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**From the Institute of Neurobiology
of the University of Lübeck
Director: Prof. Dr. rer. nat. Henrik Oster**

“The influence of zeitgeber intervention on the circadian clock system in mice”

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Isabel Heyde
from Forst, Germany

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First referee: Prof. Dr. rer. nat. Henrik Oster

Second referee: Prof. Dr. rer. nat. Jens Mittag

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Declaration

Herewith, I confirm that I have written the present PhD thesis independently and with no other sources and aids than quoted.

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Isabel Heyde

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	Curriculum Vitae	Fehler! Textmarke nicht definiert.

Abbreviations

A	Adenine (nucleobase)
AC	Adenylate cyclase
ACTH	Adrenocorticotrophic hormone
AgRP	Agouti-related peptide
ANS	Autonomic nervous system
AMPK	Adenosine monophosphate-activated protein kinase
ANOVA	Analysis of variance
ARC	Arcuate nucleus
ATP	Adenosine triphosphate
AVP	Arginine vasopressin
BAT	Brown adipose tissue
bHLH	Basic helix-loop-helix
Bhlhe40/41	Basic helix-loop-helix family, member e40/41, also known as Dec1/2
BMAL1	Brain and muscle aryl hydrocarbon receptor nuclear translocator-like protein 1 (clock protein), also known as ARNTL or MOP3
C	Cytosine (nucleobase)
Ca ²⁺	Calcium
cAMP	Cyclic adenosine monophosphate
CART	Cocaine- and amphetamine-regulated transcript
CBT	Core body temperature
CCG	Clock-controlled gene
cDNA	Complementary DNA
CK1δ/ε	Casein kinase 1 delta/epsilon
CLOCK	Circadian locomotor output cycles kaput (clock protein)
CNS	Central nervous system
cpm	Counts per minute
CRE	cAMP response element
CREB	cAMP response element-binding protein
CRY	Cryptochrome (clock protein)
DBP	D-box albumin promoter binding protein
DD	Constant darkness
ddH ₂ O	Double-distilled water
DEC1/2	Differentiated embryo chondrocyte protein 1/2
dH ₂ O	Distilled water
DLAN	Dim light at night
DMH	Dorsomedial hypothalamus
DNA	Deoxyribonucleic acid
dNTP	2'-Desoxyribonucleoside-5'triphosphate
<i>e.g.</i>	<i>exempli gratia</i> (Latin), for example
E4BP4	E4 promoter-binding protein 4, also known as NFIL3

E-box	Enhancer box (clock protein-binding DNA sequence)
EDTA	Ethylenediaminetetraacetic acid
eIF4E	Eukaryotic translation initiation factor 4 E
ELISA	Enzyme-linked immunosorbent assay
eWAT	Epididymal white adipose tissue
FAA	Food-anticipatory activity
FBXL3	F-box and leucine-rich repeat protein 3
FEO	Food-entrainable oscillator
FGF21	Fibroblast growth factor 21
FOXO3	Forkhead box class O3
G	Guanine (nucleobase)
G6pc	Glucose-6-phosphatase
GABA	γ -aminobutyric acid
GCs	Glucocorticoids
GCGR	Glucagon receptor
GH	Growth hormone
GLP1R	Glucagon-like peptide-1 receptor
GLUT4	Facilitated glucose transporter member 4
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
GRP	Gastrin-releasing peptide
GSK3 β	Glycogen synthase kinase 3 beta
h	hour
HDL	High-density lipoprotein
HFD	High-fat diet
HPA	Hypothalamus-pituitary-adrenal axis
<i>i.e.</i>	<i>id est</i> (Latin), that is
IGL	Intergeniculate leaflet
IL-6	Interleukin-6
i.p.	Intraperitoneal
ipRGC	Intrinsically photosensitive retinal ganglion cell
ISH	<i>in situ</i> hybridization
KO	Knockout
KPNB1	Karyopherin subunit beta 1
LD	Light: dark cycle
LD-12	Light: dark ('LD 12:12': 12 h light: 12 h dark)
LD-14	Light: dark ('LD 14:14': 14 h light: 14 h dark)
LH	Lateral hypothalamus
LHA	Lateral hypothalamic area
LL	Constant light
LTB	Lateral parabrachial nucleus
MAPK	Mitogen activated protein kinase

MCH	Melanin-concentrating hormone
min	Minute
mRNA	Messenger ribonucleic acid
mTOR	Mechanistic target of rapamycin kinase
mTORC1	Mechanistic target of rapamycin kinase complex 1
NAD ⁺	Nicotinamide adenine dinucleotide
Nampt	Nicotinamide phosphoribosyltransferase
NPAS2	Neuronal PAS domain protein 2 (clock protein), also known as MOP4
NPY	Neuropeptide Y
NTE	NaCl-Tris-EDTA buffer
NTS	Nucleus of the solitary tract
<i>ob</i>	Obese, gene encoding for leptin
OPN4	Melanopsin (photopigment)
PACAP	Pituitary adenylate cyclase-activating polypeptide
PAS	Per-Arnt-Sim
PBS	Phosphate buffered saline
Pck1	Phosphoenolpyruvate carboxykinase 1
PCR	Polymerase chain reaction
PER	Period (clock protein)
PFA	Paraformaldehyde
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
POA	Preoptical area
POMC	Proopiomelanocortin
PPAR α	Peroxisome proliferator-activated receptor α
PRKC α	Protein kinase C α
PVN	Paraventricular nucleus
qPCR	Quantitative real-time PCR
rATP	riboadenosine triphosphate
rCTP	ribocytidine triphosphate
REV-ERB α/β	Reverse-erythroblastosis virus alpha/beta (clock protein)
rGTP	riboguanosine triphosphate
RF	Restricted feeding cycles
RF-12	Restricted feeding ('RF 12:12': 12 h feeding: 12 h fasting)
RF-14	Restricted feeding ('RF 14:14': 14 h feeding: 14 h fasting)
RHT	Retinohypothalamic tract
RNA	Ribonucleic acid
ROR $\alpha/\beta/\gamma$	Retinoic acid receptor-related orphan receptor alpha/beta/gamma (clock protein)
RORE	Retinoid orphan receptor response element (clock protein-binding DNA sequence)
rpm	Revolutions per minute
s	Second

SCN	Suprachiasmatic nucleus
Sirt1	Sirtuin 1
SP	Substance P
SPVZ	Sub-paraventricular zone
SSC	Saline sodium citrate buffer
StAR	Steroidogenic acute regulatory protein
STAT3/5	Signal transducer and activator of transcription 3/5
S6K1/2	Ribosomal protein S6 kinase1/2
T	Thymine (nucleobase)
TAE	Tris-acetate-EDTA buffer
TRF	Time restricted feeding
TTL	Transcriptional-translational feedback loop
UTP	Uridine triphosphate
v/v	Volume-to-volume
VIP	Vasoactive intestinal polypeptide
VMH	Ventromedial hypothalamus
VLPO	Ventrolateral preoptic nucleus
w/v	Weight-to-volume
WAT	White adipose tissue
ZT	Zeitgeber Time, ZT 0 = lights on, ZT 0 = food out
β -MeSH	β -mercaptoethanol
β -TrCP	Beta-transducin repeat-containing E3 ubiquitin protein ligase
τ	Tau, symbol for the period length
θ	Phase, time reference in a circadian oscillation
χ^2	Chi ² , periodogram analysis method
°	Degree
4E-BP	4E-binding proteins

Summary

Life on Earth is characterized by recurrent changes in environmental conditions, *e.g.* the light:dark cycle or fluctuations in food availability. The circadian clock system evolved to anticipate those changes. In mammals, circadian clocks are present in nearly all cells of the body and regulate functions in a tissue-specific manner along the 24-hour day. Although circadian clocks are self-sustained, showing periods around 24 h, it is essential that the circadian clock system is synchronized to geophysical time to generate coherent rhythms in behavior and physiology. External time cues that entrain the circadian system are called *zeitgebers*. While light is the most potent *zeitgeber* synchronizing the circadian master pacemaker in the hypothalamic suprachiasmatic nucleus (SCN), food timing is the most potent *zeitgeber* for many peripheral tissues such as the liver. In industrialized countries, people are frequently exposed to disrupted *zeitgeber* conditions including light pollution, shift work, and the constant availability of food. This leads to an uncoupling of external time and endogenous rhythms which may ultimately result in internal desynchronization, *i.e.* a misalignment between different tissue clocks. Internal desynchronization is thought to contribute to the development of diseases such as obesity, metabolic syndrome, cardiovascular disorders, and various types of cancer. In this PhD thesis, I quantitatively investigated the differential contribution of the two main *zeitgebers*, light and food, on tissue clock resetting and metabolic state as well as the development of internal desynchronization in mice.

I established different protocols in which *zeitgeber* periods were either running at 24 h ('*zeitgeber* X'-12) or 28 h ('*zeitgeber* X'-14). Hence, it was possible to assess the effects of chronodisruptive conditions on locomotor activity and the development of internal desynchrony. To evaluate internal (de)synchrony, I analyzed phase shifts of endogenous rhythms between conditions of minimal and maximal *zeitgeber* phase alignment across different tissues. Locomotor activity period lengthened under 28-hour light:dark (14 h light: 14 h darkness (LD-14)) cycles independent of the *zeitgeber* food (14 h fasting: 14 h feeding (Dark-RF) or 12 h fasting: 12 h feeding (RF-12)). However, food timing strongly impacted the phasing of peripheral tissue clocks. Internal synchrony between locomotor activity – reflecting SCN phase – and peripheral tissue clocks was maintained under LD-14/Dark-RF conditions. In contrast, LD-14/RF-12 conditions induced internal desynchrony between locomotor activity and peripheral tissue clocks. In a long-term experiment, mice showed cyclic variations in locomotor activity period and metabolic function depending on *zeitgeber* phase angle under LD-14/RF-12 conditions. Rhythms in locomotor activity, clock gene expression, and hormones were phase-shifted in a tissue- and rhythm-specific manner under LD-14/RF-12 conditions. In contrast, diverging *zeitgeber* input under LD-12/RF-14 conditions did not differentially impact peripheral tissue clocks. Nevertheless, animals showed cyclic changes in metabolic state depending on the *zeitgeber* phase angle under these conditions.

In summary, my data show that especially the LD-14/RF-12 protocol makes it possible to disentangle differential *zeitgeber* contribution and study the physiological consequences of internal desynchronization. Here, clock gene expression rhythms in liver and epididymal

white adipose tissue (eWAT) primarily aligned with food timing whereas rhythms in SCN and adrenal glands were affected by both *zeitgebers*, light and food, as were rhythms of the adipocyte-derived hormone leptin. Corticosterone rhythms, in contrast, were predominantly driven by the light:dark cycle. Such distinct tissue susceptibility to light- and food-related *zeitgeber* signals might be responsible for the internal desynchronization underlying the disease-promoting effects of circadian disruption.

Zusammenfassung

Das Leben auf der Erde ist durch wiederkehrende Veränderungen in den Umweltbedingungen wie den Tag:Nacht-Zyklus oder die wechselnde Verfügbarkeit von Nahrung gekennzeichnet. Im Laufe der Evolution entwickelte sich das zirkadiane Uhrensystem, um solche tagesrhythmischen Veränderungen zu antizipieren. Bei Säugetieren existieren zirkadiane Uhren in fast allen Körperzellen. Sie regulieren physiologische Funktionen in einer gewebespezifischen Weise im Verlauf des 24-Stunden Tages. Obwohl zirkadiane Uhren selbsterhaltend sind und Freilaufperioden von ca. 24 h zeigen, ist es wichtig, dass das zirkadiane Uhrensystem mit der geophysikalischen Zeit synchronisiert wird, um kohärente Rhythmen in Verhalten und Physiologie zu erzeugen. Externe Stimuli, die das zirkadiane System beeinflussen, werden *Zeitgeber* genannt. Während Licht der stärkste Zeitgeber für die Synchronisierung des zirkadianen Hauptschrittmachers im hypothalamischen *Nucleus suprachiasmaticus* (SCN) ist, ist die Zeit der Nahrungsaufnahme der dominante Zeitgeber für die Synchronisierung vieler peripherer Gewebe, z.B. der Leber. In Industrieländern sind Menschen häufig gestörten Umweltrhythmen – sei es durch Lichtverschmutzung, Schichtarbeit oder die ständige Verfügbarkeit von Nahrung – ausgesetzt. Dies führt zu *Chronodisruption*, eine Entkopplung von äußerer Zeit und den endogenen Rhythmen. Dies wiederum fördert die *interne Desynchronisation*, d.h. eine Aufhebung der Phasenkohärenz innerhalb des zirkadianen Uhrensystems. Man geht davon aus, dass die interne Desynchronisation maßgeblich zur Entstehung von Krankheiten, wie Fettleibigkeit, metabolischem Syndrom, Herz-Kreislauf-Erkrankungen und verschiedenen Krebsarten, beiträgt. In dieser Doktorarbeit habe ich quantitativ den Beitrag der beiden Hauptzeitgeber, Licht und Nahrung, auf die Synchronisierung verschiedener Gewebeuhren, auf den Energiestoffwechsel sowie die Entwicklung der internen Desynchronisation bei Mäusen untersucht.

Mithilfe verschiedener Protokolle, in denen die Zeitgeber-Perioden entweder 24 h (,Zeitgeber X'-12) oder 28 h (,Zeitgeber X'-14) betrugen, war es möglich, die Auswirkungen chronodisruptiver Zustände auf die Laufradaktivität und die Entwicklung interner Desynchronisation zu bestimmen. Um die innere (De-)Synchronizität zu quantifizieren, analysierte ich die Phasenverschiebungen endogener Rhythmen zwischen Bedingungen mit minimaler und maximaler Zeitgeber-Phasenausrichtung über mehrere Gewebe. Die Periode der Laufradaktivität verlängerte sich unter 28-stündigen Hell:Dunkel-Zyklen (14 h Hellphase: 14 h Dunkelphase (LD-14)) unabhängig von der Periode des Zeitgebers Nahrung (14 h Fasten: 14 h Futterzugang (Dark-RF) oder 12 h Fasten: 12 h Futterzugang (RF-12)). Der Zeitgeber Nahrung hatte jedoch einen starken Einfluss auf die peripheren Gewebeuhren. Unter LD-14/Dark-RF-Bedingungen blieb die interne Synchronität zwischen der Laufradaktivität – d.h. dem SCN – und den peripheren Gewebeuhren erhalten. Im Gegensatz dazu wurde unter LD-14/RF-12-Bedingungen eine interne Desynchronisation zwischen der Laufradaktivität und den peripheren Gewebeuhren induziert. Im LD-14/RF-12-Langzeitexperiment zeigten die Mäuse vom Zeitgeber-Phasenwinkel abhängige Änderungen in ihren Laufradaktivitäts-

Perioden und Stoffwechselfunktionen. Uhrengenexpressions-, Aktivitäts- und Hormonrhythmen erschienen in gewebe- und rhythmusspezifischer Weise phasenverschoben. Im Gegensatz dazu hatten die divergierenden Zeitgeber-Zustände unter LD-12/RF-14-Bedingungen keinen differentiellen Einfluss auf die peripheren Gewebeuhren. Allerdings zeigten die Tiere auch hier phasenwinkelabhängige Änderungen in ihren Stoffwechselzuständen.

Zusammenfassend zeigen meine Daten, dass insbesondere das LD-14/RF-12-Protokoll geeignet ist, um den jeweiligen Zeitgeberbeitrag auf interne Rhythmen zu differenzieren und die physiologischen Folgen der inneren Desynchronisation zu untersuchen. Die Rhythmen der Uhrengenexpression in der Leber und im epididymalen weißen Fettgewebe (eWAT) zeigten eine sehr starke Beeinflussung durch den Zeitgeber Nahrung. Rhythmen im SCN und den Nebennieren werden ähnlich durch Licht und Nahrung beeinflusst. Ähnlich verhält es sich mit dem Fettgewebshormon Leptin. Der Kortikosteron-Rhythmus dagegen wird hauptsächlich durch den Zeitgeber Licht gesteuert. Diese differentielle Suszeptibilität einzelner Gewebe für licht- und nahrungsvermittelte Zeitgeber-Signale könnte für die den krankheitsbegünstigenden Effekten der Chronodisruption zugrundeliegenden interne Desynchronisation verantwortlich sein.

1 Introduction

Under natural conditions, organisms experience recurrent changes in the environment like day:night cycles and fluctuations in the availability of food. In modern societies, these changes are disrupted by artificial lighting and the constant availability of consumer products. Modern lifestyles strongly impact on behavioral rhythms like sleep:wake cycles or meal timing. Moreover, the percentage of people working outside normal office hours – and, thus outside their natural activity phase – has increased for decades. Recent studies show that the prevalence of shift work is about 20 % among people older than 50 and about 26 % among people younger than 35 years (Sixth European working conditions survey, 2015). An increasing number of epidemiological studies show adverse health effects of such a lifestyle. Shift work is associated with an increased prevalence of obesity, type 2 diabetes, metabolic syndrome, coronary heart diseases, but also mood disorders and certain types of cancer (Bacquer et al., 2009; Di Lorenzo et al., 2003; Karlsson et al., 2003; Kawachi et al., 1995; Mokdad et al., 2003).

Studies of the last decades show that so-called circadian clocks (from Latin: *circa*-approximately, *dies*- day) control behavioral changes across the day, *e.g.* the sleep:wake cycle and the food intake rhythm but they also regulate numerous physiological processes to adapt to recurrent changes in environmental conditions (Takahashi et al., 2008). Chronic disruption of the circadian rhythm is associated with various diseases like those observed in shift workers (Karatsoreos, 2012). Therefore, it is fundamental to understand how non-natural environmental conditions, which humans are exposed to in modern societies, affect circadian clocks throughout the body. This may help to find preventive treatments against the negative consequences of circadian disruption.

1.1 Biological rhythms

Besides circadian rhythms, life is characterized by various other repetitive biological processes. The periodicity of such biological rhythms can range from milliseconds to years. The fastest known biological rhythms are neuronal transmissions which are depending on precisely regulated activation and inactivation of ion channels (Bean, 2007). A bit slower, mitosis cycles occur in the range of minutes to hours and follow a strict sequence of chromatin condensation, aligning, and dividing regulated by cyclin-dependent kinases (Graña and Reddy, 1995). The ovarian cycle is controlled by hormonal networks linking the brain and reproductive organs leading to the menstrual cycle with a length of about 28 days in women (Karsch, 1987). Spontaneous locomotor activity in marine organisms, *e.g.* crabs, is regulated by circalunar clocks and is composed of intertwined circadian and circatidal rhythms (Bennett et al., 1957; Chabot et al., 2016). Circannual rhythms, *e.g.* torpor, hibernation or fertility, are most likely induced by external factors like changes in photoperiod, temperature, and food availability. They are controlled by complex feedback mechanisms that are not fully understood to date (Gwinner and Dittami, 1990; Körtner and Geiser, 2000; Woodfill et al., 1991). In migratory birds, migratory restlessness and molting are driven by endogenous

mechanisms occurring in a circannual manner even under constant conditions (Gwinner, 1977; Holberton and Able, 1992). Nymphs of the periodical cicadas of the genus *Magicicada* feed on roots and the cicadas emerge synchronously from the ground every 13 or 17 years (Williams and Simon, 1995).

Arguably, the most influential biological rhythms for life on Earth are circadian rhythms. Life on Earth is characterized by daily recurrent changes in environmental conditions, *e.g.* light:dark, and concomitant with that, high:low temperatures, which are caused by the Earth's rotation around its axis. Food availability and predator occurrence vary over the course of the day, too. Hence, organisms increased their chance of survival by developing a system to anticipate such recurring events – the circadian clock system which coordinates ~24-hour rhythms in behavior and physiology. Circadian rhythms are the most investigated form of repetitive biological processes and have been described in all *phyla* from protists, algae, bacteria and fungi to multicellular organisms such as animals and plants (Dunlap and Loros, 2006; Harmer, 2009; Iwasaki and Kondo, 2004; Panda et al., 2002a).

An essential property of biological rhythms, especially in higher organisms, is the regulation by complex feedback mechanisms. These mechanisms include, *inter alia*, activation and inhibition of signaling pathways and transcription as well as synthesis and breakdown of proteins and hormones. Moreover, feedback loops are often redundant and interlocked and, thereby, stabilized against perturbations.

1.2 The circadian clock

The circadian clock system is highly conserved and has been described for very diverse species. Nearly all organisms are exposed to changing environmental conditions and, thus, the circadian clock most likely evolved several times in response to evolutionary pressure (Bhadra et al., 2017; Dvornyk et al., 2003; Jindrich et al., 2017). Despite this, all circadian systems share common properties which also leaves the possibility of one common unknown progenitor. Of note, the earliest form of a time-keeping system probably exists in cyanobacteria consisting only of the three genes *kaiA/B/C* (Ishiura et al., 1998). From an evolutionary perspective, the circadian clock coordinates the organism's behavior and physiology with the geophysical time to enhance the chance of survival and reproduction. Furthermore, it facilitates temporal compartmentalization of opposite physiological processes, *e.g.* energy storage and breakdown at high and low energy states, respectively. Thus, it anticipates changes in the organism's needs in advance of recurrent environmental alterations.

Circadian clocks are defined by three characteristics. They are self-sustained, entrainable and temperature compensated. The first recorded observational study of endogenous rhythms was performed in *Mimosa pudica* in 1729. The plant continued to show nearly 24-hour rhythms of leaf movements under constant darkness (DD) conditions proving the self-sustained property (de Mairan, 1729). Rhythms of spontaneous activity under constant conditions were also proven in various animals (Aschoff, 1966; Richter, 1922). In humans, endogenous circadian rhythms were shown in the famous bunker experiments performed by Jürgen Aschoff.

Participants were socially isolated without any information about external time for several weeks. Under those constant conditions, their sleep:wake cycle, body temperature and urine excretion were still rhythmic, *i.e.* self-sustained, although free-running with a period a little longer than 24 h (Aschoff, 1965; Aschoff et al., 1967). In mice, the free-running period is shorter than 24 h in constant darkness (Bunger et al., 2000). The period length (τ) is defined by the time to complete one cycle. Self-sustained circadian clocks must be synchronized – *entrained* – with the external time of day. Phase (θ) refers to the fixed time interval between two reference points of an internal (*e.g.* activity onset) and an external rhythm (*e.g.* sun rise) and serves as a measure for the entrainment of an endogenous rhythm. Stimuli which can entrain the circadian clock are called *zeitgebers* (German for ‘time giver’) and will be addressed in detail in section 1.3. Last but not least, circadian rhythms are temperature compensated, *i.e.* maintain constant kinetics over a wide range of temperatures, which is contrary to most biological reactions (Barrett and Takahashi, 1995; Menaker and Wisner, 1983; Pittendrigh, 1954; Reyes et al., 2008).

1.2.1 The mammalian molecular clock

At the molecular level, the circadian clock of mammals is based on interlocked transcriptional-translational feedback loops (TTLs) (**Figure 1-1**). In the core loop, the positive limb is formed by the activating transcription factors brain and muscle aryl hydrocarbon receptor nuclear translocator-like protein 1 (BMAL1, also called ARNTL or MOP3) and circadian locomotor output cycle kaput (CLOCK) (or its paralogue neuronal PAS domain protein 2 (NPAS2, also called MOP4) (Bunger et al., 2000; DeBruyne et al., 2007; Ikeda and Nomura, 1997; Shearman et al., 2000; Vitaterna et al., 1994). These transcription factors form heterodimers *via* their PER-ARNT-SIM (PAS) domains and bind to enhancer boxes (E-boxes, a CACGTT-sequence motif) in promotor regions *via* their basic-helix-loop-helix (bHLH) domains (Antoch et al., 1997; Gekakis et al., 1998; King et al., 1997). Subsequently, transcription of *Period1-3* (*Per1-3*), *Cryptochrome1/2* (*Cry1/2*), and so-called *clock-controlled* genes (CCGs) with E-box containing promoters is induced (Kume et al., 1999; Sun et al., 1997; Tei et al., 1997). PER and CRY proteins form the negative limb of the core TTL. The stability and degradation of PER and CRY is regulated by post-translational modifications. In the cytoplasm, casein kinases 1 δ and ϵ (CK1 δ/ϵ) phosphorylate PER proteins targeting these for ubiquitination by beta-transducin repeat-containing E3 ubiquitin protein ligase (β TRP) (Camacho et al., 2001; Eide et al., 2005; Keesler et al., 2000; Shirogane et al., 2005). CRY proteins are phosphorylated by adenosine monophosphate-activated protein kinase (AMPK) and glycogen synthase kinase 3 (GSK3) which promotes ubiquitination by F-box and leucine-rich repeat protein 21 (FBXL21) (Harada et al., 2005; Lamia et al., 2009; Yoo et al., 2013). Ubiquitinated PER and CRY are rapidly degraded by the proteasome. Though, with a temporal delay, PER and CRY are accumulating in the cytoplasm, forming heterodimers and interacting with CK1 δ/ϵ (Akashi et al., 2002; Takano et al., 2004). Nuclear translocation of phosphorylated PER:CRY complexes is regulated by karyopherin subunit beta 1 (KPNB1) which mainly interacts with PER proteins (Lee et al., 2015; Miyazaki et al., 2001). In the nucleus, PER:CRY complexes are interacting with

BMAL1:CLOCK or BMAL1:NPAS2 (hereafter always referred to as BMAL1:CLOCK) complexes downregulating their own transcription (Griffin et al., 1999; Sangoram et al., 1998). While CRY physically interacts with the C-Terminus of BMAL1, PER2 recruits repressive chromatin modifiers (Duong and Weitz, 2014; Kim et al., 2014; Sato et al., 2006; Xu et al., 2015; Ye et al., 2014). As time progresses, nuclear PER and CRY are ubiquitinated and degraded by the proteasome and BMAL1:CLOCK can again bind to E-box containing promoters to start a new cycle of transcription (Busino et al., 2007; Yoo et al., 2013).

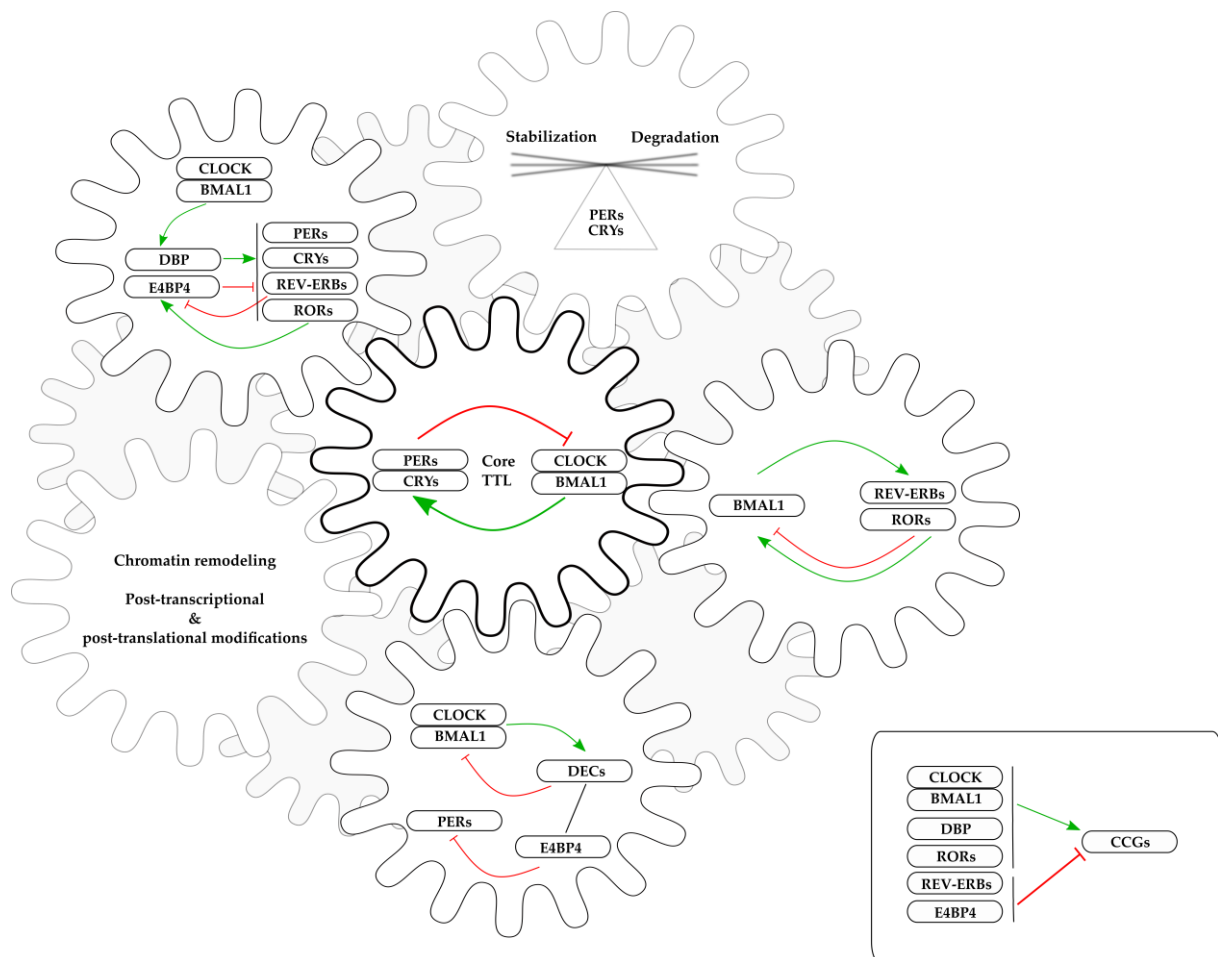


Figure 1-1: The mammalian molecular circadian clock. The core TTL is comprised of the transcription factors **CLOCK** and **BMAL1** which bind as heterodimers to E-Boxes in the promotor regions of the clock genes *Per1-3* and *Cry1/2*. **CRYs** and **PERs** form heterodimers and repress **BMAL1:CLOCK**-mediated transcription. Stability and degradation of **PER** and **CRY** proteins are controlled by kinases and E3 ubiquitin ligase complexes. Phosphorylated **PER** and **CRY** proteins are destabilized and **BMAL1:CLOCK**-mediated transcription is reinitiated when **PER** and **CRY** are degraded. The core TTL is stabilized by auxiliary feedback loops. **BMAL1:CLOCK** activate expression of *Rev-Erba/β* and *Rora/β/γ*. **REV-ERBs** and **RORs** compete for binding to **RORE** motifs and repress or induce *Bmal1* transcription, respectively. **BMAL1:CLOCK** activate *Dec1/2* expression which in turn inhibit **BMAL1:CLOCK**-mediated transcription by competing for binding sites or interaction with **BMAL1**. Additionally, **DEC2** interacts with **E4BP4** to inhibit *Per2* expression. Gene expression of *Dbp* is activated by **BMAL1:CLOCK**, and gene expression of *E4bp4* is regulated by **REV-ERBs** and **RORs**. **DBP** and **E4BP4** compete for binding to D-boxes in the promoters of *Per1-3*, *Cry1*, *Rev-Erba/β* and *Rora/β/γ*. **DBP** and

E4BP4 activate or inhibit target gene expression, respectively. Furthermore, chromatin remodeling, post-transcriptional and post-translational modifications modulate the dynamics of the interlocked feedback loops. Altogether, the intertwined TTLs generate ~24-hour rhythms in the expression of thousands of CCGs. Of note, besides the depicted feedback loops, many other modulating factors are known to impinge on the molecular circadian clock system.

The core TTL is stabilized by auxiliary intertwined feedback loops to keep ~24 h oscillations and allow fine-tuning of the circadian clock system. One of the best investigated loops is comprised of reverse-erythroblastosis virus α/β (REV-ERB α/β , also called NR1D1/2) and retinoic acid receptor-related orphan receptors $\alpha/\beta/\gamma$ (ROR $\alpha/\beta/\gamma$, also called NR1F1/2/3). BMAL1:CLOCK bind to E-boxes in the *RevErb* and *Ror* genes to activate gene expression (André et al., 1998; Triqueneaux et al., 2004). In turn, REV-ERB and ROR compete for binding to retinoic acid-related orphan receptor response elements (ROREs) to repress or activate target gene expression, e.g. *Bmal1* (Akashi and Takumi, 2005; Nakajima et al., 2004; Preitner et al., 2002; Sato et al., 2004; Ueda et al., 2002). Of note, REV-ERBs are functionally redundant and essential for rhythmic *Bmal1* expression whereas RORs modulate the amplitude but are not required for *Bmal1* rhythms (Liu et al., 2008). A second auxiliary loop is formed by the feedback of E4 promotor-binding protein 4 (E4BP4, also called NFIL3) and D-site albumin promotor binding protein (DBP) on gene expression. *E4bp4* expression is regulated by REV-ERB/ROR and *Dbp* transcription is activated by BMAL1:CLOCK (Ripperger and Schibler, 2006; Stratmann et al., 2012; Takeda et al., 2012). DBP and E4BP4 compete for binding to D-boxes in gene promoters, e.g. *Per1-3*, *RevErb* α/β , *Rora* α/β and *Cry1*, to activate or repress transcription, respectively (Mitsui et al., 2001; Ohno et al., 2007; Yamaguchi et al., 2000; Yamajuku et al., 2011). Additionally, other genes with D-box and RORE containing promoters are regulated in a circadian manner and are, thus, CCGs. A third auxiliary loop is comprised of the bHLH proteins differentiated embryo chondrocyte protein 1/2 (DEC1/2, also known as basic helix-loop-helix family member e40/41 (Bhlhe40/41) (Rossner et al., 2008). *Dec1/2* expression is activated by binding of BMAL1:CLOCK complexes to E-boxes. In turn, DEC1/2 inhibit BMAL1:CLOCK-mediated transcription by interacting with BMAL1 or competing for binding to E-boxes (Honma et al., 2002). Interestingly, the DEC1/2-mediated regulation on rhythmicity and period is dependent on the tissue and on the time of day (Bode et al., 2011; Tsang et al., 2012). Furthermore, DEC2 can interact with E4BP4 to repress *Per2* transcription (Tanoue et al., 2015).

In addition, circadian oscillations of core clock genes and CCGs are further regulated by chromatin remodeling, and post-transcriptional and post-translational modifications (Doi et al., 2006; Gallego and Virshup, 2007; Grimaldi et al., 2007, 2009; Kojima et al., 2011; Nakahata et al., 2008; Preußner et al., 2014). Transcriptome analyses show that over 40 % of all protein-coding genes in mice and over 80 % in baboons exhibit daily oscillations in at least one tissue (Mure et al., 2018; Zhang et al., 2014).

1.2.2 Hierarchical organization of the circadian clock system

Circadian oscillations can be found in nearly every cell in the organism with individual period lengths (Nagoshi et al., 2004; Welsh et al., 1995). These single circadian clocks are coordinated and synchronized to prevent desynchronization within and between tissues. In mammals, this clock network is organized in a hierarchical manner with the central pacemaker, the suprachiasmatic nucleus (SCN), at the top of the hierarchy. As the name suggests, the SCN resides above the optic chiasm in the hypothalamus. It receives external cues about the time of day and synchronizes subordinated clocks *via* humoral outputs, the autonomic nervous system (ANS), and *via* regulation of behavior and core body temperature. The central role of the SCN for the circadian system was initially demonstrated in lesion studies. Bilateral SCN lesions in rats lead to arrhythmic locomotor and drinking behavior and result in a loss of endocrine rhythms such as those of corticosterone (Moore and Eichler, 1972; Stephan and Zucker, 1972). In addition, transplantation of SCN grafts to SCN lesioned animals restores behavioral circadian rhythms (Lehman et al., 1987; Sawaki et al., 1984; Tousson and Meissl, 2004). Further evidence for the pacemaker role of the SCN came from SCN cross-transplantation studies using hamster mutants with short locomotor activity periods. Here, host activity rhythms always reflect those of the graft genotype (Ralph et al., 1990). However, this dominant role of the SCN in driving circadian rhythms across the body was recently challenged by studies using genetic ablation of SCN clock function (see 1.4.1).

1.2.3 Clock communication

Tissue clocks communicate with each other to generate coherent rhythms in physiology and behavior. The SCN clock is divided into a dorsomedial shell and a ventrolateral core based on retinal innervation and neurotransmitter expression (Romijn et al., 1997). Neurons in the core are synchronized by photic input and release the neurotransmitters vasoactive intestinal polypeptide (VIP), substance P (SP), gastrin-releasing peptide (GRP) and γ -aminobutyric acid (GABA) to synchronize the SCN shell (Abe et al., 1996; Aida et al., 2002; Albus et al., 2005; Meyer-Spasche and Piggins, 2004). SCN shell neurons secrete arginine vasopressin (AVP), GABA and glutamate to communicate with extra-SCN target regions (Hermes et al., 1996; Moore and Speh, 1993; Reghunandanan et al., 1998). Interestingly, extra-SCN clocks are entrained by the SCN shell but not the SCN core (Evans et al., 2015). A recent study uncovered novel neuronal phenotypes and communication pathways adding a new level of complexity within the SCN (Park et al., 2016). Astrocytes also play an important role in the maintenance of robust circadian oscillations but the underlying mechanisms are not yet understood (Brancaccio et al., 2017; Tso et al., 2017).

Communication between the SCN clock and numerous extra-SCN oscillators is dependent on neuronal connections and tightly regulated release of neurotransmitter such as AVP, GABA and other diffusible signals. In turn, projections from the arcuate nucleus (ARC), the dorsomedial hypothalamus (DMH), the intergeniculate leaflet (IGL) and the nucleus of the solitary tract (NTS) to the SCN are described. Disruption of the crosstalk between those brain structures and the SCN eliminates corticosterone rhythms, alters locomotor activity rhythms

and peripheral metabolic feedback, and change blood pressure responses (Acosta-Galvan et al., 2011; Buijs et al., 2014, 2017; Janik and Mrosovsky, 1994; Saderi et al., 2013; Yi et al., 2006).

Hormonal rhythms constitute major synchronizing pathways within the circadian clock network. The pineal gland-derived hormone melatonin is exclusively secreted in the dark phase (Adrendt et al., 1977; Kennaway et al., 1977; Perreau-Lenz et al., 2003). Melatonin is able to reset the SCN clock *in vitro* (McArthur et al., 1991; Starkey et al., 1995). *In vivo*, it is assumed that melatonin strengthens behavioral rhythms by a feedback mechanism to the SCN under LD conditions (Cassone et al., 1993; Yanovski et al., 1990). Melatonin levels in the blood are suppressed by light and, thus, function as an integrator of seasonal changes in day length to regulate seasonal physiology. However, melatonin is not essential for the generation of circadian rhythmicity *per se* as several inbred mouse strains, including the strain used in this project, show robust circadian rhythms despite being melatonin-deficient (Ebihara et al., 1986; Roseboom et al., 1998). Another systemic synchronizer is the hypothalamic-pituitary-adrenal (HPA) axis. The SCN stimulates rhythmic glucocorticoid (GC) synthesis in the adrenal cortex *via* hormonal output from the hypothalamus and the pituitary. At the same time, the SCN synchronizes adrenal clocks *via* the ANS which gates the sensitivity for incoming signals, *i.e.* adrenocorticotrophic hormone (ACTH) (Oster et al., 2006a). GCs are secreted in a circadian manner as well as in response to a stress stimulus and bind to intracellular receptors. Upon hormone binding, glucocorticoid receptors (GR) translocate into the nucleus and activate target gene transcription *via* binding to glucocorticoid response elements (GREs) in their promotor region (Balsalobre et al., 2000; Reddy et al., 2007; So et al., 2009). GCs are able to synchronize about 60 % of the liver transcriptome either directly by regulating their target genes, including clock genes, or indirectly *via* transcriptional regulation of other transcription factors (Reddy et al., 2007). Interestingly, GCs regulate many genes involved in glucose and lipid metabolism. High GC secretion in the beginning of the active phase thereby elevates plasma glucose levels through stimulation of gluconeogenesis in the liver, thus providing the body with the required energy. Furthermore, GCs promote lipolysis but also insulin-mediated lipogenic functions in adipose tissue (Guia et al., 2014).

Metabolic homeostasis is regulated by peripheral hormones transmitting information on the metabolic state to other peripheral tissues and to the brain. Hence, those hormones promote or inhibit food intake and activate anabolic or catabolic pathways to maintain energy balance. Most of those hormones are also able to reset peripheral clocks which reinforces internal synchrony between peripheral oscillators. These mechanisms may mediate the entrainment of peripheral tissue clocks to enforced feeding:fasting cycles. The pancreas releases insulin to activate glucose uptake by metabolic tissues such as muscle, liver or adipose tissue. On the molecular level, insulin induces hepatic *Per2* while it downregulates *Rev-Erba* transcription (Tahara et al., 2011). Furthermore, insulin indirectly regulates *Clock* expression in the liver (Chaves et al., 2014). *Bmal1* transcription is repressed by insulin while it is induced by glucagon, one of the major fasting hormones (Sun et al., 2015). The gut derived hormone oxyntomodulin can also reset the liver clock by inducing *Per1* and *Per2* transcription (Landgraf

et al., 2015). Leptin, released by adipocytes in a circadian manner, acts as a satiety hormone in the brain. Leptin levels correlate with body fat content and decrease during fasting. This hormone can induce phase shifts *in vitro* but by itself cannot entrain the SCN clock under constant conditions *in vivo* (Martinez-Merlos et al., 2004; Prosser and Bergeron, 2003). The hormone ghrelin increases during fasting and stimulates food intake. Ghrelin can reset the SCN *in vitro* and induces phase shifts in behavioral rhythms after long-term food deprivation (Yannielli et al., 2007). Expression of fibroblast growth factor 21 (FGF21) is induced by fasting in a peroxisome proliferator-activated receptor α (PPAR α)-dependent manner, but its expression is also regulated by the circadian clock *via* RevERB, ROR and E4BP4 (Chavan et al., 2016; Oishi et al., 2008, 2011; Tong et al., 2010). In periphery, FGF21 decreases energy expenditure and increases ketone production to maintain energy homeostasis. Together with β -Klotho, FGF21 increases GC levels, suppresses locomotor activity, and alters locomotor activity patterns (Bookout et al., 2013).

In summary, the SCN coordinates synchronicity within the circadian clock network. The integrated signal of the SCN clock and extra-SCN oscillators is transduced to peripheral tissue clocks *via* hormones and the ANS but also *via* regulation of behavioral rhythms and core body temperature (see 1.4.1). In the periphery, these signals are processed and peripheral clocks are synchronized. This may involve circadian gating of incoming signals as shown for the adrenal. Hormonal outputs of peripheral oscillators reset or modulate other peripheral tissue clocks and feedback to the brain to stabilize the circadian network. However, the rules of communication between tissue clocks are complex and remain largely unknown. They are, however, essential for the clock system to cope with fluctuations in environmental conditions.

1.3 Zeitgebers

Endogenous rhythms must be synchronized to the external environment to be biologically useful. Such entrainment is necessary because the endogenous periods of most circadian rhythms are a little longer or shorter than 24 h. Several *zeitgebers* impact and reset the circadian clock system (**Figure 1-2**). In accordance, circadian oscillations are often measured along *zeitgeber* times (ZT) referring to specific events in the external *zeitgeber* cycle. In nocturnal rodents, *e.g.*, ZT0 corresponds to the time of “lights on” (or “food out” in a restricted feeding paradigm) and ZT12 to “lights off” (or “food in”).

1.3.1 Entrainment of the circadian network by the zeitgeber light

Light is the most potent *zeitgeber* for entrainment of the circadian clock. In the early 20th century, preservation of the pupillary reflex in blind mice suggested the presence of another class of retinal photoreceptor system apart from rods and cones (Keeler, 1928). Later, a class of intrinsically photosensitive retinal ganglion cells (ipRGCs) was described (Berson et al., 2002; Foster et al., 1991; Freedman et al., 1999; Lucas et al., 1999). ipRGCs express the photopigment melanopsin (OPN4) which is most sensitive to blue light of 480 nm. They transmit light information directly to the SCN *via* the retinohypothalamic tract (RHT) (Gooley et al., 2001; Hattar et al., 2002; Moore et al., 1995; Mrosovsky and Hattar, 2003; Provencio et al., 2000; Zaidi

et al., 2007). Light stimulates the release of pituitary adenylate cyclase-activating polypeptide (PACAP) and glutamate from ipRGC terminals in the SCN leading to membrane depolarization in SCN core neurons (Hannibal et al., 2000; Meijer et al., 1993; Michel et al., 2006). Subsequently, intracellular calcium (Ca^{2+}) levels are increased activating Ca^{2+} /calmodulin-dependent protein kinase (CamK) which activates mitogen-activated protein kinase (MAPK). Additionally, glutamate-activated adenylate cyclase (AC) increases 3',5'-cyclic adenosine monophosphate (cAMP) levels which activates protein kinase A (PKA). PKA can also activate MAPK. Thereby, cAMP response element-binding protein (CREB) is activated (Butcher et al., 2002, 2005; Ding et al., 1997; Dziema and Obrietan, 2002; Ginty et al., 1993; Obrietan et al., 1998; Prosser and Gillette, 1989; Tischkau et al., 2000; Yokota et al., 2001). Phosphorylated CREB binds to cAMP-response elements (CREs) in the promoters of *Per1* and *Per2* to induce transcription (Nielsen et al., 2001; Travnickova-Bendova et al., 2002).

A light pulse at any time during subjective night under DD conditions rapidly induces the transcription of *Per1*, but not *Per2*, in the SCN; the latter being light responsive only during the first half of the dark phase (Albrecht et al., 1997; Shigeyoshi et al., 1997). In another study, light pulses in the early and late subjective night induced *Per1* and *Per2*, but not *Per3*, expression (Shearman et al., 1997; Zylka et al., 1998). SCN *Per* gene expression also rapidly reacts and re-entrains under shifted light:dark (LD) cycle (*jetlag*) conditions, whereas re-entrainment of *Cry* expression is slow (Reddy et al., 2002). *In vivo*, rapid *Per* and slower *Bmal1* entrainment is associated with rapid entrainment of activity onset and slower entrainment in activity offset, respectively (Ono et al., 2017). Furthermore, protein kinase C α (PRKCa) is activated by light and transiently stabilizes PER2 retaining the protein in the cytoplasm (Jakubcaková et al., 2007). Exposure to light during the subjective day phase under DD conditions does not induce *Per* transcription (Shigeyoshi et al., 1997). Thus, resetting of the SCN clock by light is time of day dependent. Behavioral rhythms are phase delayed upon light exposure in the early subjective night phase whereas they are phase advanced when light is applied in the late subjective night. Exposure to light in the subjective day phase has no behavioral phase-shifting effect (Daan and Pittendrigh, 1976; Khalsa et al., 2003; Minors et al., 1991).

1.3.2 *Entrainment of the circadian network by the zeitgeber food*

Time restricted feeding (TRF) induces increased locomotor activity shortly before meal timing, so-called food anticipatory activity (FAA) (Krieger, 1974; Richter, 1922). In SCN-lesioned rats, FAA persists for several days under food deprivation conditions (Stephan et al., 1979). FAA cannot be observed in SCN lesioned mice after 6 days of restricted feeding, but it develops when time-restricted feeding is pursued for more than a week (Hara et al., 2001; Marchant and Mistlberger, 1997; Stephan and Becker, 1989; Storch and Weitz, 2009). This suggests that FAA is driven by an SCN-independent food-entrainable oscillator (FEO), a network of, yet unknown, interconnected oscillators residing in the brain and in the periphery. The importance of the DMH, an important behavioral output center, for developing FAA is controversial (Fuller et al., 2008; Gooley et al., 2006; Landry et al., 2006, 2007). Contradictory results were obtained for other brain regions, too (Davidson, 2009). Moreover, recent studies

suggest that peripheral tissues and their secreted hormones, *e.g.* stomach-derived ghrelin, are an important part of the FEO (LeSauter et al., 2009). The FEO probably senses the organisms' metabolic state inducing activity and foraging to ensure survival. Interestingly, FAA does not necessarily rely on a functional circadian clock machinery. *Bmal1* knockout (KO), *Per1/2* double mutant, *Per2* mutant and *Clock* mutant mice exhibit robust FAA whereas *Cry1/2* double KOs show instable FAA (Iijima et al., 2005; Pendergast et al., 2009; Pitts et al., 2003; Storch and Weitz, 2009).

Although the circadian clock system is not needed for FAA, peripheral tissue clocks and hormonal rhythms are highly impacted by food intake timing. In rats, light phase-restricted feeding (ZT2.5-4.5) induces 12-hour phase shifts in corticosterone excretion, hippocampal norepinephrine and serotonin levels (Krieger, 1974). Daytime-restricted feeding entrains extra-SCN and peripheral tissue clocks, *e.g.* in hippocampus, liver, kidney, and pancreas, without phase shifting the SCN clock (Damiola et al., 2000; Hara et al., 2001; Inouye, 1982; Stokkan et al., 2001; Wakamatsu et al., 2001). Under constant light (LL) or DD conditions, restricted feeding can entrain the SCN clock to the restricted-feeding schedule after several weeks (Castillo et al., 2004; Lamont et al., 2005). However, daytime-restricted feeding under rhythmic LD cycles leads to an uncoupling of the SCN and peripheral tissue clocks resulting in internal desynchrony. In consequence, this may affect energy metabolism and increase the risk of metabolic diseases. Upon changing to daytime-restricted feeding, phase shifts in clock gene expression in peripheral tissues occur gradually with full entrainment being reached within a week (Damiola et al., 2000; Stokkan et al., 2001). In SCN lesioned mice, however, peripheral tissue clocks entrain to daytime-restricted feeding within 1-2 days suggesting that the SCN counteracts food-driven entrainment signals (Saini et al., 2013). Besides peripheral tissue clocks, corticosterone and core body temperature rhythms can also be entrained by daytime-restricted feeding in SCN lesioned mice (Hara et al., 2001; Krieger et al., 1977). Additionally, time-restricted feeding can partially restore rhythmic gene expression in the liver of *Cry1/2* double KO mice. Interestingly, number of rhythmic transcripts are suppressed by 80 % by prolonged fasting (24 h) (Vollmers et al., 2009). Thus, rhythmic food intake is a strong *zeitgeber* inducing circadian transcription programs in peripheral tissues (Greenwell et al., 2019).

Circadian clock resetting by food involves integration of numerous signals. It is likely that hormones involved in metabolic regulation, *i.e.* insulin, leptin, ghrelin and others, play an important role (see 1.2.3.). The GC rhythm entrains to scheduled feeding after long-lasting restricted feeding and further modulates tissue clock gene expression (Balsalobre et al., 2000; Krieger, 1974). However, GC rhythms counteract rapid entrainment of peripheral clocks to daytime-restricted feeding in nocturnal animals. *Vice versa*, GCs promote the fast re-entrainment of peripheral tissue clocks upon changing back to nighttime-restricting feeding (Le Minh et al., 2001). Several clock genes have functional GREs and, thus, are targets for GC-mediated clock resetting (So et al., 2009).

1.3.3 *Entrainment of the circadian network by other zeitgebers*

Besides light and food, being the most potent *zeitgebers* for the circadian network, other stimuli are known to modulate the circadian system.

Although body temperature in homeothermic animals is rather stable and unaffected by outside temperature, mammalian cellular rhythms can be entrained by temperature cycles *in vitro* (Brown et al., 2002; Hoffmann, 1969; Saini et al., 2012). While the SCN clock itself is resistant to temperature entrainment, it regulates body temperature rhythms to affect peripheral clocks. In the periphery, the heat shock pathway is essential for temperature-mediated clock resetting but also for temperature compensation (Buhr et al., 2010; Reinke et al., 2008; Saini et al., 2012). Temperature changes also impact on mRNA splicing and mRNA stability. These effects are independent of the cell's circadian clock and, thus, add a new level to the diurnal control of mRNA abundance (Gotic et al., 2016).

Changes in the rest:activity cycle or exercise require elevated energy supply which effects the body's physiology. Scheduled exercise in the rest phase advances the phase of the circadian clock in skeletal muscles and the lung without phase shifting the SCN clock (Wolff and Esser, 2012). In line with phase-shifted clock gene expression, it is very likely that also CCGs involved in metabolic functions are phase-shifted by exercise to cope with increased energy demands. In aged animals, behavioral rhythms are phase-advanced and overall dampened which might be caused by disrupted outputs of the SCN (Leise et al., 2013; Nakamura et al., 2011; Weinert, 2000). Recent studies show that voluntary and scheduled exercise can induce phase shifts and improve re-entrainment to new LD cycles in young but also in old organisms (Leise et al., 2013; Yamanaka et al., 2008, 2013; Youngstedt et al., 2016). Interestingly, scheduled exercise-mediated phase shifts are dependent on the circadian phase (Reebs and Mrosovsky, 1989). Despite the effects on the circadian clock, voluntary exercise prevents body weight gain under dim light at night (DLAN) conditions, although light-phase food intake is increased (Fonken et al., 2014). Together, the data show that exercise affects the circadian clock. However, the underlying mechanisms are not yet well understood. It is very likely that hyperthermia, excretion of glucocorticoids and activation of the sympathetic nervous system are involved (Atkinson et al., 2007; Tahara et al., 2017). In line with that, acute stress induces *Per1* transcription probably *via* glucocorticoid signaling (Yamamoto et al., 2005).

Changes in rest:activity cycles and poor sleep quality are associated with a disturbed circadian system, as observed in shift workers, in elderly, and in mentally ill people (Mishima et al., 1994; Rajaratnam et al., 2013; Wulff et al., 2010, 2012). Exercise, rest:activity rhythms, and social interaction with others influence the circadian system and are thereby defined as social *zeitgebers*. In healthy people, cortisol levels and activity are high at the start and decrease towards the end of the activity phase. In elderly people suffering from depression this association is lost, suggesting that the ability of social activities to entrain the circadian system is weakened (Stetler et al., 2004). On the other hand, social activation and physical exercise improve subjective sleep quality and behavioral rhythms in elderly (Kuck et al., 2014). Similar

to the effects of light and exercise, social interaction phase-shifts behavioral rhythms in a circadian phase-dependent manner. Under constant conditions, humans can be entrained by social interactions showing that social cues can have a strong influence (Aschoff et al., 1971). In hamsters, locomotor activity rhythms are phase advanced and phase delayed when social interaction takes place in the middle of the subjective day and late in the subjective night, respectively (Mrosovsky, 1988). Besides such modulating effects on behavioral rhythms, the efficacy of social *zeitgebers* on the circadian clock system are controversial suggesting, if any, a minor role in circadian clock entrainment. Studies investigating the efficacy of social *zeitgebers* are often poorly controlled for confounding factors (Favreau et al., 2009; Mistlberger and Skene, 2004).

In summary, several external and internal stimuli affect the circadian system. The circadian system integrates external *zeitgebers*, e.g. light and food intake timing, and internal *zeitgebers* – changes in body temperature, arousal, nervous system activation, and hormonal secretion – to temporally adapt and synchronize the whole organism.

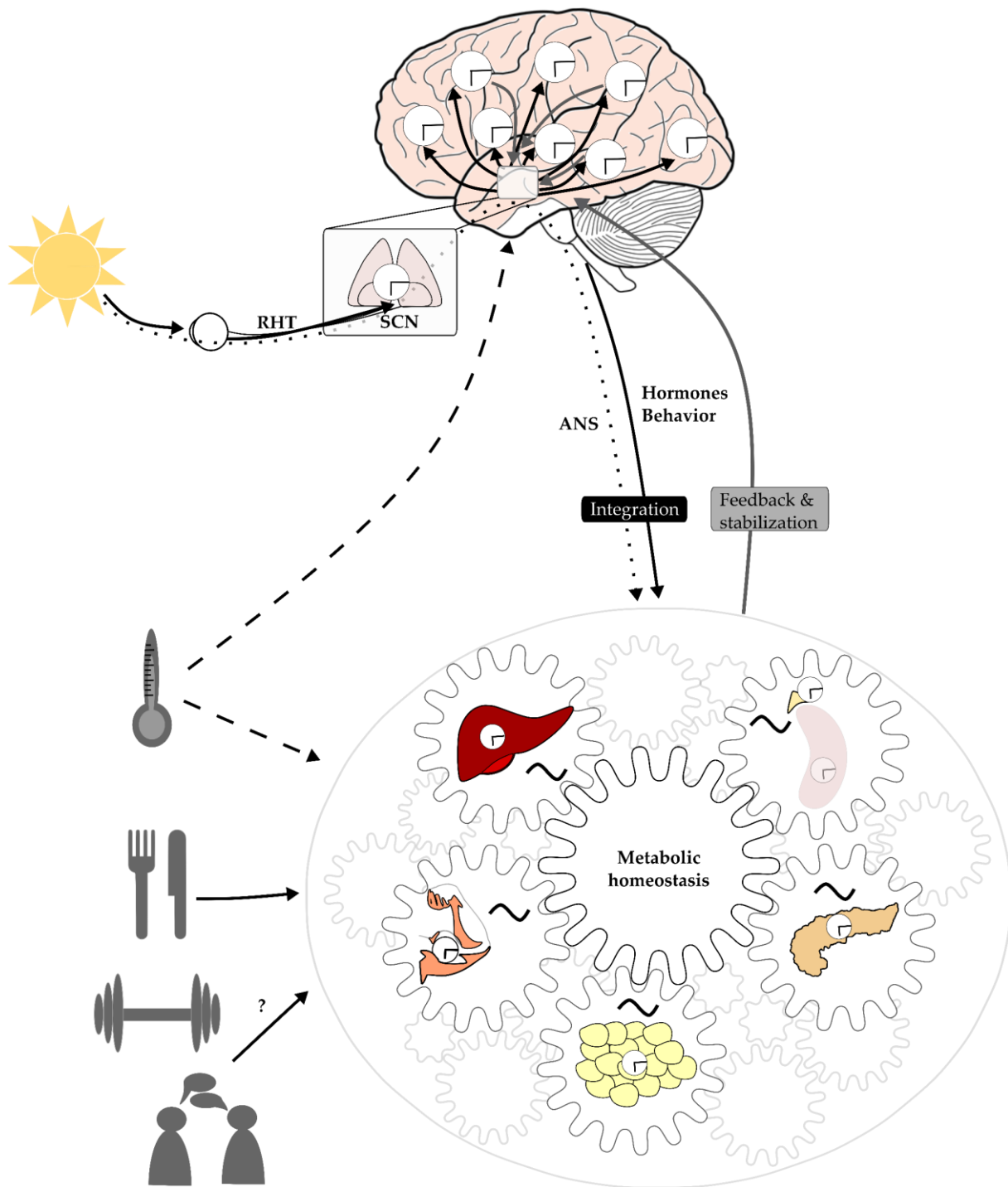


Figure 1-2: Zeitgebers impact the circadian system on several levels. Light is the most potent *zeitgeber* in mammals. Light information is received by ipRGCs in the retina. Subsequently, the information is transmitted to the SCN *via* the RHT. The SCN synchronizes extra-SCN oscillators in the brain and, thereby, regulates behavior and hormonal secretion (black lines). Moreover, light can entrain peripheral tissues independent of the SCN clock *via* the ANS (dotted line). In this way, peripheral tissue clocks are synchronized with the geophysical time. Besides synchronization by the SCN, peripheral tissues clocks are also influenced by food intake timing. Environmental temperature also impacts the circadian system, although, this is a neglectable stimulus on the circadian system in mammals. Body temperature rhythms, driven by the SCN, are important for entrainment of peripheral tissues. The efficacy of social *zeitgebers* like exercise and social interaction is debatable. Peripheral tissue clocks regulate the secretion

of hormones which then influence other peripheral tissue clocks. Outputs of peripheral tissue clocks feedback to the brain to further stabilize the circadian clock network (grey lines). Together, synchronized tissue clocks generate coherent rhythms to regulate metabolic homeostasis.

1.4 Functional role of tissue clocks

1.4.1 Suprachiasmatic nucleus (SCN), the central pacemaker

The SCN comprises two nuclei of densely packed neurons in the ventral hypothalamus atop the optic chiasm. In mice, the SCN consists of ca. 10,000 neurons *per* hemisphere. The neurons of the SCN are predominantly GABAergic. Anatomically, the SCN can be divided in a ventral core and dorsal shell region defined by their distinct neuropeptide expression. Furthermore, there are also changes apparent in neuropeptide expression and clock gene localization along the rostral-caudal axis (Park et al., 2016; Riddle et al., 2017; Yan et al., 2007). The core region contains VIP and GRP neurons whereas the shell region predominantly consists of AVP positive neurons (Welsh et al., 2010). As described in chapter 1.3.1, the core region receives photic input *via* the RHT and synchronizes the SCN shell. VIP neurons play an important role in mediating temporal signals to the shell. Furthermore, VIPR2, the VIP receptor, is necessary to maintain synchrony within individual SCN neurons but also between the SCN and other tissues (Maywood et al., 2006). *Vipr2* KO mice exhibit disrupted behavioral and metabolic rhythms in line with altered SCN neuronal activity and clock gene expression (Harmar et al., 2002). Maywood *et al.* also show that the neuropeptide GRP acutely enhances and synchronizes the molecular clock in SCN neurons with deleted *Vipr2* gene. However, these rhythms are not sustained. VIP and GRP further act as paracrine signals within the SCN and are able to drive circadian oscillations in arrhythmic *Cry*-null SCN tissue (Maywood et al., 2011). The main communication route within the SCN is from VIP expressing core neurons to AVP containing shell neurons. There is little reciprocal communication. In addition, VIP-deficient mice exhibit reduced *Avp* expression in the SCN underpinning the importance of VIP-to-AVP communication (Varadarajan et al., 2018).

As outlined above, synchrony within the SCN is achieved by inter-neuronal coupling and paracrine signals. AVP containing SCN shell neurons also connect the SCN with hypothalamic and extra-hypothalamic brain regions to regulate behavior and physiology. Electrical lesions of the SCN lead to behavioral arrhythmicity and internal desynchronization which can be restored by SCN graft transplantation (Ralph et al., 1990). This led to the accepted opinion that the SCN clock is the central pacemaker driving all subordinated clocks and rhythms. However, this view has been challenged in the recent years. Genetic SCN clock knockout (SCN-KO) mice exhibit rhythmic locomotor activity and circadian oscillations in peripheral tissue clocks under LD conditions (Husse et al., 2011, 2014; Kolbe et al., 2019). These findings are further supported using other mouse lines which inherit a non-functional SCN clock (Izumo et al., 2014; Weaver et al., 2018). It is plausible, that light directly affects behavioral and food intake rhythms by an effect called light masking. This term describes the effect that light acutely suppresses activity in nocturnal animals. Mice with a genetically ablated clock in the SCN show strong light

masking which becomes apparent under unnaturally short LD cycles (Husse et al., 2011). However, in DD conditions, SCN-KO mice become behaviorally arrhythmic and tissue clock as well as hormonal rhythms are rapidly dampened and desynchronized. Additionally, these mice exhibit metabolic impairments (Husse et al., 2014; Izumo et al., 2014; Kolbe et al., 2016, 2019; Weaver et al., 2018). Taken together, this shows that the SCN clock is essential to maintain synchrony between tissue clocks under *zeitgeber*-free (free-running) conditions. In contrast, it is dispensable under LD conditions.

Entrainment to the LD cycle is mediated by the SCN which directly and indirectly innervates the sub-paraventricular zone (SPVZ) and the ventrolateral preoptic nucleus (VLPO) regulating rest:activity rhythms (Abrahamson and Moore, 2006; Deurveilher et al., 2002). Projections from the SCN to the DMH, to the lateral hypothalamus (LH) and to the *locus coeruleus* are driving rhythms of locomotor activity and feeding (Aston-Jones et al., 2001; Chou et al., 2003; Mahoney et al., 2013). The ARC is innervated by the SCN and regulates activity, temperature and corticosterone rhythms (Buijs et al., 2017). The SCN projects to the paraventricular nucleus (PVN) controlling several hormonal axes (Isobe and Nishino, 2004; Vrang et al., 1995). Additional SCN projections are also described for other brain regions (Begemann et al., 2020; Kalsbeek et al., 1993; Kriegsfeld et al., 2004). Peripheral tissues are synchronized by hormonal outputs controlled by the SCN but they can also be affected *via* the ANS (Cailotto et al., 2009; Ishida et al., 2005). Interestingly, light exposure affects adrenal clock gene expression and corticosterone release without a photic response in the SCN clock (Kiessling et al., 2014). The behavioral range of entrainment is investigated using T-cycles, *i.e.* rhythmic LD schedules with a period different from 24 h. The range of entrainment is defined as the range of *zeitgeber* periods to which an organism, tissue or cell is able to entrain (Aschoff and Pohl, 1978). The range of entrainment for light is between 21 h and 27 h, but behavioral entrainment to 28-hour cycles has also been observed (Aton et al., 2004; Erzberger et al., 2013; Stephan, 1983; Usui et al., 2000; West et al., 2017). These studies, however, report high variances in entrainment ability and strength under extremely short and long T-cycles. In contrast, various clock gene mutant mice are able to entrain to a broad range of T-cycles indicating that a fully functional circadian system narrows the entrainment range (Erzberger et al., 2013).

The SCN regulates body temperature rhythms by regulating behavior, *e.g.* sleep:wake cycles and the timing of food intake (Abe et al., 1979; Osborne and Refinetti, 1995). In contrast, baseline thermoregulation is controlled by the preoptic area (POA). Lesions of the POA increase body temperature independent of the ambient temperature but without disrupting its diurnal rhythm (Osborne and Refinetti, 1995). Indirect and direct reciprocal projections have been described between the SCN and the POA indicating that feedback mechanisms are involved in body temperature regulation (Chou et al., 2002; Deurveilher et al., 2002). In short, thermoregulation is controlled by interconnected brain regions which integrate information from thermoreceptors residing in the skin. The lateral parabrachial nucleus (LTB) integrates information about ambient temperature and projects to the POA. The information is processed within the POA sub-nuclei and thermogenesis-promoting or -inhibiting signals are

transmitted to the DMH. The DMH regulates peripheral heat production and heat loss in brown adipose tissue (BAT), muscle, skin, and vasculature (Morrison, 2016). Thereby, short term temperature fluctuations are counterbalanced by heat production and loss and, thus, overall body temperature maintains relatively constant.

As pointed out earlier (see 1.2.3), the SCN receives non-photic input from different brain regions including the PVN, ARC, DMH, the intergeniculate leaflet (IGL) and the NTS. These projections feed back to the SCN mediating the organism's metabolic, behavioral, and arousal state. Thus, the SCN receives information from the environment as well as from the body and integrates all signals to temporally adjust systemic physiology.

1.4.2 *Peripheral clocks*

Peripheral tissue clocks retain circadian oscillations for many days in isolation (Yamazaki et al., 2000; Yoo et al., 2004). This proves that peripheral tissues possess self-sustained circadian clocks. However, peripheral clocks desynchronize faster than the SCN clock which may be caused by weaker – or absent – inter-cellular coupling. Considering their function, peripheral tissues regulate the energy state of the body and, thus, are dependent on the interaction with other tissues and the ability to react to stimuli from those.

Bmal1 is the only non-redundant clock gene known so far. Its deletion immediately abrogates molecular circadian rhythms. Therefore, most of the studies target this gene to investigate the role of specific tissue clocks. Genetical clock ablation in the pancreas results in a type 1 diabetes-like phenotype caused by defective β -cell function and, thus, impaired insulin secretion (Marcheva et al., 2010). *Bmal1* deletion in adipose tissue leads to obesity which is triggered by a reduction of adipocytic poly-unsaturated fatty acid release and resultant changes in hypothalamic neuropeptide expression. This causes a shift in the timing of food intake and reveals an important role of the adipose clock in the temporal regulation of energy intake (Paschos et al., 2012). Liver specific *Bmal1* KO mice are hypoglycemic in fasting phases and show a loss in hepatic glucose regulatory genes. This highlights the importance of the liver clock for balancing glucose export especially in the resting phase (Lamia et al., 2008). In contrast, myocyte-specific loss of *Bmal1* impairs glucose import by reducing protein levels of the main glucose uptake transporter (GLUT4). Furthermore, expression of key enzymes involved in glucose metabolism are altered which affects muscle glucose metabolism (Dyar et al., 2014). There are only minor effects on body weight regulation in liver- and adipose-specific *Bmal1* KOs. Altogether, in most cases tissue-specific clock ablation leads to much milder phenotypes compared to global clock ablation. This suggests compensatory effects by other tissue clocks balancing metabolic homeostasis. Adrenal cortex-specific clock ablation abolishes behavioral and GC rhythms under DD conditions. Loss of GC rhythms are likely caused by the absence of circadian rhythm in the steroidogenic acute regulatory protein (StAR) which is the rate-limiting enzyme of steroidogenesis (Son et al., 2008). Disruption of rhythmic steroidogenic gene expression, including *StAR*, is also seen in another transgenic mouse strain with an ablated adrenal cortex clock. In contrast to the study of Son *et al.*, these mice remain

behaviorally rhythmic under DD conditions (Dumbell et al., 2016). Under short T-cycles, the adrenal clock stabilizes the circadian GC rhythm and prevents hyperadrenocorticism (Engeland et al., 2018). Contradictory results are shown for the role of the adrenal clock in acute stress responses. Recent studies observe increased GC release in response to stress in adrenal clock mutants (Engeland et al., 2018, 2019) whereas earlier studies demonstrate normal stress responses (Dumbell et al., 2016; Son et al., 2008).

Reconstitution of the liver clock in *Clock* mutant mice normalizes body weight under high-fat diet (HFD) conditions (Meyer-Kovac et al., 2017). The liver clock alone, in otherwise *Bmal1* KO mice, can drive rhythmic oscillations in metabolic pathways like nicotinamide adenine dinucleotide (NAD⁺) salvage and glycogen turnover. Interestingly, the liver clock alone can only reconstitute about 10 % of the normally rhythmic transcripts under LD conditions but these rhythms are abolished under DD conditions. The restoration of the liver clock has no beneficial effect on body weight development or glucose homeostasis under standard diet conditions (Koronowski et al., 2019).

Taken together, data from the SCN and peripheral tissue-specific clock ablation studies contradicts the traditional view of the SCN clock being the master pacemaker and the peripheral tissue clocks being the “slaves” of the circadian system. On the other hand, tissue-specific clock restoration can reconstitute some circadian oscillations but is not sufficient to keep synchrony under *zeitgeber*-free conditions. These data suggest that the circadian clock system is rather organized like a federate network (Heyde and Oster, 2020). *Zeitgeber* information needs to be processed, integrated, and revaluated on several levels to ensure optimal metabolic homeostasis.

1.4.3 Regulation of metabolism

Clock-controlled mechanisms have evolved to regulate metabolite uptake and release. These mechanisms prevent dramatic fluctuations and, thus, ensure metabolic homeostasis.

Appetite and feeding are controlled by the central nervous system driving homeostatic food intake (Schwartz et al., 2000). Circadian rhythms are detectable in interconnected brain regions which regulate food intake, *e.g.* ARC, DMH and VMH (Feillet et al., 2008; Guilding et al., 2009; Orozco-Solis et al., 2016). These brain regions express orexigenic or anorexigenic neurotransmitter and neuropeptides, *e.g.* orexin, neuropeptide Y (NPY), agouti-related protein (AgRP), melanin-concentrating hormone (MCH), pro-opiomelanocortin (POMC) and cocaine and amphetamine regulated transcript (CART), some of which show circadian oscillations (Oppenhuizen et al., 2016; Stütz et al., 2007). Altogether, the interplay of orexigenic and anorexigenic factors controls food ingestion (Oppenhuizen et al., 2016; Schwartz et al., 2000; Stütz et al., 2007). Food intake increases blood glucose levels. In a response to high glucose levels, the pancreatic hormone insulin is secreted into the bloodstream to reduce the blood glucose level. Upon binding of insulin to its receptor in various peripheral tissues, glucose transporters are translocated into the plasma membrane to reduce blood glucose levels (Agouni et al., 2010). Levels of leptin, a hormone secreted by adipocytes, correlates with insulin

levels and with body fat content (Ahrén et al., 1997). Insulin and leptin can cross the blood brain barrier to stimulate responsive brain regions which reduce energy intake (Campfield et al., 1995; Woods et al., 1979). Levels of the stomach-derived peptide ghrelin decrease during feeding and increases during fasting. Elevated ghrelin levels induce food intake by stimulating susceptible brain regions (Kojima et al., 1999; Nakazato et al., 2001). The ARC integrates peripheral signals on energy homeostasis, *e.g.* insulin, leptin, and ghrelin levels. It projects to neurons of the VMH, DMH, PVN and lateral hypothalamic area (LHA) controlling feeding behavior (Bouret et al., 2004; Yi et al., 2006). The VMH projects to the SCN communicating the peripheral metabolic signals to the master pacemaker (Yi et al., 2006). Furthermore, the circadian clock in the VMH regulates cyclic thermogenesis in BAT and modulates basal energy expenditure (Orozco-Solis et al., 2016).

The function of the circadian clock is important for metabolic homeostasis. *Clock* mutant mice are hyperphagic, obese and under HFD conditions develop the metabolic syndrome (Turek et al., 2005). *Bmal1* KO and *Clock* mutant mice display impaired glucose tolerance and reduced insulin sensitivity. This is probably due to the loss of circadian rhythms in the production of rate-limiting enzymes which are important for metabolic homeostasis (Rudic et al., 2004). *Per2* KO mice show altered lipid metabolism and hypotriglyceridemia (Grimaldi et al., 2010). *Per1/2* double KO mice show higher body weight and constantly high leptin levels compared to wildtype (WT) mice resulting in leptin resistance. In contrast, *Cry1/2* double KOs also show constant leptin levels which are increased during the inactive phase and lower during the active phase compared to WT leptin levels (Kettner et al., 2015). Furthermore, it becomes apparent, that coherent circadian timing between the different tissue clocks is needed for optimal energy homeostasis (see 1.4.2). From an evolutionary perspective, temporal compartmentalization is necessary to separate opposing metabolic pathways. Clock deficiency results in arrhythmicity in behavior and feeding patterns. Clock gene mutant animals often develop obesity paired with a loss of rhythmic output of energy homeostasis-related hormones (Kiehn et al., 2017). Mice consuming HFD develop diet-induced obesity. These obese mice show disrupted behavioral rhythms and dampened clock gene rhythms in the liver and white adipose tissue (WAT) (Chaix et al., 2014; Hatori et al., 2012; Kohsaka et al., 2007). Moreover, obese mice display altered glucose and fatty acid metabolism. Thus, loss of rhythm promotes adverse health outcomes. Restricting food intake to the dark phase in nocturnal mice prevents the detrimental effects of a HFD (Chaix et al., 2014; Hatori et al., 2012). These studies show that the time of feeding, instead of the food composition, impacts the transcriptome and alterations in the timing of food ingestion lead to negative metabolic outcomes.

On the tissue level, differences in energy state are anticipated and expression of important transporters and enzymes is under circadian control. In the liver, expression levels of glucose transporters and glycogen synthase peak in the beginning of the active phase preparing the liver for glucose uptake and energy storage (Doi et al., 2010; Panda et al., 2002b). On the other hand, in the fasting (*i.e.* rest) phase, glucagon stimulates the expression of two key enzymes of gluconeogenesis, phosphoenopyruvate carboxykinase 1 (*Pck1*) and glucose-6-phosphatase

(*G6pc*), by CREB-mediated transcriptional activation (Zhang et al., 2010). Furthermore, GCs also activate *Pck1* expression (Lamia et al., 2011). In contrast, CRY proteins block glucagon-mediated increases in cAMP and inhibit GR activity and, thus, inhibit gluconeogenesis towards the end of the fasting period (Lamia et al., 2011; Zhang et al., 2010). Additionally, GCs regulate the transcription of numerous metabolic genes of glucose metabolism and therefore play an important role in energy homeostasis (Weger et al., 2016). However, GCs can also promote negative health outcomes. Chronic treatment with a GC analogue leads to glucose and insulin intolerance in WT mice. In contrast, deletion of the functional GRE in the *Per2* gene leads to elevated leptin levels, but mice are protected against the development of glucose and insulin intolerance (So et al., 2009). This suggests that GCs regulate glucose homeostasis, in part, directly *via* control of the circadian clock.

On the molecular level, metabolic state and the circadian clock are intertwined *via* a number of feedback mechanisms. NAD⁺ levels oscillate in a circadian manner which is driven by the circadian clock. BMAL1:CLOCK regulate the circadian expression of nicotinamide phosphoribosyltransferase (*Nampt*), the rate-limiting enzyme in the NAD⁺ salvage pathway (Nakahata et al., 2009; Ramsey et al., 2009). High NAD⁺ levels activate the histone deacetylase sirtuin 1 (SIRT1) which counteracts the histone acetyltransferase function of CLOCK. Thereby, SIRT1 inhibits BMAL1:CLOCK-mediated transcription including that of *Nampt* (Asher et al., 2008; Nakahata et al., 2008). In consequence, SIRT1 activity is repressed by decreasing NAD⁺ levels and BMAL1:CLOCK can re-activate the expression of CCGs. Mice with constant high levels of NAD⁺ show altered behavioral rhythms which might be caused by deregulated clock gene expression. Furthermore, these animals display altered gene expression involved in amino acid metabolism underpinning the essential role of oscillating NAD⁺ levels (Sahar et al., 2011). A recent study suggests that NAD⁺ regulates PER2 acetylation and nuclear translocation. Thereby, BMAL1:CLOCK-mediated transcription is controlled (Levine et al., 2020). Another feedback loop mechanism is comprised of the adenosine monophosphate (AMP)/ adenosine triphosphate (ATP) ratio within the cell. AMP activates AMPK which induces a metabolic switch activating catabolic pathways in the periphery. At the same time, activated AMPK stimulates food intake in the hypothalamus (Minokoshi et al., 2004). *In vitro*, AMPK phosphorylates CK1 α and CRY1 affecting the circadian rhythm (Um et al., 2011). Furthermore, AMPK has been shown to promote degradation of CRY1 and PER2 the latter of which is indirectly mediated by AMPK-mediated phosphorylation of CK1 ϵ which then phosphorylates PER2 (Lamia et al., 2009; Um et al., 2007). Tissue clocks are further affected by extracellular metabolites, *e.g.* glucose, glutamine, lactate and polyamines (Reinke and Asher, 2019).

1.5 Impact of chronodisruption on the circadian clock network

The circadian network modulates behavior and physiology to anticipate recurrent changes in environmental demands. Abrupt or unforeseeable changes in external conditions, in turn, may lead to circadian disruption. On the other hand, changes in behavior leading to physiological changes can result in circadian disruption, too. Industrialization led to changes of daily life

and shift work is common in modern societies. On a long-term scale, those changes favor chronodisruption, *i.e.* a misalignment of external and internal time which ultimately results in internal desynchronization. This internal desynchronization is suggested to promote various diseases which are common in modern societies (**Figure 1-3**).

1.5.1 Chronodisruption and its health effects

Chronodisruption can be provoked by sleep deprivation, shift work, light pollution, and social jetlag. All of those are common in modern societies. Sleep:wake cycles are one of the major behavioral outputs of the circadian system and, thus, a powerful pathway of regulating food intake timing. Sleep deprivation increases food intake and craving for hedonic food (Brondel et al., 2010; Greer et al., 2013; Hogenkamp et al., 2013). In line with this, the abundance of circulating metabolites is changed which acts on the circadian clock as previously described. Interestingly, sleep deprivation impairs memory formation, a process which also is regulated in a circadian fashion (A.Kondratova et al., 2010; Binder et al., 2012; Eckel-Mahan et al., 2008; Krishnan and Lyons, 2015; Lewis et al., 2020). Chronic jetlag interferes with learning in humans and animals and has long-lasting effects on memory formation (Cho et al., 2000; Gibson et al., 2010).

Shift work is associated with mistimed sleeping and sleep curtailment, but naturally also with altered behavioral rhythms. Obesity, type 2 diabetes, metabolic syndrome, cardiovascular diseases but also certain types of cancer are more prevalent in shift than in day workers (Bacquer et al., 2009; Biggi et al., 2008; Karlsson et al., 2003; Kawachi et al., 1995; Szosland, 2010; Zimberg et al., 2012). Altered sleep:wake rhythms are highly prevalent in modern societies and growing evidence shows an association between disturbed sleep and the development of diabetes (Karthikeyan et al., 2019).

99 % of the population in the USA and Europe live in areas where the night sky is brighter than the light pollution threshold (Cinzano et al., 2001). Studies in mice show that LL conditions lead to body weight gain and impaired glucose tolerance. However, food intake and locomotor activity levels are comparable to mice housed under 12 h light: 12 h darkness (LD-12) conditions. Under LL conditions, mice shift their time of food intake which likely causes a disruption in metabolic homeostasis. Restricting food access time to the normal active phase under LL conditions, in turn, prevents body weight gain in mice (Fonken et al., 2010). This shows the importance of the regulation of food intake timing which must happen in synchrony with the SCN clock to prevent metabolic disruption. Furthermore, constant light increases anxiety-like and depression-like behavior in rats. Under these conditions, GC concentrations were reduced suggesting an impaired HPA axis regulation (Fonken et al., 2009).

Social jetlag is defined as the time difference between the midpoint of sleep on workdays and on free days. It is an assumption of the misalignment between the internal and social time. In Europe, one third of the population suffers from 2 h or more of social jetlag and increased social jetlag correlates with increased BMI in overweight people (Roenneberg et al., 2012). Other studies show an association between 2 h or more of social jetlag and an increased risk

for metabolic syndrome, type 2 diabetes and cardiovascular disease (Koopman et al., 2017; Parsons et al., 2015; Wong et al., 2015).

Together, chronodisruption is associated with adverse health outcomes likely caused by internal desynchrony. Understanding the underlying mechanisms is important to develop therapeutic approaches so this is the aim of ongoing research (see 1.5.3).

1.5.2 Mechanistic model of internal desynchronization

Light is the most potent *zeitgeber* for our circadian system. However, in modern societies disruptive *zeitgeber* input is common, *i.e.* receiving light input in the normal dark phase. Early studies show that even low illumination can cause phase shifts in the circadian system (Boivin and Czeisler, 1998). Shift workers are exposed to sunlight during the day and artificial light at night. Thus, the SCN receives photic input at all circadian phases which likely disrupts the circadian system. Furthermore, it is evident that shift work, but also modern lifestyles in general, are accompanied by changes in food intake timing (Gill and Panda, 2015). Moreover, food intake timing varies from day to day and, thus, the circadian system cannot anticipate the food intake time. Because concentrations of circulating metabolites and metabolism-related hormones correlate with food intake they are altered in chronodisruptive conditions.

Another disruptive *zeitgeber* input is mediated by social *zeitgebers*, *e.g.* exercise and social activities. In humans, these are also frequently phase shifted and the daily duration of exposure is extended in modern 24-hour societies. It is plausible that these changes correlate with a shortening in total sleep durations, but results are controversial (Hoyos et al., 2015). It has not been investigated yet if disruptions in ambient temperature can cause internal desynchronization. The impact on humans, if any, might be overall low as the *zeitgeber* temperature has only minor effects in homeothermic animals.

In summary, *zeitgebers* differentially influence circadian clocks throughout the body and disruptive inputs cause loss of phase coherence within the clock network, *i.e.* internal desynchrony. In the end, this causes disruptions in metabolic homeostasis which promote the development of numerous diseases, *e.g.* obesity, metabolic syndrome, cancer, cardiovascular diseases, cognitive and mood disorders.

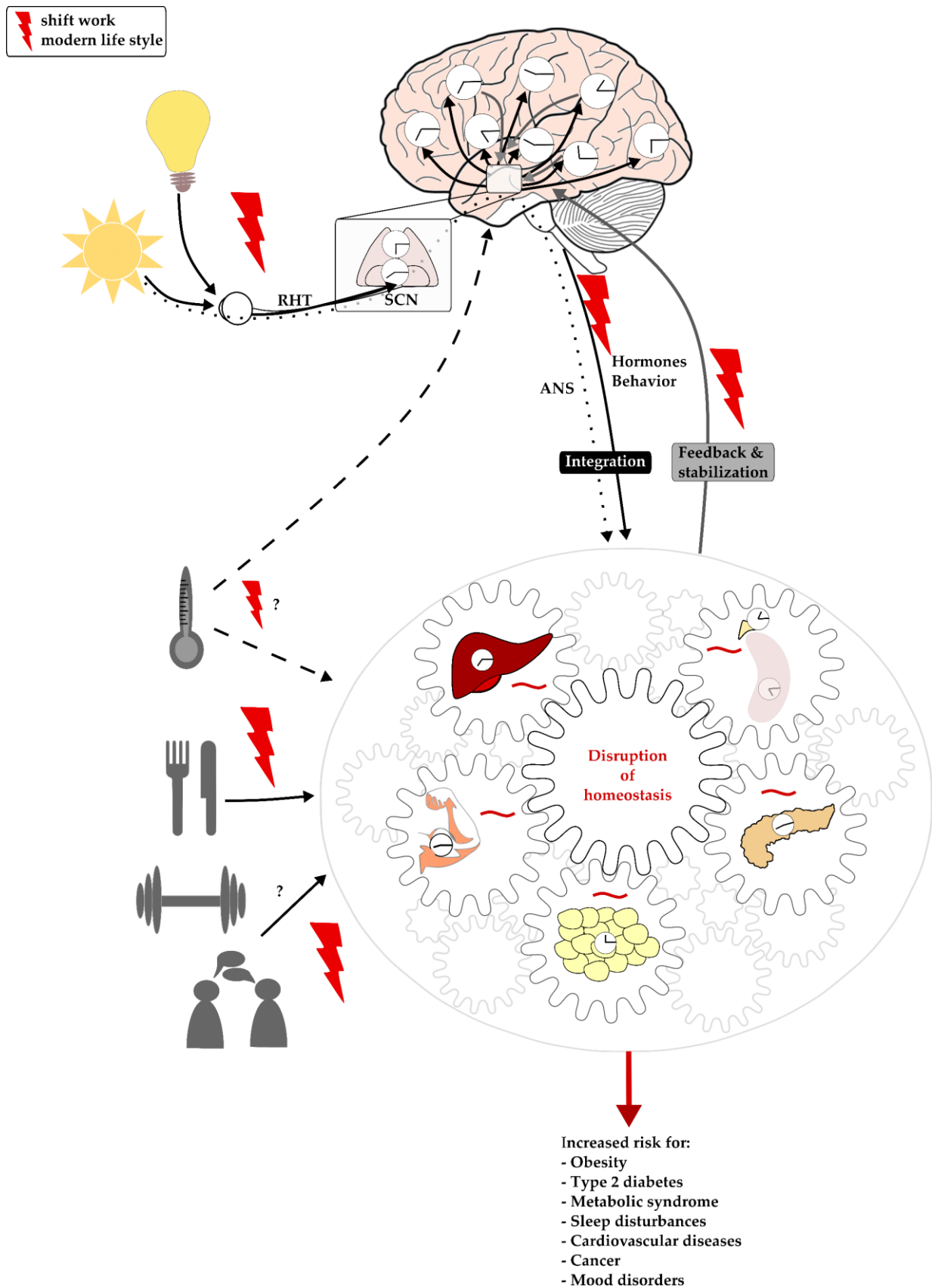


Figure 1-3: Chronodisruption leads to internal desynchronization disrupting metabolic homeostasis. Unnatural light input to the SCN disrupts the SCN clock leading to an internal desynchronization within the SCN nuclei. In consequence, SCN outputs are disrupted and subordinated central nervous system (CNS) clocks are misaligned with each other. Behavioral and hormonal outputs are perturbed,

and synchronization of peripheral tissue clocks is impaired. Moreover, changes in food intake time primarily impact peripheral tissue clocks leading to internal desynchrony within the peripheral tissue clocks but also between peripheral and central tissue clocks. Changes in the social *zeitgebers*, e.g. physical activity and social interactions, may exacerbate internal desynchrony. The chronodisruptive potential of the *zeitgeber* temperature is not yet investigated in humans. Altogether, *zeitgeber* disruptions lead to an internal desynchrony of the circadian clock network which results in a disruption of metabolic homeostasis. Ultimately, these disruptions promote the development of various diseases.

1.5.3 Effects on the circadian clock and metabolism

1.5.3.1 Effects on the systemic level

Humans with 2 h or more of social jetlag display higher cortisol levels and increased resting heart rates compared to participants with minor social jet lag. These parameters suggest a state of chronodisruption that may contribute to the development of metabolic diseases (Rutters et al., 2014). Shift workers show dampened amplitude of cortisol rhythm its circadian pattern remains entrained to the LD cycle (Touitou et al., 1990). Elevated cortisol levels at the cortisol nadir may be explained by changes in activity patterns. Similar effects are observed in a rat model of night work. In rats, night work is simulated by forced activity for 8 hours in the middle of the resting phase on weekdays. Simulated night work shifts the temporal pattern of activity and food intake. In consequence, daily rhythms in glucose are abolished, and those of triglyceride are reversed. Corticosterone and SCN clock gene rhythms stayed aligned to the LD cycle. Additionally, forced activity induces a transient increase in corticosterone levels (Salgado-Delgado et al., 2008). Forced activity and feeding are uncoupled from the circadian clock inducing internal desynchronization which may cause adverse health outcomes. Interestingly, time restricted feeding to the normal active phase prevents the development of obesity and circadian desynchrony in this model of night work. Nighttime-restricted feeding maintains the circadian variation in glucose and triglycerides which are lost in forced-activity rats with daytime-restricted or *ad libitum* food access. Corticosterone rhythms are not influenced by TRF (Salgado-Delgado et al., 2010). The data show that TRF to the normal food intake time can prevent internal desynchronization even under chronodisruptive *zeitgeber* input conditions.

Shift workers show higher levels of triglycerides and lower levels of high-density lipoprotein (HDL) cholesterol. Furthermore, they display higher abdominal obesity compared to day workers (Karlsson et al., 2003). Interestingly, social jetlag is also associated with lower HDL cholesterol, higher triglycerides and higher fasting insulin levels as well as insulin resistance besides adiposity (Wong et al., 2015). In addition, unhealthy obese people show an association between social jetlag and inflammation which may further promote adverse health outcomes (Parsons et al., 2015). One day of total sleep deprivation enhances nighttime melatonin but has no influence on cortisol rhythms in humans (Ackermann et al., 2013; Goh et al., 2001; Honma et al., 2020). It also suppresses the release of growth hormone (GH) and decreases the levels of the pro-inflammatory cytokine interleukin-6 (IL-6) in the blood (Redwine et al., 2000). Sleep curtailment decreases insulin sensitivity and increases inflammation in a laboratory study (5 h of sleep at night, aligned group). Misaligned sleep restriction, i.e. a delay of sleep timing by

8.5 h on 4 of the 8 laboratory days, aggravates insulin resistance and inflammatory markers compared to the aligned group (Leproult et al., 2014). Another study in humans shows that a 4-hour delay in sleep time for 3 consecutive days leads to a dramatic reduction of detectable circadian transcripts in the blood (Archer et al., 2014).

In humans, the forced desynchrony protocol has been developed to induce chronodisruption. In the laboratory, daylength is extended to 28 h and time spent awake or asleep is proportionally adjusted in a ratio of 2:1 (18.66 h light/wake and 9.33 h dark/sleep) and meals are provided 1 h, 5 h, 11.5 h and 15.5 h post-wakening. Thus, participants eat and sleep at all circadian phases. On the day of full circadian misalignment, when participants eat and sleep 12 h out of phase compared to their aligned day (first day of experiment), leptin levels are decreased by 17 % whereas glucose and insulin levels are increased by 6 % and 22 %, respectively. Most participants exhibit increased postprandial glucose response on the misaligned day compared to the aligned day (Scheer et al., 2009). In this study, participants could proportionally sleep the same amount of time as under pre-experimental conditions. Thus, in another study the forced desynchrony protocol was combined with sleep curtailment (5.6 h/ 24 h). Averaged over the 19 experimental days, participants exhibit reduced resting metabolic rates. This could contribute to the weight gain seen in shift workers. Furthermore, postprandial glucose is increased (+ 15 %) and postprandial insulin secretion is decreased (- 27 %) compared to baseline measurements. These findings suggest an impaired pancreatic β -cell response. Interestingly, postprandial leptin and ghrelin responses are comparable between baseline and chronic chronodisruption measures (Buxton et al., 2012). In the laboratory, internal desynchronization is evoked by the free-running circadian clock which is uncoupled from the enforced sleep:wake and eating:fasting cycles.

Altogether, chronodisruption changes hormonal levels, food intake timing and thereby metabolite occurrence which likely affects tissue transcriptomes. Temporal patterns of corticosterone stay aligned with the LD cycle, although some effects on the amplitude are observed. In consequence, misalignment of peripheral tissue clocks and corticosterone rhythm, as one of the major circadian outputs, aggravates desynchrony between peripheral and central clocks. Altogether this leads to disruption of metabolic homeostasis.

1.5.3.2 *Effects on the cellular level*

Experimental jetlag, *i.e.* an abrupt phase shift in the LD cycle, causes desynchrony within the SCN clock. In the SCN core region, *Per1*, *Per2* and *Cry1* expression rhythms rapidly re-entrain to the new LD cycle whereas re-entrainment in the non-photoreceptive shell is relatively slow. Furthermore, re-entrainment is faster for phase delays than for advances (Nagano et al., 2003).

In mice, timed-sleep restriction (TSR, 2 x 5 days of 6 h sleep restriction) induces internal desynchrony and by this metabolic homeostasis is disrupted. TSR elevates the overall expression of clock genes in the SCN but the temporal pattern remains entrained to the LD cycle. In the liver, rhythmic transcription of glucose metabolism-related genes is abolished (Barclay et al., 2012). The same experimental approach reveals the induction of lipogenic and

glycolytic genes in WAT. Some of the transcriptional changes last for more than a week after the last sleep restriction. Interestingly, *Per1/2* double KO mice are protected from adverse metabolic changes which suggests that sleep restriction perturbs the circadian clock which then disrupts metabolic homeostasis (Husse et al., 2012). Sleep restriction changes activity patterns and influences food intake timing. This causes the reprogramming of clock- and food-driven transcription. This interaction makes it hard to unravel the contribution of each parameter. This is addressed in another study. Here, nighttime-restricted feeding during TSR prevents the transcriptional reprogramming which again highlights that time-restricted feeding may be a therapeutic approach for shift workers (Barclay et al., 2012). In leukocytes, one day of total sleep deprivation suppresses *Bmal1* expression but has no effect on other core clock genes (Ackermann et al., 2013).

Daytime-restricted feeding phase-shifts or abolishes rhythmic expression of clock genes and genes involved in energy metabolism in the LH, liver, and muscle. Thus, food intake during the rest phase induces desynchronization between the tissues (Opperhuizen et al., 2016). All tissues of the HPA axis show circadian oscillations synchronized with the SCN. However, HPA tissue clocks are entrained to the scheduled feeding (Girotti et al., 2009). GC secretion is also entrained to time restricted feeding and may promote changes in metabolic homeostasis by the regulation of target gene expression.

Under LD-11 (11 h light:11 h darkness) conditions, it is suggested that the SCN clock desynchronizes. In this protocol, rats display two activity periods. One activity period is entrained to the short LD cycle, the other free-runs with a period greater than 24 h. It is proposed that this is caused by different entrainment capacities in the SCN core and shell which leads to distinct periodicities (de la Iglesia et al., 2004). Internal desynchrony is not only apparent in the locomotor activity period but also in core body temperature and sleep stages (Cambras et al., 2007). Chronic internal desynchrony further manifests in de-compression and compression in hormonal release and patterns *e.g.* for melatonin and corticosterone (Schwartz et al., 2009; Wotus et al., 2013). Of note, such short LD cycles also result in depression-like phenotypes which reflects the human pathology under chronodisruptive conditions (Ben-Hamo et al., 2017).

Taken together, modern societies are prone to chronodisruption which results in internal desynchrony. This promotes adverse health outcomes. It is suggested that changes in behavior further aggravate internal desynchrony. Furthermore, it is proposed that TRF can improve internal synchrony under chronodisruptive conditions. The effects of the two *zeitgebers*, light and food, on the circadian clock system are extensively studied. It is widely accepted that light is the most potent *zeitgeber* for the SCN while food is the most potent *zeitgeber* for peripheral tissues. To date, there is little knowledge about the differential contributions of the two *zeitgebers* to the circadian system under conflicting *zeitgeber* conditions.

1.6 Hypothesis

In this PhD project, the contribution of different *zeitgeber* input on tissue clock resetting was investigated. Therefore, mice were kept under diverging *zeitgeber* input and expression of clock genes in different tissues, as well as behavioral and endocrine rhythms were investigated under maximal and minimal *zeitgeber* (light and food) alignment. It was hypothesized that conflicting *zeitgeber* input differently impacts distinct tissue clocks.

Furthermore, metabolic state was studied under long-term diverging *zeitgeber* input conditions. To this end, daily food intake and body weight were measured. Additionally, the metabolic state was further explored under minimal and maximal *zeitgeber* alignment conditions. It was hypothesized, that conflicting *zeitgeber* input impairs metabolic homeostasis through internal desynchronization.

2 Materials and Methods

2.1 *Animal experiments*

All animal experiments were performed in accordance with the German Law on Animal Welfare and the FELASA guidelines for animal research. They were ethically assessed and legally approved by the 'Ministerium für Energiewende, Landwirtschaft, Umwelt und ländliche Räume (MELUR)' of the State Schleswig-Holstein, Germany.

For this PhD thesis only adult male C57BL/6J wild-type mice were used. Mice were either bred in the animal facility of the University of Lübeck or obtained from the commercial mouse breeder Janvier (C57BL/6JRj).

2.2 *Animal housing*

Animals were kept in the animal facility of the University of Lübeck. Prior to experiments, mice were housed in small groups (up to 5 animals *per* cage) in individually ventilated cages (SealSafe Plus GM500, Tecniplast). In all animal rooms, temperature and humidity were kept at 21-24 °C and 55-65 %, respectively. Mice were kept under LD-12 conditions with 250-300 lux illumination in the light phase. The animals had access to regular chow (breeding diet #1314, Altromin) and tap water *ad libitum*.

During the experiments, mice were singly housed in transparent plastic cages (1155M or 1284L, Tecniplast) with built-in steel running-wheels (11.5 cm diameter, #6083, TRIXIE GmbH & Co. KG 6083) placed in isolation cabinets fitting 12 cages (Jud et al., 2005). Cages were changed every 2-3 weeks primarily in the active phase to minimize disturbances of the mice's daily behavior.

2.3 *Zeitgeber intervention paradigms*

Distinct *zeitgeber* intervention paradigms were established to quantify the differential contribution of the two main *zeitgebers*, light and food, to the resetting of specific tissue clocks and the effect of *zeitgeber* desynchrony on metabolic homeostasis. Prior to all experiments, mice were acclimatized to single-housing and running-wheel cages under LD-12 conditions with access to regular chow and tap water *ad libitum*. Running-wheel usage was recorded during acclimatization and throughout the experiments. If weighing of food and mice took place in the dark phase, the weighing was conducted under dim red light. Of note, results of *zeitgeber* desynchrony (ZD) and inverse *zeitgeber* desynchrony (iZD) and the corresponding methods have been published in December 2019 (see Heyde and Oster, 2019). In the ZD paradigm, peripheral gene expression data of two of the three anti-phase profiles as well as food and body weight data were generated by two students. For this thesis, the raw data or tissue samples were reanalyzed or processed, respectively.

2.3.1 Acute zeitgeber intervention (ZI) paradigm

This paradigm was designed to investigate the acute effects of *zeitgeber* intervention on the circadian clock system. The light intensity was reduced to 3 lux at the start of the acute *zeitgeber* intervention experiment. In a first approach, mice were put under 14 h light: 14 h darkness (LD-14) conditions while food access was restricted to the dark phase (Dark-RF). Food was removed from the hoppers in the beginning of the light phase (ZT0) and put back in the beginning of the dark phase (ZT14) (**Figure 2-1 a**). To investigate the effects of conflicting zeitgeber input on the circadian system, animals were put under LD-14 conditions while food was not accessible for 12 h followed by 12 h food access every 24 h (RF-12) intervals. On the first day of the experiment food was removed 1 h after “lights on” (ZT1) and available again 1 h before “lights off” (ZT13) (**Figure 2-1 b**).

2.3.1.1 Daily profile generation in the acute ZI paradigm

Tissue samples and trunk blood were collected at evenly spaced intervals throughout the first (baseline) and fourth (misalignment) day as described in (**Figure 2-1**, asterisks). In the LD-14/Dark-RF paradigm, mice were sacrificed at ZT0, 7, 14, 21 and 28 spanning the LD cycle (ZT0 is “lights on”) on the baseline and misaligned day. Under LD-14/RF-12 conditions, mice were sacrificed at ZT3, 9, 15, 21 and 27 relative to the LD cycle on the misaligned day.

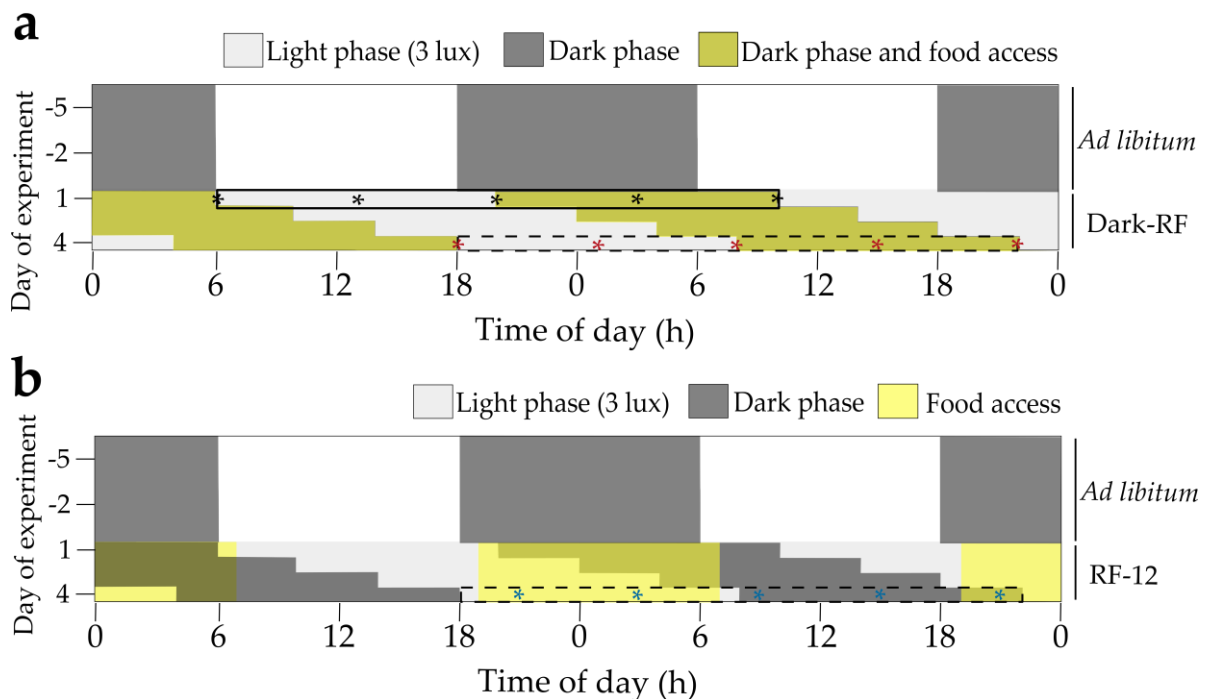


Figure 2-1: Acute zeitgeber intervention paradigms. After one week of acclimatization under LD-12 conditions with 250-300 lux illumination and food access *ad libitum*, the LD cycle was extended to LD-14 and light intensity was reduced to 3 lux in the light phase (light grey shading). Food access was restricted to either the dark phases (a) or to 12 h (b). Dark grey and yellow shadings indicate dark phases and food access times, respectively. Tissue and serum samples were collected on the first (baseline day, solid bar) and fourth (misalignment day, dashed bar) experimental day. Asterisks indicate timepoints of sacrificing.

2.3.2 Zeitgeber desynchrony (ZD) paradigm

This paradigm was designed to investigate the long-term effects of *zeitgeber* desynchrony on the circadian clock system and metabolic homeostasis. In the ZD paradigm, the effects of an abnormal (28 h) LD cycle combined with a normal (24-hour) feeding:fasting cycle were examined. The illumination was left unchanged throughout the whole experiment. Mice were handled daily during the acclimatization phase. At the start of the experiment mice were kept under 14 h light: 14 h darkness (LD-14) conditions while food access was changed to 12 h fasting followed by 12 h food access every 24 h (RF-12). On the first experimental day, food was removed from the hopper one hour after “lights on” (ZT1) and returned one hour before “lights off” (ZT13). Afterwards, food was removed and accessible in 12-hour time intervals (Figure 2-2). Every 6th experimental day, mice experienced conditions with food access coinciding with the dark phase (Figure 2-2, solid bars). These days were defined as *in-phase* days since under standard housing conditions mice, as a nocturnal species, consume ~70 % of their daily food intake in the dark phase (Greenwell et al., 2019). On those days, mice were exposed to maximal aligned *zeitgeber* input. Accordingly, the days at which the food was exclusively accessible in the light phase were defined as *anti-phase* days (Figure 2-2, dashed bars). Here, mice experienced maximal conflicting *zeitgeber* input. Each day mice and food were weighed prior to food access. Tissue samples and trunk blood were evenly collected throughout one in-phase and three anti-phase days in 6-hour time intervals (Figure 2-2, asterisks) (see 2.3.4). One experiment lasted between 4-9 weeks.

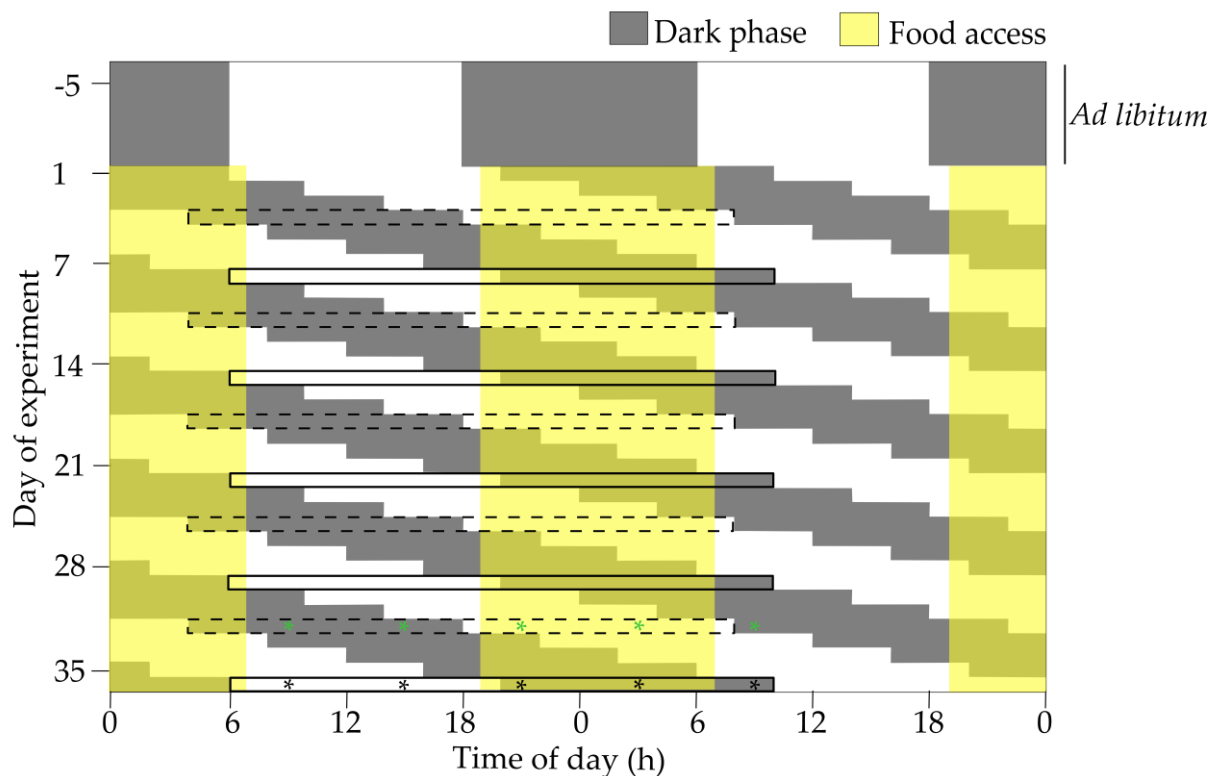


Figure 2-2: Scheme of the *zeitgeber* desynchrony (ZD) paradigm. Mice were acclimatized to single-housing and running-wheel cages for one week under LD-12 conditions and food access *ad libitum*. At the start of the experiment the LD cycle was extended to 14 h light: 14 h darkness (LD-14)

and food access was restricted to 12 h fasting: 12 h feeding. On the first day of the experiment food was removed one hour after “lights on” and accessible again one hour before “lights off”. Grey and yellow shadings indicate dark phases and food access times, respectively. Solid and dashed bars indicate in-phase and anti-phase days, respectively. Daily tissue and serum profiles were collected on selected in-phase and anti-phase days. Representative sacrificing timepoints for in-phase and anti-phase profiles are indicated by asterisks.

2.3.3 Inverse zeitgeber desynchrony (iZD)

This paradigm was complementary to the ZD paradigm, but with inverted *zeitgeber* periods. Like the ZD paradigm, it was designed to investigate the long-term effects *zeitgeber* desynchrony on the circadian clock system and metabolic homeostasis. In other words, in the iZD paradigm, the effects of an extended feeding:fasting cycle combined with a normal LD cycle were examined. During the acclimatization week, mice were accustomed to handling. Light schedule and intensity were left unchanged throughout the whole experiment (LD-12). On the first day of the experiment food was removed one hour before “lights on” (ZT23) and returned to the food hoppers one hour after “lights off” (ZT13). Thereafter, food was removed and accessible in 14-hour time intervals (RF-14) (**Figure 2-3**). Accordingly, every 6th experimental day mice experienced conditions with food access coinciding almost exclusively with the dark phase (**Figure 2-3**, solid bars, in-phase days). Again, days on which the food was mostly accessible in the light phase were defined as anti-phase days (**Figure 2-3**, dashed bars). Tissue samples and trunk blood were collected at evenly spaced intervals throughout in-phase and anti-phase days (**Figure 2-3**, asterisks) (see 2.3.4). The daily profiles were collected in the iZD 4th cycle.

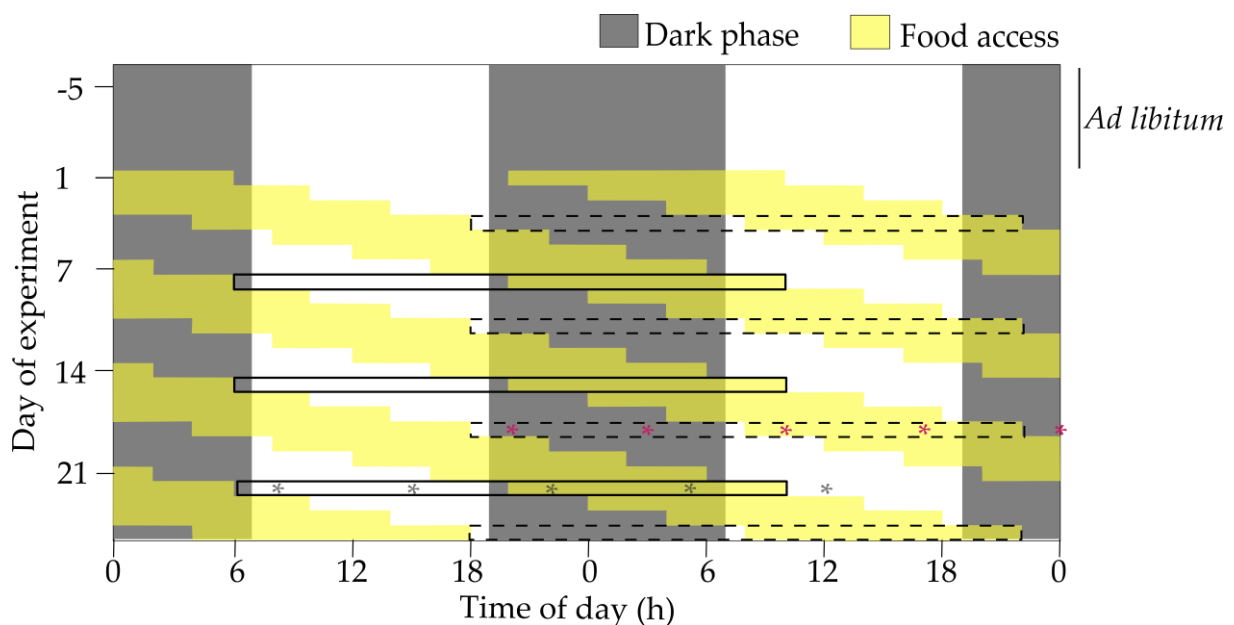


Figure 2-3: Scheme of the inverse *zeitgeber* desynchrony (iZD) paradigm. Mice were acclimatized to single-housing and running-wheel cages for one week under LD-12 conditions and food access *ad libitum*. At the start of the experiment the food access cycle was extended to 14 h fasting followed by 14 h food access (RF-14) while the LD cycle was left unchanged. On the first day of the experiment food was removed one hour before “lights on” and accessible again one hour after “lights off”. Grey and

yellow shadings indicate dark phases and food access times, respectively. Solid and dashed bars indicate in-phase and anti-phase days, respectively. Daily tissue and serum profiles were collected on selected in-phase and anti-phase days. Asterisks indicate timepoints of sacrificing.

2.3.4 Daily profiles in the ZD and iZD paradigms

In the ZD paradigm, animals were sacrificed on in-phase and anti-phase days at specified times relative to food access (“food in” at ZT12). Mice were sacrificed at ZT2, 8, 14, 20 and again at ZT2 on the following day. The latter time point was not included in the analysis. Under iZD conditions animals were sacrificed at comparable time points along the feeding:fasting cycle (e.g. “ZT2”= 2.3 h after “food out”).

2.4 Behavioral measurements

2.4.1 Locomotor activity

To investigate locomotor activity under different *zeitgeber* intervention paradigms, running-wheel usage of single-housed mice was assessed. Here, a running-wheel revolution induced an opening and closing of a magnetic switch which was recorded and subsequently analyzed by the ClockLab system and software (ClockLab version 6.0.34, Actimetrics). Activity recordings started during acclimatization to ensure that mice were adapted to LD-12 conditions and actually used the running-wheels. During the ZD and iZD experiments, mice and food were weighed daily prior to food access which induced a 2-minute gap in the activity recordings.

2.4.1.1 Analysis of locomotor activity in the acute ZI paradigm

Activity profiles were generated for the four pre-experimental days and for the days of acute ZI. Therefore, 20-minute bins of running-wheel activity were extracted. Average total activity over 24 h was calculated for the four pre-experimental days and used for determination of relative activity per bin size for the pre-experimental and experimental days. The obtained relative activity profiles were smoothed using a 3-neighbour running average in GraphPad Prism 7 (GraphPad Software).

To calculate the activity period under acute ZI conditions, activity onsets were determined daily by the experimenter. Subsequently, a straight line was fitted to the activity onsets to determine the activity period. This activity period was used as time reference in the gene expression analysis (see 2.11.1).

2.4.1.2 Analysis of locomotor activity in the ZD and iZD paradigms

For the ZD and iZD paradigms, activity rhythmicity and period lengths were determined by χ^2 periodogram analysis excluding the first 3 experimental days using ClockLab. For the χ^2 periodogram analysis, individual activity data were subjected to fast Fourier transformations with weighted least-squares analysis for a period range of 6-30 h. The standard deviation (SD) of individual periods was normalized to the SD of the entire data set and tested for statistical difference (Sokolove and Bushell, 1978). Peaks above $p < 0.05$ were considered as statistically significant.

For the ZD and iZD paradigms, activity profiles (presented in 7.5-degree bins with 3-neighbour running-average smoothing) were analyzed for in-phase and anti-phase conditions. Raw data were normalized to daily running-wheel activity averaged over 4-6 in-phase or anti-phase days with one day (360 °) lasting for 28 h (relative to light) or 24 h (relative to food) for each animal. 0 ° represents “lights on”/“food out” and 180 ° represents “lights off”/“food in”, respectively. Note, that for LD cycle profiles light and dark activity sections of anti-phase days were plotted in reverse order for easier comparison of phase angles. Feeding/fasting and light/dark distributions were calculated from individual normalized activity profiles and averaged for in-phase and anti-phase days for statistical testing.

For the ZD paradigm, activity onset of experimental days 1 to 36 were determined by three independent, experienced investigators on double-plotted actograms. Investigators were blind to the phase relation of the ZD cycle. If investigators disagreed, two additional investigators were asked to determine activity onsets. The activity onset times of at least two investigators had to match for inclusion in further analysis. Activity phase shifts were averaged over 3-5 in-phase to anti-phase transitions of individual activity onsets.

2.4.2 Core body temperature

Core body temperature measurements were conducted in the ZD and the iZD paradigm on a cohort which was not used for activity and gene expression analysis. Mice were intraperitoneally (i.p.) equipped with E-Mitter telemetry probes (G2 E-Mitter, STARR Life Science Corp.) which continuously recorded core body temperature. Implantation was performed under sterile conditions. Mice were anesthetized with isoflurane (Vetflurane, Virbac) (4 % in air; flow rate 400 mL/min) and with additional i.p. injections of the analgesic carprofen (Rimadyl, Zoetis; dose: 4 mg/kg in 0.9 % saline in a volume of 20 µL). Eyes were covered with eye ointment (Bepanthen, Bayer) to avoid dehydration. The depth of anesthesia was checked regularly by testing the toe reflex. A lack of the reflex indicated an adequate depth of anesthesia. The ventral part of the abdomen was shaved (ISIS Aesculap GT421, Braun Melsungen) and disinfected with 70 % ethanol (EtOH) (Ethanol ROTIPURAN ≥ 99.8 % p.a., Roth). Mice were secured on the table with adhesive tape. The abdomen was opened by a 1.5-centimeter incision on the left side of the midline. The probe was cleaned in 70 % EtOH and dried before placing it into the abdominal cavity. The abdomen skin was sutured and mice were placed in a heating chamber at 32 °C for 40-60 min to recover from surgery. Then, mice were returned to their home cages. Core body temperature recordings were started using VitalView Software (STARR Life Science Corp.) after 7 recovery days. G2 E-Mitter data were exported and analyzed using Excel (Microsoft).

2.5 Metabolic measurements

Changes in metabolic homeostasis were investigated in the ZD and the iZD paradigm under in-phase and anti-phase conditions. The metabolic measurements were performed in a separate cohort which was not used in the long-term recordings of locomotor activity, body weight and food intake measures, gene expression profiles, and body temperature recordings.

Glucose tolerance tests were conducted at the end of a 12-hour fasting period followed by body composition measurements.

2.5.1 *Glucose tolerance test (GTT)*

GTTs were conducted in the 4th cycle of the ZD and the iZD paradigm. Animals were only used once and fasted for 12 h according to their fasting intervals on in-phase or anti-phase days prior to GTT. A stock solution of 0.125 g/mL glucose (D(+)-glucose, Roth) in 0.9 % saline (NaCl 0.9 %, Fresenius Kabi) was prepared and sterile filtered (syringe filter, 0.2 µm, Pall). Baseline blood glucose levels were measured before the start of the GTT using a glucometer (ACCU-CHECK Aviva, Roche). For this, mice were secured in a restrainer and blood was taken from a small incision of the tail tip. Intraperitoneal injection of 1.5 g glucose/kg body weight was followed by blood glucose determinations at 15, 30, 60, 90 and 120 min after injection. Incremental areas under the curve were individually calculated and compared using GraphPad Prism 7 (GraphPad).

2.5.2 *Body composition*

Body composition was analyzed by nuclear magnetic resonance measurements (minispec LF110, Bruker). The measurement was conducted after the GTT in the fasted mice. Mice were sacrificed shortly before measuring free body fluid, fat, and lean mass. Fat and lean mass were normalized to body weight.

2.6 *Tissue and serum collections*

Mice were sacrificed by cervical dislocation followed by immediate decapitation. In the dark phase sacrificing was performed under dim red light and eyes were removed before room lights were switched on for tissue dissection. Trunk blood was collected and kept at room temperature for 30 min for clotting and then centrifuged for 30 min at 664 rcf at 4 °C. The supernatant (serum) was transferred to a new tube and stored at -20 °C until further use (see 2.9 and 2.10). Liver, epididymal white adipose tissue (eWAT), adrenals and in the acute *zeitgeber* intervention pancreas were collected and stored in a RNA-stabilizing buffer (RNAlater, ThermoFisher) at -20 °C until further use (see 2.7). Whole brains were removed and paraffin embedded as described in 2.6.1.

2.6.1 *Paraffin embedding of brains*

Whole brains were carefully isolated from the skull and fixed in 4 % paraformaldehyde (PFA, Roth) in phosphate-buffered saline (1 x PBS; 137.00 mM NaCl, 2.68 mM KCl, 10.14 mM Na₂HPO₄ (anhydrous), 1.76 mM KH₂PO₄ (anhydrous), (Roth) in ddH₂O, pH 7.4) for 12-18 h at 4 °C. Next, brains were dehydrated in ascending ethanol series (3 h in 30 % EtOH, 3 h in 50 % EtOH, 8-12 h in 70% EtOH) at 4 °C. Dehydrated brains were stored in 100 % EtOH at -20 °C. For paraffin embedding, brains were soaked for 2 h in 50 % EtOH/ 50 % xylene substitute (Shandon, ThermoFisher Scientific) followed by 2 h in xylene substitute at room temperature. This was followed by 1 h in 50 % xylene substitute/ 50 % paraffin (Histoplast pelletised paraffin wax, ThermoFisher Scientific) at 60 °C. Brains were then saturated with paraffin by three 100 %

paraffin washes (8-12 h followed two 1 h rinses) at 60 °C. Subsequently, brains were embedded in hexagonal weighing dishes (hexagonal LLG-Wägeschälchen, Gr. S, Heathrow Scientific, Th. Geyer) and stored at room temperature until further use.

2.7 RNA isolation and quantitative real-time (q)PCR

Total RNA was isolated from liver, eWAT, adrenal, and pancreas tissue. Frozen tissue samples were transferred to screw cap tubes containing 0.3 (adrenal) or 1 mL (liver, eWAT and pancreas) TRIzol (ThermoFisher Scientific) and 6-15 ceramic beads (1.4 mm ceramic beads, Omni International) and homogenized using a bench homogenizer (Omni Bead Ruptor, Omni International) (program: 4.5 m/s speed, 3 cycles, 20 s, 3 s decay). The homogenate was incubated at room temperature for 5 min. Then, 60 or 200 µL of high-purity chloroform ($\geq 99.5\%$, Honeywell, ThermoFisher Scientific) were added and samples were shaken vigorously for 10-15 s and kept on ice for 5 min. Samples were centrifuged at 20,817 rcf for 15 min at 4 °C. The upper, RNA-containing phase was mixed with 150 or 500 µL of ice-cold isopropanol (Roth). The mixture was kept on ice or at -20 °C for 0.5-2 h and centrifuged at 20,817 rcf for 30 min at 4 °C. The precipitated RNA pellets were washed with 600 µL or 1 mL 75 % EtOH, centrifuged at 20,817 rcf for 10 min at 4 °C and air-dried for 5-10 min. Pellets were dissolved in an appropriate volume of nuclease-free water (Go-Taq qPCR Master Mix Kit, Promega). RNA concentration was measured using a microplate spectrophotometer (Epoch Microplate Spectrophotometer, BioTek Instruments). The 260 nm to 280 nm absorbance ratio was used for quantification and analyzed using the Gen5 software, version 2.0 (BioTek Instruments). Samples were frozen at -80 °C until further use.

For reverse transcription, the RNA samples were thawed on ice. cDNA was obtained from 2-3 µg total RNA according to the manufacturer's protocol and random hexamer RT primers (High-capacity cDNA Reverse Transcription (RT) Kit, Applied Biosystems). The amount of total RNA used for RT reaction was the same within one tissue. The appropriate volume of RNA was topped up to 10 µL with nuclease-free water (Go-Taq qPCR Master Mix Kit, Promega). 10 µL of RT master mix was added before the RT reaction was started.

RT master mix:	<u>Substance</u>	<u>Volume (µL)</u>
	10 x RT buffer	2
	25 x dNTP mix	0.8
	10 x RT primers	2
	MultiScribe RT	1
	nuclease-free water	4.2
RT reaction protocol:	<u>Temperature (°C)</u>	<u>Time (min)</u>
	25	10
	37	120
	85	5

cDNA was diluted 1:5 (pancreas), 1:10 (adrenal, eWAT), or 1:20 (liver) with nuclease-free water (Go-Taq qPCR Master Mix Kit, Promega) and stored at -20 °C. qPCR was conducted using

Go-Taq qPCR Master Mix Kit (Promega) on a real-time PCR detection system (CFX96 thermocycler, Bio-Rad). An appropriate amount of cDNA:SYBR master mix (5 μ L cDNA and 10 μ L SYBR Green Pre-Mix per reaction) was prepared. 15 μ L cDNA:SYBR mix was pipetted into appropriate wells and 5 μ L of 1.4- μ M primer mix (see Table 1) was added. The plate (Multiplate PCR plates 96-well, Bio-Rad) was sealed with adhesive tape (adhesive clear PCR seal, Biozym Biotech Trading) and centrifuged for 1 min at 1,890 rcf. Temperature cycling conditions were as described below.

qPCR protocol:	<u>Temperature (°C)</u>	<u>Time</u>	
	94	5 min	
	94	15 s	} 40 cycles
	60	15 s	
	72	20 s	
	72	5 min	

Table 1: Primers used for qPCR

Gene	Forward primer 5' $\rightarrow$ 3'	Reverse primer 5' $\rightarrow$ 3'
<i>eEF1α</i>	TGCCCCAGGACACAGAGACTTCA	AATTCACCAACACCAGCAGCAA
<i>Bmal1</i>	CCTAATTCTCAGGGCAGCAGAT	TCCAGTCTTGGCATCAATGAGT
<i>Per2</i>	GCCAAGTTTGTGGAGATTCCTG	CTTGACCTTGACCAGGTAGG
<i>Dbp</i>	AATGACCTTTGAACCTTGATCCCGCT	GCTCCAGTACTTCTCATCCTTCTGT
<i>Nr1d1</i>	AGCTCAACTCCCTGGCACTTAC	CTTCTCGGAATGCATGTTGTTC
<i>Cry1</i>	GTCATTGCAGGAAAATGGGAAG	TAAAGAGGCGGAGAGACAAAGG

Gene expression (Table 1) was normalized to the housekeeping gene *eukaryotic elongation factor-1 α* (*eEF1 α*). Relative expression ratios were calculated using the $\Delta\Delta$ CT method (Pfaffl, 2001) and normalized to the corresponding mean expression ratio of the in-phase profile.

2.8 Radioactive in situ hybridization (ISH)

Radioactive *in situ* hybridization was conducted to investigate circadian *Bmal1* and *Per2* expression in the SCN. mRNA levels were quantified by densitometric detection of bound ^{35} S-coupled complementary RNA probes on SCN tissue sections.

2.8.1 Template preparation

Plasmid stocks of previously designed and described m*Bmal1* and m*Per2*-ISH probes were used (Albrecht et al., 1997; Oster et al., 2002). m*Bmal1* and m*Per2* probes were complementary to base positions 654-1290 (NCBI accession number: AF015953) and 229-768 (NCBI accession number: AF036893), respectively.

2.8.1.1 *Plasmid amplification*

50 µL DH5α competent *Escherichia coli* (MAX efficiency DH5α competent cells, ThermoFisher Scientific) were transformed with 1 ng *mBmal1*-ISH plasmid (pCR Script vector) and incubated on ice for 30 min. Cells were heat-shocked at 42 °C for 90 s and then placed on ice for 2 min. After adding 200 µL of super optimal broth with catabolite repression (S.O.C.) medium (Invitrogen, ThermoFisher Scientific), cells were incubated at 37 °C for 45 min. Cells were evenly spread on lysogeny broth (LB)/agar plates (10 g/L NaCl (Roth), 10 g/L tryptone (Roth), 5 g/L yeast extract (Roth), 15 g/L agar-agar (Roth) (autoclaved)) containing 0.1 mg/mL ampicillin (Gibco, ThermoFisher Scientific). Plates were incubated upside-down overnight at 37 °C. Single colonies were picked and plated to a new LB/agar plate which was incubated as described above. The rest of the colony was transferred to a tube containing 3 mL LB medium (10 g/L NaCl, 10 g/L tryptone, 5 g/L yeast extract (autoclaved) and 0.1 mg/mL ampicillin). The cells were shaken at 200 revolutions *per* minute (rpm) at 37 °C for several hours. For plasmid sequencing, cells were lysed and plasmid DNA was bound to the column membrane of the used kit (QuickLyse Miniprep kit, Qiagen) and eluted in 40 µL nuclease-free water. Sequencing was performed by Eurofins using T7 promotor primer. Sequencing results were validated using FASTA *mBmal1* sequence and alignment in BLAST (NIH). Subsequently, a colony from the corresponding second LB/agar plate was picked and transferred to 3 mL LB medium (+0.1 mg/mL ampicillin). Cells were shaken at 200 rpm at 37 °C for 8 h (pre-culture). The pre-culture was transferred to 400 mL LB medium (+0.1 mg/mL ampicillin) and incubated overnight at 37 °C at 200 rpm. Before the bacteria culture was pelleted for plasmid purification (see 2.8.1.2), bacterial glycerol stock was prepared by mixing 1.5 mL bacteria culture with 0.5 mL of 60 % glycerol (autoclaved) (Merck). Glycerol stocks were shock-frozen in liquid nitrogen and stored at -80 °C.

2.8.1.2 *Plasmid purification*

Plasmid DNA was purified using a kit (NucleoBond Xtra Midi/Maxi, Machery-Nagel) following the manufacturer's protocol. In brief, the bacteria culture was pelleted by centrifugation at 6,000 rcf for 10 min at 4 °C. The bacteria pellet was completely resuspended in resuspension buffer and RNA was degraded by RNase A treatment. Bacteria were lysed and transferred to the column. The plasmid DNA was bound to the column membrane while the rest was eluted. Afterwards, plasmid DNA was eluted by adding elution buffer. Plasmid DNA was precipitated with isopropanol and pelleted by centrifugation at 15,000 rcf for 30 min at 4 °C. DNA pellet was washed with 70 % EtOH and centrifuged at 15,000 rcf for 5 min at room temperature. The DNA pellet was air-dried and dissolved in an appropriate volume of nuclease-free water. The DNA concentration was measured using the microplate spectrophotometer (Epoch Microplate Spectrophotometer, BioTek Instruments). 260-to-280 nm absorbance ratios were determined and analyzed using the Gen5 software (BioTek Instruments). The plasmid DNA was stored at -20 °C until further use.

2.8.1.3 Template linearization and precipitation

For linearization, 20 µg of the purified plasmid was mixed with 1 µL of the applicable restriction enzyme (*Bmal1*-plasmid: NotI HF (New England Biolabs (NEB)), *Per2*-plasmid: EcoRI HF (NEB)), 5 µL of the appropriate NEB buffer and 0.5 µL of 100 x BSA. The mixture was topped up to 50 µL with ddH₂O and incubated overnight at 37 °C. Restriction enzymes were digested by adding 5 µL of 10 x proteinase K buffer (0.5 M Tris/HCl (pH 7.6); Roth, 50 mM EDTA (pH 8); Roth, adjusted to pH 8.5) and 2,5 µL proteinase K (1 mg/mL; Roche). After incubation for 1 h at 37 °C, the mixture was topped up to 200 µL with ddH₂O. DNA was extracted by mixing with an equal volume of phenol:chloroform:isoamyl alcohol (12:24:1; Sigma Aldrich) until formation of a milky emulsion. The mixture was centrifuged at 1,000 rcf for 5 min. The upper aqueous phase was transferred to a new tube and the step was repeated. Afterwards, the aqueous phase was mixed with an equal volume of chloroform and centrifuged at 12,000 rcf for 5 min. The upper aqueous phase was transferred to a new tube and the DNA was precipitated with the double volume of 100 % EtOH. The mixture was centrifuged at 12,000 rcf for 10 min. The DNA pellet was washed with an equal volume of 70 % EtOH followed by a final centrifugation at 12,000 rcf for 10 min. All centrifugation steps were performed at room temperature. The DNA pellet was dissolved in 20 µL nuclease-free water and concentration was measured in a microplate spectrophotometer (Epoch Microplate Spectrophotometer, BioTek Instruments) using the program Gen5.

2.8.1.4 Riboprobe preparation

The radioactive-labelled riboprobe was synthesized by *in-vitro* transcription of 1 µg linearized DNA template using an RNA transcription kit (Ambion MAXIscript SP6/T7 Transcription Kit, ThermoFisher Scientific) and α³⁵S-UTP (1 Ci/mol, 19 µCi/µL, Perkin Elmer).

Transcription protocol:	<u>Substance</u>	<u>Volume (µL)</u>
	1 µg linearized DNA template	x
	10 x Transcription buffer (kit)	3
	RiboLock RNase inhibitor (40 U/µL, ThermoFisher)	1
	rATP (10 mM, kit)	1
	rGTP (10 mM, kit)	1
	rCTP (10 mM, kit)	1
	α ³⁵ S-UTP	5
	RNA polymerase:	1
	- <i>Bmal1</i> : T7 (15 U/µL, Kit)	
	- <i>Per2</i> : T3 (20 U/µL, Thermo Scientific)	
	0.75 M dithiothreitol (DTT) (Roth)	1
	Nuclease-free water	ad 30

DNA template and substances were gently mixed and incubated for 2 h at 37 °C and shaking at 400 rpm. Subsequently, the DNA template was digested by adding 19 µL nuclease-free

water, 1.7 μL 0.3 M MgCl_2 (Roth) and 1 μL DNase I (2 U/ μL , Ambion TURBO DNase, ThermoFisher Scientific) and incubation for 15 min at 37 °C and shaking at 400 rpm. The riboprobe was purified using riboprobe columns (Illustra ProbeQuant G-50 Micro columns, GE Healthcare Life Sciences) following the manufacturer's protocol. In short, the samples were loaded on the Sephadex G-50 DNA Grade resin spin column. Fragments greater than 20 bases were eluted by centrifugation. Smaller fragments were bound to the resin, including unincorporated $\alpha^{35}\text{S}$ -UTP. The amount of incorporated radioactive $\alpha^{35}\text{S}$ -UTP was measured in a β -counter (1400 DSA, Wallac) by diluting 1 μL of the purified riboprobe in 4 μL scintillator cocktail (Rotizint eco plus, Roth). The riboprobes were diluted in ISH hybridization buffer (Ambion ISH hybridisation buffer, ThermoFisher Scientific) to 3-4 million counts *per* minute (cpm) *per* slide (3-4 million cpm/100 μL hybridization buffer) for subsequent *in situ* hybridization. Riboprobes were either directly used or stored for up to 2 days at -20 °C.

2.8.2 Slide preparation

Paraffin embedded brains (see 2.6.1) were cut in 8 μm -thick coronal sections. Sections were collected from -0.22 mm to -0.82 mm relative to Bregma and split into 4 series of comparable sections for the different genes investigated. Sections were mounted on glass slides (Polylysine Menzel-Gläser, ThermoFisher Scientific) and stored at room temperature.

One day before the slide preparation, tanks were filled with 1 M NaOH (sodium hydroxide, Roth) to destroy RNases. Before hybridization, paraffin was removed from the sections to make mRNA accessible. Paraffin was removed by two 10-minute washes in xylene substitute followed by hydration in a descending ethanol series (2 x 2 min in 100 % EtOH, 1 min each in 95 %, 70 %, 50 %, and 30 % EtOH). Thereafter, slides were equilibrated for 5 min each in 0.9 % NaCl and 1 x PBS. Tissues were fixed in 4 % PFA in 1 x PBS for 10 min and washed in 1 x PBS for 5 min. Permeabilized tissue sections were treated for 5 min with proteinase K in 1 x proteinase K buffer (20 $\mu\text{g}/\text{mL}$ proteinase K in 50 mM Tris/HCl (pH 7.6), 5 mM EDTA (pH 8), adjusted to pH 8.5) and the reaction was stopped by a 5-minute wash in 0.2 M HCl (Roth). The slides were equilibrated in 1 x PBS for 5 min and post-fixed in 4 % PFA in 1 x PBS for 20 min. Background signal was reduced by an acetylation step. Slides were incubated in freshly produced 0.1 M triethanolamine (TEA) (Sigma Aldrich) containing 600 μL acetic anhydride (25 mM; Sigma Aldrich). After 5 min, another 600 μL of acetic anhydride (25 mM) was added and slides were incubated for an additional 5 min. Sections were washed for 5 min each in 1 x PBS and 0.9 % NaCl. Tissue sections were dehydrated in an ascending ethanol series (30 % and 50 % for 1 min each, 70 % for 5 min, and 80 %, 95 % and 2 times 100 % for 1 min each) and air-dried in an RNase-free Perspex chamber. All solutions were prepared with autoclaved ddH₂O and steps were performed at room temperature.

2.8.3 Hybridization and post-hybridization washes

Dry slides were covered with 100 μL hybridization solution containing the ^{35}S -labeled riboprobe (see 2.8.1.4) and carefully coverslipped avoiding any air bubbles. Slides were placed in a humidified chamber (5 x saline sodium citrate (5 x SSC, 0.75 M NaCl, 75mM sodium

citrate, pH 7, in dd H₂O)/ 50 % formamide (v/v; VWR International) in ddH₂O). Slides were incubated overnight at 50 °C.

Post-hybridization washes were performed to remove unbound and non-specifically bound riboprobes. Coverslips were carefully removed after a 10-minute removal wash (5 x SSC / 40 mM β -mercaptoethanol (β -MeSH, Sigma Aldrich)) at 64 °C. Slides were incubated in the same washing solution for another 20 min. Slides were then treated with 2 x SSC / 50 % formamide/ 40 mM β -MeSH for 30 min at 64 °C. Sections were washed twice in 1 x NTE (0.5 M NaCl, 10 mM Tris/HCl (pH 8.0), 5 mM EDTA, adjusted to pH 8.0)/ 40 mM β -MeSH at 37 °C. Non-ligated RNA (mRNA and riboprobes) were degraded by RNase A (20 μ g/mL in 1 x NTE ; ThermoFisher Scientific) treatment for 30 min at 37 °C. Slides were re-equilibrated by a 1 x NTE washing step for 15 min at 37 °C followed by another wash step with 2 x SSC/ 50 % formamide/ 40 mM β -MeSH for 30 min at 64 °C. Tissue sections were washed in 2 x SSC followed by 0.1 x SSC for 15 min each at 37 °C. Sections were dehydrated in an ascending ethanol series (30 %, 50 % and 70% EtOH with 0.3 M ammonium acetate followed by 95 % EtOH (without acetate) and two times 100 % EtOH for 30 s each) at room temperature. Slides were air-dried in a RNase-free chamber. Steps at 64 °C and 37 °C were performed in a shaking and non-shaking water bath, respectively. All solutions were prepared with autoclaved ddH₂O. β -MeSH was added shortly before the respective washing solution was used.

2.8.4 Visualization and quantification of the radioactive signal

Slides were placed in a light-tight film cassette (Hypercassette, Amersham Biosciences) and covered with an autoradiography film (Amersham Hyperfilm MP, GE Healthcare Life Sciences). The film was developed in an automatic developing machine (Kodak X-OMAT 1000 Processor, Kodak) using fixer (Carestream Kodak autoradiography HBX fixer replenisher, Kodak) and developer (Carestream Kodak autoradiography HBX developer replenisher, Kodak) after 8-16 d exposure time. Subsequently, the film was scanned in high resolution (GS-800 Calibrated Imaging Densitometer, Bio-Rad). Greyscale intensity was measured using the program Quantity One (Bio-Rad) to quantify the hybridization signal and, thus, expression level of *Bmal1* and *Per2* mRNA in the SCN. Background signal of the individual films was subtracted from hybridization signal of the region of interest before further analysis.

2.9 Leptin enzyme-linked immunosorbent assay (ELISA)

Serum leptin levels were quantified using a sandwich ELISA (mouse leptin ELISA Kit, Crystal Chem) according to the manufacturer's protocol. In short, leptin was immobilized by rabbit anti-leptin antibodies and simultaneously bound by anti-leptin immunoglobulins G (IgG) from guinea pig anti-serum. The latter was bound by horse radish peroxidase (HRP)-conjugated anti-guinea pig IgG antibody. Non-bound components were washed off. Bound HRP-conjugated antibodies were detected by adding 3, 3', 5, 5'-tetramethylbenzidine. The enzymatic reaction was stopped by adding sulfuric acid and absorbance was measured at 450 nm and 630 nm in a microplate spectrophotometer (Epoch Microplate Spectrophotometer,

BioTek Instruments) using the program Gen5 (BioTek Instruments). Serum leptin levels were interpolated using a standard curve of known leptin concentrations. Leptin level of the anti-phase profiles were normalized to the mean leptin level of the in-phase profile.

2.10 Corticosterone radioimmunoassay (RIA)

Serum corticosterone levels were determined by radioimmunoassay (ImmuChem Double Antibody Corticosterone ^{125}I RIA Kit, MP Biomedicals) according to the manufacturer's protocol. Diluted serum samples were mixed with a specific amount of ^{125}I -labeled corticosterone and anti-corticosterone antibodies (rabbit). The mixture was incubated for 2 h at room temperature. Antibody-bound corticosterone was precipitated by adding anti-rabbit IgG. Precipitate was centrifuged at 2,300 rcf for 15 min and free corticosterone (supernatant) was discarded. Radioactivity was measured in a gamma-counter (Wizard² 2470 Automatic Gamma Counter, Perkin Elmer) and serum corticosterone levels were interpolated using a standard curve of known corticosterone concentrations. Corticosterone level of the anti-phase profiles were normalized to the mean corticosterone level of the in-phase profile.

2.11 Data analysis and statistics

All data are shown as means $\pm$ standard errors of the mean (SEMs). Statistical analysis was performed using GraphPad Prism 7 software (GraphPad). P-values below 0.05 were considered significant. CircWave 1.4 (EUCLOCK) was used to test for rhythmicity in diurnal/circadian gene expression profiles. Here, rhythmicity is analyzed by sine/cosine fitting with Monte-Carlo statistical testing assuming a period of 24 h. Diurnal profiles were defined as rhythmic with p-values below 0.05.

2.11.1 Acute ZI

Analysis of activity was performed as described in 2.4.1.1. Activity period was calculated from the phase shift of the activity onset between the first (baseline) day and the fourth (misalignment) day. The resulting activity period was taken as wavelength for subsequent non-zero sine wave fitting of gene expression profiles (using GraphPad software).

For gene expression analysis, outliers were detected by the Dean Dixon test with $\alpha = 0.01$ and excluded from presentation and analysis. Non-rhythmically expressed genes were excluded from statistical tests. Changes in amplitude were tested for significance in one-sample *t*-tests against the hypothetical value of 100 % (mean of the baseline expression). Because mice behaved uniformly between and within the paradigms, maximum of the gene expression was calculated from the modeled sine wave. Subsequent, phase shifts of maximum gene expression were calculated between baseline and misaligned conditions under two different *zeitgeber* intervention strategies. Statistical differences of phase shifts between the two intervention strategies (Dark-RF vs. RF-12) were tested in a two-way ANOVA with Sidak's post-tests.

2.11.2 ZD and iZD

For the ZD and iZD paradigms, body weight development was analyzed by comparing fasted body weights between two consecutive days for each animal. Daily food intake was normalized to the mouse's fasted body weight. Food conversion rate was calculated by the ratio of the body weight change to the food intake on the same experimental day. For comparison of body weight changes, food intake and food conversion of 3-6 in-phase and anti-phase days were averaged for each mouse and tested for significant differences using two-tailed, paired *t*-tests.

For the ZD and iZD paradigms, telemetric recorded core body temperature and locomotor activity data were analyzed in 1-minute bins with 200-neighbour running-average smoothing (GraphPad). The data were normalized to the average of three pre-experimental days. Data of the ZD and iZD paradigm are presented as average over two experimental weeks relative to the phase relation of LD and feeding:fasting (LD-TRF phase angle).

For GTTs analysis in the ZD and iZD paradigms, two-way ANOVAs with Sidak's post-tests were used to test for significant differences between the conditions of the same paradigm. Incremental areas under the curve (iAUCs) were analyzed using two-tailed, unpaired *t*-tests. Differences in body composition were tested for significant differences using one-way ANOVA (testing between paradigms) or two-tailed, unpaired *t*-tests (between in-phase and anti-phase conditions of the same paradigm).

For the ZD and iZD paradigms, activity analysis was performed as described in 2.4.1.2. Two-way ANOVAs with Sidak's post-tests were used to test for significant differences in locomotor activity distribution relative to LD or feeding:fasting cycle. For the ZD paradigm, locomotor activity peaks were determined from averaged activity curves under in-phase and anti-phase conditions. Differences in activity peaks between the conditions relative to light or food phase were tested for statistical differences using two-tailed, paired *t*-tests.

For the ZD and iZD paradigms, gene expression was normalized as described in 2.7. Hormone excretion and gene expression amplitudes were calculated by subtracting the minimal from the maximal measured hormone level or relative expression. Amplitudes of the excreted hormones or gene expression of the anti-phase days were normalized to corresponding amplitudes of the in-phase day and subsequent averaged over the genes/excretion of the anti-phase day. Normalized amplitudes of the anti-phase days were then tested for statistical differences using one-sample *t*-tests against 100 % (in-phase amplitude). In the long-term experiment, mice were behaviorally free-running and therefore centers of gravity (COGs), instead of the maxima of a fitted sine wave, were determined in the ZD and iZD paradigm. Biological replicates of each timepoint of the individual profiles were averaged and COGs were determined from the obtained expression curves using CircWave 1.4 (Oster et al., 2006b). In the ZD paradigm, three independent anti-phase and one in-phase gene expression profiles were compared for phase shifts using the COGs as reference. COG phase shifts of analyzed genes (*Bmal1*, *Per2* and *Dbp* for peripheral tissues and *Bmal1* and *Per2* for SCN) were averaged

for each anti-phase profile to estimate overall phase shifts for each tissue clock individually. Likewise, differences of hormonal COGs between in-phase and anti-phase profiles were calculated. Subsequently, the COG phase shifts between the three anti-phase days and the in-phase day were tested for statistical differences. Contrary, only one anti-phase gene expression profile was generated in the iZD paradigm. Therefore, COG phase shifts of the gene expression curves between the one in-phase and the one anti-phase day were calculated. Here, COG phase shifts of analyzed genes in one tissue between the in-phase and the anti-phase day were tested for statistical differences. In the ZD and iZD, statistical differences of COG phase shifts, in gene expression, hormones and activity, between in-phase and anti-phase conditions were tested in one-sample *t*-tests against hypothetical values of minimal (0 h) and maximal (12 h) phase shifts.

3 Results

In this PhD project, the contribution of different *zeitgeber* input on tissue clock resetting was investigated. Therefore, mice were exposed to conflicting *zeitgeber* input for short-term and long-term and effects on locomotor activity, metabolic homeostasis and the circadian clock system were investigated. In the acute *zeitgeber* intervention experiments, effects on circadian clock system were compared between LD-14/Dark-RF and LD-14/RF-12. In long-term experiments, therapeutic effect of TRF was investigated under chronodisruptive conditions. To explore this, mice were kept under LD-14/RF12 (ZD) or LD-12/RF-14 (iZD) conditions and effects on circadian clock system as well as on metabolic state were studied.

3.1 Acute *zeitgeber* intervention

3.1.1 Activity periods are comparable between both acute ZI paradigms

To investigate the effects of ZI on behavior, wheel-running activity of single-housed mice was recorded. Prior to the start of acute ZI paradigms at experimental days -3 – 0, when animals were kept under LD-12 cycles with *ad libitum* food access, mice were predominantly active in the dark phases (Figure 3-1 a, c).

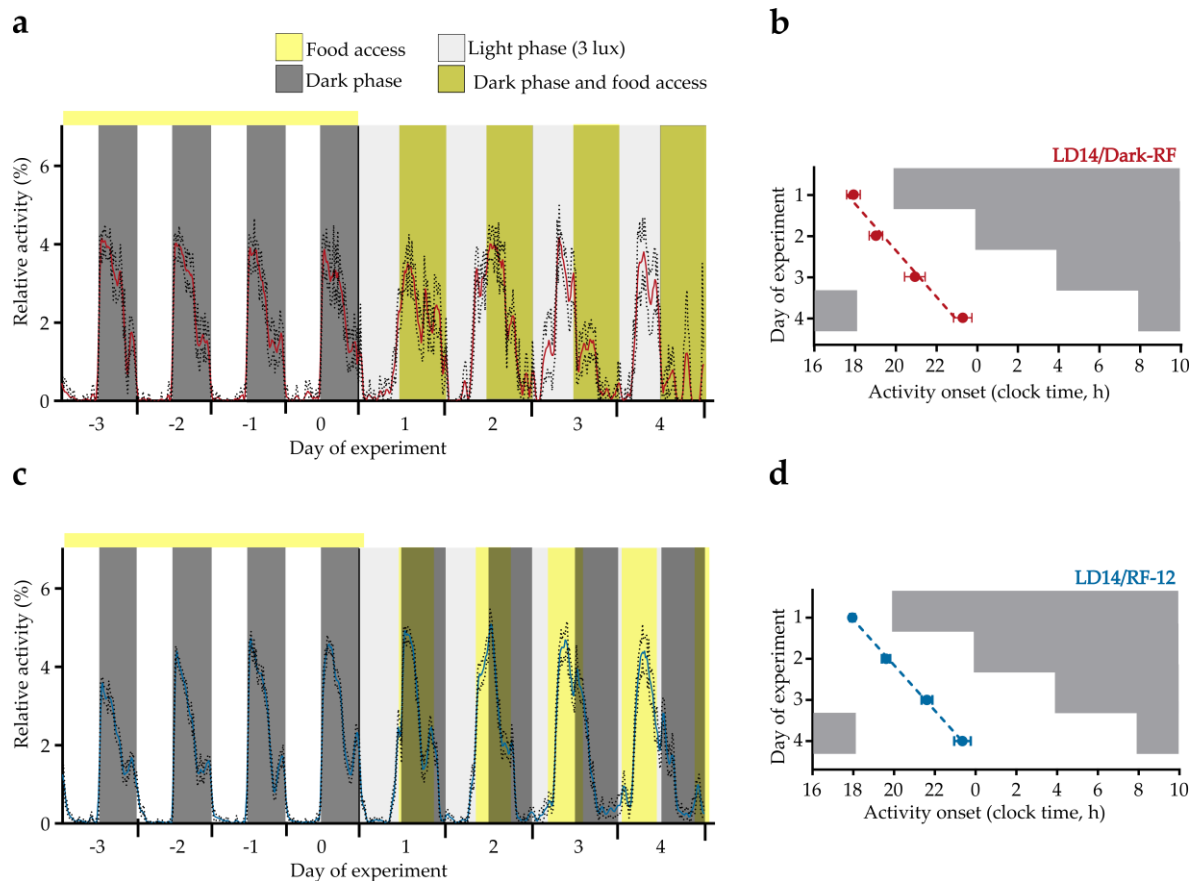


Figure 3-1: Locomotor activity does not entrain to LD or feeding:fasting cycles under acute ZI conditions. Running-wheel activity and daily activity onsets under (a, b) LD-14/Dark-RF (n = 8-15, red) and (c, d) LD-14/RF-12 (n = 58-66, blue) conditions. Mice which showed interrupted activity recordings were excluded for the respective time periods. Dark phases, food access times and experimental light

phases are indicated in dark grey, yellow and light grey shadings, respectively. Data are shown as means. Dotted lines (a, c) and error bars (b, d) indicate SEMs.

Locomotor activity is regulated by the SCN (Houben et al., 2009). Therefore, activity onsets were determined for both acute ZI approaches to indirectly investigate the phasing of the SCN clock. At the beginning of the acute ZI paradigm (day 1), food was removed from the hoppers at ZT0 (LD-14/Dark-RF) or ZT1 (LD-14/RF-12) relative to the LD cycle. The animals' activity was slightly increased by this disturbance and mice started being active ~2 h before the onset of the dark phase on the first day of the experiment in both setups (**Figure 3-1 b, d**). Mice showed a broadened activity phase for the first 3 days of the experiment under LD14/Dark-RF conditions compared to mice under LD-14/RF-12 conditions (**Figure 3-1 a, c**). Daily total activity was comparable between the paradigms (data not shown). Over the four days under LD-14/Dark-RF, mice showed a total phase delay of 5.38 h (with activity onsets at 17.90 ± 0.3 h (day 1, n=12), 19.00 ± 0.33 h (day 2, n=11), 20.78 ± 0.54 h (day 3, n=10) and 23.28 ± 0.45 h (day 4, n=8) (**Figure 3-1 b**). In the LD-14/RF-12 paradigm, mice showed a total phase shift of 5.40 h over the four experimental days (with activity onsets at 17.96 ± 0.12 h (day 1, n=65), 19.61 ± 0.20 h (day 2, n=64), 21.62 ± 0.27 h (day 3, n=64) and 23.36 ± 0.41 h (day 4, n=58) (**Figure 3-1 d**). Straight lines were fitted through the activity onsets yielding highly comparable slopes of 1.75 and 1.82 h/d for LD-14/Dark-RF and LD-14/RF-12, respectively (**Figure 3-1 b, d**). Consequently, mice phase-delayed their locomotor behavior by ~1.8 h per day resulting in a modeled activity period of 25.8 h which was used for clock gene expression phase shift analyses.

In summary, locomotor activity did not entrain to *zeitgeber* input and the two time-restricted feeding schedules did not differentially affect activity periods. In both acute ZI paradigms, mice showed comparable activity period lengthening over the course of the experiment.

3.1.2 Chronodisruption of clock gene expression is independent of the timing of restricted feeding

Besides their synchronization by the SCN, peripheral clocks are strongly impacted by the *zeitgeber* food. To investigate the acute effects of diverging *zeitgeber* input on molecular clock resetting, peripheral clock gene expression was measured under baseline (first day of the experiment) and misaligned (fourth day of the experiment) conditions. The LD cycle was phase-shifted by 12 h between the baseline and the misaligned day in both paradigms and tissue sampling was performed spanning the LD cycle (28 h).

Under LD-14/Dark-RF conditions, *Bmal1* expression in the liver showed a U-shaped expression profile along the LD cycle on the baseline day (ZT0: 1.59 ± 0.06 , ZT7: 0.12 ± 0.01 ; ZT14: 0.25 ± 0.05 , ZT21: 1.59 ± 0.16 , ZT28: 1.40 ± 0.01). On the misaligned day, *Bmal1* expression showed a bell-shaped expression profile with a low amplitude (ZT0: 0.40 ± 0.12 , ZT7: 0.20 ± 0.11 , ZT14: 1.01 ± 0.09 , ZT21: 1.80 ± 0.13 , ZT28: 0.16 ± 0.02). The phase difference between the two fitted sine wave maxima was 6.94 h (**Figure 3-2 a, Table S1**). *Per2* gene expression was highest in the first half of the dark phase and in the middle of the light phase

under baseline and misaligned conditions, respectively (ZT0: 0.44 ± 0.12 (baseline) *vs.* 0.8 ± 0.12 (misaligned), ZT7: 0.50 ± 0.04 *vs.* 1.19 ± 0.15 , ZT14: 2.01 ± 0.07 *vs.* 0.85 ± 0.20 , ZT21: 1.91 ± 0.43 *vs.* 0.58 ± 0.11 , ZT28: 0.14 ± 0.01 *vs.* 1.18 ± 0.11). The sine fit phase difference between baseline and misaligned conditions was 1.92 h. Under baseline conditions, hepatic *Dbp* gene expression was highest in the middle of the light phase (ZT0: 0.03 ± 0.004 , ZT7: 3.59 ± 0.18 , ZT14: 1.29 ± 0.19 , ZT21: 0.09 ± 0.01 , ZT28: 0.01 ± 0.001). Under misaligned conditions, peak *Dbp* expression levels were measured at the dark:light phase transition (ZT0: 1.76 ± 0.37 , ZT7: 1.45 ± 0.44 , ZT14: 0.17 ± 0.01 , ZT21: 0.02 ± 0.003 , ZT28: 1.89 ± 0.29) resulting in a sine fit phase delay of 6.54 h. *Cry1* and *Nr1d1* gene expression rhythms were anti-phasic on the same day and both genes were phase-shifted between baseline and misaligned days (with *Cry1* levels at ZT0: 1.26 ± 0.27 (baseline) *vs.* 0.17 ± 0.02 (misaligned), ZT7: 0.24 ± 0.07 *vs.* 0.41 ± 0.13 , ZT14: 1.01 ± 0.09 *vs.* 1.63 ± 0.44 , ZT21: 2.00 ± 0.32 *vs.* 0.87 ± 0.11 , ZT28: 0.49 ± 0.13 *vs.* 0.16 ± 0.02 and *Nr1d1* levels at ZT0: 0.04 ± 0.006 *vs.* 3.18 ± 0.84 , ZT7: 4.04 ± 1.90 *vs.* 0.49 ± 0.12 , ZT14: 0.36 ± 0.07 *vs.* 0.09 ± 0.03 , ZT21: 0.02 ± 0.003 *vs.* 0.18 ± 0.02 , ZT28: 0.55 ± 0.11 *vs.* 1.09 ± 0.15). Sine fit of *Cry1* expression rhythms were phase-shifted by 7.09 h, whereas sine fit of *Nr1d1* rhythms were shifted by 4.76 h between baseline and misaligned conditions (**Figure 3-2 a**).

In the adrenal, *Bmal1* expression showed a U-shaped curve progression along the LD cycle on the baseline day (ZT0: 1.73 ± 0.04 , ZT7: 0.16 ± 0.01 , ZT14: 0.41 ± 0.11 , ZT21: 2.16 ± 0.22 , ZT28: 0.54 ± 0.18). On the misaligned day, *Bmal1* expression was highest at the light:dark transition (ZT0: 0.16 ± 0.04 , ZT7: 0.92 ± 0.26 , ZT14: 1.90 ± 0.17 , ZT21: 1.36 ± 0.22 , ZT28: 0.10 ± 0.003). The sine fit phase delay between baseline and misaligned conditions was 5.26 h (**Figure 3-2 b**). *Per2* gene expression was highest at ZT14 and ZT7 under baseline and misaligned conditions, respectively (ZT0: 0.19 ± 0.01 (baseline) *vs.* 1.01 ± 0.22 (misaligned), ZT7: 0.90 ± 0.09 *vs.* 2.21 ± 0.27 , ZT14: 2.45 ± 0.10 *vs.* 0.152 ± 0.32 , ZT21: 1.01 ± 0.27 *vs.* 0.20 ± 0.06 , ZT28: 0.46 ± 0.22 *vs.* 1.02 ± 0.067). The *Per2* expression rhythms were phase-delayed by 6.58 h between baseline and misaligned conditions. Under baseline conditions, *Cry1* gene expression peaked in the middle of the dark phase (ZT0: 0.79 ± 0.25 , ZT7: 0.30 ± 0.06 , ZT14: 1.56 ± 0.11 , ZT21: 2.09 ± 0.28 , ZT28: 0.26 ± 0.23). Under misaligned conditions, highest *Cry1* expression levels were measured around transition between the light and the dark phase (ZT0: 0.27 ± 0.02 , ZT7: 1.62 ± 0.08 , ZT14: 2.34 ± 0.13 , ZT21: 0.31 ± 0.04 , ZT28: 0.20 ± 0.003). *Dbp* and *Nr1d1* gene expression rhythms were similar phased on the same days and phase-delayed between the baseline and the misaligned days (with *Dbp* level at ZT0: 0.24 ± 0.15 (baseline) *vs.* 2.69 ± 0.35 (misaligned), ZT7: 2.66 ± 0.27 *vs.* 0.65 ± 0.18 , ZT14: 0.91 ± 0.31 *vs.* 0.05 ± 0.007 , ZT21: 0.06 ± 0.01 *vs.* 0.16 ± 0.07 , ZT28: 1.32 ± 0.50 *vs.* 1.95 ± 0.27 and *Nr1d1* level at ZT0: 0.46 ± 0.16 *vs.* 2.12 ± 0.21 , ZT7: 2.33 ± 0.25 *vs.* 0.30 ± 0.08 , ZT14: 0.52 ± 0.21 *vs.* 0.06 ± 0.02 , ZT21: 0.11 ± 0.02 *vs.* 0.32 ± 0.08 , ZT28: 1.58 ± 0.23 *vs.* 1.34 ± 0.33). The phase shifts in the sine fits between baseline and misaligned conditions for *Dbp*, *Cry1* and *Nr1d1* expression were similar with 5.61 h, 5.61 h and 5.93 h, respectively (**Figure 3-2 b**).

Phase shifts in gene expression rhythms between the baseline and the misaligned day under LD-14/Dark-RF conditions were most prominent in eWAT. The expression curve of *Bmal1* was U-shaped along the LD cycle on the baseline day (ZT0: 1.98 ± 0.25 , ZT7: 0.22 ± 0.02 , ZT14: 0.07 ± 0.02 , ZT21: 1.56 ± 0.40 , ZT28: 1.17 ± 0.21). On the misaligned day, *Bmal1* expression levels were lowest in the middle of the light phase and peaked in the middle of the dark phase

(ZT0: 0.69 ± 0.08 , ZT7: 0.04 ± 0.004 , ZT14: 1.04 ± 0.23 , ZT21: 1.78 ± 0.20 , ZT28: 0.27 ± 0.10). The sine fit for *Bmal1* expression was phase-delayed by 7.30 h on the misaligned compared to the baseline days (**Figure 3-2 c**). *Per2* gene expression showed its highest expression levels at the light:dark transition under baseline conditions (ZT0: 0.36 ± 0.11 , ZT7: 0.59 ± 0.12 , ZT14: 2.74 ± 0.67 , ZT21: 1.11 ± 0.49 , ZT28: 0.20 ± 0.05). In contrast, it was highest in the middle of the light phase under misaligned conditions (ZT0: 0.57 ± 0.07 , ZT7: 2.32 ± 0.23 , ZT14: 1.66 ± 0.10 , ZT21: 0.11 ± 0.03 , ZT28: 0.66 ± 0.07). *Dbp* expression was high in the second and first half of the light phase on baseline and misaligned days, respectively (ZT0: 0.13 ± 0.04 (baseline) vs. 1.41 ± 0.21 (misaligned), ZT7: 2.40 ± 0.25 vs. 2.68 ± 0.04 , ZT14: 2.18 ± 0.30 vs. 0.32 ± 0.04 , ZT21: 0.17 ± 0.03 vs. 0.12 ± 0.02 , ZT28: 0.14 ± 0.02 vs. 1.56 ± 0.21). Sine fit phase delays between baseline and misaligned days were similar for *Per2* and *Dbp* with 6.61 h and 6.90 h, respectively. *Cry1* gene expression showed a rhythm with a low amplitude under baseline conditions and was not found to be significantly rhythmic under misaligned conditions (ZT0: 1.24 ± 0.36 , ZT7: 0.91 ± 0.18 , ZT14: 0.65 ± 0.26 , ZT21: 1.78 ± 0.26 , ZT28: 0.42 ± 0.03 on the baseline day and ZT0: 0.99 ± 0.22 , ZT7: 0.97 ± 0.17 , ZT14: 0.88 ± 0.24 , ZT21: 0.89 ± 0.25 , ZT28: 0.26 ± 0.09 on the misaligned day). *Nr1d1* expression was highest in the middle of the light phase on the baseline day and in the beginning of the light phase on the misaligned day (ZT0: 0.17 ± 0.05 (baseline) vs. 1.82 ± 0.03 (misaligned), ZT7: 3.34 ± 0.18 vs. 1.43 ± 0.24 , ZT14: 0.65 ± 0.15 vs. 0.19 ± 0.08 , ZT21: 0.08 ± 0.04 vs. 0.34 ± 0.06 , ZT28: 0.76 ± 0.24 vs. 0.94 ± 0.33). The sine fit phase delay was 7.70 h for *Nr1d1* between baseline and misaligned days (**Figure 3-2 c**).

In the pancreas, *Bmal1* gene expression showed high expression levels at ZT0 and ZT21 on the baseline day under LD-14/Dark-RF conditions (ZT0: 1.69 ± 0.15 , ZT7: 0.46 ± 0.08 , ZT14: 0.25 ± 0.02 , ZT21: 1.61 ± 0.42 , ZT28: 1.16 ± 0.38) (**Figure 3-2 d**). On the misaligned day, *Bmal1* expression was highest in the beginning of the dark phase (ZT0: 0.85 ± 0.17 , ZT7: 0.33 ± 0.06 , ZT14: 3.08 ± 1.37 , ZT21: 2.12 ± 0.35 , ZT28: 0.53 ± 0.25). *Bmal1* expression rhythms were phase-delayed by 5.04 h between baseline and misaligned days. *Per2* expression profiles were rhythmic with high expression levels in the second half of the light phase on the baseline day but not significantly rhythmic on the misaligned day (ZT0: 0.39 ± 0.21 (baseline) vs. 0.25 ± 0.01 (misaligned), ZT7: 1.86 ± 0.82 vs. 0.41 ± 0.07 , ZT14: 1.79 ± 0.39 vs. 0.67 ± 0.07 , ZT21: 0.59 ± 0.09 vs. 0.11 ± 0.01 , ZT28: 0.06 ± 0.01 vs. 1.51 ± 0.80). *Dbp* showed its highest expression at the light:dark transition on the baseline day (ZT0: 0.11 ± 0.01 , ZT7: 1.57 ± 0.70 , ZT14: 2.53 ± 1.39 , ZT21: 0.18 ± 0.06 , ZT28: 0.34 ± 0.25). On the misaligned day, the amplitude of *Dbp* gene expression was doubled compared to baseline and showed highest expression levels at the transition of the dark to light phase at ZT28 (ZT0: 3.65 ± 2.36 ZT7: 1.37 ± 0.40 , ZT14: 0.21 ± 0.05 , ZT21: 0.07 ± 0.02 , ZT28: 7.10 ± 1.58). The sine fit phase delay for *Dbp* was moderate with 2.76 h between baseline and misaligned conditions (**Figure 3-2 d**). Pancreatic *Cry1* gene expression was not rhythmic on the baseline day nor on the misaligned day and, thus, was excluded from the phase shift analysis. *Nr1d1* expression showed highest expression at ZT7 on the baseline day (ZT0: 0.04 ± 0.03 , ZT7: 5.63 ± 0.75 , ZT14: 0.48 ± 0.10 , ZT21: 0.03 ± 0.00 , ZT28: 0.26 ± 0.01). On the misaligned day under LD-14/Dark-RF conditions, *Nr1d1* expression showed two peaks at ZT0 and ZT28 (ZT0: 1.97 ± 0.29 , ZT7: 0.19 ± 0.05 , ZT14: 0.04 ± 0.01 ,

ZT21: 0.09 ± 0.002 , ZT28: 2.18 ± 0.29). The phase delay for *Nr1d1* expression rhythms was 5.26 h between the baseline and the misaligned day (Figure 3-2 d).

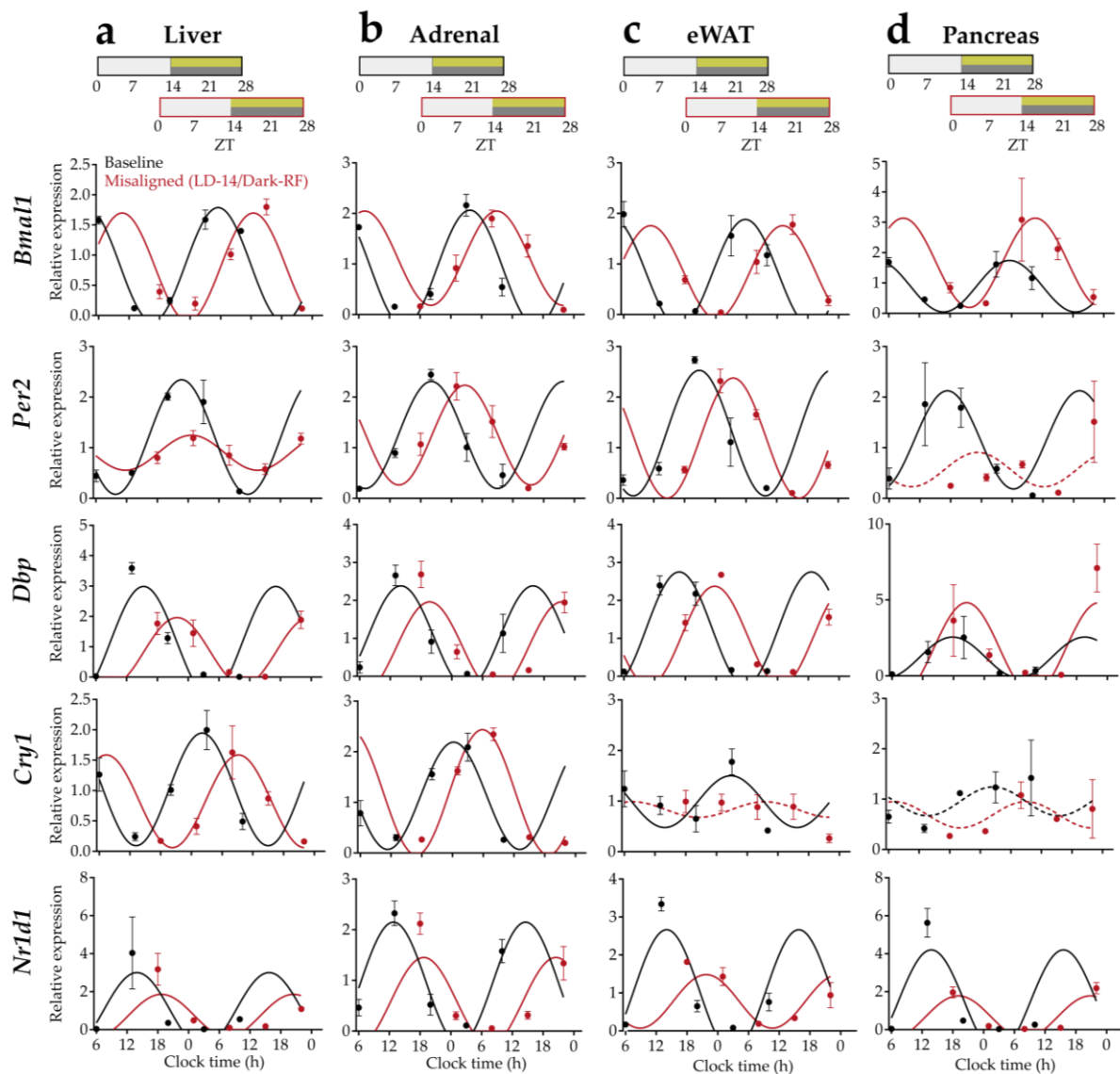


Figure 3-2 : Peripheral clock gene expression is phase-shifted between the baseline and the misaligned day in the LD-14/Dark-RF paradigm. Gene expression of *Bmal1*, *Per2*, *Dbp*, *Cry1*, and *Nr1d1* (top to bottom) was measured in (a) liver, (b) adrenal, (c) eWAT and (d) pancreas by qPCR under baseline (black) and misaligned (red) conditions over 28 h (n = 3-5 animals *per* timepoint). Data are shown as means ± SEMs. Solid and dashed lines indicate significant 24-h rhythmic and non-rhythmic gene expression profiles (as tested in CircWave), respectively. Light grey, dark grey and yellow bars on top indicate light phase, dark phase and food access time, respectively.

In summary, all investigated genes in the examined tissues showed phase shifts in the gene expression profiles between the baseline day and the misaligned day under LD-14/Dark-RF conditions. *Cry1* expression profiles were not found to be rhythmic for the misaligned day in eWAT and pancreas as well as for the baseline day in the pancreas. Pancreatic *Per2* expression profile was not rhythmic on the misaligned day.

Food intake is the dominant *zeitgeber* for peripheral tissues and peripheral clocks are shown to entrain to TRF within a couple of days (Damiola et al., 2000). Therefore, it was tested if TRF within in the entrainment range of 24 h would prevent phase shifts in peripheral clock gene expression under extended LD cycles. Mice were released into LD-14 and food was restricted to a 12-hour time window which roughly coincided with the pre-experimental dark phase. Tissue samples were taken on the 4th day (misaligned) under LD-14/RF-12 conditions when the LD cycle was phase-shifted by 12 h compared to the first day of the experiment. The profile of this misaligned day under LD-14/RF-12 conditions was compared to the above described baseline day under LD-14/Dark-RF conditions.

Hepatic *Bmal1* gene expression rose in the second half of the light phase and peaked at ZT15 on the misaligned day under LD-14/RF-12 conditions (ZT3: 0.07 ± 0.02 , ZT9: 1.91 ± 0.23 , ZT15: 2.78 ± 0.29 , ZT21: 1.36 ± 0.20 , ZT27: 0.05 ± 0.01). The sine fit phase delay between the baseline and the misaligned days was 2.72 h (**Figure 3-3 a**, **Table S1**). *Per2* showed highest expression levels at ZT9 and ZT27 on the misaligned day (ZT3: 2.14 ± 0.22 , ZT9: 3.41 ± 0.56 , ZT15: 0.50 ± 0.11 , ZT21: 0.14 ± 0.01 , ZT27: 3.20 ± 0.44) and the sine fit phase delay was 1.24 h compared to the baseline expression rhythm. *Dbp* and *Cry1* expression rhythms were anti-phasic on the misaligned day and the sine fits were phase-delayed compared to the rhythms on the baseline day (with *Dbp* level at ZT3: 0.427 ± 0.99 , ZT9: 0.25 ± 0.04 , ZT15: 0.04 ± 0.01 , ZT21: 0.56 ± 0.18 , ZT27: 8.49 ± 0.78 and *Cry1* level at ZT3: 1.92 ± 0.56 , ZT9: 3.66 ± 0.29 , ZT15: 5.14 ± 1.35 , ZT21: 0.96 ± 0.30 , ZT27: 1.36 ± 0.27). The largest sine fit phase delays within this tissue were calculated for *Dbp* and *Cry1* with 4.22 h and 3.88 h, respectively. *Nr1d1* expression showed its highest expression levels in the second half of the dark phase on the misaligned day (ZT3: 0.92 ± 0.33 , ZT9: 0.05 ± 0.02 , ZT15: 0.25 ± 0.16 , ZT21: 2.60 ± 0.83 , ZT27: 1.41 ± 0.46). The sine fit phase delay between the baseline and the misaligned days was 0.72 h (**Figure 3-3 a**).

In the adrenal, clock gene expression rhythms were phase-delayed on the misaligned condition (LD-14/RF-12) compared to the baseline condition (LD-14/Dark-RF). *Bmal1* expression levels were 0.34 ± 0.11 (ZT3), 2.34 ± 0.51 (ZT9), 2.48 ± 0.43 (ZT15), 0.81 ± 0.31 (ZT21), 0.24 ± 0.07 (ZT27) on the misaligned day. The sine fit phase delay between the baseline and the misaligned days was 2.88 h. *Per2* expression levels were 2.63 ± 0.28 (ZT3), 3.38 ± 0.76 (ZT9), 1.42 ± 0.45 (ZT15), 0.63 ± 0.10 (ZT21), 3.43 ± 1.06 (ZT27) on the misaligned day and sine fit phase delay was 3.94 h compared to the baseline *Per2* expression rhythm. *Dbp* expression was U-shaped along the LD cycle (ZT3: 2.96 ± 0.62 , ZT9: 0.18 ± 0.03 , ZT15: 0.15 ± 0.02 , ZT21: 2.21 ± 0.33 , ZT27: 9.79 ± 3.80) and the sine fit was phase-delayed by 4.32 h compared to the baseline day (**Figure 3-3 b**). Furthermore, *Cry1* and *Nr1d1* expression rhythms were anti-phasic on the misaligned day and sine fits were phase-shifted compared to the baseline day in the adrenal (with *Cry1* level at ZT3: 1.00 ± 0.31 , ZT9: 2.50 ± 0.23 , ZT15: 1.68 ± 0.37 , ZT21: 0.41 ± 0.05 , ZT27: 0.92 ± 0.17 and *Nr1d1* level at ZT3: 0.56 ± 0.09 , ZT9: 0.06 ± 0.01 , ZT15: 0.20 ± 0.05 , ZT21: 1.86 ± 0.80 , ZT27: 1.25 ± 0.25). The sine fit phase delays were 3.76 h and 2.12 h for *Cry1* and *Nr1d1*, respectively (**Figure 3-3 b**).

In the eWAT, all investigated gene expression rhythms were similarly phase-delayed between the baseline and the misaligned (LD-14/RF-12) days. *Bmal1* and *Per2* expression rhythms were anti-phasic on the misaligned day (with *Bmal1* level at ZT3: 0.08 ± 0.01 , ZT9: 0.75 ± 0.12 , ZT15: 1.49 ± 0.23 , ZT21: 0.46 ± 0.10 , ZT27: 0.05 ± 0.004 and *Per2* level at ZT3: 1.63 ± 0.64 , ZT9: 1.16 ± 0.37 , ZT15: 0.43 ± 0.04 , ZT21: 0.15 ± 0.01 , ZT27: 1.65 ± 0.04). The sine fit phase delays were 2.40 h for *Bmal1* and 1.64 h for *Per2* expression rhythms between baseline and misaligned days (**Figure 3-3 c**). On the misaligned day under LD-14/RF-12 conditions, adipose *Dbp* expression rhythm was U-shaped (ZT3: 2.38 ± 0.86 , ZT9: 0.31 ± 0.07 , ZT15: 0.12 ± 0.07 , ZT21: 0.85 ± 0.25 , ZT27: 3.61 ± 0.24) and the expression rhythm was phase-delayed by 2.30 h compared the baseline day. *Cry1* expression rhythm was not rhythmic on the misaligned day and, thus, excluded from the analysis. On the misaligned day, *Nr1d1* expression peaked in the second half of the dark phase in the eWAT (ZT3: 0.96 ± 0.36 , ZT9: 0.07 ± 0.02 , ZT15: 0.12 ± 0.09 , ZT21: 1.60 ± 0.73 , ZT27: 1.27 ± 0.26). The expression rhythm of *Nr1d1* was phase-delayed by 1.72 h compared to the expression rhythm on the baseline day (**Figure 3-3 c**).

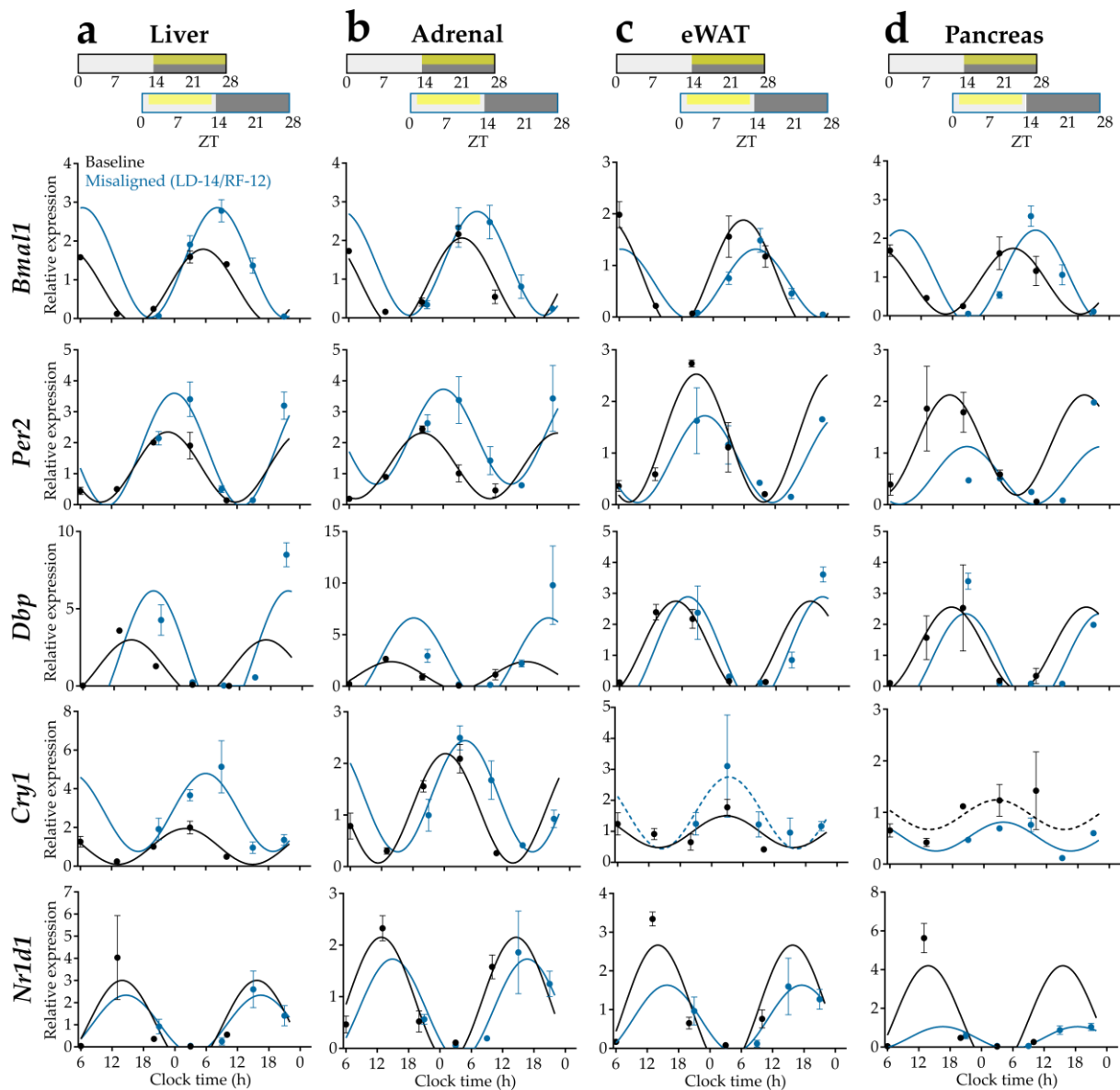


Figure 3-3: Peripheral clock gene expression is phase-shifted between baseline and misaligned day in the LD-14/RF-12 paradigm. Gene expression of *Bmal1*, *Per2*, *Dbp*, *Cry1*, and *Nr1d1* (top to bottom) was measured in (a) liver, (b) adrenal, (c) eWAT and (d) pancreas by qPCR under baseline (LD-14/Dark-RF, black) and misaligned (blue) conditions over 28 h ($n = 3-5$ animals *per* timepoint). Data are shown as means $\pm$ SEMs. Solid and dashed lines indicate significant 24-h rhythmic and non-rhythmic gene expression profiles (as tested in CircWave), respectively. Fitted curves are sine waves with a wavelength of 25.8 h. Light grey, dark grey and yellow bars on top indicate light phase, dark phase and food access time, respectively.

On the baseline day, pancreatic *Bmal1* expression peaked in the middle of the dark phase. On the misaligned day under LD-14/RF-12 conditions, *Bmal1* expression peaked in the beginning of the dark phase (ZT3: 0.05 ± 0.02 , ZT9: 0.54 ± 0.09 , ZT15: 2.58 ± 0.26 , ZT21: 1.06 ± 0.26 , ZT27: 0.11 ± 0.06) and the sine fit was phase-shifted by 4.32 h compared to the baseline *Bmal1* expression rhythm (**Figure 3-3 d**). *Per2* gene showed highest expression levels at the end of the dark phase (ZT3: 0.47 ± 0.03 , ZT9: 0.51 ± 0.04 , ZT15: 0.25 ± 0.05 , ZT21: 0.08 ± 0.02 , ZT27: 1.98 ± 0.42). The *Per2* sine fit phase delay was 3.32 h between the baseline and the misaligned days. *Dbp* levels were 3.39 ± 0.26 (ZT3), 0.10 ± 0.02 (ZT9), 0.09 ± 0.01 (ZT15), 0.08 ± 0.03 (ZT21), 1.99 ± 0.09 (ZT27) on the misaligned day and the sine fit showed only a moderate phase delay of 2.64 h compared to the baseline rhythm. In contrast to the pancreatic *Cry1* expression profiles on the baseline and the misaligned days under LD-14/Dark-RF conditions, *Cry1* expression was rhythmic with low amplitude under LD-14/RF-12 conditions (ZT3: 0.47 ± 0.05 , ZT9: 0.69 ± 0.04 , ZT15: 0.76 ± 0.13 , ZT21: 0.12 ± 0.02 , ZT27: 0.60 ± 0.02). *Nr1d1* expression rhythm was dampened by 80 % compared to the baseline *Nr1d1* rhythm and showed its highest expression levels at ZT27 (ZT3: 0.59 ± 0.16 , ZT9: 0.06 ± 0.01 , ZT15: 0.06 ± 0.02 , ZT21: 0.88 ± 0.23 , ZT27: 1.04 ± 0.19). *Nr1d1* expression rhythms were phase-shifted by 2.80 h between the baseline and the misaligned days (**Figure 3-3 d**).

In summary, phase shifts in clock gene expression profiles between baseline and misaligned (LD-14/RF-12) conditions were smaller in liver and eWAT and larger in adrenal and pancreas. On the misaligned day under LD-14/RF-12 conditions, *Cry1* expression levels in eWAT showed high variation, thus reducing statistical power so that significance was lost for rhythmicity.

3.1.3 Peripheral clock gene expression is phase-delayed with little effect on the amplitude in both zeitgeber intervention paradigms

To quantify the effect of different *zeitgeber* intervention strategies on peripheral clock rhythms, phase shifts and amplitude changes between baseline and misaligned days were calculated from sine waves fitted to the different clock gene expression profiles (matches the activity period, see 3.1.1). The wavelength of sine fitting was set to the locomotor activity period (see 3.1.1) and calculated maxima are described in detail in 3.1.2.

In the liver, clock gene expression rhythms were phase-delayed by 5.5 ± 1.0 h under LD-14/Dark-RF conditions and by 2.6 ± 0.7 h under LD-14/12-RF conditions. Furthermore,

clock gene expression amplitude was significantly reduced by 35 % on misaligned days compared to amplitude on the baseline day under LD-14/Dark-RF conditions ($p = 0.03$, one-sample t -test). Under LD-14/RF-12 conditions, the amplitude was slightly, but not significantly, elevated (164.8 ± 28.9 % of baseline amplitude) (**Figure 3-4 a**). The adrenal clock rhythms were phase-delayed by 5.8 ± 0.2 h and 3.4 ± 0.4 h under LD-14/Dark-RF and LD-14/RF-12 conditions, respectively. The clock gene expression amplitudes on misaligned days were comparable to the baseline day with 89.8 ± 7.7 % and 148.2 ± 38.5 % for LD-14/Dark--RF and LD-14/RF-12 conditions, respectively (**Figure 3-4 b**). The biggest effects between the different *zeitgeber* intervention strategies were observable in eWAT. Here, clock gene expression rhythms were phase-delayed by 7.1 ± 0.2 h under LD-14/Dark-RF but only 2.0 ± 0.2 h under LD-14/RF-12 conditions between the baseline and the misaligned days. Clock gene expression amplitudes were slightly decreased in both paradigms, but the reduction was not significant (78.2 ± 11.8 % (LD-14/Dark-RF) and 74.9 ± 13.9 % (LD-14/RF-12)) (**Figure 3-4 c**). The pancreatic tissue clock was impacted to similar extents by the two different time-restricted feeding regimes. Calculated phase shifts between baseline and misaligned days were 4.4 ± 1.4 h and 3.3 ± 0.4 h under LD-14/Dark-RF and LD-14/RF-12 conditions, respectively. There was no significant change in the amplitude of clock gene expression compared to baseline in any of the two paradigms (LD-14/Dark-RF: 146.7 ± 54.9 %, LD-14/RF-12: 82.2 ± 26.5 %) (**Figure 3-4 d**).

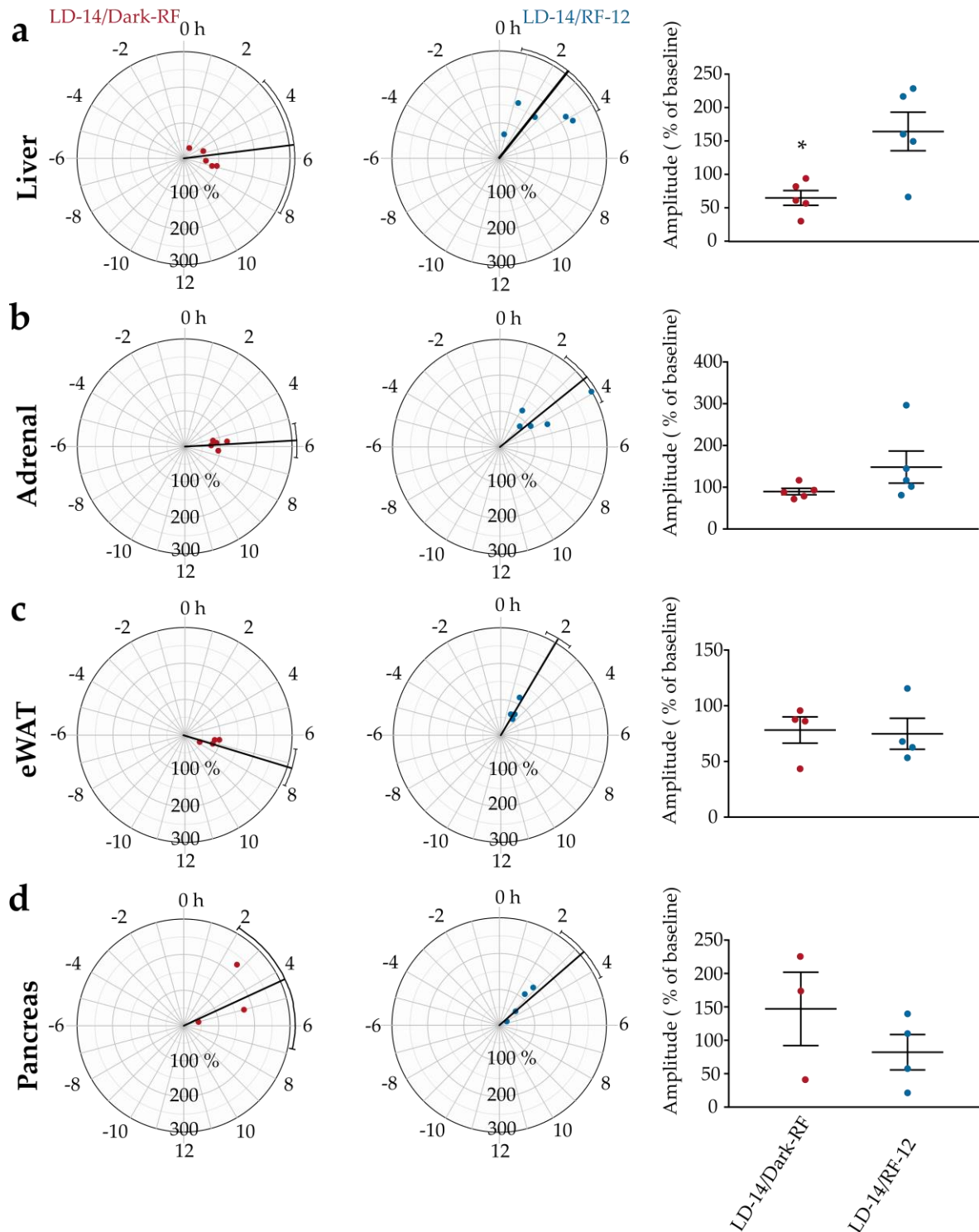


Figure 3-4: Clock gene expression rhythms are phase-delayed between baseline and misaligned days under LD-14/Dark-RF and LD-14/RF-12 with little effect on amplitude. Phase shifts of clock gene expression calculated from maxima of fitted sine waves for LD-14/Dark-RF (red) and LD-14/RF-12 (blue) conditions in (a) liver, (b) adrenal, (c) eWAT and (d) pancreas. Clock gene expression rhythm phase shifts (in hours) and normalized amplitudes relative to baseline (in % of baseline) are shown on radial and axial axis, respectively. Black lines indicate the averages $\pm$ 95 % confidence interval. Right: Normalized amplitudes relative to baseline for LD-14/Dark-RF (red) and LD-14/RF-12 (blue) conditions. $n = 3-5$, * $p < 0.05$ in one-sample t -test against 100 %. Data are shown as means $\pm$ SEMs.

Taken together, *zeitgeber* intervention phase-delays clock gene expression within four days independent from the feeding regime. Phase shifts in tissue clocks between baseline and misaligned days were smaller under LD-14/RF-12 compared to LD-14/Dark-RF conditions. The amplitudes of clock gene expression profiles were, on average, unaffected by the *zeitgeber* intervention except for the liver under LD-14/Dark-RF. Here, amplitudes were reduced compared to the baseline clock gene expression amplitudes.

3.1.4 The circadian clock network is internally desynchronized under LD14/RF-12 ZI conditions

Period lengths of tissue clock rhythms (based on shifts in clock gene expression) rhythms and locomotor activity were calculated from total phase shifts between baseline and misaligned day (see **Figure 3-4**) to quantify the magnitude of internal desynchronization. Internal desynchronization is defined as a disturbance in the circadian organization of physiology and behavior. Activity period was 25.8 h for both feeding regimes and indirectly represents the phasing of the SCN pacemaker. The period of the liver clock was close to that of locomotor activity under LD-14/Dark-RF conditions with 25.8 ± 0.3 h and significantly shorter under LD-14/RF-12 conditions (24.9 ± 0.2 h) ($p = 0.0034$, two-way ANOVA) (**Figure 3-5**). The adrenal clock showed a period of 25.1 ± 0.1 h under LD14/RF-12 conditions and ran significantly slower under LD-14/Dark-RF conditions (25.9 ± 0.1 h) ($p = 0.0220$, two-way ANOVA). The eWAT clock was impacted the strongest by the different feeding regimes. Under LD-14/RF-12 conditions, the eWAT clock period was 24.7 ± 0.1 h and, thus, almost 2 h faster than under LD-14/Dark-RF conditions (26.4 ± 0.1 h) ($p < 0.0001$, two-way ANOVA). Pancreas clock periods were comparable under both feeding regimes with 25.4 ± 0.3 h and 25.1 ± 0.1 h under LD-14/Dark-RF and LD-14/RF-12 conditions, respectively ($p = 0.4445$, two-way ANOVA).

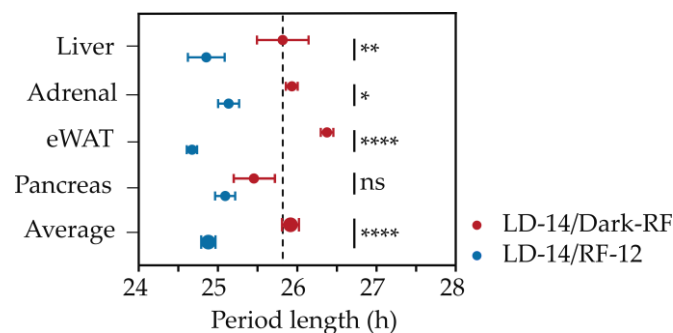


Figure 3-5: Time of feeding differentially impacts the period length of peripheral clocks. Periods of tissue clock rhythms were calculated from clock gene expression phase shifts between baseline and misaligned conditions under LD-14/Dark-RF (red) and LD-14/RF-12 (blue) conditions. Dashed line indicates locomotor activity period. $n = 3-5$, ns: not significant, * $p < 0.05$, ** $p < 0.001$, **** $p < 0.0001$ between the feeding regimes, two-way ANOVA. Data are shown as means $\pm$ SEMs.

Peripheral tissue clocks did not entrain to the respective feeding regimes within the four days of experiment. Overall, RF-12 induced less period lengthening in peripheral tissue clocks than Dark-RF ($p < 0.0001$, two-way ANOVA, 24.9 ± 0.1 h (RF-12) *vs.* 25.9 ± 0.1 h (Dark-RF)). Under

LD-14/Dark-RF conditions, the average tissue clock period was closer to that of activity indicating less tissue clock dissociation, *i.e.* internal desynchronization.

3.2 Long-term zeitgeber desynchrony

The above described acute zeitgeber intervention with different TRF strategies showed differential phase shifts within 4 days of experiment. Mice fed in the dark phase (Dark-RF) did not show an internal desynchronization between peripheral tissue clocks and behavior under LD-14 conditions. In contrast, time restricted feeding of 12 h fasting: 12 h feeding (RF-12) led to an internal desynchronization under LD-14 conditions but a smaller chronodisruption comparing to the feeding:fasting cycle. Internal desynchronization it thought to contribute to adverse health effects seen in shift workers. On the other hand, it has been suggested to apply TRF as a therapeutic approach to improve health in shift workers who are suffering from chronodisruption (Chaix et al., 2014; Gill and Panda, 2015). Therefore, effects of recurring zeitgeber desynchrony conditions were studied in a long-term experiment with 14 h light: 14 h dark cycles combined with 12 h feeding: 12 h fasting cycles (LD-14/RF-12) to disentangle the differential zeitgeber input into the circadian system.

3.2.1 Effects under ZD conditions

3.2.1.1 Running-wheel behavior diverges and re-converges under recurring ZD conditions

Under recurring ZD conditions, roughly ~ 13 % of the animals showed 28-hour activity rhythms phase locked to the LD cycle (**Figure 3-6 a**). All other mice showed activity rhythms with a main period length between 24 h and 28 h (median 25.6 h, **Figure 3-6 b**). Individual mice displayed a range of different activity rhythms throughout the ZD paradigm. X² periodogram analysis of a representative mouse revealed four distinct activity period peaks (at 22-23 h, 24 h, 25-26 h and 28 h) with a characteristic long period-short period sequence (**Figure 3-6 c, d, Figure S1**). This was also reflected in the determined activity onsets relative to food access time over the course of the ZD experiment and averaged over 5 ZD cycles. Mice lengthened their locomotor activity period when phase angles between zeitgebers became larger (45 ° to ±180 ° phase angle), but “snapped back” (*i.e.* shortened their activity periods) when zeitgeber phases re-converged (-135 ° to -45 °) throughout the ZD cycle (**Figure 3-6 e, f**) (45 °: -3.1 ± 0.3 h, 90 °: -0.6 ± 0.4 h, 135 °: 2.3 ± 0.4 h, ±180 °: 3.2 ± 0.6 h, -135 °: -0.3 ± 0.8 h, -90 °: 2.7 ± 0.7 h, -45 °: 4.5 ± 0.4 h). The maximum phase angle difference (45 ° vs. 180 °) was 7.7 h.

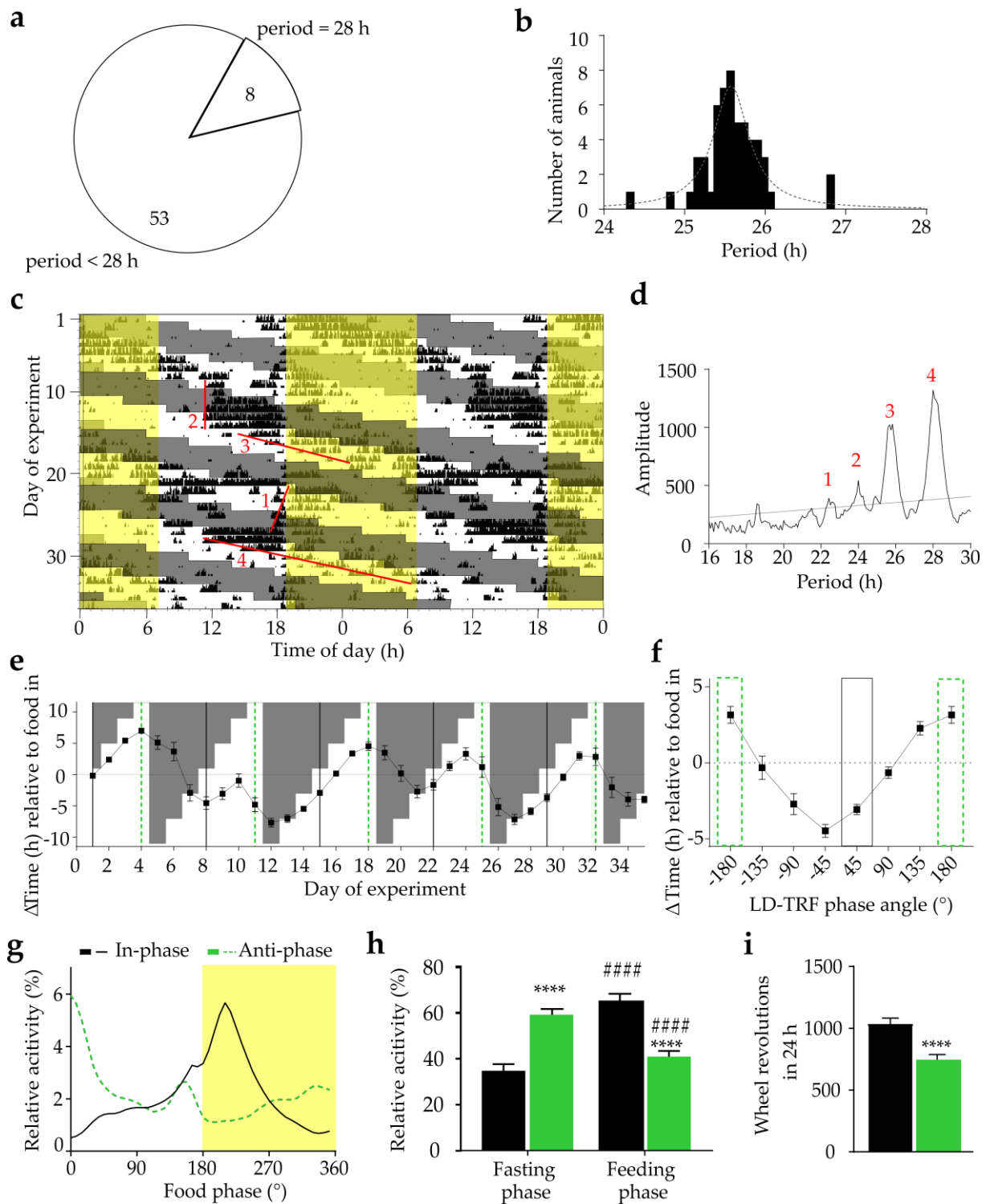


Figure 3-6: Locomotor activity period length depends on the phase angle difference between the two zeitgebers in the ZD paradigm. a) Fractions of animals that adapted to the 28-hour LD cycle ($n = 8$) and of free-running animals with periods < 28 h ($n = 53$) under ZD conditions. b) Distribution of dominant free-running activity periods (main periodogram peaks). The dashed curve shows a Lorentzian distribution fit (peak at 25.6 h). c) Representative long-term activity recording of one mouse over the course of five ZD cycles. Red lines depict different free-running rhythms as indicated in (d). d) X²periodogram analysis of running-wheel activity of the representative mouse and corresponding free-running period components indicated by red numbers as depicted in (c). e, f) Daily activity onsets relative to the feeding intervals (0 h represents “food in”) (e) throughout the experiment and (f)

averaged over one ZD cycle relative to the phase angle of the two *zeitgeber* (-180° and 180° represent maximal misalignment of food access and dark phase intervals, $n = 58$). Three mice were excluded due to highly fragmented activity patterns. Solid black and dashed green lines indicate in-phase and anti-phase days, respectively. g) Mean activity profiles at in-phase (solid black line) and anti-phase (dashed green line) days relative to food access ($180^\circ = \text{"food in"}$). h) Feeding/fasting phase activity distribution at in-phase (black) and anti-phase (green) days. **** $p < 0.0001$ between days, #### $p < 0.0001$ between feeding and fasting phase on the same days, two-way ANOVA. i) Total running-wheel activity at in-phase and anti-phase days. **** $p < 0.0001$, two-tailed, paired t -test. g-i) One animal was excluded due to missing values in the activity recording ($n = 60$). e, f, i, h) Data are shown as means $\pm$ SEMs.

On in-phase days, running-wheel activity peaked at the beginning of the feeding/dark phase. Of note, there was also activity in the fasting/light phase, especially towards the end of this phase, which was above the activity levels seen under standard LD cycles (data not shown). On anti-phase days, mice showed increased activity in the first half of the fasting phase, coinciding the dark phase, and showed another small activity peak at the end of the fasting phase. Activity levels were relatively low in the first half of the feeding phase and increased towards the end of the feeding phase (**Figure 3-6 g**). To quantify the change in activity distribution, feeding and fasting phase running-wheel activity were compared between in-phase and anti-phase days. Locomotor activity in the feeding phase was significantly higher on in-phase days compared to anti-phase days ($p < 0.0001$, two-way ANOVA, in-phase: $65.2 \pm 2.9\%$ vs. anti-phase: $40.9 \pm 2.5\%$). *Vice versa*, activity was much lower in the fasting phase on in-phase days compared to anti-phase days ($p < 0.0001$, two-way ANOVA, in-phase: $34.8 \pm 2.9\%$ vs. anti-phase: $59.1 \pm 2.5\%$). Additionally, there was a significant difference in the feeding-to-fasting phase locomotor activity distribution under in-phase and anti-phase conditions ($p < 0.0001$, two-way ANOVA) (**Figure 3-6 h**). Relative to the 24 h feeding/fasting cycle, mice were 30 % less active on anti-phase days than on in-phase days ($p < 0.0001$, two-tailed paired t -test, 747 ± 41 vs. $1,031 \pm 47$ revolutions in 24 h) (**Figure 3-6 i**).

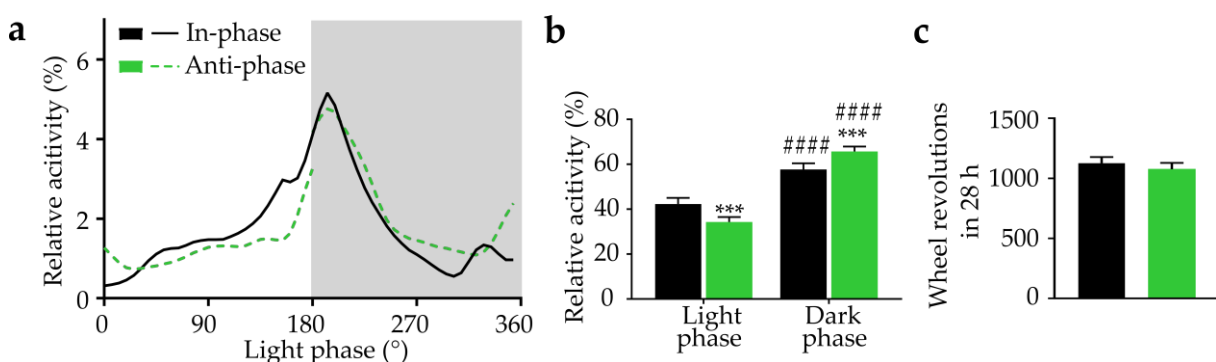


Figure 3-7: Mice were more active in the dark phase on in-phase and anti-phase days under ZD conditions. a) Mean activity profiles on in-phase (solid black line) and anti-phase (dashed green line) days relative to the LD cycle ($180^\circ = \text{"lights off"}$). Grey shading indicates dark phase. b) Light/dark phase activity distributions on in-phase (black) compared to anti-phase (green) days, *** $p = 0.0002$ between days, #### $p < 0.0001$ between feeding and fasting phase on the same day, two-way ANOVA. c) Total running-wheel revolutions in 28 h. b, c) Data are shown as means $\pm$ SEMs.

In contrast, mice showed comparable total activity level relative to the LD cycle (28 h) (**Figure 3-7 a, c**). Mice were more active during the dark phase on anti-phase days compared to in-phase days ($p < 0.0001$, two-way ANOVA, $65.7 \pm 2.2\%$ vs. $57.7 \pm 2.7\%$) relative to the 28-hour LD cycle. Furthermore, mice were significantly more active in the dark phase than in the light phase at both in-phase and anti-phase days ($p < 0.0001$, two-tailed paired t -test) (**Figure 3-7 b**).

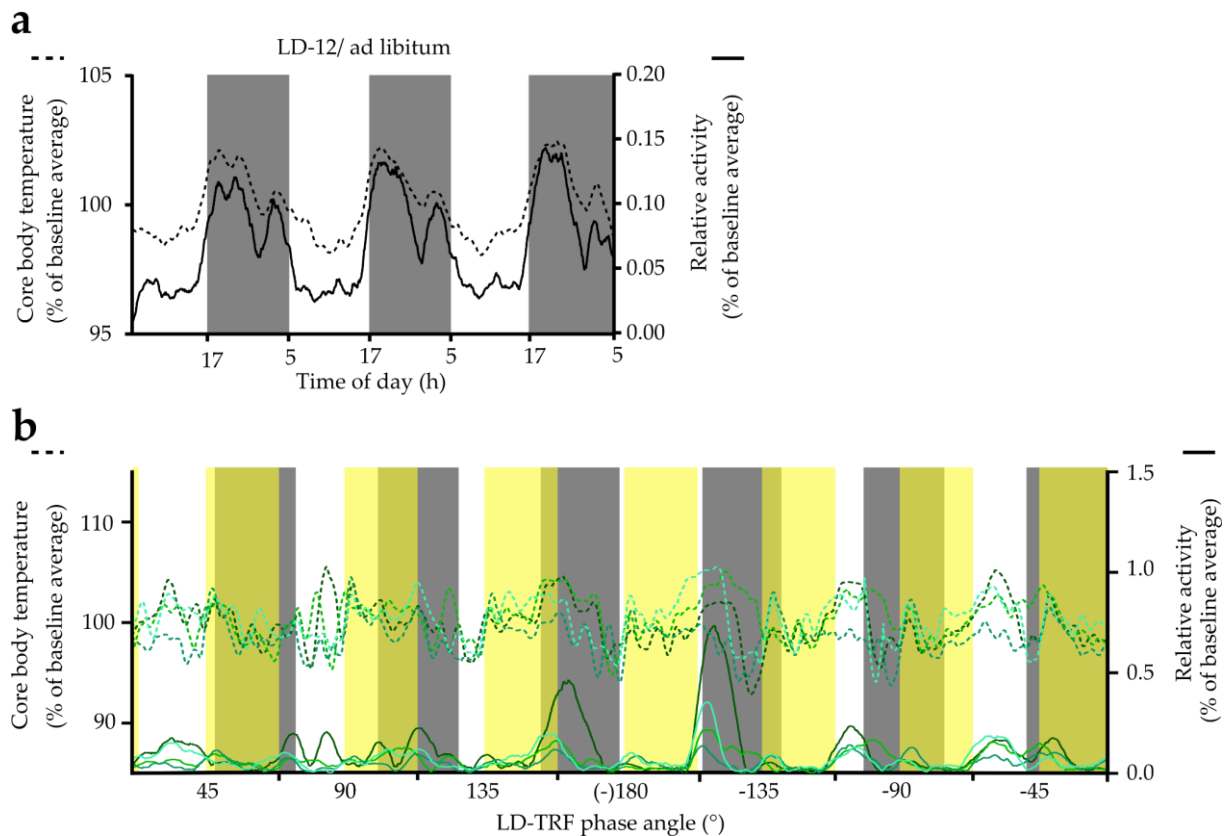


Figure 3-8: Normalized core body temperature and locomotor activity under baseline and ZD conditions. Core body temperature (dashed lines) and locomotor activity (solid lines) under (a) LD12/*ad libitum* feeding and (b) ZD conditions, $n = 4$. a) Data are means of four animals. b) Data of single mice are averaged and smoothed over two experimental cycles. Dark grey and yellow shadings indicate dark phase and food access time. Solid lines indicate relative locomotor activity (right y-axis) and dashed lines indicate normalized core body temperature (left y-axis).

Behavioral rhythms and core body temperature (CBT) are tightly linked and regulated by the SCN. It is shown, however, that CBT uncouples from rest:activity cycle under short light T-cycles conditions with *ad libitum* food access (Cambras et al., 2007). To test this under extended T-cycles and TRF, mice were equipped with telemetry probes. CBT was low throughout the light phase and increased in the dark phase under LD-12 with *ad libitum* food access (baseline conditions) (**Figure 3-8 a**). Under ZD conditions, CBT showed a variable pattern along 28-hour day. For most days, a peak in CBT coincided with an increase in activity measured by telemetry and another increase in CBT occurred in the beginning of each feeding interval. The latter increase did not always coincide with increased activity measured by telemetry (**Figure 3-8 b**). Total activity levels, measured by telemetry, were higher on anti-phase days (180°) than on in-phase days (45°) which differed markedly from the results

obtained from running-wheel recordings. Overall, core body temperature showed a complex curve progression throughout the experiment (**Figure 3-8 b**).

In summary, the regulation of activity was impacted by both *zeitgebers*, light and food, and was a function of *zeitgeber* phase angle. CBT was also impacted by diverging *zeitgeber* input, however patterns appeared very complex. CBT and activity were not completely uncoupled by recurrent ZD conditions, although, did show some discrepancies. Both outputs were not entrained to one *zeitgeber* rhythm and, thus were chronodisrupted under recurrent ZD conditions.

3.2.1.2 Metabolic states cycle under ZD conditions

The behavioral data implied a state of chronodisruption under ZD conditions which is also a hallmark of shift work. Chronodisruption has been suggested to contribute to the increased risk of obesity and metabolic disorders seen in shift workers (Reiter et al., 2012). Under ZD conditions, mice were exposed to LD shifts and forced to consume food at different LD-TRF phase angles. To determine to which extent divergent *zeitgeber* input affects metabolic state, mice were weighed daily prior to feeding time intervals. Additionally, daily food intake was measured. Similar to what was seen for locomotor activity, animals showed cyclic changes in food consumption and body weight regulation under recurrent ZD conditions (**Figure 3-9 a, b**). Over one ZD cycle, metabolic states showed two phases – one catabolic and one anabolic phase. Mice were in a catabolic state losing weight when the *zeitgeber* were maximal out-of-phase until the day prior to the in-phase day ($\pm 180^\circ$ - -45°). During these days, food intake was stable. Mice increased their food intake by ~14 % on the day prior to the in-phase day and then switched from the catabolic to the anabolic state increasing their body weight by 13 % on the in-phase day. Thereafter, mice stayed in the anabolic state until the anti-phase day. Food intake then decreased on the day before the anti-phase day in the ZD cycle prefiguring the switch to the catabolic state (**Figure 3-9 b**). When comparing in-phase to anti-phase days, mice increased their food intake and body weight by 14 % and 24 %, respectively (relative food intake: 18.9 ± 0.3 % (in-phase) *vs.* 16.2 ± 0.2 % (anti-phase) and relative weight gain: 1.9 ± 0.3 % *vs.* -0.6 ± 0.2 %; $p < 0.0001$, two-tailed, paired *t*-test for both) (**Figure 3-9 c, d**). This resulted in a markedly higher relative energy conversion rate, the ratio of weight gain to food intake, on in-phase days compared to anti-phase days (7.4 ± 1.5 % *vs.* -2.5 ± 0.8 %, $p < 0.0001$ two-tailed, paired *t*-test) (**Figure 3-9 e**).

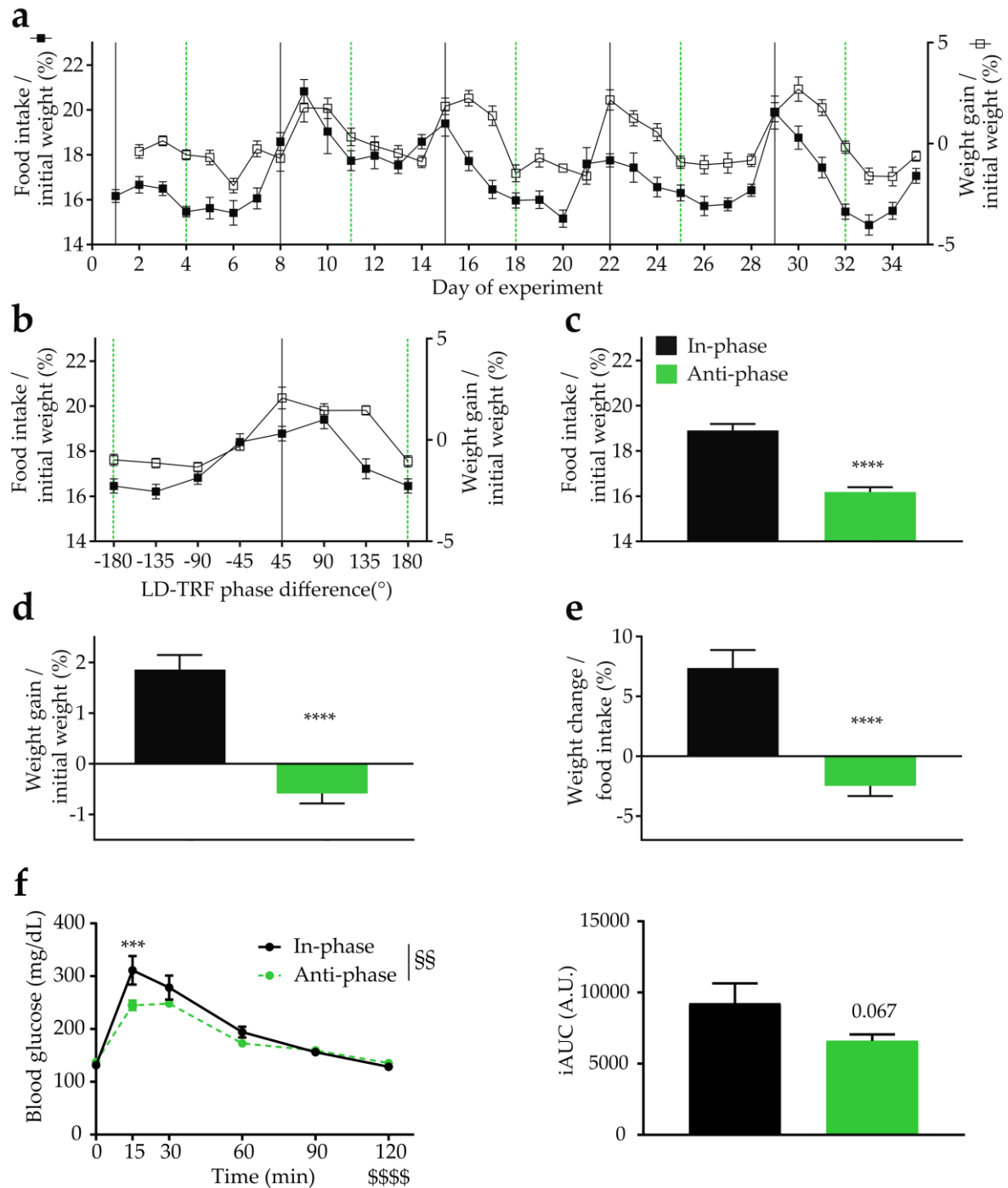


Figure 3-9: Mice show increased body weight and food intake and decreased glucose tolerance on in-phase days under ZD conditions. a) Food intake (closed squares, left y-axis) and body weight gain (open squares, right y-axis) throughout 5 cycles of the ZD paradigm. In-phase (solid black line) and anti-phase (dashed green line) days are indicated. b) Averaged normalized food intake (closed square, left y-axis) and body weight change (open squares, right y-axis) across one ZD cycle. (c) Normalized food consumption, (d) weight change and (e) energy conversion on in-phase (black) and anti-phase (green) days, $n = 45$. **** $p < 0.0001$, two-tailed, paired t -tests. f) Glucose tolerance test over time (left) and calculated incremental area under the curve (right) on in-phase ($n = 4$) and anti-phase ($n = 6$) days. Due to technical problems, two animals in the in-phase condition had to be excluded. *** $p < 0.001$,

condition effect ^{ss} $p = 0.0049$, time effect $^{$$$}$ $p < 0.0001$, two-way ANOVA. Data are shown as means $\pm$ SEMs.

Mice were metabolically challenged in the 4th cycle of the ZD paradigm by a GTT. Fasted blood glucose levels rose within 15 min after a glucose bolus was given and declined gradually to reach baseline levels again after two hours at both in-phase and anti-phase days ($p < 0.0001$, time effect, two-way ANOVA). Animals showed an improved glucose tolerance on anti-phase compared to in-phase days ($p = 0.0049$, two-way ANOVA) with significantly lower blood glucose levels at 15 min after glucose injection (0 min: 131 ± 6 mg/dL (in-phase day) *vs.* 138 ± 2 mg/dL (anti-phase day), 15 min: 311 ± 27 mg/dL *vs.* 245 ± 9 mg/dL, $p < 0.0001$ Sidak's post-test, two-way ANOVA, 30 min: 279 ± 23 mg/dL *vs.* 248 ± 2 mg/dL, 60 min: 194 ± 10 mg/dL *vs.* 173 ± 5 mg/dL, 90 min: 156 ± 4 mg/dL *vs.* 160 ± 5 mg/dL, 120 min: 129 ± 5 mg/dL *vs.* 136 ± 5 mg/dL). Overall, the incremental area under the curve showed a trend towards lower values on anti-phase compared to in-phase days ($6,602 \pm 458$ (mg $\times$ min)/dL *vs.* $9,244 \pm 1,404$ (mg $\times$ min)/dL, $p = 0.067$, two-tailed, unpaired *t*-test) (**Figure 3-9 f**). Body composition was comparable between the conditions (fat mass: 20.9 ± 0.9 % (in-phase) *vs.* 20.5 ± 0.7 % (anti-phase), lean mass: 75.6 ± 0.9 % *vs.* 74.2 ± 0.5 %, and free body fluid: 3.5 ± 0.4 % *vs.* 5.3 ± 0.3 %) (**Figure 3-9**, black and green bars).

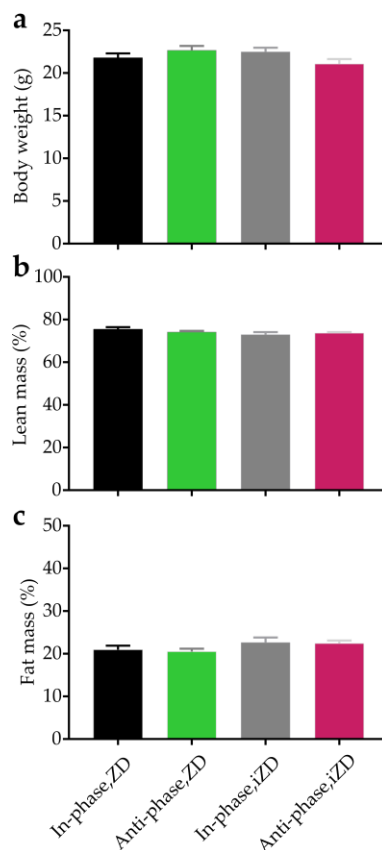


Figure 3-10: Body composition is comparable on in-phase and anti-phase days under ZD and iZD conditions. a) Body weight, b) normalized lean mass, and c) normalized fat mass ($n = 6$ for in-phase ZD, anti-phase ZD and in-phase iZD, $n = 5$ for anti-phase iZD conditions). Data are shown as means $\pm$ SEMs.

In summary, mice showed cyclic variations in metabolic state with increased food intake and body weight on in-phase compared to anti-phase days. Overall, mice did not gain weight throughout the experiment (data not shown). Glucose tolerance was impaired on the in-phase compared to the anti-phase condition. Taken together, cyclic changes in the behavioral rhythms and metabolic readouts substantiated a possible chronodisruption on the molecular level.

3.2.1.3 Hormonal rhythms are phase-shifted in the ZD paradigm

Behavior and metabolism are partly hormonally regulated. Therefore, serum corticosterone and leptin levels were determined by RIA and ELISA, respectively, at in-phase and anti-phase days (**Figure 3-11 a**).

Serum corticosterone levels rose during the fasting period and reached their peak at ZT14 on in-phase days. On anti-phase days, corticosterone levels were highest at ZT14 and ZT20 relative to the food (ZT2: 70 ± 27 ng/mL (in-phase) vs. 50 ± 14 ng/mL (anti-phase), ZT8: 130 ± 55 ng/mL vs. 58 ± 20 ng/mL, ZT14: 167 ± 54 ng/mL vs. 112 ± 37 ng/mL, ZT20: 40 ± 22 ng/mL vs. 124 ± 29 ng/mL, ZT2: 123 ± 63 ng/mL vs. 104 ± 29 ng/mL) (**Figure 3-11 b**). COGs of corticosterone excretion patterns were at 183.2° on the in-phase day and 290.4° , 1.0° and 233.3° on the three anti-phase days. On average, corticosterone excretion was phase-delayed by $112 \pm 37^\circ$ (**Figure 3-11 d**). Corticosterone excretion was not rhythmic on in-phase and anti-phase days as determined by CircWave.

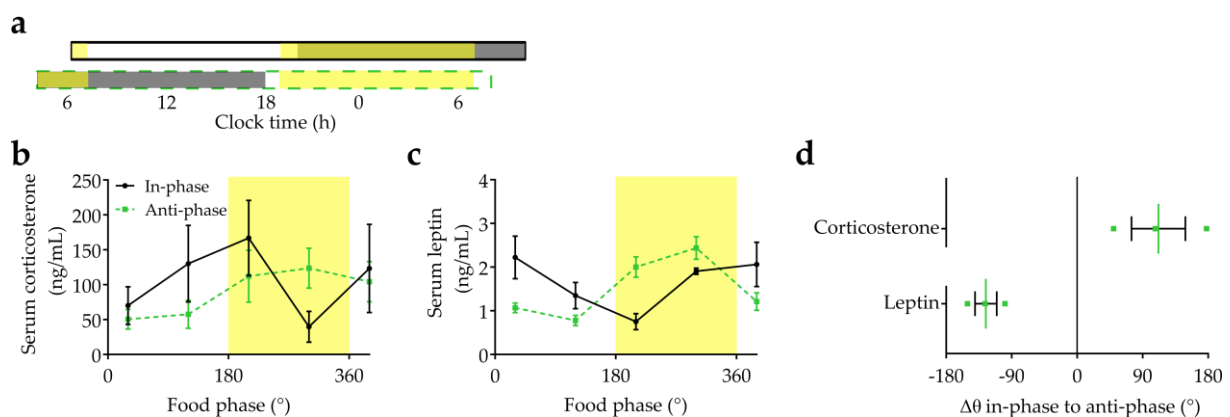


Figure 3-11: Corticosterone and leptin rhythms are phase-shifted between in-phase and anti-phase days under ZD conditions. a) Synopsis of in-phase and anti-phase condition relative to external clock time. Diurnal serum (b) corticosterone and (c) leptin profiles on in-phase (solid black line) and anti-phase (dashed green line) days. Anti-phase expression profile represents the 3 independent experiments. Yellow shading indicates time of food access (with 180° = “food in”, 360° correspond to 24 h) ($n = 2-4$ animals *per* timepoint *per* profile). d) Phase shifts of corticosterone and leptin rhythms in the serum between in-phase and anti-phase days. Data are shown as means $\pm$ SEMs.

On in-phase days, serum leptin showed a U-shaped excretion profile along the feeding:fasting cycle. Serum leptin rhythms were almost anti-phasic on anti-phase days compared to the in-phase profile with highest levels in the feeding phase (ZT2: 2.20 ± 0.49 (in-phase) vs. 1.07 ± 0.11 (anti-phase), ZT8: 1.35 ± 0.30 vs. 0.78 ± 0.12 , ZT14: 0.75 ± 0.18 vs. 2.00 ± 0.24 ,

ZT20: 1.91 ± 0.07 vs. 2.44 ± 0.26 , ZT2: 2.06 ± 0.51 vs. 1.21 ± 0.20) (**Figure 3-11 c**). For leptin excretion, COGs were at 33.6° (in-phase condition) and 267.0° , 294.6° and 242.9° on anti-phase days. Excretion of leptin was phase-advanced by $-125 \pm 15^\circ$ between in-phase and anti-phase days (**Figure 3-11 d**). All leptin excretion profiles were significantly rhythmic.

3.2.1.4 Clock gene expression rhythms in the SCN and peripheral tissues are phase-shifted in the ZD paradigm

The changes in behavior and metabolism as well as phase-shifted hormonal excretion rhythms are likely caused by alterations on the molecular level. First, SCN mRNA expression was investigated by *in situ* hybridization. Clock gene mRNA profiles in the SCN were phase-delayed relative to the feeding time on anti-phase days compared to in-phase days (**Figure 3-12**). In general, *Bmal1* expression patterns were not rhythmic on in-phase and two of the three anti-phase days. *Bmal1* expression was low during the fasting phase and elevated in the feeding phase at in-phase and anti-phase days (ZT2: 0.71 ± 0.71 (in-phase) vs. 2.00 ± 0.59 (anti-phase), ZT8: 0.12 ± 0.10 vs. 1.08 ± 0.47 , ZT14: 2.97 ± 0.39 vs. 2.42 ± 0.28 , ZT20: 0.74 ± 0.64 vs. 2.56 ± 0.41 , ZT2: 0.21 ± 0.23 vs. 2.23 ± 0.59 , greyscale units). In addition, mean amplitude of *Bmal1* was dampened by 48 % with overall high levels on anti-phase days (**Figure 3-12 a**). The COGs were at 225.0° for the in-phase and 238.2° , 297.0° and 332.6° for anti-phase conditions (**Figure 3-12 b**). *Per2* gene expression was highest in the fasting phase on the in-phase day whereas it peaked in the beginning of the feeding phase on anti-phase days (ZT2: 0.70 ± 0.39 (in-phase) vs. 0.48 ± 0.09 (anti-phase), ZT8: 1.81 ± 0.55 vs. 1.37 ± 0.25 , ZT14: 0.85 ± 0.14 vs. 2.13 ± 0.18 , ZT20: 0.33 ± 0.15 vs. 1.33 ± 0.26 , ZT2: 1.7 ± 0.68 vs. 0.93 ± 0.23) (**Figure 3-12 a**). Individual COGs for *Per2* expression profiles were at 125.9° for the in-phase and 198.5° , 225.5° and 180.6° for the anti-phase days. Mean COG phase shifts between the in-phase and the anti-phase days were $64 \pm 28^\circ$ for *Bmal1* and $75 \pm 12^\circ$ for *Per2* (**Figure 3-12 b**).

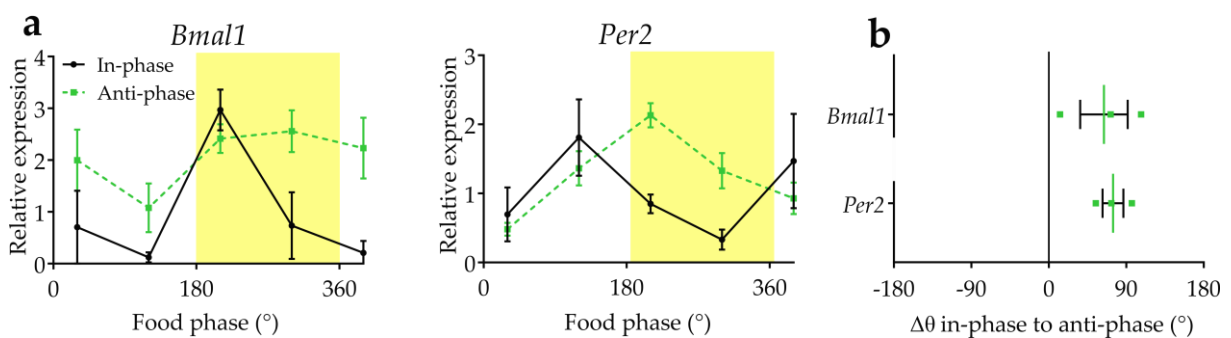


Figure 3-12: Clock gene expression in the SCN is phase-delayed between in-phase and anti-phase days under ZD conditions. a) Diurnal mRNA expression profiles for *Bmal1* and *Per2* on in-phase (solid black line) and anti-phase (dashed green line) days determined by ^{35}S -UTP *in situ* hybridization. Anti-phase expression profile represents 3 independent experiments. Yellow shading indicates time of food access (with $180^\circ =$ “food in”, 360° correspond to 24 h) ($n = 2\text{--}3$ animals *per* timepoint *per* profile). b) Phase shifts of clock gene expression rhythms in the SCN between in-phase and anti-phase days. Data are shown as means $\pm$ SEMs.

Next, clock gene expression profiles were investigated in peripheral tissues liver, eWAT, and adrenals. Hepatic *Bmal1* expression was high in the beginning of the fasting phase to then decline with lowest expression levels around the end of the fasting phase. In the feeding phase *Bmal1* expression rose again. This profile was seen at both in-phase and anti-phase days (ZT2: 1.27 ± 0.65 (in-phase) *vs.* 0.99 ± 0.12 (anti-phase), ZT8: 0.02 ± 0.004 *vs.* 0.09 ± 0.02 , ZT14: 0.23 ± 0.11 *vs.* 0.08 ± 0.04 , ZT20: 1.23 ± 0.27 *vs.* 0.68 ± 0.11 , ZT2: 1.86 ± 0.58 *vs.* 1.51 ± 0.37) (**Figure 3-13 a**). COGs of *Bmal1* expression patterns were at 330.3° (in-phase day) and 5.2° , 357.0° and 346.0° for anti-phase days. *Per2* expression patterns were almost anti-phasic to *Bmal1* with highest expression values in the beginning of the feeding phase on in-phase and anti-phase days (ZT2: 0.10 ± 0.04 (in-phase) *vs.* 0.21 ± 0.03 (anti-phase), ZT8: 0.73 ± 0.06 *vs.* 0.24 ± 0.02 , ZT14: 1.97 ± 0.52 *vs.* 1.74 ± 0.13 , ZT20: 1.85 ± 0.29 *vs.* 1.44 ± 0.16 , ZT2: 0.25 ± 0.07 *vs.* 0.29 ± 0.05). *Per2* COGs were at 244.6° (in-phase condition) and 251.8° , 245.6° and 231.9° for the anti-phase conditions (**Figure 3-13 d**). *Dbp* expression peaked at ZT8 relative to the food phase on in-phase days and anti-phase days but was significantly lower on anti-phase days at this timepoint (ZT2: 0.03 ± 0.01 (in-phase) *vs.* 0.03 ± 0.01 (anti-phase), ZT8: 4.85 ± 0.31 *vs.* 2.27 ± 0.34 , $p < 0.0001$, ZT14: 0.75 ± 0.24 *vs.* 1.53 ± 0.30 , ZT20: 0.08 ± 0.01 *vs.* 0.11 ± 0.02 , ZT2: 0.21 ± 0.09 *vs.* 0.02 ± 0.002) (**Figure 3-13 a**). *Dbp* COGs were at 131.5° on the in-phase day and 167.7° , 161.7° and 140.8° on anti-phase days. Phase shifts of individual hepatic clock genes were similar between the in-phase and each anti-phase condition. On average, the phase delays were $28 \pm 4^\circ$, $7 \pm 3^\circ$ and $25 \pm 8^\circ$ for *Bmal1*, *Per2* and *Dbp*, respectively (**Figure 3-13 d**). On average, clock gene expression amplitudes were slightly, but not significantly, decreased by 25 % on anti-phase conditions compared to in-phase conditions ($74.9 \pm 14.0\%$, data not shown).

In eWAT, *Bmal1* expression started high in the beginning of the fasting phase and declined with lowest expression levels in the beginning of the feeding phase to rise thereafter at in-phase and anti-phase days (ZT2: 1.14 ± 0.67 (in-phase) *vs.* 0.93 ± 0.17 (anti-phase), ZT8: 0.27 ± 0.10 *vs.* 0.11 ± 0.01 , ZT14: 0.16 ± 0.07 *vs.* 0.11 ± 0.03 , ZT20: 1.91 ± 0.93 *vs.* 0.72 ± 0.19 , $p = 0.0183$, and ZT2: 1.53 ± 0.11 *vs.* 1.43 ± 0.28) (**Figure 3-13 b**). *Bmal1* expression was non-rhythmic on the in-phase day. The individual COGs were at 321.8° on the in-phase day and 4.5° , 357.6° and 346.0° on the anti-phase days. *Per2* expression patterns were anti-phasic to *Bmal1* with highest expression values at ZT14 on in-phase and anti-phase days (ZT2: 0.34 ± 0.19 (in-phase) *vs.* 0.26 ± 0.04 (anti-phase), ZT8: 1.61 ± 0.18 *vs.* 0.49 ± 0.10 , ZT14: 1.84 ± 0.18 *vs.* 3.06 ± 1.07 , ZT20: 0.72 ± 0.13 *vs.* 0.87 ± 0.28 , ZT2: 0.31 ± 0.03 *vs.* 0.33 ± 0.05). *Per2* COGs were at 192.8° on the in-phase and 223.7° , 216.5° and 198.0° on the anti-phase days (**Figure 3-13 e**). *Dbp* expression peaked at ZT8 on in-phase and ZT14 on anti-phase days (ZT2: 0.30 ± 0.11 (in-phase) *vs.* 0.19 ± 0.09 (anti-phase), ZT8: 3.38 ± 0.78 *vs.* 1.61 ± 0.14 , ZT14: 1.11 ± 0.20 *vs.* 3.06 ± 1.17 , ZT20: 0.13 ± 0.02 *vs.* 0.52 ± 0.32 , ZT2: 0.35 ± 0.09 *vs.* 0.05 ± 0.006) (**Figure 3-13 b**). *Dbp* COGs were at 131.5° (in-phase condition), 167.7° , 161.3° and 140.8° (anti-phase conditions). COG phase shifts in the adipose tissue clock between the in-phase and anti-phase days were $33 \pm 5^\circ$, $20 \pm 8^\circ$ and $31 \pm 19^\circ$ for *Bmal1*, *Per2* and *Dbp*, respectively (**Figure**

3-13 e). Overall, clock gene expression amplitudes were comparable between the conditions (112.1 ± 55.3 % (anti-phase) *vs.* 100 % (in-phase), data not shown).

In the adrenal glands, *Bmal1* expression showed no significant circadian rhythm on the in-phase day but elevated gene expression in the feeding phase (ZT2: 1.21 ± 0.73 , ZT8: 0.45 ± 0.38 , ZT14: 1.17 ± 0.51 , ZT20: 1.38 ± 0.41 , ZT2: 0.80 ± 0.17). The *Bmal1* expression pattern was U-shaped along the feeding:fasting cycle on anti-phase days (ZT2: 1.26 ± 0.17 , ZT8: 0.24 ± 0.07 , ZT14: 0.33 ± 0.14 , ZT20: 1.06 ± 0.30 , ZT2: 2.53 ± 0.48). One of the three anti-phase expression profiles was not significantly rhythmic (**Figure 3-13 c**). COGs of *Bmal1* expression patterns were at 302.3° on the in-phase day and 14.0° , 357.9° and 329.0° on the anti-phase days. The *Per2* expression peaked in the beginning of the feeding phase on the in-phase day. The different phasing of individual *Per2* expression rhythms on anti-phase days led to an average expression curve with a peak towards the end of the feeding phase. On individual level, all *Per2* expression profiles were significantly rhythmic (ZT2: 0.53 ± 0.38 (in-phase) *vs.* 0.44 ± 0.08 (anti-phase), ZT8: 0.63 ± 0.13 *vs.* 0.51 ± 0.09 , ZT14: 2.79 ± 0.48 *vs.* 1.08 ± 0.17 , $p < 0.0001$, ZT20: 0.46 ± 0.17 *vs.* 1.21 ± 0.12 , ZT2: 0.60 ± 0.15 *vs.* 1.51 ± 0.42). For *Per2*, COGs were at 205.7° (in-phase day) and 267.4° , 265.5° and 243.3° (anti-phase days) (**Figure 3-13 e**). *Dbp* expression peaked at ZT8 relative to the food phase on in-phase days and anti-phase days (ZT2: 0.61 ± 0.41 (in-phase) *vs.* 0.35 ± 0.08 (anti-phase), ZT8: 2.15 ± 0.29 *vs.* 1.57 ± 0.14 , ZT14: 0.40 ± 0.10 *vs.* 1.39 ± 0.37 , ZT20: 0.19 ± 0.05 *vs.* 0.45 ± 0.23 , ZT2: 1.66 ± 0.25 *vs.* 0.40 ± 0.12) (**Figure 3-13 c**). One of the anti-phase *Dbp* expression patterns was not significantly rhythmic. *Dbp* COGs were at 113.9° on the in-phase day and 184.4° , 165.5° and 139.1° on anti-phase days. On average, COG phase shifts between the in-phase and anti-phase days were $51 \pm 13^\circ$ (*Bmal1*), $53 \pm 8^\circ$ (*Per2*) and $49 \pm 13^\circ$ (*Dbp*) which were the largest phase shifts observed in peripheral tissue clocks in this setup (**Figure 3-13 f**). On average, clock gene expression amplitudes were slightly, but not significantly, decreased (75.6 ± 13.8 %, data not shown).

In summary, overall phase shifts were smaller in peripheral tissue clocks than in the SCN. Peripheral clock gene expression profiles were more similarly phased relative to feeding time between in-phase and anti-phase days. The SCN clock was more impacted by the LD-14 cycle.

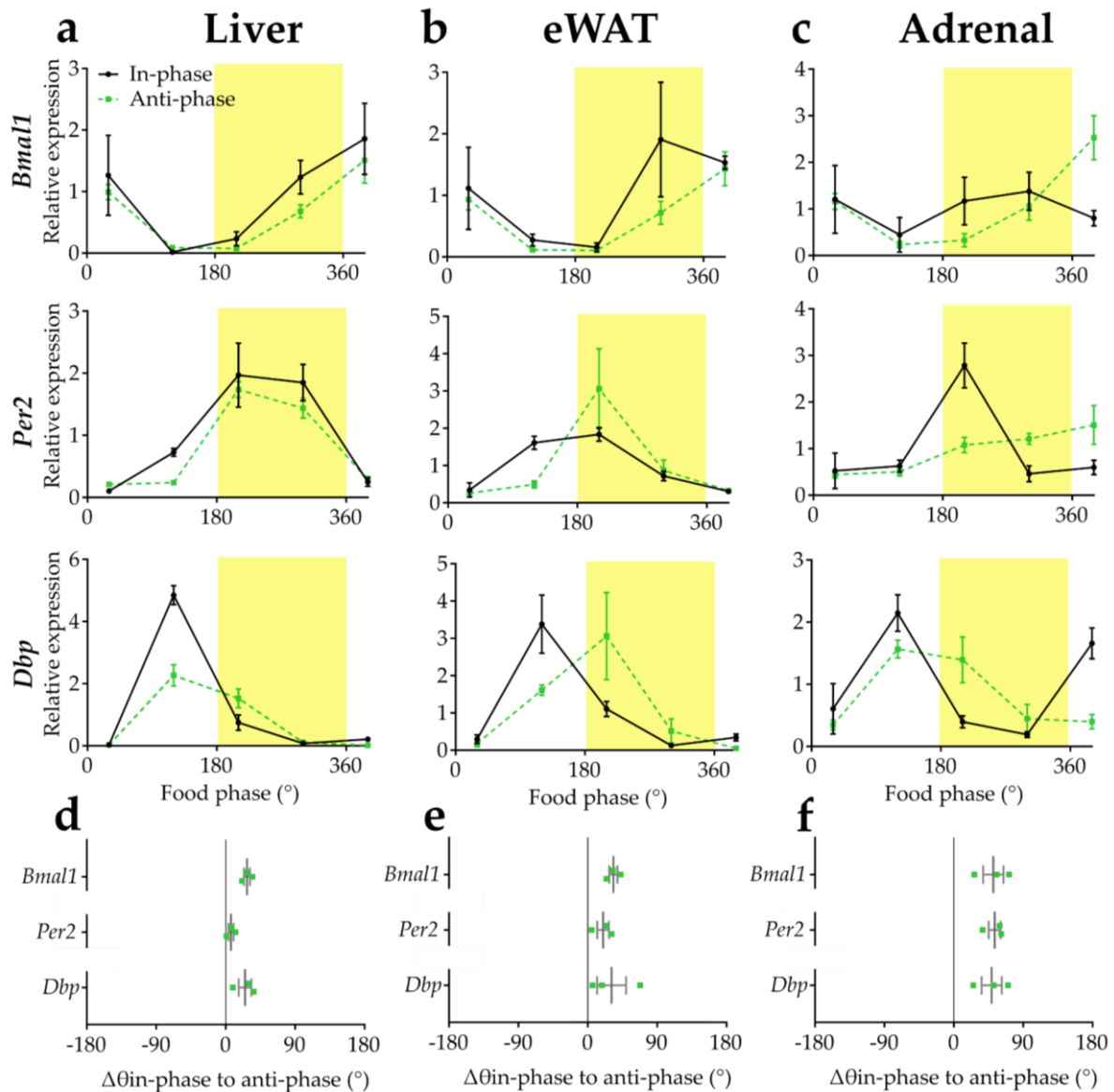


Figure 3-13: Peripheral clock gene expression is phase-delayed between in-phase and anti-phase days under ZD conditions. a) Diurnal mRNA expression profiles for *Bmal1* (top), *Per2* (middle) and *Dbp* (bottom) on in-phase (black solid line) and anti-phase (green dashed line) days determined by qPCR in (a) liver, (b) eWAT and (c) adrenal. The anti-phase expression profile represents 3 independent experiments, $n = 2-4$ animals *per* timepoint *per* profile. Yellow shading indicates time of food access (with $180^\circ = \text{“food in”}$, 360° correspond to 24 h). Phase shifts of tissue clock gene expression rhythms in (d) liver, (e) eWAT and (f) adrenal between in-phase and anti-phase days. Data are shown as means $\pm$ SEMs.

3.2.1.5 Chronodisruption is tissue- and rhythm-specific under ZD conditions

In order to differentially quantify *zeitgeber* input into specific molecular and behavioral rhythms, in-phase-to-anti-phase phase shifts were compared across tissues and outputs. For easier comparison, external clock time was used as reference frame and phase shifts for each tissue were averaged across genes. Thus, a phase shift of 0 h would mean that the rhythms stayed aligned with the feeding:fasting cycle (RF-12) while a phase shift of ± 12 h would signify a rhythm phase locked to the light:dark cycle (LD-14). Leptin was phase-advanced by

7.08 ± 0.70 h and significantly different from, both, RF-12 and LD-14 ($p = 0.0076$ *vs.* light and $p = 0.0097$ *vs.* food, one-sample *t*-tests) (**Figure 3-14**). Liver and eWAT tissue clocks stayed phase-locked to RF-12 with a small phase delay of 1.06 ± 0.39 h ($p = 0.0013$ *vs.* light) and 2.12 ± 0.68 h ($p = 0.0048$ *vs.* light), respectively. The adrenal clock was phase shifted by 3.76 ± 0.90 h between in-phase and anti-phase days ($p = 0.0111$ *vs.* light and $p = 0.0492$ *vs.* food). The SCN showed a phase shift of 5.00 ± 1.13 h between in-phase and anti-phase days and was significantly different from, both, light and food rhythms ($p = 0.0250$ *vs.* light and $p = 0.0475$ *vs.* food). Activity onset was similarly impacted by food and light and showed a phase shift of 6.29 ± 0.49 h ($p < 0.0001$ *vs.* light and food). Corticosterone showed the largest phase shift with 8.40 ± 1.79 h and was significantly different from food rhythms ($p = 0.0426$ *vs.* food).

In summary, except for leptin, all rhythms were phase-delayed. Liver and eWAT aligned with feeding:fasting cycle, while adrenal was impacted by light and food. Phase shifts did not significantly differ between peripheral tissues. In contrast, SCN, activity onsets, and corticosterone excretion aligned more closely with the light:dark cycle. These data reveal a marked tissue, hormonal, and behavioral rhythm specificity of the two *zeitgebers* and allow a comparison of *zeitgeber* resetting capacity across different biological functions.

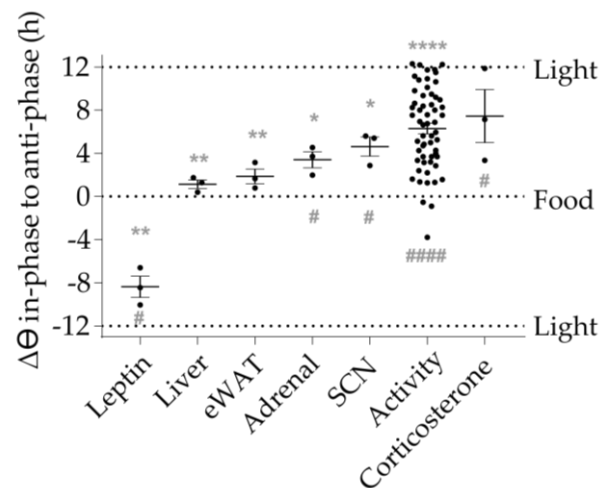


Figure 3-14: Under ZD conditions, diverging *zeitgebers* differentially impact specific circadian rhythms. Phase shifts between in-phase and anti-phase days under ZD conditions for different endocrine, molecular tissue clock, and behavioral rhythms, $n = 3$ for tissues and hormones, $n = 58$ for activity. 0 h = aligned with the feeding:fasting cycle, ± 12 h = aligned with the light:dark cycle, * $p < 0.05$, ** $p < 0.005$, **** $p < 0.0001$ *vs.* light, # $p < 0.05$, #### $p < 0.0001$ *vs.* food, one-sample *t*-tests. Data are shown as means $\pm$ SEMs.

3.2.2 Effects under *iZD* conditions

The ZD data showed a distinct tissue specificity to recurrent *zeitgeber* desynchrony under LD-14/RF-12 conditions that allowed a comparison of *zeitgeber* resetting capacities. In this paradigm, peripheral tissues were most impacted by the *zeitgeber* food while the SCN and the activity was influenced by both *zeitgebers*. To further test the concept of tissue-specific resetting

by differentiating *zeitgeber* conditions, an inverse *zeitgeber* desynchrony experiment with LD-12/RF-14 conditions was conducted.

3.2.2.1 *Locomotor activity shows 24-hour rhythms and metabolic state cycles under iZD conditions*

In the iZD paradigm, under LD-12/RF-14 conditions, 95 % of the mice ($n = 40$) adapted to the 12 h light: 12 h dark cycles. Only two animals (5 %) showed a dominant 28-hour adapted activity period but also revealed additional 24-hour activity rhythms. Three of the forty-two animals showed additional periods between the 24-hour and 28-hour *zeitgeber* rhythms (**Figure 3-15 a, Figure S2**). Mice showed increased activity during anti-phase days compared to in-phase days ($p < 0.0001$ for 24 h and 28 h, two-tailed, paired *t*-tests, 28 h: $7,516 \pm 502$ wheel revolutions (in-phase days) *vs.* $11,918 \pm 745$ wheel revolutions (anti-phase days), 24 h: $6,992 \pm 456$ wheel revolutions (in-phase days) *vs.* $10,225 \pm 627$ wheel revolutions (anti-phase days)) (**Figure 3-15 c, e**). More precisely, the animals were more active in the dark phase on anti-phase days than on in-phase days ($p < 0.0001$, two-tailed, paired *t*-test, 93.6 ± 1.0 % (anti-phase days) *vs.* 83.5 ± 2.6 % (in-phase days)) (**Figure 3-15 b, d**).

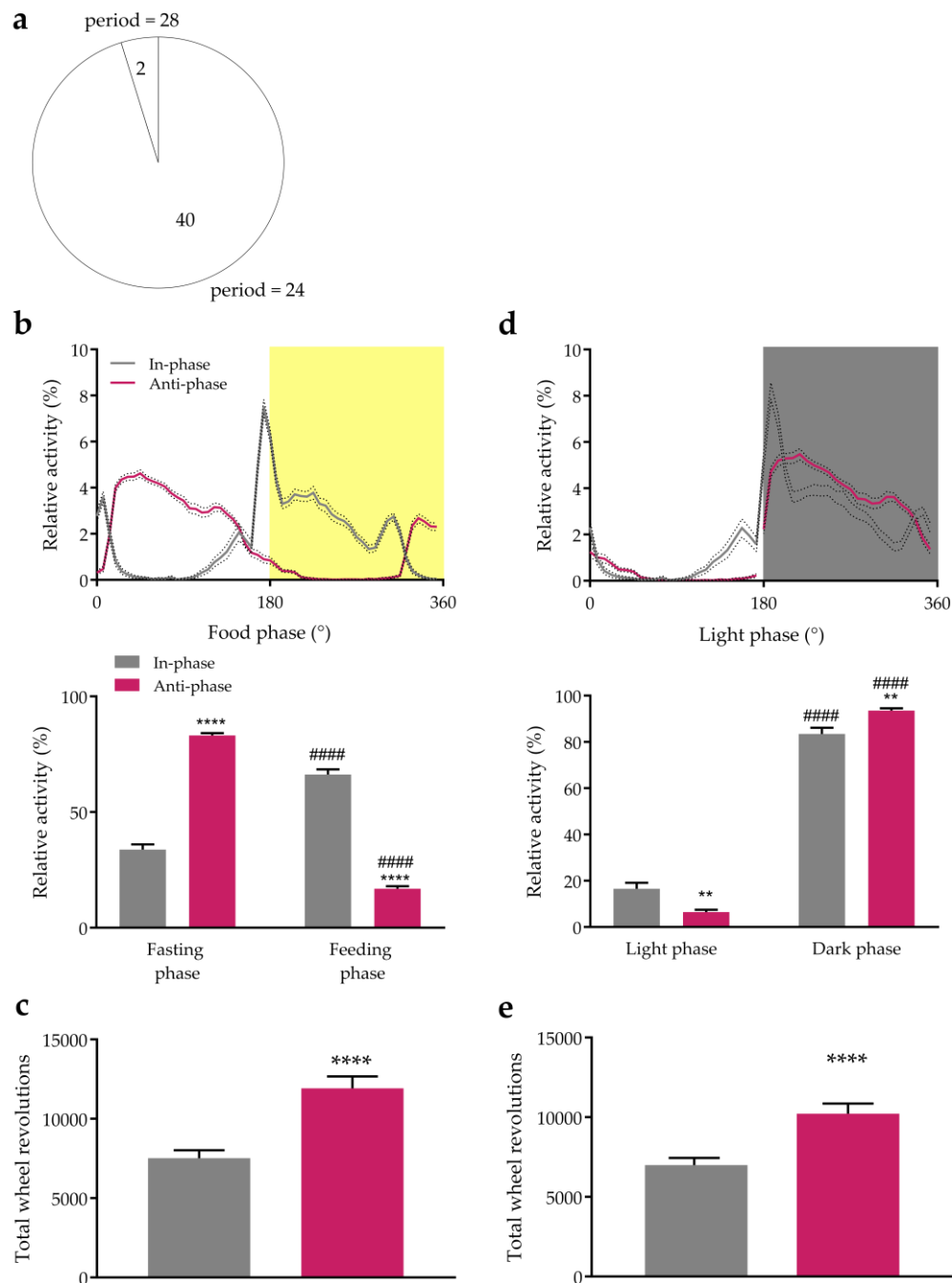


Figure 3-15: Mice were more active on anti-phase days under iZD conditions. a) Fractions of animals that entrained to the 24 h LD cycle (n = 40) or showed a main activity period of 28 h (n = 2) determined by X² periodogram analysis. b) Mean activity profiles on the in-phase (solid grey line) and the anti-phase (solid pink line) days relative to the feeding:fasting cycle (180 ° = “food in”, 360 ° correspond to 28 h). Yellow shading indicates food phase (top). Feeding/fastig phase activity distribution on in-phase and anti-phase conditions (bottom), **** p < 0.0001 between days, #### p < 0.0001 between feeding and fasting phase on the same day, two-way ANOVA. c) Total running-wheel revolutions in 28 h, **** p < 0.0001 two-tailed, paired t-test. d) Mean activity profiles on in-phase (solid grey line) and anti-phase (solid pink line) days relative to the LD cycle (180 ° = “lights off”, 360 ° correspond to 24 h). Grey shading indicates dark phase (top). Light/dark phase activity distributions on in-phase compared to anti-phase days, ** p = 0.0025 between days, #### p < 0.0001 between feeding and fasting phase on the same day,

two-way ANOVA. e) Total running-wheel revolutions in 24 h, **** $p < 0.0001$ two-tailed, paired t -test. Data are shown as means $\pm$ SEMs.

Core body temperature rhythms stayed aligned with the LD cycle, showing low temperature during the light phases and high temperature during the dark phases throughout the iZD cycle (**Figure 3-16 c**). These observations mirrored the telemetrically recorded activity. It further confirmed the observations from running-wheel recordings that mice were more active on anti-phase days (180°) than on in-phase days (45°), in particular during the dark phase (**Figure 3-16 c**).

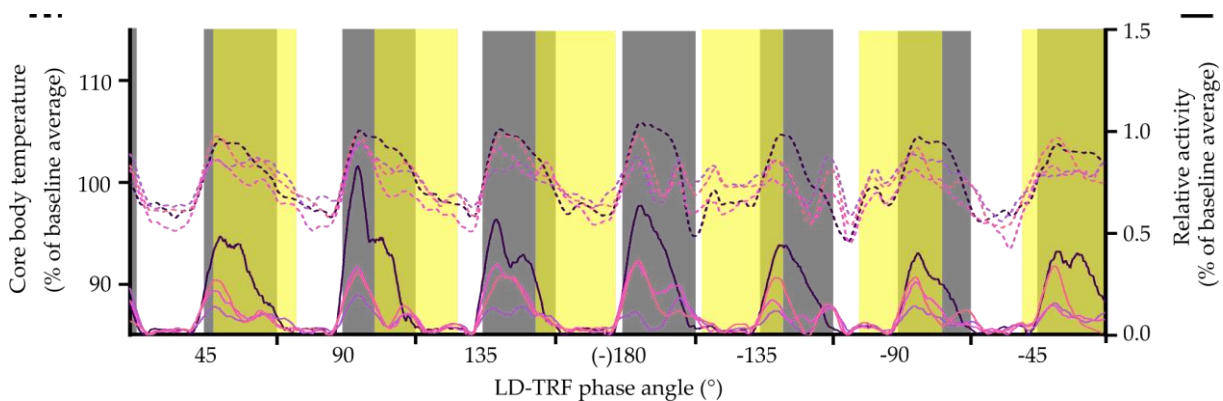


Figure 3-16: Normalized core body temperature under iZD conditions. Core body temperature (dashed lines) and locomotor activity (solid lines) under iZD conditions, $n = 4$. Data of single mice are averaged and smoothed over two experimental cycle. Dark grey and yellow shadings indicate dark phase and food access time.

Comparable to the ZD experiment, food intake and body weight were measured prior to food access time. Similar to the ZD paradigm, food consumption and body weight were cyclic throughout recurrent iZD conditions (**Figure 3-17 a**). In contrast to the ZD experiment, relative food intake and body weight were increased by 28 % and 100 % on anti-phase days compared to in-phase days under iZD conditions (food intake: 18.3 ± 0.6 % (in-phase) *vs.* 23.4 ± 0.5 % (anti-phase), body weight gain: -0.8 ± 0.3 % *vs.* 0.8 ± 0.2 %, both $p < 0.0001$, two-tailed, paired t -tests) (**Figure 3-17 b, c**). This resulted in a significantly higher energy conversion on anti-phase days compared to in-phase days (3.8 ± 0.9 % *vs.* -6.0 ± 1.5 %, $p < 0.0001$, two-tailed, paired t -test) (**Figure 3-17 d**). Thus, mice were in a catabolic state on and around in-phase days and in an anabolic state on and around anti-phase days.

Glucose handling was assessed in the 4th cycle of the iZD paradigm by a GTT. Fasted blood glucose level were elevated 15 min after the glucose bolus and decreased to baseline levels within two hours at, both, in-phase and anti-phase days ($p < 0.0001$, two-way ANOVA). Glucose tolerance was comparable between in-phase and anti-phase days under iZD conditions (0 min: 125 ± 10 mg/dL (in-phase day) *vs.* 119 ± 4 mg/dL (anti-phase day), 15 min: 243 ± 14 mg/dL *vs.* 256 ± 24 mg/dL, 30 min: 217 ± 12 mg/dL *vs.* 262 ± 27 mg/dL, 60 min: 169 ± 7 mg/dL *vs.* 185 ± 15 mg/dL, 90 min: 154 ± 5 mg/dL *vs.* 160 ± 3 mg/dL, 120 min: 140 ± 10 mg/dL *vs.* 130 ± 5 mg/dL). Overall, incremental areas under the curve were

similar between both days ($6,969 \pm 896$ (mg $\times$ min)/dL *vs.* $8,782 \pm 1,722$ (mg $\times$ min)/dL) (**Figure 3-17 e**). Additionally, body composition was analyzed but no differences were detected between the two conditions (fat mass: 22.7 ± 1.2 % (in-phase day) *vs.* 22.5 ± 1.5 % (anti-phase), lean mass: 72.9 ± 1.2 % *vs.* 73.7 ± 0.4 %, and free body fluid: 4.4 ± 0.2 % *vs.* 3.8 ± 0.1 %) (**Figure 3-10**, grey and pink bars). Body composition between ZD and iZD conditions was comparable (**Figure 3-10**).

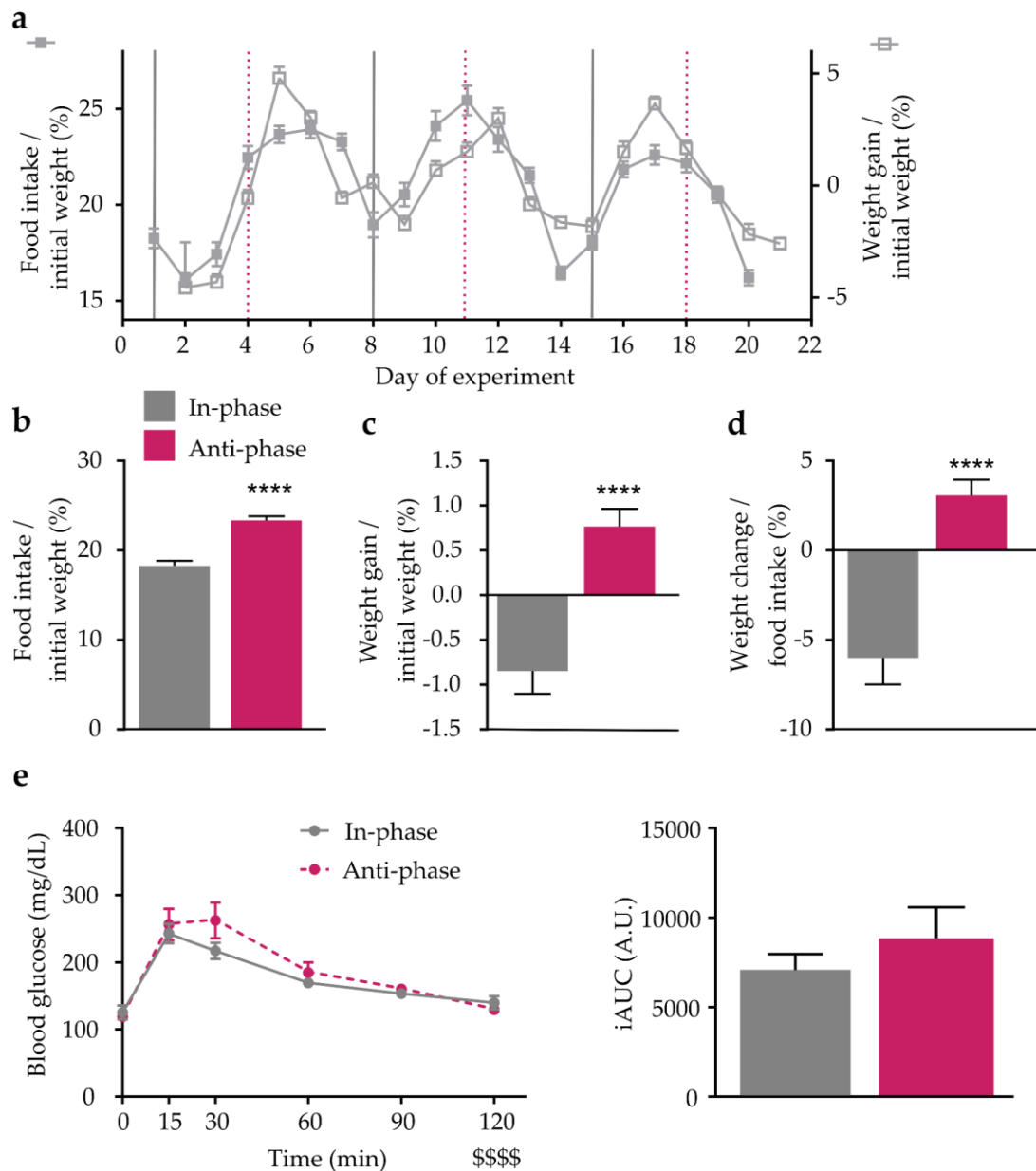


Figure 3-17: Mice show increased body weight, food intake and moderate metabolic impairments on anti-phase days under iZD conditions. a) Normalized food consumption (closed squares, left y-axis) and body weight gain (open squares, right y-axis) throughout 3 cycles of the iZD paradigm. In-phase (solid grey line) and anti-phase (dashed pink line) days are indicated. (b) Normalized food intake, (c) weight change and (d) energy conversion on in-phase (grey) and anti-phase (pink) days ($n = 48$, **** $p < 0.0001$, two-tailed, paired t-tests). e) Glucose tolerance test over time (left) and calculated incremental area under the curve (right) on in-phase ($n = 6$) and anti-phase ($n = 5$) days. Due to technical

problems, one animal in the anti-phase condition had to be excluded. Time effect \$\$\$\$ $p < 0.0001$, two-way ANOVA. Data are shown as means $\pm$ SEMs.

3.2.2.2 Clock gene expression in peripheral tissues is phase-shifted in the iZD paradigm

Under ZD conditions, variations between in gene expression rhythms between different anti-phase days were small. Therefore, only one anti-phase profile was investigated under iZD conditions.

Profiles were obtained at timepoints spanning the feeding:fasting cycle (28 h) which were comparable to those under ZD conditions. Under iZD conditions, feeding:fasting cycles were phase-shifted by 12 h between in-phase and anti-phase days (**Figure 3-18 a**).

On in-phase days, hepatic *Bmal1* gene expression showed a U-shaped expression pattern along the feeding:fasting cycle. *Bmal1* expression peaked around the fasting-feeding transition on anti-phase days (ZT2.3: 2.03 ± 0.17 (in-phase) vs. 0.10 ± 0.01 (anti-phase), ZT9.3: 0.33 ± 0.01 vs. 0.91 ± 0.15 , ZT16.3: 0.47 ± 0.08 vs. 1.07 ± 0.04 , ZT23.3: 1.83 ± 0.13 vs. 0.19 ± 0.06 , ZT2.3: 0.64 ± 0.13 vs. 0.59 ± 0.06). *Bmal1* COGs were at 0.48 h external clock time (in-phase day) and 1.93 h (anti-phase day) (**Figure 3-18 b, e, Table S3**). *Per2* expression patterns were almost anti-phasic to *Bmal1* both at in-phase and anti-phase days (ZT2.3: 0.13 ± 0.18 (in-phase) vs. 1.42 ± 0.19 (anti-phase), ZT9.3: 1.16 ± 0.09 vs. 0.34 ± 0.04 , ZT16.3: 2.62 ± 0.25 vs. 0.42 ± 0.04 , ZT23.3: 0.94 ± 0.22 vs. 0.77 ± 0.02 , ZT2.3: 0.15 ± 0.02 vs. 0.93 ± 0.20 , COGs at 16.02 h external clock time (in-phase day) and 13.11 h (anti-phase day)). *Dbp* expression peaked in the middle of the fasting phase on the in-phase day whereas two peaks, in the beginning of the fasting phase and in the middle of the feeding phase, were observed for the anti-phase condition (ZT2.3: 0.03 ± 0.01 (in-phase) vs. 1.78 ± 0.07 (anti-phase), ZT9.3: 3.81 ± 0.46 vs. 0.13 ± 0.03 , ZT16.3: 0.25 ± 0.002 vs. 0.04 ± 0.01 , ZT23.3: 0.03 ± 0.01 vs. 1.14 ± 0.15 , ZT2.3: 0.88 ± 0.13 vs. 0.36 ± 0.08). *Dbp* COGs were found at 9.50 h external time on the in-phase and at 13.23 h on the anti-phase condition (**Figure 3-18 b, Table S3**). On average, the phase shift across the genes between the in-phase day and the anti-phase day was $10 \pm 25^\circ$ in the liver (**Figure 3-18 e**). Overall the amplitude of clock gene expression was significantly decreased by 54 % on anti-phase days compared to in-phase days ($46.1 \pm 1.5\%$, $p = 0.000813$, one-sample *t*-test against 100 % (in-phase amplitude), data not shown).

In eWAT, *Bmal1* expression was highest in the first and second half of the feeding phase on the anti-phase and the in-phase day, respectively (ZT2.3: 1.66 ± 0.10 (in-phase) vs. 0.13 ± 0.02 (anti-phase), ZT9.3: 0.07 ± 0.02 vs. 0.54 ± 0.10 , ZT16.3: 0.60 ± 0.11 vs. 1.66 ± 0.09 , ZT23.3: 2.15 ± 0.06 vs. 1.10 ± 0.14 , ZT2.3: 0.52 ± 0.08 vs. 0.37 ± 0.07 , COGs were at 0.06 h external clock time (in-phase day) and 5.97 h (anti-phase day)) (**Figure 3-18 c, f**). *Per2* expression profiles were anti-phasic to *Bmal1* expression patterns with high levels at the beginning of the fasting phase on the anti-phase day and at the fasting-feeding transition on the in-phase day (ZT2.3: 0.13 ± 0.02 (in-phase) vs. 2.79 ± 0.33 (anti-phase), ZT9.3: 1.86 ± 0.15 vs. 1.65 ± 0.15 , ZT16.3: 2.27 ± 0.38 vs. 0.69 ± 0.04 , ZT23.3: 0.42 ± 0.04 vs. 0.56 ± 0.08 , ZT2.3: 0.32 ± 0.03 vs. 0.97 ± 0.11). *Per2* COGs were at 13.36 h on the in-phase day and at 15.73 h

on the anti-phase day. *Dbp* expression patterns in eWAT were similar to the ones seen in liver (ZT2.3: 0.15 ± 0.04 (in-phase) vs. 1.95 ± 0.15 (anti-phase), ZT9.3: 3.04 ± 0.16 vs. 0.70 ± 0.24 , ZT16.3: 0.51 ± 0.06 vs. 0.17 ± 0.01 , ZT23.3: 0.07 ± 0.002 vs. 0.63 ± 0.09 , ZT2.3: 1.23 ± 0.26 vs. 0.61 ± 0.06). The COGs were at 9.73 h external clock time on the in-phase day and at 14.49 h on the anti-phase day (**Table S3**). On average, clock gene expression rhythms were phase-shifted between the in-phase day and the anti-phase day by $55 \pm 15^\circ$ (**Figure 3-18 f**) and clock gene expression amplitude was slightly, but not significantly, decreased on the anti-phase day ($79.1 \pm 1.3\%$, data not shown).

Adrenal *Bmal1* expression peaked at the fasting:feeding transition on the anti-phase day and in the second half of the feeding phase on the in-phase day (ZT2.3: 1.32 ± 0.18 (in-phase) vs. 0.23 ± 0.03 (anti-phase), ZT9.3: 0.17 ± 0.09 vs. 1.53 ± 0.13 , ZT16.3: 1.49 ± 0.16 vs. 1.47 ± 0.10 , ZT23.3: 1.84 ± 0.26 vs. 0.19 ± 0.05 , ZT2.3: 0.18 ± 0.04 vs. 0.79 ± 0.08 , COGs were found at 22.59 h external clock time (in-phase day) and 1.29 h (anti-phase day)) (**Figure 3-18 d**). *Per2* expression was highest at the beginning of the fasting phase on the anti-phase day and the beginning of the feeding phase on the in-phase day (ZT2.3: 0.31 ± 0.02 (in-phase) vs. 2.66 ± 0.54 (anti-phase), ZT9.3: 1.43 ± 0.16 vs. 1.72 ± 0.22 , ZT16.3: 2.15 ± 0.11 vs. 0.24 ± 0.02 , ZT23.3: 0.54 ± 0.10 vs. 0.94 ± 0.07 , ZT2.3: 0.57 ± 0.04 vs. 1.36 ± 0.27). *Per2* COGs were at 14.51 h external clock time (in-phase day) and 15.46 h (anti-phase day). *Dbp* expression peaked at ZT9 on the in-phase day (ZT2.3: 0.49 ± 0.06 , ZT9.3: 2.30 ± 0.29 , ZT16.3: 0.11 ± 0.01 , ZT23.3: 0.08 ± 0.01 , ZT2.3: 2.01 ± 0.12). On the anti-phase day, the *Dbp* expression profile was U-shaped along the feeding:fasting cycle (ZT2.3: 1.48 ± 0.12 , ZT10.3: 0.10 ± 0.03 , ZT16.3: 0.21 ± 0.04 , ZT23.3: 1.44 ± 0.20 , ZT2.3: 0.42 ± 0.05) (**Figure 3-18 d**). *Dbp* COGs were at 8.55 h external clock time on the in-phase and at 12.81 h on the anti-phase day (**Table S3**). The mean phase shift over all gene profiles was $34 \pm 12^\circ$ between the in-phase and the anti-phase day in the adrenal gland (**Figure 3-18 g**).

In summary, the liver showed the highest variance in the phase shifts between the genes but the overall smallest phase shift between in-phase and anti-phase conditions under iZD conditions. eWAT and adrenal clock gene expression were both moderately phase-delayed between the in-phase and the anti-phase day.

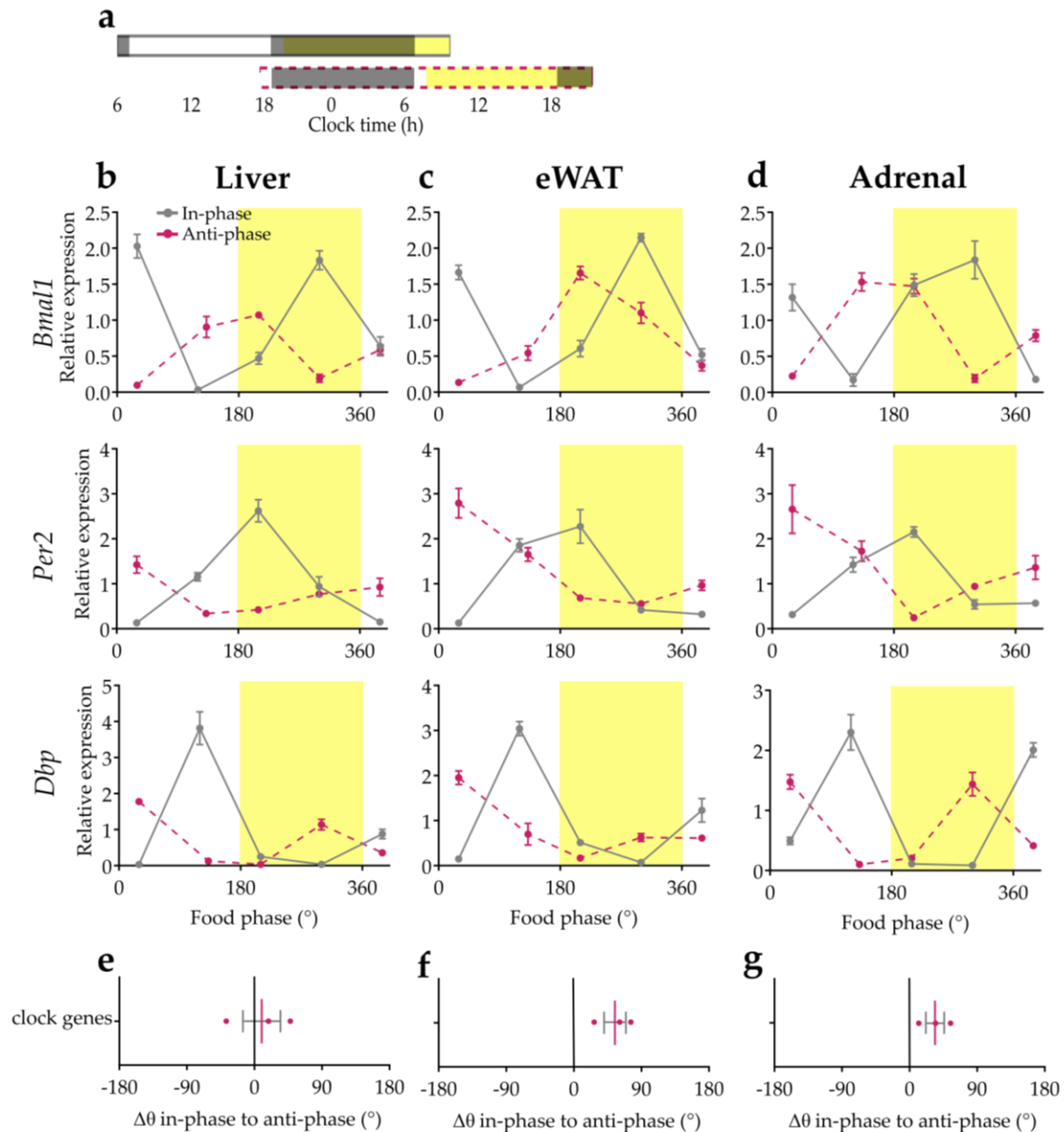


Figure 3-18: Peripheral clock gene expression rhythms show small phase-shifts between in-phase and anti-phase days under iZD conditions. a) Synopsis of in-phase and anti-phase conditions relative to external clock time. mRNA expression profiles for *Bmal1* (top), *Per2* (middle) and *Dbp* (bottom) determined by qPCR in (b) liver, (c) eWAT and (d) adrenal on in-phase (solid grey line) and anti-phase (dashed pink line) days, $n = 3-5$ per timepoint. Yellow shading indicates time of food access ($180^\circ =$ “food in”, 360° corresponds to 28 h). Phase shifts of individual tissue clocks are shown for (e) liver, (f) eWAT, and (g) adrenal between the in-phase and the anti-phase day. Data are shown as means $\pm$ SEMs.

3.2.2.3 Peripheral tissue clocks stay aligned to the LD cycle under iZD conditions

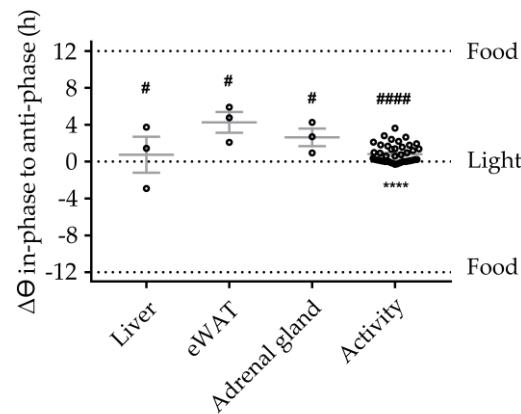


Figure 3-19: Peripheral tissue clocks align to the LD cycle under iZD conditions. Phase shifts between in-phase and anti-phase days under iZD conditions for different tissue clocks and behavioral rhythms, $n = 3$ for tissues, $n = 42$ for activity. $0 \text{ h} = \text{light}$, $\pm 12 \text{ h} = \text{feeding}$, # $p < 0.05$, #### $p < 0.0001$ *vs.* food, **** $p < 0.0001$ *vs.* light, one-sample t -tests. Data are shown as means $\pm$ SEMs.

Under inversed *zeitgeber* desynchrony conditions (iZD, LD-12/RF-14) liver and adrenal tissue clocks showed smaller phase shifts compared to ZD conditions – but higher within-tissue variance between the genes investigated – between the in-phase day and the anti-phase day. All peripheral tissue clocks stayed aligned to the LD cycle with a mean COG phase shift over all genes of $0.76 \pm 1.95 \text{ h}$ (liver), $4.26 \pm 1.13 \text{ h}$ (eWAT), and $2.64 \pm 0.96 \text{ h}$ (adrenal). These phase shifts were significantly different from the food phase with $p = 0.0287$, $p = 0.0206$, and $p = 0.0103$ for liver, eWAT, and adrenal, respectively (one sample t -tests). Activity onset phase shifts were significantly different from, both, light and food rhythms ($0.81 \pm 0.15 \text{ h}$, $p < 0.0001$ *vs.* food and light rhythms, one-sample t -tests) (**Figure 3-19**). However, those phase shifts were much smaller under iZD compared to ZD conditions (0.81 h (iZD) *vs.* 6.29 h (ZD), see **Figure 3-14**) and mice showed high amplitude 24 h locomotor activity period by X^2 periodogram analysis.

Thus, normal light:dark cycles are more potent to entrain behavioral rhythms and the entrained SCN may stabilize peripheral clocks at a 24-hour period when feeding:fasting cycles are out of the range of entrainment. Therefore, the iZD paradigm does not allow the investigation of differential *zeitgeber* impact on tissue clock resetting.

4 Discussion

The circadian clock system evolved to anticipate recurrent changes in environmental conditions and, thus, increases the organism's chance of survival. It is hierarchically organized with a master pacemaker residing in the SCN (Ralph et al., 1990). Recent studies, however, provide evidence that the circadian clock system has a more federated organization of semi-dependent tissue clocks, in which the SCN clock becomes essential in orchestrating subordinate clocks under noisy *zeitgeber* or *zeitgeber*-free conditions (Heyde and Oster, 2020; Husse et al., 2014; Kolbe et al., 2016, 2019). Together, local tissue clocks form an interconnected network coordinating ~24-hour rhythms in behavior and physiology. At the same time, the circadian clock provides temporal compartmentalization of opposing physiological processes and, thereby, balances metabolic homeostasis. Circadian clocks must be entrained to external time in order to provide an adaptive function. To achieve this, the SCN receives light input *via* the RHT which resets the SCN clock to align with the LD cycle (Albrecht et al., 1997; Freedman et al., 1999; Hattar et al., 2002). Temporal information is processed and transmitted to subordinate tissue clocks *via* neurotransmitter release, humoral output, the ANS, and regulation of behavioral rhythms (Astiz et al., 2019; Cailotto et al., 2009; Dibner et al., 2010; Yoo et al., 2004). Besides this SCN-mediated photic entrainment, peripheral tissue clocks can be uncoupled from the master pacemaker by rest phase-restricted feeding (Damiola et al., 2000; Le Minh et al., 2001; Stokkan et al., 2001). Thus, food intake timing is a potent non-photoc *zeitgeber* for peripheral tissues while the SCN is primarily entrained by the LD cycle.

In industrialized countries, the population is exposed to unnatural environmental conditions including irregular LD cycles, nocturnal light pollution, and the continuous availability of food products. Furthermore, the number of people working outside the 9-to-5-time window is increasing (Sixth European working conditions survey, 2015). Altogether, this induces chronodisruption, *i.e.* a misalignment of external and internal time. Substantial evidence have demonstrated that chronic circadian misalignment increases the risk for developing various diseases, *e.g.* obesity, type 2 diabetes, metabolic syndrome, cardiovascular diseases, and various types of cancer (Bacquer et al., 2009; Di Lorenzo et al., 2003; Karlsson et al., 2003; Kawachi et al., 1995; Mokdad et al., 2003). Furthermore, sleep disturbances and curtailment likely contribute to those diseases (Buxton et al., 2012; Reiter et al., 2012).

In laboratory studies, forced desynchrony protocols are used to investigate the cause and consequence of chronodisruption on physiological processes (Morris et al., 2016; Scheer et al., 2009). However, in those studies the *zeitgebers* light, food, and behavioral rhythms are changed with the same kinetics. In these paradigms it is impossible to unravel the individual contribution of the main *zeitgebers*, light and food, on the circadian clock system. Therefore, I established *zeitgeber* desynchrony protocols in which LD cycles and feeding:fasting cycles were running with different periods of 24 h or 28 h. This allowed the quantification of differential contributions of the two *zeitgebers* to the resetting of distinct tissue clocks. It also allowed investigation of the effects of *zeitgeber* uncoupling on the metabolic state. I found that diverging

zeitgeber input, under LD-14/RF-12 conditions, induced internal desynchronization in a short-term and long-term experiment. Furthermore, conflicting *zeitgeber* input phase-shifted circadian oscillations in a tissue- and rhythm-specific manner in a long-term experiment. The data suggests that distinct tissue susceptibility to light- and food-related signals might be responsible for differences in clock resetting under diverging *zeitgeber* input. However, under LD-12/RF-14 conditions, an entrained SCN stabilized peripheral tissue clocks when the LD cycle was within the entrainment range. Metabolic state cycled throughout both *zeitgeber* desynchrony protocols and was dependent on the *zeitgeber* phase angle.

4.1 Locomotor activity period is lengthened under acute ZI conditions independent of time of restricted feeding

To investigate acute effects of *zeitgeber* intervention on the circadian system, mice were put under LD-14/Dark-RF or LD-14/RF-12 conditions. At the beginning of the experiment, light intensity was reduced to 3 lux. This light intensity is able to entrain the circadian system in mice (Ebihara and Tsuji, 1980). In human studies, low light intensity has been used during the experimental light phases to reduce light effects on the circadian system to explore effects of (behavioral) forced desynchrony on endogenous circadian parameters (Carskadon et al., 1999; Hilton et al., 2000; Morris et al., 2015; Scheer et al., 2009). Human forced desynchrony protocols comprise enforced sleep:wake cycles in 1:2 ratio with 20-hour or 28-hour experimental days and corresponding adjustment of mealtimes. Over the course of the experiment, thus, participants are forced to sleep and eat at different phases of the circadian cycle. This allows the investigation of effects of aligned and misaligned conditions between externally controlled behavioral and free-running endogenous circadian cycles (Morris et al., 2015; Scheer et al., 2009, 2010). Nonetheless, a residual influence of light cannot be excluded and it is a limitation of these protocols. In contrast, my project focused on the effect of *zeitgeber* intervention on the murine circadian clock system and mice were not forced to follow set sleep:wake cycles during the experiment. In such a way, impact of the *zeitgebers* light and food could be investigated.

On the first day under extended light:dark conditions, mice started their activity phase 2 h before lights turned off at about the same time as under LD-12 conditions with *ad libitum* food access (Figure 3-1). From day 2 to day 4, locomotor activity onsets phase-delayed by ~1.8 h per day. This observation was independent of the restricted feeding schedule. Thus, the fasting period of 12 h (LD-14/RF-12) or 14 h (LD-14/Dark-RF) itself did not impact locomotor activity. This is in line with other studies that show that only fasting periods above 18 h increase activity likely indicating food seeking (Cao et al., 2009; Gelegen et al., 2006; Williams et al., 2002). In consequence, it is reasonable that the locomotor activity period observed in the acute ZI paradigms represents the period of the SCN (Houben et al., 2009; Ralph et al., 1990). The deviation of the ZI locomotor activity period with 25.8 h from standard DD free-running periods (23.8 h, (Schwartz and Zimmerman, 1990)) suggests that the SCN was impacted by the *zeitgeber* light but could not entrain to LD-14 cycles (Figure 3-1). A lengthening of the activity period was expected since light is the most potent *zeitgeber* for the SCN which controls

behavioral rhythms (Daan and Pittendrigh, 1976; Damiola et al., 2000; Hara et al., 2001; Husse et al., 2014; Yamaguchi et al., 2013) and increasing light intensities have been shown to lengthen locomotor activity period in constant light (LL) conditions (Aschoff's rule, (Aschoff, 1979). Furthermore, it was expected that mice would not entrain to LD-14 since previous studies showed that the LD entrainment range is limited to T-cycles between 21 h and 27 h (Erzberger et al., 2013; Stephan, 1983; West et al., 2017). While the entrainment range for LD was addressed in several studies, the range of entrainment to feeding:fasting cycles was only recently investigated. Hamaguchi *et al.* showed that clock gene expression in peripheral tissues such as kidney, liver and submandibular gland entrains to feeding T-cycles of 22 – 24 h under LD-12 conditions (Hamaguchi et al., 2015). The range of entrainment for a specific *zeitgeber* can be calculated mathematically using the Poincaré model in which the entrainment range is defined by the relationship between *zeitgeber* strength and oscillator amplitude for a given oscillator period and its relaxation rate. Thus, the entrainment range is large for a strong *zeitgeber* and a weak oscillator and it is narrow for a weak *zeitgeber* and a strong oscillator. In an *ex vivo* study, this relationship was tested, and it was shown that the lung has a larger entrainment range to temperature cycles than the SCN. Thus, in accordance with previous publications temperature cycles are a strong *zeitgeber* for peripheral tissues while the SCN is not susceptible to temperature changes (Buhr et al., 2010). It can also be speculated, based on the mathematical model that the lung is a weak oscillator while the SCN is a strong oscillator. It was proven that intra-tissue coupling plays an important role in determining oscillator amplitude and strong coupling was shown for the SCN (Abraham et al., 2010; Liu et al., 2007; Yamazaki et al., 2000; Yoo et al., 2004). A limitation of the mathematical model, suggested by Abraham *et al.*, is that only one *zeitgeber* and one oscillator can be addressed. Thus, the mathematical model is suitable for precisely controlled *ex vivo* experiments, but it cannot predict the *in vivo* situation. On the other hand, *in vivo* studies assessing the entrainment range of LD or feeding T-cycles lack the discussion that food and light will impact the organism's behavior and tissue clock resetting. The acute ZI paradigms combined extended LD cycles and TRF. It might be possible that TRF – especially RF-12, which is in the range of entrainment – could influence the SCN clock and, thus, behavioral output under non-entrainable LD T-cycles in a long-term experiment. While under standard (LD) TRF conditions the SCN clock is not susceptible to the *zeitgeber* food, it has been shown that long-term TRF under constant light conditions or hypocaloric feeding can entrain the SCN clock (Damiola et al., 2000; Hara et al., 2001; Inouye, 1982; Lamont et al., 2005; Mendoza et al., 2005; Nováková et al., 2011). A long-term experiment investigating the effects of RF-12 under LD-14 cycles was performed (see 3.2.1) and will be discussed in chapter 4.3.

4.2 *Internal desynchronization is induced by acute LD-14/RF-12 ZI conditions*

The SCN synchronizes peripheral tissue clocks to external LD cycles by regulating behavior, e.g. food intake and activity. Peripheral clocks are strongly impacted by the *zeitgeber* food (Damiola et al., 2000; Hara et al., 2001; Stokkan et al., 2001). To investigate the acute effects of

diverging *zeitgeber* input, *i.e.* LD-14/Dark-RF or LD-14/RF-12, on molecular clock resetting, clock gene expression of peripheral tissues was measured under baseline (1st day of the experiment) and misaligned (4th day of the experiment) conditions. Consequently, the LD cycle was phase-shifted by 12 h between baseline and misaligned days. Tissue samples were collected spanning the 28-hour LD cycle. Under LD-14/RF-12 conditions, periods of liver, adrenal, and eWAT clock gene expression were shorter, *i.e.* showed smaller phase shifts between baseline and misaligned days, compared to periods determined for LD-14/Dark-RF conditions (on average: 24.9 h *vs.* 25.9 h) (Figure 3-1 - 3-5). Pancreas clock gene rhythms were not differentially impacted by the feeding schedule (RF-12 and Dark-RF) under LD-14 conditions which might be in part caused by high variations in qPCR results for different genes. In fact, qPCR results of pancreas RNA yielded unusually high CT values, probably due to increased RNA degradation during RNA isolation. Pancreas is known as an RNase-rich tissue which leads to fast degradation of RNAs (Azevedo-Pouly et al., 2014; Griffin et al., 2012; Li et al., 2009). On average, tissue clock gene expression periods were 25.9 h under LD-14/Dark-RF conditions and 24.9 h under LD-14/RF-12 conditions. Together, small period differences between peripheral clock gene rhythms and activity onsets (25.8 h) indicated a high level of internal synchrony under LD-14/Dark-RF conditions. In contrast, peripheral tissue clocks and activity onsets exhibited relatively large period differences under LD-14/RF-12 conditions and, thus, suggested a high level of internal desynchrony which could result in metabolic impairments. This was addressed in a long-term experiment (ZD (LD-14/RF-12), discussed in 4.4).

In the acute ZI paradigm, it is very likely that differences in peripheral clock rhythms were predominantly caused by food-mediated signals. Food is the main *zeitgeber* for peripheral tissue clocks under standard LD-12 or constant conditions. However, it is not yet investigated whether peripheral tissue clocks entrain to TRF under unnatural LD T-cycles combined with feeding:fasting cycles within or outside the range of entrainment. Additionally, the underlying mechanisms of how peripheral tissue clocks entrain to feeding:fasting cycles are yet not well understood but it is plausible that various food-related signals are integrated to generate and maintain coherent circadian rhythms. Leptin and ghrelin are involved in signaling the organism's metabolic state to the brain to inhibit and promote food intake, respectively (Shintani et al., 2001). There is evidence that both hormones affect the circadian clock. Receptors for both hormones are expressed in the SCN and their stimulation can induce phase shifts in clock gene expression *in vitro* (Prosser and Bergeron, 2003; Yannielli et al., 2007). Leptin shows circadian oscillations and is released after food ingestion (Martinez-Merlos et al., 2004; Prosser and Bergeron, 2003; Schoeller et al., 1997). Leptin-deficient (*ob/ob*) mice and leptin receptor-deficient *Zucker* diabetic fatty rats display dampened amplitudes in clock gene expression in various peripheral tissues including liver and WAT. Leptin treatment can restore peripheral clock gene rhythms in *ob/ob* mice (Ando et al., 2011; Motosugi et al., 2011). Although the underlying mechanism is not fully investigated, leptin seems to play an important role in modulating clock gene expression in the periphery. It has been shown that leptin activates signal transducer and activator of transcription (STAT3/5) but also signals *via* the

phosphoinositide 3-kinase (PI3K)/mechanistic target of rapamycin kinase complex 1 (mTORC1)/ribosomal protein S6 kinases (S6Ks) pathway. mTORC1 is an activator of 4E-binding proteins (4E-BPs) and S6K1/2. In consequence, 4E-BP1-mediated repression of eukaryotic translation initiation factor 4 (eIF4E) is inhibited and S6K is activated. Activation of S6Ks and eIF4E results in upregulation of protein synthesis (Ramanathan et al., 2018). In fibroblasts, constitutive activation of mTOR elevates levels of core clock proteins including CRY1, BMAL1 and CLOCK (Ramanathan et al., 2018). This pathway was also shown to be involved in photic resetting of the SCN (Cao, 2018; Cao and Obrietan, 2010; Cao et al., 2008). Thus, although not yet directly shown, the leptin-mediated activation of mTOR/S6K could upregulate translation of clock gene mRNAs. Besides its effects on core clock genes, mTOR is also involved in the regulation of food intake by promoting hypothalamic leptin response *via* S6K (Cota et al., 2006). Together, leptin is a promising target mediating food-related changes on central and peripheral clocks. Interestingly, in a human study investigating the effect of circadian misalignment (behavioral cycle is 12-hour out-of-phase), overall leptin levels were decreased by 17 % (Scheer et al., 2009). Ghrelin, the counteracting regulator of leptin in the brain, displays a circadian variation with higher levels in the biological evening and lower levels in the morning. Additionally, ghrelin levels decrease postprandially. In humans, circadian misalignment dampens circadian variation and alters postprandial responses in ghrelin levels between measures in biological morning and evening (Qian et al., 2019). This suggests that ghrelin's feedback to the brain is impaired which might contribute to metabolic imbalance seen in shift workers. Interestingly, ghrelin levels are blunted in mice fed a HFD and ghrelin treatment can restore clock gene expression and amplitudes in the liver *in vitro* and *in vivo*. Additionally, ghrelin induces phase shifts in some clock genes *via* the mTOR/S6Ks pathway (Wang et al., 2018). Other food-related signals mediate their effects predominantly on peripheral tissues. Insulin is released postprandially by pancreatic β -cells and binds to its receptor, expressed in various tissues, promoting glucose uptake. Beside that main function, various publications describe an insulin-mediated effect on the circadian clock. In liver, insulin activates the expression of *Per2* while it inhibits *Rev-Erba* within two hours after food intake (Tahara et al., 2011). Furthermore, insulin indirectly regulates *Clock* expression and represses *Bmal1* expression (Chaves et al., 2014; Sun et al., 2015). *Clock* expression is induced by forkhead box class O3 (FOXO3), a protein which is inactivated by insulin-mediated PI3K signaling (Chaves et al., 2014). Yamajuku *et al.* show that insulin mediates clock resetting in a circadian phase-dependent manner (Yamajuku et al., 2012). Besides this indirect regulation, insulin affects the circadian clocks by further pathways. One study shows that insulin rather increases translation of PER than affecting its *de novo* transcription (Crosby et al., 2019). This is consistent with the circadian abundance of *Per* mRNA and the phase-dependency of the insulin-mediated effects seen in the study of Yamajuku *et al.*. Moreover, the study of Crosby *et al.* shows that elevated translation is mediated by activation of PI3K/mTORC1 pathway (Crosby et al., 2019). Interestingly, Dang *et al.* found that insulin promotes BMAL1 dissociation from the DNA and subsequent enhanced nuclear export (Dang et al., 2016). However, studies in insulin-resistant mice show that insulin is not essential for phase shifting the liver clock and,

thus, proves that other food-related signals must exist to mediate clock resetting (Oishi et al., 2004). Secretion of the gut-derived hormone oxyntomodulin is stimulated by food intake. Oxyntomodulin activates glucagon-like peptide-1 receptor (GLP1R) and glucagon receptor (GCGR) and can reset the liver clock by induction of *Per1* and *Per2* expression (Jorgensen et al., 2007; Landgraf et al., 2015; Pocai, 2013). Oxyntomodulin-mediated phase-resetting is dependent on GCGR but not on GLP1R. Apart from the described effectors, there are many more food-related signals and metabolites which may affect tissue clocks (Reinke and Asher, 2019). Thus, it is difficult to unravel their differential contribution to clock resetting in *in vivo* experiments like the conducted experiments in this PhD project.

It is very likely that food-independent signaling is involved in clock resetting observed in the acute ZI paradigms. GCs have been shown to induce expression of *Per1/2*, *E4bp4* and *Npas2* while inhibiting transcription of *Rev-Erb β* and *Dec2*. These effects are mediated by GR binding to GRE motifs in the respective promoters (So et al., 2009). Furthermore, corticosterone and insulin rhythms are phase shifted to each other with corticosterone peaking some hours before insulin under LD-12 cycles and *ad libitum* food access. It is postulated that this temporal relation is disrupted under shift work conditions. This is based on the assumption that insulin rhythms, which are dependent on food intake, will be phase-shifted together with activity. Corticosterone rhythms are often unchanged in shift workers. Thus, insulin would peak some hours before corticosterone. In consequence, such conflicting temporal relation between insulin and corticosterone might cause dampening of clock gene expression amplitudes. This idea was confirmed in *in vitro* studies (Crosby et al., 2019). Given that corticosterone rhythms would be unchanged under extended light:dark cycles and insulin rhythms would be mainly driven by food intake, I would expect that the temporal relation of corticosterone and insulin rhythms would be disrupted in under LD-14/Dark-RF conditions. As a result, I should observe dampening in clock gene expression rhythms in this paradigm. However, I only observe dampening in liver clock gene expression rhythms under LD-14/RF-12 conditions. In part, this might be due to the low time resolution of tissue sampling in this paradigm (7 h between sampling time points). It could also be speculated that, corticosterone rhythms phase-shifted like the SCN leading to a temporal misalignment especially under LD-14/RF-12 conditions. Hence, under LD-14/Dark-RF corticosterone and insulin rhythms may have phase-shifted with the same kinetics preventing conflicting temporal relation and, thus, preventing dampening in clock gene expression amplitude.

I did not investigate food-related signals, metabolites or corticosterone levels and, thus, can only speculate that these signals are integrated and responsible for differential phase shifts seen under LD-14/Dark-RF and LD-14/RF-12 conditions. It is very challenging to disentangle and rate the importance of single signals based on the described publications. Further experiments using transgenic mouse strains (e.g. *ob/ob* mice), mouse models of type 1 diabetes or adrenalectomized mice, could provide more insight into specific contributions of individual signals on clock resetting under diverging *zeitgeber* input.

Very few studies investigate the effect of jet lag combined with restricted feeding. Re-entrainment of peripheral tissue clocks to a reversed feeding schedule is accelerated if the LD cycle is also reversed (Wu et al., 2008). Thus, it cannot be excluded that the LD-14 cycle promotes food-driven phase shifts in peripheral tissue clocks under LD-14/Dark-RF conditions. It is important to mention that the study of Wu *et al.* was conducted under 200 lux. In contrast, both acute ZI paradigms were performed under 3 lux light intensity which was shown to entrain mice under LD-12 cycles. However, it was also shown that using such low light intensity weakens the *zeitgeber* strength (Ebihara and Tsuji, 1980). In consequence, I would only expect minor effects of the LD cycle on food-driven changes under LD-14/Dark-RF conditions.

In summary, LD-14/Dark-RF led to comparable phase shifts in locomotor activity – thus, presumably, the SCN clock – and peripheral tissue clocks. This indicates a high level of internal synchrony. In contrast, under LD-14/RF-12 conditions, peripheral tissue clock rhythms and behavioral rhythms showed large phase differences when compared to each other which suggests a high degree of internal desynchrony. The underlying mechanisms responsible for these effects require further investigation, but it is very likely that food-related signals drive peripheral resetting in the acute ZI paradigms.

4.3 *ZD conditions impact behavioral rhythms and metabolic state to a larger extent than iZD conditions*

In the long-term *zeitgeber* desynchrony paradigms, the impact of diverging *zeitgebers* on behavioral rhythms, metabolic state, tissue clock resetting, and humoral output was investigated. To avoid entrainment to the LD cycle, a long T-cycle was applied. A 28-hour T-cycle was chosen speculating that this allows the quantification of *zeitgeber* impact on individual tissues. Under ZD conditions (LD-14/RF-12), most mice displayed activity patterns which were in-between the two *zeitgeber* periods (Figure 3-6 a, b). This is in line with previous studies defining the range of entrainment to LD T-cycles as 21 - 27 h in nocturnal rodents (Stephan, 1983; West et al., 2017). Especially in the upper limit mice show behavioral entrainment but shift their rest:activity rhythms relative to the LD cycle. In a 27-hour T-cycle (LD-13.5), for example, mice are active in the second half of the light phase and first half of the dark phase (West et al., 2017). In other studies, behaviorally entrainment to LD-14 was successful in rats and mice when LD period lengthening occurred gradually (Aton et al., 2004; Usui et al., 2000). Surprisingly, in my experiments, although the LD-12 cycle was abruptly lengthened to LD-14, 13 % of mice (8 of 61) showed very strong adaption to LD-14 under ZD conditions and did not show a second activity period peak in the X^2 periodogram analysis (Figure 3-6 a, Figure S1). There is only one study where behavioral entrainment to abruptly lengthened LD-14 cycles is observed in about 50 % of WT mice (Erzberger et al., 2013). Unfortunately, the period strength or occurrence of further periods was not reported which makes it difficult to evaluate and compare results of Erzberger *et al.* to results obtained in the ZD paradigm. However, behavioral entrainment to LD-14 contrasted earlier findings on the

entrainment range and might be a rare phenomenon as most publications determine a limit of the entrainment range to LD T-cycles at below 28 h. A mathematical model, using the Poincaré model, suggests that the range of entrainment is determined by the relationship of *zeitgeber* strength to oscillator amplitude for a given oscillator period and its relaxation rate (Abraham et al., 2010). Thus, it is possible that the light intensity of 250-300 lux increased the *zeitgeber* strength to an extent that mice with a relatively weak SCN oscillator – *i.e.* weaker coupling – could adapt to such a long T-cycle. The coupling strength and dispersion of intrinsic periods among SCN neurons influence the range of entrainment and might be slightly different between individual mice (Gu et al., 2009, 2013). Adaption to LD-14 could also suggest that those mice showed stronger light masking, *i.e.* acutely suppression of activity caused by light. This phenomenon is observed for, *e.g.*, melanopsin-deficient mice and mice with a genetically ablated SCN clock (Husse et al., 2011; Izumo et al., 2014; Mrosovsky and Hattar, 2003). However, the underlying mechanisms of light masking in general and light masking under extended light:dark cycles have not been investigated yet. My data showed that most mice (87 %) did not entrain to LD-14 and showed various periods over the course of the ZD experiment (Figure 3-6 b). Distinct locomotor activity periods are observed in studies using short T-cycles (Ben-Hamo et al., 2017; de la Iglesia et al., 2004; Schwartz et al., 2009; Wotus et al., 2013). In these studies, animals show an activity period phase-locked to LD-11 cycles, *i.e.* 22 h, and an activity period of 25 h (LD-dissociated). The authors suggest that the LD-dependent and LD-dissociated activity rhythms are driven by the SCN core and shell, respectively. Laasko *et al.* observe two distinct activity periods under LD-14 conditions. X² periodogram analysis reveal that one activity period is phase-locked to the LD cycle (*i.e.* 28 h) and the other one is at 26 h (Laakso et al., 1995). Thus, intra-SCN alignment and misalignment could contribute to the various periods of activity seen under ZD conditions. In all mentioned studies investigating the range of entrainment animals had *ad libitum* access to food. In contrast, in my ZD experiment food access time was restricted. Although TRF is not able to entrain locomotor activity periods under constant conditions, it can induce FAA and, thus, influence activity rhythms to a certain extent (Abe et al., 1989; Honma et al., 1983). Under constant DD or LL conditions, TRF is able to entrain the SCN and behavioral rhythms (Castillo et al., 2004; Lamont et al., 2005). However, TRF was restricted to 3-6 h which probably increases *zeitgeber* strength due to the narrow time window. Thus, this might increase the organism's anticipation. Whether RF-12 could entrain the SCN and behavioral rhythms under constant conditions is questionable since the SCN has a strong intrinsic period regulating behavioral output. On the other hand, range of entrainment to LD T-cycles was shown to be limited to 27 h, although Erzberger *et al.* reported behavioral entrainment in 50 % of WT mice under *ad libitum* food access (Erzberger et al., 2013). Thus, I would expect that light and food would impact behavioral rhythms under ZD conditions. Indeed, most mice displayed periods of activity dependent on the phase angle of the two *zeitgebers* (Figure 3-6 c-f). The ZD data suggest that both *zeitgebers*, light and food, impact behavioral output and that diverging *zeitgeber* alignment may compress and de-compress behavioral adaption as seen for hormonal output under short LD T-cycles (Schwartz et al., 2009; Wotus et al., 2013). Thus, conflicting

zeitgeber input prevented the formation of stable activity periods and, thus, entrainment to one *zeitgeber* or expression of free-running activity periods in most mice. In the iZD paradigm (LD-12/RF-14) only 5 % of mice (2 of 42) responded to extended feeding:fasting cycles by behavioral entrainment to 28 h. Nonetheless, those animals showed an additional significant activity period of 24 h. In consequence, it appears that LD-12 entrained the SCN pacemaker in all animals. Interestingly, over 50 % of all mice displayed a second activity period of 28 h (or slightly shorter) under iZD conditions. This is very likely caused by extended fasting periods which led to increased hunger and enhanced search for food (Williams et al., 2002).

Locomotor activity was compared in more detail on days with in-phase (*i.e.* dark phase equals feeding phase) and anti-phase (*i.e.* light phase equals feeding phase) *zeitgeber* input to quantify the pull of each *zeitgeber* on behavioral rhythms. Under ZD conditions, analysis of the RF-12 timeframe revealed a reduction of total activity on anti-phase days due to reduced running-wheel activity especially during the feeding (light) phase (Figure 3-6 g-i, Figure 3-7 a, b). Analysis of the 28-hour timeframe (LD-14) revealed comparable total running-wheel activity (Figure 3-7 c). Furthermore, mice displayed a small activity peak at the end of the fasting period on anti-phase days (Figure 3-6 g). That could be interpreted as FAA. However, this activity peak does not occur at the same time prior to the feeding interval throughout the experiment as it would be expected for FAA. Additionally, mice had access to food for 12 h per 24 h and FAA is usually only observed under restricted feeding with 2 - 8 h food access per day (Stephan and Becker, 1989; Wakamatsu et al., 2001). Thus, increased activity prior to feeding phases might reflect increased hunger under ZD conditions. However, it must be mentioned that FAA is usually investigated under LD-12 or constant conditions. Thus, it is possible that mice develop some sort of FAA under ZD conditions due to entrainable feeding:fasting cycles and non-entrainable LD cycles. Under iZD conditions, analysis of the LD-12 time frame yielded increased total activity on anti-phase days which is due to elevated running-wheel usage in the dark (fasting) phase (Figure 3-15 e). The difference in total activity persisted over the 28-hour timeframe (RF-14) (Figure 3-15 c). This could reflect increased search for food and elevated arousal driven by the entrained SCN. On in-phase days, mice exhibited increased activity in the late light phase (Figure 3-15 b, d). Potentially, mice were seeking for food and were generally more aroused as their activity phase was about to start. As expected, FAA-like activity did not occur under LD-12 cycles and food availability for 14 h. Fasting intervals above 16 h seem to be important for the development of FAA (Stephan and Becker, 1989).

Shift work and jetlag are associated with adverse health outcomes including obesity and type 2 diabetes, in part, presumably, by changing food intake timing and, thus, contributing to internal desynchrony between different tissue clocks (Espitia-Bautista et al., 2017; Karlsson et al., 2003; Koopman et al., 2017; Salgado-Delgado et al., 2008; Schilperoort et al., 2019; Szosland, 2010). Metabolic state was investigated in the ZD and iZD paradigms by analyzing daily changes in food intake and bodyweight. Furthermore, glucose tolerance tests were conducted at the end of the fasting phase on in-phase and anti-phase days. Mice showed cycles

in metabolic states under ZD and iZD conditions. Under ZD conditions, mice gained weight on in-phase days and lost weight on anti-phase days (Figure 3-9 a-e). In contrast, mice showed increased body weight on anti-phase days compared to in-phase days under iZD conditions (Figure 3-17 a-d). Body weight reduction or increase was transient as mice consumed less or more food when getting closer to the anti-phase days in the ZD or iZD paradigm, respectively. Daytime food intake is unusual for nocturnal animals and might have caused a reduction of food intake and, consequently, weight under ZD conditions on anti-phase days. Under iZD conditions, longer fasting periods might have induced overeating on anti-phase days resulting in a transient body weight gain. There was no net body weight gain throughout the experiment neither under ZD nor under iZD conditions. Furthermore, body composition was similar between the paradigms (ZD *vs.* iZD) and in-between the days (in-phase day *vs.* anti-phase day) (Figure 3-10). Usually, chronodisruptive conditions are associated with body weight gain leading to obesity in mice and men (Antunes et al., 2010; Di Lorenzo et al., 2003; Karatsoreos et al., 2011). Under ZD and iZD conditions, mice were forced to eat in the light phase especially on anti-phase days. Several studies show that long-term daytime restricted feeding increases body weight on chow and HFD (Arble et al., 2009; Bray et al., 2013; De Araujo et al., 2016). *Ad libitum* food access promotes weight gain during simulated night-work due to changes in food intake pattern with increased food ingestion during the light-phase and, thus, circadian disruption (Salgado-Delgado et al., 2008). On the other hand, TRF during the biological night has previously been shown to prevent weight gain even under chronodisruptive conditions (Chaix et al., 2014; Jang et al., 2012; Oike et al., 2015; Salgado-Delgado et al., 2010). In consequence, TRF and changes in daily food intake presumably obviated weight gain under ZD and iZD conditions. Furthermore, under LD-13.5 conditions, mice increased their food consumption especially in the light phase but exhibited similar body weight compared to mice under LD-12. This is surprising since energy expenditure is similar between LD-12 and LD-13.5 cohorts. Mice exhibited decreased energy efficiency under LD-13.5 conditions (West et al., 2017). Thus, although not tested in this PhD project, daily changes in energy expenditure and energy efficiency might prevent net body weight gain under ZD and iZD conditions and should be investigated in future experiments. Surprisingly, glucose metabolism was impaired on the in-phase day compared to the anti-phase day under ZD conditions. Other studies observed adverse effects on glucose metabolism under misaligned (*i.e.* anti-phase) conditions (Morris et al., 2015; Scheer et al., 2009). However, these studies were performed in humans using a 28-hour forced desynchrony protocol where the misaligned condition is defined by sleep:wake cycle being 12 h out of phase with the pre-experimental sleep:wake cycle. In contrast, the anti-phase day under ZD and iZD condition was defined by food access time and dark phase being 12 h out of phase while sleep:wake cycles were not restricted. It might be expected that peripheral tissue clocks are entrained to the feeding:fasting cycle under ZD conditions and, thus, glucose tolerance should be comparable under in-phase and anti-phase conditions. However, this was not observed and the underlying mechanism would need further insight. It could be speculated that dynamic *zeitgeber* phase relations in the ZD paradigm changed insulin secretion by differential impact on the pancreas tissue clock. It is

also possible that glucose clearance was delayed by reduced insulin sensitivity in liver or muscle. Furthermore, mice spent more energy, *i.e.* were more active, in the fasting period on anti-phase days and might be in need of glucose to replenish energy stores. Under iZD conditions, glucose tolerance was slightly, but not significantly, decreased on the anti-phase day compared to the in-phase day. However, GTT curve progression on anti-phase days was comparable under iZD and ZD conditions. This further supports the idea that TRF may prevent metabolic impairments (Chaix et al., 2014). It is important to mention that GTTs were performed at different circadian phases under iZD conditions which was due to the experimental setup. More precisely, GTTs were performed at the end of the fasting phase on the respective days, *i.e.* close to the biological active phase on the in-phase day and close to the biological inactive phase on the anti-phase day. In humans, it is observed that the circadian phase has an effect on glucose metabolism with improved glucose tolerance in the biological morning (active phase) than evening (inactive phase) (Morris et al., 2015). This fits to my data indicating that mice showed slightly decreased glucose tolerance on anti-phase days, *i.e.* in their inactive phase, under iZD conditions. Thus, it cannot be excluded that small differences in glucose tolerance might result from testing at different circadian phases rather than testing at different *zeitgeber* phase relations.

Under baseline conditions with LD-12 and *ad libitum* food access, core body temperature showed a circadian rhythm with decreased temperatures in the light phase and increased temperatures in the dark phase (Figure 3-8 a). This rhythm is expected for nocturnal species (West et al., 2017). Under ZD conditions, core body temperature rhythms dissociated from baseline rhythms and revealed a complex pattern (Figure 3-8 b). Increases in body temperature were detected after food intake suggesting diet-induced thermogenesis. This is also observed by other research groups (Sen et al., 2017). Increased activity was associated with increased body temperature. This has also been shown in other studies (Jhaveri et al., 2007; Refinetti, 1999). Furthermore, small temperature peaks were observed which coincided neither with increased activity nor with food access times. These peaks probably represent the endogenous component of core body temperature rhythms. Core body temperature exhibits a circadian variation which is regulated by the SCN and other brain nuclei (Abe et al., 1979; Osborne and Refinetti, 1995; West et al., 2017). Under iZD conditions, rhythms in core body temperature and activity stayed aligned to the LD-12 cycle (Figure 3-16). This suggests that LD cycle-entrained endogenous rhythms such as activity maintain normal body temperature rhythms. Small diet-induced increases in core body temperature were only seen on the two days following the anti-phase day (at -135° and -90° LD-TRF phase angle).

In summary, behavioral rhythms and metabolic state were dependent on the phase angle of the two *zeitgebers*, light and food, under ZD conditions. Mice showed various activity periods throughout the experiment. Metabolism of the animals was in an anabolic state on in-phase days and in a catabolic state on anti-phase days. Glucose tolerance was negatively affected on in-phase days. Core body temperature patterns were complex suggesting a mixture of activity-mediated, diet-mediated, and endogenous-mediated output. Under iZD conditions,

behavior was entrained to the LD-12 cycle, but metabolic state was dependent on the phase angle of the two *zeitgebers*. In contrast to the ZD paradigm, mice were in a catabolic state on in-phase days and in an anabolic state on in-phase days under iZD conditions. However, this did not result in changes in glucose tolerance. Core body temperature rhythms stayed aligned to the LD cycle and, thus, to endogenous rhythms such as rest:activity rhythms.

4.4 *The ZD but not the iZD paradigm allows for quantification of differential zeitgeber impact on distinct tissue clocks*

To investigate the contribution of diverging *zeitgeber* input on molecular clock resetting and humoral output, mice were sacrificed under in-phase and anti-phase conditions spanning 28-hour experimental days in the ZD and iZD paradigms. Under ZD conditions, COGs of *Per2* and *Bmal1* expression in the SCN were about 5 h phase-shifted between in-phase and anti-phase days and, thus, showed the largest phase shift in clock gene expression of investigated tissues (Figure 3-12, Figure 3-14). This was in line with studies of other groups using single LD phase advances or delays. In these studies, the SCN clock is the first to re-entrain to the new LD cycle (Kiehl et al., 2010; Yamazaki et al., 2000). Although average phase shifts between in-phase and anti-phase days were similar for *Per2* and *Bmal1*, variations in *Per2* COGs were smaller than in *Bmal1* COGs between different anti-phase days (Figure 3-12). This is expected since *Per2* is directly light inducible leading to fast adaption under jetlag conditions (Albrecht et al., 1997; Kiehl et al., 2010; Ono et al., 2017; Reddy et al., 2002; Shearman et al., 1997). After a 6-hour LD phase delay, *Per1/2* and *Cry1* were re-entrained to the new LD cycle within two days (Reddy et al., 2002). Observed higher variability in *Bmal1* peak expression might be a result of slow adaption rates of *Bmal1* expression following a light pulse (Ono et al., 2017). After a single 9-hour light pulse in the beginning of the dark phase, kinetics of re-entrainment of *Per1* and *Bmal1* are tightly associated with kinetics of phase shifts in locomotor activity onsets and offsets, respectively (Ono et al., 2017). Due to the low temporal resolution of tissue sampling, this could not be investigated in detail in the ZD paradigm. Phase shifts between in-phase and anti-phase days in SCN clock gene expression (5 h) and activity onset (6.3 h) were similar under ZD conditions. This underlines the importance of the SCN in driving behavioral output. It is known, that the SCN is mainly impacted by light but it cannot be excluded that food might impact the SCN clock *via* various food-mediated signals discussed in detail in 4.2. In short, two peripheral food-related hormones, leptin and ghrelin, can induce phase shifts in SCN tissue in *in vitro* studies and both can cross the blood brain barrier (Banks et al., 1996, 2002; Burguera et al., 2000; Prosser and Bergeron, 2003; Yannielli et al., 2007). Moreover, the respective hormone receptors are expressed in the SCN (Guan et al., 1997; Zigman et al., 2006). Additionally, indirect effects would be conceivable inducing changes in the phasing of the SCN *via* afferents from the VMH and the ARC, brain regions involved in maintaining metabolic homeostasis receiving feedback from periphery about metabolic state (Buijs et al., 2017; Yi et al., 2006). Food-mediated impact on the SCN clock could be uncovered by inhibiting the transport of food-related hormones, blocking hormone receptors or lesioning communication pathways between different brain regions. So far, there

are no studies combining daily jetlag with time-restricted feeding, but one study investigated weekly 12-hour LD phase shifts combined with 12-hour time-restricted feeding. The researchers could show that restricted feeding to the pre-experimental dark phase decelerates behavioral re-entrainment to weekly changes in the LD cycle compared to *ad libitum* food access or time-restricted feeding to current dark phases (Schilperoort et al., 2019). This suggests that RF-12 may have prevented larger phase shifts in the SCN clock under ZD conditions. Under iZD conditions, it is very likely that the SCN stayed entrained to the LD cycle because 95 % of mice showed high-amplitude 24-hour locomotor activity rhythms (Figure 3-15, Figure S2). Thus, phase shifts in activity onsets between in-phase and anti-phase days (0.8 h) were likely an effect of an earlier activity onset, especially on in-phase days (Figure 3-19, Figure S2). As pointed out earlier this might originate from increased arousal and hunger. A marginal effect of food-mediated signals on the SCN clock cannot be excluded; however, it is not expected since published data show that the SCN clock is resistant to changes in food access times under LD-12 conditions (Damiola et al., 2000; Hara et al., 2001).

Food is the dominant *zeitgeber* for peripheral tissues and time-restricted feeding can uncouple peripheral tissue clocks from the SCN clock (Damiola et al., 2000; Hara et al., 2001; Stokkan et al., 2001). In line with this, peripheral tissues showed overall smaller phase shifts between in-phase and anti-phase days compared to the SCN under ZD conditions. Phase shifts were not significantly different when peripheral tissues were directly compared with each other, but a significant differential regulation relative to the two *zeitgebers* was observed. Liver showed the strongest correlation with the feeding cycle with only 1 h phase shift between in-phase and anti-phase conditions. eWAT clock gene expression rhythms shifted a little more, namely by 2 h, but were still well aligned with feeding cycles (Figure 3-13 a, b, Figure 3-14). This fits with previous publications showing that the liver clock is highly susceptible to changes in feeding:fasting cycles (Damiola et al., 2000; Hara et al., 2001; Stokkan et al., 2001). Adrenal glands were rather influenced by light and clock gene expression rhythms were phase shifted by almost 4 h between in-phase and anti-phase days (Figure 3-13 c, Figure 3-14). It was previously shown that adrenal clocks are directly impacted by light and food which might explain the more balanced impact of both *zeitgebers* under ZD conditions (Girotti et al., 2009; Kiessling et al., 2014; Son et al., 2018). After a single 6-hour phase advance, the adrenal clock shows a slightly faster re-entrainment to the new LD cycle than the liver clock. Moreover, *Per2* is the fastest and *Bmal1* the slowest to adapt in both tissues while *Dbp* shows an intermediate speed in re-entrainment (Kiessling et al., 2010). My data also revealed a tissue-specific effect on clock resetting. In line with Kiessling *et al.*, adrenal clocks showed the largest and liver clocks the smallest phase shift between in-phase and anti-phase conditions. In contrast to Kiessling *et al.*, however, I did not observe strong differences in resetting kinetics between different genes. This might be due to exposure of repeated delays in LD cycles in the ZD paradigm instead of a single 6-hour advance in the LD cycle as used in the Kiessling study. Moreover, in that study mice had *ad libitum* access to food whereas mice were on TRF in the ZD paradigm. Food intake induces *Per1*, *Per2* and *Dec1* expression within 1 h in liver but not in lung after a 24-hour fasting period (Oike et al., 2011; Wu et al., 2010). In liver, I also observed

the highest expression in *Per2* in the beginning of the feeding phase on in-phase and anti-phase days. Moreover, liver showed the smallest phase shifts in *Per2* expression rhythms of all investigated tissues which might be due to high sensitivity of the liver to RF-12. Small phase shifts between in-phase and anti-phase days were determined for eWAT indicating that this tissue is also highly susceptible to food-related signals. Oike et al. studied the food effects on clock gene expression by injecting glucose and amino acids which suggests that nutrients are driving peripheral clock resetting. Investigations on the crosstalk between metabolism and the circadian clock are of high interest and more evidence is emerging (Adamovich et al., 2014; Asher and Sassone-Corsi, 2015; Chaix et al., 2014; Hatori et al., 2012; Hirota et al., 2002; Reinke and Asher, 2019; Schilperoort et al., 2019). Under ZD conditions, phase shifts in clock gene expression rhythms were a result of integrated light-driven and food-driven signals. Food-related hormones which might contribute to clock resetting are described in 4.2.

Under iZD conditions, activity – and, thus, presumably the SCN – stayed entrained to LD cycle and peripheral tissue clocks were stabilized at a 24-hour period, too. The liver clock showed a phase shift of a little less than 1 h between in-phase and anti-phase days. The largest phase shift was seen in eWAT clock with slightly more than 4 h. This is surprising and would suggest that eWAT is more susceptible to food-related signals than liver. This further suggests that the range of entrainment to food T-cycles might be tissue specific. A recent publication suggests a narrow range of entrainment to food being 22 h to 24 h in peripheral tissues (Hamaguchi et al., 2015). However, the researchers did not investigate eWAT and also did not test extended feeding:fasting cycles. Therefore, more experiments are needed to further understand this phenomenon. An intermediate phase shift of about 2.5 h was calculated for the adrenal clock. All phase shifts in peripheral tissues were indifferent to the LD cycle under iZD conditions. This supports the idea that the entrained SCN has a stabilizing effect on peripheral tissue clocks. Furthermore, regular LD schedules can synchronize peripheral tissues without participation of the SCN clock which further stabilizes alignment of peripheral tissue clocks with LD-12 cycles (Husse et al., 2014; Koronowski et al., 2019; Meyer-Kovac et al., 2017). Together, my data support the idea that peripheral tissue clocks entrain to a *zeitgeber* which is in the range of entrainment (*i.e.* 24-hour) in order to optimize metabolic yield. Nevertheless, minor phase shifts in peripheral tissue clocks were seen in the iZD paradigm suggesting that food-related signals impact tissue clocks to some extent (possible effectors are discussed in chapter 4.2). In the iZD paradigm, tissue-specific differences in clock resetting were not observed. iZD, therefore, is not a suitable paradigm to disentangle differential *zeitgeber* impact on the circadian clock network.

On average, COGs in corticosterone rhythms were phase-delayed by almost 8.5 h between in-phase and anti-phase days under ZD conditions (Figure 3-11 b, d, Figure 3-14). There was, however, a substantial variability in determined COGs between the different anti-phase days. Higher variations in single sampling time points were seen on the in-phase day. Although no difference in overall released corticosterone was found between the days, it appeared that the amplitude of corticosterone was slightly, but not significantly, dampened on anti-phase days.

This suggests that corticosterone release from the adrenal might be impaired on anti-phase days when *Per2* gene expression in this tissue is dampened (Oster et al., 2006a). GCs may themselves play a role in tissue clock entrainment. Dexamethasone, a GC analogue, can reset the liver clock *in vivo* (Balsalobre et al., 2000). On the other hand, phase shifts in locomotor activity cannot be induced by repeated daily corticosterone injections (Stokkan et al., 2001). However, GC signalling seems to be important in resetting peripheral tissue clocks (Balsalobre et al., 2000; Ikeda et al., 2015; Le Minh et al., 2001). GCs attenuate TRF-induced uncoupling of SCN and peripheral tissue clocks. Peripheral tissue clocks adapt to daytime-restricted feeding within one week while GC rhythms are unchanged. Adrenalectomy leads to faster adaption of peripheral tissue clocks upon daytime-restricted feeding suggesting that GCs may stabilize coupling between SCN and peripheral tissues (Le Minh et al., 2001). In the ZD paradigm, phase shifts of SCN and corticosterone were different from phase shifts seen in peripheral tissues. This suggests that GCs, although playing an important role in peripheral clock resetting, did not drive clock resetting of peripheral tissues under ZD conditions. However, unnatural long T-cycles impacted GC rhythms dramatically and, thereby, possibly disabled their function on coupling SCN and peripheral tissue clocks. Regarding the impact of GCs on the SCN clock, it is very unlikely that GC rhythms directly impacted SCN clock because the SCN only show low levels of GR expression (Balsalobre et al., 2000; Rosenfeld et al., 1988). Nevertheless, GCs might impact the SCN clock indirectly *via* numerous afferents from other brain nuclei which express GRs (Han et al., 2005; Roland et al., 1995). The effects of GCs on peripheral clock resetting could be investigated in more detail in adrenalectomized animals.

Leptin rhythms were phase-advanced by 7 h between in-phase and anti-phase conditions in the ZD paradigm with comparable amplitudes (Figure 3-11 c, d, Figure 3-14). The latter was expected since leptin levels correlate with body fat content which was similar between in-phase and anti-phase conditions (Figure 3-10) (Ahrén et al., 1997; Havel et al., 1996; Sinha et al., 1996). Moreover, leptin patterns under all conditions were rhythmic which is also in line with the literature (Ahima et al., 1998; Ahrén et al., 1997; Kalsbeek et al., 2001; Sinha et al., 1996). Besides central regulation of leptin rhythms, its secretion is regulated by meal timing (Schoeller et al., 1997). Leptin levels decrease during fasting and increase following food ingestion independent of the time of day (Dallongeville et al., 1998). This fits with my data since lowest leptin levels were measured in the beginning of the feeding phase under in-phase conditions and in the end of the fasting phase under anti-phase conditions. Elevated leptin levels in the beginning of the fasting phase under in-phase conditions would suggest that mice ate late in the feeding phase on days before in-phase days. It is important to note that the phase-advance in leptin rhythms arose from the assumption that in a circadian system the maximum phase shift possible would be 12 h. However, in a human study, leptin rhythms shifted almost 14 h within 4 days after a single 12-hour LD phase shift, although meal timings were phase shifted, too (Schoeller et al., 1997). Thus, it cannot be excluded that leptin rhythms were phase-delayed by 17 h between in-phase and anti-phase days under ZD conditions. This could be investigated by sampling blood on additional days, *e.g.* at 90 ° and 135 ° LD-TRF phase angles. Furthermore, leptin rhythms and corticosterone rhythms were anti-phasic under

in-phase conditions whereas these rhythms showed similar phasing under anti-phase conditions. An anti-phasic relationship between these hormones was shown under LD-12 conditions and *ad libitum* food access and maintained when daytime-restricted feeding was applied (Ahima et al., 1998). Conflicting inputs from the SCN and feeding signals may counteract humoral outputs leading to internal desynchronization but the underlining mechanisms will need further investigation.

Together, it is reasonable to suggest that the changes I observe on behavioral, molecular, and humoral level are a result of various inputs from central and peripheral tissue clocks (Astiz et al., 2019; Heyde and Oster, 2020). In the ZD paradigm, liver and eWAT clocks were primarily impacted by feeding:fasting cycles (RF-12). Adrenal, SCN and activity were impacted by both *zeitgebers* showing phase shifts of 4 - 6 h between in-phase and anti-phase days. Corticosterone release was mostly impacted by light and might therefore contribute to shifts in behavioral and peripheral clock outputs. Leptin rhythms were phase advanced by 7 h and, thus, impacted by both *zeitgebers*. A limitation of the ZD experiment is that some timepoints in the profile of gene expression in the SCN and hormone levels were based on low replicate numbers (n = 2 for some timepoints). This was due to technical issues. Nevertheless, the data suggest a model of circadian rhythm adaption under dynamically conflicting *zeitgeber* conditions in which the circadian clock network de- and reconverges along the ZD cycle. In line with this, food intake and body weight cycle throughout the ZD sequence reaching their troughs on days of maximal *zeitgeber* misalignment and peaking on days of maximal *zeitgeber* alignment. Effects of ZD on food intake, body weight, and glucose tolerance might be a result of dynamic changes in phase relations of feeding:fasting and light:dark cycles and corresponding changes in the expression of key enzymes, hormones and nutrients (Sinturel et al., 2020). The ZD paradigm allowed for a much more fine-grained assessment of different *zeitgeber* inputs to circadian clocks and behavior. In contrast, central and peripheral tissue clocks were not different from LD-12 cycles in the iZD paradigm. Thus, normal light:dark cycles were more potent to entrain behavioral rhythms and might stabilize peripheral tissue clocks at a 24-hour period when feeding:fasting cycles were out of the range of entrainment. In consequence, the iZD paradigm was not suitable to disentangle differential *zeitgeber* impact on various clock resetting. Future experiments combining shorter LD T-cycles and lengthened food T-cycles may further disentangle differential impact on different tissues.

4.5 Conclusion and outlook

In this PhD thesis, it was investigated for the first time how diverging *zeitgeber* input impacts output of tissue clocks, hormonal rhythms, and behavior. Dark-phase restricted feeding prevented internal desynchronization under extended light:dark cycles but led to chronodisruption with regard to normal 24-hour rhythms. In contrast, RF-12 promoted development of internal desynchronization between central and peripheral tissue clocks under extended light:dark cycles. Internal desynchronization in clock gene expression rhythms and metabolic state were transient and de- and resynchronized dependent on *zeitgeber* phase relation (Figure 4-1: Model of circadian rhythm adaptation under conflicting

zeitgeber input. **For details see text.**Figure 4-1). In conclusion, the ZD paradigm (LD-14/RF-12) provides a powerful tool to study differences in *zeitgeber* impact on tissue clock function as well as on physiological and behavioral outputs.

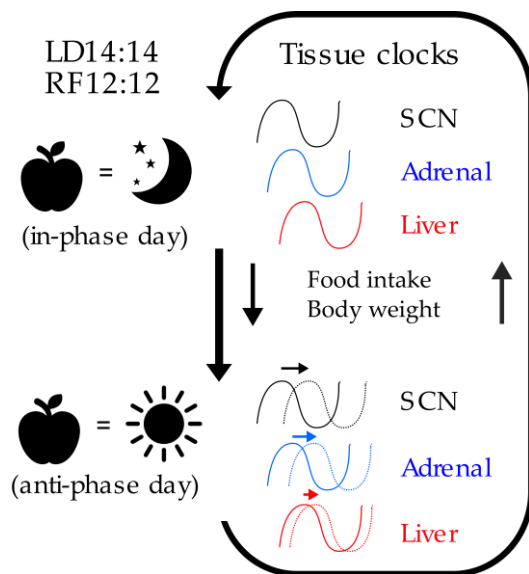


Figure 4-1: Model of circadian rhythm adaptation under conflicting *zeitgeber* input. For details see text.

Glucose clearance was delayed on in-phase compared to anti-phase days under ZD conditions. Thus, although clock gene expression rhythms in liver and eWAT stayed aligned with feeding:fasting cycles, metabolism was disrupted. It is possible that extra-SCN clocks show slower re-entrainment capacities than the SCN and, thus, are de-regulated. This may induce overeating or metabolic switching which leads to weight gain under re-aligning and aligned *zeitgeber* conditions. Thus, aligned peripheral tissue clocks may not be sufficient to prevent metabolic disruptions which possibly lead to adverse health outcomes under chronodisruptive conditions. Future experiments are needed to investigate the phenomenon of overeating under such conditions. At the present state of knowledge, my data may suggest that TRF together with caloric restriction, especially around days of maximal *zeitgeber* alignment, may help to prevent metabolic impairments. The short-term LD-14/Dark-RF experiment revealed a high degree of internal synchrony between behavioral output and peripheral tissue clock which was lost under LD-14/RF-12 conditions. Long-term experiments should further investigate the metabolic homeostasis under LD-14/Dark-14 conditions. If internal desynchrony persist under long-term LD-14/Dark-RF conditions this may be translated to the human situation. Activity phase-restricted meal timing could easily be applied in shift worker.

Understanding the tissue-specific mechanisms of how *zeitgebers* facilitate clock resetting will be fundamental and help devising tissue-specific manipulations of clock/rhythm function. This will be of special interest since an increasing number of people live and work under chronodisruptive conditions which promotes internal desynchronization and adverse metabolic outcomes.

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Supplements

Table S1: Fitted sine wave maxima of clock gene rhythms in peripheral tissues under acute ZI conditions. Maxima are presented in decimal hours (external clock time). NR: not significantly rhythmic by CircWave analysis

		Baseline	Misaligned (LD-14/Dark-RF)	Misaligned (LD-14/RF-12)
Liver	<i>Bmal1</i>	5.50	12.44	8.23
	<i>Per2</i>	22.74	0.66	23.98
	<i>Dbp</i>	15.32	21.86	19.53
	<i>Cry1</i>	2.10	9.19	5.98
	<i>Nr1d1</i>	13.91	18.68	14.63
Adrenal	<i>Bmal1</i>	3.70	8.96	6.58
	<i>Per2</i>	20.08	2.66	0.02
	<i>Dbp</i>	14.05	19.65	18.37
	<i>Cry1</i>	0.22	5.82	3.98
	<i>Nr1d1</i>	12.79	18.72	14.91
eWAT	<i>Bmal1</i>	5.78	13.09	8.19
	<i>Per2</i>	20.89	3.50	22.54
	<i>Dbp</i>	16.80	23.70	19.09
	<i>Cry1</i>	2.78	NR	NR
	<i>Nr1d1</i>	14.03	21.74	15.76
Pancreas	<i>Bmal1</i>	5.58	10.63	9.91
	<i>Per2</i>	17.37	NR	20.69
	<i>Dbp</i>	17.81	20.57	20.45
	<i>Cry1</i>	NR	NR	3.70
	<i>Nr1d1</i>	13.79	19.05	16.60

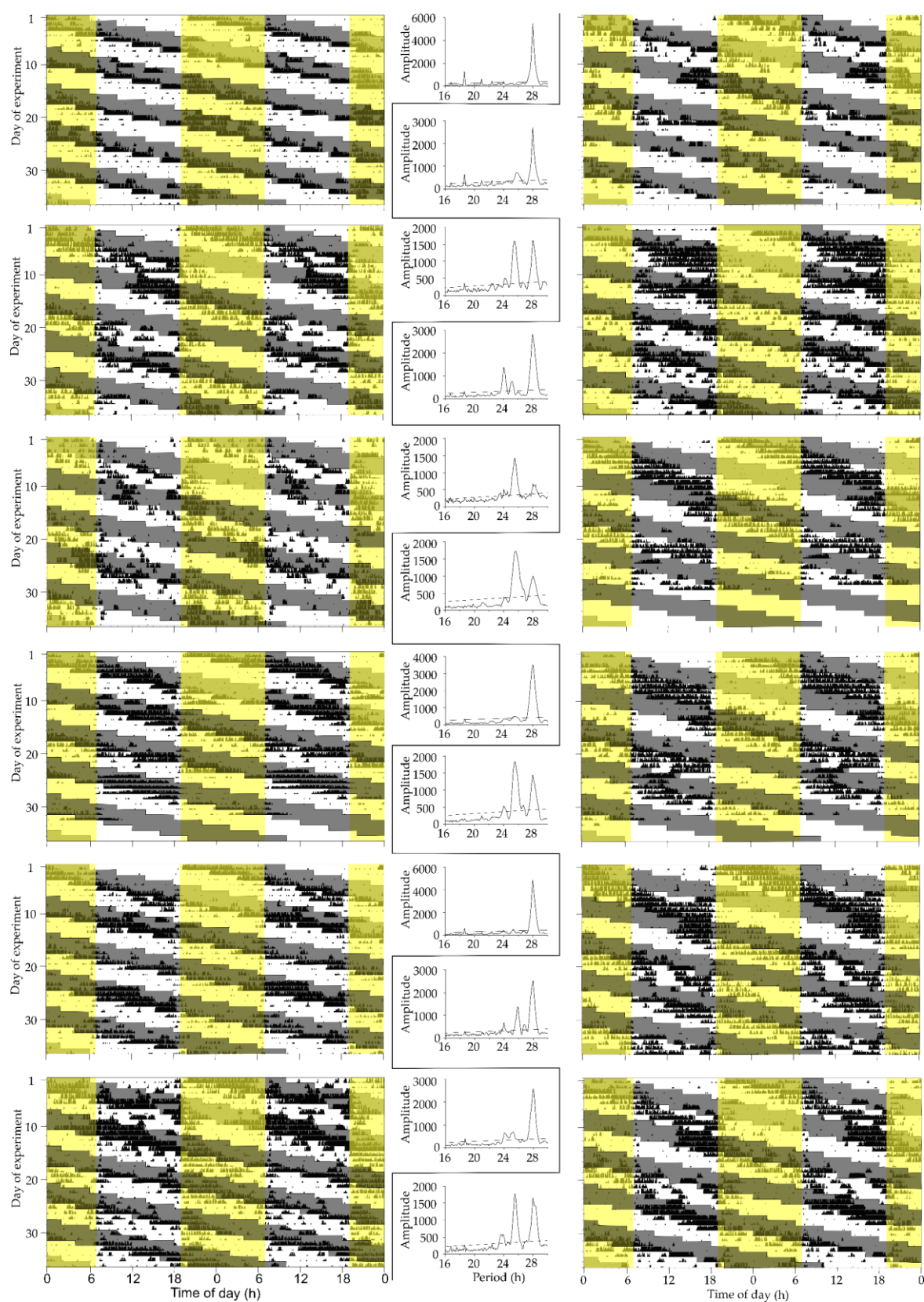


Figure S1: Representative double-plotted actograms and corresponding X^2 periodogram analyses of mice under ZD conditions. Grey and yellow shadings indicate dark phases and feeding intervals, respectively.

Table S2: COGs of clock gene and hormone levels in peripheral and central tissue and serum under ZD conditions, respectively. COGs are presented as decimal hours (external clock time).

		In-phase	Anti-phase 1	Anti-phase 2	Anti-phase 3
Liver	<i>Bmal1</i>	22.02	0.35	23.80	23.45
	<i>Per2</i>	16.30	16.79	16.37	15.46
	<i>Dbp</i>	8.77	11.18	10.76	9.38
eWAT	<i>Bmal1</i>	21.45	0.30	23.64	23.07
	<i>Per2</i>	12.85	14.91	14.43	13.20
	<i>Dbp</i>	9.34	13.87	10.57	9.77
Adrenal	<i>Bmal1</i>	20.16	0.93	23.86	21.93
	<i>Per2</i>	13.71	17.83	17.70	16.22
	<i>Dbp</i>	7.59	12.30	11.03	9.27
SCN	<i>Bmal1</i>	15.01	15.88	19.80	22.17
	<i>Per2</i>	8.39	13.24	14.83	12.04
Corticosterone		12.21	19.36	0.07	15.55
Leptin		2.24	17.80	19.64	16.20

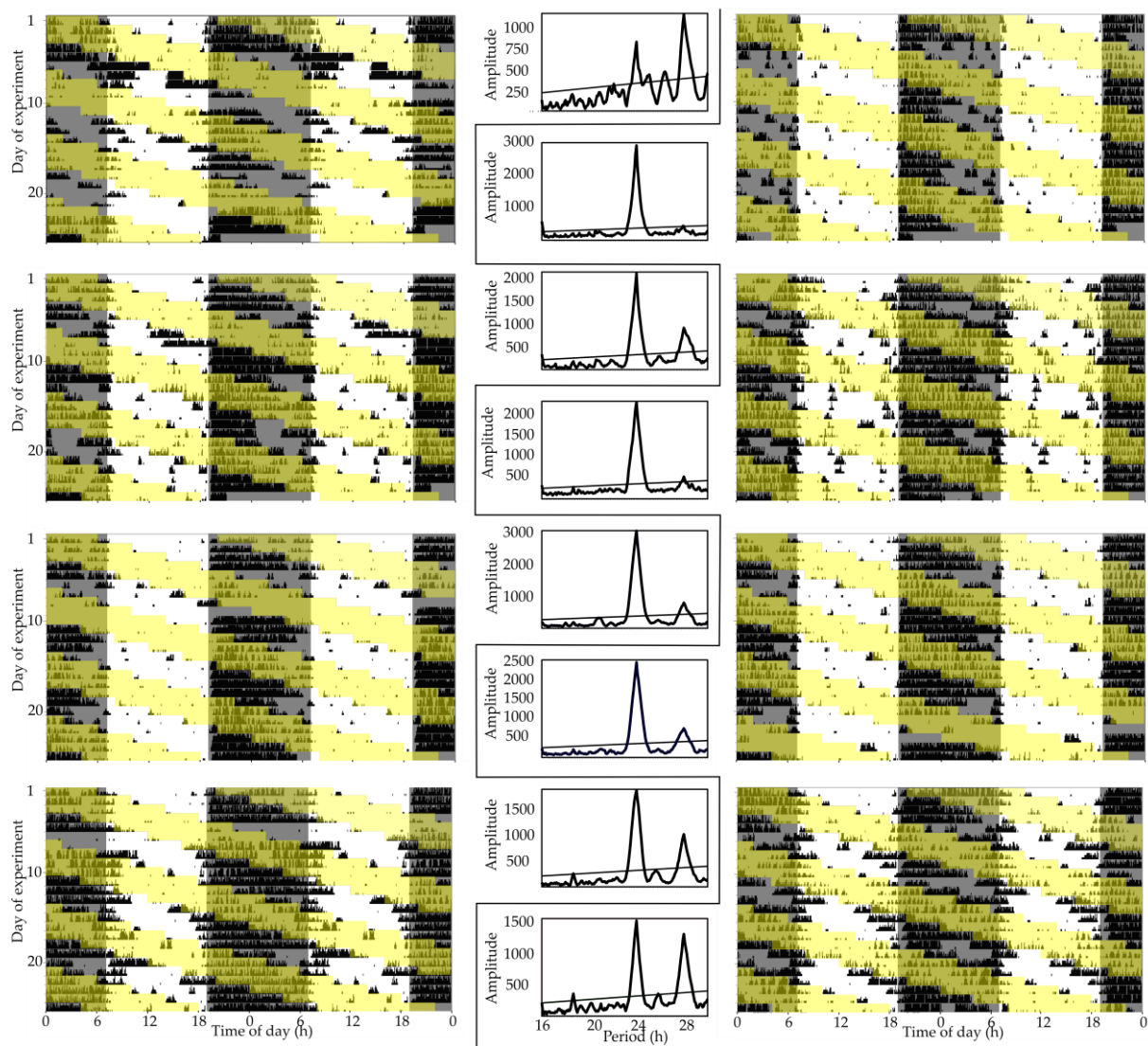


Figure S2: Representative double-plotted actograms and corresponding X^2 periodogram analyses of mice under iZD conditions. Grey and yellow shadings indicate dark phases and feeding intervals, respectively.

Table S3: COGs of gene expression in peripheral tissues under iZD conditions. COGs are presented as decimal hours (external clock time).

		In-phase	Anti-phase
Liver	<i>Bmal1</i>	7.48	8.93
	<i>Per2</i>	23.023	20.11
	<i>Dbp</i>	16.50	20.23
eWAT	<i>Bmal1</i>	7.06	12.97
	<i>Per2</i>	20.63	22.73
	<i>Dbp</i>	16.73	21.49
Adrenal	<i>Bmal1</i>	5.59	8.29
	<i>Per2</i>	21.51	22.46
	<i>Dbp</i>	15.55	19.81

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