UiT The Arctic University of Norway

ActFaculty of Bioscience, Fisheries and Economics

Department of Arctic and Marine Biology

The Suprachiasmatic Nucleus of The Reindeer (*Rangifer tarandus tarandus*) and it's Circadian Outputs

Mariel Wictoria Holmen

Master's thesis in Biology, BIO-3950, May 2023

Faculty of Biosciences, Fisheries and Economics
Department of Arctic and Marine Biology

The Suprachiasmatic Nucleus of The Reindeer (*Rangifer tarandus tarandus*) and it's Circadian Outputs

---

Mariel Wictoria Holmen

BIO-3950 Master's thesis in Biology

Arctic chronobiology and physiology

May 2023

Supervisors

Main supervisor: Dr Shona Wood, Associate professor, AMB

Co-supervisor: Dr David Hazlerigg, Professor, AMB

Co-supervisor: Dr Daniel Appenroth, ASTI post-doc, AMB

Cover photo: Mariel Wictoria Holmen

Picture of *Rangifer tarandus tarandus*

Acknowledgements

I would like to thank my incredible supervisors, associate professor Dr. Shona Wood, Professor Dr. David Hazlerigg and Dr. Daniel Appenroth for giving me this intriguing project to work on. Thank you for all the guiding, discussions, the help you have provided in the lab and during the experiments and all the useful feedback. I could not have done this without you. I would like to extend a thanks to the technicians working at the animal facility, Hans Lian, Renate Thorvaldsen and Hans Arne Solvang for all the help you have provided me in the caretaking of reindeer. I would also like to thank Magdalena, Laureen, Sara and Marte for helping me feed the reindeer at awkward hours. I would like to thank associate professor Alina Lynn Evans and senior researcher Ingebjørg Nymo for first dedicating hours and hours to make sure that the rumen loggers worked and then for inserting them in the reindeer. Thanks to all the people in the Arctic chronobiology and Physiology group for answering questions from time to time, participating in various discussion which both helped me in planning the project and gave me greater understanding of my subject and for lending me a hand when I needed it. A special thanks goes to my officemates Sona and Silje for all the long hours, discussions and laughs. Finally, my deepest gratitude goes to my partner Øystein Falkeid for being there for all the ups and downs during these past years.

Abstract

The Earth is in constant movement around the Sun – this has created environments with changing light throughout the day and year. Through evolution animals have adapted to this change in light and circadian and circannual rhythms have evolved to enhance fitness and survival. Animals living in the Arctic region have a different relationship to light changes as for several months a year the Sun is either above or below the horizon all day creating only a minimum amplitude of light change throughout the day. Consequently, circadian organization may be less important is these animals. However, there are limited research on animals in the Arctic concerning circadian rhythms.

This thesis took a closer look at the Norwegian reindeer (*Rangifer tarandus*). The reindeer suprachiasmatic nucleus (SCN) was characterized with the use of DIG in-situ hybridization and found that arginine vasopressin (AVP) and vasoactive intestinal polypeptide (VIP) are expressed in the SCN, similar to what is seen in sheep. To measure the output physiology – locomotor activity, feeding behavior, rumen temperature, heart rate and cortisol – a forced desynchrony protocol was developed. The reindeer had a weak 24-hour diurnal locomotor activity pattern when a light-dark cycle is present. In constant light this rhythm breaks down and this study did not provide any evidence of a strong circadian rhythm in locomotor activity, feeding behavior or rumen temperature. The most dominant rhythm appears to be ultradian. The limitations of the set-up and devices, and the ultradian rhythmicity in reindeer, resulted in no signs of uncoupling of locomotor activity and feeding behavior from rumen temperature. The three parameters, locomotor activity, feeding behavior and rumen temperature were correlated and this correlation was most apparent at an ultradian level, and the relationship between the parameters did not change throughout the protocol. The reindeer have strong social organization and becomes stressed by being indoors for a longer period of time.

As reindeer are ruminants living in the Arctic, both the environment and feeding demands can have contributed to a less important circadian organization.

Contents

1 Introduction ... 1
1.1 Light and biological rhythms ... 1
1.2 Circadian rhythms ... 2
1.3 Circadian organization ... 3
1.4 Suprachiasmatic nucleus ... 5
1.4.1 Afferent pathways ... 7
1.4.2 Neuropeptides ... 7
1.4.3 Efferent pathways ... 10
1.5 Output physiology ... 11
1.5.1 Glucocorticoids ... 11
1.5.2 Body temperature ... 12
1.5.3 Melatonin ... 13
1.5.4 Experimental designs to measure circadian outputs ... 14
1.6 Circadian clocks in the Arctic ... 15
1.7 Research questions and hypotheses ... 17
2 Methods ... 19
2.1 Characterization of the suprachiasmatic nucleus ... 19
2.1.1 Research animals ... 19
2.1.2 Sample preparation ... 19
2.1.3 Sectioning ... 19
2.1.4 Digoxigenin in situ hybridization ... 20
2.1.5 Nissl staining ... 22
2.1.6 Imaging & measurement ... 22
2.2 Circadian output physiology ... 23
2.2.1 Research animals ... 23
2.2.2 Experimental set-up ... 23

2.2.3 Measurements 27

2.2.4 Analyses 30

3 Results 33

3.1 The SCN of the reindeer expressed AVP and VIP 33

3.2 An alternating feeding protocol in constant darkness conditions confounds the investigation of circadian rhythms in reindeer 38

3.3 Forced desynchrony 42

3.3.1 No evidence of a circadian pattern of locomotor activity, feeding behavior and rumen temperature in forced desynchrony protocol 43

3.3.2 Ultradian rhythms are the strongest component in reindeer 48

3.3.3 The strong ultradian patterning in reindeer prevents desynchrony of internal from external rhythms 50

3.3.4 Testing for confounding factors 54

4 Discussion 63

4.1 Summary 63

4.2 AVP and VIP are expressed in reindeer 63

4.3 Could phylogeny be an explanation for the difference in SCN across animal groups? 64

4.4 Forced desynchrony – did it work? 65

4.5 A presence of a 24-hour rhythm 66

4.6 Ultradian cycles are more important in ruminants 67

4.7 The presence of a cortisol rhythm 68

4.8 Ruminant adaption to life in the Arctic 69

4.9 Notes on possible improvements 69

5 Conclusion 71

Works cited 72

Appendix 79

Appendix A: Supplementary figures for SCN characterization 79

Appendix B: Supplementary figures for pilot study 81

Appendix C: Supplementary figures for forced desynchrony 84

Appendix D: R-codes 90

List of figures

Figure 1: The rotation and orbit of the earth creates a daily and seasonal variation in the amount of light available and the tilt of the earth makes this variation most extreme at the poles……………………………………………………………………………………... 1

Figure 2: Actogram of a nocturnal animal demonstrating fundamental properties of circadian rhythmicity……………………………………………………………….…. 3

Figure 3: Eskinogram framework to understand the circadian pathway……………………. 4

Figure 4: Two transcriptional-translational feedback loops of the circadian organization... 5

Figure 5: The location and structure of the rodent SCN…………………………………… 6

Figure 6: Schematic representation of the expression pattern of neuropeptides in the rodent SCN……………………………………………………………………………………... 8

Figure 7: Expression of arginine vasopressin (AVP) and vasoactive intestinal polypeptide (VIP) rostral and caudal in the SCN of Guinea Pig, Human and Sheep…………………… 9

Figure 8: A diagram of the oscillation of the glucocorticoids cortisol and corticosterone….. 12

Figure 9: A diagram of the oscillation of body temperature for a human………………….. 13

Figure 10: A diagram of the oscillation of melatonin in rats……………………………….... 14

Figure 11: Activity rhythm of Norwegian reindeer (*Rangifer tarandus tarandus*) during short photoperiod (SP; December), long photoperiod (LP; March) and continuous light (LL; June)………………………………………………………………………………. 16

Figure 12: Reindeer room set-up…………………………………………………………….. 24

Figure 13: Forced desynchrony protocol…………………………………………………….. 25

Figure 14: Actiwatch attached to reindeer harness………………………………………….... 27

Figure 15: HOBO logger set-up to measure feeding behavior (FB)……………………….. 28

Figure 16: RumiLogger collar placed on reindeer………………………………………… 29

Figure 17: Illustration demonstrating the most rostral part of reindeer SCN……………….. 33

Figure 18: Representative pictures (reindeer 12/10) of stained reindeer SCN and the areas around it…………………………………………………................................... 35

Figure 19: Pictures of Nissl, AVP, VIP and GRP staining and the rostral caudal extent of that staining in the region of the reindeer SCN..... 36
Figure 20: Locomotor activity (LA) rhythm of reindeer (1/21) during pilot study..... 39
Figure 21: Surface body temperature (T_s) rhythm of reindeer (1/21) during pilot study..... 40
Figure 22: Circular barplot illustrating how the locomotor activity (LA) of reindeer (1/21) increased during the constant darkness in the pilot study..... 41
Figure 23: Actogram and periodograms of locomotor activity (LA) measurements from reindeer 2/22..... 44
Figure 24: Actogram and periodograms of feeding behavior (FB) from reindeer 2/22..... 45
Figure 25: Actogram and periodograms of rumen temperature (T_r) from reindeer 1/22..... 46
Figure 26: Actogram and periodograms of rumen temperature (T_r) from reindeer 2/22..... 47
Figure 27: Wavelet analysis of locomotor activity (LA), feeding behavior (FB), rumen temperature (T_r) and heart rate (HR) in the three reindeer 1/22, 2/22 and 3/22..... 51
Figure 28: Locomotor activity (LA) and feeding behavior (FB) actograms with heatmap of rumen temperature (T_r) 51
Figure 29: Cross-correlation between parameters of reindeer (2/22) during the control and forced desynchrony..... 52
Figure 30: Cross-wavelets of locomotor activity (LA), feeding behavior (FB) and rumen temperature (T_r)..... 53
Figure 31: Locomotor activity (LA) actograms from three reindeer..... 55
Figure 32: Cross-correlation of locomotor activity (LA) between reindeer..... 56
Figure 33: Wavelet analysis of ambient temperature..... 57
Figure 34: Average ambient temperature (T_a) for each hour of the days during the forced desynchrony..... 58
Figure 35: Cortisol levels in reindeer 1/22..... 59
Figure 36: Cortisol levels in reindeer 2/22..... 60
Figure 37: Cortisol levels in reindeer 3/22..... 61
Figure 38: Average heart rate (HR) (BPM) each day during the protocol (reindeer 1/22)..... 62
Figure 39: Average heart rate (HR) (BPM) each day during the protocol (reindeer 2/22)..... 62

List of tables

Table 1: Disturbance routine of the forced desynchrony part of the protocol …………….. 26

Table 2: Summary of the extent of the suprachiasmatic nucleus (SCN) and the extent of arginine vasopressin (AVP), vasoactive intestinal polypeptide (VIP) and gastrin-releasing peptide (GRP)…………………………………………………………………... 37

List of abbreviations

3V: Third ventricle

AVP: Arginine vasopressin

D1: Disturbance-1

D2: Disturbance-2

FB: Feeding behavior

GRP: Gastrin-releasing peptide

HR: Heart rate

LA: Locomotor activity

LD: Light-dark cycle

OC: Optic chiasm

PVN: Paraventricular nucleus

SCN: Suprachiasmatic nucleus

SON: Supraoptic nucleus

T_a: Ambient temperature

T_r: Rumen temperature

T_s: Surface body temperature

VIP: Vasoactive intestinal polypeptide

1 Introduction

1.1 Light and biological rhythms

All planets in the universe are in constant movement, and our planet – the Earth is no exception. The movement of the Earth relative to the Sun creates changes in light and, consequently, a temperature change. With a period of 24 hours, the Earth rotates on its axis. The rotation gives rise to light changes throughout the day, creating what we familiarly recognize as day and night. The Earth's axis is tilted 23.4^{o} relative to the sun (Figure 1a). Since the Earth uses a year to orbit around the sun, this gives rise to an even more significant change in light throughout the year and creates seasons. Due to the tilt, some parts of the planet have less light when the Earth is tilted away from the Sun (winter solstice) and more light when the Earth is tilted toward the Sun (summer solstice). The poles experience the most extreme changes in the light environment throughout the year, with the daily availably of the light (photoperiod) ranging from 24 hours in the "polar day", to 0 hours in the "polar night" (Figure 1b). However, the environment in the Arctic is not constant as there exist a daily variation in insolation, position of the Sun and temperature.

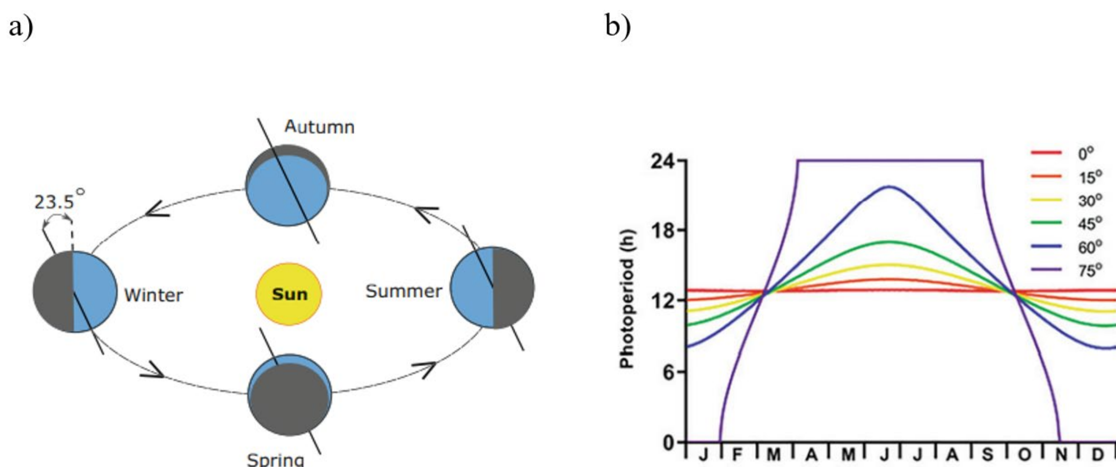

Figure 1:The rotation and orbit of the Earth creates a daily and seasonal variation in the amount of light available and the tilt of the Earth makes this variation most extreme at the poles. *a) The earth tilts 23.5^{p} on its axis. Therefore, some parts of the planet have less light (shorter days) when the Earth is tilted away from the Sun and more light (longer days) when the Earth is tilted toward the Sun. b) graph showing how the photoperiod changes during the year based on latitude. Pictures from the book "Neuroendocrine clocks and calendars" figures 2.1 and 7.2 (Ebling & Piggins, 2020).*

Animals have evolved to use the daily and seasonal changes in photoperiod to anticipate changes in their environment and adapt their physiology. This physiological optimization based on time of year or day is beneficial to health and fitness (Helm & Lincoln, 2017; Scheer et al., 2009). Organisms achieve this through synchronizing their internal rhythms with the external

light environment. These light synchronized internal or endogenous rhythms include circadian rhythms (approximately 24 hours) and-circannual rhythms (approximately 1 year) (Ebling & Piggins, 2020).

This thesis will focus on circadian rhythms, in my introduction I will review the state of knowledge on the location of the circadian clock in mammals, the inputs this receives, and the output physiology the circadian clock generates, and how to test for the presence of circadian rhythms. However, the unusual light environment of the Arctic suggests that there may not be a fitness benefit to maintaining a circadian clockwork in the polar night and day, or, that there may be differences in how the circadian rhythms are synchronized or function. Therefore, I will also introduce the main findings from the limited number of studies on circadian clocks in the Arctic.

1.2 Circadian rhythms

The term circadian is derived from Latin, circa meaning 'approximately' and dies meaning 'day.' For a rhythm to be characterized as circadian, three fundamental properties must be present (Figure 2: Edery (2000)).

1) First, the internal rhythm must free-run with a period of approximately 24 hours in constant conditions. A free running rhythm is the rhythm an organism internally has when there are no environmental cue present. As this is an internal rhythm it is not exactly 24 hours, but near enough that it can synchronize to an environmental oscillation like light. Which brings us the to the second property.
2) Environmental cues can entrain the rhythm. Because the rhythm of the animal is not exactly 24 hours, environmental cues – referred to as zeitgebers – is needed to entrain and ensure that the animal's rhythm is in sync with the environment around it. A shift in the environmental cue will create a shift in the animal's internal rhythm. This is referred to as phase-shifting. A phase advance is when the rhythm shifts backwards in time. A phase delay is when the rhythms shift forward in time. The most potent environmental cue is light and the most used in studies about biological clocks. Under a light-dark cycle 'masking' may be a confounding factor - a light-dark cycle may affect the activity of an animal, this may occur circadian, but is in fact a direct result of how the animal is affected by the zeitgeber.
3) The third fundamental property is temperature compensation. Biological reactions are chemical based, and all chemical reactions change rate with temperature; this is the Q10

effect (reviewed in: Blix (2005)). Most biochemical processes increase their factor by 2 or 3 with every 10ºC. The circadian clock, however, has compensation for this to maintain its rhythm in changing temperature, so Q10 values are approximately 1 (Ruby & Heller, 1996).

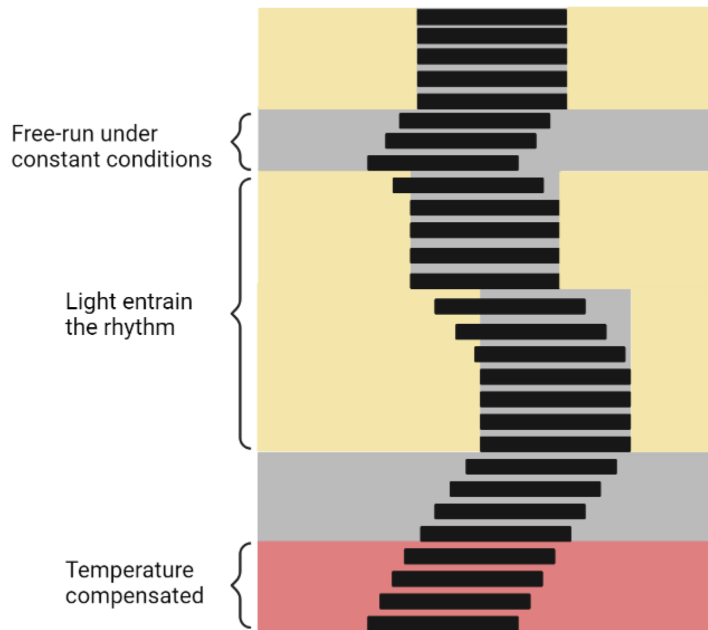

Figure 2: Actogram of a nocturnal animal demonstrating fundamental properties of circadian rhythmicity*. The top part demonstrates the animal in a light-dark cycle where the animal is active during night. When the light changes to constant darkness the internal rhythm of the animal starts to free-run, with a period less but close to 24-hours. When the lights come on again, the animal synchronize to the light conditions and therefore phase-delays its rhythm, and when the light changes again the same happens. When the conditions become constant the animal's rhythm free-runs again and when the temperature changes it maintains it internal rhythm.*

1.3 Circadian organization

The circadian system comprises three parts 1) input signals, 2) a central pacemaker, and 3) outputs (Figure 3; Edery (2000)). The input signals can come from the environment, and are used as zeitgebers, light is the strongest zeitgeber. The central pacemaker interprets the input signals and then delivers a timing signal to the peripheral tissue, which in turns coordinates output physiology.

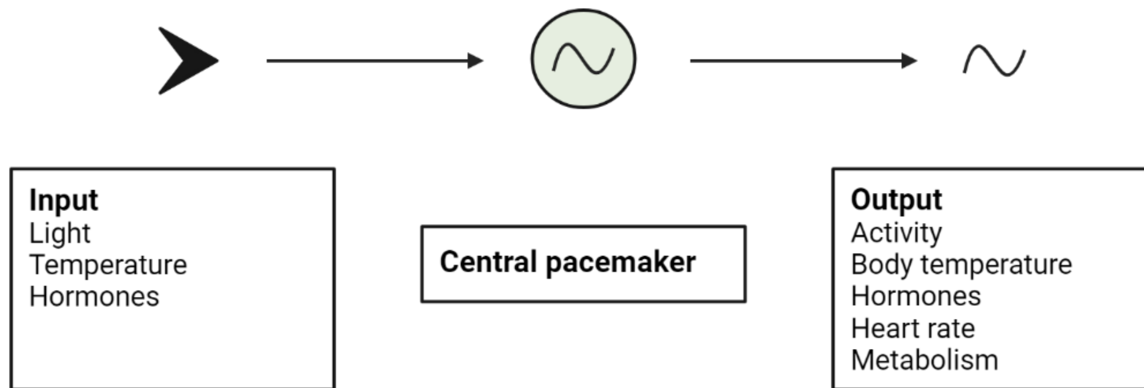

Figure 3: Eskinogram framework to understand the circadian pathway. *The circadian system receives input from the environment (for example light or temperature), or internally (for example hormones). The central pacemaker interprets the input signals and then delivers a timing signal to the peripheral tissue, which in turns coordinates output physiology like activity, body temperature, hormones, heart rate and/or metabolism.*

The clockwork in mammals is hierarchically organized with a central pacemaker and subsidiary peripheral oscillators. The central pacemaker must periodically re-entrain the peripheral tissue to prevent dampening of the molecular clock (Yamazaki et al., 2000). In mammals, the central pacemaker has neural connections to other brain areas, which may serve to entrain the tissue. However, it is likely that it also uses a multiple-signal pathway. For example, both glucocorticoids and temperature have been shown to reset the oscillation in the peripheral tissue (Balsalobre et al., 2000; Buhr et al., 2010).

The circadian system impacts many physiological functions. One of the most obvious and probably easiest outputs to observe is the activity/rest cycle, which is frequently used in studying circadian clocks. Other physiological functions that the circadian system impacts are body temperature, feeding habits, hormone levels, metabolism, and possibly many more. A standard view is that the circadian system aims to adjust homeostatic control to meet daily changes in activity and energy demand.

Every cell in the body oscillates, and the mechanism that drives the oscillation in the cells is a molecular clock. The molecular clock is similar in the central pacemaker and in the cells in the peripheral tissue (reviewed in: Ko and Takahashi (2006)). The circadian molecular clock consists of several genes that form transcription-translation feedback loops (Figure 4). The core loops consist of the genes brain and muscle ARNT-Like 1 (*Bmal1*), circadian locomotor output cycles kaput (*clock*), Cryptochrome (*Cry1, Cry2*), and period (*Per1, Per2, Per3*). In the nucleus, transcription of the genes *Bmal1* and *Clock* takes place. After translation, the proteins translocate back to the nucleus where BMAL1 and CLOCK heterodimerize and make the

transcriptional factor BMAL1:CLOCK that targets genes through binding to e-boxes and promotes transcription, including the genes *Per* and *Cry*. In the cytoplasm, PER and CRY heterodimerize. The PER:CRY heterodimers are translocated back to the nucleus and repress the activity of the BMAL1:CLOCK complex, therefore acting as a negative factor on their transcription. The heterodimers BMAL1:CLOCK is also promoting the genes *Rev-erba* and *Ror-α* through e-boxes. The proteins ROR and REV-ERB are translocated back to the nucleus and affect the transcription of *Bmal1* by competing for binding to the RORE receptor. ROR activates the transcription of *Bmal1*, whereas REV-ERB suppresses the transcription of *Bmal1*. The ~24 periodicities are generated by modification of the proteins through phosphorylation and ubiquitination. The heterodimers BMAL1:CLOCK also attach to e-boxes that promotes transcription of clock controlled genes, which subsequently leads to biological rhythmic processes. Zeitgebers affect the molecular clock. For example, light affects the molecular clock by induction of *Per1* and *Per2* expression (Pando et al., 2001) and therefore works to synchronize the molecular clock through the central pacemaker.

Figure 4: Two transcription-translation feedback loops of the circadian clockwork. *The transcriptional factor BMAL1:CLOCK binds to e-boxes and promotes transcription of the genes* Per, Cry, Rev-erba *and* Ror-α. *PER:CRY heterodimers repress the activity of the BMAL1:CLOCK complex. The proteins ROR and REV-ERB affect the transcription of* Bmal1 *by competing for binding to the RORE receptor. The proteins are degraded during the process, generating the ~24 periodicity. Figure adapted from the book "neuroendocrine clock and calendars" figure 11.1 (Ebling & Piggins, 2020).*

1.4 Suprachiasmatic nucleus

In mammals, the central pacemaker is the suprachiasmatic nucleus (SCN). The SCN was first anatomically described in the 1920s (Gurdjian, 1927), but the role of the SCN was not

recognized before the 1970s when experiments showed that a lesion of the SCN resulted in the loss of circadian corticosterone rhythm (Moore & Eichler, 1972).

The SCN was then transplanted between a tau mutant animal (i.e., an animal which have a mutant which makes its periodicity to be ≠ 24 hours) and a wild type animal (i.e., an animal which have a free-running rhythm of approximately 24 hours). The transplantation resulted in a change in circadian behavior, tau mutant animal that received the SCN of the wild type expressed a 24 hour rhythm and the wild type animal that received the tau mutant SCN expressed the rhythm of the donor (Ralph et al., 1990).

Since circadian rhythms seem to affect many biological processes, the structure and function of the SCN have been well characterized, usually with rodents as model animals (Figure 5). The SCN is located close to the third ventricle of the anterior hypothalamus and immediately above the optic chiasm (Abrahamson & Moore, 2001). The SCN consist of densely packed neurons and is a bilateral structure that consists of two nuclei (Figure 5). Each nucleus can be divided into two subregions, a ventrolateral and a dorsomedial, often referred to as core and shell respectively. The subregions differ in their anatomical and cytochemical properties. The division is very distinct in rodents, but in other mammals, the distinction is not always as clear (Cassone et al., 1988; Dardente et al., 2016)

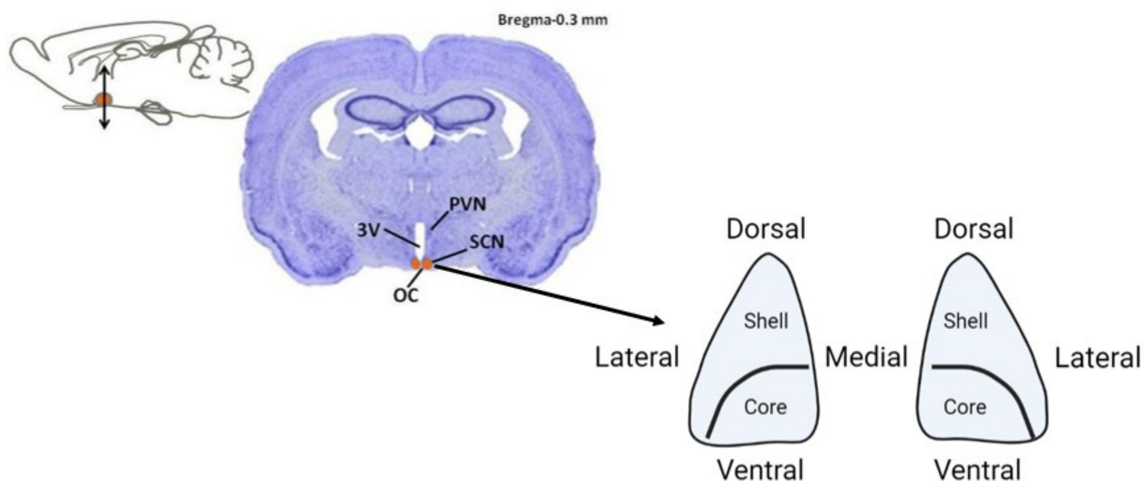

***Figure 5**: The location and structure of the rodent SCN.* *In a coronal section of the rodent brain the SCN is located at the bottom of the brain, close to the third ventricle (3V) and right above the optic chiasm (OC). When zooming in on the structure of the SCN it is organized in two nuclei which each have two distinct subregions. One located ventral-medial, often referred to as the core, and one located dorsal-lateral, often referred to as the shell. Figure adapted from: Jha (2016) and created in biorender.com.*

1.4.1 Afferent pathways

Afferent pathways to the SCN conveys information about the outside environment and state in the body. The SCN has three major afferent pathways, the retinohypothalamic tract (RHT), the median raphe serotonergic pathway, and the geniculohypothalamic (GHT), NPY-containing pathway from the thalamic intergeniculate leaflet (IGL) (Moga & Moore, 1997). However, anatomical routes that theoretically could be involved in rhythm regulation are enormous. About 35 regions directly innervate the SCN. If one considers multi-synaptic projections, the number expands to about 85 areas that could potentially provide input to the SCN (reviewed in Morin (2013)).

The three major pathways mainly terminate in the ventromedial area of the SCN and convey information like light, locomotor activity and metabolic information (Janik & Mrosovsky, 1994; Moga & Moore, 1997; Saderi et al., 2013). Light is the strongest zeitgeber and in this information is mainly transmitted via specialized photopigments melanopsin, a subset of photosensitive retinal ganglion cells (pRGCs) in the RHT, and is coded by the *Opn4* gene (Panda et al., 2002). The signal conveys through the neurotransmitters glutamate and pituitary adenylate cyclase-activating peptide (PACAP) (Hannibal et al., 2000). However, the SCN in *Opn4-deficient* organisms can receive information about light, which has been proved to be mediated through rod and cone photoreceptors (Panda et al., 2003). In addition to neural projection, it has been shown that the ventrolateral region has androgen receptors (Karatsoreos et al., 2007), meaning that the SCN potentially also receives endocrine signals.

1.4.2 Neuropeptides

Cytochemical, the ventromedial and dorsolateral subregions in rodents like mouse and rat clearly differ in their neuropeptide expression. Well characterized neuropeptides that are believed to be part of the circadian organization are gastrin-releasing peptide (GRP), neurotensin (NT), calretinin (CALR) and vasoactive intestinal polypeptide (VIP) which is expressed in the ventromedial area, met-enkephalin (mENK), angiotensin II (AII), arginine vasopressin (AVP) which is expressed in the dorsolateral area and Gamma-aminobutyric acid (GABA) and neuromedin S (NMS) which is expressed throughout the whole SCN (Abrahamson & Moore, 2001; Ono et al., 2021) (Figure 6). Yet, many of the neuropeptides localized in the SCN are still unidentified (reviewed in Morgado et al. (2015)).

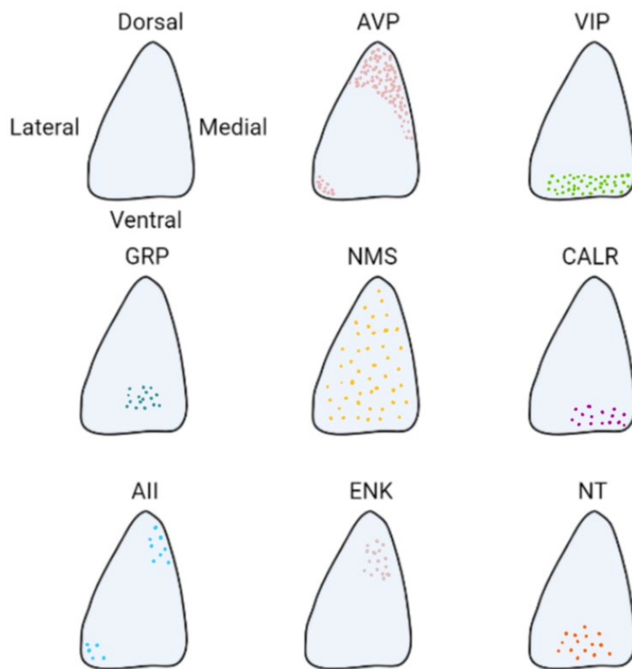

***Figure 6: Schematic representation of the expression pattern of neuropeptides in the rodent SCN**. These neuropeptides are believed to be a part of the circadian organization. In the upper left corner, the orientation of the SCN is displayed. The neuropeptides that are illustrated are arginine vasopressin (AVP), vasoactive intestinal polypeptide (VIP), gastrin-releasing peptide (GRP), neuromedin S (NMS), calretinin (CALR), angiotensin II (AII), met-enkephalin (mENK), neurotensin (NT). Modified from (Ono et al., 2021).*

In other animals the distinction in neuropeptide expression between core and shell is not as clear. In marsupials, AVP and VIP expressions are more co-distributed than being in distinct subregions (Cassone et al., 1988). In many other cases, the distribution of AVP and VIP varies throughout the rostral-caudal extent (Figure 7); for example, in the guinea pig, AVP is distributed throughout the whole SCN in the rostral area, but in the caudal area, it is restricted to a thin dorsomedial subdivision (Cassone et al., 1988). In humans, VIP is in the rostral area scattered among AVP, but in the caudal area, it is surrounded by AVP (Hofman et al., 1996). In sheep, there are less distinct subregions as VIP is distributed throughout the whole SCN but more densely in the ventral portion and AVP is in the caudal part most densely dorsally but in the rostral part, AVP is evenly distributed throughout the nucleus, and GRP is not expressed in the SCN (Dardente et al., 2016; Tillet et al., 1989).

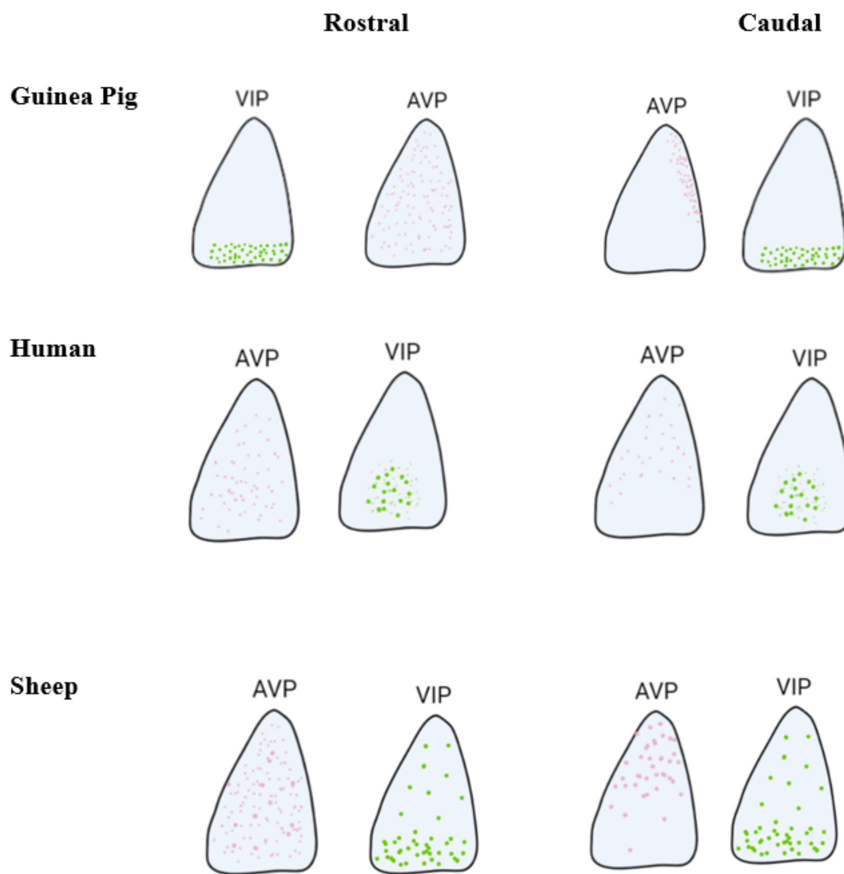

Figure 7: Expression of arginine vasopressin (AVP) and vasoactive intestinal polypeptide (VIP) rostral and caudal in the SCN of Guinea Pig, Human and Sheep. *The illustration demonstrates that AVP and VIP may be differently expressed through the rostral-caudal extent of the SCN in addition to differ between animal groups. Modified from (Cassone et al., 1988; Dardente et al., 2016; Hofman et al., 1996).*

VIP is believed to be essential in transmitting the light-dark cycle to other cells. In Syrian hamster VIP follows the light-dark cycle and this rhythm abolish in constant darkness (Francl et al., 2010). The activity of VIP is strongest during light (Romijn et al., 1996). If the pathway of VIP is blocked, as in a VPAC2 knockout mice, where the VIP receptor VPAC2 is not functional, the animal is unable to sustain a circadian activity rhythm (Harmar et al., 2002). VIP can phase-shift the release of AVP and the clockwork in the SCN (Reed et al., 2001; Watanabe et al., 2000). AVP is clearly affected by VIP and in mice it has been seen that AVP peaks during subjective day (Silver et al., 1999). AVP is believed to be important in transmitting circadian information to cells outside the SCN. Application of AVP can increase firing rate in SCN neurons, which have been seen from studies in SCN slices from Brattleboro rats (Ingram et al., 1996). When mice are deficient of AVP or one of its receptors (e.g. V1a), they show weakened locomotor rhythms and dampened secretion of prokineticin 2 (PK2), a neuropeptide important for transmitting circadian output (Li et al., 2009; Silver et al., 1999). GRP is co-expressed with VIP both in function and location (Kawamoto et al., 2003). GRP neurons in mice receive retinal

input, and GRP can induce expression in c-fos, *Per1* and *Per2*, in addition to phase-shift the circadian clock (Aida et al., 2002; Gamble et al., 2007; Karatsoreos et al., 2004). In VPAC2 knockout mice, GRP can create coordinated rhythmicity – meaning that GRP can substitute for VIPs role in transmitting the light-dark cycle (Brown et al., 2005).

The other neuropeptides of the SCN are less studied, but it has been seen that calretinin, in mice, can act as a Ca^{2+}- buffering protein in retinorecipient neurons (Ikeda & Allen, 2003) and neurotensin can phase-shift the SCN neurons' firing rate in rats (Meyer-Spasche et al., 2002).

1.4.3 Efferent pathways

According to Kalsbeek et al. (2006), the SCN outputs can be organized into three main types: connection with endocrine neurons, preautonomic neurons and intermediate neurons. Monosynaptic projections from the SCN mostly reach the nearby hypothalamic targets (Abrahamson & Moore, 2001), which include, but are not limited to, the subparaventricular zone (sPVZ), dorsomedial hypothalamic nucleus (DMH), the medial pre-optic nucleus (MPN), and the paraventricular hypothalamic nucleus (PVN).

In addition to neural projections, the SCN also signals through diffusible signals. Signal like prokineticin 2 (PK2), transforming growth factor-α (TGF-α) and cardiotrophin-like cytokine (CLC) is linked to the circadian system. PK2 is, as mentioned earlier, affected by AVP and is found in the SCN and many SCN output targets. PK2 oscillates in a circadian fashion with a peak at daytime and a low at night-time in nocturnal animals and maintains its rhythm in constant darkness and can be entrained (Cheng et al., 2002). The circadian clock genes affect PK2, the CLOCK and BMAL1 heterodimers activate the transcription of PK2 through an e-box, and PER1, PER2, PER3, CRY1 and CRY2 can inhibit the transcription. Circadian outputs are affected by PK2. It suppresses locomotor activity (Cheng et al., 2002). Furthermore, null mutation of PK2 led to a disturb coordination of the circadian thermoregulatory function in mice (Prosser et al., 2007). TGF-α is rhythmically expressed in the SCN. TGF-α suppresses locomotor activity and disrupts the circadian sleep-wake cycle. When the TGF-α pathway is disrupted in mice, the animal shows excessive daytime locomotor activity and failure to suppress activity when exposed to light (Kramer et al., 2001). CLC is expressed in a subpopulation of AVP neurons in the SCN, which have a circadian rhythm that peaks when locomotor activity is low. CLC also suppresses locomotor activity, but does not affect the circadian clockwork (Kraves & Weitz, 2006).

1.5 Output physiology

The SCN connects to many areas in the brain in addition to signaling through diffusible signals. These pathways eventually create the output physiology of the SCN either directly or indirectly. I will discuss the glucocorticoid axis, body temperature, and melatonin as key examples of commonly measured circadian output physiological parameters. I will also describe appropriate experimental designs to measure for circadian variation in these factors.

1.5.1 Glucocorticoids

Glucocorticoids are important for many physiological processes such as metabolism, stress, DNA-synthesis, inflammation and also have an effect on other hormones (Hill et al., 2016). Glucocorticoids are mainly secreted from the ACTH receptor-expressing cells of the zona fasciculata in the adrenal cortex and is primarily regulated through the limbic-hypothalamic-pituitary adrenal system (HPA-axis). Corticotrophin–releasing hormone (CRH) is produced in the PVN of the hypothalamus and stimulates the secretion of adrenocorticotrophic hormones (ACTH) from the anterior pituitary (Chung et al., 2011). ACTH stimulates the production of glucocorticoids in the adrenal gland (Hill et al., 2016).

To produce glucocorticoids, free cholesterol must be delivered to the inner membrane of the mitochondria in the cells of the zona fasciculata. This is the first and rate-limiting step of the biosynthesis of glucocorticoids (reviewed in Manna et al. (2009). The delivery of free cholesterol is mediated by the steroidogenic acute regulatory protein (StAR). The increase in steroidogenesis is mediated through induced StAR expression, and hormones like ACTH mainly affect the activity of StAR. Cholesterol delivered to the inner mitochondrial membrane is subject to sequential steroidogenic processing by a subset of steroidogenic genes. Corticosterone is a primary glucocorticoid in rodents, while cortisol is the most important glucocorticoid in primates, as it is responsible for 95% of the glucocorticoid effects (Chung et al., 2011; Sand et al., 2014).

The plasma levels of cortisol in humans typically shows a rise during the night with a peak withing the first hours after awakening, then it drops quickly, has a slight increase after mid-day, and then declines slowly to a low during the night (Figure 8) (Sand et al., 2014). The levels of corticosterone typically rise toward late afternoon to early evening just prior to the onset of darkness in laboratory mice (Figure 8) (Kakihana & Moore, 1976).

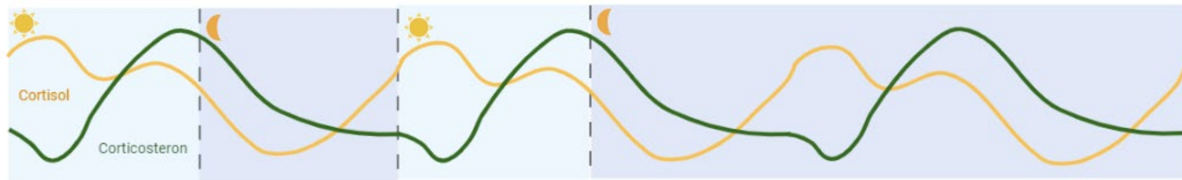

Figure 8: A diagram of the oscillation of the glucocorticoid's cortisol and corticosterone. *Cortisol (yellow) show a 24-hour rhythm in light-dark conditions, with a peak at morning, a quick drop and then a slight increase after mid-day before it declines slowly to a low during the night and follow the same pattern if conditions become constant. Corticosterone (green) rise during late afternoon and reaches its high during darkness and follow the same pattern if conditions become constant.*

Lesions of the SCN abolish the circadian rhythmicity of corticosterone secretion, which strongly indicates that the SCN controls the rhythmic release (Moore & Eichler, 1972). Anatomically the SCN connects to the PVN (Abrahamson & Moore, 2001), which could be the pathway by which SCN controls the rhythmic release of glucocorticoids. When AVP (SCN neuropeptide, see above), is infused into the hypothalamus, it inhibits ACTH and glucocorticoid secretion, and an AVP antagonist effectively increases glucocorticoid secretion (Buijs et al., 2003; Kalsbeek et al., 1996), these studies would support the view of a direct connection.

However, studies have also suggested that glucocorticoid levels may be regulated by a polysynaptic projection from the SCN to the adrenal gland. This projection goes from the SCN to the sPVZ, through the spinal cord to the splanchnic nerves, which innervates the adrenal gland. Photic signals that are transmitted through the splanchnic nerve induce a delayed increase in plasma corticosterone levels (Ishida et al., 2005). This inducement is probably mediated through catecholamines from the adrenal medulla, and a study done in primates showed that epinephrine might be of importance in entraining the release of glucocorticoids (Lemos et al., 2006). The splanchnic nerve has been shown to modulate the adrenal sensitivity to ACTH during the day, leading to a decrease in corticosterone release in the evening (Ulrich-Lai et al., 2006).

It is wildly accepted that these two pathways create a circadian cortisol rhythm and this is commonly used output measure for circadian rhythms (Murphy et al., 2011; Scheer et al., 2009).

1.5.2 Body temperature

One of the homeostatic functions the SCN contributes to regulating is body temperature. All organisms produce heat through metabolic processes, and animals can have different relations to the ambient temperature. Endotherms warm their tissue from the inside, while ectotherms depend on the environment. Endotherms that thermoregulate are called homeotherms, as all

their tissue is the same temperature (Hill et al., 2016). The thermoregulation depends on afferent input, central control and efferent control (Nakamura, 2011). The central control is in the brain in the pre-optic area (POA), which is a part of the hypothalamus. The role of the POA in the circadian regulation of body temperature is not well understood. The SCN projects directly to the MPN (Abrahamson & Moore, 2001), which is a nuclei in the POA (Tsuneoka & Funato, 2021) and indirectly to the POA via the sPVZ and DMH (Chou et al., 2002; Deurveilher et al., 2002), which all could be pathways that the SCN regulate body temperature. Lesions of the SCN established that the SCN regulates the circadian variation of body temperature, but not the homeostatic control (Refinetti, 1995). The POA receives information about the internal temperature through sensory neurons. The thermoregulation of body temperature appears to behave as if there is a 'set point' - to maintain body temperature withing specific limits. When the sensory input perceive a deviation from this 'set point', thermoregulatory responses like thermogenesis, tachycardia, vasoconstriction or vasodilatation is conducted (Nakamura, 2011). Circadian regulation appears to shift the thermoregulatory "set-point" of the core body temperature (Kräuchi, 2007). The body temperature reaches its maximum in the early evening and minimum in the early morning hours with a range of 0.9ºC in humans (Figure 9) (Kräuchi, 2007).

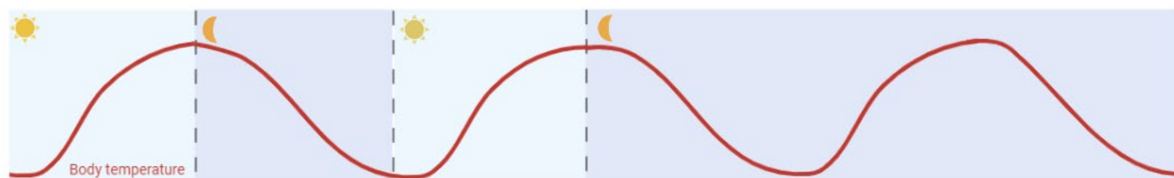

Figure 9: A diagram of the oscillation of core body temperature for a human. *Core body temperature reaches its maximum during early evening and minimum in early morning and maintains its rhythm in constant conditions.*

1.5.3 Melatonin

Melatonin is secreted from the pineal gland, with elevated plasma concentration during the dark period (Figure 10). Melatonin secretion in rat remains rhythmic in constant conditions, but is abolished if the SCN is lesioned (Klein & Moore, 1979). When melatonin is produced, it is released into the capillaries and the cerebrospinal fluid and then distributed to the body tissue (Pandi-Perumal et al., 2008). Melatonin regulates many physiological bodily activities like seasonal reproduction, sleep regulation, cancer and diabetes (Borjigin et al. (2012)).

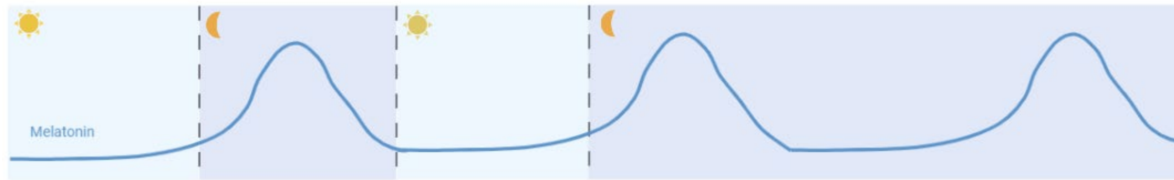

***Figure 10: A diagram of the oscillation of melatonin in rats**. Melatonin rises during darkness and is suppressed by light. Melatonin maintains its rhythm in constant darkness in rodents.*

A poly-synaptic projection from the SCN to the pineal gland have been identified in rats. Projections go from the SCN to the PVN and from the PVN, neurons project to the medial forebrain bundle and the reticular formation; from here, fibers go to the intermediolateral horns of the cervical segments of the spinal cord. Finally, postganglionic sympathetic fibers from the superior cervical ganglia project to the pineal gland (Buijs et al., 2003; Pandi-Perumal et al., 2008). Presynaptic neurons release norepinephrine (NE) which regulates melatonin biosynthesis and NE release occurs during the subjective night of the circadian pacemaker (Pandi-Perumal et al., 2008). NE binds to β-adrenergic receptors on pinealocytes, increasing 3’,5’-cylclic adenosine monophosphate (cAMP) concentration. cAMP stimulates enzyme arylalkylamine–N–acetyl transferase (AANAT) expression and phosphorylation (Klein et al., 1971; Pandi-Perumal et al., 2008).

Melatonin is synthesized from tryptophan which is taken up from the circulation. Tryptophan is first converted to serotonin which is then converted into N-acetylseretonin by AANAT (Pandi-Perumal et al., 2008). N-acetylserotonin is metabolized to melatonin by the enzyme hydroxyindole-O-methyltransferase (HIOMT). If there is exposure to bright light during the night, melatonin production will be suppressed through the degradation of AANAT.

1.5.4 Experimental designs to measure circadian outputs

One way to study circadian rhythms is to place the organism of study in a constant routine protocol. In this protocol, the aim is to keep all environmental factors constant. The organism is most commonly provided with food and water *ad libitum*, the light does not change, and the temperature is constant. The most beneficial is if the organism is kept undisturbed or visited completely randomly throughout the day (Hofstra & De Weerd, 2008). One problem that may arise from this protocol is if the organism needs occasional visits due to husbandry, health or other reasons, and the random visit is insufficient. If one wants to study circadian rhythms outside the sleep-wake pattern, it can be beneficial to hold the organism awake during the protocol because sleeping can itself exert a ‘masking’ effect (Hofstra & De Weerd, 2008). This

may prove difficult if one wants to have a solid evidence base for the data, as this may lead to sleep deprivation and cause other kinds of problems (Hofstra & De Weerd, 2008).

Another possibility, that may overcome the difficulties in a constant routine protocol, is to uncouple internal and external rhythms using a forced desynchrony protocol. In this protocol, the study organism is exposed to a light-dark cycle or an activity-rest schedule much longer or shorter than the 24 hour which the endogenous circadian rhythm cannot entrain to. This allows the period to free-run according to its internal rhythm. This makes it possible to assess how external factors influence the free-running rhythm (Binder et al., 2009). Both protocols that are longer – 28 hours, and shorter – 20 or 22 hours, than 24 hours have been seen to successfully desynchronize the external and internal rhythm (Hiddinga & Van Den Hoofdakker, 1997; Scheer et al., 2009).

Depending on the organism of study, there are several ways to set up the protocol to desynchronize. Scheer et al. (2009) did a study with humans to investigate how a desynchronized protocol affected health. In this protocol, humans were kept in dim light, and their sleeping times and meals were scheduled with a period of 28 hours. This will nevertheless most likely not work for organisms that are not as civilized as humans – as animals cannot understand and follow the concept of going to bed at a specific time in constant conditions. Another option is forcing them to run in a wheel during the activity period (Strijkstra et al., 1999). Another possibility is to use the light to desynchronize Cambras et al. (2007) placed rats in a light-dark cycle that was 22 hours long, and successfully desynchronized their temperature internal rhythm from the external activity pattern. Internal rhythms that have previously been successfully desynchronized from the external rhythm in this type of protocol are, among others, body temperature, cortisol, glucose, insulin, epinephrine and norepinephrine (Dijk & Czeisler, 1995; Hiddinga & Van Den Hoofdakker, 1997; Scheer et al., 2009).

1.6 Circadian clocks in the Arctic

True arctic species are defined as "animals that have winter residency and their main area of distribution within the arctic region" (Blix, 2005). The circadian clockwork has been studied in several of these animals, like the reindeer (*Rangifer Tarandus*) (Arnold et al., 2018; Lin et al., 2019; Lu et al., 2010; Stokkan et al., 1994; Van Oort et al., 2005; van Oort et al., 2007), the Svalbard rock ptarmigan (*Lagopus muta*) (Appenroth, Nord, et al., 2021), the Arctic Ground squirrel (*Urocitellus parryii*) (Williams et al., 2012), and the polar bear (*Ursus maritimus*)

(Ware et al., 2020). This thesis will focus on the reindeer, as this is the most studied in terms of the circadian clockwork.

Reindeer are ruminant species belonging to the order of even-toed ungulates (*Artiodactyla)*. The reindeer is divided into seven subspecies, with a circumpolar distribution in the northern hemisphere (Blix, 2005). Reindeer show diel activity rhythms under a light-dark regime, but this activity rhythm breaks down during the times of the year when the zeitgeber light is minimal (Van Oort et al., 2005). This was also confirmed in a later study (van Oort et al., 2007), where they also noted that the reindeer had strong ultradian organization but that this organization altered throughout the year. The ultradian bouts of activity had a shorter period in constant light, five hours, compared to eight when a light-dark cycle is present (Figure 11; van Oort et al. (2007)) Other physiological circadian outputs of reindeer have been poorly tested. One study investigating rumen temperature, heart rate and activity claims that even during mid-summer and mid-winter there is a circadian organization – however, this organization is very weak (Arnold et al., 2018). These three studies are done in reindeer free-ranging in their natural habitat, in northern Norway at 70° N (Van Oort et al., 2005; van Oort et al., 2007) and at Svalbard at 78° N (Arnold et al., 2018; Van Oort et al., 2005; van Oort et al., 2007). This means that at least one of the criteria for determining if a rhythm is circadian is not met – as natural habitats do not provide constant conditions and there is presence of a range of zeitgebers, like small light changes, temperature and solar position, even during polar night and midnight sun.

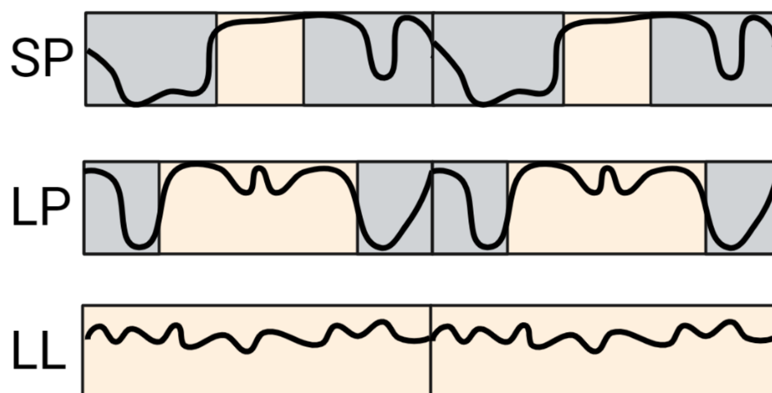

Figure 11: Activity rhythm of Norwegian reindeer (Rangifer tarandus tarandus) during short photoperiod (SP; December) long photoperiod (LP; march) and continuous light (LL; June). *Activity bouts are longer when there is a light-dark cycle (8 hours) compared to constant light (5 hours). Activity is plotted as activity (min.h^{-1}). Redrawn and modified from (van Oort et al., 2007).*

The only hormone studied in reindeer is melatonin. In captive Norwegian animals held in Tromsø, melatonin had a clear 24-hour light-dark melatonin rhythm during late winter and

autumn (Stokkan et al., 1994). However, during the summer and middle of winter, the melatonin concentration had no daily rhythm (Stokkan et al., 1994). Another study from 2010 (Lu et al., 2010), also in captive Norwegian animals in Tromsø, investigated how melatonin responded to abrupt changes in light. They introduced reindeer to an artificial light-dark cycle of 2.5 hours of light followed by 2.5 hours of dark. They showed that melatonin was suppressed by light and immediately raised when they entered the dark phase and then again suppressed by light, having no circadian properties but only following light passively (Lu et al., 2010).

This study also investigated the circadian properties of the molecular clock in reindeer. They introduced *luc* gene attached to *Bmal1* and *Per2* promoters into cultured reindeer fibroblast using lentiviral vectors. They then measured luminescence when fibroblasts were in constant darkness and showed that reindeer, in contrast to mice, showed no circadian oscillation (Lu et al., 2010), implicating a lack of organization in the reindeer circadian clockwork.

Of genes that are involved in circadian pathways, Lin et al. (2019) found eight genes in reindeer that exhibit reindeer-specific mutations in functional domains (PER2, NOCT, GRIA1, GRIN2B, GRIN2C, ITPR3, ADCY5 and NOSIAP) in addition, four genes were identified as rapidly evolving (ADCY2, ADCY8, CALML4 and CAMK2) where two of the genes had reindeer-specific mutations. Of most interest, three reindeer-specific mutations were found in the *Per2* gene, where one of the mutations (P1172T) was identified to affect the binding of PER2 and CRY1 (Lin et al., 2019). This mutation was, however, not adequately tested to determine if it affected the circadian clockwork in reindeer. Nonetheless, this study provides insight into the understanding that there are adaptations in the reindeer on a molecular level to the Arctic region when it concerns the circadian clock – and this should be investigated further.

1.7 Research questions and hypotheses

Even though reindeer is the most studied arctic animal in terms of circadian organization there is clearly a gap in knowledge. First, the appearance of circadian rhythms has not been studied in controlled protocols – and with this in mind one cannot state that the rhythms observed are truly circadian and not the effect of masking. Second, the area that controls the circadian rhythmicity has not been studied – the SCN. Third, only one hormone has been studied in the terms of circadian outputs and, finally, the circadian genes have not been studied in the SCN of the reindeer and the previous fibroblast work is limited.

I will, in this master's project, aim to fill some gaps of knowledge and therefore look at the output physiology in controlled conditions and try to characterize the SCN in reindeer. I will ask the following research questions and have the following hypothesis:

Research question 1: Do we observe the same characteristics in the central pacemaker (SCN) as in other mammals?

Hypothesis 2: I expect the reindeer's suprachiasmatic nucleus to have the same anatomical and cytochemical properties as other mammals. That means that the suprachiasmatic nucleus is found immediately above the optic chiasm and lateral to the third ventricle. I further expect that the suprachiasmatic nucleus contains two subregions – one located ventral medial and the other located dorsal lateral. I expect that the vasoactive intestinal polypeptide (VIP) and gastrin-releasing peptide (GRP) are mainly expressed in the ventral medial area and that the arginine vasopressin (AVP) are mainly expressed in the dorsal lateral area.

Research question 2: Can you observe circadian outputs (hormones, locomotor activity, heart rate, body temperature and cortisol) in reindeer in controlled constant conditions?

Hypothesis 2: As earlier studies have indicated that reindeer have a weak circadian organization, I expect they will not show a circadian rhythm in locomotor activity, body temperature, heart rate, or cortisol under constant conditions.

Research question 3: Will different outputs maintain circadian rhythms in a forced desynchrony protocol?

Hypothesis 3: It is expected that the reindeer will not maintain a circadian rhythm in locomotor activity, body temperature, heart rate or cortisol under a forced desynchrony protocol.

2 Methods

2.1 Characterization of the suprachiasmatic nucleus

For the characterization of the reindeer suprachiasmatic nucleus (SCN) a digoxigenin (DIG)-labeled RNA probe in situ hybridization was decided to be used. In situ hybridizations are a good method for localization and detection of specific RNA sequences in a cell (Jensen, 2014).

2.1.1 Research animals

The animals used in this study are part of the reindeer herd owned and maintained by The University of Tromsø - the Arctic University of Norway. The reindeer used in this study were two males approximately 1 year of age (IDs: 4/21 and 6/21), one male of 6 months old (ID: 5/22), one female of 11 years (ID: 12/10), and a pre-term female who was euthanized for welfare reasons 6 hours after birth in 2021.

2.1.2 Sample preparation

All animals, except the pre-term female, were euthanized by a captive bolt to the head, positioned to avoid the midline and hypothalamic region, by technical staff at the department of Arctic and Marine biology. The pre-term female was euthanized by injection of 0.25ml medotomidin/100mg/ml zalopinc.

On confirmation of death (no respiration for one minute) the animals were decapitated. The antlers were removed using a handsaw, if necessary, then a 35-degree cut was made from the back of the orbit down to the foramen magnum with a bandsaw to expose the brain. The hypothalamic region was identified, and a hypothalamic block was dissected and lowered into isopentane cooled to approximately -35°C on dry ice. The frozen block was stored at -80ºC. The process from death to freezing took on average 15 minutes.

2.1.3 Sectioning

The hypothalamic block was sectioned from rostral to caudal with sections of 20 micrometers with a cryostat (Leica CM3050 S). The slides were placed on SuperFrost®Plus microscopic

slides (VWR) or Superfrost Plus^{TM} Gold Adhesion Microscope slides (Epredia). Sections were stored at -80ºC until the next step.

2.1.4 Digoxigenin in situ hybridization

Plasmids containing in situ hybridization probe sequences for vasoactive intestinal polypeptide (VIP), arginine vasopressin (AVP) and gastrin-releasing peptide (GRP) were kindly designed, prepared and given to us by Hugues Dardente, Université de Tours. All probes are designed specific to reindeer (*Rangifer tarandus*) by using available genomic data (NBCI) and RNA samples from reindeer.

Riboprobe synthesis

The riboprobe was synthesized by adding 1 µg linearized DNA template, 2 µl DIG RNA labelling mix (11277073910, Roche), 4 µl 5X transcription buffer (P1460, Promega) and 1 µl of RNA polymerase SP6/T7 (P1460, Promega) in a solution with a total volume of 20 µl before incubated at 37ºC for two hours. After incubation, 1 µl of DNase (P1460, Promega) (1 U/µl) was added and then incubated for 15 min at 37ºC. 3 µl of tRNA (10109525001, Roche) with a concentration of 10mg/ml, 1.5 µl of 8M lithium chloride (L7026, Sigma) and 70 µl of cold 100% ethanol (EtOH) was added. The solution was incubated at -20ºC overnight.

On the second day, the probe was centrifuged at 14000 rpm (Himac CT15RE) at 8ºC for 45 minutes. EtOH was carefully removed from the pellet. 300 µl of 70% cold EtOH was added, and the probe was centrifuged at 14000 rpm (Himac CT15RE) at 8ºC for 15 minutes. The EtOH was then carefully removed, and the pellet was left to dry for 5 minutes until the rest of the EtOH evaporated. The pellet was resuspended with 100 µl of diethylpyrocarbonate-treated water (1 mL DEPC/L). To confirm that the probe had been synthesized, it was checked on a tape station instrument (Agilent 4200 TapeStation, Agilent Technologies, Inc. 2015) and analyzed in the Agilent Tapestation Analysis Software (Agilent Technologies, Inc. 2015).

Acetylation and fixation

The sections were transferred from -80ºC to -20ºC for slowly thawing overnight. Fixation was done using 4% paraformaldehyde (PFA; P 6148, Sigma), with 10N sodium hydroxide (NaOH) in 0.1M phosphate buffer (PB), for 20 minutes in the fridge. After that, it was washed two times for five minutes in 0.1M PB. Acetylation was done using 0.1M triethanolamine (TEA; T 1502, Sigma) with 5 µl/ml 10N NaOH for 2 minutes. Next, the slides were immersed in 0.1M TEA

with 2.5 µl/ml acetic anhydride (A604, Sigma) for 10 minutes. The slides were washed with 0.1M PB for two minutes two times.

Hybridization

In advance a hybridization buffer (ISH buffer) were made by dissolving 10 g dextran sulphate (D8906, Sigma) in 15 mL DEPC water in a sterile 50 mL centrifuge tube. This was transferred to an empty DEPC-treated 100 mL bottle. The tube was rinse with a mix of the following: 6 ml 5M sodium chloride (NaCl), 2 ml 50x Denhardts (30915, Sigma), 1 ml 1M tromethamine (TRIS; pH 8), 200 µl 0.5M etylendiamintetraacetat (EDTA; pH 8 adjusted by NaOH, E7889 Sigma), 800 µl DEPC water and finally 50 mL 8M Urea (51457, Sigma).

80 of µl a hybridization mixture made of the hybridization buffer and the following components 15.625 µl probe/mL ISH buffer, 62.5 µl tRNA/mL ISH buffer, 171.875 µl DEPC-treated water/mL ISH buffer were pipetted on the slides, coverslipped, and the process ran overnight at 56ºC.

RNase treatment

Before RNase treatment, the sections were washed with 5x saline-sodium citrate buffer (SSC) three times five minutes. The sections were RNase treated with an RNase solution which consisted of 100 µL/mL 5M NaCl, 10 µL/mL 1M Tris with pH eight adjusted by hydrogen chloride (HCl), 2 µL/mL 0.5 M EDTA (E7889 Sigma), 2 µL/mL RNase A (10 mg/mL; R 5125, Sigma) for 30 minutes at 37ºC.

Stringency washes

After RNase treatment, stringency washes were performed. The sections were first immersed in 2xSSC with 1 µl/mL 1M dithiothreitol (DTT; D 9779, Sigma) for two times five minutes. Second, they were immersed in 1xSSC with 1 µl/mL 1M DTT for ten minutes. Third, they were immersed in 0.5xSSC with 1 µl/mL 1M DTT for ten minutes. Fourth, they were immersed in 0.1xSSC with 1 µl/mL 1M DTT for 30 minutes at 60ºC. Fifth, they were rinsed with 0.1xSSC with 1 µl/mL 1M DTT at room temperature. Lastly, they were immersed in 1xA-DIG (10xA-DIG; 1M Tris, 1.5M NaCl, 50mM Magnesium chloride ($MgCl_2$), pH 7.5) for two times five minutes.

Probe detection

After stringency washes, the reaction was blocked with 0.5% blocking reagent (11096176001, Roche) in A-DIG buffer, after one hour, the blocking reagent was exchanged for antibody anti-DIG coupled to AP (11093274910, Roche) at 1/5000 in blocking reagent and the reaction run overnight. On the fourth day, the slides were rinsed with 1xA-DIG three times for ten minutes and with 1xAP (10xAP; 1M Tris, 1M NaCl, 50nM $MgCl_2$, pH 9.5) three times for five minutes.

For staining the antibodies, a solution of 6.6 µl/mL of nitrotetrazolium blue chloride (N6639, Sigma) of 50 mg/mL in 70% dimethylformamide (DMF) and 6.6 µl/mL of 5-Bromo-4-chloro-3-indolyl phosphate disodium salt hydrate (B6149, Sigma) of 50 mg/mL in DMF in a buffer made of 4% polyvinyl alcohol (PVA; P1763, Sigma) in 1xAP with 2.5 mM tetramisole hydrochloride (T1512, Sigma) were used. The staining took place in the dark overnight.

The sections were rinsed with tap water, mounted with CC/Mount aqueous mounting solution (C9368, Sigma), and finished off with nail polish or eukitt quick-hardening mounting media (03989, Fluka/Sigma).

2.1.5 Nissl staining

The slides were fixated in 4% PFA for 10 minutes on ice and then washed with 1x phosphate-buffered salin (PBS) three times for two minutes, followed by three washes in water. The slides were immersed in 1% Cresyl violet (Nissl solution) for 1.5 minutes. The slides were immersed in increasing ethanol concentrations; 50%, 75%, 95%, 100%, 100%, and finally in toluene for 30 seconds each. The slides were coverslipped with eukitt quick-hardening mounting media (03989, Fluka/Sigma).

2.1.6 Imaging & measurement

The slides were imaged with AXIO Zoom.V16 (ZEISS) using PlanNeoFluar Z 2.3x/0.57 FWD 10.6mm objective (ZEISS) for overview pictures and PlanApo Z 0.5x/0.125 FWD 114 mm objective for zoomed in pictures (ZEISS). Images were processed using ZEN 3.5 (ZEN pro).

The SCNs size was measured using the program ZEISS ZEN 3.7 and the graphic measurement tool 'line'. The medial-lateral and dorsal-ventral extent were estimated based on the AVP and VIP expression extent. With the start measurement with the first expression in the photo and the end measurement with the last expression in the photo. Measurements were taken each 460 µm or 580 µm in the rostral-caudal extent. The rostral-caudal extent was calculated based on which slide the expression started and which slide the expression ended. The average and standard deviation were calculated.

2.2 Circadian output physiology

This project is approved by the Norwegian Food Safety Authority (FOTS ID: 28929). The project complies with the Norwegian and European legalization for animal research.

2.2.1 Research animals

The animals used in this study are part of the reindeer herd owned and maintained by The University of Tromsø - the Arctic University of Norway. For this part of the project, it was essential to have animals that tolerated both human presence and staying indoors for a couple of weeks. For this purpose, calves were tamed with bottle feeding. This study used two male calves born in May 2021 (ID: 1/21 and ID: 2/21) and three calves – one male and two females (ID: 1/22, ID: 2/22 and ID: 3/22) born in May 2022. One to two weeks after birth, they were brought inside. They were frequently fed by bottles with a milk mix – which at the start consisted of milk powder replacement for cow calves and heavy cream. After a while, the heavy cream was replaced with sour milk. In the start the reindeer calves were fed five times a day distributed throughout the day (08:00 – 23:00), by technical staff, volunteers, and me. After approximately three months the feeding frequency was slowly reduced based on how much milk the reindeer drank. After four months the frequency was reduced to once a day, after this the reindeer were given the bottle until they stopped wanting it. Reindeer were also habituated to walking in leash and using a halter during the months in which they were tamed.

2.2.2 Experimental set-up

The aims of this study are to investigate circadian control of behavioral outputs. Hence, according to the definition of circadian rhythms (see introduction), controlled experimental conditions are essential. The tamed animals were, for this part, placed inside for several weeks in an experimental light and feeding set-up.

2.2.2.1 Pilot study 1: Body temperature and locomotor activity rhythms

During April 2022, reindeer 1/21 and 2/21 were kept indoors in a light-dark cycle of 16 hours of light and 8 hours of dark (LD 16:8) for 8 days and transferred to constant dim red light for 3 days. They were fed once a day, alternating between 9 am and 3 pm to reduce clues about the time of day.

Locomotor activity (LA), surface body temperature (T_s), light conditions and ambient temperature (T_a) were measured as described in 2.3.3 Measurements. This experiment was cut

short – as they were supposed to stay in dim red light for 8 days - because the feeding was shown to confound the dampening of circadian rhythms. These data informed the main experimental design.

2.2.2.2 Study design: forced desynchrony

Based on the pilot study, a forced desynchrony protocol was adopted. The protocol aims to desynchronize internal rhythms (rumen temperature (T_r), heart rate (HR) and cortisol levels) from external rhythms (feeding behavior (FB) and LA). This protocol was carried out on three reindeer (1/22, 2/22 and 3/22) for 20 days in March 2023 inside a room with three separate reindeer stalls with see-through walls (Figure 12).

***Figure 12: Reindeer room set-up**. The reindeer room were set up with three separate stalls. In each stall there fere a food-station, water bucket and a mat.*

The protocol which was designed and used (Figure 13) consists of four parts; 1) entrainment, 2) control, 3) re-entrainment and 4) forced desynchrony.

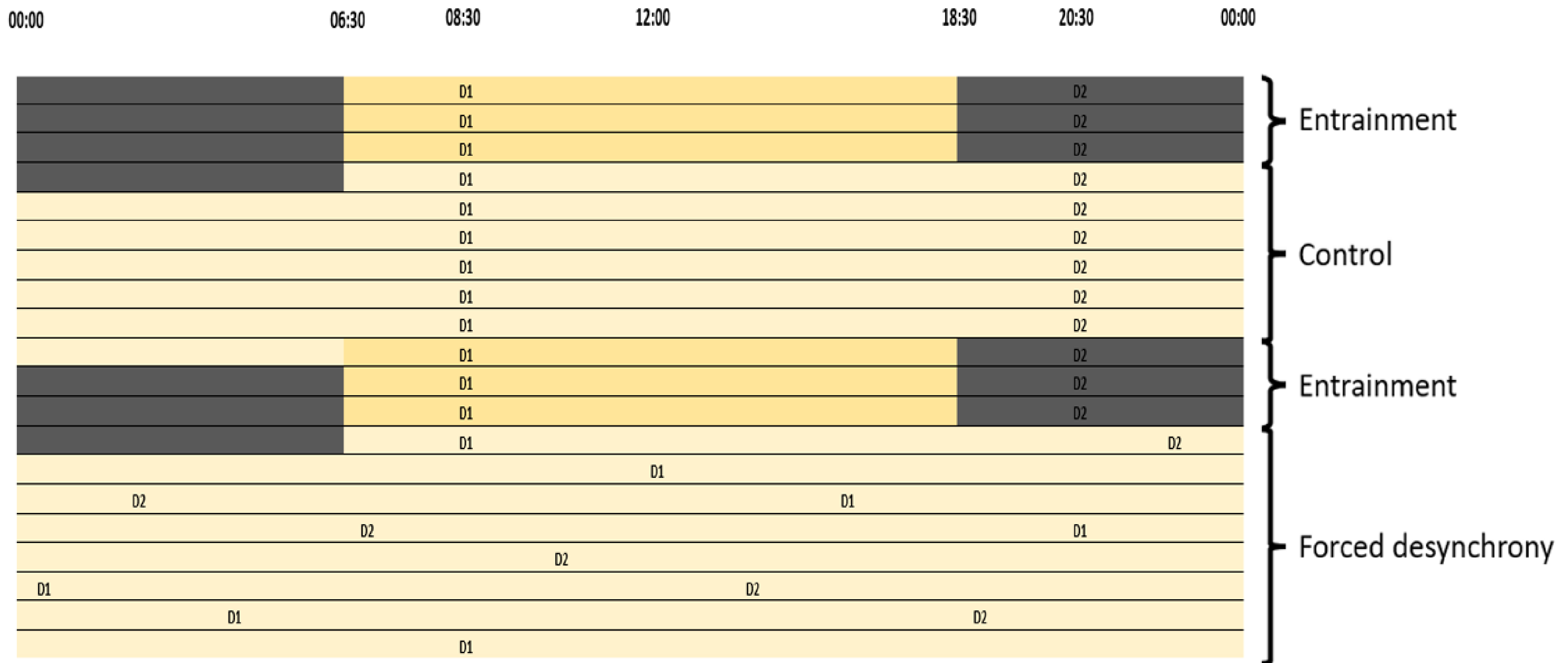

Figure 13: Forced desynchrony protocol. *The first 3 days of the protocol are entrainment with a light-dark cycle of 12 hours light and 12 hours dark (LD 12:12), with lights on at 06:30 and lights of at 18:30. The next 6 days is a control which consists of 6 days of dim light. The following 3 days are a second entrainment with LD 12:12 with lights on at 06:30 and light off at 18:30. During the two entrainment periods and the control, the reindeer are disturbed two times a day with a 12-hour interval. These disturbances are at 08:30 and 20:30. During disturbance-1 (D1; 08:30), salvia samples are taken, the stalls are cleaned, and food and water are changed. During disturbance-2 (D2; 20:30), saliva samples are taken, and the reindeer are given lichen. The last 8 days of the protocol consist of the forced desynchrony part with dim lights and disturbance every 14th hour. The disturbance occurs chronologically through the 8 days at the following hours: 08:30, 22:30, 12:30, 02:30, 16:30, 06:30, 20:30, 10:30, 00:30, 14:30, 04:30, 18:30 and 08:30, where the first disturbance (D1) corresponds to disturbance-1 and every second is the same. The second disturbance (D2) corresponds to disturbance-2; every second is the same.*

The first entrainment consisted of 3 days of 12 hours of light and 12 hours of darkness (LD 12:12), with lights on at 06:30 and lights off at 18:30 –similar to the light length of the spring equinox. During entrainment, the reindeer were disturbed twice a day at intervals of 12 hours. These disturbances were either in the morning at 08:30 (D1) or evening at 20:30 (D2). In disturbance 1 (D1), saliva samples were collected, the stalls cleaned, and food and water changed. Food and water were given ad libitum during the whole experiment. In disturbance 2 (D2), salvia samples were collected, and lichen was given. The following 6 days of the protocol, days 4 to 9, serve as the control. During the control, the light is constant at ~12 lux. The disturbance routine is the same as during the entrainment. Subsequently, there are 3 days, days 10 to 12, of re-entrainment, which has the same set-up as the first entrainment. Afterwards, the experimental part of the protocol takes place - the forced desynchrony part. The forced desynchrony part extends over 8 days, from day 13 to 20, in the protocol. The light is dim and constant (~12 lux) during the forced desynchrony part, and disturbance occurs every 14th hour. Every other disturbance corresponds to the routines of disturbance 1, and the rest of the disturbances correspond to the routines of disturbance 2 (Table 1).

Table 1: Disturbance routine of the forced desynchrony part of the protocol*. the forced desynchrony part extended for 8 days, corresponding to day 13 to day 20 of the protocol. Disturbance 1 corresponds to disturbance 1 for the rest of the protocol. Saliva samples were taken, stalls cleaned, and food and water changed. Disturbance 2 corresponds to disturbance 2 for the rest of the protocol. Saliva samples were taken, and lichen was given.*

Day of protocol	Hour	Disturbance routine
Day 13	08:30	D1
Day 13	22:30	D2
Day 14	12:30	D1
Day 15	02:30	D2
Day 15	16:30	D1
Day 16	06:30	D2
Day 16	20:30	D1
Day 17	10:30	D2
Day 18	00:30	D1
Day 18	14:30	D2
Day 19	04:30	D1
Day 19	18:30	D2
Day 20	08:30	D1

During the protocol LA, FB, T_r, HR, saliva samples, T_a and light conditions were collected as described in 2.2.3 Measurements.

2.2.3 Measurements

During the experimental set-ups, several different parameters were measured, namely: LA, FB, body temperature (T_s and T_r), HR, cortisol, T_a, door movement and light conditions.

2.2.3.1 Locomotor activity

Actiwatches (MotionWatch 8, CamNtech Ltd, camtech.com) were set to measure LA per minute. Actiwatches uses accelerometery to measure activity counts. The actiwatches were placed on the reindeer's harness or collar (Figure 14). The data was retrieved using the program MotionWare 1.3.17 (camtech.com).

Figure 14: Actiwatch attached to reindeer harness. *Attached with duct tape to ensure that it stays on the reindeer for the whole experiment.*

2.2.3.2 Feeding behavior

FB was measured with HOBO loggers (Onset Brand) placed on their food station. HOBO loggers are passive infrared sensors that measures occupancy, and these were set-up to measure occupancy for every 10 seconds. HOBO loggers were placed so that it only registered when the

animal went into the bowl to feed. This was done by using a feeding station with walls (Figure 15a), and then the HOBO loggers were placed under a wooden edge (Figure 15b) with a cap on the sensor to ensure that it only registered below the wooden edge. The data was retrieved using the software HOBOware (Onset Brand).

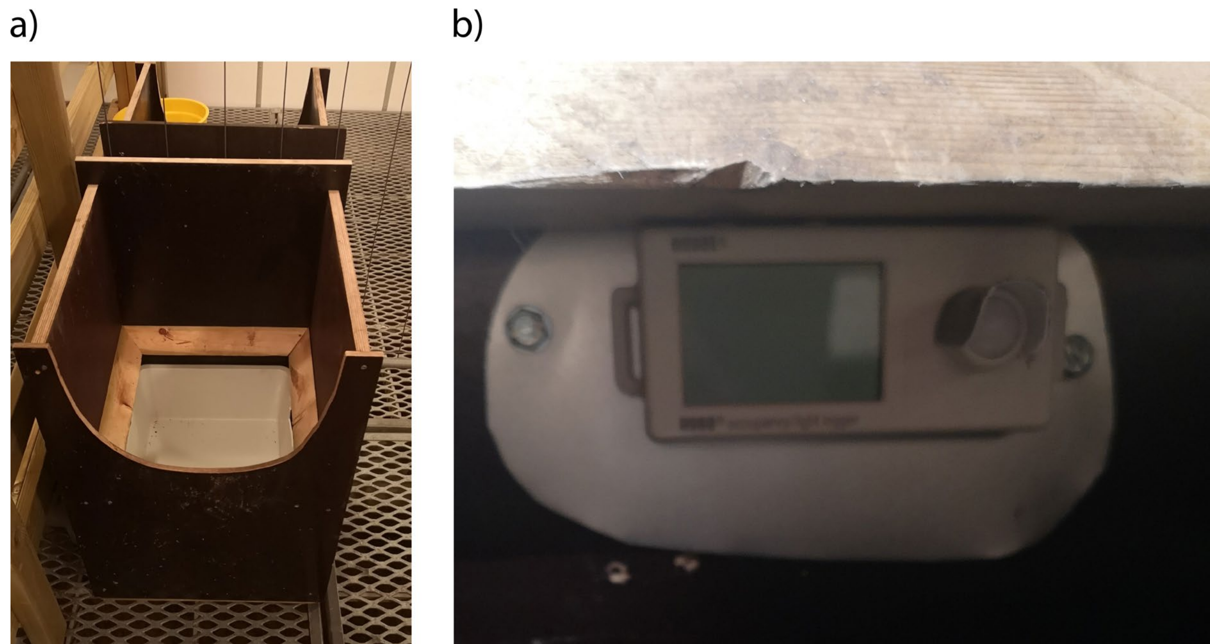

Figure 15: HOBO logger set-up to measure feeding behavior (FB). *a) Feeding station inside the reindeer stall. Made up of wood plates. Possibility to take food box in and out from behind. Wooden edges on the insideside so foodbox cannot be taken out by the reindeer and to ensure correct measurement of feeding intervals. b) HOBO logger set-up under the wooden edge. A steel board is screwed into the wooden plate, and the sensor with magnets is placed on it. A cap made of duct tape to ensure correct measurement.*

2.2.3.3 Body temperature and heart rate

The body temperature was measured by two means. For T_s, used during the pilot, an anipill® (BodyCap) was placed on the harness close to the skin with padding surrounding it to reduce the influence of T_a. In addition, a small part of the fur was shaved to get closer to the skin. The activation was done using the anipill activator (BodyCap) and reading of the data was done using the AniLogger® Monitor (BodyCap) and read via the Anilogger manager (BodyCap).

For T_r and HR, used during forced desynchrony, rumiloggers (Vectronic Aerospace) were administrated orally in two reindeer (1/22 and 2/22) by associate professor Alina Lynn Evans, Inland Norway University of Applied Sciences, and senior researcher Ingebjørg Helena Nymo, Norwegian Veterinary Institute. The rumiloggers have temperature sensors and measure heart rate by acceleration sensor. The rumiloggers (22×80mm, 100g) is about 1-2% of the reindeer's

body weight and transmit data to a collar placed on the reindeer (Vectronic Aerospace; Figure 16). The data was retrieved using the software Rumilog (Vectronic Aerospace).

***Figure 16: Rumilogger collar placed on reindeer**. The collar receives data about heart rate (HR) and rumen temperature (T_r) from an ingested rumilogger that measure HR and T_r.*

2.2.3.4 Cortisol

At every disturbance, saliva samples were collected for cortisol analyses using the SalivaBio Children's Swab (Salimetrics, State College, PA), a synthetic swab specifically designed to improve volume collection and increase participant compliance and validated for use with salivary cortisol. The salvia was retrieved before the reindeer were given food to reduce sample contamination. After sampling, the swab was placed into a tube, immediately placed on ice, and then transferred to a freezer at -20ºC. The samples were stored at -20ºC until the assay.

Samples were taken out of the freezer, thawed, and centrifuged at 1500 rpm (SL 40, Thermo Scientific™) for 15 minutes. An enzyme-linked immunosorbent assay (ELISA) kit (Salimetrics Assay #1-3102) was used for these analyses. 25 µl of assay diluent to serve as zero, standards,

controls and saliva samples were pipetted into wells. The enzyme conjugate was diluted 1:1600 in assay diluent, and 200 µl of the dilution was added to each well. The plate was mixed for 5 minutes at 500 rpm (SL 40, Thermo Scientific™) before being incubated for 1 hour. The plate was washed 4 times with 1:10 diluted 10xWash buffer. 200 µl of 3,3',5,5'-Tetramethylbenzidine (TMB) substrate solution was added to each well before mixing for 5 minutes at 500 rpm (SL 40, Thermo Scientific™) and incubated in the dark for 25 minutes. The reaction was stopped by adding 50 µl of stop solution and mixed for 3 minutes at 500 rpm (SL 40, Thermo Scientific™). The plate was read at 450 nm with 490 nm as reference.

2.2.3.5 Ambient temperature and door movement

T_a and door movement was recorded using a Ruuvi tag (RuuviTag Bluetooth Sensor, Ruuvi, https://ruuvi.com/ruuvitag/) which was placed inside the room. The tag uses Bluetooth, and the data was therefore automatically uploaded to Grafana.

2.2.3.6 Light conditions

Light conditions were recorded using an actiwatch (MotionWatch 8, CamNtech Ltd, camtech.com) placed on the wall of the room and retrieved using MotionWare 1.3.17 (camtech.com). Light control was via the Hugo Muller light control system and the save n carry software.

2.2.4 Analyses

T_r and HR data were filtered with FIWI filtering program (https://rumina.fiwi.at/). All raw data were handled in excel (Microsoft 365 personal). Several analyses do not allow missing data. Therefore, for T_r missing values were replaced with average T_r in RStudio (2022.07.2 + 576). The HR data contained about 80% missing data, therefore, these data were averaged for each hour for further analysis, and any missing data after averaging per hour was replaced with the HR average in RStudio (2022.07.2 + 576). FB was converted to a binary file (0: no eating and 1: eating) in RStudio (2022.07.2 + 576). Graphs made for illustration, except actograms, were made in RStudio (2022.07.2 + 576).

2.2.4.1 Actograms and periodograms

All actograms were made with Image J and plugin ActogramJ. For T_s the limits of the visual actogram were set to 30ºC - 34 ºC (1/21) and 28ºC - 32 ºC (2/21), to give a good representation of the fluctuations of in T_s. For T_r limits of the visual actogram was set to 38ºC and 39ºC, here also to give a good representation of the fluctuations. For LA the lower limit was set to 0

(activity counts/min) and upper limit to 300 (activity counts/min). For FB limits was set to 0 and 1. Chi-square periodogram (Sokolove & Bushell, 1978) in ActogramJ were used to analyze the periods. The periodograms were set to analyze for periods between 60 or 960 minutes and 1920 minutes and the significance level were set to 0.05.

2.2.4.2 Wavelets

Wavelets were made in RStudio (2022.07.2 + 576) with the WaveletComp package. A wavelet analysis was done on each dataset (see appendix D for R-code). The analysis performs a Mortlet wavelet transform and conduct a set of wavelet coefficients which gives an estimate on how much of the time series matches each scale and position of the wavelet. Power is directly linked to the coefficients as the power is calculated by squaring the coefficients. The power in the analysis therefore provide a measure of the amount of intensity or strength at each frequency and time in that timeseries (Rösch & Schmidbauer, 2016). The wavelet analysis was set to look for periods between 1 and 48 hours. The images are displayed with power spectrums were red indicates a stronger power and blue indicates no power.

2.2.4.3 Correlation and cross-correlation

Pearson's correlation analysis was done in RStudio (2022.07.2 + 576; see appendix D for R-code). which gives a correlation value ranging from 0 to 1, where 1 is means fully correlated and 0 means no correlation.

Cross-correlation analyses were performed in RStudio (2022.07.2 + 576; see appendix D for R-code). Cross-correlation analysis is a method used to see how similar two signals are by measuring how much they overlap when one is shifted over time. The cross-correlation analysis gives correlation values at different time lags where the value ranges from 0 to 1, where 1 is means fully correlated and 0 means no correlation.

Cross-wavelet analysis were performed with WaveletComp package in RStudio (2022.07.2 + 576). It uses the same principle as the wavelet analysis and analyze both series with a wavelet, then it gives a cross-wavelet coefficients which gives a measure of the phase angle and strength of the correlation between the two timeseries at different scales and positions in time (Rösch & Schmidbauer, 2016). The cross-wavelet analysis was set to look for correlation between periods of 1 and 48 hours and 22 and 28 hours. The images are displayed with cross-correlation power spectrums were red indicates a stronger correlation and blue indicates no correlation. Arrows

are displayed in the images to indicate a significant correlation ($p<0.05$) and the direction of the arrows indicate the phase angel.

2.2.4.4 Repeated measures ANOVA and paired t-test

Cortisol data was analyzed in RStudio (2022.07.2 + 576). A repeated analysis of variance (ANOVA) was conducted by fitting a model to the cortisol data with the disturbance (D1/D2), conditions (entrainment, control, second entrainment and forced desynchrony) and each reindeer as factor and all measurement was paired and used as the identifier (see appendix D for R-code). In addition, a paired Student's t-test were conducted between disturbance for each reindeer in each part of the protocol (see appendix D for R-code).

3 Results

3.1 The SCN of the reindeer expressed AVP and VIP

To investigate the anatomical characteristics of the reindeer's suprachiasmatic nucleus (SCN), I used digoxigenin in-situ hybridization to stain the mRNA of the proteins arginine vasopressin (AVP), vasoactive intestinal polypeptide (VIP), and gastrin-releasing peptide (GRP). The most rostral part of the SCN is located approximately where the optic chiasm merge with the hypothalamus (Figure 17). It is right next to the third ventricle (3V), and nearby areas include the paraventricular nucleus of the hypothalamus (PVN) and supraoptic nucleus (SON) (Figure 18).

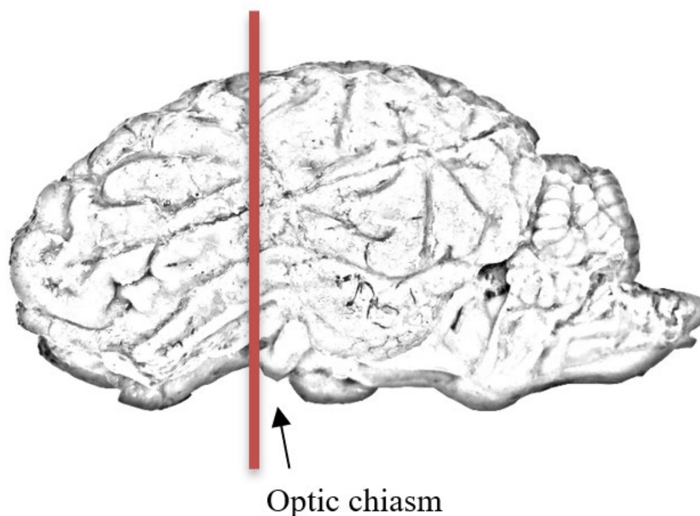

***Figure 17: Illustration demonstrating the most rostral part of the reindeer SCN.** The first sections of reindeer brain occurred approximately where the optic chiasm fuse with the hypothalamus and continued in 20 μm sections for about 2000 μm. Picture of sagittal view of sheep brain adapted from (Ashik, 2020).*

I found expression of AVP and VIP in the SCN (Figure 18; Appendix A). Additionally, I found that AVP and VIP were expressed in the supraoptic nucleus (SON) and that AVP was expressed in the paraventricular nucleus (PVN) (Figure 18; Appendix A). I did not find expression of GRP in the SCN or the immediate area around it (Figure 18). AVP and VIP are scattered throughout the SCN (Figure 19; Appendix A). The size of the SCN was estimated based on AVP and VIP expression.

I found that the rostral-caudal (RC) extent of the SCN was 1400 μm ± 522.54 μm, the medial-lateral (ML) extent was 1104.21 μm ± 361.21 μm, and the dorsal-ventral (DV) extent was 387.78 μm ± 186.72 μm. Table 2 compares the reindeer SCN to SCN in mouse (*Mus musculus)*,

hamster (*Mesocricetus auratus*), marsupials, cat (*Felis catus*), pig (*Sus domesticus*), guinea pig (*Cavia porcellus*), human (*Homo sapiens*), rat (*Rattus novegicus domestica*), and sheep (*Ovis aries*). Scaled to body size the SCN of reindeer is in (assuming a size of 90 kg) the RC extent 16 µm/kg, the ML extent 12 µm/kg, and the DV extent 4 µm/kg, compared to a mouse where (assuming a weight of 20 g) the RC extent is 30 000 µm/kg, the ML extent is 15 000 µm/kg and the DV extent is 17 500 µm/kg, it is 2000 times smaller.

In conclusion, the reindeer SCN express the neuropeptides AVP and VIP but not GRP, similar to the sheep (Dardente et al., 2016).

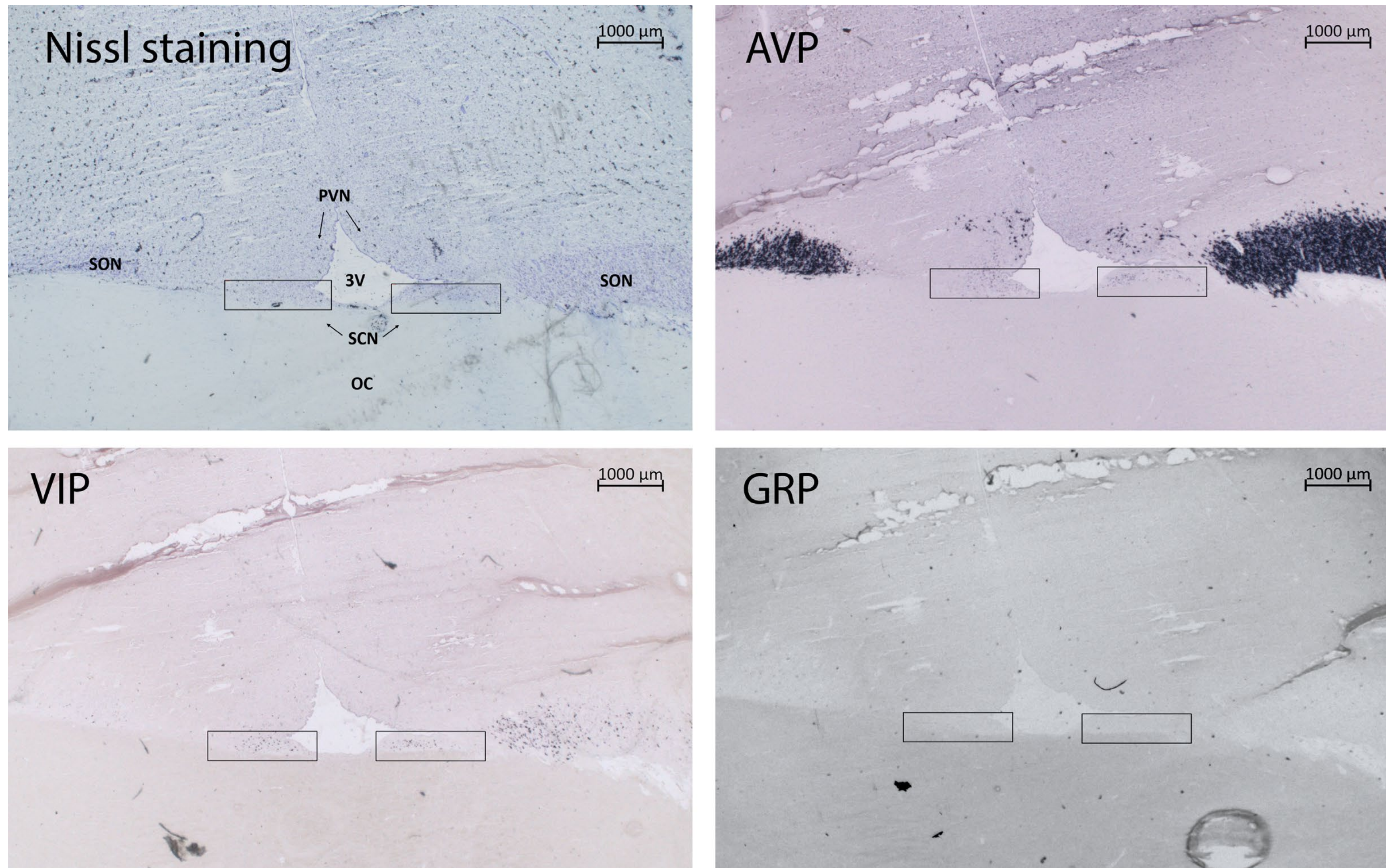

Figure 18: Representative pictures (Reindeer 12/10) of stained reindeer SCN and the areas around it*. Pictures showing nissl staining and staining of the mRNA of the proteins arginine vasopressin (AVP), vasoactive intestinal polypeptide (VIP) and gastrin-releasing polypeptide (GRP). The pictures include the anatomical structures; suprachiasmatic nucleus (SCN), indicated by black boxes; optic chiasm (OC); supraoptic nucleus (SON); paraventricular nucleus (PVN), indicated by arrows and the third ventricle (3V). The size of the structures is indicated by the scale bar in the upper right conor.*

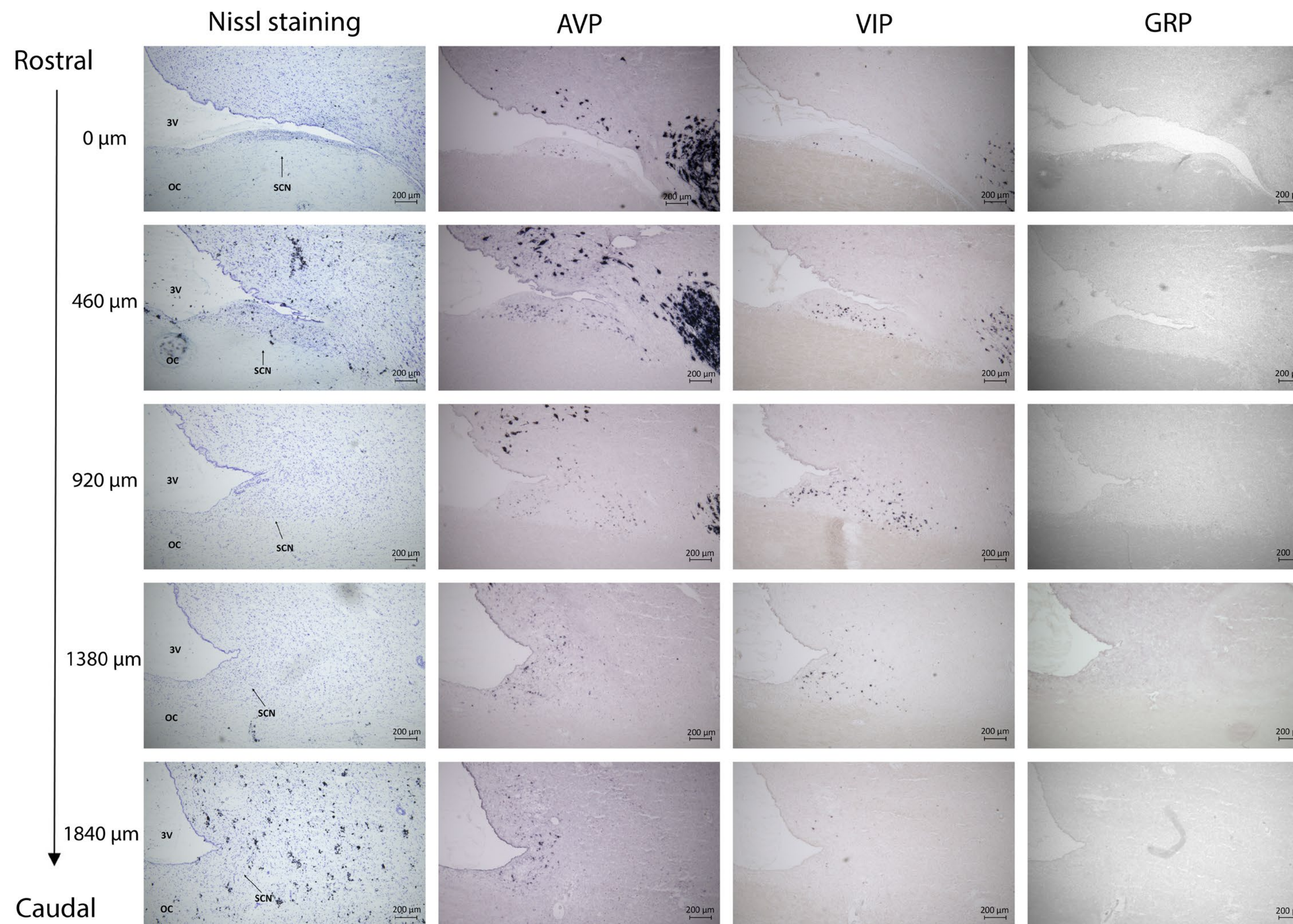

Figure 19: Pictures of Nissl, AVP, VIP and GRP staining and the rostral caudal extent of that staining in the region of the reindeer SCN. *Representaive pictures (Reindeer 12/10) showing nissl staining and staining of the mRNA of the proteins arginine vasopressin (AVP), vasoactive intestinal polypeptide (VIP) and gastrin-releasing polypeptide (GRP). The pictures include the anatomical structures; suprachiasmatic nucleus (SCN), indicated by arrows, optic chiasm (OC), and the third ventricle (3V). The size of the structures is indicated by the scale bar in the lower right conor. Pictures represent how the SCN changes from rostral to caudal. Each line is consecutive slides with different staining, and downwards it is moving through the SCN. The distance between sections in each line is 460 µm.*

Table 2: Summary of the extent of the suprachiasmatic nucleus (SCN) and the extent of arginine vasopressin (AVP), vasoactive intestinal polypeptide (VIP) and gastrin-releasing peptide (GRP). *Abbreviations: RC = Rostral-caudal, ML = Medial-lateral, DV = Dorsal-ventral. Ventral =V, Dorsal=D, Medial=M, Lateral=L, Scattered =S, Rostral = R, Caudal = C, Intermediate = I, Not expressed in the SCN = NE, NA = not available.*

	Size (µm ± STD)	*AVP*	*VIP*	*GRP*
Mouse (Mus musculus) *Abrahamson and Moore (2001)*	RC: 600 ML: 300 DV: 350	R: DM I: DM & VL C: S	V	V
Hamster (Mesocricetus auratus) *Card and Moore (1984)*	RC: 650 ML: 300 DV: 150-600	R: S & DM I: DM	I: VL C: S	NA
Marsupials *Cassone et al. (1988)*	RC: 950 – 1650 ± 25-106 ML: 145-340 ± 7 – 42 DV: 187-620 ± 7-70	S	S	NA
Cat (Felis catus) *Cassone et al. (1988)*	RC: 2150 ± 66 ML: 616 ± 18 DV: 805 ± 27	D	V	NA
Pig (Sus domesticus) *Cassone et al. (1988)*	RC: 1740 ± 25 ML: 425 ± 11 DV: 725 ± 8	D	V	NA
Guinea pig (Cavia porcellus) *Cassone et al. (1988)*	RC: 755 ± 10 ML: 256 ± 7 DV: 316 ± 10	R: S C: DM	VM	NA
Human (Homo sapiens) *Hofman et al. (1996)*	NA	D	R: S C: V	NA
Rat (Rattus norvegicus domestica) *Moore et al. (2002)*	RC: 730 ± 68 ML: 300	DM	V	V
Sheep (Ovis aries) *Dardente et al. (2016) & Tillet et al. (1989)*	NA	S	S	NE
Reindeer (Rangifer tarandus) *Figure 18*	RC: 1400 ± 522.54 ML: 1104.21 ± 361.21 DV: 387.78 ± 186.72	S	S	NA

3.2 An alternating feeding protocol in constant darkness conditions confounds the investigation of circadian rhythms in reindeer

The pilot study aimed to investigate whether an alternating feeding protocol was sufficient to investigate circadian rhythms. During the protocol, there was clear evidence of circadian entrainment of the animal under a light-dark regime of 16 hours light and 8 hours dark (LD 16:8), both seen in locomotor activity (LA; $p<0.05$) and surface body temperature (T_s; $p<0.05$) (Figure 20a-b; Figure 21a-b). However, this rhythm seems to dampen throughout the LD 16:8. Under the constant darkness, there was evidence of a free-running circadian rhythm both for LA at 30 hours, and a small peak at 24 hours ($p<0.05$) and for T_s at 25 hours ($p<0.05$) (Figure 20a-b; Figure 21a-b). A LA of 30 hours can be the result of the protocol as there is 30 hours between 09:00 one day to 15:00 the next. Patterns were similar for both animals in LD 16:8 (Appendix B), in the constant darkness however, for one (1/21) the LA rhythm breaks down faster than the T_s rhythm and for the other (2/22) there was no evidence of a 24-hour T_s component during constant darkness (Appendix B). This may be due to the way we measured T_s – as it is surface body temperature it can be affected by the ambient temperature if it is not insulated enough. For both animals the ultradian component is very strong and the frequency of this rhythm is between 2 and 8 hours (Figure 20; Figure 21).

When taking a closer look at the data, it was noticed that during the constant darkness, there were signs of patterns that showed a peak around 09:00 and 16:00 (Figure 22), suggesting that the feeding times could be masking the underlying circadian rhythm. Due to the limitation and potential masking effects of this protocol, a forced desynchrony protocol was designed.

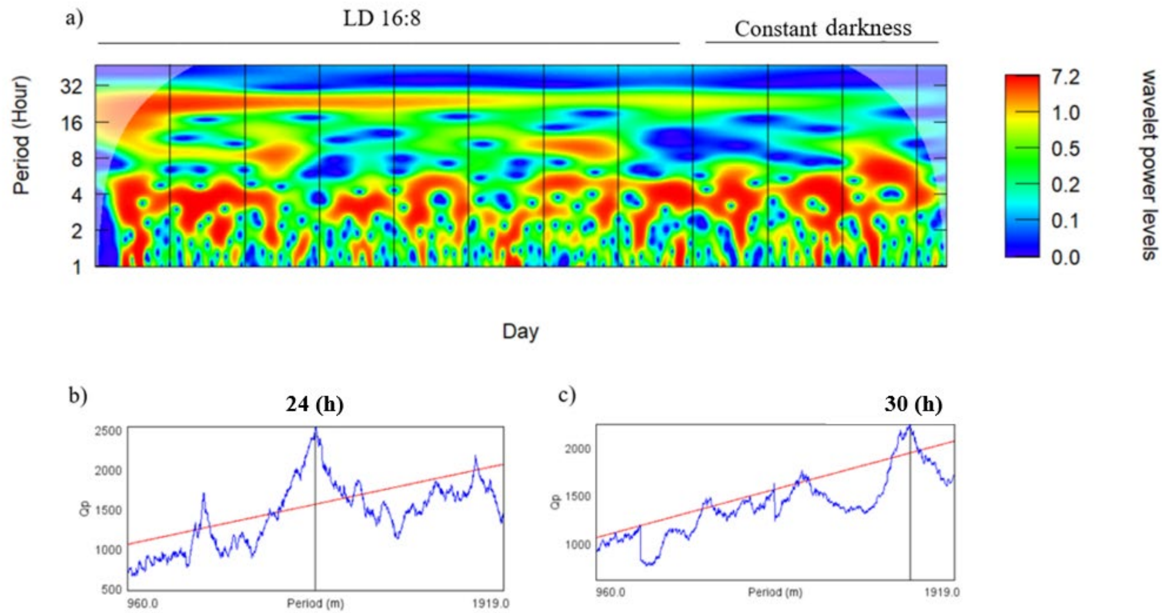

***Figure 20: Locomotor activity (LA) rhythm of reindeer (1/21) during pilot study**. The study is divided into two parts: an entrainment phase (8 days) with a light-dark cycle of 16 hours light and 8 hours dark (LD 16:8) and a period of constant darkness (3 days). a) wavelet presenting the power of the periods. The wavelet power goes from 0 (dark blue) to 7.2 (red). A stronger power indicates a stronger rhythm. The wavelet shows a strong 24-hour period during LD 16:8 with a subsequently dampening during constant darkness. The wavelet shows strong ultradian components in the LA rhythm of reindeer. b) chi-square test of LA during LD 16:8 shows a significant peak (p<0.05) at 24 hours. c) chi-square test of LA during constant darkness shows a significant peak (p<0.05) at 30 hours. Actogram for periodograms is shown in Appendix B (figure B-3).*

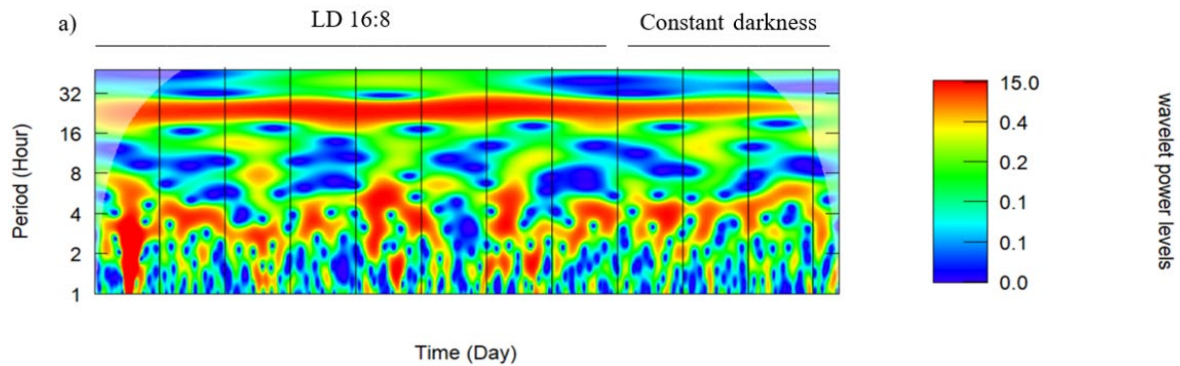

***Figure 21*: *Surface body temperature (T_s) rhythm of reindeer (1/21) during pilot study*.** *The study is divided into two parts: an entrainment phase (8 days) with a light-dark cycle of 16 hours light and 8 hours dark (LD 16:8) and a period of constant darkness (3 days). a) wavelet presenting the power of the periods. The wavelet power goes from 0 (dark blue) to 15 (red). A stronger power indicates a stronger rhythm. The wavelet shows a strong 24-hour period during LD 16:8 with a subsequently dampening during constant darkness. The wavelet shows strong ultradian components in the T_s rhythm of reindeer. b) chi-square test of T_s during LD 16:8 shows a significant peak ($p<0.05$) at 23.75 hours. c) chi-square test of T_s during constant darkness shows a significant peak ($p<0.05$) at 25 hours. Actogram for periodograms is shown in Appendix B (figure B-3).*

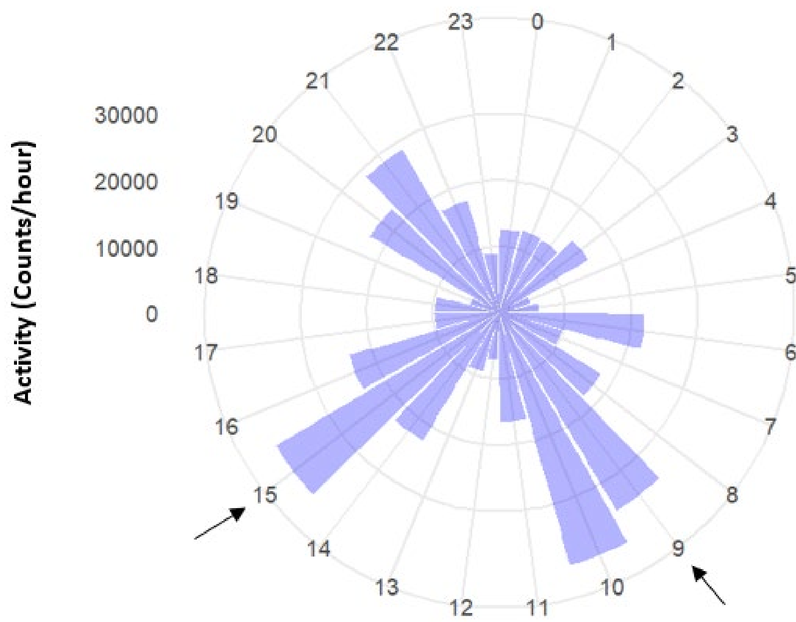

Figure 22: Cicular barplot illulstrating how the locomotor activity (LA) of reindeer (1/21) increased during the constant darkness in the pilot study. *The LA show a high increase when the animal is fed at 09:00 or 15:00 – indicated by arrrows in the plot, creating a potential masking effect. The circular barplot show the hours throughout the day and the bars show the summed activity (count/hour), going from 0 to 40000, of reindeer for each hours during the day of the dark in the protocol. Each cicular line in the circular barplot represents the numbers displayed at the top left cornor.*

3.3 Forced desynchrony

The forced desynchrony protocol was designed based on the pilot study. The pilot study gave evidence of visits affecting the LA of the animals (Figure 22) in addition to a potential sustained T_s (Figure 21). The forced desynchrony protocol were therefore designed to uncouple external rhythms and internal rhythms.

LA were recorded for investigating how the external rhythm of reindeer were expressed in the forced desynchrony. Feeding behavior (FB) was recorded for the purpose of investigating if FB could be a driver of rhythms in reindeer. During the course of the protocol the reindeer unfortunately started to interfere with (i.e. play with) the set-up of the feeding monitors. As the monitor were attached with magnet, they were at risk of falling – which they started to do in the middle of the protocol. The first monitor was discovered outside the feeding station of reindeer 3/22 at day 10. The further we got into the experiment, the more frequently the feeding monitors were found outside the feeding station or in the food box of the animals. These monitors stop recording if the battery dies, which it can if it gets too cold or if it gets wet. The feeding monitors were, among other places, found in the water bucket of the reindeer. Two of the monitors therefore stopped working during the experiment, at day 14 and day 17, for reindeer 1/22 and 3/22 respectively.

Rumen temperature (T_r), heart rate (HR) and saliva cortisol levels were recorded to investigate how the internal rhythm of reindeer were expressed during the forced desynchrony. The HR data had about 80% missing date because it only display the resting HR of the animal – this was confirmed by checking that there only were HR data when the animal was resting (appendix C; Figure C-1 & Figure C-2). Reindeer are ruminant and uses a major part of the day on grazing/eating meaning that there is little info in the HR data for the purpose it was recorded. HR data will therefore not be displayed as a parameter of its own and is not used in the assessment of forced desynchrony, it is however included in chapters of the results to explain stress level and ultradian rhythmicity. Cortisol was only measured two times a day and there is not enough data to give a good assessment of if there exist an internal rhythm. It is therefore not either included in assessment of forced desynchrony but is commented on and included in the chapter to look at stress levels.

Therefore, in this result section LA, FB and T_r will first be presented independently to assess how each parameter were affected by the protocol. Then a comparison of these parameters will follow to see if the forced desynchrony protocol did separate the external and internal rhythms.

Lastly, I will present data on the confounding factors social entrainment, ambient temperature (T_a), and stress.

3.3.1 No evidence of a circadian pattern of locomotor activity, feeding behavior and rumen temperature in forced desynchrony protocol

LA, FB and T_r were independently assessed with the use of actograms and periodograms. To give a visual view of the data actograms were made for LA, FB, and T_r. LA and FB were similar across animals, therefore actograms and periodograms of reindeer 2/22 is displayed here, the others are found in appendix C. For the period analyses each part of the experiment were tested with chi-square periodograms that were set to test for rhythm between 1 hour and 32 hours. From the actograms LA seem to be affected by human disturbances. It looks like the FB increases most after disturbance-1 (D1), and it is worth noticing that at this disturbance food is changed. Water intake have previously been seen to affect T_r (Herberg et al., 2018) and a threshold of 37.54°C were conducted on the datasets, however, this filtering only resulted in a few datapoints being removed and did not affect the results.

During the first entrainment (LD 12:12) there is a clear LA pattern around 24 hours ($p<0.05$; Figure 23), this is also true for FB ($p<0.05$; Figure 24) and T_r ($p<0.05$; Figure 25; Figure 26). During the control these pattern breaks down for LA, FB and T_r ($p<0.05$; Figure 23; Figure 24; Figure 25; Figure 26). For LA and T_r for one animal (1/22) these rhythm bouts come in four hours intervals (Figure 23; Figure 25). The second entrainment is sufficient in re-entraining to a 24-hour rhythmicity for LA, FB for all animals and T_r for one reindeer (1/22) ($p<0.05$; Figure 23; Figure 24; Figure 25). The forced desynchrony affects the rhythms and LA, FB and T_r (1/22) shows peaks around 14 hour and 28 hour ($p<0.05$; Figure 23; Figure 24; Figure 25). In addition, LA, FB and T_r have an approximately 24 hour significant peak during the forced desynchrony ($p<0.05$; Figure 23; Figure 24; Figure 25; Figure 26).

LA, FB and T_r show a diel 24-hour pattern in a light dark cycle. This diel pattern breaks down in constant dim light, even with 12 hourly disturbances, and in the forced desynchrony phase of the experiment. Therefore, there is no strong evidence for circadian rhythms and the defining time component in these factors appears to be ultradian.

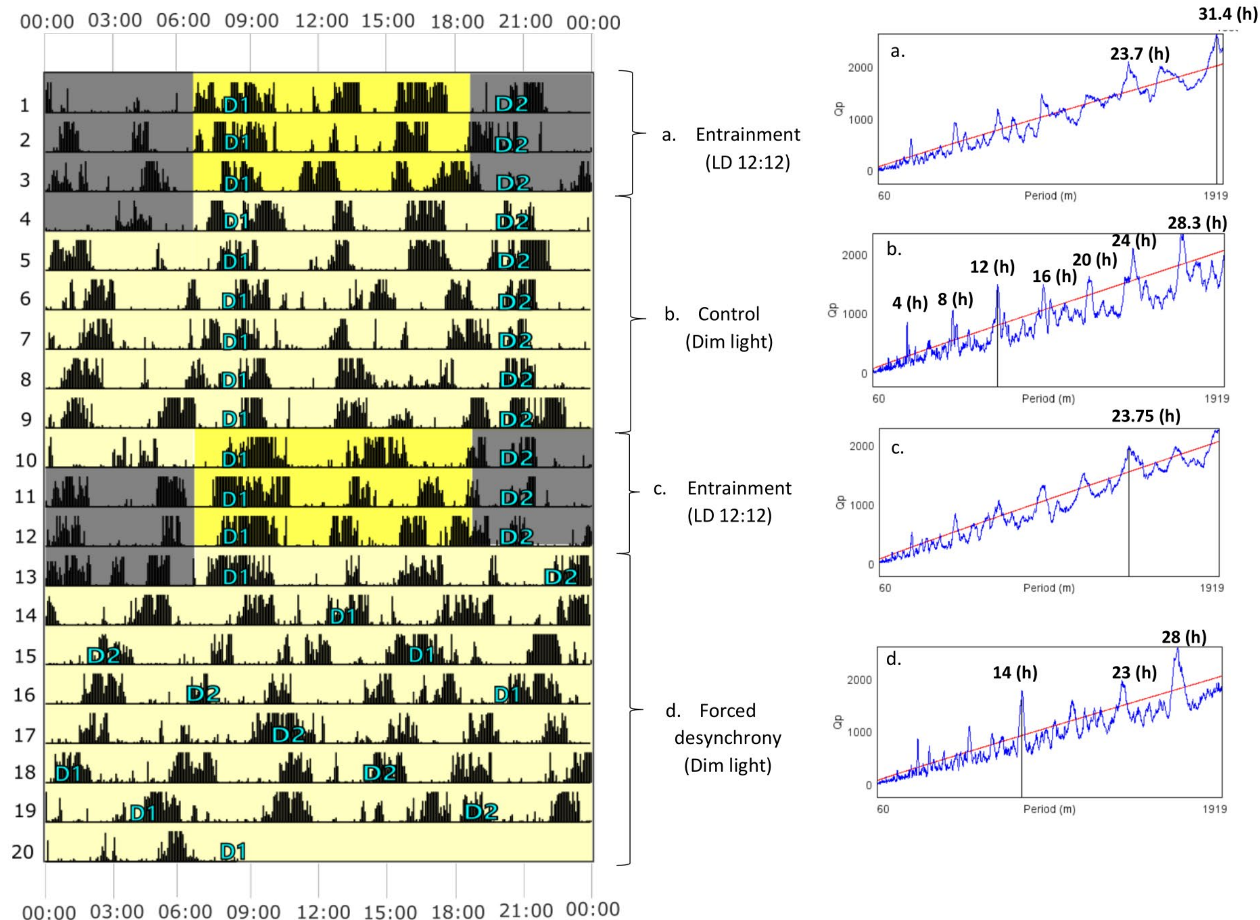

Figure 23: Actogram and periodograms of locomotor activity (LA) measurements from reindeer 2/22. *LA is measured with Actiwacth attached to the harness of the reindeer. On the left panel the actogram with raw data is displayed. Each line represents one day in the experiment as is represented by the numbers on the left of the actogram. On the top and bottom of the actogram the time of day is displayed. On the right of the actogram each part of the protocol is demonstrated. a) the first entrainment with a light regime of 12 hours dark and 12 hours light, lights on at 06:30 and lights of at 18:30 (LD 12:12) with disturbances every 12 hours at 08:30 and 20:30. b) The second part is the control with constant dim light and disturbances every 12 hours at 08:30 and 20:30. C) the second entrainment follows the same regime as a). d) the forced desynchrony protocol with constant dim light and disturbances every 14 hours as described in Table 1. D1 and D2 represent disturbance-1 and disturbance-2, respectively. The lower limit of the actogram was set to 0 and the upper limit set to 300. The right panel show the respectively periodograms for each part of the protocol. Tested with a chi-square periodogram with a significance level of 0.05.*

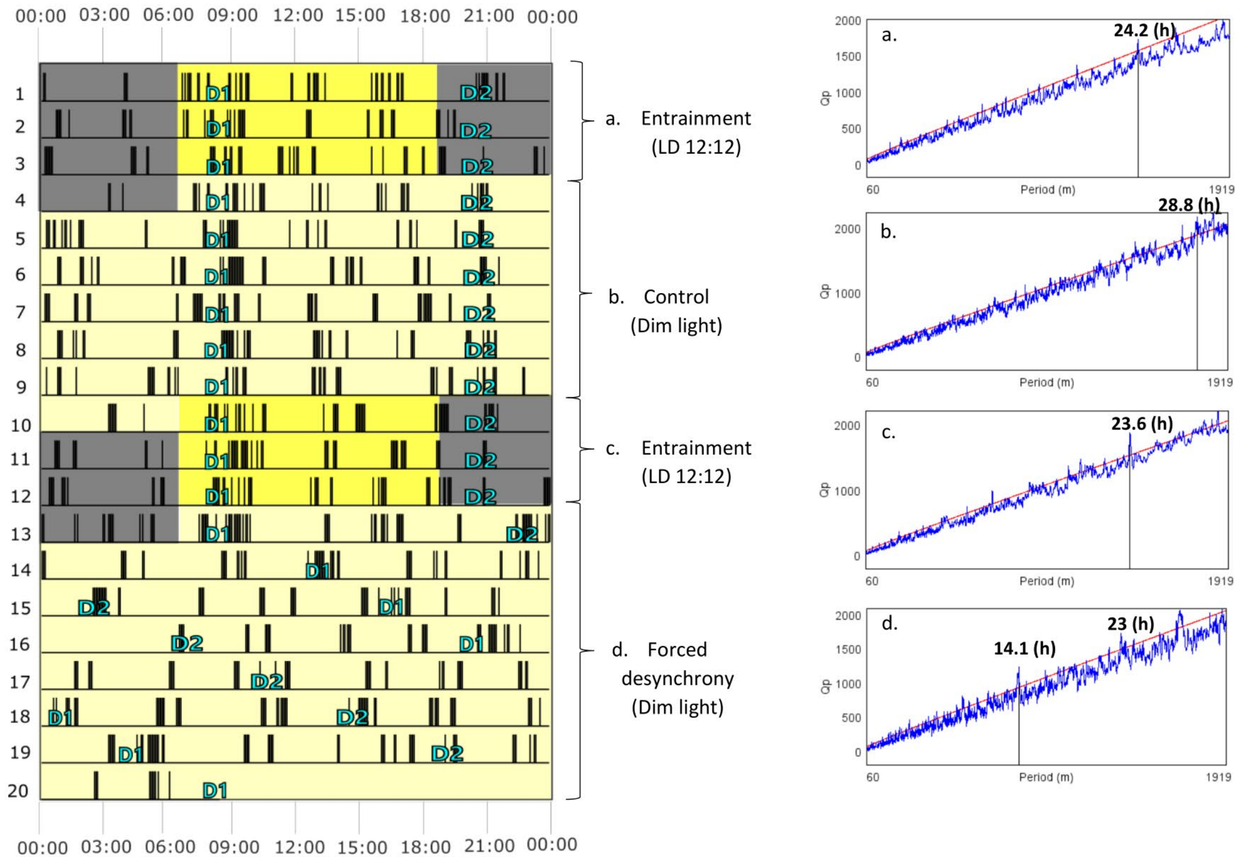

Figure 24: Actogram and periodograms of feeding behavior (FB) from reindeer 2/22. *FB is measured with HOBO loggers in feeding station. On the left panel the actogram with the binary data is displayed. Each line represents one day in the experiment as is represented by the numbers on the left of the actogram. On the top and bottom of the actogram the time of day is displayed. On the right of the actogram each part of the protocol is demonstrated. a) the first entrainment with a light regime of 12 hours dark and 12 hours light, lights on at 06:30 and lights of at 18:30 (LD 12:12) with disturbances every 12 hours at 08:30 and 20:30. b) The second part is the control with constant dim light and disturbances every 12 hours at 08:30 and 20:30. C) the second entrainment follows the same regime as a). d) the forced desynchrony protocol with constant dim light and disturbances every 14 hours as described in Table 1. D1 and D2 represent disturbance-1 and disturbance-2, respectively. The lower limit of the actogram was set to 0 and the upper limit set to 1. The right panel show the respectively periodograms for each part of the protocol. Tested with a chi-square periodogram with a significance level of 0.05.*

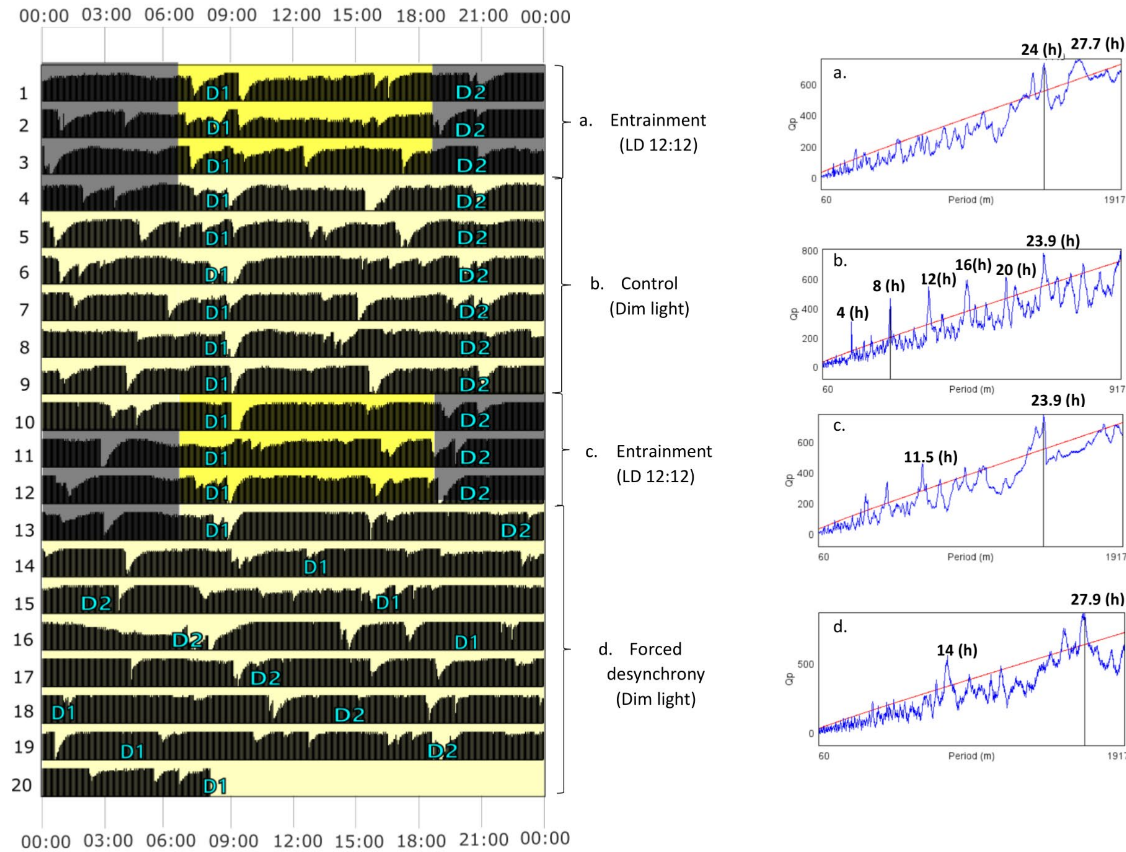

Figure 25: Actogram and periodograms of rumen temperature (T_r) from reindeer 1/22*. T_r recorded by implanted rumiloggers. On the left panel the actogram with data is displayed. Each line represents one day in the experiment as is represented by the numbers on the left of the actogram. On the top and bottom of the actogram the time of day is displayed. On the right of the actogram each part of the protocol is demonstrated. a) the first entrainment with a light regime of 12 hours dark and 12 hours light, lights on at 06:30 and lights of at 18:30 (LD 12:12) with disturbances every 12 hours at 08:30 and 20:30. b) The second part is the control with constant dim light and disturbances every 12 hours at 08:30 and 20:30. C) the second entrainment follows the same regime as a). d) the forced desynchrony protocol with constant dim light and disturbances every 14 hours as described in Table 1. D1 and D2 represent disturbance-1 and disturbance-2, respectively. The lower limit of the actogram was set to 38 and the upper limit set to 39. The right panel show the respectively periodograms for each part of the protocol. Tested with a chi-square periodogram with a significance level of 0.05.*

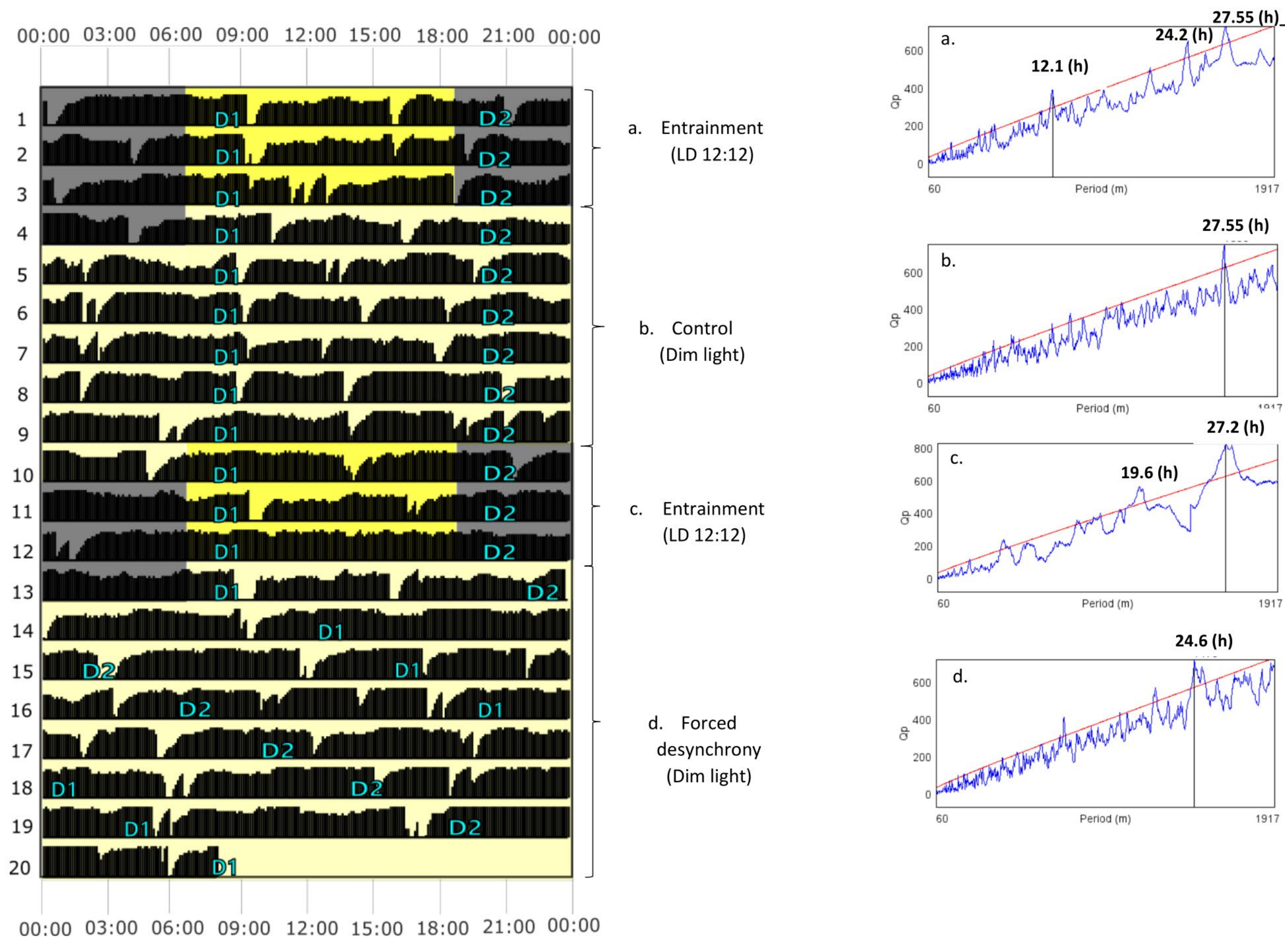

Figure 26: Actogram and periodograms of rumen temperature (T_r) from reindeer 2/22*. T_r recorded by implanted rumiloggers. On the left panel the actogram with data is displayed. Each line represents one day in the experiment as is represented by the numbers on the left of the actogram. On the top and bottom of the actogram the time of day is displayed. On the right of the actogram each part of the protocol is demonstrated. a) the first entrainment with a light regime of 12 hours dark and 12 hours light, lights on at 06:30 and lights of at 18:30 (LD 12:12) with disturabances every 12 hours at 08:30 and 20:30. b) The second part is the control with constant dim light and disturbances every 12 hours at 08:30 and 20:30. C) the second entrainment follows the same regime as a). d) the forced desynchrony protocol with constant dim light and disturbances every 14 hours as described in Table 1. D1 and D2 represent disturbance-1 and disturbance-2, respectively. The lower limit of the actogram was set to 38 and the upper limit set to 39. The right panel show the respectively periodograms for each part of the protocol. Tested with a chi-square periodogram with a significance level of 0.05.*

3.3.2 Ultradian rhythms are the strongest component in reindeer

Reindeer have previously been proven to have strong ultradian rhythms (van Oort et al., 2007), and in the periodograms analyses it was clear that several rhythms are present in animals. To investigate the difference in power of the rhythms in these reindeer I analyzed all components with the use of wavelet analyses (Figure 27). Wavelet analyses of LA, FB, T_r and HR reveals that the 24-hour diel rhythm when a light-dark cycle is present is very weak and that it dampens quickly in constant light, even with 12-hour disturbances present. The strongest rhythmic component in reindeer is ultradian rhythms and holds periods between 2 hours and 8 hours.

In conclusion, wavelet analysis gives further evidence of a lack of circadian rhythmicity in reindeer in constant conditions as wavelets of LA, FB, T_r and HR have a very strong ultradian component with a very rapidly dampening of any 24-hour rhythm in constant light.

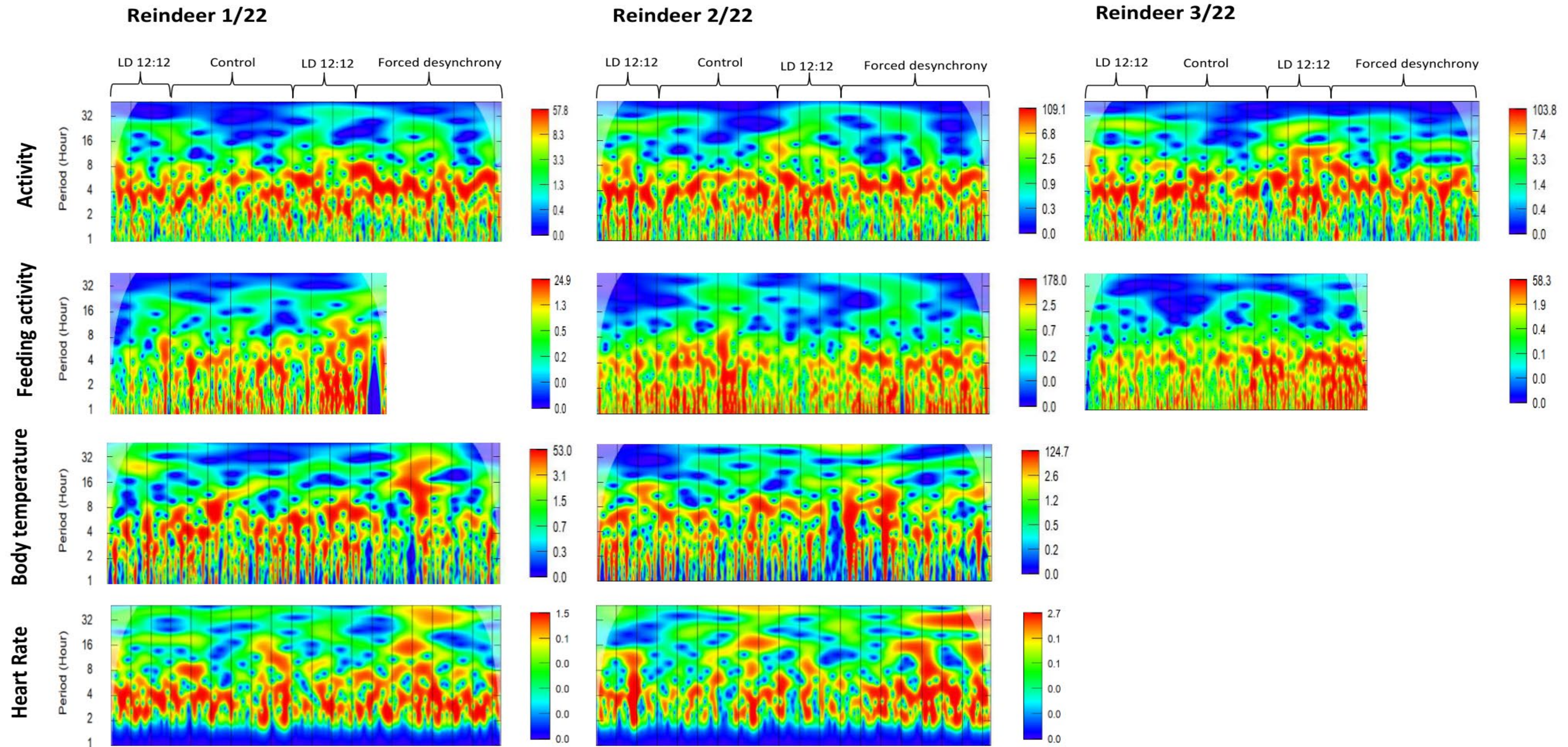

Figure 27: Wavelet analysis of locomotor activity (LA), feeding behavior (FB), rumen temperature (T_r) and heart rate (HR) in the three reindeer 1/22, 2/22 and 3/22. *From left to right in the figure the three animals are represented and from top to bottom the different parameters is displayed. The y-axis shows the periods that are analyzed in the power analyses, going from 1 to 48. On the top the protocol is illustrated. First, the first entrainment with a light regime of 12 hours dark and 12 hours light, lights on at 06:30 and lights of at 18:30 (LD 12:12) with disturbances every 12 hours at 08:30 and 20:30. The second part is the control with constant dim light and disturbances every 12 hours at 08:30 and 20:30. The second entrainment follows the same regime as the first and lastly the forced desynchrony protocol with constant dim light and disturbances every 14 hours as described in Table 1. Each line in the wavelets represents the days in the protocol. The power of the wavelet is displayed to the right of the figures. Blue indicates a low power and red indicates a strong power in the rhythm.*

3.3.3 The strong ultradian patterning in reindeer prevents desynchrony of internal from external rhythms

To investigate how the relationship between LA, FB and T_r changed during the course of the protocol analysis was done on the data from one of the reindeer (2/22) – for which a full dataset for FB was obtained. A visual representation was first made with a heatmap of T_r overlaying the actograms of LA and FB (Figure 28). What seems to be the case when looking at it, is that T_r decreases when the animal is active and feeding. To look how the relationship of the parameters is correlated I did cross-correlation analysis for all the parameters (Figure 29). These analyses were done both between the parameters during the control and during the forced desynchrony to investigate if the relationship between the parameters changed. The relationship between the parameters is very similar during the control and the forced desynchrony – and all parameters are correlated with T_r and FB of highest significance. Further cross-wavelet correlations were done to determine at which level the correlation appear (Figure 30). The cross-wavelet analyses reveal a significant correlation during forced desynchrony at periods between 22 and 28 hours, but compared to the cross-wavelet analysis for periods between 1 and 48 hours it becomes clear that that the cross-correlation between the parameters is most apparent at an ultradian level.

The strong ultradian component in reindeer prevents desynchronization of LA, FB and T_r. T_r is correlated to both LA and FB, with highest correlation to FB, indicating that feeding lowers the T_r or reindeer – which also prevents desynchronization of T_r from FB.

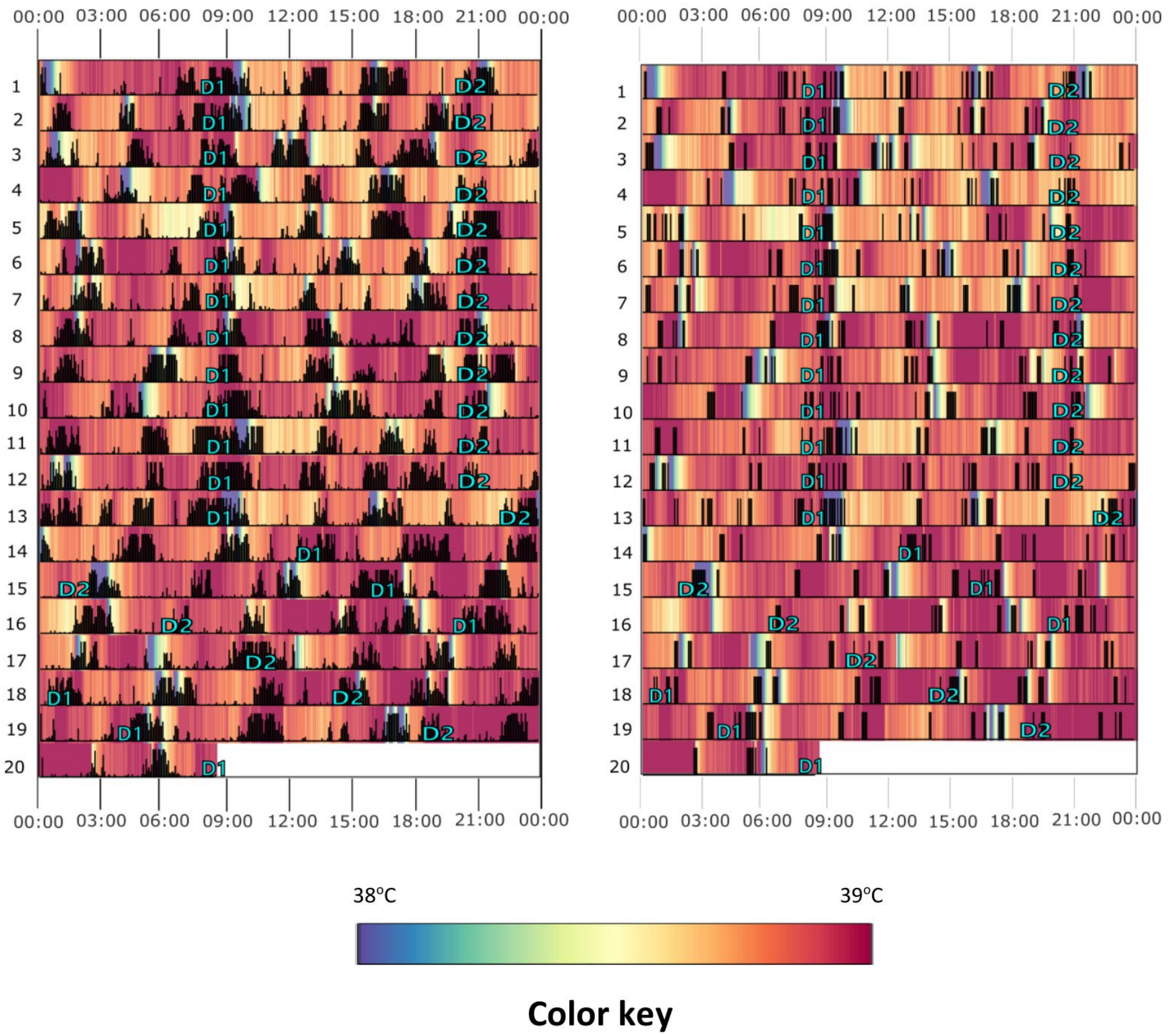

Figure 28: Locomotor activity (LA) and feeding behavior (FB) actograms with heatmaps of rumen temperature (T_r). *The left actogram shows LA and the right show FB. Each line represents one day in the experiment as is represented by the numbers on the left of the actogram. On the top and bottom of the actogram the time of day is displayed. The D1 and D2 represent disturbance-1 and disturbance-2, respectively. The lower limit of the actogram was set to 0 and the upper limit set to 300 for LA and lower limit was set to 0 and the upper limit was set to 1 for FB. The heatmap color key is displayed in on the bottom of the figure with blue as the lower value color and dark red as the upper value color. The values range from 38°C to 39°C.*

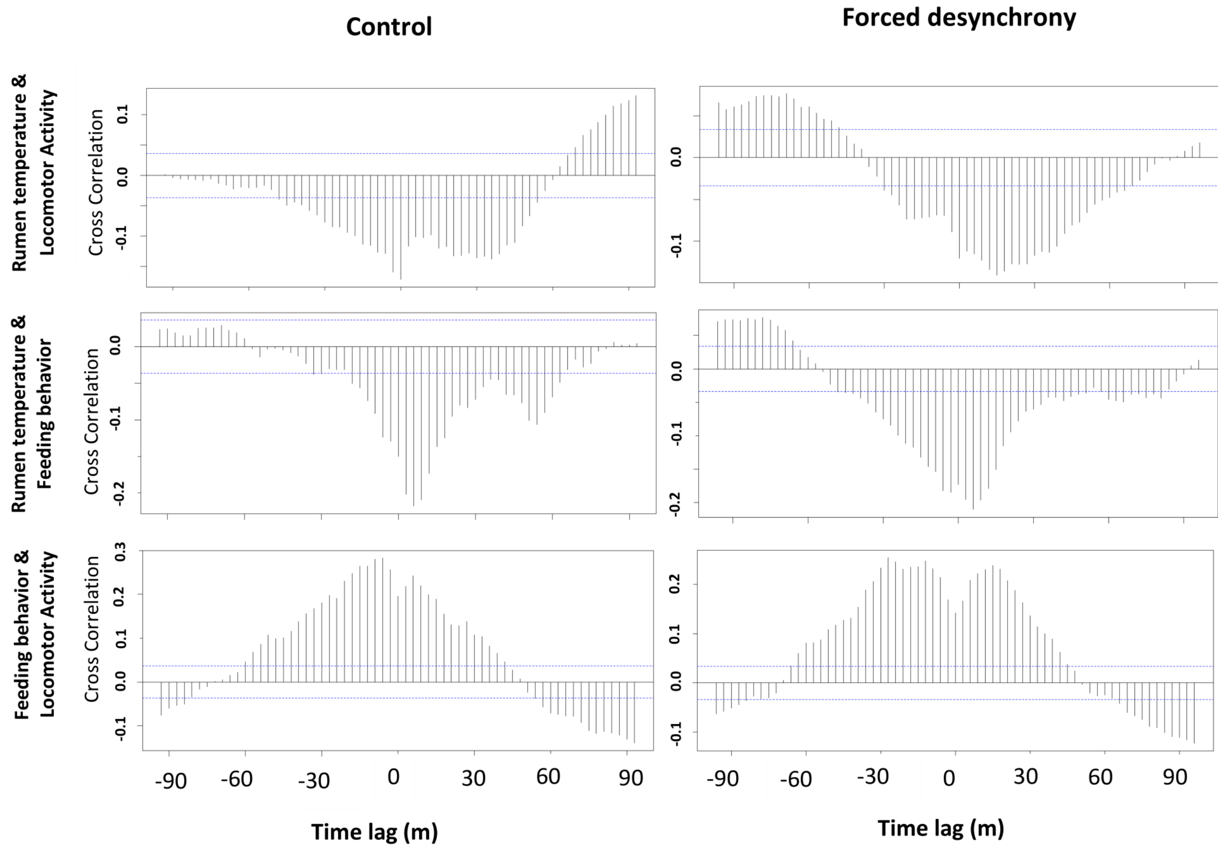

Figure 29: Cross-correlation between parameters of reindeer (2/22) during the control and forced desynchrony. *During the control, the highest significant value between rumen temperature and locomotor activity was at time lag 0 minutes (correlation: -0.171). The highest significant value between rumen temperature and feeding behavior was at time lag 6 minutes (correlation: -0.218). The highest significant value between feeding behavior and locomotor activity was at time lag -6 minutes (correlation: -0.283). During forced desynchrony the highest significant value between rumen temperature and locomotor activity was at time lag 15 minutes (correlation: -0.141). The highest significant value between rumen temperature and feeding behavior was at time lag 6 minutes (correlation: -0.210). The highest significant value between feeding behavior and locomotor activity was at time lag -27 minutes (correlation: -0.254).*

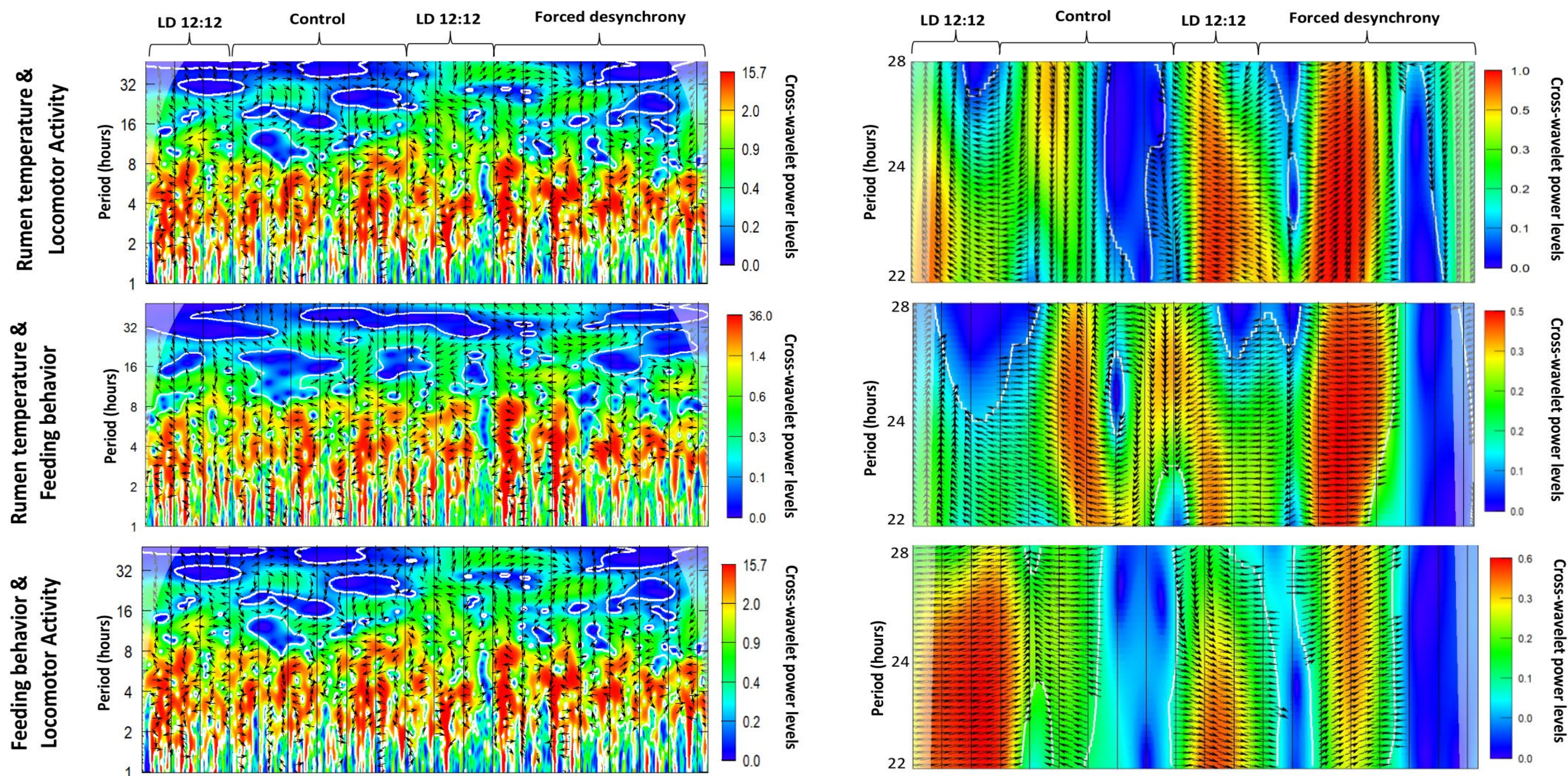

Figure 30: Cross-wavelets of locomotor activity (LA), feeding behavior (FB) and rumen temperature (T_r). *The left panel show cross-wavelets of periods between 1 and 48 hours. The right panel show cross-wavelets of periods between 22 and 28 hours. The y-axis shows the periods that are analyzed in the power analyses. On the top the protocol is illustrated. First, the first entrainment with a light regime of 12 hours dark and 12 hours light, lights on at 06:30 and lights of at 18:30 (LD 12:12) with disturbances every 12 hours at 08:30 and 20:30. The second part is the control with constant dim light and disturbances every 12 hours at 08:30 and 20:30. The second entrainment follows the same regime as the first and lastly the forced desynchrony protocol with constant dim light and disturbances every 14 hours as described in Table 1. Each line in the wavelets represents the days in the protocol. The power of the wavelet is displayed to the right of the figures. Blue indicates a low cross-correlation and red indicates a strong cross-correlation in the rhythms. Black arrows indicate a significant cross-correlation ($p<0.05$), and the direction of the arrow indicates at which phase the correlation appears.*

3.3.4 Testing for confounding factors

3.3.4.1 Reindeer show strong social organization of behavior

Reindeer have been noticed to be very social and follow the same actvity pattern in bigger groups (Russell et al., 1993). To see if social cues were an organiziser of LA in these animals actograms were made to visually represent the LA (Figure 31). Correlation between the LA of the animals were checked by using Pearson's correlation test. Between reindeer 1/22 and reindeer 2/22 there is a strong positive correlarion (0.51 $p<0.05$, CI (0.50 – 0.52)). Between reindeer 1/22 and 3/22 there is a strong positive correlation (0.52 $p<0.05$, CI (0.51 – 0.53)). Between reindeer 2/22 and 3/22 there is a strong positive correlation (0.44 $p<0.05$, CI (0.43 – 0.45)). To further understand the relationship of this correlation I did cross-correlation analyses between the LA of the animals, this revealed that the LA is highest correlated with zero time lag. This correlation is the same correlation as values from Pearson's correlation test (Figure 32).

Social entrainment is strong in reindeer and could therefore mask any underlying individual rhythm.

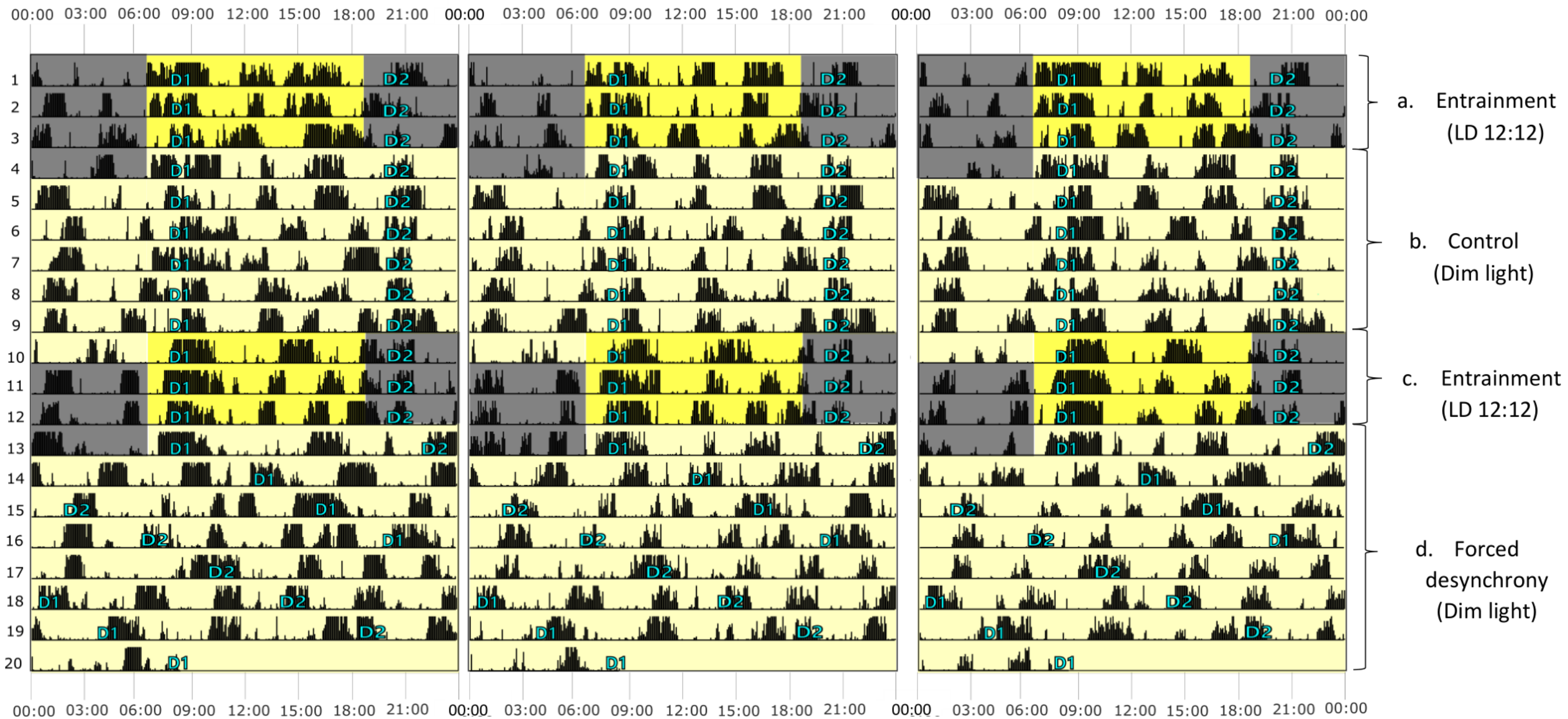

Figure 31: Locomotor activity (LA) actograms from three reindeer*. From left to right: 1/22, 2/22, 3/22. LA is measured with actiwacthes attached to the harness of the reindeer and the raw data from the actiwathes is displayed. On the top and the bottom of the actograms the time of day is displayed. Each line represents one day in the protocol. On the right of the actograms each part of the protocol is demonstrated. a) the first entrainment with a light regime of 12 hours dark and 12 hours light, lights on at 06:30 and lights of at 18:30 (LD 12:12) with disturbances every 12 hours at 08:30 and 20:30. b) The second part is the control with constant dim light and disturbances every 12 hours at 08:30 and 20:30. C) the second entrainment follows the same regime as a). d) the forced desynchrony protocol with constant dim light and disturbances every 14 hours as described in Table 1. D1 and D2 represent disturbance-1 and disturbance-2, respectively. The lower limit of the actogram was set to 0 and the upper limit set to 30*

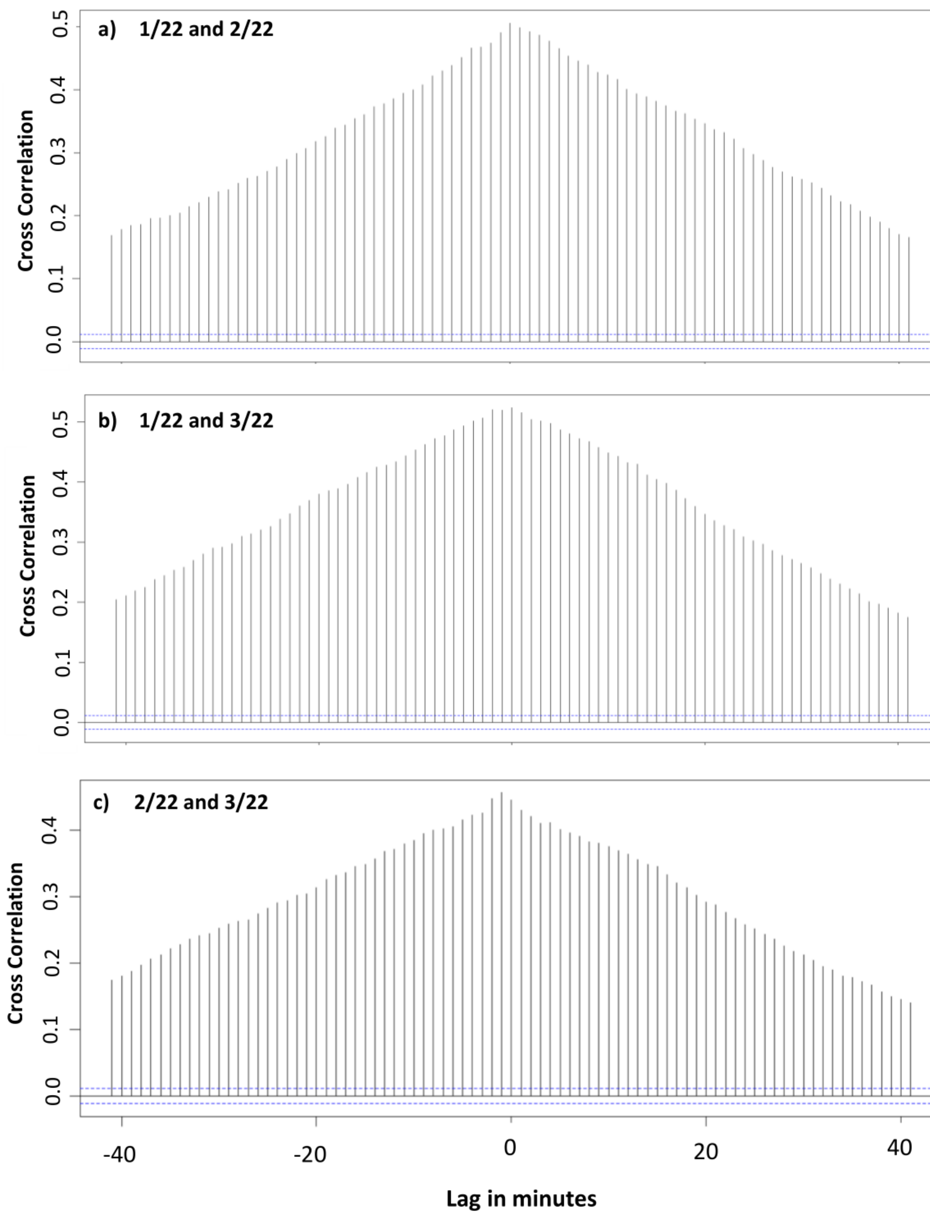

Figure 32: Cross-correlation of locomotor activity (LA) between reindeer. *Graphs representing the cross-correlation analyses between the LA of the three reindeer. The x-axis represents the time lag in minutes at where correlation occurs. The y-axis shows how large the correlation is at different time lags. The graphs show that the correlation is strongest at time lag 0 between all reindeer. The correlation at time lag 0 between reindeer 1/22 and 2/22 is 0.51. The correlation at time lag 0 between reindeer 1/22 and 3/22 is 0.52. The correlation at time lag 0 between reindeer 2/22 and 3/22 is 0.44.*

3.3.4.2 Ambient temperature may have provided a weak zeitgeber in the forced desynchrony phase of the experiment

I wanted to know if ambient temperature (T_a) affected the T_r of the reindeer. To get a visual view if there existed any rhythms in the T_a and how strong these rhythms are I analysed the T_a with a wavelet (Figure 33). There seem to be a strong 24 hour T_a rhythm in the experiment. I analysed the periods of the the T_a with chi-square periodograms and found a significant peak around 24 hours during the last three parts of the experiment ($p<0.05$). With the use of a Pearson's correlation test I found no significant correlation between T_a and reindeer T_r when looking at the whole experiment. However, with the use of a Pearson's correlation test I found a weak negative correlation between the T_r of reindeer 1/22 and T_a during the forced desynchrony part (correlation = -0.14, $p<0.05$, CI[-0.21, -0.06]) and a weak positive correlation between the T_r of reindeer 2/22 and T_a during the forced desynchrony part (correlation = 0.1, $p<0.05$, CI[0.02, 0.17]. It may not be that the T_a directly affected the body temperature of the reindeer, but T_a might work as a zeitgeber (Appendix C; figure C-7). To look at how much the T_a changed duing the forced desynchonry the average for each hour during the days of the forced desynchonry were plotted and it shows a clear pattern of rising about 0.5 C^o during the day (Figure 34).

In conclusion, the ambient temperature does not affect the body temperature directly, but may have functioned as a weak zeitgeber in the experiement, however, given the weak observation of a 24 hour rhythm in the parameters measured in the reindeer during the forced desynchrony phase of the protocol it is unlikely to have had a confounding effect.

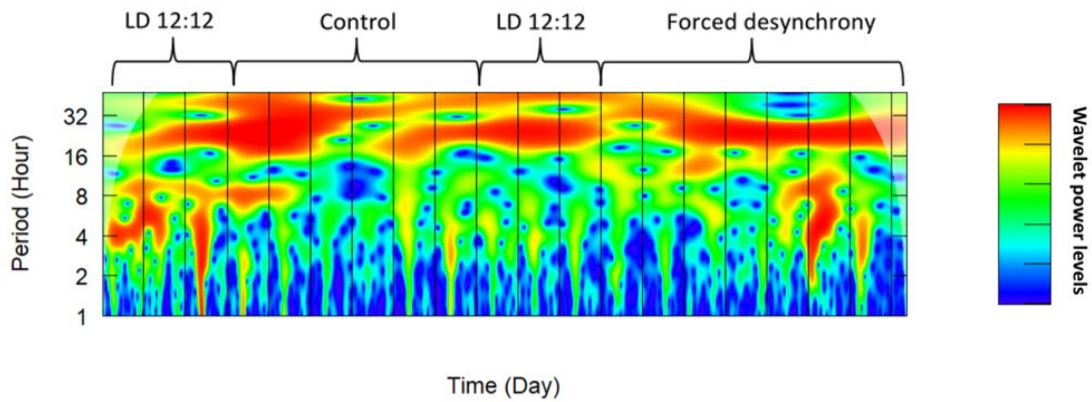

Figure 33: Wavelet analyses of ambient temperature*. The y-axis shows the periods that are analyzed in the power analyses, going from 1 to 32. On the top the protocol is illustrated. First, the first entrainment with a light regime of 12 hours dark and 12 hours light, lights on at 06:30 and lights of at 18:30 (LD 12:12) with disturbances every 12 hours at 08:30 and 20:30. The second part is the control with constant dim light and disturbances every 12 hours at 08:30 and 20:30. The second entrainment follows the same regime as the first and lastly the forced desynchrony protocol with constant dim light and disturbances every 14 hours as described in Table 1. Each line in the wavelets represents the days in the protocol. The power of the wavelet is displayed on the right side in the figure. Blue indicates a low power and red indicates a strong power in the rhythm.*

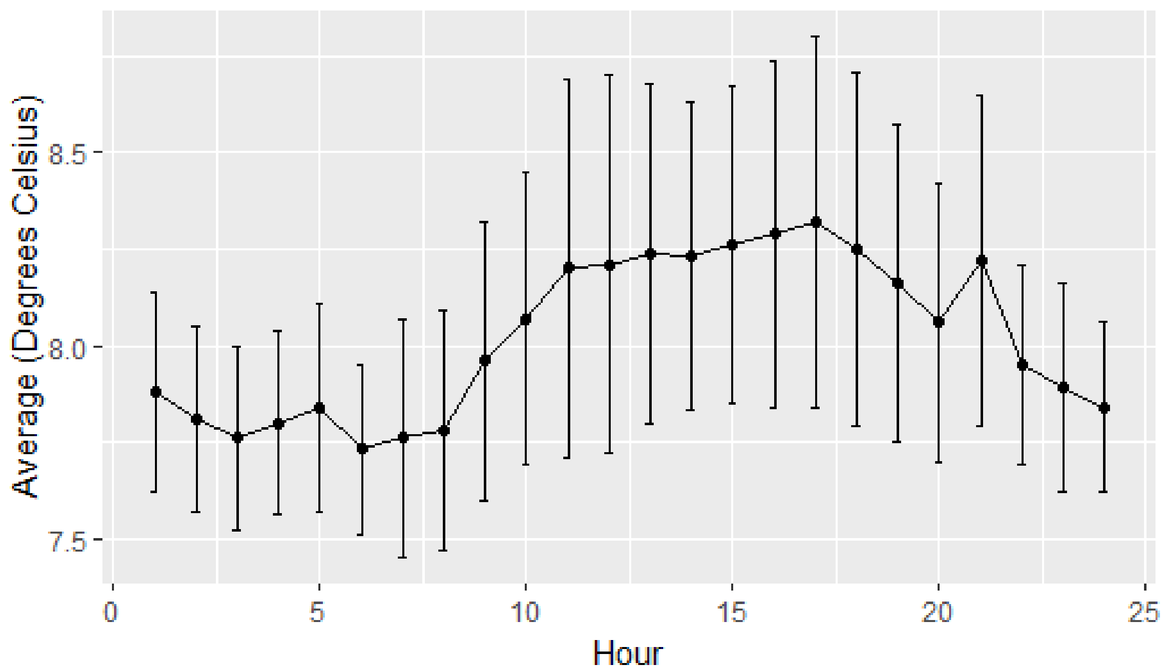

Figure 34: Average ambient temperature (T_a) for each hour of the days during forced desynchrony*. The average of each hour is plotted with standard deviation as error bars. The x-axis shows the hours and y-axis show the average in degrees Celsius.*

3.3.4.3 Heart rate and cortisol rose over the duration of the experiment suggesting that the Reindeer became stressed

Cortisol levels have never been looked at in reindeer before and cortisol was investigated to look if the reindeer display a cortisol rhythm and if it there was a presence of an internal rhythm of cortisol during the forced desynchrony. Due to missing points and the fact that this were only measure two times a day it could not be used to determine if cortisol remained a rhythm. There seem to be a trend in the rhythm of cortisol in presence of a light-dark cycle and during the entrainment (Figure 35; Figure 36; Figure 37), however, there is large variability in these data. A repeated measures ANOVA analysis showed that the only variance in the dataset that had significant impact ($p<0.05$) on the cortisol levels was at which conditions the animals were held in. As can be seen in the graphs (Figure 35; Figure 36; Figure 37) the cortisol levels rises during the course of the experiment, the same is true for HR (Figure 38; Figure 39) – which also were measured to investigate internal rhythms, but had missing values. Indicating that animals became stressed during by either the forced desynchrony protocol or by staying indoors for several weeks. A paired student t-tests were conducted on each part of the protocol for each reindeer between disturbance-1 and disturbance-2 for cortisol levels and there were no significant results ($p>0.05$).

In conclusion, cortisol is possible to measure in reindeer saliva, the trend seems to be that there is a rhythm during the entrainment and control. However, the tests show no significance between the two disturbances, this may be because the data measured in this experiment is of high variability and possibly due to few data points. The conditions had a significant effect on the cortisol levels – and by taken this together with HR the reindeer show indications of higher stress levels at the end of the experiment.

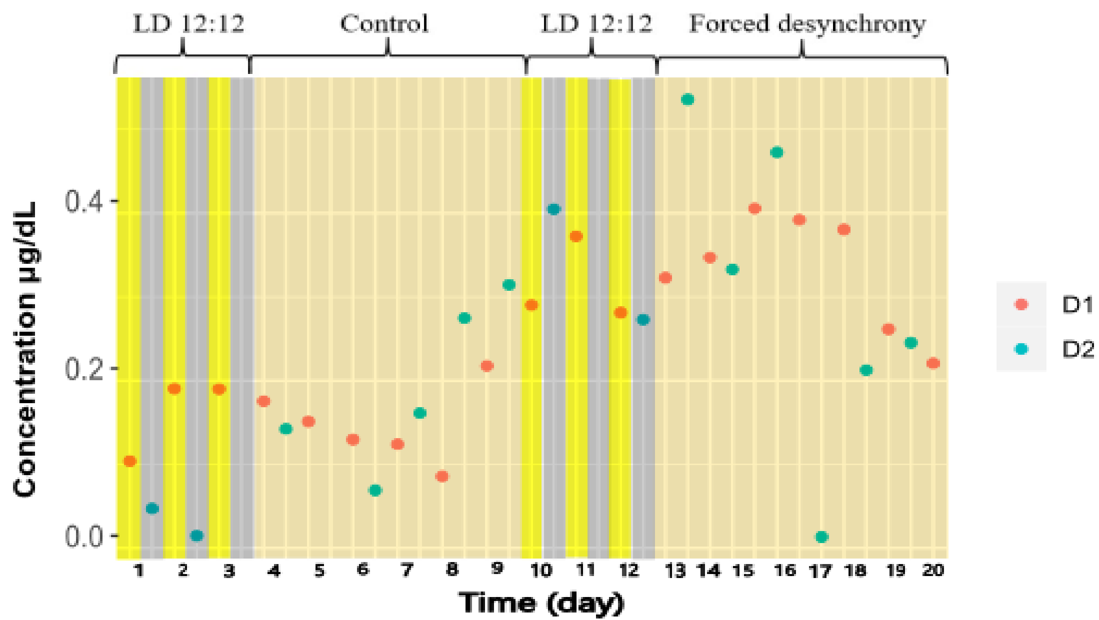

***Figure 35: Cortisol levels in reindeer 1/22**. The y-axis shows the concentration of cortisol (µg/dL) and the x-axis show the time course of the experiment. The graph is colored in with the light-regime conducted, light-dark cycle of 12-hour light (yellow) and 12-hour dark (grey) – entrainments and dim light (bright yellow) for the control and the forced desynchrony. Each dot represents one measurement, and the two different colors represent at which disturbance the measurement was taken, disturbance-1 (D1; red) and disturbance-2 (D2; blue). During the entrainments and the control, the samples were taken at 08:30 (D1) and 20:30 (D2) and during the forced desynchrony it was taken every 14th hour as described in Table 1.*

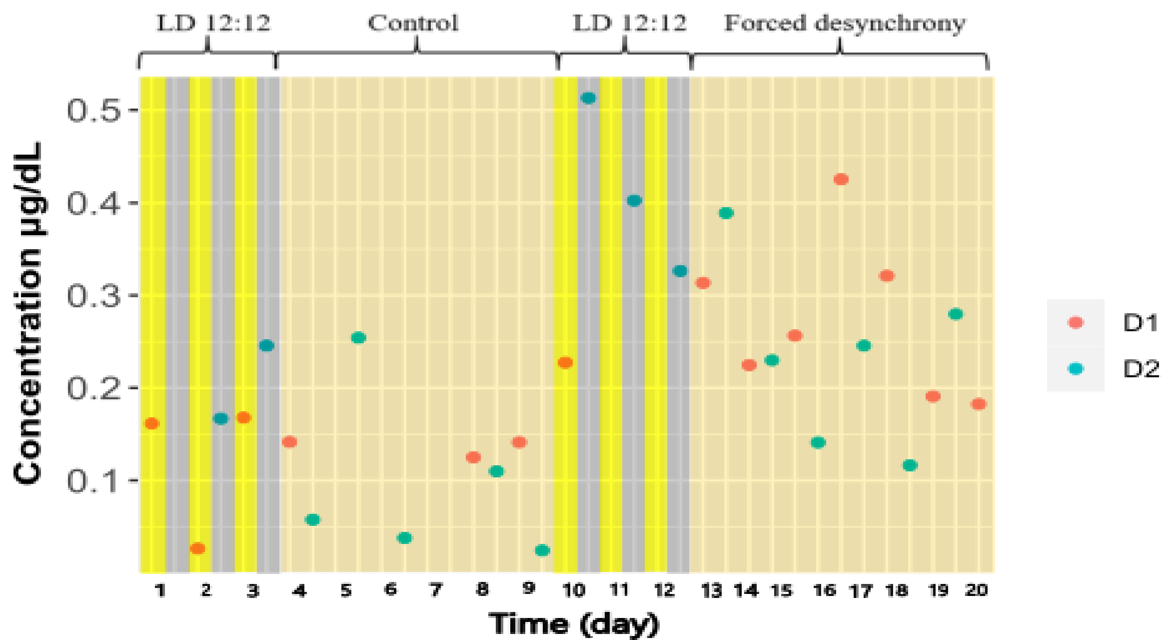

***Figure 36: Cortisol levels in reindeer 2/22**. The y-axis shows the concentration of cortisol (µg/dL) and the x-axis show the time course of the experiment. The graph is colored in with the light-regime conducted, light-dark cycle of 12-hour light (yellow) and 12-hour dark (grey) – entrainments and dim light (bright yellow) for the control and the forced desynchrony. Each dot represents one measurement, and the two different colors represent at which disturbance the measurement was taken, disturbance-1 (D1; red) and disturbance-2 (D2; blue). During the entrainments and the control, the samples were taken at 08:30 (D1) and 20:30 (D2) and during the forced desynchrony it was taken every 14th hour as described in Table 1.*

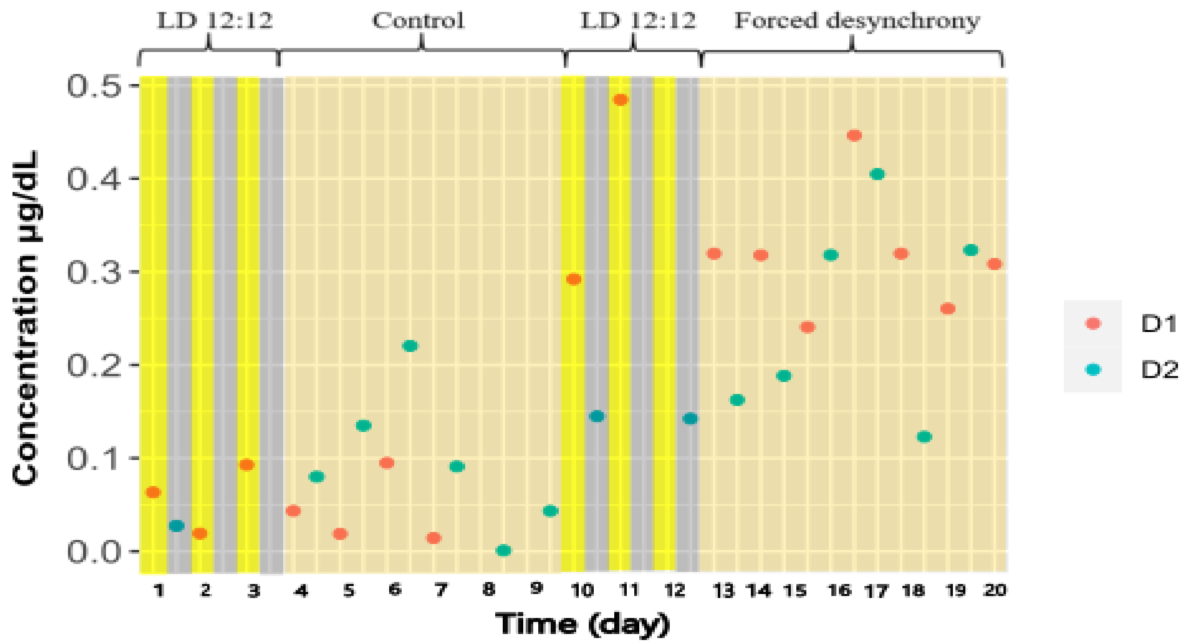

Figure 37: Cortisol levels in reindeer 3/22*. The y-axis shows the concentration of cortisol (µg/dL) and the x-axis show the time course of the experiment. The graph is colored in with the light-regime conducted, light-dark cycle of 12-hour light (yellow) and 12-hour dark (grey) – entrainments and dim light (bright yellow) for the control and the forced desynchrony. Each dot represents one measurement, and the two different colors represent at which disturbance the measurement was taken, disturbance-1 (D1; red) and disturbance-2 (D2; blue). During the entrainments and the control, the samples were taken at 08:30 (D1) and 20:30 (D2) and during the forced desynchrony it was taken every 14th hour as described in Table 1.*

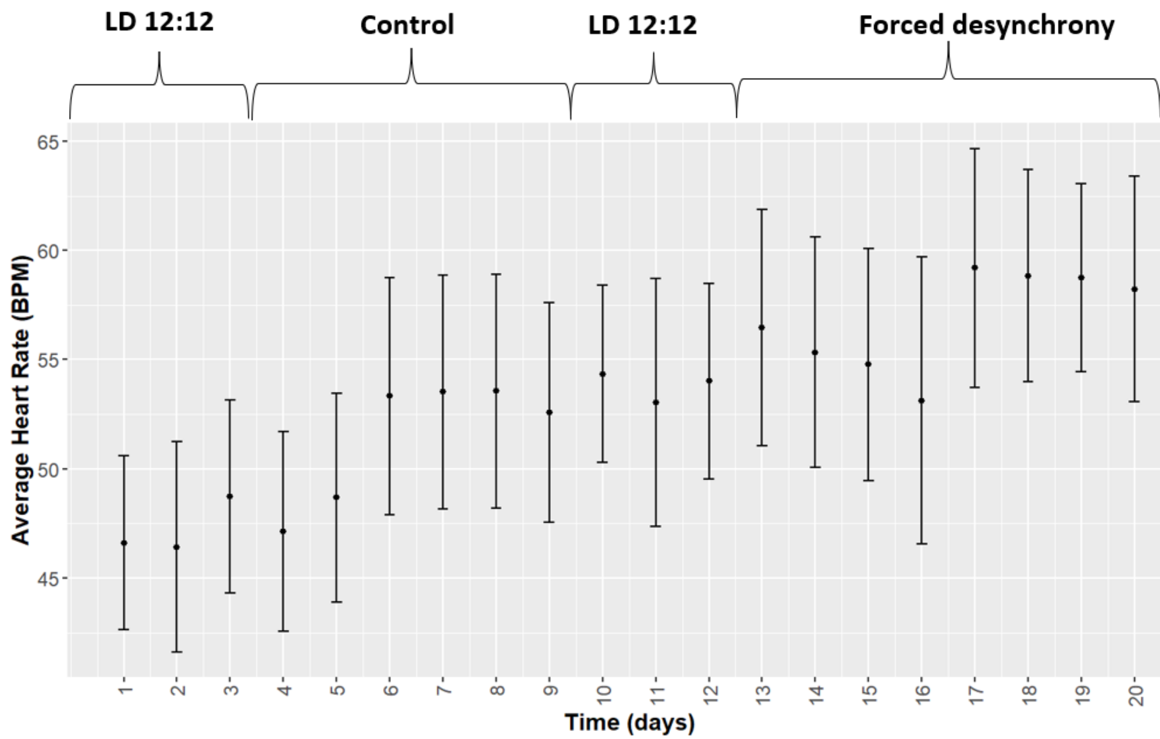

Figure 38: Average heart rate (HR) (BMP) each day during the protocol (reindeer 1/22)*. The y-axis show the average HR (BPM) and the x-axis show the time in days. Each dot represents the average and the whiskers represent the standard deviation. The HR value increases at the end of the protocol.*

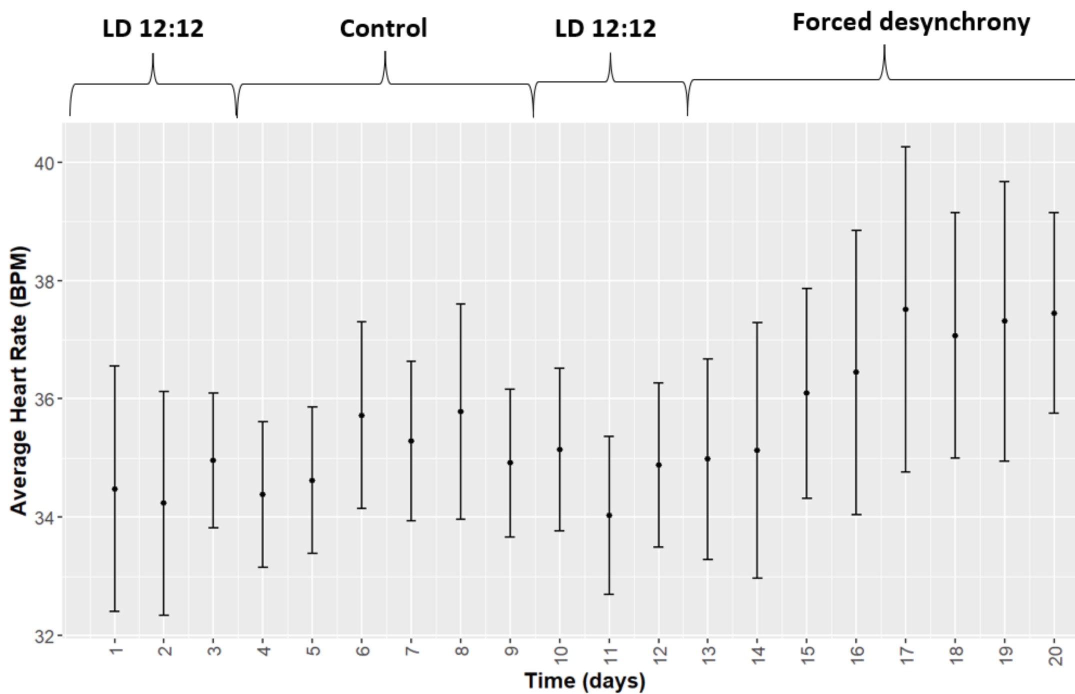

Figure 39: Average heart rate (HR) (BMP) each day during the protocol (reindeer 2/22)*. The y-axis show the average HR (BPM) and the x-axis show the time in days. Each dot represents the average and the whiskers represent the standard deviation. The HR value increases at the end of the protocol.*

4 Discussion

4.1 Summary

The expected anatomical distribution of arginine vasopressin (AVP), vasoactive intestinal polypeptide (VIP) and gastrin-releasing peptide (GRP) was that VIP and GRP is mainly in the ventral medial area and that AVP is mainly in the dorsal lateral area. The reindeer do not have a distinct separation of the suprachiasmatic nucleus (SCN), and expression AVP and VIP are scattered in the SCN. GRP expression was not detected in this thesis.

The expected output rhythms of the reindeer were that they will not show any signs of circadian rhythmicity under constant conditions in the forced desynchrony. The forced desynchrony protocol was not effective in uncoupling behavior from internal rhythms in the parameters investigated. The protocol did, however, provide a method for investigating whether there is a presence of circadian rhythms as it was successful in not giving a 24-hour time clue. The reindeer show diurnal patterns in the presence of a light-dark cycle, this rhythm breaks down in constant light and the study gave no strong evidence of a circadian rhythm in in locomotor activity (LA), feeding behavior (FB) and rumen temperature (T_r) under constant conditions and the rhythm that appears most dominant is ultradian. The reindeer became stressed as a consequence to a long experiment as can be seen in cortisol and heart rate (HR) data.

4.2 AVP and VIP are expressed in reindeer

Almost all studies on anatomical description of the SCN comes from rodents. The few studies that have looked outside rodents have found different patterns than the traditional rodent based view on how the SCN is organized (Cassone et al., 1988; Dardente et al., 2016). Table 2 summarizes the extent of the SCN and how the three neuropeptides AVP, VIP and GRP as localized in the SCN in mouse (*Mus musculus)*, hamster (*Mesocricetus auratus*), marsupials, cat (*Felis catus*), pig (*Sus domesticus*), guinea pig (*Cavia porcellus*), human (*Homo sapiens*), rat (*Rattus novegicus domestica*), and sheep (*Ovis aries*) and results from my own study, reindeer. As can be seen, most species investigated have to some extent a separation of AVP and VIP in the SCN, with AVP mostly distributed dorsal and VIP mostly distributed ventral. A few species investigated earlier, the group marsupials and the sheep, have co-distribution of AVP and VIP. This is similar to what I have seen in reindeer (Figure 19). In addition, I did not find any evidence of GRP expression in the reindeer SCN (Figure 19), which is similar to what is seen in sheep. However, I did not find any GRP in the immediate areas around the SCN either (Figure 18)– as can be seen in sheep. The reason for this may be simply because GRP is not

expressed in this area in reindeer – we do not know that. It may be that the staining is too faint with non-radioactive in-situ hybridization (ISH), so that it is not possible to see. Or maybe something went wrong in the process. Other more sensitive approaches could be used in the future such as radioactive ISH or RNAscope ISH to check for low expression of GRP. A new ISH of GRP in reindeer SCN and brain should be done in the future, this should also include a positive control of tissue with high expression of GRP, e.g. sheep SON.

The functional consequence of this less distinct separation in the SCN is to this date not investigated. The traditional rodent-based model holds that VIP receives the light input and communicates this through the SCN, including, AVP expressing neurons (Francl et al., 2010; Reed et al., 2001; Watanabe et al., 2000). This may be just as true in the species with less distinct sub-regions. The role of GRP is less studied and has, to my knowledge, only been looked at in relation to the expression in SCN, in rodents and sheep. The role of GRP in circadian regulation is very much alike the role of VIP (Kawamoto et al., 2003) and can substitute for VIPs role in transmitting the light-dark cycle (Brown et al., 2005). Why sheep and potentially also reindeer have a loss of GRP is a question unanswered.

4.3 Could phylogeny be an explanation for the difference in SCN across animal groups?

The reason for a less distinct subregions is unknown and can have many explanations. As the reindeer and sheep SCN is similar there is reason to speculate if phylogeny may be the reason for this. Ungulates have been separated from rodents and primates for around 80 million years – it may simply just be that the two different animal groups have evolved differently. This is also supported by the fact that marsupials do not have distinct subregions and they have been separated from placental mammals for about 160 million years (Upham et al., 2019).

Reindeer are diurnal animals while most rodents investigated are nocturnal. In diurnal rodents VIP and AVP is not as strongly separated as in nocturnal rodents. A study on murid Rodent from East Africa (*Arvicanthis niloticus*) shows that AVP is distributed throughout the whole SCN through all parts – however most dense in the dorsal area (Smale & Boverhof, 1999). A diurnal lifestyle may have resulted in a difference in organization in the SCN.

A much larger portion of the mouse brain (assuming body weight of 20 g) is dedicated to the SCN than in bigger mammals, like pig and reindeer (assuming body weights of 100 kg and 90 kg respectively) (Table 2), if scaled to body size the SCN of the mouse is about 2000 times

bigger than the reindeer. A question that arises is if the size of the SCN relates to circadian dominance, but little is known about this. Although, other brain areas which differ in scaled size has a link to functionality, like the cerebral cortex (reviewed in: Hofman (2014)). It is therefore not unreasonable to speculate that this may also is the case for the SCN.

4.4 Forced desynchrony – did it work?

This was the first attempt to do a forced desynchrony protocol in reindeer. A question that arises from this is – did it work? A forced desynchrony protocol aims to uncouple internal rhythms (i.e. T_r, HR and cortisol) from behavioral rhythms (i.e. FB and LA) (Binder et al., 2009).

This protocol was altered to fit the study organism and is therefore unlike forced desynchrony protocols used previously. Other forced desynchrony protocol have used scheduled eating and sleeping times (Scheer et al., 2009), forced activity (Strijkstra et al., 1999) and a light-dark cycle shorter than 24-hour (Cambras et al., 2007) to desynchronize the two components. None of these alternatives were decided to be a good alternative for reindeer. You cannot tell reindeer when to eat – of course one could have restricted food availability and only given food at certain times of day, but as reindeer are ruminants this would have constricted their natural behavior of eating and was deemed unethical. As with eating you cannot tell reindeer when they should sleep. Forced activity rhythm were done on rodents in running wheel – setting up some sort of forced activity for reindeer would have proven extremely difficult. Lastly, it could have been considered having a protocol with a light-dark cycle longer or shorter than 24 hours, but as reindeer have been seen to follow a light-dark cycle rather passively (Lu et al., 2010) it was decided that this may mask any underlying rhythm in reindeer. Therefore, it was decided to adapt the protocol of Scheer et al. (2009) and the reindeer were exposed to a disturbance protocol with two disturbances each day (D1 and D2) which hopefully gave a clue about the time of day.

The protocol successfully affected behavioral rhythms in terms of creating a 28-hour rhythm in LA and FB (Figure 23; Figure 24). This rhythm is, however, not very strong (Figure 27). The T_r of the reindeer does not seem to have an internal rhythm that fluctuate independently of the LA and FB (Figure 29). This is most likely because feeding and drinking affects the rumen temperature, as seen in my result (Figure 28; Figure 29) and earlier studies (Herberg et al., 2018). During the control part and forced desynchrony part the cross-correlation in rhythms between 22 and 28 hours is strong at first but rapidly dampens after one to two days (Figure 30) this may be a sign of desynchrony, but it may also just be a lack of rhythms at these periods.

The cross-correlation at the ultradian level is, however, still very strong. The ultradian rhythmicity of the reindeer makes analyzes of circadian rhythms difficult in reindeer.

To conclude if the protocol worked or not is therefore difficult as the only parameters that I have been able to compare is T_r with LA and FB, these parameters are clearly linked and if there is an internal rhythm in the reindeer it is probably too weak to recognize when the T_r is affected to such a degree by feeding and the ultradian rhythmicity. This experiment aimed to use heart rate and cortisol data to further investigate the internal rhythm, these parameters is probably less linked to feeding and drinking and the story might have been different if these parameters had given results that were possible to include. This is the first time a forced desynchrony protocol have been used in an animal which are believed to have a weak circadian organization – other organism that have been studied is first and foremost rodents and humans, and it may be that this uncoupling of T_r from LA and FB, is only possible in organisms with strong circadian organization. However, even if this protocol does not work for uncoupling , it did, in contrast to the pilot study (Figure 20; Figure 21) not give any clues about a 24-hour day and still gave sufficient visits for checking up on the animals.

4.5 A presence of a 24-hour rhythm

During forced desynchrony, in addition to the 14-hour and 28-hour rhythms, a rhythm which is about 24-hours were expressed in LA (Figure 23), FB (Figure 24) and T_r (Figure 25; Figure 26). This was not expected and contradicts most earlier studies on reindeer LA in their natural habitat (Lu et al., 2010; Van Oort et al., 2005), only one study have seen a 24-hour rhythm present during polar day (Arnold et al., 2018).

Why this rhythm is present here can have several explanations. One may be that the reindeer in fact have a circadian organization. However, the T_r of reindeer 2/22 do not show any signs of 24-hour rhythmicity during the control and the second entrainment. During these two parts of the experiment the reindeer was given clues about time in form of a light-dark cycle and/or disturbances. Considering that the T_r do not hold a 24-hour rhythmicity during these parts it is unlikely that the T_r starts to free-run with a 24-hour cycle after one week without. Another possibility is that there is a presence of a weak zeitgeber. Unfortunately, the climate in Tromsø had a very drastic change during the last week of my protocol, leading to very low temperatures at night. As the reindeer room is not isolated enough the T_a in the room became low at night and rise during the day with a range of 0.5ºC (Figure 34). Ectotherm animals and some small mammals have been seen to entrain to small T_a changes (Rensing & Ruoff, 2002). If this is the

case for reindeer is unlikely as it usually takes stronger temperature changes to entrain a large mammal (Rensing & Ruoff, 2002).

Even if the hallway just outside the reindeer room were closed off, sound may travel inside the room and activity around the hallway occurred at working hours (08:00 – 16:00) every day – and one must consider if this affected the LA. Social cues can act as a powerful organizer of activity in addition to amplify ambiguous cues (Davidson & Menaker, 2003). Reindeer are herding animals and the grazing activity of caribou have been seen to be synchronized (Russell et al., 1993), and also in this experiment the reindeer were clearly affected by each other (Figure 32) and the presence of humans. If temperature or the sound of activity outside the closed of hallway is an ambiguous cue, the social entrainment of the animal may have amplified the rhythm.

These are all factors which were tried taking into consideration when setting up the experiment – but the facility may just not be good enough to conduct this kind of truly closed environment that is needed to exclude all possible disturbance.

4.6 Ultradian cycles are more important in ruminants

The results seen in this experiment reflects what one has seen of the reindeer in their natural habitat. The reindeer express a diurnal rhythmicity in LA when light is present (Figure 23), this is consistent with what earlier studies have seen in terms of LA with a light-dark cycle present (Arnold et al., 2018; Van Oort et al., 2005; van Oort et al., 2007). The T_r holds a weaker organization - a weak organization in T_r have also been reported before (Arnold et al., 2018).

The most dominant rhythm in constant light is ultradian (Figure 27) and the period of the ultradian bouts is similar to what is seen earlier in constant light, short ultradian bouts of around 4-5 hours (Arnold et al., 2018; van Oort et al., 2007). Reindeer are ruminants and these animals may not need circadian clocks because of the way their physiology functions. Their ultradian cycles of digestion and frequent feeding could be drives. Other ruminant species, like red deer (Cervus elaphus) and horse have been seen to have weak circadian organization (Ensing et al., 2014; Murphy et al., 2011). The sheep's amplitude of circadian rhythmicity is much weaker then rodents (Andersson et al., 2005; Balsalobre et al., 2000) and only show sustained circadian rhythmicity for one to two weeks in constant conditions (Ebling et al., 1988), amplifying the fact that ruminants may rely more of their ultradian cycles of feeding rather than an circadian cycle.

Another interesting point made by Hazlerigg and Tyler (2019) is that the strong circadian organization of small mammals may be due to thermal balance. This argument comes from small animals needing to withdrawal into nests, burrow or other places where they are shielded to maintains its high metabolic costs of holding a euthermic body temperature. In these places it is dark, and animals need some internal clock to have a sense of time – to know when they can emerge. Larger mammals – like ruminants - never had this pressure to have a sense of time as they always are exposed to a light-dark cycle. Considering the anatomical differences in the SCN as well, it may be that a difference in evolutionary adaptation may be a significant cause in the differences seen in circadian rhythmicity.

4.7 The presence of a cortisol rhythm

The cortisol rhythm of the reindeer seems to be affected differently during the different phases of the protocol. In the entrainment phases it seems that the cortisol levels are highest during morning (D1) and lowest in the late evening (D2), this rhythm is maintained for reindeer 2/22 (Figure 36) and halfway through the control in 1/22 before it shifts to the opposite (Figure 35), low in the morning (D1) and high in the late evening (D2) – this rhythm is what is seen throughout the whole protocol in reindeer 3/22 (Figure 37). The reindeer may switch their rhythms to D1 being subjective evening and D2 being subjective morning during the control phase as there is no indication on what time of day there is.

However, the few data points and values obtained do not lead to statistically robust statements. During the forced desynchrony the cortisol rhythm seems to disappear, which is the opposite of what have been seen in horse – they have sustained circadian rhythmicity in cortisol levels during darkness (Murphy et al., 2011). However, in this study the samples were taken every 2-hour compared to my study where samples are taken every 12th or 14th hour. In a future study cortisol sample should be taken more frequently to be able to determine if there is presence of a cortisol rhythm. If one take into consideration that glucocorticoids can act as a tissue synchronizer (Balsalobre et al., 2000) it would be interesting to see if the cortisol rhythm disappear during constant conditions, this and may point to an early desynchronization in the animal's circadian system.

This is the first-time cortisol analyses have been done in reindeer, that itself is a result worth acknowledging, and as samples only are taken two times a day it is difficult to say anything more about the rhythm. In addition, these samples have been of high risk of contamination as the protocol originally says that animals should not eat the hour before samples are taken, this

was not possible to do in this protocol as it would have interfered with the other parameters. Either way, such preliminary results of reindeer cortisol levels is promising for further studies, and in the future, the use of a microdialysis device would give even more information about the internal rhythms of the reindeer.

4.8 Ruminant adaption to life in the Arctic

Van Oort et al. (2005) suggests there is little advantage for an herbivore in polar regions to maintain a strong internal clock. The only other herbivore living in the Arctic is Svalbard rock ptarmigans (*Lagopus muta*). Svalbard rock ptarmigan shows a rhythm when a light-dark cycle is present, but the rhythm is abolished during 'polar day' and 'polar night' and in laboratory conditions of constant light and constant darkness (Appenroth, Nord, et al., 2021; Reierth & Stokkan, 1998). In Svalbard rock ptarmigan it has, however, been seen a that it maintains its circadian clock in tissues essential for seasonal timekeeping (Appenroth, Wagner, et al., 2021). This circadian rhythmicity is suggested to be maintained for measuring photoperiod, but that the circadian system in uncoupled from the output physiology. Circadian rhythmicity in clock genes have in reindeer only been measured in fibroblasts (Lu et al., 2010), it would be interesting to see how the genes act in tissue essential for seasonality, and of course in the SCN. However, melatonin is important for seasonality and in reindeer it have been suggested that melatonin directly entrains a seasonality clock as melatonin follows the light-dark cycle passively (Lu et al., 2010), meaning that seasonality would be uncoupled from the circadian system.

Other species living the Arctic have been found to have persistent rhythms during 'polar day' and 'polar night'. In polar bears (*Ursus maritimus*) there is a sustained 24-hour rhythm during 'polar night' and 'polar day' (Ware et al., 2020). Circadian rhythm of Arctic ground squirrels (*Urocitellus parryii*) disappears during hibernation and is re-established when they emerge and persist during constant light (Williams et al., 2012; Williams et al., 2017). Free-Living Lapland Longspurs (*Calcarius lapponicus*) remain a diurnal rhythm during 'polar day'. Several animals living in other kind of arrhythmic environments keeps a circadian organization (reviewed in Beale et al. (2016)), but some does not. The difference in circadian organization can reflect a difference in evolutionary adaptations.

4.9 Notes on possible improvements

If this experiment were to be repeated, I would like to share some notes on possible improvements of the experimental design in addition to experimental set-up. The second

entrainment period should be longer, as this period do not seem to be sufficient to re-entrain all parameters (i.e., body temperature and cortisol; Figure 26; Figure 35; Figure 36; Figure 37). It would also be worth considering doing this experiment in a two-part protocol, with this I mean that the animal should be considered to be re-entrained outside – as the 24-hour rhythm seem to dampen already in the inside entrainment phase (Figure 20; Figure 21). It would also be worth considering having a control outside in their natural habitat to understand further if the inside environment affects their behavior. It would also be advantageous to have a longer protocol to give more robust data.

The facility and the set-up of the experiment needs some improvement for future studies. Feeding monitors should be set-up differently so that it is not possible for the experimental animal to interfere (i.e. play) with the monitors. HR and cortisol levels rise during the course of the experiment (Figure 35; Figure 36; Figure 37; Figure 38; Figure 39) indicating that the reindeer have increased levels of stress. This is something that should be taken into consideration if this kind of experiment were to be undertaken in the future. Even with tamed animal raised inside and habituated to humans, there are factors that makes them stressed. Environmental enrichment reduces stress (Moncek et al., 2004) and should be implemented in future studies.

One should consider having room that is climate regulated, so that even if the outside temperature has drastic changes, it will not affect the ambient temperature inside the room. For more information about internal rhythms, it would be interesting to have core body temperature, it would also be advantageous to have a microdialysis device to measure cortisol – and possible other parameters, for example melatonin (Lu et al., 2010) and norepinephrine (Scheer et al., 2009) to investigate how the internal rhythm of the reindeer is expressed.

5 Conclusion

To conclude, both the Arctic environment and the ruminant feeding demands seem to have contributed to a less important circadian organization in reindeer. The expression of neuropeptides in the suprachiasmatic nucleus (SCN) is similar to another ruminant, sheep, with arginine vasopressin (AVP) and vasoactive intestinal polypeptide (VIP) expressed in the reindeer SCN and the SCN is located right above the optic chiasm and close to the third ventricle. The size of the SCN compared to mouse and scaled to body size is about 2000 times smaller – maybe indicating a less important function of the SCN. A forced desynchrony protocol was successively conducted on reindeer, but the limitations this study provided in resources resulted in no signs of uncoupling of internal and external rhythms. It was, however, successful in giving an alternative to a constant condition protocol in making sure there were no 24-hour component present. This study gave no strong evidence of a circadian component in reindeer and ultradian rhythms appears to be dominant. Other ruminants, and animals living in the Arctic have also been seen to have less important circadian organization.

Works cited

Abrahamson, E. E., & Moore, R. Y. (2001). Suprachiasmatic nucleus in the mouse: retinal innervation, intrinsic organization and efferent projections. *Brain research*, *916*(1-2), 172-191.

Aida, R., Moriya, T., Araki, M., Akiyama, M., Wada, K., Wada, E., & Shibata, S. (2002). Gastrin-Releasing Peptide Mediates Photic Entrainable Signals to Dorsal Subsets of Suprachiasmatic Nucleus via Induction ofPeriod Gene in Mice. *Molecular pharmacology*, *61*(1), 26-34.

Andersson, H., Johnston, J. D., Messager, S., Hazlerigg, D., & Lincoln, G. (2005). Photoperiod regulates clock gene rhythms in the ovine liver. *General and comparative endocrinology*, *142*(3), 357-363.

Appenroth, D., Nord, A., Hazlerigg, D. G., & Wagner, G. C. (2021). Body temperature and activity rhythms under different photoperiods in high arctic Svalbard ptarmigan (Lagopus muta hyperborea). *Frontiers in physiology*, *12*, 633866.

Appenroth, D., Wagner, G. C., Hazlerigg, D. G., & West, A. C. (2021). Evidence for circadian-based photoperiodic timekeeping in Svalbard ptarmigan, the northernmost resident bird. *Current Biology*, *31*(12), 2720-2727. e2725.

Arnold, W., Ruf, T., Loe, L. E., Irvine, R. J., Ropstad, E., Veiberg, V., & Albon, S. D. (2018). Circadian rhythmicity persists through the Polar night and midnight sun in Svalbard reindeer. *Scientific Reports*, *8*(1), 1-12.

Ashik, D. B. (2020). Sheep brain lateral view. *Zenodo*. https://doi.org/10.5281/zenodo.3926573

Balsalobre, A., Brown, S. A., Marcacci, L., Tronche, F., Kellendonk, C., Reichardt, H. M., Schutz, G., & Schibler, U. (2000). Resetting of circadian time in peripheral tissues by glucocorticoid signaling. *Science*, *289*(5488), 2344-2347.

Beale, A. D., Whitmore, D., & Moran, D. (2016). Life in a dark biosphere: a review of circadian physiology in “arrhythmic” environments. *Journal of Comparative Physiology B*, *186*, 947-968.

Binder, M. D., Hirokawa, N., & Windhorst, U. (2009). Forced Desynchrony. In *Encyclopedia of Neuroscience* (pp. 1615-1615). Springer Berlin Heidelberg. https://doi.org/10.1007/978-3-540-29678-2_1820

Blix, A. S. (2005). *Arctic animals and their adaptations to life on the edge*. Tapir Academic Press.

Borjigin, J., Zhang, L. S., & Calinescu, A.-A. (2012). Circadian regulation of pineal gland rhythmicity. *Molecular and cellular endocrinology*, *349*(1), 13-19.

Brown, T. M., Hughes, A. T., & Piggins, H. D. (2005). Gastrin-releasing peptide promotes suprachiasmatic nuclei cellular rhythmicity in the absence of vasoactive intestinal polypeptide-VPAC2 receptor signaling. *Journal of Neuroscience*, *25*(48), 11155-11164.

Buhr, E. D., Yoo, S.-H., & Takahashi, J. S. (2010). Temperature as a universal resetting cue for mammalian circadian oscillators. *Science*, *330*(6002), 379-385.

Buijs, R., Eden, C. v., Goncharuk, V., & Kalsbeek, A. (2003). Circadian and seasonal rhythms-the biological clock tunes the organs of the body: timing by hormones and the autonomic nervous system. *Journal of Endocrinology*, *177*(1), 17-26.

Cambras, T., Weller, J. R., Anglès-Pujoràs, M., Lee, M. L., Christopher, A., Díez-Noguera, A., Krueger, J. M., & de la Iglesia, H. O. (2007). Circadian desynchronization of core body temperature and sleep stages in the rat. *Proceedings of the National Academy of Sciences*, *104*(18), 7634-7639.

Card, J., & Moore, R. (1984). The suprachiasmatic nucleus of the golden hamster: immunohistochemical analysis of cell and fiber distribution. *Neuroscience*, *13*(2), 415-431.
Cassone, V. M., Speh, J. C., Card, J. P., & Moore, R. Y. (1988). Comparative anatomy of the mammalian hypothalamic suprachiasmatic nucleus. *Journal of biological rhythms*, *3*(1), 71-91.
Cheng, M. Y., Bullock, C. M., Li, C., Lee, A. G., Bermak, J. C., Belluzzi, J., Weaver, D. R., Leslie, F. M., & Zhou, Q.-Y. (2002). Prokineticin 2 transmits the behavioural circadian rhythm of the suprachiasmatic nucleus. *Nature*, *417*(6887), 405-410.
Chou, T. C., Bjorkum, A. A., Gaus, S. E., Lu, J., Scammell, T. E., & Saper, C. B. (2002). Afferents to the ventrolateral preoptic nucleus. *Journal of Neuroscience*, *22*(3), 977-990.
Chung, S., Son, G. H., & Kim, K. (2011). Adrenal peripheral oscillator in generating the circadian glucocorticoid rhythm. *Annals of the New York Academy of Sciences*, *1220*(1), 71-81.
Dardente, H., Wyse, C. A., Lincoln, G. A., Wagner, G. C., & Hazlerigg, D. G. (2016). Effects of photoperiod extension on clock gene and neuropeptide RNA expression in the SCN of the Soay Sheep. *PLoS One*, *11*(7), e0159201.
Davidson, A. J., & Menaker, M. (2003). Birds of a feather clock together–sometimes: social synchronization of circadian rhythms. *Current opinion in neurobiology*, *13*(6), 765-769.
Deurveilher, S., Burns, J., & Semba, K. (2002). Indirect projections from the suprachiasmatic nucleus to the ventrolateral preoptic nucleus: a dual tract ‐ tracing study in rat. *European Journal of Neuroscience*, *16*(7), 1195-1213.
Dijk, D.-J., & Czeisler, C. A. (1995). Contribution of the circadian pacemaker and the sleep homeostat to sleep propensity, sleep structure, electroencephalographic slow waves, and sleep spindle activity in humans. *Journal of Neuroscience*, *15*(5), 3526-3538.
Ebling, F. J., Lincoln, G. A., Wollnik, F., & Anderson, N. (1988). Effects of constant darkness and constant light on circadian organization and reproductive responses in the ram. *Journal of biological rhythms*, *3*(4), 365-384.
Ebling, F. J., & Piggins, H. D. (2020). *Neuroendocrine Clocks and Calendars*. Springer.
Edery, I. (2000). Circadian rhythms in a nutshell. *Physiological genomics*, *3*(2), 59-74.
Ensing, E. P., Ciuti, S., de Wijs, F. A., Lentferink, D. H., Ten Hoedt, A., Boyce, M. S., & Hut, R. A. (2014). GPS based daily activity patterns in European red deer and North American elk (Cervus elaphus): indication for a weak circadian clock in ungulates. *PLoS One*, *9*(9), e106997.
Francl, J. M., Kaur, G., & Glass, J. D. (2010). Regulation of vasoactive intestinal polypeptide release in the SCN circadian clock. *Neuroreport*, *21*(16), 1055.
Gamble, K. L., Allen, G. C., Zhou, T., & McMahon, D. G. (2007). Gastrin-releasing peptide mediates light-like resetting of the suprachiasmatic nucleus circadian pacemaker through cAMP response element-binding protein and Per1 activation. *Journal of Neuroscience*, *27*(44), 12078-12087.
Gurdjian, E. S. (1927). The diencephalon of the albino rat. Studies on the brain of the rat. No. 2. *Journal of Comparative Neurology*, *43*(1), 1-114.
Hannibal, J., Moller, M., Ottersen, O. P., & Fahrenkrug, J. (2000). PACAP and glutamate are co ‐ stored in the retinohypothalamic tract. *Journal of Comparative Neurology*, *418*(2), 147-155.
Harmar, A. J., Marston, H. M., Shen, S., Spratt, C., West, K. M., Sheward, W. J., Morrison, C. F., Dorin, J. R., Piggins, H. D., & Reubi, J.-C. (2002). The VPAC2 receptor is

essential for circadian function in the mouse suprachiasmatic nuclei. *Cell*, *109*(4), 497-508.
Hazlerigg, D. G., & Tyler, N. J. (2019). Activity patterns in mammals: Circadian dominance challenged. *PLoS biology*, *17*(7), e3000360.
Helm, B., & Lincoln, G. A. (2017). *Circannual rhythms anticipate the earth's annual periodicity*. Springer.
Herberg, A. M., St‐Louis, V., Carstensen, M., Fieberg, J., Thompson, D. P., Crouse, J. A., & Forester, J. D. (2018). Calibration of a rumen bolus to measure continuous internal body temperature in moose. *Wildlife Society Bulletin*, *42*(2), 328-337.
Hiddinga, A., & Van Den Hoofdakker, R. (1997). Endogenous and exogenous components in the circadian variation of core body temperature in humans. *Journal of sleep research*, *6*(3), 156-163.
Hill, R. W., Wyse, G. A., & Anderson, M. (2016). *Animal physiology* (Fourth edition. ed.). Sinauer Associates, Inc. Publishers.
Hofman, M., Zhou, J. N., & Swaab, D. (1996). Suprachiasmatic nucleus of the human brain: an immunocytochemical and morphometric analysis. *The Anatomical Record: An Official Publication of the American Association of Anatomists*, *244*(4), 552-562.
Hofman, M. A. (2014). Evolution of the human brain: when bigger is better. *Frontiers in neuroanatomy*, *8*, 15.
Hofstra, W. A., & De Weerd, A. W. (2008). How to assess circadian rhythm in humans: a review of literature. *Epilepsy & behavior*, *13*(3), 438-444.
Ikeda, M., & Allen, C. N. (2003). Developmental changes in calbindin‐D28k and calretinin expression in the mouse suprachiasmatic nucleus. *European Journal of Neuroscience*, *17*(5), 1111-1118.
Ingram, C., Snowball, R., & Mihai, R. (1996). Circadian rhythm of neuronal activity in suprachiasmatic nucleus slices from the vasopressin-deficient Brattleboro rat. *Neuroscience*, *75*(2), 635-641.
Ishida, A., Mutoh, T., Ueyama, T., Bando, H., Masubuchi, S., Nakahara, D., Tsujimoto, G., & Okamura, H. (2005). Light activates the adrenal gland: timing of gene expression and glucocorticoid release. *Cell metabolism*, *2*(5), 297-307.
Janik, D., & Mrosovsky, N. (1994). Intergeniculate leaflet lesions and behaviorally-induced shifts of circadian rhythms. *Brain research*, *651*(1-2), 174-182.
Jensen, E. (2014). Technical review: In situ hybridization. *The Anatomical Record*, *297*(8), 1349-1353.
Jha, P. K. (2016). *Sleep deprivation and its impact on circadian rhythms and glucose metabolism* Strasbourg].
Kakihana, R., & Moore, J. A. (1976). Circadian rhythm of corticosterone in mice: the effect of chronic consumption of alcohol. *Psychopharmacologia*, *46*, 301-305.
Kalsbeek, A., Palm, I. F., La Fleur, S. E., Scheer, F. A., Perreau-Lenz, S., Ruiter, M., Kreier, F., Cailotto, C., & Buijs, R. M. (2006). SCN outputs and the hypothalamic balance of life. *J Biol Rhythms*, *21*(6), 458-469. https://doi.org/10.1177/0748730406293854
Kalsbeek, A., Van Heerikhuize, J. J., Wortel, J., & Buijs, R. M. (1996). A diurnal rhythm of stimulatory input to the hypothalamo–pituitary–adrenal system as revealed by timed intrahypothalamic administration of the vasopressin V1Antagonist. *Journal of Neuroscience*, *16*(17), 5555-5565.
Karatsoreos, I. N., Wang, A., Sasanian, J., & Silver, R. (2007). A role for androgens in regulating circadian behavior and the suprachiasmatic nucleus. *Endocrinology*, *148*(11), 5487-5495.

Karatsoreos, I. N., Yan, L., LeSauter, J., & Silver, R. (2004). Phenotype matters: identification of light-responsive cells in the mouse suprachiasmatic nucleus. *Journal of Neuroscience*, *24*(1), 68-75.
Kawamoto, K., Nagano, M., Kanda, F., Chihara, K., Shigeyoshi, Y., & Okamura, H. (2003). Two types of VIP neuronal components in rat suprachiasmatic nucleus. *Journal of neuroscience research*, *74*(6), 852-857.
Klein, D. C., & Moore, R. Y. (1979). Pineal N-acetyltransferase and hydroxyindole-O-methyltransferase: control by the retinohypothalamic tract and the suprachiasmatic nucleus. *Brain Res*, *174*(2), 245-262. https://doi.org/10.1016/0006-8993(79)90848-5
Klein, D. C., Weller, J. L., & Moore, R. Y. (1971). Melatonin metabolism: neural regulation of pineal serotonin: acetyl coenzyme AN-acetyltransferase activity. *Proceedings of the National Academy of Sciences*, *68*(12), 3107-3110.
Ko, C. H., & Takahashi, J. S. (2006). Molecular components of the mammalian circadian clock. *Human molecular genetics*, *15*(suppl_2), R271-R277.
Kramer, A., Yang, F.-C., Snodgrass, P., Li, X., Scammell, T. E., Davis, F. C., & Weitz, C. J. (2001). Regulation of daily locomotor activity and sleep by hypothalamic EGF receptor signaling. *Science*, *294*(5551), 2511-2515.
Kräuchi, K. (2007). The thermophysiological cascade leading to sleep initiation in relation to phase of entrainment. *Sleep medicine reviews*, *11*(6), 439-451.
Kraves, S., & Weitz, C. J. (2006). A role for cardiotrophin-like cytokine in the circadian control of mammalian locomotor activity. *Nature neuroscience*, *9*(2), 212-219.
Lemos, D. R., Downs, J. L., & Urbanski, H. F. (2006). Twenty-four-hour rhythmic gene expression in the rhesus macaque adrenal gland. *Molecular Endocrinology*, *20*(5), 1164-1176.
Li, J.-D., Burton, K. J., Zhang, C., Hu, S.-B., & Zhou, Q.-Y. (2009). Vasopressin receptor V1a regulates circadian rhythms of locomotor activity and expression of clock-controlled genes in the suprachiasmatic nuclei. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *296*(3), R824-R830.
Lin, Z., Chen, L., Chen, X., Zhong, Y., Yang, Y., Xia, W., Liu, C., Zhu, W., Wang, H., & Yan, B. (2019). Biological adaptations in the Arctic cervid, the reindeer (Rangifer tarandus). *Science*, *364*(6446), eaav6312.
Lu, W., Meng, Q.-J., Tyler, N. J., Stokkan, K.-A., & Loudon, A. S. (2010). A circadian clock is not required in an arctic mammal. *Current Biology*, *20*(6), 533-537.
Manna, P. R., Dyson, M. T., & Stocco, D. M. (2009). Regulation of the steroidogenic acute regulatory protein gene expression: present and future perspectives. *Molecular human reproduction*, *15*(6), 321-333.
Meyer‐Spasche, A., Reed, H. E., & Piggins, H. D. (2002). Neurotensin phase‐shifts the firing rate rhythm of neurons in the rat suprachiasmatic nuclei in vitro. *European Journal of Neuroscience*, *16*(2), 339-344.
Moga, M. M., & Moore, R. Y. (1997). Organization of neural inputs to the suprachiasmatic nucleus in the rat. *Journal of Comparative Neurology*, *389*(3), 508-534.
Moncek, F., Duncko, R., Johansson, B., & Jezova, D. (2004). Effect of environmental enrichment on stress related systems in rats. *Journal of neuroendocrinology*, *16*(5), 423-431.
Moore, R. Y., & Eichler, V. B. (1972). Loss of a circadian adrenal corticosterone rhythm following suprachiasmatic lesions in the rat. *Brain research*.
Moore, R. Y., Speh, J. C., & Leak, R. K. (2002). Suprachiasmatic nucleus organization. *Cell and tissue research*, *309*(1), 89-98.

Morgado, E., Juárez-Portilla, C., Silverman, A.-J., & Silver, R. (2015). Relevance of network organization in SCN clock function. In *Mechanisms of Circadian Systems in Animals and Their Clinical Relevance* (pp. 149-175). Springer.

Morin, L. P. (2013). Neuroanatomy of the extended circadian rhythm system. *Experimental neurology*, *243*, 4-20.

Murphy, B. A., Martin, A.-M., Furney, P., & Elliott, J. A. (2011). Absence of a serum melatonin rhythm under acutely extended darkness in the horse. *Journal of Circadian Rhythms*, *9*(1), 1-8.

Nakamura, K. (2011). Central circuitries for body temperature regulation and fever. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *301*(5), R1207-R1228.

Ono, D., Honma, K.-i., & Honma, S. (2021). Roles of Neuropeptides, VIP and AVP, in the Mammalian Central Circadian Clock. *Frontiers in neuroscience*, *15*, 650154.

Panda, S., Provencio, I., Tu, D. C., Pires, S. S., Rollag, M. D., Castrucci, A. M., Pletcher, M. T., Sato, T. K., Wiltshire, T., & Andahazy, M. (2003). Melanopsin is required for non-image-forming photic responses in blind mice. *Science*, *301*(5632), 525-527.

Panda, S., Sato, T. K., Castrucci, A. M., Rollag, M. D., DeGrip, W. J., Hogenesch, J. B., Provencio, I., & Kay, S. A. (2002). Melanopsin (Opn4) requirement for normal light-induced circadian phase shifting. *Science*, *298*(5601), 2213-2216.

Pandi-Perumal, S. R., Trakht, I., Srinivasan, V., Spence, D. W., Maestroni, G. J., Zisapel, N., & Cardinali, D. P. (2008). Physiological effects of melatonin: role of melatonin receptors and signal transduction pathways. *Progress in neurobiology*, *85*(3), 335-353.

Pando, M. P., Pinchak, A. B., Cermakian, N., & Sassone-Corsi, P. (2001). A cell-based system that recapitulates the dynamic light-dependent regulation of the vertebrate clock. *Proceedings of the National Academy of Sciences*, *98*(18), 10178-10183.

Prosser, H. M., Bradley, A., Chesham, J. E., Ebling, F. J., Hastings, M. H., & Maywood, E. S. (2007). Prokineticin receptor 2 (Prokr2) is essential for the regulation of circadian behavior by the suprachiasmatic nuclei. *Proceedings of the National Academy of Sciences*, *104*(2), 648-653.

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

Reed, H. E., Meyer‐Spasche, A., Cutler, D. J., Coen, C. W., & Piggins, H. D. (2001). Vasoactive intestinal polypeptide (VIP) phase‐shifts the rat suprachiasmatic nucleus clock in vitro. *European Journal of Neuroscience*, *13*(4), 839-843.

Refinetti, R. (1995). Effects of suprachiasmatic lesions on temperature regulation in the golden hamster. *Brain research bulletin*, *36*(1), 81-84.

Reierth, E., & Stokkan, K.-A. (1998). Activity rhythm in High Arctic Svalbard ptarmigan (Lagopus mutus hyperboreus). *Canadian Journal of Zoology*, *76*(11), 2031-2039.

Rensing, L., & Ruoff, P. (2002). Temperature effect on entrainment, phase shifting, and amplitude of circadian clocks and its molecular bases. *Chronobiology international*, *19*(5), 807-864.

Romijn, H., Sluiter, A., Pool, C., Wortel, J., & Buijs, R. (1996). Differences in colocalization between Fos and PHI, GRP, VIP and VP in neurons of the rat suprachiasmatic nucleus after a light stimulus during the phase delay versus the phase advance period of the night. *Journal of Comparative Neurology*, *372*(1), 1-8.

Rösch, A., & Schmidbauer, H. (2016). WaveletComp 1.1: A guided tour through the R package. *URL: http://www. hsstat. com/projects/WaveletComp/WaveletComp_guided_tour. pdf*.

Ruby, N. F., & Heller, H. C. (1996). Temperature sensitivity of the suprachiasmatic nucleus of ground squirrels and rats in vitro. *Journal of biological rhythms*, *11*(2), 126-136.

Russell, D. E., Martell, A. M., & Nixon, W. A. (1993). Range ecology of the Porcupine caribou herd in Canada. *Rangifer*, 1-168.

Saderi, N., Cazarez-Marquez, F., Buijs, F., Salgado-Delgado, R., Guzman-Ruiz, M., del Carmen Basualdo, M., Escobar, C., & Buijs, R. (2013). The NPY intergeniculate leaflet projections to the suprachiasmatic nucleus transmit metabolic conditions. *Neuroscience*, *246*, 291-300.

Sand, O., Sjaastad, Ø. V., Haug, E., & Toverud, K. C. (2014). *Menneskets fysiologi* (2. utg. ed.). Gyldendal akademisk.

Scheer, F. A., Hilton, M. F., Mantzoros, C. S., & Shea, S. A. (2009). Adverse metabolic and cardiovascular consequences of circadian misalignment. *Proceedings of the National Academy of Sciences*, *106*(11), 4453-4458.

Silver, R., Sookhoo, A. I., LeSauter, J., Stevens, P., Jansen, H. T., & Lehman, M. N. (1999). Multiple regulatory elements result in regional specificity in circadian rhythms of neuropeptide expression in mouse SCN. *Neuroreport*, *10*(15), 3165-3174.

Smale, L., & Boverhof, J. (1999). The suprachiasmatic nucleus and intergeniculate leaflet of Arvicanthis niloticus, a diurnal murid rodent from East Africa. *J Comp Neurol*, *403*(2), 190-208. https://doi.org/10.1002/(sici)1096-9861(19990111)403:2<190::aid-cne4>3.0.co;2-k

Sokolove, P. G., & Bushell, W. N. (1978). The chi square periodogram: Its utility for analysis of circadian rhythms. *Journal of Theoretical Biology*, *72*(1), 131-160. https://doi.org/https://doi.org/10.1016/0022-5193(78)90022-X

Stokkan, K.-A., Tyler, N. J. C., & Reiter, R. J. (1994). The pineal gland signals autumn to reindeer (Rangifer tarandus tarandus) exposed to the continuous daylight of the Arctic summer. *Canadian Journal of Zoology*, *72*(5), 904-909. https://doi.org/10.1139/z94-123

Strijkstra, A. M., Meerlo, P., & Beersma, D. G. (1999). Forced desynchrony of orcadian rhythms of body temperature and activity in rats. *Chronobiology international*, *16*(4), 431-440.

Tillet, Y., Caldani, M., & Tramu, G. (1989). Immunohistochemical characterization of the sheep suprachiasmatic nucleus. *Journal of Chemical Neuroanatomy*, *2*(4), 215-226.

Tsuneoka, Y., & Funato, H. (2021). Cellular composition of the preoptic area regulating sleep, parental, and sexual behavior. *Frontiers in neuroscience*, *15*, 649159.

Ulrich-Lai, Y. M., Arnhold, M. M., & Engeland, W. C. (2006). Adrenal splanchnic innervation contributes to the diurnal rhythm of plasma corticosterone in rats by modulating adrenal sensitivity to ACTH. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *290*(4), R1128-R1135.

Upham, N. S., Esselstyn, J. A., & Jetz, W. (2019). Inferring the mammal tree: species-level sets of phylogenies for questions in ecology, evolution, and conservation. *PLoS biology*, *17*(12), e3000494.

Van Oort, B. E., Tyler, N. J., Gerkema, M. P., Folkow, L., Blix, A. S., & Stokkan, K.-A. (2005). Circadian organization in reindeer. *Nature*, *438*(7071), 1095-1096.

van Oort, B. E., Tyler, N. J., Gerkema, M. P., Folkow, L., & Stokkan, K.-A. (2007). Where clocks are redundant: weak circadian mechanisms in reindeer living under polar photic conditions. *Naturwissenschaften*, *94*(3), 183-194.

Ware, J. V., Rode, K. D., Robbins, C. T., Leise, T., Weil, C. R., & Jansen, H. T. (2020). The clock keeps ticking: circadian rhythms of free-ranging polar bears. *Journal of biological rhythms*, *35*(2), 180-194.

Watanabe, K., Vanecek, J., & Yamaoka, S. (2000). In vitro entrainment of the circadian rhythm of vasopressin-releasing cells in suprachiasmatic nucleus by vasoactive intestinal polypeptide. *Brain research*, *877*(2), 361-366.

Williams, C. T., Barnes, B. M., Richter, M., & Buck, C. L. (2012). Hibernation and circadian rhythms of body temperature in free-living Arctic ground squirrels. *Physiological and Biochemical Zoology*, *85*(4), 397-404.
Williams, C. T., Barnes, B. M., Yan, L., & Buck, C. L. (2017). Entraining to the polar day: circadian rhythms in arctic ground squirrels. *Journal of Experimental Biology*, *220*(17), 3095-3102.
Yamazaki, S., Numano, R., Abe, M., Hida, A., Takahashi, R.-i., Ueda, M., Block, G. D., Sakaki, Y., Menaker, M., & Tei, H. (2000). Resetting central and peripheral circadian oscillators in transgenic rats. *Science*, *288*(5466), 682-685.

Appendix

Appendix A: Supplementary figures for SCN characterization

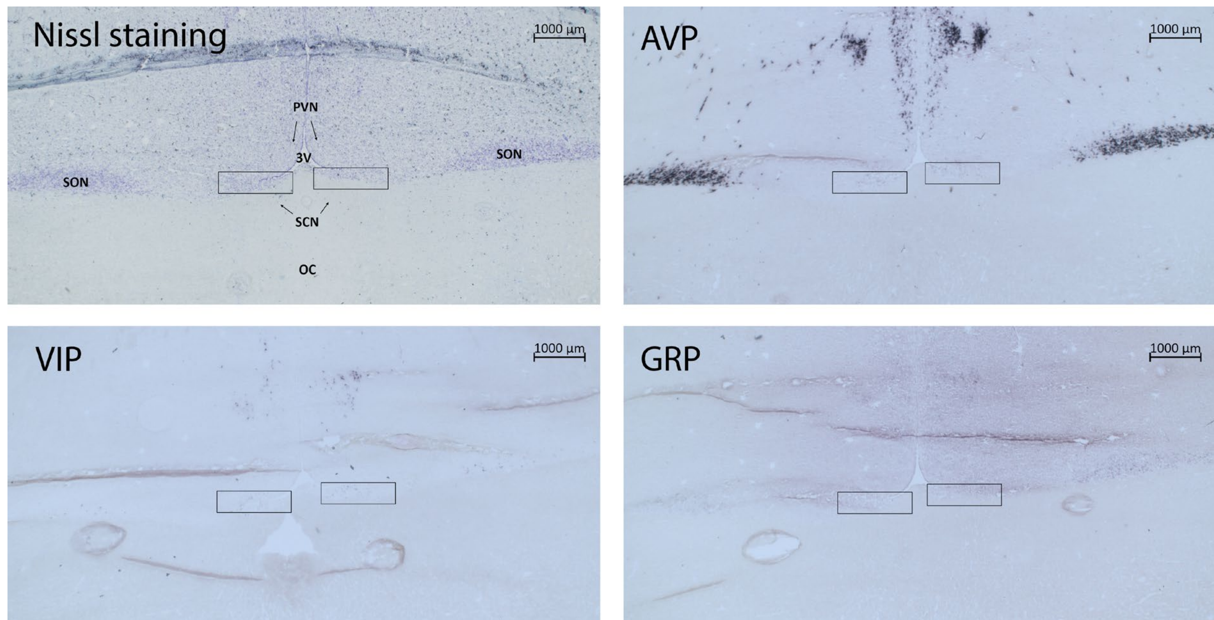

***Figure A-1: Pictures of stained reindeer (5/22) SCN and the areas around it**. Pictures showing nissl staining and staining of the mRNA of the proteins arginine vasopressin (AVP), vasoactive intestinal polypeptide (VIP) and gastrin-releasing polypeptide (GRP). The pictures include the anatomical structures; suprachiasmatic nucleus (SCN), indicated by black boxes; optic chiasm (OC); supraoptic nucleus (SON); paraventricular nucleus (PVN), indicated by arrows and the third ventricle (3V). The size of the structures is indicated by the scale bar in the upper right conor.*

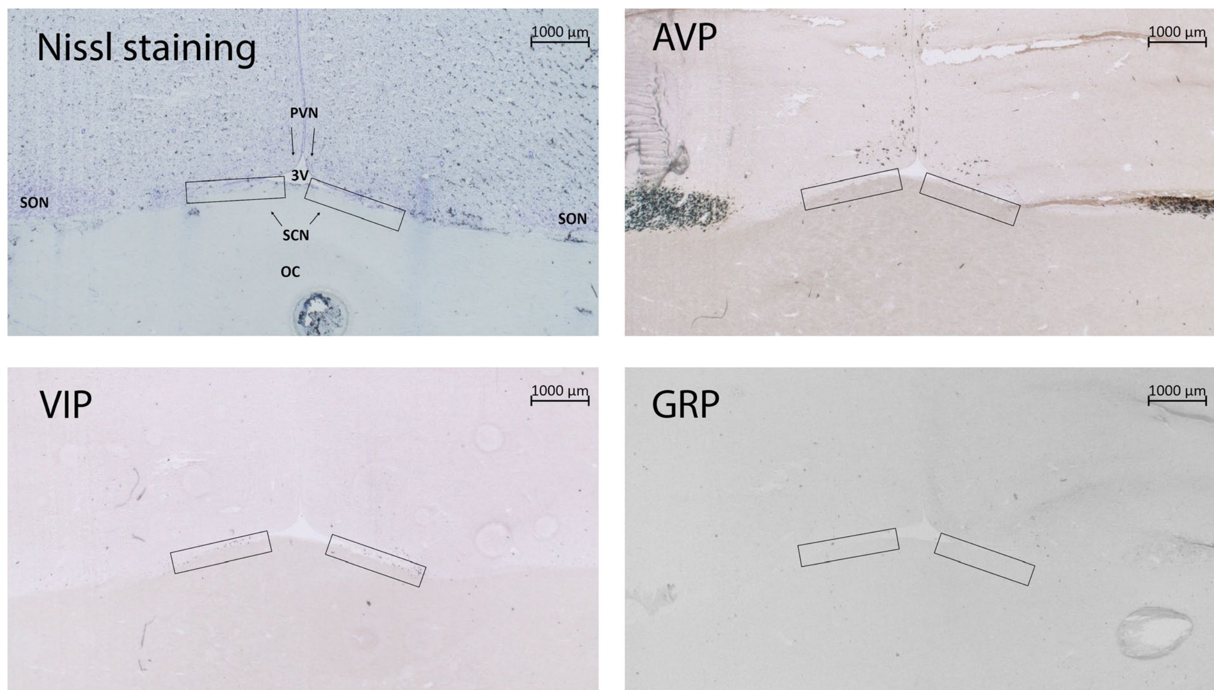

Figure A-2: Pictures of stained reindeer (4/21) SCN and the areas around it*. Pictures showing nissl staining and staining of the mRNA of the proteins arginine vasopressin (AVP), vasoactive intestinal polypeptide (VIP) and gastrin-releasing polypeptide (GRP). The pictures include the anatomical structures; suprachiasmatic nucleus (SCN), indicated by black boxes; optic chiasm (OC); supraoptic nucleus (SON); paraventricular nucleus (PVN), indicated by arrows and the third ventricle (3V). The size of the structures is indicated by the scale bar in the upper right conor.*

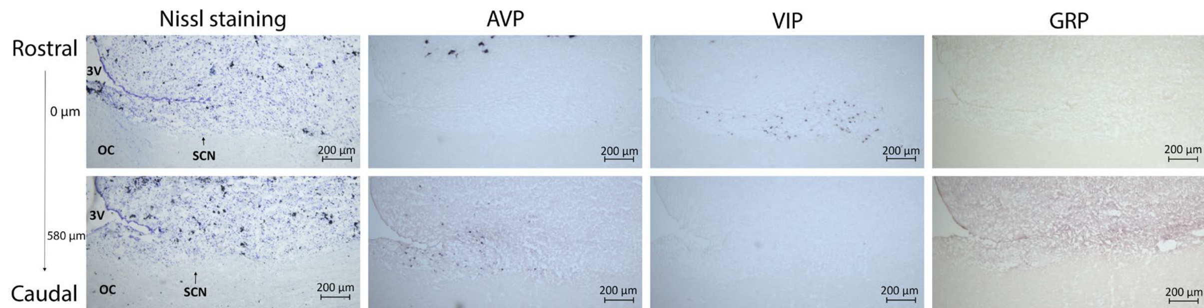

Figure A-3: Pictures of stained reindeer (5/22) SCN. *Pictures showing nissl staining and staining of the mRNA of the proteins arginine vasopressin (AVP), vasoactive intestinal polypeptide (VIP) and gastrin-releasing polypeptide (GRP). The pictures include the anatomical structures; suprachiasmatic nucleus (SCN), indicated by arrows, optic chiasm (OC), and the third ventricle (3V). The size of the structures is indicated by the scale bar in the lower right conor. Pictures represent how the SCN changes from rostral to caudal. Each line is consecutive slides with different staining, and downwards it is moving through the SCN. The distance between sections in each line is 580 μm.*

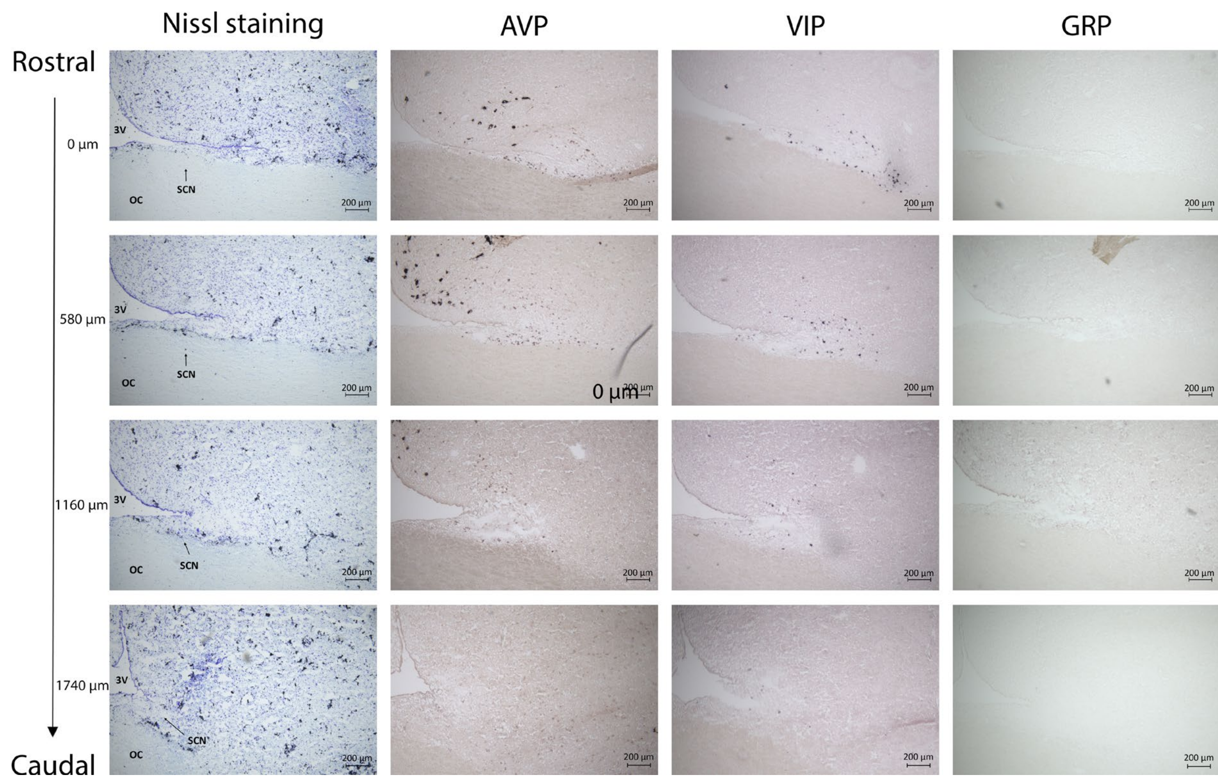

Figure A-4: Pictures of stained reindeer (4/21) SCN. *Pictures showing nissl staining and staining of the mRNA of the proteins arginine vasopressin (AVP), vasoactive intestinal polypeptide (VIP) and gastrin-releasing polypeptide (GRP). The pictures include the anatomical structures; suprachiasmatic nucleus (SCN), indicated by arrows, optic chiasm (OC), and the third ventricle (3V). The size of the structures is indicated by the scale bar in the lower right conor. Pictures represent how the SCN changes from rostral to caudal. Each line is consecutive slides with different staining, and downwards it is moving through the SCN. The distance between sections in each line is 580 μm.*

Appendix B: Supplementary figures for pilot study

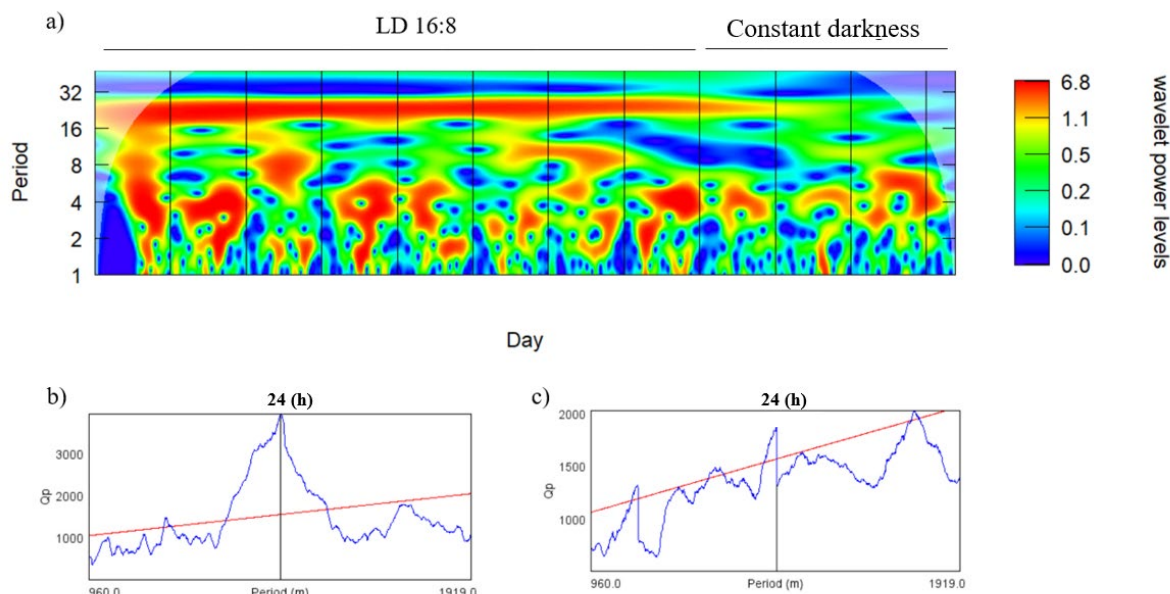

Figure B-1: Locomotor activity rhythm of reindeer (2/21) during pilot study*. The study is divided into two parts: an entrainment phase (8 days) with a light-dark cycle of 16 hours light and 8 hours dark (LD 16:8) and a period of constant darkness (3 days). a) wavelet presenting the power of the periods. The wavelet power goes from 0 (dark blue) to 6.8 (red). A stronger power indicates a stronger rhythm. The wavelet shows a strong 24-hour period during LD 16:8 with a subsequently dampening during constant darkness. The wavelet shows strong ultradian components in the activity rhythm of reindeer. b) chi-square test of activity pattern during LD 16:8 shows a significant peak (p<0.05) at 24 hours. c) chi-square test of activity pattern during constant darkness shows a significant peak (p<0.05) at 24 hours. Actogram for periodograms is shown in figure B-4.*

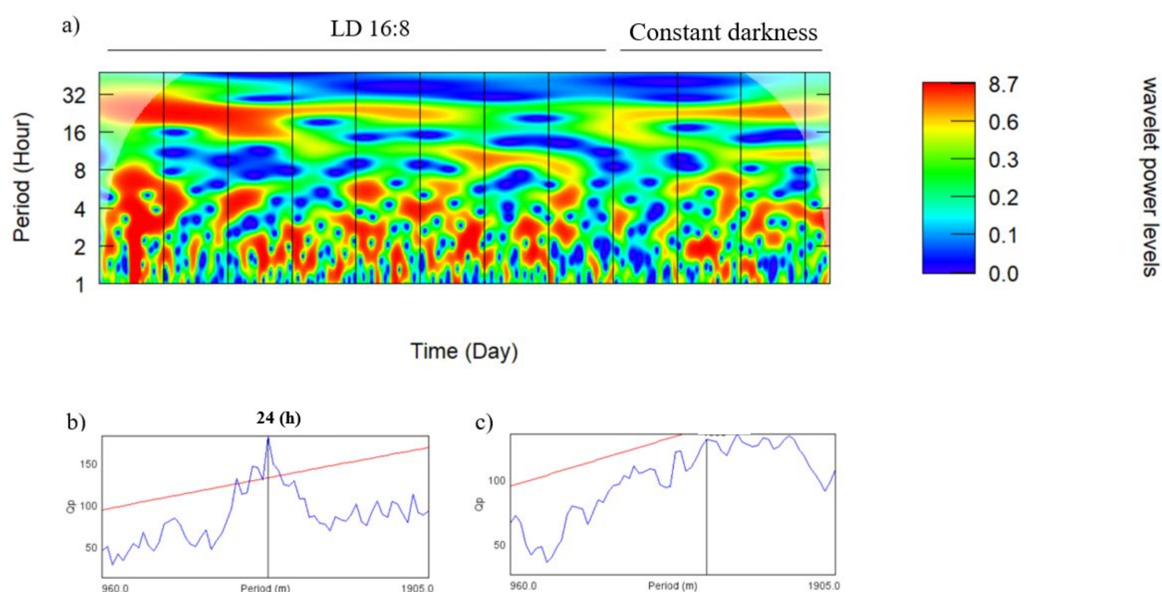

Figure B-2: Surface body temperature rhythm of reindeer (2/21) during pilot study*. The study is divided into two parts: an entrainment phase (8 days) with a light-dark cycle of 16 hours light and 8 hours dark (LD 16:8) and a period of constant darkness (3 days). a) wavelet presenting the power of the periods. The wavelet power goes from 0 (dark blue) to 15 (red). A stronger power indicates a stronger rhythm. The wavelet shows a strong 24-hour period during LD 16:8 with a subsequently dampening during constant darkness. The wavelet shows strong ultradian*

components in the surface body temperature rhythm of reindeer. b) chi-square test of surface body temperature pattern during LD 16:8 shows a significant peak (p<0.05) at 24 hours. c) chi-square test of surface body temperature pattern during constant darkness shows no significant peak. Actogram for periodograms is shown in figure B-4.

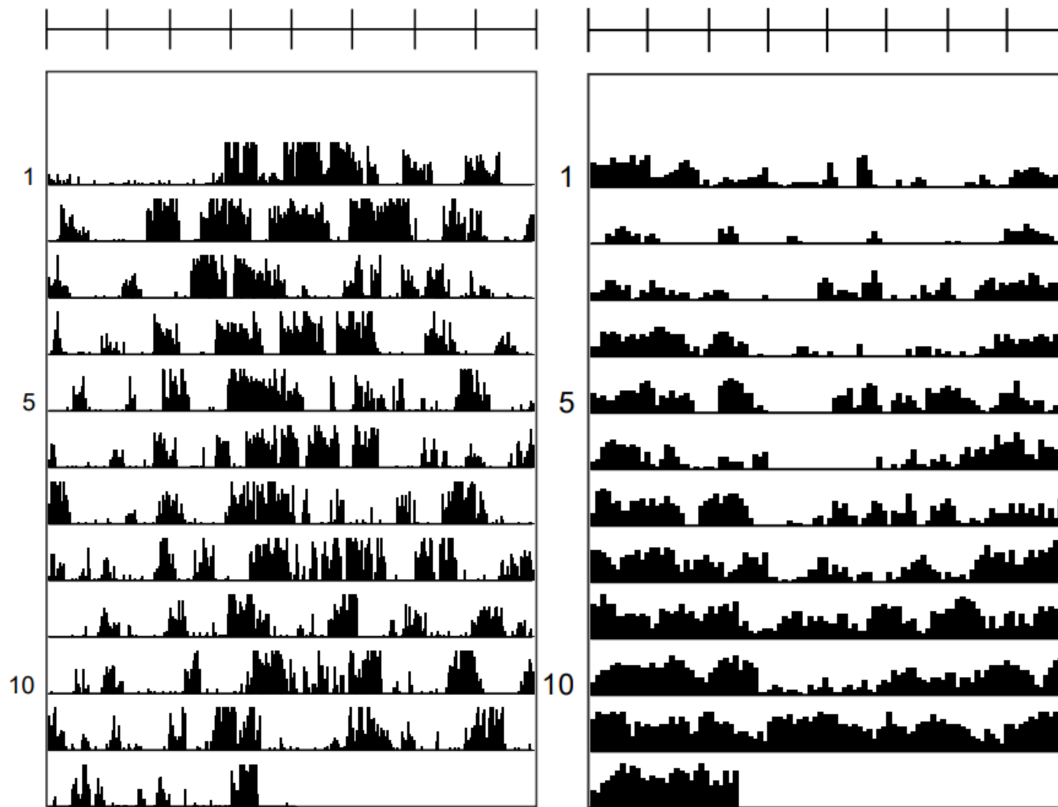

***Figure B-3: Actograms for locomotor activity (left) and surface body temperature (right) for reindeer 1/21 during the pilot study**. Each line represents one day in the protocol. The study is divided into two parts: an entrainment phase (the first 8 days) with a light-dark cycle of 16 hours light and 8 hours dark (LD 16:8) and a period of constant darkness (the last 3 days). On the top of the actogram a scale is present, the scale represents the 24-hour during the day with start at 00:00. The upper limit for visual representation in the actogram was for locomotor activity set at 300 and for surface body temperature at 34°C and the lower limits were set to 0 for locomotor activity and 30°C for surface body temperature.*

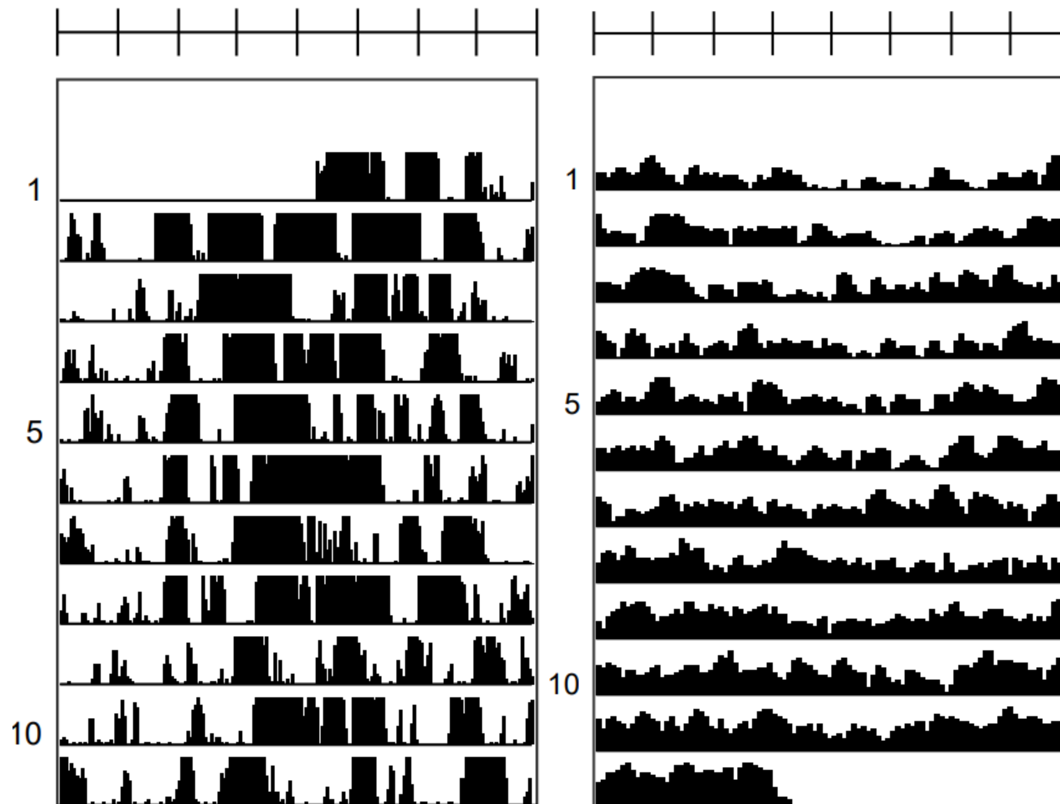

Figure B-4: Actograms for locmotor activity (left) and surface body temperature (right) for reindeer 2/21 during the pilot study*. Each line represents one day in the protocol. The study is divided into two parts: an entrainment phase (the first 8 days) with a light-dark cycle of 16 hours light and 8 hours dark (LD 16:8) and a period of constant darkness (the last 3 days). On the top of the actogram a scale is present, the scale represents the 24-hour during the day with start at 00:00. The upper limit for visual representation in the actogram was for locomotor activity set at 300 and for surface body temperature at 32°C and the lower limits were set to 0 for locomotor activity and 28°C for surface body temperature.*

Appendix C: Supplementary figures for forced desynchrony

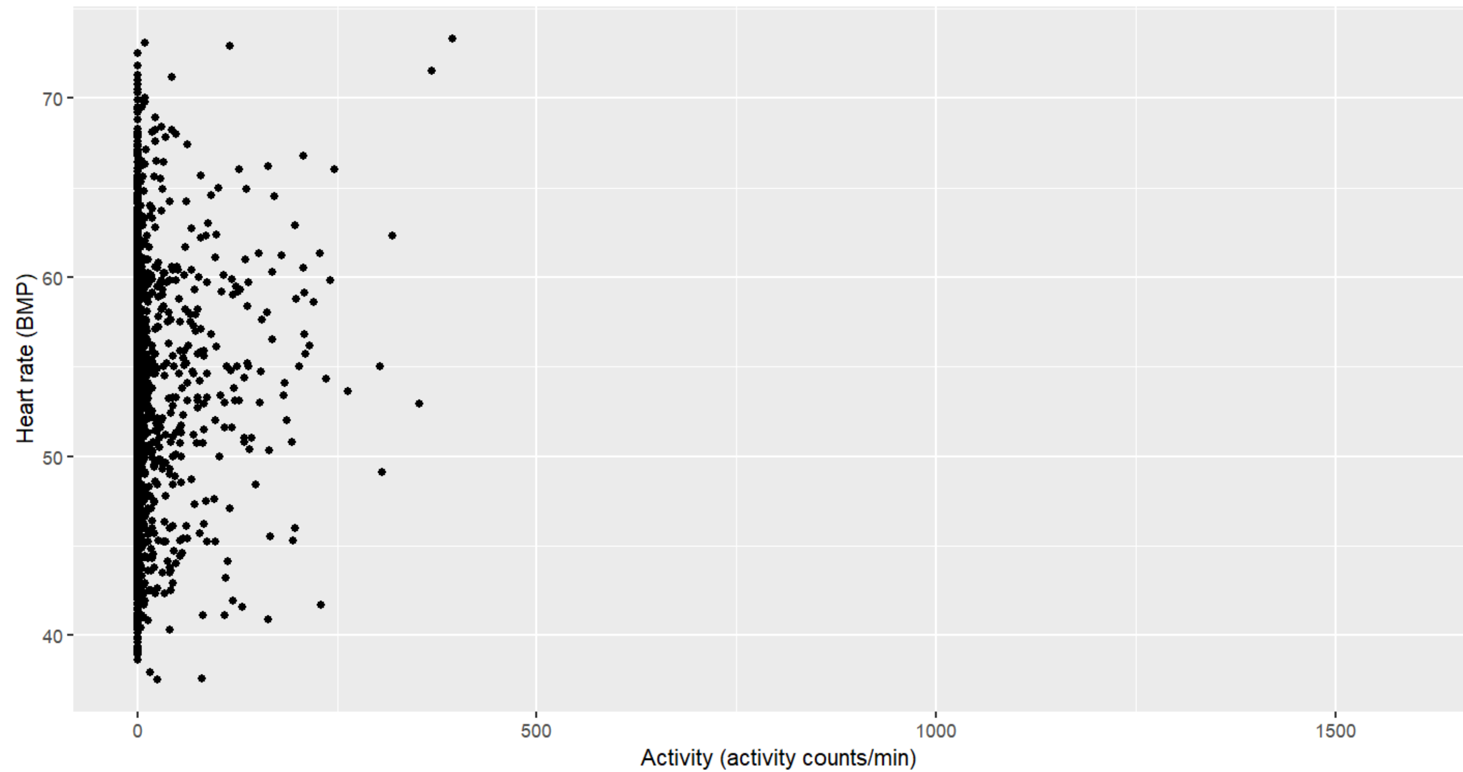

Figure C-1: Plot illustrating that heart rate (HR) and resting locomotor activity (LA) is correlated for reindeer (1/22). *The x-axis shows the LA (activity counts/min) and the y-axis show the ~20% remaining datapoints of HR after filtering. HR data is concentrated when there are 0 or very little activity of the reindeer.*

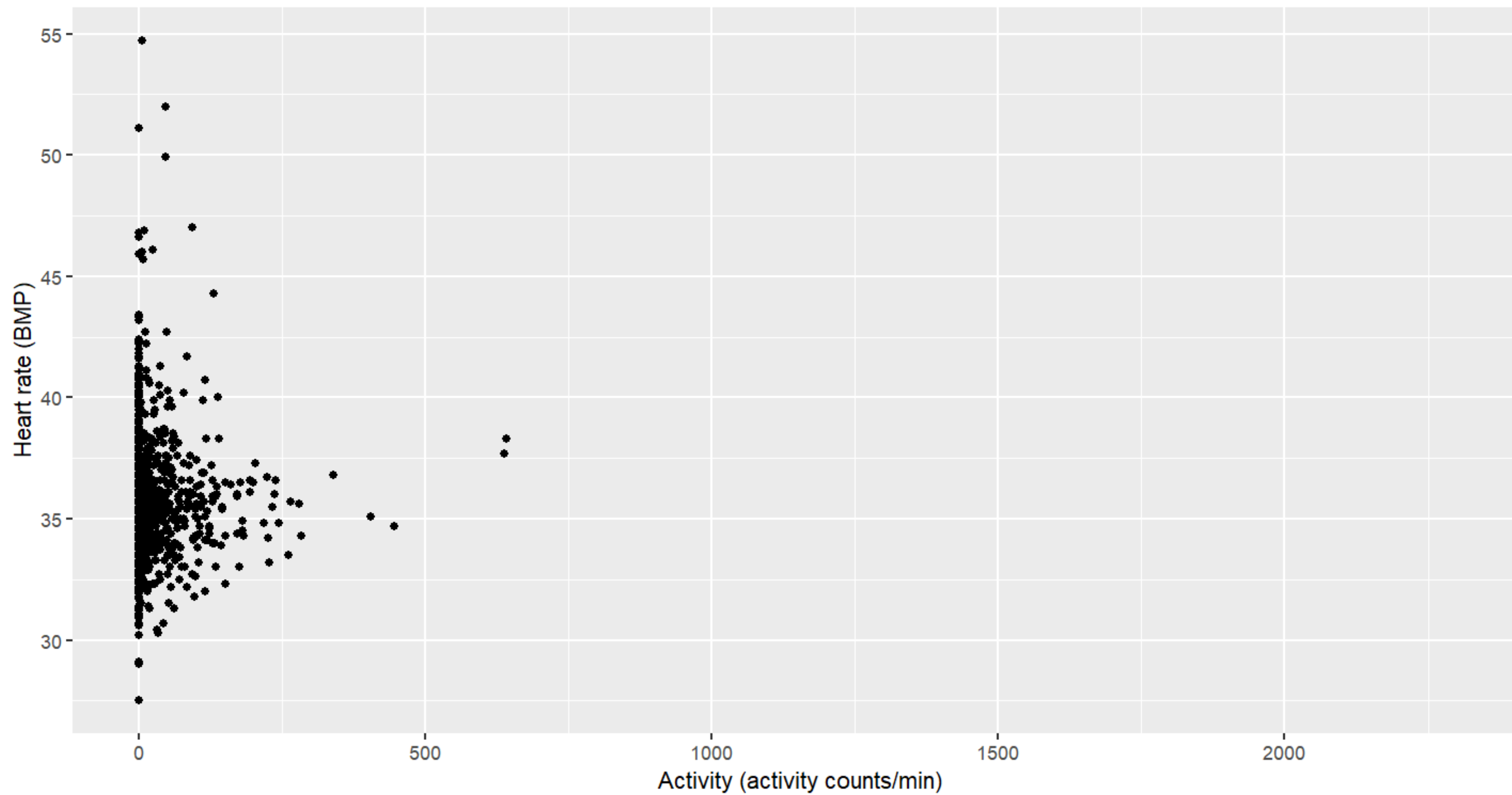

Figure C-2: Plot illustrating that heart rate (HR) and resting locomotor activity (LA) is correlated for reindeer (2/22). *The x-axis shows the LA (activity counts/min) and the y-axis show the ~20% remaining datapoints of HR after filtering. HR data is concentrated when there are 0 or very little activity of the reindeer.*

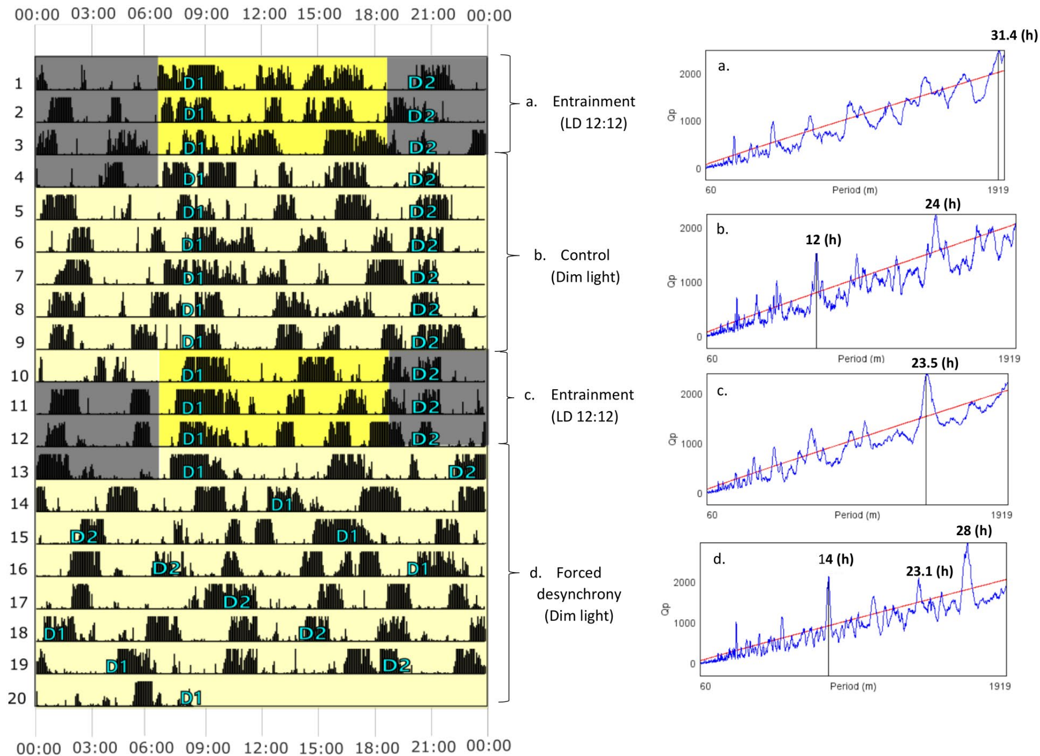

***Figure C-3: Actogram and periodograms of locomotor activity (LA) measurements from reindeer 1/22**. LA is measured with Actiwacth attached to the harness of the reindeer. On the left panel the actogram with raw data is displayed. Each line represents one day in the experiment as is represented by the numbers on the left of the actogram. On the top and bottom of the actogram the time of day is displayed. On the right of the actogram each part of the protocol is demonstrated. a) the first entrainment with a light regime of 12 hours dark and 12 hours light, lights on at 06:30 and lights of at 18:30 (LD 12:12) with disturbances every 12 hours at 08:30 and 20:30. b) The second part is the control with constant dim light and disturbances every 12 hours at 08:30 and 20:30. C) the second entrainment follows the same regime as a). d) the forced desynchrony protocol with constant dim light and disturbances every 14 hours as described in Table 1. D1 and D2 represent disturbance-1 and disturbance-2, respectively. The lower limit of the actogram was set to 0 and the upper limit set to 300. The right panel show the respectively periodograms for each part of the protocol. Tested with a chi-square periodogram with a significance level of 0.05.*

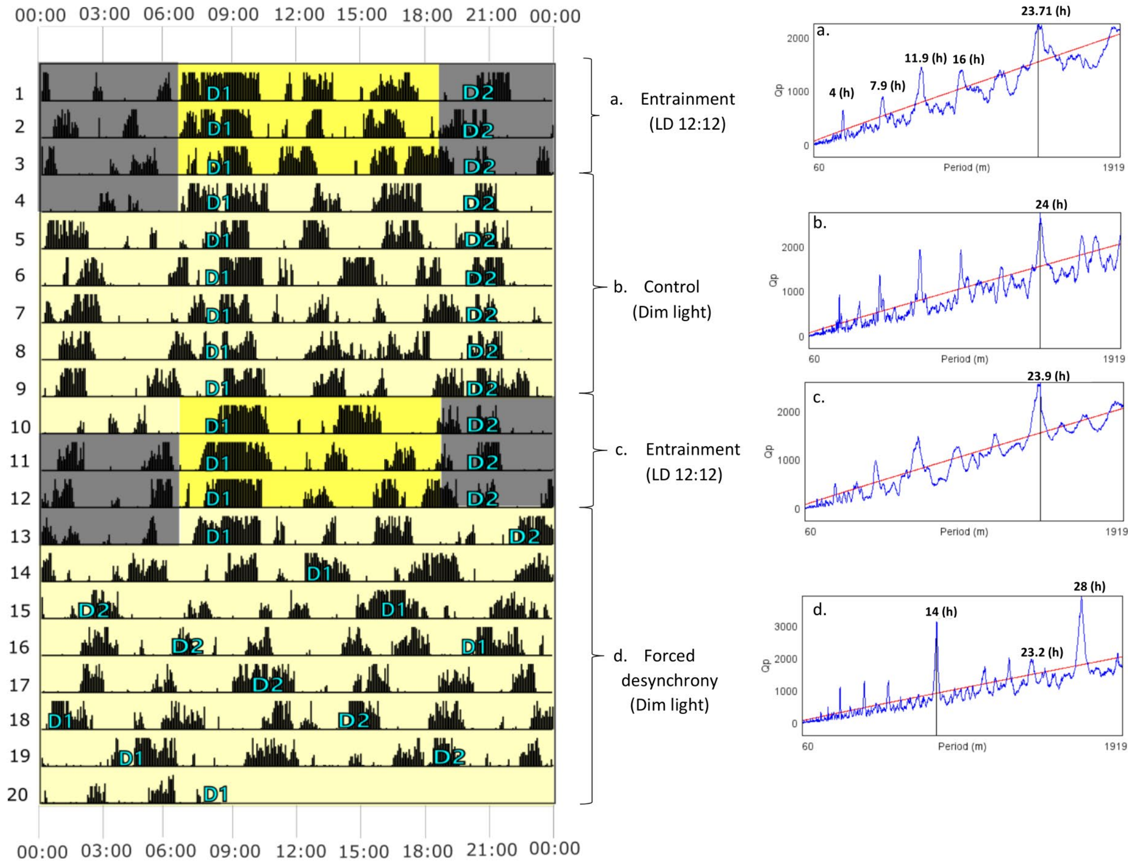

Figure C-4: Actogram and periodograms of locomotor activity (LA) measurements from reindeer 3/22. *LA is measured with Actiwacth attached to the harness of the reindeer. On the left panel the actogram with raw data is displayed. Each line represents one day in the experiment as is represented by the numbers on the left of the actogram. On the top and bottom of the actogram the time of day is displayed. On the right of the actogram each part of the protocol is demonstrated. a) the first entrainment with a light regime of 12 hours dark and 12 hours light, lights on at 06:30 and lights of at 18:30 (LD 12:12) with disturbances every 12 hours at 08:30 and 20:30. b) The second part is the control with constant dim light and disturbances every 12 hours at 08:30 and 20:30. C) the second entrainment follows the same regime as a). d) the forced desynchrony protocol with constant dim light and disturbances every 14 hours as described in Table 1. D1 and D2 represent disturbance-1 and disturbance-2, respectively. The lower limit of the actogram was set to 0 and the upper limit set to 300. The right panel show the respectively periodograms for each part of the protocol. Tested with a chi-square periodogram with a significance level of 0.05.*

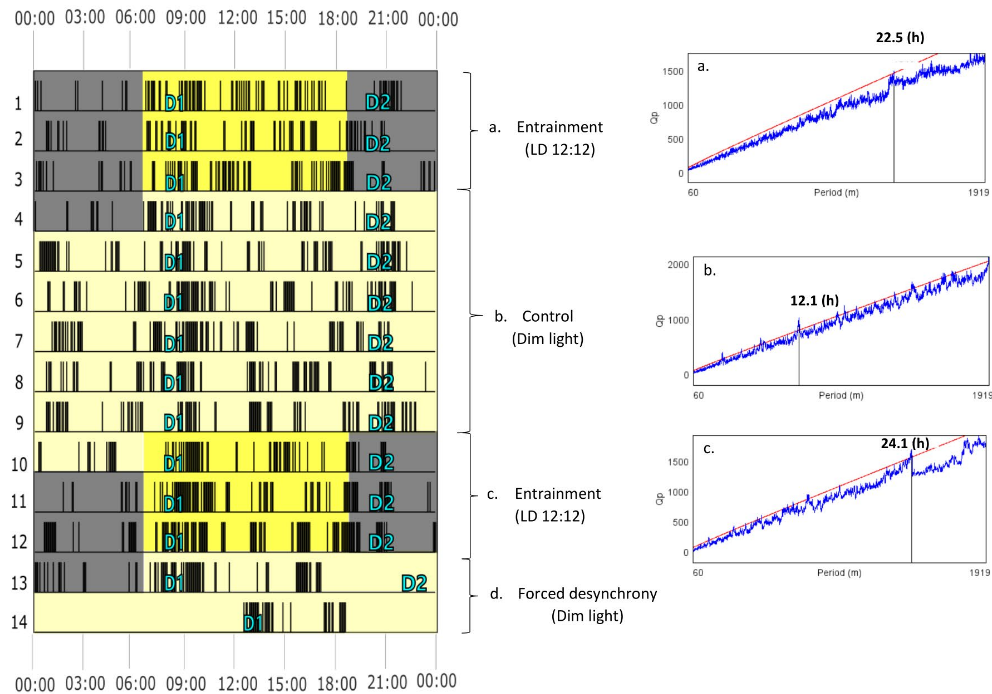

***Figure C-5: Actogram and periodograms of feeding behavior (FB) from reindeer 1/22**. FB is measured with HOBO loggers in feeding station. On the left panel the actogram with the binary data is displayed. Each line represents one day in the experiment as is represented by the numbers on the left of the actogram. On the top and bottom of the actogram the time of day is displayed. On the right of the actogram each part of the protocol is demonstrateda) the first entrainment with a light regime of 12 hours dark and 12 hours light, lights on at 06:30 and lights of at 18:30 (LD 12:12) with disturbances every 12 hours at 08:30 and 20:30. b) The second part is the control with constant dim light and disturbances every 12 hours at 08:30 and 20:30. C) the second entrainment follows the same regime as a) – since the HOBO logger stopped working a periodogram of forced desynchrony is not included. D1 and D2 represent disturbance-1 and disturbance-2, respectively. The lower limit of the actogram was set to 0 and the upper limit set to 1. The right panel show the respectively periodograms for each part of the protocol. Tested with a chi-square periodogram with a significance level of 0.05.*

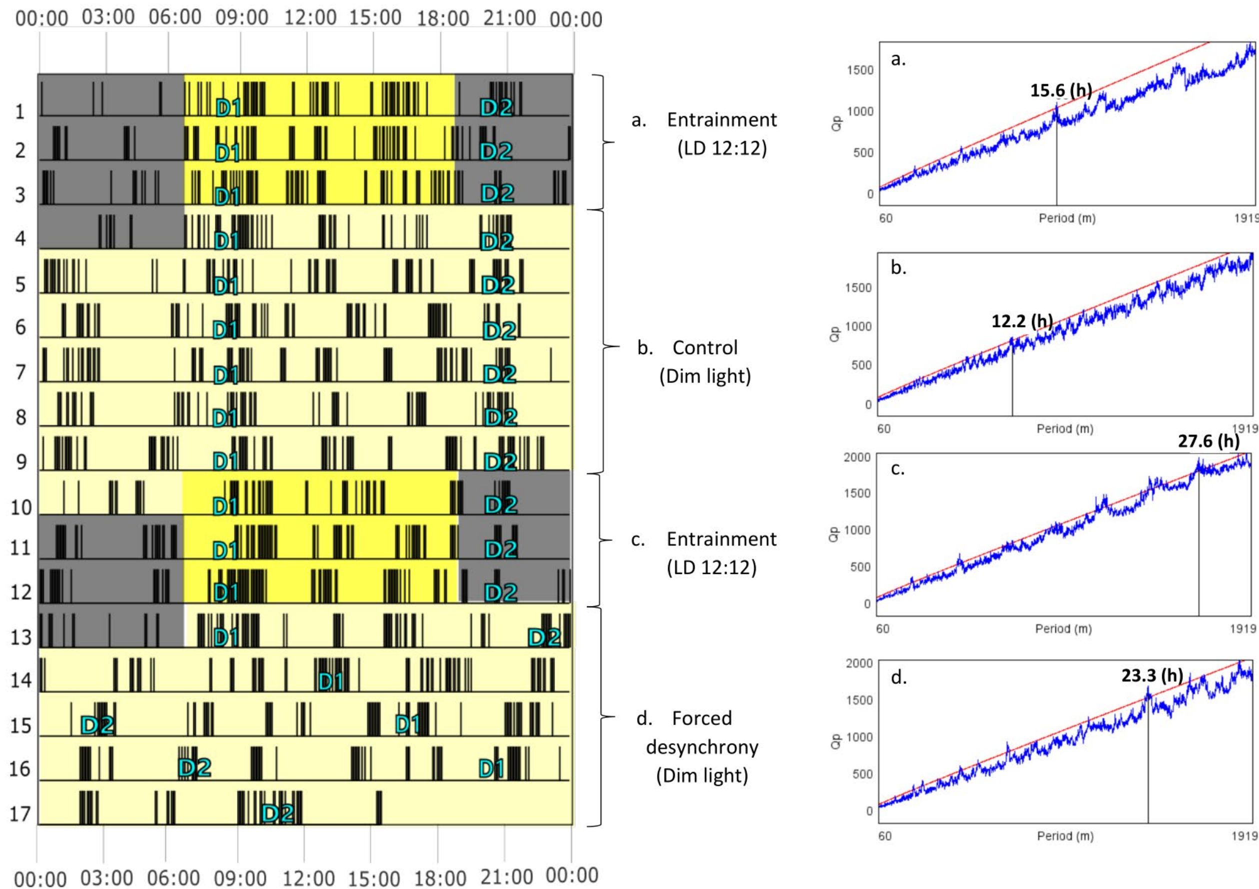

Figure C-6: Actogram and periodograms of feeding behavior (FB) from reindeer 3/22*. FB is measured with HOBO loggers in feeding station. On the left panel the actogram with the binary data is displayed. Each line represents one day in the experiment as is represented by the numbers on the left of the actogram. On the top and bottom of the actogram the time of day is displayed. On the right of the actogram each part of the protocol is demonstrated. a) the first entrainment with a light regime of 12 hours dark and 12 hours light, lights on at 06:30 and lights of at 18:30 (LD 12:12) with disturbances every 12 hours at 08:30 and 20:30. b) The second part is the control with constant dim light and disturbances every 12 hours at 08:30 and 20:30. C) the second entrainment follows the same regime as a). d) the forced desynchrony protocol with constant dim light and disturbances every 14 hours as described in Table 1. D1 and D2 represent disturbance-1 and disturbance-2, respectively. The lower limit of the actogram was set to 0 and the upper limit set to 1. The right panel show the respectively periodograms for each part of the protocol. Tested with a chi-square periodogram with a significance level of 0.05.*

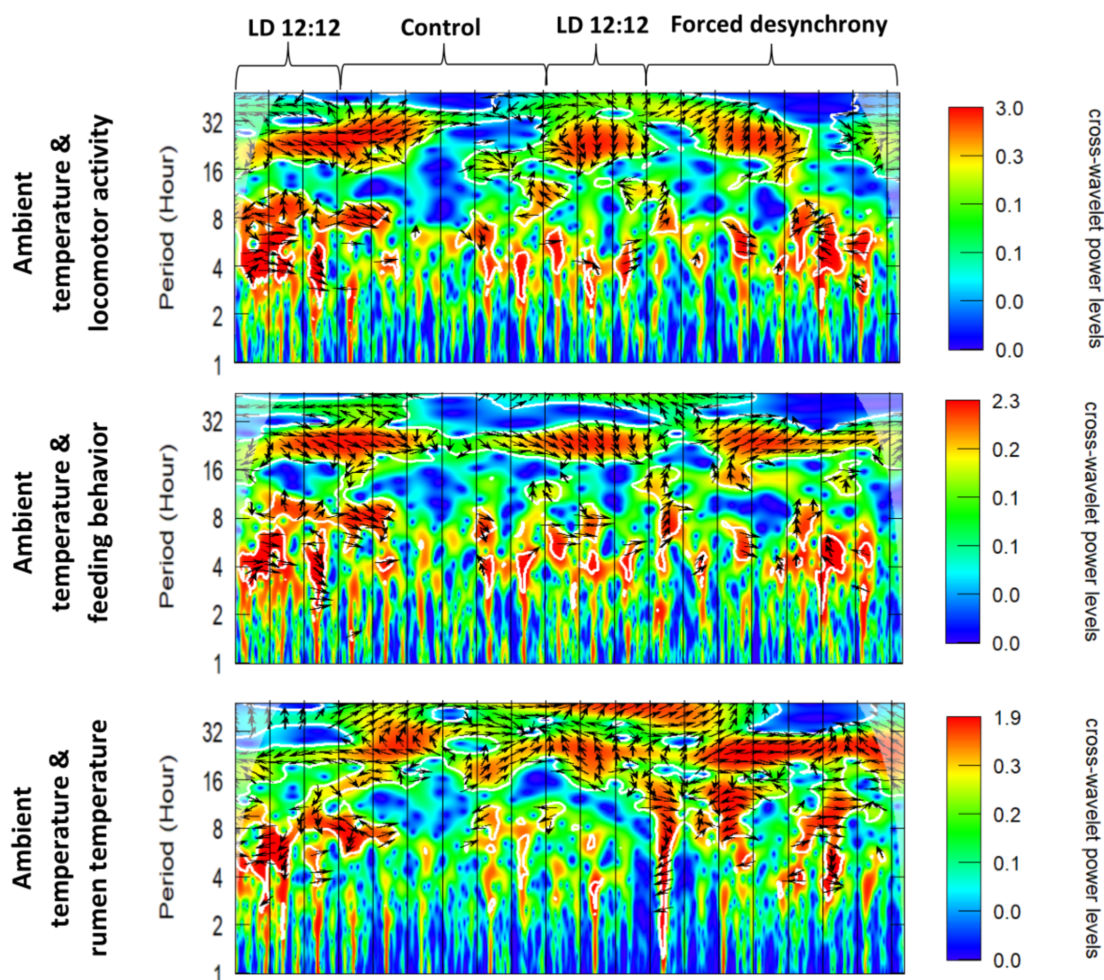

Figure C-7: Cross-wavelet analyses of ambient temperature. *Figures displaying cross-wavelet analysis of ambient temperature with the parameter's locomotor activity, feeding behavior and rumen temperature. The y-axis shows the periods that are analyzed in the power analyses, going from 1 to 48. On the top the protocol is illustrated. First, the first entrainment with a light regime of 12 hours dark and 12 hours light, lights on at 06:30 and lights of at 18:30 (LD 12:12) with disturbances every 12 hours at 08:30 and 20:30. The second part is the control with constant dim light and disturbances every 12 hours at 08:30 and 20:30. The second entrainment follows the same regime as the first and lastly the forced desynchrony protocol with constant dim light and disturbances every 14 hours as described in Table 1. Each line in the wavelets represents the days in the protocol. The power of the wavelets is displayed on the right side in the figure. Blue indicates a low power and red indicates a strong power in the rhythm. The black arrows indicate significant cross-correlation (p<0.05).*

Appendix D: R-codes

Same R-code was used for all analyses, it this appendix the general R-code for these analyses is provided.

Wavelet analyze

#df = dataframe used

```
dfW <- analyze.wavelet(df, "*", #* parameter to be analyzed
                loess.span = 0,
                dt = *, dj = 1/250, #dt = * frequency of data points/60 minutes
                lowerPeriod = 1,
                upperPeriod = 48,
                make.pval = TRUE, n.sim = 10)
```

Correlation

```
cor.test(parameter1, parameter2) #parameter that are tested for correlation
```

Cross-correlation

```
ccf(parameter1, parameter2) #parameter that are tested
print(ccf(parameter1, parameter2)) #parameter that are tested
```

Cross-wavelet

```
dfW <- analyze.coherency(df, my.pair = c("x","y"), #x & y = the objects for cross-correlation
                    loess.span = 0,
                    dt = *, dj = 1/100, #dt = * frequency of data points/60 minutes
                     loess.span = 0,
                    make.pval = TRUE, n.sim = 10,
                    lowerPeriod = *, #1 or 22
                    upperPeriod = *) #28 or 48
```

Repeated measures ANOVA

```
anova_result <- df %>% #df used
  anova_test(Value ~ Conditions * Disturbance * Reindeer ID + Error(Paired
samples/Disturbance))
get_anova_table(anova_result)
```

Paired t-test

```
t.test(Values, Values, paired = T) #values of cortisol
```