating glucocorticoids stays raised conferring an anxiety-related phenotype to the mutant.

Zebrafish *gr*$^{-/-}$ has been built and kindly provided by Dr. Luisa Dalla Valle, University of Padua.

Systematic behavioral analysis in *gr*$^{-/-}$ larvae and adults showed that the light entrainable locomotor activity is synchronized to the zeitgeber and maintain its oscillatory properties in absence of any cue.

The onset of daily locomotor activity occurred one day later in mutants with respects to the wild type. This delay is linked to the slower striated muscle development in the *gr*$^{-/-}$ which recover regular fiber density at 6 days post fertilization.

Furthermore, *gr*$^{-/-}$ larvae showed differences in the expression levels or in the peak phase of positive (*arntl1a* and *clock1a*) and negative (*per1*, *per2a* and *cry1a*) elements of the molecular clock.

Outside the core clock network, an analysis on *gr*$^{-/-}$ adult livers reported an abolished daily expression of *pck2*, a gene involved in gluconeogenesis. In addition, *srebp1* expression level has an anticipated acrophase in *gr*$^{-/-}$.

Feeding entrainment fails to occur in the mutants. Larvae and adults produced abnormal profiles of circadian locomotor activity.

Further molecular investigation revealed this behavioral disruption wasn't associated with a breakdown of molecular rhythms in the core clock genes. Nevertheless, the molecular phenotypes observed during feeding entrainment underlined a *cry1a* lack of rhythmicity.

These data suggest the existence of a blurred boundary between the circadian-glucocorticoids crosstalk. A complex organization of the two produces an altered behavioral output in a food entrained schedule in *gr*$^{-/-}$ zebrafish.

The proximate cause of input and output misalignment underlying food entrained locomotion has not been provided, but a step towards a more exhaustive comprehension about the circadian-glucocorticoids interaction paves the way for an in-depth investigation.

Riassunto

Il comportamento è concepito come una relazione dipendente stimolo-risposta tra un input sensoriale e una risposta motoria. Nel passaggio da input a output, l'omeostasi interna è continuamente modellata per mantenere un equilibrio ottimale della spesa energetica. Lo scopo ultimo di mantenere l'omeostasi in relazione al mondo circostante viene raggiunto attraverso la produzione di comportamenti adattativi che permettono di incrementare la fitness alla luce della selezione naturale.

L'ambiente circostante può essere sia prevedibile sia imprevedibile.

La prima condizione ha portato all'evoluzione del ritmo circadiano che promuove la fase di attività durante il momento più favorevole della giornata, mentre la seconda si serve dei glucocorticoidi per affrontare le sfide imprevedibili. Quindi, un dialogo tra il sistema circadiano e il sistema dei glucocorticoidi è mantenuto allo scopo di ottenere una regolazione ottimale dell'attività animale.

Il mio obiettivo è quello di capire il dialogo tra i due sistemi monitorando il comportamento giornaliero e circadiano, e la sua controparte molecolare, in fase a differenti cicli di luce e cibo.

La mia specie modello è lo zebrafish (*Danio rerio*), in particolare, ho utilizzano un mutante costruito con la tecnica CRISPR/Cas9 che non possiede la capacità di coordinare la via di trascrizione dei glucocorticoidi a causa della mancata funzionalità del loro recettore cognato tale che l'interazione ligando recettore non è mantenuta. Di conseguenza, i livelli circolanti di glucocorticoidi restano elevati, conferendo al mutante un fenotipo ansioso.

Zebrafish $gr^{-/-}$ è stato costruito e gentilmente fornito dal laboratorio della Prof.ssa Dalla Valle, Università degli studi di Padova.

L'analisi sistematica del comportamento in larve e adulti di $gr^{-/-}$ ha mostrato che l'attività locomotoria sincronizzata alla luce mantiene le sue proprietà oscillatorie endogene. Tuttavia, l'attività locomotoria giornaliera insorge con un ritardo di un giorno nei mutanti rispetto ai wild type. Questa insorgenza ritardata è associata a un rallentamento nello sviluppo del tessuto muscolare striato, la normale densità delle fibre muscolari viene ripristinata nei $gr^{-/-}$ al sesto giorno dopo la fertilizzazione.

Inoltre, le larve *gr*$^{-/-}$ hanno mostrato differenze nei livelli di espressione e nelle relative acrofasi di elementi positivi (*arntl1a* and *clock1a*) e negativi (*per1*, *per2a* and *cry1a*) dell'orologio molecolare.

Al di là degli elementi del cuore dell'orologio circadiano, un'analisi nel fegato di adullti *gr*$^{-/-}$ rivela un'abolizione dell'espressione di *pck2*, un gene implicato nella gluconeogenesi. In aggiunta, *srebp1* ha un'acrofase anticipata nei mutanti.

La sincronizzazione circadiana al cibo fallisce nei *gr*$^{-/-}$, sia larve sia adulti producono profili anomali dell'attività locomotoria. L'analisi molecolare non associa la disfunzionalità comportamentale con quella genetica, infatti i geni orologio non mostrano alterate oscillazioni ad eccezione di *cry1a.*

Questi dati suggeriscono l'esistenza di un confine sfuocato tra il sistema circadiano e quello dei glucocorticoidi e una complessa organizzazione dei due ha prodotto un alterato output comportamentale negli zebrafish *gr*$^{-/-}$.

La causa prossima del disallineamento tra lo stimolo cibo e la locomozione non è stata chiarita sebbene un passo avanti verso una maggiore comprensione del dialogo tra glucocorticoidi e orologio circadiano getta le basi per un'indagine più profonda.

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Nomenclature

Abbreviation	*Description*
AANAT	arylalkylamine-N-acetyltransferase
ACSS3	acyl-CoA synthetase short-chain member 3
ACTH	adrenocorticotropic hormone
AKT	protein kinase B
AGPAT	1-acyl-glycerol-3-phosphate acyltransferase
AgRP	agouti-related peptide
AMPK	AMP-activated protein kinase
ARC	arcuate nuclei
arntl1	aryl hydrocarbon receptor nuclear translocator-like 1a
ATP	adenosine triphosphate
bHLH-PAS	basic helix-loop-helix,
bmal	brain and muscle ARNT-like
cAMP	cyclic adenosine monophosphate
CCG	clock-controlled gene
c-FOS	cellular FOS proto-oncogene
CLOCK	circadian locomoter output cycles protein kaput
cGMP	cyclic guanosine monophosphate
CK1ε	casein kinase-1ε
CREB	cAMP response element binding protein
CRH	corticotropin release hormone
CRTC2	CREB regulated transcription co-activator
cry	cryptochrome

CT	circadian time
CYC	cycle
D-box	distal zinc finger
DBS	DNA binding sequence
DMH	dorsomedial hypothalamic nucleus
DNA	deoxyribonucleic acid
dpf	days post fertilization
E-box	enhancer box
ELVOL	elongation of very long chain fatty acids
EGFP	enhanced-GFP
FAA	food-anticipatory activity
FEO	food entrainable oscillator
FGF3	fibroblast growth factors 3
frq	frequency
FW	fish water
HPA	hypothalamic-pituitary-adrenal
HPI	hypothalamic-pituitary-interrenal
ipRGCs	intrinsically photosensitive retinal ganglion cells
GABA	gamma amino butyric acid
gapdh	gliceraldeide fosfato deidrogenasi
GC	glucocorticoid
GLP1	glucagon- like peptide 1
GPCR	G protein-coupled receptor
GR	glucocorticoid receptor
GRE	glucocorticoid responsive element

GSK3β	glycogen synthase kinase 3β
HDAC3	histone deacetylase 3
LD	light-dark cycle
LEO	light entrainable oscillator
LL	constant light
LH	lateral hindbrain
MAPK	mitogen-activated protein kinase
MCH	melanin concentrating hormone
MC2R	melanocortin 2 receptor
MC3	melanocortin receptor 3
MSH	melanocyte-stimulating hormone
mTOR	mammalian target of rapamycin
NAD+	oxidized form of nicotinamide adenine dinucleotide
NADH	reduced form of nicotinamide adenindinucleotide
NPAS2	neuronal PAS domain protein 2
NPY	neuropeptide Y
ndrn1	nuclear receptor subfamily 1 group D member 1
NTS	nuclei of the solitary tract
opn4	opsin 4
opn4m	opsin 4 mammalian-like
OXM	oxyntomodulin
PACAP	pituitary adenylate cyclase-activating polypeptide
PARP-1	poly (ADP-ribose) polymerase 1
PCK1	phosphoenolpyruvate carboxykinase 1
PCK2	phosphoenolpyruvate carboxykinase 2

PCR	polymerase chain reaction
per	period
POMC	pro-opiomelanocortin
PPARα	peroxisome proliferator-activated receptor alpha
PVN	paraventricular nuclei
qPCR	quantitative PCR
REV-ERBα	nuclear receptor subfamily 1, group D, member
RF	restricted feeding
RGCs	retinal ganglion cells
RHT	retinohypothalamic tract
RNA	ribonucleic acid
RORα	RAR-related orphan receptor alpha
RORE	retinoic acid-related orphan receptor response element
ROS	reactive oxygen species
rplp0	ribosomal protein lateral stalk subunit P0
rpl13a	ribosomal protein L13a
rx3	retinal homeobox gene 3
SCN	suprachiasmatic nucleus
SEM	standard error of mean
Srebp1	sterol regulatory element-binding transcription factor 1
SSRI	selective serotonin reuptake inhibitor
StAR	steroidogenic acute regulatory protein
TEF	thyrotroph embryonic factor
Tim	timeless
TMN	tuberomammillary nuclei

TMT	teleost multiple tissue
TPA	tissue plasminogen activator
TRH	thyrotropin-releasing hormone neurons
TTFLs	transcription-translation feedback loops
UV	ultraviolet
VMH	ventromedial hypothalamic nuclei
wcc	white collar complex
WT	wild type
ZT	zeitgeber time

Publications

Morbiato E., Frigato E., Dinarello A., Maradonna F., Facchinello N., Argenton F., Carnevali O., Dalla Valle L., Bertolucci C. Feeding Entrainment of the zebrafish circadian clock is regulated by the glucocorticoid receptor. Cells 2019, 8, 1342.

Morbiato E., Bilel S., Tirri M., Arfè R., Fantinati A., Savchuk S., Appolonova S., Tagliaro F., Neri M., Grignolio S., Bertolucci C., Marti M. Potential of the zebrafish model for the forensic toxicology screening of NPS: a comparative study of the effects of APINAC and Methiopropamine on the behavior of zebrafish larvae and mice. Neurotoxicology 2020

1. Introduction

A metronome, from Greek: *métron* " to measure" and *némo*, "to lead", is a device that produces an audible click at a regular interval. Musicians use the device to practice playing to a regular pulse, namely the ability to stick to a tempo. A tempo is the speed or pace of a given piece. While the ability to hold a steady tempo is a vital skill for a musical performer, tempo is changeable. Depending on the genre of a piece of music, a piece may be played with slight “*tempo rubato*” or drastic “*accelerando*”.

Musical and biological rhythms adopt the same strategy to keep on time.

1.1 *Components of the biological clock*

Life on earth has been shaped by environmental cues: exogenous signals trigger the capability of organisms to entrain their activities in order to exploit the most favorable environmental condition. The rotation of our planet around itself and its revolution around the Sun is the strongest rhythm imposed on living organisms who evolved a biologic clock system which coordinate behavioral and physiologic functions in a circadian fashion by anticipating and coordinating the required metabolic programme to maintain a dynamic homeostasis. Homeostasis itself can be perturbed by exogenous or endogenous stressors which influence the capability of organisms to adapt their responses to sustain the optimal equilibrium.
A heuristic technique called Eksinogram, taking the name by his founder, Arnold Eksin [1], has the aim to simplify the interaction between the environment and the circadian clock. The model implies three characters: 1. the *input*, or zeitgeber (German for time-giver), namely light-dark alternation, food availability, temperature fluctuation as exogenous sources of rhythmicity, or endogenous signals such as hormones and neurotransmitters. These variables set the circadian (about a day) clock by an active process called entrainment. The signals are collected by receptors and then transduced and sent to the 2. rhythm generator: an autonomous *oscillator* that ticks in a circadian fashion also in absence of environmental cues, the so-called free-running period. The free-running period is slightly different within individuals (slightly longer or shorter than 24 h), but the differences in the phase of entrainment belong also to the nature and strength of the zeitgeber. The rhythm generator triggers another transduction cascade that results as 3. the behavioral and metabolic *output* (Figure 1.1)

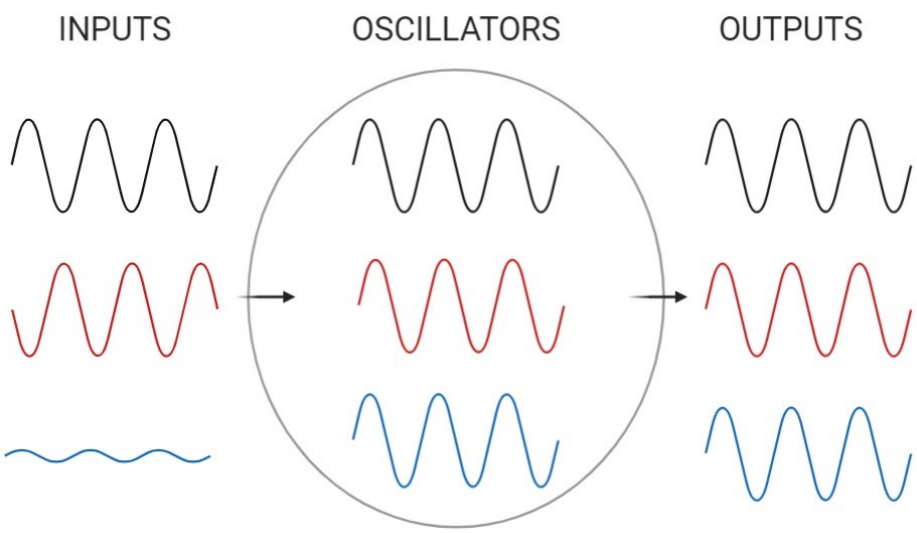

Figure 1.1 Schematic representation of the circadian timekeeping system. Inputs may have different phases and amplitudes. Each input influences internal oscillators which typically work to align the phases in order to synchronize the outputs to a period of around 24h.

1.2 *The oscillators tick in a circadian fashion in absence of environmental cues*

An oscillator is a circuit which produces a continuous, repeated, alternating waveform of the desired frequency without any input. In the biological systems, time is computed at cellular level where oscillation is achieved by a molecular clock machinery that is driven by the 24-hour rhythm of the so-called clock genes. The first insight into the circadian oscillator properties came from Seymour Benzer and Ronald Konopka work carried out in *Drosophila melanogaster* in 1971. By coupling an eclosion rhythm assay with a mutagenesis screen they identified a *period* (*per*) gene alteration which provokes a defects in the timing of locomotor activity and eclosion in flies mutants [2]. The pioneer studies on flies lead to the discovery of the first biological oscillator, a gene belonging to the clock core oscillator: *per*.

1.3 *The core oscillator is self-sustained and has a free running period of about 24 h*

The core oscillator is a mechanism intrinsic of every cell, tissue and organ, ubiquitous to almost all the living beings.

The mammals clock core oscillator, also known as the molecular clock, is a 24-hour transcriptional/translational feedback loop (TTFL) in which BMAL1(Brain Muscle ARNT-Like) and CLOCK (Circadian Locomoter Output Cycles Protein Kaput), regulate the cyclic expression of two repressors families, the *period* (*per*1-3) and *cryptochrome* (*cry*1-2) genes. CLOCK and BMAL transcription factors form a heterodimer which binds to a cis-regulatory element, the E-box, and activates transcription of *per* and *cry* genes. The PER-CRY heterocomplexe translocates back to the nucleus where it inhibits the transcriptional activity of the CLOCK-BMAL complex and thus their own transcription. The inhibitory PER-CRY complexes are subsequently degraded in the proteasome following phosphorylation by casein kinase Iε (CKIε) and then ubiquitination which removes the inhibition on CLOCK and BMAL, allowing the feedback loop to restart again in a 24 h cycle.

A finer tuning is accomplished by the ROR-REV-ERB accessory feedback loop. The transcriptional expression of these two elements is controlled by the CLOCK-BMAL1 heterodimer. The retinoic acid receptor-related orphan receptors, REV-ERBα/β (nuclear receptor subfamily 1, group D, member 1) and RORα (RAR-related orphan receptor alpha) compete to bind the RORE (retinoic acid-related orphan receptor response element), the enhancer elements on the *bmal1* promoter to inhibit or promote its transcription, respectively. Oscillations in transcription of REV-ERBα and RORα thus drive the rhythmic expression of BMAL1[3]. The core clock machinery oscillation is self-sustained, in absence of any cue it has a free running period of about 24 h [4] (Figure 1.2).

The TTFL mechanism is a convergent evolved trait shared between distinct lineages. If the phenotype linked to the TTFL is common to different phyla, at molecular levels, genetic variations has been detected. In cyanobacteria, KaiA, KaiB and KaiC comprise the feedback circuit elements [5]. In *Neurospora*, circadian rhythm is kept by the balance between the positive regulator WCC (white

collar complex), and the negative regulator FRQ(frequency) [6]. In *Drosophila*, feedback loops are composed by CLK (Clock) and CYC (Cycle), homologous to CLK:BMAL, activators of the transcription of *per* (*period*) and *tim* (*timeless*), homologous to *per* and *cry*, with PER and TIM heterodimer acting as a suppressor of the transcriptional activity of the CLK:CYC complex [7].

Even though the core clock machinery is present at cellular level in any tissue of an organism, a hierarchical structure of the oscillators has been identified with a master clock oscillator which can impose his rhythm to the peripheral clocks, depending on the input extent and periodicity.

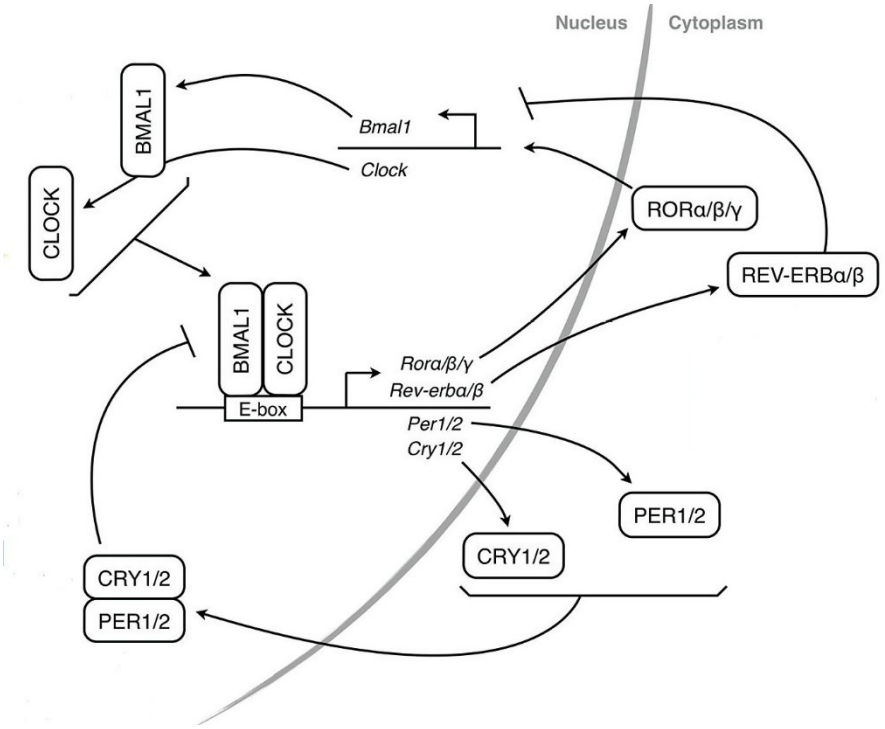

Figure 1.2 Simplify molecular clock machinery scheme. Illustrated are the components of transcription-translation feedback loops (TTFLs) in mammals and fish. The molecular circadian clock is constituted by the core loop (BMAL, CLOCK, PERs, CRYs) and the accessory loop (BMAL, CLOCK, REV-ERBα, RORα) which together mediate the 24 h self-sustained rhythm. Adapted from: Stojkovic et al. 2014 [8].

1.4 *The master oscillator leads the phase*

1.4.1 *Mammals*

In 1980, a study conducted in Menaker's laboratory, provided the basis for a hypothetical multioscillator framework underlying circadian locomotor activity. By delivering two conflicting zeitgeber to hamster: food at noon, wheel at night, they found out that animals showed a bimodal locomotor activity: a peak appears at meal time, the actual rest phase, and another one at night time, the typical wheeling phase in this species [9]. This experiment has been the groundwork for establish the existence of multiple oscillators.

Later, in 1990, has been proved that the anatomical region responsible for the light-dark phase maintenance is the master clock and it is located within the neurons of the suprachiasmatic nucleus (SCN) in the hypothalamus [10].

The SCN receives signals from the environment and provides the principal timing cues for synchronize the daily oscillations in peripheral tissues. Light is received by the intrinsically photosensitive retinal ganglion cells (ipRGCs), located in the retina and then transmitted to the SNC through two pathways: directly via the retino-hypotalamic tract, using glutamate and pituitary adenylate cyclase activating polypeptide (PACAP) neurotransmitter, and indirectly, via the intergeniculate leaflet and the hindbrain, using gamma amino butyric acid (GABA), neuropeptide Y (NPY), and serotonin as transmitters [11]. Then, in turn, timing information are conveyed to the peripheral clocks via a complex set of systemic signals [12]. The master and the peripheral clocks usually act in phase, but in specific conditions, their oscillation can be decoupled [13].

1.4.2 *Fish*

In fish, the existence of a hierarchically organized clock machinery analogous to the mammals one is still debated. However, the putative evolutionary conserved

circadian master district has been identified as the pineal gland [14]. This is a photosensitive gland, located on the dorsal surface of the diencephalon (in the zebrafish), which controls the release of the melatonin hormone. Four enzymes are involved in its biosynthetic pathway, with arylalkylamine N-acetyltransferase (AANAT) representing the rate-limiting step [15]. Melatonin has been baptized the *Dracula* hormone because its secretion occurs in darkness. As a result, melatonin levels in the blood rise sharply at night and alert levels go down allowing the sleep phase incoming. Melatonin levels in the blood stay elevated for about 12 h and then they fall back to low daytime levels at sunrise when melatonin inhibition is directly triggered by the light. Melatonin is also produced by the retina, providing a local paracrine signal [16].

In zebrafish, the pineal gland develops by 19 hours post-fertilization and immediately becomes responsive to light and starts secreting melatonin [17; 18]. Pineal glands in some teleosts (e.g., zebrafish and pike) maintain intrinsic circadian clocks. Pinealectomy can significantly disrupt circadian rhythmicity in these species, presumably because their pineal glands serve as master circadian clocks [19]. Conversely, pineal glands in other species such as salmonids, are photosensitive but lack an intrinsic circadian oscillator [20; 21; 22; 23]. Accordingly, pinealectomy does not dramatically alter their circadian rhythmicity.

It is worth mentioning that evolution led to a genome duplication in teleosts, thus each mammal gene has multiple counterparts in fish. After duplication a gene may be lost, recombined, modified or retained in an unaltered state. Such duplication has indeed provided the context for an expanded repertoire of molecular variability underscoring the overall plasticity of the fish circadian system.

1.5 *The peripheral oscillators. Rather than slaves, fundamental timekeepers.*

1.5.1 *Mammals*

The evolution of larger and opaque organisms triggered the development of different, nonphotic entrainment pathways. In mammals, a compartmentalized

district evolved by segregating a highly specialized master pacemaker in the SCN, which receives photic input from the retina via the retinohypothalamic tract and conveys timing information to the entire organism via direct and indirect signaling pathways is combined with peripheral circadian oscillators sensitive to a variety of chemical cues [12].

Thus, abandoned the classical view of a unique circadian pacemaker orchestrating all over rhythms in physiology and behavior, investigations on peripheral oscillators began.

Clock gene transcripts properties in peripheral tissues are equalized with the ones of the SCN [24]. However, the phase of the peripheral clock oscillations is typically delayed by 3–9 h, suggesting that the peripheral tissues might be receiving timing cues from the master oscillator in the SCN in order to keep the phase over time [12]. Furthermore, the SCN pacemaker and peripheral clocks are differentially responsive to entrainment by different zeitgebers. In mammals, light directly synchronize the master pacemaker, but peripheral clocks in the subsidiary organs cannot be independently synchronized by light (Figure 1.3). Non-photic signals such as food and temperature are capable to reset the phase of circadian gene expression in some peripheral tissues independently of the SCN rhythms [25; 26]. Indeed, if cultured, peripheral cells can be easily synchronized by several non-photic zeitgeber, such as serum [26] glucocorticoids [27], dexamethasone [28], TPA (activator of protein kinase C) [29], glucose [30], oxygen [31], rhythmic blood-borne signals [32], temperature cycles [26], the SNC ticking is strongly light-dependent. The way which ensure a temporal coordinated physiology between the two systems is still poorly understood.

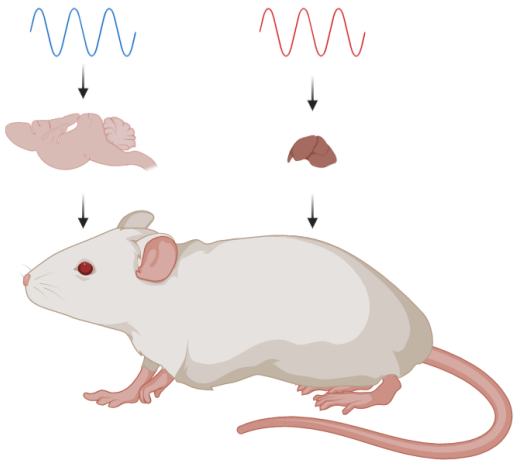

Figure 1.3 Compartmentalized oscillators in mammals. The LEO (blue) is located in the SNC, it leads the phase and convey the information to the other tissues. Peripheral oscillators (red) can be entrained by different type of cues except for the light. Arrows indicate brain and liver oscillators.

1.5.2 *Fish*

Considering fish evolving environment it must be taken into account that light affects this phylum differentially from mammals not only because of the shape of their integumentary system, but also for the different nature of the light diffusion medium.

Thus, even though there's a high circadian homology within all the living beings, adaptive variations evolved.

By using gene cloning technique, it turned out that all zebrafish tissues tested both *in vivo* and *in vitro* oscillate in a circadian fashion. They hold clearly independent circadian pacemakers which show high-amplitude and robust clock rhythms at the transcriptional level [33; 34].

A striking difference between mammals and teleosts peripheral clocks lies to the capability of the latter to be rhythmic and directly light responsive without any apparent need for eyes or pineal gland. As a result, zebrafish cell types do not require a centralized photosensitive structure to turn on light-induced transcription (Figure 1.4). Indeed, cells and organs can be entrained directly by light stimuli

through visual and non-visual peripheral opsins [35]. Vertebrate photoreception is often linked to the visual system, and even though visual light detection trough rods and/or cones is engaged in most vertebrates, non-visual photoreception and the use of non-visual opsins also plays a role in many critical biological processes, such as seasonality, photoperiodism, circadian entrainment and DNA repair [36; 37; 38; 39].

The peripheral clock can be entrained not only by light, but it can set its phase in response to a wide range of cues such as temperature fluctuations [40], feeding time [41], glucocorticoids fluctuations [28] and glucose levels[42].

This highly decentralized model of fish circadian biology, with independent, light-responsive circadian pacemakers in all tissues and cells, put the need of a master clock no longer necessary.

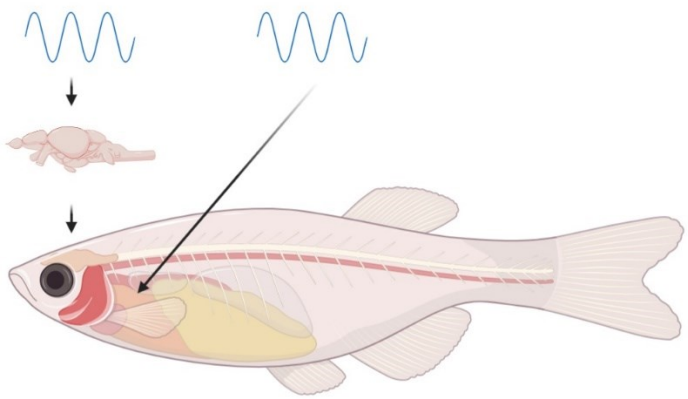

Figure 1.4 Decentralized oscillators in fish. Any tissue owns a LEO (blue). Brain and liver oscillators are indicated by the arrows. The same tissues are capable of entrainment to a large set of stimuli.

1.6 *Inputs set the activity phase*

Any information capable of convey a message to a subject can be considered as an input. In chronobiology, an input owns oscillatory properties, that's why it is termed *zeitgeber*. *Zeitgebers* are a multitude: light-dark alternation, food availability, predation risk, social interactions, seasonal changes, cellular cycle, hormone peaks and so on.

The living beings adapt their rhythm in order to be aligned with these cues to better exploit the resources. Usually, the activity phase of a species is synchronized with every zeitgeber, i.e. humans are active during daytime when light, food and social interactions occur. If zeitgebers are aligned within each other, the active phase of a species would look like a modal curve, if zeitgebers are in conflict, namely they are present in both active and resting phase of a species, the activity phase can be adjusted in different ways: by following one unique phase (favoring a zeitgeber rhythm over the others), or by assuming a bimodal distribution of its activity (synchronizing to each zeitgeber). The distribution of activity depends on the nature of the zeitgeber (light vs food) and on its strength (ad libitum vs restricted food).

1.7 *Exogenous inputs: the light entrainable oscillator*

1.7.1 *Mammals*

The light-dark alternation deriving from the Earth rotation and revolution around the Sun and its repeatability led the light to become the strongest zeitgeber acting on the living beings, thus light entrainable oscillators (LEO) evolved in order to keep this rhythm.

Light entrainment occurs through two different pathways: the visual and the non-visual.

Two photoreceptor cells are involved in vision in vertebrates: rod and cone. The rod and cone cells contain different opsins. Opsins are light sensors which belong to the G protein-coupled receptor (GPCR) superfamily of proteins, they signal and thereby activate light-induced clock genes, using the classic, well-established downstream pathways: the excited pigments activate the G-protein transducin, which stimulates cGMP phosphodiesterase, resulting in a decrease in intracellular cGMP concentration. This decrease leads to closure of a cGMP-gated cation channel with consequent hyperpolarization of the visual photoreceptor cell rhodopsin, which underlies twilight vision, and cone opsins, which underlie daylight (color) vision [43]. The photic entrainment of the mammalian SCN occurs through the retina, where the classical rod and cone photoreceptors located in the outer retinal layer are not required for photic entrainment [44; 45]. Instead, a cluster of retinal ganglion cells that are intrinsically photoreceptive (ipRGCs) expresses the photopigment melanopsin (Opn4), and act as photoreceptor in the circadian photic entrainment. Melanopsin knockout mice (*opn4*$^{-/-}$) exhibit severely attenuated phase shifting, a lower magnitude of photic responses, incomplete pupillary light reflex, and loss of direct photosensitivity in ipRGCs. A partial response to light is maintained in both *opn4*$^{-/-}$, and rodents lacking rods and cones photoreceptors suggesting that the ipRGCs, rods and cones can all together to some extent participate in keeping circadian photoreception function pointing evidence of a close interactions between these classical visual photoreceptors and the non-visual photoreceptor melanopsin in visual input to the SCN [46; 47; 48; 49; 50; 51].
Non-visual opsins were discovered in the early 1990s. These genes play roles in circadian rhythm in mammals, seasonal reproduction in birds, light avoidance in amphibian larvae, and neural development in fish [52].
Teleost-specific genome duplication lead to the evolution of two genes coding for parapinopsin: *PP1* and *PP2* where *PP1* is a UV-sensitive pigment, similar to lamprey parapinopsin, and *PP2* is a blue-sensitive pigment. Moreover, *PP2* influence melatonin secretion via a neofunctionalization event in the zebrafish [53].

1.7.2 *Fish*

The zebrafish pineal gland contains photoreceptor cells with structural and functional similarities to mammalian retinal photoreceptors [54]. The pinealocytes in the zebrafish pineal gland detects light via the photoreceptor rhodopsin, which transduces the photic information into melatonin synthesis. Melatonin secretion, in turn, regulate circadian rhythmicity. Like in mammals, the zebrafish retina expresses the photopigment melanopsin, involved in light perception it directly responds of the clock to light entrainment [55]. As mentioned earlier, a wide range of non-visual photoreceptors are also expressed in the zebrafish pinealocytes which role is linked to the detection of blue and UV wavelengths as well as circadian control through melatonin regulation.

Not only the zebrafish brain, but all its peripheral organs and cells are directly photosensitive, even though the light pathways to the cellular environment are not fully understood yet.

To date, three different peripheral photoreceptors which allow the light perception and clock synchronization have been described: 1. non-visual opsins, 2. cryptochromes and 3. flavin-containing oxidase.

1. Non-visual opsin transduce the photic information into melatonin synthesis and then regulate circadian rhythmicity through melatonin secretion [55; 56]. Zebrafish own 42 different opsin genes (10 visual and 32 non-visual), currently the highest reported number of opsins in any animal, with multiple non visual opsins expressed in every organ [35]. Among them, TMT (teleost multiple tissue) opsin, *opn4m1* and *opn4m2* are responsible for the lack of light entrainment in the blind cavefish *Phreatichthys andruzzii* [57].

2. Six cryptochrome genes has been identified in zebrafish: *cry1a*, *cry1b*, *cry2a*, *cry2b*, *cry3*, *cry4*. They exhibit distinct patterns of temporal and spatial expression. *Cry1aa*, *cry1ab*, *cry1ba* and *cry1bb* are able to repress CLOCK-BMAL mediated transcription, while *cry3* and *cry1b* have been described as light photoreceptors engaged in clock entrainment by activating the MAPK signaling pathway [58].

3. Flavin-containing oxidase activity in response to blue light exposure leads to an increase in cellular H_2O_2 levels, which in turn act as a second messenger to activate expression of core circadian clock genes [59].

Teleosts lineage genome duplication lead to the appearance of a wide genetic variability. Within the clock genes, four *period* (*per1a*, *per1b*, *per2*, and *per3*), six *cryptochrome* (*cry1a*, *cry1b*, *cry2a*, *cry2b*, *cry3*, and *cry4*), three *clock* (*clock1a*, *clock1b*, and *clock2*), and three *bmal* (*bmal1a*, *bmal1b*, and *bmal2*) genes have been cloned from zebrafish (Table 1.1).

Light exposure dramatically increases expression of the clock genes *per2* and *cry1a* in all tissues and cell lines, triggering the transcriptional/translational feedback loop activity. Light regulates the transcription of these two key genes through a light responsive module consisting of promoters E- and D-box elements spaced close together and in proximity to the transcriptional start site of *per2* and *cry1a* [60]. The D-box confers light-driven expression through the bind with the thyrotroph embryonic factor (TEF) zebrafish homologue, whilst the E-box directs circadian clock regulation by mediating CLOCK-BMAL activity [61].

The zebrafish whole body widespread LEO support the hypothesis of a double role of cellular light sensitivity: rhythm keeper and UV damage control machinery. Indeed, several genes are either light-inducible expressed, and either DNA UV damaged engaged [62].

Nomenclature	Other names	Closest homologs	Role	Ref.
bmal1a	*bmal1, arntl1a*	*mbmal1*	Heterodimerizes with Clock	[63]
bmal1b	*bmal3, arntl1b*	*mbmal1*	Heterodimerizes with Clock	[64]
bmal2	*bmal2, arntl2*	*mbmal1*	Heterodimerizes with Clock	[63]
clock1a	*clock1*	*mclock*		[34]
clock1b	*clock3*	*mclock*		[64]
clock2	*clock2, npas2*	*mclock*		[64]
cry1a		*mcry1*	Light-induced gene. Represses Clock:Bmal activation	[65]
cry1b		*mcry1*	Represses Clock:Bmal activation	[65]
cry2a		*mcry1*	Represses Clock:Bmal activation	[65]
cry2b		*mcry1*	Represses Clock:Bmal activation	[65]
cry3		*mcry*	Doesn't repress Clock:Bmal activation	[65]
cry4		*dcry*	Doesn't repress Clock:Bmal activation. Photoreceptor function	[65]
per1a	*per1*	*mper1*		[66]
per1b	*per4*	*mper1*	Repressed by light. CLOCK is essential for its transcriptional regulation	[67]
per2		*mper2*	Light-induced gene. Necessary for the onset of the circadian clock	[68]
per3		*mper3*		[69]

Table 1.1 Zebrafish clock genes. List of zebrafish clock genes flanked by the closest homologs. Role of the zebrafish genes in the circadian keeping system. Adapted from: Vatine et al. 2011 [70].

1.8 *Exogenous inputs: the food entrainable oscillator*

1.8.1 *Mammals*

Food availability is an essential resource for survival and reproduction, so, the living beings evolved a food entrainable oscillator (FEO) which allows them to be active during the phase of incoming nutrients.

The molecular mechanisms underlying food anticipation in mammals remain controversial.

It has been shown that daily feeding–fasting cycle is altered in mice with impaired clock machinery, precisely, a global deletion of clock genes, including *per1*, *per2*, *cry1* and *cry2* or *rev-erbα* provokes dampened day–night variations of food intake in mice [71; 72; 73].

Despite intensive investigation, the brain location of the food clock is still elusive. The putative structures underlying the food clock might be located in hypothalamus areas such as the arcuate nuclei (ARC), the lateral hypothalamus (LH), the hindbrain (parabrachial nuclei), but also in the dorsal striatum and the cerebellum [72].

The proposed humoral-neural pathway engaged in food consumption homeostasis is the following: while fasting, levels of ghrelin in the plasma rise, at a certain threshold, ghrelin in the ARC stimulates the release of neuropeptide Y (NPY) an Agouti-related peptide (AgRP). AgRP is co-expressed with NPY and acts to increase appetite and decrease metabolism and energy expenditure. On one hand, neurons expressing AgRP and NYP activate the lateral hypotalamus where they triggered the release of orexin (a neuropeptide that regulates arousal, wakefulness, and appetite) and melanin concentrating hormone (MCH), and on the other hand, they inhibit oxitocin releasing neurons in the PVN. These mechanisms cooperate to trigger orexinergic effects. In turn, excitatory input from thyrotropin-releasing hormone neurons (TRH) located in the PVN feedback to AgRP neurons and induces intense feeding (Figure 1.5).

Lesion to the AgRP-NPY neurons results in reduced food anticipatory activity. Furthermore, the ARC receives anorexigenic humoral signals which activate pro-

opiomelanocortin (POMC) neurons and the release of alfa-melanocyte-stimulating hormone (MSH) which bind melanocortin receptors in the PVN and lateral hypotalamus. These mechanisms together participate in keeping satiation. Deletion of the melanocortin receptor 3 (MC3) gene in mice disrupted cortical expression of clock genes such as *bmal1*, *npas2*, and *per2* and attenuated food anticipatory activity during restricted feeding [72].

Interestingly, the magnitude of response observed in NPY/AGRP and POMC neurons to feeding related cues (such as ghrelin administration or fasting), as well as during genetic or diet-induced obesity, has been demonstrated to be dependent on levels of ROS production [74]. Indeed, the NAD+ dependent PARP-1 (poly ADP-ribose polymerase 1) activity is cycled and regulated by feeding entrainment in the mouse liver. PARP-1 deficient mice show abnormal peripheral clock and locomotor activity in response to the scheduled feeding cycle [75]. Furthermore, metabolites can mediate food intake through metabolic reprogramming of the phase via multiple signaling pathways. Under restricted food access, the accumulated free fatty acids and glucagon activate PPARα (peroxisome proliferator-activated receptor alpha) and CREB (cAMP response element-binding protein) signaling pathways leading to anti-phase rhythmic expression of *rev-erbα*, *per1* and *per2* in the liver [76]. Glucagon is released by pancreatic α cells and flows into the plasma during fasting. As the levels of glucagon increase, liver glucogenesis starts and transcription of *per1*, *per2*, *bmal1* is activated by CREB and CREB regulated transcription co-activator (CRTC2) [76; 77]. In addition, time-restricted feeding regimen stimulates GSK3β (glycogen synthase kinase 3β), DBS (RORE DNA binding sequence), AMPK (AMP-activated protein kinase), mTOR (mammalian target of rapamycin) pathways to mediate FEO related outputs [78]. Insulin signalling can reset peripheral clocks by triggering *per1* and *per2* hepatic transcription and repressing *rev-erbα* expression. At post transcriptional level, insulin also stimulate AKT (protein kinase B) mediated phosphorylation of *bmal1* [79; 80].

Humoral signals are also engaged in feeding entrainment. For instance circulating levels of gherlin oscillate accordingly to feed time: they rise and fall before and after a meal time and the rise again all along the fasting phase [81]. Moreover, impairment of gherlin receptors outcome in a reduced food anticipatory activity [82]. Oxyntomodulin (OXM) stimulates food intake, its secretion from the gut resets

liver transcription rhythms via induction of the core clock genes *per1* and *per2*. Inhibition of OXM signaling blocks food-mediated resetting of hepatocyte clocks [83]. *Rev-erbα* is involved in the modulation of the transcription of metabolic, lipidic, and glucidic genes. *Rev-erbα* regulates metabolic genes primarily by recruiting the HDAC3 (histone deacetylase 3) co-repressor to sites to which it is tethered by cell type-specific transcription factors. REVERBα can modulate the transcription of: ELVOL (elongation of very long chain fatty acids), ELOVL5 (fatty acids elongase 5) and ACSS3 (acyl-CoA synthetase short-chain member 3) [84]. Glycerol and lipid metabolism are modulated by REVERBα and HCAD3 coordinating binding which influence the expression of AGPAT(1-acyl-glycerol-3-phosphate acyltransferase) and LPIN (phosphotidate phosphatase lipin), ultimately limiting lipidic biosynthesis and toxic lipidic accumulation during the feeding phase [85]. *Rev-erbα*, controls the timing of cyclic accumulation in the nucleus of *srebp1*, a key transcription factor that regulates genes in the *de novo* lipogenesis and glycolysis pathways [86; 87]. Interestingly, not only REVERBα, but half of the nuclear receptors are rhythmically expressed in the liver [88]. Together, they cooperate to shape metabolism through the regulation of gene expression in concert with core clock transcription factors PER2, CRY1 and CRY2 [89; 90].

In SCN-lesioned rats, restricted feeding (RF) is capable of trigger rhythmic pineal melatonin synthesis and rhythmic transcription of AANAT and a rhythmic expression of c-FOS [91].

The circadian clocks resetting may occur by peripheral metabolic and neuronal signals which coordinate food- anticipatory rhythms controlled by the food clock. Moreover, a daily palatable food reward can trigger an anticipatory bout of locomotor activity and arousal [92], suggesting that, in addition to metabolic cues, the dopaminergic reward pathways play a modulatory role in food anticipation [93]. Such framework highlight how far are from clear the food entrainable oscillator properties and it stressed out on the idea than rather than a simple defined pathway, a complex dialogue between clock, neuronal and humoral factors is engaged in maintaining the feeding related phase.

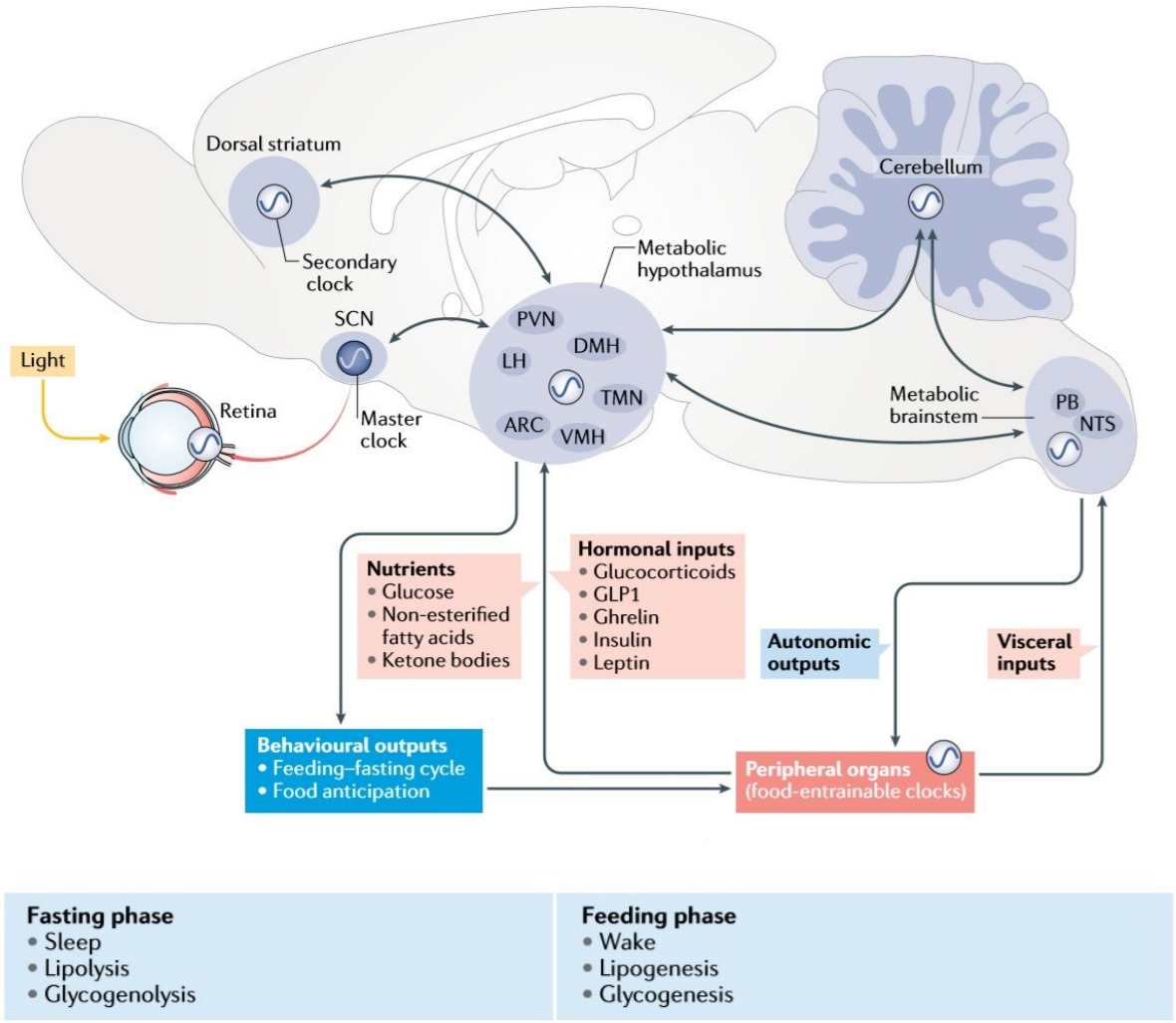

Figure 1.5 Putative FEO districts in rodent's brain. The brain locations of the food clock are: the arcuate nuclei (ARC), the metabolic hypothalamus, the parabrachial nuclei, the dorsal striatum and the cerebellum. Feeding and fasting alternations are gated by the peripheral organs which send information on energy status and mealtime to the brain via circulating nutrients and hormonal inputs as well as visceral neural inputs.

ARC, arcuate nuclei; DMH, dorsomedial hypothalamic nuclei; GLP1, glucagon- like peptide 1; LH, hypothalamic lateral areas; NTS, nuclei of the solitary tract; PVN, paraventricular nuclei; TMN, tuberomammillary nuclei; VMH, ventromedial hypothalamic nuclei. Adapted from: Challet 2019 [72].

1.8.2 *Fish*

The investigation on the FEO is at an embryonal stage in fish. As in mammals, fish LEO and FEO usually phase together and locomotor activity stays aligned to them. If the feeding cue is the only zeitgeber available, fish synchronize their locomotor activity and their clock genes expression (*per1, per2*, *cry, bmal1, clock*) in the liver, but not in the brain, to it. Instead, if light and feeding stimuli are copresent, genes expression (*per1*) in the brain stays always synchronized to the light-dark cycle regardless of the feeding timing in the zebrafish and in the sea bream [94; 95; 96; 97]. This is not true for the goldfish, where the liver but also the hypothalamus and the optic tectum express genes (*per1*, *cry3*) in phase with the feeding schedule [97]. Another study conducted on goldfish highlighted the capability of the hindgut genes *per1*, *per2*, *per3* to tick in phase with the feeding time [98]. In cavefish, a troglomorphic fish adapted to constant dark environment, FEO can oscillate with a 47 h period despite lacking entrainment by LD cycles [57].
Within the endogenous synchronizers, gherlin and orexin has been proved to entrain goldfish hepatic cells. This hormones trigger entrainment of *bmal*, *clock*, *per*, *reverbα* in cultured liver [99].
FEO anatomical district and physiological features are at the beginning of their investigation, and, as mentioned above, fish FEO properties can be different between species, so, a wide spectrum of research must be done to clarify this subject.

1.9 *Endogenous inputs: the role of Glucocorticoids*

1.9.1 *Mammals*

Glucocorticoids (GCs) are steroid hormones involved in several physiological processes including metabolism homeostasis, brain functions, immune regulation, and stress response.

The circulating levels of GCs are normally regulated by the hypothalamus–pituitary–adrenal (HPA or hypothalamus–pituitary–interrenal, HPI in fish) neuroendocrine axis in response to stress stimuli. Beside variations due to stress, a peak of GCs can be measured prior or in correspondence to the onset of the active phase of the day (early morning in human and zebrafish and early evening in rodents), or as part of the so-called circadian GCs rhythm [100]. Furthermore, also a pulsatile, ultradian secretion of GCs is present.

Based on chronobiological research, regulation of GCs circadian rhythm is believed to rely on multiple control mechanisms [101]. Primary, the regulation of this hormone concentration can be adjusted by the suprachiasmatic nucleus (SCN) alias the circadian central pacemaker in the mammal's brain. Modulation of the HPA axis activity has been demonstrated by ablation of SNC and the resulting loss of adrenocorticotropic hormone (ACTH) and GCs daily rhythms [102]. Moreover, the central pacemaker can control the adrenal gland also by splanchnic nerve innervation [103]. Other mechanisms are adrenal-intrinsic and linked to the adrenal local oscillator [101] that controls the sensitivity of the gland to ACTH [104] and the cyclic expression of steroidogenic acute regulatory protein (StAR), a rate-limiting gene of steroid biosynthesis [105] and an adrenal-specific clock-controlled gene [106]. Interestingly, *StAR* cyclic expression, and thus GCs concentration, seems to be mainly regulated by the adrenal local clock itself, indeed its mRNA persists after splanchnic denervation [106].

Glucocorticoids act as internal time-giver which coordinate circadian signals within peripheral tissues that express their cognate receptor. Once GCs bind the glucocorticoid receptor (GR), the complex translocates into the nucleus, where it directly binds to GCs responsive elements (GREs) in the promoter region of target genes, thus regulating their transcription in positive or negative (nGREs) ways. GRE elements are located also upstream to circadian genes responsive elements which transcription can be in turn up or down regulated depending on the amount of circulating GCs (Figure 1.6). These secondary clocks in the peripheral tissues are reset by glucocorticoid through the transcriptional modulation of the clock gene *per1*, *per2* and *reverbα* [107; 108].

Glucocorticoids interaction with peripheral oscillator influence also the FEO. In rats fed during the day, their peripheral organ such as liver, kidney, heart, and pancreas show circadian gene expression in phase with the feeding schedule

despite the SNC stays synchronized to the light cue [13]. The phase adaptation of peripheral organs to daytime feeding is slow and this kinetics of phase adaptation is glucocorticoid dependent. In the absence of the glucocorticoid receptor or of glucocorticoid hormones, the phases of peripheral oscillators adapt very quickly to the daytime feeding regimens. Hence, the rhythmic secretion of corticosterone appears to inhibit the uncoupling of peripheral clocks from the central pacemaker [109].

The pre-prandial peak of GCs positively modulates food-anticipatory activity and ultimate provide for glucose demand control by synchronize behavioral and physiological functions. The increased concentration of circulating GCs before food intake is not mediated by ACTH, but it might be triggered by noradrenaline in the paraventricular nuclei (PVN) and in the pituitary gland. PVN neurons release corticotropin release hormone (CRH) typically in response to stress, nonetheless, in mice with CRH impaired signalling, bout activity related to food consumption is shifted to the resting phase. Feeding during the active phase can be restore after exogenous glucocorticoid treatment supporting the idea about this hormone capability to maintain feeding entrainment [110]. Furthermore, it has been shown that during the active phase onset, rodents prefer carbohydrate based meals which foster the release of glucocorticoid and noradrenaline in the PVN [111].

A coordination between circadian clock and glucocorticoids is also engaged in the glucose metabolism. Extensively explored has been the glucose homeostasis in the liver where blood glucose fluctuations are buffered in a time-of-day dependent manner [112].

Indeed, glucose transporters and glucagon receptors peak at the beginning of the activity phase, when food consumption occur after a phase of fasting, etymologically speaking: the breakfast. At this time of the day, the synthesis of glycogen polymers is anticipated by CLOCK dependent expression of liver glycogen synthase [113]. Conversely, if glucose levels are low, gluconeogenesis during the rest phase is stimulated by glucocorticoids thanks to a protein-protein interaction between CRY and the glucocorticoid receptors [114; 115]. Cryptochromes also regulate gluconeogenesis mediating glucagon receptors signaling. Activated glucagon receptors trigger cAMP production which in turn regulate circadian cAMP phosphorylation of CREB and its transcriptional activity which include the expression of a key gluconeogenic gene, phosphoenolpyruvate

carboxykinase (*pck1*). cAMP accumulation is indeed inhibited by cryptochromes negative modulation of the heterotrimeric protein g which transduce the signal from the glucocorticoid receptor resulting in a lowered expression of PCK1 [116; 117]. Overall, the major glucocorticoid role is to maintain glucose homeostasis with the ultimate goal of preserving plasma glucose storage to be deliver to the brain during stress, as transiently raising blood glucose is important to promote maximal brain function.

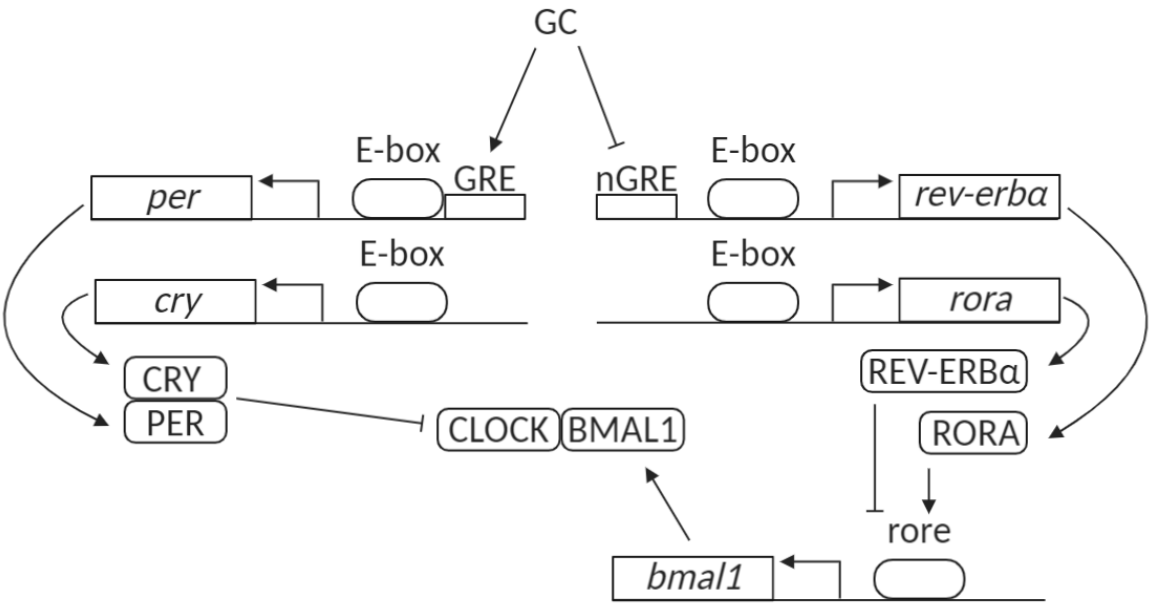

Figure 1.6 Glucocorticoids interaction with the core clock genes. Glucocorticoid responsive elements upstream to the core and the accessory loop can in turn up or down regulate the transcription of clock genes.

1.9.2 *Fish*

The main glucocorticoid produced by fish is cortisol which, besides its main role in the regulation of the stress response, influences many other processes such as behavior, growth, reproduction, and osmoregulation [118].

As mentioned earlier, a genome duplication occurred around 450 million years ago before the radiation of teleosts and after the tetrapods divergence from fish lineages [119]. Duplicates GR genes are present in salmonoids (rainbow trout)

and percomorphs (fugu, tetraodon, Burton's mouthbrooder) [120; 121]. The two receptors encoded by these genes show a high amino acid sequence identity that range from 49 to 51% in the overall sequence and from 80 to 86% in the C-terminal domain (DNA binding, hinge and ligand binding region). These genes are also subject of alternative splicing [122; 123]. GR duplicated and spliced variants are differentially expressed in fish tissue and show a different affinity for cortisol [123; 124].

Surprisingly, in zebrafish, the genome contains a unique *gr* gene [125], which show high similarity to the human gene, *hGR* [126]. In humans, two main protein isoforms, GRα and GRβ, are produced by means of alternative splicing processes. Although the splicing events diverge in the two species, the zebrafish Grβ is comparable to its human equivalent in structure and expression level [127], but it differs in function, lacking the dominant-negative inhibitor activity of zGrβ in either cultured cells or zebrafish larvae [128].

Thanks to molecular genetic tools, the role of GR has been explored in numerous zebrafish mutants.

The zebrafish mutant gr^{s357} has a single base pair change which disrupts the GR transcriptional regulation of its target genes. This mutation lead to a hyperactivated HPA axis, such as increased expression of POMC, blunted suppression of cortisol by dexamethasone (a glucocorticoid receptor agonists) and increased depression-like behavior in response to mild stress. Diazepam and fluoxetine (antidepressant of the selective serotonin reuptake inhibitor (SSRI) class) treatments, as well as social interactions, reverse the abnormal behavior [129]. Besides, visual behavior is abolished following a period of darkness and recovers sluggishly after return to the light. Indeed, GRs are expressed in the inner nuclear and ganglion cell layers of the larval zebrafish retina. As a results, a mutation in the retinal homeobox gene 3 (*rx3*) provokes a loss of corticotrope cells in the pituitary gland as well as reduced cortisol levels [130]. Specifically, this mutation alters the basal fluctuation but not the rhythmic variation of the hypothalamic *crh* suggesting its independency from circadian glucocorticoid signaling, however, the dampening of the cortisol rhythm is accompanied by the alteration of the core circadian regulating gene *per4* [131].

Genes designated in the dopamine signaling (an important neuromodulator engaged in the light adaptation) are affected by gr^{s357} mutation [132]. Furthermore,

gr^{s357} mutant larvae have larger auditory-evoked startle responses compared to wildtype sibling controls, despite having lower spontaneous activity levels. Fluoxetine (Prozac) treatment in mutants decreases startle response and increases spontaneous activity, making them behaviorally close to the wildtype. These results mirror known effects of selective serotonin reuptake inhibitors (SSRIs) in modifying glucocorticoid signaling and alleviating stress disorders in human patients [133] and underline GR role in the integration of neuroendocrine and sensory responses to environmental changes. Again, *gr*^{s357} cortisol signaling through the GR receptor was thought to be a main determinant of exercise-enhanced growth. However, RNA sequence analysis revealed modulatory effects of cortisol, mediated by GR, at the transcriptional level in white skeletal muscle tissue during exercise-enhanced growth, which apparently do not affect growth but most likely alter the physiology of the muscle tissue [134]. Mutants in exon 2 of *mc2r* (adrenocorticotropic hormone receptor), and in exon 2 and exon 5 of *nr3c1* (glucocorticoid receptor), showed significantly decreased locomotor responses in light and hyperosmotic stress assays. The locomotor response phenotypes of homozygous mutant fish in the corticosteroid receptors can be switched to the wild type phenotype by treating them with anxiolytic drugs (SSRIs) or anxiogenic compounds (phenylephrine) [135]. The examination of pituitary mutant larvae, which corticotrope cells contained lower cortisol levels than wild type, revealed that circadian cell cycle rhythms depend on systemic input from this gland [130]. This study pinpointed the corticotrope lineage as the source for the required signal demonstrating that cell cycle rhythms can be rescued by tonic treatment with dexamethasone. In addition, the fibroblast growth factors (FGF3) has been shown to be a key factor during pituitary development in the zebrafish, *lia*/*fgf3* null mutants [136].

Also, transient knock down of steroid biosynthesis by meaning of morpholino have been investigated. When subjected to hypoxia, embryos morphants (an antisense morpholino (Mo) targeted towards the zebrafish GR gene) showed delayed physical development, in particular: slower hatching, straightening of head–trunk angle and shorter body length. They also showed a lowered locomotor activity, reduced tactile responses and anxiogenic activity which persists through adulthood [137].

The existence of rhythms in the HPI axis components of fish has scarcely been investigated. Since the feeding–fasting cycle is a powerful non-photic signal that may synchronize the daily cortisol rhythms in fish, pre-prandial cortisol changes have been observed in some teleosts, such as rainbow trout [138; 139], brown trout [140], goldfish [141; 142] and gilthead seabream [143]. A daily rhythm in circulating adrenocorticotropic hormone was demonstrated in goldfish [144] as well as a rhythmic expression of genes encoding pro-opiomelanocortin [145], and the *crh* receptor 1 [146]. Furthermore, *crh* and plasma cortisol fluctuation in sole suggests that the daily cortisol rhythm may be a direct consequence of hypothalamic CRH production [147]. In the gold fish, the interrenal tissue owns a 24 h rhythmicity of *per1a*, *per3* and *cry3* in antiphase to the *clock1a*, as expected in a functional oscillator [146]. Moreover, dexamethasone represses the positive elements of the liver clock (*bmal1a* and *clock1a*) in the gold fish cell culture [148], suggesting a putative resetting function of GC in fish. Ghrelin hormone is engaged in clock genes regulation via the PLC-PKC pathway and, to a lesser extent, the AC-PKA pathway [99]. In zebrafish and cavefish cell lines transfected with zfper1b-luc, dexamethasone entrains bioluminescence rhythms [57]. Apparently, the molecular mechanism underlying this entrainment is the GC induction of *per1*, a process already reported in the goldfish liver both *in vivo* and *in vitro* [148] and in cavefish cell lines [57]. Dexamethasone can trigger a phase shift in *opn4m1* in zebrafish cell culture whereas *opn4m2* oscillation is not affect by dex pulses. The presence of GRE element upstream to *opn4m1*, and its absence on *opn4m2* may explain dex mechanism of interaction with only one melanopsin gene. Exposure to exogenous corticosteroid modulate core clock genes transcriptome in zebrafish which shows either down (*rorca*, *rorcb*, *arntl1a*, *arntl1b*, *arntl2*) or up (*clock1b*, *per1a*, *per2*, *cry1b*, *ndr1*, *ndr2a*) regulation. Transient upregulation of *per1* and *per2* and downregulation of *reverbα* expression occur by meaning of GC bind to GRE and nGRE [149; 150]. These effects mirror the impaired locomotor activity detected in treated zebrafish larvae [151]. Glucocorticoid interaction with the circadian system has been proven to impair behavioral, genetic and humoral factors in fish, increasing the curiosity for more exploration.

1.10 *Outputs*

In experimental sciences, the values of dependent variables depend on the values of independent variables. The dependent variables represent the output whose variation is being studied.

1.11 *Locomotor activity as read out of the circadian rhythm*

Although it seems anachronistic considering the current knowledge on circadian rhythms, the output has historically been the first evidence of rhythmicity recorded in nature. Specifically, behaviors such as foraging, mating, predation and social interaction are strongly rhythmic and triggered scientist curiosity on chronobiology. The standard circadian behavioral readout in zebrafish, and indeed in many other laboratory organisms, is the locomotor activity.

1.12 *Light entrained locomotor activity*

The story about circadian rhythm started a very long time ago, before inputs, oscillators and outputs were linked together to tell us the entire process of entraining. Interestingly, the first record of circadian rhythmicity come from an astronomer called Jean-Jacques d'Ortous De Mairan [152]. He noticed that plants maintained in constant darkness and at a relatively constant temperature demonstrate diurnally periodic leaf movements in much the same way as if the plants were exposed to the normal light-dark alternation. This discovery is to date in 1729, when the observation of the output, namely the behavior, laid the foundation of circadian rhythm concept.

Since then, chronobiology focused its studies on organism movements, and the gold standard to describe animal chronotypes became the recording of their

spontaneous locomotor activity as it is a highly correlated behavioral output of the circadian clock.

Light-entrainable locomotor activity must be the outcome of three networked process: photoreception, it allows to synchronize and to adjust the locomotor activity to the dark-light cycle; intrinsic oscillation: the locomotor activity persists in absence of the stimulus, namely the free-running period; and the capability of convey the phase to local oscillators by keeping locomotor activity period set to the phase when all others resources are delivered.

Within 120 h post fertilization, zebrafish fully develop a complex nervous system [153; 154]. After 1 week of development, zebrafish larvae already display behavioral patterns as complex as adults: they show a defined locomotor repertoire such as swim, turn and pray capture. The swim pattern is initially infrequent and in bursts, namely, slowly transitions into beat-and-glide swimming mode after swim bladder inflation and before feeding at 4 dpf [155].

From around 20 h post fertilization zebrafish react to light [18]. After the first day of development, they respond with heavy tail twitches to a short light flash [156], and at 4 dpf, a distinct day-night activity pattern is expressed [157]. This daily locomotor activity has been shown to be linked to a robust circadian rhythm. Larvae maintained in constant conditions are rhythmic and the timing of peak activity in constant conditions is determined by the prior LD cycle, with the highest activity during the subjective day. The mean freerunning period of the activity rhythms is 25.6 h [158]. The same pattern has been assessed also in zebrafish adults (Figure 1.7) [159].

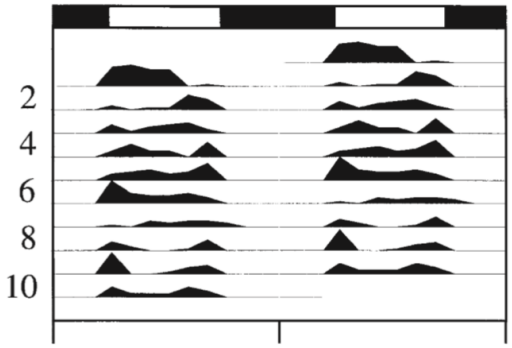

Figure 1.7 Zebrafish adult locomotor activity. Representative double plotted actograms of zebrafish locomotor activity in a 12:12 LD cycle (light and dark phases are indicated by top white and dark bars). The y axis shows 11 days of light-dark entrainment. Each day reports activity bouts as dark shadow. Adapted from: Hurd et al. 1998 [159].

1.13 *Food entrained locomotor activity*

The feeding entrainment phenomenon is detected by a distinctive behavioral trait: the food anticipatory activity (FAA). An anticipatory system can be defined as one containing a predictive model of itself and/or its environment, which allows it to change state in accordance with the model's predictions pertaining to a latter instant [160]. FAA can be described as a rising bout of activity before food access is allowed, indeed, when food availability is restricted, animals adjust their behavior according to the timing of food access (Figure 1.8). In 1910, August Forel realized he was visited by bees that came to feed on his marmalade while he was eating breakfast on the patio of his vacation apartment. The number of bees increased over a number of days until they were so numerous that he could no longer eat outdoors. On subsequent days, Forel noted that the bees continued to arrive at breakfast time even though he and his marmalade were safely indoors. He reported that the bees appeared to have a time-memory (zeitgedachtnis) for when breakfast was served. Hugo Berthold von Buttel-Reepen (1900) had earlier reported that bees exhibited a time-sense (zeitsinn) for foraging, they visited a buckwheat field only in the morning while the blossoms were open and secreting

nectar. These studies constitute the earliest descriptions of food anticipatory activity (FAA) [161]. Curt Richter (1922) conducted the earliest systematic analysis in mammals of timing behavior [162]. Among his many findings there was the observation that rats housed in constant illumination anticipated daily feeding with an increase in activity in the two to three hours preceding food presentation. About a century ago FAA has already been described in phylogenetic distant species, all of them share food entrainable clocks that are self-sustaining [163; 164], exhibit circadian limits to entrainment [165], and generate transient cycles in response to phase shifts of the entraining schedule [166], exactly like the light entrainable oscillators. It is possible that different motivational states promote different behavioral strategies for acquiring necessary resources. Given that food sources differ in nutrient composition, it might be reasonable to hypothesize that that FAA may change dramatically if the food source is rewarding. Indeed, FAA has been demonstrated to be more robust when highly palatable meal is delivered [92] or when food is accompanied with a freely rotating wheel [167]. These results highlight the engagement of dopamine-sensitive circadian oscillators in FAA because dopamine pathways are critical for processing of reward stimuli and for expression of motivated behavior [93]. Ablation experiments investigated the brain structures depicted as FAA keeping structures. Among them, SCN [168], hypothalamus [169], hippocampus, amygdale and nucleus accumbens [170], area postrema [171] and olfactory bulb [172] are not required for FAA emergence. Core clock genes investigations related to FAA entrainment shown that FAA is persistent in clock mutant mice which lack circadian function in all tissues [173; 174]. Nevertheless, FAA closely interacts with the circadian clock machinery: *per2* mutant mice fail to display FAA [175]. FAA gherlin dependent is significantly reduced in mutant mice [81]. Furthermore, *rev-erbα* global knockout mice subjected to restricted feeding showed reduced elevations of locomotor activity prior to mealtime, regardless of the lighting conditions [176]. Restricted periodic food availability induces FAA also in zebrafish. Rhythmic expression of the core clock genes (*per1*, *cry1*) in the zebrafish liver has been reported to adjust its circadian phase according to inverted feeding regimes [94], whereas these genes are insensitive to the feeding schedules in the zebrafish brain suggesting an uncoupled activity of the brain and peripheral oscillators [95].

The mechanisms of FAA generation and regulation analyzed so far underpin a complex scenario, where clocks oscillators might not be the only characters involved in maintaining this behavioral trait. Rather, a conjunction of multiple factors, such as predictions, expectations, learning, memory, reward, punishment, cognition, arousal and homeostatic feedback can be associated to a multifaceted control of anticipation.

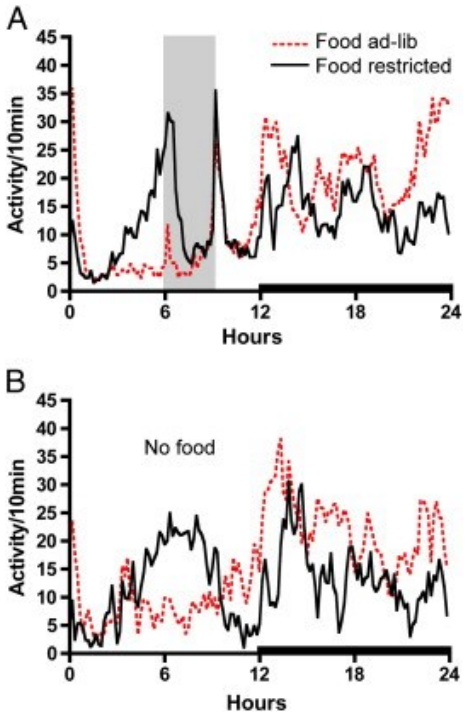

Figure 1.8 Food anticipatory activity in mice. Restricted food availability triggers an increased locomotor activity few hours before the forthcoming meal (Panel A, black line). Feed entrainment persists in absence of the input demonstrating the self-sustained properties of the FEO. Adapted from: Mistlberger 2011 [177].

1.14 *The zebrafish as a model system in chronobiology*

During the last decades, zebrafish (*Danio rerio*) became a valuable model for the study of the vertebrate circadian system thanks to several features. This species owns the classical traits belonging to a good organism model such as: short generation time (3 months), large number of eggs production (hundreds per week), large egg diameter (0.7 mm), transparent eggs and larvae, well recognized behavioral stereotypes and cheap costs of rearing and maintenance. Moreover, the 70% of human genes have at least one obvious zebrafish orthologue, of these genes, the 84% are known to be associated with human disease [178]. In addition, zebrafish owns unique features for the study of circadian rhythm: as well as the eyes, teleosts own a photosensitive pineal gland, deep brain photoreceptors, and dermal melanophores that are light responsive [179]. Furthermore, the peripheral clocks of fish are directly entrained by light [33]. This led to the advantage of set internal clocks by simply exposing tissue to time-givers into a culture dish. Zebrafish exhibits robust circadian rhythms of locomotor activity both in larvae [158] and in adults [159], with high locomotor activity in the daytime and low activity in the nighttime. The organization of the zebrafish circadian timing system has a high degree of homology with the mammalian one. The core mechanism underlying the circadian clock is based on TTFLs. However, because of the genome duplications during early teleost evolution [119], the TTFLs are constituted by more homologues of clock components.

Zebrafish null glucocorticoid receptor $gr^{ia30/ia30}$ (hereafter referred to as $gr^{-/-}$ line) mutant line previously established [180] is engaged in my research on GCs interaction with the clock circadian. Differently from GR-null mice [181], zebrafish $gr^{-/-}$ mutants survive till adulthood, although with reduced fitness than wild-type (WT) fish, and thus can provide a useful model to *in vivo* study GCs functions in an integrated system. Our zebrafish line $gr^{-/-}$ displays physiological responses linked to GC-resistance, such as overstimulation of basal HPI due to failure of the negative feedback loop mediated by the GC-GR complex, in particular, *pomca* and *crh* fail to increase after stress induction. Moreover, cortisol concentration is higher in $gr^{-/-}$ with respect to WT and mutant are unresponsive to a mechanical stressor [180]. The evaluation of genes expressing for cytokines showed that the $gr^{-/-}$

mutant line does not respond to an inflammatory stimulus. For what concern the phenotype, *gr*$^{-/-}$ bulb epithelium was rather thin and less elaborated into folds than in WT suggesting lesser digestive capacity and nutrient absorption. The *gr*$^{-/-}$ heart ventricle has reduced trabecular network, the subcutaneous adipose tissue is significantly increased, and the pancreas extension is reduced.

Dysregulation of the daily GC rhythm occurs in various human diseases likes Cushing's syndrome, mood disorders, Alzheimer's disease, and metabolic syndrome in which GCs were found to deviate from the norm [182]. Understanding the importance of GCs rhythm would be of primary interest to better design GCs replacement therapies. Spatial and temporal variations of glucocorticoid activity have been firstly detected in the whole larvae using the ia20 zebrafish transgenic line [27]. In this line, Enhanced-GFP (EGFP) expression is driven by nine GRE tandem repeats allowing to dynamically trace GC transcriptional activity during development and adult life. We focalized our analysis on *egfp* mRNA WMISH in ia20 larvae during the day starting from ZT21, three hours before the lights on, until ZT5, in order to visualize the highly dynamic daytime GCs activity. GC activity was low and mainly limited to the digestive tract at the end of the scotophase (from 5 to 7 am) but was markedly intensified just before the lights on in the liver and the intestine, where it remained high till 10 am. In the eyes and the brain, the signal increased after lights on from ZT1 to ZT4 and then decreased (Figure 1.9).

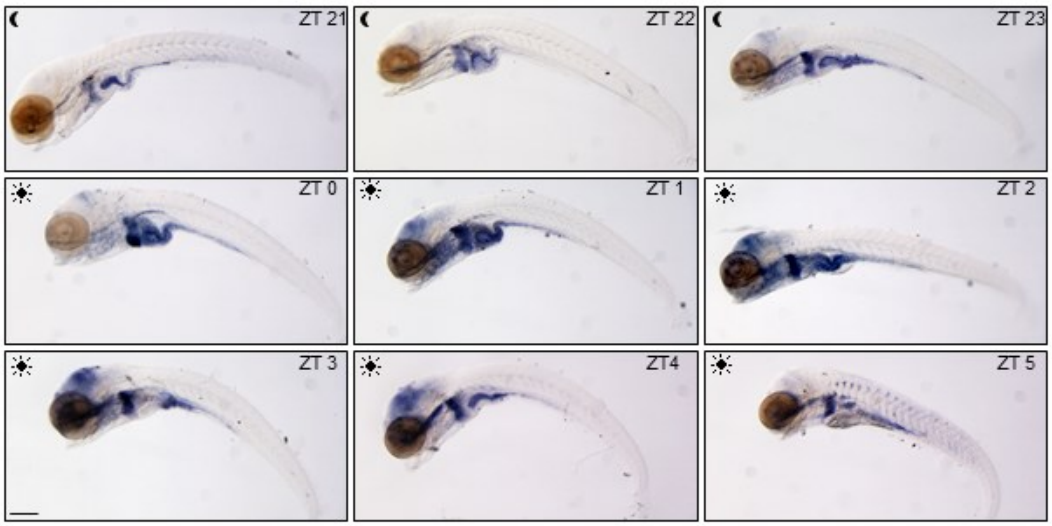

Figure 1.9 Spatial and temporal variations of glucocorticoid activity. WMISH of egfp mRNA in 5-dpf ia20 larvae exposed to standard photoperiodic regime and analyzed from 3 h before to 5 h after light onset (from ZT21 to ZT5). All larvae are lateral views with head pointing to the left. Scale bar: 200 μM. Adapted from: Morbiato et al. 2019 [183].

1.15 *Main aims*

The zebrafish circadian clock is a multioscillatory system capable of processing multiple sensory signals to drive complex temporally-coordinated outputs. To lock the activity phase in the light of adaptation, the oscillators are coupled by meaning of an extensive interaction between genetic, hormonal and humoral signals. In between them, GC interplay with the circadian clock has a major role in keeping oscillators aligned. When a mismatch appears, animals report several diseases.
My research goals attempt to tackle some key questions regarding the existence and relationship of multiple clock mechanisms in zebrafish. Particularly, I tried to dissect, using *gr*$^{-/-}$ zebrafish: a mutant built and kindly provided by Prof. Dalla Valle lab, the behavioral and the genetic features of this mutant by meaning of a classical chronobiology approach.

1. I've conducted a systematic study of circadian locomotor behavior under differentially phased light cycle. This will provide a more complete assessment of clock function in a GC impaired system.
2. I've linked behavioral responses of the clock network to their underlying molecular oscillations to elucidate the relationship between internal and external representations of the time of day
3. I've linked behavioral responses of the clock network to the underlying molecular oscillations of metabolism genes to elucidate the relationship between internal and external representations of the time of day
4. To gain further insight on the peripheral clock network architecture, I've investigated 1. and 2. in the light of the FEO. This would contribute to a fuller picture of the multioscillatory processing by the clock system.
5. I've investigated 1. 2. and 4. In both larvae and adult of *gr*$^{-/-}$ zebrafish to explore GC and circadian clock interplay during the ontogeny.

2. Materials and methods

2.1 *Ethics Statement*

All husbandry and experimental procedures were performed in accordance with European Legislation for the Protection of Animals used for Scientific Purposes (Directive 2010/63/EU) and the Italian (D.lgs. 26/2014) animal protection standards. Research was approved by the University of Ferrara Institutional Animal Care and Use Committee and the Italian Ministry of Health (auth. num. 801/2017-PR).

2.2 *Zebrafish rearing*

Wild-type (WT) and mutant zebrafish as well as zebrafish of the (9xGCRE-HSV.Ul23:EGFP) ia20 transgenic line (hereafter referred to as ia20 line) [184] were staged and maintained according to standard procedures [185]. Embryos were obtained by natural mating and raised at 28°C in Petri dishes containing fish water (FW, 50X: 25 g Instant Ocean, 39.25 g $CaSO_4$ and 5 g $NaHCO_3$ for 1 l) and kept in a 12:12 light-dark (LD) cycle. Larvae and adults were euthanized with an overdose of tricaine methane sulfonate (Sigma-Aldrich, E10521).

2.3 *Novel tank test*

Novel tank test has been applied in adult zebrafish [186]. Zebrafish $gr^{-/-}$ and $gr^{+/+}$ are exposed to the experimental challenge in a pretreatment beaker before being transferred into the novel tank for behavioral observation and phenotyping. After pretreatment, video recording begin and the fish are quickly transferred to the novel tank by gently scooping them with a net (one fish per net) and then gently

placing the net in the novel tank, maneuvering it to allow the fish to swim out. Behavioral data endpoints are generated by video-tracking analyses in the 6-min novel tank test by Ethovision 11 software (Noldus Information Technology, NL). To avoid experimenter-induced behavioral alteration in fish, their behavior has been recorded for an additional 2 min, which will be excluded from behavioral analyses. The IR-sensitive camera was set to 25 frames per second. Irradiance was set at 0.6 W/m2 and the temperature was held constant at 28°C. Locomotor activity of each individual was calculated during a 6 min time window in a trapezoidal tank (15.2-cm height × 27.9-cm top × 22.5-cm bottom × 7.1-cm width,1.5-liter tank Techniplast). The tank has been divided into two equal virtual horizontal portions, marked by a dividing line on the outside walls. Behavioral endpoints analyzed are: mean distance moved, frequency spent to the top of the tank, frequency spent to the bottom of the tank, frequency of transitions from top to bottom or vice versa.

2.4 *Recording of adult locomotor activity*

Adult $gr^{-/-}$ and $gr^{+/+}$ zebrafish locomotor activity was recorded continuously by means of an infrared photocell (E3S-AD62, Omron) placed at the aquarium wall. The photocell was placed 2 cm from the water surface and 8 cm from the bottom. The number of light-beam interruptions was counted and stored every 10 min by a computer connected to the photocell. Zebrafish (n=6-8 per aquarium; 3 aquaria per genotype) were exposed under 12:12 light-dark (12:12 LD) cycle or constant dim light (LL). It is conventional to divide the 24-hour LD cycle into 24-hour zeitgeber time (ZT) units and to indicate the time of lights on as ZT0 and the time of lights off as ZT plus the number of hours till light switch off, in our case, light and dark phases are both of 12 hours, then ZT12 indicates the beginning of the dark phase. The 24-hour circadian cycle is divided into 24-hour circadian time (CT) units in absence of a zeitgeber. As a reference point in LL, the time that would normally correspond to the onset of activity (i.e., in LD 12:12 when light switch on) is the reference point for the beginning of a circadian cycle, and it is termed CT0. In all tests a light-emitting diodes (Superlight Technology Co. Ltd., China) sources were used. Irradiance was measured with a radiometer (DO9721, Probe LP9021

RAD, Spectral range 400-950 nm, DeltaOHM, Italy) and set at 0.6 W/m^2 for the LD tests and at 0.05 W/m^2 for the LL tests. The temperature was held constant at 28°C by means of water heaters (50 W, Sera GmbH, Germany) and recorded every 10 minutes with data loggers (Hobo Pendant, Onset Computer Corporation, Massachusetts, USA). The representation of actograms was performed using the chronobiology software El Temps (version 1.228). For each genotype, tests were conducted three times.

2.5 *Recording of larvae locomotor activity*

Gr$^{-/-}$ and *gr*$^{+/+}$ embryos were collected immediately after spawning and raised in FW in a light- and temperature-controlled incubator. At 4 dpf larvae were placed in 48-well plate (1 larva per well, 1.6 mL of FW medium) in the observation chamber of the DanioVision tracking system (Noldus Information Technology, NL). DanioVision is equipped with an IR-sensitive camera, a temperature controller unit and a power supply to control light intensity. From 5 dpf, larvae locomotor activity was tracked for 4 consecutive days and then analyzed by Ethovision 11 software (Noldus Information Technology, NL). The IR-sensitive camera was set to 25 frames per second. Locomotor activity of each larva was calculated as the total distance moved during a 6 min time window. A minimal distance moved of 0.2 mm was used. Larvae were kept under 12:12 LD cycles (lights on at 06:00, lights of 18:00) or under LL conditions. For light sources, an array of LED strips was used, and irradiances were set at 0.17 W/m^2 for the 12:12 LD cycle and at 0.015 W/m^2 for the LL conditions. For each genotype, three biological repeats were conducted.

2.6 *Feeding entrainment*

To study the effect of feeding time as an exogenous zeitgeber [57] on adult, *gr*$^{-/-}$ and *gr*$^{+/+}$ zebrafish (N=8/aquarium; 3 aquaria for each genotype) were maintained under DD and fed once a day (0.3% of body weight) at a fixed time (i.e. 06:00,

12:00 or 24:00) with dry food (TetraMin, Tetra GmbH, D) using a programmable feeder (Eihem, D). Fasting and shifting of the feeding time were applied to confirm the entrainment. Locomotor activity was recorded continuously by means of an infrared photocell (E3S-AD62, Omron) for 30 days. Two biological repeats were conducted.

The feeding entrainment has been also tested in juvenile *gr*$^{-/-}$ and *gr*$^{+/+}$ zebrafish. 15 dpf old larvae from both genotypes were maintained in 10 liters aquaria under LL (0.015 W/m^2) and constant temperature (28°C). Juveniles were daily fed at midday with frozen Artemia nauplii and powered food (GEMMA Micro 75, Skretting, USA). Juveniles had free access to the food for one hour. After 20 days of feeding entrainment juveniles were placed in 9-well plate (1 larva per well, 5 ml of FW medium) in the DanioVision tracking system (Noldus Information Technology, NL) in the same lighting and temperature conditions. Locomotor activity was videotracked for 84 consecutive hours and then analyzed by Ethovision 11 software (Noldus Information Technology, NL). The IR-sensitive camera was set to 25 frames per second. Locomotor activity of each juvenile was calculated as the total distance moved during a 6 min time window. A minimal distance moved of 0.2 mm was used. For each genotype, five biological repeats were conducted.

2.7 *Gene Expression Analysis*

Gr$^{-/-}$ and *gr*$^{+/+}$ larvae were maintained under 12:12 LD cycles and sampled at different time points (ZT3, 9, 15 and 21; ZT0 = lights on, ZT12 = lights off), during day 5, 6 and 12 dpf. For each ZT, 15 larvae were sampled and pooled (n=4 pooled samples per ZT). Juveniles from both genotypes were sampled at CT3, 9, 15 and 21. For each CT, 15 larvae were sampled and pooled (n=4 pooled samples per CT). *gr*$^{-/-}$ and *gr*$^{+/+}$ adult zebrafish livers and eyes were harvested from ZT3 every 6 hours for a day (n=5 per ZT). Total RNA was isolated from zebrafish larvae and tissues using Trizol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. The amount, quality and composition of isolated RNA were analyzed by BioSpec-nano (Shimadzu, Kyoto, Japan). One microgram of

total RNA was incubated with DNase I (Invitrogen, Carlsbad, CA, USA) at room temperature for 30 min and then at 85°C for 15 min to inactivate the enzyme. DNase-treated RNA was used to perform cDNA synthesis in a final volume of 20 µl, using iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA). The reaction was performed at 42°C for 30 min, followed by an inactivation step of 5 min at 85°C. Three microliters of 1:10 diluted first-strand cDNA was PCR amplified with a Chromo4 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) using SsoFast EvaGreen Supermix (Bio-Rad Laboratories, Hercules, CA, USA). Thermal cycling conditions were as follows: 3 min denaturation at 95°C, followed by 40 cycles of a 15 s denaturation step at 95°C and an annealing-elongation step for 20 s at 60°C. After amplification, a melting curve analysis to confirm the specificity of the amplicon was performed. Gene-specific primers for *per1b*, *per2*, *clock1a*, *cry1a*, *arntl1a*, *nr1d1*, *pck2* and *srebp1* are reported in Table 2.1. We verified the efficiency of the primers by constructing standard curves for all genes investigated. The relative levels of each sample were calculated by the $2^{-\Delta\Delta CT}$ method (where CT is the cycle number at which the signal reaches the threshold of detection [187]). As housekeeping genes, we used *gapdh*, *rplp0*, *18S* and *rpl13a*, validated reference genes for zebrafish [188; 189]. Primers for housekeeping genes are reported in Table 2.1. Nearly identical results were observed with all housekeeping genes. Each CT value used for these calculations is the mean of three replicates of the same reaction.

Gene	Forward (5'-3')	Reverse (5'-3')	Genbank acc. no.
clock1a	CTGGAGGATCAGCTGGGTAG	CACACACAGGCACAGACACA	NM_130957
arntl1a	TAGAGCGCTGTTTGCTGATG	GACCCGTGGACTTCAGTGAC	NM_131578
per1b	CCGTCAGTTTCGCTTTTCTC	ATGTGCAGGCTGTAGATCCC	NM_001030183
per2	ATGTCGATGGCTTTAGGCAG	CGAGACATCCAGAAGGTGCT	NM_182857
cry1a	TCCGCTGTGTGTACATCCTC	CAAACACTGCAGCAAAAACC	NM_001077297
nr1d1	GCAATTCACCCAACAAATCAG	CAGGCATGGACGCCATAGT	NM_001130592
pck2	CTGTGTGCTCATCCAAACTCC	GATCTCATAGCTGCACCAACG	NM_213192
srebp1	GACACTTCTCTGGACACTCTG	ATCGAACAGCCCAAACTCC	NM_001105129
rpl13a	TCTGGAGGACTGTAAGAGGTATGC	AGACGCACAATCTTGAGAGCAG	NM_198143
gapdh	GTGGAGTCTACTGGTGTCTTC	GTGCAGGAGGCATTGCTTACA	NM_001115114.1
rplp0	CTGAACATCTCGCCCTTCTC	TAGCCGATCTGCAGACACAC	NM_001161350
18s	ACCACCCACAGAATCGAGAAA	GCCTGCGGCTTAATTTGACT	KY486501

Table 2.1 qPCR primers sequences.

2.8 *Statistical Analysis*

All the results were expressed as means ± SEM. Data were analyzed by parametric and non-parametric tests to determine significant differences using the software Prism 5 (GraphPad Inc., La Jolla, CA, USA) or R 3.6.1 (https://www.R-project.org/). p values <0.05 were considered statistically significant. The novel tank test data are presented as mean ± SEM, **P < 0.01, ***P < 0.005, trend (U-test). Daily and circadian expression gene expression profile (periodicity and phase of oscillation) was evaluated by RAIN algorithm [190]. The presence of daily and circadian periodicity in the activity rhythms was determined by means of Cosinor [191] or χ^2 periodogram analysis (ActogramJ 1.0). Periodogram analyses were performed with intervals of 6-10 days. The daily acrophase (i.e. the time at which the peak of a rhythm occurs) of the locomotor activity rhythm was calculated (ActogramJ 1.0) and the average acrophase was determined by vector addition. The Rayleigh test was used to test whether the acrophases deviated from uniform

and whether they were concentrated at a given time of the day ($p<0.05$). Hotelling's Paired test was performed to test for differences among average acrophases of different periods ($p < 0.05$) [192].

3. Results

3.1 *Explorative phenotype*

The behavioral phenotype of the mutant has been explored trough the novel tank test [186]. No difference has been detected between the WT and the *gr*$^{-/-}$ in the total distance moved (Figure 3.1, A), however, the two genotypes showed statistically significant difference of the tank occupancy and exploration (Figure 3.2). *Gr*$^{-/-}$ spent 1/3 less time on the top of the tank ($p < 0.0001$) with respect to the wild type. The occupancy of the bottom is increased by 16% (p 0.005) in the mutant which also showed a 41% (p 0.0001) lowered transition frequency from top to bottom or viceversa (Figure 3.1, B, C, D).

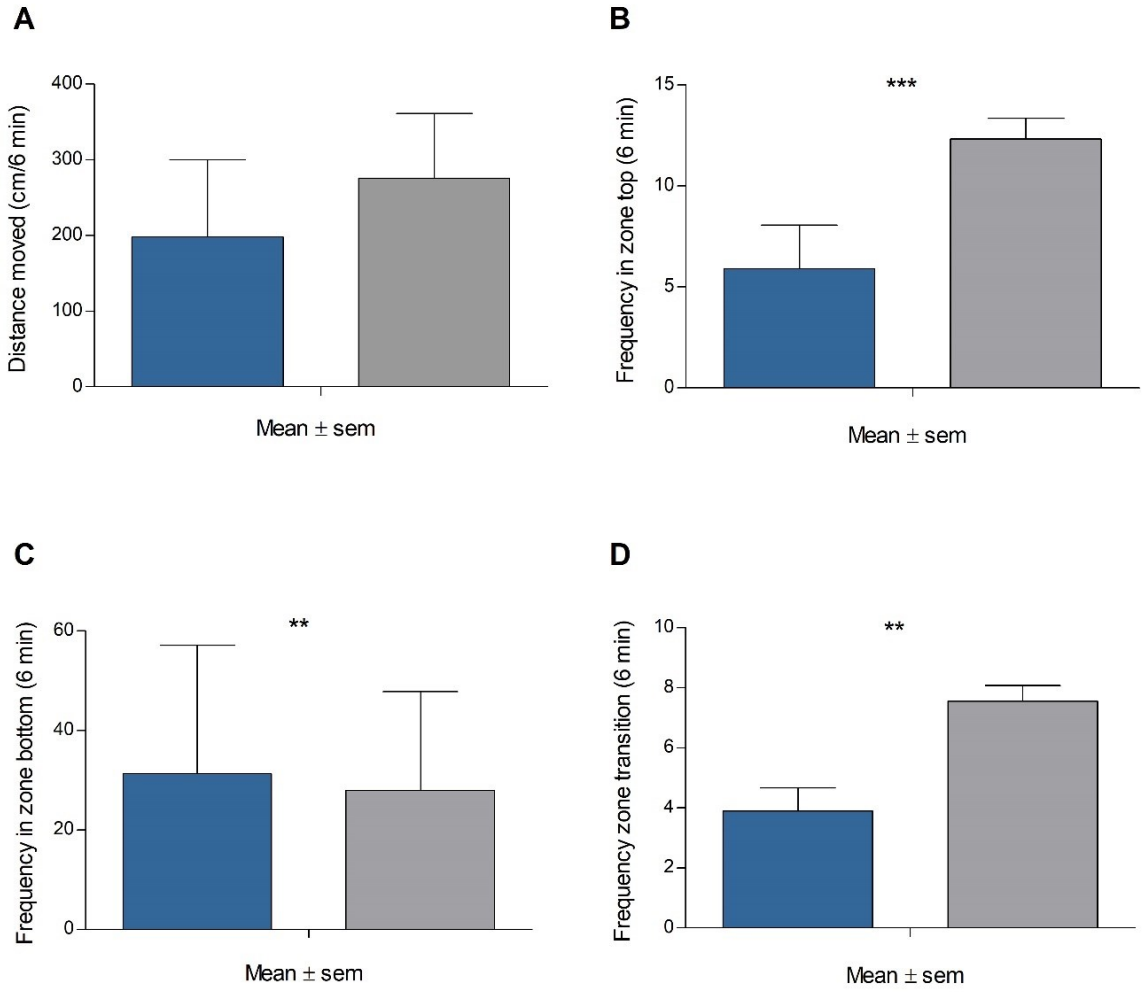

Figure 3.1 The novel tank test. Mean distance moved ± SEM (cm/6min) of WT (grey) and *gr*$^{-/-}$ (blue) adult zebrafish (A). Frequency in zone top: mean of events of center body point on the top of the tank (B). Frequency in zone bottom: mean of events of center body point on the bottom of the tank (C). Frequency of zone transition: mean of events of center body point crosses the virtual line which separates the tank into two equal zones (D). The test has been performed on 10 individuals per genotype.

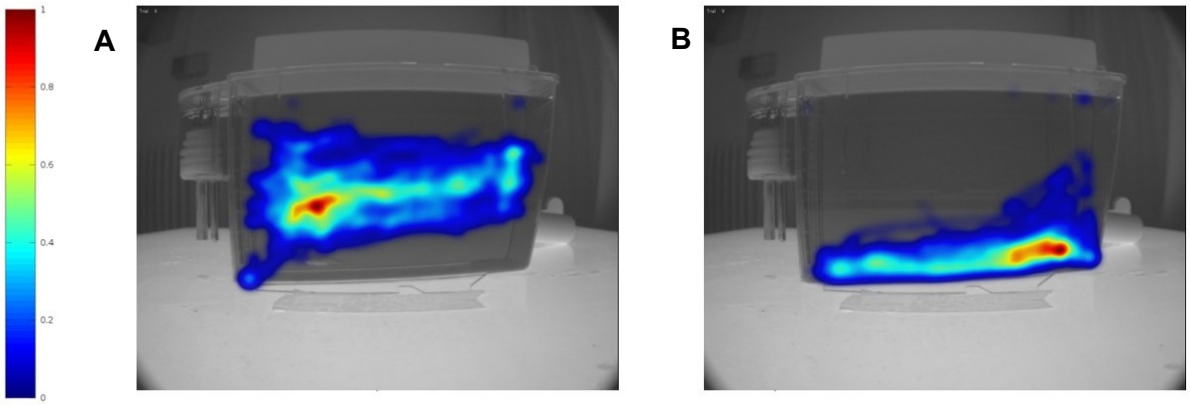

Figure 3.2 Novel tank heat map. Track visualization of the novel tank test. Shown is the actual path of the animals during the test. WT (A) and *gr*$^{-/-}$ (B). Time spent in each spot of the tank varies from blue (the less amount of time spent) and red (the maximum amount of time spent).

3.2 *Larvae light entrained locomotor activity*

To determine the role of GR signalling in generating and regulating circadian rhythms, I firstly analyzed the rhythmic locomotor activity of *gr*$^{-/-}$ and control larvae under various photic conditions using an automated high-throughput videotracking system. Control larvae displayed a daily rhythm of locomotor activity from 5 dpf (Cosinor, $p<0.001$) and the typical diurnal pattern of zebrafish, with higher activity during the light phase (Figure 3.3 A, D, E). Differently, daily rhythms of activity in *gr*$^{-/-}$ larvae became significant one day later, at 6 dpf (Figure 3.3 A-C; Cosinor, $p<0.001$). Indeed, the difference in the quantity of activity between light and dark phase in mutant appeared from 6 dpf (Tukey's Multiple Comparison Test, $p<0.05$), while in control larvae it was already present at 5 dpf (Tukey's Multiple Comparison Test, $p<0.001$; Figure 3.3 E). Furthermore, larvae showed differences in the overall daily amount of activity (Figure 3.3 E; Kruskall-Wallis one-way ANOVA, *gr*$^{-/-}$: $F(7,959) = 640.8$, $p<0.0001$, *gr*$^{+/+}$: $F(7,959) = 804.9$, $p<0.0001$) for the whole recording.

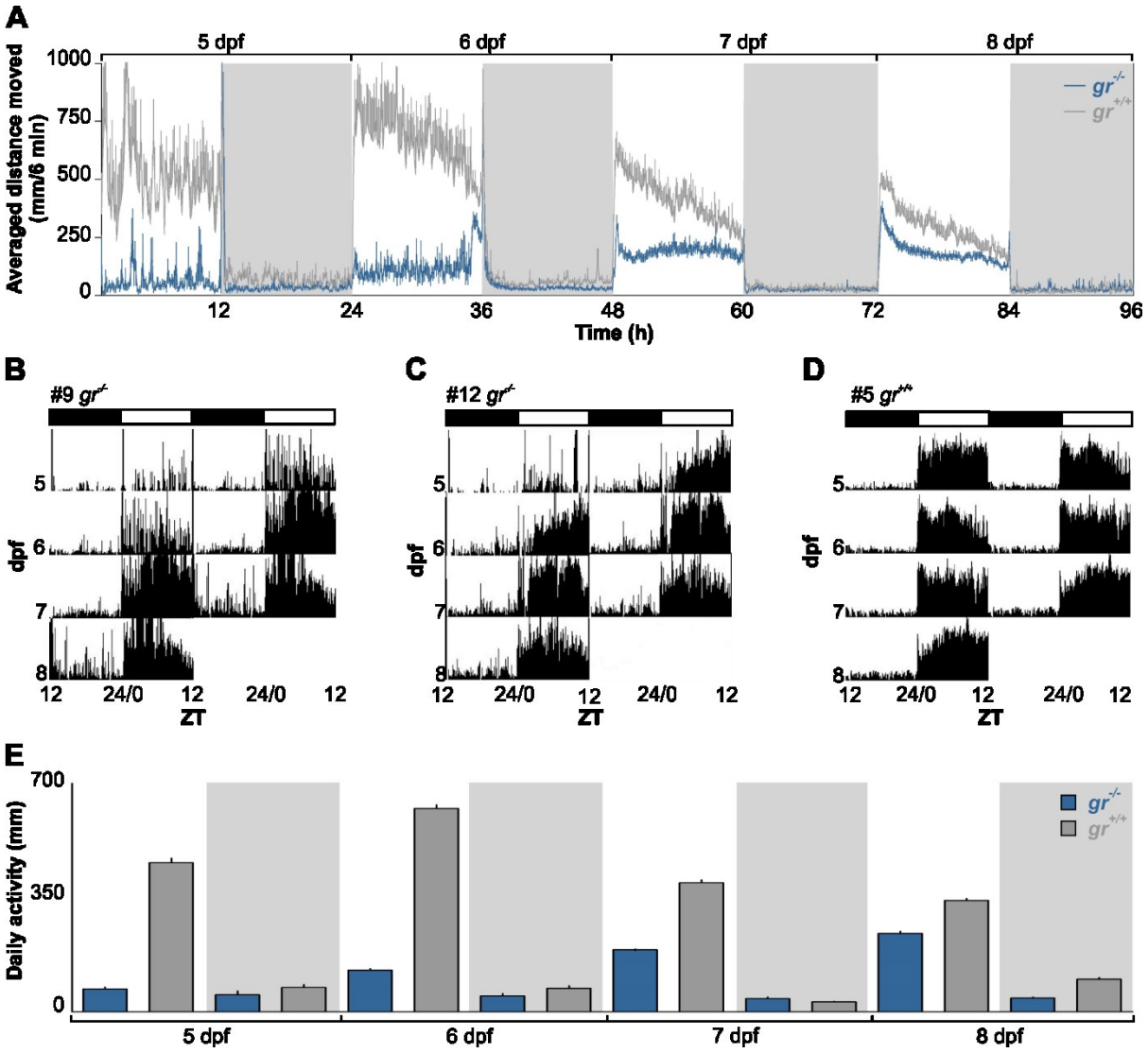

Figure 3.3 Daily activity rhythms of *gr*$^{+/+}$ and *gr*$^{-/-}$ larvae zebrafish. (A) Mean waveform of locomotor activity under 12:12 LD cycles from 5 to 8 dpf (n=120/genotype). Vertical axis shows averaged distance moved (mm/6 min) while x axis indicates time in recording. White and grey bars show light and dark phase, respectively. Data are expressed as mean ± SEM. (B-D) Representative actograms of 3 zebrafish (*gr*$^{-/-}$ B-C; *gr*$^{+/+}$ D). Actograms are double plotted on a 48-h time scale to help the interpretation. The height of each point represents the distance travelled in 6 min. Bars at the top of each actogram indicate light (white) and dark (black) phases the LD cycles. The age of zebrafish is indicated on the left and the zeitgeber time (ZT) is plotted on the bottom of each actogram. (E) Mean activity (n=120/genotype) in the light and dark phases from 5 to 8 dpf. Data are expressed as mean ± SEM.

3.3 *Larvae light entrained oscillator*

To confirm the presence of a circadian timekeeping system in zebrafish lacking GR I recorded circadian locomotor activity in larvae kept in constant dim light (LL). Larvae were exposed to 12:12 LD cycle until 6 dpf and then to LL for 3 days (Figure 3.4 A). I recorded the locomotor activity the second and the third day in constant condition and found a clear circadian rhythmicity with a period of about 24 hours in both genotypes (Figure 3.4 B; Cosinor, $p<0.05$).

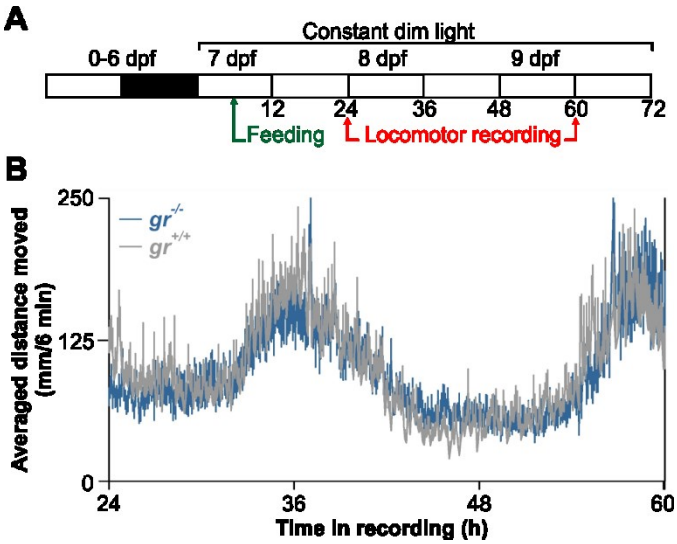

Figure 3.4 Circadian activity rhythms of $gr^{+/+}$ and $gr^{-/-}$ larvae zebrafish. (A) Larvae were reared under LD from 0 to 6 dpf and then maintained for 3 days in constant dim light (LL). Locomotor activity were recording from 24 to 60 hrs in LL. (B) Mean waveform of locomotor activity under LL for 36 hrs (n=144/genotype).

3.4 *Larvae light entrained clock core genes expression*

To check at the molecular level the lack of behavioral rhythmicity in mutant zebrafish at 5 dpf, I examined expression pattern of a set of clock and clock-related genes at different stages (5, 6 and 12 dpf) under LD cycles. I firstly

measured the expression of clock-regulated (*clock1a*, *arntl1a*, *per1b*) and light-regulated (*per2*, *cry1a*) genes [34; 36]. Larvae from both genotypes showed daily changes of expression levels in all genes investigated (Figure 3.5; Table 3.1). However, differences in the expression levels (Figure 3.5 A, E, G, H and J) or in the phase of peaks (Figure 3.5 B and D) have been found for both positive (*arntl1a* and *clock1a*) and negative (*per1*, *per2a* and *cry1a*) elements of the molecular clock. Interestingly, at 12 dpf, the expression level at ZT3 is significantly dampened in two light-inducible genes, *per2* and *cry1a* (Figure 3.5 H and J; Pairwise comparison after Kruskal-Wallis test, $p<0.05$), and in *per1b* (Figure 3.5 F; Pairwise comparison after Kruskal-Wallis test, $p<0.05$).

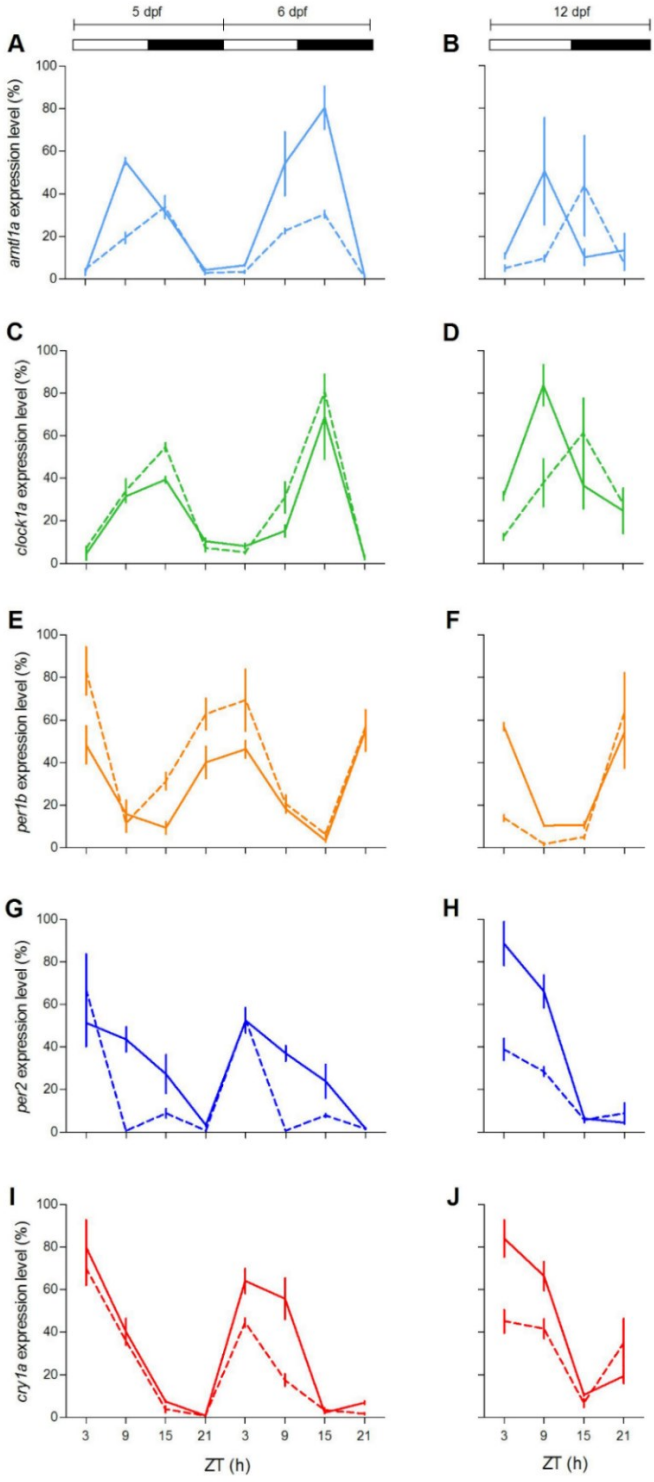

Figure 3.5 Daily expression levels of clock genes in zebrafish larvae. qPCR analysis of clock and light-regulated clock gene expression at 5-6 dpf (A, C, E, G, I) and 12 dpf (B, D, F, H, J) in zebrafish larvae exposed to LD cycles. For all panels, each point represents the mean ± SEM (n=4). On the y-axes are plotted relative expression levels (100% is the maximum level detected for each gene in all dpf), while on the x-axes time is expressed as zeitgeber time (ZT, where ZT0 represents lights on). White and black bars represent light and dark periods, respectively. $gr^{+/+}$= solid line; $gr^{-/-}$ = dotted line.

3.5 *Adults light entrained locomotor activity*

To verify the presence of behavioral and molecular rhythmicity in adult zebrafish lacking GR I performed behavioral and molecular analysis in adults (Figure 3.6-3.10). I recorded the locomotor activity in adult zebrafish from both genotypes exposed to two consecutives 12:12 LD cycles (LD1: lights-on at 06:00, LD2: lights-on at 12:00) (Figure 3.6). Both mutants and controls showed a clear rhythmic, diurnal pattern (Figure 3.6 A, F and 3.7), but the mean of daily activity in $gr^{-/-}$ was significantly lower than in the control (Mann-Whitney U-test, $p<0.001$; Figure 3.7). Periodogram analysis indicated a strong entrainment to both LD cycles (Figure 3.6 C, E, H, J). The 6-h shift of the lights-on from LD1 to LD2 induced shift of the activity onsets, which resulted, after 4-5 days, in entrainment to the new light schedule (Figure 3.6 A, E, F, J). To verify the accuracy of the entrained rhythm I estimated the time of acrophase (the time at which the rhythm peak occurred) with respect to the lights on (ZT0) for the last 10 days of recording in each LD cycle. Using a circular statistic approach, I showed that the distribution of acrophases deviated from uniform (Rayleigh test, $p<0.002$), and the mean acrophases fell between ZT2 and 4 (Figure 3.6 B, D, G, I). As expected, the mean acrophases significantly changed after the 6 hours shift of the lights on ($gr^{-/-}$ from ZT2 to ZT11; $gr^{+/+}$ from ZT2.5 to ZT10.8; Hotelling's Paired test; $p<0.0001$).

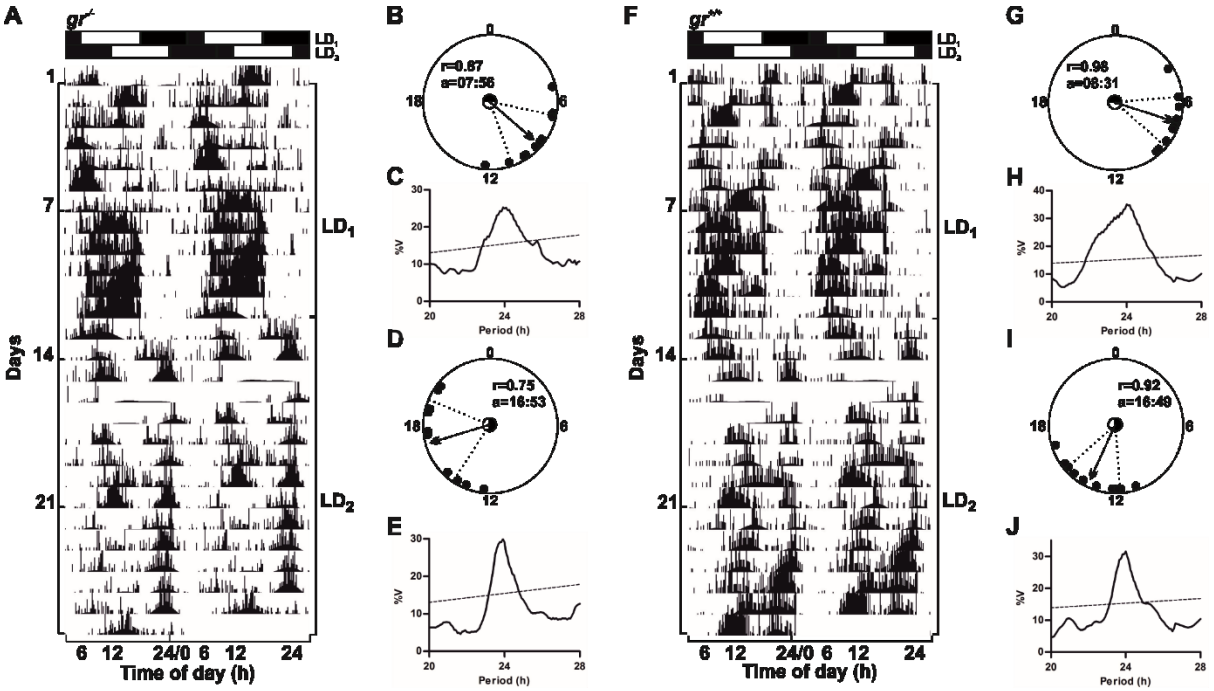

Figure 3.6 Daily activity rhythms of $gr^{+/+}$ and $gr^{-/-}$ adult zebrafish. Representative actograms (A, F), circular diagrams (B, D, G, I) and χ2 periodogram analysis (C, E, H, J) of adult zebrafish $gr^{+/+}$ and $gr^{-/-}$ subjected to 12:12 LD cycles. In actograms the height of each point represents the number of infrared light-beam interruptions in 10 min. Starting and ending day of each LD cycle (LD1 and LD2) is shown on the right of the actogram. The number of days is indicated on the left and the time of day is plotted on the bottom of each actogram. Circular diagrams showing acrophases for the last 10 days of each LD period are plotted (B; D; G; I). Each black dot shows the daily acrophase, while arrows indicate the average acrophase represented as a vector. In each circle the mean vector length (r) and mean acrophase (a) are shown (B and D for $gr^{-/-}$; G and I for $gr^{+/+}$, for LD1 and LD2, respectively). The circle inside each circular diagram represents critical values of the Rayleigh test ($p<0.05$) and the black part of the circle shows the duration of dark phase. The dotted lines represent the confidence intervals. Activity records in the last 10 days of each LD cycle were also subjected to χ^2 periodogram analysis (C and E for $gr^{-/-}$, H and J for $gr^{+/+}$). Confidence limits were chosen at the 99% level.

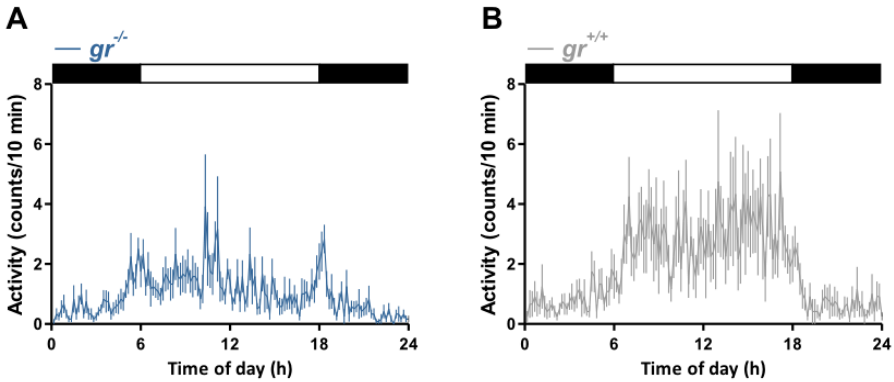

Figure 3.7 Daily activity rhythms of $gr^{+/+}$ and $gr^{-/-}$ adult zebrafish. Adult zebrafish $gr^{-/-}$ (A) and $gr^{+/+}$ (B) subjected to 12:12 LD cycle. Mean waveform of locomotor activity under LD for 24 h.

3.6 *Adults light entrained oscillator*

To confirm the presence of a circadian timekeeping system also in adult, I recorded locomotor activity in $gr^{-/-}$ mutant and WT kept in LL after 20 days of entrainment in a 12:12 LD cycle (Figure 3.8). In LL both genotypes displayed a rhythm of locomotor activity demonstrating the circadian nature of the rhythmicity (Figure 3.8). As previously reported [158], the locomotor activity in constant lighting conditions exhibited different patterns as a splitting of activity in different periodicity within the circadian range (20-30 hours; representative example in Figure 3.8 A) or a unimodal activity with a period close to 24 hours (representative example in Figure 3.8 C). Periodogram analysis showed the entrainment to both LD cycles and the significant rhythmicity in LL (Figure 3.8 B, D, E).

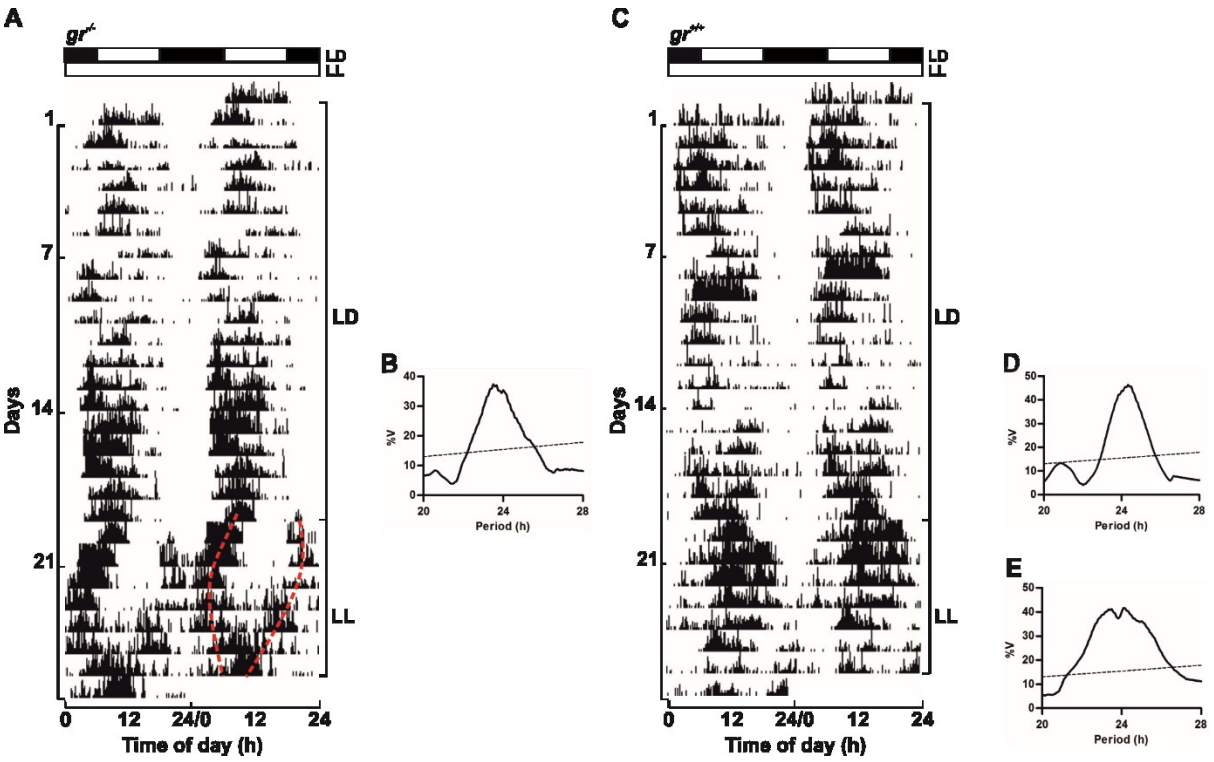

Figure 3.8 Circadian activity rhythms of $gr^{+/+}$ and $gr^{-/-}$ adult zebrafish. Representative actograms (A, C) of adult zebrafish $gr^{+/+}$ and $gr^{-/-}$ subjected to a 12:12 LD cycles and then to LL. Activity records in the last 10 days of LD cycles (B and D) and of LL (E) were subjected to χ^2 periodogram analysis. Dotted lines marked the splitting of the rhythm into two independent components with periodicity in the circadian range. For more details see Figure 3.6.

3.7 *Adults core clock genes expression*

In order to explore at molecular level the circadian clock of both zebrafish genotypes, I examined the expression pattern under LD cycles of a set of clock and clock-related genes in two peripheral oscillators, the eyes and the liver, in adults. In agreement with previous reports, exposure of zebrafish WT to a LD cycle results in robust rhythmic expression of these genes (Figure 3.9-3.10, Table 3.1), except for *clock1a* in the liver. Remarkably, rhythmic gene expression was also encountered in $gr^{-/-}$ tissues (Figure 3.9-3.10, Table 3.1). Interestingly, the comparison of rhythmic clock gene expression between genotypes show differences in expression level at some ZTs in both tissues (Figure 3.9 C, D: *per1b*

and *per2*; Figure 3.10 B, E: *clock1a* and *per2*; Pairwise comparison after Kruskal-Wallis test, $p<0.05$).

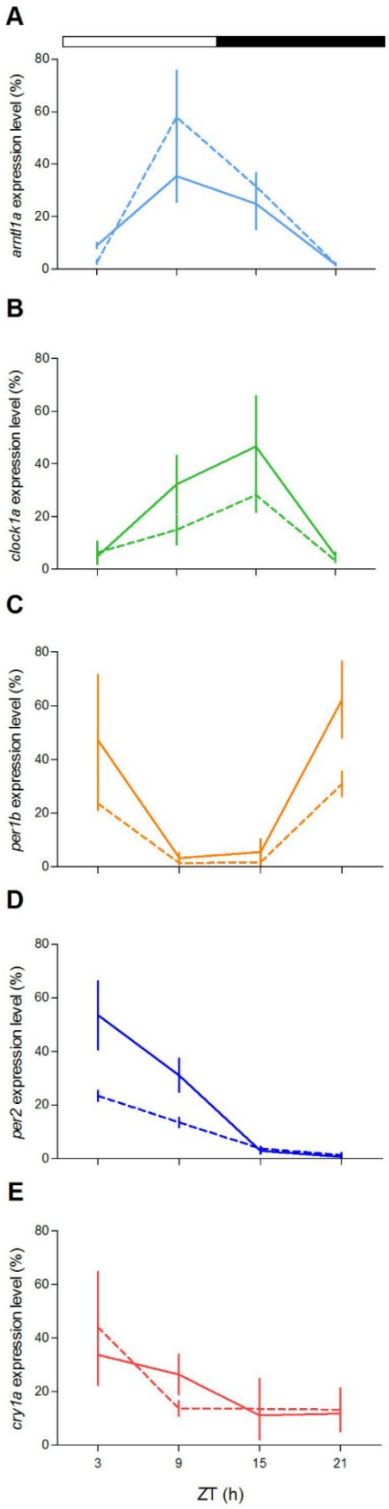

Figure 3.9 Daily expression levels of clock genes in zebrafish adult eye. qPCR analysis of clock and light-regulated clock gene expression in the eye of zebrafish exposed to LD cycles. For all panels, each point represents the mean ± SEM (n=4). $gr^{+/+}$= solid line; $gr^{-/-}$ = dotted line. For more details see Figure 3.5.

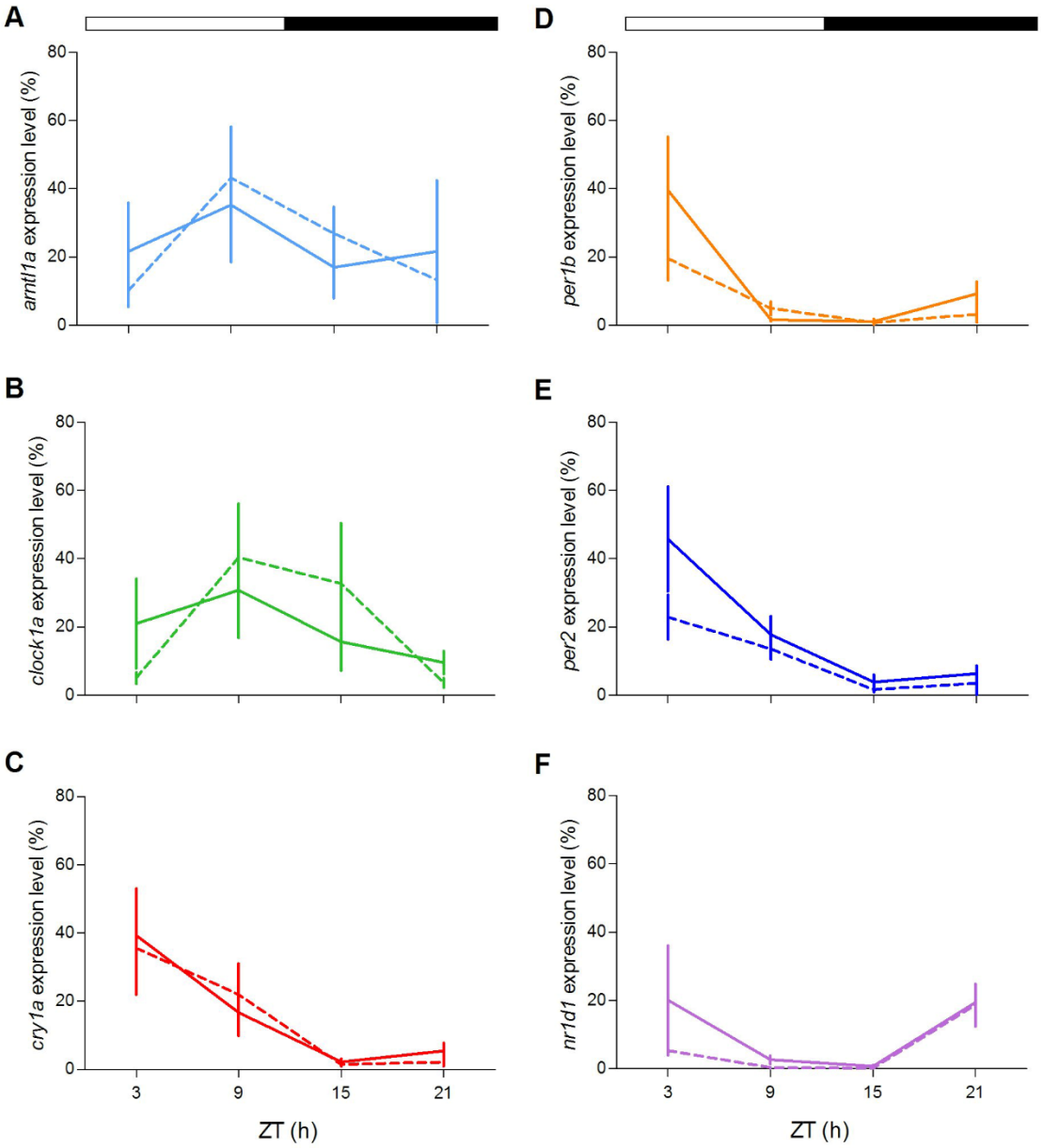

Figure 3.10 Daily expression levels of clock genes in zebrafish adult liver. qPCR analysis of clock and light-regulated clock gene expression in the liver of zebrafish exposed to LD cycles. For all panels, each point represents the mean ± SEM (n=4). *gr*$^{+/+}$= solid line; *gr*$^{-/-}$ = dotted line. For more details see Figure 3.5.

3.8 *Adults light entrained metabolic genes*

Furthermore, I tested the daily expression of *srebp1* and *pck2* in the liver, two genes involved in the lipidic and glucidic metabolism, respectively. *srebp1*, of which the timing of cyclic accumulation in the nucleus is under the control of *nr1d1*, is a key transcription factor that regulates genes in the *de novo* lipogenesis and glycolysis pathways [193; 194], pck2 codifies for an enzyme essential in gluconeogenesis, a process which is directly regulated by glucocorticoids [195]. Interestingly, while *pck2* is rhythmically expressed only in the $gr^{+/+}$ ($gr^{+/+}$: p<0.0001, $gr^{-/-}$: p>0.7; Figure 3.11 A), *srebp1* changed its expression during the day in both genotypes (p<0.0001; Figure 3.11 B) but peaked at different ZTs ($gr^{+/+}$: ZT15, $gr^{-/-}$: ZT9).

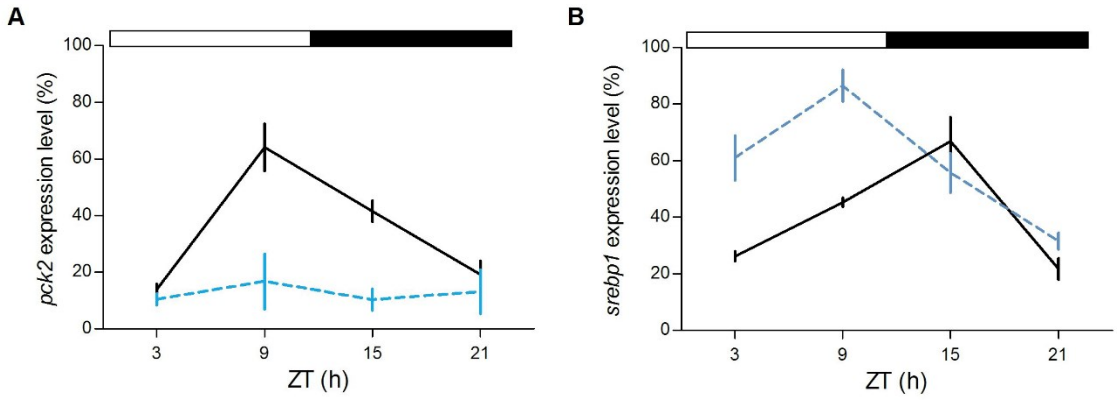

Figure 3.11 Daily expression levels of genes involved in metabolism in zebrafish adult liver. qPCR analysis of *pck2* and *srebp1* in the liver of zebrafish exposed to LD cycles. For both panels, each point represents the mean ± SEM (n=4). $gr^{+/+}$ = solid line; $gr^{-/-}$ = dotted line. For more details see Figure 3.5.

3.9 *Adults food entrained locomotor activity*

To confirm that GC/GR is not critical for the regulation of circadian rhythmicity in zebrafish I assessed whether the *gr*$^{-/-}$ zebrafish circadian clock could be entrained by periodic food availability, a second crucial environmental zeitgeber [196]. *Gr*$^{-/-}$ and control adults were fed once at the same time each day under constant lighting conditions (Figure 3.12). Surprisingly, I did not observe an entrainment of the locomotor activity in *gr*$^{-/-}$ (Figure 13.12 A-C, 3.13 A-D). Indeed, mutant adult zebrafish sometimes responded to food administration with an increase of activity at the mealtime, but the food anticipatory activity (FAA) [57], a characteristic increase in locomotor activity encountered a few hours prior to mealtime, was almost always absent. Periodogram analysis confirmed the arrhythmicity (Figure 3.12 B), also when the time of periodic food availability changed (Figure 3.13 B-D). Furthermore, during starvation, the arrhythmic pattern of locomotor activity showed by adult mutants suggests the absence of regulation by a food-entrainable circadian oscillator (Figure 3.12 A, C). Conversely, in controls we observed the canonical strong entrainment of rhythmic locomotor activity and the FAA (Figure 3.12 D, E). Furthermore, during starvation after feeding entrainment, zebrafish showed a significant circadian rhythmicity (Figure 3.12 D, F).

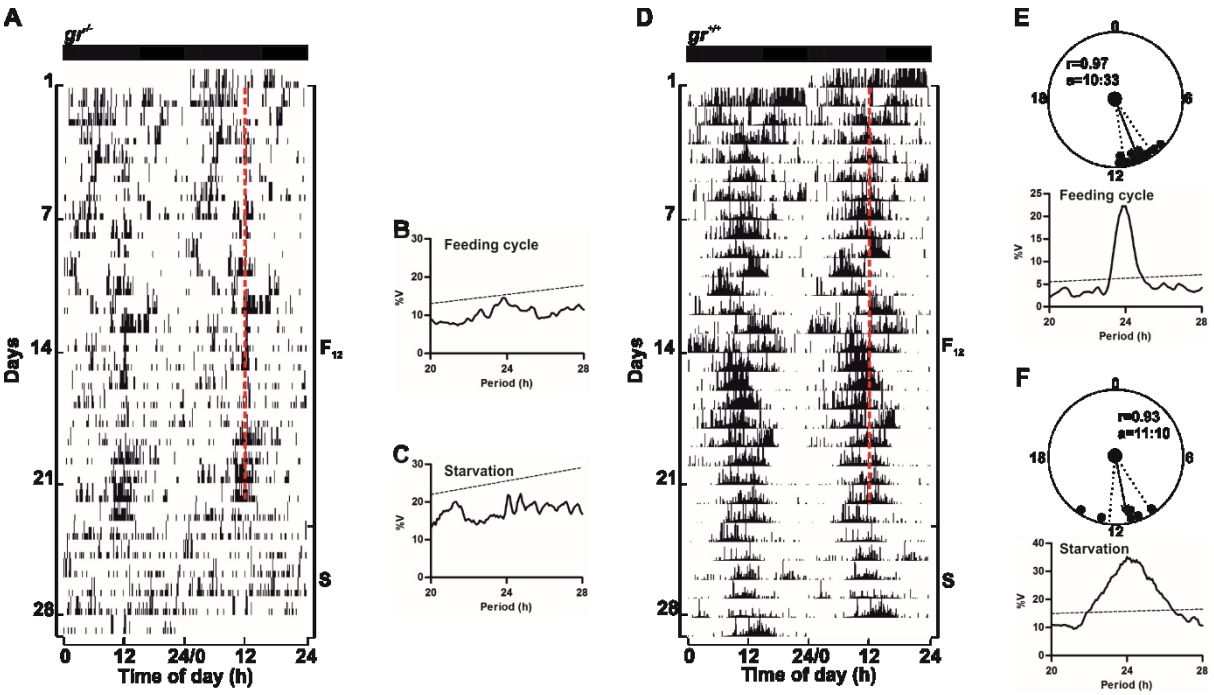

Figure 3.12 Behavioral entrainment by periodic food availability of *gr*$^{-/-}$ and *gr*$^{+/+}$ adult zebrafish. Representative actograms (A, D), χ2 periodogram analysis and circular diagrams (B-C, E-F) of adult zebrafish *gr*$^{-/-}$ and *gr*$^{+/+}$ maintained under constant darkness and fed once a day at a fixed time (12:00). Starting and ending day of each feeding cycle (F12) and starvation (S) is shown on the right of the actogram. The number of days is indicated on the left and the time of day is plotted on the bottom of each actogram. Red dotted lines indicate the time of feeding. Circular diagrams showing acrophases for F12 and S period are plotted (E-F). Activity records of each F12 or S cycle were subjected to χ2 periodogram analysis (B-C, E-F). For more details see Figure 3.6

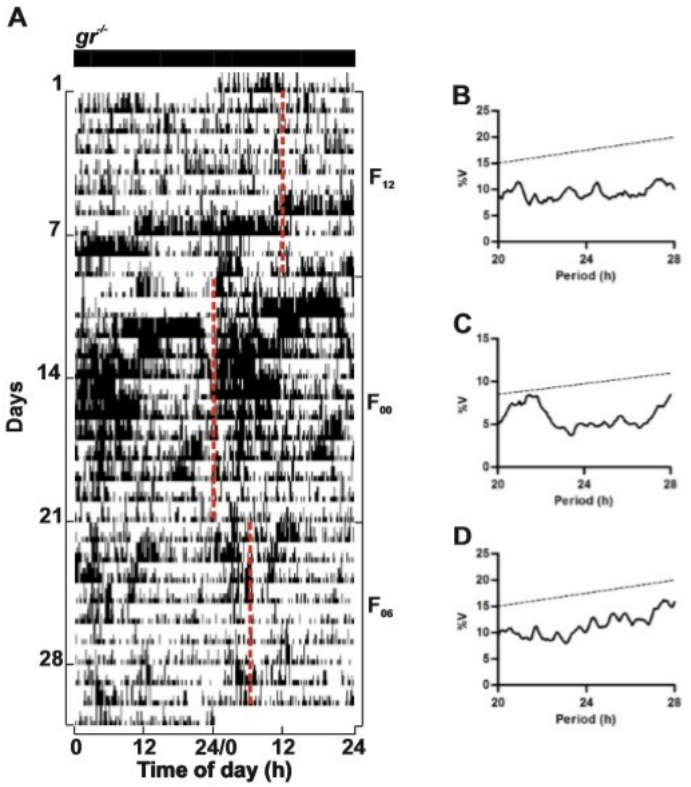

Figure 3.13 Behavioral entrainment by periodic food availability of *gr*$^{-/-}$ adult zebrafish. Representative actograms (A) and χ2 periodogram analysis (B-D) of *gr*$^{-/-}$ adult zebrafish maintained under constant darkness and fed once a day at a fixed time (06:00, 12:00, 24:00). Starting and ending day of each feeding cycle (F_{12}, F_{00}, and F_{06}) are shown on the right of the actogram. The number of days is indicated on the left and the time of day is plotted on the bottom of each actogram. Activity records of each F cycle were subjected to χ2 periodogram analysis (B-D). Red dotted line indicates the time of feeding. For more details see Fig. 3.6 and 3.12.

3.10 *Larvae food entrained locomotor activity*

This original result motivated me to verify, to my knowledge for the first time, the feeding entrainment also in juveniles. After 20 days of daily feeding at midday (Figure 3.14), WT juvenile showed a strong circadian rhythm of locomotor activity (Figure 3.14 B), with a period of 24 h (Cosinor, $p < 0.001$) and a peak at the subjective midday (CT12). By contrast, a weak feeding entrainment has been found in *gr*$^{-/-}$ juvenile (Figure 3.14 B); the circadian activity is dampened in the first

two days in constant conditions in comparison with the WT pattern and it became arrhythmic on the third day of recording (Cosinor, $p > 0.05$; Figure).

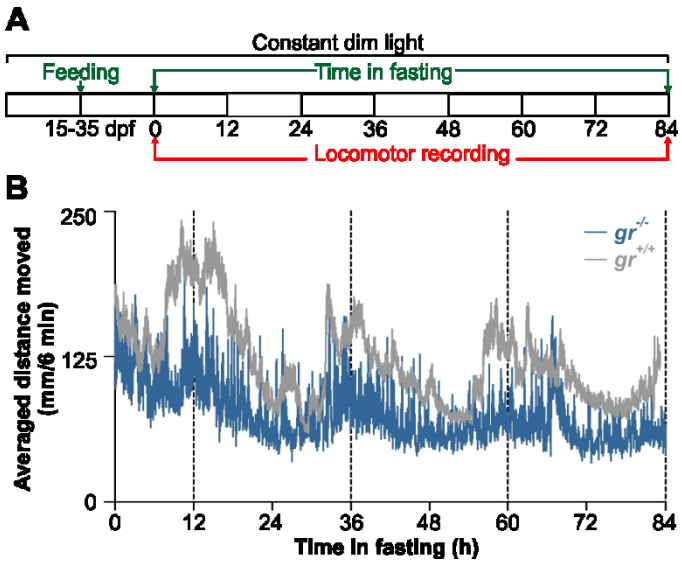

Figure 3.14 Behavioral entrainment by periodic food availability of *gr*$^{+/+}$ and *gr*$^{-/-}$ zebrafish juvenile. (A) Larvae were reared under LL from 15 to 35 dpf and fed once a day at midday for 20 days. After then larvae were starved for 84 hours and the locomotor activity has been recorded. (B) Mean waveform of locomotor activity under LL and starvation for 84 hrs (n=45/genotype). Vertical dotted lines denote when the feeding would have normally occurred according to the previous regular feeding regime.

3.11 *Larvae food entrained clock core genes expression*

I next tested circadian clock gene expression in juveniles of both genotypes during the third day of fasting after feeding entrainment (Figure 3.15; Table 3.1). Rhythmic clock gene expression was observed for all clock genes (Figure 3.15 A, B, D-F), with the only exception of *cry1a* (Figure 3.15 C). Furthermore, the level of expression of *clock1a* and *per1b* was significantly dampened during the subjective day (Figure 3.15 B, D; pairwise comparison after Kruskal–Wallis test, $p < 0.05$).

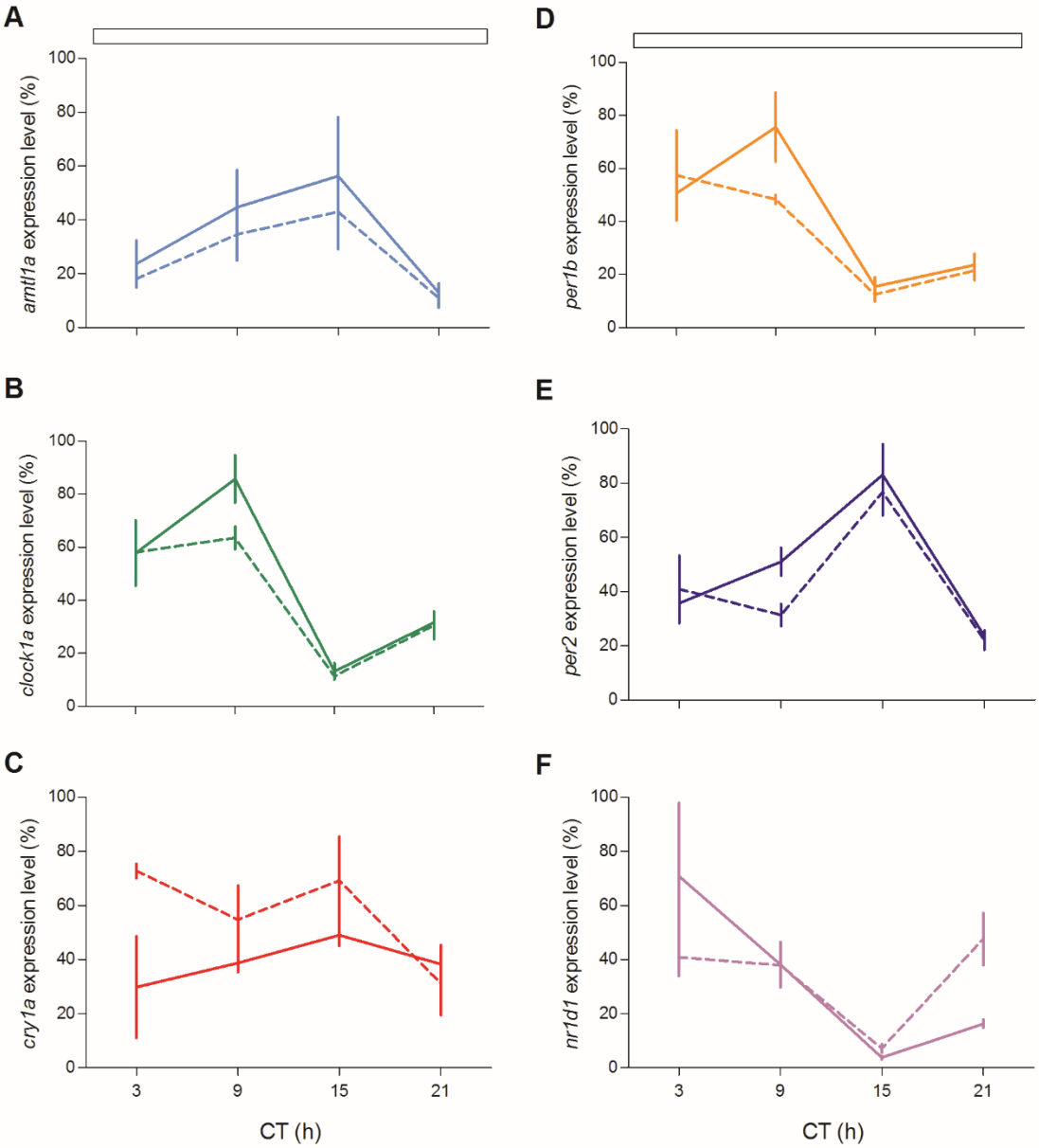

Figure 3.15 Circadian expression levels of clock genes in zebrafish juvenile. qPCR analysis of clock and light-regulated clock gene expression in zebrafish juvenile at the third day of starvation after feeding entrainment. White bars represent LL. For more details see Figure 3.5.

Gene expression statistical analysis table

LE Adults				
Eyes				
	gr$^{+/+}$		*gr*$^{-/-}$	
	P	A (ZT)	P	A (ZT)
arntl1	<0.00001	9	<0.00001	9
clock1a	0.003	15	0.008	15
per1b	<0.00001	21	<0.00001	21
per2	<0.00001	3	<0.00001	3
cry1a	<0.00001	3	<0.00001	3
Liver				
	gr$^{+/+}$		*gr*$^{-/-}$	
	P	A (ZT)	P	A (ZT)
arntl1	-	6	-	6
clock1a	-	9	0.011	9
per1b	<0.00001	3	<0.00001	3
per2	<0.00001	3	<0.00001	3
cry1a	<0.00001	3	<0.00001	3

LE Larvae				
5 dpf				
	gr$^{+/+}$		*gr*$^{-/-}$	
	P	A (ZT)	P	A (ZT)
arntl1	<0.00001	9	<0.00001	15
clock1a	<0.00001	15	<0.00001	15
per1b	0.007	3	<0.00001	3
per2	0.003	3	-	3
cry1a	<0.00001	3	<0.00001	3
6 dpf				
	gr$^{+/+}$		*gr*$^{-/-}$	
	P	A (ZT)	P	A (ZT)
arntl1	<0.00001	15	<0.00001	15
clock1a	<0.00001	15	<0.00001	15
per1b	<0.00001	21	0.001	3
per2	-	3	<0.00001	3
cry1a	0.001	3	<0.00001	3

	12dpf			
	gr$^{+/+}$		*gr*$^{-/-}$	
	P	A (ZT)	P	A (ZT)
arntl1	0.001	9	<0.00001	15
clock1a	0.005	9	<0.00001	15
per1b	0.006	3	<0.00001	21
per2	<0.00001	3	0.002	3
cry1a	<0.00001	3	0.013	3
	FE Larvae			
	gr$^{+/+}$		*gr*$^{-/-}$	
	P	A (CT)	P	A (CT)
arntl1	0.023	15	0.006	15
clock1a	<0.00001	9	<0.00001	9
per1b	<0.00001	9	0.002	3
per2	<0.00001	15	0.015	15
cry1a	-	15	-	3
nr1d1	-	21	0.006	3

Table 3.1 Gene expression statistic. Circadian gene expression profile has been analyzed as transcript level (P=p value) and level peak (A=acrophase) by RAIN algorithm.

4. Discussion

Fish are valuable complementary animal models for studying various aspects of clock biology. Although many clock genes are present in multiple copies, the molecular core clock mechanism is fundamentally conserved and the most important environmental zeitgebers, such as light and food, are able to entrain the circadian clock [196; 197]. Furthermore, since their peripheral clocks are directly entrained by light in almost all tissues investigated [198; 199; 200], and cell lines, and they can be synchronized by metabolic shock as a serum treatment [57], fish have become interesting models for studying how clocks respond to environmental cues and endogenous inputs, as neuropeptides and hormones. Here we explored the role of the GR/GCs complex in the zebrafish circadian clock system.

Gr$^{-/-}$ behavioral phenotype has been evaluated by the novel tank test. The two genotypes showed no difference in the total distance moved, however, when placed into an unfamiliar aquarium (the novel tank), mutant showed a reduced exploratory behavior. This is consistent with the typical anxious phenotype observed in HPA (or HPI) impaired axes animals, thus an incapacity of modulate the stress response was expected to be observed. This abnormal stress-related behavior response to a mildly anxiogenic environment has been reported also by Ziv and colleagues whom demonstrated that *gr*$^{s357/s357}$ mutant abnormal behavior can be restored by fluoxetine, diazepam treatment and conspecific exposure [129]. Using zebrafish GR null mutant, I showed that GR/GCs did not play a role in the generation of the circadian rhythmicity and in the photic entrainment. Indeed, both larvae and adults behavioral activity and clock gene expression were entrained to the LD cycle and rhythmic in constant conditions. In *gr*$^{-/-}$ larvae, the daily locomotor rhythmicity onset results to be one day delayed with respect to the control. This delay could be related, at least partially, to a general slowdown in mutant muscle development as suggested by birefringence analysis of *gr*$^{-/-}$ larvae muscle during development. Indeed, the delay of the onset of the daily locomotor rhythmicity of *gr*$^{-/-}$ larvae is linked to physical characteristics of the mutant larvae, as shown by their body length and muscle birefringence (Figure 4.1). Together with very faint differences of larvae length, is reported a reduction of striated muscle formation in mutants between 3 and 5 dpf with respect to the WT. Birefringence levels were

instead comparable between WT and mutant larvae at 6 and 10 dpf. The reduced birefringence was not associated to the patch-like pattern that is normally linked to disorganization of skeletal muscle [183].

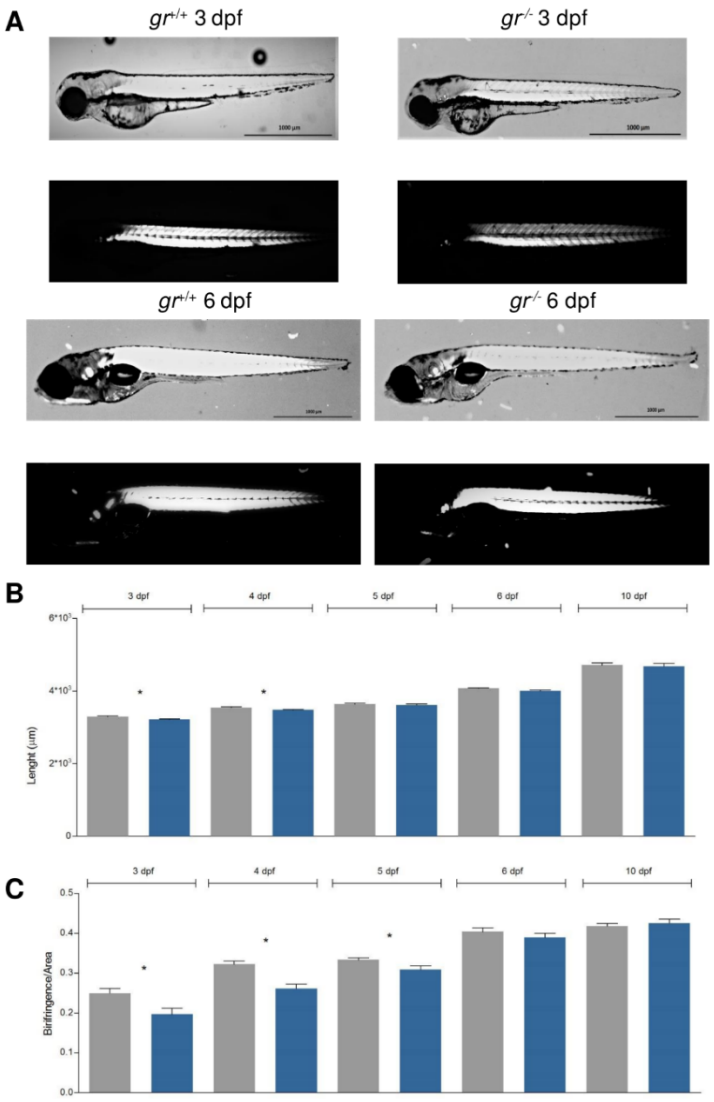

Figure 4.1 Birifrangence essay. The birifrangence essay shows differences of larvae length (A, B) ($p<0.05$ only at 3 and 4 dpf; Mann-Whitney U-test), particularly, a reduction of striated muscle formation in mutants between 3 and 5 dpf with respect to the WT (A, C) ($p<0.05$; Mann-Whitney U-test). Birefringence levels are not significantly different between WT and mutant larvae at 6 and 10 dpf (A, C) ($p>0.1$; Mann-Whitney U-test).
Muscle morphology. Birifrangence (A, C) and body length (B) analyzed from 3 to 10 dpf. All larvae are lateral views with head pointing to the left. Scale bar: 1000 µM. Adapted from: Morbiato et al. 2019 [183].

This result agrees with previous *gr* knockdown studies [201; 202] showing an involvement of GCs on mesoderm development. Moreover, delayed physical development leading to reduced locomotor activity was also found at 5 dpf by Wilson and coworkers after *gr* morpholino treatment [137]. A reduction in spontaneous activities was detected in gr^{s357} mutant which DNA binding activity of the GR has been abolished by a single base-pair substitution in the DNA-binding domain. In addition to muscle fiber organization, as previously reported, the decrease in locomotor activity has been associated also in larvae, to behavioral changes linked to depression or anxiety-like states [133]. Locomotor activity was partially restored by treatment with the antidepressant fluoxetine, possibly through modulation of HPA axis hyperactivity of the mutant line [133].

Interestingly, differences in the pattern of expression of clock genes in $gr^{-/-}$ larvae and adult tissues indicate a modulatory role of GCs on both positive and negative elements of the circadian feedback loops [16]. Previous investigation in the liver of goldfish (*Carassius auratus*) indicated that *per1*, *clock1a* and *bmal1a* are glucocorticoid targets and GREs have been identified in clock gene promoters [148]. We found that expression of the light-inducible gene *per2* in $gr^{-/-}$ is dampened. Both *per2* and *cry1a* expression is light-induced via the D-box and E-box elements [196; 203; 204], but their promoters contain GRE and negative GRE that may enhance or repress their transcription [204].

Previous analysis of liver transcriptome showed a daily and rhythmic expression pattern of many genes related to metabolic processes [205]. Among them I examined *pck2* and *srebp1*, two clock-regulated genes involved in glucidic and lipidic metabolism [206; 207]. *Pck2* catalyzes the conversion of oxaloacetate to phosphoenolpyruvate and its transcription is enhanced by GC [195]. In $gr^{-/-}$ zebrafish liver I found that the daily expression of *pck2* is abolished and its levels are markedly reduced confirming the positive effect of GCs on hepatic gluconeogenesis also in fish. By contrast, the expression of *srebp1* is rhythmic in both zebrafish genotypes, but the acrophase was anticipated in $gr^{-/-}$. This change in the peak of expression from the beginning of the dark phase to the end of the light phase could be related to the significant dampening of *nr1d1* expression at ZT3 found in the mutant. *Rev-erbα* (*nr1d1*) is member of the secondary loop and acts as repressor of *bmal1* (*arntl1*) transcription [3]. It is highly expressed in metabolic tissues, with known functions in conferring circadian clock integration to

glucose, lipoprotein and bile acid metabolism [208]. A previous investigation in mice showed that *rev-erbα* is a key player in the circadian regulation of cholesterol synthesis by influencing rhythmic *srebp* activity [86]. I could propose that the anticipation in the acrophase of *srebp1* depends to the reduction of the *nr1d1* expression levels at the beginning of the light phase. The link between metabolic signals and circadian system is also demonstrated by the fact that the feeding is temporally organized during the day and it is regulated by the circadian clock [77; 99].

Feeding entrainment has been found in many marine and freshwater fish species, including blind cavefish which lack the LEO, but maintain the FEO [20]. My investigation in *gr*$^{-/-}$ confirm the importance of GCs in the feeding entrainment also in fish. As previously shown, adult WT activity was entrained to daily food availability and showed an evident FAA. Conversely, adult *gr*$^{-/-}$ were not synchronized by daily food administration. To confirm this novel result, I adapted the adult zebrafish feeding protocol to larvae/juveniles. Also at this developmental stage, a strong entrainment was present only in WT. To my knowledge, this is the first evidence of the existence of feeding entrainment of locomotor activity in early juvenile zebrafish. It confirms the importance of feeding signal also during the juvenile stages. In addition, the absence of feeding entrainment in adult *gr*$^{-/-}$ clearly points to the role of GC in the regulation of the feeding entrainment of the circadian clock in fish. However, we showed that, after one month of regular daily food administration, clock genes in *gr*$^{-/-}$ early juveniles are rhythmically expressed, with the exception of *cry1a*, indicating that the role of GCs in the zebrafish feeding entrainment could be not linked directly to the clock gene expression. A mismatch between behavioral activity and gene expression has been revealed also by a study conducted on the goldfish [97]. Goldfish food entrained individuals showed a FAA and a robust clock gene oscillation in the hypothalamus, optic tectum and the liver, while, random feeding is not linked to clock gene circadian variation in the brain, neither to FAA. Interestingly, liver gene showed oscillating amplitude food schedule related. Taken together, these data suggest that in the absence of any LD cycle, a feeding schedule regime is sufficient to maintain both the rhythmic expression of several clock genes in the optic tectum and in the hypothalamus and locomotor activity rhythms. However, in the liver, most of the clock genes presented significant daily rhythms in phase with the last food supply, suggesting a

key role of this organ in keeping feeding synchronization, even if FAA is absent. Preoptic region fish brain is homologue to the mouse PVH/PVN, while the hypothalamic region is homologue to the mammalian arcuate nucleus. These two areas have been proved to be strongly engaged in the rodents feeding entrainment, thus, this result is encouraging to further investigate *gr*$^{-/-}$ gene expression considering the brain as a target for the FAA impairment observed.

Differently, in mammals, the GC rhythmicity is an internal periodic signal to the peripheral tissues that, through GR, modulates the transcription of clock genes as *per1*, *per2* and *rev-erbα* [149]. However, as the role of GC/GR could be limited to particular brain nuclei, further detailed investigations are necessary to clarify the neural control on the feeding entrainment in fish.

The circadian system of vertebrates is a multioscillatory network comprising a master clock often located in the hypothalamus and many peripheral clocks in different organs and tissues. Light and food availability oscillate in a periodic and predictable manner in the natural environment and are the most widespread zeitgeber that entrain circadian clocks. Taken together, mine results confirmed in a non-mammalian species, the zebrafish, the role of GCs as endocrine signals related to the feeding synchronization of circadian clocks. Although the mechanism remains to be discerned, our results confirmed the role of GCs-GR mediated signaling in the feeding entrainment in a non-mammalian species, the zebrafish. Thus, zebrafish *gr*$^{-/-}$ mutant is an excellent model to study the role of GCs and GR in the circadian timekeeping system and to deepen our knowledge on the pathways involved in feeding behavior.

5. Conclusions and perspectives

The strongest evidence reported in my research is about the lack of feeding entrainment in a model species impaired for a functional glucocorticoid-cognate receptor interaction.

Despite the failed food synchronization in *gr*$^{-/-}$ is a fact, and a simple cause-effect model can explain that *gr*$^{-/-}$ mutation (cause) provokes an impairment of food entrained locomotor activity (effect), the proximate mechanism which is involved in this relationship is still undiscovered. Indeed, core clock gene expression is almost completely functional.

The most critical step of my experimental protocol is related to the impossibility to discern between organs the gene expression analysis in larvae, so that a masking effect of some tissue might have hidden the gene profile expression of the others.

I believe that a tissue-specific analysis on adults gene profile could give a higher resolution about the interaction between GCs and GRE elements upstream to the clock genes helping to select the elements involved in the food entrainment process.

Furthermore, as suggested by the metabolic genes analysis results, I propose an extended network between circadian clock, metabolic and humoral systems is engaged in the FAA outcome. Concluding, the zebrafish *gr*$^{-/-}$ has revealed as a powerful model to dissect this intricate multifactorial communication.

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Biologia evoluzionistica ed ecologia

Ciclo di Dottorato

32

Titolo della tesi:

Modulation of circadian rhythms by glucocorticoids

Titolo della tesi (traduzione):

Modulazione dei ritmi circadiani da parte dei glucocorticoidi

Tutore: Prof. (Cognome e Nome)

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BIO/05

Parole chiave della tesi (max 10):

circadian rhythms, ritmi circadiani, glucocorticoids, glucocorticoidi, metabolism, metabolismo, zebrafish, pesce zebra, behavior, comportamento, gene expression, espressione genica

Consapevole, dichiara

CONSAPEVOLE: (1) del fatto che in caso di dichiarazioni mendaci, oltre alle sanzioni previste dal codice penale e dalle Leggi speciali per l'ipotesi di falsità in atti ed uso di atti falsi, decade fin dall'inizio e senza necessità di alcuna formalità dai benefici conseguenti al provvedimento emanato sulla base di tali dichiarazioni; (2) dell'obbligo per l'Università di provvedere al deposito di legge delle tesi di dottorato al fine di assicurarne la conservazione e la consultabilità da parte di terzi; (3) della procedura adottata dall'Università di Ferrara ove si richiede che la tesi sia consegnata dal dottorando in 2 copie di cui una in formato cartaceo e una in formato pdf non modificabile su idonei supporti (CD-ROM, DVD) secondo le istruzioni pubblicate sul sito: http://www.unife.it/studenti/dottorato alla voce ESAME FINALE – disposizioni e modulistica; (4) del fatto che l'Università, sulla base dei dati forniti, archivierà e renderà consultabile in rete il testo completo della tesi di dottorato di cui alla presente dichiarazione attraverso l'Archivio istituzionale ad accesso aperto "EPRINTS.unife.it" oltre che attraverso i Cataloghi delle Biblioteche Nazionali Centrali di Roma e Firenze; DICHIARO SOTTO LA MIA RESPONSABILITA': (1) che la copia della tesi depositata presso l'Università di Ferrara in formato cartaceo è del tutto identica a quella

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