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Linking circadian clock with metabolism

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Abbreviation

AA	amino acids
Aanat2	arylalkylamine-N-acetyltransferase 2
ABCA1	ATP-binding cassette transporter A1
ADP	adenosine diphosphate
ACTH	adrenocorticotropic hormone
Ala	alanine
AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase
ANOVA	analysis of variance
ANS	autonomic nervous system
AP-1	activator protein 1
Arg	arginine
ARNT	aryl hydrocarbon receptor nuclear translocator protein
AARE	amino acid response element
Asn	asparagine
Asns	asparagine synthetase
Asp	aspartic acid
ATF4	activating transcription factor 4
ATP	adenosine triphosphate
AVP	arginine vasopressin
bHLH-PAS	basic helix-loop-helix, Per-Arnt-Single minded
Bmal, BMAL	brain and muscle ARNT-like
bp	base pair
BrdU	bromodeoxyuridine
BSA	bovine serum albumin
bZIP	basic leucine zipper
$CaCl_2$	calcium chloride
cAMP	cyclic adenosine monophosphate
CCGs	clock controlled genes
Cdc2	cell division cycle 2
CDCA	chenodeoxycholic acid
CDK	cyclin-dependent kinases

Abbreviation

cDNA	complementary deoxyribonucleic acid
C/EBPβ	CCAAT /enhancer-binding protein β
CHOP	C/EBP homologous protein
CIP	calf intestinal alkaline phosphatase
CIRP	cold-inducible RNA-binding proteins
CKI	cyclin-dependent kinase inhibitor
CLOCK, CLK	circadian locomotor output cycles kaput
Δclock1, ΔCLK	dominant negative form of the CLOCK1
cm^2	square centimetre
CNS	central nervous system
CRE	cAMP response element
CREB	cAMP response element binding protein
CRH	Corticotropin-realising hormone
Cry,CRY	cryptochrome
CT	circadian time
CYC	cycle
Cyp27c1	cytochrome P450, family 27, subfamily c, polypeptide 1
D-box	D-box enhancer element
DBP	D-site of albumin promoter Binding Protein
DPT	dermatopontin
DCA	deoxycholic acid
DD	constant darkness
DDF	feeding in constant darkness
DDM	medium change in constant darkness
DN	dominant negative
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleosidtriphosphate
DMH	dorsomedial hypothalamic nucleus
dpf	days post fertilization
DTT	dithiothreitol
EAAs	essential amino acids
E-box	E-box enhancer element
E4bp4 (Nfil3)	nuclear factor, interleukin 3, regulated
EDTA	ethylenediaminetetraacetic acid

Abbreviation

eIF2α	eukaryotic translation initiation factor 2α
FAA	food-anticipatory activity
FEO	food entrainable oscillator
FOXO1	forkhead box O1
Frq	frequency
GABA	gamma-aminobutyric acid
GC	glucocorticoid
GCN2	general control nonderepressible-2 kinase
Gln	glutamine
Glu	glutamate
Gly	glycine
GR	glucocorticoid receptor
GRE	glucocorticoid response element
GRIZLY	Glucocorticoid Responsive *In vivo* Zebrafish Luciferase activitY assay
GRP	gastrin-releasing peptide
FCS	fetal calf serum
FEO	food entrainable oscillator
FOXO1	forkhead box O1
Frq	frequency
HCl	hydrogen chloride
His	histidine
HIF-1	hypoxia inducible factor-1
HLF	hepatic leukemia factor
HLH	helix-loop-helix motif
HNF-4α	hepatocyte nuclear factor 4, alpha
HRE	hypoxia response element
HSF1	heat shock transcription factor 1
NMR	nuclear magnetic resonance spectroscopy
HLF	hepatic leukemia factor
H2O2	hydrogen peroxide
HPA	hypothalamic-pituitary-adrenal
HSF1	heat shock factor 1
ipRGCs	intrinsically photosensitive retinal ganglion cells
IR	infrared

Abbreviation

Ile	isoleucine
kb	kilobase
KCl	potassium chloride
KO	knock out
LC3-I /LC3-II	microtubule-associated protein1 light chain 3 I, II
LD	light-dark cycle
LDfIR	far infrared light-dark cycle
LDR	red light-dark cycle
LDW	white light-dark cycle
LED	light-emitting diode
LEO	light entrainable oscillator
Leu	leucine
LLS	Lateral-line system
LRM	light responsive module
Luc	luciferase
lws	long-wavelength sensitive
LXR	liver X receptor
Lys	lysine
Map1lc3b	Microtubule Associated Protein 1 Light Chain 3 Beta
MD	middle of dark period
Met	methionine
MetOH	methanol
ML	middle of light period
ml	milliliter
mM	millimolar
MgCl2	magnesium chloride
MgSO4	magnesium sulphate
MRM	multiple reaction monitoring
mRNA	messenger ribonucleic acid
mTOR	mammalian target of rapamycin
NaCl	sodium chloride
NAD+	oxidized form of nicotinamide adenine dinucleotide
NADH	reduced form of nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form of NADP+)

Abbreviation

NAM	nicotinamide
NAMPT, *Nampt*	nicotinamide phosphoribosyltransferase
Nampta	nicotinamide phosphoribosyltransferase a
NEAAs	non essential amino acids
ng	nanogram
nm	nanometre
NMN	nicotinamide mononucleotide
NMNAT1-3	nicotinamide mononucleotide adenylyltransferase enzymes 1-3
NSRU	nutrient sensing response unit
OPN	olivary pretecal nucleus
Opn4	opsin4
Opn4m2	opsin 4 mammalian-like 2
OXM	oxyntomodulin
P21	Cyclin-dependent kinase inhibitor 1
PACAP	pituitary adenylate cyclase-activating polypeptide
PAR	proline and acidic amino-acid-residue rich
PARP-1	poly (ADP-ribose) polymerase 1
PAS	per-arnt-sim domain
PBS	phosphate buffered saline
PCR	polymerase chain reaction
per, PER	period
PGC1α	PPARγ coactivator α
Phe	phenylalanine
PK2	prokineticin 2
PKC	protein kinase C
PPARα	peroxisome proliferator-activated receptor alpha
PRXs	peroxiredoxins
PRX-SO2/3	overoxidised/hyperoxidised peroxiredoxin
PVN	paraventricular nucleus
qRT-PCR	quantitative RT-PCR
REV-ERBα	nuclear receptor subfamily 1, group D, member 1
RGCs	retinal ganglion cells
rh	rhodopsin-like
RHT	retinohypothalamic tract

RNA	ribonucleic acid
ROR	RAR-related orphan receptor
RORα	RAR-related orphan receptor alpha
RORE	retinoic acid-related orphan receptor response element
Rorγ	RAR-related orphan receptor gamma
ROS	reactive oxygen species
RREs	ROR elements
rpm	revolutions per minute
RT	reverse transcription
SCN	suprachiasmatic nucleus
SCFA	short chain fatty acids
SD	standard deviation
Ser	serine
sws	long-wavelength sensitive
Sirt1	sirtuin 1
SLC	solute-linked carriers (SLC)
SNAT2, SLC38A2	Sodium coupled neutral amino acid transporter 2
S-phase	synthesis phase
sPVZ	subparaventricular zone
SDS	sodium dodecyl sulphate
Tef, TEF	thyrotroph embryonic factor
TH	thyroid hormone
Thr	threonine
Tim, TIM	timeless
TMT	teleost multiple tissue
Tris	Tris(hydroxymethyl)-aminomethane
TRPCs	transient receptor potential canonical
tRNA	transfer RNA
Tue	Tübingen
TTFLs	transcription-translation feedback loops
Tyr	tyrosine
UPLC/MS-MS	ultraperformance liquid chromatography-tandem mass spectrometry
UV	ultra-violet light
Val	valine

Abbreviation

VIP	vasoactive intestinal peptide
Wcc	white collar complex
Wee1	WEE1 G2 checkpoint kinase
WT	wild type
ZT	zeitgeber time
μg	microgram
μl	microliter
μW	microwatt

Abstract

Nutrient and energy metabolism in organisms oscillates in a time-of the-day-dependent manner under the control of an endogenous timing mechanism called the circadian clock. This is a cell autonomous, self-sustained molecular mechanism, which is synchronized by a key environmental signals, notably light and food availability. There is a wealth of evidence showing a bidirectional interaction between food-regulated clocks and the rhythmic expression of metabolic genes in peripheral tissues, notably the liver. For example, genetic or environmental disruption of the circadian clock is linked with metabolic disease, such as obesity and type 2 diabetes. Furthermore, cycling changes in cellular redox potential impact on the expression of circadian clock genes and influence energy metabolism. Therefore, it is vital to understand how animals integrate input from lighting conditions and food availability to ultimately coordinate their daily metabolic rhythms. In this regard, one key issue is whether there are genetically distinct light and food regulated circadian clock mechanisms. The Foulkes group has used zebrafish and blind cavefish models to demonstrate that certain metabolic pathways cycle according to the light dark cycle and are unaffected by the timing of feeding activity, while other pathways are predominantly feeding time regulated. Based on these preliminary data, this thesis project used fish models and fish-derived cell lines to explore the genetic mechanisms linking metabolism with light and food regulated circadian clocks.

The first part of this project aimed to explore at which stage during early zebrafish development a feeding-regulated clock first appears. Due to reduced feeding activity in constant darkness it was not feasible to examine the impact of feeding on clock gene expression. However, it was revealed that regular handling and disturbance of fish larvae, under otherwise constant environmental conditions results in the emergence of circadian clock rhythmicity. Several lines of evidence indicate that stress serves as a Zeitgeber and results in the emergence of rhythmicity in clock gene expression as well as clock outputs such as the cell cycle.

The second part of this thesis explored whether genetically distinct light and food regulated clocks coexist in fish cells and which transcriptional control mechanisms link food-regulated circadian clocks with metabolism. It was demonstrated that during restricted feeding in zebrafish, rhythmic expression of core clock genes in the liver is regulated according to the timing of light-dark cycles, whereas the expression of genes involved in the control of metabolism are influenced by feeding time. However, this study was unable to confirm previous data obtained using NMR, where it was shown that circadian rhythmicity in the levels of essential amino acids is regulated by the light-dark cycle while rhythmic non-essential amino

acid levels are influenced by feeding time. Instead, by UPLC-MS/MS analysis, daily changes in the concentration of both essential and non-essential amino acids were shown to be set by the phase of regular timed feeding and not by the light dark cycle. Furthermore, the NAD+ biosynthesis pathway and autophagy were affected by a clock which is set by feeding time and not by light-dark cycles. In addition, regular nocturnal feeding resulted in an increase in obesity. These findings point to the presence of at least two distinct clock mechanism in the zebrafish liver.

In order to explore in more detail, the nature of the multiple clock mechanisms in zebrafish cells, the next part of this project employed multi-omics approaches and revealed infradian rhythmicity in amino acid concentrations in cultured fish cell lines. However, neither the expression of amino acid transporters nor autophagy exhibited infradian rhythmicity, instead showing circadian rhythmicity. In order to explore the involvement of the classical circadian clock mechanism in generating infradian rhythmicity, a cell line expressing a dominant negative form of *clock1* gene was examined and shown to lack infradian rhythmicity in amino acid levels. Interestingly, the mRNA expression of Asparagine synthetase (asns) shows infradian rhythmicity, which are disrupted in *Δclock1* cells. These data lead to the hypothesis that asns may be involved in the regulation of infradian rhythms in amino acid levels and point to a complex interplay between circadian and infradian rhythmicity.

Keywords: circadian clock, zeitgeber, stress, zebrafish, infradian rhythms, amino acid metabolism

Zusammenfassung

Der Nährstoff- und Energiestoffwechsel in den Organismen oszilliert in einer zeitabhängigen Weise unter der Kontrolle eines endogenen Zeitmechanismus, der zirkadianen Uhr. Diese ist ein zell-autonomer, selbst erhaltender, molekularer Mechanismus, der durch die externen Zeitgeber, insbesondere Licht oder Nahrungsverfügbarkeit, mit dem 24-stündigen Umweltzyklus synchronisiert wird. Zahlreiche Hinweise belegen, dass eine bidirektionale Wechselwirkung zwischen Nahrungsmittel-regulierten Uhren und der rhythmischen Expression von Stoffwechselgenen in peripheren Geweben, insbesondere in der Leber, besteht. Zum Beispiel werden genetische oder umweltbedingte Störungen der zirkadianen Uhr mit Stoffwechselerkrankungen wie Fettleibigkeit und Typ-2-Diabetes assoziiert. Darüber hinaus beeinflussen rhythmische Änderungen in zellulären Redoxpotentialen, die zirkadiane Expression von Uhr-Gene. Dies hat auch eine Wirkung auf den Energiestoffwechsel. Aus diesem Grund ist es wichtig zu verstehen, wie Tiere die Umweltsignale, wie Lichtverhältnisse und Nahrungsverfügbarkeit, integrieren um ihren täglichen Stoffwechselrhythmus zu koordinieren. In diesem Zusammenhang entsteht eine wichtige Frage: Sind Licht- und Nahrungsmittel-regulierte zirkadiane Uhrmechanismen genetisch unterschiedlich? Die Forschungsgruppe Foulkes hat Zebrafische und blinde Höhlenfische als Modellorgansimen verwendet, um zu demonstrieren, dass bestimmte Stoffwechselwege gemäß dem Hell-Dunkel-Zyklus ablaufen und vom Zeitpunkt der Fütterung nicht beeinflusst sind, während andere Stoffwechselwege über die Fütterungszeiten reguliert werden. Basierend auf diesen vorläufigen Ergebnissen wurden in diesem Projekt Fischmodelle und von Fischen gewonnene Zelllinien verwendet, um die genetischen Mechanismen zu untersuchen, die den Stoffwechsel mit den Licht- und Nahrungs-regulierten zirkadianen Uhren verbinden.

Im ersten Teil des Projekts wurde untersucht, in welchem Stadium während der frühen Zebrafischentwicklung zum ersten Mal eine durch Fütterung regulierte Uhr erscheint. Aufgrund sehr geringer Fütterungsaktivität in konstanter Dunkelheit war es nicht möglich, den Einfluss der Fütterung auf die Expression von Uhr-Genen zu untersuchen. Allerdings wurde festgestellt, dass das regelmäßige Handling und Stimulation von Fischlarven unter konstanten Umgebungsbedingungen zur Entstehung der zirkadianen Rhythmen von Uhr-Genen führt. Mehrere Hinweise deuten darauf hin, dass Stress als Zeitgeber dient und zur Entstehung von Rhythmizität in der Expression von Uhr-Gene, sowie Uhr-Outputs wie dem Zellzyklus führt.

Der zweite Teil des Projekts befasst sich mit der Frage, ob genetisch unterschiedliche Licht- und Nahrungs-regulierte Uhren in Fischzellen koexistieren und welche Transkriptionskontroll-

mechanismen die Nahrungs-regulierte zirkadiane Uhren mit dem Stoffwechsel verbinden. Es wurde gezeigt, dass während der eingeschränkten Fütterung von Zebrafischen die rhythmische Expression von Kernuhrgenen in der Leber gemäß dem Zeitpunkt der Hell-Dunkel-Zyklen reguliert wird, während die Expression von Genen, die an der Kontrolle des Stoffwechsels beteiligt sind, durch die Fütterungszeit beeinflusst wird. Diese Studie konnte jedoch nicht die vorherigen, mittels NMR-Spektrometrie erhaltenen Versuchsergebnisse, bestätigen. Die NMR-Analyse hatte gezeigt, dass der zirkadiane Rhythmus der essentiellen Aminosäuren-Konzentration durch den Hell-Dunkel-Zyklus reguliert wird, während die Fütterungszeit die rhythmische Änderung von nicht-essentiellen Aminosäuren beeinflusst. Stattdessen wurde mittels UPLC-MS/MS-Analyse gezeigt, dass tägliche Änderungen in der Konzentration sowohl essentieller, als auch nicht-essentieller Aminosäuren durch die Phase der regelmäßigen zeitkontrollierten Fütterung und nicht durch den Hell-Dunkel-Zyklus eingestellt werden. Darüber hinaus wurden gezeigt, dass der NAD+ -Biosyntheseweg und die Autophagie durch eine Uhr beeinflusst ist, die durch die Fütterungszeit und nicht durch Hell-Dunkel-Zyklen bestimmt wird. Weiterhin führte die regelmäßige nächtliche Fütterung zu einer Zunahme der Fettleibigkeit. Diese Befunde weisen auf das Vorhandensein von mindestens zwei unterschiedlichen Uhrmechanismen in der Zebrafischleber hin.

Um die Ursprünge der multiplen Uhr-mechanismen in Zebrafischzellen genauer zu untersuchen, wurden im nächste Teil dieses Projekts Multi-Omics-Ansätze verwendet, welche zur Entdeckung der infradianen Rhythmen der Aminosäurekonzentrationen in kultivierten Fischzelllinien führte. Weder die Expression von Aminosäuretransportern, noch die Autophagie zeigten eine infradiane Rhythmizität. Stattdessen zeigte sich eine zirkadiane Rhythmizität. Um die Beteiligung des klassischen zirkadianen Uhr-Mechanismus an der Erzeugung des infradianen Rhythmus zu untersuchen, wurde eine Zelllinie begutachtet, die eine dominante negative Form des *clock1*-Gens exprimiert. In diesen Zellen wurde keine infradiane Rhythmik in der Aminosäuren-Konzentration detektiert. Interessanterweise zeigt die mRNA-Expression von Asparaginsynthetase (*asns*) eine infradiane Rhythmizität, die in *Δclock1*-Zellen gestört ist. Diese Ergebnisse führen zu der Hypothese, dass Asns an der Regulation des infradianen Rhythmus in Aminosäuren beteiligt sein könnten und weisen auf ein komplexes Zusammenspiel zwischen zirkadianer- und infradianer-Rhythmizität hin.

Stichwörter: innere Uhr, Zeitgeber, Stress, Zebrafisch, infradiane Rhythmen, Aminosäuren Stoffwechsel

1. Introduction

The daily Earth´s rotation on its axis generates predictable environmental patterns, notably light and temperature cycles as part of the day-night cycle. In order to anticipate these changes and to ensure optimal fitness most organisms evolved endogenous timers, so called circadian clocks. The term circadian is derived from the Latin *circa*, which means "about", and *diem*, which means "day". These clocks are not driven by daily environmental cycles; however, they are synchronized with the day-night cycle through a process called entrainment. The most potent environmental entraining signals, so called Zeitgebers (for "time givers" in German), are the changes in light and temperature, as well as food availability (Pittendrigh, 1993). The daily cycle of light and dark is the most important and well-studied environmental cue that influences circadian biology in organisms. However, non-photic Zeitgebers like feeding also play crucial roles in the synchronization of brain and peripheral clocks and are responsible for coordinating food anticipatory activity, changes in behavior and physiology that occur just before feeding time. The circadian clock mechanism has been implicated in the regulation of a number of biological processes, including metabolism, the endocrine system and development. On the other hand, the circadian clock machinery can be regulated by its own effectors. For example, via metabolic inputs and cortisol, bidirectional connections exist between the circadian clock, glucocorticoids and metabolic signals.

In general the circadian clock system is subdivided into three parts (Figure 1) (Menaker et al., 1978):

1. An input pathway, which transfers timing information from external environment cues, for example the light-dark cycle, to the central pacemaker;
2. A central pacemaker (or oscillator) that generates circadian rhythmicity in the organism;
3. An output pathway, which conveys temporal signals from the central oscillator to regulate various physiological and behavioural process, including the sleep-wake cycle and metabolism.

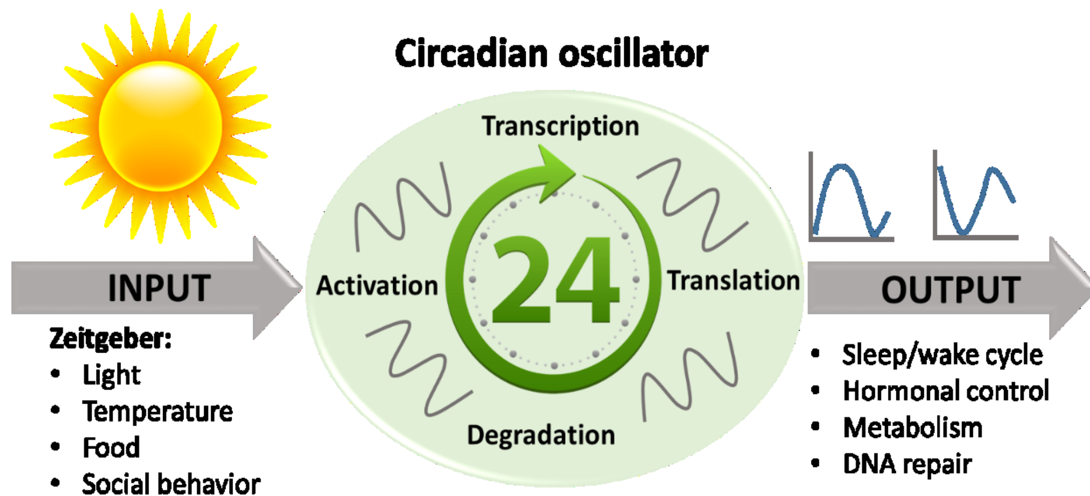


Figure 1: Model of the circadian timing system. External zeitgebers such as light, temperature and food synchronize the central pacemaker through an input pathway. The central pacemaker generates a self-sustaining rhythm and drives the regulation of various aspects of physiology and behavior via output pathways. (Source: mpi-dortmund.mpg.de)

In the modern world, the classical environmental signals which regulate the circadian clock mechanism, are dramatically altered by human activities. For example, artificial light at night, constant temperature during the day, continuous accesses to high fat food, frequent traveling across time zones and shift work. These changes have led to the development of life-style influenced pathologies, including sleep disturbance, depression and various metabolic disorders (Evans and Davidson, 2013). Furthermore, the frequent disruption of circadian rhythms, for example caused through regular night-shift work, has been linked with an increased risk of the development of type 2 diabetes, obesity and cancer (Antunes et al., 2010; Mason et al., 2020; Sigurdardottir et al., 2012).

Understanding the circadian clock mechanism and how it regulates physiology is vital because of the way in which it can also influence medical treatment. The field which focuses on the timing of medical treatment is called chronotherapy. The first evidence that the timing of medical treatment was a crucial factor influencing its efficacity came from the 1970s when scientists observed that the timing of chemotherapeutic drug administration optimised cancer treatment efficiency in mice (Haus et al., 1972). Furthermore, over the past decade it has also become evident that the risk and/ or intensity of diseases such as stroke, hypertension, asthma and arthritis varies predictably according to the time of day (Cutolo and Masi, 2005; Durrington et al., 2014; Elliott, 1998). The careful consideration of the time of day in therapeutic regimes (so-called personalized chronotherapy) may therefore provide benefits for the treatment of various diseases by increasing drugs' effectiveness, minimizing toxicity and decreasing adverse effects in patients.

1.1 General aspects of the circadian clock

In circadian clock research, the biggest initial milestone was the identification of the location and cellular organisation of the circadian pacemaker. It soon became apparent that the circadian clock system is organised as a hierarchy of multiple clocks, that function at the cellular, tissue, and systems levels. In mammals, a central circadian clock, the so called "master circadian pacemaker", is located in the brain and is entrained indirectly by environmental zeitgebers via sensory organs which communicate with the SCN clock through neuronal pathways. The master pacemaker in turn plays a key role in orchestrating the rhythms of the multiple clocks localised in most peripheral tissues. Despite increasing knowledge of the mechanism of the circadian clock, the molecular pathways through which the central clock communicates with peripheral clocks are far from clear. Indeed, a major challenge remains to understand the way in which clockwork controls circadian physiology and gene expression and how it remains synchronised with the environment at the whole animal level.

1.1.1 Circadian pacemakers

In 1960 Colin Pittendrigh, regarded as the "father of Chronobiology", theorized that independent but coupled, circadian oscillators, which are sensitive to light, exist (Pittendrigh, 1960). In the following years, numerous studies were conducted to identify the structure that could serve as a master pacemaker in different organisms. Lesion and transplant studies led to the identification that the suprachiasmatic nucleus (SCN) is the site of the master circadian pacemaker in mammals. Complete destruction of the SCN caused a loss of circadian rhythms in locomotor activity, the endocrine system and metabolism (Moore and Eichler, 1972; Stephan and Zucker, 1972). Circadian rhythms in animals bearing SCN lesions could be restored by implantation of fetal SCN tissue (Lehman et al., 1987). Importantly, the restored rhythms showed the period characteristic of the donor animal and not the SCN-lesioned host (Ralph et al., 1990). Additionally, isolated SCN tissue *in vivo* and *in vitro* maintain circadian rhythms in electrical and metabolic activity (Groos and Hendriks, 1982; Ralph et al., 1990). Taken together, these findings indicate that the circadian timing system is hierarchically organised.
In mammals, the paired bilateral SCN is located in the anteroventral hypothalamus lying dorsal to the optic chiasma and bilateral to the third ventricle (Mohawk et al., 2012). Each unilateral SCN is constituted by about 10 000 neurons and subdivided into a ventrolateral core and a dorsomedial shell region (Figure 2A) (Abrahamson and Moore, 2001). Both regions are distinguished based on their anatomy and neurochemistry. The core region is made up of light-

responsive neurons that receive photic input from the retina via the retinohypothalamic tract (RHT) to confer intrinsic synchronization of SCN neurons (Welsh et al., 2010). Neurons within the core produce the neuropeptide vasoactive intestinal polypeptide (VIP) and gastrin-releasing peptide (GRP), as well as the neurotransmitter gamma-aminobutyric acid GABA (Abrahamson and Moore, 2001). On the other hand the shell region generates robust circadian gene expression and produce the neuropeptides vasopressin (AVP) and prokineticin 2 (PK2), as well as GABA (Figure 2 B) (Colwell, 2011; Ramkisoensing and Meijer, 2015; Yan et al., 2007).

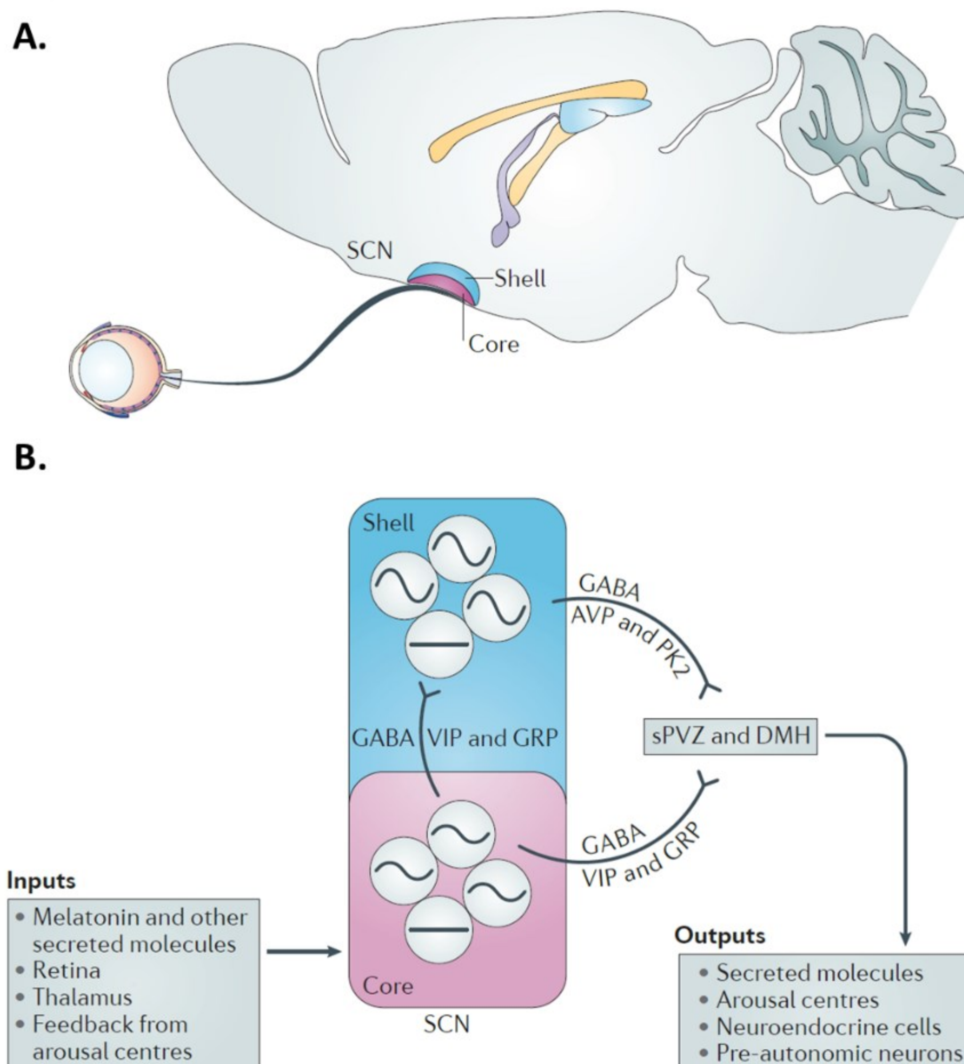


Figure 2: Mammalian central pacemaker organization. (A) The SCN in the hypothalamus is divided into two subregions: dorsal (shell) and ventral (core). (B) The SCN clock can be entrained by light signals via an indirect interaction with the retina via the RHT or by receiving inputs from various parts of the brain, such as the thalamus. This information is transmitted by the core to the shell using GABA, VIP and GRP. In turn, the shell sends output signals to other hypothalamic regions using GABA, AVP and PK2. SCN - suprachiasmatic nucleus, RHT - retinohypothalamic tract, GABA - ionotropic γ-aminobutyric acid receptor, VIP - vasoactive intestinal polypeptide, GRP - gastrin-releasing peptide, sPVZ –subparaventricular zone, DMH - dorsal medial hypothalamus, AVP - arginine vasopressin, PK2 - prokineticin 2. (Colwell, 2011)

A key neuroendocrine organ that is closely linked with the function of master clock in vertebrates is the pineal gland. This gland is connected to the dorsal epithalamus by a stalk and is responsible for controlling the local synthesis of the hormone melatonin (N-acetyl-5-methoxytryptamine) with a characteristic day – night rhythm ((Falcon, 1999; Li et al., 2012)). In mammals, diurnal rhythms of melatonin are controlled by the SCN and are considered as an output signal of the clock. However, it is though that in non-mammalian vertebrates a central circadian pacemaker is located in the pineal gland. For example, in adult fish the pineal gland is located just below the skull, which often appears thinner in this area, and the skin covering it is less pigmented, allowing direct illumination. Indeed, the pineal organ has photoreceptive properties. The pineal epithelium contains photoreceptor cells that resemble retinal cone photoreceptors, both from a structural and functional point of view. These photoreceptors establish synaptic contacts with neurons sending their axons to the brain. Melatonin, is synthesized and secreted exclusively at night by these photoreceptor cells and is released into the cerebro-spinal fluid or blood thereby providing the organism with endocrine information about the light dark cycle and in particular the duration of the night period.

Circadian clocks in mammals exist not just in the SCN, but they are also present in peripheral tissues, such as the liver kidney and heart. The basic properties of the cellular oscillators in the SCN and peripheral tissues are very similar. For example, Pagani et al. reported that the period length of SCN-controlled behaviour is directly proportional to the period length of clock-generated gene expression rhythms in peripheral skin fibroblasts in humans (Pagani et al., 2010). However, *s*tudies in transgenic rats have shown that SCN explants maintain robust rhythms over a period of up to four weeks, whereas peripheral oscillators loose rhythmicity after several cycles under the same conditions (Yamazaki et al., 2000). The most fundamental differences between central and peripheral oscillators lies in their responses to environmental signals. The SCN clock is synchronized with the day night cycle through the perception of light by the retina, whereas the peripheral clocks of mammals are not light sensitive. Indeed, the timing of food intake may affect the circadian clock independently of the SCN. For example, restricted feeding during the rest phase (*i.e.* the night in diurnal animals or the day in nocturnal animals) leads to uncoupling of peripheral clocks from the SCN oscillator (Damiola et al., 2000). However, the mechanism which desynchronises the phase of the peripheral clocks from the SCN clock is poorly understood.

In some lower vertebrate species including zebrafish, light sensitive oscillators in peripheral tissues, organs and even in cultured cells are present. The expression of clock genes can be

directly synchronized with the light-dark cycle, suggesting that clock systems are highly decentralised in zebrafish (Cermakian et al., 2000; Whitmore et al., 1998).

1.1.2 The interactions between central pacemaker and peripheral clocks

Numerous studies indicated that the circadian timing system in mammals is organised in a hierarchical manner, where the brain and liver takes on the most important function in coordinating this system. However, the way in which external signals regulate the circadian clock mechanism is far from clear. It is supposed that direct communication between master and peripheral clocks takes place via the nervous system as well as a humoral system which includes circulating levels of glucocorticoids cyclic changes in body temperature and food-derived metabolites (Figure 3) (Brown et al., 2002; Damiola et al., 2000; Le Minh et al., 2001; Stokkan et al., 2001).

(1) Autonomic nervous system

The SCN is composed of thousands of oscillating neurons that integrate information from the environment and transmit timing signals directly to peripheral tissues through the sympathetic and parasympathetic motor systems of the autonomic nervous system (Kalsbeek et al., 2010; Ueyama et al., 1999).

(2) Endocrine system

The SCN plays a major role in regulating rhythms of hormone release. These hormones in turn entrain the phase of peripheral clocks. One of the best studied humoral mediators transmitting the information between SCN and peripheral clocks is adrenal glucocorticoid (GC) levels (Balsalobre et al., 2000). The rhythms of GC are driven by the SCN via the hypothalamic-pituitary-adrenal (HPA) axis (Oster et al., 2006). GC cycles entrain the phase of peripheral clocks by targeting the glucocorticoid nuclear hormone receptor (GR). Interestingly, GR is present in almost tissues except the SCN (Balsalobre et al., 2000).

(3) Body temperature

Rhythms in body temperature are driven by the SCN. However, the precise mechanism underlying this control still remains elusive. Recent studies have indicated that clocks are entrained by temperature-cycles in peripheral organs and cultured cells, whereas the SCN master clock is resistant to temperature rhythms *in vivo* and *in vitro* (Brown et al., 2002; Buhr

et al., 2010). It has been proposed that heat shock factor 1 (HSF1) and cold-inducible RNA-binding proteins (e.g., CIRP) regulates the synchronisation of peripheral oscillators by body temperature (Morf et al., 2012; Saini et al., 2012).

(4) Feeding-fasting cycle

Daily feeding-fasting cycles represent important zeitgebers, which entrain circadian clocks in peripheral organs, including the liver. Regular restricted feeding can shift the phase of peripheral clocks without affecting the phase of the SCN (Damiola et al., 2000). The mechanism which regulates the entrainment of peripheral clocks by food is still poorly understood. However it is supposed that glucose and insulin play a role by regulating this mechanism (Hirota et al., 2002).

Taken together the mechanisms whereby the SCN and peripheral clocks communicate are complex, highly interconnected and present at all levels—from intracellular signalling to intercellular and systemic coordination.

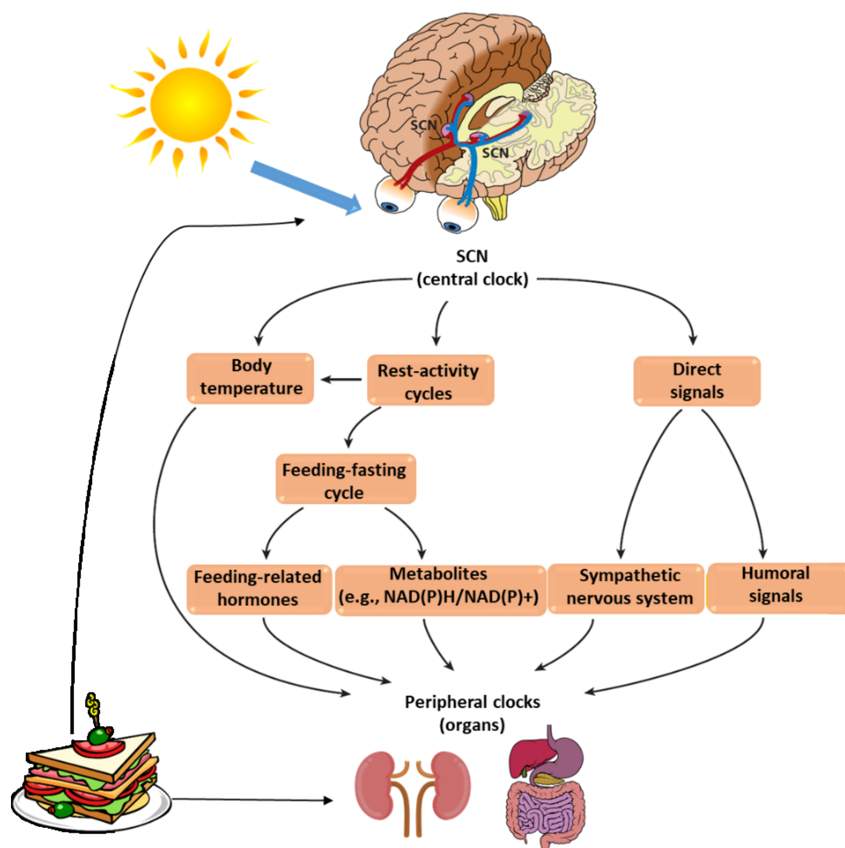


Figure 3: Hierarchical organisation of circadian clock system in mammals. The master clock localised in the brain can be entrained by external signals, including light and food. In turn, the SCN clock synchronises peripheral clocks with environmental cycles through a range of direct and indirect cues, such as sympathetic nervous system output, humoral signals, feeding-related cues and body temperature etc. Peripheral clocks can also be directly entrained by non photic cues, e.g. feeding time. (Dibner et al., 2010)

1.1.3 Molecular clock mechanisms

In the past decades genetic analysis has identified a number of genes that are essential for circadian clock function. In 1971, by forward genetic analysis Konopka and Benzer identified the period (per) locus in *Drosophila* (Konopka and Benzer, 1971). A decade later the gene was cloned and characterised by Jeffery C. Hall, Michael Rosbash and Michael Young (Bargiello et al., 1984; Bargiello and Young, 1984; Reddy et al., 1984; Zehring et al., 1984). In 2017 their discovery was honoured with the Nobel Prize in Physiology or Medicine. In the following years additional clock genes were identified and characterised. These findings represent the basis for our current understanding of the circadian clock mechanism. Nowadays it is considered that the molecular clock mechanism consists of autoregulatory transcription-translation feedback loops (TTFLs), composed of so-called clock genes (Dunlap, 1999; Hardin et al., 1990; Lowrey and Takahashi, 2004). However, recent studies have established that additional timekeeping mechanisms in eukaryotic cells exist, which function independently of transcription and translation (O'Neill and Reddy, 2011).

1.1.3.1 Transcriptional translation feedback loops

At the molecular level, circadian clock rhythms in vertebrates are generated by a transcription–translation gene regulatory feedback loop (TTFL) (Reppert and Weaver, 2002). The positive elements in this loop are comprised of a set of transcriptional activators, the CLOCK (circadian locomotor output cycles kaput) and BMAL1 (brain and muscle ARNT-like protein 1) transcription factors (Gekakis et al., 1998), while the negative loop is constituted by repressors, the PER (period) and CRY (cryptochrome) proteins (Figure 4) (Dunlap, 1999). CLOCK and BMAL1 are bHLH-PAS (basic helix–loop–helix; Per-Arnt-Single minded) transcription factors which bind as heterodimeric complexes to E-box enhancer elements (5’-CACGTG-3’) in the promoter regions of clock controlled genes (CCGs). Thereby, the CLOCK/BMAL1 complex drives the expression of three *Period* (*per1*, *per2, per3*) and two *Cryptochrome* (*cry1* and *cry2*) genes. Subsequently, the PER and CRY proteins heterodimerize, translocate from the cytoplasm to the nucleus and inhibit their own transcription by physically interacting with and thereby blocking transcriptional activation by the CLOCK/BMAL1 complex (Shearman et al., 2000).

Additional feedback loops exist that serve to stabilize the core loop described above. One such loop is formed by the CLOCK/BMAL1 heterodimer complex binding to and regulating the promoter of retinoic acid-related orphan nuclear receptors, including the RORs and REV-ERBs (Figure 4) (Preitner et al., 2002). REV-ERBα represses the transcription of the *Bmal1* gene by

binding to an RORE elements (retinoic acid-related orphan receptor response element) in its promoter region, while RORα activates the transcription of *Bmal1* (Buhr and Takahashi, 2013; Guillaumond et al., 2005; Preitner et al., 2002). This regulation results in clock-driven rhythmic expression of BMAL1.

A third feedback loop in mammals involves PAR-bZip (proline- and acidic amino acid-rich, basic leucine zipper) transcription factors including DBP (albumin D-site-binding protein), TEF (thyrotroph embryonic factor), HLF (hepatic leukemia factor) and E4BP4. In mammals, the expression of DBP, TEF and HLF is regulated by the circadian clock and in turn these factors bind to and regulate the D-box enhancer with a circadian rhythm. This enhancer is located in the promoters of certain clock genes and so contributes to shaping their rhythmic expression pattern. Thereby, the D-box in mammals serves as part of a regulatory feedback loop within the clock mechanism itself.

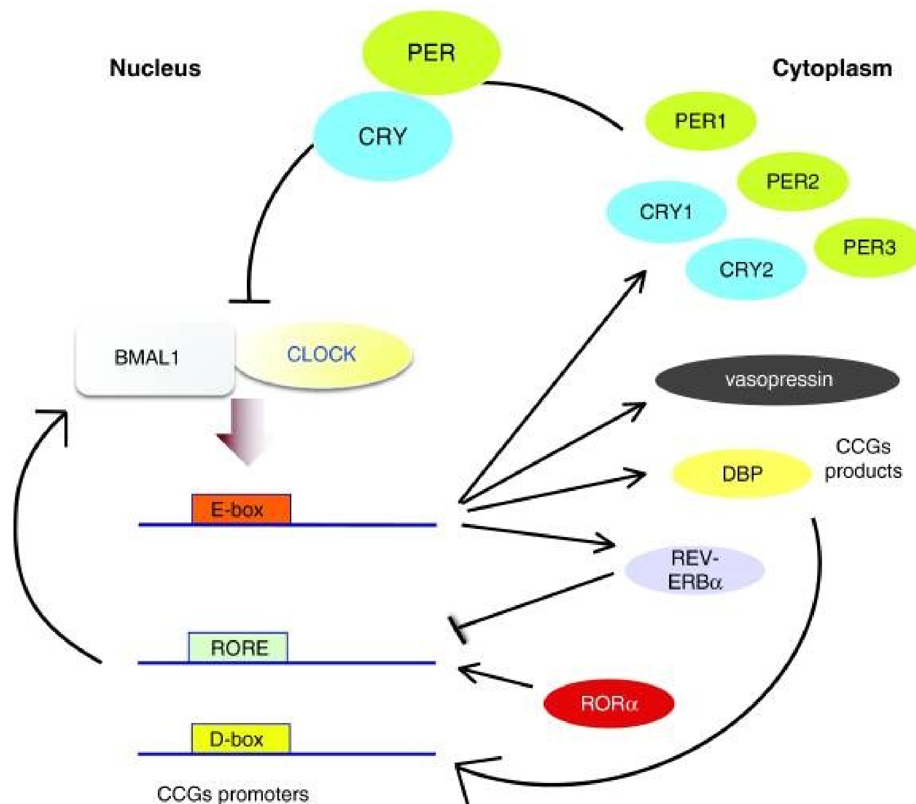


Figure 4: Core clock machinery in mammals. The molecular clock mechanism consists of positive elements, BMAL1 and CLOCK, and negative elements, PER and CRY. CLOCK and BMAL1 form a heterodimer complex which interacts with E-box enhancer elements in the promoter regions of per and cry genes to activate their transcription. The PER and CRY proteins heterodimerize and translocate from the cytoplasm to the nucleus, where they inhibit their own transcription. The core clock loop is stabilized by a secondary REV-ERBα/RORα loop, that regulates the activity of BMAL1. The expression of D-box enhancers is regulated by PAR-bZIP factors, including DBP, that acts as a clock output pathway as well as a feedback loop within the core clock machinery. (Bellet and Sassone-Corsi, 2010)

The basic architecture of the TTFL-based clock mechanism is high conserved across species and present in almost all cells and tissues. However, there are some differences in the core elements of the TTFL between the species from different phyla, suggesting that this timekeeping mechanism may have evolved independently multiple times during evolution. The simplest circadian clock mechanism is found in cyanobacteria. Here, the clock is composed of three proteins: KaiA, KaiB and KaiC (Ishiura et al., 1998). In the filamentous fungus *Neurospora crassa* the central element of circadian oscillator is *Frq* (frequency), which functions as negative element in the feedback loop (Aronson et al., 1994). The transcription of *Frq* is driven by the positive elements, *Wcc* (white collar complex) (Crosthwaite et al., 1997). The molecular mechanism of the circadian clock in *Drosophila* is very similar to the mammalian system. The transcription of negative elements, *Per* (Period) and *Tim* (Timeless) is regulated by activator complex CLK (Clock) and CYC (Cycle) (Rosbash et al., 1996). In turn *Per* and *Tim* interacts negatively with CLK and CYC, attenuating the activation of their own genes. This fundamental similarity between the *Drosophila* and mammalian clock mechanisms played an important role in advancing our understanding of the clock mechanism in mammals, based on insight gained in *Drosophila* studies.

1.1.3.2 Non-transcriptional mechanisms

For decades it has been considered that a TTFL mechanism is essential for generating circadian rhythms in various model organisms. However, mounting evidence suggests that non-transcriptional processes are also sufficient to sustain circadian timekeeping, and that they work in parallel with transcriptional elements. The key evidence for the existence of an alternative cellular timekeeping model was reported in 2005, when Kondo and colleges observed robust, temperature-compensated circadian rhythms in phosphorylation of KaiC in cyanobacteria (*Synechococcus elongates*) under conditions of constant darkness and upon inhibition of the TTFL mechanism (Nakajima et al., 2005; Tomita et al., 2005). Furthermore, a cell-free mixture of the KaiA, KaiB and KaiC proteins together with ATP, was sufficient to establish a circadian rhythm of Kai C phosphorylation (Akiyama et al., 2008). A few years later, rhythmic oscillation in the redox status of perorodoxin (PRX) proteins was identified in human red blood cells, which lack a nucleus and do not produce new RNA or protein, and in unicellular algae *Ostreococcus tauri* (O'Neill and Reddy, 2011; O'Neill et al., 2011). Phylogenetic studies show that oscillation of the PRX redox state is highly conserved across all organisms, suggesting that non-transcriptional timekeeping mechanisms play an important role in the maintenance of circadian rhythms in cells (Edgar et al., 2012).

PRX represent a family of antioxidant enzymes that are involved in the regulation of reactive oxygen species (ROS) homeostasis within the cells. PRX activity is dependent upon oxidation-reduction cycles. The reduced PRXs catalyse the conversation of hydrogen peroxide (H_2O_2) to water (H_2O), where cysteine residues on the PRXs are oxidized to disulphide. Then the disulphides of oxidized PRXs are reduced by an NADPH-dependent thioredoxin reductase-thioredoxin system to complete the catalytic cycle (Chae et al., 1994; Hoyle and O'Neill, 2015). In the catalytic cycle PRXs may be present in different forms, which depends upon the redox status of cysteine residues in the active site: namely they can have a reduced form (PRX-Cys-SH), oxidized form (PRX-Cys- SOH), over-oxidised (PRX-Cys-SO_2) or hyper-oxidised (PRX-Cys-SO_3) forms (Figure 5) (Reddy and Rey, 2014). A number of studies have indicated that levels of over- and hyper-oxidized PRX exhibit robust endogenous circadian oscillations, even under constant darkness, in a range of eukaryotic and prokaryotic models, most likely reflecting an endogenous rhythm in the production of ROS (Edgar et al., 2012; O'Neill and Reddy, 2011). Moreover, the period of PRX rhythms is temperature-compensated and entrainable by temperature cycles, suggesting that non-transcriptional clock mechanisms are sufficient to sustain ~24 h rhythms (O'Neill and Reddy, 2011). Nevertheless, the relationship between TTFL and non-transcriptional oscillators is still poorly understood.

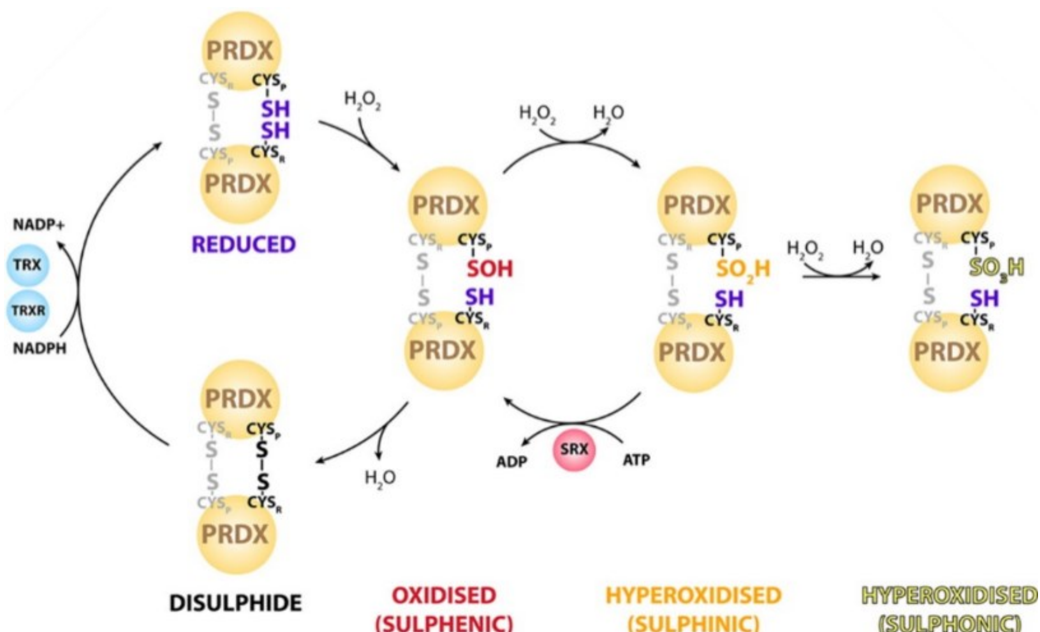


Figure 5: Peroxiredoxin system. 2-Cys PRDXs exist in dimers and form intramolecular disulfide bridges between the peroxidatic (CysP) and resolving (CysR) cysteines. Disulfides are reduced by NADPH-dependent thioredoxin reductase-thioredoxin system (TRX). The sulphenic moiety of PRDXs (Cys-SOH) can be hyperoxidized to form sulphinic PRDX. Sulphinic PRDX is recycled by sulphiredoxin (SRX). Further oxidation (termed hyperoxidation) of sulphinic PRDX to sulphonic PRDX is thought to be irreversible. (Hoyle and O'Neill, 2015; Reddy and Rey, 2014)

1.1.4 The light input pathway

The principal and best characterised Zeitgeber that synchronizes endogenous circadian rhythms with the local time is light. In mammals, the integrity of the eye and the retina is crucial for photic entrainment of the clock. Until recently, all ocular photoreception was attributed to the rods and cones of the retina and involved visual opsin photoreceptors. However, in the early 1990s mice with progressive outer retinal degeneration were examined to determine the contribution of rod and cones to photoentrainment. Remarkably, mice completely lacking rods and cones still retained the capacity of light to regulate their circadian rhythms (Freedman et al., 1999; Lucas et al., 1999). Subsequent studies indicated that the circadian and visual systems process photic information in different ways, suggesting that the retina contains additional non-visual photoreceptors that are involved in the photic entrainment of the clock (Czeisler et al., 1995; David-Gray et al., 1998). Subsequently, Provencio et al. identified a non-visual opsin photoreceptor termed melanopsin (Opn4) in the dermal melanophores of *Xenopus* (Provencio et al., 1998). The orthologue of melanopsin was also found in mammals, namely the mouse and humans. *In situ* hybridisation histochemistry showed that melanopsin is expressed in a small subset (1-2%) of retinal ganglion cells, namely the intrinsically photosensitive retinal ganglion cells (ipRGCs)(Figure 6) (Provencio et al., 2000; Sexton et al., 2012). The role of melanopsin in circadian photoentrainment was explored in *Opn4* $^{-/-}$ mice that lack melanopsin and in *Opn4*$^{-/-}$ triple knockout mice lacking melanopsin, cones and rods. The experiments showed that the loss of melanopsin alone did not drastically alter circadian photosensitivity, but rather led to attenuated circadian responses to light (Panda et al., 2002; Ruby et al., 2002). These studies suggest that rods and cones are able to partially compensate for the loss of melanopsin function. However, loss of all visual cone and rod photoreceptors as well as melanopsin completely abolishes circadian entrainment of clocks by light (Golombek and Rosenstein, 2010). This evidence suggests that all three retinal photoreceptor cell types together play a vital role in the regulation of the circadian clock by light. The photopigment melanopsin, like rod and cones photopigments, belongs to the G-protein-coupled receptor family and is covalently linked to a chromophore consisting of 11-cis-retinal. Light-induced isomerization of the chromophore is the first step in the phototransduction process that leads to activation of melanopsin (Walker et al., 2008). The subsequent chromophore-induced changes in opsin structure lead to the activation of a membrane bound signalling cascade, where retinal specific G proteins activate phospholipase C and induce the opening of transient receptor potential channels (TRPCs) (Nickle and Robinson, 2007; Schmidt et al., 2011). Light activation of

melanopsin also triggers the activation of calcium-sensitive protein kinase (*PKC),* which phosphorylates downstream targets (Isoldi et al., 2005).

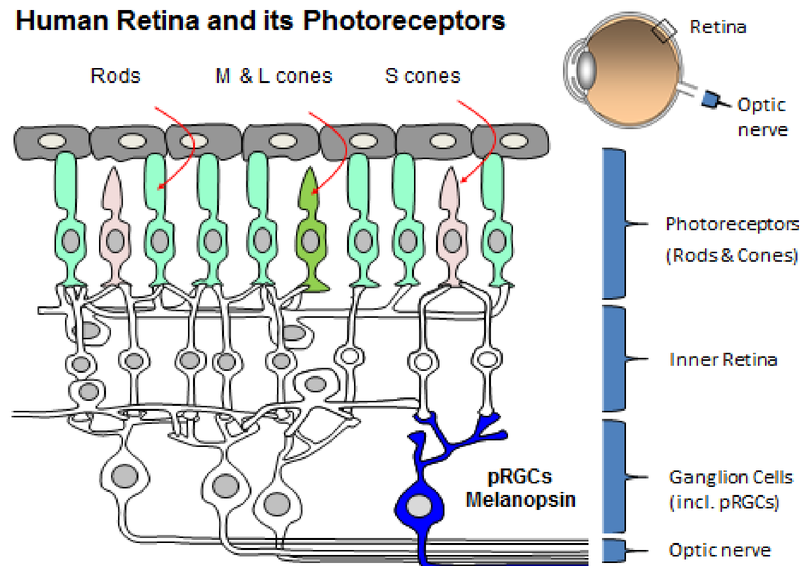


Figure 6: Visual and non-visual photoreceptors in the retina. Rods and cones are classical visual photoreceptros. Rods are responsible for night vision, whereas the cones are used for color vision. L and M cones are specialized for the detection of long and medium wavelengths; S cones are sensitive to short wavelength light. The photoreceptive ganglion cells respond to light, due to the expression of melanopsin, and provide non-visual photoreception function and play a key role in circadian photoentrainment. Source: (Oxford Sparks, University of Oxford web site).

Light-induced neuronal signals initiated by melanopsin-expressing ipRGCs are transmitted to specific areas of the brain, including the SCN and olivary pretecal nucleus (OPN), controlling circadian photoentrainment and the pupil light reflex (Hattar et al., 2002). ipRGCs regulate non-image-forming visual functions by releasing the neurotransmitters glutamate and pituitary adenylate cyclase-activating peptide (PACAP) via the RHT (Hannibal et al., 2004). This leads to an increase in intracellular calcium concentration, which results in the phosphorylation and activation of cyclic adenosine monophosphate (cAMP)-response-element-binding protein (CREB). Activated CREB binds to calcium/cAMP response elements (CREs) in the promoter of certain clock genes including *Per1* and thereby induces their expression as an indirect response to light exposure (Albrecht, 2002; Welsh et al., 2010) .

1.1.5 Food input pathway (FEO)

Primarily, molecular oscillators in the SCN are entrained by light. However, significant evidence points to the existence of an SCN-independent circadian system that regulates rhythms in behaviour and physiology in response to timed intake of food (Mistlberger, 1994). This

system is termed the food-entrainable oscillator (FEO). (Blum et al., 2012). The first evidence for the existence of food regulated circadian clocks came from the studies exploring the role of timed feeding in regulating behaviour. Curt Richter observed that rats anticipate feeding time by increasing levels of their locomotor activity a few hours before mealtime, and this activity pattern persists for several days even during total food deprivation (Richter CP. (1922). This rhythmic pattern of food anticipation was named food-anticipatory activity (FAA) and is also observed in many non-mammalian species, including zebrafish (Davis and Bardach, 1965). In subsequent studies, restricted-feeding was also shown to affect body temperature, serum corticosterone levels and gastrointestinal motility, suggesting that food serves as a Zeitgeber which competes with the entraining effects of light signals (Comperatore and Stephan, 1987; Krieger, 1974; Moberg et al., 1975).

Investigation of the effects of timed feeding on physiological processes demonstrated that the SCN is not required for the regulation of FAA. In SCN lesioned rodents FAA was still present and temperature and corticosterone rhythms entrained by feeding time were not abolished (Krieger et al., 1977; Stephan, 2002). These findings led to the idea that multiple timing systems exist: one system is specialized for entrainment by light (the light entrainable oscillator, LEO), and the other - to feeding time (FEO). Due to the fact that the SCN is localised in the hypothalamus and is entrained by light via the retina, it was assumed that the FEO should also be localised in the hypothalamus, within the cell groups that respond to peripheral metabolic signals and which regulate feeding and metabolism. Brain lesion experiments and gene knockouts (KOs) revealed that the integrity of other sites within the central nervous system, such as the hypothalamus (ventromedial, paraventricular and lateral parts), hippocampus, amygdale and nucleus accumbens, area postrema and olfactory bulb were also not necessary for FAA (Davidson et al., 2001a; Davidson et al., 2001b; Mistlberger and Mumby, 1992; Stephan et al., 1979). Accumulating evidence also indicates that various neural and neuroendocrine pathways as well as peripheral organs play a role in the regulation of FAA, but are not essential (Mistlberger, 1994; Stephan, 2002). Furthermore, hormones that regulate food intake and energy homeostasis appear to play a role in, but are not necessary for circadian anticipatory behaviour (Gunapala et al., 2011). Recent studies have suggested that the dorsomedial hypothalamic nucleus (DMH), which plays a role in regulating feeding behaviour, is one of the key structures contributing to the FEO (Gooley et al., 2006; Mieda et al., 2006). Rhythmic expression of clock genes in the DMH is entrained by restricted feeding (Mieda et al., 2006). However, a local DMH circadian clock is not essential for FAA or for maintaining temperature rhythms (Landry et al., 2006; Moriya et al., 2009). This evidence suggests that the

system that underlies the FEO is composed of complex, multiple, interconnected structures and pathways.

The mechanism of the FEO still remains poorly understood. Some studies indicated that FAA was under the control of the core circadian clock mechanism. These observations lead to the prediction that circadian clock mutations should also affect FAA. Mice lacking either *Clock*, *Bmal1* or *Cry1/2* genes, have disrupted behavioural rhythms in constant darkness (Bunger et al., 2000; van der Horst et al., 1999; Vitaterna et al., 1994). However, restricted feeding entrains normal FAA in these mutant mice (Iijima et al., 2005; Pendergast et al., 2009; Storch and Weitz, 2009). This suggests that *Clock*, *Bmal1* or *Cry1/2* functions are not necessary for FAA. Subsequently, it was revealed that *Npas2*, which is able to heterodimerize with *Bmal1*, is able to compensate for the loss of *Clock,* and therefore maintains circadian oscillation in the SCN (Debruyne et al., 2006) of *Clock* mutants. In parallel it was shown that in *Npas2* knockout mice under restricted feeding, FAA was abnormal (Dudley et al., 2003). Furthermore, mutation of the *Per2* and *Rev-erbα* genes led to abnormal FAA (Delezie et al., 2016; Feillet et al., 2006). These findings indicate that *Npas2* and *Per2* are important genes in the regulation of FAA.

Restricted feeding plays a major role in regulating rhythms in the liver transcriptome (Atger et al., 2015). However, the signal transduction pathways involved in the synchronisation of peripheral clocks by feeding remain unknown. Restricted feeding also has a major impact on the maintenance of metabolic rhythms, suggesting that peripheral clocks may be entrained through metabolic pathways. Changes in metabolic status also involve regulation of the redox status within cells. Nicotinamide adenine dinucleotide NAD^+, a key molecule involved in the maintenance of redox state in the cells, is involved in the synchronisation of the FEO (Rutter et al., 2002). The NAD^+ dependent ADP-ribosyltransferase, Poly (ADP-ribose) polymerase 1 (PARP-1), that regulates the activity of transcriptional regulatory proteins, is rhythmically expressed in the liver and is affected by the feeding time (Asher et al., 2010). PARP-1 directly interacts with CLOCK, and mutation of the PARP-1 gene leads to disruptions in circadian clock gene expression in the liver (Asher et al., 2010). Sirtuin 1 (Sirt1), that deacetylates the CLOCK/BMAL1 complex, is another regulatory factor that is controlled by NAD+ cycles (Jung-Hynes and Ahmad, 2009). The knockout of *Sirt1* leads to attenuation of FAA in mice, while overexpression of *Sirt1* increases FAA (Satoh et al., 2010). The rhythms of adenosine monophosphate (AMP)–activated protein kinase (AMPK), that is a metabolic sensor and regulates redox homeostasis within cells, are synchronized with feeding-fasting cycles and disruptions in the AMPK pathway alter circadian clocks in the liver (Lamia et al., 2009).

In addition to energy metabolism, some hormones may also play a role in synchronising FEO activity in peripheral tissues. One of the key candidates is oxyntomodulin (OXM), an intestinal peptide hormone, that regulates resetting of liver transcriptional rhythms via induction of the core clock genes *Per1* and *Per2* (Landgraf et al., 2015). Restricted feeding regulates periodic availability of many circulating macronutrients, which plays an important role in the regulation of clock gene expression in peripheral tissues (Woods, 2005). For example, restricted feeding may shift the phase of daily insulin rhythms that regulates *Per* gene expression by modulating the nuclear accumulation of *Bmal1* (Dang et al., 2016). Changes in body temperature associated with restricted feeding could also be a relevant factor resetting clock gene expression in peripheral tissues. Studies have shown that circadian clock gene expression in fibroblasts can be entrained by low-amplitude temperature cycles (Brown et al., 2002). However, the mechanism by which temperature cycles might entrain peripheral clocks remains poorly understood. Signalling molecules may also be involved in the regulation of FAA. For example, the dynamics of expression of RGS16, that regulates G-protein coupled receptor signalling, is affected by fasting-feeding cycles (Hayasaka et al., 2011; Huang et al., 2006). In Rgs16 knockdown mice, FAA was shown to be attenuated during restricted feeding (Hayasaka et al., 2011; Huang et al., 2006).

All these findings point to the FEO representing a network composed of multiple neuronal structures and that it is entrained by fluctuation of humoral signals that are regulated by feeding time.

1.1.6 Clock output pathways in vertebrates

The circadian system plays a pivotal role in regulating numerous behavioural and physiological processes, including the wake-sleep cycle, locomotor activity and metabolic homeostasis. It is assumed that the circadian regulation of physiological processes confers a survival advantage on organisms. Therefore, key components of the circadian timing system are output pathways whereby various physiological and behavioural aspects are linked with the molecular clock machinery. Clock outputs act at the organismal level to allow the organism to adapt to ambient conditions and at the cellular level where peripheral clocks are able to differentially and directly regulate the expression of clock-controlled genes (Schibler and Sassone-Corsi, 2002). Output pathways are controlled by direct or indirect signals from the SCN. Disruptions of the circadian clock system appear to contribute to the development of pathologies, including cancer, cardiovascular disease and type 2 diabetes (Klerman, 2005).

In the case of peripheral clocks, direct regulation of gene expression by the core clock factors CLOCK and BMAL1 via E-box enhancer promoter elements represents a major clock output pathway (Schibler and Sassone-Corsi, 2002). By this cell autonomous mechanism, so-called clock controlled genes (CCGs) are expressed with rhythms established by the core clock feedback loops. Via this mechanism, key cell cycle regulatory steps are clock regulated (Ko and Takahashi, 2006). Furthermore, clock disruption leads to aberrant expression of cell cycle genes and is associated with an increased risk of cancer (Sahar and Sassone-Corsi, 2009). In a similar fashion, D-box enhancers also represent key clock output elements in mammals by relaying circadian timing information to changes in gene expression. The clock-controlled, D-box binding transcription factors are involved in the regulation of biochemical pathways such as the metabolism of glucose and lipids and the synthesis of cholesterol and bile acids (Staels, 2006). In addition, ROR elements (RREs) and cAMP response elements (CREs) also play important roles in regulating the transcription of CCGs as well as the metabolic state within the cell (Bozek et al., 2009; Ueda et al., 2005). Dysregulation of these pathways results in an increased incidence of several metabolic syndromes (Staels, 2006).

1.1.6.1 Interplay between regulators of the circadian clock and metabolism

A number of studies have shown that the circadian clock orchestrates metabolic functions. This is achieved by its regulation of rate-limiting enzyme expression (e.g. *Nampt*), via the integration of nuclear receptor and nutrient-sensor input with metabolic pathways (e.g. RORα, SIRT1), or by gating the abundance of several metabolites (e.g. amino acids) (Sahar and Sassone-Corsi, 2012). The connections between the circadian clock and metabolism are bidirectional. Although the circadian clock system coordinates multiple metabolic pathways, in turn, metabolite levels also affect the circadian clock (Damiola et al., 2000). Here I focus on the NAD+ biosynthesis pathway as a representative example of a feedback loop linking the circadian clock with metabolism.

Circadian regulation of the NAD^+ biosynthesis and salvage pathway is highly conserved from yeast to vertebrates (Nakahata et al., 2009). In mammals, the rhythmic expression of NAMPT (nicotinamide phosphoribosyl transferase), which is a key rate-limiting enzyme in the salvage pathway, is driven by direct binding of the CLOCK/BMAL1 complex to E-boxes in the promoter (Figure 7) (Nakahata et al., 2009; Ramsey et al., 2009). NAMPT catalyses the conversion of nicotinamide (NAM) to β-nicotinamide mononucleotide (NMN). In the following step NMN is converted to NAD^+ by the nicotinamide mononucleotide adenylyltransferase enzymes 1-3 (NMNAT1-3). NAD^+ is a key molecule in metabolism and maintenance of redox

status, activating a number of enzymes and transcription factors involved in multiple metabolic pathways. One of these factors is deacetylase SIRT1(Sirtuin1), which catalyses the conversion of NAD^+ to form NAM and O-acetyl-ADP-ribose. In addition SIRT1 feeds back on the core clock by rhythmic deacetylation and destabilisation of BMAL1 and PER2, thus contributing to the synthesis of its own coenzyme (Asher et al., 2008; Nakahata et al., 2008). Ablation of the *Sirt1* gene or pharmacological inhibition of SIRT1 enzymatic activity leads to disruption of the circadian clock system (Nakahata et al., 2008). Importantly, whereas no circadian oscillation in SIRT1 protein levels and mRNA expression was detected, the enzymatic activity of SIRT1 oscillates in a time dependent manner (Nakahata et al., 2008). A possible explanation could be that SIRT1 plays an important role in controlling the stability of certain clock proteins, including PER2, and transducing signals originating from metabolites to the circadian clock (Asher et al., 2008; Nakahata et al., 2008).

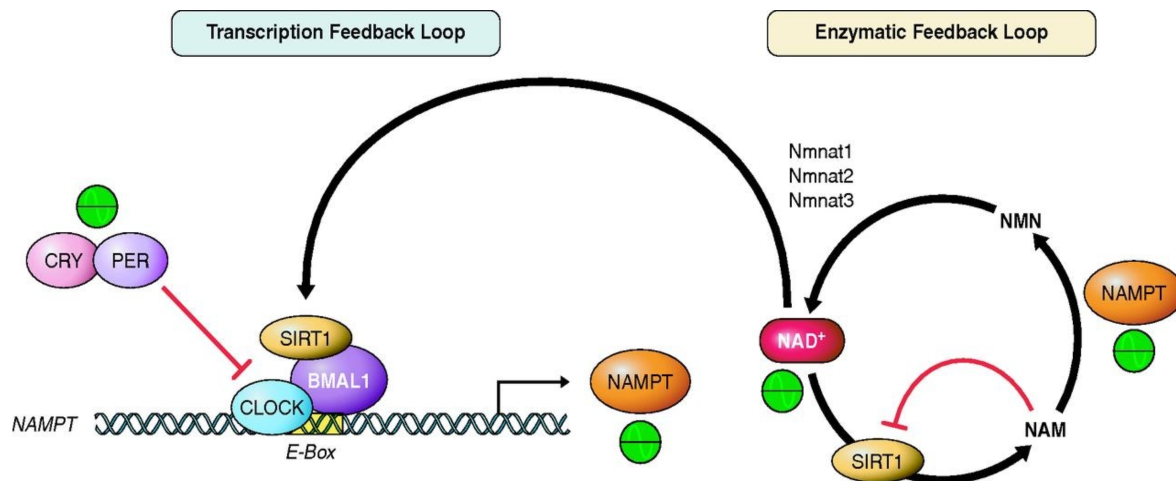


Figure 7: Interplay between circadian clock elements and the enzymatic feedback loop. The molecular circadian oscillator in cells relies on a TTFL mechanism. The transcription activator complex is composed of CLOCK and BMAL1. This complex binds on the E-box element in the promoter region of target genes and drives the expression of negative loop elements, PER and CRY, as well as regulating circadian expression of NAMPT. NAMPT in turn regulates the circadian clock by the targeting CLOCK/BMAL1 heterodimer via NAD+ and SIRT1. (Eckel-Mahan and Sassone-Corsi, 2013)

Besides clock genes, SIRT1 deacetylates a number of transcriptional regulatory proteins that are involved in multiple metabolic pathways. SIRT1 controls gluconeogenesis by deacetylating PPARγ-coactivator α (PGC1α) and Forkhead box O1 (FOXO1) (Schwer and Verdin, 2008). PGC1α coactivates glucocorticoid receptors and hepatic nuclear factor 4-alpha (HNF-4α) to induce the expression of gluconeogenic genes (Yoon et al., 2001). FOXO1 directly regulates the expression of several gluconeogenic genes (Frescas et al., 2005). SIRT1 is also involved in the regulation of cholesterol metabolism by deacetylating Liver X receptor (LXR) (Li et al.,

2007). LXR plays a key role by regulation of cholesterol homeostasis by driving the expression of the ATP-binding cassette transporter A1 (ABCA1), which promotes cholesterol efflux (Oram and Heinecke, 2005). SIRT1 is also involved in the regulation of feeding behaviour through the regulation of POMC and NF-Y neurons in hypothalamus (Satoh et al., 2010). Taken together SIRT1 is an important element which regulates circadian clock gene expression and functions as a molecular link between the circadian clock system and metabolism.

1.1.6.2 The circadian clock and amino acid metabolism

Amino acids (AAs) are not just fundamental elements for protein synthesis, but they are also important biomolecules playing a pivotal role in signalling pathways and metabolism. In the human body there 20 AAs that are classified as either essential amino acids (EAAs) or nonessential amino acids (NEAAs) according to nutritional requirements (Wu, 2009). EAAs cannot be synthesized by cells and must be obtained from dietary sources, whereas NEAAs can be synthesized from precursor molecules by simple steps. Each AA has the same fundamental structure, which contains an N-terminus, a C-terminus, and a central carbon atom with an attached side chain. Their precise chemical properties are determined by the chemical and physical properties of their particular side chain.

Metabolic homeostasis in cells is coordinated by the integration of different pathways that detect intra- and extracellular levels of amino acids (Figure 8). The ability of the body to sense and respond to daily changes in extracellular and intracellular AA levels is mediated by a complex regulatory network consisting of various dynamic players, which are necessary for initiating downstream effectors at the cellular level (Kilberg et al., 2005). Dietary intake is a major source of amino acids in the body. Transport of AA into cells is driven by plasma membrane localized solute-linked carriers (SLC), specifically by SLC1, SLC6, SLC7 (in co-operation with SLC3), SLC16, SLC36, SLC38 and SLC43 (Poncet and Taylor, 2013). AAs bind to the extracellular side of these transporters and induce conformational changes. These changes result in the delivery of AA into the cytoplasm of the cell. The transport across a membrane is driven by the electrochemical gradient (Poncet and Taylor, 2013). The expression of AA transporters can be driven by the availability of AA via a process considered as adaptive regulation. For example AA deprivation activates a number of transporters, especially those that are involved in the regulation of the GCN2/eIF2α pathway, including SLC38A2 (SNAT2/System A) and SLC7A1 (CAT1) (Malmberg and Adams, 2008). SNAT2 activity is

also regulated by the circadian clock system through REV-ERBα. The expression of SNAT2 is repressed in REV-ERBα knockout mice (Dyar et al., 2018).

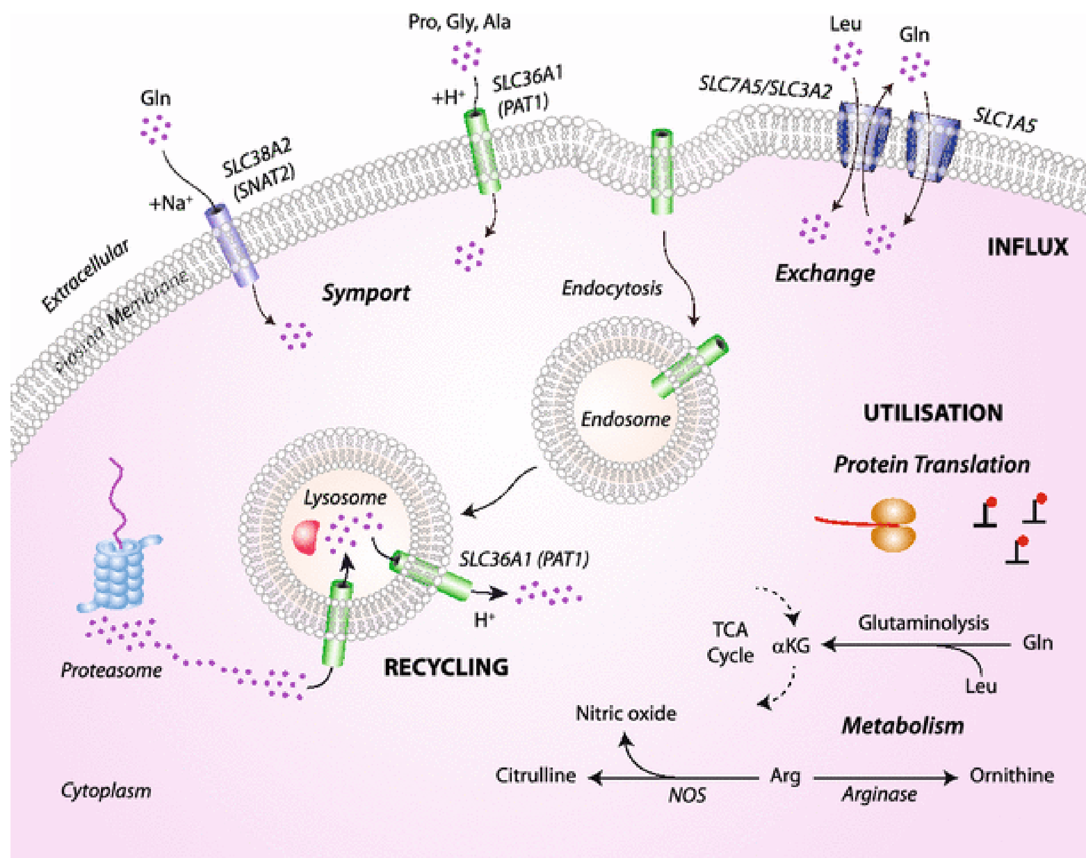


Figure 8: Amino acid homeostasis. Intracellular levels of amino acids are maintained by a constant influx via the transporters localized on the plasma membrane, e.g. SLC7A5/SLC3A2, protein translation and metabolism as well as via recycling, e.g. autophagy). (Carroll et al., 2015)

Besides special transporters, intracellular AA levels are also regulated by two major signal transduction mechanisms, involving mammalian target of rapamycin (mTOR) and general control nonderepressible-2 kinase (GCN2) pathways (Gonzalez and Hall, 2017). mTOR is a key regulator of cellular metabolism, which integrates signals from amino acids, growth factors and energy status to mediate anabolic processes such as protein synthesis and at the same time it supress catabolic processes, including autophagy (Peng et al., 2012). The mechanism through which mTOR senses intracellular amino acid levels is unknown. However, it seems that a key aspect of this process is that amino acid sufficiency activates the mTOR kinase cascade that leads to phosphorylation of the ribosome-associated S6 kinase and promotion of protein synthesis (Hara et al., 1998). The second amino acid sensing pathway is important for the detection of amino acid deficiency and regulation of catabolic processes including amino acid biosynthesis and autophagy (Bunpo et al., 2010). Limiting availability of amino acids supresses the synthesis of proteins and leads to an increase in uncharged tRNAs that bind to GCN2

(Sonenberg and Hinnebusch, 2009). Activated GCN2 phosphorylates eukaryotic translation initiation factor eIF2α, which stimulates the activation of transcription factor ATF4. ATF4, in turn, drives the expression of asparagine synthetase (ASNS) and CCAAT/enhancer-binding protein (C/EBP) homology protein (CHOP) by binding to the amino acid response element AARE or the nutrient sensing response unit (NSRU) enhancer elements (Averous et al., 2004; Zhong et al., 2003). ATF4 also regulates autophagy through driving the transcription of the essential autophagy gene Map1lc3b, that is used as a biomarker of autophagy (Rzymski et al., 2009). Autophagy is an intracellular degradation system and a key mechanism involved in the regulation of cellular homeostasis. Accumulating evidence suggests that autophagic degradation of cellular components varies over the course of the day, with the peak during the rest phase of animals (Ma et al., 2012; Pfeifer and Scheller, 1975; Pfeifer and Strauss, 1981). These observations indicate that the FEO is also involved in the regulation of autophagy in peripheral tissues. The coupling of autophagic degradation to the circadian clock system provides a distinct advantage to achieve temporal compartmentalization of cellular and metabolic processes within tissues (Ma et al., 2012). Additionally it has been shown that ATF4 directly regulates the circadian clock system, through binding to the TTGCAGCA motif in the *Per2* promoter and in turn enhancing its transcription (Pathak et al., 2019).

1.1.6.3 Glucocorticoids and the circadian clock

Glucocorticoids (GCs) are steroid hormones synthesised by the adrenal gland (*zona fasciculata*). GCs play an important role in the regulation of biological processes including cell cycle, development, metabolism and stress response. In mammals the major GC is Cortisol. Cortisol release is controlled by the hypothalamic-pituitary-adrenal (HPA) axis via complex interactive mechanisms (Capper et al., 2016). The HPA axis plays an important role in the regulation of metabolic homeostasis and mediates daily changes of Cortisol levels in the blood. The highest levels of Cortisol are detected early in the morning, when we wake up, and declines throughout the day with lowest concentrations around midnight (Chan and Debono, 2010; Dickmeis, 2009). Glucocorticoids are not just regulated by the circadian clock system, but in turn they can affect the circadian clock itself (Dickmeis, 2009).

Endogenous GC levels are controlled by physiologic processes within the organism, as well as by external stimuli, for example stress (Figure 9). Stress cues stimulate production of endogenous Cortisol through activation of the HPA axis. In the first step, stress signals stimulate the synthesis of neuropeptides including the corticotropin-releasing hormone (CRH) from neuroendocrine cells localized within the paraventricular nucleus (PVN) of the hypothalamus

(Simpson and Waterman, 1988). Then CRH is released into the hypophyseal portal system that connects the hypothalamus with the anterior pituitary. The portal system carries CRH to the anterior pituitary, where it stimulates the production of adrenocorticotropic hormone (ACTH) within corticotropic cells (Simpson and Waterman, 1988). ACTH travels to the adrenal gland via the bloodstream and mediates the biosynthesis and the release of GCs within the adrenal cortex. In turn Cortisol is transported via the blood stream to target organs, where it mediates the response to physiological stress (Oakley and Cidlowski, 1993; Papadimitriou and Priftis, 2009; Simpson and Waterman, 1988). The endogenous Cortisol levels are reduced by the HPA axis via a negative feedback-loop based mechanism. For example, high levels of Cortisol lead to repression of CRX secretion and ACTH production from the hypothalamus and the pituitary gland (Kyrylov et al., 2005).
The levels of GC are also directly controlled by the master clock localised in the SCN via two pathways, the HPA axis and autonomic nervous system (ANS) (Figure 9) (Dickmeis, 2009; Kalsbeek et al., 2012). Briefly, SCN directs CRH release from the PVN, that in turn induces the production of ACTH, that regulates circadian GC release from the adrenal cortex. In addition, SCN generated neuronal signals mediate circadian GC synthesis through ANS (Buijs et al., 2003). The rhythmic changes in GC levels are eliminated in SCN lesioned rats (Moore and Eichler, 1972). However, the precise mechanism whereby ANS regulates GC synthesis remains poorly understood.
GCs mediate their effect on various physiological functions through the glucocorticoid receptor (GR). GR is ligand-inducible transcription factor, which belongs to the nuclear receptor superfamily. The activity of GR can be induced either by Cortisol or other natural and synthetic GC ligands. In the absence of ligands, GR is inactive and resides in the cytoplasm in a complex with chaperone proteins. Upon GC binding, the GR becomes active and translocates from the cytoplasm to the nucleus, where it regulates the transcription of GR-dependent genes, including core clock genes such as *Per, Npas2*, and *Reverbβ*, through the binding to the specific glucocorticoid response elements (GREs) in the promoter (Balsalobre et al., 2000; So et al., 2009). Depending on the type of GRE and the context of other enhancer elements, the ligand-receptor complex can activate (transactivation) or reduce (transrepression) gene expression. GR also regulates target gene transcription without direct interaction with DNA, but via tethering, a physical interaction with other transcription factors (De Bosscher et al., 2003; Oakley and Cidlowski, 2013; Ratman et al., 2013; Schaaf and Cidlowski, 2002).

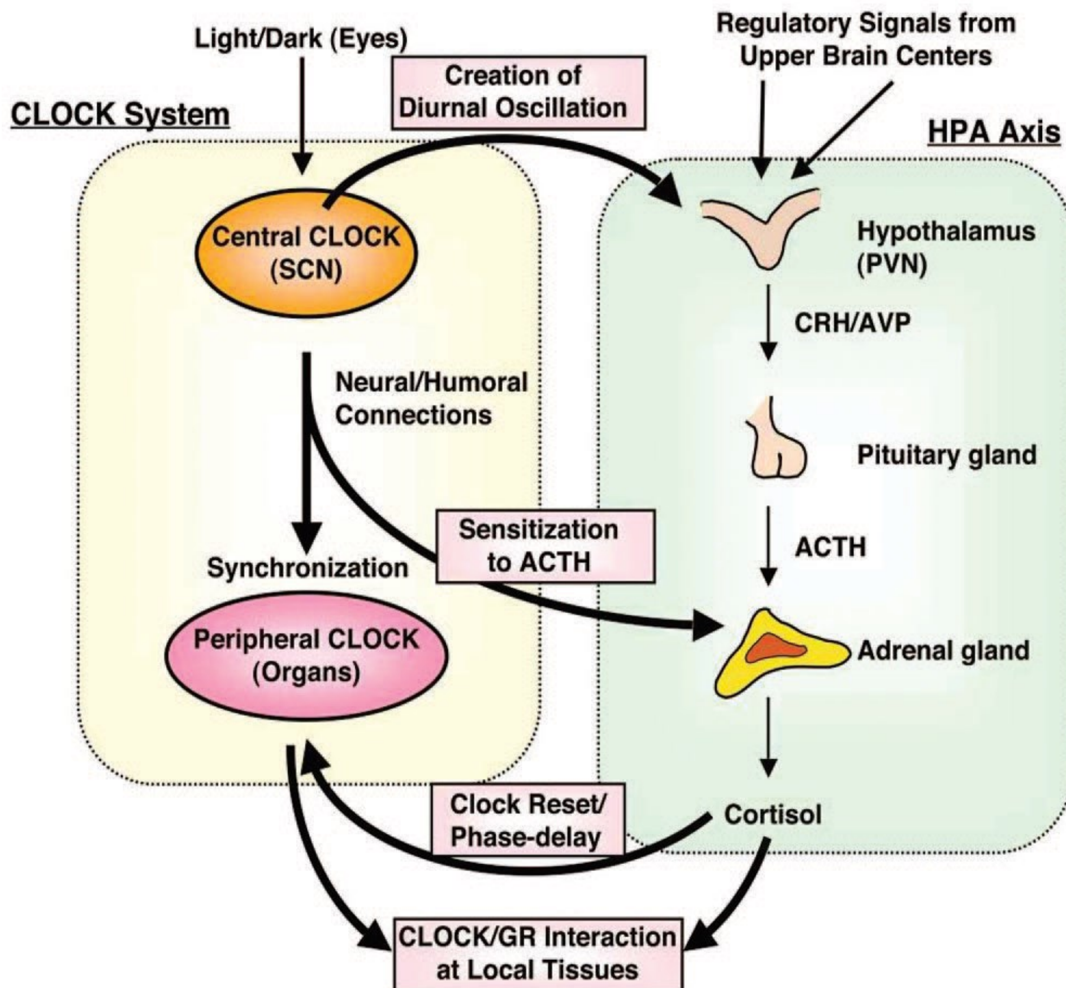


Figure 9: Interaction between the circadian clock system and glucocorticoids. The synthesis of glucocorticoids can be regulated by the endogenous and external cues, e.g. stress, via the HPA axis. Circadian clock system can also directly mediate rhythmic synthesis of cortisol either via the HPA axis or the SCN-ANS axis. Secreted glucocorticoids in turn regulate the transcription of clock genes in peripheral tissues. (Kassi and Chrousos, 2013)

1.1.6.4 Circadian rhythms and the cell cycle

Cell proliferation and differentiation are crucial for normal cell growth, development and for tissue regeneration. Several lines of evidence have shown that the circadian clock system plays a fundamental role in the regulation of cell cycle from bacteria to vertebrates (Johnson, 2010; Mori et al., 1996). This interaction is necessary for the reduction of UV exposure damage on DNA replication and cell division (Geyfman et al., 2012). While DNA replication is observed when exposure to UV is at its lowest, during the night phase, DNA repair takes place during the light phase.

The timing of cell proliferation is regulated by the expression of circadian clock genes, including PER, CLOCK and BMAL1, through cell cycle related genes, such as *p21* and *Wee-1*

(Figure 10) (Grechez-Cassiau et al., 2008; Matsuo et al., 2003). *p21* regulates the G1/S transition in the cells by inactivating the cyclin E-cdk2 complex in a time dependent manner, whereas circadian expression of Wee-1 mediates the timing of cell entry into mitosis through phosphorylation of CDC2/cyclin B kinase complex (Grechez-Cassiau et al., 2008; Matsuo et al., 2003). For example, knockout of BMAL1 leads to cell cycle disruption by deregulation of *p21* and *wee1* in hepatocytes (Elshazley et al., 2012; Grechez-Cassiau et al., 2008). Some evidence suggests that the interaction between the circadian clock system and cell cycle is bidirectional. For example, Nagoshi and colleges have shown that the length of the circadian period is longer in proliferating fibroblasts, than in non-proliferating fibroblasts (Nagoshi et al., 2004). This study suggests that cell division alters the circadian clock system through regulation of PER/CRY levels. However, the mechanism regulating this interaction is still not fully understood.

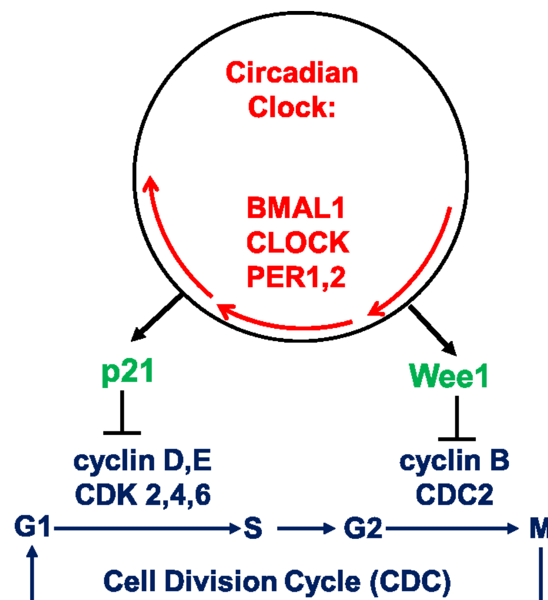


Figure 10: Interaction between the circadian clock and the cell cycle. The circadian system gates the Cell Division Cycle (CDC) through control of key CDC regulators such as p21 and wee1. p21 induces G1 arrest and block entry into S phase by inactivating Cdks. Wee1, kinase negatively regulates entry into mitosis by catalyzing the inhibitory tyrosine phosphorylation of CDC2/cyclin B kinase. (Johnson, 2010)

1.2 Fish as circadian clock models

1.2.1 Zebrafish as a model for studying circadian clocks

Laboratory zebrafish (*Danio rerio*) strains are derived from wild type populations native to the subtropical waters of southeast Asia (Bangladesh, India and Pakistan), where they live in slow moving or stagnant water such as rice fields (Figure 11). Zebrafish is used as one of the most important animal models for studying embryogenesis of vertebrates, development and even toxicology using high-throughput screening. The maintenance of zebrafish is easy and cheap compared with mammalian models. The high fertility and fecundity, short generation cycle and external development make it well-suited for *high-throughput* screening (Dahm and Geisler, 2006; Delvecchio et al., 2011). The transparency of eggs and embryos enables observation of individual cells during early developmental stages and embryogenesis which can be easily monitored by live imaging techniques. Zebrafish embryonic development is rapid; hatching occurs between 48 and 72 hours post fertilization (hpf) and organogenesis is mostly completed between 96 and 120 hpf (Gustafson et al., 2012). This rapid external development, a fully sequenced genome and the availability of many tools and techniques for manipulating gene expression makes the zebrafish highly suitable for large-scale mutagenesis, forward/reverse genetic screens and the generation of transgenic lines (Chatterjee and Lufkin, 2012; Haffter et al., 1996; Mullins and Nusslein-Volhard, 1993).

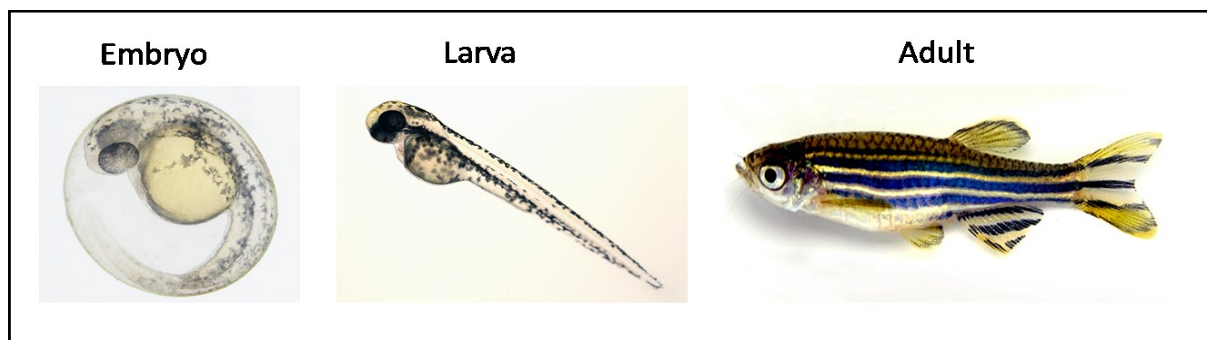


Figure 11: Danio rerio. Zebrafish, a freshwater fish belonging to the Cyprinidae family.

The zebrafish has received considerable attention as a model for studying the circadian clock. An anatomical counterpart of the mammalian central pacemaker, the SCN, has been described in zebrafish (Moore and Whitmore, 2014). However, there is little evidence regarding its function in regulating circadian rhythms in zebrafish (Moore and Whitmore, 2014). Instead, it appears that the pineal gland may serve as a central pacemaker in zebrafish (Noche et al., 2011). Importantly, a key early discovery was that zebrafish cells and tissues are intrinsically light

responsive, meaning that direct light exposure is able to reset and synchronize the peripheral clocks (Whitmore et al., 2000). Additionally studies showed that also cultured cells possess directly light entrainable clocks, making them a powerful *in vitro* model to study light input and output pathways (Vallone et al., 2004). These findings together with the potential for large scale genetic analysis make zebrafish an important vertebrate genetic model for studying the ontogenesis of circadian clock system as well as the basic mechanisms and organization of the vertebrate circadian timing system.

1.2.2 Origin of the circadian clock during early development

The importance of the circadian clock system in the regulation of behavioural and physiological processes in adults is well established. However, the knowledge of the circadian clock system ontogenesis and its role during embryogenetic development is still limited. Previous studies have established that expression of clock genes in zebrafish larvae raised under constant conditions, such as constant darkness and constant temperature, is arrhythmic. These results led to the hypothesis that for the normal development of circadian rhythms, exposure to zeitgebers, such as light-dark or temperature cycles, during the early stages of development are required (Dekens and Whitmore, 2008). In parallel it was shown that maternal inheritance does not play a role in the development of circadian clock rhythms at the whole animal level. Analysis of clock gene expression using single cell imaging has indicated that in zebrafish embryos, raised in constant darkness under constant temperature, transcription of *per1b* is rhythmic in each cell, but comparing different cells, this rhythmicity is asynchronous (Dekens and Whitmore, 2008). These findings indicate that even in the absence of environmental signals, functional circadian clock rhythms are established. However, for synchronisation of clock gene expression, exposure to external Zeitgebers is essential. The light-independent rhythmic transcription of *per1* is observed already in 1 dpf embryos, whereas the rhythmic expression of *clk1* and *bmal1* is only detected at later stages of development, around 4-5 dpf (Dekens and Whitmore, 2008). These *clk1* and *bmal1* rhythms appear together with other key clock outputs such as behaviour activity (Hurd and Cahill, 2002) implying a differential and progressive maturation of circadian clock elements during early stages of development.

1.2.3 Circadian clock genes in zebrafish

Following the cloning and functional characterization of the first mammalian clock genes, considerable progress has been made toward elucidating the core clock mechanism in other vertebrates, including zebrafish (Shearman et al., 1999). During early teleost evolution a

genome duplication occurred, leading to the emergence of extra copies of many of the clock genes (Postlethwait et al., 1998). Through comparative analysis of vertebrate genomes and yeast two-hybrid *(Y2H)* screens, the following zebrafish clock genes have been identified: three *clock* genes (*clock1a, 1b, 2*) (Wang, 2008b; Whitmore et al., 1998), four *period* genes (*period 1a, 1b, 2, 3*) (Vallone et al., 2004; Wang, 2008a), three *bmal* genes (*bmal1a, 1b, 2*)(Wang, 2009), six *cryptochrome* genes (*cry1a, cry1b, cry2a, cry2b, cry3, cry4*) (Kobayashi et al., 2000), three *Ror* genes (*Ror*α*, Ror*β*, Ror*γ) (Flores et al., 2007) and one *Rev-erbα* gene (Kakizawa et al., 2007). Phylogenetic studies indicated that zebrafish *cryptochrome1a*, *1b*, *2a* and *2b* share most sequence homology with mammalian *mcry2* and are able to repress CLOCK/BMAL transcription, whereas *Cry3* and *Cry4* are homologous to *Drosophila cry* and do not affect CLOCK/BMAL activity (Kobayashi et al., 2000). Interestingly the transcription of *clock1* in zebrafish tissues is rhythmic, while *clock* expression in mammalian cells shows no rhythmicity (Vatine et al., 2011; Whitmore et al., 1998). Moreover, it has emerged that light inducibility of gene expression is not a property restricted to circadian clock genes. Also, many genes with different cellular functions, including the DNA repair enzyme *Cry5* (*6-4 photolyase*) can also be directly induced upon light exposure (Gavriouchkina et al., 2010; Weger et al., 2011).

1.2.4 Central pacemakers in zebrafish

In mammals, the central pacemaker is located in the SCN. However, an anatomical *structure* equivalent to the SCN has not been identified in zebrafish. It is considered that the pineal gland, a neuroendocrine organ, acts as master clock in zebrafish, since it contains three key functional elements of the circadian timing system. Namely, it is directly light sensitive and so contains a clock input pathway, it contains a self-sustaining clock function based on the expression of core clock genes and finally, as a clock output, it synthesises and secretes the hormone, melatonin under the control of the endogenous circadian clock as well as the direct effect of light exposure (Ziv et al., 2005). The zebrafish retina is also photosensitive, locally produces melatonin in response to light- and clock-regulation and also many components of visual phototransduction pathways are expressed under circadian clock control (Li et al., 2012). Interestingly, while both the zebrafish retina and pineal gland synthesize and secrete melatonin under the control of their local circadian clocks, (Cahill, 1996), in mammals melatonin secretion by the pineal gland is regulated indirectly via the SCN and light perception occurs also indirectly through the retina. The highest levels of melatonin are detected during the night period and the lowest – during the day. Rhythmic melatonin synthesis is mediated by the rate-limiting enzyme Aanat2 (arylalkylamine-N-acetyltransferase 2), which is expressed in pineal and retinal photoreceptors

under clock control (Gothilf et al., 1999). Melatonin is an important output signal, playing a role in the temporal regulation of a multitude of physiological functions, including the sleep-wake cycle (Zhdanova, 2011).

1.2.5 Light-entrainable oscillators (LEOs) in zebrafish

Light represents the most important environmental zeitgeber for the clock in most species. Most of what we currently know about the circadian clock in a variety of different organisms concerns a clock mechanism that is regulated by light signals, and has therefore been termed the light-entrainable oscillator (LEO). In non-mammalian vertebrates such as the zebrafish, photic entrainment and circadian rhythm generation involves specialized photoreceptive organs, which develop from the embryonic forebrain including the eyes, pineal gland and deep brain photoreceptors (Figure 12) (Peirson et al., 2009). However, most zebrafish tissues and cells also contain directly light-responsive circadian clocks pointing to a general tissue photosensitivity in zebrafish and thereby making this species a powerful model for studying the light signalling pathway to the clock (Vatine et al., 2009; Whitmore et al., 2000). However, the precise nature of photoreceptors in the zebrafish still remains poorly understood.

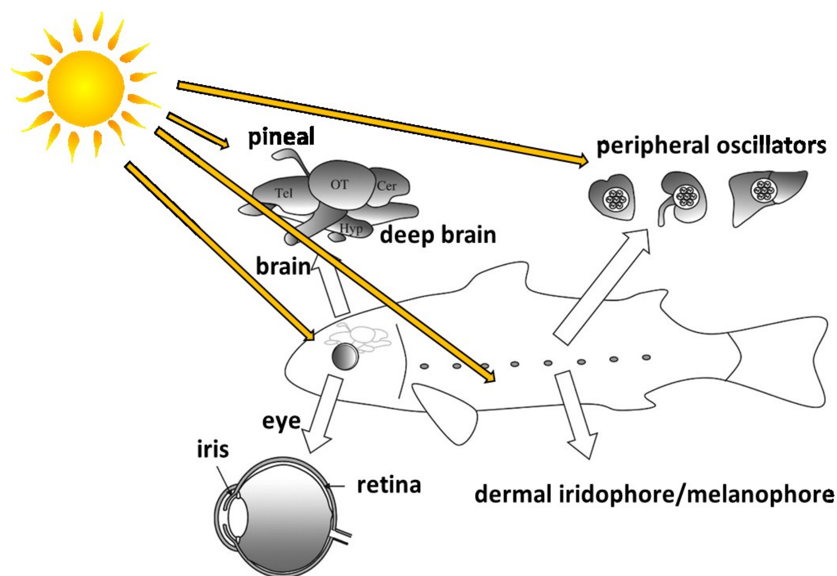


Figure 12: Localization of circadian clock photoreceptors in zebrafish. Zebrafish tissues possess directly light responsive circadian clocks. Photo-receptors in zebrafish are expressed in the eye, brain, peripheral tissues, e.g. kidney, heart, liver and dermal cells. (Peirson et al., 2009)

The key function of photopigments is to absorb photons and then to translate this light signal into neural signals, to regulate biological processes. In zebrafish as in other vertebrates, phototransduction is typically a G-protein-coupled signalling pathway (Zang and Neuhauss, 2021). Opsins are photosensitive seven-transmembrane (7TM) G-protein-coupled receptors

covalently bound to a vitamin A based chromophore, retinal via a protonated Schiff base linkage. Following photon absorption, the chromophore photoisomerises from the 11-cis-retinal to the all-trans-retinal state, thereby inducing a conformational change in the opsin, which leads to activation of the phototransduction signalling cascade via the selective recruitment of a subset of G-proteins (Figure 13). The regeneration of opsin occurs through replacement of the all trans-retinal with a newly synthesized 11-cis-retinal chromophore.

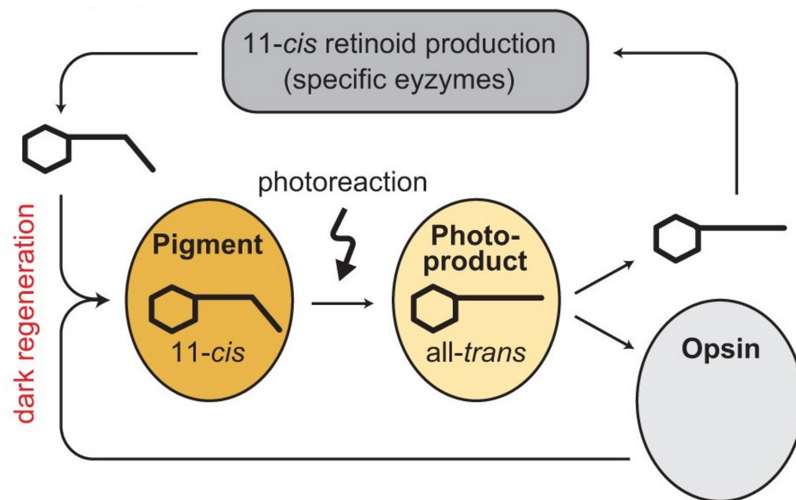


Figure 13: Schematic diagram of the photoreaction and regeneration of opsin-based pigments. A. In the dark condition, retinaldehyde is present as 11-cis retinal which upon light absorbtion is isomerized to the all-trans form. The all-trans isomer changes the conformation of the opsin and activates a G-protein which in turn triggers a specific phototransduction cascade. The regeneration of opsin occurs in the dark through newly synthesized 11-cis-retinal chromophore. (Terakita and Nagata, 2014)

Functional genomic analysis in zebrafish has surprisingly identified more than 40 opsins: 10 classical visual opsins and 32 nonvisual opsins (Davies et al., 2015). The visual photopigments are responsible for image-forming colour vision and are localized in rods and cones of the retina (Peirson et al., 2009). In this opsin group there is one class of rod opsins (*rhodopsin-like-1; rh1*) and four classes of cone opsins (*long-wavelength-sensitive, lws; short-wave-length-sensitive -1, sws1; short-wavelength-sensitive-2, sws2; and rhodopsin-like-2, rh2*)(Davies et al., 2015). Zebrafish also possess duplicated green-red-sensitive *lws* and green-sensitive *rh1* opsin genes, and four copies of the green-sensitive *rh2* opsin genes (Davies et al., 2015; Tsujimura et al., 2015). These multiple opsin variants have slightly different light wavelength detection spectra and so it has been speculated that this leads to a broadening of the range of wavelengths that fish are able to respond to.

The non-visual opsins play an important role in the regulation of the circadian clock, photoperiodism, locomotion, cell cycle and DNA repair (Davies et al., 2015; Peirson et al., 2009). Non-visual opsin classes consist of the cone-like opsins, encephalopsin/ panopsin

(*opn3*), teleost multiple tissue (*tmt*) opsins, retinal pigment epithelium (RPE) retinal G protein receptor (*rgr*), retinal pigment epithelium-derived rhodopsin homolog (*rrh*), neuropsin (*opn5*), melanopsin (*opn4*) and novel opsins (*opn6-9*) (Davies et al., 2015). In zebrafish, non-visual opsins are expressed widely, in the pineal gland, deep brain, dermal cells and in peripheral organs, including the heart, kidney and liver (Figure 12) (Peirson et al., 2009).
Besides opsins, other candidate circadian clock photoreceptors are the cryptochrome proteins (Sancar et al., 2000). Cryptochromes belong to the family of DNA photolyase/cryptochrome flavoproteins and present in all unicellular and multicellular organisms (Kobayashi et al., 2000). In zebrafish six cryptochrome homologs have been identified (Liu et al., 2015). One group of zebrafish cryptochromes shares more similarity with the mammalian cryptochromes, that serve as core clock elements by repressing CLOCK/BMAL-mediated transcription but have no photoreception function (Kobayashi et al., 2000). However, one zebrafish cryptochrome gene, *zfCry4*, does not play a role in repressing CLOCK/BMAL and instead shares higher homology with the *Drosophila* cryptochrome, has been implicated in mediating circadian photoreception (Kobayashi et al., 2000).
One characteristic feature of the circadian clock mechanism in zebrafish is that transcription of clock genes is induced upon direct exposure of cells and tissues to light. Much effort has been focused on identifying the light responsive transcription control mechanisms, which by definition serve as part of the clock input pathway. The Foulkes laboratory has discovered a Light Responsive Module (LRM) within the promoter region of the light-induced gene, *period 2* (*per2*) (Vatine et al., 2009) as well as the *cry1a* and cry5 genes (Mracek et al., 2012; Zhao et al., 2018). The LRMs contain D-box enhancer elements, which are essential for their light regulation in zebrafish. In contrast, in mammals, the D-box enhancer serves as a clock output element (Bozek et al., 2009). Consistent with the teleost genome duplication, there are many zebrafish homologs of D-box binding factors: TEF1, TEF2, HLF1, HLF2, DBP1, DBP2 and 6 members of the E4BP4 family (E4BP4-1 to 6) (Ben-Moshe et al., 2010; Vatine et al., 2009). Hirayama et al. implicated the Activator Protein-1 (AP-1) complex in driving the expression of photosensitive genes (Hirayama et al., 2005). However, more recent research from our group was not able to confirm the role of AP-1 in the regulation of light-driven gene expression (Mracek et al., 2012).

1.2.6 The food-entrainable oscillator (FEO) in zebrafish

In zebrafish, as in mammals, in addition to light signals, regular feeding also plays an important role in synchronizing endogenous circadian clock systems with the environment (Lopez-

Olmeda et al., 2010). Upon restricted feeding, independently of light cues, fish develop feeding anticipatory activity (FAA), which is one of the best studied outputs of the circadian food-entrainable oscillator (FEO). Interestingly, the timing of regular feeding has little effect on clock gene expression in the central pacemaker (Sanchez and Sanchez-Vazquez, 2009). Indeed in the zebrafish liver, the phase of cycling core circadian genes (*Per* and *Cry*) is adjusted to match the regular feeding time (Lopez-Olmeda et al., 2010). Furthermore, feeding cycles play a key role in the regulation of cell cycle timing in the intestine (Peyric et al., 2013). The neuroanatomical location of the FEO in fish and its molecular mechanism is still unknown. Moreover, it is still not known how the LEO and FEO interact with each other in order to maintain metabolic homeostasis in cells.

1.2.7 Zebrafish clock output pathways

Initial studies addressing the zebrafish circadian clock mechanism focused on the rhythmic synthesis of melatonin by the pineal gland and retina (Begay et al., 1998). Melatonin has been implicated in many aspects of zebrafish physiology including the regulation of sleep, development and nutrition (Lima-Cabello et al., 2014). In addition, locomotor activity rhythms with a predominantly daytime (diurnal) activity is another important circadian clock driven rhythm in adult zebrafish as well as in larvae (Hurd et al., 1998). Interestingly, under constant light conditions, fish are constantly active, while under constant darkness, their overall activity is reduced (Tovin et al., 2012). Under both sets of constant conditions, fish activity is arrhythmic. The circadian clock mechanism also plays an important role in the regulation of the cell cycle and cell proliferation. The clock regulates the timing of S-phase with a peak during the night period (Dekens et al., 2003). Moreover, consistent with the control of cell cycle regulators by the core clock machinery, the overexpression of an engineered, dominant negative form of CLOCK1 results in arrhythmicity of the cell cycle even under light-dark conditions (Tamai et al., 2012). This data confirms that the cell cycle is modulated by the circadian pacemaker rather than directly by light (Tamai et al., 2012).

1.2.8 Comparative studies involving zebrafish and blind cavefish

Fish represent the largest and most diverse of vertebrate groups, with many fish species inhabiting even extreme environments. In this regard, a diverse set of so-called cavefish species occupy perpetually dark environments and present a striking set of adaptations to life without light, including notably eye loss. Comparison of cavefish with zebrafish represents a powerful approach for exploring the basic mechanisms involved in light perception and light-dependent

aspects of physiology. *Phreatichthys andruzzii* is one extreme example of blind cavefish from Somalia, that has adapted to life in constant darkness in subterranean bodies of water beneath the Somalian desert (Figure 14) (Ercolini and Berti, 1975). During evolution they have evolved regressive and constructive phenotypes, such as complete loss of eyes, pigmentation and importantly, they also possess an aberrant circadian clock that is no longer entrainable by light (Stemmer et al., 2015). *P. andruzzii* belongs to the Cyprinidae family and so is a close relative to the zebrafish (Tarttelin et al., 2012). This allows many of the tools, experimental approaches and information that have been developed for zebrafish studies to be applied to the Somalian cavefish. The Somalian cavefish can be regarded as a "natural knockout" of the light entrainable circadian clock and this novel fish model allows us to study the light input pathway as part of a comparative analysis with zebrafish.

Figure 14: *Phreatichthys andruzzii.* The blind Somalian cavefish belongs to the Cyprinidae family. During evolution for at least 3 million years in complete darkness, they have loss eyes, scales and all body pigmentation. (Picture: Matthew Niemiller)

Recent behavioural studies have shown that *P. andruzzii* does still possess some degree of photic sensitivity: specifically, they tend to avoid light areas and prefer hiding in dark areas. This so-called photophobic effect was observed predominantly in response to green (539nm), light (Ercolini and Berti, 1975). It has been shown that rhodopsin (521nm) and exorhodopsin (520nm) are most likely responsible for these light-responsive, behavioral responses in Somalian cavefish (Tarttelin et al., 2012). A comparative study using Somalian cavefish and zebrafish revealed a striking loss of light inducibility clock gene expression in the cavefish, that could be partially rescued by the ectopic expression in cavefish cells of two zebrafish opsins, Melanopsin Opn4m2 and TMT opsin (Cavallari et al., 2011). The cavefish homologs of these two opsins were found to be mutated and non-functional, pointing to the involvement of these two opsins in the perception of light for synchronizing the clock in fish. Interestingly, this study also observed that a circadian clock in *P. andruzzii* is maintained *in vivo* and is strongly regulated by food.

2. Methods and Materials

2.1 Animal experiments

2.1.1 Ethics Statement

All zebrafish husbandry and experimental procedures were performed in accordance with the German animal protection standards (Animal Protection Law, BGBl. I, 1934 (2010) and were approved by the Local Government of Baden-Wurttemberg, Karlsruhe, Germany (Az.: 35-9185.81/G-130/12). General license for fish maintenance and breeding: Az.: 35-9185.64).

2.1.2 Animals

Zebrafish (*D. rerio*), wild type (WT) Wik and Tübingen (Tue) strains, were maintained and crossed according to standard procedures (Christiane Nüsslein-Volhard and Ralf Dahm 2002, Zebrafish Book; International Zebrafish and Medaka course (IZMC)) in a re-circulating water system at 28°C, under 14:10 light-dark (LD) cycles and fed twice daily. Fertilized eggs were collected within 2 hours and aliquots of 20 embryos were transferred into 5 ml of E3 media (5mM NaCl, 0.17mM KCl, 0.33mM $CaCl_2$, 0.33$MgSO_4$, 10-5 % Methylene Blue) in petri dishes (35x10 mm; Greiner Bio-one). Petri dishes were incubated in a thermostatically controlled incubator (Lovibond). Where necessary, larvae were illuminated with LED or Tungsten light sources (see section 2.2) or alternatively treated in constant darkness conditions (DD). Light sources were controlled by a programmable timer.

2.1.3 Feeding entrainment of adult fish and sample collection

6-12 months old Tue strain fish (only male) were used for the feeding entrainment experiments. The fish were maintained in transparent glass aquaria with a re-circulating water system under 14h:10h LD cycles. Each aquarium had 12-13 adult fish. Usually, ten aquaria were needed for each experiment. According to the feeding time, all the fish were classified into two groups: fish fed once a day at mid-light (ML, 15:00, ZT7) and fish fed once a day at mid-dark (MD, 3:00, ZT19). Fish were fed with commercial flake (Preis aquaristik) by an automatic feeding system. The amount of flake given to the fish was fixed at approximately 1% of the fish body weight per day. Before the feeding entrainment, we weighed the fish in a beaker with water and then subtracted the weight of the beaker only with the water. Then, we calibrated the food provided by the automatic feeders (Eheim). For this, we rotated the feeder several times (8-10) and then calculated the mean amount of food per rotation. The food was given from a middle

position over the top of the aquaria. When all the fish were fully adapted (after ca. one month) livers were collected at 6h intervals starting at ZT0 (zeitgeber time 0, the time of lights on). 5 fish were sacrificed per time point per group. The fish were anesthetized by rapid cooling with ice slush, then tissues were dissected out and frozen immediately in liquid nitrogen and stored at -80°C. The sample collection in darkness was performed under dim red light.

2.1.4 Feeding of zebrafish larvae with *Paramecia*

In order to observe feeding behaviour an automated, high-throughput robotic imaging platform, equipped with a Stäubli robot arm (https://www.staubli.com/en/robotics/) and MEye camera (https:// en.ids-imaging.com/) was used.
To study feeding behaviour, 7 dpf zebrafish hatchlings were used. Firstly, petri dishes containing 3 ml of E3-medium without methyl blue with a defined number of *Paramecia* were prepared. Then one hatchling was transferred into each petri dish. Videos of 30 s duration per petri-dish were acquired at different time points and the remaining number of *Paramecia* was quantified using an automated algorithm and Matlab (Probst et al., 2020).

2.2 Light sources

Cells or embryos were illuminated at a constant temperature of 25 °C using one of the following light-sources:

- Tungsten white-light source (20 μW/cm^2)
- Monochromatic red-light-emitting diodes (LED, Kopa, 657nm)
- Monochromatic far infrared light (LED, INSTAR IN-905, 850nm)

2.3 Cell culture maintenance

2.3.1 Zebrafish and cavefish cell lines

The zebrafish embryonic cell line PAC-2 (Lin et al., 1994) was propagated at 26°C in L-15 medium (Gibco BRL) supplemented with 15% Fetal Calf Serum (Gibco BRL), 100 units/ml penicillin (Gibco BRL), 100 µg/ml streptomycin (Gibco BRL) and 50 µg/ml gentamycin (Gibco BRL) in an atmospheric CO_2, non-humidified cell culture incubator. PAC-2 cells were passaged once every week by washing twice with phosphate buffered saline (PBS 1x; Gibco BRL) without calcium and magnesium and detaching from the flask surface by treatment with Trypsin-EDTA (Gibco BRL). Subsequently, the cells were seeded in fresh culture flasks (Greiner Bio-one) at a dilution ratio of 1:4. The cavefish cell line CF-1, isolated from

regenerating caudal fins of adult *Phreatichthys andruzzii* was cultured under equivalent conditions as the zebrafish cell lines (Cavallari et al., 2011). However, these cavefish cell lines were cultured in medium containing 20% Fetal Calf Serum. CF-1 cells were passaged once every two weeks with a dilution ratio of 1:2.

2.3.2 Cell counting and transfection

Dissociated cells were re-suspended in cell culture medium. 10 µl of the cell suspension were placed in a Neubauer counting chamber. The total number of cells in 4 squares was counted. The average of counted cells divided by 4, gives the number x 10^4 of cells present in 1 ml of medium.
$5x10^4$ cells were seeded into each well of a 96-well fluoro-assay-plate (Perkin Elmer 6005680). The plate was then incubated overnight at 25°C to ensure attachment of the cells to the surface of the well. On the following day, the cell culture medium was replaced with serum free L-15 medium and transfected with promoter-luciferase-reporter vectors using Fugene HDTM transfection reagent (Roche Diagnostics) according to the manufacturer's protocol.

2.4 Molecular biology experiments

2.4.1 RNA extraction

Total RNA was extracted from a confluent monolayer of cells by lysing in TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. The samples were homogenized and frozen at -80 °C for at least 24 hours. Then the samples were dissociated by repeated passage through a 1 ml syringe with a 25G needle. Addition of 100 µl chloroform, vigorous shaking and subsequent centrifugation for 15 minutes at 12000 rpm and 4°C lead to phase separation under conditions where RNA remained water-soluble and proteins or DNA were partitioned in the lower, organic phase or at its interface. Total RNA from the aqueous phase was recovered by isopropanol precipitation followed by centrifugation for 15 min at 12000 rpm and 4°C. Thereafter, the pellets were washed with 80% Ethanol by centrifugation for 5 min at 12000 rpm, 4°C. Pellets were resuspended in 30 µl of water and the concentration of the RNA was measured using Nano Drop.

2.4.2 Reverse transcription

For RNA reverse transcription, 1000 ng of total RNA were used. The appropriate amount of RNA was diluted in H_20 in a volume of 7 µl. 1 µl of RQ1 DNase buffer 10x (Promega), 1 µl of DNase (Promega, 1u/1µl) and 1 µl of RNase inhibitor (Promega ,40U/µl) were added. The samples were incubated for 30 minutes at 37 °C. After addition of 1 µl of DNase Stop solution (Promega), the samples were incubated for 10 minutes at 65 °C. Then, after addition of 1 µl of Random primers (Sigma Aldrich 200ng/µl) and incubation for 5 minutes at 70 °C a cDNA synthesis mix was added composed of 3 µl H_2O, 4 µl M-MLV RT buffer 5x (Thermo Scientific), 2 µl of 10 mM dNTPs (Sigma Aldrich) and 1 µl of M-MLV reverse transcriptase (Thermo Scientific 200U/µl). The samples were incubated with the cDNA synthesis mix for 10 minutes at 25 °C, followed by 60 minutes at 42 °C and 10 minutes at 70 °C. Finally, the cDNAs were diluted 1:10 with distilled sterile water and stored at -20°C until use.
A control PCR for the quality of the cDNA synthesis was performed by testing *β-actin* levels using 2 µl of the cDNA in 10 µl reaction with 0,125 µl GoldTaq polymerase (Promega), 0,25 µl of dNTP (10 mM) and 0,5 µl of *zf β-actin* forward and reverse primers (10 µM) (see table 3 for primers sequences).

2.4.3 Real-time PCR

Quantitative PCR (qPCR) was performed with the cDNA synthesized as described above. 3 µl of a 1:10 diluted cDNA was pipetted into each well of a 96 well plate together with 10,5 µl SYBRgreen-Primer-MasterMix (Promega). qPCR was performed in a StepOnePlus Real-Time RT-PCR System with the followed PCR conditions: 15 min at 95°C, then 40 cycles of 15 sec at 95°C, 30 sec at 60°C. The relative RNA level was calculated by the $2^{-\Delta\Delta CT}$ method (CT standing for the cycle number at which the signal reaches the threshold of detection). The results obtained were subsequently standardized using the relative mRNA expression of *zf β-actin*

Table 1: qRT-PCR primers sequences

Gene:	**Forward oligo**	**Reverse oligo**
zf β -actin	F: GCCTGACGGACAGGTCAT	R: ACCGCAAGATTCCATACCC
zf per1b	F: CCGTCAGTTTCGCTTTTCTC	R: ATGTGCAGGCTGTAGATCCC
zf per2	F: CTTCACCACACCATACAGG	R: GTCTGACGGGGACGAGTCT
zf cry1a	F: CAAACACTGCAGCAAAAACC	R: TCCGCTGTGTGTACATCCTC
zf clock1	F: CTGGAGGATCAGCTGGGTAG	R: CACACACAGGCACAGACACA
zf cry2a	F: TGCCAGTGGTAACAGGAGTG	R: AAATCTACTGTTGTGGCCCG
zf p21	F: TGACATCAGCGGGTTTACAG	R: TTCTGCTGCTTTTCCTGACA
zf *cyclinA2*	F: TAGATTGCGATCCCTTCCTC	R: CCTGTTGAGCGTGTTGAGAA
zf nampta	F: GTGTTCAGGAACGGCAAGAT	R: GGACAGAAACCGTGTGTCCT
zf sirt1	F: GCACCGAACCGTTATGTTTT	R: GTCCGTTTCCTTATCCGTCA
zf Slc38a2	F: CCATCTGCTGCTCAAAACGG	R: ACTCGCCATAGCTCCAAAGT
zf mTORC1	F: TTATCGTGCTGGTCCGAGCT	R: AAGTGGGCCCTTATCGCTGT
zf m*ap1lc3b*	F: AATGTGACGATTGGACACGAGT	R: AGTACAACAGCTCACGGTTATGC
zf asns	F: AGGAGCACATCGAGTCTGAG	R: CTTGGCCAGGGTAATGCTTC

2.4.4 BrdU labeling of larvae

Larvae were incubated for 15 min in E3 buffer with a final concentration of 10mM BrdU. Larvae were fixed in 4% formaldehyde for a few hours, washed with 50% methanol (MetOH)/PBST (1xPBS- 0.2% Triton) and incubated in 100% MetOH overnight at -20°C. After 1 wash in 50% MetOH/PBST and 2 washes in PBST they were incubated in 4M HCl for 20 min Subsequently samples were washed in PBST several times and incubated in 1%BSA, 0.4% Triton, 1xPBS for at least 1 hour at RT. Larvae were labeled with the primary mouse a-BrdU antibody (1:50 dilution-ICN; clone II5B; Cappel 11200) followed by the secondary horse α-mouse alkaline phosphatase-conjugated (AP) antibody (1:500, Vector Laboratories). After washing in 100mM Tris pH 9.5, 50mM $MgCl_2$, 100mM NaCl, 0.1% Tween, larvae were stained with a mixture of Nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP, Roche). Samples were then washed several times in PBST and fixed in 4% PFA for 20 min. Samples were stored in PBST at 4°C. Images were taken using a Zeiss STEMI SV11 microscope and an AxioVision camera and software (Zeiss).

2.4.5 Cortisol ELISA

Twenty larvae for each sample time point were raised in petri dishes (35x10mm) with 5 ml of E3 medium in DD under constant temperature of 26°C. At day 7 in one group the medium was changed while in the second group no change of fish water occurred. At each sampling time point, larvae were rapidly transferred to 2-ml Eppendorf tubes, the medium was removed, and the larvae were snap frozen in liquid nitrogen and stored a -80°C until further processing.
Larvae were homogenized on ice with a microgrinder (Eppendorf, Hamburg, Germany), then 500 µl of cold ethanol was added and centrifuged for 10 min at 3,000 rpm at 4°C. The supernatant was transferred to a new Eppendorf tube and evaporated in a SpeedVac. The resulting pellet was suspended in 20 µl of standard A buffer of the IBL cortisol ELISA KIT (RE52611; IBL International GmbH) and measured following the instructions provided by the supplier. The plate was read by a luminescence plate reader (Spectra Max iD3, Molecular Devices), and the raw data were analysed using the OriginLab software (Origin 2020b).

2.4.6 Luciferase reporter constructs

6xD-box$_{cry1a}$-Luc

This reporter construct contains six copies of the D-box sequence (5'–AAGTTATACAACAGC –3') from the zebrafish *cry1a* gene promoter (position -154 bp relative to the ATG) cloned into the minimal promoter, luciferase reporter vector pTal-Luc (Addgene).

4xE-box$_{per4}$-Luc

This reporter construct contains four copies of the E-box element (5′–CACGTG–3) from the *zfper1b* promoter (Vallone et al., 2004) (position -7 bp canonical E-box) cloned into a minimal promoter, luciferase reporter vector pLucMCS (Stratagene).

zf *nampta*-Luc

The zf *nampta*-Luc reporter plasmid containing a 1492 bp *nampta* promoter fragment was constructed as previously described (Vallone et al., 2004). First, a promoter fragment from position -161 to -1651 bp of *nampta* was amplified from PAC-2 cell genomic DNA by PCR using the following primers: FW: ATTGCAGCATAGCACACGTA,
RV: TGACTGAGACGTCATGGCAA. The promoter fragment was initially cloned into the vector pGEM-T easy (Promega), and then subcloned into the vector pGL3 basic (Promega) using specific primers incorporating a 5' SacI (5'- CAGCAGAGCTCACGTACAATAAG–3') and a 3' HindIII restriction site (5'-GCCGCGAATTCACTAAAGCTTTGACT–3')

zf *asns*-Luc

The zf *asns*-Luc plasmid incorporates a 1,199 kb fragment of the zebrafish *asns* promoter (position -1,703 to -2,850 kb) that was amplified from PAC-2 cell genomic DNA by PCR using the following primers: FW: AACAAGTTTTCTCAAACCCATACCT, RV: TACTTGTTTAGTCGGAGGCTGT. The promoter fragment was cloned into the vector pGEM-T easy (Promega) initially, and then subcloned into the vector pGL3 basic (Promega) using specific primers incorporating a
5' KpnI (5'-GATTAACAAGGTACCTCAAACCCATA–3') and a 3' HindIII restriction site (5'–CTAGTGATAAGCTTGTTTAGTCGGA–3').

3xAARE-Luc

The AARE reporter construct contains three multimerized copies of a consensus AARE sequence (5'–GTTTCATCA-3') from the zebrafish *asns* promoter (position -1,857 kb relative to the ATG) cloned into the minimal promoter, luciferase reporter vector pT2Luci:MCS (Weger et al., 2012).

2.4.7 *In vivo* luciferase assay

24 h after transfection, the culture medium was replaced with 200 µl fresh culture medium containing 0.5 mM beetle luciferin potassium salt solution (Promega). Plates were subsequently sealed using an adhesive "Top Seal" sealing sheet (Perkin Elmer) and transferred into an EnVision multilabel counter (Perkin Elmer) or a Topcount NXT automatic scintillation counter (2-detector model) (Perkin Elmer) and exposed to LD cycles (12:12) or maintained in constant darkness conditions, depending on the experiment. The counter was located in a thermostatically controlled dark room and illuminated using a LED light source connected to a programmable timer. Bioluminescence was measured and expressed as the frequency of photon emission (cps) counted during 4-6 sec/well at intervals of 30-60 min. Luciferase assay data were analysed and graphically plotted using the Microsoft Excel macro software I&A (S. Kay, Scripps Research Institute).

2.4.8 Detection of glucocorticoid signaling in *Tg(GRE:Luc)* larvae

The luciferase-expressing transgenic zebrafish line *Tg(GRE:Luc)* carries a transgenic construct that expresses a luciferase reporter gene under the control of four concatenated GREs and was used to analyse stress levels after handling. Fertilized eggs were collected within 2 hours and transferred into petri dishes (145x20 mm) containing 25 ml of E3 media. The embryos were

incubated in DD under constant temperature (26°C). The following day, embryos were transferred into 96-well plates and incubated in E3-luciferin medium (0.5 mM) in DD. The embryos were divided into two groups. In the first group the medium was change at regular time points, while in the second group no regular change of fish water occurred. Under exposure to stress the endogenous GRs are activated and ligand-receptor binding to the 4xGREs is induced. Subsequently, reporter gene transcription and expression of luciferase is induced. Luciferase catalyzes the oxidation of luciferin (supplemented in the medium) that leads to the emission of a bioluminescent signal. Luciferase reporter activity was measured on a Topcount NXT automatic scintillation counter. Luciferase assay data were analysed and graphically plotted using the Microsoft Excel macro software I&A (S. Kay, Scripps Research Institute).

2.5 Western blotting

2.5.1 Preparation of protein extracts from fish cells

Whole liver protein extracts were prepared from zebrafish liver by direct addition of 200 µl of 1x Laemmli buffer (6% SDS, 20% glycerol, 125mM Tris pH6.8, 0.01% bromophenol blue, 100mM DTT) including a cocktail of phosphatase and protease inhibitors (Sigma Aldrich), lysis *in situ* at room temperature and then transfer into 1,5 ml eppendorf tubes. Lysate samples were then boiled for 5 min to denature the proteins before storage at –20°C.

2.5.2 SDS-PAGE, transfer and immunodetection

Samples were loaded on a 15 % SDS-PAGE gel for electrophoresis. The run was performed at 80 V for circa 120 min. After the proteins were transferred from the polyacrylamide gel onto an Immobilon-P-membrane (Millipore) using a wet electro blotting system. Transferred membranes were then blocked by incubation for one hour at room temperature in 5 % BSA (10 g Bovine Serum Albumin, 200 ml TBS (1x) + Tween 20 (0,1%)) buffer to prevent non-specific binding of antibodies. Primary antibodies (Table 2) were used at a dilution of 1:1000 in 5 % BSA buffer and incubated with the membrane in a plastic bag at 4˚C overnight. After three times washing at room temperature for 10 minutes in TBS-T (TBS + 0,1% Tween), membranes were incubated at room temperature for one hour with secondary antibodies (Table 2). We used HRP conjugated with either anti-rabbit or anti-mouse antibodies at a dilution of 1:7500 in 5% BSA buffer. Binding of each antibody was visualized using the ECL detection system (Biorad). All images were acquired and analysed using the ChemiDoc Touch imaging system. Images were then quantified using ImageJ software.

Table 2: List of antibodies used

Primary Antibody	Details
Anti-β-actin (Sigma A5441)	Monoclonal Anti β-actin clone AC-15
LC3B (ThermoFisher Scientific, PA1-46286)	Polyclonal, N-terminal region of the human LC3, isoform B protein.1:1000
Secondary Antibody	**Details**
Rabbit Polyclonal, Goat, HRP	Cell Signaling (7074S)
α- Mouse Monoclonal,Goat,HRP	Cell Signaling (7076S)

2.6 UPLC-MS/MS Assay

2.6.1 Homogenization of zebrafish liver

According to different experimental designs, time series of adult zebrafish livers were collected and frozen quickly in liquid nitrogen and stored at -80°C. For the extraction of AAs zebrafish livers were then homogenized. The samples were taken from -80°C, put on ice and immediately 0,3 ml H_2O were added to each tube containing one liver. The samples were mixed on a vortex mixer (Vortex-Genie 2, Scientific industries, Inc.) for 1-2 min, sonicated: 3 x 20 s, using highest power (*Diagenode's Bioruptor®*). Samples were centrifuged 15 minutes at 12.000 x g at 4°C and the supernatants were transferred to new tubes. The samples were stored at -20°C until derivatization.

2.6.2 Preparation of cultured cells for UPLC-MS/MS

$2x10^6$ PAC-2, *Δclock1* cells and $6x10^6$ CF-1 cells were seeded petri dishes (145x20mm). The petri dishes were wrapped in aluminium foil and after 2 days were either exposed to LD cycles or incubated further in DD. At day 7 the sampling was started. First, the culture medium was discarded and the cells were washed once with ice cold 10 ml 1×PBS. Then the cells were scraped and cell suspension were removed to 2 ml Eppendorf tubes by pipetting. 400µl of 80%MetOH were added, vortexed 20 s and sonicated: 3 x 20 s, using highest power. 15 minutes at 12.000 x g at 4°C and the supernatants were transferred into new tubes. The samples were stored at -20°C until derivatization.

2.6.3 Derivatization

The liver or cell homogenates were vortexed for 20 s and 50 µl of liver or 5 µl of cell supernatant extract was transferred into new 1.5 ml screw-thread brown glass vials (art. no.: VT1101211, Dr. R. Forche Chromatographie) containing 300 µl MetOH and 2µl labelled amino acid standard Mix (2.5 mmol/l) (Set A, Cambridge isotope laboratories Inc.). Vials were closed with silicone/ PTFE caps (art. no.: CT11S3015, Dr. R. Forche Chromatographie), vortexed for 20 s and dried under a liquid nitrogen stream at 45°C on an evaporator (Vapotherm basis mobil II, Barkey GmbH & Co. KG). Then 300µl butanol:acetylchloride (4:1) was added to each vial and incubated 20min at 65°C with centrifugation at 450 rpm. Then the reagent was blown with nitrogen to dryness and the residue taken up in 500 µl of an aqueous solution containing 0,1% trifluoroacetic acid (TFA; CAS 76-05-1, Merck).

2.6.4 UPLC-MS/MS

Detection and quantification of amino acids in zebrafish liver and cultured cells were conducted by Ultra-performance liquid chromatography tandem mass spectrometry UPLC-MS/MS. Chromatographic separation was performed by an ExionLC™ AD system (AB Sciex). From each sample, a volume of 1 µl was injected and fractionated by a Kinetex 2,6µM XB-C18 100A (100 x 2,1 mm) column (Phenomenex). In direct analyses, the mobile phase consisted of solution A (acetonitrile (# 83.640.320, VWR International):water: TFA, *v*: *v*: *v* = 95: 5: 0.1) and solution B (0,1% TFA in water). The gradient condition 0 - 0.40 min, 90% B; 0.40 – 1.9 min, 72% B; 1.9 – 2.60 min; 72% B; 2.60 – 3.3 min, 20 - 60% B; 3.3 – 3.6 min, 60% B; 3.6 – 3.8 min, 90% B; 3.8 – 5 min, 90% B was applied for the elution of amino acids from the column. The flow rate was 0.70 ml/min, and the column was maintained at 45°C using a column oven. Mass spectrometry was conducted using an API 4000™ tandem mass spectrometer with a TurboIonSpray source (AB Sciex) in the positive mode. Mass detection was achieved operating in the multiple reaction monitoring (MRM) mode analyzing precursor ion/product ion mass transitions. The optimized MS parameters and the MRM mass transitions for each compound are given in the appendix as Supplementary Figure A,B. Data acquisition and analysis were all performed with Analyst 1.6.3 software (AB SCIEX).

2.7 Statistical Analysis

Data were analyzed by T-Test using Excel software, one-way or two-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison tests with GraphPad Prism 4.0 (http://www.graphpad.com). All the results were expressed as means ± SD of three independent experiments. In the statistical tests $p<0.05$ was considered statistically significant. In each figure, $p<0.05$, $p<0.001$ and $p<0.0001$ are represented by *, ** and *** respectively.

3. Experimental Aims

A major challenge is to unravel the molecular pathways through which the circadian clock is controlled by light and food and how it in turn regulates metabolism. My research project attempts to extend the existing comparative studies from a previous PhD student in our lab, Ying Li, that explored the existence of genetically distinct feeding and light-entrainable circadian clocks in fish cells. Within this context, my proposed project attempted to use zebrafish as a model to tackle the following major questions:

When can non-photic zeitgebers entrain the clock during early development?
The initial objective of the present work was to investigate how light and feeding-regulated clocks first appear during early development. Specifically, my aim was to define at which stage during zebrafish development regular feeding is able to initiate circadian rhythmicity at the whole animal level. However, based on the results of our pilot experiments, we realized that this was technically problematic. We thereby chose to adjust our aims to specifically explore the role of regular stress in the establishment of circadian clock function during early larval development by studying clock gene expression patterns and the timing of cell cycle.

How are feeding- and light-regulated clocks differentially coupled with metabolism?
Based on preliminary data from LI Ying obtained by exposing zebrafish to combinations of lighting and feeding regimes, we aimed to perform a detailed Mass Spectrometry (MS) analysis to confirm the original NMR results, which showed that metabolites differentially cycle according to the timing of light dark and feeding cycles in the zebrafish liver. A major goal of our study was to use a combination of *in vivo* and *in vitro* cell culture models to identify key gene regulatory networks that differentially couple food and light regulated circadian clocks with amino acid metabolism.

4. Results

4.1 Ontogenetic development of the zebrafish circadian clock

The following section focuses on the question: whether feeding plays a role in clock entrainment during early zebrafish development? This aspect of circadian clock research is relatively poorly understood and to tackle this question, firstly, we decided to analyse feeding behaviour under different lighting conditions.

4.1.1 Feeding behavior

Environmental signals, such as light, temperature and feeding time play an important role in regulating zebrafish behavior. Periods of resting and activity exhibit circadian rhythms, which are regulated by the light-dark cycle. Feeding related cues, including feeding time and diet composition, also act as a prevailing timing cue for circadian synchronization and thereby direct circadian locomotor activity. Thereby, zebrafish exhibit food anticipatory activity (FAA), an increase in locomotor activity occurring just in anticipation of a regular feeding time. In zebrafish larvae, visual guided prey capture behavior is already observed at five days post fertilization upon feeding on the unicellular protozoan *Paramecium*, as soon as active swimming begins. However, it is still not known how light and feeding time regulate the development of FAA and how it affects the ontogenesis of rhythmic clock gene expression during early zebrafish development.

4.1.1.1 Feeding behavior in constant darkness and under exposure to light

In order to investigate the emergence of FAA as the larvae mature in constant darkness (DD), in collaboration with Dr. Ravindra Peravali at the Screening Center IBCS-BIP, KIT, we employed a robotic imaging system. Zebrafish eggs were divided into two groups immediately after fertilization. One group was exposed to a light-dark-cycle (LD) and the second group was maintained in constant darkness (DD). The feeding behavior was examined on the seventh day post-fertilization (dpf). The hatchlings were provided with a defined number of *Paramecia* and subsequently recorded for about 28 hours. The remaining number of *Paramecia* was calculated after each period of imaging. The rate at which zebrafish larvae consume *Paramecia* in a given time is used as a measure to quantify feeding behavior. The experimental scheme is visualized in Figure 15.

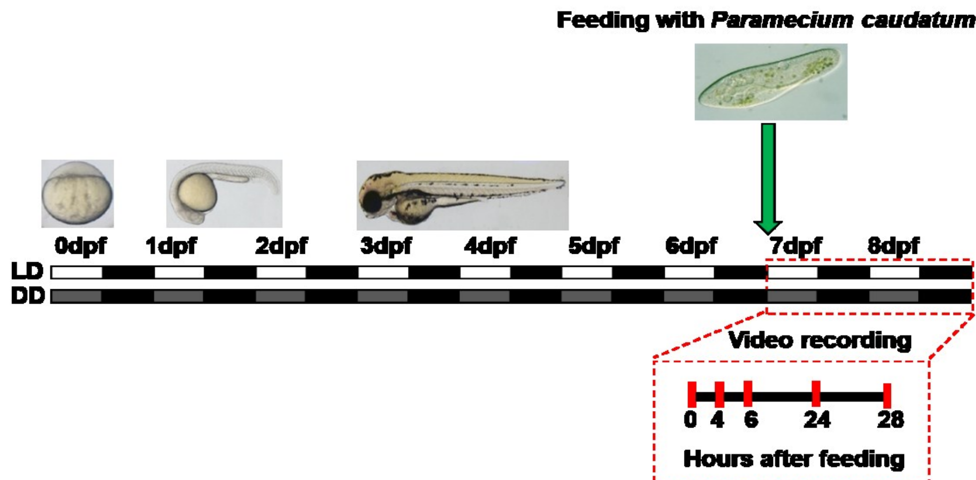


Figure 15: Overview of the experimental design. One group of zebrafish embryos was raised under a light-dark (12:12 hours) (LD) cycle and the second group in constant darkness (DD). At day 7, larvae were fed with a defined number of *Paramecia*. The imaging of the petri dishes with *Paramecia* and hatchlings was performed at different time points and the remaining number of *Paramecia* was calculated. dpf-days post fertilisation.

We observed significant differences in the consumption rate of *Paramecia* between the LD and DD groups (P<0,001). Under DD, reduced feeding activity was already evident 4 hours after addition of *Paramecia* compared with the LD group (P<0,001). In the LD group, almost all the *Paramecia* were consumed within 28 hours (Figure 16), whereas in the DD group just 20% of the *Paramecia* were consumed within the same period (Figure 16). These results suggest that visible light plays an important role in supporting feeding behavior in zebrafish larvae. However, our basic question was to investigate whether regular feeding time is able to entrain the clock from the very onset of feeding behaviour in DD and how this compares with the emergence of light entrainable clock function. For this reason, we chose to test whether illumination under “dark-room” lighting conditions (a commonly used experimental strategy in the circadian rhythm field to practically facilitate handling animals in constant darkness conditions), could be used as a substitute for constant darkness and thereby maintain normal levels of feeding behaviour. Therefore, we next wished to explore whether hatchlings exposed to dim red or infrared light, were still able to detect and to consume *Paramecia*.

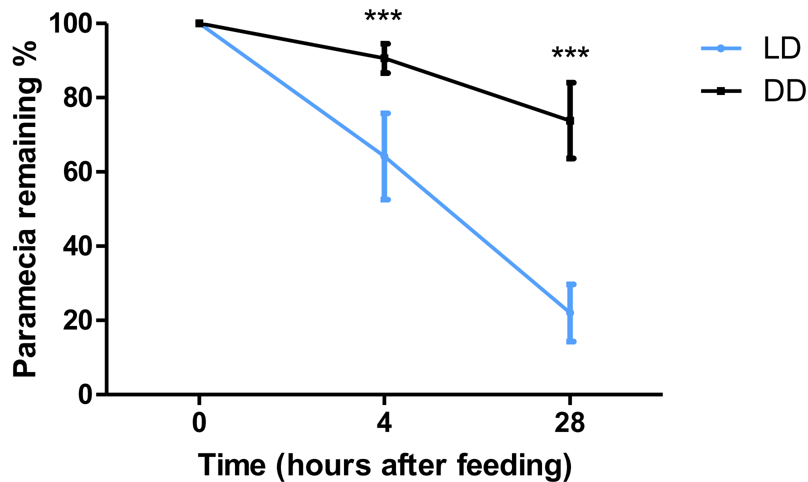


Figure 16: Comparison of feeding rate between LD and DD groups. 7 dpf hatchlings consumed almost all *Paramecia* within 28 hours under white light-dark cycle (LDW) exposure. In constant darkness (DD) around 20% of *Paramecia* were consumed. Mean of the remaining number of Paramecia expressed in % (n=5) ± SD is plotted on the y-axis. Time after the addition of *Paramecia* to the petri dish is plotted on the x-axis. Statistical analysis was performed using 2 way ANOVA (Bonferroni post test), where asterisks (*) indicate statistical significance; * P<0,05; **: P<0,01; *** P<0,001.

4.1.1.2 Feeding behavior under exposure to red and infrared light.

Typically, in the circadian clock field, samples adapted to DD conditions are collected under dim red light, supposing that it has minimal impact on free-running circadian clock function. Furthermore, some studies have shown that infrared (IR) light does not affect the circadian clock system and for this reason it is used for recording locomotor activity in darkness (Dekens et al., 2017). Based on this background we decided to explore whether zebrafish larvae are able to detect and consume *Paramecia* under exposure to light sources of dim red light at 657 nm or far infrared (IR) light at 850 nm wavelength.

Zebrafish embryos were raised under constant dim red or constant far IR LED light sources and as in our previous experiment the feeding behavior was analyzed in 7 dpf larvae. Our data analysis showed that zebrafish larvae were able to detect and to consume *Paramecia* under constant exposure to red light (Figure 17). Surprisingly, prey capture behavior was comparable with the group which was exposed to white light-dark cycles (P>0,05) (Figure 17). In both groups almost all *Paramecia* was consumed within 27 hours. On the other hand, the consumption rate of the prey under constant exposure to far IR light was significantly lower (P<0,001) and comparable with the DD group (P>0,05) (Figure 17). Collectively, our results

are consistent with hatchlings being able to detect and capture food under dim visible red as well as white light but are unable to feed under far IR lighting conditions.

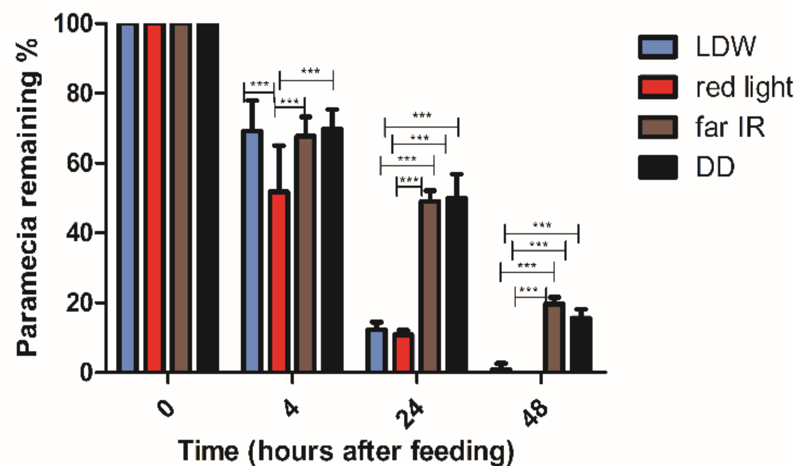


Figure 17: Comparison of feeding rate between LD, red light, far IR and DD groups. 7 dpf hatchlings consumed almost all *Paramecia* within 24 hours under white light-dark cycle (LDW) conditions and constant red light (red light) exposure. Under constant exposure to far infrared (far IR) and in constant darkness (DD), around 40% of *Paramecia* was consumed. The number of *Paramecia* in % (n=5) ± SD is plotted on the y-axis. Time after addition of the *Paramecia* to the petri dish is plotted on the x-axis. Statistical analysis was performed using 2way ANOVA (Bonferroni post test), where asterisks (*) indicate statistical significance; * P<0,05; **: P<0,01; *** P<0,001.

4.1.1.3 Effect of red and infrared light on entraining clock gene expression in zebrafish larvae

We next wished to explore the effect of exposure to red light and far IR light on circadian clock entrainment. For this reason, instead of constant illumination with red or IR light, we exposed 5 - 6 dpf old zebrafish larvae to alternating 12 hours light- 12 hours dark cycles, using either red or far IR light in comparison with white light and examined the ability of each LD cycle condition to entrain circadian rhythms of clock gene expression (Figure 18A). A control group remained in DD to compare with the effects of complete light deprivation on clock gene expression (Figure 18A). Our results revealed comparable high amplitude rhythmic expression of the light-inducible clock genes, *per2* and *cry1a* under both white and red light-dark cycles (P<0,001). Furthermore, we observed rhythmic expression of the *clock1* gene under white light (P<0,001) and under red light (P<0,01) (Figure 18B). Interestingly, higher amplitude cycling of clock-regulated *per1b* expression was observed under red light-dark cycles compared with white light-dark cycles (P<0,001). Surprisingly, we revealed that exposure to far IR entrains rhythmic expression of the clock-regulated *per1b* and *clock1* genes as well as the light-inducible

clock gene *cry1a* (P<0,001) (Figure 18B). However, there was no significant rhythm in expression of *per2* under far IR cycles (P>0,05). Consistent with previous reports, no rhythmic expression of clock genes was detected in the DD group (P>0,05) (Figure 18B). These findings reveal that dim red light has the capacity to entrain the zebrafish larval circadian clock while interestingly, far IR light differentially entrains rhythmic clock gene expression.

Figure 18: Daily expression levels of clock genes in 5 dpf zebrafish larvae under different light-dark cycle conditions. A: Schematic representation of fish entrainment and sample collection. The horizontal bars represent the lighting conditions (white light-dark cycle (LDW), red light-dark cycle (LDR), far infrared light-dark cycle (LDfIR) and constant darkness (DD)) before and during sampling; white boxes represent light, grey boxes represent subjective light and black boxes represent dark periods; the arrows represent the sampling times (0,6,12,18,24,6). B: Expression patterns of circadian core clock genes *per1b, per2, cry1a*, *clock1* under exposure to white-, red-, far IR light-dark cycles and DD sampled every 6 hours in 5 dpf larvae. Relative mRNA levels are plotted on the y-axis. Each time point on the x-axis represents the mean ± SD (n=3). Statistical analysis was performed using one and 2way ANOVA (Bonferroni post test), where asterisks (*) indicate statistical significance; * P<0,05; **: P<0,01; *** P<0,001. dpf – days postfertilization, ZT – zeitgeber time, CT – circadian time.

How does exposure to dim red or far IR light affect clock gene expression in cultured zebrafish cells? This specifically addresses the important issue of whether these wavelengths of light have the capacity to entrain the directly light sensitive peripheral clocks in fish. In order to explore this question, the zebrafish embryonic cell line (PAC-2) was exposed to the same light cycles as larvae (white-, red- and far IR- light cycles). A control group was maintained in DD. The expression of clock genes was analysed after 5 LD cycles (Figure 19A). We observed that rhythmic expression of *per1b* is synchronised by red and white light-dark cycles ($P<0,001$) (Figure 19B). However, exposure to far IR light-dark cycles led to lower amplitude rhythmic *per1b* expression ($P<0,05$) (Figure 19B). Interestingly, in contrast to the hatchlings, in PAC-2 cells exposed to red light-dark cycles a lower amplitude of rhythmic *per2* expression ($P<0,05$) but a comparable high amplitude of rhythmic *cry1a* expression was observed ($P<0,001$) (Figure 19B). Exposure to far IR light-dark cycles (Figure 19B) resulted in low amplitude rhythms of per1b ($P<0,05$) expression but no significant rhythmicity of *clock1*, *cry1a* or *per2* expression (Figure 19B). These data suggest that there are important differences in the regulation of the circadian clock machinery by dim red light and far IR light between live animals and cultured cell lines. We predict that opsins which may be sensitive to red light or particularly far IR light, which are normally expressed in the eyes or possibly the skin, may be missing or non-functional in cultured cells.

Figure 19: Daily expression levels of clock genes in PAC-2 cells under different light-dark cycles. A: Schematic illustration of fish entrainment and sample collection. The horizontal bars represent the lighting conditions (LDW, LDR, LDfIR, DD) before and during sampling; white boxes represent light, grey boxes represent subjective light and black boxes represent dark periods; the arrows represent the sampling times (0,6,12,18,24,6). B: Expression patterns of circadian core clock genes *clock1, per1b, per2, cry2a* after exposure to five white-, red-, far IR cycles and DD sampled every 6 hours. Relative mRNA levels are plotted on the y-axis. Each time point on the x-axis represents the mean ± SD (n=3), where asterisks (*) indicate statistical significance; * P<0,05; **: P<0,01; *** P<0,001. Statistical analysis was performed using one and 2way ANOVA (Bonferroni post test). LD – light dark cycles, DD – constant darkness, LDW – white light, LDR – red light, LDfIR – far infrared light, ZT – zeitgeber time, CT – circadian time.

4.1.1.4 When does a feeding entrained clock first mature during early development?

The initial major objective of this project was to determine the onset of feeding regulated clock gene expression. In order to investigate this question, it was essential to study the feeding behaviour in the absence of light- and temperature cycles, because previous studies have shown that exposure either to a light-dark cycle or temperature cycles is essential for the ontogeny of daily rhythms of clock gene expression during larval development (Dekens and Whitmore, 2008; Di Rosa et al., 2015; Lahiri et al., 2005). This means that the studies should be done in constant darkness under constant temperature. However, we observed that the consumption rate of *Paramecia* in constant darkness is 4 times lower than under exposure to white light. While

exposure to dim red light increased the consumption rate of *Paramecia*, unfortunately we observed that red light can efficiently entrain rhythmic clock gene expression. In addition, exposure to far IR light did not improve the consumption rate of *Paramecia*. Therefore, we decided to test the effect of regular feeding time on clock maturation in larvae exposed to constant darkness with the assumption that the limited feeding activity under these conditions would be sufficient to test the effects of circadian clock ontogeny.

In order to evaluate when a feeding entrained clock first matures during early development, we examined the mRNA expression of clock genes in 8 - 10 dpf larvae, at regular time points using qRT-PCR analysis. The zebrafish eggs were collected immediately after fertilization, pooled and distributed into Petri dishes. The eggs were raised in fish water at 25°C in the absence of light (DD). After the yolk sack has been depleted (at 5dpf; (Fuiman and Webb, 1988)), the embryos were divided into 2 groups and then the exogenous feeding with *Paramecia* was initiated. One group was fed with *Paramecia* (DDF), a second group received just water (DDM), as a control for whether the handling of the larvae might have an impact on the development of circadian rhythmicity of clock genes. The experiment scheme is depicted in Figure 20.

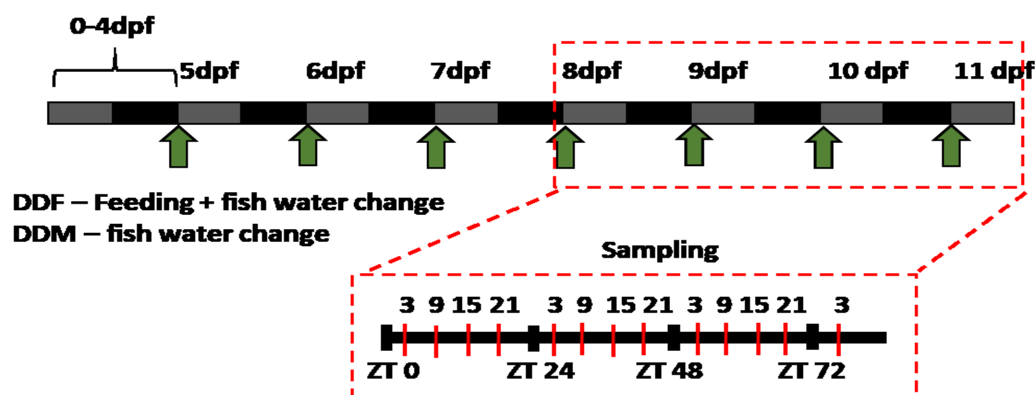


Figure 20: Overview of experimental design. The eggs were raised in fish water in the absence of light (DD). At day, 5 larvae were divided into two groups: the DDF group was fed with *Paramecia* while the DDM group did not received food. 3 hours after feeding, the fish water was changed in both groups. At day 8 the sampling was started. Samples were taken every 6 hours, RNA extracted and then gene expression was analysed by qRT-PCR.

The analysis of clock gene expression in 8-10 dpf larvae revealed that the expression levels of the clock gene *per1b* were rhythmic in both DDF and DDM groups, with a peak during the late subjective night (ZT21, P<0,001, Figure 21). Surprisingly, there was no significant difference detectable between the two groups (P>0,05). The expression of other clock gene components of the core clock feedback loop, *per2* and *cry2a*, also showed circadian rhythmicity (P<0,001, Figure 21) in their expression in both groups. Again, no significant differences between the DDF and DDM groups were observed (P>0,05). However, the expression of *clock1* was arrhythmic (Figure 21) in both groups (P>0,05).

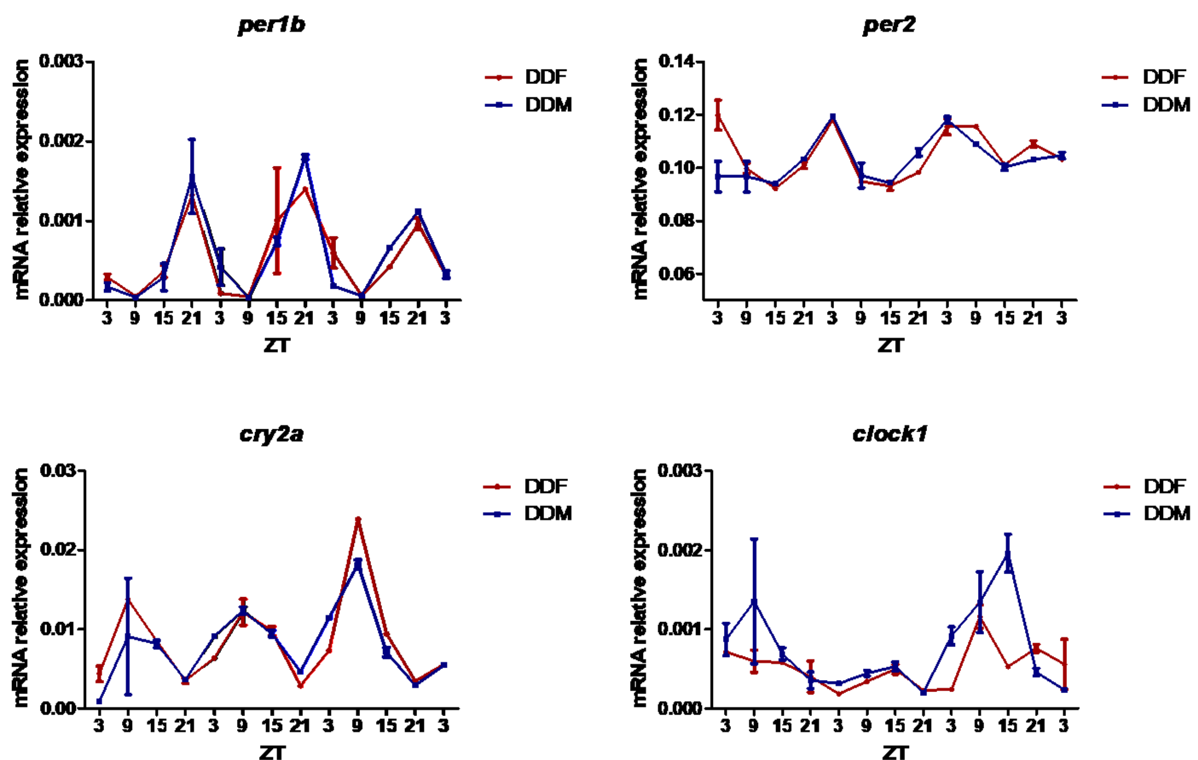


Figure 21: Daily expression levels of clock genes in 8- 10 dpf zebrafish larvae. Expression patterns of the circadian core clock genes *clock1, per1b, per2, cry2a* in the DDF (red line) and DDM (blue line) groups of 8 – 10 dpf hatchlings raised in constant darkness. Relative mRNA levels are plotted on the y-axis. Each time point on the x-axis represents the mean ± SD (n=3). Statistical analysis was performed using one and 2way ANOVA (Bonferroni post test). Prior to the water change, the DDF fish group was fed with *Paramecium caudatum* while the DDM- fish did not receive food. ZT represents zeitgeber time.

These results raise the question, what could be the signal which entrains the rhythmic expression of clock genes? Given that zebrafish embryos were raised in constant darkness under constant temperature, the regular fish water change in both the DDF and DDM groups could serve as a possible Zeitgeber entraining clock gene expression. Due to these observations we decided to change the focus of this project, in order to explore the ontogeny of the circadian

clock in our particular experimental system instead of the original goal, to focus on studying the effects of food and feeding.

4.1.2.1 Stress induction by the change of fish water

We initially hypothesised, that the action of changing the fish water, may represents an important source of stress to the young larvae and so decided to search for evidence of enhanced stress following the water change step. In order to explore in more detail whether the handling of larvae induces stress, we analysed endogenous cortisol levels after the change of fish water at the whole animal level using a competitive Cortisol ELISA Assay in a time course for a period of 18 hours. Cortisol is a stress hormone released by the inter-renal cells and is widely used as a biomarker in stress research. In this study zebrafish embryos were raised in DD under constant temperature. At day seven, the larvae were divided into two groups: in the first group the fish water was changed, while in the second group it was left unchanged. The quantified results showed a rapid increase in cortisol levels with a peak around 5 min after handling followed by a return to basal levels after 30 min in the first group (Figure 22). In the control group, where the fish water was not changed, no rapid increase in cortisol levels were observed (Figure 22).

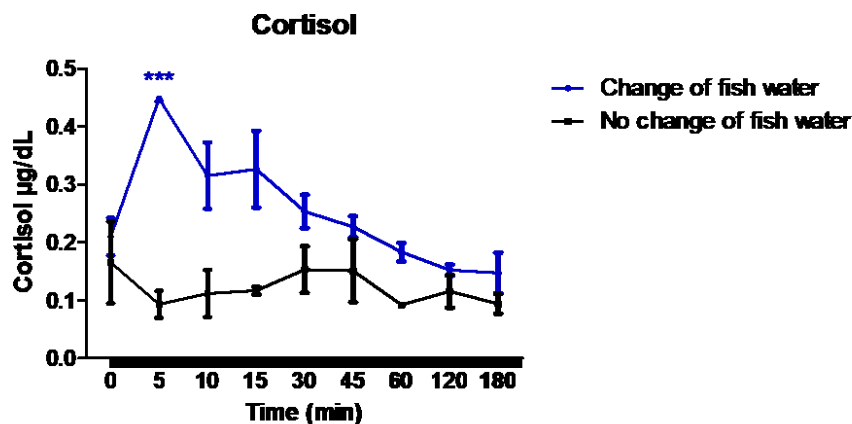


Figure 22: Cortisol ELISA Assay in 7 dpf zebrafish larvae. The analysis was performed with zebrafish larvae raised in DD. At day 7 in the 1st group, fish water was changed (blue line) while in the second group (black line) it was not changed. The sampling was started immediately. Changes in cortisol levels were observed over 18 hours. Each time point on the x-axis represents the mean ± SD (n=3), where asterisks (*) indicate statistical significance; * P<0,05; **: P<0,01; *** P<0,001. Statistical analysis was performed using one and 2way ANOVA (Bonferroni post test).

This evidence suggests that changes of fish water activate stress signalling in zebrafish larvae.

4.1.2.2 The change of fish water activates glucocorticoid signalling

Some studies have indicated that the circadian clock is affected by stress. It has been shown that stress induces the production of glucocorticoids (GCs), including cortisol. GCs bind to the glucocorticoid receptor (GR) and after ligand binding, GR migrates to the nucleus and binds to glucocorticoid response elements (GRE) thereby regulating the transcription of specific target genes, including *per1b* (Dickmeis et al., 2013). In collaboration with Dr. T. Dickmeis (IBCS-BIP, KIT) we performed a Glucocorticoid Responsive *In vivo* Zebrafish Luciferase activitY (GRIZLY) assay. This assay is based on a transgenic zebrafish line expressing a firefly luciferase reporter gene under the transcriptional control of a minimal TATA-box promotor (Pmin) and four concatenated GREs (Figure 23) (Weger et al., 2012). By activation of reporter gene expression, luciferase oxidizes luciferin (added to the fish water) leading to the production of a bioluminescent signal. This signal is measured by a bioluminescence reader.

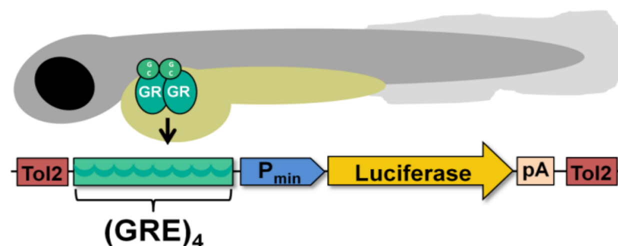


Figure 23: Transgenic zebrafish line Tg(GRE:Luc) for GRIZLY assay. A luciferase reporter gene is under the transcriptional control of a minimal TATA-box promoter (P_{min}) and 4 GREs $(GRE)_4$**.** It also contains a polyadenylation signal sequence (pA). The construct also has Tol2 transposase sites, which mediate integration of the construct into the zebrafish genome. During stress, glucocorticoids bind to GR and this ligand-receptor complex translocates to the nucleus where it activates a luciferase reporter gene through binding to multimerized GRE elements $(GRE)_4$, leading to induced expression of luciferase, a resulting increase oxidation of luciferin and emission of a bioluminescent signal. The bioluminescence signal is in turn measured by a bioluminescence reader. (Weger, B.D., et al., 2013)

To explore whether the fish water changes have an impact on the activity of the GRE-mediated reporter gene expression, we divided transgenic zebrafish eggs into 2 groups: in the first group, the fish water was changed every day at the same time point (DDM), while in the second group, no regular changes of fish water occurred (DDK). The eggs were placed individually in 96-well microtiter (mct) plates and monitored on a luminescence plate reader in DD. The data analysis showed that in the first group bioluminescence started to significantly increase about 1 h after the change of fish water (Figure 24), whereas in the second group no significant increase in

bioluminescence were observed (Figure 24). These results again confirm our hypothesis that regular change of fish water activates the stress signalling pathway in zebrafish larvae.

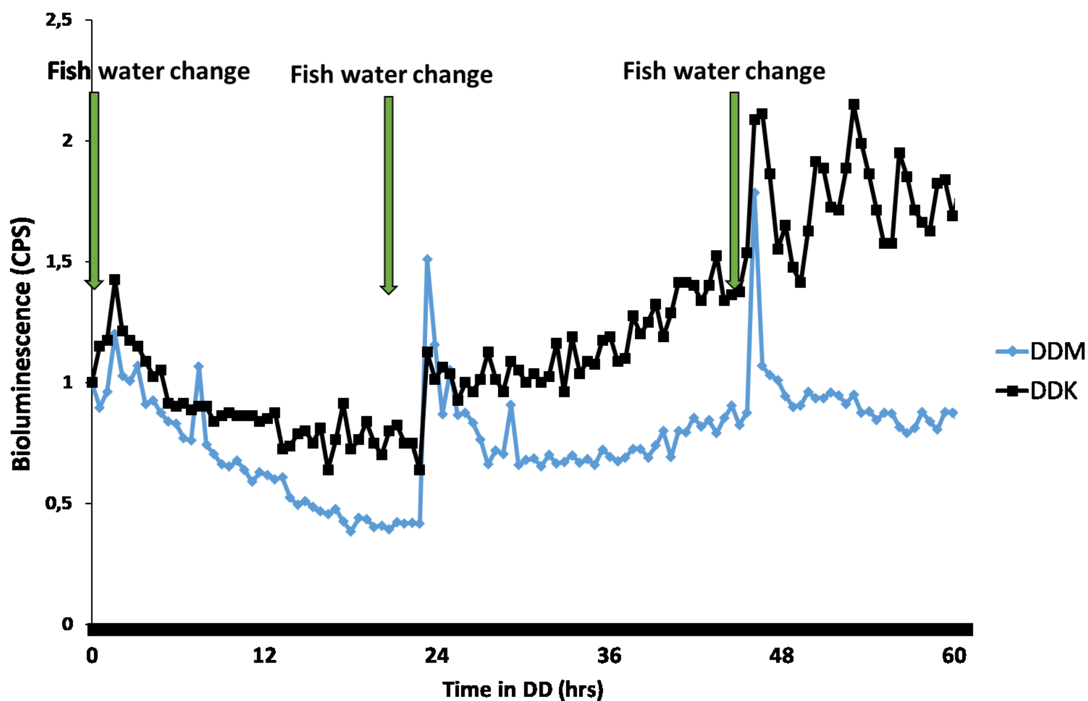


Figure 24: Activation of the glucocorticoid signalling pathway by stress in 2-4 dpf larvae of the Tg(GRE:Luc) transgenic zebrafish line. Zebrafish eggs were divided into two groups: in the first group (blue line) fish water was changed every day at the same time (green arrows), in the second group (black line) the fish water was not changed. Fold induction in bioluminescence relative to the first measurement in the **Tg(GRE:Luc)** larvae is plotted on the y-axis. The times are plotted on the x-axis.

4.1.2.3 Clock entrainment by stress in developing zebrafish larvae

Next we wondered at which stage during zebrafish development, regular stress is able to initiate circadian rhythmicity at the whole animal level. Are the patterns of stress-regulated clock gene expression comparable with the patterns of light-regulated clock gene expression? In order to tackle these questions, we compared the expression patterns of the circadian clock gene *per1b* between the DDM and DDK groups. The embryos were maintained in DD and at 3 dpf, total RNA was extracted from larvae collected at 6-h intervals for a total period of 36 hours (Figure 25A). The gene expression levels were then assayed by qRT-PCR analysis. Our results show that the expression of *per1b* is synchronised with the time of fish water change already at 4dpf (P<0,001) (Figure 25B). On the other hand, during exposure to a LD cycle the entrainment of *per1b* expression is observed in 3 dpf larvae (Di Rosa et al., 2015).

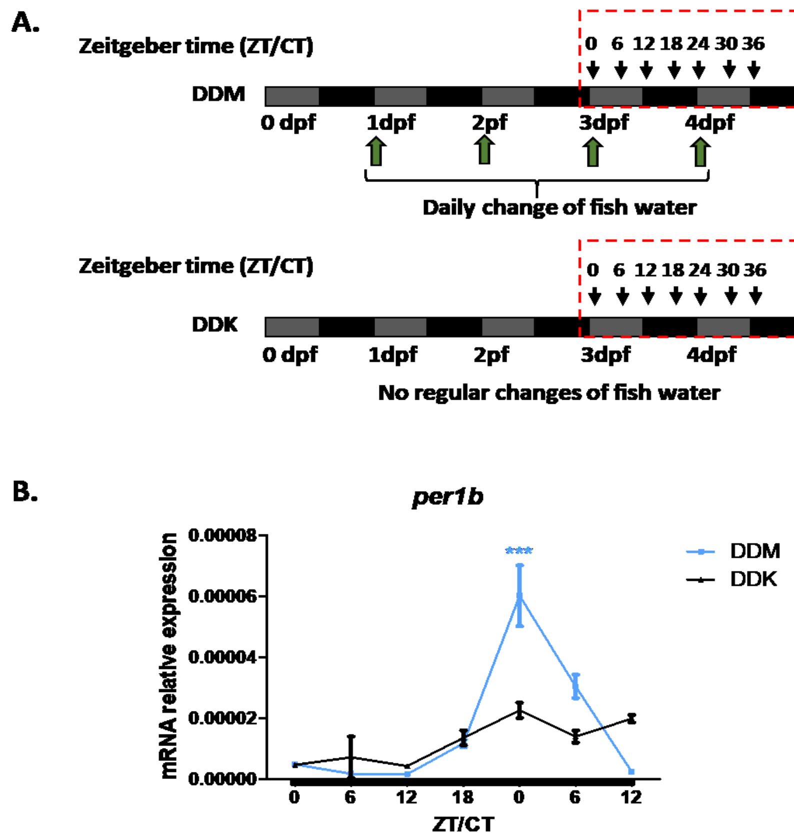


Figure 25: Daily expression levels of *per1b* in 3- 4 dpf zebrafish larvae. A: Schematic illustration of fish entrainment and sample collection. The horizontal bars represent dark period: subjective day -grey boxes, subjective night – black boxes, the black arrows represent the sampling times (0,6,12,18,24,6), the dark green arrows represent the time of fish water change. B: Expression patterns of *per1b* in DDM (blue line) and DDK (black line) groups of 3 – 4 dpf embryos raised in constant darkness. In DDM fish, water was changed every day at the same time point, while for DDK fish, there were irregular changes of fish water. Relative mRNA levels are plotted on the y-axis. Each time point on the x-axis represents the mean ± SD (n=3). Statistical analysis was performed using one and 2way ANOVA (Bonferroni post test), where asterisks (*) indicate statistical significance; * P<0,05; **: P<0,01; *** P<0,001. ZT – zeitgeber time, CT – circadian time.

4.1.2.4 Clock regulated cell cycle entrained by stress during zebrafish early development

Due to the fact that one of the best studied circadian clock outputs is the regulation of the cell cycle, we decided to explore the impact of regular stress on the dynamics of the cell cycle. The circadian system gates cell division by regulating the mRNA expression of the cyclin kinase inhibitor *p21, wee1* and *cyclin A2*. *p21* induces G1 arrest and blocks entry into S phase by inactivating cyclin D,E and Cdks. Instead, *Wee1* kinase blocks entry into mitosis by inhibiting CDC2/cyclin B kinase while *Cyclin A2* activates CDK kinases that participate in regulating S-phase as well as mitotic entry.

In our studies we chose to analyze the impact of regular fish water change on the expression of *p21*. We revealed that there were no significant differences in the expression of *p21* between DDM and DDK groups in 3 and 4 dpf larvae (P>0,05; Figure 26). However, the first significant differences in the expression levels of *p21* between DDM and DDK groups were observed in 5 dpf larvae (P<0,001; Figure 26). In contrast, we observed significant differences between DDM and DDK in *CyclinA2* expression already in 3dpf larvae (P<0,001). The entrainment of *CyclinA2* expression with the time of fish water change was observed in 4dpf larvae in the DDM group (P<0,001; Figure 26). In the DDK group, rhythmic expression of *CyclinA2* was dampened. This result argues that the regular fish water change plays a role in the regulation of the cell cycle.

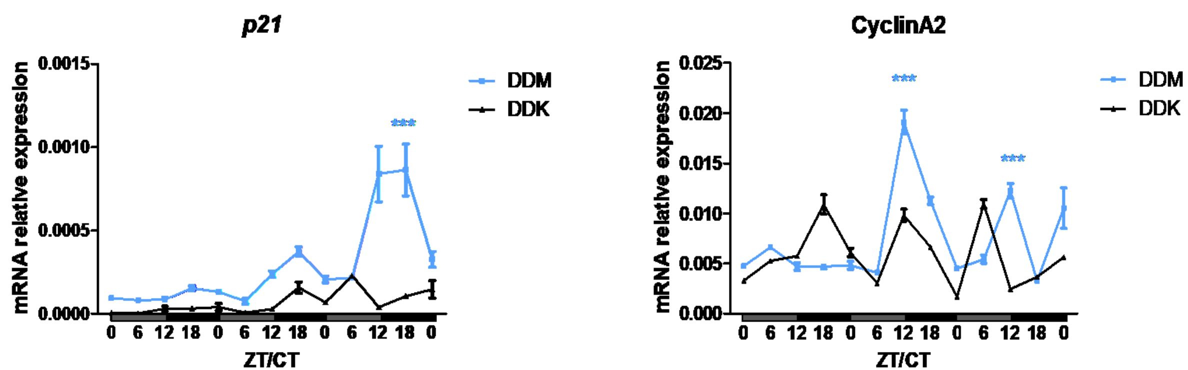


Figure 26: Daily expression levels of cell cycle control genes in 3- 5 dpf zebrafish larvae. Expression patterns of cell cycle genes *p21* and *CyclinA2* in DDM (blue line) and DDK (black line) groups of 3 – 4 dpf embryos raised in constant darkness.). In the DDM group, fish water was changed every day at the same time point, while in the case of DDK fish, there were irregular changes of fish water. Relative mRNA levels are plotted on the y-axis. Each time point on the x-axis represents the mean ± SD (n=3), where asterisks (*) indicate statistical significance; * P<0,05; **: P<0,01; *** P<0,001. Statistical analysis was performed using one and 2way ANOVA (Bonferroni post testZT – zeitgeber time, CT – circadian time.

In the next step we wished to explore whether regular stress may contribute to establishing the rhythms of entry into the cell cycle and S-phase. In order to investigate this question, we examined cell cycle rhythms in zebrafish larvae, raised in DD and under LD cycles, using bromodeoxyuridine (BrdU) incorporation as a marker for the S phase of the cell cycle. As in previous experiments, in the DDM group we changed fish water at regular time points, while in the DDK group, the fish water was changed at irregular time points. Both groups were raised in DD. In parallel as a positive control, zebrafish larvae were exposed to LD cycle without feeding. The larvae at 6 dpf were incubated for 20 minutes in BrdU–containing water and then immediately fixed at different circadian time points (ZT/CT 0, 6, 12, 18, 24, 30) for subsequent analysis. In this way, each time point represents labelling during a well-defined, short period. Positive nuclei were counted between the posterior tip of the swim bladder and the anus as

previously described (Dekens et al., 2004). Our preliminary data showed circadian rhythms in the number of S phase-positive nuclei with the peak around ZT/CT 12 in the LD and DDM groups (Figure 27). Surprisingly, in the DD group we also detected rhythmic changes in the number of S phase-positive nuclei. However, these rhythms were shorter than 24 hours (Figure 27). These results indicate that regular exposure to stress as well as LD cycles results in the entrainment of circadian cell cycle rhythms in developing larvae.

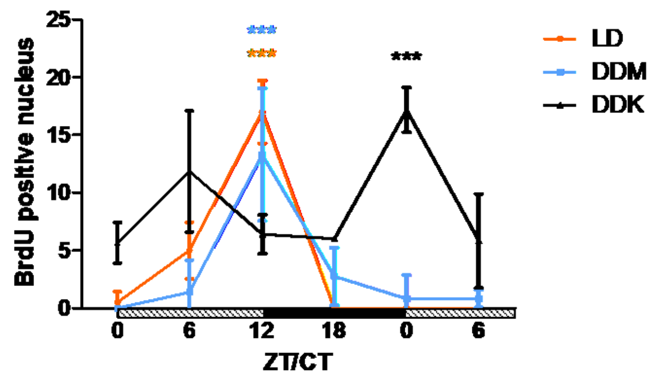


Figure 27: Circadian rhythms of S-phase in 6 dpf zebrafish larvae. Numbers of BrdU positive nuclei in zebrafish larvae exposed to LD cycles (LDK), and maintained in DD: in the DDM group, fish water was regularly changed, in DDK – no regular change of fish water occurred. All groups were not fed. The time of each sample is indicated either as zeitgeber time (ZT) or circadian time (CT). BrdU positive nucleus are plotted on the y-axis. Each time point on the x-axis represents the mean ± SD (n=3), where asterisks (*) indicate statistical significance; * P<0,05; **: P<0,01; *** P<0,001. Statistical analysis was performed using one and 2way ANOVA (Bonferroni post test).

4.2 How are feeding- and light-regulated clocks differentially coupled with metabolism?

4.2.1 Circadian clock gene expression in zebrafish liver

In order to investigate the relationship between light and feeding regulated clocks *in vivo*, there is a need for a set of experimental conditions whereby fish are exposed to combinations of zeitgebers. For this purpose, in collaboration with the group of Vazquez-Sanchez (University of Murcia, Murcia, Spain) our group has established a new approach, the so called "conflicting zeitgeber" paradigm, where adult fish are co-exposed to light-dark cycles and regular feeding regimes. Specifically, fish are maintained under 12 hours light and 12 hours dark cycles and feeding occurs once per day, either in the middle of the dark period or in the middle of the light period over the course of 3 weeks (Figure 28A). This approach enables the exploration of which circadian rhythms *in vivo* cycle according to the timing of the light cycle and which cycle according to the time of feeding. Interestingly, the feeding time and not the light-dark cycle sets

the behaviour activity of zebrafish (Li Ying PhD thesis). These previous findings are consistent with the regular timing of FAA being set by the feeding time and not by the light-dark cycle. Surprisingly, in contrast to locomotor activity rhythms, the analysis of the expressions levels of *per1b* and *per2* in zebrafish liver indicate that the cycling phase of these genes is set by the light-dark cycle independently of the feeding time (Figure 28B). It has therefore been speculated that a feeding-entrainable oscillator (FEO) is based on an independent clock mechanism.

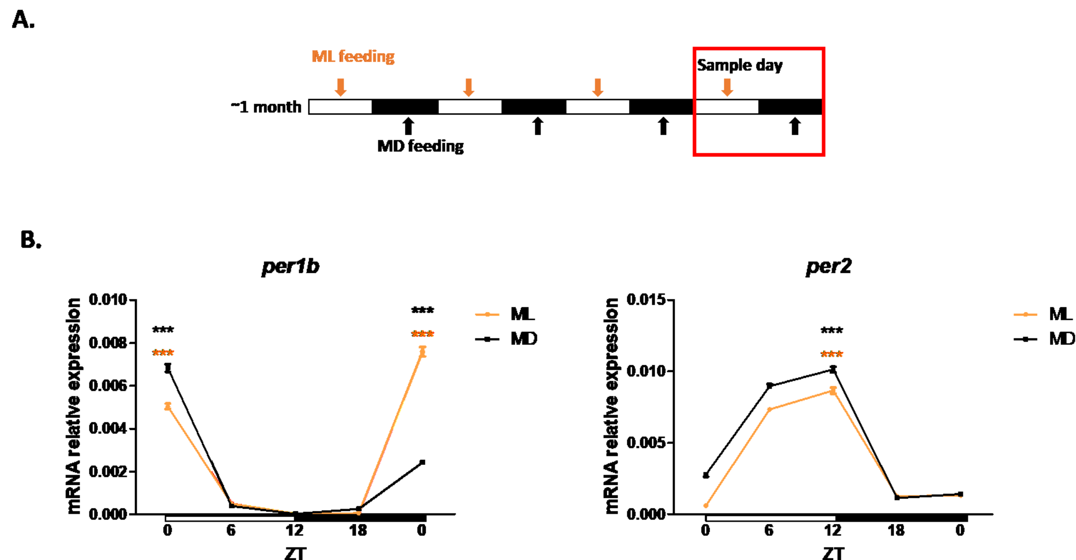


Figure 28: The rhythmic expression of core clock genes under light-dark cycles and different feeding regimes. A: Schematic illustration of fish entrainment and sample collection in our conflicting zeitgeber experiments. The horizontal bars represent light conditions: white boxes represent light, black boxes – dark. The yellow and blue arrows represent feeding time. B: Expression patterns of *per1b* and *per2* from zebrafish liver adapted to different feeding conditions. Each sample is a pool containing RNAs of three livers. Relative mRNA levels are plotted on the y-axis. Each time point on the x-axis represents the mean ± SD (n=3). Statistical analysis was performed using one and 2way ANOVA (Bonferroni post test), where asterisks (*) indicate statistical significance; * P<0,05; **: P<0,01; *** P<0,001. ML indicates midday feeding while MD indicates midnight feeding. ZT –zeitgeber time

These findings are contrary to previous studies performing using mice, which have indicated that the phase of cycling clock gene expression in the liver can be entrained by the phase of regular feeding, independently of the light-dark cycle or the master clock in the SCN (Damiola et al., 2000; Stokkan et al., 2001).

4.2.2 Entrainment of amino acid rhythms by feeding

Previous studies from Li Ying showed that circadian cycles in the levels of non-essential amino acids are regulated by the light-dark cycle and cycles of essential amino acids by the feeding time. In order to validate these findings obtained using NMR, we performed LC/MS-MS

analysis in collaboration with Dr. G. Brenner-Weiß group at IFG, KIT. Zebrafish were divided into two groups: one group was fed in the middle of the light period, the second – in the middle of the night period. After one month we dissected the liver and analysed the changes in amino acid levels during one 24-hour period. In totally we analysed 22 amino acids. Surprisingly, our results were not consistent with the previous findings from NMR analysis. We observed that the changes in the concentration of almost all analysed amino acids were affected by the feeding time and not by the light-dark cycle (Figure 29, 30).

One possible reason for this inconsistent data could be that different zebrafish strains were used in both experiments. NMR analysis was performed with Wild Indian Karyotype (WIK) line and the UPLC-MS/MS analysis with the Tübingen (TU) line. Therefore, potentially these results may point to a surprising feature that the genetic background determines which Zeitgeber sets the phase of cycling metabolites.

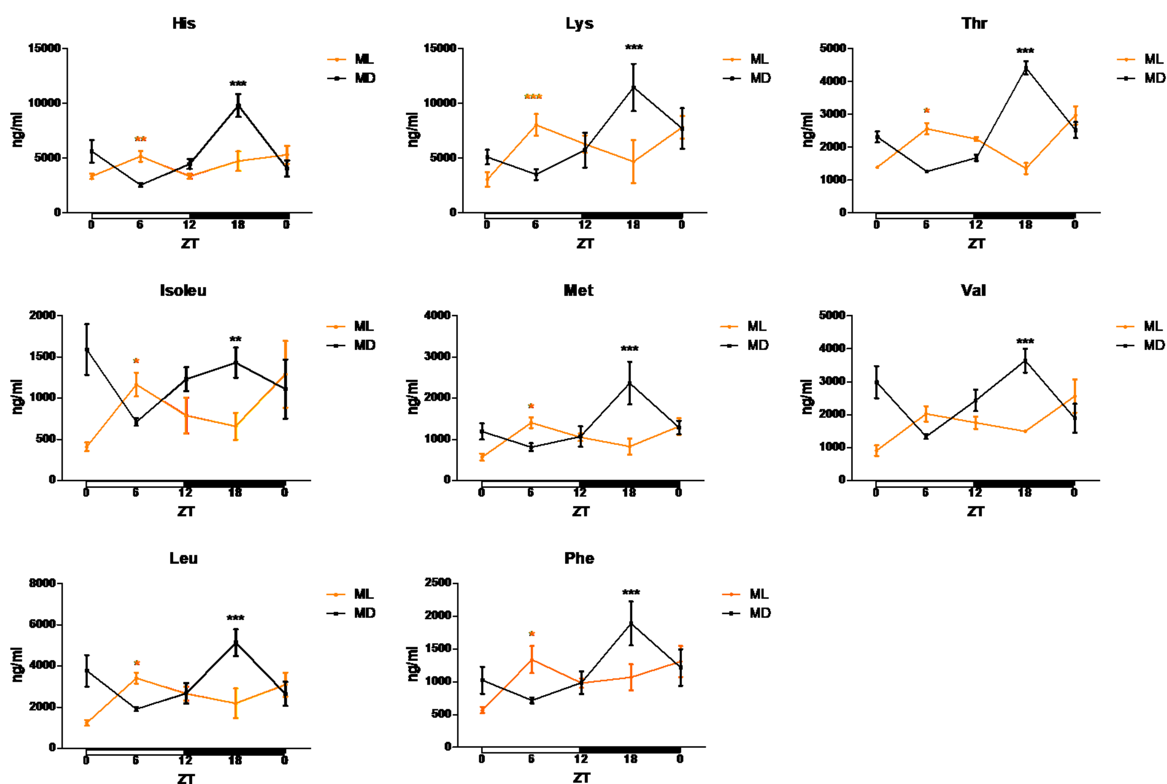


Figure 29: Daily changes in essential amino acid levels in zebrafish liver. Rhythmic changes in a representative set of essential amino acids in zebrafish liver. ML- mid light feeding, MD – mid night feeding. The time point where each sample is taken, is indicated as zeitgeber time (ZT). His – histidine, Isoleu – isoleucine, Leu – leucine, Lys – lysine, Met – methionine, Phe – phenylalanine, Thr – threonine, Val – valine. Statistical analysis was performed using one and 2way ANOVA (Bonferroni post test), where asterisks (*) indicate statistical significance; * P<0,05; **: P<0,01; *** P<0,001.

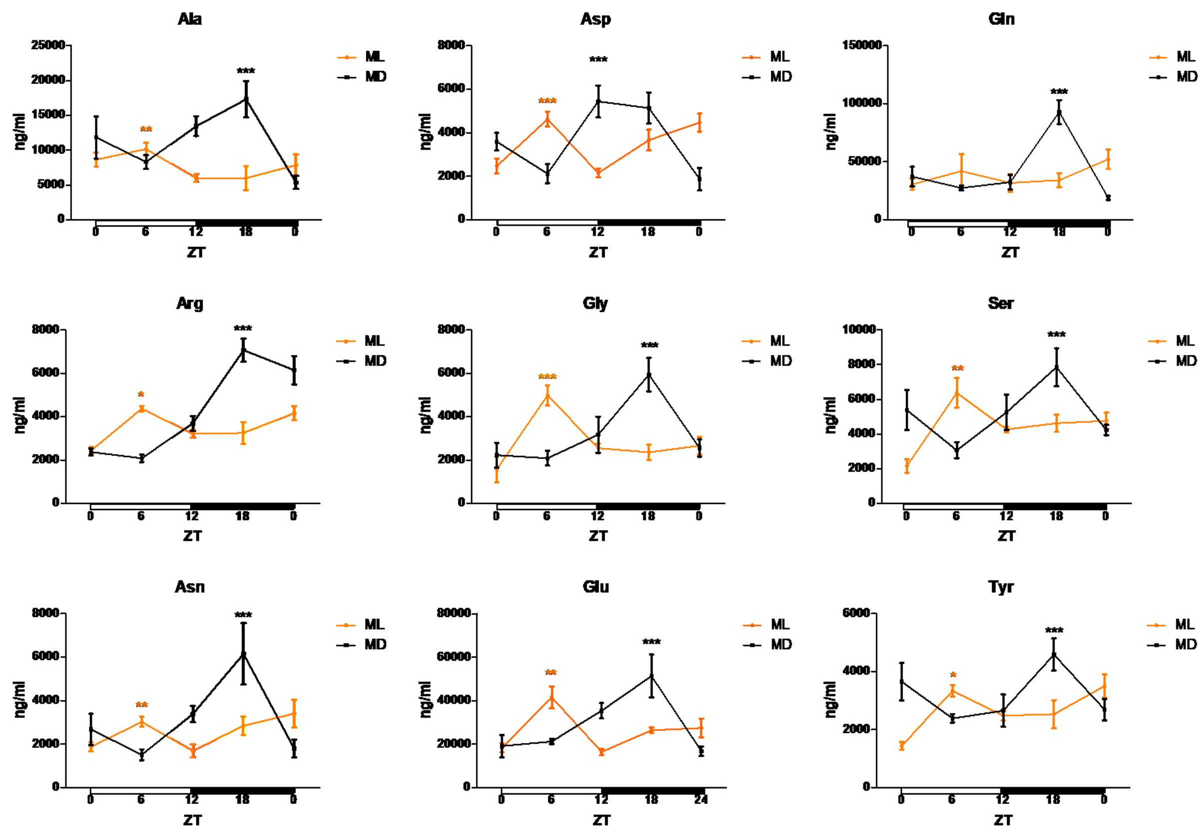


Figure 30: Daily changes in non-essential amino acid levels in zebrafish liver. Rhythmic changes in a representative set of non-essential amino acids in zebrafish liver. ML- mid light feeding, MD – mid night feeding. The time of each sample is indicated as zeitgeber time (ZT). Ala – alanine, Arg – arginine, Asn – asparagine, Asp - aspartic acid, Gly – glycine, Glu – glutamate, Gln – glutamine, Ser – serine, Tyr – tyrosine. Statistical analysis was performed using one and 2way ANOVA (Bonferroni post test), where asterisks (*) indicate statistical significance; * P<0,05; **: P<0,01; *** P<0,001

4.2.3 Feeding time controls the NAD+ biosynthesis pathway

In the next step we wished to investigate the genetic mechanism which is involved in the regulation of the dynamics of amino acid rhythms. Preliminary data from Li Ying indicated that the cycle of NAD+ undergoes circadian regulation, which is affected by the feeding time and not by the light-dark cycle. Some studies have argued that the NAD+ biosynthesis pathway connects the circadian clock system with metabolic pathways (Ramsey et al., 2009). The important players in NAD+ biosynthesis is the rate-limiting enzyme, *nampt*, and a NAD+-dependent histone deacetylase, SIRT1. It has been reported that SIRT1 directly regulates the activity of the CLOCK/BMAL1 complex in mammals. In our studies we observed that the

expression of both *nampta* and *sirt1* are affected by the feeding time and not by the light-dark cycle (Figure 31).

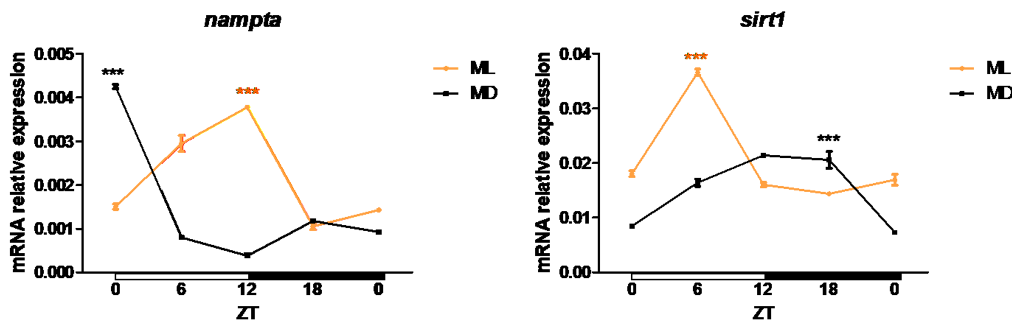


Figure 31: The rhythmic expression of *nampta* and *sirt1* under light-dark cycles and different feeding regimes. Each sample is a pool containing the RNAs of three, pooled livers. Relative mRNA levels are plotted on the y-axis. Each time point on the x-axis represents the mean ± SD (n=3). Statistical analysis was performed using one and 2way ANOVA (Bonferroni post test), where asterisks (*) indicate statistical significance; * P<0,05; **: P<0,01; *** P<0,001. ML- mid day feeding, MD – mid night feeding, ZT – zeitgeber time.

This result indicates that nampta and SIRT1 may not only represent links between energy metabolism and circadian function, but may also serve as links between the LEO and FEO.

4.2.4 Control of autophagy by feeding

We next considered the potential contribution of changes in the supply of amino acids to dynamic changes in amino acid levels in the liver. What are the possible sources of amino acids during a starvation period? Recent evidence suggests that autophagy, whereby cellular proteins are broken down into their constituent amino acids is implicated in the maintenance of amino acid levels in the liver (Ezaki et al., 2011). Moreover, it has been reported that autophagy undergoes circadian regulation in zebrafish liver (Huang et al., 2016). To explore in more detail whether the feeding time has an impact on the circadian regulation of autophagy, we analysed the expression of LC3 (microtubule-associated protein1 light chain 3), which is a widely used autophagy biomarker, in zebrafish liver using western blotting. In the ML group the lowest LC3 levels were detected during the night period at ZT18 (Figure 32 B left). In comparison when the fish were fed during the night period (MD) the lowest level of LC3 was observed at the end of the day at ZT 12 (Figure 32 B right side). The best studied and characterized negative regulator of autophagy is mTORC1 (mechanistic target of rapamycin complex I). The activity of mMTORC1 is controlled by amino acids. For example, under high amino acid levels MTORC1 is activated, whereas under amino acid deprivation it is inhibited (Rabanal-Ruiz et al., 2017). Consistently, expression analysis of mTORC1 mRNA confirmed that cycling

expression of this autophagy regulator is set by the feeding time rather than the LD cycle. (Figure 32 D).

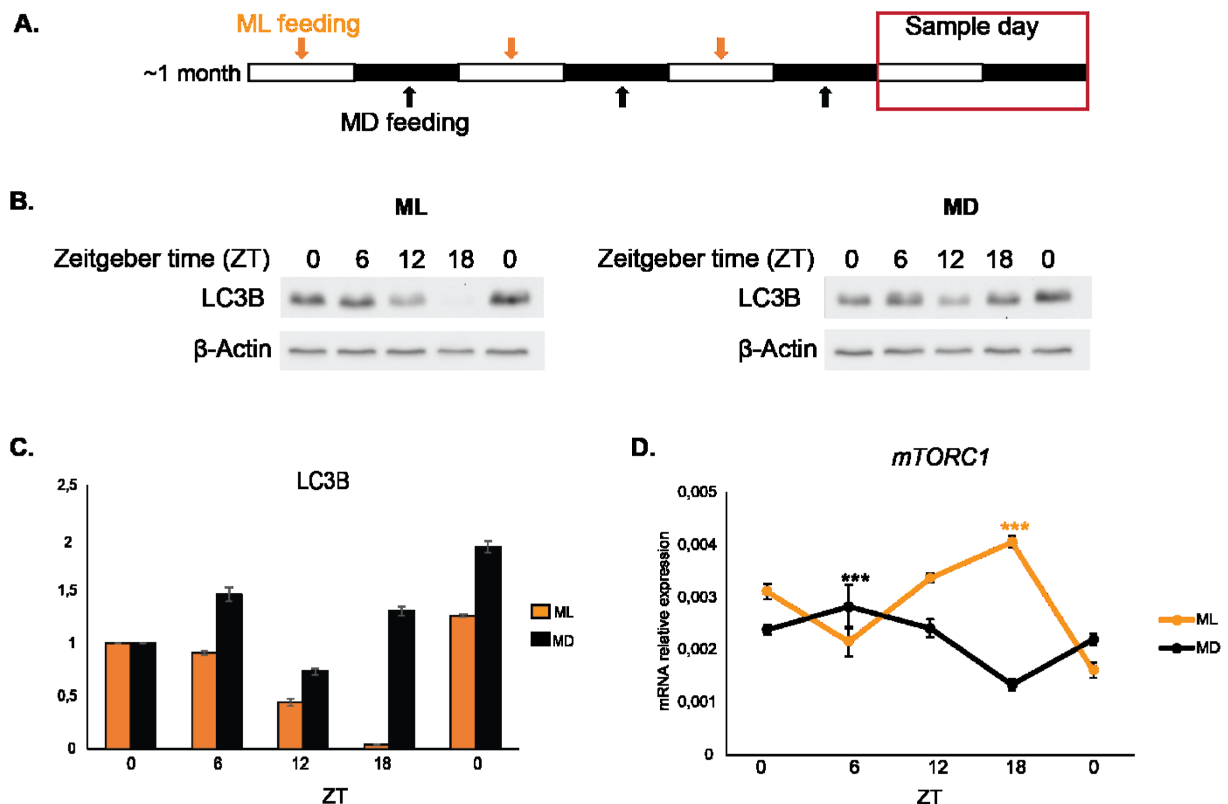


Figure 32: Regulation of autophagy by feeding. A: Schematic illustration of fish entrainment and sample collection. The horizontal bars represent light conditions: white boxes represent light, black boxes – dark. The orange and black arrows indicate feeding time. B: Western blot analysis of zebrafish liver after restricted feeding in the light period (ML) or dark period (MD). β-actin was used as a loading control. Mean values of fold induction ± SD (n=3) are plotted on the Y-axis. Times are plotted on the X-axis. C: Quantification of the western blotting performed with Image J software. D: Expression of mTORC1 mRNA. Each sample is a pool containing RNAs of three livers. Relative mRNA levels are plotted on the y-axis. Each time point on the x-axis represents the mean ± SD (n=3), where asterisks (*) indicate statistical significance; * P<0,05; **: P<0,01; *** P<0,001. Statistical analysis was performed using t-test and 2way ANOVA.

These findings argue that autophagy rhythms are influenced by the feeding regime and not by the light-dark cycle. This supports the idea that autophagy serves as a link between the circadian clock system and amino acid metabolism in the zebrafish liver.

4.2.5 Feeding time plays a role in the development of obesity

Previous studies have observed that meal timing has a major impact on the health of organisms and the development of important diseases, including obesity and type 2 diabetes. So we wondered whether feeding time might have some impact on the health of fish. In order to explore whether the time of feeding affects the gain of body mass, we categorized zebrafish

into two different groups according to the feeding time: fish fed once a day at mid-light (ML, ZT7) and fish fed once a day at mid-dark (MD, ZT19). Already after one month it was evident that the fish fed at MD gained weight faster than the fish fed at ML (Fig. 33 C). Consistently, mice studies have shown that mice which are normally nocturnally active, which are fed a high fat diet only during the 12 hour light phase, gained significantly more weight than mice fed only during the 12 hour dark phase.

Our results are consistent with the time of feeding playing a crucial role in the development of the obesity, not just in mammals but also in zebrafish.

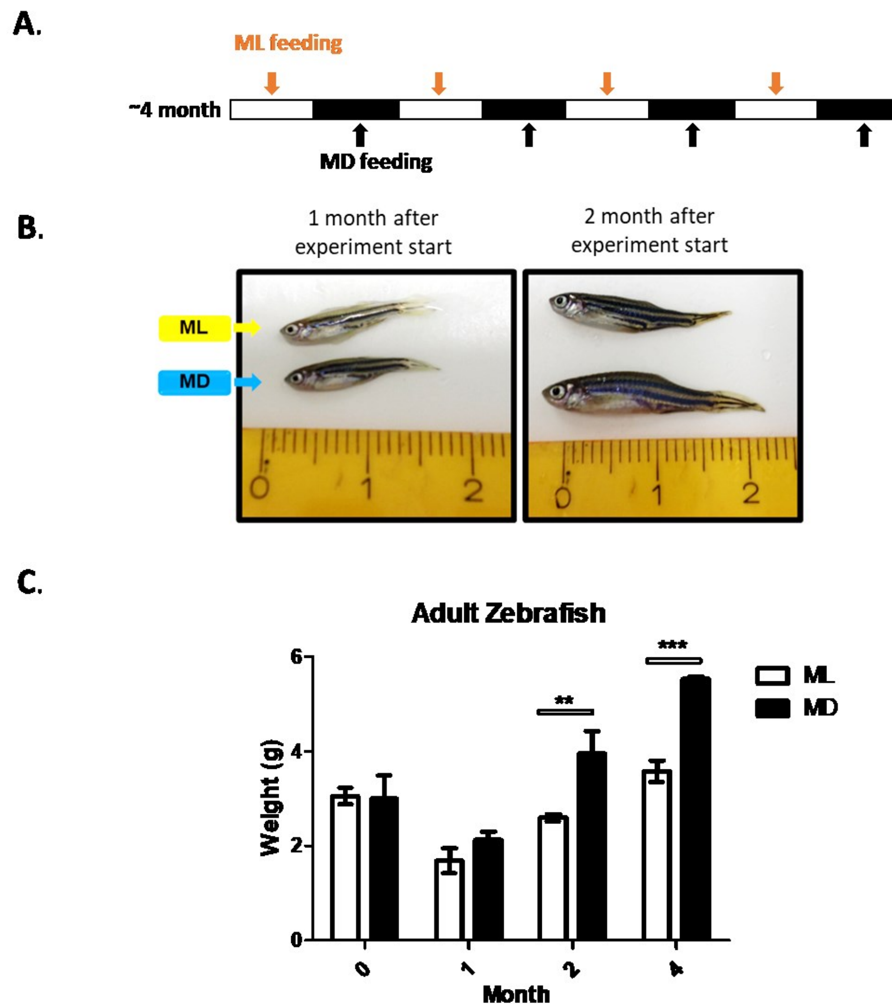


Figure 33: A: Schematic illustration of fish entrainment and sample collection. The horizontal bars represent light conditions: white boxes represent light, black boxes – dark. The orange and blue arrows represent feeding time. B: Left panel*:* zebrafish at the onset of the experiment; right panel: one month later during the experiment. C: Quantification of the zebrafish weight. Statistical analysis was performed using t-test and 2way ANOVA, where asterisks (*) indicate statistical significance; * P<0,05; **: P<0,01; *** P<0,001. ML- mid day feeding, MD – mid night feeding.

4.3 Do cell lines contain "multiple" clocks?

Our *in vivo* studies are consistent with the existence of two distinct clock mechanisms, a light entrained LEO and a feeding time regulated FEO. We next used our existing zebrafish cell lines to explore whether distinct light and food regulated clocks may also coexist in cultured fish cells. What is the evidence that multiple clocks may exist in fish cell lines? Zebrafish cell lines display a directly light entrained clock. However, acute treatments with serum can also directly entrain circadian rhythms of clock gene expression. Thus, circadian rhythmicity in zebrafish cells can respond to multiple zeitgebers.

4.3.1 Rhythmic changes in amino acid concentration in PAC-2 WT cells

We first wished to determine whether, as in the liver, levels of amino acids might be clock regulated and therefore show circadian rhythms upon exposure of the cells to light dark cycles or serum changes. In order to initially investigate whether the time of day influence amino acid levels in PAC-2 cells, we seeded the cells and maintained them for 2 days in constant darkness (DD) to ensure the loss of previous cellular clock synchronization. Then the cells were divided into two groups: the first group was exposed to a LD cycle (12:12, light-dark), the second group was left in DD. At day 7 the sampling was started. Metabolites and mRNA were isolated from the adapted cells harvested every 6 hours and subjected to UPLC-MS/MS or qRT-PCR analysis. A total of 22 amino acids were then analysed. The experiment scheme is visualized in Figure 34.

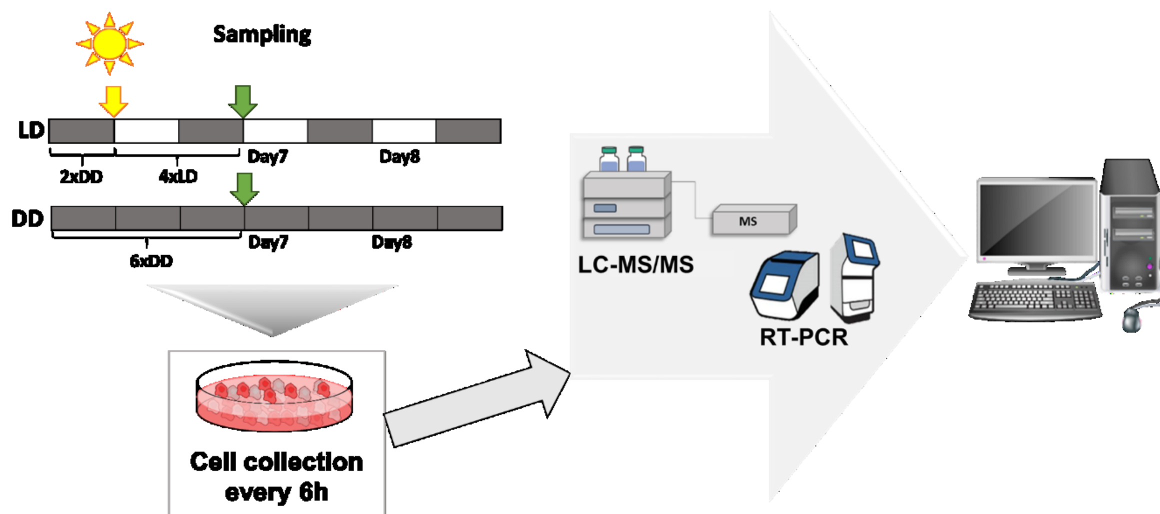


Figure 34: Experimental design. PAC-2 cells were seeded and maintained two days in DD. At day 3 one group was exposed to LD cycles (12:12), while the second group remained in DD. At day 7 the sampling was started. Metabolites or mRNA were isolated from cells harvested every 6 hours and subjected to UPLC-MS/MS and qRT-PCR.

Our data analysis showed very interesting and surprising results. First of all, we observed that the changes of almost all analysed amino acids in DD without any input of external Zeitgebers were rhythmic (Figure 35 A), whereas the expression of *per1b*, which is used as a biomarker of the circadian clock system, does not oscillate (Figure 35 A). Surprisingly, these rhythms were infradian, meaning that the period of these rhythms was significantly longer than 24 hours. In cells exposed to an LD cycle the changes in amino acid concentration were also infradian (Figure 35 B) although, rhythmic expression of *per1b* under LD cycle conditions was circadian (Figure 35 B).

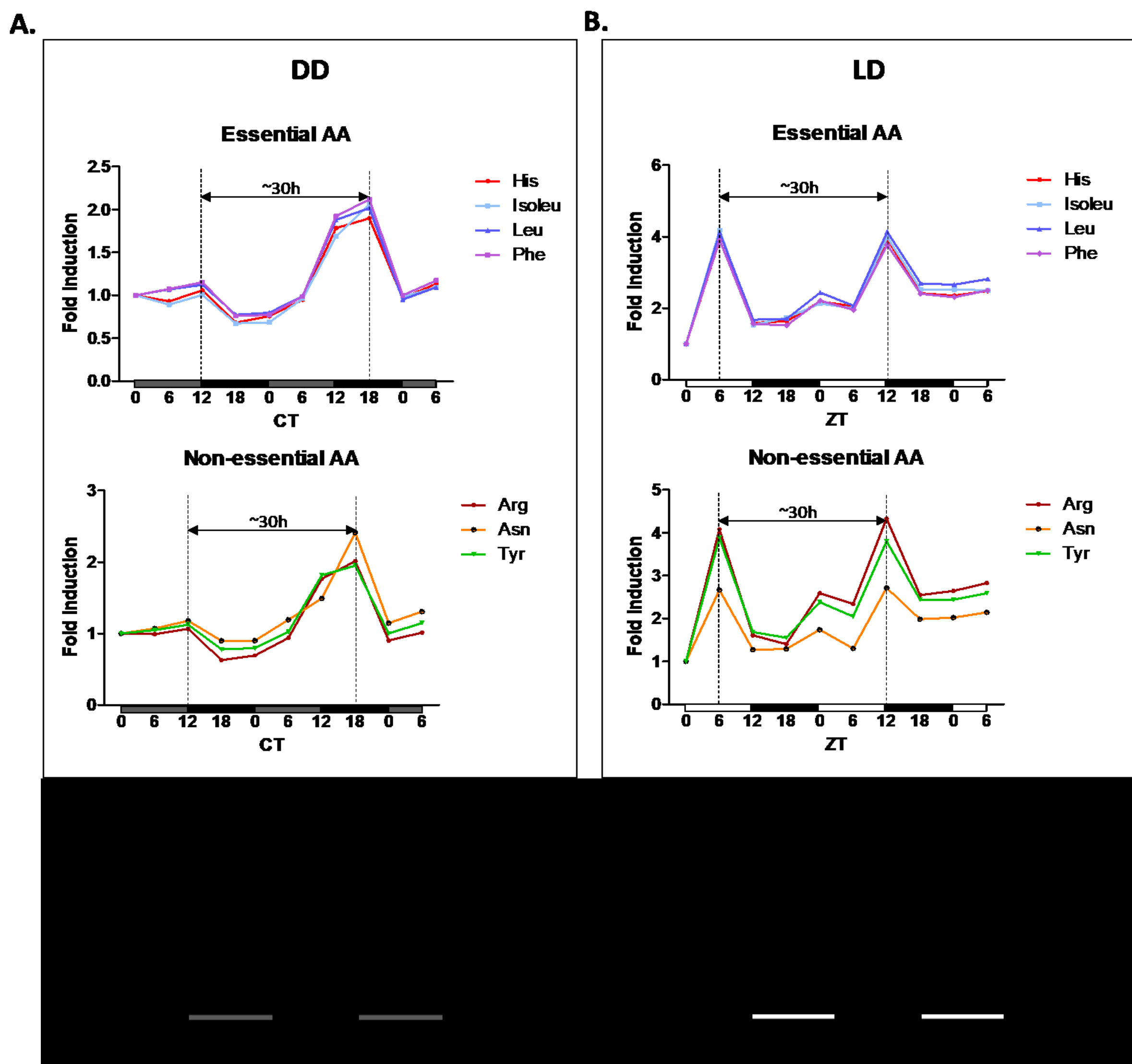


Figure 35: Rhythmic changes in amino acid concentration in PAC-2 WT cells. Quantification of amino acid levels in PAC-2 cell extracts by UPLC-MS/MS and mRNA levels of the *per1b* clock gene by qRTPCR. A: Rhythmic changes in the concentration of a representative subset of amino acids in DD. Rhythmic expression of *per1b* is abolished in DD. B: Rhythmic changes in the levels of a representative subset of amino acids under LD cycles. Expression of *per1b* shows a 24 hours, day night rhythm under LD cycles. The time of each sample is indicated either as zeitgeber time (ZT) or circadian time (CT).

From these results, it is apparent that amino acid levels in the cells are not constant, but are influenced by the time of the day and lighting conditions. Surprisingly, the changes in amino acid levels are not circadian as we expected but are infradian. We next wondered which mechanisms may be responsible for generating infradian amino acid rhythms?

4.3.2 Disrupted amino acid rhythms in Δ*clock1* cells

We first wished to explore whether the circadian clock TTFL mechanism is necessary for the regulation of cellular amino acid rhythmicity in PAC-2 cells. We analysed changes in amino acid levels in a cell line over-expressing a dominant-negative form of the CLOCK1 (*Δclock1)* gene. *Δclock1* lacks the glutamine-rich transactivation domain but still contains the bHLH DNA binding and PAS protein-protein interaction domains (Figure 36 A). Δ*clock1* can form a heterodimer with BMAL1, which binds to E-boxes in the promoter of the period gene, and thereby blocks its transcriptional activation (Figure 36 C).

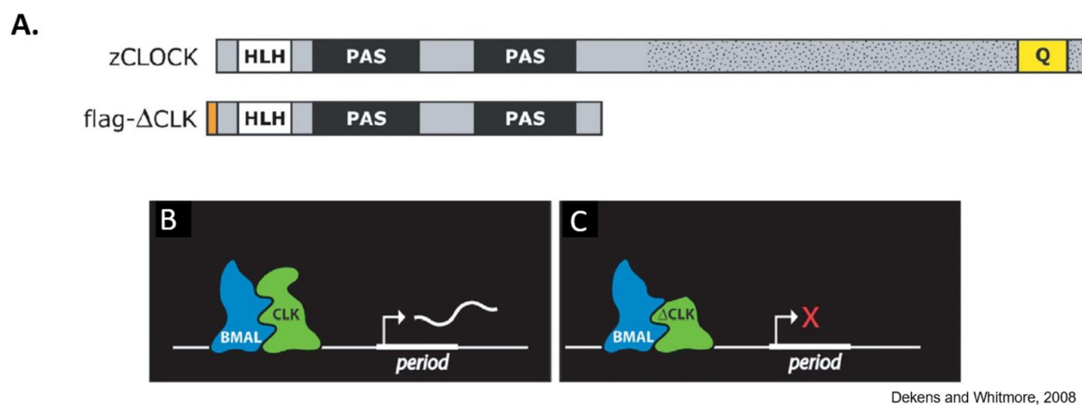


Figure 36: Zebrafish dominant negative Clock protein. A: ΔCLK lacks the glutamine-rich transactivation domain (Q) but it still contains the bHLH DNA binding and PAS protein-protein interaction domains. B: In WT cells CLK forms a heterodimer with BMAL, binds on the E-box sequence in the promoter and activates transcription of *per1b*. C.: ΔCLK is also able to form the heterodimer with BMAL, thereby inhibiting the transcription of *per1b*. (Dekens and Whitmore, 2008)

MS analysis showed that in Δ*clock1* expressing cells compared with WT cells the infradian rhythms of amino acids in constant darkness were completely disrupted (Figure 37 B). These results suggest that the core circadian clock TTFL mechanism is involved in the regulation of amino acid rhythms in PAC-2 cells.

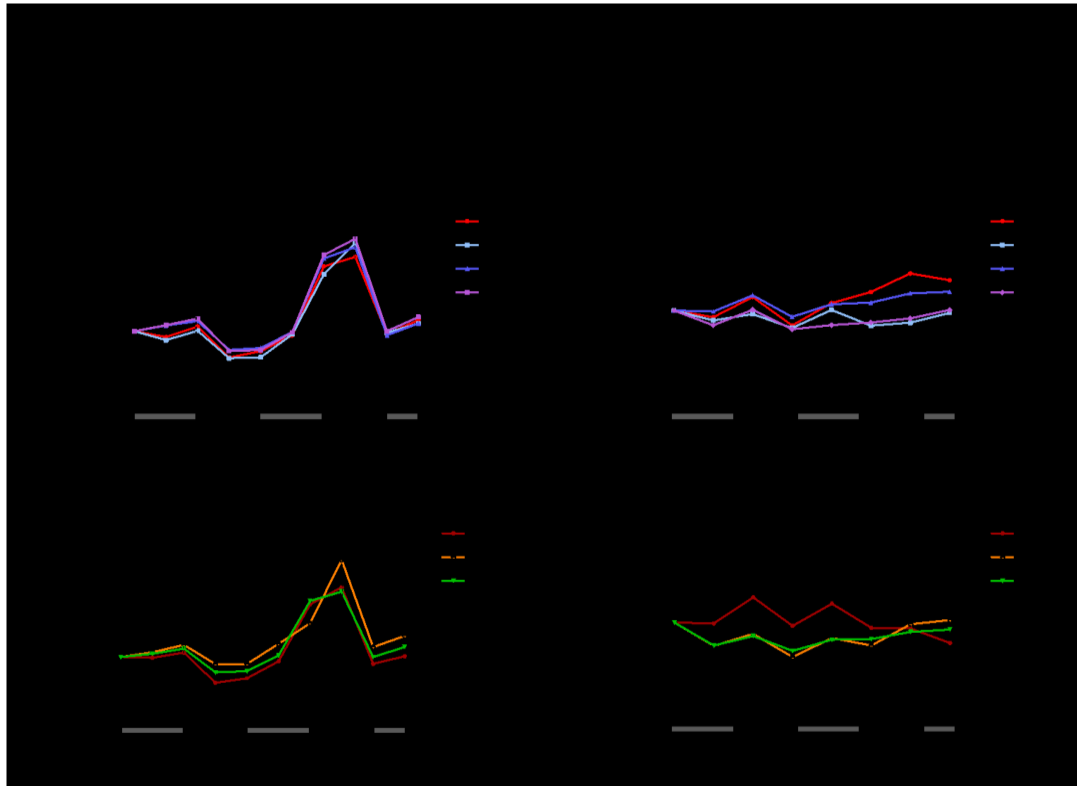


Figure 37: Daily changes in amino acid concentration in PAC-2 WT and Δclock1 cells. Quantification of the levels of a representative subset of amino acids in PAC-2 cell extracts by UPLC-MS/MS. A. Rhythmic changes in amino acid levels in WT cells in DD. B. Disrupted rhythms of amino acid concentrations in *Δclock1* cells. The time of each sample is indicated circadian time (CT).

In the next step we wish to understand which mechanism may drive these infradian rhythms.

4.3.3 Rhythmic changes in amino acid concentration in CF-1 cells

In parallel with these zebrafish based studies, the role played by regular feeding in the circadian timing system of the blind cavefish, *P. andruzzii* has also been extensively studied by the Foulkes group in collaboration with the Bertolucci group at the University of Ferrara, Italy. It was shown that cavefish possess only a food-entrainable clock, while zebrafish has both light- and food-regulated clocks. Furthermore, the period length of the circadian clock in cavefish peripheral tissues *in vitro* is infradian, around 47 hours (Cavallari et al., 2011). However, in contrast, restricted feeding can entrain circadian rhythms of cavefish locomotor activity and rhythmic clock gene expression in the liver. These features point to two distinct clock mechanisms existing in this cavefish species.

A comparative analysis of amino acid metabolism between cavefish and zebrafish could be valuable for understanding the origin of the infradian rhythms of amino acid levels in PAC-2 cells. Specifically, we first wished to explore whether changes in amino acid levels in DD are

comparable with PAC-2 cells. Aliquots of cells of the CF1 cell line derived from *P. andruzzii* were incubated in DD for 7 days. Then amino acid levels were analysed by UPLC-MS/MS in the same way as for the PAC-2 cells. Preliminary data analysis suggests amino acid levels in CF-1 cells show a robust infradian rhythmicity (Figure 38) that is comparable with the amino acid rhythms of PAC-2 cells (Figure 36).

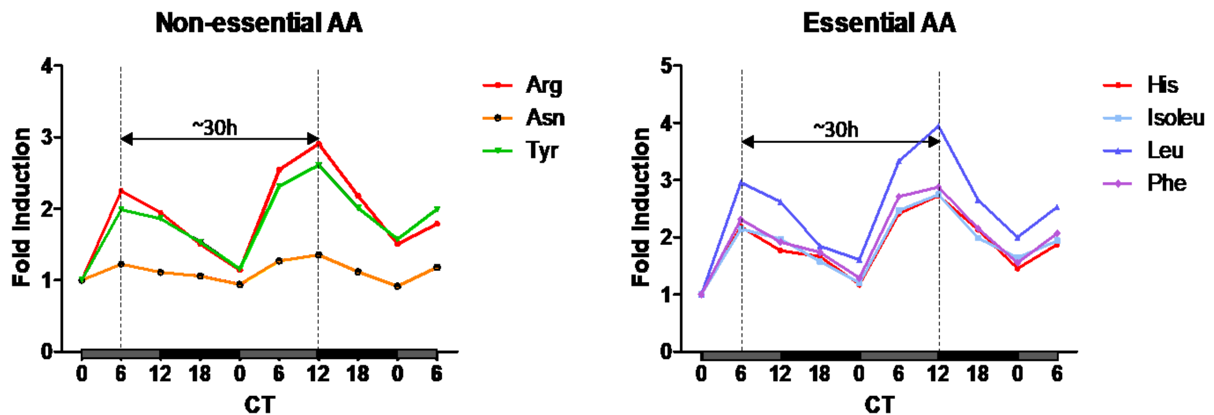


Figure 38: Rhythmic changes in amino acid concentration in CF-1 cells. Quantification of the levels of a representative subset of amino acids in CF-1 cell extracts by UPLC-MS/MS. Rhythmic changes in essential (left panel) and non-essential amino acid concentrations (right panel) in DD. The time of each sample is indicated as circadian time (CT).

4.3.4 Infradian expression of *nampta* and *sirt1* in constant darkness

A key mechanism linking the circadian clock system with metabolism is the *Nampt* dependent NAD+ biosynthesis pathway. Given that NAD+, *Nampt* and *Sirt1* undergo circadian regulation in mammals, we wondered whether the expression of these components might show any evidence of infradian rhythmicity in PAC-2 cells in DD conditions. Does the light signal have any impact on the regulation of the expression of *Nampt* and *Sirt1*? To answer these questions, we compared expression of *Nampta* and *Sirt1* between LD and DD groups of PAC-2 cells. We observed infradian rhythms of expression for *Nampta* in the DD and LD groups (Figure 39). However, the expression of S*irt1* was infradian in DD and arrhythmic in the LD group (Figure 39).

These results lead to the hypothesis that *Nampta* and are S*irt1* are possible candidates regulating infradian rhythms of amino acids in PAC-2 cells in DD.

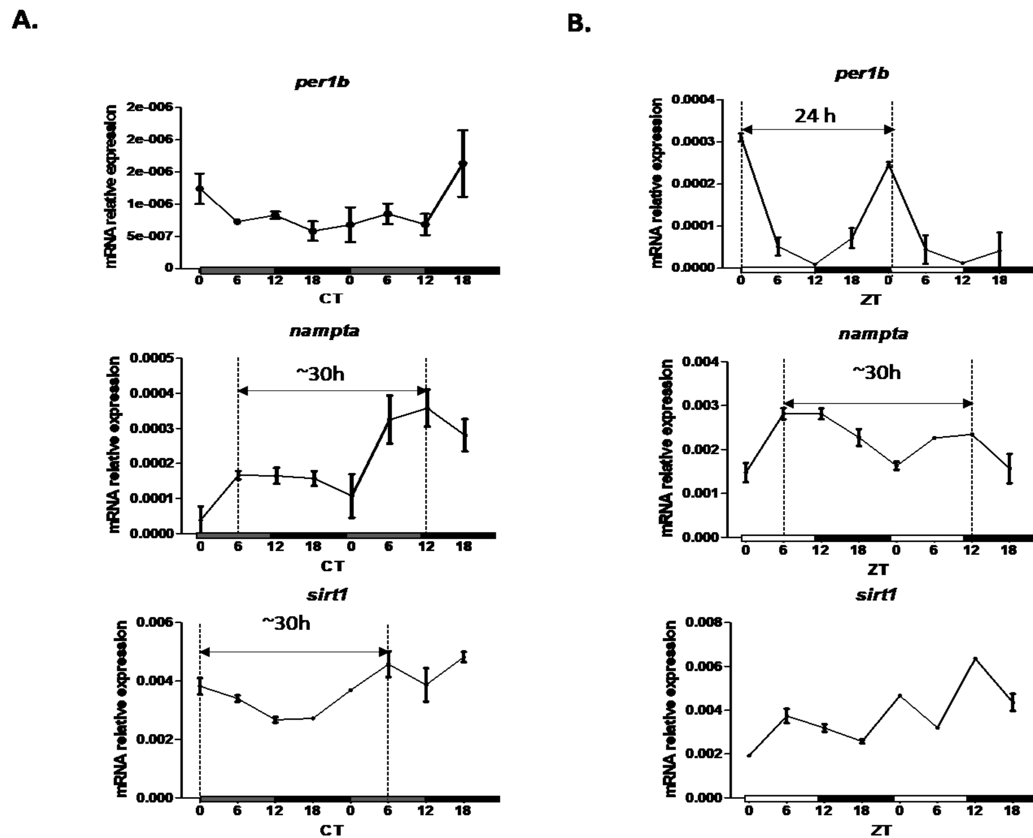


Figure 39: Expression of *nampta* and *sirt1* mRNAs in constant darkness and under light-dark cycles. Quantification of qRTPCR results measuring gene expression in PAC-2 cells under DD and LD conditions. A: Rhythmic expression of *per1b* is abolished in DD. Expression of *nampta* and *sirt1* shows infradian rhythmicity in DD. B: *per1b* shows circadian rhythmicity of expression under LD cycles. Rhythmic expression of *nampta* is infradian under LD cycles. Expression of *sirt1* appears arrhythmic under LD cycle conditions. Relative mRNA levels are plotted on the y-axis. Each time point on the x-axis represents the mean ± SD (n=3). Statistical analysis was performed using one and 2way ANOVA (Bonferroni post test).

4.3.5 Arrhythmic luciferase expression driven by the *nampta* promoter

In the next step we wished to understand better the role of *nampta* in the regulation of infradian rhythms in amino acid metabolism. Specifically, we cloned a 1,528 kb genomic DNA fragment lying 166 bp upstream of the *nampta* translation start site into a luciferase reporter vector, generating the construct, zf *nampta*-Luc. With Genomatix bioinformatics analysis, we identified one E-box sequence localised 209 bp upstream of the translation start site, whereas the mammalian *Nampt* has three E-box sequences (Nakahata et al., 2009). The expression of mammalian *Nampt* is regulated directly through the CLOCK/BMAL1 heterodimer as well as by Sirt1 (Nakahata et al., 2009). Using a transient expression assay in PAC-2 cells we were unable to detect circadian or infradian rhythms in promoter reporter expression under LD cycles or DD conditions (Figure 40 A,B). For the positive control we used a D-box luciferase reporter gene consisting of multimerized D-box enhancer elements from the *zfcry1a* promoter cloned

upstream of a minimal promoter directing luciferase reporter gene expression (*6xD-box*$_{cry1a}$-*Luc*). As expected, the expression of *6xD-box*$_{cry1a}$-*Luc* was strongly rhythmic and synchronised with the LD-cycles (Fig. 40 A), whereas in DD the expression of *6xD-box*$_{cry1a}$-*Luc* was arrhythmic (Fig. 40 B). These findings suggest that the infradian rhythms of amino acid levels are not regulated by the NAD+ salvage pathway. However it is also possible that our sequence is too short and so lacks the necessary enhancer elements that should be present in order to observe the circadian or infradian expression.

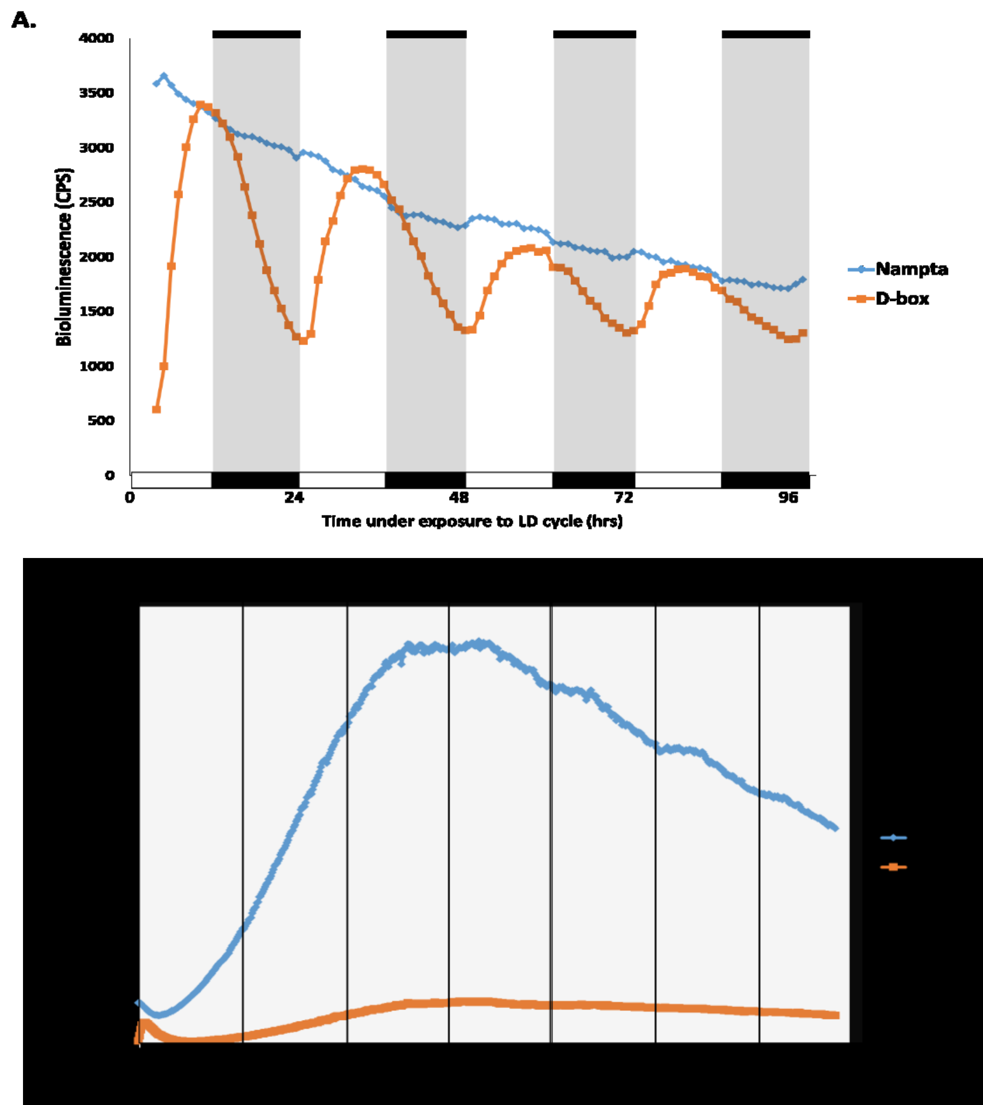


Figure 40: Arrhythmic expression of the nampta promoter in PAC-2 cells. PAC-2 cells transfected with zf nampta-Luc (blue trace) and zf 6xD-box$_{cry1a}$-Luc (orange trace, control) were exposed to LD (A) cycles or incubated in DD (B). On the y-axis are plotted the bioluminescence levels (counts per second, cps) and on the x-axis is plotted the zeitgeber time (ZT0 is lights on). Each trace is plotted as a mean of n=8 independent wells. The graphic is a representative experiment of three independent biological replicates.

4.3.6 Circadian expression of amino acid transporters and autophagy genes in DD

In order to explore which mechanism might directly drive infradian rhythms in amino acid, we decided to analyse the expression of some genes which are involved in the regulation of amino acid homeostasis in cells. One of the key regulators involved in the homeostasis of intracellular amino acid levels are amino acid transporters. The best explored amino acid transporter is Sodium-coupled neutral *amino acid transporter 2* (SNAT2), that regulates uptake of neutral alpha-amino acids, such as glutamine and alanine. Unfortunately, we could not detect any infradian rhythmicity patterns in SNAT2 expression. Instead, the rhythms of SNAT2 expression were circadian (Figure 41 C). Next, for maintenance of cellular amino acid homeostasis, autophagy plays an important role. In cells autophagy is activated during amino acid deprivation, via inhibition of mTORC1. We therefore examined the expression levels of mTORC1 and LC3b. Again, we observed that the expression of both genes showed a circadian rhythmicity and and not infradian rhythms (Figure 41 C). Taken together these data indicate that neither amino acid transporters nor autophagy are involved in the generation of infradian amino acid rhythms.

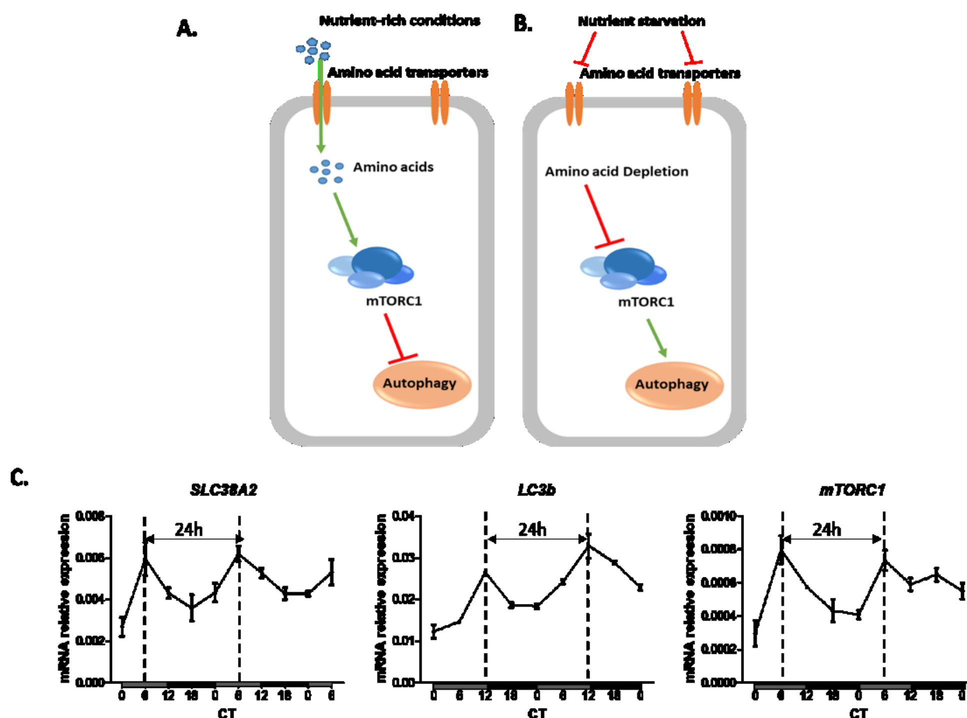


Figure 41: Circadian expression of genes involved in the regulation of amino acid homeostasis. A: Under nutrient rich conditions amino acid transporters mediate transportation of amino acids into the cell, expression of mTORC1 is activated and autophagy is inhibited. B: Under nutrient starvation the expression of mTORC1 is inhibited, whereas the expression of autophagy genes is activated. C: Expression of SNAT2, mTORC1 and LC3b1 shows circadian rhythmicity in DD. Relative mRNA levels are plotted on the y-axis. Each time point on the x-axis represents the mean ± SD (n=3). Statistical analysis was performed using t-test and 2way ANOVA.

4.3.7 Infradian expression of asparagine synthetase in DD

In the next step we investigate the role of genes encoding enzymes involved in amino acid synthesis. Specifically, we focused on asparagine synthetase (ASNS), that generates asparagine from aspartate. The transcription of *asns* gene is activated during amino acid starvation through binding of ATF4 to AARE enhancer sequences located in the *asns* gene promotor. To our surprise we observed that the expression of *asns* showed infradian rhythmicity in DD (Figure 42 B), indicating that this gene may be a possible candidate involved in the generation of infradian rhythms. Is the circadian clock mechanism involved in the regulation of *asns* expression? In order to explore this question, we analysed the expression of *asns* in the cell line expressing the *Δclock1* gene. In *Δclock1* cells the infradian expression of *asns* was disrupted (Figure 42 C), indicating that the circadian clock does indeed play a regulatory role in the control of *asns* function.

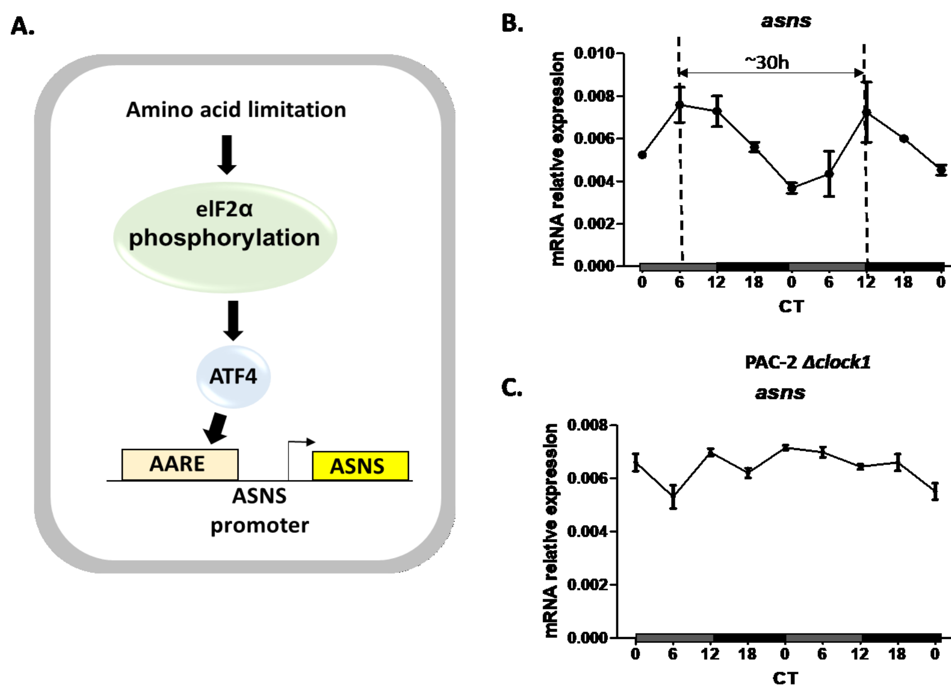
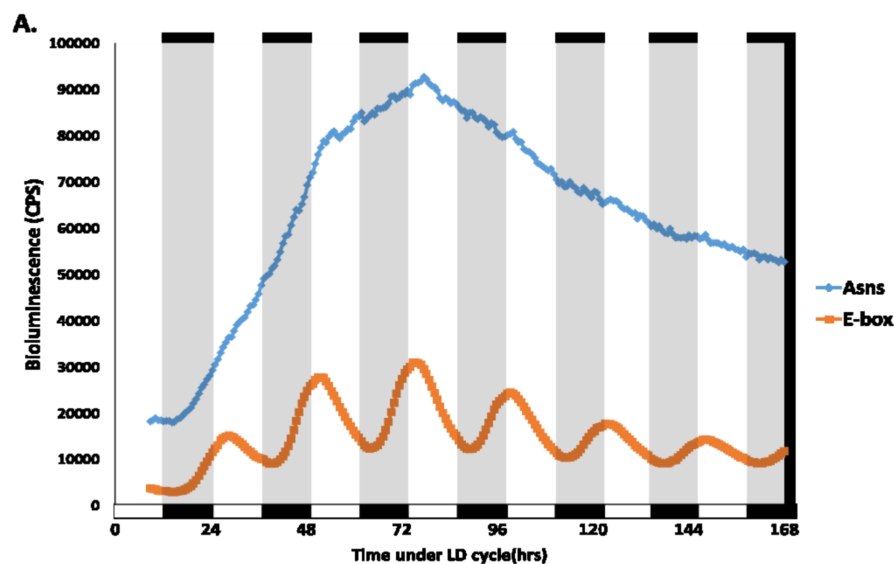


Figure 42: Expression of *asns* in PAC-2 WT and *Δclock1* cells. A. Under conditions of amino acid limition, elf2α is phosphorylated. Then it mediates the binding of ATF4 to the AARE enhancer in the promoter region of the *asns* gene, thereby activating its transcription. B: Infradian expression of *asns* in WT cells in DD conditions. C: Disrupted infradian expression of *asns* in *Δclock1* cells. The time of each sample is indicated by circadian time (CT). Relative mRNA levels are plotted on the y-axis. Each time point on the x-axis represents the mean ± SD (n=3). Statistical analysis was performed using t-test and 2way ANOVA.

4.3.8 Arrhythmic luciferase expression driven by the *asns* promoter

To explore whether the *asns* gene plays a role by the regulation of infradian rhythms in amino acid levels, we cloned a predicted promoter region of 1,199 kb lying 1,703 kb upstream of the *asns* translation start site, which is localised in exon 2, into a luciferase reporter vector, generating the construct, zf *asns*-Luc. Using Genomatix prediction software we identified an amino acid response element (AARE), the binding site of ATF4, localised in the promoter region of *asns* 1,703 kb upstream of the translation start site. The mammalian promoter contains nutrient sensing response elements I and II (NSRE-I and II), which are located 11 bp distant from each other (Barbosa-Tessmann et al., 2000). Together they function as an enhancer and regulate the transcription of *asns* in response to ATF4 binding (Chen et al., 2004). The human NSRE-I elements share high sequence identity with the AARE within the zebrafish *asns* promoter.

Using a transient transfection assay in PAC-2 cells we were not able to detect circadian or infradian rhythms in promoter expression under LD cycles or DD conditions (Figure 43). As the positive control we used *4xE-box$_{per4}$-Luc* which contains four multimerized copies of an E-box enhancer element located in the zebrafish *per1b* promoter and that is therefore transcriptionally regulated by the core mechanism of the circadian clock. These findings may indicate that our selected *asns* promoter sequence may be too short and thereby lacks key enhancer elements that are essential for directing the observed infradian expression rhythms.



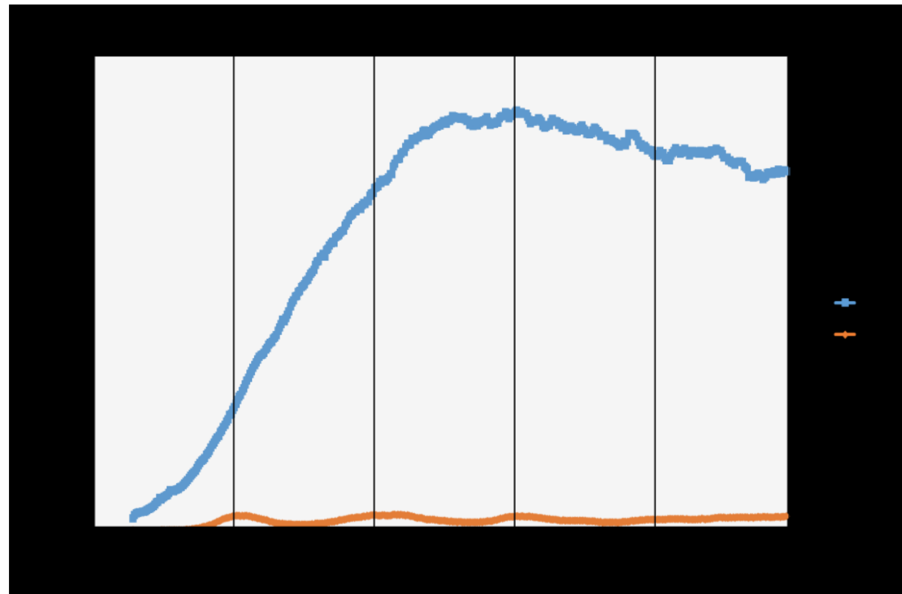


Figure 43: Arrhythmic expression of the *asns* promoter in PAC-2 cells. PAC-2 cells transfected with *zf asns*-Luc (blue trace) and *zf 4xE-box*$_{per4}$*-Luc* (orange trace, control) were exposed to LD (A) cycles and incubated in DD (B). On the y-axis are plotted the bioluminescence levels (counts per second), cps) and on the x-axis is plotted the zeitgeber time (ZT0 is lights on). Each rhythm is plotted as a mean of n=8 independent wells. The graphic is a representative experiment of three independent biological replicates.

In addition, we constructed an AARE reporter construct, which contains three multimerized copies of AARE sequence (5'–GTTTCATCA-3') from the *asns* promoter of zebrafish. The construct was transient transfected into PAC-2 cells and reporter gene expression was analysed under exposure to LD cycles and in DD. Again, we could not observed any rhythmic expression of the AARE reporter (Figure 44).

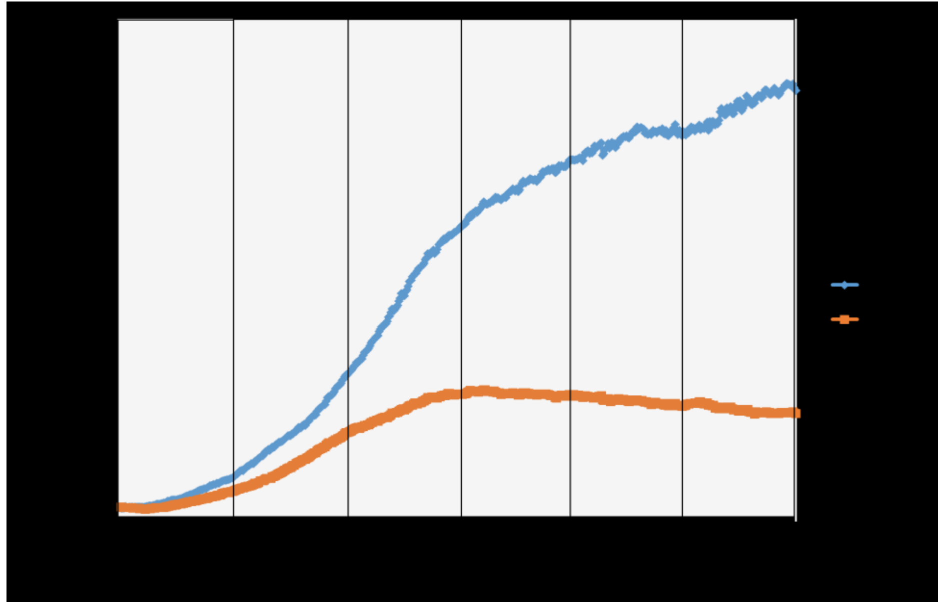


Figure 44: Arrhythmic expression of the *3xAARE$_{asns}$-Luc* in PAC-2 cells. PAC-2 cells transfected with *zf 3xAAREasns-Luc* (blue trace) and *zf 4xE-boxper4-Luc* (orange trace, control) were exposed to LD (A) cycles and incubated in DD (B). On the y-axis are plotted the bioluminescence levels (counts per second, cps) and on the x-axis is plotted the zeitgeber time (ZT0 is lights on). Each rhythm is plotted as a mean of n=8 independent wells. The graphic is a representative experiment of three independent biological replicates.

5. Discussion

5.1 Ontogeny of clock function and feeding behaviour: the influence of zeitgebers

To study the origins and regulation of the circadian clock system during early development in placental mammals is technically very challenging. For this reason, the zebrafish represents an excellent alternative model for studies of circadian clock ontogeny. However, the ontogeny of the molecular mechanism whereby the circadian clock is regulated by different zeitgebers still remains poorly understood.

By exploring the role of feeding in the development of the circadian clock system we have revealed that prey capture behaviour of zebrafish hatchlings is dependent on the wavelength of light. Moreover, by comparing clock gene expression between *in vivo* and *in vitro* models we observed that the clock system is more sensitive to long wavelength light in hatchlings than in cultured cells. Surprisingly light outside the visual range can induce clock gene expression both in zebrafish larvae as well as in cultured cells. Unfortunately, in our studies we were not able to explore how restricted feeding affects the development of circadian clock system. Indeed, we showed that the handling of zebrafish embryos plays a role in the development of clock gene expression. Furthermore, we revealed that the regular change of fish water serves as a stress factor, which activates the glucocorticoid signalling pathway and thus regulates clock gene transcription. Taken together our studies indicate that stress like light and temperature, represents an important zeitgeber for the clock during embryonic development.

5.1.1 Prey detection under exposure to different light sources

One major goal of our study was to investigate whether regular feeding time is able to entrain the clock from the very onset of feeding behaviour and how this compares with the emergence of light-entrainable clock function. Our starting point was to explore the differences in feeding behaviour between hatchlings exposed to light-dark cycles and maintained in constant darkness but with regular feeding time. A key prerequisite for this experiment is that feeding is possible in complete darkness. We explored how light influences the emergence of feeding behaviour in zebrafish larvae. Our studies revealed that feeding in larvae exposed to DD was significantly lower than in light dark cycle conditions, a potential complication for testing the effects of regular feeding on clock entrainment. An alternative strategy would be to select lighting conditions where the larvae were still able to feed, but which alone were unable to entrain the

clock. One of the most commonly used experimental approaches in circadian biology is to use exposure to dim red light (such as would be used in a photography dark room) as a way to enable the researcher to see clearly enough to accurately perform experimental work but which should not influence the clocks of the animal models under investigation. We therefore specifically tested whether exposure to long wavelengths of light, within and beyond the visible spectrum (red or infrared wavelengths), would influence feeding and whether this might also influence clock entrainment. We showed that there are no significant differences in consumption rate of *Paramecium* between hatchlings exposed to white light and dim red light (675 nm). On the other hand, under exposure to far IR light (850 nm) like DD, feeding activity was poor. In terms of clock entrainment, dim red and white light dark cycles efficiently entrained the larval clock, while far infrared light cycles resulted in rhythmicity in the expression of some but not all clock genes.

Zebrafish is a diurnal animal and their feeding behaviour is highly dependent on vision (Nikolaou and Meyer, 2015). Visual information is crucial for animals because it helps to identify food, predators and to orient in the surrounding environment. Visual light detection depends on opsins, which are G protein coupled receptors (GPCRs) covalently bound to a vitamin A-derived chromophore (Terakita, 2005). The zebrafish retina contains two photoreceptor types, rods for dim light vision and cones for bright-light vision. The zebrafish cones express 8 cone opsins. Two of them are sensitive to red light, *lws1* and *lws2*, with maximal absorbance (λ_{max} 558 nm) (Chinen et al., 2003). The maximum spectrum absorbance (λ_{max}) of cones depends on the interaction between opsins and chromophores. One mechanism which can shift spectral sensitivity to longer wavelengths is transition from vitamin A1 to vitamin A2 (Allison et al., 2004). This transition can be triggered by an endocrine signal, namely thyroid hormone (TH) through the enzyme Cyp27c1 or by colder water temperature (Allison et al., 2004; Enright et al., 2015; Mackin et al., 2019; Saszik and Bilotta, 1999). In addition, TH regulates not just transition from vitamin A1 to A2 but also the expression of long-wavelength-sensitive opsins in red cone photoreceptors (Suzuki et al., 2013). It is likely that the switch from vitamin A1 to A2 are important for the adaptation to changing environmental conditions, providing the opportunity to adjust its spectral sensitivity to more closely match natural environmental illumination.

The visual system is ranking as the most energetically costly systems in the brain. In order to save the energy zebrafish larvae reduce their visual function at night through depolarization of the photoreceptors (Emran et al., 2010), leading to a reduced rate in prey consumption in the absence of light. Moreover, visual sensitivity undergoes circadian regulation in zebrafish larvae

(Emran et al., 2010), suggesting that feeding activity is regulated not just by the external cues but also through endogenous circadian clock system. Clearly the absence of visible wavelengths of light results in an overall reduction in feeding efficiency.

However, the larvae do succeed to eat some of the paramecia under these conditions. We hypothesise that during the first hours after addition of *Paramecium*, the larvae catch and consume *Paramecium* just by accident, because of the high density of *Paramecium*. In this regard, zebrafish as many other fish possess highly developed sense organs. Besides the colour vision, they have auditory, lateral line and olfactory systems which could be used for prey detection in the darkness. The lateral-line system (LLS), which is made of a series of mechanoreceptors called neuromasts is morphologically and functionally similar to mammalian hair cells (Dijkgraaf, 1963; Nicolson, 2005; Westphal and O'Malley, 2013). The LLS is located along the body axis and is used to sense the water flow produced by the prey and to reorient towards the oncoming flow. This behaviour is better known as rheotaxis and is essential for prey detection in the absence of light (Suli et al., 2012). In addition, the olfactory system could also play an essential role by the detection the prey in darkness, where food-associated odors leads to activation of hypothalamic regions, which regulates appetite control, and in turn induce foraging behaviour (Wakisaka et al., 2017).

5.1.2 Light sensitivity is different between in vivo and in vitro models

In mammals the retina is essential for light perception and entrainment of circadian clock system. On the other hand, zebrafish can perceive photic signals not just through the retina but also directly through peripheral tissues, allowing the direct synchronisation of the internal clocks by light. Zebrafish possess multiple opsins, respectively 32 non-visual and 10 visual opsins, which sense light for visual and non-visual functions, including regulation of core clock, oxidative stress, mitochondrial function, and heme metabolism (Davies et al., 2015; Weger et al., 2011). Some non-visual photoreceptors are crucial for the integration of light signals to control behaviour, including photokinesis and photomotor responses in zebrafish larvae and locomotor activity patterns in *Xenopus* tadpoles (Currie et al., 2016; Fernandes et al., 2012). Importantly the photoreception system in zebrafish can sense not just the changes in light intensity but also different wavelengths.

By comparing the effects of illuminating zebrafish hatchlings and cultured cells with white, red and far IR light we have revealed key differential responses at the levels of gene expression. We demonstrated that red and far IR light play different roles in the entrainment of clock gene expression in *in vivo* and *in vitro*. We observed that the there are no significant differences in

the expression patterns of *per1b*, *per2* and *cry1a* between groups exposed to red- and white light cycles. Surprisingly, we observed that far IR light could also entrain rhythmic expression in a subset of clock genes. In cultured cells we could not detect significant differences in the expression patterns of *per1b* and *cry1a* upon exposure to red and white light cycles. However, the expression of *per2* was induced 4 times higher during exposure to white light compared to red light. Moreover, the effect of far IR light on *per1b* expression in PAC-2 cells is 4 times lower compare white light. How can red and even IR light differentially regulate the increase in the transcriptional levels of particular clock genes? We speculate this represents an important clue as to which photoreceptor molecules are involved in the detection of light and which clock gene targets are regulated in response to these photoreceptors. Thus our results may point to the presence of multiple light-regulated input pathways impinging on the expression of different clock genes. In this regard, how might zebrafish cells sense IR light? It could be through mitochondrial redox signalling, which could be activated by cytochrome c oxidase or light/heat gated ion channels (Hamblin, 2018). In *Drosophila* some opsins have been implicated as thermosensors (Shen et al., 2011), suggesting that in zebrafish some opsins may also act as IR or thermal sensors..

Unexpectedly, our study has also revealed that PAC-2 cells are less sensitive to red and far IR light than hatchlings. In the larval stages tested, the retina is still not completely developed with the expression of the long wavelength opsin *lws1* being absent; onset of its expression occurs in the ventral retina at approximately 6 d post-fertilization (6 dpf) (Takechi and Kawamura, 2005). Beside visual opsins, non-visual opsins clearly play an important role in the detection of light signals. Skin is one of the most important sites for photoreception in the zebrafish which directly receives light signals and presents a large surface area for photon capture. In the skin non-visual photoreceptors may occur in pigment-containing cells, the chromophores. Although, the skin pigmentation begins at approximately 24 hpf (Kazimi and Cahill, 1999), suggesting that PAC-2 cells (derived from 24 hours old embryos) may lack expression of some important non-visual opsins. However, taken together our data indicate that for the entrainment of circadian clock gene expression by long wavelength light, both visual- and non-visual photoreceptors may play an important role. However, the photosensory pigments and pathways involved in the detection of light still remains to be determined.

5.1.3 Entrainment of circadian clock system by stress

In our studies, we revealed that regular handling results in the entrainment of rhythmic clock gene expression. Handling may be sensed by fish in a similar way to predator attack, which

leads to activation of stress signalling pathways. The primary stress indicator in the fish is the GC hormone, cortisol, which typically increases immediately after exposure to various stressors (Wendelaar Bonga, 1997).

We propose that the change of fish water activates the HPA axis by signalling the hypothalamus to release CRH. Then CRH stimulates the release of cortisol from inter-renal cells. We observed that this reaction is very rapid. Already 5 min after the fish water was changed the whole-body cortisol levels reached their peak. To examine whether the changes in cortisol levels regulate the transcription of clock genes via the GC signalling pathway, we tested the impact of handling on the zebrafish transgenic reporter line, GRE:Luc. We observed that bioluminescence started to significantly increase about 2 h after the fish water was changed, showing that cortisol regulates clock gene expression via GRE localised in their promoter region. Interestingly the group of Dickmeis showed that in GRE:Luc larvae the cortisol levels increases 20 min after the treatment with 250 mM NaCl and bioluminescence increased 4 h after the treatment (Weger et al., 2012). Moreover, in this previous report the salt stress treatment increased bioluminescence in 5dpf hatchlings (Weger et al., 2012), whereas in our experiments, we observed the effects of handling stress on younger, 3dpf larvae. These results suggest that the change of fish water represents either a stronger stress factor than salt stress or during the change of fish water, additional stress pathways are activated that result in a more rapid GC-based transcriptional response.

It is interesting to speculate whether hypoxia may also play a role in the entrainment of rhythmic clock gene expression through handling. The change of fish water could lead to short-term deprivation of oxygen since this step involved the removal of almost all the water that the larvae were contained in. At this moment, the amount of oxygenated water that the larvae would be exposed to might be reduced to a minimum and so the larvae could experience a transient reduction in levels of oxygen in their cells and tissues. Subsequently, the addition of fresh water would result in a rapid restoration of oxygen levels in the larvae. Hypoxia potentially represents a pathophysiological condition. In order to prevent metabolic damage and death, the organism needs to respond very rapidly to the decreased levels of the oxygen. Oxygen levels in cells are sensed through the hypoxia inducible factor pathway. The Hypoxia Inducible Factor-1 (HIF-1) binds to the hypoxia response element (HRE) located in the promotor or enhancer regions of its target genes. HIF-1 also regulates the expression of clock genes through binding to E-box enhancers. Moreover recent studies have shown that HIF-1 interacts with glucocorticoid signalling in zebrafish (Marchi et al., 2020). However, the interaction between both pathways remains to be explored.

5.1.4 Entrainment of the clock by stress is dependent on development stage

Regular stress during the first 2 days of development fails to establish a circadian rhythm. The entrainment of *per1b* expression was observed between 3-4 dpf. In contrast LD cycles can induce the expression of *per1b* already in 27 hpf zebrafish larvae (Froland Steindal and Whitmore, 2019) . Moreover in PAC-2 cells the expression of clock genes is entrained already after one LD cycle (Whitmore et al., 2000). The ability to sense light appears very early during development, between 6-9 hpf (Tamai et al., 2004), whereas the ability to synthesize cortisol appears around 48 hpf (Alsop and Vijayan, 2008, 2009). Moreover stress-induced increase in cortisol levels occurs after the development of HPA-axis, around 97 hpf (Alsop and Vijayan, 2008, 2009). Furthermore, the sensitivity to hypoxia begins in later stages of development, around 24 hpf (Robertson et al., 2014). This evidence suggests that an interaction between both light and stress pathways may regulates the entrainment of clock gene transcription. The timing of the maturation of each pathway during early development in turn shapes the ontogeny of the entrainment of the clock by these zeitgebers during development.

5.1.5 Clock output mechanisms in the developing embryos

In the previous studies of our group, it was demonstrated that the circadian clock is involved in the regulation of the cell cycle, in particular the timing of S-phase (Dekens et al., 2003; Idda et al., 2012). Importantly the cell cycle can be synchronized not just by light-dark cycles, but also by temperature-cycles (Lahiri et al., 2014), meaning that the cell cycle is a circadian clock output. Given that stress, like light, can entrain clock function, we propose that stress may also play a role in regulation of cell cycle via its effect on the clock. The effect of stress on the rhythmic expression of *p21* was observed first in 5 dpf larvae, whereas rhythmic expression of *CyclinA2* was entrained at 4 dpf. We also analysed the effect of stress on cell proliferation. Preliminary data suggests that the effect of stress on cell proliferation is comparable with light-dark cycles. However, this conclusion should be taken with caution, because in this study the numbers of S phase-positive nuclei in 6dpf larvae were relatively low compared with previous studies from our group (Dekens et al., 2003; Lahiri et al., 2014). These differences may come either through technical problems or the reflect general differences in the rate of development of the different sets of animals. It is possible that due to repeated sibling crossing the cell proliferation properties of the offspring were altered, which affected the results of our studies. For this reason, in the future it may be important to outcross our zebrafish lines and then to repeat this study.

5.2 Metabolomics: interaction with multiple clocks.

The molecular mechanism through which the circadian clock integrates information from light and food availability and how it in turn regulates metabolism remains poorly understood. Comparative studies of circadian timing system involving the zebrafish *in vivo* and *in vitro* models, as well as a cave-adapted animal, the Somalian blind cavefish *P. andruzzii*, has enabled us to reveal that at least two distinct clock mechanism coexist in fish cells. The data presented in my thesis indicate that in zebrafish PAC-2 as well as in cavefish CF-1 cells the changes in amino acids levels are infradian. The candidate gene approach suggests that Asparagine synthetase (ASNS) and Activating transcription factor 4 (ATF4) are involved in the regulation of these rhythms. Moreover, we revealed that feeding time does not affect the timing within the classical core circadian clock mechanism TTFL. However, it does play a major role in the regulation of amino acid metabolism. We speculate that feeding regulates amino acid homeostasis through the NAD+-salvage pathway. Our results are also consistent with mammalian studies which show that desynchronization of the circadian clock leads to the development of metabolic disorders, specifically obesity. Additionally, in a comparative study involving blind cavefish and zebrafish cells as a model, we hope to explore multiple clock mechanisms underlying the temporal control of metabolism.

5.2.1 TTFL in zebrafish liver is regulated by light

In our studies we revealed that restricted feeding time plays a major role in the regulation of metabolism, whereas the light-dark cycle regulates the expression of core clock genes. However, the findings of our studies do not support previous research, which showed that restricted feeding can reset the phase of clock gene expression in mouse liver, independently of LD-cycles (Damiola et al., 2000; Mukherji et al., 2015). These data indicate that the molecular mechanisms whereby the hepatic clock responds to LD-cycles and feeding time are distinct and possibly species-specific. Furthermore, it supports the idea that in zebrafish liver the FEO and LEO are genetically and functionally distinct.

5.2.2 Amino acid metabolism is regulated by the feeding time

Previous NMR data from our lab showed that apparently cycles in the level of essential amino acids in the liver were regulated by feeding time, whereas cycles of non-essential amino acid levels appear to be regulated by the timing of the light-dark cycle. However, using UPLC-MS/MS we were not able to confirm these results. We observed that the cycles of both essential

and non-essential amino acids are regulated by feeding time and not by light-dark cycles. We speculate that one explanation for the differences between the results obtained with these two assays may be caused by genetic differences between the WIK and Tübingen lines of zebrafish used in these two sets of studies.

An alternative possible explanation may relate to differences in the gut microbiome between the two strains which might affect the interaction between TTFL and amino acid metabolism. Gut microbes use amino acids for many aspects of their metabolism, which comes from food or are synthesised by the host (Wikoff et al., 2009). Amino acids can be directly incorporated in the bacterial cells or may be catabolized (Neis et al., 2015). Moreover, the gut microbiome can produce some essential amino acids, which can be used by the host, and plays an important role in the regulation of amino acid homeostasis (Fan et al., 2015; Metges, 2000). The disruption of the gut microbiome can lead to the development of obesity and type 2 diabetes in humans (Ley et al., 2006; Qin et al., 2012). Moreover, bacterial metabolites, specifically short chain fatty acids (SCFA) and amino acids, play important roles in the regulation of host physiology (Sridharan et al., 2014). For example, studies in mice indicated that gut bacteria alter the distribution of free amino acids in the gastrointestinal tract, consequently affecting the bioavailability of amino acids for the host (Macfarlane et al., 1988). The gut microbiome can also regulate the liver circadian transcriptome through the production of a myriad of metabolites, including amino acids and nucleotides (Thaiss et al., 2016). The oscillating chromatin modifications in the liver can be regulated through butyrate, what is a short-chain fatty acid (SCFA) produced by bacterial fermentation (Leone et al., 2015). The gut microbiome can alter clock gene expression through unconjugated bile acids, specifically deoxycholic acid (DCA) and chenodeoxycholic acid (CDCA) (Govindarajan et al., 2016). Intriguingly, the circadian clock can also regulate the microbiome. For example, in *Per1*$^{-/-}$ *and Per2*$^{-/-}$ double knockout mice the function of the microbiome is dramatically altered (Thaiss et al., 2014). This evidence indicates that amino acid metabolism is regulated through a bidirectional link between the gut microbiome and the host circadian clock. An analysis of, and comparison between the microbiome of the WIK and Tübingen fish used in these experiments would ultimately be required in order to test this explanation.

5.2.3 NAD+ - biosynthesis pathway a possible link between feeding-regulated clock and amino acid metabolism

In order to investigate the mechanism which links the circadian clock with amino acid metabolism, we analysed the role of the NAD+ biosynthesis pathway. NAD+ is an essential

molecule in metabolism and maintaining the energy homeostasis in cells. Moreover, NAD+ plays an important role in regulating circadian transcription through an epigenetic mechanism and signal transduction and thereby enables the circadian clock system to adapt to changing cellular metabolic status. For example, the rhythmic changes in hepatic NAD(P)+/NAD(P)H ratio are regulated by the feeding-fasting cycling, in turn it modulates the DNA-binding activity of the core oscillators, such as CLOCK: BMAL1 and NPAS2: BMAL1 heterodimers (Rutter et al., 2001). Our data point to *nampta*, which encodes the rate-limiting enzyme in the NAD+ biosynthetic pathway, being rhythmically expressed according to the timing of feeding entrainment. SIRT1, a NAD+-dependent histone deacetylase, is another important enzyme that catalyses the conversion of NAD+ to form NAM within the NAD+ salvage pathway. It has been reported that SIRT1 can interact with the CLOCK/BMAL1 dimer directly and that the SIRT1-CLOCK/BMAL1 complex can modulate chromatin remodeling and exert NAD+-dependent regulation (e.g. Nampt expression) in mammals. In our fish study, the expression of the *sirt1* gene is also significantly influenced by feeding entrainment. This result indicates that SIRT1 and its cofactor NAD+ may not only represent links between energy metabolism and circadian function, but may also serve as links between the LEO and FEO.

5.2.4 Timed feeding regulates autophagy

Another important mechanism playing a role in the regulation of cellular amino acid homeostasis is autophagy. Autophagy is a “self-degradation” processes, which is stimulated by various stressors, including nutrition deprivation (Rabinowitz and White, 2010). Previous studies indicated that autophagy undergoes circadian regulation in both mice and zebrafish livers (Huang et al., 2016; Ma et al., 2011). The daily changes in the numbers of autophagosomes and autolysosomes as well as transcription of the genes involved in autophagic activity follow circadian rhythms, that aligns with the feeding time (Huang et al., 2016). In our studies, we analysed the expression of mTORC1, a key player of nutrient sensing and signalling, which controls the activity of autophagy. We were able to show that in the zebrafish liver the transcription of mTORC1 is regulated by the feeding-regulated clock mechanism. Circadian regulation of *LC3*-I/II, a widely used biomarker in autophagy research, was also observed. These studies are consistent with many previous reports that have demonstrated links between autophagy and the circadian clock. For example, TALEN-mediated mutation of the nuclear hormone receptor *nr1d1* has been shown to upregulate the expression of clock and autophagy genes in zebrafish liver (Huang et al., 2016). Moreover, the loss of *per1b* as well as fasting also leads to upregulation of the transcription of autophagy genes (Huang et al., 2016).

On the other hand, autophagy regulates the hepatic circadian clock system by degradation of CRY1 protein in a time-dependent manner, in turn regulating the daily rhythms of cellular glucose levels (Toledo et al., 2018). Thus, our results clearly indicate that autophagy is regulated by a clock which is set by feeding time and not by light-dark cycles. These results suggest that at least two distinct clock mechanism exist in zebrafish liver.

5.2.5 Desynchrony between FEO and LEO leads to development of metabolic disruption.

Under natural conditions, light and food regulated clocks are likely to be synchronized, since feeding time correlates with the period of maximum locomotor activity. However, the uncoupling of feeding time from the light dark cycle as happens frequently in human society, tends to uncouple light regulated clocks from food regulated clocks, leading to the development of metabolic disease. Feeding at unusual times of the day, specifically during an animal's phase of inactivity, affects body weight gain, adiposity and other metabolic parameters (Moran-Ramos et al., 2016). For example, mice fed during the sleep phase gained more weight and increased adiposity compared with mice fed during the active phase (Yasumoto et al., 2016). Consistently, fish fed during the normal rest phase (so at night) gain weight 1,5 time faster than fish fed during their normal active phase. One of the key players linking the circadian clock with metabolism is peroxisome proliferator-activated receptor α (PPAR*α*). PPAR*α* belongs to the group of nuclear receptors that function as transcription factors activating the expression of a number of genes, involved in various metabolic pathways and plays an essential role in the regulation of energy homeostasis (Wu et al., 2009). Furthermore, PPAR*α* also plays an important role in the development of obesity and other metabolic disorders (Braissant et al., 1996; Cheng and Leiter, 2008). Moreover, PPAR*α* regulates the activity of CLOCK and BMAL1 through directly binding to the E-box (Oishi et al., 2005) and it may also directly interact with PER2 (Schmutz et al., 2010). *P*PAR*α* mRNA is induced during fasting and coordinates fatty acid synthesis (Kersten et al., 1999). The restricted feeding during the rest phase uncouples clock genes, *Bmal1, Per1, Clock* from *Pparα*, within the liver and in turn it contributes to the development of obesity (Salgado-Delgado et al., 2013). In addition, feeding during the inactive phase leads to decrease in leptin sensitivity (Yasumoto et al., 2016). Leptin is involved in the regulation of energy homeostasis and regulates food intake and body weight (Klok et al., 2007). Leptin resistance leads to the development of obesity and other metabolic disorders (Yasumoto et al., 2016).

Taken together these results suggest, that not only the diet composition but also the time of feeding is a major factor contributing to the development of obesity.

5.2.6 Regulation of amino acid rhythms in PAC-2 cells

By performing UPLC-MS/MS measurements we revealed a very interesting and surprising phenomenon concerning the dynamics of amino acid metabolism in a zebrafish cell line. In PAC-2 cells lacking any regular input by external zeitgebers, we observed that levels of amino acids exhibited infradian rhythmicity. In order to understand whether the core circadian clock mechanism plays a role in the regulation of infradian rhythms, we analysed the levels of amino acids in cells expressing *Δclock1* gene. In this cell line the normal function of the clock TTFL is inhibited by over-expressing a dominant-negative (DN) mutant *CLOCK1* protein (Tamai et al., 2012). This protein lacks the C-terminal glutamine-rich activation domain, but still retains functional PAS and bHLH domains at its N terminus. *Δclock1* is able to form a heterodimer with BMAL1 and bind to the E-box sequence in the promoter region of *per1b* and in turn blocks its transcription. Our analysis showed that rhythmic changes in amino acid levels in *Δclock1* cells were disrupted, implying that the core clock may indeed play a role in the regulation of infradian rhythms. These results lead to the hypothesis that in PAC-2 cells there may exist at least two distinct mechanisms, which together regulate the interaction between metabolism and core clock.

From our *in vivo* studies, we observed that *nampta* and *sirt1* are involved in the regulation of amino acid metabolism. For this reason, we also investigated the regulation of both genes in the context of the observed infradian rhythms. Gene expression analysis revealed that both genes were expressed with infradian rhythms. However, our promoter analysis results provided inconsistent results. We were unable to detect either circadian or infradian rhythmicity in transcription of these genes. It is possible that we had not included the full gene promoters in our analysis and so testing a larger promoter fragment may be an important future step.

It is also conceivable that the enzymatic activity of Nampt and Sirt1 rather than only by their mRNA levels may be involved in the generation of infradian rhythms. The activity of Sirt1 can be controlled by altering its mRNA and protein expression as well as by mechanisms independently of its expression levels. The catalytic activity of Sirt1 is dependent on the amount of its substrate. The key co-substrate of Sirt1 is NAD+. The levels of NAD+ can be regulated by nutrient intake as well as by biosynthesis. Mammalian cells can synthesize NAD+ via a *de novo* pathway, by metabolism of the essential amino acid tryptophan, or by a salvage pathway from nicotinamide (Belenky et al., 2007). This evidence shows that there is a bidirectional interaction between amino acid metabolism and Sirt1. It leads to a prediction that rhythms in amino acids regulate infradian expression of Sirt1 and Nampt.

It is important to consider that in mammals two distinct forms of NAMPT exist, intra- and extracellular (iNampt and eNampt, respectively) (Revollo et al., 2007). The role of iNampt as a rate limiting enzyme in the NAD+ salvage pathway is firmly established. However, its enzymatic activity in the extracellular place remains poorly understood. In the future we would like to perform a more detailed investigation of the function of eNampt in the regulation of infradian rhythms in amino acids.

5.2.7 Expression of amino acid homeostasis regulation genes

Cellular amino acid homeostasis is maintained by constant influx via the transporters localized on the plasma membrane, protein translation and metabolism as well as via autophagy. Our studies imply that neither the expression of amino acid transporters, nor autophagy plays a role in the generation of infradian rhythms in amino acid levels. Indeed, both pathways exhibit circadian regulation. In the subsequent studies we analysed the expression of the gene, which is involved in the synthesis of amino acids. In particularly we focused on the expression of Asparagine synthetase (ASNS). *asns* is expressed as a housekeeping gene and generates asparagine from aspartate. The expression of this gene is activated by amino acid starvation through the GCN2-eIF2α-ATF4 (general control nonderepressible 2-eukaryotic initiation factor 2-activating transcription factor 4) pathway (Barbosa-Tessmann et al., 2000; Siu et al., 2002). ATF4 is a transcription factor which binds on AAREs (amino acid response elements) in the promoter region of *asns* and activates its transcription (Fujita et al., 2007). We observed that the expression of *asns* shows infradian rhythmicity in DD which is abolished in *Δclock1* cells. We predict that *asns* and its transcription factor ATF4 may be involved in the regulation of amino acid levels and so predicted that transcription of this gene might display infradian rhythmicity. However, we were unable to confirm this hypothesis by our promoter analysis studies. This may reflect that the promoter fragment that we tested did not include all the necessary enhancer elements. For example, enhancers might also be located internal to the structure of the gene, in intron sequences which were not represented in our reporter construct. Alternatively, the state of the cells following the treatments that are necessary for cell transfection may have attenuated the mechanisms driving rhythmicity in amino acid levels. In our future studies we plan to clone larger promoter fragments of the *asns* gene and to study its regulation in transiently transfected zebrafish cell lines to at least exclude the possibility that we have not yet characterized the complete promoter.

5.2.8 Alternative clock mechanisms

Much attention has been focused on the possibility that TTFL mechanisms are not essential for the generation of circadian rhythmicity and that other mechanisms which do not involve transcription, may also play a role. Thus, it has been described that circadian oscillations in the redox state of peroxiredoxin represents an additional non-transcriptional timekeeping mechanism (O'Neill et al., 2011). We speculate that the observed infradian rhythms could be regulated by such a non-canonical mechanism involved in redox homeostasis. Interestingly, recent studies using a *Drosophila* clock mutant line where the function of the core circadian clock TTFL mechanism has been disrupted, indicated that besides redox rhythms a number of metabolites and transcription factors continued to show circadian oscillation independently of TTFL (Rey et al., 2018). However, the novel mechanism which regulates these rhythms remains to be identified. A potential candidate could be the kinase DBT and its mammalian homolog CKI, which is not only critical for period determination of the canonical TTFL-based clock but which also controls the peroxiredoxin cycle period (O'Neill et al., 2011). Interestingly multi-omic analysis in mouse liver indicated that the links between rhythmic transcripts and rhythmic proteins can be uncoupled. Specifically, some proteins encoded by mRNAs which are not rhythmically expressed, still showed daily oscillation in their levels, suggesting that translational or posttranslational regulation also plays an important role in generating rhythmicity (Mauvoisin et al., 2014). It is interesting to speculate that these post transcriptional control mechanisms might also play a role in conferring the infradian rhythms in amino acid levels that we have observed in the fish cell lines.

5.2.9 Metabolic adaptations in cavefish cell lines

The Somalian blind cavefish (*Phreatichthys andruzzii*) represents another group of fish eminently well suited for studying light- and feeding-regulated clocks. During evolution in constant darkness over millions of years, these fish have developed an abnormal circadian system, with a clock which ticks with a characteristically extended period length and thereby is associated with infradian rhythms of cycling mRNA levels of core circadian genes (Cavallari et al., 2011). We have now been able to extend this original observation to reveal that cavefish-derived cell lines also show infradian rhythms of amino acid levels, interestingly comparable in timing to the infradian rhythms of these same metabolites observed in the zebrafish cell lines. To discuss the physiological significance of these dynamic changes in the cavefish metabolome, it is important to consider the biology of these cave animals. Due to the lack of plant life in their constant dark habitats, these animals have adapted to life in a nutrient-poor environment where

their very limited food supply is either periodically washed into the cave water systems from outside streams and rivers or is based on the limited ecosystem of other cave-adapted organisms that exist in the closed cave environment. These adaptations include important changes to metabolism. Specifically, the overall levels of metabolism are typically reduced as a way to economize the use of the limited food supply (Aspiras et al., 2015; Moran et al., 2014). These animals also show greatly enhanced lipogenesis and have extensive adipose tissue storage throughout their bodies (Xiong et al., 2018; Xiong et al., 2021). In this way, they are able to store excess nutrients when they are periodically available and then they can access this storage capacity when they experience periods of starvation. Loss of circadian rhythmicity may also represent a behavioural adaptation whereby the cavefish can increase the time frame over which they are able to search for food (Duboue et al., 2011; Moran et al., 2014; Yoshizawa et al., 2015). Our results add to our understanding of the metabolic adaptations in cavefish by showing that while the core circadian clock TTFL-based mechanism that is usually entrainable by light, has experienced considerable change in this cavefish, the clock mechanism which regulates infradian rhythmicity in amino acid levels in zebrafish cells, has been apparently conserved in the cavefish. Thus, the cavefish *P. andruzzii* provides more evidence for their being two genetically distinct clock mechanisms in vertebrates and provides a unique opportunity to explore the origin of infradian rhythms and to distinguish the function of the TTFL-based and non-canonical clock systems. However, the physiological significance of conserving infradian rhythmicity in amino acid levels remains completely unclear. It is tempting to speculate that there might be some evolutionary advantage for maintaining infradian rhythmicity in a constant dark cave environment – so explaining why the normal light entrainable TTFL mechanism now ticks with an infradian rhythm – but precisely why, is unclear.

5.3 Future perspectives

Our studies have provided new insight into a number of different fields and they set the stage for some new experimental approaches and projects in the future.

In our studies we have shown that feeding behaviour is strongly influenced by lighting conditions, in a wavelength-dependent manner that is presumably linked with visual function. Surprisingly we revealed that the photic spectral sensitivity of zebrafish extends beyond classical visual wavelengths of light. Interestingly, far infrared wavelengths of light are also able to differentially influence cycling clock gene expression. A surprisingly large repertoire of opsins has been identified in zebrafish, which may explain this wavelength sensitivity.

However, the functions of each opsin and how they may differentially interact with visual function and the circadian clock system remains very much unclear. Functional analysis of each opsin will clearly be an essential next step, involving possibly using CRISPR-Cas 9 to selectively generate loss of function alleles for subsets of particular opsin genes. In the future it would also be interesting to explore the mechanism, which zebrafish may detect prey independently of visual function. Using transgenic-based approach possibly with biosensors, we could investigate the relative role of the lateral line-, olfactory- and auditory systems in the regulation of feeding behaviour in darkness. Moreover, a comparative analysis between wild type animals and mutants with defects in visual function could provide us with a better understanding of the differential role of photoreception in feeding behaviour and entrainment of rhythmic clock gene expression.

In our study of the contribution of stress to the development of the circadian clock system, there are still many opportunities to refine our analysis. Our "fish handling" protocol which we use to induce stress in the zebrafish larvae, is far from ideal due to the many unavoidable problems to apply a uniform level of stress to each larva. To overcome this experimental limitation, we are planning to use a vibration assay. This involves placing the plate of larvae on top of an adapted loud speaker and thereby, being able to apply a well-defined sound frequency to the plate. This essentially involves vibrating the larvae and thereby inducing stress By this assay it would be possible to more accurately control the levels of stress experienced by each larva, and thereby allow us to explore in more depth the role of hypoxia and glucocorticoid signalling in regulating clock gene expression. To study the effects of stress on clock entrainment we also plan to use the clock-regulated luciferase reporter transgenic zebrafish line (Tg (−3.1) *per1b*::luc) where clock function can be measured more accurately and at higher resolution than by our current approach of preparing whole larval RNA extracts at fixed time points and then assaying gene expression levels by qRTPCR.

In our study of the circadian control of the zebrafish liver metabolome, we have revealed that cycling levels of amino acid levels are regulated by the feeding time, whereas the core circadian clock mechanism TTFL is regulated according to the timing of the light-dark cycle. Our next goal will be to investigate the genetic basis of these feeding- and light-entrained rhythms in the zebrafish. We plan to expand our study to document the light and food regulated transcriptome of the adult zebrafish liver. We will prepare RNA from 5 time points throughout one light dark cycle for two sets of fish, one fed exclusively at one time point in the middle of the light (day) period and the other fed exclusively in the middle of the dark (night) period in our conflicting zeitgeber experimental paradigm. Then we will compare transcriptome sequences from each

time point in the two sets of fish. In this way, we will identify which genes are rhythmically expressed with a phase set by the light dark cycle, and so which should cycle in an identical fashion identical between the mid light and mid dark fed sets of fish. Also, we will be able to identify which gene expression cycles according to when the animals have been fed – and so which may cycle out of phase between the mid day and mid night fed fish even though these animals have all been exposed to the same light – dark cycle. This invaluable dataset in comparison with the metabolome datasets, will allow us to pinpoint the key genes responsible for generating rhythmicity according to the light dark or the feeding cycle. This will provide a global view of the differential metabolome targets of the light and feeding regulated clocks. Furthermore, by comparing the promoters of genes that are rhythmically expressed according to the light-dark cycle or the feeding cycle using a bioinformatics approach, we will search for enhancer elements that are specifically associated with the promoters of genes which are expressed rhythmically according to the feeding time. Knowledge of feeding cycle associated enhancers and the transcription factors that bind to them will provide us with access to key regulatory elements downstream of the feeding regulated clock. We will then test the function of candidate feeding time-regulated enhancers by cloning multiple copies upstream of a minimal promoter directing expression of a luciferase reporter plasmid. The expression of these enhancer / reporter constructs we will initially be test by the generation of transgenic zebrafish lines.

Finally, in order to investigate the origin of the infradian rhythmicity in amino acid levels that we have observed in the zebrafish and cavefish cell lines we plan to use a similar comparative transcriptomics approach. We will collect a time-course of samples from PAC-2 and CF-1 cells over a 60 h hour sampling window at 3 h intervals under carefully controlled conditions and use these samples to perform RNA-seq, mass spectrometry-based proteomics and metabolomics analyses. By this approach we will be able to identify the genes and proteins which are regulated according to these infradian rhythms and so we would be able to better understand the broader functional significance of these metabolic rhythms. Knowledge of these infradian regulated genes may then allow us to identify shared transcriptional control mechanisms in terms of identifying "infradian enhancers" and the transcription factors which bind to them. Such an approach of course may not be informative in the event that these rhythms are generated via post transcriptional mechanisms. Additionally, by a comparative approach involving blind cavefish as a model, we hope to gain unique insight into how evolution under extreme environmental conditions can influence the mechanisms which time metabolism.

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Appendix

Table A: Optimized ESI-MSMS parameters and MRM transitions between precursor ions and product ions for each amino acid

	AA	Q1 (m/z)	Q3 (m/z)	DP (V)	CE (V)	CXP	RT (min)
Period 1							
	His	212.2	110.1	61	25	8	1.01
		212.2	83	61	45	8	
	Lys	203.23	84.1	66	31	6	1.20
		203.23	186.1	66	17	12	
	Asn	189.17	144.2	61	17	10	1.16
		189.17	74.1	61	27	6	
	Ser	162.2	60.1	56	23	4	1.31
		162.2	106	56	15	8	
	Gly	132.14	76.1	56	13	6	1.33
		132.14	57.2	56	19	10	
	Gln	203.17	186.1	41	13	12	1.31
		203.17	70	41	31	6	
	Arg	231.22	172.1	81	45	6	1.38
		231.22		81	23	10	
Period 2							
	Thr	176.22	74.1	61	21	6	1.65
			158.2	61	15	10	
	Ala	146.18	44	56	23	8	1.76
			90.1	56	13	6	
	Pro	172.2	70	66	31	6	2.02
			116.1	66	19	8	
Period 3							
	Val	174.2	72.1	56	19	6	2.48
			55.2	56	43	4	
	Tyr	238.19	136.1	66	25	10	2.47
			91.1	66	47	8	
	Met	206.2	104.1	66	19	8	2.56
		61					
Period 4							
	Isoleu	188.232	86.1	61	16	6	2.9
			69	61	33	6	
	Leu	188.21	86.1	66	19	6	2.96
			44.1	66	43	4	
	Phe	222.24	120	61	23	8	3.11
			102.9	61	49	8	
	Asp	246.22	144	76	19	8	3.28
			88	76	29	6	
	Trp	261.21	244.2	66	17	14	3.25
			132	66	39	12	
	Glu	260.19	83.9	71	37	6	3.38
			186.1	71	19	12	

Table B: Optimized MRM transitions between precursor ions and product ions for internal amino acid standards

	AA	Q1 (m/z)	Q3 (m/z)	RT (min)
Period 1				
	His-13C-15N	221.173	118.000	1.00
		221.173	89.000	
	Lys-13C-15N	211.241	90.100	1.19
		211.241	193.200	
	Asn-13C-15N	251.205	148.000	3.26
		251.205	92.000	
	Ser-13C-15N	166.161	63.100	1.31
		166.161	110.000	
	Gly-13C-15N	135.114	79.100	1.33
		135.114	57.200	
	Arg-13C-15N	241.192	75.000	1.36
		241.192	178.000	
Period 2				
	Thr-13C-15N	181.183	78.100	1.65
			60.200	
	Ala-13C-15N	150.193	47.000	1.76
			94.000	
	Pro-13C-15N	178.197	75.100	2.01
			122.000	
Period 3				
	Val-13C-15N	179.111	138.900	2.46
			60.900	
	Tyr-13C-15N	248.193	145.100	2.45
			97.900	
	Met-13C-15N	212.193	195.200	2.54
			90.1000	
Period 4				
	Isoleu/Leu-13C-15N	195.207	92.100	2.9 Isoleu 2.92 Leu
			46.400	
	Phe-13C-15N	232.164	129.100	3.08
			111.000	
	Asp-13C-15N	251.205	148.000	3.26
			92.000	
	Glu-13C-15N	266.181	89.100	3.36
			192.200	

Q – precursor ion, Q3 – produt ions, DP – declustering potential, CE – collision energy, CXP – cell exit potential, RT – retention time