2017

Age and Sex-Specific Effect of Caloric Restriction on Circadian Clock and Longevity-Associated Gene Expression

Arten Andreyevich Astafev
Cleveland State University

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AGE AND SEX - SPECIFIC EFFECT OF CALORIC RESTRICTION ON CIRCADIAN CLOCK AND LONGEVITY - ASSOCIATED GENE EXPRESSION

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Bachelor of Biology
Cleveland State University
May 2014

submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE IN BIOLOGY

at the

CLEVELAND STATE UNIVERSITY

October 2017
We hereby approve this thesis

For Artem A. Astafev

Candidate for the Master of Science in Biology Degree for the Department of

Biological, Geological and Environmental Sciences

and the CLEVELAND STATE UNIVERSITY

College of Graduate Studies by

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GRHD/BGES, Cleveland State University  
Date: 10/05/2017

Dr. Aaron Severson  
GRHD/BGES, Cleveland State University  
Date: 10/05/2017

Dr. Crystal Weyman  
GRHD/BGES, Cleveland State University  
Date: 10/05/2017

Student’s Date of Defense:

10/05/2017
DEDICATION

I dedicate this work to my family, which at the moment of my defense I am lucky to witness alive in all four generations. Since a part of my large family comes from the scientific/medical background, I am extremely glad to also make some contribution to the field with this work.
ACKNOWLEDGEMENTS

In the first place, I want to acknowledge Dr. Kondratov for his wisdom, knowledge, unbelievable patience and support. It was a pleasure to have him as my academic advisor throughout the program and the input he provided for me extended far beyond the formal educational issues. I will thoroughly value every lesson I’ve learned while being a part of Roman’s lab and I must admit – I’ve learned a lot. Whether it was an obstacle in research process or even a regular life frustration – Roman always had a wise and extremely helpful advice for all of us, lab members. What I was always amazed about when I was talking to Roman, was his vision – always two steps ahead, he demonstrated us the unmatched example of rational thinking from which we could learn (and I think every single lab member who worked with Roman will never be able to disagree on that particular part). For me personally, that was the most valuable lesson – to master the scientific thinking and a rational approach to the problem. I value the relationship that formed between me and Roman during this program – a true friendship and I hope it will continue beyond. I will always be glad to meet Roman and his family later on – just to have fun and discuss things not necessarily related to work and again, each time to learn something new from him.

I want to also acknowledge my family, especially my dad for always sharing wisdom and giving helpful advice, mom – for love and support, my wife – for love and support…and encouragement along with so much help on the daily basis even while carrying our yet unborn son. My younger brother - for the moral support during difficult
times and reminding me what matters most, my cousin and his family for believing in me.

I need to thank all of my lab mates: Sonal, Kuldeep, Nikkhil, Richa, Amol, Will, Shomo – every single one of them helped me in different aspects of my research and gave me some important tips which cannot be found in books. Special thanks to Sonal for mentoring me in the beginning. At the same time, I want to wish best of luck to our new lab members: Allan and Xhulia. The friendship which formed within our lab team will definitely continue.

I want to thank my committee members: Dr. Crystal Weyman and Dr. Aaron Severson for the knowledge they shared during lectures, for investing the time into reading my thesis and giving a valuable advice. Specifically, I want to thank Dr. Weyman for lots of help she provided me with, and Dr. Severson for providing me with a chance to teach a graduate lecture session for the first time in my life.

I need to also thank all of the professors in the department who shared so much knowledge with us, students, during lecture sessions: Dr. Shukla, Dr. Mazumder, Dr. Barik, Dr. Boerner, Dr. Li, Dr. Komar and Dr. Sam-Yellowe.
AGE AND SEX - SPECIFIC EFFECT OF CALORIC RESTRICTION ON CIRCADIAN CLOCK AND LONGEVITY - ASSOCIATED GENE EXPRESSION

ARTEM A. ASTAFEV

ABSTRACT

The rhythms in the expression of circadian clock genes are affected by calorie restriction (CR), a dietary paradigm known to increase lifespan. In our current study, we show that circadian rhythms are influenced by sex and the effects of CR are different between males and females. In particular, we found a group of clock genes which showed a sex-dependent difference in expression, as well as in response to CR (Rev-Erb α, Ror γ and both Cryptochromes: Cry1 and Cry2 genes). Two clock genes showed no difference in expression but their response to CR showed sexual dimorphism (Ror α and Rev-Erb β). Finally, we found some of the clock genes to be expressed in a sex-independent manner (Bmal1, Per1, Per2 and Per3). The response to CR for these genes did not show sexual dimorphism as well. Several genes were also previously reported to be regulated by CR. These genes showed a sex-dependent difference in expression as well as the sexual dimorphism in the response to CR in mouse liver (Fmo3, Mup4, Serpina12, Cyp4a12b and Cyp4a14a). IGF signaling plays an important role in aging and CR effects. Igf-1 expression is regulated by CR and by the circadian clock, we found that rhythms in Igf-1 expression have sexual dimorphism. We also investigated the differences in expression levels in young versus old mice to see if the effect of short-term CR differs from the effect of long-term CR. In all of the 4 analyzed genes, the expression does not
change with age. The effect of CR on the expression of 2 out of 4 genes (Bmal1 and Per2) was lost upon long-term exposure to CR. For the other 2 genes (Per1 and Per3) the effect of CR persisted over time. Thus we emphasize that sex and age are important factors for consideration when administering CR.
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<tbody>
<tr>
<td>ACTH</td>
<td>Adrenocorticotropic hormone</td>
</tr>
<tr>
<td>Akt</td>
<td>Activated Protein Kinase β</td>
</tr>
<tr>
<td>AL</td>
<td>Ad-libitum</td>
</tr>
<tr>
<td>Alas1</td>
<td>Aminolevulinate Synthase 1</td>
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<td>AMP</td>
<td>Adenosine Monophosphate</td>
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<td>AMPK</td>
<td>Adenosine Monophosphate Activated Protein Kinase</td>
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<td>ANOVA</td>
<td>Analysis of Variance</td>
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<td>ANS</td>
<td>Autonomic Nervous System</td>
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<td>AR</td>
<td>Androgen Receptor</td>
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<td>ARNTL</td>
<td>Aryl Hydrocarbon Receptor Nuclear Translocator-like Protein</td>
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<td>ATP</td>
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<td>BMAL1</td>
<td>Brain and Muscle ARNTL 1</td>
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<td>bHLH</td>
<td>Basic Helix Loop Helix</td>
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<td>D site of albumin promoter Binding Protein</td>
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<td>DEPTOR</td>
<td>DEP Domain-Containing mTOR-Interacting Protein</td>
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<td>DMH</td>
<td>Dorsomedial Hypothalamic Nucleus</td>
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<td>DNA</td>
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<td>dNTP</td>
<td>Deoxy Nucleotide Triphosphate</td>
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<td>DTT</td>
<td>Dithiothreitol</td>
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<td>Food Entrainable Oscillator</td>
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<td>FASPS</td>
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<td>Glucose 6 phosphatase</td>
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<td>GH</td>
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<td>H3 Histone</td>
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<td>HLF</td>
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<td>IGFBP</td>
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<td>mA</td>
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<td>mg</td>
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<td>Mixed Lineage Leukemia</td>
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<td>NFIL3</td>
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<td>PEPCK</td>
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<td>Rev-Erb α/β</td>
<td>Reverse erythroblastosis virus alpha/beta</td>
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<td>RORE</td>
<td>Retinoic Acid Related Orphan Response Element</td>
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<tr>
<td>ROR</td>
<td>Retinoic Acid Related Orphan Receptor</td>
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<td>Reactive Oxygen Species</td>
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<td>quantitative Polymerase Chain Reaction</td>
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<td>Reverse Transcriptase Quantitative PCR</td>
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<td>Suprachiasmatic Nucleus</td>
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<td>Shp</td>
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<td>SIRT1</td>
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CHAPTER I
INTRODUCTION

The Earth revolves around its axis with a period of 24 hours. During this cycle of rotation, a particular point on Earth has the phase of exposure to sunlight and a phase of the absence of light when that particular point on the spherical surface of Earth is directed away from the sun. This generates what in our understanding is night and day both of which at the equator are approximately equal to 12 hours with seasonal deviations the closer it is to the poles - depending on the latitude. The absolute majority of the organisms that inhabit Earth have adapted to the environmental changes that take a turn during each daily cycle in different ways, however for all of them the cycle dictates the particular patterns in behavior and physiology.

Circadian clock (from lat. *circa* (about), *diem* (day)) is the complex internal biological system within the organism that aids in the control of our physiological rhythms. At the core of the mammalian internal clock is the intricate molecular mechanism that drives the rhythms in the daily functioning of different tissues, organs and organ systems (Reppert & Weaver, 2002). At the current moment in the circadian research field, scientists subdivide the internal clock into the central (or master) clock and
the peripheral clock. The central clock is located in the suprachiasmatic nucleus (SCN) in the brain and is reset by the presence or absence of light. The peripheral clock is located in the tissues of organs that most actively contribute to the control of metabolism - nutrient turnover and detoxification (Green et al., 2008; Lowrey & Takahashi, 2004; Potter et al., 2016; Reppert & Weaver, 2002). The peripheral clock receives signals from the hypothalamic master clock however it can be uncoupled from the central clock by the external cues other than light – the major example is the feeding regimen (Green et al., 2008; Ikeda et al., 2015; Tareen & Ahmad, 2015). Healthy operation of the circadian clock is necessary for the functioning of the organism and vitality. The importance of circadian clock’s role in aging is now also recognized. Currently there are multiple evidences which show the association of the environmental disturbances to circadian rhythms and the early onset of age-related diseases (Froy & Miskin, 2010; Hatori et al., 2017; Reszka & Przybek, 2016; Toutou et al., 2017; Vinogradova et al., 2010), as well as the mutations in circadian clock genes which lead to decreased longevity (Fu et al., 2002; Kondratov et al., 2006). Therefore the current research is working on the path to find a powerful intervention which might mediate the lifespan extension and health benefits through interaction with circadian clock in order to preserve the rhythms and reduce the risk factors for the early onset of age-related diseases.

Caloric restriction (CR) is a dietary paradigm in which the daily intake of food is reduced without malnutrition. CR is more and more recognized for its benefits to health which drive the extension of lifespan (Froy & Miskin, 2010; Mitchell et al., 2016; Patel et al., 2016). There are multiple accessory pathways that mediate these effects - the interaction between the circadian clock and different aging-controlling pathways.
implicated in CR mechanism such as, the sirtuin (Chang & Guarente, 2013; Nakahata et al., 2008), insulin/IGF and mTOR signaling pathways (Khapre et al., 2014) were recognized. The exact mechanisms are still under investigation as the parts of the puzzle for CR-mediated lifespan extension keep coming together from the ongoing research work. The healthy functioning of the circadian clock is required for the benefits of CR to take place – one of the studies done earlier in our lab showed that CR fails to induce its effects in mice deficient for Bmal1 (Patel et al., 2016). In particular, more recent studies conducted in our lab showed that CR affects circadian rhythms in gene expression of core-clock and longevity-associated candidate genes (Patel et al., 2016; Patel et al., 2016). These findings provided us with the grounds for a new direction of the study – we decided to investigate the questions that were not systemically addressed before – whether the effects of CR are sex-dependent and whether CR works differently on mice of different age. These factors which are very important to consider when implementing CR in medical practice were not widely accounted for, especially when studying the effects of CR using circadian approach. We suggest this knowledge might be useful in a growing field of studies on aging considering the existing sex-based differences in longevity across species (S. Austad & Bartke, 2015). We also wanted to investigate for possible differences between short and long-term CR. The majority of previous studies on CR was done on young male mice only, however, it would be unreasonable to ignore the possible differences between the effects of CR on a young and aged organism. A lot of questions still remain unanswered: are the effects of CR on the circadian clock going to still persist over long time exposure to CR or will the effects become attenuated with time? Will CR help to address the age-related health problems if introduced at an older
age? Since it is established that molecular clock machinery is necessary to modulate CR effects, another set of questions arises: will long-term CR help to prevent the age-related disturbances in rhythm or the age-related circadian disturbance can’t be affected by such intervention? And, in the first place, what is the primary source of these disturbances – solely the age itself, or the early onset of age-related diseases provoked by the aspects of the modern lifestyle? It is well established that there are lots of factors in nowadays urban life which might contribute to chronodisruption and elevate the incidence of metabolic syndrome, cardiovascular disease, cognitive and affective impairment, sleep disorders and some cancers (Ortiz-Tudela et al., 2012). Increased nocturnal activities, systematic exposure to blue light at night, shift work, chronic jetlags – all of those factors contribute to early aging and “wear out” the internal clock in humans. On the contrary, a synchronization of lifestyle against a normal day/night cycle helps to delay the onset of the adverse health conditions (Ortiz-Tudela et al., 2012). To what extent CR might help with this synchronization and what is the optimal exposure time under CR? There are no exact answers at this point, so we took an attempt to bring one of the pieces of the puzzle into this investigation and check for the possible differences between the influence of short and long-term CR on circadian gene expression in the second part of our study.

1.1 Evolutionary relevance of circadian clock

The circadian system or circadian clock generates 24-hour rhythms in physiology and metabolism in a variety of organisms across taxa (Green et al., 2008; Lowrey & Takahashi, 2004). From cyanobacteria and plants to animals and humans circadian clock is a mechanism of major evolutionary importance for the optimization of energy expenditure, adaptation to the environment and survival. When we mention internal clock
– most often we implicate the synchronization of different processes inside the organism with a periodic 24-hour light/dark environmental cycle. The clock, however, developed to operate in so-called free-running condition as well, in order to maintain the physiological synchrony even without any external cues. In this case the physiological rhythms of an organism do not have a period of 24 hours exactly, however the length of a period can easily be entrained back to the exact 24 hours upon exposure to the cyclic environmental cues (Buhr & Takahashi, 2013; Lowrey & Takahashi, 2004). What might be the importance of intrinsic control of circadian rhythmicity even when the external cues (or Zeitgebers – from German “time givers”) are consistently present? It turns out that organisms benefit most when the environmental changes throughout the daily cycle are expected rather than repeatedly adapted to (Koch et al., 2017). The first shreds evidence track back to the ancient times when people observed the daily changes in the activity of different species simply on the level of behavior. Many plants position their leaves towards the source of light (the Sun) during the dawn in order to start photosynthetic processes, diurnal animals increase their locomotor activity a little before or shortly after the sunrise and start hunting/foraging for food. The reverse is true for nocturnal species – their elevated physiological activities happen during the night. The most ancient evidence of the presence of circadian clock known to humans is the example of cyanobacteria, in which the processes of oxygenic photosynthesis and nitrogen fixation are restricted to the light and dark phase of the day respectively. Many organisms, in turn, adapted to escape UV-light due to the ultraviolet radiation and photooxidative damage. It became apparent when people observed the specific changes on the cellular level – the S-phase of the cell cycle (DNA synthesis) was restricted to night, when UV-radiation is absent (Lowrey &
While the major zeitgeber (or external cue) for the entrainment of the circadian clock is a periodic presence and absence of light, there are other factors as well, such as daily fluctuations in temperature. Temperature changes – daily and seasonal, however sometimes can put pressure against synchronization – especially on warm-blooded animals during the periods of sharp and sudden fluctuation of the temperature. In these cases the internal rhythms on molecular and behavioral levels play an important part in temperature compensatory mechanisms, maintaining the rhythms at a homeostatic 24 hour period (Buhr & Takahashi, 2013). Therefore, the circadian clock is an ancient biological mechanism that was conserved in a variety of species on Earth in order for the organisms to optimize their physiological processes against the cyclic environmental changes which result from the rotation of the planet. It is now necessary to discuss the mechanisms underlying specifically mammalian circadian clock in more detail.

1.2 Circadian clock in mammalian physiology

In mammals, circadian clock operates in most of the tissues and organs. Circadian systems identified to date are comprised of three major components: light input pathway, central pacemaker entrainable by light and the output pathways by which the circadian pacemaker regulates the rhythms in biochemistry, physiology, and behavior throughout the organism (Lowrey & Takahashi, 2004).

*Central Clock*

In mammals, the central pacemaker is located in the suprachiasmatic nucleus (SCN) of the hypothalamus and it is entrained by the periodic light/dark cycle (Ralph et al., 1990). Retina receives the light input with the help of rods, cones and recently discovered melanopsin-containing ganglion cell photoreceptors which help to entrain
central clock even with the absence of rods and cones (Berson, 2003; Lowrey & Takahashi, 2004; Panda et al., 2003; Rollag et al., 2003). Photic information received by the retina is projected to the hypothalamus via the retinohypothalamic tract – the neural projections terminate in the bilaterally-paired suprachiasmatic nuclei of the anterior hypothalamus (Lowrey & Takahashi, 2004). Each suprachiasmatic nucleus (SCN) contains approximately 8–10,000 neurons (van den Pol, 1980). Neurons within the SCN are coupled to form an ensemble expressing synchronized circadian rhythms of spontaneous electrical activity, calcium oscillations, humoral output, metabolic activity, and gene expression, with distinct spatial and temporal properties (Lowrey & Takahashi, 2004). Central clock regulates the clocks in other organs (called peripheral clocks) (Green et al., 2008; Potter et al., 2016; Reppert & Weaver, 2002). Light information entering the SCN is transduced into neural and humoral output signals that influence various rhythms in the body including temperature, levels of activity and endocrine oscillations in hormone secretion (Lowrey & Takahashi, 2004).

**Central clock and wake/sleep cycles**

In response to photic stimuli, the SCN generates circadian signal which is transmitted down the multisynaptic pathway by the series of projections from SCN to the parvocellular autonomic component of the paraventricular nucleus, paraventricular nucleus projections to the upper thoracic intermediolateral cell column, preganglionic sympathetic fibers to the superior cervical ganglion and postganglionic sympathetic fibers from the superior cervical ganglion to the pineal gland which regulates norepinephrine release and melatonin synthesis (Klein, 2007; Moore, 1996; Potter et al., 2016). Exposure to light suppresses melatonin synthesis by the pineal gland. This effect also holds true in
case of exposure to artificial light during night time which can disrupt the rhythms in SCN clock and melatonin release (Potter et al., 2016).

Melatonin is essential for regulation of endocrine rhythms in mammals which have to adjust their physiology according to seasonal changes in day length, temperature, and other environmental changes. Melatonin conveys photoperiodic information to the pituitary *pars tuberalis* (PT), by changing the expression of some clock genes in PT which is an important relay for the regulation of seasonal rhythms in mammalian physiology – for example changes in body mass and reproductive activity (Johnston et al., 2006; Sáenz de Miera et al., 2014). Although melatonin rhythm in humans was shown to be entrained by the seasonal changes in day-night length (Slawik et al., 2016), there was a strong evidence in a later study that the presence of artificial lighting suppresses seasonal changes in melatonin circadian rhythms that might be present in natural environmental conditions (Wehr et al., 1995). Because the SCN also have the melatonin receptors, melatonin feeds back to the master clock (Potter et al., 2016). Sleep is naturally gated to specific circadian phases and the artificial shift of sleeping period to any other specific circadian phase influences the continuity, duration and architecture of sleep (Dijk & Czeisler, 1995).

Still, the interaction between sleeping cycles and circadian rhythms in central and peripheral clocks remains complex and not fully investigated. An important concept was introduced by Borbély et al. who proposed the interaction of sleep cycle with circadian rhythm as a two – process model, which posits that a homeostatic process (Process S) continuously interacts with a process controlled by the circadian pacemaker (Process C),
with time-courses derived from physiological and behavioural variables (Borbély, 1982; Borbély et al., 2016).

To sum up the described below evidences it’s important to understand that the rhythms of sleep/wake cycle and circadian rhythms are the two separate processes, however the artificial changes and shifts of sleep/wake cycle might influence systemic circadian rhythm in both SCN (through the exposure to artificial light at night and social activities) and peripheral clock, as sleeping patterns tend to also alternate time of food intake. The synchronization between sleep/wake cycle and circadian rhythm in the central and peripheral clock is essential for metabolic optimization, as sleep/fast and wake/feed phases segregate antagonistic anabolic and catabolic metabolic processes in peripheral tissues (Borbély et al., 2016).

Peripheral Clock

The peripheral clocks generate rhythms in gene expression and metabolism, they are proposed to synchronize the activity of the peripheral organs, enhancing the functional message of the central clock (Buijs & Kalsbeek, 2001). While the central clock in the SCN plays a key role in entraining circadian rhythms of the whole organism to the solar day many studies revealed the existence of the SCN-independent self-sustained clock which drives circadian rhythms in various peripheral tissues throughout the body (Green et al., 2008).

The core molecular composition of circadian clock (which is going to be discussed in detail in the next part of the chapter) is the same in all of the tissues, however it was established that the peripheral tissues are capable of generating self-sustained circadian oscillations in gene expression – transgenic animals containing
luciferase reporter constructs driven by the promoters of cycling clock genes were utilized to achieve this discovery (Yamazaki et al., 2000; Seung-hee Yoo et al., 2005). In addition, peripheral organs expressed tissue-specific differences in circadian period and phase (Seung-hee Yoo et al., 2005).

These findings suggest the existence of other regulatory pathways that could influence the rhythms in the peripheral clock. Indeed, the same idea is also supported by the study of Kornmann et al. that introduced a transgenic mouse strain in which hepatocyte clocks only operated when the tetracycline analog doxycycline was added to the food or drinking water. The genome-wide mapping analysis revealed a handful of genes which still had cyclic expression profiles even when doxycycline was not present (and hence, hepatocyte clock was not operative) (Kornmann et al., 2007). With light being the primary Zeitgeber (external cue) for the central clock in the SCN, peripheral clocks receive the inputs from the other cues, such as feeding regimen (Buijs et al., 2013; Green et al., 2008; Patel et al., 2016).

The time of access to food is essential for maintaining the synchrony between central and peripheral clocks. It was previously reported that if the access to food is provided outside of the physiologically optimal time frame (which is during the day for diurnal and during the night for nocturnal animals), the circadian rhythms in the liver cells get desynchronized with the central clock which is entrained by the solar light cycle. Desynchronization between centrally and peripherally dictated rhythms is disadvantageous for health. Chronic desynchronization promotes pathologies such as the metabolic syndrome, which is characterized by the progressive onset of hypertension, insulin resistance, and diabetes (Buijs et al., 2013). A partial explanation of these adverse
effects might be attributed to the involvement of molecular clock machinery in the regulation of the rhythms in expression of various metabolic genes and enzymes (Chaudhari et al., 2017).

Another evidence showing the effects of food entrainment on the independent peripheral oscillator is the uncoupling of the rhythms in behavior and physiology from light-dark cycles and aligning them with predictable daily mealtimes. These food-entrained rhythms persist even in mice with clock-gene mutations that disable the SCN pacemaker (Mistlberger & Antle, 2011), again suggesting the presence of complex regulation of peripheral clocks by interlocking with metabolic pathways.

*Central and peripheral clock interactions*

The complex interaction between central and peripheral clock is still remaining under investigation. The presence or absence of light, as well as daily oscillations in temperature in the environment entrain the central pacemaker in the SCN to control the circadian rhythm of body temperature, which is an important synchronizer of clocks in the peripheral tissues. The artificial changes in temperature throughout the day/night cycle from the use of thermostats can level out the daily oscillations in temperature, which might influence circadian rhythms. In addition to temperature mechanisms, the SCN resets clocks in peripheral tissues through neural signals conducted via the autonomic nervous system (ANS), as well as the timely secretion of signaling factors such as prokineticin 2. The hormonal regulation of the circadian system is controlled via the hypothalamic-pituitary-peripheral organ axes. For example, corticotropin-releasing hormone (CRH) enters the portal system through the median eminence (ME) of the hypothalamus and stimulates the secretion of adrenocorticotropic hormone (ACTH) by
the anterior pituitary gland. ACTH then regulates adrenal cortex production of cortisol, a hormone with a robust circadian oscillation and important synchronizing effects in many peripheral clocks. The timing of metabolic processes in peripheral clocks is also modified by nutritional status, and peripheral clocks relay metabolic information back to the hypothalamus through the ME (Potter et al., 2016). In modern lifestyles, arrhythmic feeding and 24-hour availability of the access to food can distort the balanced feeding/fasting cycles and desynchronize the peripheral and central clocks from their natural day/night phases. For a better visual demonstration, the interactions between central and peripheral clock are briefly summarized in Figure 1-1 and Figure 1-2.
A central pacemaker is located in the SCN of the hypothalamus. It is entrained by the phases of presence and absence of light. Retina receives the light input and transmits the signal down the RHT tract to the SCN, which generates circadian rhythms in gene expression and transmits the signals to the peripheral tissues which are responsible for metabolic turnover, such as liver, muscles, adipose tissue, spleen and kidneys. This, in turn, creates the rhythms in physiological, metabolic and behavioral activities which are
referred to as circadian output. The clock in peripheral tissues can also be entrained to a specific rhythm independently from the central clock by feeding regimen and the rhythms in peripheral clocks can be uncoupled from the central clock if the feeding is artificially restricted to the phase different from the natural solar light/dark cycle. This figure was adapted from http://www.igis.com/igis-digest/xvith-igis-symposium/i-the-circadian-system/
Figure 1-2. The interaction between wake/sleep cycles, central and peripheral clock

Top left: absence or presence of light during solar cycle entrains the central clock in the SCN via RHT. The natural environmental temperature oscillations are able to entrain central clock to the 24 hours, however, the artificial use of the thermostats can eliminate this factor. Top right: the wake/sleep cycle is controlled by melatonin release from the pineal gland. Normally, the wake/sleep cycle aligns with the respective phase of circadian rhythm and the interaction is described as a two-process model. The exposure to light at night in diurnal animals can shift the phase of circadian rhythm as well as sleep/wake cycle and desynchronize it from the natural night and day. Center/Bottom right: peripheral clocks are receiving the signals from the central clock via ANS and secretion of signaling factors (i.e. prokineticin 2), as well as through hormonal regulation via hypothalamic-pituitary-peripheral axes. The alterations in sleep/fast, wake/feed phases can result in the consumption of food outside of the physiologically optimal time-frame, which will alter circadian rhythms in peripheral tissues. Upon metabolic changes provoked by the irregular timing of food intake peripheral clocks, in turn, will feedback to the central clock in the hypothalamus through the ME (median eminence). This figure is adapted from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5142605/

Considering our interest in linking metabolism with circadian rhythms and aging, throughout our adjacent projects in previous and current studies we decided to concentrate mainly on the peripheral clock in the liver tissue. We studied the effects of different feeding regimen on circadian gene expression in liver in the previous study, and now we are comparing these effects on different variables, such as sex and age of experimental animals. But before the discussion on the relevance of this particular study
for human health as well as the relevance of between-sex/age comparisons, it is essential to look at the molecular mechanisms underlying the clock in detail.

1.3 Molecular clock

Transcriptional control

The robust 24-hour rhythms in mammalian physiology are generated by the molecular clock which operates across the tissues. The molecular clock generates rhythms in gene expression through the interlocking transcriptional/translational feedback loops. Core circadian clock components are genes the protein products of which are necessary for the generation and regulation of circadian rhythms; that is, proteins which form the primary molecular circadian oscillatory mechanism within individual cells (Lowrey & Takahashi, 2011). Transcriptional activators which comprise the positive arm of the core clock loop are the circadian locomotor output cycles kaput (CLOCK) and brain and muscle aryl hydrocarbon receptor nuclear translocator-like 1 (BMAL1). CLOCK and BMAL1 share three regions which are essential for their function: one bHLH (basic Helix- Loop- Helix) and two PAS (Period-Arnt-Single-Minded) regions which are required for DNA binding and heterodimerization (Lowrey & Takahashi, 2011; Potter et al., 2016). The core clock loop begins its cycle during the day when BMAL1 and CLOCK heterodimerize, translocate to the nucleus and initiate transcription of the genes containing E-box (5′-CACGTG-3’) or E′-box (5′-CACGTT-3’) cis-regulatory elements, including the *Period* (Per1, Per2, Per3) and *Cryptochrome* (Cry1, Cry2) genes and other clock controlled genes(CCGs) (M. K. Bunger et al., 2000; Gekakis et al., 1998; King et al., 1997; Kume et al., 1999; Seung-hee Yoo et al., 2005; Zheng et al., 2001). It is important to mention that in the forebrain the neuronal PAS-domain protein (NPAS2)
performs the function of CLOCK (Reick et al., 2001). In the SCN as well as some peripheral tissues NPAS2 was shown to at least partially substitute for CLOCK in its function (Landgraf et al., 2016).

The main components of the negative arm of circadian core clock loop are the products of CCGs named PERIOD (PER1, PER2) and CRY (CRY1, CRY2) proteins which are hypothesized to inhibit their own mRNA expression by repressing the transactivating function of the heterodimeric BMAL1-CLOCK complex (Griffin et al., 1999; Kume et al., 1999; Lee et al., 2001; Sato et al., 2006). PERs and CRYs accumulate in the cytoplasm, multimerize upon reaching a certain protein concentration and translocate to the nucleus where they physically interact with BMAL1-CLOCK heterodimer during the phase of negative transcriptional feedback (Green et al., 2008; Lee et al., 2001; Lowrey & Takahashi, 2011; Sato et al., 2006).

Besides the core clock feedback loop, there are several additional loops of control of the circadian clock. The best-characterized one is the loop controlling Bmal1 expression which involves members of the large nuclear receptor family. The promoter sequence of Bmal1 gene contains two cognate REV-ERB/ROR-binding elements (ROREs) (Lowrey & Takahashi, 2011). In mammals 2 orphan nuclear receptor families: reverse erythroblastosis virus - REV-ERB (REV-ERBα, REV-ERBβ) and retinoic acid-related orphan receptor - ROR (RORα, RORβ, RORγ) control the expression of Bmal1 by competitive binding to their specific response elements (ROREs). In this transcriptional control loop, REV-ERBs are the repressors of Bmal1 across the tissues and RORs are the activators (Guillaumond et al., 2005; Preitner et al., 2002; Sato et al., 2004). The expression of Rors and Rev-Erbs, in turn, is controlled by BMAL1-CLOCK –
mediated induction via E-boxes in their promoters, which results in rhythmic oscillations in the expression levels of Ror and Rev-Erb genes (Yang et al., 2006). Other important regulators of Bmal1 expression are peroxisome proliferator-activated receptor-α (PPARα) and PPAR coactivator - 1 alpha (PGC-1α). PPARα is hypothesized to control the expression patterns of Bmal1 by binding to the PPARα-response element in the promoter sequence of Bmal1 gene. At the same time, BMAL1 itself is an upstream regulator of Pparα gene expression. PPARα is required for rhythmic expression of Bmal1 in both SCN and liver, which shows it is an essential factor for the maintenance of circadian rhythms in both master clock and peripheral clocks (Canaple et al., 2006). PGC-1α, in turn, is enhancing the RORα–mediated Bmal1 transcription. PGC-1α was shown to stimulate the expression of Bmal1 and Reb-Erbα through the coactivation of the ROR family of orphan nuclear receptors (Liu et al., 2007).

In short, the schematic representation of the major molecular clock feedback loops is outlined below in Figure 1-3.
Figure 1-3. Molecular components of the mammalian circadian clock

On a molecular level, circadian rhythms are generated through the activity of interacting feedback loops formed by a dozen of core clock genes and their products. Transcriptional factors CLOCK and BMAL1 dimerize and drive the expression of Period (Pers) and Cryptochrome (Crys) genes, as well as other clock-controlled genes (CCGs); PERs and CRYs proteins inhibit BMAL1-CLOCK transcriptional activity and, as a result, their own expression, thus forming the negative feedback loop. The products of another group of BMAL1-CLOCK regulated genes, Rev-Erbs and Rors, in turn negatively or positively control Bmal1 transcription by binding the ROREs in the promoter sequence of Bmal1 gene, therefore forming another negative and positive feedback loop. This figure is adapted from https://doi.org/10.1093/hmg/ddl207
**Translational/Post-translational control**

It is important to recognize several levels of regulation in molecular clock: while primarily circadian clock is regulated on the transcriptional level, numerous translational and post-translational modifications are also integrated into the circadian clock operation in order to provide the necessary robustness for 24h rhythms in gene expression. What is also important, lots of post-translational modifications involved in clock regulation provide a linking point between metabolism and the clock as many of such modifications rely on oscillations in metabolite substrates that are derived from intermediary metabolism (Ribas-latre & Eckel-mahan, 2016), which is going to be further discussed in detail in the next part of this chapter. One of the major evidence that translational and post-translational modifications play an important role in clock regulation was the fact that nearly half of the proteins which showed circadian expression profiles lacked a circadian oscillation in corresponding mRNA transcript, as determined by quantitative PCR, microarray, or both. Overall, up to 20% of soluble proteins assayed in mouse liver tissue turned out to be subject to circadian control, as was reported by (Reddy et al., 2006), suggesting another evidence that specifically peripheral clocks are strongly regulated at translational and post-translational levels. Such modifications include acetylation, phosphorylation, methylation, ubiquitination and SUMOylation (Robinson & Reddy, 2014).

In order to initiate a new cycle of transcription, it is required that the components of the negative arm - PERs and CRYs get degraded. For PER proteins the phosphorylation by Casein kinase (CK)1e and CK1δ, is required for further
ubiquitination and degradation by β-transducing-repeat-containing protein (βTrCP) and 26S proteasome respectively (Eide et al., 2005). CRY1, in turn, is phosphorylated by 5’ AMP-activated protein kinase 1 (AMPK1) (Lamia et al., 2009) and CRY2 - by a sequential dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1A(DYRK1A)/glycogen synthase kinase 3beta (GSK-3β) cascade (Kurabayashi et al., 2010), which targets it for ubiquitination and degradation by F-Box and Leucine-Rich Repeat Protein 3(FBXL3) (Xing et al., 2013; Yoo et al., 2013). BMAL1 is phosphorylated by CK1ɛ and GSK3β – specifically, by GSK3b on Ser 17 and Thr 21 which primes BMAL1 for ubiquitylation. This phosphorylation is essential for BMAL1 dependent circadian gene expression which in the absence of GSK3β activity gets dampened as BMAL1 is getting stabilized (Sahar et al., 2010).

BMAL1 is subjected to acetylation by CLOCK while PER2 and BMAL1 are both subjected to deacetylation by silent mating type information regulation 2 homolog - 1 (SIRT1). Acetylation of BMAL1 by CLOCK promotes the recruitment of CRY to BMAL1-CLOCK complex (Hirayama et al., 2007). Deacetylation by SIRT1 leads to repression of BMAL1 target gene expression. By direct binding to BMAL1-CLOCK in a circadian manner, SIRT1 promotes the deacetylation and degradation of PER2 (Asher et al., 2008). The NAD(+) dependence of SIRT1 deacetylase activity is also classifying SIRT1 as one of the puzzle pieces in connection of cellular metabolism to the circadian core clockwork circuitry.

One more recently discovered component of the post-translational control of molecular clock is E3 ubiquitin ligase UBE3A which binds to and triggers BMAL1 degradation in a ubiquitin ligase-dependent manner. UBE3A is well conserved and is
involved in endogenous control in the regulation of circadian dynamics and rhythmic locomotor behavior in both flies and mammals (Gossan et al., 2014).

SUMOylation was also reported to have a part in control of the molecular circadian clock. In the mouse liver, SUMOylation of BMAL1 has a circadian pattern that parallels its transcriptional activity – conjugation with SUMO2/3 peaked at the times of maximum E-box-mediated circadian transcription (Lee et al., 2008). Conjugation with SUMO2/3 promotes nuclear localization and ubiquitin-dependent degradation of BMAL1 (Cardone et al., 2005; R. V Kondratov et al., 2003).

Finally, epigenetic modification also plays an important role in the clock regulation. The histone-remodeling enzyme mixed lineage leukemia (MLL)3 was identified as a clock-controlled factor that directly and indirectly modulates over a hundred epigenetically targeted circadian "output" genes in the liver. The histone methyltransferase activity of MLL3 is required for robust transcriptional oscillator function as catalytic inactivation of this activity significantly compromises the oscillation of "core" clock gene promoters, including Bmal1, Cry1, Per2, and Rev-erba (Valekunja et al., 2013). Another isoform, MLL1 is essential for circadian transcription and cyclic H3K4 trimethylation. MLL1 is in a complex with BMAL1-CLOCK and contributes to its rhythmic recruitment to circadian promoters and to H3 acetylation (Katada & Sassone-Corsi, 2010). In addition, CLOCK was found to have histone acetyltransferase activity (HAT) which is essential is essential for restoration of circadian rhythmicity and activation of clock genes in Clock mutant cells (Doi et al., 2006).

While in our study we don’t directly address the mechanisms of post-translational control of molecular clock, it is necessary to take these factors into account when explaining the differences between gene and protein expression profiles for some
circadian clock genes, as the levels of mRNA are not directly correlating with the protein levels for particular genes at particular time points in a daily 24-hour cycle.

1.4 Circadian rhythms and metabolism

Circadian system is essential for the regulation of metabolic homeostasis. Multiple microarray studies demonstrated the gene expression profiles throughout the daily cycle in mammalian peripheral tissues such as liver, muscle, brown and white adipose tissues and up to one-fifth of the transcriptomes within those tissues were reported to be under circadian control. Multiple genes with circadian expression profiles were involved in various metabolic processes, such as lipid/cholesterol metabolism, glycolysis and gluconeogenesis, oxidative phosphorylation, and detoxification pathways (Green et al., 2008).

The idea of a presence of the direct link between the circadian clock and metabolic homeostasis is also supported by the fact that the expressions of many rate-limiting enzymes in metabolic pathways are also under circadian control (Panda et al., 2002). The complexity of the interaction between peripheral clocks and metabolism is manifested in the fact that the circadian phase of expression profiles of many genes is tissue-specific with the different degrees of overlap between different tissues (Storch et al., 2002).

Multiple molecular feedback loops link the molecular circadian clock to metabolic status. The control of transcription by BMAL1-CLOCK complex through E-boxes is extensive. Besides earlier described PPARα/BMAL1 feedback loop, there is another regulatory loop which involves differentially expressed in chondrocytes (Dec1 and Dec2) genes and their products. Dec1 and Dec2 are the target genes of the BMAL1-CLOCK
complex. Their transcription is induced in the same manner as PERs and CRYs – by binding of BMAL1-CLOCK heterodimer to the E-Box in their promoter sequences. DEC1 and DEC2, in turn, both basic helix–loop–helix transcription factors, bind directly to BMAL1-CLOCK, inhibiting its activity. Interestingly, Dec1 and Dec2 repressed Clock/Bmal1-induced transactivation of the mouse Per1 promoter through direct protein-protein interactions with Bmal1 and/or competition for E-box elements (Honma et al., 2002). Dec1 and Dec2 also contain RORE elements in their promoters, and the additional layer of regulation was reported with RORα (but not REVERBα) binding to ROREs of Dec1 and Dec2. Besides the involvement in clock regulation Dec1 and Dec2 play a role in adipogenic cell differentiation (Ozaki et al., 2012).

There are also several other BMAL1-CLOCK target genes related to metabolism which, as well, have regulatory feedback properties on the control of the circadian clock. These genes are nicotinamide phosphoribosyltransferase (Nampt), estrogen-related receptor alpha (Errα) and proper homeobox 1 (Proxl) (Ribas-latre & Eckel-mahan, 2016). Nampt is the rate-limiting enzyme that converts Nicotinamide (NAM) to Nicotinamide Mononucleotide (NMN) – this reaction is required for the intracellular salvage of Nicotinamide Adenine Dinucleotide (NAD⁺) (Magni et al., 1999). NAD⁺, in turn, is an essential molecule in metabolism that is required for activating a number of enzymes and transcriptional factors involved in multiple metabolic pathways. The NAD⁺-dependent sirtuin deacetylase, SIRT1, is the example of such factor, which binds directly to BMAL1-CLOCK and affects its transactivating activity. Thus, BMAL1-CLOCK induced activation of Nampt forms another negative feedback loop, which couples
cellular metabolites and their targets to the core circadian clock machinery (Nakahata et al., 2009; Ramsey et al., 2009).

The additional transcriptional regulatory loop involves BMAL1, ERRα and PROX1 (products of Errα and Prox1) genes respectively. ERRα plays an essential role in the control of energy metabolism, such as the modulation of insulin sensitivity and glucose handling during the 24-hour light/dark cycle, as well as the maintenance of physiological rhythms in the oscillations of bile acid and lipid profiles. The expression of ERRα is known to be cyclic in the liver (Dufour et al., 2011). ERRα and PROX1 are involved in regulation of multiple metabolic pathways and share a handful of target genes with BMAL1. ERRα down-regulates Bmal1 expression, while its co-repressor PROX1 blocks this repression. At the same time, the interactions between ERRα and PROX1 affect the robustness in circadian rhythms of some clock target genes including Per2, Cry1, Rev-erb-α and Rev-erb-β (Dufour et al., 2011; Ribas-latre & Eckel-mahan, 2016).

It is important to mention a group of genes which are linked to metabolic pathways and are, as well, BMAL1-CLOCK targets, however, they don’t have a direct feedback on the regulation of circadian clock with one exception. Aminolevulinic acid synthase 1 (Alas1), plasminogen activator inhibitor-1 (Pai-1) and thyroid hormone receptor alpha (Tra), are involved in heme biosynthesis, thrombosis and vascular tone regulation respectively (Bailey et al., 2014; Paschos & FitzGerald, 2010; Ribas-latre & Eckel-mahan, 2016). Thyrotroph embryonic factor (Tef), hepatic leukemia factor (Hlf) and the product of nuclear factor, interleukin 3 regulated (Nfil3) gene - E4BP4 have important
regulatory functions activating downstream metabolic target genes through direct binding to D-boxes (Mitsui et al., 2001; Yoshitane et al., 2014). The above-mentioned exception in this group of genes is Dbp, the product of which is the proline and acidic amino acid–rich (PAR) basic leucine zipper (bZip) transcription factor albumin gene D-site binding protein (DBP). DBP stimulates the expression of Per1 by binding to the promoter. At the same time, Dbp transcription is regulated by BMAL1-CLOCK and PER/CRY. Therefore, a clock-controlled Dbp gene has a direct feedback in the regulation of molecular clock (Mitsui et al., 2001; Yamaguchi et al., 2000).

Further investigations on the connections of clock mechanisms with metabolism revealed that not only Bmal1 and Clock but also virtually all of the circadian genes and their products that comprise core clock regulatory loops also possess specific individual functions related to homeostatic control of metabolism (Ribas-latre & Eckel-mahan, 2016). For example, PER2 also acts as a repressor of the nuclear receptor PPARγ which plays an essential role in adipogenesis, insulin sensitivity, and inflammatory response. PER2 is required for normal lipid metabolism in white adipocyte tissue as was shown by lipidomic profiling (Grimaldi et al., 2010). REV-ERBα is playing an essential role as a heme-sensor and mediates the suppression of hepatic gluconeogenic gene expression and glucose output by heme (Yin et al., 2007). REV-ERBα directly regulates the nuclear receptor heme-binding protein (Shp) and, as well takes part in direct regulation of several gluconeogenic enzymes like glucose-6-phosphatase (G6Pase), phosphoenolpyruvate carboxykinase (Pepek), and nuclear factor interleukin 3 (Nfil3) (the gene coding for E4BP4) through RORE (Feng & Lazar, 2012).
In addition, physiological rhythms in lipid processing were also established - rate-limiting steps of fatty acid oxidation and synthesis, as well as cholesterol and bile acid biosynthesis, are under circadian control in the liver. This is reflected by distinct circadian patterns in the profiles of mRNA or protein expression of the fatty acid transporter carnitine-palmitoyl transferase 1 (CPT-1), the membrane-bound transcription factor sterol regulatory element-binding protein (SREBP)-1c and the rate-limiting enzymes 3-hydroxy-3-methyl-glutaryl-Coenzyme A reductase (HMGCR) and cholesterol 7α-hydroxylase (CYP7A1) (Gilardi et al., 2013; Gooley & Chua, 2014).

Such an interaction between circadian clock regulation and metabolism made the research advance in the direction to answer several more questions: how might the energy status, nutritional availability throughout the day and feeding regimen influence the clocks and circadian rhythms in the physiology of peripheral tissues, such as liver and adipose tissue? Will these signals influence circadian rhythms in the central oscillator in the SCN? What consequences will be caused by a disruption to the physiological rhythms in the peripheral clocks?

As mentioned earlier, the peripheral clocks can be uncoupled from the central clock by the feeding regimen. While the central pacemaker in the SCN is mainly entrained by the presence of light and remains relatively uninfluenced by the particular scheduling in food availability, the peripheral clock, especially in the liver, is relying on the pattern of the food intake as the main Zeitgeber (Green et al., 2008).

Some mechanisms of response to nutrients and metabolites have been identified to have a direct or indirect role in clock regulation. The report on nuclear receptor
expression revealed that more than half of the nuclear receptors (including RORs and REV-ERBs) which act as sensors for fat-soluble hormones, vitamins, and dietary lipids, show rhythmic expression profiles in white and brown adipose tissue, liver, and skeletal muscle (Yang et al., 2006).

One of the indirect food entrainment – related mechanisms that can influence the circadian clock comes from the changes of the cellular redox state induced by particular feeding regimen. The reduced forms of the redox cofactors, NADH and NADPH, were shown to strongly stimulate DNA binding activity of the BMAL1-CLOCK (and BMAL1-NPAS2) heterodimers, while the oxidized forms inhibited this activity. The switch from active to inactive forms of CLOCK/BMAL1 (or NPAS2/BMAL1) occurs over a specific range of reduced to oxidized NAD ratios which change during feeding/fasting phases (Rutter et al., 2001). Similarly, cellular AMP:ATP ratios change in a circadian manner throughout the wake/feeding, rest/fasting phases of the daily cycle. Adenosine monophosphate (AMP)-activated protein kinase (AMPK) functions as a detector of this ratio and promotes the rhythmic activation of metabolic pathways in response to increasing AMP levels. The molecular targets of AMPK are involved in a wide range of metabolic processes like glucose uptake, synthesis of glycogen and protein, glycolysis, gluconeogenesis and fatty acid oxidation or synthesis (Fan et al., 2011). Among these targets are also CRY and CKε proteins the phosphorylation of which is also induced by AMPK and might as well indirectly contribute to the degradation of PERs. AMPK gets activated primarily during fasting phase or during intense periods of activity when catabolic processes are essential for the maintenance of the energy balance. When enhanced rates of ATP production are required AMPK triggers ATP synthesis via
enhancing oxidative metabolism and mitochondrial biogenesis. As a result, the levels of NAD+ increase and trigger the activation of earlier mentioned NAD+ - dependent SIRT1, which in turn induces BMAL1 and PER protein deacetylation (with subsequent inactivation of BMAL1, CRY:PER destabilization and further degradation). In addition, SIRT1 indirectly activates RORα by deacetylation of PGC-1α (Cantó et al., 2009). RORα, in turn, triggers the transcription of Bmal1 by binding to RORE in the promoter sequence. Over time, increased activity of SIRT1 results in accumulation of NAM and inhibition of NAD+ formation with a subsequent decrease in the SIRT1 activity (Froy & Miskin, 2010).

Therefore we can see that the regulation of circadian rhythms on a molecular level in peripheral tissues tightly interconnects with the cellular metabolic state during a particular phase of the day, which also depends on the presence of nutrients and time of nutrient uptake. Physiological rhythms of food intake usually synchronize with the wake/activity phase of the day which is followed by sleep/fast phase. This also allows for the synchronization of the circadian rhythms between SCN and periphery. Some evidence shows that such synchronization is essential for the homeostatic optimum of energy balance and the asynchrony between the central and peripheral clock might have adverse effects on health. The modern urban lifestyle of humans, however, in many aspects does not contribute to the natural synchronization between the environmental day/night cycles and physical activity. The irregular patterns of activity provoked by social (around the clock work shifts, nightlife) as well as technological factors (artificial light, heating, artificial food) can lead to the changes in both sleep/wake cycles and timing of food consumption as well as the overall caloric intake. In order to investigate the influence of
these factors on human health, numerous animal model studies with alternate lighting conditions or feeding regimen were launched. But before reviewing the current discoveries based on those studies it is necessary to mention why disturbances to the physiological rhythms are a point of concern when we talk about human health.

1.5 Circadian clock and human health

Epidemiological data on the risk of development of many diseases such as metabolic syndrome, diabetes, cardiovascular diseases and cancer in humans (Fu et al., 2002; Reppert & Weaver, 2002; Sahar & Sassone-Corsi, 2012; Touitou et al., 2017), as well as the development of similar pathological phenotypes in circadian mutants in animal models support the importance of the circadian clocks and rhythms for health (Krizo & Mintz, 2014; Lowrey & Takahashi, 2004; Oosterman et al., 2015; Sato et al., 2006). In particular, shift work and social jetlag (which are the major desynchronizers of biological clocks in humans) have correlations with an increased risk of cancer, cardiovascular disease, sleep disturbances, impaired psychosocial health and gastrointestinal problems (Opperhuizen et al., 2015; Roenneberg et al., 2012). In most of the studies, the term “shift work” refers to the work times outside of the frame of the natural physiological phase of activity during the day – either it is work during the night time every day, or rotating shift, under which people have to switch their working timings between night and day periodically. In either scenario, shift work is creating a disturbance to circadian rhythms and this disturbance is manifested in several variables: the altered timing period of activity, the altered timing of food consumption and the number of consumed calories, the changes in duration and timing of sleep. All of these variables are considered to have a contribution to the earlier listed adverse effects.
Several health conditions which are primarily associated with chronic disturbances of the circadian clock and very often accompany each other are hypertension, obesity, and type-2 diabetes. Such changes as an increased body mass index (BMI), dyslipidemia, insulin resistance and impaired beta-cell function in the pancreas are all attributed to the risk factors for cardiovascular disease (Sirimon Reutrakul & Knutson, 2015). Interestingly, one of the studies reported that shift work is more likely to trigger the onset of type – 2 diabetes in women than in men (Eriksson et al., 2013). Another study suggested that shift work promotes insulin resistance in workers younger than 50 years old, but not in older workers, however, this relationship might be underestimated (Nagaya et al., 2002). Some experimental studies on circadian misalignment performed on human subjects with the utilization of the irregular eating and sleeping schedules linked disrupted rhythms with the increased serum glucose, despite increased insulin levels, increased mean arterial pressure, decreased leptin and reduced sleep efficiency (Scheer et al., 2009). Interestingly, prolonged sleep deprivation alone can lead to a decrease in the resting metabolic rate and increased plasma glucose concentrations after a meal - the effect which is linked to an inadequate pancreatic insulin secretion. These symptoms were shown to resolve after subsequent circadian entrainment and the recovery of normal rhythmic wake/sleep cycle (Buxton et al., 2012).

Several studies established an association between shift work and several types of cancers, such as breast, prostate, endometrial and colorectal cancers (Erren et al., 2009; Reszka & Przybek, 2016; Touitou et al., 2017; Viswanathan et al., 2007). The study on colorectal cancer and colorectal liver metastases revealed that 5 core clock genes were downregulated in colorectal cancer tissue samples (Bmal1, Per1, Per2, Per3, and Cry2).
In colorectal liver metastases samples, however, 7 clock genes were found to be significantly downregulated (Clock, Bmal1, Per1, Per2, Per3, Cry1, and Cry2) (Huisman et al., 2016). Some clock-controlled genes are involved in the control of cell cycle and proliferation, DNA damage, and apoptosis. Disturbance of circadian clock can lead to deregulation of the cell cycle which can promote carcinogenesis (Huisman et al., 2016).

Even though particular mechanisms are still under ongoing investigation, it is apparent that for each type of these cancers shift work is a multifactorial trigger which might influence the onset. Particularly, the first and foremost factor is the exposure to artificial light at night which de-regulates the rhythms in the central clock and dampens melatonin release. Melatonin has several oncostatic properties, including possible anti-estrogenic and anti-aromatase activity, as well as a role in fat metabolism. Therefore, lowered levels of melatonin might contribute to the carcinogenic effects of night time activity. In case of endometrial cancer which is influenced significantly by hormonal and metabolic factors it was established that obesity, when paired with shift work, is a particularly strong risk factor for the onset of cancer (almost doubles the risk if compared to obese women who don’t work during night time) (Viswanathan et al., 2007). At the same time night shift work can promote obesity through irregular feeding time during the biological night and the changes in duration and timing of sleep (Salgado-Delgado et al., 2010). There is also a direct correlation between time spent working on night/rotating shift schedule and the magnitude of risk for both the increased body mass index (BMI) and the onset of cancer (Kubo et al., 2011; Viswanathan et al., 2007). Also, chronic sleep deprivation which often accompanies the lifestyle under shift work can itself have a carcinogenic effect - as was shown on the nurses who worked on sustained night shift
schedule and had over 50% higher incidence of breast cancer (Touitou et al., 2017). Therefore it is fair to conclude that carcinogenic effects are significantly dependent on the de-regulation of circadian rhythms in metabolism, and rest/activity cycles.

Based on the existing evidence we can see a complex interaction of human circadian rhythms in the central and peripheral clock. The challenges to these rhythms are strongly provoked by the aspects of modern lifestyle and in long term can be detrimental to health. The around – the – clock lifestyle, exposure to artificial light and heating, reduced energy expenditure due to sedentary job types along with irregular feeding are all the factors that contribute to the desynchronization within the central and peripheral clocks and dampening of the natural biological rhythms which are supposed to be aligned with the solar night and day. While the described earlier evidence supports the concept that lots of metabolic conditions, altered activity and sleep cycles are triggered by purely artificial or modern social factors, the last, but not least, is the genetic factor, which can be responsible for the alterations in the normal operation of circadian clock in humans. For the investigations of the effects of circadian disruption on genetic level, several circadian clock mutant mouse models were used in the field of circadian research.

1.6 Mutant animal models for circadian clock studies

As it was mentioned in the previous part of the chapter, the normal physiological operation of circadian clock is essential for the maintenance of healthy state and energy balance. We also mentioned the study of human colorectal cancer and involvement of circadian clock disruption in liver metastases from that cancer – multiple clock genes showed altered circadian rhythms and downregulation in expression in original and metastatic cancer samples (Huisman et al., 2016). These findings add to the current
knowledge base, as multiple mutant studies have previously linked mutations/altered clock gene expression to various specific disorders. The deletion of each of core clock genes (or, in some cases, point mutation) resulted in a specific phenotype with a particular physiological malfunction—very often in both circadian rhythms, development and metabolic homeostasis as many genes involved in metabolic and other physiological pathways are at least partially controlled by clock genes.

The deficiency of the gene coding for the core clock factor BMAL1 in the whole body Bmal1 knock out (Bmal1-/-) mice results in complete loss of circadian rhythmicity in behavior and expression of target genes, premature aging and the early onset of age-related diseases. One of the studies which was performed earlier in our lab showed that although Bmal1-/- mouse pups are not different from wild-type (WT) littermates at birth, the early signs of aging in Bmal1-/- might start displaying already at around 16-18 weeks of age while the total lifespan of Bmal1-/- mice was ranging from 26 to 52 weeks of age (37 weeks in average) (Kondratov et al., 2006). Considering the lifespan of C57BL/6J WT mice that were used for the study is over 2 years (100-120 weeks), Bmal1-/- mice have more than two-fold shorter lifespan. The deletion of Bmal1 also leads to the development of multiple severe phenotypic changes associated with decreased longevity. Bmal1-/- mice displayed a decreased muscle and adipose tissue mass. The bone mass, mineral density, numbers of active osteoblasts and osteocytes were also decreased in Bmal1-/- mice. By 10 weeks of age testicular size in Bmal1-/- male mice was significantly reduced compared to WT males. Infertility in Bmal1-/- was observed in both males and females (Boden et al., 2010; Kondratov et al., 2006). By 40 weeks of age, the decreased size and mass was also observed in spleen, kidney, heart, and lungs in the
knockout mice without the degenerative lesions in tissue structure. This systemic decrease of organs in size without acute lesions is an event, normally observed in aged WT mice (Kondratov et al., 2006). Other effects of Bmal1 deletion are the early onset of progressive arthropathy (Bunger et al., 2005), early onset of cataracts and decreased hair re-growth (Kondratov et al., 2006), suppressed diurnal variation in glucose and triglycerides and abolished gluconeogenesis (Rudic et al., 2004).

Deficiency of Bmal1 also causes chronic oxidative stress, as BMAL1 is involved in the control of tissue homeostasis by the direct regulation of reactive oxygen species (ROS). It is established that the elevated oxidative stress contributes to the onset of many life-threatening diseases, including cardiovascular disease, cancer, and diabetes. At the same time, the administration of antioxidant N-acetyl-L-cysteine to Bmal1/- mice in drinking water throughout their entire life significantly increased average and maximal lifespan and reduced the rate of age-dependent weight loss and development of cataracts (Kondratov et al., 2009). Another earlier study performed two tissue-specific rescue experiments with Bmal1/- mice - circadian rhythms of wheel-running activity were restored in brain-rescued Bmal1/- mice in a conditional manner, however the body weight of these animals was still lower than in WT mice. Restoration of Bmal1/- expression in muscle, in turn, prevented weight loss, but, irrespectively of the normal activity levels, the behavior of these mice remained arrhythmic (Mcdearmon et al., 2006). All of these findings confirm that Bmal1 is a vital gene for circadian rhythmicity in the central and peripheral clock. Bmal1/BMAL1 is also required in the mediation of longevity through its tissue-specific ROS-protective activity and metabolic regulation.
To study the consequences of disruption of the normal Clock/CLOCK gene and protein functioning, two different mouse models were used in research – Clock-deficient mice (Clock−/−) and Clock<sup>Δ19/Δ19</sup> mutant mice (Debruyne et al., 2006; Dubrovsky et al., 2010; Gekakis et al., 1998; King et al., 1997). Clock<sup>Δ19/Δ19</sup> mutants have an intronic splice site point mutation in the Clock gene which results in excluding exon 19 from the transcript. The mutant CLOCK protein has a preserved ability to bind BMAL1, however, its transcriptional activity is halted. Complementation analysis of Clock/Clock-W19H mutant compound genotypes showed that the Clock mutation behaves as an antimorph (Gekakis et al., 1998; King et al., 1997). The Clock<sup>Δ19/Δ19</sup> mutants did not show significant difference either in longevity, or in the predisposition to neoplasia as compared to wild-type animals. Unlike in Bmal<sup>−/−</sup> mice, Clock<sup>Δ19/Δ19</sup> mutants did not show the reduction in weight, on the contrary, these mutants were 10-20% heavier than wild-type mice (Antoch et al., 2008). Also, unlike in Bmal<sup>−/−</sup> mice, Clock<sup>Δ19/Δ19</sup> mutants did not show completely abolished gluconeogenesis, however, the trend towards reduced gluconeogenesis was still present (Rudic et al., 2004). To test the possible role of the CLOCK protein in predisposition to tumor formation and the efficiency to tolerate radiation, wild-type, and Clock<sup>Δ19/Δ19</sup> mutant mice were exposed to low-dose radiation for a long period of time. Interestingly, upon long-term exposure to radiation Clock<sup>Δ19/Δ19</sup> mutant mice demonstrated a sex-specific difference in response to this treatment – female mutants had accelerated mortality and lower survival rates than their wild-type littermates but no difference in survival rates was observed between male mutants and wild-types. At the same time, the accelerated weight loss (similar to what is observed in aged wild-type mice) upon exposure to long-term radiation was observed in both sexes of mutant mice.
(Antoch et al., 2008). This finding is of particular interest for our current study which is going to be discussed further in this chapter as such sex-based difference is one of the pieces of evidence that the involvement of clock genes in a control of acute radiation response and, possibly, indirectly aging, is different between sexes. Unlike Bmal1/-mice, Clock^{Δ19/Δ19} mutants were fertile and had relatively normal reproductive capabilities in both sexes. The minor impairments involved only the lengthened period of mating and delivery, as well as slightly reduced litter size and survival to weaning (Kennaway et al., 2004). Mildly reduced male (in vivo) fertility and irregular estrous cycles in females exacerbated by continuous darkness were also observed in Clock^{Δ19/Δ19} (Dolatshad et al., 2006). In contrast to Clock^{Δ19/Δ19} mutants, Clock/CLOCK-deficient mice (Clock/-) show more severe phenotypic abnormalities. Clock/- mice have altered responses to light and even though their locomotor activity remains rhythmic, these mice have shortened lifespan, develop dermatitis and the early onset of cataracts (Dubrovsky et al., 2010). The Clock/- mice are fertile, unlike Bmal/-, but females display slightly lower reproductive success. Circadian rhythms in the SCN are still present in Clock/-, however, the alterations in rhythms are observed in peripheral tissues, which might suggest that Clock/CLOCK particularly plays an important role in the control of the peripheral clock (Debruyne et al., 2006).

Deficiency in Periods was also studied using several knock-out and mutant models, which also helped to get some knowledge on a possible non-redundancy of PER1, PER2 and PER3 functions. For instance, Per1 deficiency resulted in a 1-hour shortening of the period of locomotor activity and the delay in expression of clock genes (Per1 and Per2) in peripheral tissues (Cermakian et al., 2001). Another study used Per1
mutant mice and found that this mutation resulted in impaired daily glucocorticoid rhythm with significantly elevated levels during the day compared to control wild-type animals. In addition, Per1 (Brd) mice were significantly lighter than wild-type, however, food and water intake per gram of body mass was elevated in mutants. Interestingly, these particular symptoms were not observed in Per2 (Brd) mutants besides altered glycemia (Dallmann et al., 2006). The deficiency in Per2 gene resulted in dampening of the rhythms in mRNA profiles of clock gene expression in mouse SCN, which was not the case with the Per1 knockouts (Bae et al., 2001). It is worth noting that the phosphorylation site mutation (S662G) in the human Per2 gene specifically (hPer2) results in the familial advanced sleep phase syndrome (FASPS) – a condition in which affected individuals have a 4-hour advance in sleep, temperature, and melatonin rhythms. In this case, serine is substituted with glycine within the casein kinase I-ε (CKI-ε) binding region of hPER2, resulting in hypophosphorylation by CKI-ε in vitro (Toh et al., 2001). The deficiency in both Per1 and Per2 genes and their products results in complete loss of circadian rhythms in gene expression and behavior (Zheng et al., 2001). Per1,2-/mice also show some symptoms of accelerated aging such as decreased reproductive success in middle-aged female mice, reduction in soft tissues and impaired cell cycle regulation (Bae et al., 2001; Lee et al., 2010; Pilorz & Steinlechner, 2008; Zheng et al., 2001). Per1 and Per2 genes are considered to be essential for tumor suppression. Per1 and Per2 – deficient mice all show increased spontaneous and radiation-induced tumor development (Lee et al., 2010). Particularly, a mutation in Per2 gene was shown to promote cell proliferation and tumor formation through the deregulation of cell cycle control (L. Fu et al., 2002; Wood et al., 2014). On the other note, multiple studies showed
that the overexpression of Per2 resulted in tumor suppression (Hua et al., 2007; Oda et al., 2009). The current knowledge on the interaction between Per1/PER1 and Per2/PER2 and their involvement in circadian control suggests that PER2 regulates clock gene expression at the transcriptional level, while PER1 may instead regulate mPER2 at a posttranscriptional level. Studies of clock-controlled genes (CCGs) suggest that PER1 and PER2 proteins might independently control some output pathways (Zheng et al., 2001). Therefore PER1 and PER2 have both distinct and complementary roles in circadian clock mechanisms. Per3 gene and its product are classified outside of the core clock machinery, as no difference in behavioral rhythms was observed between Per1/Per3 or Per2/Per3 double-mutant mice and mice with mutated versions of Per1 or Per2 alone (Bae et al., 2001). However, one of the recent studies suggested that Per3 might still have a distinct role in body mass control with a connection to lipid metabolism. Targeted disruption of all three Period genes (Per1,2,3) simultaneously resulted in significant increase in body weight on high–fat diet in Per1,2,3 mutants compared to wild-type control mice on the same diet. The mice which had the same mutation in Per3 gene only, however, mimicked the phenotype of Per1,2,3 mutants under the same conditions (Dallmann & Weaver, 2010).

The deficiency in Cry1, Cry2 genes and their products result in accelerated and delayed free-running period in locomotor activity, respectively. Cry1 deficiency alone was found to promote dermatitis. The simultaneous absence of both CRY1 and CRY2 proteins results in complete loss of circadian rhythm (van der Horst et al., 1999). Cry1,2/- mice also display decreased body weight, decreased body and organ size (Chaudhari et al., 2017). The role of Cryptochromes in tumorigenesis is somewhat controversial. One of
the studies reported increased tumor incidence in *Cry1-/-* and *Cry1,2-/-* mice under both normal conditions and irradiation, suggesting *Cry1* alone might play a role in tumor suppression (Lee et al., 2010). However, in cancer-prone *p53−/−* mouse model the simultaneous deficiency in both *Cry1* and *Cry2* results in resistance to cancer and increase in lifespan (Ozturk et al., 2009), which leaves the question about Cryptochrome involvement in the control of tumorigenesis open.

Finally, a knockout and a mutant mouse model were used to study one more important factor – casein kinase I – epsilon (CKIε). The deficiency in CKIε resulted in the lengthening of circadian period, whereas CKIε(tau/tau) mutation shortened circadian period of behavior in vivo and suprachiasmatic nucleus firing rates in vitro, by accelerating PER-dependent molecular feedback loops – the degradation of PER proteins was accelerated in both SCN and in peripheral tissues (Meng et al., 2008).

The findings based on the studies involving circadian clock mutant animal models are fundamental for understanding the complex involvement of each of circadian genes and proteins in many physiological functions which are important for the homeostasis and longevity. The disruption to the circadian clock on genomic level leads to decreased lifespan due to the development of various phenotypic conditions. While the innovative directions in scientific research address the issues of the genetic effects and continue to develop the techniques for the correction of those defects, our study concentrates on conservative manipulation of the circadian clock. As mentioned earlier, the operation of the clock, especially in peripheral tissues is heavily interconnected with metabolic pathways. There is now evidence which shows that feeding is an important external cue which can influence the circadian rhythms in physiology and behavior, as well as impact
the internal circadian alignment between central and peripheral clocks. Therefore, we are going to make a brief overview of the influence of feeding patterns on circadian rhythms.

### 1.7 Feeding Regimen and Circadian Rhythms

The importance of rhythmicity in feeding and its contribution to the maintenance of metabolic homeostasis, as well as the synchronization of the central and peripheral clock was first recognized with the conceptual discovery of the food-entrainable oscillator (FEO). One of the pioneering methods in this discovery was the introduction of the time-restricted feeding regimen (TR). The amount of food normally consumed by mice throughout the day under ad-libitum condition (with the unlimited access to food throughout 24 hours) was now provided strictly at the same time every day and the period of access to food was limited to a 2-3 hour window. Within several days of adaptation to a newly introduced feeding schedule, mice started displaying elevated activity over about 2 hours before the food was provided. The physiological manifestations included increased locomotion, elevated body temperature, hepatic P450 activity, corticosterone secretion, gastrointestinal motility, and activity of digestive enzymes (Froy & Miskin, 2010). All of these changes are normally subject to daily cycling solely as the result of light-entrainment through the central clock in the SCN, as well. However, time-restricted availability of food affects the rhythms in clock gene expression in peripheral tissues, such as liver, kidney, heart, and pancreas. The phase and period in gene expression profiles can shift in those tissues accordingly to the time when the food becomes available (Froy & Miskin, 2010; Green et al., 2008). Interestingly, these food-entrained rhythms in peripheral clock persist for several days of total food deprivation and/or constant darkness or light (free - running under constant conditions). The presence of
rhythms in the food – anticipatory behavior was observed also in mice with SCN lesions, suggesting that the anatomical location of FEO is outside of the SCN (Green et al., 2008). Recent studies mapped out the possible brain regions where FEO can be located by investigating the effects of lesions in the dorsomedial hypothalamic nucleus (DMH), the brain stem parabrachial nuclei (PBN), and the core and shell regions of nucleus accumbens and concluded that these regions might be involved in FEO output, but they cannot fully account for the oscillation (Froy & Miskin, 2010).

While it was established that the rhythmic feeding drives the circadian rhythms in the peripheral clock, the ongoing research revealed that the particular feeding regimen which aid in the maintenance of central-peripheral clock synchronization might be beneficial for health. Many experiments involving feeding time and the number of calories in a daily intake as variables were performed to support this claim. Apparently, the timing of feeding was found to be very important, as the exposure to food in the wrong time of the day could promote misalignment between central and peripheral clock. Numerous studies both on human subjects and mouse model argue in support of the idea that eating in the beginning of natural activity cycle (morning for diurnal and the beginning of night after the sunset for nocturnal) is the optimal timing for maintaining healthy energy balance.

One of the first-observed consequences of feeding at the wrong time (night eating in humans and day feeding for mice) leads to weight gain and increase in obesity (Arble et al., 2009; Garaulet & Gómez-Abellán, 2014). One of the studies compared two groups of adult women who ate early lunch (1 pm) versus late lunch (4:30 pm) and found the association between eating late and decreased pre-meal resting energy expenditure,
decreased fasting carbohydrate oxidation, blunted daily profile in free cortisol concentrations and decreased thermal effect of food on as well as decreased glucose tolerance (Bandín et al., 2015). Late evening eating (after 8:00 pm) was also associated with higher BMI and increased the risk of obesity, irrespectively of the time and duration of sleep (Baron et al. 2011). It is important to note, that decreased glucose tolerance is associated with metabolic disorders, such as type 2 – diabetes and metabolic syndrome (Mekary et al., 2012).

There are multiple evidences that even long-term breakfast skipping can lead to adverse effects on cardiometabolic health. Breakfast skipping was associated with insulin resistance, higher risks of obesity and increased visceral adiposity, type 2 diabetes, and dyslipidemia in both children and adults (Smith et al., 2010). Importantly, breakfast skipping was associated with higher risk of type – 2 diabetes in both men and women (Mekary et al., 2012; Odegaard et al., 2013). Also, men who skipped breakfast had a 27% higher risk of coronary heart disease (CHD) compared with men who did not (Cahill et al., 2014). Several findings confirmed that glucose tolerance is worse in the evening and patients with type 2 diabetes who consumed a greater amount of daily calories at evening time displayed poorer glycemic control, independently of chronotype (Reutrakul et al., 2013; Van Cauter et al., 1997). On the contrary, multiple studies which addressed obesity issues and suggested weight loss strategies found larger morning meals and smaller evening meals more useful for the decrease in adiposity and reduction in fat mass, as well as improvement of insulin sensitivity (Garaulet et al., 2013; Karfopoulou et al., 2013; Lombardo et al., 2014).
While the knowledge about the timing of daily feeding expanded enough to conclude that morning eating is preferable for the healthy energy balance in humans, the next logical question is what a total daily caloric intake should be for reaching the metabolic optimum. One of the studies established that a low-calorie Mediterranean diet with a larger amount of calories consumed in the first part of the day could establish a greater reduction in fat mass and improved insulin sensitivity than a typical daily diet (Lombardo et al., 2014). Another particular example of implementation of hypoenergetic diet (or caloric restriction) was shown in the study involving type – 2 diabetes patients, showing that the feeding regimen in which two larger meals consumed in the morning time resulted in a significantly greater reduction body weight, liver fat content, fasting plasma glucose, C-peptide and glucagon, and higher insulin sensitivity than the same amount of calories split into six smaller meals across the day (Kahleova et al., 2014).

These findings indicate that, apparently, both timing and the amount of food are essential for healthy metabolic processes. As mentioned earlier, rhythmic feeding patterns in which the access to food is restricted to the particular time every day is playing a role in setting the circadian rhythms in peripheral tissues. One powerful dietary intervention gained a particular interest in research and, subsequently, in our study. Caloric restriction (CR) became very well known for its benefits for health, particularly – the extension of lifespan across species. In our studies, we also established the importance of the role of circadian clock as a mediator for CR mechanisms, which is going to be further discussed in this chapter.
1.8 Caloric Restriction

CR is a dietary paradigm in which the daily intake of food is reduced without malnutrition. For mammals, the food is typically provided in a periodic circadian manner. The increase in lifespan and delay of aging by caloric restriction (CR) is well documented in various organisms, including mammals (Froy & Miskin, 2010). CR affects many physiological systems - it causes changes in the levels various hormones, improves glucose homeostasis and mitochondrial functioning, promotes autophagy and proteostasis, increases resistance to stress and reduces the incidence of cancer. Reduction in reproductive investment and lowered body temperature also were reported upon CR and considered beneficial for health and longevity (Mitchell et al., 2016). CR in mice, rats, and monkeys delays the onset of major age-related diseases, such as cataracts, cancer, diabetes, kidney disease (Masoro, 2005; Roth et al., 2004). In humans, long-term CR helps to reduce the major risk factors for atherosclerosis, type 2 diabetes, and inflammation (Fontana, 2010).

The exact mechanisms of how CR modulates the increase in lifespan are still being under investigation. However, several signaling pathways, most of which are well conserved energy – sensing pathways, such as mammalian target of rapamycin (mTOR), sirtuin and insulin/insulin-like growth factor (IGF) signaling pathways, are implicated in the mechanisms of CR (Nakahata et al., 2008; Patel et al., 2016). Targeting some of these pathways pharmacologically might mimic the effects of CR and lead to increased lifespan (Khapre et al., 2014; Kondratov & Kondratova, 2014).

\( mTOR \) pathway
For example, one of the previous studies done in our lab showed that 24-hr rhythms in phosphorylation of mammalian target of rapamycin 1 (mTORC1) downstream targets could be entrained solely by food, persisted during prolonged food withdrawal (fasting conditions) and could be uncoupled from the rhythmic oscillation in expression of the core circadian clock genes. Furthermore, the rhythms in mTORC1 activity (phosphorylation of downstream targets the ribosomal protein S6 kinase 1 (S6K1) and ribosomal protein S6) were present in tissues of mice with the disrupted light-entrainable circadian clock. Tissue-specific rhythms in the expression of tor and its negative regulator deptor are also proposed as the molecular mechanism of the mTORC1 activity oscillation (Khapre, Kondratov, et al., 2014; Khapre, Patel, et al., 2014). Furthermore, CR downregulated the activity of mTOR and activated protein kinase B (Akt), but upregulated the activity of AMPK – these events were observed in some of the peripheral tissues in both carcinogen-treated and non-carcinogen treated rats (Jiang et al., 2008). Interestingly, deletion of the essential mTOR component - the ribosomal S6 protein kinase 1 (S6K1), led to increased lifespan and resistance to age-related pathologies, such as bone, immune, and motor dysfunction and loss of insulin sensitivity (Selman et al., 2009). Importantly, pharmacological inhibition of mTOR by rapamycin was shown to delay death without changing the distribution of presumptive causes of death in genetically heterogeneous mice and extended lifespan of cancer-prone mice, such as transgenic HER-2/neu mice (Anisimov et al., 2010; Harrison et al., 2009; Kondratov & Kondratova, 2014). Extended lifespan, prevention of age-related weight gain and suppressed carcinogenesis are all the effects observed in mice treated with rapamycin even if the treatment started in old age (Anisimov et al., 2010; Harrison et al., 2009).
Bmal1 deficiency results in increased mTOR signaling and accelerated aging, however, the administration of rapamycin increases the lifespan of Bmal1-/- mice by 50%. The main causes of death in Bmal1-/- mice treated with rapamycin are due to systemic failure due to progressive degeneration of nervous, cardiovascular and muscle systems, but virtually never cancer (Khapre et al., 2014; Kondratov & Kondratova, 2014). Caloric restriction alone, however, fails to extend lifespan in Bmal1-/- mice (Patel et al., 2016). These observations suggest that mTOR signaling has an important role in mediating both aging and carcinogenesis. The intact circadian clock is necessary for a homeostatic regulation of mTOR signaling, but pharmacological inhibition of mTOR activity extends lifespan in both wild-type mice and mice with a disrupted clock. Importantly, CR reduces mTOR signaling in wild-type mice which is considered one of the mechanisms of lifespan extension by CR.

**Sirtuins**

Sirtuins are NAD+ dependent deacetylases which, as discussed earlier, interact with circadian clock and are also proposed to regulate the aging process and mediate many beneficial effects for health induced by CR including lifespan extension (Nakahata et al., 2008). SIRT1 acts as a cellular sensor which detects energy availability and is essential for modulation of fat and glucose metabolism (Higashibata et al., 2016). CR induces Sirt1 expression and activation via increased NAD+ levels, which is a consequence of reduced energy intake. SIRT1 deacetylates target proteins and is linked to cellular metabolism, the redox state, and survival pathways. Sirt1 expression/activation is decreased in vascular tissue undergoing senescence and this event is one of the hallmarks of vascular aging and atherosclerosis. Induction of Sirt1 upon CR is proposed as a
protective therapeutic strategy against atherosclerosis as well as increasing resistance against cardiovascular diseases (CVD) triggered by the factors arising from metabolic impairment (Kitada et al., 2016). According to some evidence, Sirt1 might contribute to the neuroprotective effect of CR (Ma et al., 2016). Pharmacological activators of Sirtuin such as resveratrol, dehydroabietic acid, and natural melatonin were shown to slow down aging and increase lifespan in mice and C. Elegans (Kim et al., 2015; Ramis et al., 2015; Solon-biet et al., 2016). Resveratrol was also reported to ameliorate motor neuron degeneration and improve survival in a mouse model of amyotrophic lateral sclerosis which again demonstrates the involvement of Sirtuin signaling in neuroprotective effects (Song et al., 2014). Another SIRT1 activator - metformin has the ability to mimic CR's tumor-suppressing effects via the decrease in the levels of growth factors (IGF-1, insulin and leptin), inflammatory cytokines (MCP-1, IL-6), vascular endothelial growth factor (VEGF) in plasma and ascitic fluid (Al-Wahab et al., 2015).

**IGF-1 signaling**

Insulin/insulin-like growth factor (IGF-1) signaling is involved in multiple functions necessary for metabolism, growth and cellular proliferation, and fertility in different animal models - flies, nematodes, and mammals (Rincon et al., 2004). IGF-1 is synthesized in the liver and mainly is present in the circulation in the bound form (IGF binding protein (IGFBP)). Increased levels of free serum insulin and IGF-1 is associated with the risk of some types of cancer and the decrease in serum insulin and IGF-1 levels appear to have a protective role against cancer development (Gallagher & LeRoith, 2011). The possible direct/indirect roles of IGF-1 in tumorigenesis are still under investigation, however, increased IGF-1 signaling can indirectly induce mTOR activity
via the interaction with phosphoinositide 3-kinase (PI3K)/AKT signaling pathway which has been implicated in different types of cancers (Meynet & Ricci, 2014). CR was shown to downregulate both the Igf-1 expression in liver and serum IGF-1 levels – this effect is considered a part of CR lifespan extension mechanisms (Patel et al., 2016). However, in mammals, there are still some controversies when it comes to the question whether the chronic decrease in IGF-1 is always beneficial. While the cancer-protective effects of decreased IGF-1 signaling are contributing to the lifespan extension, decreased serum IGF-1 triggers sarcopenia which can lead to disadvantageous effects on human health at an older age (Sharples et al., 2015). Particularly, in older age attenuation in downstream IGF-1 intracellular signaling targets, including mTOR and PI3K is involved in a blunted response to resistance exercise, reduced protein synthesis and loss of muscle mass (Fry et al., 2011). One of the mouse studies suggested that reduced serum IGF-1 at 1 year of age leads to significantly deteriorated healthspan. Mice exhibited increased liver weight and inflammation and increased incidence of hepatic tumors. The skeletal muscle of those mice showed signs of increased oxidation of proteins, which indicated increased oxidative stress (Gong et al., 2014). Therefore, while some of the positive effects of reduced IGF-1 signaling on aging are evident, the overall benefits of reduced IGF-1 are debatable, as some of the age-related pathologies might actually be triggered by the decrease in serum IGF-1. Clearly, the CR-driven decrease in IGF-1 can contribute to the effects of CR on lifespan extension, however, most likely it might be not the primary factor, as many other mechanisms are involved in response to CR. Interestingly, the action of growth hormone (GH) in somatotropic signaling (collectively GH/IGF-1) is
necessary for lifespan extension mediated by pre-weaning CR in mice (based on litter-crowding observations) (Fang et al., 2017).

**Oxidative stress/reactive oxygen species**

One of the prominent theories on aging is the increasing amount of oxidative damage due to an imbalance between prooxidants and antioxidants. The reactive oxygen species (ROS) are generated in all aerobic organisms as a byproduct from the use of oxygen by cells in metabolic processes. Oxidative damage manifests in such changes as peroxidation of membrane polyunsaturated fatty acid chains, DNA modification, carbonylation and loss of sulfhydryls in proteins (Sohal & Weindruch, 1996). Recently, a particular ROS molecule - peroxide (H₂O₂), has been suggested to promote aging by activation of the TOR (target of rapamycin) pathway (Blagosklonny, 2008). CR was demonstrated to increase the resistance against oxidative stress. Importantly, ROS levels have been found to alternate during day/night cycles in circadian manner. As mentioned earlier in the chapter, several studies which utilized mice deficient in some of circadian genes found that circadian knockout mice (especially Bmal1/- KOs) had significantly higher ROS levels and diminished resistance to oxidative stress, suggesting a connection between role of CR and circadian clock in ROS homeostasis (Patel et al., 2014). An important antioxidant function in ROS homeostasis is performed by melatonin which has a strong protective effect against the generation of ROS in pancreatic β-cells, which are easily susceptible to oxidative stress. Melatonin release is controlled by the circadian clock. Disturbances to circadian rhythms, such as irregular feeding and activity cycles are triggers of type-2 diabetic phenotype in which plasma melatonin levels were shown to be
significantly decreased, which can lead to a higher oxidative damage (Jenwitheesuk et al., 2014; Ramis et al., 2015).

The tight interconnection of many metabolic pathways with circadian clock led to the question – whether circadian clocks are directly involved in the mediation of beneficial effects of CR. Recently, us and other researchers reported that CR affects the daily rhythms in the expression of several circadian clock genes in mammals and flies and proposed that circadian clocks might be involved in CR mechanisms (Katewa et al., 2016; Patel et al., 2016).

1.9 Calorie restriction effects and circadian clock

We reported previously that 30% CR affects the amplitude and absolute level of expression for several circadian clock genes (Patel et al., 2016). The effect was observed on both mRNA and protein levels in mouse liver. Importantly, our lab showed that the observed changes in many circadian gene expression profiles are caused exclusively by CR and not any other feeding regimen – the results for core clock genes are summarized in Figure 1-4.
Figure 1-4: Effect of CR on the rhythms of circadian gene expression.

On the figure: mRNA expression profiles of core clock genes – (a)Per1, (b)Per2, (c)Cry2, (d)Cry1, (e)Bmal1 and (f)Clock - were assayed in the liver of mice (n = 3 per time point) subjected to the following feeding regimens: ad libitum control (AL) – blue circles, solid line; 30% calorie restriction (CR) – red squares and solid lines; time-restricted feeding (TR) – orange triangles and solid lines, fasting (F) – green cross and solid lines. For all panels, graphs are double plotted. Data are represented as mean ± SEM. Light is on at ZT0 and off at ZT12. a, b, c, d, e, f – statistically significant difference (p<0.05) between indicated groups at particular time points. Open bars represent light and black bars represent the dark phase of the day. Food for CR and TR group was provided at ZT14. This figure is adapted from:

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4864379/
Significant upregulation in Bmal1, Cry2, Per1,2,3 gene expression and amplitude of oscillation was observed in mouse liver exclusively under CR (Figure 1-4). The expression of Rev-Erb α,β and Ror α,γ genes (not shown in the figures) was upregulated at some time-points, however, in many time points the effect of CR was similar to TR. On the protein level, CR significantly downregulated the levels of CRY1, while the expressions of CLOCK, BMAL1, PER1 and PER2 were decreased but the effect was not statistically significant (Patel et al., 2016).

It is important to note, that CR feeding regimen in our experimental set up includes two essential parameters: 1. The timing of feeding – mice on CR receive food at the same time every day. Most importantly, mice under CR receive food 2 hours after the light is off – which is physiologically optimal time for feeding in nocturnal animals. In about 2-3 hour window all of the food is consumed by mice. 2. The amount of food is reduced. The caloric intake is comprised of 70% of the number of calories consumed by AL mice daily without any differences in macronutrient ratios – all mice are feeding on the same chow (Harlan Diet – described further in “materials and methods” chapter). While other studies emphasize the importance of doing further research on macronutrient ratios, they agree with our studies on the point that both the restricted timing of feeding and reduction in calories are necessary to achieve the effects of lifespan extension (Simpson et al., 2015; Solon-biet et al., 2014). Neither time – restricted (TR) feeding without a reduction in calories, nor round-the-clock access to the daily food portion (with a reduction in calories) provides full effects of CR unless these two conditions meet together. These observations, again confirm that the optimization of metabolism relies on rhythmicity which is driven by the circadian clock. To test this interaction we previously applied CR
to the mice with the genetically disrupted circadian clock to see whether the effects of CR will be altered. We observed that CR fails to provide all physiological benefits including the effect on longevity in mice deficient for the circadian transcriptional factor BMAL1 (Patel et al., 2016). **Figure 1-5** demonstrates the survival curves of wild-type and Bmal1 deficient mice under AL-control and CR conditions.

**Figure 1-5. BMAL1 is necessary for lifespan extension in response to CR.**

*Panel A*) Kaplan-Meier survival curves of *Bmal1*−/− mice on AL (*n* = 18, gray circles), 30% CR (*n* = 36, dark gray diamonds), and CR-adjusted (*n* = 21, black squares) feeding. Mice that died during first 3 wk of 30% CR were excluded from analysis. Mice of both genders were used. Difference between survival curves of AL and CR is statistically significant by log-rank test; no statistically significant difference between AL and CR-adjusted groups was observed. *Panel B*) Kaplan-Meier survival curves of female *Bmal1*−/− mice on AL (*n* = 9, gray circles), 30% CR (*n* = 23, dark gray diamonds), and CR-adjusted (*n* = 14...
black squares) feeding. Mice that died during first 3 wk of 30% CR were excluded from analysis. Difference between survival curves of AL and CR is statistically significant according to log-rank test; no statistically significant difference between AL and CR-adjusted groups was observed. C) Kaplan-Meier survival curves of male Bmal1<sup>−/−</sup> mice on AL (n = 9, gray circles), 30% CR (n = 13, dark gray diamonds), and CR-adjusted (n = 9 black squares) feeding. Mice that died during first 3 wk of 30% CR were excluded from analysis. Difference between survival curves of AL and CR is statistically significant according to log-rank test; no statistically significant difference between AL and CR-adjusted groups was observed. D) Kaplan-Meier survival curves of WT mice on AL (n= 73, gray circles and gray dotted line) or CR (n= 31; black squares and solid black line) feeding. Mice of both genders were used. *Difference between survival curves of AL and CR statistically significant according to log-rank test. This figure is adapted from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4799504/

Similarly, CR does not have full effects on the increase in lifespan in Drosophila flies with genetic ablation of some clock genes (Katewa et al., 2016). Thus, circadian clocks might represent a conserved physiological system essential for CR mechanisms, which warrants a further study on the interaction between circadian clocks and CR.

Our lab also investigated one of the accessory pathways described earlier to see the possible response to CR. Our previous study established that the deficiency in Bmal1 also influenced CR-mediated effects on IGF-1 levels in the circulation and Igf-1 expression in liver. On mRNA level, CR downregulated Igf-1 expression at one time
point and in serum IGF-1 level was significantly decreased only in WT mice, but not in Bmal1 deficient mice, although the similar trend was observed in knock-outs. This suggests that some time-specific control, dictated by rhythmic feeding as well as circadian control is present in the regulation of Igf-1/IGF-1.

One of the comparative analyses of microarray datasets done previously also mapped out some genes that might play a role in modulation of longevity. The investigation involved the long-lived strains of dwarf mutant mice with suppressed growth hormone (GH) and insulin-like growth factor 1 (IGF-1) signaling as well as wild-type mice subjected to CR (Swindell, 2007). Some of the genes the expression of which showed significant change under CR as well as in long-lived strains were picked by us for the further analysis. The results for mRNA expression in mouse liver from the study done previously in our lab are summarized in Figure 1-6.
Figure 1-6. Effects of CR on the expression of CR-target (longevity-associated) genes

Shown on the figure: mRNA levels of several longevity associated candidate genes – (a) Fmo3, (b) Cyp4a12b, (c) Cyp4a14a, (d) Serpina12, (e) Igfals and (f) Mup4 in the liver of mice (n = 3 per time point) subjected to the following feeding regimens: ad libitum (AL) – blue circles, solid line; 30% calorie restriction (CR) – red squares and solid lines; time-restricted feeding (TR) – orange triangles and solid lines, fasting (F) – green cross and solid lines. For all panels, graphs are double plotted. Data is represented as mean ± SEM. Light is on at ZT0 and off at ZT12. a, b, c, d, e, f – statistically significant difference (p<0.05) between indicated groups at particular time points. Open bars represent light and black bars represent the dark phase of the day. Food for CR and TR group was provided at ZT14. This figure is adapted from:

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4864379/

From the subset of genes which were previously proposed to be associated with longevity and are also involved in some of the metabolic and detoxification pathways, we found that CR had differential effect on the expression. CR caused downregulation in expression of Cyp4a12b, Serpina12 and Mup4 genes and induced the expression of Fmo3 and Cyp4a14a while the expression of Igfals was not significantly affected (Patel et al., 2016).

These findings confirmed the direct effect of CR on both circadian clock as well as some metabolic pathways on molecular level in vivo and created the foundation for the extension of the study into our current project which addressed the questions that were
not systematically approached before: whether the effects of CR on circadian clock and longevity-associated genes are sex-dependent and whether the effects of CR are subjected to changes upon long-term exposure to this particular feeding regimen.

1.10 Sex as an important factor in circadian and aging research

Relevance of inclusion of both sexes in experimental studies

In the previous studies, the effect of CR on clock gene expression was investigated only in male mice. The majority of the studies on circadian clocks and rhythms either did not compare males to females or did not include females into the experimental setup at all - due to a general bias in biomedical research (Krizo & Mintz, 2014). While the NIH mandated enrollment of women in human clinical trials more than two decades ago in 1993, the situation with the inclusion of females into the experimental setups involving animal models remained without the necessary attention. For a long time, the reason for this approach was the belief that non-human female mammals are intrinsically more variable than males and too “complex” for routine inclusion, however, currently, rising number of researchers are claiming this statement is lacking foundation (Beery & Zucker, 2011).

A meta-analysis performed recently, in fact, determined that the variabilities are roughly the same between males and females based on the monitored behavioral, morphological, physiological, and molecular traits. It is important to account for sex differences which exist on all levels of biological organization – the carryover from findings obtained with males to females has significant limitations, which again, argues for the routine inclusion of female rodents into research protocols (Prendergast et al.,
Sex-differences exist in multiple traits related to circadian and aging research: those differences are present in the timing of daily onset of activity, responses to both photic (i.e. light) and non-photonic stimuli (i.e. food entrainment), and in physiological changes across the lifespan (Krizo & Mintz, 2014).

Sex-based differences in aging

The interest of our current study is also fueled by the existing knowledge on sex-differences in aging and responses to anti-aging interventions. For example, it is well established that women live longer than men irrespective of social factors – cultural, environmental and economic factors can only affect the magnitude of the difference. Interestingly, the incidence of diseases is roughly the same, however, women die at lower rates than men from all health conditions that are considered “top-ranked killers” with one exception being Alzheimer’s disease which is observed in women at much higher rates. Same differences in longevity between males and females are observed in apes, which occupy the evolutionary closest branches to humans, such as chimpanzee, gibbons, gorillas, and orangutans. Among other mammals, an interesting trend was observed: in polygynous species, females tend to live longer, in monogamous – either equally, or slightly longer lifespan is observed in males. The same pattern is observed in birds, since most of the bird species are monogamous (S. Austad & Bartke, 2015).

The situation with mice, which represent the most commonly used mammalian model for research protocols is not that clear. Out of more than hundred reports on the sex-based difference in lifespan in mice (untreated, or control mice) about half reported a male-preference for a longer lifespan, and another half – a female one. Such variation
could arise from multiple sources: the strain of mice, different genotypes used for studies, wild-derived versus inbred strains differences and so on. However, among the sole reports on the strain used in our study – C57BL/6 mice – the situation had the same degree of difference between the results (S. Austad & Bartke, 2015). Up to this date, it is not clear, which sex has a longer lifespan in mice.

The mechanisms which drive the sex-based differences are still under investigation. The main hypothesis is quite logical – the primary factor might be the involvement of sex hormones – either male reproductive hormones might increase the systemic susceptibility to common death-causing diseases, or women’s hormones might increase the resistance to these diseases via some protective physiological mechanisms. In humans, the most favored hypothesis provides the possible explanations in the fact that men’s sex hormones have a life-shortening impact on the organism. Multiple records (many of which are also quite important in terms of historical value) reported that castration in men significantly prolonged lifespan, and the earlier it was performed – the larger was the increase in lifespan. At the same time, oophorectomy does not influence lifespan in women anyhow (S. Austad & Bartke, 2015). Postmenopausal hormone replacement in women also doesn’t seem to influence lifespan, and in fact, the current knowledge suggests it does not increase longevity, as the reverse effect was reported (Michels & Manson, 2003). Hence, these evidence indicate that it is unlikely for female sex hormones to have a lifespan-extending effect, but rather the sole regulation of physiological processes by male hormones truly can contribute to life-shortening events.

Paradoxically, even while having a longer lifespan, irrespective of the economic situation in the country of residence, women display higher rates of physical illnesses and
disabilities at an older age than men (Christensen et al., 2009; Verbrugge & Wingard, 1987). Some evidence suggests that health quality versus lifespan/mortality paradox may be a result of greater connective tissue responsiveness to sex hormones in women. The specific hormonal influences on inflammatory and immunological responses might contribute to the existing differences in longevity; however, the current knowledge base for the mechanisms underlying these processes is quite scant (Austad & Fischer, 2016). On a positive note, however, such anti-aging interventions as CR were shown to increase lifespan in both sexes, but the possible difference in response to CR was not systematically studied – these events are addressed in our current study.

Sex-based differences in circadian and metabolic regulation

Some evidences demonstrate that circadian rhythms in behavior of laboratory rodents might vary between sexes. For example, in Octodon degus the free-running period is half an hour longer in females than in males, in rats and golden hamsters, on the contrary, males display longer circadian period. In mice of C57BL/6J strain used in our study up to this date no such differences have been reported – the circadian period in both males and females is approximately the same (Krizo & Mintz, 2014). While there is some difference between circadian period between sexes, we still expect them to be subtle, as all organisms are physiologically adapted to approximately 24 hours and the reported variations of sex-based differences in free-running period across species might be a result of evolutionary adaptation of different lineages to the specific stimuli determined by the geography and environmental factors of habitats. Even within human species the hypothesis of such adaptation is supported by the observed variations in free-running periods – in people of African descent (mainly involving populations originally located
close to the equator), the circadian period is shorter than in people from European/Northern descent, where, unlike along the equatorial belt, the photoperiod/day length noticeably changes with seasons. Interestingly, in people of African descent there is no difference in free running period between sexes, while in Europeans women have slightly shorter circadian period then men (Eastman et al., 2017). This is also manifested in the sex differences of a chronotype or phase of sleep. The phase of sleep changes over lifespan, during adulthood women tend to have earlier sleep phase compared against age-matched men (Roenneberg et al., 2007).

Gonadal steroids are suggested to have a role in the sex-based difference in photic sensitivity of the circadian clock. In mice, for example, females have larger phase shifts to light (Kuljis et al., 2013). Same observation was made upon gonadectomy in male mice as compared to intact males. Androgens were reported to be involved in regulating the circuitry and neural plasticity in the mouse SCN, which in turn has an effect on clock gene expression as well as responses to light and the rhythms in behavior (Karatsoreos et al., 2011). Female sex hormone profiles in turn have a specific effect on circadian rhythms - the onset of locomotor activity tends to vary in females depending on the phase of estrous cycle. This particular variability is mainly mediated by estrogen receptor β (ERβ) in mice. The locomotor activity in females advances with the elevation of estradiol levels before the ovulation and delays after. There is some evidence that these slight shifts in phase are regulated by estradiol directly via the central clock – the shortened period in activity is not observed in ovariectomized mice, but upon estradiol replacement the same shifts are observed as in intact mice (Krizo & Mintz, 2014). Some downstream signals might also be responsible for these differences in phase of activity,
for example, the upregulation of dopamine receptor 1 in the striatum by estrogens could cause an increased motivation for wheel-running activity, which results in slight advances in the onset of activity (Di Paolo, 1994; Lammers et al., 1999).

Circadian entrainment to the shifts in light–dark cycles is also influenced by the scent communication between the sexes essential for reproductive activity. For instance, ovarian hormones are necessary for both the production of and responsiveness to olfactory cues. The existing evidence from Octodon degus studies suggests the combined effect of progesterone and estrogen are essential for responsiveness to the olfactory social cues and the fastest re-entrainment to the shifts in the light cycle was displayed in females with intact gonads housed with other females (Jechura & Lee, 2004). In contrast, testosterone suppresses social cue responsiveness in terms of re-entrainment to shifts in light phase, as was confirmed by the series of experiments involving the combinations of social housing and castration/testosterone administration in rodents (Jechura et al., 2003).

Therefore, the signaling of gonadal hormones accounts for a large part of sex-based differences in the central clock. Each of the hormone receptors such as estrogen receptors (ERα, ERβ), progesterone receptor (PR), androgen receptor (AR) and G protein–coupled receptor 1 (GPER1) either alone or in a combined manner can mediate sex-differences in circadian rhythms (Krizo & Mintz, 2014). ERα, ERβ and AR are expressed in the SCN and the sexual dimorphism in expression was reported for two of these receptors – ERβ and AR, which again confirms the sex-based difference in regulation of the central clock (Vida et al., 2008).
Our interest is in seeking the possible sex-based differences in circadian entrainment to feeding, as this connection has already been established in young male mice. Interestingly, the sex-based difference in food anticipatory activity (FAA) so far was reported only in mice, which suggests the presence of sexual dimorphism in earlier discussed food-entrainable oscillator (FEO). This is a valuable finding, which indicates that peripheral clock might also be entrained differently in each sex independently of the signals emanating from the central clock. Indeed, recent findings indicate that male mice display a higher food-anticipatory activity (wheel running) than females under time-restricted feeding. The gonadectomy abolishes this difference which indicates that gonadal hormones play an essential role in the regulation of FAA in both sexes (Li et al., 2015). It is important to mention that FAA can only be displayed by animals which are receiving food in a periodic circadian manner with the interval of 24 – hours. The non-circadian availability of food (i.e. 18 hours period between receiving food) does not entrain such observable activity in animals irrespective of lighting conditions (Petersen et al., 2014). Thus the observed sex-based difference in FAA can be attributed to the part of sex-specific circadian mechanisms independent from the central clock. Interestingly, there is some evidence that the content of food might influence FAA in a sex-specific manner. The study by Hsu, et al. showed that FAA was only observed in males when mice received a high-fat snack in a time-restricted manner while the access to the regular chow was around the clock (Hsu et al., 2010). Some earlier data suggest that the estrous cycle can mediate female motivation for high fat or high sugar foods (Clarke & Ossenkopp, 1998).
While it is fair to conclude that males might display stronger FAA overall, we cannot disregard the presence of FAA in females, as females also display FAA upon complete withdrawal of food (Krizo & Mintz, 2014). Importantly, FAA is maintained under TR feeding conditions with no reduction in daily calories, as well as under CR - caloric deficit (Gallardo et al., 2012). Thus we hypothesize there might be some degree of sexual dimorphism in the expression of some circadian genes in peripheral tissues under CR.

1.11 Sex-based differences in caloric restriction effects

Our current study is aiming to determine the possible manifestations of sex-based circadian differences on molecular level particularly under CR conditions – as our previous study showed that CR specifically caused the changes in the expression of circadian genes in liver. At the moment there is some evidence showing that the response to CR might be sex-specific (Mitchell et al., 2016).

One of the most prominent differences between sexes was the response to CR in terms of lifespan – it turned out that in mice of C57BL/6J background (same mouse strain used in our study) different grades of caloric restriction might prolong lifespan in males and females with a different magnitude. It was demonstrated that 40% CR did not increase lifespan in females, but did so in males. 20% CR significantly increased survival and prolonged female lifespan up to 40% in average, whereas lifespan in males increased by 24.4% (Forster et al., 2003). In our study, we implemented 30% CR in the experimental protocol for our calorie restricted group of mice and found no significant difference in the magnitude of lifespan extension between males and females, although these results might be affected by the sample size limitations (Figure 1-5). Some other
parameters were also shown to have sex-specific changes in response to CR. For example, the reduction in body temperature (which is one of the primary responses to CR in mammals) was directly correlated with the magnitude of the caloric deficit in males. In females, there was no such correlation – females on 20% CR were reported to have higher body temperature than females under AL and 40% CR conditions (Forster et al., 2003). Some of the sex-based differences in glucose homeostasis were also observed under different grades of CR – glucose and insulin levels were lower in both sexes upon CR, however, in females they reached a plateau at 20%CR, while in males they were progressively lower under 40% CR. Serum IGF-1 levels were progressively decreased under CR with no difference between sexes. Under 20% CR, serum IGFBP-1 was lower than under AL condition in both sexes, while under 40% CR IGFBP-1 was elevated in females even compared to AL levels (Mitchell et al., 2016). This is an interesting paradox, indeed, indicating that the response to CR is not linear and is not the same in both sexes.

Based on some existing evidence we also hypothesize that one of the features of adaptation to CR in mice is the reduction in reproductive activity in order to conserve energy for somatic protection during a caloric deficit. Some of the genes encoding proteins such as major urinary protein 4 (Mup4/MUP4) are known to play role in mouse scent communication – also Mup4 was classified as a longevity-associated gene in the study of Swindell (Swindell, 2007). Other longevity genes which are also expressed in liver and were found to change the expression under CR are also involved in detoxification and lipid processing pathways. According to previous reports a lot of metabolic pathways in which those genes are involved – might be regulated differently in
males and females due to direct/indirect influence by gonadal hormones and other factors (Mode & Gustafsson, 2006).

Our previous study confirmed that besides core clock genes, a subset of longevity-associated genes has a gene-specific response to CR in young male mice; therefore we picked those genes to investigate for possible sexual dimorphism in expression. Since we’ve also established that CR downregulates \(Igf-1\) in a time-specific manner, and no difference was reported between males and females in serum IGF-1 levels under CR (Mitchell et al., 2016), we decided to check the \(Igf-1\) expression in the liver for possible sexual dimorphism.

1.12 Aging, long and short-term caloric restriction

While multiple reports state that circadian rhythms change with age in a generalized manner, it is still unclear whether the main reason is the direct disruption of circadian feedback loops on a molecular level or some of the other physiological factors that interact with the clock. Or, perhaps (and more likely) the multitude of factors combined together which accumulate with years of life? In the earlier discussion, I have mentioned that there is a direct correlation between time spent on shift work and circadian disruption in behavior and metabolism which causes adverse effects such as increased risk factors for cardiovascular diseases, obesity, type-2 diabetes, decreased neuroprotection and, as a result, decreased lifespan.

The primary manifestations of age-related circadian changes widely discussed in research are the changes in the sleep phase. Sleep/wake patterns significantly change with age and become increasingly fragmented in older people (Hood & Amir, 2017). Insomnia
and irregular sleeping patterns, which are observed very often in older age are also associated with the history of shift work and jetlag (Burman, 2017). The earlier discussed influence of gonadal hormones on the human circadian clock also has a prominent effect on the sex-specific difference in sleep phase between men and women (with women having earlier sleep phase). These differences, however, disappear after menopause/andropause which indicates that natural age-related changes in hormonal profiles might play role in the circadian changes associated with age (Cain et al., 2013; Duffy et al., 2011; Mitchell et al., 2016).

In C57BL/6J mice it was established that young mice show baseline differences in circadian rhythmicity compared to adults. These differences are manifested in advanced photocycle entrainment, larger photic phase-delays, a shorter free-running (endogenous) circadian period, and greater circadian rhythm amplitude. Interestingly, young adolescent mice are more resistant to alcohol (ethanol) induced disruption in circadian photic phase-resetting than older mice (Ruby et al., 2017). Indeed, alcohol consumption has its niche in human social culture and might affect the robustness of circadian rhythms in older age.

With the rise of interest towards anti-aging interventions, such as CR and CR-mimetics which affect circadian clock we raised the following questions in the second part of our study: will there be any changes on a molecular level in peripheral liver clock with age? We previously established that the intact clock is necessary for the mediation of CR effects, therefore, will CR have the same pronounced effect on circadian gene expression in mice of older age that it in mice of younger age? It was hard to predict the outcome, as the possible age-related changes (if present) in circadian gene expression
could also cause the changes in CR effects over time. Or, possibly, not. One of the recent studies showed that short-term CR and short-term administration of CR-mimetic resveratrol in old mice altered mitochondrial regulatory and apoptotic signaling pathways in the glycolytic muscle which might moderate protective effects against one of the age-related disorders - sarcopenia (Joseph et al., 2013). At the same time, intact mitochondrial DNA is required for CR to induce beneficial effects (Someya et al., 2017).

To the best of our knowledge, the question of the influence of long-term CR on the liver clock and possible age-related changes in the rhythms of peripheral clock remains unanswered. In this study, we attempt to at least partially find the answer.
2.1 Experimental Animals

All the animal studies were performed with approval from the Institutional Animal Care and Use Committee (IACUC) of Cleveland State University (Protocol No. 21124-KON-S). The care and use of mice were carried out in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of the Cleveland State University. All animals were wild-type (WT) mice of C57BL/6J inbred strain background, housed under standard conditions of 12 hr light/dark cycle (LD 12:12) with lights turned on at 7:00 am and turned off at 7:00 pm. All animals were fed regular 18% protein chow diet except breeders which were maintained on 19% protein diet (Harlan).

Feeding Regimen

Ad-Libitum (AL) control group had unrestricted access to food across the day. Caloric restriction (CR) was started at 3 months of age for CR experimental group of mice. For the first week of the diet, the animals were kept on 10% restriction, for the second week – on 20% restriction and on 30% restriction for the rest of their life and subsequent
experimental measurements. The CR groups received their food once per day at ZT14 (two hours after the light was turned off) as this is considered the most physiologically optimal time for food consumption, as mice are nocturnal animals and their activity peaks during the night. All groups had unrestricted access to water.

**Between-sex Comparison**

After two months of CR the tissues were collected for the analysis. For AL groups the tissues were collected at 5 months of age (age match) of the animals. Therefore, for the analysis of sex effect - 4 groups of wild-type 5-month-old mice were generated: ad-libitum males and females, and calorie-restricted males and females. Three animals of each sex and feeding regimen were used for each time point.

**Age-Based Comparison**

For the analysis of the effect of age on the gene expression and the comparison between short term and long term caloric restriction wild-type mice of different age were used. 5-month-old male and female, ad-libitum and calorie-restricted mice were used in the amount of three biological replicates per time point. For the group of old animals – male and female mice that were on caloric restriction for 2 years with age-matched ad-libitum controls were used. Only one biological replicate per time point was available for each feeding regimen and each sex in old mouse groups. Due to the lack of biological replicates mice of both sex were combined for the statistical analysis and only the genes that did not show any sexual dimorphism in expression were profiled for age-based differences. Therefore, six biological replicates were used for each feeding regimen.
within young mouse group and two biological replicates were used for each feeding regimen in old mouse group.

For all groups, the tissues were collected at six different time points across the day. Mice were euthanized using CO2 chamber followed by cervical dislocation.

The experimental design is further described in Figure 2-1.

**Figure 2-1. Experimental design**

The flowchart represents the course of life of experimental mice up to the day of tissue collection (when mice are sacrificed and their tissues are collected and preserved). C57BL/6J wild-type mice were used. The breeders were not used for experiments, mice were separated to the different cages based on sex and feeding regimen. Since birth up to
reaching adulthood (3-month-old) mice were feeding on ad-libitum diet (food present in the cage in unlimited amount 24/7). After reaching 3 months of age, mice of each sex were split into groups by feeding regimen. CR was introduced to the respective group. Tissue samples were collected from young mice after two months on CR. At the same time the samples were collected from age-matched ad-libitum control mice. From old mice, tissue samples were collected after mice had been on CR for 2 years. At the same time, the tissues from age-matched ad-libitum controls were collected. At the day of sacrifice, tissues were collected at 6 time points throughout the whole 24-hour cycle. Zeitgeber (external cue) is the light - hence the labeling “ZT” for every time point (bottom right, colored with blue, except ZT14). The light was on at ZT0 (7 am) and off at zt12 (7 pm). CR group received food at zt14 time point (bottom right, colored with red) – 2 hours after the light was off. The light bar on the bottom right represents 12 hours when the light was on. The dark bar – 12 hours when the light was off respectively.

2.2 RNA isolation and analysis of mRNA expression.

For RNA analysis, liver tissues from 3 five-month-old mice per time point on each feeding regimen (AL, CR), each sex were collected every 4 hours throughout the day, and stored at -80°C. For the age-based comparison liver tissues from 2 two-year-old mice (one male and one female) per time point were collected for each feeding regimen (AL, CR). Total RNA was isolated from the liver tissue using TriZol 48 reagent (Invitrogen, Carlsbad, CA) as per the manufacturer’s protocol. Briefly, frozen liver piece (~30mg) was minced in 1 ml TriZol reagent with a pestle on ice. RNA samples were centrifuged at 10,000 rpm for 10 mins at 4C. RNA was extracted using 200ul chloroform
and shaking vigorously. Following chloroform extraction step, total RNA was precipitated with 500μl isopropanol by centrifugation at 14,000 rpm for 10 mins at 4°C and pellet obtained was washed with 1ml 70% Ethanol. RNA pellet was air dried, diluted in 30 μl of RNase-free water and quantified on Nanodrop. RNA integrity was checked on 1% agarose gel run at 90 V for 30 minutes. 20 μl of RT mix was prepared using 1 μg of RNA, 50 ng of 50 uM random hexamer (N8080127, Invitrogen), 10 mM dNTP (DD0058, Biobasic), and 0.1 M DTT. It was then reverse transcribed by reverse transcriptase PCR machine using 200 u/μl of SuperScript® III Reverse Transcriptase (18080-044, Invitrogen) as per the manufacturer’s instructions. Incubation conditions used were: 65°C for 5 minutes followed by incubation on ice (4°C) for 1 minute; 23°C for 5 minutes; 50°C for 10 minutes; Inactivate the reaction by heating at 80°C for 10 minutes. RNA quantification was performed using qPCR with Universal SYBR Green mix (1725125, BioRad). The reaction was carried out in triplicates for the gene of interest and in duplicates for the normalizing control gene (18s rRNA), using CFX96 qPCR Detection System (BioRad) with 50 ng of cDNA. Thermal cycling conditions used were according to the instructions of SYBR Green mix protocol and relative mRNA abundance was calculated using the comparative delta-Ct method with the normalization on 18s rRNA house-keeping gene. Melting curve analysis was used for confirming the product specificity. Primer pair was tested for the efficiency that was calculated by standard curve analysis using standard serial dilutions method. Primers used for the analysis of expression are presented in Table I.
### Table I: Primers for qPCR

<table>
<thead>
<tr>
<th>Primers</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bmal1</td>
<td>5’CACTGTCCAGGCATCCA3’</td>
<td>5’TTCTCCGCGATCATCAG3’</td>
</tr>
<tr>
<td>Per1</td>
<td>5’AGGTGGCTTCTGTTGG3’</td>
<td>5’CAATCGATGGATCTGCTCGAG3’</td>
</tr>
<tr>
<td>Per2</td>
<td>5’AATCTTCCAACACCTACCC3’</td>
<td>5’CCTCAGGCTCTTACAG3’</td>
</tr>
<tr>
<td>Per3</td>
<td>5’GGTCGACATAAAGTCGAAAG</td>
<td>5’TCTTACTGGCTGCTTTTATT3’</td>
</tr>
<tr>
<td></td>
<td>A3’</td>
<td></td>
</tr>
<tr>
<td>Cry1</td>
<td>5’CGTCTGTGTCTGATCCCG3’</td>
<td>5’ATTCACGCCAGGAGTTG3’</td>
</tr>
<tr>
<td>Cry2</td>
<td>5’GGCAGACCGAGACGGACAG</td>
<td>5’ATCGATTCGGCGCCGGCG3’</td>
</tr>
<tr>
<td></td>
<td>3’</td>
<td></td>
</tr>
<tr>
<td>Rev-Erb α</td>
<td>5’TGGCATGTGCTACTGTAAG</td>
<td>5’ATATCTGTTGGATGCTCGCG3’</td>
</tr>
<tr>
<td></td>
<td>GG3’</td>
<td></td>
</tr>
<tr>
<td>Rev-Erb β</td>
<td>5’GGAGTTCTGCTTGTGAAGGC</td>
<td>5’CAGACACTTCTAAAGCGGCCTG3’</td>
</tr>
<tr>
<td></td>
<td>TGT3’</td>
<td></td>
</tr>
<tr>
<td>Ror α</td>
<td>5’GGAATCCATTATGTTGTCA</td>
<td>5’GTGGCATTGCTCTGACTT3’</td>
</tr>
<tr>
<td></td>
<td>TG3’</td>
<td></td>
</tr>
<tr>
<td>Ror γ</td>
<td>5’ACTACGGGGTTATCACC</td>
<td>5’GTGCAGGAGTAGGCCACATT3’</td>
</tr>
<tr>
<td></td>
<td>TG3’</td>
<td></td>
</tr>
</tbody>
</table>
### 2.3 Immunoblot Analysis

For analysis of protein expression tissues from three mice per time point were used for each feeding regimen and each sex. Western blotting was done on liver samples pooled together from three different mice from each of the six time points for each diet and each sex. For tissue lysates preparation, frozen liver pieces were lysed in 300μl of cell signaling lysis buffer (Tris pH 7.5, NaCl, 0.5M EGTA, 0.5 M EDTA, Triton-x 100, Na4P2O7, β-glycerophosphate, 1M Na3VO4). 10ul Protease/Phosphatase Inhibitor Cocktail (Cell Signaling Technology, Beverly, MA, USA) per ml of lysis buffer was added fresh, just before use. Tissues were first minced with a pestle until the pieces were too small to be broken down further without sonication and then were briefly lysed with
the sonicator (Fischer Scientific Model 100) with short pulses for 5-10 seconds on ice. Lysates were centrifuged at 12000 rpm for 10 mins at 4°C. The supernatant was separated and used for protein concentration measurement. Original undiluted lysates were then stored at -80°C. Protein concentration was determined by Bradford protein assay kit according to the manufacturer’s protocol using UV-spectrophotometer. 1 % BSA in cell signaling lysis buffer was used as a standard. Lysates were then diluted using 2x loading dye (300 mM Tris HCl pH 6.8, 10% SDS, 50% Glycerol, 10% 2-mercaptoethanol, 0.0025% Bromophenol blue) and cell signaling buffer to adjust the concentration of protein in all the lysates to the equal amount. Diluted lysates were vortexed, centrifuged at 12000 rpm at 4°C for 30 seconds, heated at 85°C for 3 - 4 mins, vortexed and centrifuged again. 45 ug of protein was loaded on 3–8% tris-acetate and 4–12% bis-tris gels (Invitrogen). Proteins were separated initially at 90V. After the samples fully entered the gel, the voltage was increased to 140V with a subsequent gradual decrease to 20V at the end of the run to preserve the quality of the bands. Proteins were transferred electrophoretically onto Polyvinylidene Difluoride membrane (Thermo Scientific 88518) (PVDF) at 110 mA for 70 min using transfer buffer containing 20% methanol, Tris Base 3g/L, Glycine 14.4g/L. Ponceau stain was used to check for the equality of proteins loadings. After transfer, the membranes were blocked for non-specific binding in 5% non-fat dry milk in TBS-T buffer (Tris Base 60.57 g/L, 52 NaCl 87.66 g/L PH adjusted to 7.4 with HCl and 0.1% Tween-20) on a shaker for 1 hour at room temperature. Further blots were incubated overnight on a shaker at 4°C with specific primary antibodies listed in the table below (Table II). All primary antibodies were diluted in 5% BSA dissolved in TBST with the addition of 6 ul of Sodium Azide per each milliliter of a mix. The
diluted primary antibodies were then stored at 4°C. After the incubation in primary antibodies, three 5-minute washings in TBST were performed and the blots were incubated at room temperature for 1 hr with secondary antibody. All secondary antibodies were diluted fresh in 1% non-fat dry milk dissolved in TBST right before the addition. Three 15-minute washings in TBST were performed for the elimination of the excessive binding of the secondary antibody. The imaging of the blots was performed using Clarity TM Western ECL Substrate (BIO-RAD), Scientific Imaging film and Odyssey FC imaging system (LI-COR). The analysis and quantification of the signal were done using Image Studio Lite Version 5.2. The relative protein expression was calculated by the signal ratio of the protein of interest to β-Actin, which was used as the loading control for the normalization.

**Table II: List of antibodies for immunoblot analysis**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Host</th>
<th>Company</th>
<th>Catalog number</th>
<th>Dilution</th>
<th>Exposure time (iS Ver. 5.2)</th>
<th>Chemi/700</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primary</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRY1</td>
<td>rabbit</td>
<td>Signalway Antibody</td>
<td>21414</td>
<td>1:500 in 5% BSA</td>
<td>5 min/2 min</td>
<td></td>
</tr>
<tr>
<td>CRY2</td>
<td>rabbit</td>
<td>Santa Cruz Biotechnology</td>
<td>sc-130731</td>
<td>1:200 in 5% BSA</td>
<td>5 min/2 min</td>
<td></td>
</tr>
<tr>
<td>FMO3</td>
<td>rabbit</td>
<td>Abcam</td>
<td>ab126711</td>
<td>1:1000 in 5% BSA</td>
<td>5 min/2 min</td>
<td></td>
</tr>
<tr>
<td>b-ACTIN</td>
<td>mouse</td>
<td>Sigma Aldrich</td>
<td>A5441</td>
<td>1:12000 in 5% BSA</td>
<td>2 min/30 sec</td>
<td></td>
</tr>
<tr>
<td><strong>Secondary</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-Mouse, Horse-linked</td>
<td>horse</td>
<td>Cell Signalling Technology</td>
<td>7076S</td>
<td>1:3000 in 1% milk</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-Rabbit, Goat-linked</td>
<td>goat</td>
<td>Cell-Signalling Technology</td>
<td>7074S</td>
<td>1:5000 in 1% milk</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.4 Statistical analysis.

For all experiments involving only young mice – comparisons by sex*feeding regimen, at least three male or female mice for every time point and for each feeding type were used. For all experiments involving young and old mice – comparisons by age*feeding regimen, six young mice and two old mice for every time point and for each feeding type were used. The sexes were combined for age effect analysis.

Data are shown as average +/- standard error of the mean. IBM SPSS Statistics 20 and GraphPad Prism Version 5.04 software packages were used for statistical analysis. To assay the effects of sex, diet and the time of the day, as well as the effect of age, diet and the time of the day - the analysis was performed using two-way repeated ANOVA. If the effect of feeding, time or sex was found to be statistically significant, Bonferroni correction was used to calculate the p-value for pairwise comparison. P<0.05 was considered as a statistically significant difference.

For the analysis of Circadian Rhythms in gene expression “R” Version 3.2.5 software – Cosinor Analysis package was used and the results are summarized in Tables III, IV and V.
CHAPTER III
RESULTS

3.1 Effect of sex on the circadian rhythms in expression of clock genes

In the previous study, we found that the daily rhythms in the expression of Bmal1, Per1, Per2, Per3, Cry2 and Ror γ were significantly affected by the CR in the liver of male mice (Patel et al., 2016). Our current study on the sexual dimorphism in circadian gene expression is summarized in Astafev et al. (Astafev et al., 2017). Male and female mice of the same age (3 months) were subject to 30% CR for two months. The expression of circadian clock genes was assayed in the liver of CR and control animals that have ad-libitum (AL) access to the food across the day. We found that in the liver of AL mice the expression of Bmal1 and Per1, Per2 and Per3 genes were similar between males and females, there were no significant differences in the phase of the expression (see Figure 3-1 and Table III). CR significantly induced the expression of these genes: at ZT22 and ZT2 for Bmal1; at ZT10 and ZT14 for Per1; at ZT18 for Per2 and ZT10 for Per3 in both males and females. While at some time points (ZT2 for Bmal1, ZT10 for Per1 and Per3) we observed statistically significant difference between the expression in males and females on CR, the changes in the expression were still observed for both males and
females and the changes were in the same direction, therefore, we concluded that sex of animals might have some effects on the magnitude of the response to CR but the expressions of \textit{Bmal1}, \textit{Per1}, \textit{Per2} and \textit{Per3} was similar in both sexes. CR did not affect the phase of gene expression because the food for the CR group was provided at ZT14 (2 hours after the light was off), which is the physiological time of feeding in nocturnal rodents.

Figure 3-1. Sex-independent expression and response to CR for some circadian clock genes.

The daily rhythms in expression of mRNA for \textit{Bmal1} (a), \textit{Per1} (b), \textit{Per2} (c) and \textit{Per3} (d) in the liver: blue diamonds and dashed lines - AL male mice; red squares and solid lines –
CR male mice, green triangles and dashed lines – AL female mice; purple x and solid lines – CR female mice. For all panels, graphs are double plotted. Light is on at ZT0 and off at ZT12. At every time of the day 3 mice of each sex (n=3) were used for each diet. a, b, c, d – statistically significant difference (p<0.05) between indicated groups at a particular time point. Data are represented as mean ± SEM. Open bars represent light and black bars represent the dark phase of the day. Food for CR group was provided at ZT14. Analysis of rhythmicity is summarized in Table III.

The expressions of Cry1, Cry2, Rev-Erb α and Ror γ were significantly affected by the sex of mice. In the AL group the expressions of all three genes were significantly higher in the liver of female mice: at ZT18, ZT22 and ZT2 for Cry1 (Figure 3-2a); at ZT6, ZT10 and ZT18 for Cry2 (Figure 3-2b); at ZT6 for Rev-Erb α (Figure 3-2c); at ZT22 for Ror γ (Figure 3-2d). Effect of CR on the expression of these genes was also affected by the sex. The expression of Cry1 gene was induced by CR at several time points (ZT14-ZT2) in males but CR did not have any effect on the expression of Cry1 in females (Figure 3-2a). On contrary, the expression of Cry2 was not significantly affected by CR in both males, in agreement with our previous work, and in females (Figure 3-2b). However, the mRNA levels were significantly higher in females than in males under both ad-libitum and CR conditions throughout the day. It is worth to notice, according to the rhythmic analysis, the expression of Cry2 in males was rhythmic under AL and arrhythmic upon CR, however in females, vice versa, arrhythmic expression of Cry2 under AL gained rhythm under CR (Table III). Rev-Erb α expression was induced by CR in males only at one time point (ZT6), while in females the significant induction was
detected at three time points ZT2, ZT6, ZT10 (Figure 3-2c). A similar pattern of response to CR was observed in the expression of *Ror γ* gene (Figure 3-2d). *Ror γ* expression was induced by CR in males, also, at only one time point (ZT22) while in females the significant induction was detected at three time points: ZT6, ZT14, ZT18 (Figure 3-2d). Thus, out of four clock genes, expressions of which were showing sexual dimorphism in the liver, CR affected the expression of one of them (*Cry1*) only in males; CR affected the expression of two (*Rev-Erb α, Ror γ*) in both sexes (but in females the induction was stronger and observed at several time points); and finally, CR did not affect the expression of one of these genes (*Cry2*) in both sexes.
Figure 3-2. Sexual dimorphism in the expression and response to CR for some circadian clock genes.

The daily rhythms in expression of mRNA for *Cry1* (a), *Cry2* (b), *Rev-Erb a* (c) and *Ror γ* (d) in the liver: blue diamonds and dashed lines - AL male mice; red squares and solid lines – CR male mice, green triangles and dashed lines – AL female mice; purple x and solid lines – CR female mice. For all panels, graphs are double plotted. The light is on at ZT0 and off at ZT12. At every time of the day 3 mice of each sex (n=3) were used for each diet. a, b, c, d – statistically significant difference (p<0.05) between indicated groups at a particular time point. Data are represented as mean ± SEM. Open bars represent light and black bars represent the dark phase of the day. Food for CR group was provided at ZT14. Analysis of rhythmicity is summarized in Table III.

Two clock genes – *Ror α* and *Rev-Erb β* did not show any significant difference in expression between males and females under AL conditions, however, we observed sexual dimorphism in response to CR for both of these genes. *Ror α* expression was induced in males at two time points (ZT10-ZT14) under CR. In females, the induction of expression was present throughout the whole 24-hour cycle and at ZT10-ZT14 time points the expression levels were also significantly higher in females than in males under CR condition (Figure 3-3a). It is notable that under CR condition *Ror α* expression lost circadian rhythm in males (Table III), however, based on expression pattern we can observe the rhythm still being present, but the period becomes less than 24 hours. In females, the expression remained arrhythmic under both AL and CR conditions. *Rev-Erb*
β expression was induced only in females and only at two time points (ZT10-ZT14) under CR conditions. The expression in males was not affected by CR at all. (Figure 3-3b) Therefore, for these two clock genes we can observe sex-specific difference only in the response to CR and conclude that for Ror α and Rev-Erb β CR has a significant effect on the expression in liver only in females. Even though we observed some induction in expression of Ror α in males under CR, the magnitude of the effect was still significantly smaller than in females.

![Graphs showing daily rhythms in expression of mRNA for Ror α and Rev-Erb β in the liver](image)

**Figure 3-3.** Sex-independent expression and sexual dimorphism in the response to CR for some circadian clock genes.

The daily rhythms in expression of mRNA for Ror α (a), Rev-Erb β (b) in the liver: blue diamonds and dashed lines - AL male mice; red squares and solid lines – CR male mice, green triangles and dashed lines – AL female mice; purple x and solid lines – CR female mice. For all panels, graphs are double plotted. The light is on at ZT0 and off at ZT12. At every time of the day 3 mice of each sex (n=3) were used for each diet. a, b, c, d –
statistically significant difference (p<0.05) between indicated groups at a particular time point. Data are represented as mean ± SEM. Open bars represent light and black bars represent the dark phase of the day. Food for CR group was provided at ZT14. Analysis of rhythmicity is summarized in Table III.

3.2 Effect of sex on circadian rhythms in the expression of CR regulated genes

Several genes have been identified through previous studies as targets of CR. The expression of those genes was also changed in long-lived dwarf mice which led researchers to propose them as longevity candidate genes (Swindell, 2007). We have found that in the liver their expression is regulated exclusively by CR but not by the time restricted feeding or fasting (Patel et al., 2016). It was also reported that the expression of some of them has sexual dimorphism. We analyzed the expression of Fmo3, Serpina12, Mup4, Cyp4a12b and Cyp4a14a. The results are presented in Figure 3-4 & 3-5. The expressions of all these genes were showing sexual dimorphism: Fmo3, Mup4 and Cyp4a12b expressions were different between sexes at all time points across the day; Serpina12 expression was different between males and females at ZT2, ZT10-14 (Figure 3-5b) and Cyp4a14a expression was different only at ZT6 (Figure 3-4b). Under AL conditions the Fmo3 expression was 20 - 100 folds (depending on the time of the day) higher in females than in males (Figure 3-4a). These results are in agreement with the previously reported sexual dimorphism of Fmo3 expression (Falls et al., 1997; Ripp et al., 1999). Fmo3 expression was induced by CR in both males and females, again in agreement with the previously published data (Fu & Klaassen, 2014). There was a dramatic difference between sexes in the magnitude of the CR effects on the expression. For females, Fmo3 expression was induced 2 - 5 folds, and for the males, the expression
was induced by 600 – 3000 folds. As a result of this difference in the response to CR, under CR conditions *Fmo3* expression was significantly higher in males, while under AL control conditions the expression was higher in females.

The expression of *Serpina12, Mup4, and Cyp4a12b* under AL conditions was several folds higher in males and it was significantly reduced upon CR in males only, while the tendency for the reduction in the expression was observed in females but the basal level was low and CR did not have statistically significant effect (Figure 3-5). There was no difference in the expression of *Cyp4a14a* gene between males and females at 5 time points under AL conditions, only at ZT6 the expression was higher in females (Figure 3-4b). CR induced the expression in both sexes at multiple time points suggesting the similarity in the response, however, while in males it was an overall induction upon CR, in females, it was rather a phase shift.

Thus, out of 5 tested genes four: *Fmo3, Serpina12, Mup4* and *Cyp4a12b* genes have strong sexual dimorphism in expression and for *Cyp4a14a* gene the dimorphism has been observed only at a single time point. The expression of two genes was induced by CR and three were suppressed, the four genes showing strong sexual dimorphism in the expression were also showing strong sexual dimorphism in their response to CR. *Cyp4a14a* expression showed weak sexual dimorphism in expression (with the difference only at one time point) and the response to CR was induction in both sexes with a phase shift only in females.
Figure 3-4. Sexual dimorphism in expression and response to CR for genes upregulated by CR.

The daily rhythms in expression of mRNA for *Fmo3* (a) and *Cyp4a14a* (b) in the liver: blue diamonds and dashed lines - AL male mice; red squares and solid lines – CR male mice, green triangles and dashed lines – AL female mice; purple x and solid lines – CR female mice. For all panels, graphs are double plotted. The light is on at ZT0 and off at ZT12. At every time of the day 3 mice of each sex (n=3) were used for each diet. a, b, c, d – statistically significant difference (p<0.05) between indicated groups at a particular time point. Data are represented as mean ± SEM. Open bars represent light and black bars represent the dark phase of the day. Food for CR group was provided at ZT14. Analysis of rhythmicity is summarized in Table III.
Figure 3-5. Sexual dimorphism and sex-independent expression and response to CR for genes downregulated by CR.

The daily rhythms in expression of mRNA for *Mup4* (a), *Serpina12* (b) and *Cyp4a12b* (c) and in the liver: blue diamonds and dashed lines - AL male mice; red squares and solid lines – CR male mice, green triangles and dashed lines – AL female mice; purple x and solid lines – CR female mice. For all panels, graphs are double plotted. The light is on at ZT0 and off at ZT12. At every time of the day 3 mice of each sex (n=3) were used for each diet. a, b, c, d – statistically significant difference (p<0.05) between indicated groups at a particular time point. Data are represented as mean ± SEM. Open bars
represent light and black bars represent the dark phase of the day. Food for CR group was provided at ZT14. Analysis of rhythmicity is summarized in Table III.

3.3 Effect of sex on circadian rhythms in protein expression

To assay whether the observed sexual dimorphism in mRNA expression translates into dimorphism in protein expression, we analyzed the expression of several genes by western blotting. The quantification results are presented in Figure 3-6. The respective blots are demonstrated in Figures 3-7, 3-8. In agreement with mRNA data, the expression of CRY1 protein was significantly higher in female livers under AL and CR conditions. Interestingly, under both AL and CR conditions the sex-based difference in protein levels of CRY1 was similar to the difference observed between sexes on mRNA level – females showed higher protein expression at ZT18-22 under AL and at ZT2-6; ZT18-22 under CR (Figure 3-6a, 3-6b). The expression of CRY2 was higher in female mice under CR at two time points (Figure 3-6d). Under AL conditions the expression of FMO3 protein was several folds higher in female livers than in male at all 6 time points throughout the day (Figure 3-6e), again in agreement with the mRNA data. The difference between sexes was not significant for CR samples except for ZT22 (Figure 3-6f). Thus, we observed some correlation between mRNA and protein data on sexual dimorphism in the expression of circadian clock and longevity candidate genes. At the same time, an absolute correlation between mRNA and protein data is relatively rare, the protein expression is regulated on multiple levels: translational and posttranslational. The circadian clock is involved in the posttranscriptional control of gene expression on multiple levels.
Figure 3-6. Sexual dimorphism in the expression of CRY1, CRY2 and FMO3 proteins.

The daily rhythms in expression of proteins for CRY1 (a and b), CRY2 (c and d), FMO3 (e and f) in the liver: blue diamonds and dashed lines – AL male mice; blue squares and solid lines – CR male mice, red triangles and dashed lines – AL female mice; red x and solid lines – CR female mice. For all panels, graphs are double plotted. The light is on at ZT0 and off at ZT12. Asterisk (*) indicates statistically significant difference (p<0.05) in protein levels between the sexes at a particular time point. Data are represented as mean ± SEM. Open bars represent light phase and black bars represent the dark phase of the day. Food for CR group was provided at ZT14. At every time of the day 3 mice of each sex (n=3) were used for each diet. The respective images from Western blot analysis are
shown in Figure 3-7 and Figure 3-8. Analysis of rhythmicity is summarized in Table IV.

![Figure 3-7. Sexual dimorphism in the expression of CRY1, CRY2 and FMO3 proteins.](image)

Representative Western blots for CRY1, CRY2 and FMO3 proteins assayed in the livers of mice. Liver samples from three mice (n=3) were pooled together per each time point, each sex and each feeding regimen (AL - Ad libitum and CR - Caloric Restriction). Open bars and dark bars on the top of the figure represent light and dark phase of the day. The numbers on the bottom of the figure are the time points. ZT0 is the time when light is on and ZT12 is the time when light is off. Food for CR group was provided at ZT14. Lysates (loading mix) prepared from liver samples of male and female mice of the same feeding regimen throughout 6 time points are loaded on one gel for between – sex comparison.
For each protein 2 blots are shown – with AL male and female samples, and with CR male and female samples.

Figure 3-8. Sex and diet effect on CRY1, CRY2 and FMO3 protein expression.

Representative Western blots for CRY1, CRY2 and FMO3 proteins assayed in the livers of mice. Liver samples from three mice (n=3) were pooled together per each time point, each sex and each feeding regimen (AL - Ad libitum and CR - Caloric Restriction). Open bars and dark bars on the top of the figure represent light and dark phase of the day. The numbers on the bottom of the figure are the time points. ZT0 is the time when light is on and ZT12 is the time when light is off. Food for CR group was provided at ZT14. 3 time points out of 6 are shown, lysates (loading mix) prepared from mouse liver samples from all groups – both sexes and feeding regimen from 3 time points, instead of 6, were used in order to be loaded on one gel for the demonstration of combined effect of diet and sex
on protein expression. For each protein, one blot is shown with four groups of comparison loaded back-to-back.

3.4 Effect of sex on circadian rhythms in Igf-1 expression

To extend our observations on sexual dimorphism in circadian rhythms and responses to CR we decided to investigate a signaling pathway relevant to CR, circadian clock, and aging. We recently reported that Cryptochromes are involved in the regulation of Insulin-like growth factor 1 (Igf-1) expression. We assayed the liver expression of Igf-1 gene in male and female mice on both diets. The results are presented in Figure 3-9. We found that Igf-1 mRNA expression is higher in females than in males under AL conditions. For both sexes, CR caused the reduction in the expression, but the expression in females still remained higher (Figure 3-9). Thus, the expression of Igf-1 demonstrated sexual dimorphism under both feeding regimen.
Figure 3-9. Sexual dimorphism in Igf-1 mRNA expression.

The daily rhythms in expression of mRNA for Igf-1 in the liver: blue diamonds and dashed lines - AL male mice; red squares and solid lines – CR male mice, green triangles and dashed lines – AL female mice; purple x and solid lines – CR female mice. For all panels, graphs are double plotted. The light is on at ZT0 and off at ZT12. At every time of the day 3 mice of each sex (n=3) were used for each diet. a, b, c, d – statistically significant difference (p<0.05) between indicated groups at a particular time point. Data are represented as mean ± SEM. Open bars represent light and black bars represent the dark phase of the day. Food for CR group was provided at ZT14. Analysis of rhythmicity is summarized in Table III.
3.5 Effect of age, short, and long-term CR on the circadian rhythms in expression of clock genes

The effects of CR were widely studied in the research field, however, to the best of our knowledge, there were no direct comparisons between short and long-term CR with its relation to the circadian clock. Due to lack of biological replicates for the old mouse groups, we combined sexes for this analysis and only showing the four genes which did not have any sexual dimorphism either in expression, or in response to CR. For all four genes – Bmal1, Per1, Per2, Per3 the expression did not change with age – there was no difference in expression between young and old mice under AL control condition. (Figure 3-10) All genes demonstrated induction in expression upon short-term CR: Bmal1 – at ZT22-ZT2 time points (Figure 3-10a), Per1 – at ZT10-ZT14 (Figure 3-10b), Per2 – at ZT18 (Figure 3-10c), Per3 – at ZT10 (Figure 3-10c). We found out that two of these genes retain the same response to CR with age – Per1 and Per3 genes still show the induction in expression in old age after mice have been on CR for two years. (Figure 3-10b,d) However, the other two genes – Bmal1 and Per2 showed attenuation in the response to CR – the effect disappeared with age and the expression profiles were similar in old mice under both AL and CR conditions. (Figure 3-10a,c) This result confirms the presence of underlying differences between short-term and long-term CR which might be essential for consideration. It would be interesting to further investigate if this difference is also present in the genes that show sexual dimorphism in expression and/or response to CR and whether the age-related difference between the effects of short and long-term CR is also sex-specific.
Figure 3-10. Age-independent expression, age-dependent and independent changes in response to CR for some circadian clock genes.

The daily rhythms in expression of mRNA for *Bmal1* (a), *Per1* (b), *Per2* (c) and *Per3* (d) in the liver: blue diamonds and dashed lines – young AL mice; black squares and solid lines – young CR mice, brown triangles and dashed lines – old AL mice; purple x and solid lines – old CR mice. For all panels, graphs are double plotted. The light is on at ZT0 and off at ZT12. At every time of the day 3 mice from young group (n=3) and 2 mice from old group (n=2) were used for each diet. a, b, c, d – statistically significant difference (p<0.05) between indicated groups at a particular time point. Data are represented as mean ± SEM. Open bars represent light and black bars represent dark.
phase of the day. Food for CR group was provided at ZT14. Analysis of rhythmicity is summarized in Table V.
4.1 Sex-based differences in Clock gene expression and response to CR

The roles of circadian clocks and rhythms in physiology and metabolism are well recognized. The connection between the regulation of peripheral circadian clocks and aging is also established. The clock disruption through genetic ablation of several clock genes: Bmal1, Period 2 and Clock results in reduced lifespan in mice (Dubrovsky et al., 2010; Fu et al., 2002; Kondratov et al., 2006). Similarly, clock disruption leads to the reduced lifespan in Drosophila model (Katewa et al., 2016), which suggests the conservation of clock mechanisms in aging. Recent data also support the hypothesis that circadian clocks are a part of CR mechanisms. In both Drosophila (Katewa et al., 2016) and mouse (Patel et al., 2016) models, CR significantly affects the rhythms in the clock gene expression in peripheral organs causing the change in the amplitude of the oscillations and the absolute levels of expression. Lifespan extension induced by CR is significantly compromised in flies with clock disruption (Katewa et al., 2016). In mammals the effect is even more dramatic, CR fails to increase a lifespan of mice
deficient for Bmal1 (Kondratov et al., 2006). These data together suggest a close interaction between the circadian clocks, CR, and aging.

There is more and more evidence that sex has a strong influence on many physiological processes. The circadian rhythms in physiology and physiological changes induced by CR are influenced by sex (Hadden et al., 2012; Krizo & Mintz, 2014; Mitchell et al., 2016). Indeed, there is a sex difference in the SCN morphology and signaling to and from SCN (Yan & Silver, 2016), which might contribute to the sex difference in many of the central clock controlled activities such as circadian behavior. Photic and non-photic, such as the effect of odor, responses or the timing of activity are different between males and females but the difference might be strain dependent (Mitchell et al., 2016). Sex may also affect the peripheral clocks and the entrainment of SCN independent circadian rhythms such as food anticipatory activity (Krizo & Mintz, 2014; Yan & Silver, 2016). In agreement with that in vitro and in vivo data suggest that circadian rhythms are affected by gonadotropins.

Many beneficial effects of CR including the lifespan increase are significantly affected by the sex. For example, for C57B/6J mice (the same strain that was used in our study), the 20% CR stronger increases lifespan in females than in males, while 40% CR increases lifespan in males only. Effects of CR on changes in body temperature, changes in glucose and insulin levels, changes in liver metabolism and gene expression were strongly influenced by the sex of the mice (Mitchell et al., 2016).

Here we compared the effect of CR on the expression of circadian clock genes in the liver between male and female mice, which were subjected to two months of 30%CR.
We did not find any sexual dimorphism in the expression or response to CR for such circadian clock genes as *Bmal1, Per1, Per2,* and *Per3* (Astafev et al., 2017). There are only a few reports on the effects of sex on the clock gene expression. The different phase in the expression of *Per2* gene between males and females has been reported for pituitary (Kuljis et al., 2013). Sexual dimorphism in the expression of several clock genes in response to the experimental chronic jet lag was observed in mouse lungs. After serial 8-hour advances of light/dark cycle, every 2 days for 4 weeks the expression of *Bmal1* is suppressed only in females, while the expression of *Clock* is suppressed only in males (Hadden et al., 2012). Similar to our report no sexual dimorphism in the expression of *Per1* or *Per2* was observed. We did not detect any effects of sex in the liver for *Bmal1* and *Per* genes under both AL and CR conditions, which suggests that these effects of sex on the expression might be tissue and treatment type specific.

The expression of several clocks genes: *Cry1, Cry2, Rev-Erb α and Ror γ* was significantly different between males and females on both of the diets used. In addition, the effect of CR on the expression of *Cry1, Rev-Erb α,β* and *Ror α,γ* was sex dependent too. At this moment there is little information explaining whether gonadal hormones can affect clock gene expression and if changes in levels of these hormones upon CR contribute to the observed dimorphism in response to CR.

Cryptochromes are negative regulators of circadian transcriptional-translational feedback loop, they repress circadian transcription in CLOCK:BMAL1 dependent manner. *Cry1* and *Cry2* genes have partially redundant functions in the circadian clock, animals deficient for either one of the two genes still demonstrate circadian rhythms, only the simultaneous deficiency of both genes results in the disruption of the rhythms. Both
CRY1 and CRY2 suppress CLOCK:BMAL1 transcriptional complex in a similar way. In contrast with the cell culture assays, there is more and more evidence that their functions in vivo are not identical. We found that in contrast to the other core clock genes, the expression of Cryptochromes is sex-dependent and is significantly higher in females. The response of these genes to CR is also different between males and females, Cry1 expression was affected only in males and Cry2 was not affected in both sexes. Interestingly, sexual dimorphism in Cry2 expression under the effect of chronic jet lag was detected in the above-discussed report on chronic jet lag (Hadden et al., 2012). What might be a physiological significance of this difference in Cry expression and response?

It is known that there is sexual dimorphism in gene expression in liver, which depends on transcriptional factor STAT5b. Recently CRYs have been implicated in the regulation of STAT5b activity (Chaudhari et al., 2017) and it was also previously reported that CRYs are essential for sexual dimorphism in the gene expression in liver (Bur et al., 2009). CR reduces the sexual dimorphism in Cry1 expression in liver, therefore, these results warrant further study on the crosstalk between CR, CRYs and sexual dimorphism.

Rev-Erb α and β are the isoforms of a transcriptional repressor from the family of orphan nuclear receptors, its recently found ligand is heme (Estall et al., 2009; Mazzoccoli et al., 2012). Rev-Erb α specifically binds DNA and inhibits Bmal1 gene expression. Rev-Erb α activity is important for the circadian control of metabolism (Duez & Staels, 2008; Green et al., 2008; Potter et al., 2016; Preitner et al., 2002; Sahar & Sassone-Corsi, 2012; Sato et al., 2006; Takahashi, 2016). The two isoforms: Rev-Erb α and β display a great degree of functional redundancy. Both are binding heme and acting as transcriptional repressors on RORE sites – this drives the negative feedback loop in the
regulation of Bmal1 transcription, as well as other genes possessing ROREs in their promoters. Importantly, Rev-Erb β may compensate for the deficiency of Rev-Erb α (Estall et al., 2009). The sexual dimorphism in the response to the experimental chronic jet lag (discussed above) was reported for the expression of Rev-Erb α. Upon the chronic jet lag the Rev-Erb α expression was induced in the lungs of male and suppressed in the lungs of female mice, thus, the effect was opposite in different sexes (Hadden et al., 2012). In our experiment, the effect of CR was similar (the induction of the expression) in both sexes at one time point, and observed only in females at two other time points with no difference for males. What is a physiological significance of this dimorphism in Rev-Erb α expression and response is not known, interestingly, polymorphism in Rev-Erb α promoter is linked with obesity in males but not females in humans (Ruano et al., 2014). Unlike Rev-Erb α, Rev-Erb β did not show sexual dimorphism in expression, although the trend for induction upon CR was present in males, only in females the expression was induced significantly under CR (Figure 3-2c, 3-3b). Overall, the pattern was similar to Rev-Erb α – in all of the mouse groups there was a strong circadian oscillation in expression profile with the female mice showing highest expression under CR. The only difference between the mRNA expression profiles of the two isoforms was a slightly earlier peak in expression of Rev-Erb α. This outcome was expected due to the mentioned earlier substantial degree of functional redundancy between the isoforms.

The retinoic acid-related orphan receptor γ (RORγ) is a ligand-dependent transcription factor that drives the expression of some Clock genes by binding as a monomer directly to ROR-responsive elements (ROREs) which are located in the promoter sequences of the target genes (Takeda et al., 2014). Ror γ expression is highly
tissue-specific – it is restricted to peripheral tissues which are most actively performing metabolic functions and maintain energy homeostasis, such as liver and adipose tissue, unlike the expression of Ror α/ROR α which was proposed to regulate some physiological processes distinct from the pathways regulated by ROR γ. For instance, ROR α is essential for the development of the cerebellum and is required for the maturation of photoreceptors in the retina; ROR γ, in turn, plays an important role in the development of several secondary lymphoid tissues, including lymph nodes (Jetten et al., 2009). Takeda, et al. reported that RORγ is showing strong circadian oscillation and is recruited to ROREs of circadian clock genes, such as Bmal1, Clock, Rev-Erb α, Cry1. However, in Ror γ deficient mice the moderate reduction of mRNA level in liver was observed only in Cry1 and Rev-Erb α but not in Bmal1 and Clock, which suggests that Ror γ is an essential factor to induce transcription of Cry1 and to indirectly induce Rev-Erb α transcription (Takeda et al., 2014). In the previous study performed by our lab, we found out that CR increased the mRNA levels of Ror γ at some of the time points across the day (Patel et al., 2016). To the best of our knowledge, there have been no studies investigating the effect of sex on Ror γ expression in a circadian manner. In our experiments, CR induced the expression in both sexes, however, in females, the induction was stronger. Under AL control conditions the expression was higher in females than males at one time point. Under CR Ror γ expression was significantly induced at 3 time points in females and at 1 time point in males (Figure 3-2d). The expression of Ror α was not sexually-dimorphic under AL; under CR it was induced in males at 2 time points, while in females it was induced throughout all 24 hours, demonstrating a strong dimorphism in both the response to CR and in the levels of expression under CR between
sexes (Figure 3-3a). Unlike in Ror γ, Ror α displayed a non-circadian expression profile. While some oscillation was present, the period was shortened. Some degree of functional non-redundancy and involvement of the two members of ROR family in the regulation of various tissue-specific processes might account for some of this difference. The physiological importance of sexual dimorphism in Ror expression is yet to be investigated, interestingly, the response to CR in males and females was similar in Ror γ and Rev-Erb α. A partial explanation of this particular effect might be due to the fact both Rev-Erv α and Ror γ are RORE-containing genes, therefore, CR might affect the expression of Ror γ and Rev-Erb α in a similar manner. The response to CR, however, was different between males and females in the expression of another RORE-containing target of Rev-Erb - Cry1 suggesting a more complex regulatory interaction.

4.2 Sex-based differences in longevity – associated gene expression and response to CR

We also assayed the effect of the sex on circadian rhythms in the expression of several genes, for which mRNA expressions in the liver were previously reported to be changed upon CR. For some of these genes such as Fmo3, Mup4, and Cyp4a12 the sexual dimorphism in the expression was previously known and for some, such as Serpina12, was not.

Flavin-containing monooxygenase 3 (FMO3) is responsible for the oxidation of trimethylamine (TMA) to trimethylamine-N-oxide (TMAO). This reaction is essential because TMA is a gas that has a characteristic foul “fishy” odor – this gas is produced from catabolism of dietary carnitine and choline by gut microbiota and then is absorbed
into the bloodstream. FMO3 converts TMA to TMAO - an odorless molecule, which indicates FMO3 is a vital detoxifying enzyme (Shih et al., 2015). Fmo3 is known to be one of the most strongly induced by CR gene in the liver. It is also known that Fmo3 expression is strongly affected by sex in mice, the expression is high in females and practically undetectable in males (Fu et al., 2012). In agreement with previously published data, we found that Fmo3 expression is about 20 fold higher in females than in males under AL conditions on mRNA level and about 2 fold higher in females than in males on protein level throughout the whole 24-hour cycle. In both sexes, CR induced the expression, but with a different magnitude. Fmo3 expression is significantly affected by gonadal steroids - testosterone inhibits and estrogen induces the expression. It is known that testosterone level is reduced upon CR but the reduction is about 11% in males (Müller et al., 2015), therefore, the level is still significantly higher in males than in females. At the same time, the expression of Fmo3 in males upon CR is 10 folds higher than in CR females, thus, observed changes in the expression cannot be explained only through changes in testosterone or estrogen levels, suggesting the existence of some other regulatory mechanisms. The significance of the effect of Fmo3 induction by CR on physiology and longevity is not known, but it was reported that Fmo3 homolog is involved in the control of lifespan in nematodes (Leiser et al., 2015).

Serpina12 gene encodes secreted serine protease inhibitor from serpin superfamily but the targeted protease is currently unknown. The product is known as vaspin and implicated in the control of metabolism, insulin sensitivity and glucose homeostasis (Dimova & Tankova, 2015). Treatment with recombinant vaspin was found to improve insulin sensitivity in ICR mice fed on high-fat high-fructose chow and significantly
reduce blood glucose levels 120 min after i.p. glucose injections (Hida et al., 2005). The association of elevated vaspin with obesity and type 2 diabetes mellitus was shown as well: elevated serum vaspin was reported in type 2 diabetes patients compared to healthy subjects, also there was a positive relationship between elevated serum vaspin and the presence of coronary artery disease in patients (Hao et al., 2016). In most of the studies the expression of Serpina12 gene was assayed indirectly through the serum level of vaspin protein and only a few reported the expression on mRNA level; it is known that the expression is regulated by feeding and it is changed under pathological conditions, however, the data are rather conflicting at these points (Dimova & Tankova, 2015). The sexual dimorphism in circulating vaspin level in obese individuals with women showing higher level than men has been reported (Moradi et al., 2016). Also, vaspin was found to have a dynamic increase in serum during puberty in girls, but not boys (Körner et al., 2011). The level of vaspin in human blood serum was found to be oscillating in the circadian manner (Jeong et al., 2010). The reported here strong sexual dimorphism in daily rhythms of the mRNA expression and the dimorphism in the response to CR have not been previously reported to the best of our knowledge and might contribute to the above mentioned conflicting results.

* Mup4 gene encodes major urinary protein 4, which belongs to the family of male-specific proteins that can bind odorant molecules and regulate its release from the scent marks (Sharrow et al., 2002). The observed sexual dimorphism in the expression of *Mup4* was expected and male-specific reduction in the expression was also expected as a part of a reduced physiological investment in reproduction upon CR. Our study corroborates the previously published results of Giller *et al.* (Giller et al., 2013) which report that the
mRNA levels of 8 $Mup$ isoforms are decreased in liver of C57BL/6 mice subjected to 25% CR for 6 months. $Mup$ expression is also regulated by testosterone (Cavvagioni & Mucignat-Caretta, 2000) and by growth hormone and thyroxine in a testosterone-independent manner (Knopf et al., 1983). The levels of these hormones, in turn, are affected by CR. The observed circadian rhythms in the expression might contribute to circadian rhythms in animal sexual behavior.

$Cyp4a12b$ and $Cyp4a14a$ genes encode the enzymes that belong to the cytochromeP450 superfamily. These enzymes are involved in omega-hydroxylation of fatty acids and are implicated in the regulation of blood pressure (Muller et al., 2007; Okita & Okita, 2001). $Cyp4a12b$ demonstrated a strong male-specific expression (Figure 3-5c) across the day. Similar results were reported in the liver and kidney by Jeffery et al. but it is unclear if the expression of $Cyp4a12a$ or $Cyp4a12b$ was analyzed (Jeffery et al., 2004). Zhang et al. showed that Cyp4a12b was male-predominant in liver and kidney of C57BL/6 mice which is supported by our results (Zhang & Klaassen, 2013). Muller et al. analyzed the expression of both $Cyp4a12a$ and $Cyp4a12b$ in the kidneys and found the sexual dimorphism only for $Cyp4a12a$ but not for $Cyp4a12b$ expression (Muller et al., 2007), therefore, reported here sexual dimorphism in $Cyp4a12b$ expression might be tissue specific. It was also reported by Zhang et al. that $Cyp4a14$ expression is higher in females. Our data only partially correlates with that, we observed increased expression in females only at one time point (ZT6) and the expression was similar for both sexes through the rest of the day (Figure 3-4b), thus, we concluded that unlike $Cyp4a12b$ the expression of $Cyp4a14a$ under AL conditions does not demonstrate a strong sexual dimorphism. Little is known about the effect of diet on the expression of Cyp4as. The
study of Lee et al. demonstrated the upregulation of Cyp4a14 in the liver of C57BL6 mice fed on high-fat diet upon the administration of Aliskiren (Lee et al., 2016). In our study the effect of CR on the expression of these two genes was opposite: Cyp4a12b expression was downregulated only in males and Cyp4a14a expression was upregulated in both sexes, but the response to CR was quite different – in males the induction of expression was observed throughout the day, while in females the expression profile showed rather a phase shift under CR. It will be interesting to study if this sexual dimorphism in the response to CR will differentially affect cardiovascular functions in males and females.

The expressions of some genes showing sexual dimorphism (Fmo3, Mup4 and Cyp4a12) are regulated by gonadal hormones. It is known that CR reduces the level of testosterone and, therefore, the observed changes in the expression might be a consequence of it, however, the fact that upon CR Fmo3 expression in males is significantly higher than in females (Figure 3-4a) is arguing against this simplistic explanation. It is unknown if the expression of circadian clock genes is regulated by gonadal hormones. According to our data under AL conditions the Cry1 expression is higher in females and upon the CR the expression is induced only in males, in agreement with “feminizing” effect of CR on liver gene expression. At the same time, the Cry2 expression was also higher in females and it was not affected by CR in males and the expression of Rev-Erb α and Ror γ was induced in both sexes, again suggesting a more complex mechanism of regulation than just a response to changes in gonadal hormone levels.
4.3 Sex-based differences in Igf-1 expression

It is known that some part of beneficial effects of CR is mediated by Igf-1 signaling. CR was shown to decrease Igf-1 expression levels in liver, as well as decrease serum IGF-1 levels. Decreased Igf-1 signaling is associated with a strong protective effect against some types of tumors. In our current study, we observed that CR decreased the Igf-1 expression levels in both males and females, however, under both AL and CR conditions males showed lower mRNA levels than females at several time points across the day. Recently in one of the studies done by our lab, we reported that Cryptochromes are involved in the control of Igf-1 (Chaudhari et al., 2017). We have observed sexual dimorphism in expression of Cry1 and Cry2 on both mRNA and protein levels which might be at least partially responsible for the observed difference between sexes in Igf-1 as well.

4.4 Age-based differences in Clock gene expression and response to short and long-term CR

Despite being limited in the sample size, in the analysis of short-term versus long-term CR, we managed to make two important observations: the mRNA expression profiles of analyzed core clock genes Bmal1 and Per1,2,3 do not change with age in the liver under AL conditions, and two out of four analyzed genes (Bmal1 and Per2) showed attenuation in response to CR after long-term exposure. The possible explanations for these observed events might be the following: the widely debated age-related change in circadian rhythms is mainly caused by the changes in the central clock, instead of peripheral clock, as no effect of age was observed on mRNA expression of core clock genes in liver even in mice which had round the clock access to food. The age-associated
changes might also affect the translational control and the feedback control of circadian clock on the protein level. However, in order to confirm this effect, we need to analyze the expression of BMAL1 and PER1,2,3 proteins in aged mice. Other mechanisms, such as age-associated hormonal changes might contribute to the alterations in circadian rhythms in the central clock, but not in the peripheral clock. Quite possibly, aged mice might also display habitual rhythm in their feeding and consume less food overall, which might be the reason why intact rhythms in the liver clock are preserved. The rest of the circadian genes need to be analyzed on both mRNA and protein levels in order to see whether there is any change in rhythms under AL condition. In the analysis of short term vs long term CR, we observed the unchanged response to CR in two of the genes: Per1 and Per3. Per2 expression profile in older mice which were exposed to CR for two years showed attenuation and decrease in amplitude while still retaining the peak of expression at around 4 hours after feeding – similar to the effect observed in short-term CR. This observation supports the non-redundancy of Periods. Currently there is no particular explanation why only Per2 showed dampened response under long-term exposure to CR, however it is interesting that the mutation in Per2 gene is associated with familial advanced sleep phase syndrome (FASPS) and the deficiency in Per2 causes dampened circadian rhythms in the expression of circadian genes in the central clock (Bae et al., 2001; Toh et al., 2001). At the same time, the circadian phase is observed to gradually advance with age. Also decreased levels of Per2 expression might contribute to an increased susceptibility to tumorigenesis in older age, as the overexpression of Per2 showed strong tumor-protective effect (Hua et al., 2007; Oda et al., 2009).
In case of Bmal1, we hypothesize that this core clock factor is playing an important role, particularly in the adaptation to CR conditions, which is why the response to CR attenuates with time. In the previous study where our lab reported the failure of CR to induce longevity-extending effects in Bmal1-/- mice, we also observed that the majority of these mice died shortly after CR was introduced. However, those several mice which managed to survive lived approximately as long as Bmal1-/- mice under AL. This kind of effect was not observed in wild-type C57BL/6J inbred mice, however, the similar pattern was observed in the study, where CR was introduced to a group of genetically heterogeneous mice, which were caught from the wild habitat – many of those mice failed to adapt to CR, however those that survived that initial step displayed long lifespan (Harper et al., 2006). We hypothesize that the change in Bmal1 expression under long-term CR might be due to such reason – Bmal1 expression is strongly induced upon short-term CR as a part of adaptation mechanisms, but is then dampened under long-term exposure.

4.5 Future directions

In our future studies, we are planning to assay the expression of clock and longevity-associated genes in order to see the interaction between sex and age once we have a sufficient sample size in aged mice which we lacked in the current study. We are also going to check for the sexual dimorphism in expression of some glycolytic enzymes as glucose metabolism involves the regulation by the circadian clock. Also, in order to continue one of the projects, which originated in the lab previously, we are going to further investigate the regulation of STAT5b by Cryptochromes in a sex-specific manner.
That particular direction of research might shed some light on the possible involvement of Cryptochromes in the control of the liver $Igf-1$ expression.
CHAPTER V
CONCLUSION

In summary, there is more and more understanding that sex is an important variable that affects many aspects of physiology, which are not necessarily and/or exclusively related to the reproductive functions. Our data on the sexual dimorphism in circadian rhythms in gene expression and in CR induced effects on these rhythms are in agreement with this opinion. Here we compared the circadian rhythms in the expression between male and female mice and found that out of our 10 tested genes - two (Cry1 and Cry2) have strong and two (Rev- Erb α and Ror γ) have modest sexual dimorphism in the mRNA expression in liver. The response to CR was also different between males and females for these genes – the magnitude of this difference varied with respect to each gene. Other two genes (Rev-Erb β and Ror α) did not show strong sexual dimorphism in mRNA expression in liver, but the response to CR was sexually dimorphic for this pair of genes. Out of five genes previously reported to be regulated by CR, four have shown a strong sexual dimorphism in the expression while one gene showed a modest dimorphism. All of five genes were sexually dimorphic in the response to CR. We also showed that the sexual dimorphism can extend to the pathways which interact with circadian clock and CR mechanisms – mRNA expression of Igf-1 in liver was different.
between sexes, but the response to CR was the same. It is now apparent, that these findings will need to be taken into account when designing experimental setups. The studies that involve the genes with the observed sexual dimorphism in expression will need to separate the mice by sex for sample pooling and statistical analysis. Whether these genes differently contribute to CR effects on longevity in males and females still needs to be studied. There is some controversy on the benefits of CR in short and long-term and multiple factors might influence the response to CR. We also showed that out of 4 tested genes, two (Bmal1 and Per2) showed the attenuation in response to long-term CR and the other two (Per1 and Per3) retained the response. We did not find any age-related changes in expression for any of these four genes in liver under AL conditions.

The circadian rhythms were proposed, by us and others, as an important contributor to CR mechanisms, and the present data suggest that the interaction between sex, age and the circadian rhythms must be taken into consideration for optimal CR implementation.
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**APPENDIX**

**Table III. Sex and diet effect on circadian rhythms in gene expression**

**Cosinor Wave Analysis of Clock and Longevity – Associated Genes**

Expression for Figures 3-1; 3-2; 3-3; 3-4 and 3-5. The circadian parameters were assessed by the cosinor analysis that models the circadian rhythms (Circa) as a cosine function with the following attributes: amplitude (amp), and acrophase (acr). The rhythm in gene expression pattern is considered circadian if rhythmicity fit value is greater or equal to 0.7.

*Bold italic values marked with asterisk* indicate arrhythmic expression of the respective gene for particular experimental group.

<table>
<thead>
<tr>
<th>Name of Gene</th>
<th>Rhythmicity fit</th>
<th>Acrophase</th>
<th>Amplitude</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
</tr>
<tr>
<td></td>
<td>AL  CR</td>
<td>AL  CR</td>
<td>AL  CR</td>
</tr>
<tr>
<td>Bmal1</td>
<td>0.99 0.99</td>
<td>0.95 0.97</td>
<td>22.64 22.8</td>
</tr>
<tr>
<td>Per1</td>
<td>0.89 0.81</td>
<td>0.85 0.92</td>
<td>12.12 11.22</td>
</tr>
<tr>
<td>Per2</td>
<td>0.98 0.95</td>
<td>0.99 0.94</td>
<td>14.36 15.51</td>
</tr>
<tr>
<td>Per3</td>
<td>0.9 0.82</td>
<td>0.84 0.71</td>
<td>12.07 10.26</td>
</tr>
<tr>
<td>Cry1</td>
<td>0.99 0.94</td>
<td>0.97 0.98</td>
<td>18.71 19.7</td>
</tr>
<tr>
<td>Cry2</td>
<td>0.94 <strong>0.43</strong></td>
<td><strong>0.65</strong> 0.72</td>
<td>13.51 13.86</td>
</tr>
<tr>
<td>RevErb α</td>
<td>0.97 0.94</td>
<td>0.75 0.94</td>
<td>7.58 7</td>
</tr>
<tr>
<td>Gene</td>
<td>Rev-Erb β</td>
<td>Ror α</td>
<td>Ror γ</td>
</tr>
<tr>
<td>----------</td>
<td>------------</td>
<td>-----------</td>
<td>-----------</td>
</tr>
<tr>
<td></td>
<td>0.98</td>
<td>0.74</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td>0.96</td>
<td>0.09*</td>
<td>0.98</td>
</tr>
<tr>
<td></td>
<td>0.97</td>
<td>0.06*</td>
<td>0.98</td>
</tr>
<tr>
<td></td>
<td>0.97</td>
<td>0.29*</td>
<td>0.96</td>
</tr>
<tr>
<td></td>
<td>10.21</td>
<td>20.52</td>
<td>18.72</td>
</tr>
<tr>
<td></td>
<td>10.29</td>
<td>22.89</td>
<td>18.46</td>
</tr>
<tr>
<td></td>
<td>10.66</td>
<td>17.63</td>
<td>18.76</td>
</tr>
<tr>
<td></td>
<td>6.43</td>
<td>1.45</td>
<td>4.21</td>
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<tr>
<td></td>
<td>9.74</td>
<td>1.74</td>
<td>4.52</td>
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<tr>
<td></td>
<td>11.26</td>
<td>2.61</td>
<td>6.88</td>
</tr>
<tr>
<td></td>
<td>20.76</td>
<td>1.59</td>
<td>5.17</td>
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</tbody>
</table>

Notes: * indicates statistical significance.
Table IV. Sex and diet effect on circadian rhythms in protein expression.

Cosinor Wave Analysis of CRY1, CRY2 and FMO3 protein expression for

Figure 3-6. The circadian parameters were assessed by the cosinor analysis that models the circadian rhythms (Circa) as a cosine function with the following attributes: amplitude (amp), and acrophase (acr). The rhythm in gene expression pattern is considered circadian if rhythmicity fit value is greater or equal to 0.7.

*Bold italic values marked with asterisk indicate arrhythmic expression of the respective gene for particular experimental group.

<table>
<thead>
<tr>
<th>Name of Protein</th>
<th>Rhythmicity fit</th>
<th>Acrophase</th>
<th>Amplitude</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
</tr>
<tr>
<td>AL CR</td>
<td>AL</td>
<td>CR</td>
<td>AL</td>
</tr>
<tr>
<td>CRY1</td>
<td>0.61*</td>
<td><strong>0.69</strong>*</td>
<td>0.96</td>
</tr>
<tr>
<td></td>
<td>0.89</td>
<td>0.93</td>
<td>0.37</td>
</tr>
<tr>
<td>CRY2</td>
<td>0.42*</td>
<td><strong>0.54</strong>*</td>
<td>0.78</td>
</tr>
<tr>
<td></td>
<td>0.72</td>
<td>0.72</td>
<td>0.72</td>
</tr>
<tr>
<td>FMO3</td>
<td>0.37*</td>
<td><strong>0.53</strong>*</td>
<td>0.83</td>
</tr>
<tr>
<td></td>
<td>0.54*</td>
<td><strong>0.72</strong>*</td>
<td>0.83</td>
</tr>
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</table>
Table V. Age and diet effect on circadian rhythms in gene expression

Cosinor Wave Analysis of Clock Gene Expression for Figure 3-10. The circadian parameters were assessed by the cosinor analysis that models the circadian rhythms (Circa) as a cosine function with the following attributes: amplitude (amp), and acrophase (acr). The rhythm in gene expression pattern is considered circadian if rhythmicity fit value is greater or equal to 0.7.

*Bold italic values marked with asterisk* indicate arrhythmic expression of the respective gene for particular experimental group.

<table>
<thead>
<tr>
<th>Name of Gene</th>
<th>Rhythmicity fit</th>
<th>Acrophase</th>
<th>Amplitude</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Young</td>
<td>Old</td>
<td>Young</td>
</tr>
<tr>
<td>Bmal1</td>
<td>0.99</td>
<td>0.98</td>
<td>0.98</td>
</tr>
<tr>
<td>Per1</td>
<td>0.88</td>
<td>0.88</td>
<td>0.92</td>
</tr>
<tr>
<td>Per2</td>
<td>0.99</td>
<td>0.95</td>
<td>0.92</td>
</tr>
<tr>
<td>Per3</td>
<td>0.87</td>
<td>0.75</td>
<td>0.99</td>
</tr>
</tbody>
</table>