Circadian and metabolic consequences of shift work

-a rat model

Andrea Rørvik Marti

Master's program in psychology, psychological science programme

Specialization: Behavioural Neuroscience

at

THE UNIVERSITY OF BERGEN

FACULTY OF PSYCHOLOGY

AUTUMN 2015

Main supervisor: Janne Grønli

Department of Biological and Medical Psychology, University of Bergen, Norway

Sleep and Performance Research Center, Washington State University, US

Co-supervisor: Jelena Mrdalj

Department of Biological and Medical Psychology, University of Bergen, Norway

Abstract

Shift workers are at risk for metabolic health problems. Previous research suggests circadian rhythm disruption as an underlying mechanism. In my thesis, I investigate the circadian and metabolic consequences of shift work in a rat model, and discuss mechanisms responsible for the observed changes.

To mimic human shift work, rats were kept awake/"working" in rotating wheels for 8h during resting (RW) or active (AW) phase. Body temperature, locomotor activity, food and water intake, and body weight were monitored for one shift work period (4d) and recovery (8d) in constant light conditions (12hL/D), and compared to baseline. A subset of rats was exposed to constant darkness recovery (DD) to assess endogenous rhythmicity.

AW exhibited normal circadian rhythmicity throughout the protocol. RW shifted circadian rhythm during the shift work period, and parameters recovered at different rates: body temperature nadir immediately; locomotor activity nadir after 1day; % activity rhythm remained unrecovered. This indicates internal desynchrony. Data from DD recovery demonstrate desynchronization of endogenous nature. Both groups exhibited negative energy balance during the shift work period, but RW more than AW. Only AW regained body weight during recovery. RW unexpectedly developed resting phase hypothermia in the recovery period.

In conclusion, four days of resting phase activity is sufficient to cause short- and long-lasting circadian and metabolic disruption. Findings are supported by data on circadian and metabolic gene expression, sleep and glucocorticoid levels in the same animals. This model is promising to increase our understanding of the mechanisms contributing to negative effects of shift work.

Key words: Circadian rhythms, metabolic disturbance, night work, rat model, shift work

Sammendrag

Skiftarbeid øker risiko for å utvikle metabolske forstyrrelser. En underliggende årsak kan være døgnrytmeforstyrrelser. I denne oppgaven har jeg undersøkt hvordan skiftarbeid påvirker døgnrytme og metabolisme i en rottemodell, og diskuterer mekanismene som forårsaker disse endringene.

For å etterligne skiftarbeid hos mennesker ble rotter holdt våkne («i arbeid») i roterende hjul i 8t under hvilefase (RW) eller aktiv fase (AW). Kroppstemperatur, lokomotorisk aktivitet, mat- og vanninntak, og kroppsvekt ble målt i løpet av en skiftarbeidsperiode (4d) og recovery (8d), med konstante lysforhold (12tL/D). Data ble sammenliknet med baseline. En undergruppe rotter ble eksponert for konstant mørke (DD) under recovery for å måle endogene døgnrytmeendringer.

AW opprettholdt normal døgnrytme gjennom hele protokollen. RW viste både kort- og langvarig døgnrytmeforskyvning under skiftarbeidsperioden. I løpet av recovery viste kroppstemperatur nadir en umiddelbar normalisering; lokomotorisk aktivitet nadir etter 1 dag; % aktivitetsrytme ble ikke gjenopprettet. Ulik tid for normalisering tyder på intern desynkronisering, og data fra DD-recovery viser at desynkroniseringen var endogen. Begge gruppene viste negativ energibalanse under skiftarbeidsperioden; RW mer enn AW. Kun AW gjenopprettet kroppsvekten i løpet av recovery. RW utviklet uventet hypotermi i hvilefasen i løpet av recovery.

Jeg konkluderer med at fire dager med aktivitet i hvilefasen er tilstrekkelig til å forårsake både kort- og langvarige forstyrrelser i døgnrytme og metabolisme. Funnene støttes av endringer i gendata knyttet til døgnrytme og metabolisme, samt søvn og glukokortikoidnivåer, målt hos de samme dyrene. Denne modellen kan i fremtiden brukes til å øke forståelsen av mekanismene som bidrar til negative effekter av skiftarbeid.

Nøkkelord: Døgnrytme, metabolsk forstyrrelse, nattarbeid, rottemodell, skiftarbeid

Acknowledgements

First and foremost, I want to thank my main supervisor, Janne Grønli. You have taught me so much, and given me amazing opportunities to visit sleep conferences in Estonia and Seattle, present my data, and meet some truly amazing people. Your attitudes, ideas, commitment, and enthusiasm inspire me. You have supported and challenged me through every step of the process, and I look forward to continuing working with you.

To my co-supervisor Jelena Mrdalj, I have learned so much from you in the past few years, from hours of surgeries and lab, to data analysis and thesis writing. Thank you so much for always taking the time to discuss questions and give me feedback, and of course, for the countless cups of coffee.

I want to thank my co-student Torhild Thue Pedersen. We have worked very closely over the past few years, from day and night shifts in the lab, to sharing an office during the writing process. Thank you for the constant support and coffee-breaks when they were most needed. We sure make a great team!

I also want to thank all the people who have helped me and supported me during this process, either with data collection, analyses, feedback or simply moral support; Silje Skrede, Sjoerd van Hasselt, Magne Solheim, Anne Marie, William, and my classmates Ingrid and Gustav.

I want to thank my boyfriend Vegard, for great discussions and feedback in the writing of my thesis, and for your constant love and support.

Lastly, I want to thank my family for always supporting me and believing in me. To my parents and step-parents, Linda, Thor, Sven and Janne. And of course, to the best younger brothers in the whole world, Adrian, Oliver, Jonatan and Nicolas.

My contribution to the dataset presented in this thesis

I was invited to take an active part in this project, led by Janne Grønli, in March of 2014, during the second semester of my two-year master programme. I have contributed to the design of the experiment, in surgical implantation of telemetric transmitters, post-surgical care, technical set-up of experimental equipment, collection of data, as well as the analyses of the data presented in this thesis.

The collection of data throughout the period March-June 2014 was done in close collaboration with the research team. This included daily care of animals; checking health status and providing food and water. We also weighed the rats before and after "work shifts", placed them in automatically rotating wheels, navigated through cables and connections to collect data both in the home cage and in the rotating wheels in a separate experimental room. We did this both in the daytime and during the night, with only a red lamp as a light source. At the end of each shift we cleaned the rotating wheels, collected faecal samples, and returned the rats to their home cage.

At the end of the experiment, I assisted in euthanizing animals. We collected tissue from brain, liver, adrenals, and brown and white adipose. I organized, prepared, and analysed all the circadian rhythm data, as well as data on body weight, food and water intake. I have learned a lot from being part of this project; it was hard work, but it was fun. I feel lucky to have been allowed to take an active part in all phases of the work that has lead up to the completion of this master thesis.

Gene expression analyses by use of quantitative PCR from the tissues collected were performed by exchange student Sjoerd van Hasselt from the University of Groeningen, Netherlands, supervised by post-doctoral candidate Silje Skrede at Department of Clinical Science. The gene expression data will be presented in Sjoerd's master thesis, but I will discuss the results in relation to my own findings in the discussion section of this thesis.

Table of contents

Abstract i

Sammendrag ii

Acknowledgements iii

My contribution to the dataset presented in this thesis iv

Table of contents v

List of figures ix

List of tables x

Introduction 11

Shift work 11

Circadian rhythms 11

Regulation of circadian rhythms 12

Measuring circadian rhythms 13

Endogenous nature of circadian rhythms and entrainment 17

Metabolism 18

Regulation of metabolism 19

Measuring metabolism 21

Circadian rhythms, metabolism and shift work 22

Consequences of shift work 24

Human studies 24

Methodological challenges in human shift work research 26

Animal studies 27
Establishing and evaluating animal models of shift work 29
Aims and hypotheses 30
Methods 32
Ethical approval 32
Animals and housing 32
Design 32
Surgical procedure 34
Body weight, food and water measurements 35
Shift work procedure 35
Telemetric recording and analyses 36
Statistical analyses 37
Results 39
Baseline analyses 39
Food intake, water intake, and body weight 39
Locomotor activity and body temperature 39
Consequences of one shift work period 40
Active phase workers (AW) 40
Circadian rhythmicity 40
Metabolic parameters 41
Resting phase workers (RW) 43
Circadian rhythmicity 43

Metabolic parameters 44
The recovery period 47
Active phase workers in LD recovery conditions 48
Circadian rhythmicity 48
Metabolic measures 48
Active phase workers in DD recovery conditions 48
Circadian rhythmicity 48
Metabolic measures 50
Resting phase workers in LD recovery conditions 53
Circadian rhythmicity 53
Metabolic measures 53
Resting phase workers in DD recovery conditions 54
Circadian rhythmicity 54
Metabolic measures 55
Discussion 58
Resting phase work disrupts circadian rhythmicity 58
Resting phase work disrupts circadian rhythm of metabolism 60
Circadian disruption in RW persists in the recovery period 62
Circadian disruptions after RW are of endogenous nature 66
Resting phase work disrupts metabolism 68
Resting phase work induces long-term disturbances in metabolism 73
Evaluation of the model and present experiment 75

Animal models of shift work 75

Strengths and limitations of the present experiment 76

Future perspectives 77

Conclusion 78

References 80

Appendix 90

Appendix A – Baseline analyses 90

Appendix B – One shift work period analyses 92

Appendix C – Recovery period analyses 94

Appendix D – Preliminary results on gene expression and glucocorticoid levels ... 96

List of figures

Figure 1. Theoretical schematic and parameters of body temperature in humans 15

Figure 2. Theoretical schematic of the % rhythm in three different data sets 16

Figure 3. Theoretical schematic of a person's main sleep period with and without zeitgebers 18

Figure 4. Timeline of experimental protocol. 33

Figure 5. Assignment of animals into groups 33

Figure 6. Circadian rhythmicity of locomotor activity during one shift work period .. 41

Figure 7. Circadian rhythmicity of body temperature during one shift work period ... 42

Figure 8. Food and water intake across one shift work period 43

Figure 9. Body weight change during and between shifts 45

Figure 10. Mean locomotor activity between shifts 46

Figure 11. Mean body temperature during one shift work period 47

Figure 12. Circadian rhythmicity of locomotor activity during the recovery period in active phase workers 49

Figure 13. Circadian rhythmicity of body temperature during the recovery period in active phase workers 50

Figure 14. Body weight change during the recovery period in active phase workers. 51

Figure 15. Mean locomotor activity during the recovery period in active phase workers 51

Figure 16. Mean body temperature during the recovery period in active phase workers. 52

Figure 17. Circadian rhythmicity of locomotor activity during the recovery period in resting phase workers 54

Figure 18. Circadian rhythmicity of body temperature during the recovery period for resting phase workers 55

Figure 19. Body weight change during the recovery period in resting phase workers 56

Figure 20. Mean locomotor activity during the recovery period in resting phase workers 56

Figure 21. Mean body temperature during the recovery period in resting phase workers 57

List of tables

Table 1. Animals excluded from all statistical analyses 37

Introduction

Shift work

Shift work can be defined as work hours that fall outside traditional 08:00-17:00 weekday work (Burch et al., 2009). The duration, timing and rotation of shift work schedules vary greatly. Night work is a specific type of shift work (International Agency for Research on Cancer, 2007). Definitions of what constitutes night work differ, ranging from work hours between 00:00-05:00 to 20:00-07:00 (International Agency for Research on Cancer, 2007).

Estimates of shift work prevalence vary depending on the population surveyed and the definition of shift work applied. It is estimated that only 24% of the employed European population perform "standard daytime work" (Costa et al., 2004). According to the 4th European survey on working conditions, Northern Europe (Denmark, Finland, the Netherlands and Sweden) has the highest proportion of night and evening workers in the European Union. In these countries, almost 60% of workers report to be involved in evening work and 20% report involvement in night work at least once a month (European Foundation for the Improvement of Living and Working Conditions, 2007). Interestingly, less than 15% of workers in these countries define themselves as shift workers. The majority are employed within the health sector (~35%). Other prevalent sectors are hotels and restaurants (~30%), manufacturing (~25%), and transport and communication (~25%).

Circadian rhythms

Circadian rhythms refer to any process that oscillates in an approximately 24h-fashion. The word "circadian" derives from the Latin phrase "circa diem" meaning "approximately one day". Throughout evolutionary history the rotation of the earth has imposed predictable daily rhythms of light and dark to which organisms have adapted by using precise time-

keeping mechanisms. Circadian variations are observed in virtually all species, ranging from fungus to mammals (Buijs & Kalsbeek, 2001), allowing the adaptation to daily changes in the environment. Some circadian rhythms, like the rest/activity cycle, are easily observed. However, circadian rhythms are much more widespread than that, and can be observed in several physiological systems, individual tissues, circulating hormones and at the cellular level (Albrecht, 2012).

Regulation of circadian rhythms

The Suprachiasmatic Nucleus (SCN), located in the hypothalamus of the brain, is often referred to as the "master clock" or "core oscillator". The SCN is the main driver of circadian rhythms in mammals, sending signals to other parts of the brain and to the rest of the body ensuring that rhythms of individual tissues and cells are synchronized with each other to maintain optimal physiological functioning (Albrecht, 2012). Lesion of the SCN has been found to cause loss of the circadian rhythm in locomotor activity (e.g. Sato & Kawamura, 1984). If cells from the SCN are removed from the brain and cultured in vitro, they maintain their own rhythm (Groos & Hendriks, 1982) Moreover, in a series of experiments, Ralph and colleagues replaced the SCN in normal living animals expressing a 24h rhythm with an SCN from animals expressing abnormally short rest/activity rhythm. They observed that the recipient animals adopted the rhythm of the donor animals (Ralph, Foster, Davis, & Menaker, 1990). Such an absolute interference has not been described with any other oscillating tissue (neither in the brain nor in peripheral organs such as liver or pancreas), indicating that the SCN alone maintains synchronization of the circadian rhythms within the body.

All cells in the body express so-called "clock genes," whose expression oscillate in a circadian fashion and are directly under control of SCN (Welsh, Yoo, Liu, Takahashi, & Kay, 2004). Clock genes affect the expression of tissue-specific genes, which in turn drive

circadian rhythms of behaviour and physiology (Dibner & Schibler, 2015). The first clock gene was described in the fruit fly (drosophila melanogaster) and homologues have later been identified in a number of species, including rodents and humans (Konopka & Benzer, 1971; Mohawk, Green, & Takahashi, 2012). Clock genes allow the maintenance of circadian rhythmicity in all bodily tissues.

Clock genes are involved in an auto-regulatory feedback loop which is organized into a positive and a negative element (King & Takahashi, 2000). The positive loop generates transcription factors which promote the expression of genes in the negative loop. Likewise, the negative loop generates transcription factors which inhibit the expression of genes in the positive loop. One feedback cycle takes approximately 24h, ensuring circadian rhythmicity in each cell of the body (King & Takahashi, 2000). These feedback cycles occur naturally, but when isolated from the SCN, peripheral tissues tend to only maintain their rhythm for a few oscillation cycles before the rhythm is lost (Yamazaki et al., 2000). The SCN maintains, adjusts, and synchronizes the circadian oscillations of these independent cells that can vary greatly in terms of their own period length (Dibner & Schibler, 2015).

In sum, circadian rhythms are expressed in all tissues of the body. Circadian rhythmicity is made possible through the oscillations of clock genes which control the rhythm of other genes which in turn lead to circadian oscillations in physiological functions. The synchronization and constant rhythmicity of these oscillators is ensured by the "core oscillator" located in the SCN.

Measuring circadian rhythms

Circadian rhythms can be observed as oscillations in the activity of physiological systems. There are numerous examples of circadian rhythms, such as the rest/activity cycle, the body temperature cycle and the cycles in secretion of hormones.

The rest/activity cycle is the most common way of measuring circadian rhythmicity in rodents (Whishaw & Kolb, 2004). Many studies measure activity by giving a rat or mouse access to a running wheel, and recording the times at which the animals use the wheel (which they will do for most of their active phase)(Whishaw & Kolb, 2004). Activity can also be measured by implantable radiotelemetry transmitters (Whishaw & Kolb, 2004). Such recordings allow for calculation of mean locomotor activity levels across 24h, 12h or shorter time intervals.

Core body temperature is carefully regulated and oscillates in a circadian fashion, with a minimum temperature occurring during the resting phase of the day and a maximum temperature during the active phase of the day (Refinetti, 2010). Core body temperature measurement is considered one of the most accurate ways of measuring circadian rhythmicity (Refinetti, 2010). The clock time of minimum body temperature is termed "nadir", and the clock time of the maximum body temperature is termed "acrophase" (Benloucif et al., 2005). In humans with a stable circadian rhythmicity, nadir will occur at the same time point each day; approximately 2 hours before undisturbed wake up time. In early chronotypes (persons who prefer to wake up early) nadir typically occurs at around 05:00, whereas in late chronotypes, nadir occurs at around 07:00 and later (Lack, Bailey, Lovato, & Wright, 2009). In individuals with circadian disorders, such as delayed sleep phase syndrome, nadir can occur at much more extreme time-points (Weitzman et al., 1981). Acrophase can also denote circadian phase although it is less commonly used (Refinetti, 2010).

The amplitude of the circadian rhythm is an indicator of how strongly the rhythm oscillates throughout the cycle (Benloucif et al., 2005), extracted by subtracting the minimum value from the maximum value. Figure 1 illustrates the different circadian rhythm parameters of the body temperature.

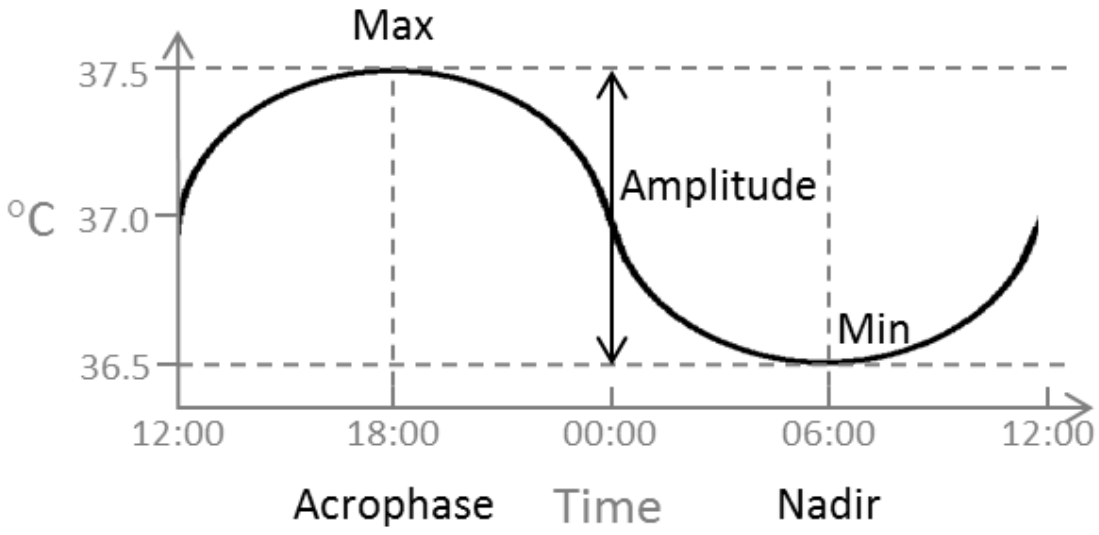

Figure 1. Theoretical schematic and parameters of body temperature in humans. Acrophase denotes the time point of the peak of the rhythm (here 18:00); nadir denotes the time point of the trough of the rhythm (here 06:00); Max denotes maximum value reached; Min denotes the minimum value reached; Amplitude denotes the difference between the maximum and minimum values (here Max – Min = 1.0).

Another circadian rhythm parameter of interest in this thesis is the % rhythm. The % rhythm indicates the degree to which the collected data points from a given marker of circadian rhythmicity across 24h fit to a perfect sinusoid curve. The % rhythm value is given on a scale of 0-100, as illustrated in figure 2.

These parameters – nadir, acrophase, amplitude and % rhythm – can be used to examine the nature of circadian rhythmicity on the individual and group level, under normal and abnormal conditions.

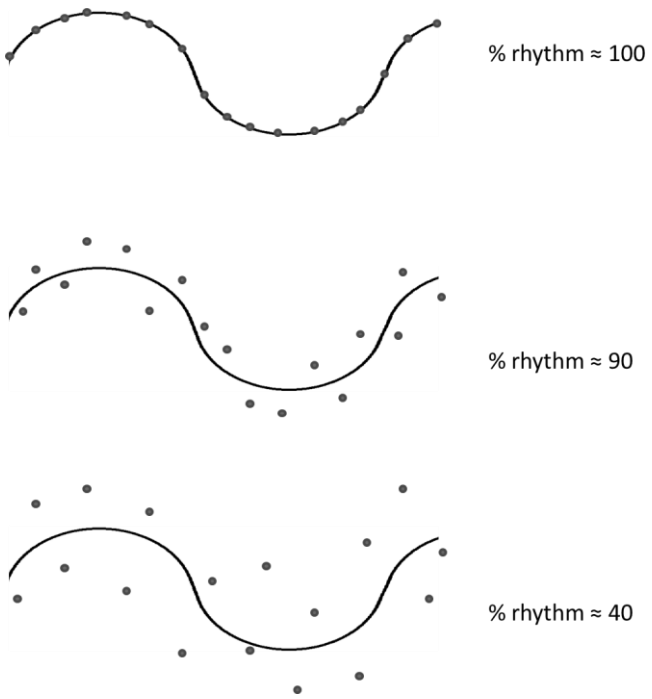

Figure 2. Theoretical schematic of the % rhythm in three different data sets. Dots indicate collected values. The line indicates the fitted sinusoid curve. The closer the data correspond to the curve, the higher the % rhythm value will be.

Hormone measurements are also commonly used in the characterization of circadian rhythmicity. Melatonin is a hormone under direct control of the SCN and is found to stabilize other circadian rhythms throughout the body (Lockley et al., 2000). Melatonin release is suppressed by light, and its evening rise (dim-light melatonin onset) is a reliable measures of the circadian phase (Pandi-Perumal et al., 2007). It is a commonly used marker of circadian rhythms in humans as it can be easily measured through saliva samples (or blood and urine), and is relatively resistant to physiological variations such as exercise and stress (Pandi-Perumal et al., 2007). Another hormone which shows a strong circadian rhythmicity is glucocorticoid (GCs; cortisol in humans; corticosterone in rodents). GCs are released in response to stress, but also act as the body's activating hormone, with its circadian acrophase just before waking (Chung, Son, & Kim, 2011). Abnormalities in the GC rhythm can indicate

disease, such as Cushing's syndrome in which the amplitude of the GC rhythm is blunted (Klerman, 2005).

Endogenous nature of circadian rhythms and entrainment

In many species (adult humans and rats) the endogenous circadian rhythm is slightly longer than 24h, but is entrained to external cues every day to generate an exact-24h rhythm (Aschoff, 1965). Such time-cues are commonly referred to as "zeitgebers", or "time-givers". The most important zeitgeber is light. Processing of light information is mediated by the photopigment melanopsin exclusively expressed in retinal photoresponsive cells (intrinisically photoresponsive ganglion cells; ipGRCs) and is directly transmitted to the SCN (Berson, 2003). Other zeitgebers, such as behaviour and food intake, can also influence circadian rhythms, particularly in peripheral organs (Oosterman, Kalsbeek, la Fleur, & Belsham, 2015). In this way, the circadian system can adapt to changes in the environment.

Zeitgebers are abundant in everyday life. However, experimental conditions allow for removal of zeitgebers, revealing the endogenous circadian rhythm generated by the SCN. A rhythm that oscillates without external cues is said to "free-run". In free-running conditions human subjects will go to sleep and wake up a little later each day (Aschoff, 1965). Similarly, all physiological functions will be gradually delayed during days spent without zeitgebers. Importantly, the circadian rhythms will stay in synchrony with each other as they are still under the control of the SCN. The study of endogenous circadian rhythms is important because it allows direct examination of the circadian rhythms generated within the body, without the interference of external factors. Figure 3 illustrates how a sleep-wake pattern may look under laboratory conditions with and without zeitgeber, 12h light/ 12h dark (LD) and constant darkness (DD) respectively.

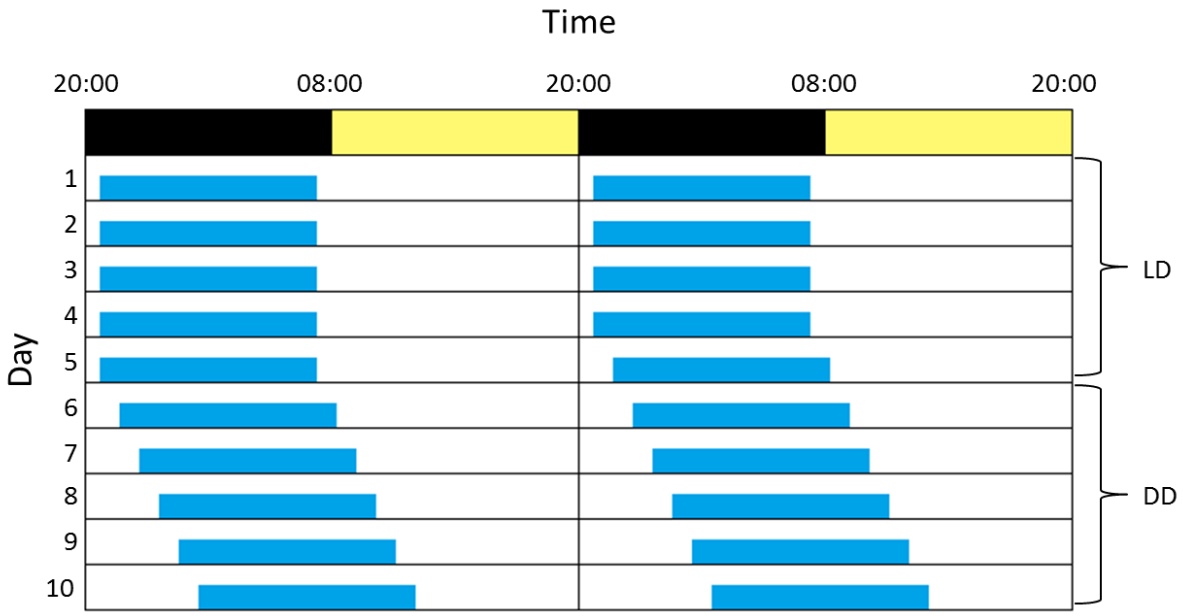

Figure 3. Theoretical schematic of a person's main sleep period with and without zeitgebers. Sleep is plotted in blue, across 10 days. The diagram is double-plotted so that each subsequent day is plotted directly below and to the right of a given day. The upper bar indicates light conditions in the room under 12h light/12h dark (LD) conditions (days 1-5). On days 6-10 the person is exposed to constant darkness (DD), which reveals the free-running sleep/wake rhythm. Since the free-running rhythm is typically longer than 24h, the individual initiates sleep slightly later each day, resulting in a gradual delay of the circadian rhythm.

Metabolism

Metabolism is an umbrella term for all processes that occur in the body in order to sustain life. These processes include the breakdown of biological substances in order to extract energy, and the use of energy to synthesize new molecules (Guyton & Hall, 2000, p. 772). To maintain metabolism, energy must be supplied through food intake, or through breakdown of energy-containing tissue. One example is the homeostasis of body weight, which is maintained by balancing energy expenditure and energy intake (Farias, Cuevas, & Rodriguez, 2011). When energy expenditure exceeds energy intake, the energy balance is

negative. Consequently, when energy expenditure is lower than energy intake, the energy balance is positive.

Regulation of metabolism

Food and water intake are important for providing essential nutrients and fluids, and for maintaining energy balance. The arcuate nucleus of the hypothalamus is considered central in the short and long term regulation of food intake (Valeur, 2007). Signals from peripheral tissues informing about the current nutritional state of the organism converge with signals from the cortex of the brain and are sent to the hypothalamus, which regulates when and how much food is ingested (Valeur, 2007). Food intake is affected by a number of physiological and psychological factors including signals about existing energy stores (long-term), total energy expenditure (short-term) and perceived food quality and palatability (Valeur, 2007). Metabolic and digestive processes such as food processing are regulated at the genetic level. The up- or downregulation of genes control the activity of metabolic tissues (Desvergne, Michalik, & Wahli, 2006). When food is consumed, carbohydrates and fats are broken down to glucose and fatty acids through the aid of insulin. Glucose and fatty acids can either be used for energy immediately, or converted to glycogen or triacylglyceride (TAG) for storage (Desvergne et al., 2006). During rest, glucose and TAG are released from the stores to provide a constant energy supply (Desvergne et al., 2006).

Water intake is important to maintain the tightly controlled balance of extracellular and intracellular fluid stores in the body. Loss of fluid, from either extra- or intracellular compartments, are sensed by osmoreceptors in the hypothalamus, which signals to stimulate the sensation of thirst and facilitates drinking (Antunes-Rodrigues, De Castro, Elias, Valenca, & McCann, 2004). As energy expenditure increases, fluid intake is also increased.

The end product of metabolic activity is heat. Hence, body temperature is sensitive to metabolic changes. Accurate thermoregulation is important for maintaining a stable core body

temperature even though the temperature outside the body can vary greatly. The preoptic area of the hypothalamus controls thermoregulation, and ensures maintenance of a stable body temperature through activation of thermoeffectors, which may induce shivering, sweating or constriction of blood vessels or other processes aimed at maintaining body temperature homeostasis (Romanovsky, 2007). Although core body temperature is carefully regulated, it increases in response to e.g. exercise (Gleeson, 1998). Additionally, when exposed to semi-starvation over time, body temperature declines to conserve energy stores (Siyamak & Macdonald, 1992). Many other factors also influence body temperature, such as the already mentioned circadian phase (active and inactive phase), and stress (acute and chronic)(Gleeson, 1998; Kataoka, Hioki, Kaneko, & Nakamura, 2014; Mrdalj et al., 2014).

Energy expenditure increases as the individual is physically active. The relationship between energy intake and locomotor activity is complex; energy intake tends to increase in response to high levels of physical activity, but not to the level needed to fully compensate for the excess energy expedited (Titchenal, 1988). In addition, during semi-starvation, hyperactivity is typically observed (Pirke, Broocks, Wilckens, Marquard, & Schweiger, 1993). This paradoxical response may have evolved to facilitate searching for food during times of famine.

Both fasting and stress can affect metabolism, through the release of GCs. Acute GC release facilitates breakdown of fat stores (lipolysis), which results in weight loss (Harris et al., 1998). However, chronic GC elevation does not cause progressive weight loss (Shibli-Rahhal, Van Beek, & Schlechte, 2006). The reasons for this are poorly understood. However, it is known that prolonged GC release promotes insulin resistance, which results in inability to clear glucose from the blood, leading to type II diabetes and metabolic dysregulation (Vegiopoulos & Herzig, 2007). Furthermore, excess GCs in the blood causes elevated levels

of free fatty acids in the bloodstream, which also alters metabolism (Vegiopoulos & Herzig, 2007).

In sum, an organism's body weight depends on the relationship between energy intake and energy expenditure. The links between factors influencing energy balance are complex. Metabolic homeostasis is ensured by closely regulating factors such as food and water intake, locomotor activity and body temperature.

Measuring metabolism

Whole-body energy metabolism is most accurately measured by calorimetry, whereby the energy liberated form the body is measured either directly through heat output, or indirectly through oxygen utilization (Guyton & Hall, 2000, p. 804).

Other measures of metabolism can be attained through measurement of e.g. food intake, water intake, body weight, locomotor activity or body temperature. These measures will not provide complete information about total energy expedited, but can be an indicator of the nutritional status of the organism (Levine, 2005). Food intake indicates amount of energy ingested. Moreover, body weight can indicate whether energy intake and expenditure are in balance across time (Hill, Wyatt, & Peters, 2012). Increases in water intake, locomotor activity and body temperature are associated with increased energy expenditure, and are indicative of energy usage (Levine, 2005).

Measurements of changes in metabolic gene expression and levels of compounds involved in metabolism can also provide valuable insight into the metabolic state of an individual (Bustin, 2002). Metabolic gene expression cannot give information about energy expenditure, but can give indirect information about the metabolic changes that occur in different tissues in response to a challenge (Bustin, 2002). Likewise, levels of glucose, fatty acids, TAG and insulin can provide information about metabolic activity, and can act as an indicator of metabolic disruption (Desvergne et al., 2006).

Circadian rhythms, metabolism and shift work

No physiological system is free from circadian variation, and metabolism is no exception. Cycles of rest and activity drives cycles of fasting and food intake (Dibner & Schibler, 2015). This makes intuitive sense as humans tend to eat and drink whilst awake, and fast during sleep. The same is true for other mammals. For example, nocturnal rats consume most of their total food (75%) and water (85%) during the dark phase (Johnson & Johnson, 1991; Rosenwasser, Boulos, & Terman, 1981). Circadian organization of metabolic tissues allows the prediction of when food will arrive, and facilitates efficiency in the metabolic system (Dibner & Schibler, 2015). Organs involved with food processing and energy metabolism like the digestive tract (Konturek, Brzozowski, & Konturek, 2011), liver (Akhtar et al., 2002; Reddy et al., 2006), pancreas (Sadacca, Lamia, deLemos, Blum, & Weitz, 2011), skeletal muscle (McCarthy et al., 2007) and adipose tissue (Zvonic et al., 2006) show circadian rhythmicity. In the same way that light is the primary zeitgeber for the SCN, food intake is the primary zeitgeber for peripheral organs involved in digestion and metabolism (Damiola et al., 2000).

Internal desynchronization occurs when individuals are exposed to environments or partake in behaviours which do not match their circadian rhythm. Shift work and jet lag are common examples (Golombek et al., 2013). Shift workers are exposed to conflicting zeitgebers; changes in food intake and activity patterns signal to peripheral organs the need to entrain to a new rhythm, and artificial lighting at a time when it is normally dark affect the rhythm of the SCN (Wyse, Biello, & Gill, 2014), as does bright daylight exposure in the morning after night work (Eastman, Stewart, Mahoney, Liu, & Fogg, 1994). Moreover, changing schedules and days off work may cause a state where the clocks within the body are constantly out of synchrony with the worker's routines. Additionally, clocks become

desynchronized as conflicting signals are sent to different tissues, and the time taken to re-entrain varies between tissues (Dibner & Schibler, 2015).

It is hypothesised that internal desynchronization causes fatigue and sleep disruptions in shift workers (Golombek et al., 2013). It is also hypothesised that internal desynchronization, particularly the lack of stable eating rhythms, contributes to shift workers' increased risk of metabolic disorders (Gangwisch, 2014). Although the link between shift work and cancer is not fully understood, it is likely that internal desynchronization plays a key role here as well (Davis, Mirick, & Stevens, 2001).

Circadian misalignment causes disruption in the metabolic system (Asher & Schibler, 2011). Clock mutant mice (mice lacking certain clock genes) show metabolic dysfunctions such as obesity, overeating, impaired lipid metabolism, impaired glucose tolerance, and hypertension (Marcheva et al., 2010; Maury, Hong, & Bass, 2014). These dysfunctions are associated with metabolic disorders such as type II diabetes, metabolic syndrome and cardiovascular disease. Such relationships may also go the other way. In one study, mice fed a high-fat diet developed impaired rhythms of free-running activity and clock genes in liver and adipose tissue (Kohsaka et al., 2007). It has also been found that men with type II diabetes show impaired melatonin rhythms (Mantele et al., 2012).

Although not discussed in detail here, sleep disruptions are also commonly associated with both circadian misalignment and metabolic disorders, and may be an important mediating factor (Reutrakul & Van Cauter, 2014). Sleep restriction impairs insulin sensitivity, contributing to the development of type II diabetes (Buxton et al., 2010). Sleep restriction also reduces the release of the satiety-inducing hormone leptin, thereby promoting food intake and increasing risk of obesity (Spiegel, Tasali, Penev, & Van Cauter, 2004). However, some studies report no association between sleep restriction and leptin-levels (Reynolds et al., 2012). Nevertheless, it is clear that circadian rhythmicity, metabolism and sleep are closely

intertwined. Hopefully, future research will aid in elucidating the mechanisms which link these processes together.

Consequences of shift work

During shift work, a large number of environmental and behavioural factors (zeitgebers) are changed at the same time. It is therefore difficult to pick apart the exact mechanisms which cause health problems for shift workers. Studies in both humans and animals are necessary to solve this problem. Some of the studies that have been performed will be reviewed in the following section.

Human studies

Studies on the effects of shift work in humans can be divided into epidemiological studies, field studies and laboratory studies. Epidemiological studies have been important in finding long-term associations between shift work and health outcomes. Such studies find that shift workers report high levels of stress, fatigue, health complaints, sleepiness, anxiety and depression (Lac & Chamoux, 2004; Oyane, Pallesen, Moen, Akerstedt, & Bjorvatn, 2013; Smith & Mason, 2001). Shift workers are also at increased risk for metabolic syndrome (Karlsson, Knutsson, & Lindahl, 2001), sleep disturbances (Tucker, Folkard, Ansiau, & Marquie, 2011), cancer (International Agency for Research on Cancer, 2007; Schernhammer et al., 2001), and death (Akerstedt, Kecklund, & Johansson, 2004). In addition, there is an association between older age and risk for negative health effects of shift work (Tucker et al., 2011). One study also showed that sleep problems associated with shift work persist even in retirement (Monk et al., 2013).

Field studies have aided in examining the physiological consequences of shift work. A number of studies have found that shift- and night workers show impaired peripheral body temperature rhythms (Ferreira, Miguel, De Martino, & Menna-Barreto, 2013), as well as

impaired rhythmicity in a number of hormones including GCs (cortisol) and insulin (Simon, Weibel, & Brandenberger, 2000; Weibel & Brandenberger, 1998). Some studies find altered melatonin rhythms in night workers, whereas others do not (Grundy et al., 2009; Sack, Blood, & Lewy, 1992). Importantly, less than 3% of permanent night workers adjust their melatonin rhythms to their work rhythms (Folkard, 2008). In one field study, Gupta and Pati (1994) measured oral temperature, heart rate, fatigue, drowsiness and cognitive task performance in shift workers and day workers throughout their waking times for several weeks. They found that the shift workers not only had impaired circadian rhythmicity within each of the parameters measured; they also found a lack of synchrony between the parameters, indicating internal desynchrony. The findings of this study highlight the importance of measuring circadian rhythmicity in several parameters in order to accurately identify the extent of circadian disruption that occurs in shift workers.

Field studies have also identified that some workers tolerate shift work schedules better than others. "Shift work tolerance" is defined as the absence of daily health complaints associated with shift work, like absence of fatigue, sleep difficulties and gastrointestinal issues (Saksvik, Bjorvatn, Hetland, Sandal, & Pallesen, 2011). Many individual factors contribute to shift work tolerance, but the design of the shift work schedule is also likely to play an important role (Saksvik et al., 2011). The characterization of schedules and routines of shift workers has so far received little focus, but is of importance when designing human laboratory studies and animal models (Opperhuizen, van Kerkhof, Proper, Rodenburg, & Kalsbeek, 2015).

Human laboratory studies have been performed either to examine effects of simulated shift work in non-shift working individuals, or to test the effects of interventions designed to alleviate some of the negative consequences of shift work. Intervention studies have focused on manipulating light exposure in order to shift or prevent shifts in circadian rhythmicity

(Eastman & Rechtschaffen, 1983; Lee, Smith, & Eastman, 2006). Other intervention studies have investigated the effects of napping during the night shift (Hilditch, Centofanti, Dorrian, Van Dongen, & Banks, 2014), or on designing daytime sleep schedules that may improve daytime sleep quality (Jackson, Banks, & Belenky, 2014). Shift work simulation studies have confirmed notions previously raised by epidemiological and observational studies; that night shifts cause an increase in snacking and choice of sweet snacks (Heath et al., 2012), and that the night-time food intake can have negative effects on the rhythmicity of hormones and other compounds involved in metabolism, such as glucose, insulin and TAG (Ribeiro, Hampton, Morgan, Deacon, & Arendt, 1998). One study also found that total energy expenditure in night workers was significantly reduced from day working controls on a 6 day simulated night work protocol (McHill et al., 2014).

Methodological challenges in human shift work research

There are multiple challenges in studying the effects of shift work in humans. In epidemiological studies there is the issue of lack of randomization. Also, it has been hypothesized that shift working populations are healthier than the general population (the healthy shift worker effect) (Knutsson, 2004). Since individuals are not randomly assigned to groups one cannot know whether changes (or lack of changes) are due to shift work or due to the groups being different at baseline (Knutsson, 2004). Moreover, shift workers engage in a wide variety of different schedules and routines. This results in variation within groups, as well as a lack of control of variables. Lack of control causes difficulty in inferring which behaviours or factors are contributing to observed effects (Opperhuizen et al., 2015).

The previously mentioned issues (lack of randomization and lack of control) can be resolved using laboratory studies of shift work. However, human laboratory studies are costly and can only be performed across short time periods. Long-term effects of shift work can be

studies in epidemiological and field studies, but results take decades to reveal (Knutsson, 2004).

Animal studies

For the above mentioned reasons, animal studies are becoming increasingly attractive in shift work research. Animal studies allow for control of variables, inference of causality, and identification of mechanisms that contribute to negative health effects of shift work. Moreover, rats' life spans are many times shorter than humans' (one rat month is equivalent to approximately three human years), meaning that long-term effects can be identified in shorter time (Nestler & Hyman, 2010; Sengupta, 2013). Animal studies have great potential when it comes to informing current research, identifying risk factors for negative effects of shift work, and also for alleviating these effects.

Animal studies have been valuable in establishing how circadian rhythmicity is regulated, how it is linked to metabolism, and the effects of circadian disruption. The recent development of animal models of shift work represents an important step from basic science to translational science. Thus far, relatively few studies have attempted to model shift work in animals. This section will give an overview of some of the studies performed.

The first attempt to model shift work in animals was performed by Carandente (1977). Most other studies have been published after 2000, and thus the field is young. The focus of shift work models has been to manipulate exposure to one or several zeitgebers, such as timing of light, food, sleep or activity. For example, some studies have aimed to model shift work by exposing mice to varying light/dark conditions (e.g. McGowan & Coogan, 2013). However, it has been argued that such manipulations more closely resemble jet lag than shift work (Salgado-Delgado, Angeles-Castellanos, Buijs, & Escobar, 2008). Timing of food intake has also been manipulated in attempts to model shift work. Damiola and colleagues (2000) allowed mice to eat only during their resting phase, which caused changes to the circadian

rhythm of the liver, and desynchrony of peripheral organs from the SCN. Timing of sleep has also been manipulated; Barclay and colleagues (2012) exposed mice to sleep restriction during the first 6 hours of their resting phase, and found disruption to circadian rhythmicity in liver, SCN, locomotor activity patterns and food intake patterns. All these studies claim to model shift work, and although they do capture some aspects, they fail to completely mimic the altered patterns of activity that characterize shift work.

A promising animal model of shift work has been created by Escobar and colleagues. In their model, rats are exposed to forced activity for 8h during their normal resting phase, five days a week for five weeks. The control group is exposed to the same amount of forced activity, but during their normal active phase. This model gives insight into the effects of chronic exposure to resting phase activity, although it does not mimic a typical human night work schedule (which usually consists of fewer night shifts and more days off each week).

In Escobar's first experiment, it was found that forced activity during resting phase caused loss of amplitude and circadian rhythmicity of locomotor activity, similar to what is observed in SCN-lesioned animals (Hsieh et al., 2014). Moreover, body weight, particularly the volume of abdominal adipose tissue, increased (Salgado-Delgado et al., 2008). Additionally, changes to the timing of food intake, abnormal corticosterone rhythms, loss of glucose rhythmicity and reversed rhythms of TAG were observed (Salgado-Delgado et al., 2008). The animals also exhibited internal desynchrony between peripheral tissues and SCN (Salgado-Delgado et al., 2008). In the latter study they also found internal desynchrony within the hypothalamus, as the SCN remained synchronized to the constant light/dark cycle, whereas other areas of the hypothalamus involved in metabolism and sleep/wake regulation showed altered rhythms (Salgado-Delgado, Nadia, Angeles-Castellanos, Buijs, & Escobar, 2010b). Moreover, in liver cells this animal model has shown an inverted rhythmicity of clock

genes and loss of rhythmicity of metabolic genes, indicating internal desynchronization (Salgado-Delgado et al., 2013).

Another research group using forced activity to model shift work aimed to examine cognitive consequences in rats (Leenaars et al., 2012). The study found no changes in cognitive abilities, and, contrary to the findings of the Escobar group, attenuated body weight gain was found in rats forced to be active in their resting phase. These contradictory findings emphasize the importance of replicating studies before drawing firm conclusions. Nevertheless, due to its apparent validity, the forced activity model has a strong potential and opens up for examination of a variety of manipulations.

Establishing and evaluating animal models of shift work

Establishing animal models for human phenomena is challenging (Nestler & Hyman, 2010). This is particularly true for complex aspects of human life, such as shift work. During shift work, a great number of factors are changed at the same time. These factors can include altered patterns of light exposure, activity, sleep, food intake and social interaction (Opperhuizen et al., 2015). Animal models are unlikely to fully mimic human shift work. However, this does not mean that animal models do not have great potential. A well-established, validated animal model of shift work can be applied to examine the mechanistic consequences of different shift work schedules and treatment strategies for attenuating the negative health effects.

When establishing an animal model of shift work, it is important to evaluate the validity of the model. To my knowledge, no present animal model of shift work has been subjected to validity criteria. Classical validation criteria for animal models with regard to psychiatric disorders have been suggested by multiple scholars. One widely cited set of criteria were proposed by Willner (1984). His criteria for a valid animal model request predictive validity, face validity and construct validity. Predictive validity assesses the extent

to which an animal model of X is able to make consistent predictions about X in humans, often with focus on effects of therapeutic interventions. Face validity assesses the extent to which symptoms induced from application of the model are similar to symptoms observed in humans. Construct validity assesses how well the model captures the phenomenon it aims to model.

In animal models of shift work, predictive validity can be assessed by examining the effects of applying interventions hypothesized to attenuate the negative effects of shift work. If the effects are attenuated in both the animal model and in later human applications, the model has high predictive validity. Likewise, interventions that are ineffective in animal models should be equally ineffective in humans. The second validation criterion for animal models is face validity. We know that humans show symptoms of circadian desynchrony, sleep disturbances, metabolic disturbances and altered meal patterns following shift work. An animal model that produces similar outcomes in the model organism would be judged to have high face validity. Lastly, as mentioned, shift work involves changes to a large number of environmental and behavioural factors, and these are likely to influence each other. Construct validity in an animal model of shift work would refer to the model's ability to capture the factor changes that it aims to examine the effects of. It is also important for the construct validity criterion that the model in reality is not capturing a different yet related phenomenon, such as jet lag.

Aims and hypotheses

The overall aim if this thesis is to use a novel rat model of shift work, and examine the effects of one shift work period of either *active phase work* (mimicking human day work) or *resting phase work* (mimicking human night work), short-term (during the shift work period; four days) and long-term (8 days after the termination of the shift work period). I aim to answer these specific questions in my master project:

1. How does one shift work period affect circadian rhythmicity?
2. How does one shift work period affect indirect measures of metabolism?

I hypothesize that active phase workers will show no shift in their circadian phase (nadir and acrophase) during and after the shift work period. The circadian rhythmicity is hypothesized to be strengthened during the shift work period due to forced activity (higher activity levels compared to the baseline), and to return to baseline early in the recovery period. Resting phase workers are expected to shift their circadian phase (either advance or delay), but not completely adapt to resting phase activity. The circadian rhythmicity is expected to be flattened. The changes are expected to return to baseline values during the 8 day recovery period recorded. The number of recovery days required will be examined. I expect a prolonged circadian disturbance in animals exposed free-running conditions during recovery, which indicates that the circadian effects are of endogenous nature.

The indirect measures of metabolism are not expected to be disrupted in active phase workers, either during the shift work period or the recovery period. Resting phase workers are expected to show metabolic disruption by a shift in the timing of food and water intake toward working hours, and increased or attenuated body weight gain. Body temperature and locomotor activity are expected to increase during work and decrease between work shifts. The number of days needed for metabolic changes to recover will be examined.

Changes in circadian processes and metabolism will be discussed and compared with changes in gene expression analysed in central and peripheral tissues as well as sleep disturbances and glucocorticoid levels in the same animals.

Methods

Ethical approval

This project was approved by the Norwegian Animal Research Authority ("Forsøksdyrutvalget", permit number: 2012463) and performed according to Norwegian laws and regulations, as well as The European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes.

Animals and housing

Male rats (n=39, Wistar, NTac:WH, Taconic, Denmark) weighing approximately 300g at arrival were acclimatized to the laboratory conditions before being group housed in individually ventilated cages (IVC, Tecniplast, Italy, 75 air changes per hour) type IV (480x375x210mm, 1500cm^2). After surgery, animals were housed individually (IVC cage type III, 425x266x185mm, 800cm^2). Food (rat and mouse no. 1 (RM1), Special Diets Services, Witham, Essex, England) and water was provided *ad libitum*. Cage bedding (BK bedding, Scanbur BK) was changed weekly, except during the course of the experiment.

The animals were kept under 12h light/12h dark (LD) cycle (lights on at 06:00, zeitgeber time 0; ZT0). Lights were gradually dimmed on and off, and were fully on at 07:00 and fully off at 19:00. A subset of rats was kept in constant darkness (DD) conditions during the recovery phase.

Animals were handled by trained and certified personnel. Gloves and lab coats/suits were worn at all times.

Design

The experimental protocol had a mixed design with independent factors (groups) and repeated measures (days). The design comprised of 4 days undisturbed baseline monitoring,

followed by 4 work days constituting one shift work period, and 8 days undisturbed recovery period. See figure 4 for a timeline of experimental protocol.

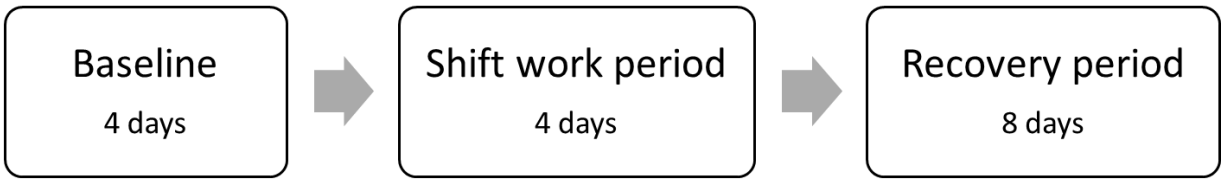

Figure 4. Timeline of experimental protocol.

Animals were randomly assigned to either resting phase work (RW, n=25) or active phase work (AW, n=16) (See figure 5). During recovery, the rats were randomly divided into two subgroups; LD or DD condition. Some animals (n=7) were first assigned to RW, then more than 30 days after, to AW.

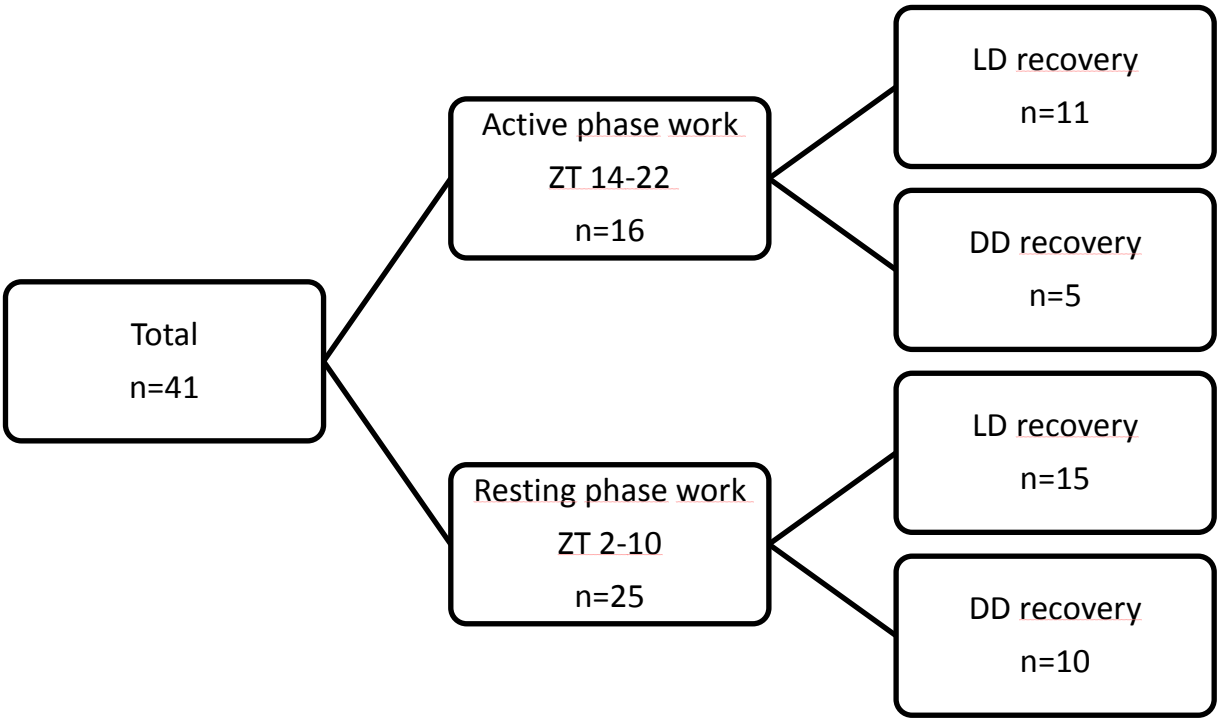

Figure 5. Assignment of animals into groups. LD: 12h light/12h dark; DD: 24h dark.

Surgical procedure

All animals were implanted with wireless transmitters for continuous recording of locomotor activity and body temperature.

Antibiotics (Bactrim, Roche; 5 ml in 250 ml drinking water) were administered up to 3 days prior to surgery. For surgery, animals were anaesthetized with a subcutaneous (s.c.) injection of a mixture of fentanyl 0.277 mg/kg, fluanizone 8.8 mg/kg, and midazolam 2.5 mg/kg (Hypnorm, Janssen; Dormicum, Roche; Midazolam Actavis, Actavis). The effects of anaesthesia were monitored by regular test of reflexes in eyes, leg and tail during the surgery. Additional anaesthesia was given when necessary, approximately every 45 minutes. The rat was placed in a stereotaxic apparatus (Kopf, USA) and laid on a heating pad in order to maintain normal body temperature. Tear gel (Viscotears, Novartis) was applied to the eyes to prevent drying.

Two different types of transmitters were used; 4ET and F40 (Physiotel®; Data Sciences International, both). Both transmitters collected body temperature and locomotor activity. In addition, electroencephalography (EEG), electromyography (EMG), and electrocardiography (ECG) were recorded, but these data are not included in this thesis. Transmitters for recording of body temperature and locomotor activity were implanted subcutaneously; the 4ET in a saddleback fashion with battery and the sensor placed in bilateral “pockets” of the dorsomedial lumbar region; and the F40 in a single pocket in the dorsolateral thoracic region. Pockets were cleaned with 0.9% sodium chloride and closed with interrupted mattress sutures, using re-sorbable thread. To allow for collection of ECG data (4ET only), one biopotential lead was attached to a muscle in the left dorsal thoracic region above the heart, and a second to a muscle in the dorsolateral lumbar region. Electrodes were fastened to the skull and in the neck muscle to monitor EEG and EMG signals, respectively.

The skin on the head was closed with interrupted mattress sutures. Otherwise, the skin was closed with clips.

Post-surgery, animals received an intraperitoneal (i.p.) injection of 5ml of Ringer Acetate (Baxter) to compensate for fluid loss during surgery. Analgesia, 0.10 ml buprenorphinum (0.30 mg/ml; Temgesic, Reckit & Benckiser) was given s.c. twice a day, and anti-inflammatory treatment, 0.30 ml meloxicam (5 mg/ml; Metacam, Boehringer Ingelheim) was given s.c. once a day for three days following surgery. Antibiotics (Bactrim, Roche; 5 ml in 250 ml drinking water) were given for two postoperative days. Daily care was provided throughout the post-operative period, and lidocaine liniment (5%; Xylocain, AstraZeneca), and a mixture of zincbacitracin (500 I.E.) and chlorhexidine acetase (5 mg), both liniment and powder (Bacimycin, Actavis) were administered when needed. A minimum of 14 days was allowed for recovery (Moscardo & Rostello, 2010).

Body weight, food and water measurements

All animals were weighed during a 24 hour time window and across the 4 baseline days (start and end of baseline period). During the same baseline days, food and water intake were monitored for 8 hours (timed to each groups' working hours) and 16 hours (timed to between shifts for each group).

During the shift work period, body weight change, food intake and water intake were monitored for each shift (8 hours) and between shifts (16 hours).

In the recovery period, body weight change was monitored every 4 days.

Shift work procedure

To mimic shift work in humans, rats were exposed to forced activity in automated running wheels (Rat Running Wheel, TSE running wheel system, Germany). The drums measured 24 cm in diameter and were set to rotate at 3 rpm. Food and water was provided *ad*

libitum. The drums were on for 8 hours, centred either during the rats' normal resting phase (08:00-16:00, ZT 2-10) or during the rats' normal active phase (20:00-04:00, ZT 14-22). Light conditions were normal (LD) throughout the shift work procedure. During the shifts, animals could see, hear and smell each other. Drums, feeders and water bottles were cleaned after each shift with 5% alcohol.

Telemetric recording and analyses

The wireless transmitter was turned on by swiping a magnet along the side of the animal. Analogue filters of 1Hz (high-pass) and 100Hz (low-pass) were used. The signals were converted and transferred to a computer (Dataquest ART, version 4.1, Data Sciences International). The sampling rate for body temperature was 50Hz and recorded every 10 seconds. Locomotor activity data was recorded as counts/minute.

Chronos-Fit software (Zuther, Gorbey, & Lemmer, 2009) was used for linear and rhythm analyses of locomotor activity and body temperature. From the linear analysis, mean values were calculated; 24h mean, 12h resting phase mean (lights on; ZT 12-24) and 12h active phase mean (lights off; ZT 0-12). For rhythm analysis, Partial Fourier analysis was applied, which generated a sine wave function fitted to the data. From this function, the following data were extracted for each 24-hour period: Nadir, acrophase, amplitude, and % rhythm.

Body temperature data were successfully recorded continuously throughout the whole experiment. However, due to limitations in the software used, mean locomotor activity and body temperature could not be calculated in other time intervals than 12h and 24h. Therefore, only 12h and 24h means are reported and not 8h during shifts and 16h between shifts. Moreover, we were unable to record reliable measures of locomotor activity during shifts. Therefore, results from analyses of mean locomotor activity during the work hours and across 24h, % rhythm and amplitude could not be reported.

Statistical analyses

Statistical analyses were conducted using STATA (release 14; StataCorp, USA) Statistical significance was accepted at $p \leq 0.05$. Outlier exclusion criteria for individual data points were set at ±3 studentized residuals from the mean. Some animals were excluded from all statistical analyses (see table 1).

Table 1

Animals excluded from all statistical analyses

N	Group	Reason for exclusion
1	RW	Technical problems with running wheel system
1	RW	Euthanized before finishing the work protocol
2	AW	Leak in bottle resulting in wet bedding; potential stressor
1	AW	Anaesthetized and sutured during baseline measurements; potential stressor

AW active phase workers
RW resting phase workers

To ensure random assignation to groups and stable circadian rhythmicity at baseline, baseline data was compared between groups and across the four baseline days. For analyses of the shift work period and recovery period, only baseline day 3 was used for comparison, as animals were completely undisturbed on this day with no human activity in the laboratory.

Baseline food intake, water intake and body weight were compared using independent-samples t-test. For all other statistical analyses, mixed model analysis using restricted maximum likelihood (REML) estimation with the unstructured covariance between random effects was used. Mixed model analysis allows the analysis of datasets that have missing data. In addition, this statistical method accounts for repeated measures and inter-individual differences at baseline. Data from baseline, the shift work period and recovery period were analysed separately. Small sample size was adjusted for by using the Satterthwaite adjustment for denominator of degrees of freedom. Where significant effects

were observed, post-hoc analyses were applied using pairwise comparisons of groups at each time point, and comparing each day to baseline.

Results

Baseline analyses

To secure that the data analysed was from a homogenous group of animals with stable circadian rhythmicity, the four baseline days were tested for differences between groups and interaction between group and baseline days on locomotor activity and body temperature (24h mean, 12h active phase mean, 12h resting phase mean, nadir, acrophase, amplitude and% rhythm). Food intake (24h), water intake (24h) and body weight (24h and 4d) were only recorded once during baseline, and group differences were tested on these parameters. See Appendix A, table I to V for overview of the statistical main effects.

Food intake, water intake, and body weight

There were no significant differences between groups in terms of food intake, water intake or body weight (p's>.09).

Locomotor activity and body temperature

There were no significant effects on parameters of locomotor activity, although some approached significance (interaction effect between baseline days and group p's>.07).

Acrophase of body temperature was significantly different on baseline day 1 compared to baseline days 2 to 4 ($p=.04$; between groups $p<.05$, all days; within-group $p<.007$, all days, both groups). Moreover, some other parameters of body temperature (active phase mean and resting phase mean) approached significance (interaction effect between baseline days and group p's>.06).

In sum, baseline day 1 differed slightly from baseline days 2 to 4. This effect was likely due to disturbance of the animals when the technology necessary to start the wireless

recording was set up. Therefore, new statistical analyses were performed for baseline days 2 to 4 only.

Baseline days 2 to 4 showed no main effect of days or interaction effects between days and group on neither locomotor activity ($p>.12$) nor body temperature parameters ($p's>.10$). Baseline day 3 was used as baseline reference in the subsequent analyses.

Consequences of one shift work period

Food intake, water intake and body weight were recorded each day across 24h, during shift (8h) and between shifts (16h). Locomotor activity was not accurately recorded during shifts (see Methods; Telemetric recording and analyses). Therefore, changes in mean value was only analysed for the 12h phase after each shift, whilst amplitude and % rhythm could not be analysed. Only nadir and acrophase were analysed. Body temperature was successfully recorded throughout the shifts, and analysed for 24h, 12h active phase and 12h resting phase, as well as nadir, acrophase, amplitude and % rhythm. See Appendix B, table V to VIII for overview of the statistical main effects.

Active phase workers (AW)

Circadian rhythmicity

The rhythmicity of locomotor activity (nadir and acrophase) showed no significant effects of work days, see figure 6 a-b.

The rhythmicity of body temperature (nadir, acrophase, amplitude and % rhythm) showed no significant effects of work days, see figure 7 a-d.

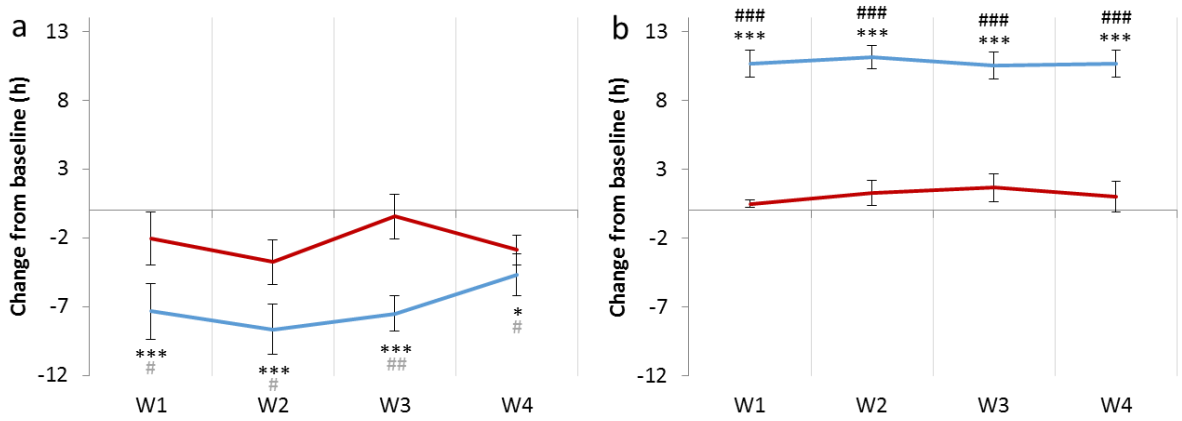

Figure 6. Circadian rhythmicity of locomotor activity during one shift work period. a) nadir; b) acrophase. active phase workers (AW); resting phase workers (RW). Data are shown as percentage change relative to baseline. Error bars indicate SEM. W1-4 indicates work days 1 to 4. * p<.05, *** p<.001, compared to baseline; # p<.05, ## p<.01, ### p<.001, compared to AW. Main effect of group for nadir of locomotor activity p=.08.

Metabolic parameters

AW reduced their food intake across 24h on work days 1-2 compared to baseline (p<.05, both work days), whilst 24h food intake was similar to baseline at work days 3-4. During shifts, food intake was reduced only on work day 3 only compared to baseline (p=.04; p<.001, shift 3). There were no significant changes in food intake between shifts. Data on food intake during the shift work period are shown in Figure 8 a-b.

Water intake did not reach significance for main effect of days across 24h (p=.06). However water intake was reduced during shifts (p<.001, all shifts) and increased between shifts (p<.001, all work days) compared to baseline. Data on water intake during the shift work period are shown in figure 8 c-d.

AW reduced their body weight across 24h (p<.001, all workdays) and during shifts (p<.001, all shifts), whilst they increased body weight between shifts (p<.001, all workdays) compared to baseline, see figure 9 a.

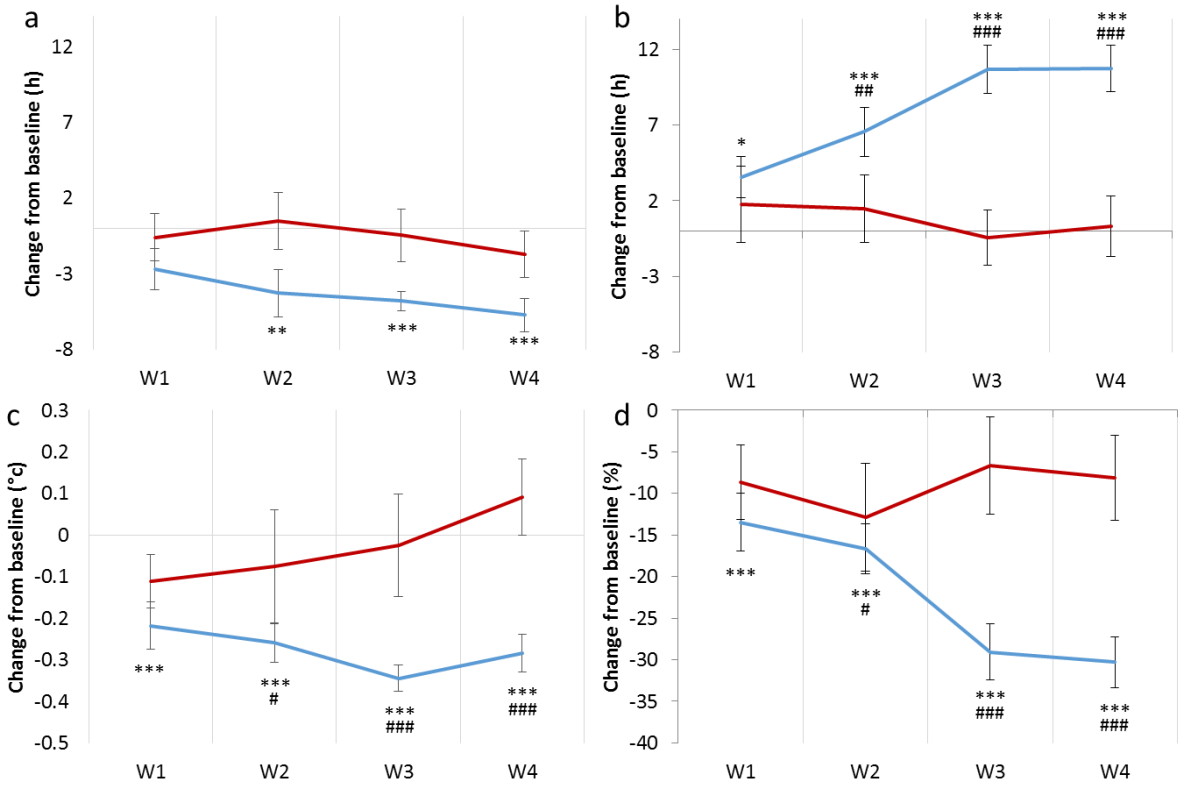

Figure 7. Circadian rhythmicity of body temperature during one shift work period. a) nadir; b) acrophase; c) amplitude; d) % rhythm. active phase workers (AW); resting phase workers (RW). Data are shown as percentage change relative to baseline. Error bars indicate SEM. W1-4 indicates work days 1 to 4. * p<.05, ** p<.01, *** p<.001, compared to baseline; # p<.05, ## p<.01, ### p<.001, compared to AW.

Locomotor activity increased after shifts on work days 3-4 (p<.001, both work days), see figure 10 a.

Mean body temperature showed no significant effect of days across 24h or in the active phase (including the 8h shift). Body temperature during resting phase (between shifts)

was increased on all work days (p<.001, all) compared to baseline. See figure 11 a for data on mean body temperature during the shift work period

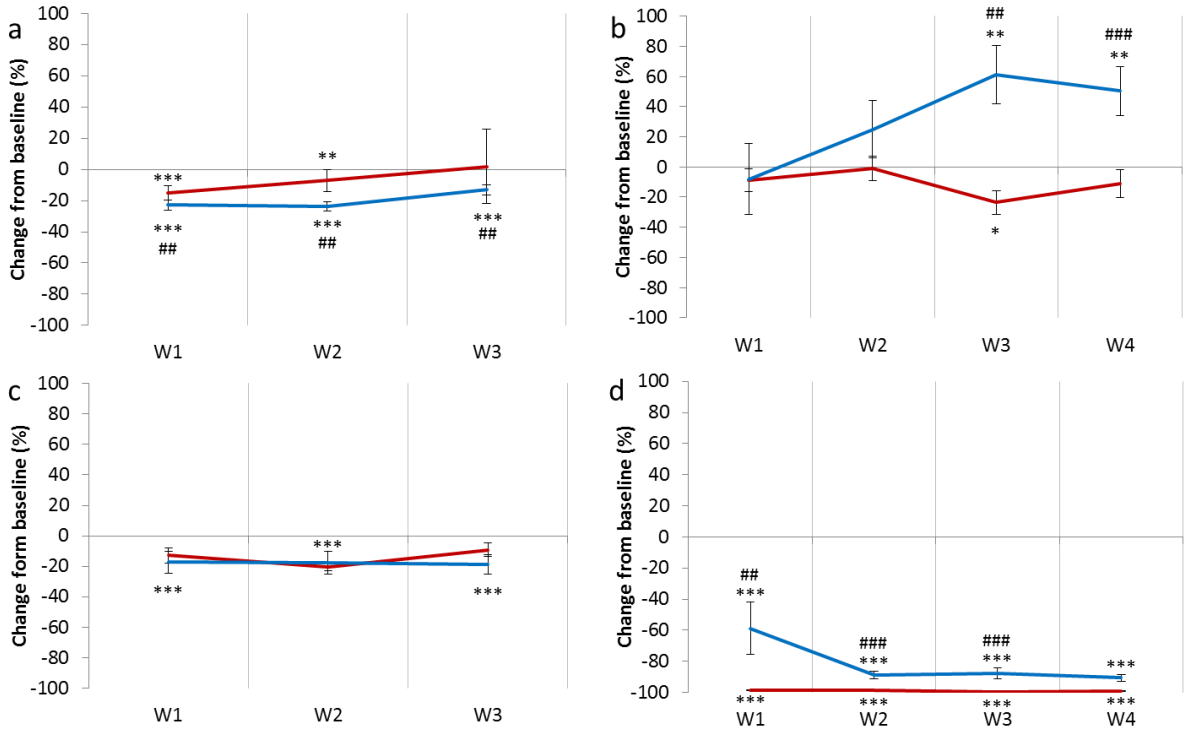

Figure 8. Food and water intake across one shift work period. a) 24h food intake; b) 8h food intake during shift; c) 24h water intake; d) 8h water intake during shift. ▬ active phase workers (AW); ▬ resting phase workers (RW). Data are shown as percentage change relative to baseline. Error bars indicate SEM. W1-4 indicates work days 1 to 4. * p<.05, ** p<.01, *** p<.001, compared to baseline. ## p<.01, ### p<.001, compared to AW.

Resting phase workers (RW)

Circadian rhythmicity

Resting phase work advanced nadir of the locomotor activity rhythm on all work days (p<.03, all) and delayed acrophase on all work days (p<.001, all) compared to baseline. Nadir approached significance compared to AW (p=.08). Acrophase was delayed in RW compared to AW on all work days (p<.001, all). Data on circadian rhythmicity are shown in figure 6 a-b.

Resting phase work advanced nadir of the body temperature during work days 2 to 4 ($p<.003$, all), and delayed acrophase during all work days ($p<.001$, all) compared to baseline. Moreover, both the % rhythm and amplitude were reduced on all work days ($p<.001$, all). Compared to AW, RW did not significantly differ in nadir, but showed delayed acrophase, reduced amplitude and reduced % rhythm during work days 2 to 4 ($p<.04$, all three work days, all parameters). See figure 7 a-d for data on circadian rhythmicity of body temperature.

Metabolic parameters

RW significantly reduced their food intake across 24h on all work days ($p<.001$, all) compared to baseline. Food intake increased during shifts on work days 3 and 4 ($p<.02$, both) and decreased between shifts on all work days ($p<.001$, all) compared to baseline. Compared to AW, RW did not differ in food intake across 24h. However, RW showed higher food intake than AW during shifts on work days 3 and 4 ($p<.01$, both shifts). No differences were observed between shifts. Data on food intake during the shift work period are shown in figure 8 a-b.

RW reduced their water intake across 24h ($p<.001$, all work days) and during shifts ($p<.001$, all shifts) compared to baseline. Water intake between shifts did not differ compared to baseline. Compared to AW, RW did not differ on 24h water intake, but drank more water during shifts on work days 1 to 3 ($p<.005$, all three shifts) and drank less between shifts ($p<.001$, all work days). See figure 8 c-d for water intake data.

RW reduced their body weight across 24h ($p<.001$, all work days) and during shifts ($p<.001$, all shifts), but gained body weight between the shifts ($p<.02$, all work days) compared to baseline. Compared to AW, RW lost more body weight across 24h ($p<.008$, all work days), during shifts ($p<.001$, all shifts) and gained less body weight between shifts ($p<.001$, all work days). Body weight data are shown in figure 9 b.

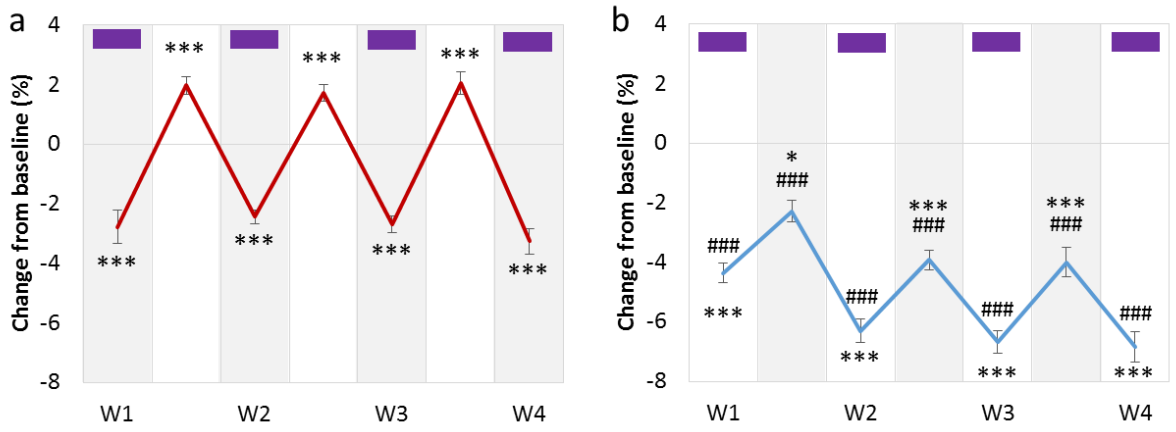

Figure 9. Body weight change during and between shifts. In a) active phase workers (AW) and b) resting phase workers (RW). Data are shown as percentage change relative to baseline. Purple squares indicate the 8h shift. Shaded bars indicate lights off (active phase). Error bars indicate SEM. W1-4 indicates work days 1 to 4. * p<.05; *** p<.001, compared to baseline. ### p<.001, compared to AW.

RW reduced locomotor activity in their active phase following the shift on work days 2 to 4 (p<.001, all three work days). Between-group comparisons were not analysed as locomotor activity means between groups were calculated from opposite circadian phases and thus were not comparable. Data on mean locomotor activity are shown in figure 10 b.

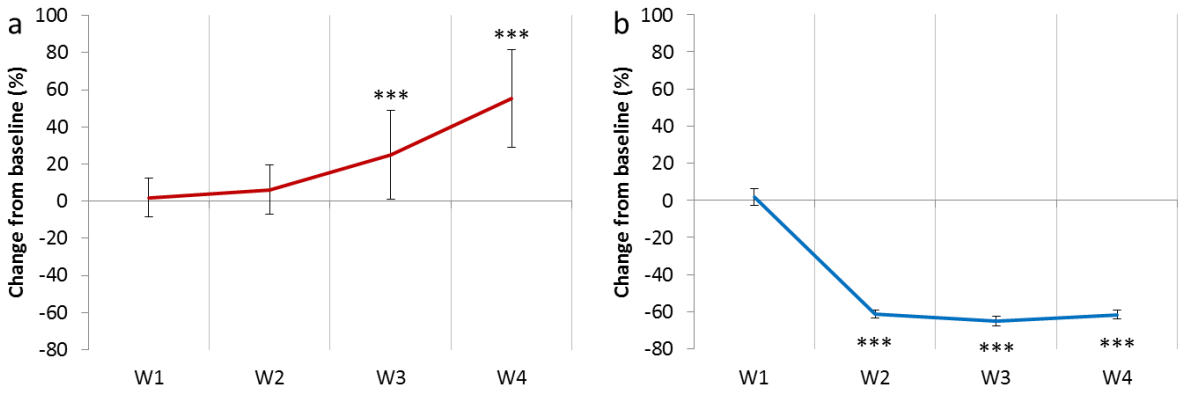

Figure 10. Mean locomotor activity between shifts. a) active phase workers (AW) (in their normal resting phase); b) resting phase workers (RW) (in their normal active phase). Data are shown as percentage change relative to baseline. Error bars indicate SEM. W1-4 indicate work days 1 to 4. * p<.05, ** p<.01, *** p<.001, compared to baseline. Between-group comparisons were not analysed as means were calculated from opposite circadian phases and thus not comparable.

Mean 24h body temperature did not differ during one shift work period compared to baseline. However, body temperature was elevated during the animals' 12h resting phase (which includes the 8h shift) on all work days (p<.001, all work days) and was reduced during the 12h active phase following the shifts on work days 2 to 4 (p<.003 all three work days) compared to baseline. Compared to AW, RW's 24h mean body temperature was not significantly different. However, the body temperature during the 12h resting phase was higher than AW's on work days 3 to 4 (p<.02 both) and lower during 12h active phase on work day 4 (p=.02). See figure 11 b for data on mean body temperature changes during the shift work period.

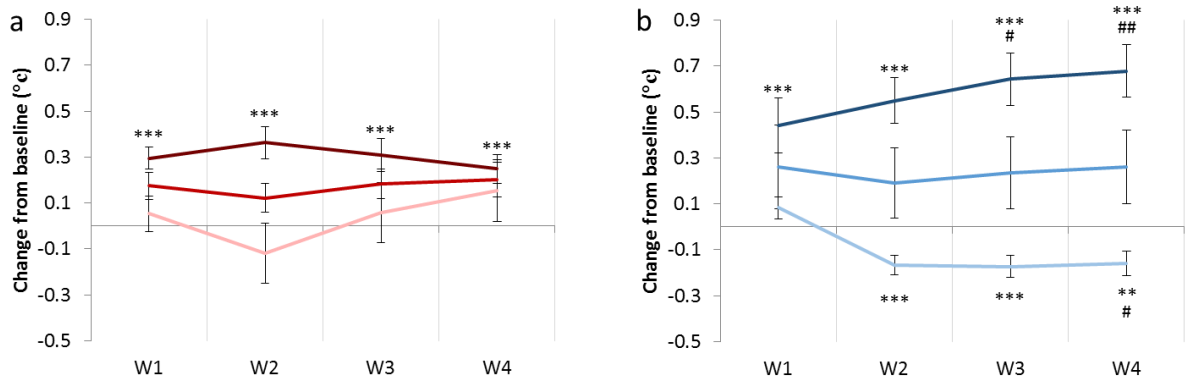

Figure 11. Mean body temperature during one shift work period. a) active phase workers (AW) 24h; AW 12h active phase; AW 12h resting phase. b) resting phase workers (RW) 24h; RW 12h active phase; RW 12h resting phase. Data are shown as mean percentage change relative to baseline. Error bars indicate SEM. W1-4 indicate work days 1 to 4. ** p<.01 *** p<.001, compared to baseline. # p<.05 ## p<.01, compared to AW.

In sum, AW showed no significant changes in circadian alignment during the shift work period. In contrast, RW shifted nadir of both the locomotor activity and body temperature rhythms to an earlier clock time, whilst acrophase of locomotor activity and body temperature were delayed. Percentage of rhythmicity of the body temperature and the amplitude were flattened for RW, both compared to baseline and to AW. Both AW and RW reduced body weight during the shift work period, but body weight loss was larger for RW. Food and water intake was reduced in both groups, but RW shifted timing of food intake toward work hours. Between shifts, RW reduced locomotor activity, whereas AW increased locomotor activity.

The recovery period

Food and water intake were not recorded during the recovery period. Body weight was recorded on recovery days 4 and 8. Body temperature and locomotor activity were recorded continuously. See Appendix C, table IX to XI for overview of the statistical main effects.

Active phase workers in LD recovery conditions

Circadian rhythmicity

Rhythmicity of locomotor activity and body temperature (nadir, acrophase, % rhythm and amplitude) during the recovery period was not different from baseline, see figures 12 a-d and 13 a-d.

Metabolic measures

The body weight of AW in LD condition was still lower compared to baseline on recovery day 4 ($p<.001$), however their body weight progressively increased throughout the recovery period and was no longer significantly different at recovery day 8 ($p=.20$), see figure 14.

Mean values of locomotor activity and body temperature in the recovery period were not different from baseline (see figures 15 a and 16 a).

Active phase workers in DD recovery conditions

Circadian rhythmicity

The locomotor activity rhythm of AW in the DD condition did not differ from baseline or from the LD condition, see figure 12 a-d.

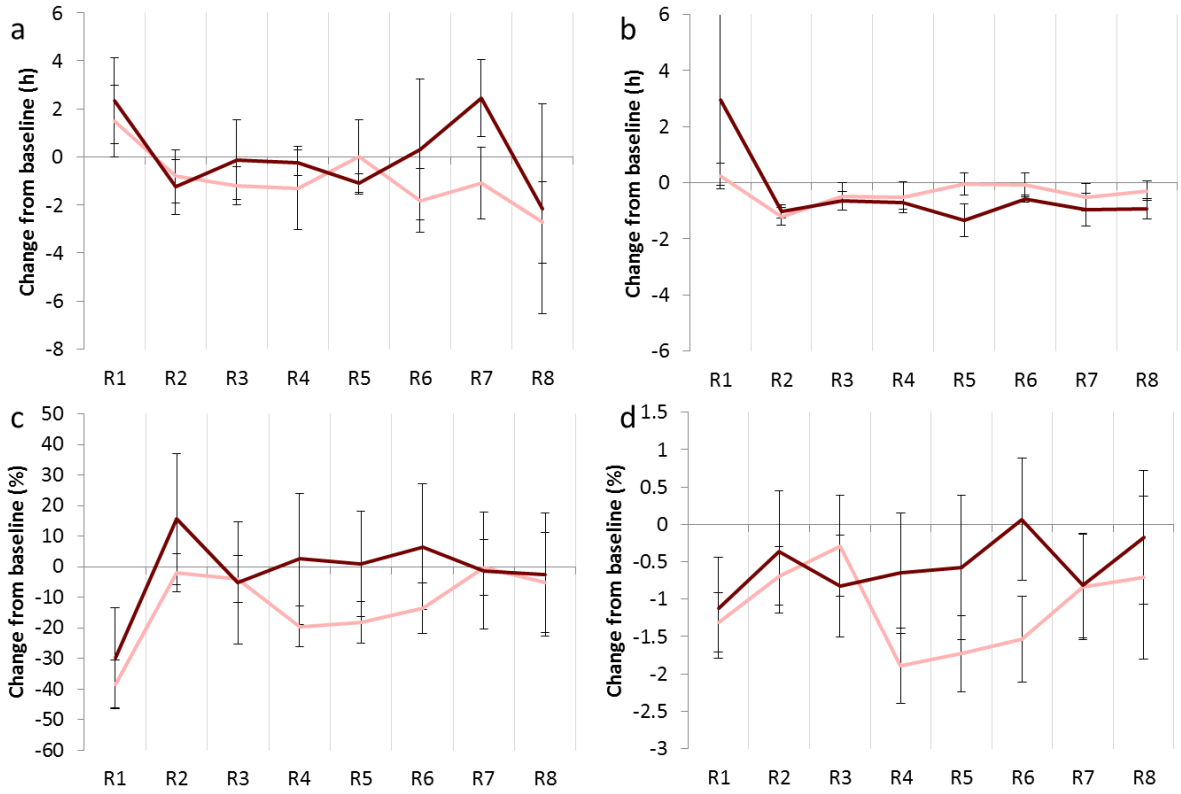

Figure 12. Circadian rhythmicity of locomotor activity during the recovery period in active phase workers. a) nadir; b) acrophase; c) amplitude; d) % rhythm. LD condition; DD condition. Data are shown as percentage change relative to baseline. Error bars indicate SEM. R1-8 indicate recovery days 1 to 8.

The body temperature rhythmicity in AW in the DD condition was not different from baseline, neither in nadir, acrophase nor % rhythm compared to baseline, however, the amplitude was reduced on recovery days 1 to 3 and 7 ($p<.02$, all four days). Compared to the LD condition, nadir and acrophase did not differ, but AW in the DD condition showed reduced % rhythm during recovery days 1 to 3 ($p<.04$, all three days) and reduced amplitude during all recovery days ($p<.03$, all days). See figure 13 a-d.

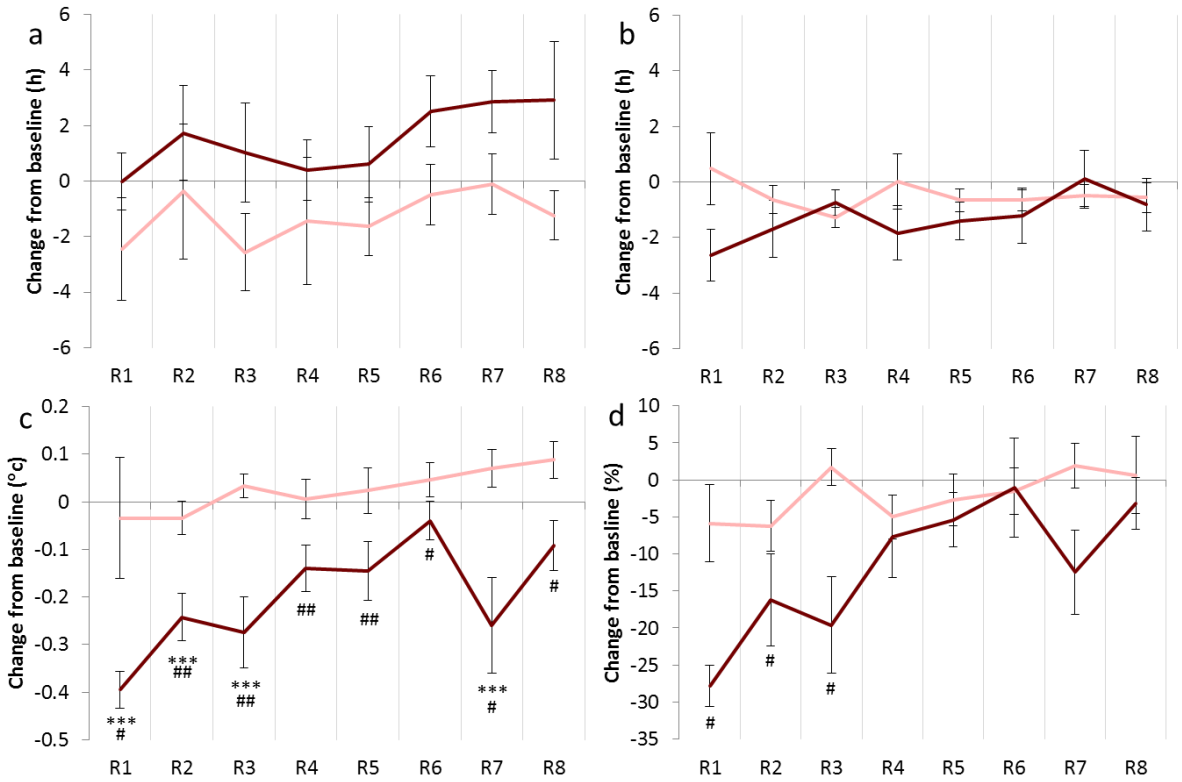

Figure 13. Circadian rhythmicity of body temperature during the recovery period in active phase workers. a) nadir; b) acrophase; c) amplitude; d) % rhythm. LD condition; DD condition. Data are shown as percentage change relative to baseline. Error bars indicate SEM. R1-8 indicate recovery days 1 to 8. * p<.05, *** p<.001, compared to baseline. # p<.05, ## p<.01, compared to LD.

Metabolic measures

Body weight change during recovery in the DD condition was not different from baseline or the LD condition. Data on body weight change during the recovery period are shown in figure 14.

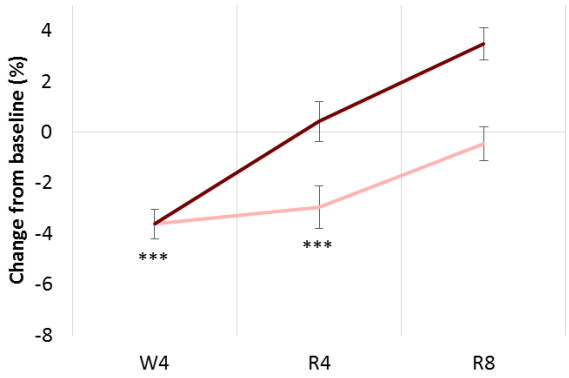

Figure 14. Body weight change during the recovery period in active phase workers. LD condition; DD condition. Data are shown as percentage change across 4 days relative to baseline. Error bars indicate SEM. W4 indicate work day 4. R4-8 indicates recovery days 4 and 8. *** p<.001, compared to baseline.

Mean locomotor activity parameters did not differ in the DD condition compared to baseline. Compared to LD, the 24h mean was elevated on recovery days 1 to 3 (p<.04, all days) due to an elevated activity in their resting phase. Resting phase activity was significantly higher compared to LD on recovery days 1 to 6 (p<.04, all days), see figure 15 b.

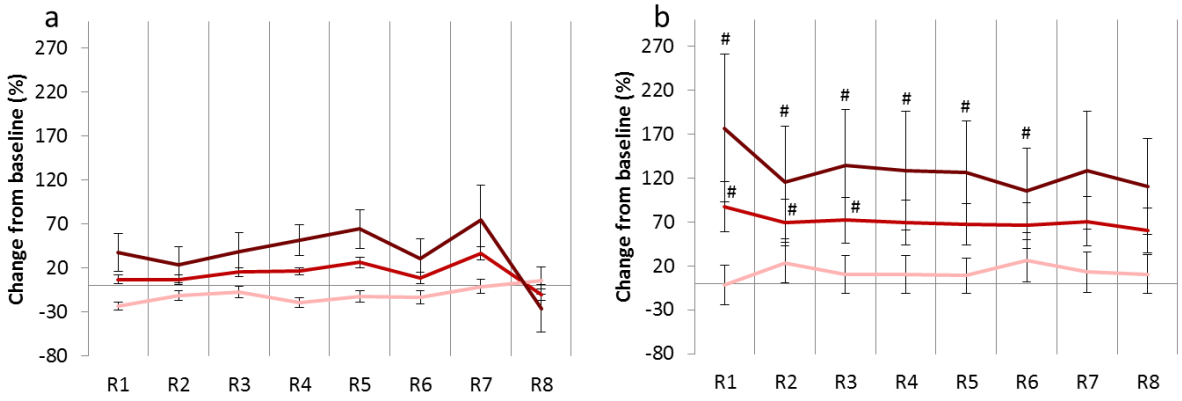

Figure 15. Mean locomotor activity during the recovery period in active phase workers. a) LD condition; b) DD condition. 24h; active phase; resting phase. Data are shown as percentage change relative to baseline. Error bars indicate SEM. R1-8 indicates recovery days 1 to 8. # p<.05, compared to LD.

Mean body temperature parameters of AW in the DD condition did not differ from baseline or from the LD condition. Data on mean body temperature during the recovery period are shown in figure 16 b.

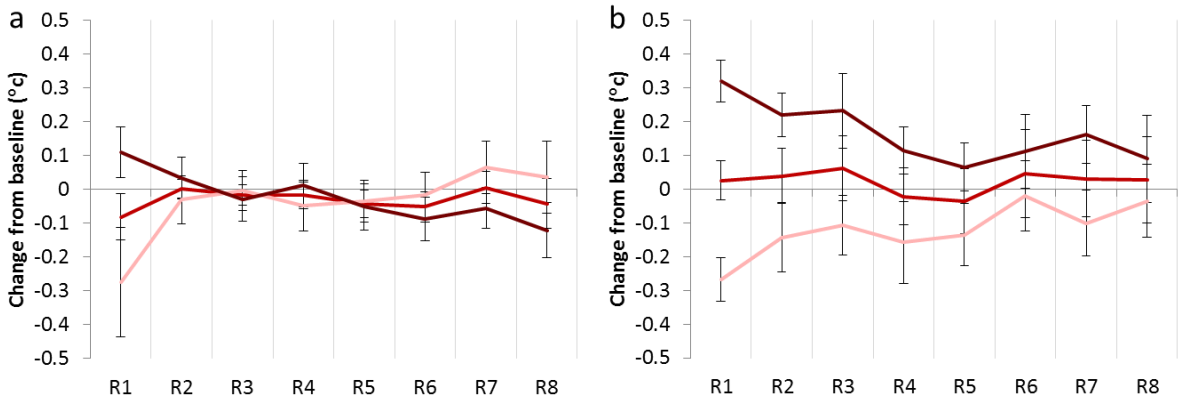

Figure 16. Mean body temperature during the recovery period in active phase workers. a) LD condition; b) DD condition. 24h; active phase; resting phase. Data are shown as percentage change relative to baseline. Error bars indicate SEM. R1-8 indicates recovery days 1 to 8.

In sum, Circadian parameters of locomotor activity as well as nadir and acrophase of the body temperature rhythm remained stable for AW throughout the recovery period in both recovery conditions. AW in LD exhibited higher amplitude and % rhythm of their body temperature compared to those in DD condition. AW in the LD condition regained body weight to baseline levels during recovery. AW in the DD condition did not significantly gain weight in their recovery period, but were not significantly different from the LD condition. Mean parameters of locomotor activity or body temperature were not changed compared to baseline in neither of the recovery conditions.

Resting phase workers in LD recovery conditions

Circadian rhythmicity

Nadir of the locomotor activity rhythm was advanced compared to baseline on recovery day 1 only ($p=.006$). The % rhythm was reduced on all recovery days ($p<.05$, all days) compared to baseline. No significant difference was observed for acrophase and amplitude. All data are shown in figure 17 a-d.

Nadir and acrophase of the body temperature rhythm showed no significant difference from baseline. The amplitude was reduced on recovery day 1 ($p<.001$) and progressively increased throughout recovery days 2-8 ($p<.02$, all seven days). Likewise, % rhythm was lower on recovery day 1 ($p<.001$) and was strengthened on recovery days 4-8 ($p<.05$, all five days). All data are shown in figure 18 a-d.

Metabolic measures

RW in the LD condition increased their body weight, but it remained significantly lower than their baseline weight throughout the recovery period ($p<.001$, both recording days), see figure 19.

Mean locomotor activity parameters in the recovery period did not differ from baseline, see figure 20 a.

Mean body temperature for 24h and 12h active phase was not significantly different from baseline. The body temperature during the 12h resting phase progressively declined throughout the recovery period with a significant reduction compared to baseline on recovery days 5 to 8 ($p<.02$, all four days). Data on mean body temperature during the recovery period are shown in figure 21 a.

Resting phase workers in DD recovery conditions

Circadian rhythmicity

None of the circadian parameters of locomotor activity differed from baseline or the LD condition. All data are shown in figure 17 a-d.

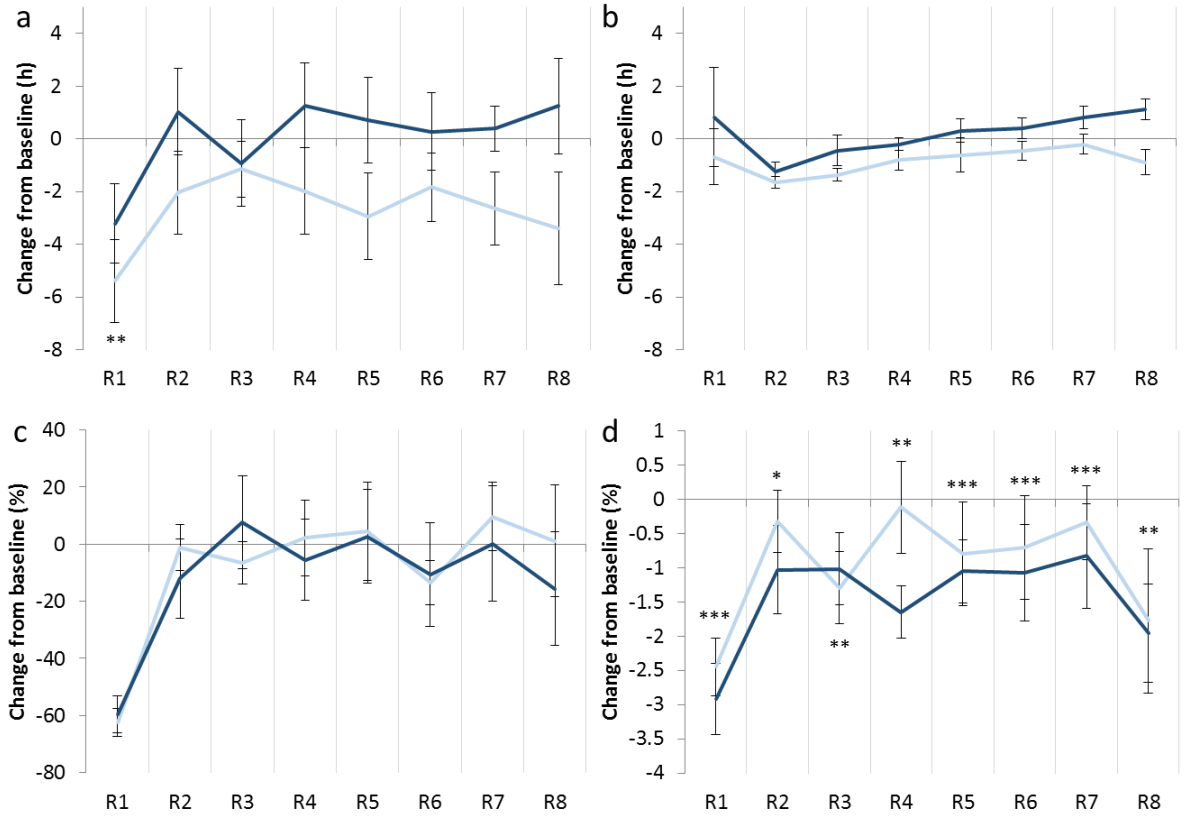

Figure 17. Circadian rhythmicity of locomotor activity during the recovery period in resting phase workers. a) nadir; b) acrophase; c) amplitude; d) % rhythm. LD condition; DD condition. Data are shown as percentage change relative to baseline. Error bars indicate SEM. R1-8 indicates recovery days. * p<.05, ** p<.01, *** p<.001, compared to baseline.

The nadir and acrophase of the body temperature rhythm did not differ from baseline or LD condition. The amplitude was lower on recovery days 1-2 (p<.001, both days) compared to baseline and lower on all recovery days (p<.009, all days) compared to the LD condition. The % rhythm was lower on recovery days 1-3 (p<.03, all three days) compared to

baseline and lower on recovery days 1-6 (p<.03, all six days) compared to the LD condition. All data are shown in figure 18 a-d.

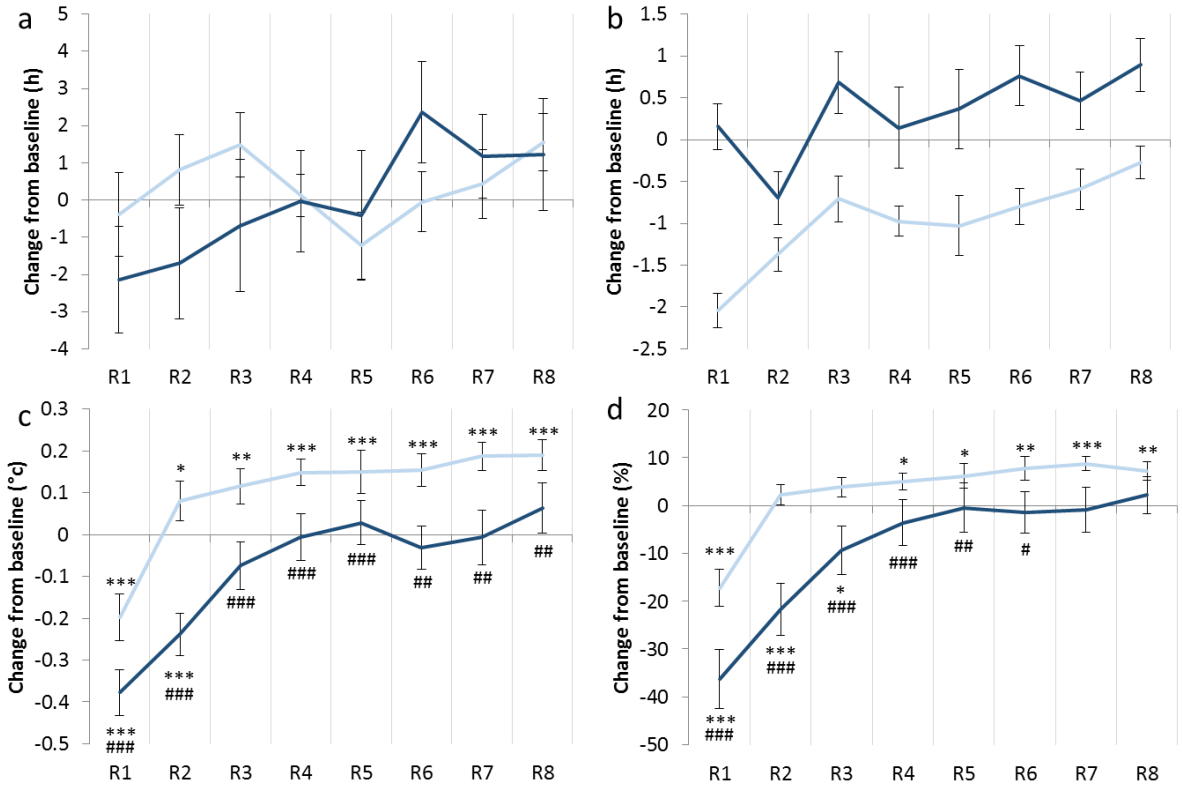

Figure 18. Circadian rhythmicity of body temperature during the recovery period for resting phase workers. a) nadir; b) acrophase; c) amplitude; d) % rhythm. LD condition; DD condition. Data are shown as percentage change relative to baseline. Error bars indicate SEM. R1-8 indicates recovery days 1 to 8. * p<.05, ** p<.01, *** p<.001, compared to baseline. # p<.05, ## p<.01, ### p<.001, compared to the LD condition.

Metabolic measures

RW in the DD condition increased body weight throughout the recovery period (p<.001, all days), but the body weight remained below baseline levels all days. Their body weight was not significantly different compared to RW in the LD condition, see figure 19.

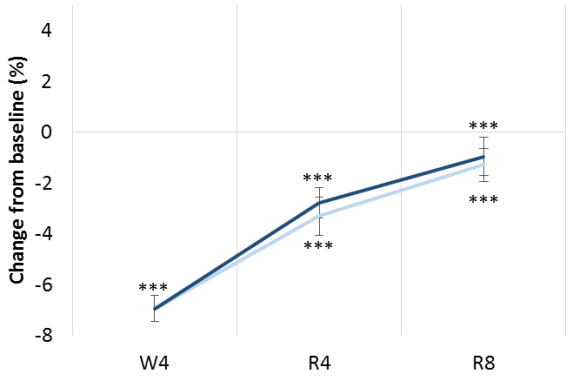

Figure 19. Body weight change during the recovery period in resting phase workers. LD condition; DD condtion. Data are shown as percentage change relative to baseline. Error bars indicate SEM. W4 indicates work day. R4-8 indicates recovery days 4 and 8. *** p<.001, compared to baseline.

Mean locomotor activity values showed no significant difference compared to baseline. Compared to LD condition, RW in the DD condition showed elevated 24h mean on recovery days 1-3 (p<.03, all three days). Furthermore, resting phase mean was elevated on all recovery days (p<.02, all days), and active phase mean was elevated on recovery day 1 only (p=.05). Figure 20 b shows data on mean locomotor activity in RW in the DD condition.

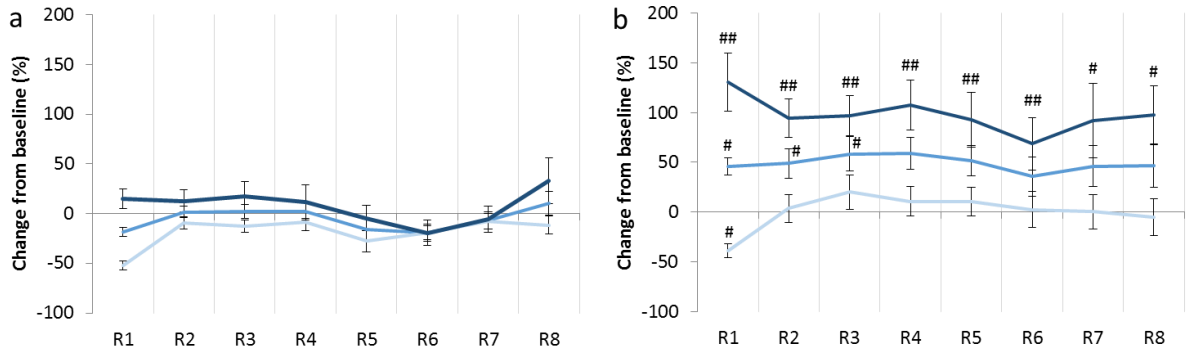

Figure 20. Mean locomotor activity during the recovery period in resting phase workers. a) LD condition; b) DD condition. 24h mean; active phase mean; resting phase mean. Data are shown as percentage change relative to baseline. Error bars indicate SEM. R1-8 indicates recovery days 1 to 8. # p<.05, ## p<.01, compared to LD condition.

Mean body temperature of RW in the DD condition differed neither compared to baseline nor the LD condition, see figure 21 b.

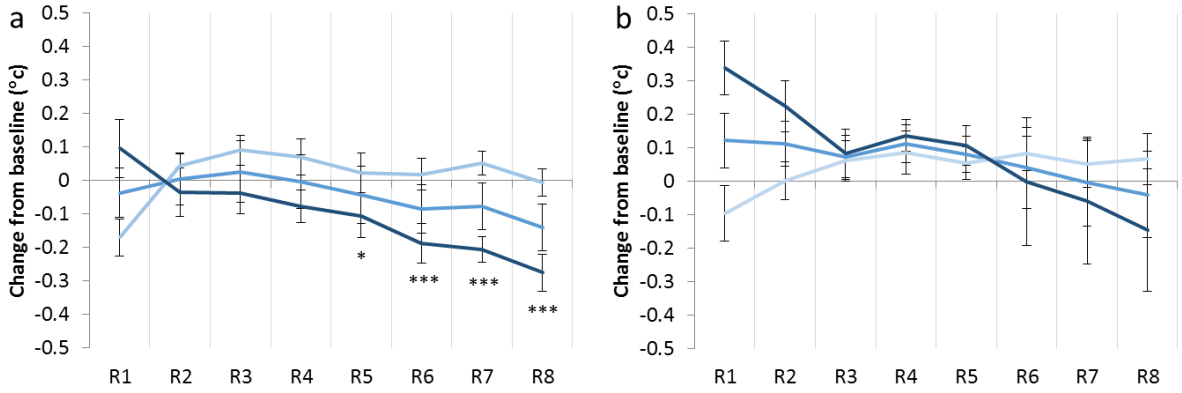

Figure 21. Mean body temperature during the recovery period in resting phase workers. a) LD condition; b) DD condition. 24h mean; active phase mean; resting phase mean. Data are shown as percentage change relative to baseline. Error bars indicate SEM. R1-8 indicates recovery days 1 to 8. * p<.01 *** p<.001, compared to baseline.

In sum, Nadir of locomotor activity and body temperature normalized within recovery day 2 for RW in the LD condition. Moreover, animals in this condition reduced % rhythm of locomotor activity, but increased amplitude and % rhythm of body temperature. In the DD condition, no significant changes were observed to circadian rhythmicity, but descriptively an endogenous shift and free-running pattern of circadian rhythmicity emerged. RW in the DD condition showed an initial reduction of amplitude and % rhythm of body temperature which normalized by recovery day 4. RW increased body weight throughout the recovery period, but did not reach baseline levels by recovery day 8 in neither of the recovery conditions. For RW in the LD condition, resting phase mean body temperature progressively declined throughout the recovery period. Compared to LD, RW in the DD condition exhibited increased resting phase mean activity levels throughout the recovery period.

Discussion

The aims of this study were to examine the extent to which one shift work period affects circadian processes and indirect measures of metabolism. Results show that only four days of resting phase work is sufficient to cause widespread disruption to both circadian rhythms and metabolism. So far, animal studies have focused on locomotor activity as an overt measure of circadian rhythmicity. The present study is the first to also record the circadian rhythmicity of body temperature following shift work. Here, I will discuss my results in relation to previous studies, and in relation to other data from the same experiment; circadian and metabolic gene-expression in peripheral and central tissues, sleep and glucocorticoid (GC) data.

Resting phase work disrupts circadian rhythmicity

Active phase workers (AWs) did not exhibit changes to circadian rhythmicity during the shift work period, whereas resting phase workers (RWs) shifted their rhythms of food intake, locomotor activity and body temperature. A reliable measure of circadian rhythmicity is the nadir of the body temperature (Benloucif et al., 2005). In RW, the nadir of body temperature gradually advanced during the shift work period, but was not completely altered to fit with the new work schedule. This indicates that four days of forced activity in the resting phase induces circadian disruption, but is not sufficient to cause full circadian adaptation to the new routine. The finding that the advance was gradual indicates that it indeed was a consequence of circadian disruption and not merely a by-product of physical activity. Circadian disruption was also evident by reduced amplitude and % rhythm of both locomotor activity and body temperature in RWs. These findings are consistent with my hypotheses and the literature on night work and circadian dysregulation. Changes in activity pattern can cause circadian shifts and weaken circadian rhythms even when light conditions

(the most powerful zeitgeber) remain constant (Folkard, 2008). Both animal and human studies show that resting phase work causes disruptions to circadian rhythmicity (e.g. Gupta & Pati, 1994; Salgado-Delgado et al., 2008). However, a complete adaptation to a night work schedule virtually never occurs (Folkard, 2008).

Our preliminary data on the expression of clock genes in this rat model of shift work support the above findings of circadian disruption after resting phase work. After work day 3, gene expression in both central (hypothalamus) and peripheral (liver) tissue were analyzed. Appendix D, figure I, shows fold-changes in clock gene expression for AW and RW relative to their respective controls (undisturbed animals euthanized at the same circadian time). Clock genes are regulated through an auto-regulatory feedback loop that consists of positive and negative elements. At the end of the active phase (ZT 24), the positive loop is normally upregulated and the negative loop is downregulated. AW (tissue harvested at ZT 24) showed a normal pattern of gene expression, but compared to their controls the pattern was more robust. This indicates that although overt measures of circadian rhythmicity (locomotor activity and body temperature) were unchanged in AW, circadian rhythms were strengthened at the genetic level in liver and hypothalamus. At the end of resting phase (ZT 12), the negative loop is normally upregulated and the positive loop is downregulated. RW (tissue harvested at ZT 12) expressed a complete inversion of normal circadian rhythmicity, as the positive loop was greatly upregulated (>11 fold-change in Bmal1) and the negative loop was downregulated. This data fits well with the overt circadian pattern expressed by RW during the shift work period, where nadir and acrophase were shifted, both for body temperature and locomotor activity. Furthermore, the data show that circadian rhythms are altered already after 3-4 days of resting phase work, not only overtly (body temperature and locomotor activity) but also at the genetic level.

Human studies have looked into clock gene expression in blood cells following night work. One study showed no changes in clock-gene expression after one single night shift (Bracci et al., 2013). However, after repeated night shifts, data on clock gene expression in plasma parallel our findings in peripheral and central tissues in the rat model of shift work; an inversion of clock-gene expression is evident, with the largest change in Bmal1 expression (Bracci et al., 2014; James, Cermakian, & Boivin, 2007). Thus, my hypothesis that resting phase activity causes circadian disruption is evident in various measurements and is consistent with the human literature.

In sum, AW showed stable circadian rhythms throughout the shift work period. In contrast, RW showed widespread circadian disruptions. The gradual shift in body temperature nadir indicates that the circadian shift indeed was a consequence of circadian disruption and not merely a byproduct of physical activity. Moreover, circadian disruptions were evident at the genetic level in both peripheral and central tissues after only three days of shift work, indicating that resting phase work causes circadian changes not only overtly (body temperature and locomotor activity) but also at the genetic level.

Resting phase work disrupts circadian rhythm of metabolism

The circadian pattern of food intake during the shift work period was maintained in AWs. In contrast, RWs showed a disruption of the circadian rhythmicity of food intake, as evidenced by a shift in the timing of food intake towards working hours. This result is consistent with my hypotheses, and results of previous studies. The Escobar group also found that rats shifted their food intake timing toward work hours following five weeks of resting phase work (Salgado-Delgado et al., 2008). Likewise, human night workers typically consume the majority of their calories during shifts (de Assis, Kupek, Nahas, & Bellisle, 2003). Altered food intake rhythms can entrain circadian rhythms, and altered activity

rhythms can entrain food intake rhythms, even when light conditions remain constant (Buijs, Salgado, Sabath, & Escobar, 2013; Mrosovsky, 1996).

The shift in food intake can be related to the findings on clock gene expression (appendix D, figure I). For both groups, clock gene changes were larger in liver compared to hypothalamus, and also larger for RW compared to AW. The modest change in the hypothalamus is likely due to the constant light conditions throughout the shift work protocol, as light is the primary zeitgeber for the hypothalamus (Berson, 2003). Nevertheless, hypothalamic clock genes did change significantly, and this finding confirms that this brain structure is also affected by other zeitgebers such as activity or food intake. The Escobar group's rat model of chronic resting phase work (five weeks) also report disruption of clock genes in peripheral tissue and in hypothalamus (Salgado-Delgado et al., 2010b). Thus, both our and previous findings that forced resting phase activity causes circadian disruption in metabolic tissues. Considering that food intake is the primary zeitgeber for peripheral tissues involved in digestion and metabolism (Damiola et al., 2000), the shift in food intake likely caused the shift in liver clock gene expression for RW.

The shift in food intake rhythm, and subsequent effects on metabolic tissues, can be of particular importance to shift work research. Several studies suggest that altered rhythms of food intake can be a key contributor to metabolic disorders. The Escobar group found that providing food only during the active phase for rats in RW prevented obesity and disruption of rhythmicity of key metabolic compounds such as TAG and glucose (Salgado-Delgado, Angeles-Castellanos, Saderi, Buijs, & Escobar, 2010a). Recent human findings also show that night-time food intake over several days results in a progressively increased glucose response to breakfast, indicating metabolic dysregulation and associated with development of type II diabetes (Banks et al., 2015; Bansal, 2015). Our finding that changes occur after such a short time (3 days) are important considering that circadian disruption is likely a key contributor to

metabolic and sleep disorders (Reddy & O'Neill, 2010). In fact, some authors recommend that human shift workers should avoid eating during the night shift (Lowden, Moreno, Holmback, Lennernas, & Tucker, 2010). Our findings support this notion. Sleep restriction may also have played a part in the increased food intake during resting phase work. Our sleep data show that RW were completely sleep deprived during each shift (resting phase, when the majority of sleep would occur)(Pedersen, 2015). Sleep restriction is known to increase hunger and craving for sweet foods in humans, and may have stimulated the increase in food intake during shifts for RW (Depner, Stothard, & Wright, 2014).

In sum, AW maintained a stable eating rhythm throughout the shift work period. In contrast, RW shifted the rhythm of food intake towards working hours. Food intake, together with forced activity and sleep restriction, likely caused an inversion of liver clock gene expression. Shift in eating rhythm may contribute to metabolic disruption in both animals, and human shift workers.

Circadian disruption in RW persists in the recovery period

During the recovery period, I aimed to investigate the long-term effects of one shift work period. AWs stable circadian rhythms of locomotor activity and body temperature throughout the shift work period remained stable in the recovery period under normal light conditions (LD). RW displayed disrupted circadian rhythmicity during their shift work period. This disruption persisted during the recovery period. Moreover, not all parameters normalized within the 8 days of recovery. Amplitude and % rhythm of body temperature were masked by hypothermia in resting phase, and thus are not suitable for interpretation as a circadian outcome (but will be discussed later). Amplitude of locomotor activity returned to baseline on recovery day 1, whereas % rhythm remained below baseline level throughout the 8 days of recovery. Thus, the locomotor activity rhythm may have been permanently affected by resting phase work. There were no significant findings for acrophase of either locomotor activity or

body temperature during the recovery period, indicating that these measures normalized within the first recovery day. The body temperature nadir exhibited a ~5h advance after 4 days of resting phase work. This parameter also returned to baseline levels already at recovery day 1. The rapid normalization is likely due to the normal light conditions, both during and after the shift work period. The nadir of locomotor activity showed an advance of almost 7h on recovery day 1 (as was also observed at the end of the shift work period), but normalized on recovery day 2. Thus, the locomotor activity nadir normalized relatively quickly, but was more affected by shift work than the body temperature rhythm.

The finding that nadir differed by 7h for body temperature and locomotor activity on recovery day 1 indicates intermittent internal desynchrony. Internal desynchrony can emerge because different circadian systems adapt to changes in zeitgeber at different rates (Dibner & Schibler, 2015). Importantly, this study shows that only four days of resting phase activity, with constant light conditions, are sufficient to cause one day of internal desynchrony between body temperature and locomotor activity rhythms. This occurred in healthy adult animals that had never before been exposed to resting phase activity. In another animal model of shift work, based on forced activity, internal desynchrony was found at the genetic level between structures within the hypothalamus (the arcuate and dorsomedial nucleus)(Salgado-Delgado et al., 2010b). Moreover, in human shift workers, internal desynchrony has been identified between circadian parameters of body temperature, heart rate, subjective fatigue and sleepiness, and cognitive ability (Gupta & Pati, 1994). Corresponding to findings in humans, our findings thus show that the rat model has face validity. Additionally, our findings highlight the importance of assessing more than one circadian rhythm measure, as disruption occurs not only within systems, but also in the synchronization between them. Importantly, internal desynchrony may be the cause of many negative health effects observed in shift

workers, such as sleep disturbances, metabolic disruption and cancer (Davis et al., 2001; Gangwisch, 2014; Golombek et al., 2013).

As mentioned, RW exhibited a significant reduction in locomotor activity % rhythm throughout the recovery period. This indicates that resting phase activity caused not only short-lasting changes to circadian rhythmicity, but also induced long-lasting effects. The previous animal study by the Escobar group, found that chronic simulated night work (5 weeks) abolished the 24h locomotor rhythmicity, and induced an ultradian pattern with cycles of maximums and minimums every 4h and 12h (Hsieh et al., 2014). The method of analysis employed in my study can only detect 24h rhythmicity. However, the development of ultradian rhythms may explain the pattern observed in our data, of reduced % activity rhythm and normal amplitude. A rhythm with several peaks and troughs across 24h would exhibit normal amplitude but reduced % rhythm since the data points would not fit well with a sinusoid curve. Moreover, I observed large intra- and inter-individual variation in the locomotor activity nadir for RW. The emergence of ultradian rhythms can explain these individual differences since the software I used identified only one nadir over a 24h period. If in fact there were several troughs in the rhythm this would increase the individual variance. Thus, the present study shows that resting phase activity caused long-term disruption of locomotor activity rhythmicity.

The indication that circadian rhythmicity is disrupted by resting phase activity, even when light conditions remain constant, is further supported by sleep data collected from the same animals (Pedersen, 2015). In rats, some sleep occurs during the normal active phase. Since AW were prevented from sleeping in the majority of this phase, sleep during their normal resting phase increased. AW's sleep was normalized after 2 days of recovery. In contrast, RW exhibited an inversion of their normal sleep/wake pattern during the shift work period. Notably, during recovery, RW exhibited an inverted sleep/wake pattern with less sleep

in their resting phase and more sleep in their active phase, another indication of circadian disruption, and needed 5 days to normalize to baseline sleep architecture. Again, internal desynchrony is evident in RW, as body temperature recovered fast, uncoupling it from the disrupted rhythms of locomotor activity and sleep. Moreover, the disrupted rhythm of sleep (normalized after 5 days) was uncoupled from the locomotor activity (% rhythm showed no signs of recovery). These findings highlight that although the body temperature rhythm is considered a reliable measure of circadian rhythmicity, other parameters should also be taken into account when investigating circadian disruption.

Effective recovery of disrupted circadian rhythmicity after shift work is a major focus in shift work research today (e.g. Eastman et al., 1994; Hilditch et al., 2014; Jackson et al., 2014). Akerstedt (2000) suggests that 3 recovery days are needed for workers to recover subjective feeling of fatigue and sleepiness after one or more night shifts. Our results indicate that certain objective measures of circadian rhythmicity (body temperature) do recover within 3 days, but others (such as sleep and locomotor activity) do not. External factors (light conditions, food intake timing etc.) both during and after the shift work period will play a major part in estimating the time needed to recover. Bear in mind that RW in this study were exposed to constant light conditions, and light is considered the primary zeitgeber for the circadian system (Mrosovsky, 1996). Thus, it is probable that forced activity combined with altered light conditions will have even more negative effects than the ones observed in this study.

In sum, one shift work period caused no short- or long term circadian disruptions in AW. Conversely, only four days of resting phase activity in constant light conditions were sufficient to cause both short- and long term circadian changes to parameters of body temperature, locomotor activity, and sleep. The locomotor activity rhythm did not return to baseline during the recovery period. Internal desynchrony was evident as parameters

recovered at different rates (body temperature nadir immediately; locomotor activity nadir 1 day; sleep disturbance 5 days; % activity rhythm at least 8 days). Based on existing literature, I hypothesize that ultradian rhythmicity in locomotor activity rhythms may have been developed in these animals.

Circadian disruptions after RW are of endogenous nature

In the present study I also aimed to examine whether the circadian disruptions after one shift work period were of an endogenous nature. When individuals are exposed to constant darkness, the effect of zeitgebers is removed, and the endogenous circadian rhythm is exposed (Aschoff, 1965). Investigation of endogenous disruptions is useful because it indicates whether changes to the internal circadian physiology have occurred in response to a challenge. When placed under constant darkness, the circadian rhythm starts to free-run, typically evident by a gradual delay of nadir, as many species (including adult humans and rats) have an endogenous circadian rhythm slightly longer than 24h (Aschoff, 1965).

In this study, AW did not exhibit circadian changes, neither during the shift work period nor in the LD recovery condition. In the DD condition, AW exhibited reduced amplitude and % rhythm, probably due to the absence of zeitgebers which would normally amplify the circadian timing signal from the SCN (Aschoff, 1965). AW also appears to develop a free-running pattern as indicated by a gradual delay of nadir and acrophase, although these changes were not significant. Lack of significant results were likely due to the small number of animals in this group (n=5). Thus, in the DD condition, AW exhibited a normal endogenous circadian rhythm.

RW showed circadian disruptions both during the shift work period and in the LD recovery condition. Data from the DD recovery condition indicate that the disruptions were of an endogenous nature. Whilst amplitude and rhythm of body temperature were masked by hypothermia in resting phase (which will be discussed later), the amplitude and % rhythm of

locomotor activity showed a similar pattern to LD recovery, indicating that the circadian disruption, and possible emergence of ultradian rhythms, was endogenous, and not affected by external light conditions. Nadir and acrophase of both body temperature and locomotor activity showed large intra- and inter-individual variation in the free-running condition. Such high variance in the data reduced the statistical power on these measures and few significant results were identified. However, descriptively, the data show an important pattern, namely a free-running pattern with a gradual delay of both nadir and acrophase of body temperature. Interestingly, locomotor activity nadir and acrophase did not develop a free-running pattern. These findings show that internal circadian physiology was altered by one shift work period.

The advanced body temperature nadir in RW in the LD condition was also present in the DD condition, showing a 2h shift on recovery day 1. In subsequent days, a free-running pattern emerged. These data indicate that resting phase work did indeed cause a long-term shift in the endogenous circadian rhythm of body temperature, which was normalized by light in the LD condition. Little is known about the effects of shift work on endogenous circadian rhythm. Some studies have examined effects of shift work by the use of dim-light melatonin onset in humans and claimed to measure endogenous rhythms (see Folkard, 2008 for review). However, this is to be questioned, as subjects were only subjected to dim light conditions for a few hours. I have not found any animal studies examining the effects of shift work on endogenous circadian rhythms. Thus, to my knowledge, our study is the first study to investigate this. The results indicate that resting phase work indeed causes a shift in the endogenous circadian rhythm, even when light conditions during the shift work period remain constant.

The locomotor activity rhythm in RW did not conform to a free-running pattern, as nadir was stable throughout recovery. This indicates that one shift work period of forced resting phase activity caused a change in the endogenous period length of the locomotor

activity rhythm. The stable nadir during the recovery period demonstrates that the circadian period must have been shortened to approximately 24h. The period length of the body temperature rhythm was clearly longer than 24h, as exemplified by the gradual delay of the nadir. Thus, the body temperature rhythm and locomotor activity rhythm exhibited different endogenous period lengths, causing gradually increasing internal desynchrony. Since these data are descriptive and based on a small number of animals, future studies have to replicate these findings before firm conclusions can be drawn. Nevertheless, our data may indicate that one shift work period of resting phase activity causes internal desynchronization of endogenous nature.

In sum, one shift work period did not affect the endogenous circadian rhythmicity in AW. However, for RW, one shift work period caused a 2h shift in their endogenous circadian rhythm of body temperature. Notably, the body temperature rhythm developed a typical free-running pattern, whereas the locomotor activity rhythm did not. This indicates that the period of the locomotor activity rhythm was shortened by shift work in RW. Additionally, the discrepancy between body temperature and locomotor activity period lengths indicate internal desynchrony of an endogenous nature.

Resting phase work disrupts metabolism

Contrary to previous results from rat models, including forced activity protocols, the animals in this study (both AW and RW) exhibited a negative energy balance during the shift work period. In our study, this was evident by an overall reduction in food intake, water intake and body weight. Weight loss was more evident in RWs than AWs, indicating that this shift work condition had more severe effects on the energy balance. Previous animal studies have reported on weight change from baseline, and compared food intake between groups following five weeks of shift work, but no previous study has reported on food intake change compared to baseline in a rat shift work model. This makes comparison of our results to

previous findings challenging. However, several human studies have examined how food intake changes when individuals are exposed to shift work. A systematic review reports that most studies find no change in total food intake in shift workers, although there are many other changes in aspects of food intake such as food intake timing, number of meals, and size and nutritional content of meals (Lowden et al., 2010). We ran a pilot experiment to test the hypothesis that food intake would increase if rats were given three "lunch breaks" during each shift. This version of the protocol was performed by stopping the rotating wheels for ten minutes, three times across the 8h shift. There was no significant difference in food intake, water intake or sleep/wakefulness under these conditions. Therefore we did not pursue this protocol further. Thus, "lunch breaks" during each shift was not an effective counter-measure for reduced total food intake. Water intake during work shifts was dramatically reduced in both groups, and was likely due to difficulty drinking from the bottles in the rotating wheels. However, animals were able to recover most of their water intake between shifts, and thus the effects of partial water restriction are likely to have been minimal. Similarly to food intake, "lunch breaks" during each shift was not an effective counter-measure for reduced water intake.

Findings on energy balance changes in animal models of shift work are ambiguous. Weight gain during periods of RW shift work (Salgado-Delgado et al., 2008) as well as attenuated weight gain in RW compared to AW has been reported (Leenaars et al., 2012). The slight reduction in water intake observed in our study may have contributed to weight loss. Moreover, we measured body weight directly after each shift. In contrast, the Escobar group report weight change only after the termination of five weeks of the shift work protocol. Similarly, the Leenaars paper reports weight change after two days off from shift work. Additionally, both studies used wheels rotating at a slower pace than the ones used in our study. Increased activity during shift work will increase energy expenditure and thus may

explain the weight loss in our study. Unfortunately, the transmitter system was unable to reliably record locomotor activity during shift work and we cannot compare activity during work to baseline. Still, the rats in our study were forced to walk approximately only 1.1 km each work shift. Considering that, on average, a rat given access to a running wheel voluntarily runs 6-9 km each day (Rodnick, Reaven, Haskell, Sims, & Mondon, 1989), the physical activity cannot be deemed strenuous. Nevertheless, forced activity may have caused an increase in energy expenditure compared to undisturbed baseline condition.

When examining measures of energy expenditure (locomotor activity and body temperature) during the shift work period, differential effects of AW and RW emerge. AW did not alter their body temperature during shifts, but increased both body temperature and locomotor activity between shifts. RW exhibited a dramatic increase in body temperature during shifts, indicating increased energy expenditure. Between shifts, RW showed a reduced locomotor activity and body temperature compared to baseline. Our data indicate a discrepancy between measures of energy intake and expenditure, and body weight change (energy balance) between shifts. We know that RW gained less weight than AW between shifts, even though energy expenditure (locomotor activity and body temperature) was lower. Moreover, total food intake (grams) was higher between shifts for RW compared to AW. Thus, all indirect measures of metabolism indicate that RW should be gaining more weight than AW between shifts.

Sleep data show that RW slept less than AW between shifts despite being less active (Pedersen, 2015). This indicates sleep disturbance and fatigue in RW, which are common complaints of human night workers, and may have an effect on metabolism (Akerstedt, 1998). I have not found any human studies addressing immediate effects of shift work on energy expenditure, but studies on sleep restriction show reduced energy expenditure in healthy individuals (Benedict et al., 2011). The reduced activity following resting phase work may

indicate a metabolic response to the acute negative energy balance. These responses may be compensatory, aiming to maintain energy balance homeostasis. The paradoxical finding that RW showed attenuated weight gain between shifts, despite indirect metabolic measures promoting energy storage, indicates metabolic disruption.

Our metabolic gene analyses performed on liver tissue after 3 work days support our indirect measures of metabolic disruption. See appendix D, figure II for metabolic gene expression results. Results show that genes involved in energy storage were more upregulated and genes involved in energy breakdown were more downregulated, in RW compared to AW. Moreover, the IRS2-gene, linked to hepatic insulin sensitivity and lipid metabolism, was upregulated in RW but not in AW (Awazawa et al., 2011; Taniguchi, Ueki, & Kahn, 2005). This indicates that at the transcriptional level, resting phase work promotes a positive energy balance and changes in insulin regulation. There are two possible explanations for why this happens. Firstly, it could be an attempt to compensate for the negative energy balance (body weight loss) and possibly other changes to glucose and lipid metabolism that occurred during the shift work period. Such metabolic compensatory mechanisms have been described in skeletal muscle, and may also occur in liver (Hood, Irrcher, Ljubicic, & Joseph, 2006). However, AWs were also under a negative energy balance (lost body weight) during the shift work period and did not show such changes. Thus, a second, more likely explanation, is that genetic changes reflect a metabolic disturbance caused by shift in the timing of activity and food intake. I hypothesise that the genetic changes evident at work day 3 may become evident in overt physiology (i.e. body weight change) at a later stage. Findings from the Escobar group supports this hypothesis, as changes both at the genetic level and at the overt physiological level (excessive weight gain) were found after five weeks of shift work (Salgado-Delgado et al., 2008). Importantly, our study shows that changes in metabolic genes occur already after 3 days of shift work.

Genetic markers of stress and inflammation (IL1-α and IL1-β) showed no changes in AW, whereas RWs showed an upregulation of these genes. Acute stress is known to cause weight loss (Harris et al., 1998). This may add to the complex picture of why RW lost more weight than AW during the shift work period. To examine this notion, we analysed glucocorticoid (GC) levels in faeces during the shifts. See appendix D, figure III for GC data. Our data show a modest increase in GC levels across days in AW. In contrast, RWs show a progressively higher concentration of faecal GCs across three shifts. GCs show circadian variation, being higher in the beginning of the active phase (when AW faeces were collected) than resting phase (when RW faeces were collected), and are excreted in both humans and animals in response to stress (Chung et al., 2011). Concentration of GC in RW indicates a circadian dysregulation of the stress marker. Both animal and human studies have shown dysregulation of GC rhythms following simulated shift work (James et al., 2007; Salgado-Delgado et al., 2008). Thus, our findings are consistent with the previous literature. Moreover, in our study, RW exhibited higher GC levels than AW during the third shift. Thus, our data may also indicate that forced activity in resting phase is more stressful than forced activity in active phase.

In sum, both AW and RW exhibited a negative energy balance during the shift work period, but RW lost more weight than AW. Overall, food intake and water intake were similar. However, between shifts, metabolic measures (food intake, gene expression, body temperature, locomotor activity) indicate that RW had a higher energy intake and lower energy expenditure compared to AW. Still, RW gained less weight between shifts, indicating a metabolic disruption. The disruption may have been caused by elevated GC levels in RW. Thus, one shift work period is sufficient to cause dysregulation of metabolism in RW.

Resting phase work induces long-term disturbances in metabolism

AW showed only modest changes in energy balance, metabolic gene expression and GC levels during shift work. By the end of recovery period, AW body weight was returned to baseline, and measures of energy expenditure (mean locomotor activity and body temperature) remained at baseline levels throughout the recovery period. RW showed increased weight gain and hypothermia in resting phase in the shift work period, persisting in the recovery period. RW did not normalize their body weight in the recovery period. However, considering that RW had lost more weight during the shift work period, they had a steeper weight gain curve than AW during recovery. This could indicate that the energy-storing changes observed in metabolic gene expression at the end of the third work day for RW persist and promote weight gain in recovery.

Mean locomotor activity normalized during recovery for both AW and RW. In the DD condition, both AW and RW exhibited increased mean locomotor activity. This is likely not an effect of shift work, but rather a response to constant darkness, as light has a direct suppressing effect on locomotor activity in rats (Nasello, Machado, Bastos, & Felicio, 1998; Reppert & Weaver, 2002).

Body temperature may be a more sensitive measure than activity for investigating energy balance in recovery from shift work. Mean body temperature levels in AW were increased following work during the shift work period, but normalized at recovery day 1. Surprisingly, RW initially recovered from the reversed mean body temperature patterns in the shift work period, but subsequently developed progressive hypothermia in their resting phase. On recovery day 8, resting phase mean body temperature was almost 0.3°C lower than at baseline. Since recordings were ended at day 8 we do not know how many days hypothermia lasted, or how severe it became. Nevertheless, the finding indicates that resting phase work induces long-term effects on body temperature.

The long-term effect on body temperature may be due to either stress or metabolic dysfunction, or a combination of both. On work day 3, the GC level was higher in RW than AW, even though GC levels are normally highest during a rats' active phase. We do not have data on GC levels during recovery, but if the pattern continued, this would constitute a chronic GC elevation. Previous results from our laboratory have shown hypothermia as an effect of chronic stress (Mrdalj et al., 2014). Thus, the hypothermia observed in RW recovery may in part be caused by stress. Another explanation is that a progressive hypothermia may be a metabolic effect of shift work, as body temperature is a measure of energy expenditure, and the lowering of body temperature, particularly peripheral body temperature (as was measured in this study), occurs in response to metabolic challenge. Rats exposed to semi-starvation exhibit a near-identical change in body temperature patterns as was seen in our experiment (Siyamak & Macdonald, 1992). Although the rats in our study were not exposed to starvation, the metabolic changes in response to a negative energy balance could be similar. Lastly, stress may itself be a metabolic response to shift work, as chronic GC elevation facilitates energy storage (Harris et al., 1998; Shibli-Rahhal et al., 2006). Taken together, I hypothesize that progressive reduction of resting phase body temperature was caused by prolonged stress and/or disturbed metabolism. Importantly, these findings show that only four days of resting phase work can induce long-lasting effects on resting phase mean body temperature.

In sum, AW returned to baseline body weight levels after 8 days of recovery. RW did not, but showed a steeper weight gain curve in the recovery period, possibly due to mechanisms promoting energy storage. RW unexpectedly developed resting phase hypothermia during the recovery period. This could be due to metabolic disruption, chronic stress, or both. Thus, one shift work period induced long-lasting effects on metabolic measures during the recovery period.

Evaluation of the model and present experiment

Animal models of shift work

Although shift work is a young area of research, several animal models of shift work have been developed in the recent years (see Opperhuizen et al., 2015 for review). Animal models allow for randomization and control of variables. Most studies to date, including the one presented here, have focused on manipulating one condition; either timing of activity, light, food or sleep. Effects have been recorded in a range of measures, but this is the first model to use telemetric recordings, and the first to measure body temperature and sleep in a rat model. In our model, light conditions were kept constant, whilst timing of activity was manipulated. Most human literature has focused on the negative effects of light exposure, and some have focused on food intake and other metabolic measures. Our study highlights the importance of activity timing as an important factor contributing to the negative effects of shift work.

Our animal model of shift work employs a forced activity paradigm to mimic certain aspects of human shift work. In terms of construct validity, this type of model captures important aspects of human shift work; the individual is kept awake (as we have confirmed through sleep measures) and active at a time when the body is producing a natural drive for rest. Animal studies which manipulate light conditions have also found disturbances to circadian and metabolic processes, but these models have been criticized for more closely resembling jet lag. A model using forced activity is therefore more appropriate as an animal model of shift work. Some have questioned whether the forced activity paradigm used in the model is problematic, considering that humans perform work voluntarily. However, one can also question whether work, and particularly work at impractical hours, is ever completely voluntary. Nevertheless, it is clear that humans act in a goal-oriented fashion, and are

compensated for their work. Although our model does not capture the goal-oriented nature of human shift work, it captures many other important aspects. Thus, the model has adequate construct validity.

Considering face validity, results from our study show sleep disturbances following shift work (Pedersen, 2015), which is one of the most common complaints in human shift workers (Oyane et al., 2013). Moreover, my results show that RW gradually delayed nadir and developed internal desynchrony both during and after the shift work period. The same patterns of circadian rhythmicity have been identified in human shift workers (Gupta & Pati, 1994). Additionally, preliminary data suggest that the animals in the experiment developed dysregulation of clock gene expression, in patterns very similar to those found in human shift workers (e.g. Bracci et al., 2014). Based on the similarity of my results to human results, I conclude that the model has good face validity.

The predictive validity of the model cannot be confirmed by the present data alone. However, the data collected so far are promising. The results of this animal model of shift work allow me to make several predictions about the effects of shift work. Firstly, results predict that rhythms of body temperature and locomotor activity are differentially affected by shift work. Secondly, it can be predicted that recovery of disrupted circadian and metabolic measures will take longer than 3 days as predicted by Akerstedt (2000). Lastly, results predict that the circadian changes are of an endogenous nature. These are all predictions that can be tested in humans, and have the potential to confirm that the animal model of shift work indeed has good predictive validity.

Strengths and limitations of the present experiment

A major strength of the present study is that through the use of telemetric devices, we have been able to measure multiple parameters (sleep, body temperature, locomotor activity, heart rate) continuously without disturbing the animals. Having more than one measure of

circadian rhythmicity and metabolism has proven important, as this allows for identification of internal desynchrony between parameters. The use of mixed model analysis is another strength of the present experiment. This statistical method is superior to the more commonly used analysis of variance (ANOVA) for data sets like the one used in this study. Mixed model analysis takes both independent factors (groups) and repeated measures (days) into accounts, while it allows for missing data (missing data does not need to be excluded or imputed). Limitations of the current experiment include the limited sample size, particularly in some recovery groups, which may have reduced statistical power and increased risk of type II errors (false negative). Moreover, the method for analyzing circadian rhythmicity has limitations as it only captures 24h sinusoid rhythms and therefore is not suitable for detecting ultradian rhythmicity or estimating period-length in free-running conditions. In future studies, different statistical methods, such as time-series analysis, could be used to allow for more detailed analysis of circadian rhythm data.

Future perspectives

Our animal model of shift work has great potential. One of the key strengths of the model is that it is flexible. For example, future studies can combine the forced activity protocol with manipulation of light conditions to examine whether there is an additive effect of combined manipulations. Food and water availability can be manipulated, as has already been done in a similar animal model (Salgado-Delgado et al., 2010a). Furthermore, as more knowledge is gained about the lifestyles and routines of human shift workers – their activity, sleep, light exposure and eating patterns – the model can be modified to mimic these routines. The model can also be used to assess the effects of long-term shift work, repeated shift work periods, various shift work schedules and so on. Animal models are likely to become an increasingly important tool when it comes to uncovering the biological mechanisms underlying negative health effects of shift work.

For specific future studies, I suggest furthering the methodology used in the present study to examine the effects of repeated shift work periods. Since one rat month is equivalent to approximately 3 human years (Sengupta, 2013), long-term effects can be examined on a short time-scale. This opens up for the possibility to examine the effects of a whole shift working career within a relatively short period of time. Furthermore, there are individual differences in human tolerance to shift work (Saksvik et al., 2011). Although I did not see clear individual differences in the present experiment, it is possible that these will become more evident in a longer-term experiment or with a larger sample size. The animal model has the potential to aid in identifying markers for predicting individual tolerance to shift work. Additionally, the model can be used to test different shift rotations, number of rest days between shift work periods etc. to design shift work schedules that may be less detrimental to health than the ones utilized today.

Conclusion

The results of the present study show that one shift work period (four shifts) of resting phase work is sufficient to induce widespread and long-lasting circadian and metabolic disturbance to physiological functions. Disruptions include internal desynchrony both during and after shift work, between a number of parameters including body temperature rhythm, locomotor activity rhythm and sleep rhythm. The circadian disturbances – phase shift, possible emergence of ultradian rhythmicity, and internal desynchrony – were of endogenous nature and, combined with the effects of stress, are likely to have contributed to the metabolic disruptions observed. This study, combined with previous studies, has elucidated some of the factors that contribute to the development of negative health effects of shift work. The results can aid in future design of experiments on both humans and animals. Moreover, this model has the potential to be used for investigating short- and long term consequences of repeated shift work periods and different shift work rotations. It can also be used to explore individual

differences in tolerance to shift work, aid in identification of individuals who may be particularly vulnerable to shift work, and in the development of methods to counteract the negative health effects of shift work.

References

Akerstedt, T. (1998). Shift work and disturbed sleep/wakefulness. *Sleep Med Rev, 2*(2), 117-128.

Akerstedt, T., Kecklund, G., Gillberg, M., Lowden, A., & Axelsson, J. (2000). Sleepiness and days of recovery. *Transportation Research Part F: Traffic Psychology and Behaviour, 3*(4), 251-261. doi: 10.1016/s1369-8478(01)00009-2

Akerstedt, T., Kecklund, G., & Johansson, S. E. (2004). Shift work and mortality. *Chronobiol Int, 21*(6), 1055-1061.

Akhtar, R. A., Reddy, A. B., Maywood, E. S., Clayton, J. D., King, V. M., Smith, A. G., . . . Kyriacou, C. P. (2002). Circadian cycling of the mouse liver transcriptome, as revealed by cDNA microarray, is driven by the suprachiasmatic nucleus. *Curr Biol, 12*(7), 540-550.

Albrecht, U. (2012). Timing to perfection: the biology of central and peripheral circadian clocks. *Neuron, 74*(2), 246-260. doi: 10.1016/j.neuron.2012.04.006

Antunes-Rodrigues, J., De Castro, M., Elias, L. L. K., Valenca, M. M., & McCann, S. M. (2004). Neuroendocrine control of body fluid metabolism. *Physiol Rev, 84*(1), 169-208. doi: 10.1152/physrev.00017.2003

Aschoff, J. (1965). Circadian Rhythms in Man - a Self-Sustained Oscillator with an Inherent Frequency Underlies Human 24-Hour Periodicity. *Science, 148*(3676), 1427-&. doi: DOI 10.1126/science.148.3676.1427

Asher, G., & Schibler, U. (2011). Crosstalk between components of circadian and metabolic cycles in mammals. *Cell Metab, 13*(2), 125-137. doi: 10.1016/j.cmet.2011.01.006

Awazawa, M., Ueki, K., Inabe, K., Yamauchi, T., Kubota, N., Kaneko, K., . . . Kadowaki, T. (2011). Adiponectin enhances insulin sensitivity by increasing hepatic IRS-2 expression via a macrophage-derived IL-6-dependent pathway. *Cell Metab, 13*(4), 401-412. doi: 10.1016/j.cmet.2011.02.010

Banks, S., Centofanti, S. A., Hilditch, C. J., Grant, C., Coates, A., & Dorrian, J. (2015). Eating On Nightshift: A Large Vs Small Snack Impairs Glucose Response To Breakfast. *Sleep, 38,* A119.

Bansal, N. (2015). Prediabetes diagnosis and treatment: A review. *World Journal of Diabetes, 6*(2), 296. doi: 10.4239/wjd.v6.i2.296

Barclay, J. L., Husse, J., Bode, B., Naujokat, N., Meyer-Kovac, J., Schmid, S. M., . . . Oster, H. (2012). Circadian desynchrony promotes metabolic disruption in a mouse model of shiftwork. *Plos One, 7*(5), e37150. doi: 10.1371/journal.pone.0037150

Benedict, C., Hallschmid, M., Lassen, A., Mahnke, C., Schultes, B., Schioth, H. B., . . . Lange, T. (2011). Acute sleep deprivation reduces energy expenditure in healthy men. *Am J Clin Nutr, 93*(6), 1229-1236. doi: 10.3945/ajcn.110.006460

Benloucif, S., Guico, M. J., Reid, K. J., Wolfe, L. F., L'Hermite-Baleriaux, M., & Zee, P. C. (2005). Stability of melatonin and temperature as circadian phase markers and their relation to sleep times in humans. *J Biol Rhythms, 20*(2), 178-188. doi: 10.1177/0748730404273983

Berson, D. M. (2003). Strange vision: ganglion cells as circadian photoreceptors. *Trends Neurosci, 26*(6), 314-320. doi: 10.1016/S0166-2236(03)00130-9

Bracci, M., Copertaro, A., Manzella, N., Staffolani, S., Strafella, E., Nocchi, L., . . . Santarelli, L. (2013). Influence of night-shift and napping at work on urinary melatonin, 17-beta-estradiol and clock gene expression in pre-menopausal nurses. *Journal of biological regulators and homeostatic agents, 27*(1), 267-274.

Bracci, M., Manzella, N., Copertaro, A., Staffolani, S., Strafella, E., Barbaresi, M., . . . Santarelli, L. (2014). Rotating-shift nurses after a day off: peripheral clock gene expression, urinary melatonin, and serum 17-beta-estradiol levels. *Scand J Work Environ Health, 40*(3), 295-304. doi: 10.5271/sjweh.3414

Buijs, R. M., & Kalsbeek, A. (2001). Hypothalamic integration of central and peripheral clocks. *Nat Rev Neurosci, 2*(7), 521-526. doi: 10.1038/35081582

Buijs, R. M., Salgado, R., Sabath, E., & Escobar, C. (2013). Peripheral circadian oscillators: time and food. *Prog Mol Biol Transl Sci, 119*, 83-103. doi: 10.1016/B978-0-12-396971-2.00004-X

Burch, J. B., Tom, J., Zhai, Y., Criswell, L., Leo, E., & Ogoussan, K. (2009). Shiftwork impacts and adaptation among health care workers. *Occup Med (Lond), 59*(3), 159-166. doi: 10.1093/occmed/kqp015

Bustin, S. A. (2002). Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. *Journal of Molecular Endocrinology, 29*(1), 23-39. doi: 10.1677/Jme.0.0290023

Buxton, O. M., Pavlova, M., Reid, E. W., Wang, W., Simonson, D. C., & Adler, G. K. (2010). Sleep restriction for 1 week reduces insulin sensitivity in healthy men. *Diabetes, 59*(9), 2126-2133. doi: 10.2337/db09-0699

Carandente, F. (1977). [Experimental research in the rat using chronobiologic methods simulating various work shifts of man]. *Ann Ist Super Sanita, 13*(1-2), 491-497.

Chung, S., Son, G. H., & Kim, K. (2011). Circadian rhythm of adrenal glucocorticoid: its regulation and clinical implications. *Biochim Biophys Acta, 1812*(5), 581-591. doi: 10.1016/j.bbadis.2011.02.003

Costa, G., Akerstedt, T., Nachreiner, F., Baltieri, F., Carvalhais, J., Folkard, S., . . . Silverio, J. (2004). Flexible working hours, health, and well-being in Europe: some considerations from a SALTSA project. *Chronobiol Int, 21*(6), 831-844.

Damiola, F., Le Minh, N., Preitner, N., Kornmann, B., Fleury-Olela, F., & Schibler, U. (2000). Restricted feeding uncouples circadian oscillators in peripheral tissues from the central pacemaker in the suprachiasmatic nucleus. *Genes Dev, 14*(23), 2950-2961.

Davis, S., Mirick, D. K., & Stevens, R. G. (2001). Night shift work, light at night, and risk of breast cancer. *J Natl Cancer Inst, 93*(20), 1557-1562.

de Assis, M. A., Kupek, E., Nahas, M. V., & Bellisle, F. (2003). Food intake and circadian rhythms in shift workers with a high workload. *Appetite, 40*(2), 175-183.

Depner, C. M., Stothard, E. R., & Wright, K. P., Jr. (2014). Metabolic consequences of sleep and circadian disorders. *Curr Diab Rep, 14*(7), 507. doi: 10.1007/s11892-014-0507-z

Desvergne, B., Michalik, L., & Wahli, W. (2006). Transcriptional regulation of metabolism. *Physiol Rev, 86*(2), 465-514. doi: 10.1152/physrev.00025.2005

Dibner, C., & Schibler, U. (2015). Circadian timing of metabolism in animal models and humans. *J Intern Med, 277*(5), 513-527. doi: 10.1111/joim.12347

Eastman, C. I., & Rechtschaffen, A. (1983). Circadian temperature and wake rhythms of rats exposed to prolonged continuous illumination. *Physiol Behav, 31*(4), 417-427.

Eastman, C. I., Stewart, K. T., Mahoney, M. P., Liu, L., & Fogg, L. F. (1994). Dark goggles and bright light improve circadian rhythm adaptation to night-shift work. *Sleep, 17*(6), 535-543.

European Foundation for the Improvement of Living and Working Conditions. (2007). Fourth european working conditions survey. Luxembourg.

Farias, M. M., Cuevas, A. M., & Rodriguez, F. (2011). Set-point theory and obesity. *Metab Syndr Relat Disord, 9*(2), 85-89. doi: 10.1089/met.2010.0090

Ferreira, L. R. C., Miguel, M. A. L., De Martino, M. M. F., & Menna-Barreto, L. (2013). Circadian rhythm of wrist temperature and night shift-work. *Biological Rhythm Research, 44*(5), 737-744. doi: Doi 10.1080/09291016.2012.739931

Folkard, S. (2008). Do permanent night workers show circadian adjustment? A review based on the endogenous melatonin rhythm. *Chronobiol Int, 25*(2), 215-224.

Gangwisch, J. E. (2014). Invited commentary: nighttime light exposure as a risk factor for obesity through disruption of circadian and circannual rhythms. *Am J Epidemiol, 180*(3), 251-253. doi: 10.1093/aje/kwu119

Gleeson, M. (1998). Temperature regulation during exercise. *Int J Sports Med, 19 Suppl 2*, S96-99. doi: 10.1055/s-2007-971967

Golombek, D. A., Casiraghi, L. P., Agostino, P. V., Paladino, N., Duhart, J. M., Plano, S. A., & Chiesa, J. J. (2013). The times they're a-changing: effects of circadian desynchronization on physiology and disease. *J Physiol Paris, 107*(4), 310-322. doi: 10.1016/j.jphysparis.2013.03.007

Groos, G., & Hendriks, J. (1982). Circadian rhythms in electrical discharge of rat suprachiasmatic neurones recorded in vitro. *Neurosci Lett, 34*(3), 283-288.

Grundy, A., Sanchez, M., Richardson, H., Tranmer, J., Borugian, M., Graham, C. H., & Aronson, K. J. (2009). Light intensity exposure, sleep duration, physical activity, and

biomarkers of melatonin among rotating shift nurses. *Chronobiol Int, 26*(7), 1443-1461. doi: 10.3109/07420520903399987

Gupta, S., & Pati, A. K. (1994). Desynchronization of circadian rhythms in a group of shift working nurses: effects of patterns of shift rotation. *J Human Ergol, 23*(2), 121-131.

Guyton, A. C., & Hall, J. E. (2000). *Textbook of Medical Physiology* (10 ed.). Philadelphia: W.B. Saunders Company.

Harris, R. B., Zhou, J., Youngblood, B. D., Rybkin, II, Smagin, G. N., & Ryan, D. H. (1998). Effect of repeated stress on body weight and body composition of rats fed low- and high-fat diets. *Am J Physiol, 275*(6 Pt 2), R1928-1938.

Heath, G., Roach, G. D., Dorrian, J., Ferguson, S. A., Darwent, D., & Sargent, C. (2012). The effect of sleep restriction on snacking behaviour during a week of simulated shiftwork. *Accid Anal Prev, 45 Suppl*, 62-67. doi: 10.1016/j.aap.2011.09.028

Hilditch, C. J., Centofanti, S. A., Dorrian, J., Van Dongen, H. P., & Banks, S. (2014). Effects of 10 min and 30 min night-time naps on sleep inertia. *J Sleep Res, 23*, 189-190.

Hill, J. O., Wyatt, H. R., & Peters, J. C. (2012). Energy balance and obesity. *Circulation, 126*(1), 126-132. doi: 10.1161/CIRCULATIONAHA.111.087213

Hood, D. A., Irrcher, I., Ljubicic, V., & Joseph, A. M. (2006). Coordination of metabolic plasticity in skeletal muscle. *Journal of Experimental Biology, 209*(12), 2265-2275. doi: 10.1242/jeb.02182

Hsieh, W. H., Escobar, C., Yugay, T., Lo, M. T., Pittman-Polletta, B., Salgado-Delgado, R., . . . Hu, K. (2014). Simulated shift work in rats perturbs multiscale regulation of locomotor activity. *J R Soc Interface, 11*(96). doi: 10.1098/rsif.2014.0318

International Agency for Research on Cancer. (2007). Painting, firefighting and shiftwork *IARC monographs on the evaluation of carcinogenic risks to humans* (Vol. 98, pp. 563-764). Lyon: World Health Organization.

Jackson, M. L., Banks, S., & Belenky, G. (2014). Investigation of the effectiveness of a split sleep schedule in sustaining sleep and maintaining performance. *Chronobiol Int, 31*(10), 1218-1230. doi: 10.3109/07420528.2014.957305

James, F. O., Cermakian, N., & Boivin, D. B. (2007). Circadian rhythms of melatonin, cortisol, and clock gene expression during simulated night shift work. *Sleep, 30*(11), 1427-1436.

Johnson, R. F., & Johnson, A. K. (1991). Drinking after osmotic challenge depends on circadian phase in rats with free-running rhythms. *Am J Physiol, 261*(2 Pt 2), R334-338.

Karlsson, B., Knutsson, A., & Lindahl, B. (2001). Is there an association between shift work and having a metabolic syndrome? results from a population based study of 27 485 people. *Occup Environ Med, 58*, 747-752.

Kataoka, N., Hioki, H., Kaneko, T., & Nakamura, K. (2014). Psychological stress activates a dorsomedial hypothalamus-medullary raphe circuit driving brown adipose tissue thermogenesis and hyperthermia. *Cell Metab, 20*(2), 346-358. doi: 10.1016/j.cmet.2014.05.018

King, D. P., & Takahashi, J. S. (2000). Molecular genetics of circadian rhythms in mammals. *Annu Rev Neurosci, 23*, 713-742. doi: 10.1146/annurev.neuro.23.1.713

Klerman, E. B. (2005). Clinical aspects of human circadian rhythms. *J Biol Rhythms, 20*(4), 375-386. doi: 10.1177/0748730405278353

Knutsson, A. (2004). Methodological aspects of shift-work research. *Chronobiol Int, 21*(6), 1037-1047.

Kohsaka, A., Laposky, A. D., Ramsey, K. M., Estrada, C., Joshu, C., Kobayashi, Y., . . . Bass, J. (2007). High-fat diet disrupts behavioral and molecular circadian rhythms in mice. *Cell Metab, 6*(5), 414-421. doi: 10.1016/j.cmet.2007.09.006

Konopka, R. J., & Benzer, S. (1971). Clock mutants of Drosophila melanogaster. *Proc Natl Acad Sci U S A, 68*(9), 2112-2116.

Konturek, P. C., Brzozowski, T., & Konturek, S. J. (2011). Gut clock: implication of circadian rhythms in the gastrointestinal tract. *J Physiol Pharmacol, 62*(2), 139-150.

Lac, G., & Chamoux, A. (2004). Biological and psychological responses to two rapid shiftwork schedules. *Ergonomics, 47*(12), 1339-1349. doi: 10.1080/00140130410001724237

Lack, L., Bailey, M., Lovato, N., & Wright, H. (2009). Chronotype differences in circadian rhythms of temperature, melatonin, and sleepiness as measured in a modified constant routine protocol. *Nature and Science of Sleep, 1*, 1-8.

Lee, C., Smith, M. R., & Eastman, C. I. (2006). A compromise phase position for permanent night shift workers: circadian phase after two night shifts with scheduled sleep and light/dark exposure. *Chronobiol Int, 23*(4), 859-875. doi: 10.1080/07420520600827160

Leenaars, C. H., Kalsbeek, A., Hanegraaf, M. A., Foppen, E., Joosten, R. N., Post, G., . . . van Someren, E. J. (2012). Unaltered instrumental learning and attenuated body-weight gain in rats during non-rotating simulated shiftwork. *Chronobiol Int, 29*(3), 344-355. doi: 10.3109/07420528.2011.654018

Levine, J. A. (2005). Measurement of energy expenditure. *Public Health Nutr, 8*(7A), 1123-1132.

Lockley, S. W., Skene, D. J., James, K., Thapan, K., Wright, J., & Arendt, J. (2000). Melatonin administration can entrain the free-running circadian system of blind subjects. *Journal of Endocrinology, 164*(1), R1-R6. doi: DOI 10.1677/joe.0.164R001

Lowden, A., Moreno, C., Holmback, U., Lennernas, M., & Tucker, P. (2010). Eating and shift work - effects on habits, metabolism and performance. *Scand J Work Environ Health, 36*(2), 150-162.

Mantele, S., Otway, D. T., Middleton, B., Bretschneider, S., Wright, J., Robertson, M. D., . . . Johnston, J. D. (2012). Daily rhythms of plasma melatonin, but not plasma leptin or leptin mRNA, vary between lean, obese and type 2 diabetic men. *Plos One, 7*(5), e37123. doi: 10.1371/journal.pone.0037123

Marcheva, B., Ramsey, K. M., Buhr, E. D., Kobayashi, Y., Su, H., Ko, C. H., . . . Bass, J. (2010). Disruption of the clock components CLOCK and BMAL1 leads to hypoinsulinaemia and diabetes. *Nature, 466*(7306), 627-631. doi: 10.1038/nature09253

Maury, E., Hong, H. K., & Bass, J. (2014). Circadian disruption in the pathogenesis of metabolic syndrome. *Diabetes Metab, 40*(5), 338-346. doi: 10.1016/j.diabet.2013.12.005

McCarthy, J. J., Andrews, J. L., McDearmon, E. L., Campbell, K. S., Barber, B. K., Miller, B. H., . . . Esser, K. A. (2007). Identification of the circadian transcriptome in adult mouse skeletal muscle. *Physiol Genomics, 31*(1), 86-95. doi: 10.1152/physiolgenomics.00066.2007

McGowan, N., & Coogan, A. (2013). Circadian and behavioural responses to shift work-like schedules of light/dark in the mouse. *Journal of Molecular Psychiatry, 1*(1), 7.

McHill, A. W., Melanson, E. L., Higgins, J., Connick, E., Moehlman, T. M., Stothard, E. R., & Wright, K. P., Jr. (2014). Impact of circadian misalignment on energy metabolism during simulated nightshift work. *Proc Natl Acad Sci U S A, 111*(48), 17302-17307. doi: 10.1073/pnas.1412021111

Mohawk, J. A., Green, C. B., & Takahashi, J. S. (2012). Central and peripheral circadian clocks in mammals. *Annu Rev Neurosci, 35*, 445-462. doi: 10.1146/annurev-neuro-060909-153128

Monk, T. H., Buysse, D. J., Billy, B. D., Fletcher, M. E., Kennedy, K. S., Begley, A. E., . . . Beach, S. R. (2013). Shiftworkers report worse sleep than day workers, even in retirement. *J Sleep Res, 22*(2), 201-208. doi: 10.1111/jsr.12003

Moscardo, E., & Rostello, C. (2010). An integrated system for video and telemetric electroencephalographic recording to measure behavioural and physiological parameters. *J Pharmacol Toxicol Methods, 62*(1), 64-71. doi: 10.1016/j.vascn.2010.04.004

Mrdalj, J., Lundegaard Mattson, A., Murison, R., Konow Jellestad, F., Milde, A. M., Pallesen, S., . . . Gronli, J. (2014). Hypothermia after chronic mild stress exposure in rats with a history of postnatal maternal separations. *Chronobiol Int, 31*(2), 252-264. doi: 10.3109/07420528.2013.846351

Mrosovsky, N. (1996). Locomotor activity and non-photic influences on circadian clocks. *Biol Rev Camb Philos Soc, 71*(3), 343-372. doi: 10.1111/j.1469-185X.1996.tb01278.x

Nasello, A. G., Machado, C., Bastos, J. F., & Felicio, L. F. (1998). Sudden darkness induces a high activity-low anxiety state in male and female rats. *Physiol Behav, 63*(3), 451-454. doi: 10.1016/S0031-9384(97)00462-9

Nestler, E. J., & Hyman, S. E. (2010). Animal models of neuropsychiatric disorders. *Nat Neurosci, 13*(10), 1161-1169. doi: 10.1038/nn.2647

Oosterman, J. E., Kalsbeek, A., la Fleur, S. E., & Belsham, D. D. (2015). Impact of nutrients on circadian rhythmicity. *Am J Physiol Regul Integr Comp Physiol, 308*(5), R337-R350. doi: 10.1152/ajpregu.00322.2014

Opperhuizen, A. L., van Kerkhof, L. W., Proper, K. I., Rodenburg, W., & Kalsbeek, A. (2015). Rodent models to study the metabolic effects of shiftwork in humans. *Front Pharmacol, 6*, 50. doi: 10.3389/fphar.2015.00050

Oyane, N. M., Pallesen, S., Moen, B. E., Akerstedt, T., & Bjorvatn, B. (2013). Associations between night work and anxiety, depression, insomnia, sleepiness and fatigue in a sample of Norwegian nurses. *Plos One, 8*(8), e70228. doi: 10.1371/journal.pone.0070228

Pandi-Perumal, S. R., Smits, M., Spence, W., Srinivasan, V., Cardinali, D. P., Lowe, A. D., & Kayumov, L. (2007). Dim light melatonin onset (DLMO): a tool for the analysis of circadian phase in human sleep and chronobiological disorders. *Prog Neuropsychopharmacol Biol Psychiatry, 31*(1), 1-11. doi: 10.1016/j.pnpbp.2006.06.020

Pedersen, T. (2015). *Night work and disturbances in sleep - an animal model.* (Master thesis), University of Bergen, Bergen.

Pirke, K. M., Broocks, A., Wilckens, T., Marquard, R., & Schweiger, U. (1993). Starvation-induced hyperactivity in the rat: the role of endocrine and neurotransmitter changes. *Neurosci Biobehav Rev, 17*(3), 287-294.

Ralph, M. R., Foster, R. G., Davis, F. C., & Menaker, M. (1990). Transplanted suprachiasmatic nucleus determines circadian period. *Science, 247*(4945), 975-978. doi: 10.1126/science.2305266

Reddy, A. B., Karp, N. A., Maywood, E. S., Sage, E. A., Deery, M., O'Neill, J. S., . . . Hastings, M. H. (2006). Circadian orchestration of the hepatic proteome. *Curr Biol, 16*(11), 1107-1115. doi: 10.1016/j.cub.2006.04.026

Reddy, A. B., & O'Neill, J. S. (2010). Healthy clocks, healthy body, healthy mind. *Trends Cell Biol, 20*(1), 36-44. doi: 10.1016/j.tcb.2009.10.005

Refinetti, R. (2010). The circadian rhythm of body temperature. *Front Biosci (Landmark Ed), 15*, 564-594.

Reppert, S. M., & Weaver, D. R. (2002). Coordination of circadian timing in mammals. *Nature, 418*(6901), 935-941. doi: 10.1038/nature00965

Reutrakul, S., & Van Cauter, E. (2014). Interactions between sleep, circadian function, and glucose metabolism: implications for risk and severity of diabetes. *Ann N Y Acad Sci, 1311*, 151-173. doi: 10.1111/nyas.12355

Reynolds, A. C., Dorrian, J., Liu, P. Y., Van Dongen, H. P., Wittert, G. A., Harmer, L. J., & Banks, S. (2012). Impact of five nights of sleep restriction on glucose metabolism,

leptin and testosterone in young adult men. *Plos One, 7*(7), e41218. doi: 10.1371/journal.pone.0041218

Ribeiro, D. C., Hampton, S. M., Morgan, L., Deacon, S., & Arendt, J. (1998). Altered postprandial hormone and metabolic responses in a simulated shift work environment. *J Endocrinol, 158*(3), 305-310.

Rodnick, K. J., Reaven, G. M., Haskell, W. L., Sims, C. R., & Mondon, C. E. (1989). Variations in running activity and enzymatic adaptations in voluntary running rats. *J Appl Physiol (1985), 66*(3), 1250-1257.

Romanovsky, A. A. (2007). Thermoregulation: some concepts have changed. Functional architecture of the thermoregulatory system. *Am J Physiol Regul Integr Comp Physiol, 292*(1), R37-46. doi: 10.1152/ajpregu.00668.2006

Rosenwasser, A. M., Boulos, Z., & Terman, M. (1981). Circadian organization of food intake and meal patterns in the rat. *Physiol Behav, 27*(1), 33-39.

Sack, R. L., Blood, M. L., & Lewy, A. J. (1992). Melatonin rhythms in night shift workers. *Sleep, 15*(5), 434-441.

Sadacca, L. A., Lamia, K. A., deLemos, A. S., Blum, B., & Weitz, C. J. (2011). An intrinsic circadian clock of the pancreas is required for normal insulin release and glucose homeostasis in mice. *Diabetologia, 54*(1), 120-124. doi: 10.1007/s00125-010-1920-8

Saksvik, I. B., Bjorvatn, B., Hetland, H., Sandal, G. M., & Pallesen, S. (2011). Individual differences in tolerance to shift work--a systematic review. *Sleep Med Rev, 15*(4), 221-235. doi: 10.1016/j.smrv.2010.07.002

Salgado-Delgado, R., Angeles-Castellanos, M., Buijs, R. M., & Escobar, C. (2008). Internal desynchronization in a model of night-work by forced activity in rats. *Neuroscience, 154*(3), 922-931. doi: 10.1016/j.neuroscience.2008.03.066

Salgado-Delgado, R., Angeles-Castellanos, M., Saderi, N., Buijs, R. M., & Escobar, C. (2010a). Food intake during the normal activity phase prevents obesity and circadian desynchrony in a rat model of night work. *Endocrinology, 151*(3), 1019-1029. doi: 10.1210/en.2009-0864

Salgado-Delgado, R., Nadia, S., Angeles-Castellanos, M., Buijs, R. M., & Escobar, C. (2010b). In a rat model of night work, activity during the normal resting phase produces desynchrony in the hypothalamus. *J Biol Rhythms, 25*(6), 421-431. doi: 10.1177/0748730410383403

Salgado-Delgado, R., Saderi, N., Basualdo Mdel, C., Guerrero-Vargas, N. N., Escobar, C., & Buijs, R. M. (2013). Shift work or food intake during the rest phase promotes metabolic disruption and desynchrony of liver genes in male rats. *Plos One, 8*(4), e60052. doi: 10.1371/journal.pone.0060052

Sato, T., & Kawamura, H. (1984). Effects of Bilateral Suprachiasmatic Nucleus Lesions on the Circadian-Rhythms in a Diurnal Rodent, the Siberian Chipmunk (Eutamias-

Sibiricus). *Journal of Comparative Physiology, 155*(6), 745-752. doi: Doi 10.1007/Bf00611591

Schernhammer, E. S., Laden, F., Speizer, F. E., Willett, W. C., Hunter, D. J., Kawachi, I., & Colditz, G. A. (2001). Rotating night shifts and risk of breast cancer in women participating in the nurses' health study. *J Natl Cancer Inst, 93*(20), 1563-1568. doi: 10.1093/jnci/93.20.1563

Sengupta, P. (2013). The Laboratory Rat: Relating Its Age With Human's. *Int J Prev Med, 4*(6), 624-630.

Shibli-Rahhal, A., Van Beek, M., & Schlechte, J. A. (2006). Cushing's syndrome. *Clin Dermatol, 24*(4), 260-265. doi: 10.1016/j.clindermatol.2006.04.012

Simon, C., Weibel, L., & Brandenberger, G. (2000). Twenty-four-hour rhythms of plasma glucose and insulin secretion rate in regular night workers. *Am J Physiol Endocrinol Metab, 278*(3), E413-420.

Siyamak, A. Y., & Macdonald, I. A. (1992). Sub-acute underfeeding but not acute starvation reduces noradrenaline induced thermogenesis in the conscious rat. *Int J Obes Relat Metab Disord, 16*(2), 113-117.

Smith, L., & Mason, C. (2001). Age and the subjective experience of shiftwork. *J Hum Ergol (Tokyo), 30*(1-2), 307-313.

Spiegel, K., Tasali, E., Penev, P., & Van Cauter, E. (2004). Brief communication: Sleep curtailment in healthy young men is associated with decreased leptin levels, elevated ghrelin levels, and increased hunger and appetite. *Annals of Internal Medicine, 141*(11), 846-850. doi: 10.7326/0003-4819-141-11-200412070-00008

Taniguchi, C. M., Ueki, K., & Kahn, R. (2005). Complementary roles of IRS-1 and IRS-2 in the hepatic regulation of metabolism. *J Clin Invest, 115*(3), 718-727. doi: 10.1172/JCI23187

Titchenal, C. A. (1988). Exercise and food intake. What is the relationship? *Sports Med, 6*(3), 135-145.

Tucker, P., Folkard, S., Ansiau, D., & Marquie, J. C. (2011). The effects of age and shiftwork on perceived sleep problems: results from the VISAT combined longitudinal and cross-sectional study. *J Occup Environ Med, 53*(7), 794-798. doi: 10.1097/JOM.0b013e318221c64c

Valeur, J. (2007). Sult og metthet - en biologisk forklaringsmodell. *Tidsskr Nor Lægeforen, 127*(19), 2546-2548.

Vegiopoulos, A., & Herzig, S. (2007). Glucocorticoids, metabolism and metabolic diseases. *Mol Cell Endocrinol, 275*(1-2), 43-61. doi: 10.1016/j.mce.2007.05.015

Weibel, L., & Brandenberger, G. (1998). Disturbances in hormonal profiles of night workers during their usual sleep and work times. *J Biol Rhythms, 13*(3), 202-208.

Weitzman, E. D., Czeisler, C. A., Coleman, R. M., Spielman, A. J., Zimmerman, J. C., Dement, W., . . . Pollak, C. P. (1981). Delayed sleep phase syndrome. A chronobiological disorder with sleep-onset insomnia. *Arch Gen Psychiatry, 38*(7), 737-746. doi: 10.1001/archpsyc.1981.01780320017001

Welsh, D. K., Yoo, S. H., Liu, A. C., Takahashi, J. S., & Kay, S. A. (2004). Bioluminescence imaging of individual fibroblasts reveals persistent, independently phased circadian rhythms of clock gene expression. *Curr Biol, 14*(24), 2289-2295. doi: 10.1016/j.cub.2004.11.057

Whishaw, I. Q., & Kolb, B. (2004). *The behavior of the laboratory rat: A handbook with tests*. Oxford: Oxford University Press.

Willner, P. (1984). The validity of animal models of depression. *Psychopharmacology (Berl), 83*(1), 1-16.

Wyse, C. A., Biello, S. M., & Gill, J. M. (2014). The bright-nights and dim-days of the urban photoperiod: implications for circadian rhythmicity, metabolism and obesity. *Ann Med, 46*(5), 253-263. doi: 10.3109/07853890.2014.913422

Yamazaki, S., Numano, R., Abe, M., Hida, A., Takahashi, R., Ueda, M., . . . Tei, H. (2000). Resetting central and peripheral circadian oscillators in transgenic rats. *Science, 288*(5466), 682-685.

Zuther, P., Gorbey, S., & Lemmer, B. (2009). Chronos-Fit 1.05, http://www.ma.uni-heidelberg.de/inst/phar/lehre/chrono.html.

Zvonic, S., Ptitsyn, A. A., Conrad, S. A., Scott, L. K., Floyd, Z. E., Kilroy, G., . . . Gimble, J. M. (2006). Characterization of peripheral circadian clocks in adipose tissues. *Diabetes, 55*(4), 962-970.

Appendix

Appendix A – Baseline analyses

Table I

Results from statistical analyses on food intake, water intake and body weight (BW) during baseline.

		t	df	p
4d	BW	-1.22	31	NS
24h	Food	1.23	22	NS
	Water	1.76	32	NS
	BW	.09	32	NS

For detail on statistical tests, see the methods section

Table II

Results from initial statistical analyses on locomotor activity parameters during baseline (baseline days 1 to 4).

	AW (effect of day)			RW (effect of day)			Between groups (effect of group)			Interaction effect (day x group)		
	F	df	p	F	df	p	F	df	p	F	df	p
Mean	2.14	1,13	NS	2.23	1,24	NS	0.72	1,38	NS	4.0	1,38	NS
Resting mean	2.04	1,13	NS	3.04	1,24	NS	0.01	1,38	NS	1.58	1,38	NS
Active mean	0.46	1,13	NS	1.22	1,24	NS	1.16	1,38	NS	2.98	1,38	NS
Nadir	0.01	1,13	NS	0.22	1,24	NS	1.68	1,38	NS	0.16	1,38	NS
Acrophase	2.13	1,13	NS	0.01	1,24	NS	0.93	1,38	NS	0.09	1,38	NS
Amplitude	1.35	1,13	NS	1.94	1,24	NS	1.06	1,38	NS	1.68	1,38	NS
Rhythm	1.04	1,13	NS	2.30	1,24	NS	0.87	1,38	NS	1.23	1,38	NS

AW active phase workers

RW resting phase workers

For details on statistical tests, see the methods section

Table III

Results from initial statistical analyses on body temperature parameters during baseline (Baseline days 1 to 4).

	AW (effect of day)			RW (effect of day)			Between groups (effect of group)			Interaction effect (day x group)		
	F	df	p	F	df	p	F	df	p	F	df	p
Mean	1.18	1,15	NS	0.08	1,23	NS	1.84	1,38	NS	2.91	1,38	NS
Resting mean	2.17	1,15	NS	1.73	1,23	NS	3.42	1,38	NS	4.21	1,38	NS
Active mean	3.07	1,15	NS	1.57	1,23	NS	3.39	1,38	NS	3.0	1,38	NS
Nadir	0.43	1,15	NS	0.57	1,23	NS	0.06	1,38	NS	0.01	1,38	NS
Acrophase	3.49	1,15	NS	2.02	1,23	NS	**4.32**	**1,38**	**.04**	2.10	1,38	NS
Amplitude	0.69	1,15	NS	1.96	1,23	NS	2.06	1,38	NS	2.06	1,38	NS
Rhythm	0.01	1,15	NS	2.05	1,23	NS	0.39	1,38	NS	0.39	1,38	NS

AW active phase workers
RW resting phase workers
For details on statistical tests, see the methods section

Table IV

Results from final statistical analyses on locomotor activity parameters during baseline (baseline days 2 to 4).

	AW (effect of day)			RW (effect of day)			Between groups (effect of group)			Interaction effect (day x group)		
	F	df	p	F	df	p	F	df	p	F	df	p
Mean	1.91	1,15	NS	0.04	1,23	NS	0.98	1,38	NS	2.57	1,38	NS
Resting mean	2.33	1,15	NS	2.04	1,23	NS	0.01	1,38	NS	0.49	1,38	NS
Active mean	1.21	1,15	NS	1.45	1,23	NS	1.66	1,38	NS	2.52	1,38	NS
Nadir	0.45	1,15	NS	0.40	1,23	NS	0.75	1,38	NS	0.16	1,38	NS
Acrophase	0.49	1,15	NS	0.70	1,23	NS	0.22	1,38	NS	0.06	1,38	NS
Amplitude	1.44	1,15	NS	0.89	1,23	NS	0.62	1,38	NS	0.94	1,38	NS
Rhythm	0.85	1,15	NS	1.13	1,23	NS	0.96	1,38	NS	1.06	1,38	NS

AW active phase workers
RW resting phase workers
For details on statistical tests, see the methods section

Table V

Results from final statistical analyses on body temperature parameters during baseline (baseline days 2 to 4).

	AW (effect of day)			RW (effect of day)			Between groups (effect of group)			Interaction effect (day x group)		
	F	df	p	F	df	p	F	df	p	F	df	p
Mean	1.94	1,15	NS	1.55	1,23	NS	2.70	1,38	NS	2.80	1,38	NS
Resting mean	0.82	1,15	NS	0.09	1,23	NS	2.74	1,38	NS	0.38	1,38	NS
Active mean	1.60	1,15	NS	1.23	1,23	NS	2.40	1,38	NS	1.66	1,38	NS
Nadir	2.20	1,15	NS	0.70	1,23	NS	0.46	1,38	NS	0.20	1,38	NS
Acrophase	1.61	1,15	NS	1.27	1,23	NS	2.38	1,38	NS	0.67	1,38	NS
Amplitude	1.53	1,15	NS	1.20	1,23	NS	0.13	1,38	NS	0.04	1,38	NS
Rhythm	0.02	1,15	NS	0.06	1,23	NS	0.16	1,38	NS	0.01	1,38	NS

AW active phase workers
RW resting phase workers
For details on statistical tests, see the methods section

Appendix B – One shift work period analyses

Table VI

Results from statistical analyses on food intake, water intake and body weight (BW) during one shift work period.

		AW (effect of day)			RW (effect of day)			Between groups (effect of group)			Interaction effect (day x group)		
		F	df	p	F	df	p	F	df	p	F	df	p
24h	Food	**11.57**	**1,13**	**.005**	**7.55**	**1,19**	**.01**	0.12	1,32	NS	0.13	1,32	NS
	Water	4.16	1,13	NS	**11.61**	**1,19**	**.003**	<0.01	1,32	NS	0.32	1,32	NS
	BW	**8.43**	**1,13**	**.01**	**36.78**	**1,19**	**<.001**	**7.77**	**1,32**	**.009**	0.5	1,32	NS
8h	Food	**5.51**	**1,13**	**.04**	**12.62**	**1,19**	**.002**	0.26	1,31	NS	**7.5**	**1,31**	**.01**
	Water	**57.58**	**1,13**	**<.001**	**47.35**	**1,19**	**<.001**	**6.01**	**1,31**	**.02**	2.26	1,31	NS
	BW	**54.66**	**1,13**	**<.001**	**61.92**	**1,19**	**<.001**	**9.46**	**1,31**	**.004**	3.9	1,31	NS
16h	Food	0.01	1,13	NS	**19.90**	**1,19**	**<.001**	3.27	1,29	NS	0.45	1,29	NS
	Water	**66.72**	**1,13**	**<.001**	1.16	1,19	NS	**14.8**	**1,29**	**<.001**	0.27	1,29	NS
	BW	**47.34**	**1,13**	**<.001**	**18.53**	**1,19**	**<.001**	**38.02**	**1,29**	**<.001**	**5.46**	**1,29**	**.03**

AW active phase workers
RW resting phase workers
For details on statistical tests, see the methods section

Table VII

Results from statistical analyses on locomotor activity parameters during one shift work period.

	AW (effect of day)			RW (effect of day)			Between groups (effect of group)			Interaction effect (day x group)		
	F	df	p	F	df	p	F	df	p	F	df	p
Mean	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Resting mean	**4.08**	**1,15**	**.05**	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Active mean	N/A	N/A	N/A	**140.79**	**1,24**	**<.001**	N/A	N/A	N/A	N/A	N/A	N/A
Nadir	3.12	1,15	NS	**6.51**	**1,24**	**<.001**	3.24	1,39	NS	0.61	1,39	NS
Acrophase	3.46	1,15	NS	**94.65**	**1,24**	**<.001**	**59.49**	**1,39**	**<.001**	0.44	1,39	NS
Amplitude	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Rhythm	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

AW active phase workers

RW resting phase workers

For details on statistical tests, see the methods section

Table VIII

Results from statistical analyses on body temperature parameters during one shift work period.

	AW (effect of day)			RW (effect of day)			Between groups (effect of group)			Interaction effect (day x group)		
	F	df	p	F	df	p	F	df	p	F	df	p
Mean	1.24	1,13	NS	**13.02**	**1,19**	**.002**	0.01	1,35	NS	2.05	1,35	NS
Resting mean	**7.42**	**1,13**	**.02**	**33.49**	**1,19**	**<.001**	0.02	1,35	NS	**5.73**	**1,35**	**.02**
Active mean	0.68	1,13	NS	**13.02**	**1,19**	**.002**	1.37	1,35	NS	**7.72**	**1,35**	**.001**
Nadir	0.5	1,13	NS	**19.38**	**1,19**	**<.001**	0.86	1,36	NS	0.40	1,36	NS
Acrophase	0.11	1,13	NS	**40.66**	**1,19**	**<.001**	0.07	1,36	NS	**11.56**	**1,36**	**.002**
Amplitude	1.24	1,13	NS	**26.21**	**1,19**	**<.001**	0.01	1,36	NS	**7.24**	**1,36**	**.01**
Rhythm	1.0	1,13	NS	**77.19**	**1,19**	**<.001**	0.40	1,36	NS	**9.82**	**1,36**	**.003**

AW active phase workers

RW resting phase workers

For details on statistical tests, see the methods section

Appendix C – Recovery period analyses

Table IX

Results from statistical analyses on body weight (BW) during the recovery period.

	AWLD (effect of day)			AWDD (effect of day)			RWLD (effect of day)			RWDD (effect of day)			Between groups (effect of group)			Interaction effect (day x group)		
	F	df	p	F	df	p	F	df	p	F	df	p	F	df	p	F	df	p
BW	**28.34**	**1,8**	**<.001**	2,63	1,4	NS	**40.73**	**1,9**	**<.001**	**37.56**	**1,9**	**<.001**	1.22	3,32	NS	0.26	3,32	NS

AWLD active phase workers in LD recovery condition
AWDD active phase workers in DD recovery condition
RWLD resting phase workers in LD recovery condition
RWDD resting phase workers in DD recovery condition
For details on statistical tests, see the methods section

Table X

Results from statistical analyses on body temperature parameters during the recovery period.

	AWLD (effect of day)			AWDD (effect of day)			RWLD (effect of day)			RWDD (effect of day)			Between groups (effect of group)			Interaction effect (day x group)		
	F	df	p	F	df	p	F	df	p	F	df	p	F	df	p	F	df	p
Mean	1.09	1,9	NS	0.01	1,3	NS	1.6	1,9	NS	1.29	1,8	NS	0.7	3,35	NS	1.14	3,35	NS
Resting mean	3.06	1,9	NS	0.01	1,3	NS	**45.33**	**1,9**	**<.001**	1.16	1,8	NS	2.41	3,35	NS	1.44	3,35	NS
Active mean	2.09	1,9	NS	0.002	1,3	NS	1.24	1,9	NS	1.54	1,8	NS	1.12	3,35	NS	0.71	3,35	NS
Nadir	0.03	1,10	NS	2.41	1,4	NS	1.18	1,13	NS	3.51	1,8	NS	0.62	3,34	NS	0.77	3,34	NS
Acrophase	1.99	1,10	NS	0.01	1,4	NS	0.02	1,13	NS	0.01	1,8	NS	1.55	3,34	NS	1.60	3,34	NS
Amplitude	0.64	1,10	NS	**8.9**	**1,4**	**<.001**	**49.45**	**1,13**	**<.001**	**7.97**	**1,8**	**.02**	**4.59**	**3,34**	**.009**	**3.65**	**3,34**	**.02**
Rhythm	0.06	1,10	NS	0.37	1,4	NS	**25.0**	**1,13**	**<.001**	**6.92**	**1,8**	**.03**	**7.08**	**3,34**	**<.001**	**6.02**	**3,34**	**.002**

AWLD active phase workers in LD recovery condition
AWDD active phase workers in DD recovery condition
RWLD resting phase workers in LD recovery condition
RWDD resting phase workers in DD recovery condition
For details on statistical tests, see the methods section

Table XI

Results from statistical analyses on locomotor activity parameters during the recovery period.

	AWLD (effect of day)			AWDD (effect of day)			RWLD (effect of day)			RWDD (effect of day)			Between groups (effect of group)			Interaction effect (day x group)		
	F	df	p	F	df	p	F	df	p	F	df	p	F	df	p	F	df	p
Mean	4.10	1,9	NS	0.23	1,4	NS	0.45	1,13	NS	0.24	1,9	NS	**4.56**	**3,35**	**.01**	0.67	3,35	NS
Resting mean	0.05	1,9	NS	0.38	1,4	NS	0.44	1,13	NS	0.33	1,9	NS	**2.98**	**3,35**	**.04**	0.22	3,35	NS
Active mean	1.34	1,9	NS	0.14	1,4	NS	0.31	1,13	NS	0.11	1,9	NS	**3.90**	**3,35**	**.02**	0.83	3,35	NS
Nadir	1.9	1,10	NS	0.08	1,4	NS	**6.33**	**1,14**	**.03**	4.36	1,9	NS	1.82	3,34	NS	1.61	3,34	NS
Acrophase	0.96	1,10	NS	1.54	1,4	NS	2.15	1,14	NS	0.19	1,9	NS	1.08	3,34	NS	1.35	3,34	NS
Amplitude	1.12	1,10	NS	0.71	1,4	NS	0.59	1,14	NS	1.56	1,9	NS	2.13	3,34	NS	1.24	3,34	NS
Rhythm	0.73	1,10	NS	0.11	1,4	NS	**5.02**	**1,14**	**<.001**	2.36	1,9	NS	0.23	3,34	NS	0.12	3,34	NS

AWLD active phase workers in LD recovery condition
AWDD active phase workers in DD recovery condition
RWLD resting phase workers in LD recovery condition
RWDD resting phase workers in DD recovery condition
For details on statistical tests, see the methods section

Appendix D – Preliminary results on gene expression and glucocorticoid levels

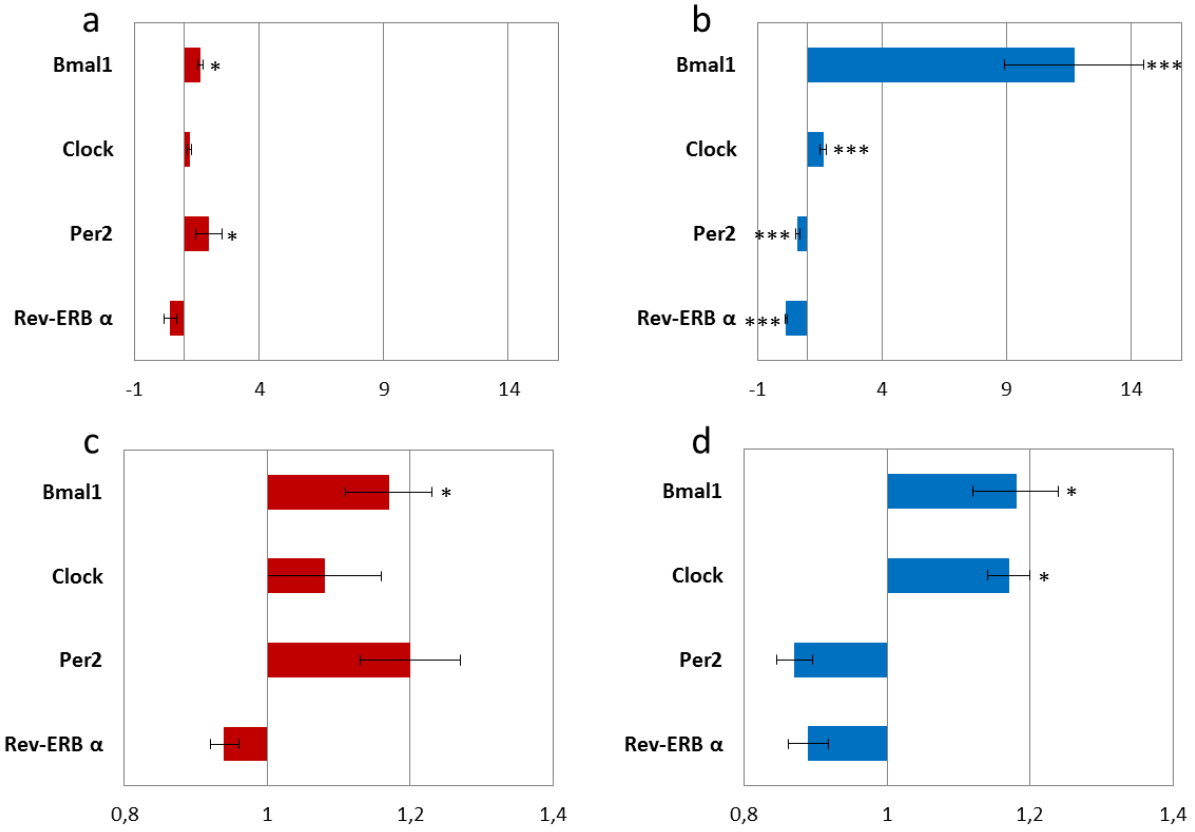

Figure I. Fold-change of **c**lock genes mRNA in active phase workers (AW; red) and resting phase workers (RW; blue) after 3 work days. a and b) peripheral tissue (liver); c and d) central tissue (hypothalamus). Bmal1 and Clock are part of the positive loop. Per2 and Rev-ERB α are part of the negative loop. Animals were euthanized 2h after end of their third work shift (AW: ZT 24 and RW; ZT 12). Error bars indicate SEM. * p<.05, *** p<.001, compared to control condition.

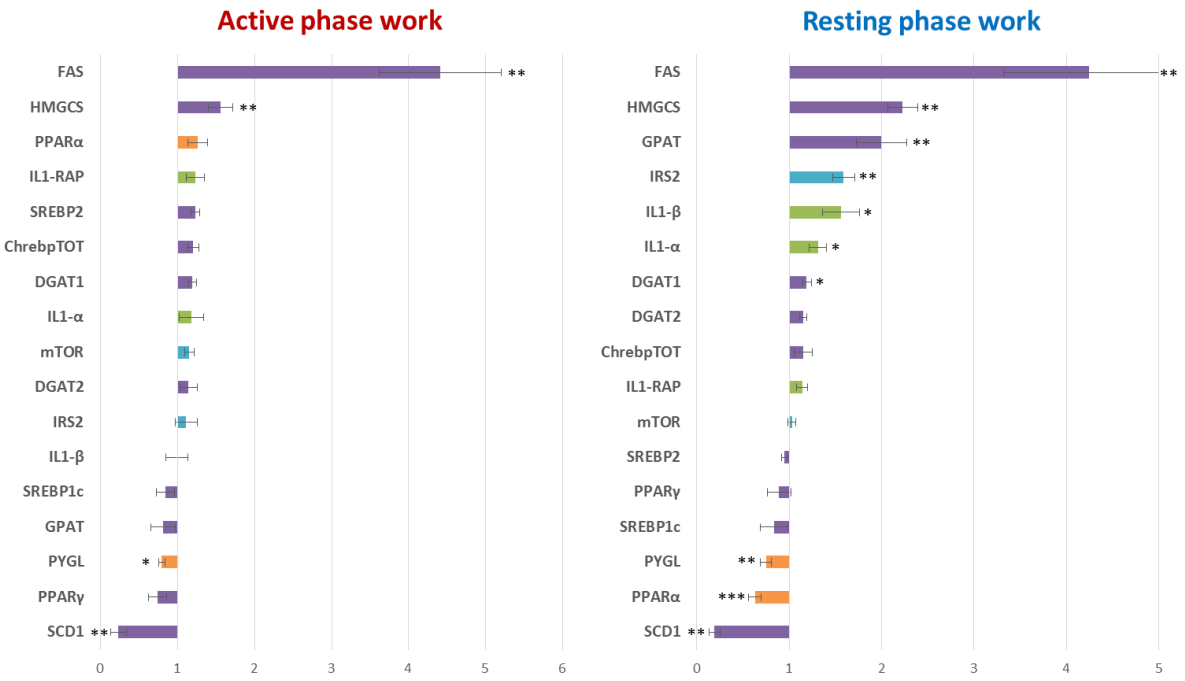

Figure II. Fold-change of metabolic genes mRNA in active phase workers (AW) and resting phase workers (RW) in liver tissue, after 3 work shifts. Colours indicate primary functional role of genes in liver: Energy storage; Energy breakdown; Insulin function; Stress and inflammation. Animals were euthanized 2h after end of their third work shift (AW: ZT 24 and RW; ZT 12). Error bars indicate SEM. * p<.05; ** p<.01; *** p<.001, compared to control condition.

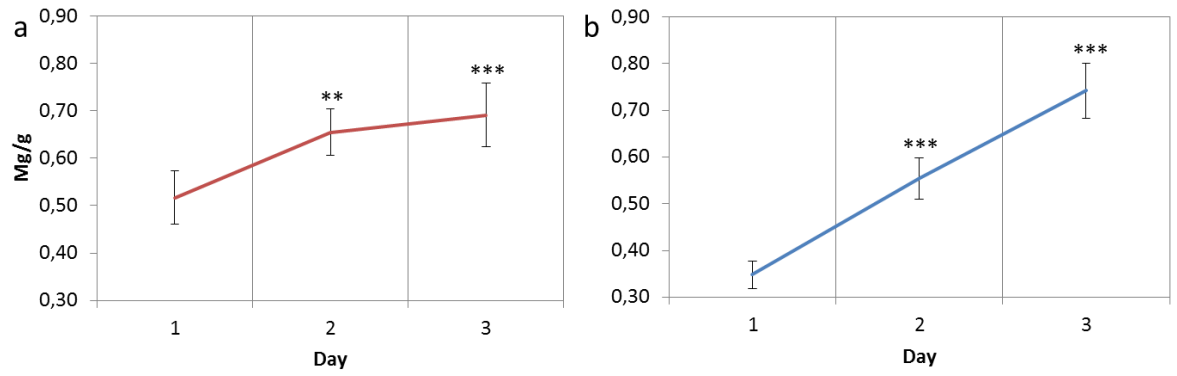

Figure III. Figure showing corticosterone levels in feces (mg/g) during work for AW and RW. a) active phase workers; b) resting phase workers. Error bars indicate SEM. W1-3 indicate work shift 1 to 3. ** p<.01; *** p<.001, compared to work shift 1.