Balancing expectations

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DOI:
10.33612/diss.208878689

Balancing Expectations

Adaptive flexibility of mammalian circadian organisation

Sjaak J Riede

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The research described in this thesis was performed at the department of Chronobiology, embedded within the Groningen Institute for evolutionary life sciences (GELIFES) – part of the University of Groningen, The Netherlands. The funding for my studies was provided by the University of Groningen, specifically the Groningen Graduate School of Science (GGSS) in the form of an Ubbo-Emmius Scholarship, the research school of Behavioural and Cognitive Neuroscience (BCN) and the departments of Chronobiology and Neuroscience, for which I am grateful.

Cover design: Sjaak J. Riede

Lay-out: Sjaak J. Riede

Printing: Gildeprint (www.gildeprint.nl)

university of groningen

Balancing Expectations

Adaptive flexibility in mammalian circadian organisation

PhD thesis

to obtain the degree of PhD at the
University of Groningen
on the authority of the
Rector Magnificus Prof. C. Wijmenga
and in accordance with
the decision by the College of Deans.

This thesis will be defended in public on

Tuesday 12 April 2022 at 12.45 hours

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Preface

“Disorder is inherent in stability. Civilized man doesn't understand stability. He's confused it with rigidity. Our political and economic and social leaders drool about stability constantly. It's their favorite word, next to 'power. Gotta stabilize the political situation in Southeast Asia, gotta stabilize oil production and consumption, gotta stabilize student opposition to the government' and so forth.

Stabilization to them means order, uniformity, control. And that's a half-witted and potentially genocidal misconception. No matter how thoroughly they control a system, disorder invariably leaks into it. Then the managers panic, rush to plug the leak and endeavor to tighten the controls. Therefore, totalitarianism grows in viciousness and scope. And the blind pity is, rigidity isn't the same as stability at all.

True stability results when presumed order and presumed disorder are balanced. A truly stable system expects the unexpected, is prepared to be disrupted, waits to be transformed.”

— Tom Robbins, Even Cowgirls Get the Blues, novel 1976

Glossary

Terminology

Acclimatize Refers in our experiments to the time needed to become familiar with the light-environment (synchronizing internal rhythms with the environment)

Adaptation Adjustment to a situation for the better, making use of opportunities and/or avoiding negative consequences. Mal-adaptation is the opposite, in which the adjustment is actually detrimental and hampering fitness.

Anticipation Preparing for an external event that is expected to happen

After-effects Lasting alterations in behaviour and/or physiological measures that persist after withdrawal from the inducing conditions

Circadian A feature or pattern that reoccurs roughly every 24 hours in and environment with constant conditions (circadian = "*about a day*") that is driven by endogenous oscillators.

Endogenous Coming from one-self, not triggered by something in the environment

Entrainment A situation under which environmental and internal rhythms have a stable relationship with matching periods and having a stable phase angle. The phase of entrainment reflects to how the rhythms are aligned with each other.

Fitness "The unit of life"; commonly refers to number of successfully surviving offspring, which is influenced by factors like survival/life-span and reproductive success (including parental care). Abstract and hard to measure as it involved also principles like chance (e.g., likelihood of getting predated). One trait might improve one aspect (bright red beak increasing chances of acquiring mate) but hamper others (being more conspicuous to predators) making net effects on fitness complex.

Free-run The behavioural or physiological rhythm of an animal that is displayed in a constant environment. Since the endogenous clock can deviate from 24, a free-running pattern when plotted on a 24h

time scale will lead to a forward slanted (tau < 24 h) or backward-slanted (tau > 24 h) pattern.

Masking Direct influences from the environment on physiology and behaviour. In chapter 4 we focus on light masking and the direct effects on activity levels in mice. Positive light masking refers to a light-induced increase in activity whereas negative light masking refers to reductions in activity level caused by light.

Oscillator Something that alternates between two states with a stable periodicity, like a swing or a (biological) rhythm generator.

Synchronize Adjusting phase and period of internal rhythms to rhythms from the environment (or rhythms in other tissues) which leads to stable constant phase-relations. See also entrainment.

Tau The period or the duration of a pattern, i.e., the amount of time before a rhythm repeats itself.

List of abbreviations

Anatomical structures

SCN *Suprachiasmatic nucleus*; hypothalamic clusters of neurons directly above the optic chiasm that are considered the mammalian master oscillator. In chapter 6 we compared two treatment groups that had their SCN-structure either surgically lesioned (SCN_X) or left intact following sham-surgery (SCN_{SHAM})

PVN *Paraventricular nucleus* of the hypothalamus. Considered a main projection-target for SCN cells. The PVN regulates amongst other things the HPA-reactivity (influencing, e.g., corticosterone)

PVT *Paraventricular nucleus of the thalamus*, midline thalamic nucleus receiving many peripheral and hypothalamic signals with bilateral connections with cortical areas involved in cognitive processing. Sometimes used with the prefix "a" to specify the anterior portion of the structure. In chapter 6 we compared mice with lesions to this structure ($aPVT_X$) with mice with the structure left intact following sham surgery ($aPVT_{SHAM}$)

AD *adrenal;* referring to the adrenal glands. In chapter 6 we have compared for instance animals in which the adrenals were surgically removed (AD_X) versus mice in which the adrenals were left intact after sham surgery (AD_{SHAM})

Terminology

CT Circadian time. Time of day based on the length of the endogenous rhythm an animal displays under constant conditions. A circadian hour is 1/24 of this free-running period. CTo is the start of activity phase of a diurnal animal or roughly projected sunrise.

CTE Circadian Thermo-Energetics Hypothesis, posed by our lab. The hypothesis states that the circadian phase of entrainment has a large influence on daily energy expenditure and the need for thermogenesis. Adverse conditions that induce a negative energy balance (cold, hunger) can promote a shift of the phase of entrainment towards a day-active phenotype to counter the negative energetic state by reducing thermogenesis costs.

ZT *Zeitgeber* Time; scale on which time of day is projected with ZTo defining the start of the light phase of an LD cycle.

Treatment and protocol related

WFF Work-for-food paradigm, in which mice have to obtain food by completing set amounts of wheel revolutions

HWL High workload, during which each food pellet requires a high number of wheel revolution, simulating food scarcity.

LWL Low workload, during which each food pellets requires a low number of wheel revolutions, generally set at a level by which spontaneous wheel running activity levels are sufficient to food in surplus of what is eaten by the individual on a daily basis.

AL From Latin *ad libitum* (at one's pleasure) also *ad lib.*; round-the-clock and unconditional access to food (or water) in quantities exceeding the daily intake.

LD Light:dark cycle. Sometimes followed by a number to indicate the period length in hours; like LD_{24} or LD_7 in chapter 4. When providing cycles in which the photoperiod differs from 50 % of the cycle duration, numbers preceding this abbreviation denote light phase duration and dark phase duration respectively (e.g., a 16:8 LD cycle having a period of 24 hours, in which 16 hours of light are followed by 8 hours of darkness).

LL In contrast to LD, LL denotes constant light exposure

DD In contrast to LD, DD denotes constant darkness (or constant dim-red light in some experiments, in which case it is specified explicitly in the text).

Table of Contents

Short Summary (Lay-audience)

Circadian rhythms are internally generated oscillations of approximately 24 hours that synchronize with the environments day-night cycle, which drive and modulate countless behavioural and physiological processes. In this thesis we use a novel behavioural work-for-food paradigm which allows to study how changes in energy balance cause a change in the temporal niche of mice, making them adopt a day-active activity pattern. We show and discuss data supporting a functional role of circadian flexibility; diurnal activity patterns requiring less energy versus remaining night-active - for burrowing small mammals in temperate climate. We build on this by showing the rearrangements in temporal niche are associated with plasticity in the direct light response (photic masking) and explore differences between male and female mice. Further we show data that neither the adrenals nor the Paraventricular thalamic Nucleus are essential for circadian niche adaptation whereas the central circadian clock located in the suprachiasmatic nucleus remains of vital importance, despite itself not appearing to change phase. The rigidity of the SCN-timing might be linked to its role in measuring daylength and guiding seasonal rhythms. Which processes make behavioural and physiological rhythms obtain a different phase angle to the SCN during simulated food shortage remains largely elusive. The work in this thesis provides a solid scientific basis to re-address circadian flexibility and it's relation to energy balance in future studies. Gaining more insights in circadian rhythm flexibility might solve the poorly understood mechanisms behind metabolic risks associated with human shift-work and how to cope with circadian disruptions.

Korte samenvatting (Breed publiek)

Circadiane ritmen zijn intern gegenereerde oscillaties van ongeveer 24 uur die synchroon lopen met de dag-nacht cyclus van de omgeving en die talloze gedragsmatige en fysiologische processen aansturen en moduleren. In dit proefschrift gebruiken we een nieuw gedragsparadigma dat ons toelaat om te bestuderen hoe veranderingen in de energiebalans een verandering veroorzaken in de temporele niche van muizen waarmee ze een dag-actief activiteitspatroon aannemen. We tonen en bespreken gegevens die een functionele rol van circadiane flexibiliteit ondersteunen – dagactieve patronen kostten kleine zoogdieren doorgaans minder energie dan nachtactief blijven. We bouwen hierop voort door aan te tonen dat de herschikkingen in de temporele niche geassocieerd zijn met plasticiteit van de directe lichtrespons (fotomaskering) en onderzoeken verschillen tussen mannelijke en vrouwelijke muizen. Verder tonen we aan dat noch de bijnieren, noch de paraventriculaire thalamusnucleus essentieel zijn voor de aanpassing van de circadiane niche, terwijl de centrale circadiane klok in de suprachiasmatische kern van vitaal belang blijft hoewel deze zelf niet lijkt te

verschuiven in fase. De rigiditeit van de SCN-timing zou verband kunnen houden met diens rol in het meten van de daglengte en het sturen van seizoensgebonden ritmiek. Welke processen ervoor zorgen dat gedrags- en fysiologische ritmen een andere fase-hoek verkrijgen t.o.v. de SCN tijdens gesimuleerde voedselschaarste blijft grotendeels onopgehelderd. Het werk in dit proefschrift biedt echter een solide wetenschappelijke basis om circadiane flexibiliteit en het verband met energiebalans in toekomstige studies opnieuw aan de orde te stellen. Meer inzicht in de flexibiliteit van het circadiane ritme kan een oplossing bieden voor de onbegrepen mechanismen achter metabolische risico's van ploegendienst en hoe om te gaan met circadiane verstoringen.

Kurtze Zusammenfassung (Breit Publikum)

Circadiane Rhythmen sind intern erzeugte Rhythmen von etwa 24 Stunden, die sich mit dem Tag-Nacht-Zyklus der Umwelt synchronisieren und zahllose verhaltensbezogene und physiologische Prozesse steuern und regulieren. In dieser Arbeit verwenden wir ein neuartiges Verhaltensparadigma, das es uns ermöglicht zu untersuchen, wie Veränderungen im Energiehaushalt eine Veränderung in der zeitlichen Nische von Mäusen bewirken, so dass sie ein tagaktives Aktivitätsmuster annehmen. Wir zeigen und erörtern Daten, die eine funktionelle Rolle der zirkadianen Flexibilität - d. h. tagaktive Aktivitätsmuster, die weniger Energie benötigen, im Gegensatz zu nächtlichen Aktivitätsmustern - für wühlende kleine Säugetiere in gemäßigtem Klima belegen. Wir bauen darauf auf, indem wir zeigen, dass die Veränderungen in der zeitlichen Nische mit der Plastizität der direkten Lichtreaktion (photische Maskierung) verbunden sind, und untersuchen Unterschiede zwischen männlichen und weiblichen Mäusen. Ferner zeigen wir, dass weder die Nebennieren noch der paraventrikuläre thalamische Kern für die Anpassung der zirkadianen Nische wesentlich sind, während die zentrale zirkadiane Uhr im suprachiasmatischen Kern von entscheidender Bedeutung bleibt, obwohl sie selbst die Phase nicht zu ändern scheint. Die Starrheit der SCN-Uhr könnte mit ihrer Rolle bei der Messung der Tageslänge und der Steuerung saisonaler Rhythmen zusammenhängen. Welche Prozesse dazu führen, dass Verhaltens- und physiologische Rhythmen während simulierter Nahrungsknappheit einen anderen Phasenwinkel als der SCN einnehmen, bleibt weitgehend ungeklärt. Die Arbeit in dieser Dissertation bietet eine solide wissenschaftliche Grundlage, um die zirkadiane Flexibilität und ihre Beziehung zum Energiehaushalt in künftigen Studien erneut zu untersuchen. Weitere Erkenntnisse über die Flexibilität des zirkadianen Rhythmus könnten zur Klärung der kaum verstandenen Mechanismen beitragen, die den metabolischen Risiken im Zusammenhang mit der menschlichen Schichtarbeit zugrunde liegen, und zeigen, wie man mit zirkadianen Störungen umgehen kann.

Chapter 1

Introduction to the field of chronobiology and outline of this thesis

Sjaak J Riede

Balancing expectations

The natural world can be a thing of beauty, a theatre in which all organisms perform their daily rope acts balancing and navigating a thin path between risks that threaten their survival whilst capitalizing on the opportunities their habitat provides. A central theme within this thesis is how the circadian system organizes the timing of behaviour and physiology to accommodate differences in habitat qualities; how its output (in our case, the phase of the rest-activity cycle) is adaptive and flexible. One of the cornerstones of life is the ability to adapt to changes in the local environment. It is an integral part of the theory of evolution in which those individuals best adapted to their surroundings tend to have the highest fitness, thus contributing more to the future genetic pool of their species. As aptly worded by "The Streets" in the song "On the edge of a cliff": *For billions of years since the onset of time, every single one of your ancestors has survived – successfully looked after and passed on to your life*. Yet, adapting to your environment remains somewhat challenging to study at the more fundamental level.

To understand biological processes in a detailed and mechanistic manner, the majority of research experiments focus on a very limited number of model species. Rodents are used most extensively as a mammalian model organism, specifically mice and rats. Variation is further reduced by selecting young and healthy individuals of a tight age range or focusing exclusively on the male sex. On top, typically inbred strains of mice and rats are used, making the pool of genetic diversity as small as possible. Whereas this offers important benefits (lower variation between the subjects allows you to draw significant conclusions based on fewer data points, reducing the number of subjects and amount of work required). Like with the subjects, also the conditions in which animals are kept have been greatly standardized. The food quality is more uniform, each bite providing the same ratio of nutrients. Food and water are furthermore freely accessible and "all you can eat" (*ad libitum*) for the animals in most studies. Room temperatures are generally monitored and controlled, pathogens and parasites are activity removed, predation or the chance of failing to obtain food do not exist. Social structures are greatly reduced in comparison to the diversity they will have in the natural world. Chance and randomness leaking into the experimental design, and affecting outcomes, are generally undesirable and actively avoided. These changes have had a massive impact on the reproducibility and efficiency of research resources. Newer (and often expensive) techniques allowed us to look at processes at the molecular and genetic level further reducing inter-individual variation and omitting variation in treatment.

Yet, it is sometimes important to take a step back and try to reconnect how these discovered mechanisms serve their function within the evolutionary context. An

important aspect in this is asking how a given system can cope under a variety of conditions, how it responds to change, responds when challenged, deals with unexpectedness, how it can settle conflicting situations. In this thesis, we address how the circadian system is modulated by energy balance, building on our group's previous work showing that simulated food shortage alters the daily timing of behaviour and physiology in nocturnal rodents to become active predominantly during the day. We consider this diurnality to be adaptive, an attempt to re-balance energy homeostasis by reducing the costs of maintaining body temperature. The "expectations" part of the title comes from the observation that we see flexibility in circadian phase even in the absence of an actual (energetic) benefit. We even see mice becoming day-active when housed in a reversed temperature cycle (van der Vinne et al., 2014b), meaning they would actually increase their daily energy requirements. Based on the time of day, which is internally represented by the phase of a light-sensitive master clock, it seems that animals make predications and have expectations of their surroundings and the expected risks and benefits associated with different circadian patterns of activity.

Chronobiology is a sub-discipline of biology that studies the timing of biological processes that occur in a cyclic (i.e., repeating) manner. Many aspects of life are of cyclic nature. The alternation of day and night, patterns of ambient temperature, the change of seasons and tidal movements are some examples of rhythmically changing variables in the environment. To survive and successfully compete for resources, organisms must accommodate these encountered environmental rhythms and try to minimize the risks they pose whilst capitalizing on the opportunities they offer. An important aspect of dealing with these rhythmic changes is the ability to predict their timing and anticipate future conditions. Which factors are relevant for such prediction, and which signals can be used as reliable time cues, differs between species, habitats and might even depend the current condition or priorities of an individual organism.

The regulation in cyclic biological process can be studies in almost all forms of life, from humans and other mammals across plants and fungi and down to single-celled organisms. Unicellular organisms like photosynthetic dinoflagellates for example depend on light to harvest energy from their environment and are shown to be most capable of doing so during a daily window of several hours around mid-day (Doty and Oguri, 1957; Prézelin et al., 1977). Interestingly, these rhythms are not merely direct responses to environmental cues; even when kept in constant light (or dark) conditions, these rhythms are observable and continue to persist for several cycles. They depend, for a large extend, on internal and self-sustaining (**endogenous**) oscillations within the organism. It is these endogenous timing mechanisms and how

they operate, how they lock onto environmental rhythms and how they drive physiology, biochemical pathways and behaviours that remains a truly intriguing aspect of life and is the main focus of chronobiologists.

Diversity in rhythms; classifications based on cycle duration

Endogenous timing mechanisms can be observed in almost all phyla, an indirect indication of their importance, but the mechanisms that drive them as well as the duration of the generated rhythms can differ markedly. A first distinction between endogenous rhythms can be made based on their **period** (cycle length or duration). Rhythms that repeat daily are termed **circadian**, after the Latin words for *about* (*circa*) and *day* (*dies*). As most lifeforms are on or near the surface of the earth, they are exposed to the influences of sunlight across the 24-hour day. Differences of night and day can pose important limitations for a given species and circadian rhythms are hence ubiquitously found. Circadian rhythms are the main focus of this thesis. Other rhythms, especially in longer-lived species, might repeat themselves every year. Examples are migration patterns in birds, the mating season in deer, or winter hibernation in hamsters. Also, these yearly-recurrent behaviours and the associated physiological changes are driven to a large extend by self-sustaining timing mechanisms (persisting in constant conditions) and called **circannual.** Although not covered in this thesis, some organisms (or rather: certain aspects of their physiology or behaviour) might display rhythms that are matched in period to the moon phase (**circa-lunar**) or the tides (**circa-tidal**). The terminology of above-mentioned rhythms is related to the environmental rhythm they seem to lock onto in natural conditions. Two important other classifications related to the period of an endogenously generated rhythm, are **ultradian** and **infradian** – referring to periods shorter (multiple cycles within a day) and longer (one cycle spanning multiple days) than a day, respectively. The feeding-fasting cycle in some species of voles is showing a robust period of around 3-5 hours, which is an example of an ultradian rhythm - whereas the 4-5-day estrous cycle in rats is an example of infradian organisation.

Diversity in rhythms; classifications based on phase

Since a cycle has no end or beginning it is important to identify which part or point within a rhythm you are referring to when you describe its timing. To indicate the stage of a cyclic process we talk about its **phase,** and to do so we first must define a **phase-marker** of a given rhythmic process. A phase marker can describe a **point** within the rhythm such as a peak, trough or midpoint or can describe an **interval** such as a rising or declining portion of the rhythm. Full moon, new moon or the waning or waxing phases of the moon are examples of how we can indicate the phase of the lunar cycle. The same can be done for many biological processes. The active- and resting-phase or feeding- and fasting-phase are indications of intervals within a

rhythm whereas peak and trough (e.g., core body temperature minimum) can be used to denote discrete time points with higher temporal resolution. In addition to peak and troughs we can use derived phase markers to define additional discrete points within the rhythm. Three examples of phase markers which are commonly used within the field (and within this thesis) are the **onset**, **offset** and the **centre of gravity** (CoG). Fig. 1 depicts the terminology covered here and indicates these phase markers.

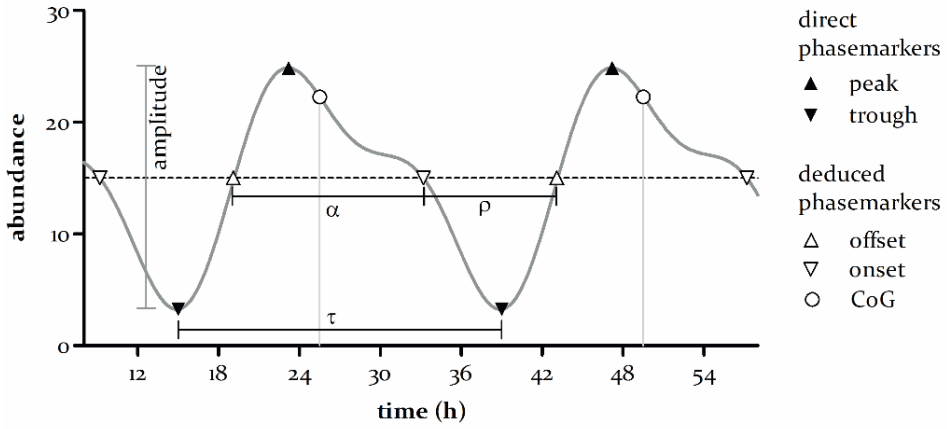

Figure 1: Indication of phase markers and associated terminology. *The curve depicts a daily rhythm in the abundance of a variable "x". From this rhythm, the maximal and minimal (▲peak / ▼through) values can be directly measured and used as phase markers to describe the phase of the rhythm. Other, often used, phase markers can be deduced by calculating the intercepts with for example the daily average (as depicted above by the dotted horizontal line) but, alternatively, one can also use a fraction of the peak or a fixed value in these deductions [melatonin onset is often set as the time at which melatonin levels exceed a concentration of 3 pg/ml in plasma in the evening]. Crossings during the rising phase are called onsets (Δ), whereas the crossing in the declining phase is referred to as offset (∇). The time between two identical phase markers is called the period, denoted by the Greek letter tau (τ). When sub-dividing the period, one often refers to the interval between onset and offset as the active-phase (α) and the remainder as the rest phase (ρ). The vertical distance between peak and through is the amplitude. The center of gravity (CoG, ○) is calculated as the circular average of the data; the area under the curve in the ½ τ hours before this point being equal to the area under the curve in the ½ τ following it.*

After establishing a way to refer to the phase of a biological rhythm, one can start to describe its phase relationships, either to an external timing cue or relative to other rhythmic variables. After defining the phase of a rhythm, one can also start to describe the effects manipulations can have on the timing of rhythms. If after a treatment (e.g., a light pulse preceding the morning dawn) the same phase marker

starts to occur earlier in the day, one speaks of a phase-advance, the reverse being a phase-delay.

When it comes to describing the phasing of rest-activity rhythms, we generally focus on the intervals of the day-night cycle in which most of the locomotor/foraging activity occurs. There is wide diversity between species, and even within species, in what their activity patterns look like. They can be highly **consolidated** (long bouts of continued rest or activity of up to several hours) or extremely **fragmented** (interruptions of activity, sometimes with micro-sleep episodes of mere seconds or minutes). Some terminology is worthwhile to introduce with respect to the shape of rhythmic patterns: **unimodal** refers to the simplest shapes of rhythms, showing one distinct peak and trough over the cycle. The sleep-wake cycle of humans, on the whole, is an example of a unimodal rhythm with one distinct and consolidated resting-phase every day. **Bimodal** rhythms, on the other hand, show two distinct peaks and include for example the activity-rhythms of animals that are most active around both sunrise and sunset. This brings us to classifications based on the timing relative to the external day-night cycle. **Crepuscular** rhythms are timed around the twilights (two peaks of activity every 24-hour cycle). **Nocturnal** is related to peaking during the night whereas **diurnal** is when some organism or process is being most active or expressed during daytime. It is important to realize that within a single organism, divergent rhythms can occur simultaneously. Foraging behaviour of shore birds can be partly modulated by the tidal rhythm (mudflat foraging grounds becoming accessible) and partly circadian (requiring sufficient illumination to fly and navigate). Meanwhile, circannual rhythms are present in the timing of their migration whereas the synthesis of certain hormones like melatonin only occurs at night (nocturnal). Figure 2 illustrates some of the terminology covered here.

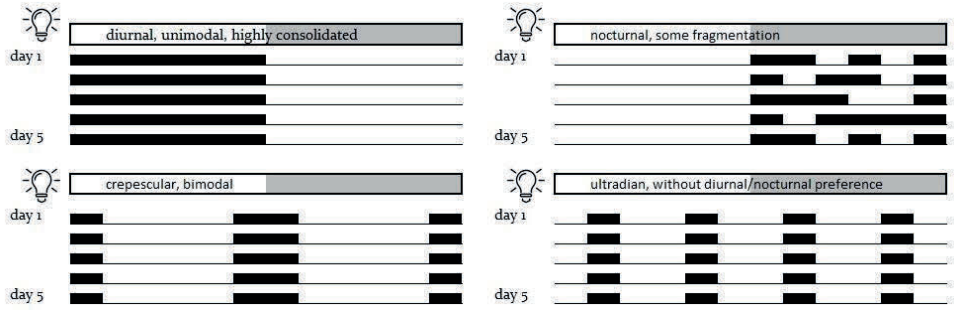

Figure 2: examples of diversity of circadian rhythms. *The bars on top of each plot denote a 12:12 LD cycle. Below are 5 consecutive days of fictive activity recordings, black bars denoting high levels of activity. This representation of rhythmic data is called an actogram. Examples include a diurnal (top left), nocturnal (top right), crepuscular (bottom, left) and an ultradian (lower, right) type of behavioural rhythm.*

Synchronization of rhythms with the environment

As mentioned, the phase-relation between endogenous rhythms and environmental time is important as it ensures biological processes are timed in ways that help to deal with expected perturbations. The endogenous timing systems have a period close to (hence circa-), but not equal to, the environmental rhythm(s) they are tracking. In constant conditions such as continued darkness, the period of the rest-activity rhythm in mice can be for example ~23.5 hours, which is referred to as the **free-running period (tau; τ)**. When housed in a 24-hour environment, the period measured from the displayed sleep-wake rhythms is fixed to 24h – matching the environment. This synchronization with environmental rhythms is called **entrainment**. Rhythmic cues from the environment that are capable to synchronize endogenous rhythms are called ***Zeitgeber*** (from German, meaning "time giver"). The effect of a Zeitgeber on the timing of endogenous rhythms depends on the phase at which it is perceived. This relationship is described in the **phase-response curve** (**PRC**) as schematically illustrated in figure 3A. The shape of the example in this figure resembles the PRC of many mammals to light. Light perceived in the late night or around dawn will induce phase-advances of the circadian system whereas light stimulation around and following dusk will lead to phase-delays. The endogenous free-running period of most mammals is not exactly 24 h and the system needs a small daily adjustment by processing Zeitgeber(s) to remain entrained. In the presented examples in figure 3B an organism with an endogenous period < 24 h (free running period) needs a small delay on a daily basis to synchronize with a 24-hour Zeitgeber period. Thus, when only providing a brief light pulse every cycle in otherwise constant dark conditions, the endogenous circadian system would align its subjective dusk with this light pulse. In contrast, an individual with a free-running period longer than 24 h would stabilize with the light pulse around its subjective morning, thus achieving the daily advance to entrain to the 24-hour period.

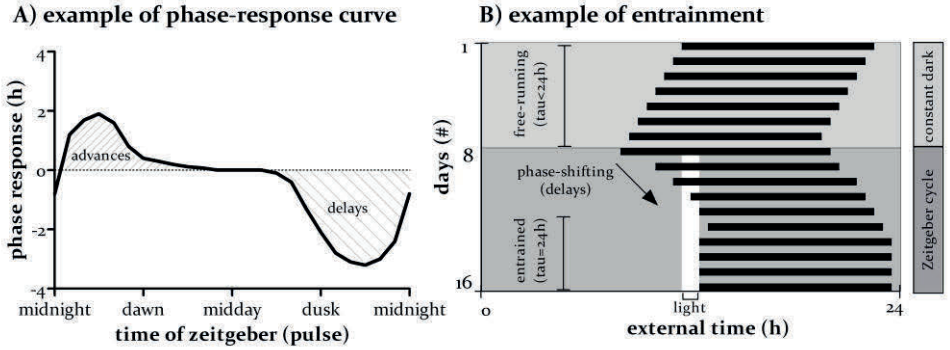

Figure 3: Illustrating the concepts of the phase-response curve (A) and the process of entrainment (B).

In (most) mammals, light is considered the dominant environmental variable for entrainment. Besides this process of **photic entrainment**, it is also possible to entrain the circadian system by other, non-photic cues including (forced) physical activity, social cues like vocalizations, or through temperature cycles. Such **non-photic entrainment** mechanisms are less well understood, but it is generally acknowledged that (timed) exposure to rewards (food, drugs, activity) or negative stimuli (foot-shocks, predation cues) can entrain circadian rhythms, or even modulate and reshape the default rhythms in relation to the LD cycle. The non-photic entrainment machinery seems partly independent from the photic, as non-photic cues can induce or restore circadian rhythmicity when the known components of the photic entrainment pathways are damaged or absent.

Aspects of light in circadian entrainment

The amplitude and shape of the light-PRC depend on both the duration of stimulation (Comas et al., 2006) as well as on the intensity (which in turn is modulated by the spectral composition of the light). The input-pathway of light into the circadian system is mediated for a large extent by specialized intrinsically photosensitive retinal ganglion cells (ipRGCs) that express the photopigment melanopsin (OPN4). The peak-sensitivity of melanopsin lies around 480 nm (which is perceived as blue), but in addition the activation of ipRGCs can be mediated by wavelengths outside their own sensitivity window as they receive both stimulation and inhibition from the classical photopigment (rods and cones) (van Diepen et al., 2013; Woelders et al., 2018). Of note in the context of humans, avoiding blue light exposure in the late evening (e.g., from smartphone screens in bed prior to sleep) is a potent and recommendable strategy for people experiencing difficulties to fall asleep at night or having problems to wake up and get out of bed in the morning - as the delaying effects of this evening light support a late chronotype and can, when in conflict with the social schedule of work or school, induce social jetlag (Wittmann et al., 2006; Zerbini et al., 2018). The later, defined as the discrepancy between midpoint of sleep on free-days compared to work-days, in turn being implied in association with health risks; contributing to conditions like sleep deprivation and loss of concentration during daytime hours (Roenneberg et al., 2019; Touitou et al., 2016). Especially adolescents are vulnerable to the negative consequences of blue evening light as their mean chronotype is (on average) already later than other age groups and their use of electronic screens (tablets, phones and monitors) exceeds that of most other population groups. Consequently, a larger social jetlag and/or later chronotype is likely to exert negative consequences on school performance (van der Vinne et al., 2015b).

Genetic clockworks: the transcription-translation feedback cycle

We previously stated that most cells in the body have the endogenous capacity to produce self-sustaining rhythms with a period close to 24 h, even in the absence of rhythmic cues in their environment. In mammals, a select repertoire of genes has been found to drive such rhythms, collectively referred to as **clock genes**. In turn, the products of many of these clock genes are serving as transcriptional regulators themselves, inducing rhythmic expression of many other – tissue specific – clock-controlled genes (**ccg**'s). To briefly describe the genetic basis of the circadian clockwork we will highlight a couple of its components. First, two positive regulators called *brain and muscle ARNT-like 1* (**BMAL1**) and *circadian locomotor cycles kaput* (**CLOCK**) can dimerize in the nucleus and bind with E-BOX promotor sequences in the DNA. Their binding stimulates gene expression of these E-BOX regulated genes (Ripperger and Schibler, 2006). Among these are clock-genes such as the *period*-genes (***PER1-3***) and the *cryptochromes* (***CRY1,2***). After their transcription and translation in the cytoplasm, CRY and PER proteins form dimers and enter the nucleus where they in turn interact with the BMAL1:CLOCK complex, causing a halt to the transcriptional drive of E-BOX-controlled genes. The CRYs and PERs are therefore referred to as the negative limb of the circadian clock. After nuclear degradation of the PER/CRY dimers (which in turn is regulated by clock-auxiliary enzymes like the casein kinases (**CK1δ,ε**)), the BMAL1:CLOCK dimers can re-establish their transcriptional activity and the next cycle starts. The time that passes with each cycle forms the backbone of the generated circadian cycle, but fine-tuning and modulation can occur at different levels. In addition, this molecular clockwork has been extensively expanded by additional transcriptional regulators, such as the REV-ERB proteins (nuclear receptor on the reverse strand of the ERBA gene, encoded by *NR1D1* and *NR1D2* genes) and *ROR* genes (retinoic-acid related orphan receptors, which, apart from modifying BMAL1 expression (Guillaumond, 2005), also exert transcriptional influence over many other clock controlled genes (Delezie et al., 2012; Fang and Lazar, 2016). Many of these secondary clock-genes or clock-modulators serve bidirectional functions: they not only allow a circadian clock influence over key transcriptional events, but can also serve as input-hubs for physiological feedback shaping the cellular clockwork (Astafev et al., 2017; Krueger and Feldman, 2013). Examples of such genes are the *NR1D's, Sirtuins* and genes like *PPARs* and *C-EBP* which form an interface between the circadian clock and metabolism by guiding transcription of tissue specific genes in rhythmic fashion (Asher and Schibler, 2011; Bahrami-Nejad et al., 2018; Kawasaki et al., 2013; Nakahata et al., 2008). Besides serving as transcriptional regulators directly, circadian gene products can also modify, stabilize or induce rhythms on other levels. This includes **epigenetic modulation**, a process by proteins can modify and alter the chromatin structure of

sections of the genome, making the DNA-strand become less or more readily available for transcriptional machinery. The core clock gene **CLOCK** (mentioned above) is a prime example of a protein with histone acetyl transferase (HAT) activity (Doi et al., 2006; Valekunja et al., 2013), thus capable of regulating chromatin structure and modifying the DNA-superstructure changes across the daily cycle. The functional connections and regulation pathways of these clock modulators by extracellular cues can differ widely for the plethora of different cell types in our body, hence a more detailed description is beyond the scope of this introductory chapter.

Non-transcriptional molecular clockworks

Besides genetic and epigenetic rhythms, also **protein-protein interactions** can facilitate rhythmic changes in cell-function. Circadian rhythms in protein-phosphorylation and redox-potential for example have been observed in human red blood cells (erythrocytes) which lack a nucleus and, thus, do not rely on gene transcription (O'Neill et al., 2011; Stangherlin and Reddy, 2013). The contribution and relative importance of such non-genetic oscillations in mammals is, however, not yet clear and is still an aspect of daily rhythm generation often neglected or overlooked. The combination of advanced and time-specific proteomics, genomics and metabolomics studies has pointed out that there is a huge variation in the temporal organisation of key processes with often variable and perhaps even tuneable intervals between transcription, translocation, translation, modification and degradation – often with tissue specific differences (Masri et al., 2013; McClung, 2011; Menet et al., 2012).

Dispersed clockworks – hierarchy of rhythms

A much-debated concept within circadian rhythm generation is how cells and tissues cooperate with another and synchronize their cellular clockworks. In the early days of chronobiology, and still upheld by some today, circadian rhythms were thought to be mainly organised by the central nervous system. To be more specific, the mammalian daily timing system has been centred on a pair of small pear-shaped nuclei in the ventral hypothalamus; the **suprachiasmatic nuclei** (SCN). This structure receives direct projections from the ipRGCs (discussed above under the header "aspects of light-entrainment") to get information on the day-night cycle. The SCN shows pronounced daily rhythms in neuronal firing frequency and genetic or physical ablation of the SCN leads to loss of circadian rest-activity and feeding patterns in mice and rats housed in constant environments. Transplantation studies furthermore showed that receiving a graft of SCN tissue can restore rhythmic behaviours with a period equal to that of the SCN-donor animal (Ralph et al., 1990). For many years, the dominant view was that the SCN in mammals serves as the master clock – receiving and aligning strong circadian rhythms that arise in the SCN-

network with the environmental day-night cycle, and relaying time of day through humoral and neuronal outputs to tissues across the body. Nowadays, this concept of a master clock starts to become questioned more often. Whereas the SCN continues to serve as an important nucleus related to circadian timekeeping, its influence over the timing of behaviours and physiological rhythms seems more flexible and context dependent. For instance, when the timing of food availability is in conflict with the "natural" rest-activity rhythm, food timing can become a dominant predictor of foraging activity and metabolic rhythms. It is this flexible alignment of circadian output rhythms with regards to the SCN/LD-cycle which is the primary topic of this thesis.

Flexibility of daily output rhythms: functional benefit of diurnality

Prior to starting the work described in this thesis, Hut and co-workers found that simulating food scarcity (increasing the efforts needed to obtain food) is sufficient to induce an advanced phase of entrainment in mice, making them predominantly diurnal (Hut et al., 2011). This altered re-alignment of the rest-activity rhythm towards the LD-cycle was observed for both activity patterns as well as in the body temperature rhythms of the mice. Their method was straight-forward: mice were housed in individual running-wheel cages and had to obtain their food pellets by completing a set number of wheel revolutions which triggered the release of a food pellet by an automated feeder setup. The number of rotations per reward was set by the experimenter allowing the comparison of low- and high-workload conditions. The paradigm was dubbed the **work-for-food (WFF) method**. In this thesis, we employed the WFF method in various ways aimed at gaining a better understanding regarding why (function) and how (mechanism) changes in food availability can alter the circadian niche of mice. Part of the experiments conducted became part of the thesis of my colleague, Vincent van der Vinne, with whom we established that the shift towards diurnality seems to depend on metabolic balance (van der Vinne et al., 2015c). When the WFF paradigm was performed at lower room temperature (hence increasing the daily energy expenditure) the shift occurred at lower workloads. Furthermore, van der Vinne and Hut developed a hypothetical framework which would explain the functional benefit of the circadian realignment of rest-activity towards the day-night cycle during food scarcity. Their theory was set on paper as the **circadian thermo-energetics hypothesis** (CTE) stating that nocturnal animals reduce their daily energy expenditure when they align their foraging behaviour with the warmer day and their resting phase (including insulating and energy saving strategies like torpor) with the cold night (Hut et al., 2012). In 2016, this led to our group's publication using energy expenditure measurements under various housing and environmental conditions such as simulated ambient temperature cycles, social

burrowing strategies (huddling, buffering potential of the nest) and factors like wind chill and entering a state of torpor – combined with meteorological temperature data for a wide range of natural habitats from locations across Europe (van der Vinne et al., 2015c). This work showed that a diurnal lifestyle of mice could reduce energy (i.e., food) requirements up to 30 % compared to maintaining a nocturnal phenotype. This however poses the question as to why mice (and many other rodent species) do not become day active all the time? The most reasonable assumption perhaps lies in the non-energetic costs of a diurnal lifestyle. Although hard to test empirically, it is likely that leaving the burrow in broad daylight places mice at a larger risk for **predation**. It is conceivable that mice as a prey species avoid daytime activity as long as their energetic situation allows it. We attempted to find support for this hypothesis by conducting some experiments in our outside mice enclosures in which we monitored mouse activity near a food hopper in large but relatively barren field enclosures (Vinne et al., 2019). Manipulation of food density (accomplished by a slowly rotating conveyor system suppling the equivalent of 3-7 grams of chow per mice per day) induced more diurnal activity with reduced food availability – in agreement with the results from the indoor WFF experiments. Furthermore, the presence of runway cover to the feeder location had a major impact on mouse behaviour. More cover (interpretable as a shelter reducing the subjective predation risk) led to more diurnal feeding activity of the populations (especially in female populations). Combining the field data with the indoor WFF results seemed to be in agreement with the CTE hypothesis, in which diurnality is energetically beneficial but increases predation risk.

Outline of this thesis

To explore general advantages that flexibility in circadian rhythms can have, we summarized and interpreted literature to clarify how rhythms can be seen in relation to predictable habitat variation in **Chapter 2** (van der Veen et al., 2017). The timing information contained within the environment shapes, modulates and synchronizes circadian outputs, which may allow for fitness benefits. The discrepancies between field and laboratory studies emphasize the importance of studying circadian organization in a (semi-)natural context.

An important part of the natural environment is how it influences energy balance. The two sides of energy balance, energy expenditure and energy intake, change over time of day. Ambient temperature rises during daytime and decreases during the night, while food intake only occurs when the animals are active, either during the day or the night. These effects are evaluated in **Chapter 3** using a classic Scholander curve approach while taking into account changing ambient temperatures across the day and the advantage of nest insulation during the rest phase (Riede et al., 2017).

We use this approach to evaluate the collective findings from the work-for-food paradigm used to study the degree of adaptive flexibility in circadian behaviour. We thereby clarify how diurnality to small euthermic and burrowing animals would be energetically beneficial.

In the three original data chapters, we explore which mechanisms might underlie the adaptive change to diurnality in energetically challenged mice during the work-for-food protocol. Classically, nocturnal species (including *ad libitum* fed mice) show a direct reduction in activity levels during a light stimulation, whereas diurnal species (like humans) tend to show increased activity levels during light stimulation. For nocturnal mice to become diurnal, the activity suppressing effect of light should be alleviated or perhaps even inversed. We tested for changes in activity modulating effects of light under high workloads in **Chapter 4**. We show that when mice change towards a (more) diurnal phenotype, the suppression of activity by light is greatly diminished.

Previous publications, including our own work in outdoor enclosures (Vinne et al., 2019), suggest that female and male mice may have different behavioural responses to food scarcity. This was further explored in a direct comparison in **Chapter 5**, where we describe sex differences in response to the work-for-food paradigm. The data indicate that under the work-for-food protocol, female mice show flexibility in the *amount* of activity, but males show a higher degree of flexibility with regard to *when* they are active.

Finally, we report in **Chapter 6** our efforts to identify the structures that are crucial for the nocturnal-to-diurnal switch under the work-for-food protocol. The adrenal glands are an important factor in coordinating circadian rhythms in metabolic tissues and energy balance. Adrenalectomized mice did, however, continue to show a change to a more diurnal phenotype when facing high workloads, suggesting that adrenal hormones are not necessary for circadian plasticity in foraging behaviour. In contrast, the central clock in the SCN remains an integral component, as high workload did not induce a diurnal phenotype in SCN lesioned mice, which – combined with the fact that clock gene rhythms in the SCN of intact animals remain locked and unaltered in phase during WFF - suggest that high workloads act downstream of the SCN to alter the alignment of behaviour and physiology. Comparisons of neuronal activity patterns between diurnal (HWL) and nocturnal (AL-fed) animals indicated a role for the anterior region of the paraventricular thalamic nucleus (aPVT). This region showed marked differences in both amplitude and phase of neuronal activity patterns, making it a potential candidate to alter the alignment of behaviour during negative energy balance. Lesions to the aPVT, however, did not prevent the behavioural change to diurnality under high workloads,

suggesting that this region is not essential for food scarcity induced circadian plasticity.

The necessity of the SCN is not limited at structuring daily activity patterns, be it diurnal or nocturnal. The SCN also plays an essential role in annual rhythms and the response to changing day length. **Chapter 7** highlights and hypothesizes how different hypothalamic structures collectively shape photoperiodic responses in both short- and long-day breeders (Hut et al., 2014). Crosstalk between regions that measure energy stores, food intake, temperature, and day length may help to trigger hormonal cascades in order to be ready for reproduction or hibernation at the right time of year. This small review is included in this thesis, because it illustrates that the hypothalamus can use the same structures to generate biological rhythms in opposing phases, be it on a daily or on an annual time scale.

This thesis ends with a summarizing discussion in **Chapter 8**, where the various results and findings in the data chapters are evaluated and combined to a concise narrative that presents the current state of understanding of mechanisms of flexibility in circadian and daily organization of physiology and behaviour.

Image: Striped field mouse (*Apodemus agrarius*) in its habitat, with filter

Chapter 2

Flexible clock systems:

adjusting the temporal programme

REVIEW PAPER

Aimed to bring the fields of ecology and chronobiology closer together, a conference on clocks and timing systems under natural conditions took place on Texel in 2016. Besides novel ideas, new friends and collaborators and a great time, this conference resulted in the submission of several papers to a themed issue of Philosophical Transactions of the Royal Society B, one of the oldest scientific journals around (1665). I had the pleasure to enjoy this meeting and the honor of collaborating on this review.

Chapter 2

Flexible clock systems: adjusting the temporal programme

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Paper published in
Philosophical Transactions of the Royal Society B,
November 19, 2017 in theme issue 'Wild clocks: integrating chronobiology and ecology to understand timekeeping in free-living animals'.
DOI: 10.1098/rstb.2016.0254

Abstract

Under natural conditions, many aspects of the abiotic and biotic environment vary with time of day, season or even era, whilst these conditions are typically kept constant in laboratory settings. The timing information contained within the environment serve as critical timing cues for the internal biological timing system, but how this system drives daily rhythms in behaviour and physiology may also depend on the internal state of the animal. The disparity between timing of these cues in natural and laboratory conditions can result in substantial differences in the scheduling of behaviour and physiology under these conditions. In nature, temporal coordination of biological processes is critical to maximise fitness because they optimise the balance between reproduction, foraging, and predation risk. Here we focus on the role of peripheral circadian clocks, and the rhythms that they drive, in enabling adaptive phenotypes. We discuss how reproduction, endocrine activity and metabolism interact with peripheral clocks, and outline the complex phenotypes arising from changes in this system. We conclude that peripheral timing is critical to adaptive plasticity of circadian organisation in the field, and that we must abandon standard laboratory conditions to understand the mechanisms that underlie this plasticity which maximises fitness under natural conditions.

Introduction

The temporal program of behaviours and physiology expressed by an organism is driven by a vast network of clocks and rhythms distributed across tissues throughout the body (Albrecht, 2012). The interactions within this network and its response to external time cues have been intensively studied in laboratory experiments. Here we want to assess how this network would operate under natural conditions, where a plethora of potential time cues are – often simultaneously - acting on it. The hierarchical view of the circadian system in mammals is that the clock in the Suprachiasmatic Nuclei (SCN) of the hypothalamus is entrained by the environmental light-dark conditions, and that this timing information is transmitted to clocks in other brain regions and tissues of the body (Fig.1). All non-SCN clocks are collectively known as the 'peripheral timing system', and we know that almost every cell in the body can expresses a circadian clock, making the peripheral timing system a large and complex system which expresses many rhythms with different phases. The current hierarchical view that the SCN acts as the conductor of this orchestra of peripheral clocks underplays the independence of most peripheral clocks (Davidson et al., 2003), which can autonomously entrain to many non-photic timing cues (Zeitgebers) such as food availability (Damiola et al., 2000; Stokkan et al., 2001), temperature (Buhr et al., 2010), arousal (van der Veen et al., 2006) and

internal glucocorticoid levels (Balsalobre et al., 2000), independently of SCN derived timing.

Perhaps one of the most intriguing questions is how timing of all the clocks in the peripheral timing system can be mapped against the abundance in rhythms in physiology and behaviour. Several clocks will contribute to the timing of a single process, which is for example clear from the simultaneous expression of both light and food driven timing of behavioural activity (Stephan et al., 1979; van der Veen et al., 2011). In laboratory conditions, photic and non-photic cues may be altered independently, whereas in the field they are not always independent. For example, daily temperature cycles can be offered in antiphase with the light-dark cycle in the laboratory, but in nature temperature is typically higher during the light phase due to the heat that radiates from the sun. Similarly, laboratory experiments offering food exclusively during the rest phase have taught us a lot about the food entrainable oscillator (FEO), but under natural conditions food intake will mostly happen in the active phase, and timing of food availability may in fact be one of the reasons why an organism is active at that time. These dependencies among Zeitgebers in nature are complex, and our laboratory experiments do not necessarily take these complex relationships into account, which reduces the translation value of our laboratory experiments.

An added complication is that phase relationships between Zeitgebers can be variable in nature. For instance predation risk for small rodents may be inflicted by both nocturnal and diurnal predators, and depending on season, vegetation cover, or habitat high nocturnal predation risk may be replaced by high diurnal predation risk. Such changes may also lead to adaptations in prey species, leading to changed phase angles of peripheral oscillators in the body. In general, Zeitgebers to which peripheral clocks entrain, such as food intake, light exposure, temperature and arousal can differ in their temporal relationships due to factors such as predation, seasons, climate, social interactions and ongoing day-to-day variation. In addition, animals can often self-regulate light exposure in the field by retreating into burrows, a condition that is not always available in laboratory conditions. Adaptation to, and anticipation of these timed events requires a flexible phenotype that responds to daily, seasonal and annual changes in the environment, and given that different peripheral clocks (in e.g. the liver, heart and adipose tissue) may respond differently to each of the Zeitgebers, this flexible phenotype must rely heavily on the peripheral timing system.

The complexity of the changes in this peripheral, multi-clock timing system that result from altering phase relationships between photic and non-photic cues, are nicely demonstrated by studies on humans and rodent models in the laboratory, in

which photic and non-photic cues are misaligned in conditions such as shift-work and sleep restriction. For instance, shift work in mice or humans leads to severe, and complex disruption of timing of gene transcription, and the timing of gene expression may not simply shift in line with a single oscillator, but exposes genes in which rhythmicity is lost or altered in phase and amplitude, and new rhythms even appear where transcription was nonrhythmic before (Archer et al., 2014; Barclay et al., 2012a). This leads to the view of the biological timing system as a 4-dimensional landscape of loosely delineated, resonating tissue clocks and rhythms which drive timing of behaviour and physiology. Perturbations in this system may be associated with several adverse health conditions such as obesity, diabetes, cancer, problems with cognitive performance and mood disorders in humans, but almost nothing is known about the implications of this complex system on timing and survival of non-humans in nature.

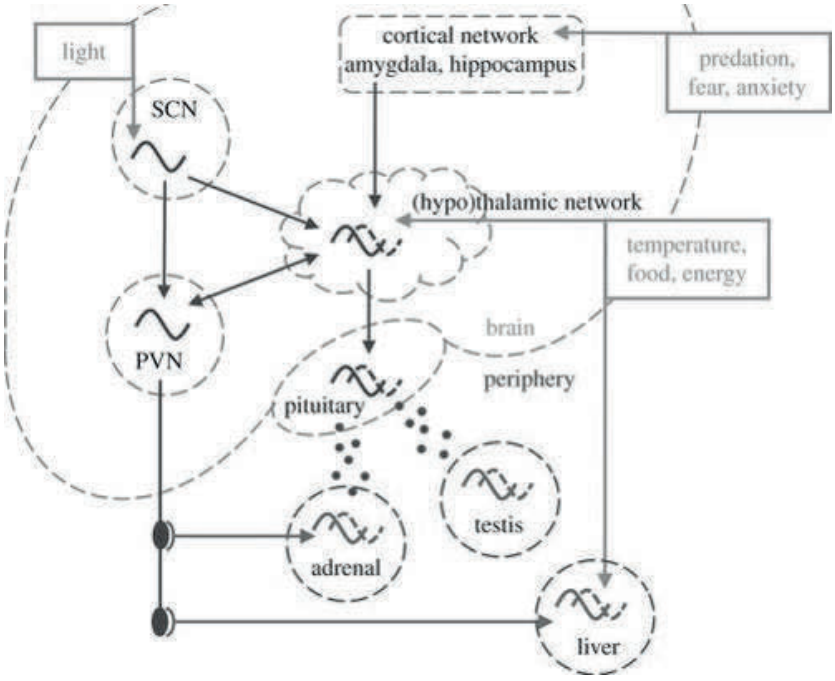

Figure 1: Schematic representation of the circadian timing system, relevant to this chapter.

Here, we hypothesise that in natural conditions the peripheral timing system is key to the flexible phenotype, and that it cannot be attributed to the dominance of a single 'master' oscillator in the brain. We provide evidence for this hypothesis in four aspects of the temporal niche in the field: reproduction, endocrine activity, feeding and chronotype and argue that, given this evidence, we must abandon standard

laboratory conditions if we are to understand the regulatory mechanisms that underlie the flexible timing on the field, where it is essential to survival.

Mechanisms of orchestrating peripheral timing in the field

Endogenous circadian rhythms and their alignment with the external world provide an adaptive advantage when they help animals to predict upcoming events or conditions in their (a)biotic environment. Such anticipation of environmental risks and opportunities can increase fitness in many ways such as limiting predation risk, generating offspring at times of plentiful food availability, and by increasing energy efficiency. In nature however, the temporal occurrence of Zeitgebers may be irregular, and can vary among Zeitgebers such as light, food and predation, which can cause a reduction in fitness due to misalignment of endogenous and environmental rhythms. Moreover, such misalignment may not only exist between the environment and the endogenous timing system as a whole, but because clocks in different tissue synchronise (entrain) to different Zeitgebers, misalignment may also occur among oscillators within the body. Therefore, optimal timing in nature faces two challenges: 1) how to align circadian rhythms of multiple tissues correctly towards each other and 2) what is the most beneficial internal alignment pattern for any given environmental temporal niche? These questions remain mostly unanswered, and in this section we will discuss these by taking a bottom-up approach, starting at the cellular circadian oscillator.

The molecular circadian clockwork is expressed in almost all cells

A key feature of circadian rhythms is that they are endogenously driven with a period close to 24h in constant conditions. To a large extend, these rhythms are considered to be generated by cellular molecular oscillators, which are observed in almost every nucleated cell of the body. These oscillators drive widespread temporal gene expression patterns that are tissue specific (Zhang et al., 2014), forming the foundation of rhythmic physiology and behaviour. In mammals, this clockwork consists of two interlocking transcription-translation feedback loops in which products serve as negative feedback on their own transcription. This molecular oscillator can explain the rhythmic expression of numerous tissue specific genes through rhythmic interactions with the E-Box, D-Box, and RRE elements in the promotor regions of many genes throughout the genome (Yan et al., 2008). However, this molecular oscillator can also have more subtle effects on downstream gene expression, for example through opening up the chromatin structure, which 'gates' the ability of circadian transcription factors to bind to the promotor region of target genes (Fustin et al., 2009; Menet et al., 2012; Menet et al., 2014). It is easily conceivable that many clock controlled genes are influenced in their final phase of expression by

the combined actions of these rhythmic core transcription factors (Korenčič et al., 2015).

It is estimated that the cellular molecular clock drives thousands of different protein coding genes to exhibit daily rhythms in expression (Zhang et al., 2014). These circadian transcripts contribute significantly to the physiological functions of a cell. However, because circadian rhythmicity is so widespread, the origin of circadian rhythmicity in a given processes can be complex. For example, the response to a constant signal (e.g. a stable plasma glucose level) may still be rhythmic if there is a daily rhythm in the sensitivity of a cell due to a daily rhythm in receptor expression. In line with the different physiological roles of different cell types and tissues, we typically observe different combinations of transcripts of Clock Controlled Genes (CCG's) across tissues, and therefore each tissue possesses its own, unique rhythmic transcriptome (Hughes et al., 2009; Zhang et al., 2014). Moreover cellular clocks, and their accompanying rhythmic transcriptome are sensitive to entrainment by Zeitgeber in a tissue-specific way; the liver is primarily entrained by food intake, whereas the Suprachiasmatic Nucleus (SCN) of the hypothalamus is almost exclusively entrained by environmental light cycles.

The SCN as central light entrainable clock

In mammals, neurons in the SCN display a robust and high amplitude circadian rhythm in neuronal activity, with high frequency action-potentials trains during daytime and relatively few neurons firing at night (Inouye and Kawamura, 1979; Schaap et al., 2003). Neurons of the SCN are highly coupled, increasing both the robustness as well as the accuracy of the generated rhythms (for review see Mohawk and Takahashi, 2011; Welsh et al., 2010). Light is the main Zeitgeber that determines the phase, period and amplitude of the SCN (for review see Colwell, 2011). The SCN is often regarded as the main circadian pacemaker that sets the phase of peripheral oscillators throughout the body, but - while this might be valid under stable laboratory conditions - natural conditions might reveal the SCN's role as less prominent. SCN lesions in rodents lead to a loss of circadian rhythmicity in physiology and behaviour in constant environments, a finding that first led to the (now outdated) title of "master clock" for the SCN (Moore and Eichler, 1972; Richter, 1967; Stephan and Zucker, 1972). However, housing animals in more natural rhythmic environments, in which factors such as light-level, food abundance, social cues, ambient temperature, or exposure to rewarding or fearful experiences show daily variation, can reinstate rhythms in behaviour and physiology in SCN-lesioned individuals (DeCoursey and Krulas, 1998; Pellman et al., 2015; Stephan et al., 1979). These rhythmic phenotypes in SCN lesioned animals are testimony to the fact that circadian responses that animals display can (in part) be independent of SCN timing,

and that rhythmicity in physiology and behaviour may also be a direct response to environmental cues (masking; see Mrosovsky, 1999) or may even trigger extra-SCN circadian oscillators to drive rhythmicity in the body (Pezuk et al., 2010; Stephan et al., 1979). Rhythmicity in peripheral organs therefore does not require an intact SCN, and instead, it seems more appropriate to denote the SCN as an entity which provides the body and brain with an internal representation of the external light-dark cycle, rather than a 'master' clock.

Linking the SCN and peripheral timing

Under constant laboratory conditions, the (dorsal) SCN provides a phase reference through direct neuronal projections, releasing glutamate, GABA, AVP and other factors. These pathways regulate target (thalamic and hypothalamic) brain regions through neuronal projections (Buijs and Kalsbeek, 2001; Kalsbeek et al., 2006; Vujovic et al., 2015) and paracrine output (Silver et al., 1996). A major neuronal output pathway is the SCN's projection to the paraventricular nucleus (PVN); driving the autonomous nervous system that reaches peripheral organs like liver, kidney and adrenal gland (Kalsbeek et al., 2004; Oster et al., 2006b). The SCN-PVN-adrenal projection offers a potentially important role for glucocorticoids to mediate circadian organisation in the body (Balsalobre et al., 2000; Oster et al., 2006a; Oster et al., 2006b), although they can also encode stressful events.

Glucocorticoids have been shown to be a key Zeitgebers for peripheral tissues (Balsalobre et al., 2000), which can interfere with other Zeitgebers such as food availability (Le Minh et al., 2001) as described below. There are also several interactions between circadian and glucocorticoid systems during early development that are still not well understood (Cagampang et al., 2011). For example, foetuses exposed to increased maternal glucocorticoid concentrations display phase-advances in locomotor activity rhythms later in life (citations in Cagampang et al., 2011). This effect is likely mediated through glucocorticoid receptors, which are present in the foetal SCN (Rosenfeld et al., 1988). Further blood borne Zeitgebers could include several cytokines – including TNFα and IL-6, which were suggested to have regulatory effects on peripheral clocks (Cavadini et al., 2007; Dudek et al., 2017). As these anti-inflammatory cytokines are also produced by glial cells, it remains to be tested whether glial communication might also be involved in the principles of entraining and maintaining rhythmicity in neuronal tissues (a theory already proposed more than 20 years ago (Van Den Pol and Dudek, 1993)).

Modulation of SCN-rhythmicity by peripheral feedback

Whereas the SCN seems primarily entrained by photic input (both directly through the RHT and indirectly through the GHT – NPY pathway) it has also been shown that

non-photic inputs can modulate the circadian properties of the SCN-clock (see for review Wams et al., 2017). Inducing activity during the day was shown to induce NPY from IGL terminals onto SCN-cells at the time of induced running (Glass et al., 2010). Indeed, running wheel access and scheduled activity can have considerable impact on light entrained activity patterns in rodents and also in humans (Wams et al., 2017). Another level of SCN modulation by peripheral feedback may be through sex hormones such as androgens and estrogens, for which the SCN expresses receptors (Model et al., 2015; Mong et al., 2011), as is described later.

Non-SCN derived Zeitgebers of peripheral clocks

Physiological output rhythms of one tissue (e.g. body temperature or corticosterone release) can serve as rhythmic input to other cellular clocks. For example, all peripheral tissues can entrain to body temperature cycles in vitro (Buhr et al., 2010). Often, peripheral tissue clocks are sensitive to (rhythmic) inputs from multiple sources (e.g. expressing both melatonin and glucocorticoid receptors and receiving both sympathetic and parasympathetic input). In addition, it is well known that behavioural rhythms such as feeding and fasting can have a strong effect on the circadian phase of local tissue clock-gene rhythms. The wide diversity of signalling routes makes it a complex and daunting task to understand the phase relationship between cellular clocks in multiple tissues, and grasp the flexibility between circadian rhythms in the SCN versus those in non-SCN tissues. For most tissues we still need to establish which specific inputs determine the phase of the local cellular clock. Local clock-phase is likely the result of checks and balances between multiple inputs collectively, and the relative contributions of the involved signals may be altered depending on the environmental or internal condition at the time that the Zeitgebers are perceived.

A flexible timing system would be essential for evolutionary reasons, as circadian organisation may need to alter alignment when the environment or the animal's internal state changes over time, requiring adaptive phasing of behaviour and physiology. We are only just starting to comprehend the flexibility between the phase of SCN and organ clocks and in order to obtain a useful concept it is important to understand the dynamics and variability of the natural habitats. Understanding the relevance and limitations of circadian flexibility may bear highly relevant insights into human health (e.g. shift work, jet-lag recovery, performance and productivity, timed medicine, and healthy lifestyles). Similarly, understanding circadian flexibility and the adaptive incorporation of past experiences into future predictions is a mayor insight that would help conservation biology in predicting the detrimental effects of global warming.

Contribution of peripheral clocks to flexible reproductive timing in the field

Reproductive activity is typically observed during specific seasons, and circadian timing of Zeitgebers such as dusk and dawn (photoperiod) predictably vary between these seasons. Indeed, in the laboratory, rodents from the temperate zone often respond robustly to short photoperiod by suppression of reproductive physiology and activity, and respond to long photoperiod by stimulating reproduction (Follett, 2015; Prendergast et al., 2001). These photoperiodic responses depend upon nocturnal secretion of melatonin by the pineal, driven by a functioning central circadian clock (Coomans et al., 2015). In the field, however, the patterns can be more complex than explainable by reference solely to melatonin and the central clock (Bronson and Heideman, 1994). For example, in some species of *Peromyscus*, long photoperiods in the laboratory trigger fertility and breeding (Prendergast et al., 2001), but the same species - and often the same populations in the field - exhibit a summer, long-day hiatus in breeding (Boyer and Terman, 1993; Bronson and Heideman, 1994; Heideman et al., 1999; Terman, 1998). The causes of the summer interruption in breeding are debated, with lines of evidence emerging that parasites, disease, and food availability all have significant roles in this behaviour (Pedersen and Greives, 2008; Terman, 1999; Vandegrift and Hudson, 2009; Vandegrift et al., 2008).

The mismatches between laboratory and field data are conceivably due to (1) different inputs to the central clock in the SCN, (2) difference in other environmental factors that act as gatekeepers for the reproductive axis by affecting hypothalamic reproductive neurons, and (3) environmental factors that act on peripheral clocks in reproductive tissues. The presence of robust peripheral circadian rhythms have been implicated in reproductive function of the hypothalamus (Chassard et al., 2015), pituitary, gonads, and reproductive organs (Richards and Gumz, 2012; Sellix, 2013). The single exception is the testis, which exhibits relatively weak circadian rhythms (Liu et al., 2007; Mazzoccoli et al.; Meyer and Lerchl, 2014; Richards and Gumz, 2012; Singh et al., 2013). In the testis, the peripheral clock is blocked by the testis-specific repressor PASD1 acting against CLOCK:BMAL1 (Michael et al., 2015). However, testicular Leydig cells and their secretory rhythm of testosterone appear to be controlled at least partially by a peripheral clock (Alvarez et al., 2008). Disruption of peripheral clocks may contribute to infertility (Alvarez et al., 2008; Sellix, 2013; Sellix et al., 2010), suggesting that these peripheral clocks are potential targets for environmental modification of reproduction.

A role for peripheral clocks in incorporating food availability and disease-loads into reproductive functions

In males, even in the absence of strong peripheral clocks in the testis, peripheral clocks have the potential to alter fertility. Dissociating feeding cycles form the light-dark cycle (known as temporal food restriction; described below) has been reported to cause changes in androgen-dependent male reproductive tissues, including changes that inhibit fertility (Go and Lee, 2014). In wild mammals, this response to temporal food restriction could inhibit fertility when unusual conditions force a male to feed outside of the normal activity period. This response to temporal food restriction may increase fitness by reducing the costs of reproduction until conditions return a male to a normal feeding cycle.

Peripheral clocks appear to be present in reproductive tissues across mammals, birds, other vertebrates, and invertebrates (Pezuk et al., 2010). Some of these clocks may have little direct effect on reproduction; instead, for example, they could be coordinating a daily cycle of nutrient input from gut and liver with body-wide cycles of nutrient demand (Gerhart-Hines and Lazar, 2015; Krueger and Feldman, 2013; Zwighaft et al., 2016). However, there is evidence for relevance of these peripheral clocks to fertility. There is a growing body of evidence that peripheral circadian clocks in the ovaries of vertebrate females can adjust the sensitivity of the ovary to luteinizing hormone (LH), thereby modifying the LH surge leading to ovulation (and, in many species, mating behaviours). This will alter the timing of ovulation in mammals (Pezuk et al., 2010; Sen and Sellix, 2016) and birds (Nakao et al., 2007). Such alterations in the timing of mating and conception could affect predation risk during vulnerable periods of mating, as well as the timing of subsequent events such as implantation and parturition. This suggests that the peripheral ovarian clock may serve an important ecological function.

Ovarian clocks may affect both the development of embryos and sex steroid secretion. After fertilisation, the oviduct supports the developing embryo for a period of days, and the timing of mating affects the proportion of morphologically normal embryos through the embryo. An influence of the timing of mating on the frequency of morphologically defective embryos in the oviduct suggests that a peripheral clock in the oviduct could affect fertility (Kennaway, 2004). Elsewhere in the ovary, clock gene knockouts restricted to steroidogenic tissues disrupt the secretion of steroids, altering stages of the female reproductive cycle from ovulation to parturition (Liu et al., 2014), further indicating the importance of peripheral clocks for fertility and reproduction. Following conception, the corpus luteum produces progesterone to maintain the uterus in a condition to support embryos. *Clock* and *Bmal1* knockouts that are either systemic, ovary-specific, or even clock-gene knockdowns within the

ovary, can all result in implantation failures. These failures may be caused by a deficient clock in the corpus luteum, which can contribute to reduced progesterone secretion and failure of implantation or reabsorption of an embryo (Li et al., 2015; Liu et al., 2014; Miller et al., 2004; Ratajczak et al., 2012). In nature, disruption of a peripheral clock in the corpus luteum could be an ecologically relevant mechanism for post-fertilisation blockage of pregnancy, in response to an environment hostile to raising offspring. Finally, uterine myometrial deletion of *Bmal1* can alter the timing of parturition (Li et al., 2015), further evidence for peripheral clocks as modulators of reproductive events. In mammals, the timing of birth is often linked to specific times of day that minimize risk to mother and offspring during this vulnerable period, suggesting an important ecological role for a myometrial uterine clock.

How the reproductive state might affect peripheral rhythms of other organs

Peripheral reproductive clocks respond to ecologically relevant signals that include availability of metabolic fuels. These include glucose-sensing (Barclay et al., 2012b) and responses to free fatty acids (Furutani et al., 2015) by specific aspects of the molecular cellular clock. Peripheral clocks in the adrenal glands, and elsewhere, have been shown to contribute to the control of blood glucose (Barclay et al., 2012b). Neurons in the central circadian clock alter function in response to glucose-sensing (Oosterman and Belsham, 2016), and glucose-sensing alters the function of peripheral clocks in the liver (Kaasik et al., 2013) and isolated fibroblasts (Hirota et al., 2002). There is evidence for feedback from reproductive tissues to the liver, as pregnancy alters circadian expression of clock genes in the liver (Wharfe et al., 2011), raising the intriguing possibility that synchronisation between peripheral clocks in the liver and placenta are a normal part of pregnancy. One function of peripheral reproductive clocks may be to stimulate or inhibit activity in reproductive organs based upon availability of metabolic fuels as well as to stimulate other peripheral clocks to increase nutrient availability.

Fertility is affected by parasites (Blackwell et al., 2015) and disease, and there are ecological trade-offs between immunity and fertility (Lochmiller and Deerenberg, 2000; Sheldon and Verhulst, 1996). It seems likely that there are interactions between parasites, disease, and inflammation with peripheral clocks involved in fertility. Peripheral clocks exist in immune tissues (see Table 1 in Martinez-Bakker and Helm, 2015), and the immune system has multiple mechanisms of signalling to the circadian system (Geiger et al., 2015). These local responses may be a particularly rich area for investigation. Pathogen associated molecular patterns (PAMPs) and damage associated molecular patterns (DAMPs) are major molecular inputs to pattern-recognition receptors in signalling pathways that respond to damage or disease (Labrecque and Cermakian, 2015). Peripheral clocks can control the timing of

expression of receptors for DAMPs or PAMPs (e.g. Silver et al., 2012), and immune cells that respond to DAMPs and PAMPs produce circulating signals that can affect peripheral clocks in other tissues (de Juan et al., 2016; Labrecque and Cermakian, 2015; Scheiermann et al., 2013). For example, specific regions of the ovary, oviduct, and uterus (all of which contain peripheral clocks) might respond to local signals of damage by modifying their clock function. Firstly, peripheral clocks in reproductive tissues might be impacted by regions of damage to reduce, for example, the receptivity of dysfunctional areas within a uterus to implantation. Secondly, systemic signalling induced by parasites or disease may affect peripheral reproductive clocks. Systemic effects of rhythms in the peripheral immune system include the circadian rhythm of TNF-alpha, driven in part by peripheral clocks in lymphocytes of the immune system (Hashiramoto et al., 2010), as well as proinflammatory cytokines regulated by the clock protein CRYPTOCHROME (Narasimamurthy et al., 2012). The role of cytokine signalling from peripheral clocks to the reproductive system is not well studied, but there are indications that cytokines affect fertility. Local proinflammatory signals may be a normal aspect of mammalian implantation (Mathew et al., 2016), while excessive proinflammatory signals may disrupt fertility and pregnancy (Banerjee et al., 2013; Vannuccini et al., 2016) and contribute to infertility in males (Fraczek and Kurpisz, 2015; Leisegang et al., 2016). Here, there are profound gaps: could PAMPS and DAMPS affect peripheral clocks and rhythms in the reproductive system and fertility, either directly, or indirectly via signals from clock-driven lymphocytes? It seems reasonable that circadian clocks may integrate information about damage or parasite infestation to cause adaptive, localised responses that allow the reproductive system to bypass areas of damage, and direct activities and embryos to areas that are undamaged. One could speculate that signals from the immune system modify peripheral clocks involved in pregnancy, in order to alter the rate of development during responses to parasites or disease. In such a case, peripheral clocks may permit adaptive changes in fertility in response to systemic signals of parasitism or disease.

It is plausible that these peripheral clocks are sensitive to additional environmental inputs that in nature either enhance or suppress fertility to increase fitness. For example, high levels of androgens from either endogenous or exogenous sources may disrupt peripheral clocks and alter fertility (Amaral et al., 2014). Both central and peripheral clocks contain elements for glucose sensing (Kaasik et al., 2013) and free fatty acid sensing (Furutani et al., 2015), suggesting that peripheral clocks in reproductive tissues might modify fertility based upon the availability of nutrients. Decreases in Leydig cell metabolism may be linked via the cell energy-sensing protein SIRT-1 to the peripheral clock and reduced secretion of testosterone, suggesting a potential link between the nutritional environment and fertility in

males (Baburski et al., 2016). Variation in nutritional input may be due to external ecological factors, such as drought or competition for resources, or due to internal signals, such as nutritional modulation by social cues, parasites, or disease.

It is clear from laboratory studies that peripheral clocks are necessary for normal fertility, and that modulation of peripheral clocks may enhance or inhibit fertility. However, whether such involvement of peripheral timing benefits fertility and reproduction in field condition remains mostly unclear. As it stands, there is insufficient evidence arguing either for or against such benefits, but given the many roles of peripheral clocks in regulating fertility, particularly in females, it is reasonable to expect that environmental signals act on fertility in many animals in part by modifying peripheral clocks.

Contributions of sex hormones to flexibility timing in the field

Reproductive hormones such as sex steroids convey information on biotic or abiotic variation in the ecology of an organism to the internal central and peripheral clock systems. It is well established that social and environmental factors can modulate plasma concentrations of sex steroids both in the field and in the laboratory (Adkins-Regan, 2005; Nelson, 2011), as well as cause ubiquitous changes in many other hormones related to reproduction in vertebrates (Ball and Bently, 2000; Bronson, 1989; Stevenson et al., 2012). For example, in the majority of seasonal vertebrate species circulating concentrations of sex steroids increase dramatically when the reproductive axis becomes activated as a result of photic stimulation. Such elevated levels of sex steroids serve to promote the expression of behaviours associated with reproduction, including territorial aggression, courtship, competitive and mating behaviour (Ball and Balthazart, 2004; Floody, 1983; Monaghan and Glickman, 1992; Woolley et al., 2004). Not only photic signals, but also social stimuli can serve as potent modulators of sex steroid concentrations (Hirschenhauser and Oliveira, 2006; Wingfield et al., 1990).

In many species, plasma sex steroid concentrations undergo diel variations, with testosterone typically being elevated at night and/or during the early morning (Hau et al., 2002; Kriegsfeld et al., 2002; Laucht et al., 2011). While such diel rhythms in sex steroid concentrations are clearly present in the lab, at present we lack evidence for their existence under natural conditions (though diel rhythms in glucocorticoids are present in wild populations (Murray et al., 2013; Tarlow et al., 2003)). As a result, specific characteristics of diel rhythms in sex steroids in wild populations such as phase angles and amplitudes, as well as their variation with season, local environmental conditions and social circumstances still have not received much attention, and remain mostly unknown. Partly, this lack of knowledge may arise from

logistical issues associated with sampling wild populations repeatedly at specific times of day. However, non-invasive techniques such as hormone analyses from excrements and new technologies including implantable biosensors may open up new opportunities for closing this gap (Gumus et al., 2015; Lynch et al., 2003); but still has drawbacks and concerns (Goymann, 2012).

A topic that has received more attention, at least in select species, are the actions of sex steroids on circadian timing. In various vertebrates, experimental manipulation of sex steroid concentrations and/or their rhythms has profound effects on circadian characteristics, with consequences for entrainment properties including altered phase angles and activity timing (Turek and Gwinner, 1982). For example, in male mice a reduction of testosterone concentrations through gonadectomy results in lengthening of the free-running period (τ) of circadian activity rhythms, and reduces the precision of activity onset as well as overall activity levels, while androgen replacement restores these changes (Daan et al., 1975; Model et al., 2015). Estrogens also can affect circadian properties in rodents (Blattner and Mahoney, 2015). For example, natural increases in estrogen concentrations during estrous as well as experimental estrogen administration phase-advances behavioural activity rhythms in some rodent species (Albers et al., 1981; Labyak and Lee, 1995; Morin et al., 1977). Estrogens can also increase overall behavioural locomotor activity, and estrogens may also modulate the effectiveness of nonphotic stimuli mediated through e.g. wheel-running on circadian rhythms (Legan et al., 2015). However, the proximate mechanism underlying these estrogenic actions in female rodents are still debated, and may involve changes in activity levels, arousal, sleep and responsiveness to non-photic stimuli rather than changes in τ- although species-differences may also account for some divergences in mechanisms (Labyak and Lee, 1995; Mong et al., 2011; Yan and Silver, 2016).

In terms of mechanism, the effects of androgens on τ in rodents is likely mediated by androgen receptors, which are present in the SCN (Model et al., 2015). The SCN also expresses estrogen receptors, although only sparsely in female rodents (Mong et al., 2011). Hence, effects of estrogens on circadian functioning may occur primarily through peripheral sites outside of the SCN, and mechanisms of actions may differ between the two sexes (Mong et al., 2011). Both androgen and estrogen receptors have been located in brain regions that communicate with the SCN, as well as other parts of the body including the gonads (Dart et al., 2013).

Taken together, the available evidence from laboratory-based studies suggests that sex steroids can have substantial modulatory effects on circadian rhythms, although there exist large variation in these effects, and the mechanism they employ among taxa. It has been hypothesized that these actions are adaptive, serving to fine-tune

activity times to match altered needs during the reproductive period. For example, altered phase-angles during estrous may promote encounter rates between males and females, thus increasing mating opportunities (Morin et al., 1977). Rigid field tests of this appealing idea are thus far still lacking. However, some recent field studies in avian species have corroborated fitness benefits associated with altered circadian function during the reproductive period (Hau et al., 2017). For example, in blue and great tits, males with earlier activity and song onset during the mating period gain more extra-pair fertilizations than males that become active later (Kempenaers et al., 2010; Poesel et al., 2006). Further, experimentally induced delays in male activity onset increases the rate of cuckoldry of focal individuals, suggesting that mate-guarding abilities are also impaired by mistimed activity (Greives et al., 2015). It is tempting to speculate that sex steroids, which are increased during the reproductive season, are involved in this circadian reorganization, but direct tests of this idea have not yet been carried out.

Clearly more work under natural conditions is needed to understand why there exists variation in the effects of sex steroids on circadian functioning among taxa and between the sexes. Likewise, the functional reason for the existence of diel variations in circulating sex steroid concentrations are also still unclear. Why is testosterone elevated at night in diurnal taxa like birds? Does that pattern represent a phylogenetic constraint, since it appears to be present across taxa or does it have specific functions, for example for the reorganization of circadian patterns?

Contribution of peripheral food entrainment to flexible timing in the field

Food availability has long been known as the 'other' circadian Zeitgeber (Stephan, 2002) which serves side-by-side with the light-dark Zeitgeber as the dominant entraining cue for the circadian timing system. The feeding ecology of most animals in the field includes temporal restrictions on food availability for reasons such as daily prey availability/predation risk, environmental darkness and of course one's own sleep-wake cycle. Daily feeding constraints lead to demonstrable changes in timing of overt behaviour in laboratory rodents, most notably as daily bouts of Food Anticipatory Activity (FAA) (Mistlberger, 2009). Many studies have exposed that ability of food to entrain circadian feeding patterns in e.g. rodents (Hatori and Panda, 2015), omnivores (Ware et al., 2012), herbivores (Tucker, 2009), carnivores (Zielinski, 1986), primates (Boulos et al., 1989), marsupials (Kennedy et al., 1996; O'Reilly et al., 1986) and birds (Hau and Gwinner, 1992), but the 'strength' of feeding as a timing cue for FAA varies between species and studies.

It has been firmly established in laboratory conditions that food timing directly entrains molecular peripheral clocks and the transcriptome of tissues such as in the

liver, adipose tissue, gastrointestinal tract, kidney, heart and pancreas, without shifting the clock in the SCN (Damiola et al., 2000; Hoogerwerf et al., 2007; Stokkan et al., 2001; Zvonic et al., 2006), which means that food entrainment is restricted to the peripheral timing system, including non-SCN brain regions (Mistlberger, 2009; Mistlberger, 2011). Food entrainment has been conceptualised to be driven by a 'Food Entrainable Oscillator', but the exact nature and location of this oscillator is not fully understood (Mistlberger, 2009). Even though it is unclear what the critical clocks are, the mechanisms that encode food timing into cellular circadian oscillators in mammals are being identified as bi-directional interactions between the genes and proteins involved with the circadian clock and biochemical markers of cellular cata- and anabolism. Known examples include intracellular (1) NAD+, (2) AMP, (3) haem and (4) ROS which participate in molecular interactions with clock genes, thereby altering their expression and protein stability (Asher and Schibler, 2011; Bass, 2012). (1) BMAL1 and PER2 are subject to NAD+ dependent deacetylation by SIRT1, altering their stability and thereby altering their transcriptional control over other clock genes (Nakahata et al., 2008). (2) AMP-activated protein kinase phosphorylation destabilises CRY1, altering the repressive feedback on Clock and Bmal1 (Lamia et al., 2009). (3) REV-ERBα non-covalently binds haem, which promotes binding of REV-ERBα with a co-repressor complex resulting in repression of Bmal1 expression (Yin et al., 2007). (4) Hyperoxidised moieties of peroxiredoxins (PRX-SO2/3H) exhibit circadian rhythms, reflecting ROS production, which is proposed as a novel, non-genetic circadian clock (O'Neill and Reddy, 2011).

In field conditions, food availability is paramount to survival, especially for species that feed only at limited times of the day such as hunting species. However, many species, including grazers have been shown to forage at several, and not a single, specific times of day, which can be synchronized among conspecifics within the same group (Rands et al., 2008). Animals eat in many different feeding patterns, which are often multi-modal, with 2 or more 'meals' per day (Rijnsdorp et al., 1981). Such multi-modal patterns in feeding are different from what is normally assumed and/or enforced in laboratory conditions and therefore the translation of laboratory studies to field conditions is constrained. The clock in the liver can entrain to a sequence of several meals, rather than single meal, possibly taking into account both timing and size of each meal (Kuroda et al., 2012; Luby et al., 2012). However, the consequences of several meals on peripheral timing has been reported to dissociate timing of rhythms such as ghrelin and cortisol, as well as the phase of several peripheral brain clocks (Patton et al., 2014).

Bi, or tri-modal feeding patterns are distinctly different from ultradian feeding patterns, which are defined by semi-equidistant meals throughout day and night. In

the field, ultradian feeding and sleep cycles are particularly evident in the common vole (*Microtus arvalis*) (Daan and Slopsema, 1978). This herbivore hindgut fermenter feeds every 2-3 hours throughout the day and night, whilst simultaneously expressing weak circadian modulation of behaviour (van der Veen et al., 2011). In the vole liver, there are no detectable circadian expression patterns of clock genes, while in the very same animals these genes exhibit clear circadian expression rhythms in the SCN (van der Veen et al., 2006). Imposing daily, 8-hour fasting episodes results in strengthened circadian timing of behaviour in the vole, and the emergence of circadian cycles of clock gene expression in the liver (van der Veen et al., 2006). Interestingly, it was recently shown that widespread ultradian gene expression patterns in the mouse liver transcriptome in vivo, as well as fibroblast in cell culture in vitro, are strongly associated with cellular metabolism (van der Veen and Gerkema, 2017), suggesting a relevance of ultradian timing of metabolism irrespective of feeding patterns.

Next to the multimodal feeding patterns often observed in nature, a second constraint in translating our laboratory-based data to the field is the daily variation in feeding time. For example, in a group of Kerry Cows, day-to-day variation in feeding time is larger than between-individual variation (Linnane et al., 2001), but for many species these levels of variation are unknown. The ability of the food entrainment system to deal with such daily variability is mostly unexplored, although Escobar and colleagues showed that rats receiving daily changes in food access were able to shift their FAA each day corresponding to the food availability the day before (Escobar et al., 2007).

Food entrainment can even serve to drive behavioural and physiological cycles that are independent of the SCN, or light-entrained activity patterns (Krieger et al., 1977; Stephan et al., 1979; van der Veen et al., 2011), which indicates that besides the primary benefit of anticipating food availability and post-prandial anabolic metabolism, food entrainment may also aid entrainment in conditions where the light-dark cycle is a weak or absent Zeitgeber. Food cycles hasten re-entrainment to a changing phase in the light-dark cycle (Angeles-Castellanos et al., 2011; Carneiro and Araujo, 2011), which may aid entrainment of species that live in covered or underground habitats such as the ground squirrel (Hut et al., 1999a; Hut et al., 1999b). It has also been suggested that food availability can substitute for photoperiod as the primary signal driving seasonal timing at latitudes where the seasonal change in photoperiod is minimal, or in temperate ecosystems where food is more critical than season for initiation reproductive effort (Bronson, 2009).

One interesting aspects of food entrainment of the peripheral timing system is the relationship between diet and chronotype (temporal niche). For example in fish, in which major shifts in daily timing and chronotype occur with changes in diet and

juvenile and adult phase (Amundsen et al., 1999), and the cheetah varies the number and duration of feeding bouts in response to the lunar cycle (visibility) and wet versus dry season (Broekhuis et al., 2014). These changes in chronotype are directly related to availability of the food in terms of accessibility and abundance, but have also been suggested to relate to energy balance and thermoregulation.

Temporal niche switching

When daily activity patterns of several species have been measured in the field, these patterns appear to be decidedly different from the nocturnal activity patterns of these same animals in the laboratory (Calisi and Bentley, 2009). A landmark study that exposed switches in the temporal niche of overt behaviour in the golden hamster (*Mesocricetus auratus*), reported that in their native habitat in Turkey these animals exhibited crepuscular activity patterns, which contrasts the laboratory-conditions in which they are almost completely nocturnal (Gattermann et al., 2008). Similarly, when laboratory mice were housed in large outdoor enclosures exposing them to natural weather conditions they reverted their nocturnal behavioural activity rhythms, and showed multiple temporal niche switches between nocturnality and diurnality over the two-year study period (Daan et al., 2011). The functional factors underlying temporal niche switching in these studies are mostly unknown but multiple studies have suggested changes in predation risk (Bakker et al., 2005; Fenn and Macdonald, 1995), interspecific competition (Levy et al., 2007) and challenges to the energetic balance (Daan et al., 2011; Hut et al., 2012) as driving forces.

The effect of energetic challenges on the daily timing of behaviour in mice has been shown under controlled laboratory conditions. Challenging mice by housing them at lowered ambient temperatures and/or letting them run in a wheel to earn food pellets results in phase advances of the active behavioural phase, where the magnitude of the shift into the light phase depends on the severity of the energetic challenge (Hut et al., 2011; Perrigo, 1987; van der Vinne et al., 2014b). Phase-shifting the light-dark cycle results in a corresponding shift of activity, demonstrating continued light-dark entrainment under energetically challenging conditions (van der Vinne et al., 2014b). The phase-shifted behavioural active phase does not depend on a phase shift of the central circadian clock in the SCN (van der Vinne et al., 2014b) but likely results from a shifted downstream mechanism controlling the timing of behaviour. Such an altered coupling of behavioural timing to the SCN phase suggests a role for the SCN as an internal representation of the external light-dark cycle, with other mechanisms linking behavioural timing to the SCN. Such a mechanism would be beneficial to animals that spent large parts of the day hiding in dark locations, to distribute physiological and behavioural activity into the appropriate daily phase without the need to constantly assess the external light-dark cycle. A further benefit

of using the SCN as an internal representation of the light-dark cycle is that it can make the daily timing of activity more robust to daily variability in light exposure. The self-sustained nature of the SCN clock makes it robust against day-to-day variability in light exposure, thereby preventing dramatic shifts in daily activity timing as a result of daily variability in the timing of light exposure.

The role of the peripheral timing system in temporal niche switching

The behavioural shift to diurnality in response to energetic challenges is mirrored by simultaneous phase advances of peripheral oscillations (van der Vinne et al., 2014b). Peripheral rhythms of corticosterone and *Period2* clock gene expression in the liver and adrenal are shifted by 4-6h under conditions of simulated food shortage (van der Vinne et al., 2014b), resulting in an internal phase angle between SCN and peripheral clocks that is similar to that seen in diurnal rodents (Lambert and Weaver, 2006; Lambert et al., 2005). The shifted phase of peripheral oscillators likely reflects a shift of the overall physiology to remain synchronized with the shifted timing of activity in energetically challenged mice. The mechanisms responsible for the circadian re-organization of internal clocks and behaviour in response to energetic challenges are however unknown. The shift in the phase of peripheral clocks under these conditions is likely a direct consequence of the shift in behavioural timing and clocks in the brain, and not dependent on altered characteristics of peripheral oscillators.

Peripheral clocks are entrained by the combined influence of multiple factors that act as Zeitgebers to peripheral oscillators, and under natural conditions, all of these timing signals are expected to peak at roughly the same time of day since environmental and brain-controlled rhythms will be synchronized to the stable environmental day-night cycle. The brain mechanisms involved in resynchronising the timing of behaviour and timing cues originating from SCN phase are unknown. An oscillator downstream of the SCN that alters its phase relationship with the SCN depending on the energetic state of an animal and controls the timing of behaviour and systemic timing signals would be sufficient to optimize the timing of physiology and behaviour to the encountered environmental conditions. However, at the current time it is unclear whether a change in timing of peripheral clocks precedes, or follows, the shift in behavioural timing.

Ultimate consequences of temporal niche switching

Avoiding prolonged periods of negative energy balance is an important and often challenging requirement for animals living under natural conditions in the field. Maintaining energy balance requires balancing energy intake and expenditure and both of these components are influenced by the daily timing of activity and rest. A substantial part of energy expenditure of small endotherms living in temperate

climates is used for thermoregulation (Speakman, 1997). The circadian thermo-energetics hypothesis proposes that endotherms can reduce thermoregulatory costs by shifting activity to the day (Hut et al., 2012). Because ambient temperatures are high during the day and low at night and the resting phase is associated with energy saving strategies, being active during the warmer day allows animals to optimize the energetic benefits of insulation and reduce daily energy expenditure. Quantification of different environmental factors modulating energy expenditure identifies thermal buffering provided by a sheltered nesting location as the dominant factor in determining the energetic consequences of temporal niche switching (van der Vinne et al., 2015c). The daily temperature cycle inside a sheltered nesting location has a reduced amplitude compared to the outside ambient temperature cycle. Diurnality therefore allows animals to use the difference in nest and outside temperatures to encounter the higher nest temperature during the night and go outside during the warmer day, thereby encountering higher ambient temperature during both day and night (van der Veen et al., 2011). Daily energy expenditure of small endotherms living in temperate climates is therefore expected to be reduced by 6-10% as a result of a shift of the active phase to the day (van der Vinne et al., 2015c).

Optimizing the daily timing of activity in relation to the timing of other animals is a second major factor in determining the ultimate consequences of a selected temporal niche (Kronfeld-Schor and Dayan, 2003). One obvious example of the importance of finding the appropriate temporal niche is during the search for a mate, since a mate can only be found when both animals are active at the same time. Similarly, avoiding activity at times when predators are present can increase survival while activity should be synchronized with prey species. An illustrative example of how temporal niche selection depends on the activity timing of both predator and prey has been documented in two species of hawks (Roth and Lima, 2007). This study recorded the activity timing of the sharp-shinned hawk (*Accipiter striatus)* and the larger Cooper's hawks *(Accipiter cooperii)* and their relation to the activity patterns of their primary prey; small songbirds (Roth and Lima, 2007). The activity and most dense period of attacks of the Cooper's hawks followed the peak in prey abundance in the hours around sunrise and sunset. For the smaller *A. striatus* the morning peak of hunting activity was largely absent, the hawks leaving their roosts after the time of densest prey abundance. Of the 12 sharp-shinned hawks that were killed by predators by the end of the study, one fell prey to a Cooper's hawk and the remaining 11 were caught by nocturnal owls (Roth and Lima, 2007; Roth et al., 2005). Hence, the presence of higher order predators might relieve some of the predation pressures on the small song-birds in this habitat; indicating that predator-prey relationships might operate directly and indirectly between the different species that occupy a certain habitat. In general, there is not a specific temporal niche that will minimize predation risks and

maximize food availability for all habitats, but whenever daily rhythms in predation risk/food availability are present it is to be expected that a specific temporal niche exists that would be optimal. Plasticity in the daily timing of activity allows animals to respond to environmental changes and be able to cope with different ecological niches.

Selecting the optimal temporal niche in a complex environment

In order to increase fitness, temporal niche selection should ultimately optimize the sum of costs and benefits of all possible fitness components, such as energy expenditure, survival, and reproduction. These different fitness components are influenced by rhythms in environmental factors such as ambient temperature, predation risk and the availability of food and mates. Since the optimal time of day to be active will often be different for different environmental factors (e.g. predation risk might be lowest at night but this is also the energetically worst time to be active), trade-offs between fitness components have to be made. The mechanisms involved in making this trade-off are unknown but are important for understanding temporal niche selection in the field.

Although the ultimate benefits of temporal niche switching might be reductions in energy expenditure or predation risks, the proximate mechanisms responsible for these shifts can be unrelated to the specific benefits. In order to assess the roles of the light-dark and ambient temperature cycles for selecting a temporal niche, energetically challenged mice were housed under laboratory conditions with temperature cycles either in phase or in anti-phase with the light-dark cycle, which altered their daily distribution of activity, including a switch from nocturnality to diurnality (van der Vinne et al., 2014b). Similarly, common voles (*Microtus arvalis*) exposed to constant high ambient temperatures during lactation shifted their nursing behaviour to the night (van der Vinne et al., 2014a). Both of these examples illustrate that it is the light-dark cycle and not the temperature cycle that is used to determine the energetically optimal timing of activity and rest. Since daytime temperatures are reliably higher compared to the night (diurnality is energetically beneficial on 95-98% of days (van der Vinne et al., 2015c)), the light-dark cycle can be used as a proxy for the energetically optimal time of day to be active. The high predictability of higher temperatures during the light phase thus makes more complex regulatory mechanisms involving direct feedback from the ambient temperature cycles unnecessary.

Identification of the proximate factors responsible for temporal niche switching in response to changes in predation risk is hampered by the difficulties in systematically studying predator-prey interactions. Field studies assessing changes in daily activity

timing in response to changes in (perceived) predation risk have shown that prey species can respond to increased night-time predation by becoming diurnal (Bakker et al., 2005; Fenn and Macdonald, 1995) and vice versa following increased daytime predation (Kitchen et al., 2000). This suggests that prey species, at the very least, incorporate the temporal niche of the predator in their temporal niche selection. The greater variability in the temporal organization of predation risk compared to the high predictability of ambient temperature rhythms would be an argument for more complex regulatory mechanisms being responsible for temporal niche switching in response to changes in predation risk.

Putting it all together

Under natural conditions, animals are exposed to a variety of environmental opportunities, challenges and threats that require adaptive responses to maximize fitness. Some of these environmental variables will exhibit predictable variation over the day (e.g. light, temperature), some will be constantly present (e.g. food for grazers), and some will have erratic timing over the day or season (e.g. rain, drought). Moreover, the diurnal patterns of variables such as predation risk can vary strongly between days. Thus, in order to maximize fitness, animals must find the optimal balance between foraging, predation risk, and reproduction, and finding such a balance requires orchestrating optimal timing of these behaviours across the day.

The mammalian SCN provides an internal representation of the light-dark cycle (Riede et al., 2017) and therefore provides an essential timing signal for target tissues in the central nervous system and peripheral organs (Fig 1). The SCN has therefore been historically coined as the 'conductor' of the clocks in the rest of the body (Davidson et al., 2003). However, this paradigm is primarily based on evidence gathered under laboratory conditions, when most peripheral Zeitgebers are aligned with the light-dark cycle. Under natural conditions not all relevant environmental variables show stable, aligned daily cycles, and the notion of the SCN as orchestrator is stretched to its limits. In fact, maximisation of fitness is reliant on plasticity in the timing of these peripheral rhythms, as well as behavioural activity that is associated with non-photic cycles and patterns. These real-live considerations imply that a SCN-centric view is in direct contradiction with maximizing fitness.

We have shown here that timing of behavioural activity associated with e.g. the endocrine system, food availability and other factors is associated with peripheral clocks and rhythms. Moreover, there are many examples of non-photic Zeitgebers driving clocks and rhythms in the periphery, often dissociated from the light-dark cycle. Even a lack of food per se will have a dramatic impact on overt and endogenous rhythms. Similarly, ambient temperature and predation risk can lead to substantial changes in rhythmicity in mice, and voles seem especially adapted to change their

rhythmicity from more circadian to more ultradian forms, whereby a noticeable shift to more diurnal activity occurs especially when it is cold and food is scarce.

Based on these tangible, real-live considerations - rather than those originating from lab-based observations - is does not seem appropriate describe the SCN as the conductor of the orchestra of body tissues. It seems much more pertinent to consider the SCN primarily as the internal representation of the external light-dark cycle, providing a signal that can be consulted by other brain areas and peripheral tissues (Fig 1). This change in viewpoint provides a prominent role for peripheral tissues, also because the degree to which peripheral clocks follow the SCN signal or other signals depends – at least in part - on the state of each of these individual clocks. The palette of hormonal and neurotransmitter receptors expressed by each tissue in the body, including hypothalamic nuclei downstream from the SCN, will therefore determine their phase angle relative to that of the SCN, as well as their response to environmental cues.

Some functions of peripheral tissues, however, require a tight phase angle with the light-dark cycle by means of a tight coupling with the SCN. Melatonin production may be one of them. The direct coupling of the melatonin producing pinealocytes to the SCN (Buijs and Kalsbeek, 2001; Kalsbeek et al., 2006) can be functionally understood from its role in the photoperiodic response, driving seasonal gonadal development (Hau et al., 2017). The peripheral clock in the ovaries for example will detect the carefully timed hypothalamic drive on gonadotropin release by the pituitary as a response to photoperiod (Sellix et al., 2010). This hypothalamic drive depends on the measurement of photoperiod, requiring the SCN-melatonin axis and circadian clocks in the pars tuberalis to precisely work together (Dardente et al., 2010). A series of circadian clock systems need to be precisely tuned to the environment and to each other to get successful ovulation: day length > SCN > melatonin > pars tuberalis > pituitary > ovary. But even here it is clear that internal state (fat reserves) and environmental variables (e.g. temperature and food) play an important role in modifying the hypothalamic drive to the pituitary, although the precise mechanisms by which these modifiers act remain unclear (Hut et al., 2014).

Understanding the adaptive value of the mammalian circadian system thus requires a better understanding of the mechanisms by which peripheral clocks are timed by the environmental cues in combination with SCN derived signals. To obtain such insights, we must abandon standard 12h light: 12h dark light-regimes at room temperature, because these artificial conditions hide, or mask, the independence of peripheral clocks. The plasticity of circadian organisation, as provided by the contribution of peripheral clocks under a range of real environmental conditions should be studied to provide insight in the heterogeneity of regulatory mechanisms

in circadian organisation. Eventually we should be able to understand the variety of circadian patterns observed under natural conditions, how they evolve, to which signals they respond, what the functions of those responses are, and how they contribute to increasing survival and reproduction. Eventually we will learn how a range of mechanisms will let the environment interact with a complex internal circadian landscape, to allow an optimal timing relationship between the SCN and the multitude of peripheral clocks which maximises fitness under natural conditions.

Chapter 3

The flexible clock: predicative and reactive homeostasis, energy balance and the circadian regulation of sleep-wake timing.

REVIEW PAPER

As a follow-up on the publication about the energetic benefits of diurnal behaviour in mice by van der Vinne in the Journal of Experimental Biology (2014) we were invited to provide an extended review paper to explain the concepts behind the circadian energetics hypothesis and integrate the findings of our working-for-food paradigm with available literature. This review was published in 2017 in the Journal of Experimental Biology and was the first publication with myself as the lead author.

Chapter 3

The flexible clock:

Predictive and reactive homeostasis, energy balance and the circadian regulation of sleep–wake timing

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Paper is published in the
Journal of Experimental Biology
March 1, 2017; 220 (pt5): 738-749
DOI: 10.1232/jeb.130757

Abstract

This Review aims to assess how the Darwinian fitness of mammals living in a rhythmic environment depends on endogenous daily (circadian) rhythms in behaviour and physiology. First, mechanisms underlying the circadian regulation of physiology and behaviour in mammals are elucidated. Second, we review recent efforts to understand circadian flexibility (e.g. how the phase of activity and rest is altered depending on the encountered environment). We explain why shifting activity to the day is an adaptive strategy to cope with energetic challenges, and we show how this can reduce thermoregulatory costs. A framework is provided to make predictions about the optimal timing of activity and rest of non-model species for a wide range of habitats. This Review illustrates how the timing of daily rhythms is reciprocally linked to energy homeostasis, and it highlights the importance of this link in understanding daily rhythms in physiology and behaviour.

Introduction

The mammalian circadian system is a collection of intrinsic timekeeping mechanisms, regulating biological processes in a time-of-day dependent manner. The circadian system plays an important role in the daily variation of homeostatic set-points, including fluctuations in plasma glucose, body temperature (T_{body}) and hormone levels (termed rheostasis; see Mrosovsky, 1990). Furthermore, it allows animals to organize their behaviour and physiology in anticipation of expected risks and opportunities in their environment and to anticipate predicted perturbations of their internal state (also referred to as predictive homeostasis; see Glossary; Moore-Ede 1986). To drive these rhythms, most cells possess molecular clocks of interacting clock genes that are expressed in an alternating manner. Besides their capacity to promote or inhibit each other's expression and function, these clock genes also regulate the expression of various tissue-specific genes – referred to as clock-controlled genes (Storch et al., 2002) – thereby regulating tissue activity in a time-of-day dependent manner. The current consensus is that, in order to produce functional anticipatory rhythms, cells within a tissue have to work together (adjust their timing relative to the timing of other cells) as well as obtain the correct alignment to their (extracellular and extra-organismal) environment. To do so, a light-sensitive master-clock in the suprachiasmatic nucleus (SCN) in the hypothalamus sends out signals that can be used to convey time-of-day information to local tissue clocks or to induce behavioural rhythms at a stable phase relative to the environmental solar day (Dibner et al., 2010, and references therein).

Manipulations such as time-restricted feeding, forced activity and certain (bio)chemical signals can alter the phase of output rhythms relative to that of the light–dark-tracking SCN. As these manipulations also induce self-sustaining

rhythms in SCN-lesioned animals, they are considered to be partly or fully reliant on alternative circadian pacemakers (for more detail see Pezuk et al., 2010). Despite many attempts, the (neuro)anatomical locations and mechanisms of these alternative circadian oscillators have not been identified. It has been proposed that these alternative pacemakers might actually be the result of a network feature of multiple hypothalamic clocks, possibly interacting with local tissue clocks (Acosta-Galvan et al., 2011; Blum et al., 2012; Buijs and Kalsbeek, 2001).

Although we do not fully comprehend how, the organization of circadian rhythms in animals living in the wild is evidently much more flexible than the well-characterized rhythms of laboratory-housed animals. The phase of output rhythms is not enslaved to the phase of the SCN and light–dark cycles alone, but is capable of adjusting to both the experienced and expected environment. Understanding the nature of the flexible timing of output rhythms relative to the SCN might yield important implications for human shiftwork, chronotherapy (see Glossary), athletic performance and general well-being and health.

Here, we review our recent progress in understanding the adaptive flexibility of circadian rhythms. We discuss the idea that, in animals exposed to the same external timing cue [or Zeitgeber (see Glossary), e.g. light–dark cycle], differences in energy balance can lead to different circadian phenotypes in a single individual. The data seem to support a model in which, during these adjustments in circadian phenotype, the SCN continues to guide the timing of output rhythms to the most favourable time of day (in contrast to uncoupling from the SCN with rhythmicity being driven by extra-SCN pacemakers). Here, we evaluate our proposed hypothesis explaining why such responses would be ecologically and evolutionarily relevant (the circadian thermo-energetics hypothesis), and our findings that support this hypothesis. We further highlight that divergent phasing between output rhythms and the SCN does not require external timing cues but can be mediated through alterations in an animal's homeostatic state. The exposure of nocturnal mice to an environment with a constant low food density, or to constant cool ambient temperature ($T_{ambient}$), can alter their behavioural, physiological and local tissue clock phases relative to the phase of the SCN. This suggests the circadian system is not only the facilitator of predictive homeostasis, but that it itself is also subject to homeostatic fine-tuning. As the timing (phase) of circadian output rhythms can be modulated by homeostatic changes, we suggest a novel perspective on the flexibility of circadian rhythm generation. Circadian time-keeping mechanisms enable predictive homeostasis, but the circadian machinery itself can be modulated through principles of reactive homeostasis (see Glossary). This changes the view of circadian phase from being a rigid response to Zeitgeber stimuli to being an adaptive system that actively reshapes

how Zeitgebers are processed depending on homeostatic outcomes and internal states.

The regulation of daily rhythms in mammals

The SCN coordinates output rhythms

Daily rhythms in sleep–wake behaviour, feeding–fasting cycles, drinking behaviour, hormone levels, melatonin synthesis and locomotor activity are examples of circadian output rhythms in mammals. They are endogenously driven, as they persist in constant environments with periods close to 24h (i.e. they are circadian; meaning 'about a day'). Lesions of the hypothalamic SCN abolish these rhythms (Eastman et al., 1984; Klein and Moore, 1979; Moore and Eichler, 1972; Stephan and Zucker, 1972), whereas transplanting SCN tissue into arrhythmic hosts reintroduces circadian rhythms with a circadian period equal to that of the donor (Ralph et al., 1990; Sujino et al., 2003). The development of novel methods for labelling, imaging, measuring and manipulating neuronal functions has allowed researchers to develop a detailed understanding of how the SCN generates daily rhythms in neuronal firing patterns and how it can match these in their periodicity and in a fixed phase relative to the environment (Abrahamson & Moore 2001, and references therein). Individual neurons of the SCN show ~24h rhythms in their spontaneous firing frequency which – in turn – arise from (and feedback on) self-sustaining molecular feedback loops of clock gene expression (Colwell, 2011; Jones et al., 2015; Nakamura et al., 2002; Shearman et al., 2000). SCN neurons are highly interconnected and can influence each other's electrical and molecular rhythmicity (Colwell, 2011; Mohawk and Takahashi, 2011; Van Den Pol and Dudek, 1993). Input derived from rhythmic external cues as well as rhythmic internal signals (feedback) can modulate the molecular and electrophysiological rhythms of SCN neurons, altering the phase, period and waveform of the generated rhythm (see reviews by Evans & Gorman 2016; Yannielli & Harrington 2004 for details).

In a natural environment, the rhythm of the SCN is considered to be influenced primarily by the light–dark cycle. First, direct and indirect projections from light-capturing retinal ganglion cells reach neurons of the SCN, modulating their activity (Abrahamson and Moore, 2001; Edelstein and Amir, 1999; Fernandez et al., 2016; Michel et al., 2006; Nakamura et al., 2004). Depending on the phase of the receiving SCN neuron, light input can stimulate active firing of the neuron and modulate clock gene expression (Brown & Piggins 2007; Hamada et al., 2001; but also see Drouyer et al., 2007, highlighting SCN heterogenetity in this response). Due to cellular communication between SCN neurons, adjusting the phase of individual cells can subsequently induce a shift in the activity of the whole SCN (reviewed by Welsh et

al. 2010; Belle 2015). In short, light perceived around dawn will advance clock phase, such that the next day cells increase their firing frequency earlier. Conversely, light perceived around dusk will delay clock phase. Through these principles of photic entrainment (see Glossary), the SCN can serve as an accurate clock that generates an internal representation of the external solar day. SCN neurons are most active during daytime, and relatively few cells fire during the night (Schaap et al. 2003); this is true for both diurnal and nocturnal mammals (Challet, 2007; Hut et al., 2012; Inouye and Kawamura, 1979; Kurumiya and Kawamura, 1988; Sato and Kawamura, 1984). Increasing day length widens the distribution of phases of individual SCN neurons, making the SCN an internal representation of both time-of-day and season (Inagaki et al., 2007; Mrugala et al., 2000; Sosniyenko et al., 2009; Sumova et al., 1995; VanderLeest et al., 2007). Neuronal and paracrine output signals from the SCN are thought to be important cues to sustain, phase and pace cellular rhythms in downstream targets, allowing them to align optimally with each other and with the external world (Bartness et al., 2001; Song and Bartness, 1998). Apart from light-derived stimulation, neuronal NPY and serotonin projections can also provide input to the SCN cells; these are collectively referred to as 'non-photic' inputs (Glass et al., 2010; Meyer-Bernstein et al., 1997; Prosser, 2003). In the absence of a light–dark cycle, non-photic signals, such as a timed palatable meal or timed access to a running wheel, can be sufficient to entrain the phase of the SCN (Castillo et al., 2004; Edgar and Dement, 1991; Hut et al., 1999a; Mendoza et al., 2005). Different types of non-photic cues can be distinguished, and different non-photic cues may yield completely different phase response curves and, hence, different modes of circadian entrainment (see for review Wams et al., 2017).

Local tissue clocks

The discovery that clock genes identical or orthologous to those responsible for circadian rhythms in SCN neurons are also expressed in non-SCN cells suggested that most mammalian cells may express endogenous circadian rhythms (Balsalobre et al., 1998; Zylka et al., 1998). Instead of having a single well-characterized clock responsible for the control of daily timing (i.e. the SCN), clocks are everywhere in the body. Furthermore, it was shown using *in vitro* explants that these local tissue clocks can sustain their circadian rhythm in isolation and that they retain rhythmicity in SCN-lesioned animals, albeit with different phases between tissues and with phase differences between animals (Yamazaki et al., 2000; Yoo et al., 2004). Under standardized housing conditions, most peripheral clocks are considered to have a similar phase and bear a stable phase relation to the rhythms produced by the SCN, as shown *in vivo* (Tahara et al., 2012). Lesioning of the SCN results in severely dampened organ-level oscillations, showing the importance of the SCN in

maintaining synchrony within peripheral tissues (Tahara et al., 2012), although this was only established for laboratory-housed animals, devoid of natural timing cues. Furthermore, the phase of cellular clocks has been linked to tissue-specific rhythms in gene expression (Storch et al., 2002), giving rise to circadian rhythms in cell function and sensitivity, and leading to rhythmic activation of tissue-specific pathways (Oster et al., 2006b; Zhang et al., 2014). The importance of maintaining an appropriate phase of peripheral oscillators is illustrated by the observation that the phase of several local tissue clocks in night-active (nocturnal) species is approximately 12h shifted compared with that of day-active (diurnal) species (Challet, 2007; Hut et al., 2012; Lambert and Weaver, 2006; Ramanathan et al., 2010).

Phase-control of local tissue clocks by the SCN

As discussed above, light and non-photic feedback cues can set the phase of the circadian rhythm generated by the SCN. The phase of peripheral clocks and the timing of behavioural and physiological output rhythms are controlled through multiple mechanisms. The widely accepted view is that daily rhythms are controlled in a hierarchical manner (summarized in Fig. 1). At the top of this hierarchy is the light-entrainable oscillator (LEO) in the SCN, which generates a time-of-day signal capable of entraining the phase of local tissue clocks both in and outside the CNS (Fig.1, red box). This time-of-day cue can be transmitted from the SCN via direct neuronal efferent projections (Bartness et al., 2001; Kalsbeek et al., 2004) as well as by diffuse humoral signals (Guo et al., 2005; Silver et al., 1996; Song and Bartness, 1998). It reaches targets in the brain that are responsible for selecting behaviour (e.g. sleep, arousal, feeding), as well as peripheral tissues involved in the maintenance of homeostasis. The resulting rhythms in tissue activity and behaviour cause rhythmic changes to physiological parameters such as T_{body}, heart rate and the levels of circulating nutrients, hormones and metabolites. These physiological rhythms in turn can serve as reinforcing feedback to both the SCN (non-photic cues) and to the CNS and local targets (Balsalobre et al., 2000; Buhr et al., 2010; Stokkan et al., 2001; Tataroglu et al., 2015). Under this model, the phase relations between the SCN and peripheral targets are generally regarded as hard-wired and rigid, ensuring the rhythms are accurately timed relative to the light–dark cycle.

Phase control of local tissue clocks by non-SCN pacemakers

Non-SCN circadian timing systems can, under certain conditions, determine the phase of local tissue clocks and output rhythms that are otherwise controlled by the SCN (Damiola et al., 2000; Pezuk et al., 2010). Animals with SCN lesions become arrhythmic in constant environments (Eastman et al., 1984; Moore and Eichler, 1972; Stephan and Zucker, 1972). However, timed access to food, reward or methamphetamine infusion can re-introduce daily rhythms in SCN-ablated

mammals; alternative (non-SCN) circadian pacemakers have been proposed in order to explain this (Honma et al., 1988; Krieger et al., 1977). The food-entrainable oscillator (FEO) and the methamphetamine-induced circadian oscillator [MAO; also known as the methamphetamine-sensitive circadian oscillator (MASCO); Fig. 1] can induce phase coherence between tissues, as well as allowing (in the case of food) anticipation of daily events that recur at ~24h periods (reviewed in Mistlberger 2011). Recently, daily timed exposure to treats, threats and exercise were also shown to induce self-sustaining circadian responses at phases deviating from the SCN-directed rhythm, suggesting that more pacemakers might exist (Flôres et al., 2016; Pellman et al., 2015). The underlying timekeeping mechanisms of non-SCN circadian pacemakers have not been identified (Mistlberger, 2011). However, the dominant view is that non-SCN oscillators either can be slaves to the SCN (thus supporting the robustness of the circadian rhythm) or can become independent in their phase when they receive specific cues, such as methamphetamine or rhythmic food availability (Pezuk et al., 2010).

Most cells and tissues – including many brain regions – possess clock gene cycles and could thus potentially serve as extra-SCN circadian rhythm generators. Multiple interactions between the clocks in different tissues might explain why identifying the locations of extra-SCN pacemakers has proven difficult; many of the involved components may show some degree of redundancy. Hierarchical organization of the communication pathways between the clock gene rhythms in local tissues remains largely unestablished, although it has been shown that various output rhythms (including corticosterone, melatonin and T_{body} patterns) can play important roles in the entrainment of circadian oscillations at the tissue level (Balsalobre et al., 2000; Buhr et al., 2010; Torres-Farfan et al., 2011). It is thought that these 'output as input' interactions provide robustness to the circadian system, but they might also facilitate collective flexibility in the timing of overt rhythms in behaviour and physiology relative to the SCN phase. Rhythms in behaviour and physiology can thus be regarded as both cause and consequence of peripheral clock phasing (Fig.1, black and blue arrows).

Circadian flexibility: the 'work for food' paradigm

Ecological relevance of circadian flexibility in the phase of daily rhythms

Under 24h entrainment, circadian rhythms are routines that repeat themselves on a daily basis. The purpose of these routines is twofold; first, they allow animals to prepare and anticipate their actions, thereby utilizing time and resources more efficiently. Second, routines provide safety – 'if it worked for me yesterday, it will probably work today'. As for most traits found in nature; the performance of routine

behaviour has emerged under, and has been shaped by, the pressures of natural selection. Thus, the phase of the daily routines is optimized for an animal's natural

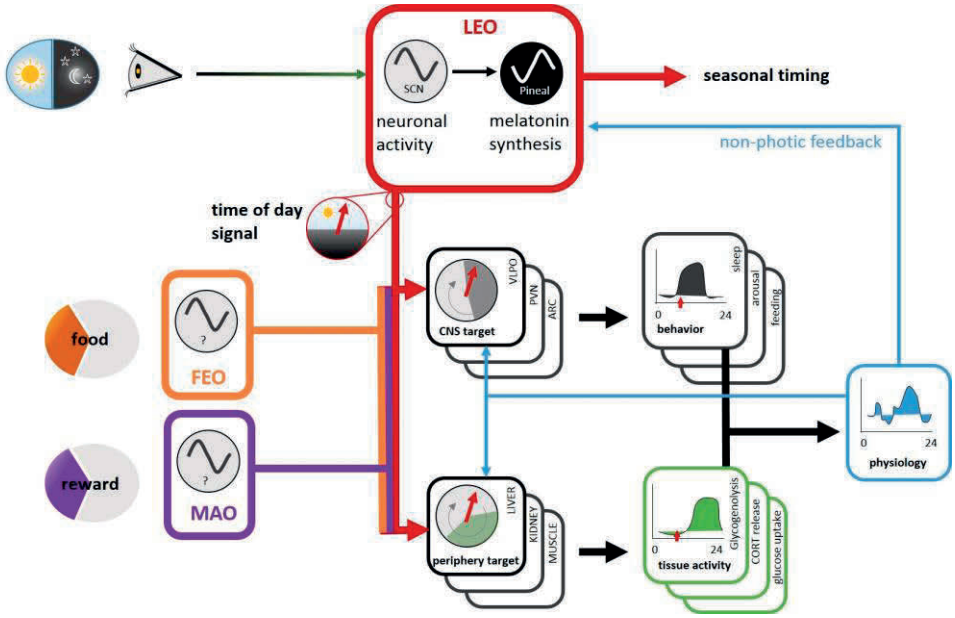


Figure 1. Overview of the proposed hierarchical control of circadian rhythms in mammals. *The high-amplitude endogenous rhythm in the firing rate of suprachiasmatic nucleus (SCN) neurons is entrained to the external light–dark cycle through direct projections of specialized neurons in the retina. The SCN rhythm directly regulates melatonin synthesis in the pineal gland, which, in turn, is involved in seasonal rhythmicity (red box). Together, neuronal and humoral signals from the SCN and melatonin provide the basis for the time-of-day signal to downstream targets in the brain and body (black boxes). The phase of cellular clocks in these targets relates to rhythmic patterns in behaviour and tissue activity (green boxes), collectively inducing daily rhythms in physiological variables (blue box). These physiological rhythms, in turn, provide non-photic feedback to both the SCN and local clocks (blue arrows), which can modulate their phase and accuracy. Timed access to food and rewards (indicated by the orange and purple circle segments, respectively) induces rhythmicity in alternative circadian oscillators – the food-entrainable oscillator (FEO) and the methamphetamine-sensitive circadian oscillator (MAO), respectively. Their locations and mechanisms are poorly understood (indicated by the question marks), but they are proposed to guide the circadian phase of CNS and peripheral clocks independent of the SCN phase. The depicted CNS and peripheral targets and their functional output rhythms are chosen as arbitrary examples, and include the arcuate nucleus (ARC), ventrolateral preoptic area (VLPO) and the paraventricular nucleus of the hypothalamus (PVN), the latter linked with rhythmic corticosteroid (CORT) release. LEO, light-entrainable oscillator.*

habitat. These habitats are often complex and diverse (e.g. arctic versus tropical, desert versus wetland), providing the basis for the large diversity in the phase of the circadian rhythms found in their inhabitants. Generally, some of the environmental

parameters relevant for survival of a given species are bound to show daily variation. These can be direct and abiotic (e.g. light, temperature, UV radiation) or indirect and biotic factors (e.g. the presence of conspecifics, prey, predators and competing species). Furthermore, habitats themselves can be dynamic (e.g. seasonal or sudden changes in weather, food and predator density, parasite and disease loads), resulting in differences in the optimal circadian phenotype. Often, animals will have to make trade-offs in their selection of daily temporal niche (see Glossary), as the safest time to forage might coincide with the least efficient time for finding food, for example. Field observations clearly indicate that daily rhythms of free-living animals can differ from their rhythms when kept under laboratory conditions, or can change within individuals in response to the dynamics of their habitat or to their specific priorities (reviewed in Hut et al., 2012). The mechanisms underlying this flexibility are poorly understood, but seem to be regulated by modifiers downstream of the SCN (Kas and Edgar, 1999b; van der Vinne et al., 2014b). This line of investigation might eventually bear relevant insights for targeted treatment of humans who are facing frequent disruptions of their daily routines (e.g. shiftwork, travelling time zones, irregular lifestyles) or who have reduced exposure to natural light–dark cycles (Woelders et al., 2017).

Developing the 'work for food' model to study circadian flexibility

The relevance of the flexible timing of circadian rhythms was apparent when our lab attempted to unite the results of several lines of research involving behavioural energetics, circadian rhythms and ecological timing. Firstly, Daan et al. (2011) found that, for extended periods of time (up to several months), house mice kept under natural conditions in outdoor field enclosures were exclusively active during the day, whereas during other periods the mice were more nocturnal (Daan et al., 2011). Around the same time, Schubert et al. (2008) performed lab studies on the effect of habitat quality and energy balance on physiological responses in CD1 mice. They compared female mice exposed to high foraging costs (incurred by wheel running) with mice receiving a matched food intake without the need to forage for it. Mice that had to work hard to obtain their food showed reduced daily energy expenditure, had lower resting metabolic rate and ceased estrous cyclicity. Under these conditions, mice started to show daily torpor – a state in which metabolism is greatly reduced for several hours, resulting in a pronounced drop in T_{body} (Schubert et al., 2010).

Schubert's work inspired Pilorz to use the same setup to develop a paradigm for simulated shift work, where mice could obtain food rewards only at pre-scheduled times of day. During training, mice could earn food during the dark and their workload per food reward was increased, but, surprisingly, the mice phase-advanced

their activity period to the extent that they were resting during the scheduled shift work window. This resulted in reduced foraging yields and dramatic weight loss (Pilorz and Hut, personal communication). We subsequently performed this experiment without time constraints to the reward window (mice could work for food for the full 24h cycle), and found that increased workload (i.e. increased travelling distance in the wheel per unit food reward) shifted the activity of CBA/CaJ mice from the night into the day (Hut et al., 2011). With this paradigm, there are no external Zeitgebers provided other than the light–dark cycle. The pre-set workload is equal over the 24h cycle and temperature and humidity are tightly controlled at stable values. This 'work for food' (WFF) protocol induced robust and repeatable temporal niche switching (see Glossary) in response to increased work load – every mouse tested showed a shift from nocturnal to diurnal activity rhythms when the food reward ratio was decreased.

To establish whether the change in circadian organization was related to reduced food intake *per se* or negative energy balance in general, we tested whether lowering $T_{ambient}$ would yield similar effects. Indeed, mice fed *ad libitum* showed a significantly higher proportion of their activity during the light phase at lower $T_{ambient}$ (~10% at 25ºC and up to 32% at 10ºC). In addition, lower $T_{ambient}$ enhanced the effect of WFF on diurnality (van der Vinne et al., 2014b). Collectively, these results suggest that negative energy balance, caused either by lowering energy intake or by increasing energy expenditure, favours a diurnal phenotype. Reviewing the available literature indicates that temporal niche switching is widespread in nature, but mainly in predominantly nocturnal mammals, where it often occurs in response to changes in energy consumption or availability (Hut et al., 2012). In summary, factors that have a negative impact on energy balance seem to be associated with increased daytime activity, whereas energy surplus (such as *ad libitum* feeding in the lab) or high daytime temperatures can lead to increased nocturnal activity (Hut et al., 2012).

Functional consequences of flexible circadian phenotype: the circadian thermo-energetics hypothesis

Benefits of flexible output rhythms: a diurnal lifestyle reduces energetic needs

The adaptive significance of temporal niche switching from nocturnal to diurnal activity may involve a reduction in energy consumption. This is the basis of the circadian thermo-energetics (CTE) hypothesis, which builds on two basic features: 1) the night is colder than the day, and 2) sleep and rest are associated with better thermal insulation and therefore reduced heat loss (Hut et al., 2012). Examples of such heat loss-preventing strategies include postural changes, reduced breathing

rate, reduced blood flow to (and temperature of) the extremities, huddling with conspecifics or sleeping in an insulated temperature-buffered location like a burrow or nest (see Table 1). Because thermal insulation reduces a fraction of the energetic costs required to maintain T_{body}, it will return higher energy savings in absolute terms at low $T_{ambient}$. Aligning the rest phase with the night and actively foraging during the day is therefore more energy-efficient under most natural conditions (van der Vinne et al., 2015c). Nocturnality is therefore energetically costly, as discussed below.

Table 1. Variables that determine steepness of Scholander Slopes

Variable	Phase	Effect on slope
Insulation material around the body	Rest	↓
Huddling with conspecifics	Rest	↓
Avoiding wind chill (sheltered location)	Rest	↓
Vasoconstriction in periphery	Rest	↓
Reduced surface area (curled up sleeping postures)	Rest	↓
Low maintenance costs of posture (e.g. lying down)	Rest	↓
Torpor*	Rest	←/↓
Wind chill	Active	↑
Humidity and rain	Active	↑
Vasodilation in periphery	Active	↑
Higher breathing rate	Active	↑
Activity-related thermogenesis	Active	↓
Sun basking‡	Active	↓
Increased subcutaneous fat mass	Active/Rest	↓
Increased fur thickness	Active/Rest	↓
Digestion-related thermogenesis	Active/Rest	↓
Increased body size	Active/Rest	↓

**It is debated whether torpor can been seen as resting, but it does rule out physical activity. As it is a severe reduction of the defended Tb, it causes a leftward shift of the Scholander curve, often bringing the TLC to below Tambient, thereby reducing thermogenesis to very low values. The net result is lower energy expenditure at the same $T_{ambient}$.*
‡Sun basking is only available during the daytime and on the surface. As resting in such an exposed location is dangerous for many prey species, it is most commonly utilized in the active phase. However, some species (e.g. larger carnivores) might utilize sun basking in the rest phase.

Burrowing animals: diurnality provides energetic benefits by altering daily ambient temperature exposure

$T_{ambient}$ influences the energy expenditure of endothermic animals, as it affects the rate of heat loss and, thus, thermoregulation costs. Daily patterns in surface temperature ($T_{surface}$) are common in many habitats, with temperature peaking during the daytime and being lowest at night (Fig. 2A; Δ). Many species are not

continuously exposed to the $T_{surface}$ cycle, but instead retreat into the safety and thermal comfort of a burrow when resting. Burrows are buffered from the $T_{surface}$, having a reduced daily amplitude of temperature change and – in some cases – a slightly delayed phase (Fig. 2A; ∇). Retreating into a burrow at night provides an energetic benefit, as burrowing animals will encounter a higher average 24h temperature (Fig. 2A, orange curve) compared to when they are active during the cold night and rest in their burrow during daytime (Fig. 2A, green curve).

In our example (Fig. 2A), the mean burrow temperature (T_{burrow}) falls within the $T_{surface}$ range. It is conceivable that in some ecological habitats this is not always the case. For example, the mean T_{burrow} of an arctic ground squirrel might be low and fairly constant in a frozen tundra, well below the daily average of the air temperature (Long et al., 2005). Conversely, the mean 24h T_{burrow} temperature in a dark-soiled rocky location, or near geothermally active sites, might exceed the average 24h air temperature (e.g. see Tomotani et al. 2012). However, a burrowing animal would also spend less energy on thermoregulation under these conditions when diurnal, as long as T_{burrow} and $T_{surface}$ fall below the animals' thermoneutral zone (TNZ; see below). Provided that the animal needs to surface for foraging, aligning this activity with the warmest $T_{surface}$ is energetically favourable as long as the T_{burrow} amplitude is lower than the $T_{surface}$ amplitude. Reversely, when $T_{ambient}$ exceeds the TNZ of an animal, as might be the case in hot climates, predictions are that – in order to conserve energy – the warmest part of the day is better avoided for activity.

Relationship between ambient temperature and energy expenditure

The relationship between energy consumption and $T_{ambient}$ is described by Scholander curves (Scholander et al., 1950) (Fig. 2B). Endothermic animals actively maintain their T_{body}, investing energy to generate heat or to lose it by panting and sweating. The range of temperatures where no energy investment is needed for thermoregulation is called the TNZ; in this temperature range, energy expenditure is considered nearly constant. The borders of the TNZ are called the lower and upper critical temperature (T_{LC} and T_{UC}, respectively) (Fig. 2B). For each degree away from the thermoneutral border (below T_{LC} or above T_{UC}), energy expenditure increases in a linear fashion (the 'Scholander slopes'), representing higher thermoregulation costs (Scholander et al., 1950). When the $T_{ambient}$ falls below T_{LC}, metabolic rate increases so that heat produced by the body matches the heat loss to the environment, thereby maintaining T_{body}. The TNZ metabolic rate, defended T_{body} (see Glossary) and heat dissipation rate of an animal are generally different for the active and rest phase. For example, reduced blood flow towards skin and limbs during resting will decrease heat dissipation from the skin to surrounding air, reducing the

Scholander slope (Fig. 2B; 4). Therefore, the Scholander curves of the active (A') and resting (R') phase are different in shape (Fig. 2C).

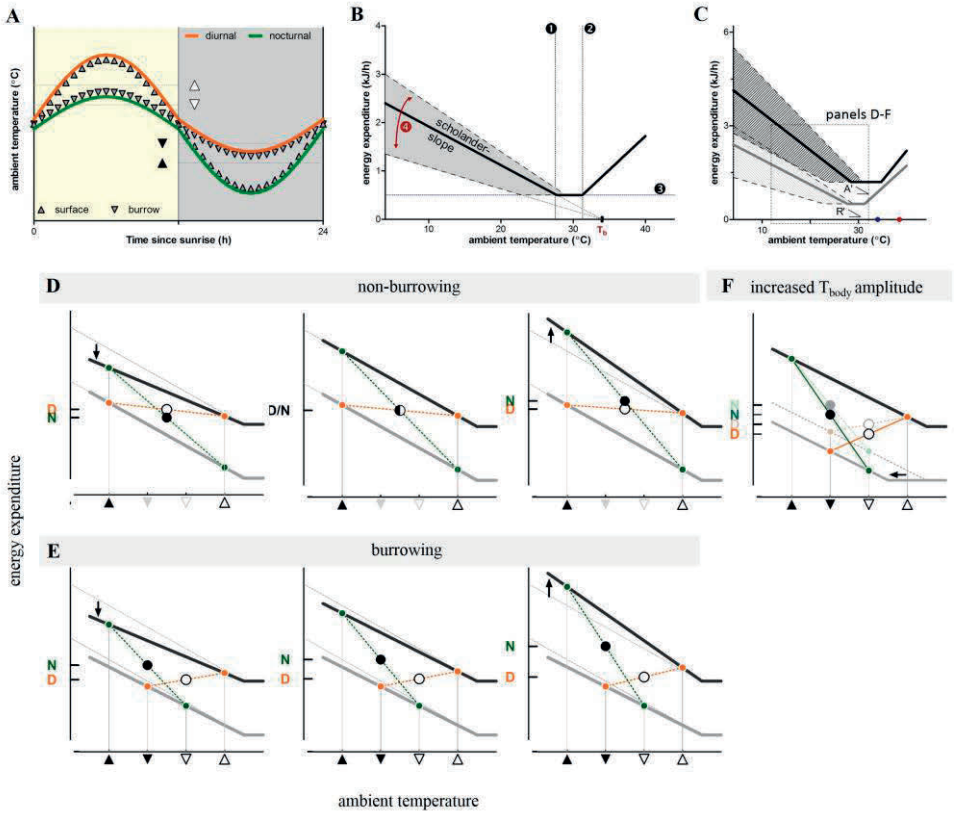


Figure 2. The energetic benefit of diurnality depends on the encountered temperature profile and the difference between the rest and active phase Scholander slopes. *(A) Surface (Δ) and burrow (∇) temperatures across the solar day are modelled by a sinusoidal pattern, with the amplitude difference depending on the insulation constant of the burrow. Diurnal burrowing animals (orange line) encounter higher ambient temperatures than nocturnal animals (green line). The larger triangles represent the mean temperatures for daytime (when white) and night-time (when black) for surface (Δ) and burrow (∇), respectively. The light–dark cycle is represented by the background colours (yellow for daytime, gray for night). (B) As a result of thermoregulation costs, mammals increase their energy expenditure to above their thermoneutral resting metabolic rate (3) at temperatures further removed from their thermoneutral zone [below the lower critical temperature (1) and above the upper critical temperature (2)]. The Scholander slope depends on heat conductance and (4) can be modulated by physiological, anatomical and behavioural adaptations (indicated by the gray shaded zone). The Scholander slope intersects the horizontal axis at the defended body temperature (Tb). (C) During rest and active phases, differences in defended Tb (blue and red dots, respectively) and*

physical/physiological activity mean that the rest phase and active phase are classified by separate slopes (R' and A') and different basal rates of energy expenditure (horizontal plateaus). (D)Without a burrow, the phase with the lowest slope (resting or active, represented by gray and black lines, respectively) is best performed at night, in order to conserve energy. (E) Burrows, by changing the encountered temperature, reduce the energy expenditure when they are utilized during the cold night. In addition, they reduce the rest-phase Scholander slope. (F) Besides the slopes, TNZ metabolic rates and defended Tb also have an impact on energy expenditure. However, these horizontal or vertical shifts in one or both of the curves do not in themselves favor diurnal or nocturnal behaviour per se, as the absolute difference in energy expenditure between a diurnal and nocturnal circadian phenotype is maintained. The graph in F shows an example where the defended Tb is reduced during the rest phase. Plots are stylized for clarity but rely on measures of energy consumption of mice kept under various ambient temperatures and housing conditions (original data in van der Vinne et al., 2014b, 2015b). The x-axis triangle symbols in D–F are explained by figure and text of A. The coloured 'D' and 'N' reflect the mean hourly energy consumption for fully diurnal (open circle) and nocturnal (closed circle) animals under the indicated condition (equal rest and active phase duration).

Differences in the active and resting phase Scholander slope predict energetic costs of diurnal versus nocturnal rest–activity rhythms

Numerous behavioural, anatomical and physiological adaptations play a role in reducing energetic costs by reducing conductance (the rate of heat loss per degree of difference between T_{body} and $T_{ambient}$). Despite this, for small endothermic mammals facing naturally encountered low temperatures, thermogenesis costs can comprise more than two-thirds of their daily energy budget (Chappell et al., 2004). Any adjustment in temporal organization of rest and activity that reduces these costs could therefore be an ecologically relevant strategy for survival when facing adverse energetic circumstances (e.g. food scarcity or cold weather). The slope of the Scholander curves of the rest and active phase can be modulated by numerous external and internal variables for animals living in their natural habitat (Table 1). For instance, social animals might reduce the steepness of the rest phase slope by huddling to share body heat during their rest phase (Fleming, 1980). The steepness of the active phase slope might be reduced by utilizing sunlight to warm the body (sun basking; Geiser et al., 2016) or utilizing the heat generated by physical activity for thermogenesis (Weinert and Waterhouse, 1998). Conversely, exposure to wind and rain steepens slope A' significantly. An overview of some relevant variables, and how they influence the slopes (A', R' or both) is provided in Table 1. Some of these variables might not be relevant for all species, whereas additional unmentioned variables might apply to others. Due to these influences, the rest and active phase slopes can differ in steepness. As illustrated in Fig. 2, in order to conserve energy, it would be advantageous to perform the behavioural phase (either activity or rest) associated with the lowest steepness during the coldest part of the daily $T_{ambient}$ cycle

(Fig. 2D). These effects add to the energetic effects of altered $T_{ambient}$ exposure by burrowing (Fig. 2E), as discussed above.

Body temperature amplitude and TNZ metabolic rates have an impact on energy expenditure, but do not favor diurnal or nocturnal behaviour in itself

An additional strategy used by mammals to reduce energy expenditure when facing harsh conditions might be to reduce their metabolic rate by reducing their investments in immune functions or reproduction (Martin et al., 2008), or by reducing their defended T_{body} during rest phase (Yoda et al., 2000). These responses will cause a vertical or horizontal shift of one or both of the Scholander curves. However, as long as these shifts have no influence on the steepness of the slopes, and the ambient temperature remains below the T_{LC}, they will not favor diurnal or nocturnal behaviour *per se* (illustrated in Fig. 2F). The absolute difference between a nocturnal and diurnal phenotype in response to these shifts stays constant. This means they can alter the size of a relative benefit (e.g. diurnal energy expenditure might change from 75% to 60% in relation to nocturnal energy expenditure), but never the direction. In summary, the slopes (R' and A') and the $T_{ambient}$ to which an animal is exposed determine the energetically optimal phase for activity.

Energy expenditure measures confirm that diurnality in mice would save energy in their natural habitat

Recently, we used an indirect calorimetry set-up to assess the minimal hourly energy expenditure in resting and active mice (CBA-CaJ strain) for a range of temperatures in and below the TNZ (10–30°C), and constructed the Scholander curves of their rest and active phase (van der Vinne et al. 2015b). Additionally, we measured the resting phase energy consumption for the same range of temperatures when mice were given increasing amounts of nest material (cotton wool) or cage partners to allow huddling (in groups of 1–3 mice) during rest. To estimate the effect of activity-related thermogenesis, we measured energy expenditure during the active phase both with and without access to a running wheel. To incorporate the effect of wind chill, we compared the rate of heat loss of a warmed-up dead mouse in both the open field (in shade) and indoors. To incorporate the temperature-buffering capacity of a burrow, we simultaneously measured $T_{surface}$ rhythms and the temperature rhythm in a hay-filled nest box. Additional measures regarding the thermal buffering capacity of natural nests and burrows were obtained from the literature. Lastly, we measured T_{body} and energy expenditure during the resting and active phase for mice that were working for their food and were thus required to minimize their energy expenditure (van der Vinne et al., 2014b). Combining these measurements, we showed that mice can indeed reduce their daily energy expenditure by adopting a diurnal phenotype. By incorporating a buffered nest temperature cycle and the thermal benefit of

nesting (huddling and insulation) into the rest phase Scholander curve, and considering the effect of wind chill on the active phase Scholander curve, we calculated that there is a steeper slope for the active phase than for the resting phase Scholander curve. This provides a situation where diurnality reduces energy expenditure (Fig. 2E, right plot). We estimate that diurnality in mice would reduce their total daily energy consumption by ~6–10%. This does not include the energy-saving effect of torpor at the end of the night (the coldest phase of the day), which would reduce their total daily energy consumption by an additional ~10%. Differences in the amplitude and shape of the $T_{surface}$, as well as differences in the duration of the active phase of the mice can reduce or increase this energetic benefit. Projecting our model data to wide a range of habitats – utilizing hourly temperature data from across Europe – the diurnal benefit holds for all geographical locations with daily $T_{surface}$ rhythms that are below the TNZ of the species (van der Vinne et al., 2015c). Furthermore, it is very consistent over days; a diurnal benefit would be present on 98.5% of all individual days within a single location (van der Vinne et al., 2015c). The fact that a diurnal phenotype reliably provides energetic savings relies on thermodynamics and would therefore extend to other mammals, but the precise magnitude of energetic savings would depend on factors like individual life style, specific habitat characteristics and body size.

Diurnality: cheap but risky?

In nature, animals face important trade-offs to ensure survival and successful reproduction. Obtaining sufficient resources whilst guarding against pathogens, injury and predators is often critical for increasing fitness. As small endothermic burrowing mammals require a lower energy intake when they exhibit a diurnal phenotype, the question arises why many small mammals are nocturnal. First, we would like to stress that, in their natural habitat, many small rodent species typically considered as nocturnal can indeed show extensive periods in which a substantial or even the dominant fraction of their daily activity occurs during the light phase. This includes rats, mice, hamsters and even several subterranean species (Daan et al., 2011; Gattermann et al., 2008; Harper and Bunbury, 2015; Levy et al., 2007; Tomotani et al., 2012; Urrejola et al., 2005; reviewed in Hut et al. 2012). We propose that the danger of predation might be an important factor in favouring nocturnality in rodents and other herbivorous prey species whenever they can afford it. Indeed, some studies report high amounts of diurnal activity in rats on islands devoid of natural predators (e.g. Harper & Bunbury, 2015). Diurnality in these rats seems especially high in years with high population densities when food becomes scarce, which would indeed increase the pressure to reduce energy expenditure. Additionally, some evidence suggests that prey species can actively alter their activity patterns in response to

changes in the perceived nocturnal or diurnal predation risk (Bakker et al., 2005; Fenn and Macdonald, 1995). Conversely, black bears, which lack natural (non-human) predators and are largely herbivorous (85% of their diet consists of plant material), are generally diurnal, but can alter the timing of their activity during the hunting season, when bears show more movement and road-crossing behaviour during the night when hunters are absent (Stillfried et al., 2015). It is hard to measure and compare the relative predation risks in a certain habitat for both day and night. Some insights can be acquired indirectly by observing vigilance levels (time and effort expended to monitor the environment for danger). Variation between species and habitats is large when it comes to diurnal and nocturnal predation risks, but in most species that forage both during the day and the night, vigilance levels appear higher during the daytime, suggesting that the night might be the safer daily temporal niche for foraging (Beauchamp, 2007). In addition to high diurnal predation risk, the risk of overheating when daytime temperatures approach or exceed T_{UC} (van der Vinne et al., 2014a) or the risk of dehydration (Levy et al., 2016) might facilitate nocturnality in some habitats (Fig. 3). For some species, the risk of UV damage to the eyes or skin might also prevent daytime activity (Hut et al., 2012). Ultimately, the best time of day to become active is the phase that optimizes the benefit-to-cost ratio of all fitness components.

Circadian flexibility is regulated by robust and flexible clocks

In mice, energetic challenges result in a phase advance of behaviour and most physiological rhythms (T_{body}, food intake, plasma corticosterone, peripheral clock gene expression), while the SCN phase remains unaltered (Hut et al., 2011; van der Vinne et al., 2014b). The experiments leading to this conclusion were performed under standard laboratory conditions (stable $T_{ambient}$, no burrowing, no predation) where light–dark cues were the only available Zeitgeber. Subjecting mice to phase shifts of the light–dark cycle revealed that the activity of mice remained entrained to this cycle (van der Vinne et al., 2014b). When exposed to competing Zeitgeber signals (a 10°C temperature cycle that was in anti-phase with light–dark cues), mice also remained entrained to the light–dark cycle (van der Vinne et al., 2014b). In the absence of all timing cues in constant darkness, a main bout of locomotor activity appeared to free-run with a constant period, while a shifting activity bout phase-advanced during the energetic challenge of the workload regime (Hut et al., 2011). Collectively, these observations show that 1) the SCN remains entrained to the external light–dark cycle under energetically challenging conditions and 2) the phase of activity and peripheral clocks shifts around the timing information provided by the SCN (and light–dark cycle), depending on an animal's energetic state.

Although the benefit of diurnal activity in small nocturnal mammals is to reduce thermogenesis costs, shifting physiology and behaviour to the light phase will also occur when energetic benefits are absent (e.g. when $T_{ambient}$ is constant or in anti-phase; van der Vinne et al., 2014b). Under natural conditions, weather changes that similarly result in reduced energetic benefits of diurnality rarely occur (van der Vinne et al., 2015b). Conversely, clocks facilitate the anticipation of frequent (and therefore predictable) conditions. The high probability that daytime temperatures will be higher than nighttime temperatures explains why the SCN, entrained by the light–dark cycle, is used as a stable and reliable proximate cue to predict the energetically optimal phase of activity. The flexible phase of output rhythms is thus always influenced by the stable (entrained) phase of the SCN, to ensure that the flexible circadian phenotype can be adjusted to different (optimal) phases of the external day. This novel view, that extra-SCN oscillators are flexibly coupled to the SCN phase, collectively determining output rhythms, is gaining more support (Pendergast and Yamazaki, 2014). Experiments aiming to isolate the role of the SCN in regulating this flexible circadian phenotype in mice are currently ongoing.

Homeostatic feedback on peripheral circadian phase

Alternative (extra-SCN) circadian pacemakers such as the FEO are often considered to rely on environmental timing cues to produce a certain (novel) phase. In our experiments, such timing cues are absent, revealing that the change to diurnality is endogenous. In free-living animals, food intake is often the result of foraging activity, and mechanisms underlying food entrainment might naturally emerge when foraging success is high at specific times of day. However, running leading to food reward alone is not sufficient to induce diurnality; mice that can earn palatable chocolate-flavoured pellets with running (whilst being fed with *ad libitum* chow) do not shift their circadian phase (van der Vinne et al., 2015a). Similarly, working for food at low workloads (small distance of running per pellet) does not induce significant changes to the circadian phase of the rest–activity rhythm (Hut et al., 2011). Weight loss over multiple days seems to be necessary before the diurnal phenotype emerges but, once induced, the diurnality can be maintained in mice with stable bodyweights (ranging from ~18 to >30 grams, suggesting that absolute bodyweight might be of lesser importance). Daily torpor does occur in some, but not all, shifted mice, and the advance of the circadian phenotype always precedes the occurrence of the first daily torpor, making it an unlikely component of the mechanism to induce a diurnal phenotype (Hut et al., 2011). Anatomically, the shifted mice show reduced deposits of white adipose tissue, both abdominally and subcutaneously. Male mice have reduced testes size and lower basal plasma glucose levels (unpublished data, Hut, Pilorz, Eijer and Riede), whereas their corticosterone

levels are higher than those of non-shifted males (van der Vinne et al., 2014b). These alterations to homeostatic parameters might provide a signal to change the phase alignment of extra-SCN clock gene cycles in relation to the SCN. Glucose sensing in particular might be an integral component of circadian phase responses to prolonged reduced food intake (Challet et al., 1998). Hypoglycemia induces the release of adrenalin and alters circulating insulin levels, both of which modulate the phase of peripheral clock gene rhythms (Terazono et al., 2003). Inducing hyperglycemia without increasing bodyweight – by feeding mice a diet with a very high fat content – was shown to delay the clock in peripheral tissues and to phase-delay rest–activity rhythms (Genzer et al., 2015; Honma et al., 2016). Furthermore, recently it was reported that liver-derived ketone bodies (resulting from converting endogenous fat reserves to supply energy) play an important role in the rhythmicity of food anticipation (see Glossary; Chavan et al., 2016). Besides these energy homeostasis-related variables, the responses to hypoxia, hyper- or hypothermia, inflammation and other cellular stressors also provide feedback to (or modulate) the phase of peripheral clock-gene expression rhythms (Cavadini et al., 2007; DeBruyne et al., 2015; Gerber et al., 2015; Tamaru and Ikeda, 2016). Collectively, these findings point towards a system in which failure to maintain physiological variables within their usual homeostatic range can alter the circadian timing of output rhythms.

To maximize fitness, animals must optimally interact with their environment in order to maintain homeostatic balance over a prolonged timespan. The temporal organization of physiology and behaviour are key to successfully maintaining this balance. We argue here that the circadian timing of output rhythms (peripheral clocks regulating cellular activity and behaviours) is reciprocally linked to homeostasis. If the phenotype is successful (i.e. 'in balance'), it strengthens the daily routines, leading to a robust phase. By contrast, prolonged failure to maintain homeostatic balance can alter the phase-angle between peripheral clocks and the SCN (Fig. 4). We think that flexibility in output rhythm timing is an essential feature of the circadian system, allowing it to cope with variations in the quality or temporal dynamics of the habitat.

Factors influencing circadian niche in the wild

Field data indicates circadian niche depends on context

Our circadian thermo-energetics hypothesis aims to explain the relevance of temporal niche switching; connecting energy balance, circadian timing and predation risks. Recently, Stawski et al. (2016) studied the effects of a forest fire on the behaviour and thermal rhythms of wild brown antechinus (*Antechinus stuartii*), a small insectivorous Australian marsupial mammal living in densely vegetated

forests. When fires destroy much of their habitat these animals survive by retreating into burrows or rock crevices, but are subsequently faced with a harsh and dangerous environment with little cover, increased predation pressure and low food abundance. In response, they reduce their activity levels post-fire and show reduced day-time foraging (proposed to alleviate their predation risk), which is energetically compensated for by spending more time in daily torpor. Similarly, nocturnal grey mouse lemurs (*Microcebus murinus*) spend a larger portion of the night in torpor when they are energetically challenged in a controlled laboratory experiment (Canale et al., 2011), which can thus advance the timing of foraging behaviour, in some cases forcing it into the day.

The opposite response, temporal niche switching of diurnal species towards nocturnality, is also not uncommon. Some rodent species that are diurnal in the field become nocturnal under certain laboratory conditions, including degus (Ebensperger et al., 2004; Kas and Edgar, 1999b), hamsters (Gattermann et al., 2008), tuco-tucos (Tomotani et al., 2012), cururos (Urrejola et al., 2005), nile grass rats (Blanchong et al., 1999) and golden spiny mice (Cohen et al., 2010a). These laboratory-to-field circadian discrepancies raise the question of whether a 'default' circadian phenotype actually exists. Does the animals' natural habitat present constant threats or challenges that drive them to become diurnal (e.g. low food abundance, lack of water) or are the unnatural laboratory conditions changing the phase of entrainment? Regardless, these examples point out that circadian phasing of daily rhythms can be flexibly aligned based on the context and quality of the habitat or state of the animal.

Food, water and shelter can modulate circadian phase

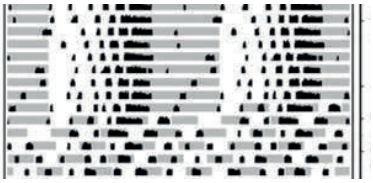
To further exemplify the complexity of circadian niche, fat-tailed sand rats (*Psammomys obesus*), which live in arid desert conditions, are diurnal in the field but show a nocturnal phase of general activity and peak body temperatures in the lab when housed with *ad libitum* food (Barak and Kronfeld-Schor, 2013). Unlike wild animals, captive animals are not exposed to UV radiation, lack a natural daily fluctuation in $T_{ambient}$ and humidity, and often have *ad libitum* access to food and water. In addition, laboratory housed animals generally lack the opportunity to burrow. Burrows provide the animal with a safe shelter, and lack thereof might increase anxiety, potentially contributing to the shift in activity to the night, which might be perceived as safer. In addition, burrows can have a strong effect on evaporative water loss, another abiotic factor that can have a strong daily rhythm, and which is especially relevant for survival in arid environments. Furthermore, $T_{ambient}$ values that overlap or exceed the TNZ can dramatically alter the relationship between activity timing and energy expenditure. Also, water loss was recently

proposed as a mechanism that can modulate the foraging patterns of spiny mice, a species that shows a high degree of circadian flexibility (Levy et al., 2016). Interestingly, Tachinardi et al., (2015) recently reported that tuco-tucos (*Ctenomys aff. Knighti*), which are diurnal in the field and nocturnal in the laboratory, spontaneously revert to a diurnal phenotype when placed in a respirometry set-up. The authors propose that tuco-tucos, which live in sealed underground tunnels, might be sensitive to small changes in air composition and humidity which can affect their circadian behaviour. Another interesting finding, also in captive tuco-tucos (*Ctenomys talarum*) is that diet quality can change the daily temporal organization of behaviour in this species. Comparing the distribution of *ad libitum* grass intake over the day, they found that animals maintained on a hard-to-digest grass of low energetic quality (*Cortaderia selloana*) predominantly fed during daytime, whereas the bulk of easy-to-digest grass (*Bromus unioloides*) intake occurred during the dark hours (Martino et al., 2007). Also of note – a recent study compared the daily distribution of activity in kaluta (*Dasykaluta rosamondae*), a small Australian marsupial, which is diurnal during the colder winter (proposed to save energy) and nocturnal during the warm summer (proposed to avoid predation and dehydration), indicating that factors influencing daily temporal niche selection can be subject to seasonal change (Pavey et al., 2016). Thus, food abundance, food quality, water balance, thermoregulation, energy homeostasis and predation might all be determinants of the circadian phenotype.

Conclusions

Here, we have reviewed the WFF-paradigm as a method to explore the flexibility of daily rhythms in mice. For small endothermic animals, such as many rodents, thermoregulatory costs can consume a significant portion of the daily energy budget. Our findings that negative energy balance (i.e. a change in the homeostatic balance) induces a diurnal phenotype illustrates that daily rhythms can be shaped and flexibly aligned to the light–dark environment in a way that would improve fitness in nature. Endothermic burrowing mammals can reduce their daily thermo-energetic costs significantly by becoming diurnal, as they avoid the coldest ambient temperatures at night, thus encountering higher average temperatures throughout their circadian cycle. Also, non-burrowing animals, when exposed to $T_{surface}$ rhythms that remain below thermo-neutrality, gain an energetic benefit from diurnality when they employ heat-conserving behavioural and physiological mechanisms during the cold night (Fig. 2). A shift towards a diurnal phase might come at the cost of increased predation risk, and the optimal phasing of circadian rest–activity cycles in a natural environment thus involves the integration of multiple trade-offs, rather than being set by single timing cues (like light) alone (Fig. 3). The undisturbed light entrainment

of the SCN will remain a reliable predictor of the thermal environment, which is especially important in burrowing animals that do not have continuous information on surface light and temperature. The SCN, as an internal representation of the external light–dark cycle, thus plays a critical role in optimizing activity patterns, such that predation risks are balanced against thermo-regulatory costs. For humans, understanding the flexibility in the timing of output rhythms might reduce the negative consequences of non-conventional life styles (like shiftwork) or help athletes to optimize their physical performance to the time of an event. Also, the intimate link between metabolic balance and circadian rhythms could suggest interesting new approaches to the prevention and treatment of obesity or metabolic problems. In general, we also hope that our data indicate that habitat and context (of humans and animals alike) actively regulate our functioning; a factor frequently underestimated or ignored. Understanding response diversity in complex natural situations can in some cases can provide novel insights that are easily missed in controlled experiments. Bringing the fields of ecology, physiology and neuroscience closer together would be of interest to all parties involved.



Chapter 4

Energy balance modulates the behavioural response to light

DATA CHAPTER

In this chapter we describe our experiments in which we determined light masking effects on behavioural activity levels of mice when they become diurnal in the work-for-food protocol. We hope it will be published after further editing in the near future.

Chapter 4

Energy balance modulates the behavioural response to light

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Chapter is being prepared and edited for future submission

Abstract

Light exposure modulates the behavioural activity patterns by two distinct routes; entraining the circadian clock that drives 24-hour cycles in rest and activity, as well as by exerting more direct behavioural effects termed light masking. In previous experiments we identified that the circadian rest-activity pattern of energetically challenged mice phase-advanced towards diurnality. Here we tested the question whether this shift to diurnal activity is facilitated by changes in the masking response to light. We therefore analysed direct masking effects of light under normal and negative energy balance conditions. In experiment 1, mice forced to work for their food by wheel running (diurnal high workload group) were compared with a group of *ad libitum*-fed controls in the behavioural changes during 1-hour light pulses applied at 4 circadian time points. In experiment 2, mice were exposed to a 7-day period in which they were subjected to a non-circadian LD cycle (LD_7; 3.5h light: 3.5h darkness), first under *ad lib.* (nocturnal) conditions and later when facing a high workload (diurnal). Masking effects in experiment 1 were not strong in either group and no significant changes were found. In experiment 2, however, *ad lib.*-fed mice were less active when lights were turned on for most of their activity phase (strongest effects at the early subjective night) whereas light failed to modulate wheel running intensity in mice during high workload conditions (diurnal, work for food). These results indicate that – like the adaptive plasticity in circadian patterns – behavioural modulation by light (light masking) can be reshaped depending on the energetic status of mice. Reducing the behavioural suppression by light might be a prerequisite for the diurnal behavioural phenotype during negative energy balance, thereby facilitating this previously shown energy saving diurnal activity pattern when food availability is low.

Introduction

The regulation of daily rhythms in mammals

Light and darkness influence mammalian activity patterns by entrainment of the circadian system, but also by a more direct modulation of behavioural activity called 'light masking' (Aschoff, 1960; Mrosovsky, 1999). Generally, the masking effect of light and the circadian drive complement each other: in nocturnal animals light generally suppresses activity (negative masking), whereas in diurnal animals it stimulates behavioural activity (positive masking) (Aschoff and von Goetz, 1988; Aschoff and von Goetz, 1989; Mrosovsky, 1994).

Adaptive plasticity in circadian activity timing

Classically, species are often attributed labels like diurnal (selectively or predominantly active during the day) and nocturnal (selectively or predominantly

active at night) by observing their behaviour, for instance by monitoring the intensity of wheel running behaviour under a light-dark cycle (LD). Mice, rats and hamsters are examples of nocturnal species defined in such a way. However, numerous studies report a change in behavioural activity patterns when species are studied in their natural habitat (for review see Hut et al., 2012). Laboratory defined nocturnal species may show two main bouts of activity during the twilights (crepuscular) or more dispersed modes of activity in their natural habitat (Daan et al., 2011; Gattermann et al., 2008). More importantly, the ratio between diurnal and nocturnal activity for most species seems far from constant, but tailored to food availability, temperature, energy balance or predation risk (Barak and Kronfeld-Schor, 2013; Cohen et al., 2010a; Hut et al., 2012; Katona and Smale, 1997; Levy et al., 2007; Refinetti, 2006; Riede et al., 2017; van der Veen et al., 2017). One major influence on the circadian phenotype seems to be the availability of nutrients. We previously found that mice respond to simulated food scarcity by becoming increasingly diurnal (Hut et al., 2011). In many habitats, nutrient availability might be sufficiently low to favor diurnality. In most natural habitats, temperature increases during the day and drops during the night. As mammals are endotherms, they have the capacity to raise and maintain their body temperature at levels above ambient temperature, showing a generally linear increase of thermogenic costs with decreasing ambient temperature provided the encountered temperatures are below thermoneutrality (Riede et al., 2017; Scholander et al., 1950; Speakman, 1997). Because usually nights are colder than the days, this means that in general nocturnally is energetically costly, especially in burrowing animals that can profit from nest insulation during the rest phase (Riede et al., 2017; van der Vinne et al., 2015c). This rationale explains why diurnality might be advantageous under energetically challenging conditions.

Circadian plasticity and masking

Adaptive changes in the timing of activity rhythms, like nocturnal to diurnal temporal niche switches, seem to be linked to the circadian system and regulated downstream from the main pacemaker in the suprachiasmatic nucleus (SCN; Van der Vinne et al 2014). However, the precise mechanisms responsible for altering circadian activity patterns in response to changes in habitat quality remain largely elusive. Similarly, the consequences of changing the circadian niche imply that animals perceive environmental stimuli at opposite behavioural states, like their light-masking responses. However, in order to be effective, such circadian plasticity needs to be accompanied by changes in the masking effect of light. For example, light has been shown to induce sleep in mice (Lupi et al., 2008; Pilorz et al., 2016; Rupp et al., 2019) which can be seen as an extreme form of negative masking on activity levels. When adaptive circadian flexibility causes mice to adopt a diurnal chronotype, one would expect the masking effect of light to become blocked or even inversed. Circadian flexibility can enable adaptive optimization of activity timing, but only when it is accompanied by adaptive flexibility in the masking response to light.

We therefore aimed to study the adaptive capacity of the light masking response by comparing the light masking responses in mice with a predominantly nocturnal phenotype (fed *ad lib.*) with those of mice that adopted a diurnal phenotype under the work for food paradigm. To do so, we quantified behavioural activity during light pulses at four different times of day (experiment 1) and quantified activity levels during non-circadian ultra-short LD cycles of 3.5 h light and 3.5 h darkness ($LD_{3.5:3.5}$, experiment 2).

Materials and Methods

Animals and housing conditions

Male mice (CBA/CaJ, ~4 months old), bred in our animal facility in Groningen, were housed individually in standard running-wheel cages (w*l*h = 13.5*33*16 cm). In both the experimental work-for-food (WFF), and the control *ad libitum*-fed group, wheel rotations (14cm diameter) were registered using a magnetic switch connected to a computer system that recorded actigraphy data (CAMS, developed by H. Cooper and INSERM associates, Lyon, France; 1-min resolution). For the working-for-food group a second switch controlled an automated food dispenser (Med associates Inc, Fairfax, VT, #ENV-203-45) delivering precision food pellets (Bio Serve, Flemington NJ USA, type #F0165, 45 mg) upon completion of a set number of revolutions. WFF and *ad lib.*-fed controls were housed together in a climate-controlled room (20-21 °C, relative humidity ~55 %) to expose them simultaneously to any (disturbing) stimuli such as daily welfare checks, cage cleaning or body weight measurements. Two pairs of ceiling-mounted double fluorescent polychromatic standard white tube lights provided around 300 lux at cage level. Light, temperature and relative humidity were recorded. Continuous dim red light (< 2 lux at cage level) was provided throughout the studies to enable daily checks of equipment and animal welfare at any time of day.

Working for food paradigm

A work for food (WFF) protocol to make mice adopt a diurnal phenotype was utilized as described before (Hut et al., 2011; Schubert et al., 2008). The WFF paradigm starts with mice receiving a 45mg food pellet every 75 rotations (~33m/150cal), which surmounts in obtaining about 120 pellets per day for spontaneous wheel running activity (18.1 kCal or 75KJ day^{-1}), which exceeds food intake of *ad lib.* fed mice of the same strain and age (van der Vinne et al., 2015c). Next, the number of wheel revolutions needed per pellet (workload in revs/pellet) was increased with daily increments (10-20 rev/pellet), whilst weight-loss was carefully monitored. Ultimately, animals in the WFF treatment increase their total wheel running activity during these experiments in order to maintain a daily energy intake of around 80

pellets (12 kCal or 50KJ day^{-1}). Typically, mice plateau at high workloads (HWL) of 260-300 revs/pellet (~125m/150cal). Bodyweights decrease in the initial stages of these experiments compared to *ad lib.*-fed mice, but generally stabilize around 25-27 grams which they can maintain without obvious signs of stress, discomfort or strain for extended periods (weeks to months). Over the duration of this WFF-protocol mice phase-advance their daily rhythms of wheel running activity into the light phase, allowing us to compare diurnal and nocturnal mice across various conditions such as, in the current study, their behavioural responses to light and darkness. *Ad lib.* control groups were provided the same food pellets in excess of their daily intake (AL pellets) or supplied with regular chow feed (AL chow controls).

Experiment 1: Pulses

In the first experiment we measured the behavioural responses to 1-h light pulses provided at four circadian phases (ZT2, 9, 14 and 21). Mice received repeating blocks of 2 days with a standard 12h light - 12h darkness LD-cycles (LD_{24}) to maintain entrainment, followed by two days of continues dim red light with a 1-h ~300 lux white light pulse provided on this last day. Twenty mice were randomly divided into two groups, 10 mice entering a WFF paradigm and 10 mice being fed the same dispenser pellets *ad lib.*, serving as nocturnal controls. After collecting the data for each of the four pulse times in duplicate, we changed the diet of all mice to standard chow, provided *ad lib*. After a two week refeeding period behavioural responses for two of the pulses (ZT9 and 14) were repeated, allowing us to establish intra-individual differences between HWL_{chow} and AL_{chow}, and to evaluate if the behavioural responses of the *ad lib.*-fed controls obtained during pulse period 1 were influenced by being housed with diurnal mice within the same experimental room (see timeline Fig. 1).

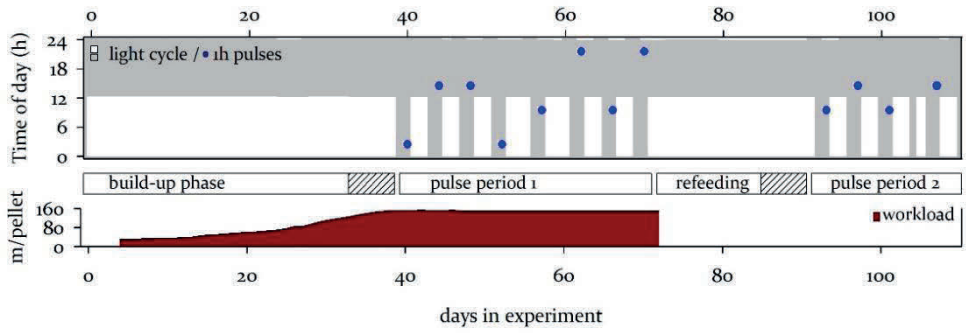


Figure 1. Timeline of experiment 1 in which 1h light pulses were applied at four circadian time points. *One hour light pulses (300-400 lux) were given on the second day of two consecutive days in continuous dim red light as indicated by the blue circles, starting at ZT2, 14, 9 or 21 respectively. Between pulses, two to three days of the 12:12 LD cycle were provided to*

maintain light-entrainment. Half of the twenty mice were in a WFF paradigm, having to obtain their food by wheel running (workload in m/pellet) as shown in lower panel. The other ten mice served as controls and had ad lib. access to the same food dispenser pellets during the build-up and pulse period 1. During refeeding and pulse period 2, all twenty mice were fed ad lib. with standard chow. The last days in $LD_{12:12}$ prior to starting the pulses were used to calculate circadian characteristics of the groups as indicated by the hatched bars.

Experiment 2: Ultrashort LD-cycles

To further characterize masking responses by light, we exposed ten naive mice to an ultra-short LD cycle of 3.5h of light and 3.5h darkness (LD_7) in a second experiment. This approach can provide information on behavioural light responses across the full circadian cycle. From every individual we compared one week of $LD_{12:12}$ running wheel data with a week of LD_7 cycles, both under *ad lib.*-feeding and under a high workload. Ten mice were habituated for two weeks to consume food dispenser pellets (provided *ad lib.*) and use their running wheels. After the daily spontaneous wheel running counts stabilized, 7 consecutive days of LD_{24} activity data were used to calculate the circadian characteristics (onset, COG and offset) of the wheel running behaviour (LD-AL). Subsequently, we changed to an ultra–short LD_7-cycle (US-AL), which was repeated 24 times (7 days). As this 7h light-dark cycle and its multiples fall outside the range of entrainment, the mice exhibit circadian free-running behaviour. We calculated the endogenous period (tau) over these 7 days of wheel running data for each individual. The free-running periods were than used to express the wheel-running data on a circadian time scale, extrapolating from the preceding 12h: 12h LD cycle with the last lights on (ZT0) used to define CT0.

Light masking was quantified by converting the time of every activity bin to a circadian time using each individual's free running period, and subsequently segregated the dataset for lights on or off. Next, data were binned to 1 circadian hour. Across the 7 days, each circadian bin was encountered about 7 times, so generally 3 or 4 times in light and darkness respectively. Activity was averaged ~10 minute bins (tau in h/144) bins for light and darkness separately, and the difference used to establish the effect of light exposure (paired t-test). As a baseline measurement, the behaviour during the US-cycles was first quantified under *ad lib.*-fed conditions. After this, the LD_{24} cycle was re-instated and mice were exposed to WFF paradigm. After 4 days, all but ~20 dispenser pellets were removed, making mice fully reliant on their daily wheel running activity to obtain their food. Workloads were increased gradually across a 5 week period. At stable high workload, 7 days of actigraphy were used to calculate circadian characteristics in LD_{24} (LD-HWL). Finally, the high workload was extended for one more week, but now combined with the LD_7 to assess light modulation across circadian phases for HWL (predominantly diurnal) mice.

Statistics and software used

Running wheel data was stored in 2 minute bins by the CAMS system and used to plot actigraphy records using a custom Excel-based software plug-in (called ACTOVIEW, developed by C.K. Mulder, freely available on request) and Sigmaplot

10.0. Behavioural onsets and offsets were defined by the intersections of the smoothed data (1h running average) with a running 24h activity mean, followed by visual validation based on the actograms. Center of gravity (COG) of wheel running behaviour is calculated by circular statistics approach, where. In this method, all data of a circadian or 24h cycle are plotted on a circle to determine the average vector direction. The level of activity of each bin is than converted into a horizontal and vertical component using the cosine and sin functions. Next, we used the arctangent of those x and y coordinates to find the time of the center of gravity in radians. In addition, the length of the resulting vector divided by the summed daily activity $\frac{\sqrt{(\sum \cos^2 + \sum \sin^2}}{\sum daily\ activity}$ was used as a measure of clustering of daily activity, whereas day-to-day-variability (SD) in the time of the COG was used as an indicator for rhythm stability. Individual circadian free-running periods (tau) were established by Lomb-Scargle periodogram analysis (Ruf, 1999; Chronoshop 1.04 Spoelstra, 2015). Paired and unpaired T-tests were used for statistical evaluation of the data obtained.

Results

Inducing diurnality with a work-for-food paradigm

In experiment 1, HWL successfully advanced wheel running patterns into the light phase (Fig. 2), WFF-mice displaying 69.9 (±9.1) percent of their daily wheel running activity during the day, significantly more than the *ad lib.* pellet fed control mice ($AL_{pellets}$) at 45.3±10.5% ($p<0.001$, t-test with Welch's-correction). Three weeks refeeding with *ad lib.* standard chow returned WFF individuals to a predominantly nocturnal phenotype (41.5 ±8.5% of activity during the light phase, $p<0.001$, paired t-test). Phase of entrainment of mice fed *ad lib.* did not differ depending on the food provided ($AL_{pellets}$ vs AL_{chow}, Fig. 2). In addition, the mice that had experienced HWL during the first stage resumed their nocturnal phenotype once refed *ad lib.* (see Fig. 2). Similar shifts to diurnality were found for experiment 2. COG-timing was advanced during HWL by around 3.4h ($p<0.0001$, paired t-test) in experiment 1 and by 4.7h for experiment 2. In addition to the phase advance of daily wheel running rhythms, the WFF protocol increased the daily sum of wheel-rotations (distance/day) and causes wheel running patterns to become more fragmented (example actogram see Fig. S1). This fragmentation resulted in an increase in both the within- and between-individual variation. The stability of COG (intra-individual SD) decreased in mice during high workload in experiment 1; from an average of 26.7 minutes under AL_{chow} conditions to 46.1 minutes under HWL ($p=0.03$, paired t-test). In experiment 2, COG stability did not decrease by the WFF condition (25 versus 31 min, $p=0.44$). During HWL, the length of the active-phase increased (longer interval between onset and offset, Fig. 2). The increased fragmentation and expended active

phase length contributed to a decrease in clustering during HWL; from 76 to 56 ($AL_{pellets}$ vs $WFF_{pellets}$, experiment 1) and from 62 to 43 (AL vs WFF, experiment 2) respectively (see Fig. S3-S6 for behavioural activity profiles of the individual mice in all groups).

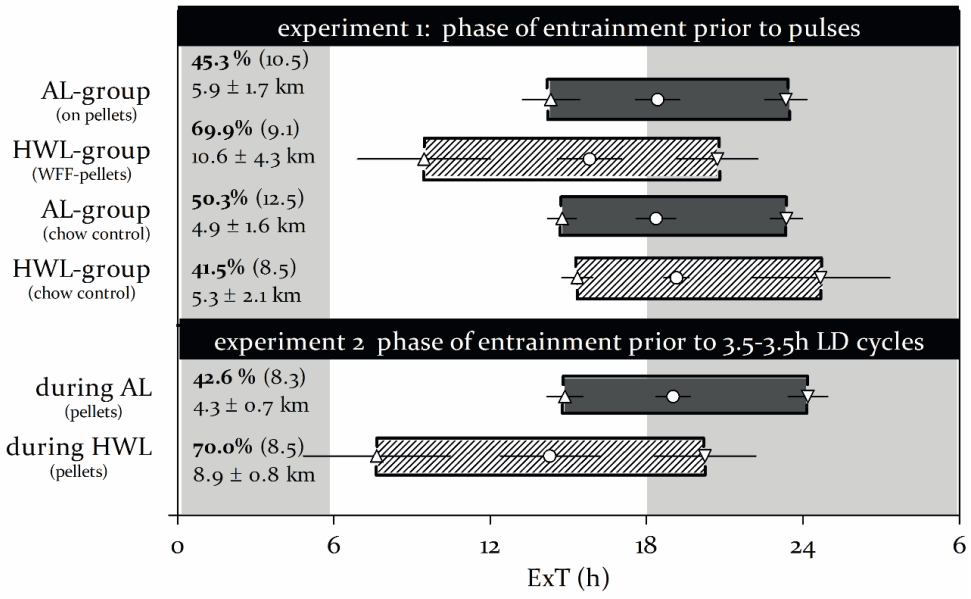


Figure 2. High workload induces a diurnal phenotype in mice. *Phase of wheel running activity in 12:12 LD prior to applying light pulses in experiment 1 (upper part) and preceding the 3.5:3.5h LD-cycles in experiment 2 (lower part). Indicated are the group means (with SD) for the onset (Δ), center of gravity (○) and offsets (▽) of running wheel activity, derived from 7 days of actigraphy data, n=10 for all groups. Percentages on left side of the graph indicate the mean (± SD) percentage of daily activity displayed during the light phase and subsequently show the average distance run (in km) per day (±SD). In the first experiment, two groups of 10 mice were measured during both feeding with pellets (WFF and AL) and when fed chow ad lib. (chow control). In experiment 2, a third group of 10 mice was measured under both an AL-feeding and during HWL. For all groups; n=10.*

Experiment 1: Masking responses to short light pulses

Comparing activity levels during a 1-h light pulse interval with the activity in the same hour on the preceding day (dark control) revealed no consistent effect of light masking for each of the four time-points tested. For the *ad lib.* control group, activity during ZT2-3 and ZT21-22 was almost absent - regardless of these hours being dark or light exposed (Fig. 3 A1, A4). Mean levels of activity were not significantly reduced during the light pulse versus the dark control for the remaining time points ZT9-10 (t=-1.74, p=0.44, Fig. 3-A2) and ZT14-15 (t=-0.24, p=0.82, Fig. 3-A3), with mixed responses on the individual level (Fig. 3, C2-C3). For the mice on HWL, there were

no significant overall effects (Fig 3, B1-B4), but slightly lower activity levels for the pulses during subjective daytime at ZT2-3 (t=-1.08, p=0.31) and ZT9-10 (t=-0.43, p=0.67) and slightly higher activity levels during light pulses applied during the subjective night at ZT14-15 (t=1.4, p=0.19) and ZT21-22 (t=1.79, p=0.11). Individual responses of WFF mice again showed considerable variation in both the magnitude and the direction of these responses (Fig. 3, C1-C4), explaining the lack of significant light effects with this sample size (see Fig. S2 for detailed actigraphy). The response to light pulses at CT9 and CT14 after switching all mice back to *ad lib.* chow did not show consistent significant light masking effects (Fig. 3; D-F, see Fig. S2 for actigraphy).

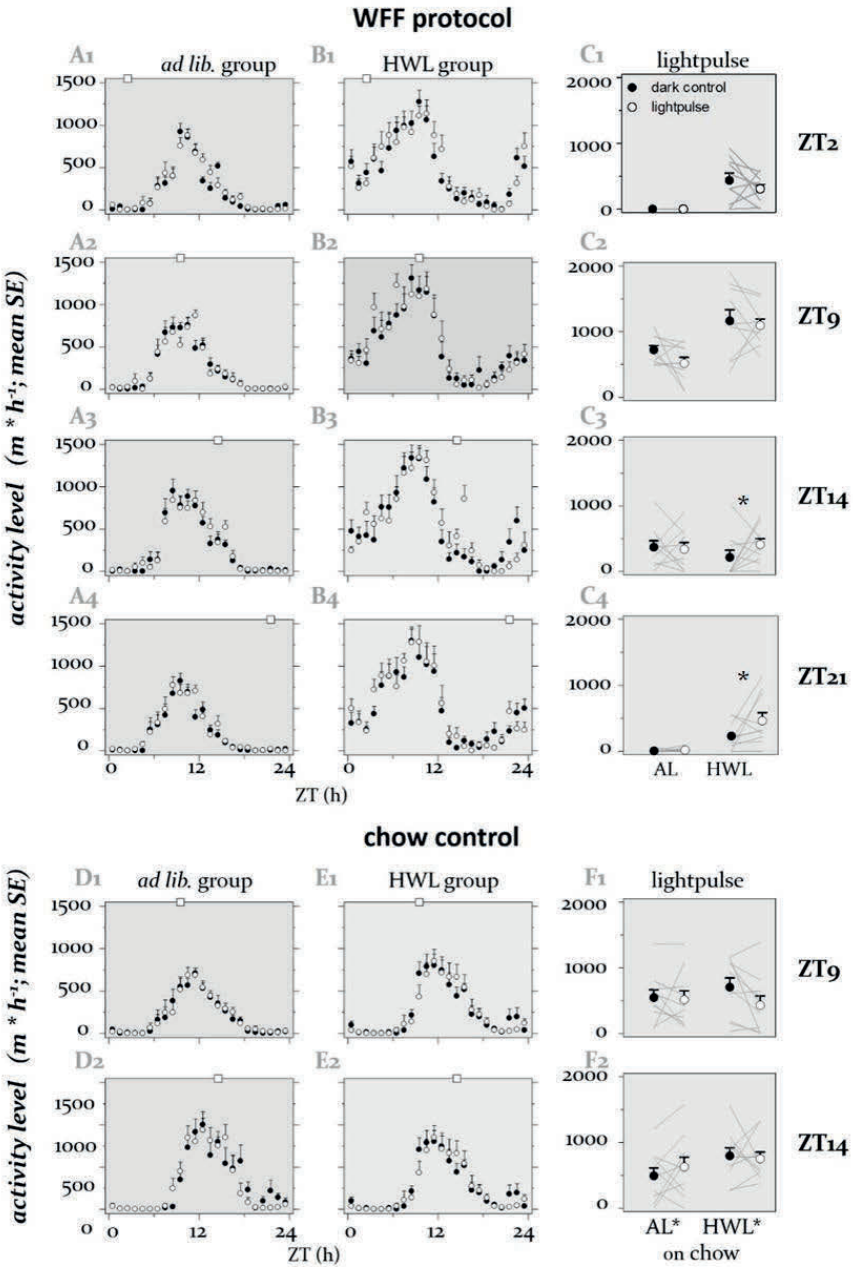


Figure 3. Effect of 1-h light pulses on wheel running activity of diurnal and nocturnal mice. *Activity profiles of mice during the pre-pulse day in constant dim (●, dark-control) and the subsequent day (○) during which a 1-h light pulse (□, indicated on top X-axis) was provided at one of 4 circadian phases (Pulse start time in ZT shown on right margin). Right panels (C,F) show average activity levels during the two pulse-days (○) and the their dark control days (●) as lines, with group mean and SD indicated by the symbols. Lower plots show the same responses once all mice in both groups had been refed with ad lib. chow, serving as internal controls. * indicates significantly higher levels of activity during light pulse (paired t-test).*

Experiment 2: Masking Quantification by ultrashort light-dark cycles

LD_7-cyles were used to further test for changes in light responses. One week of LD_{24} running wheel data was compared with that from a week of LD_7-cycles, both under *ad lib.* and under HWL (Fig. 4). In order to analyse the mean effects of light on activity levels across the circadian cycle we first determined the dominant circadian period (*Tau*) across the 7d US-AL and US-HWL periods for each individual (Tau_{AL}= 23.66±0.39 / Tau_{HWL} = 23.78±0.70; mean±SD, p=0.6 by paired t-test). Mean activity level for all circadian phases across these 7 days was than calculated and plotted as circadian activity profiles for the light and dark conditions separately (Fig. 5).

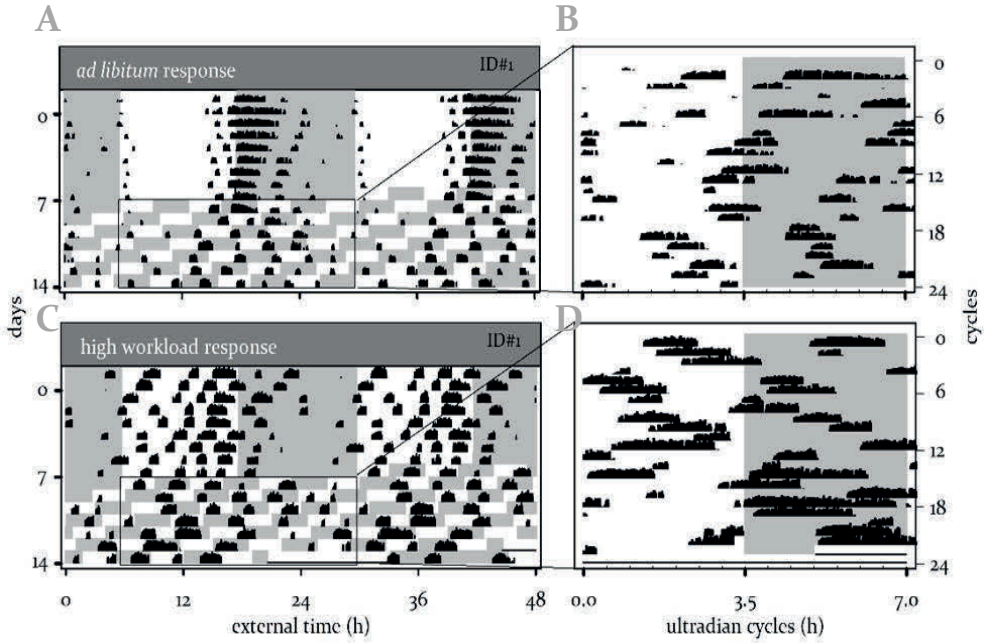


Figure 4. ***Example of wheel running recordings obtained in experiment 2.*** *Left panels (A,C) show a double-plot of the activity. In the first week, animals were exposed to a 12:12 LD cycle. In the second week, mice were exposed to 24 repeats of an ultra-short of 3.5:3.5h LD cycle (marked with box outline). This second week is replotted in the panels on the right (B,D) on a 7h time axis (24 cycles of 7h). The bottom panels show the same individual when it was exposed to a high workload (having a diurnal phenotype). Dark horizontal line at end of ultradian LD-period 2 denotes missing data.*

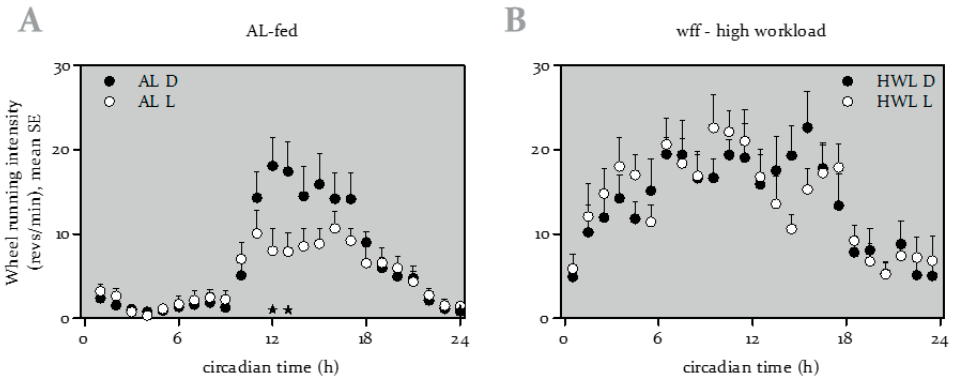


Figure 5. ***Mean activity profiles (n=10) constructed from the 7 days of 3.5h L - 3.5h D periods.*** *Timestamps of actigraphy were converted to CT using the individual's tau, extrapolated from the last LD cycle using lights on as CT 0, and subsequently classified for occurring in light or darkness respectively. In ad lib.-fed mice most activity occurs between CT 9 and 19 (subjectively in the late light and early dark phase, i.e. predominantly nocturnal). Less activity was noted when the light is turned on; indicating that light in general has an inhibitory effect on their wheel running intensity; or negative masking. During HWL, mice are generally more active throughout the circadian day, with notably more active during subjective daytime hours (CT0-12). The presence or absence of light seems to make little to no difference for the expression of wheel running activity in WFF mice; suggesting an absence of light masking under high workload/negative energy balance conditions. Stars denote significant differences for the indicated time-point as tested by paired t-test (two-tailed): * p<0.05 / ** p<0.01).*

Overall, AL mice ran more in darkness during the 7 days of the LD_7-cycles, averaging 56% of their activity displayed during darkness, with one outlier showing a clear diurnal preference (Fig. 6a). For WFF mice, the overall distribution was not significantly different from 50%, with one animal showing a strong diurnal preference (Fig. 6b). Within animal comparisons revealed that challenging mice with WFF resulted in a significant reduction of dark preference, making their distribution of activity over the light and dark pulses closer to 50% (Fig. 6c) and suggesting that the negative masking effect of light is reduced when energetically challenged. The outlier with light preference under AL retained a similar light preference under HWL.

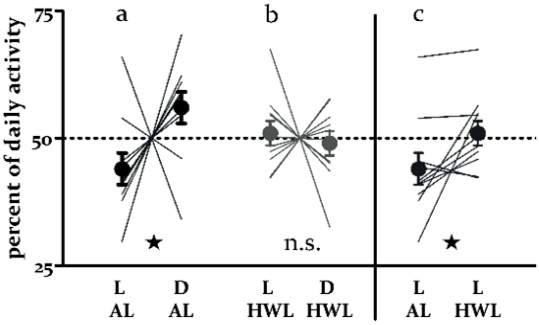


Figure 6. Activity distribution across the light (L) and dark-phase (D) in the 3.5-3.5h LD cycles. *(a) In ad lib. fed mice a higher proportion of activity was observed during darkness (56.01%, SD=9.83; p=0.04 by one-sided paired t-test). (b) Under high workload (HWL) no preference for light or darkness was observed. (c) Comparing individuals across the two conditions revealed a significant reduction in dark preference by the high workload. Lines represent individuals, symbols reflect mean ±SE.*

Discussion

Answer to main hypothesis

In the two experiments described here, our main aim was to establish if the light-masking responses of mice change when they switch from a nocturnal to a more diurnal circadian activity pattern, as triggered by the WFF-paradigm. In experiment 1, where we applied 1h pulses of light at four circadian phases, we observed no consistent changes in behavioural activity levels, neither the (nocturnal) *ad lib.* fed control group nor the (diurnal) mice facing high-workload. In experiment 2, using LD_7–cycles, we quantified activity-modulating effects of light throughout the circadian cycle and found nocturnal controls to suppress activity when experiencing light during their subjective night, whereas such a decrease was absent in the same individuals when facing negative energy balance. Instead, diurnal HWL-mice displayed a tendency to have more intense wheel running behaviour when lights were on, especially during their subjective day (Fig. 6). Together, the data are a first hint that the light-masking responses of mice, like their circadian phenotype, might be subject to adaptive plasticity when they face negative energy balance, allowing a more diurnal behavioural pattern.

Yet, compared with previous findings by others (Hattar et al., 2003; Mrosovsky, 1994; Mrosovsky and Thompson, 2008; Pendergast and Yamazaki, 2011; Shuboni et al., 2012), we were surprised that mice fed *ad lib.* showed as little evidence for negative light-masking as they did in experiment 1. Even with the limited sample size (n=10), we anticipated that performing each pulse in duplicate would yield ample data to see a clear decrease in wheel running behaviour during light exposure. A possible explanation of reduced negative masking was the result of wheel-running activity in neighbouring cages (by mice of the opposite treatment) provided auditory or other stimuli that enhanced activity seems unlikely, as repeating pulses when all animals where fed *ad lib.* (Chow-control) yielded the same lack of negative light-masking on

wheel running activity (Fig. 3, D-F). Second, as the sensitivity to light, and the magnitude of masking response in wild-type mice is known to depend on the time of day (Pendergast and Yamazaki, 2011), an alternative explanation might be that we (involuntarily) had selected times for our pulses at which the CBA/CaJ system is relatively unresponsive. Though we did not of light-masking in CBA/CaJ mice was found Even though we targeted the ZT14 pulse so it would coincide with the strongest suppression of activity in other mice strains (Pendergast and Yamazaki, 2011; Shuboni et al., 2012).

We addressed the question whether light masking responses of mice is changing when their circadian phenotype shifts from nocturnal towards predominantly diurnal due to an energetic challenge. For mice from the high workload group, which had a diurnal phenotype in LD_{24} cycles, negative light-masking was absent, with pulses provided during subjective night (ZT14-15 and ZT21-22) suggesting a slight but significant increase in locomotor activity during light stimulation (Fig $3_{C3,C4}$). This suggests that diurnal mice indeed show alterations in their masking response to light, which would allow or facilitate diurnal behaviour.

The behavioural patterns of free-running mice exposed to an LD_7 cycle in experiment 2 showed suppression of activity by light when they were *ad lib.*-fed, especially during their subjective night (significant for CT12 and 13, Fig 5a). This activity suppressing effect of light was clearly absent in the same mice when they were diurnal under HWL (Fig. 5b). Combined, these two experiments suggest that negative energy balance decreases the magnitude of activity suppression by light exposure.

These findings are similar to some previous studies by others. Comparing the masking responses of nocturnal common spiny mice (*Acomys cahirinus*) and temporal niche switching golden spiny mice (*Acomys russatus*) to 3h long 100 lux light pulses at ZT14 indicated clear negative masking in the former, but no consistent effects in the latter (despite their nocturnal phenotype), due to high intra- and inter-individual variation (Cohen et al., 2010a).

Limited negative masking in AL fed mice

In this study, the negative masking effect of light in the *ad lib.* fed control groups was low or absent, in contrast to previous publications in which *ad lib.* fed mice show robust negative masking (Mrosovsky and Hattar, 2003; Shuboni et al., 2012). This may be due to potential limitations. First, the maximum intensity of the ceiling mounted light armatures, which applied 280 to 400lux at cage level, was not bright enough to elicit strong behavioural masking effects in the *ad lib.* fed control groups. In terms of the biological relevance, light levels in open space on a clear summer's day might be as high as 100.000 lux. Cloud cover can reduce this with about 1 log unit (Woelders et al., 2017). Natural shading like buildings or sparse trees tend to reduce another log unit, making the natural range of daylight intensities typically encountered around 1.000-100.000 lux. As the habitat of *Mus musculus* is associated with human habitation, their natural foraging grounds can include even more light

sheltered areas, including indoor areas like attics, basements, floor-spaces, in houses, barns and food-storage facilitates. Furthermore mice tend to utilize routes with provide cover from (aerial) predators when available (Vinne et al., 2019). Therefore, we consider that illumination levels of 300-400 lux do reflect a relevant range that might be encountered by mice when they would display a diurnal phenotype in their natural habitat. Their capability to become active during these levels can therefore be considered as biologically relevant.

Second, we tested the masking responses in this study by the quantification of wheel running behaviour. In order to prevent wheel blockades and ensure light exposure, mice in the WFF paradigm in general lack nesting material or other abilities to avoid the light. In comparison, classical methods to quantify masking in mice can use general cage motility and in some cases do have sheltered nesting locations available to their animals (in which they might withdraw during light stimulation). Furthermore, the running wheel behaviour within our experiments, specifically in HWL-groups, might no longer spontaneous voluntary exercise but rather the expression of goal oriented (foraging) behaviour. Motivational systems are likely to modulate the expression of wheel running behaviour in the context of HWL treatments, potentially influencing risk-reward trade-off decisions. It could be that prioritized or strongly motivated behavioural outputs are less sensitive to masking than spontaneous but non-functional behaviours, such as wheel running without food reward.

Third, during experiment 1, the animals from the WFF protocol and the ad-lib fed controls were housed in the same experimental room in alternating cage order. This was done in order to ensure that we exposed both groups to identical light conditions and other stimuli. As a consequence, every WFF-mouse and AL-fed mouse had at least one direct neighbour with a different circadian phenotype. As mice are social animals, it could have been that *ad lib.*-fed mice be more inclined to run if their noticed their WFF-neighbour becoming active in its wheel, and *vice-versa;* hence reducing the differences between the two treatment groups. To tackle this issue, we decided to extend experiment 1 to include an internal control, (re)feeding the mice of both groups standard chow *ad lib.* for four weeks with a repeat of the pulses for ZT9-10 and ZT14-15) in the last week. The responses from AL-pellet fed mice during pulse period 1 was not significantly different than the response of AL-chow fed mice of either group during pulse period 2. This at least suggest that the measured light responses for the AL group were representative of this strain's responsiveness to these light stimulations.

For the pulses applied during the resting period of AL fed mice (ZT2-3 and ZT 21-22) there was a lack of activity during the darkness control day and during the pulse. Therefore, only the data for the late light phase (ZT9-10) and early night (ZT14-15) were considered relevant for AL fed mice. Mice on the WFF paradigm, tended to show substantially more activity during daytime hours (ZT0-12) than AL counterparts, but in addition retain a portion of activity during the early night.

Masking response mechanisms

Behavioural responses to light stimulation may depend on the wavelength used, with monochromatic light sources capable of generating opposing immediate effects depending on the wavelength used (Pilorz et al., 2016). In our experiments we used the same polychromatic light source, thus potentially eliciting both stimulating and inhibiting effects. It could be theorized that HWL causes physiological changes that effect the downstream sensitivity to these contrasting signals, thereby altering the dominant result of light stimulation on the level of behavioural activity. One such change might be glucocorticoid signalling. In WFF mice, the circadian pattern and peak expression of plasma corticosterone are both phase advanced and drastically elevated (van der Vinne et al., 2014b). Blocking glucocorticoid signalling can prevent positive light masking effects by preventing sympathic system activation (Pilorz et al., 2016), suggesting a heightened sympathetic tone might increase positive (activity stimulating) masking effects by light. In addition, metabolic markers such as decreases in circulating insulin or glucose might similarly affect how light modulates behavioural activity. In the mammalian retina, rods and cones are primarily involved in transferring visual information whereas specialized intrinsically photosensitive retinal ganglion cells (ipRGC's) are required for non-image forming (NIF) effects of light, including circadian light entrainment, pineal melatonin synthesis, pupillary and masking responses (Peirson et al., 2017). Genetic ablations have indicated that omitting the photopigment melanopsin from these ipRCG's greatly dampens these NIF-responses, whereas deleting the ganglion cells entirely completely abolishes them (Hattar et al., 2003; Panda, 2003). This indicates that the ipRGC's rely both on their own light-detection and receive additional signals from the rods and cones that can potentially modulate, augment or stabilize their outputs. In turn, ipRGCs project light information to many different brain regions (Hattar et al., 2006; Martersteck et al., 2017). It is generally thought that ipRGCs mediate downstream NIF-responses mainly by the release of (excitatory) glutamate, but recently studies highlight that a substantial proportion of their cellular targets receive inhibitory or both GABAergic and Glutamatergic inputs – at least on the level of the SCN (Sonoda et al., 2020). Interestingly, the same authors found that by blocking the actions of glucocorticoids by a systemic injection of glucocorticoid-receptor antagonist mifepristone (RU-486). sleep induction was enhanced following blue light exposure (Pilorz et al., 2016). The suggestion that systemic levels of corticosterone might modulate how light can regulate sleep and wakefulness is interesting, especially since we have previously established that corticosterone levels of mice during the WFF-paradigm become both elevated and phase advanced (van der Vinne et al., 2014b).Therefore we conclude that the HWL induced increase in systemic glucocorticoids may attenuate the ipRGC's signalling and its associated negative behavioural masking response to light.

Masking responses in crepuscular and temporal niche switching species

Access to running wheels can induce enhanced nocturnal activity in some 'diurnal' species, including Nile grass rats and the south American Degu (Kas and Edgar, 1999b; Redlin and Mrosovsky, 2004). With enhanced nocturnal running induced by wheel access, these species displayed more activity during dark or dimmed light pulses during the day (Redlin and Mrosovsky, 2004) or reduced activity during nocturnal illumination (Kas and Edgar, 1999a) respectively, indicating negative masking in diurnal species as they change their circadian niche.

Quantification of masking responses to light in diurnal species indicates that the presence of light is capable of modulating activity levels, but does have many nuances, partly due to a lack of diurnal rodent models. Mongolian gerbils display a diurnal bimodal activity rhythm (with a first peak in the early light phase and a second peak around the light to dark transition, with the majority of all activity in the light phase) but become nocturnal when they are housed with access to running wheels. Similar effects with increases in nocturnal activity are seen for other diurnal species, including degu and Nile Grass rats when provided access to a running wheel. In (diurnal) Mongolian Gerbils light pulses during the night almost completely abolished wheel running behaviour but light and dark pulses had little effect on general cage activity (Weinert et al., 2007). A comparison of masking responses for the (strictly) nocturnal common spiny mice and the more diurnal golden spiny mouse revealed that dark pulses during the daytime increased (general) activity in both (Cohen et al., 2010a). In contrast, light pulses during the night consistently suppressed activity in the common spiny mouse and had non-significant overall effects on the golden variant, with some decreasing, some increasing and some displaying a lack in activity level changes which were non-consistent within individuals (Cohen et al., 2010a), resembling the HWL-mice masking results we obtained in experiment 1. Furthermore, most light masking responses are performed on backgrounds of continues dark or dim light. In natural situations, diurnal species are more likely to perceive light with variable intensity levels depending on their location, cover, activity or weather conditions. It is therefore perhaps unsurprising that diurnal animals tend to be less sensitive to changes in illumination during the day.

Non-circadian light-dark cycles show lack of light masking in diurnal HWL mice

In experiment 2, we evaluated how light modulated the expression of behavioural activity across the circadian day by applying an ultrashort (non-circadian) $LD_{3.5:3.5}$ cycle over a series a 7 days. For each individual we expressed a mean daily profile for both the presence and the absence of light which were subsequently averaged for the treatment group, as shown in Fig. 5. For *ad lib.* fed control mice, the expression of behavioural wheel running activity was notably reduced during the early subjective night which corresponds with the interval in which they display the bulk of their daily activity. In comparison, we did not observe a reduction of activity by light in

mice that were subject to high workloads. In general, HWL mice display more of their daily activity during the subjective day and the presence or absence of light in this interval did not modulate the overall level of activity. These data suggest that light exposure during the (subjective) day raise activity levels in HWL individuals. This shows that in diurnal mice under HWL, the magnitude of negative light masking is either greatly reduced or even opposite in direction from that of nocturnal *ad lib.* fed animals.

Modes of activity measurement

Using the intensity of behavioural activity patterns as the primary output parameter in explaining the effects of masking stimuli is a widely accepted method of quantification. However, behavioural activity in itself is a complex behaviour that can reflect a wide array of underlying motivations. To illustrate potential problems; physical activity can be either motivated by foraging urges, by general excitation, but can also reflect that the subject wants to actively avoid the situation it is in. It is therefore important to be aware of the full set of criteria in any masking study. In our experiments, the mice did not have a nesting box or other means to actively avoid the light which they experienced. In studies that include nest boxes and assess general cage movements the effect of negative light masking might be easier to identify. In addition, the patterns of wheel running activity, especially in HWL mice, are not blocks of continuous wheel running, but rather this behaviour is organized in separated bouts of high running activity separated by bouts of non-wheel running activity; which might either be rest or non-foraging activity (See S3-S6). As these bout-lengths are highly variable between and within individuals, the signal to noise ratio of using wheel running activity as a sole behaviour output parameter, especially if the number of repeated measures is limited (and each measure requires four days of measurements as in experiment 1). Assessment of general cage movements, or direct measuring of sleep and or (neuronal) activity – for example by in vivo-EEG or electrophysiology - in addition to the wheel running measurements would be recommendable when future experiments addressing the research question would be performed.

Concluding remarks

The ability to adapt to a more diurnal phenotype might increase survival changes of endotherm nocturnal rodents in natural habitats, especially when limited food or low temperatures pose an energetic challenge. In the current sets of experiments, we report that, along with the reorganization of their circadian activity patterns, the activity suppressing effect of light exposure is reduced or reversed during high workload. The precise mechanisms underlying adaptive flexibility in light masking responses remains to be addressed in future research. Such mechanisms may be relevant to understand effects of light stimulation on alertness and mental performance when a diurnal species occupy a nocturnal niche, as for instance would be the case for humans involved in night shift work.

Supplementary information

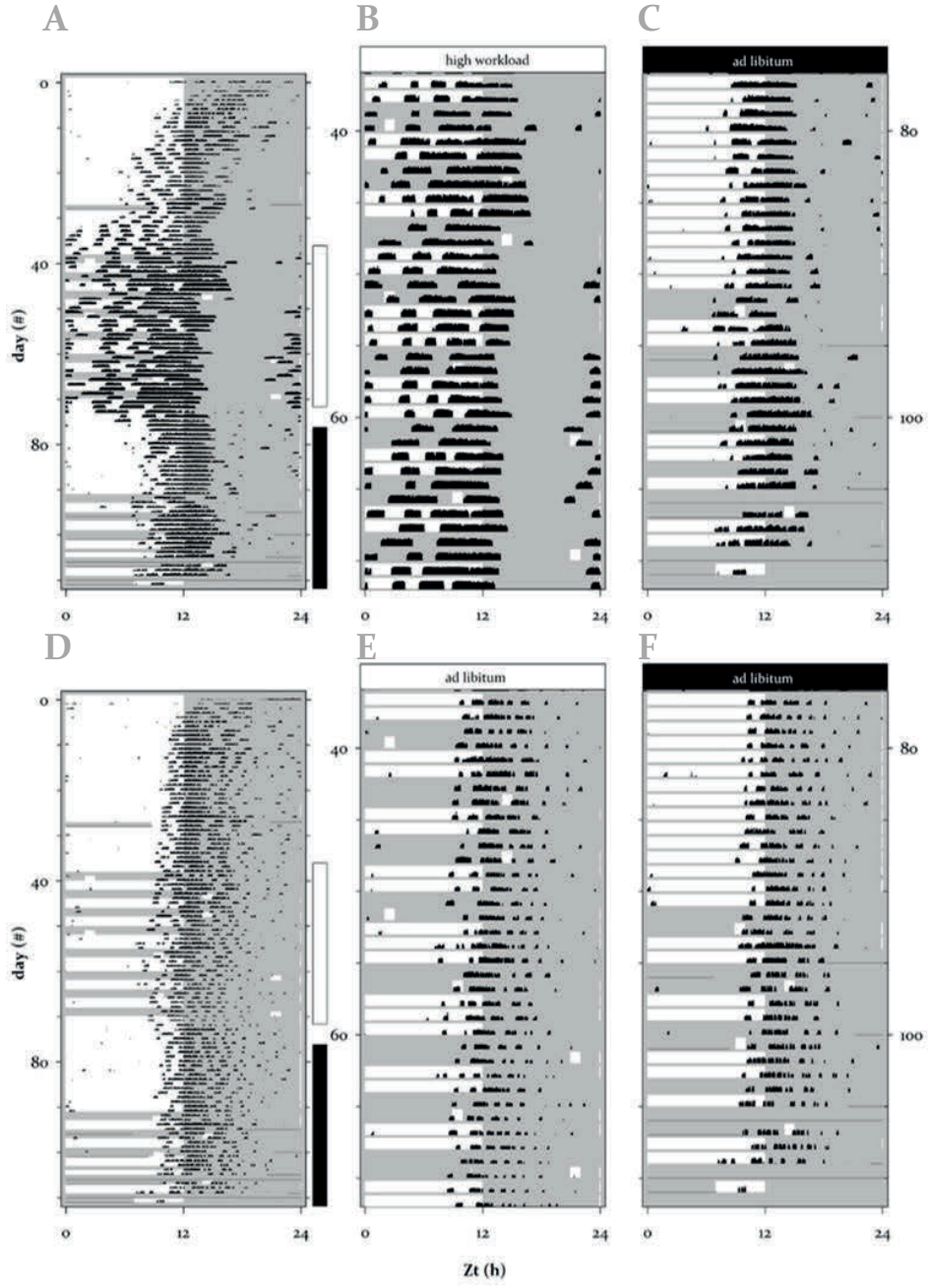


Fig. S 1: Example actograms from experiment 1*. Top Panels (A-C) show a mouse from the WFF group, in full (A) and with close-up of the pulse period on high workload (B) and after*

refeeding with ad lib. chow (C). Lower plots (D-F) show the same for a mouse from the ad lib. control group, on pellets (E) and on chow (F).

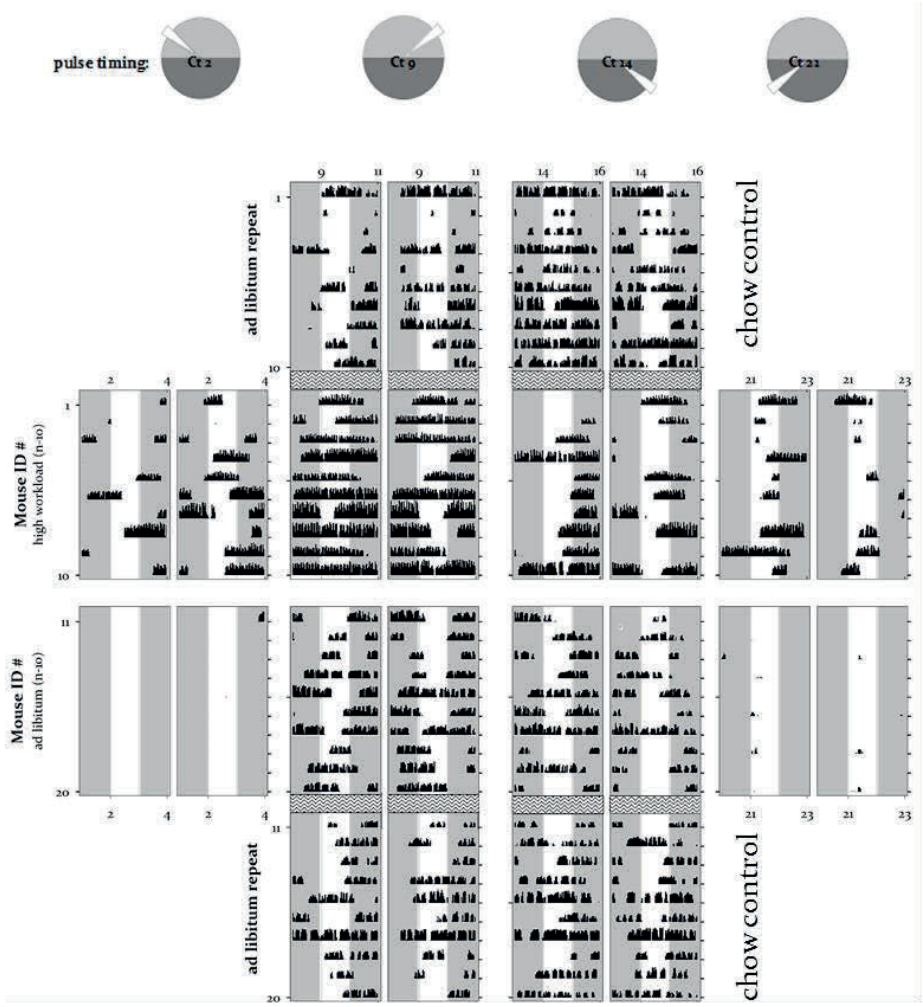


Fig. S 2. Direct responses to light stimulation in the pulse intervals of experiment 1. *Behavioural wheel running activity of all the mice in experiment 1 in the 3h interval surrounding each pulse; with pulse period 1 shown (8 pulses) on the second and third row, and AL chow control measurements of the same individuals during pulse period 2 show on top and bottom rows. The time (CT) at which each pulse was applied is indicated by top pictograms.*

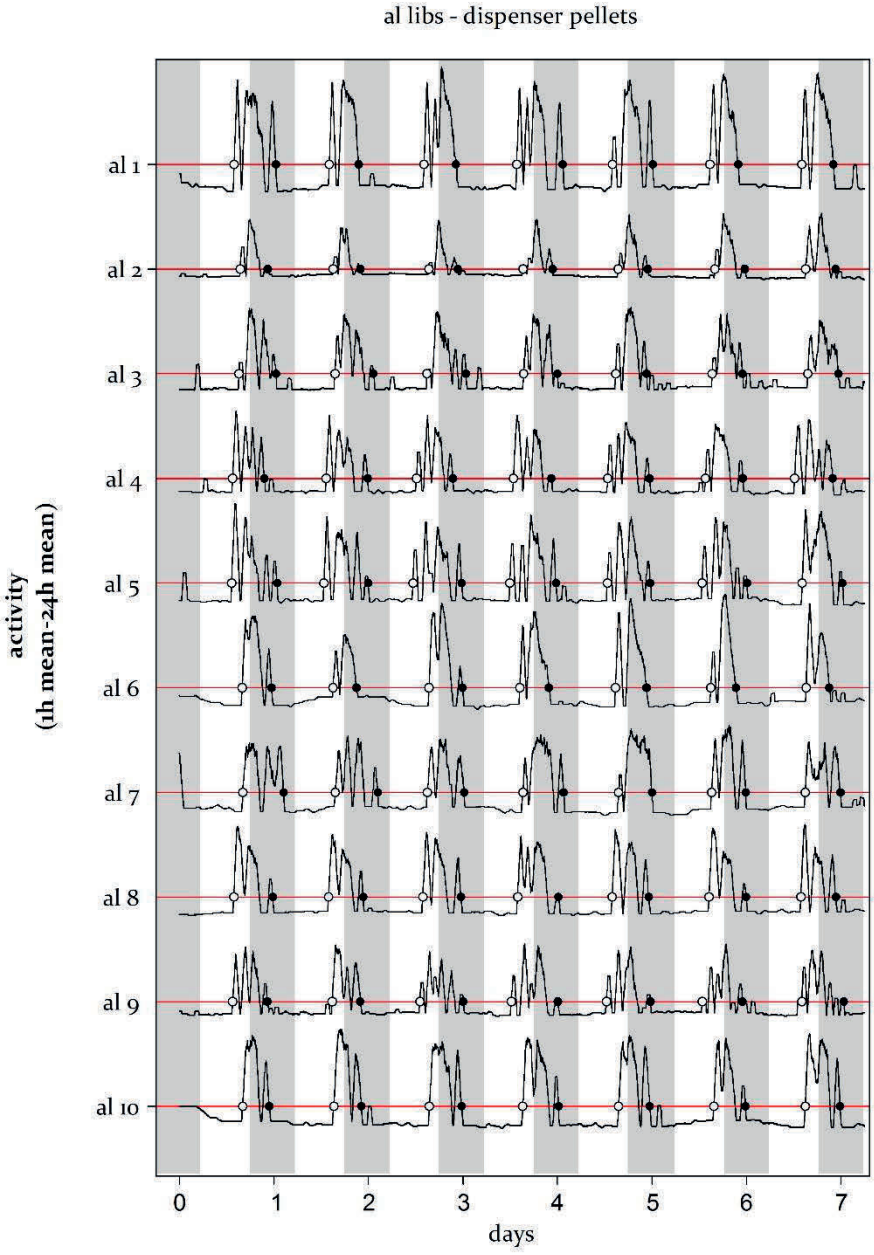


Fig. S 3; Activity profiles of AL-fed (pellets) control group, prior to starting pulse period 1 of experiment 1.

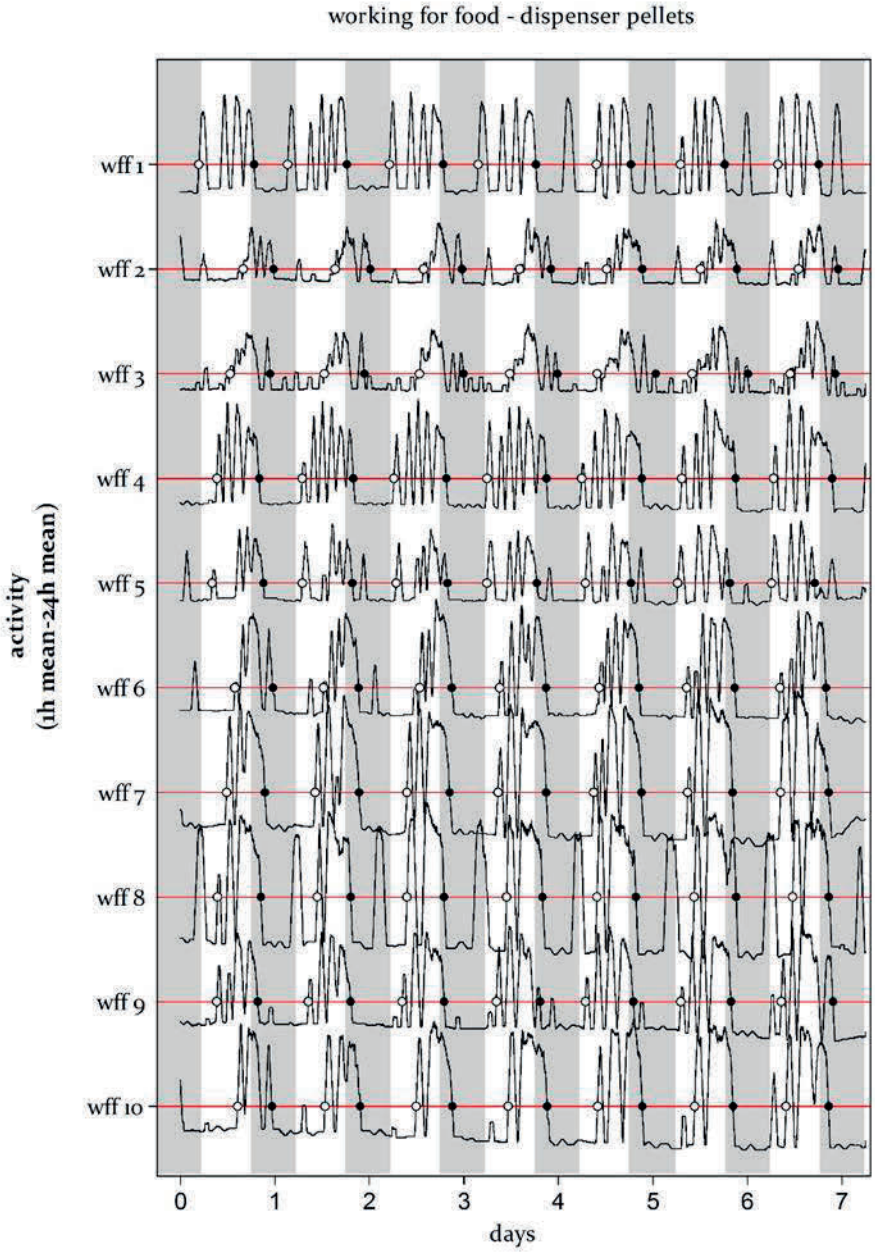


Fig. S 4; Activity profiles of mice on high workload (HWL- group), prior to starting pulse period 1 of experiment 1.

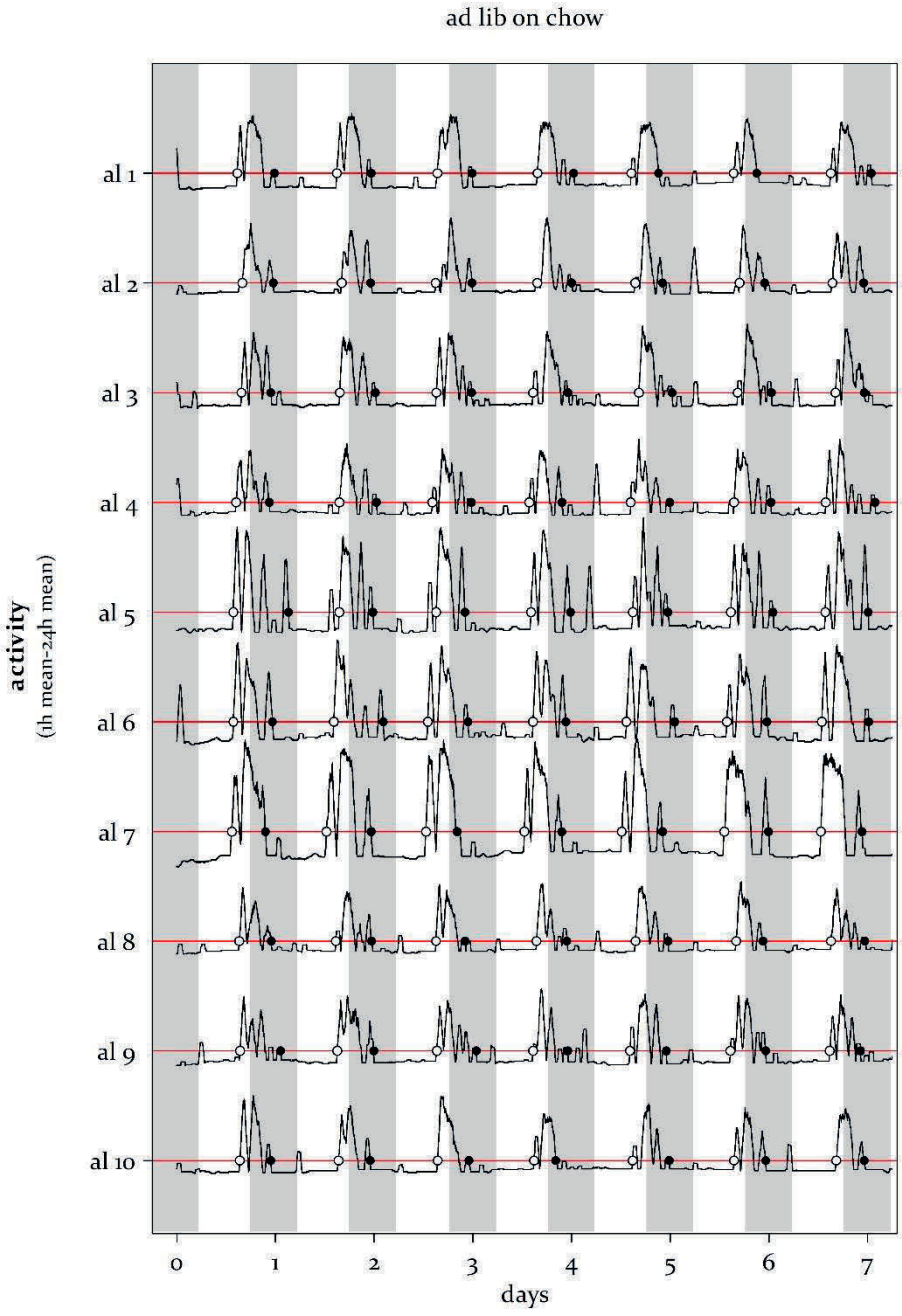


Fig. S 5; Activity profiles of the mice in the AL-group when fed ad lib. regular chow, prior to pulse period 2 of experiment 1.

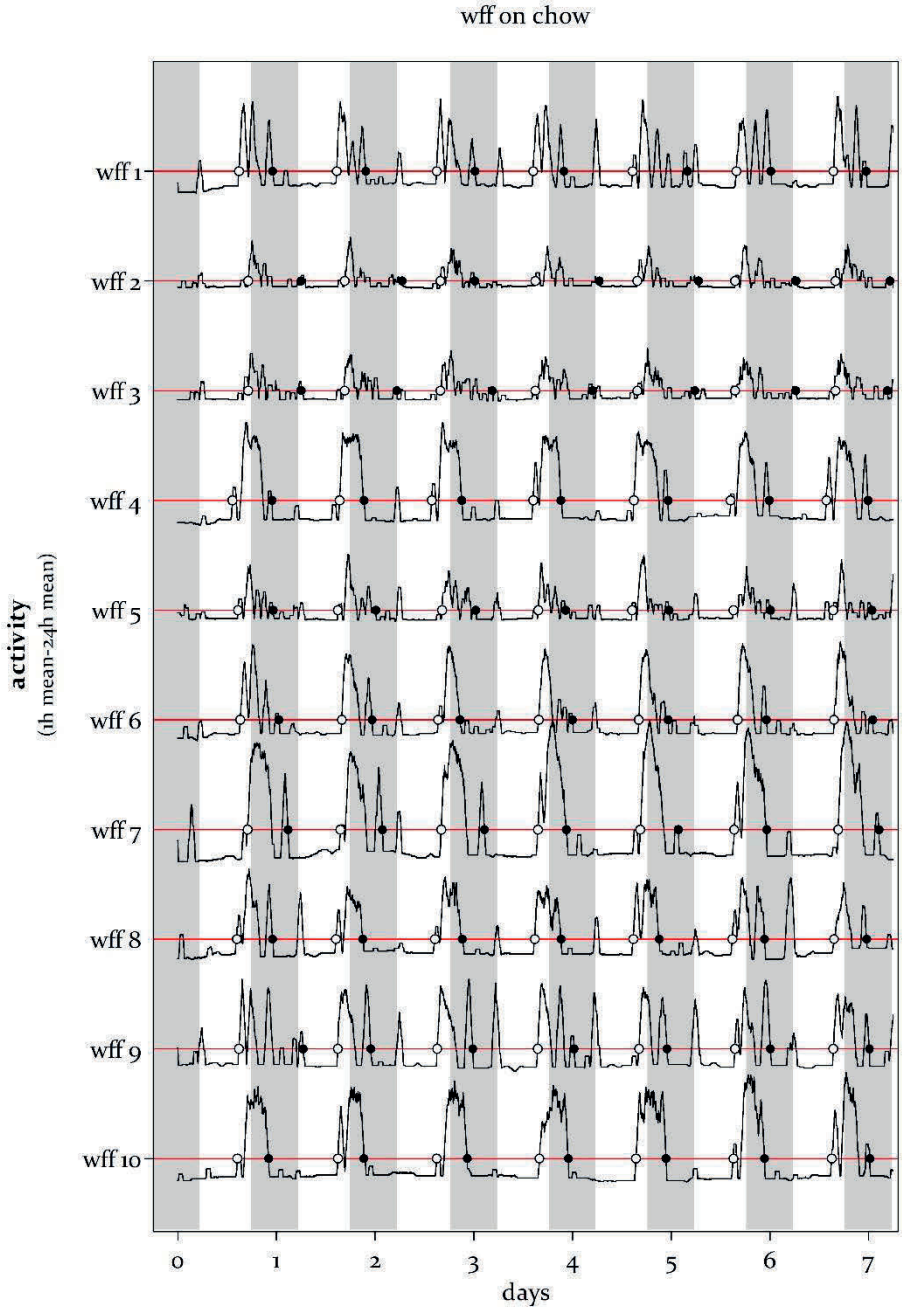
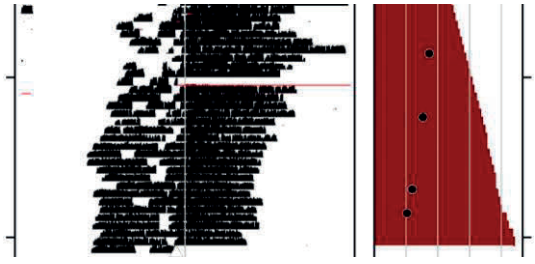


Fig. S 6; Activity profiles of the mice in the HWL-group when fed ad lib. regular chow, prior to pulse period 2 of experiment 1.



Chapter 5

Sex differences in WFF response

DATA CHAPTER

In this chapter we directly compare how male and female CBA/CaJ mice respond to increasing workloads and negative energy balance in the work-for-food paradigm.

Chapter 5

Handling high workload: females work harder but males show more circadian flexibility

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Chapter is being prepared and edited for future submitting

Abstract

When facing conditions of food scarcity, animals must sustain themselves by reducing energy expenditure and increasing foraging efforts. Under negative energy balance, small burrowing mammals can reduce their energy expenditure by shifting their activity phase into the day, which will reduce thermoregulatory costs under natural conditions by 10-20%. Previous empirical work indeed confirmed that male mice readily shift to diurnality under negative energy balance. Other studies suggest, however, that female mice maintain their nocturnal activity phase under conditions of high foraging costs, thereby apparently failing to capitalize on the energetic benefit of diurnality. This suggests different behavioural strategies between males and females in response to food shortage, but such conclusions can only be drawn when both sexes of the same mouse strain are directly compared under identical experimental conditions. Here we compare female and male responses to simulated food shortage in CBA/CaJ mice using the work-for-food protocol. Our data indicate that female mice showed substantially more wheel running efforts compared to male mice in order to obtain sufficient food. Female mice also shifted to diurnal activity, but at much higher workloads than male mice. These results support the view that the order of behavioural coping strategies to food scarcity differs between sexes, while weight-loss enhances diurnality in both male and female mice.

Introduction

Male CBA-CaJ mice respond to simulated food shortage by becoming increasingly diurnal in their overt wheel-running behaviour (Hut et al., 2011). Alongside this altered alignment of their sleep-wake cycle to the environmental light-dark cycle, changes in the phase of clock gene expression in metabolically relevant tissues (more specifically liver and adrenal period-2 gene expression) have also been described, as well as phase changes in plasma corticosterone levels and body temperature rhythms (van der Vinne et al., 2014b). This response to become diurnal following simulated food shortage in a work-for-food protocol (WFF) might offer benefits for survival in their natural habitat. When natural ambient temperature rhythms are present, mice displaying a diurnal phenotypes would require 9 to 20 % less energy for thermoregulation than nocturnal counterparts (Hut et al., 2012; Riede et al., 2017; van der Vinne et al., 2015c).

Earlier work by Perrigo et al (1985), studied how high foraging costs (up to 275 revs/pellets) and low ambient temperatures affect growth and reproductive maturation (and seasonal breeding strategies) in juvenile female mice. Specifically, they showed the combination of colder ambient temperatures and high foraging costs prevented sexual maturation in female mice, but the circadian aspects of foraging behaviour where not described in detail in this work (Perrigo and Bronson,

1985). In later work, the same researchers compared daily activity rhythms of female house mice (*Mus domesticus – musculus*) with female deer mice (*Peromyseus maniculatus*) under various foraging costs (Perrigo, 1987). They reported behavioural shifts and enhanced diurnal wheel running activity in the house mice during lactation (when energy expenditure is highest) for the high foraging costs group, whereas animals remained nocturnal at this same high workload for the other reproductive stages (i.e. during early pregnancy and gestation). The female deer mice at the same foraging costs level increased their wheel running speed, but remained nocturnal while reducing their litter-size by infanticide during the high energetic challenge of high foraging costs and lactation, thereby preventing negative energy balance (Perrigo, 1987).

Previously, female CD1-mice under high workload conditions did not show profound changes in their circadian activity patterns, but rather reduction in their energy expenditure; including the cessation of estrous cyclicity, lowering of resting body temperature (T_b) along with the occurrence of daily torpor (Schubert et al., 2008; Schubert et al., 2010). In males, we also described the incidental occurrence of daily torpor, but only after a profound advance of the activity phase into the day had occurred (Hut et al., 2011). As behavioural and physiological data from females is underrepresented in scientific literature, the potential differences between the sexes in their responses to negative energy balance poses an intriguing question that deserves to be tested in a direct comparison. In the experiment described here, we aim to test if the (potentially) energy-saving response of becoming day-active during simulated food shortage is sex-specific for mice, or whether the previously described differences between males and females can be attributed to differences in experimental design.

Secondary to measuring sex differences in the work-for-food protocol, we also wanted to validate that the mice we use in most of our experiments on diurnality (CBA/CaJ) maintained a stable response to the work-for-food protocol or whether possible transgenerational epigenetic mechanisms may have changed their response. Although we can with exclude that any of the mothers of mice used in our experiments were ever exposed to WFF prior to breeding, some males used in behavioural WFF-experiments had been used prior to fathering offspring. As the circadian phenotype of our *ad libitum*-fed CBA/CaJ mice can include relatively early behavioural onsets (up to several hours prior to lights-off), we wanted to establish if any diurnality-influencing effects could have be obtained through life-experiences of previous generations. Paternal life experiences epigenetically altering the metabolic phenotype of offspring has been previously shown to occur in mice, with fathers enrolled in long term exercise paradigms producing offspring with lower energy

expenditure (Murashov et al., 2015). Potentially, paternally imprinted genes such as Mage-like2 (an ubiquitin ligase where only the paternal copy is expressed in offspring) might alter the circadian characteristics of the offspring (Kozlov et al., 2007; Mercer et al., 2013), but other epigenetic routes are also conceivable. Independent from the mechanism, the adaptive benefit would hypothetically allow life-experience of the father to influence the phenotype of the offspring, increasing their diurnality and reducing their daily energy requirements. We tested for such cross-generational effects by comparing offspring from WFF-experienced fathers with the offspring of naïve fathers that never encountered energy shortages nested within our assessment of sex differences in behavioural response to high work load.

Materials and Methods

Animals & Housing

Mice were housed individually in wheel running cages (wheel diameter 0.14m) and habituated for a minimum of five days, prior to the start of the experiments. Food pellets (Bio-Serv #F0165; 45mg/pellet, contents 21.3% protein / 3.8% fat / 54.0% carbohydrates; 3.35kcal/g) were initially available *ad libitum* in PVC cups (diameter 0.04m) mounted to the cage wall. Mice were housed in climate-controlled chambers (20-21°C, relative humidity 50-60%) with a 12:12LD cycle provided by ceiling mounted lights (200-300 lux at cage level, broad spectrum polychromatic white light source, light on at 8:00 local time). Male and female mice were housed within the same room.

Work for food protocol

Following habituation, spontaneous levels of wheel running activity were determined for each individual over a period of 7 to 14 days during which the food pellets were provided *ad libitum*. The amount of activity (in revolutions/day) was divided by the estimated spontaneous food intake under *ad lib.* (120-140 pellets/day) to determine the starting workload (in revs/pellet). Next, food pellets were removed from the cage and the automated feeder system was engaged, supplying a single 45mg food pellet after a pre-determined number of wheel rotations. The workloads (revs/pellet) were increased remotely with daily increments, simulating a slow decline in food abundance. Workloads were increased around mid-day, at which time the number of revolutions completed and pellets earned in the last 24-h interval where noted. Room entry during the light phase was kept to a minimum, with welfare checks, cage cleaning and weighing all done during the late light phase (ZT10-12) to minimize sleep-disturbance to the mice. The protocol was ended when the mice reached a stable phase of wheel running activity and a relatively stable bodyweight. We used 4 subgroups in our experiments; 5 nests (18 males, 12 females)

were the direct offspring of males that had experienced a prolonged (>8 weeks) WFF-protocol prior to breeding. These 30 mice were run in the WFF set-up in parallel (with sexes and nests distributed evenly across two climate controlled experimental rooms). In a follow-up cohort, mice from 4 nests (8 males, 8 females) were derived from our SPF- CBA/CaJ breeding colony to serve as (naïve) controls (housed in a single experimental room). All mice were between 3 and 4 months of age at the start of the experiments.

Registration and analysis of activity rhythms

Wheel-running activity was recorded by an automated circadian activity monitoring system (CAMS; developed by the group of Howard Cooper, Lyon, France) and stored for analysis in 2-minute bins. As activity records of mice under high workload can show a considerable amount of fragmentation, automated methods to identify phase markers yielded highly variable onsets and offsets which in many cases were a poor reflection to the underlying data. To identify the onsets and offsets of the activity rhythm in this study, we therefore took the 4-hour running average (of the wheel running data) divided by the 24-hour running mean to obtain relative activity level patterns that are comparable through the protocol. Days with more than 30 minutes of missing data were omitted (>15 of the 720 bins), as well as days in which less than a total of 4000 wheel running counts were registered (indicative of sensor problems). After this procedure we did a baseline correction and determined the zero-crossings; positive crossings classified as onsets and negative crossings as the offset. We took a conservative approach by taking the first positive crossing followed by 5 hours of "predominant activity" as a true onset. Similar, the offsets were selected when followed by 5h of "predominant inactivity". Calculated phase markers were visually verified to properly match the onsets and offsets in the underlying activity records. In addition, we expressed the distribution of activity over the light- and dark phase: with diurnality index (DI) defined as percentage of the daily activity that occurs in the light phase.

Ethical approval

The procedures reported here were approved by the University of Groningen animal-ethics-committee (DEC#6545) and in according with international standards on animal experiments. The authors declare no conflict of interests.

Results

Lack of cross-generation effects of the WFF protocol

Comparing behavioural responses to HWL of mice that were either offspring of WFF-experienced fathers (m=18, f=12), or naïve fathers from the SPF CBA/CaJ mice (m=8,

f=8), revealed no differences between the groups (Supplemental figures S1-S4). For subsequent experiments, data of progeny of naïve and WFF-exposed males were therefore pooled.

Outlier due technical issues

One of our male mice (progeny of WFF subgroup) showed a behavioural response very unlike that of all other mice (Supplemental figure S1, ID #300). Whereas an initial decrease in BW and shift to diurnality was observed in this individual during the first two weeks, a subsequent return to nocturnality despite continuous increases in the workload and severely reduced number of (registered) food pellets obtained was seen (Fig S1, ID #300). Most probably, technical issues with the automated feeder (dropping multiples of pellets instead of single pellets) of this individual might have contributed to this abnormal response. The individual was therefore considered as outlier and removed from the subsequent analysis. Final analysis was thus performed for 25 males and 20 females respectively.

Sex differences in activity levels require tuning of workload protocol

Monitoring the food intake and spontaneous amount of running wheel revolutions per day during the *ad libitum* baseline period revealed large differences in activity level between individuals, and specifically between the sexes. On average, females showed double amounts of wheel rotations as males (13k ±4k revolutions for males, 28k ±8k for females, mean ± SD). The quantity of food pellets eaten in this baseline period seemed similar between the two sexes (estimated at 120-140 pellets = 5.4 to 6.3 gram/day). We therefore decided to set the starting workloads at tailored starting values, supporting a manageable pellet-surplus and starting from a similar level of challenge. The starting workloads for most females were around 180 revs/pellet whereas this initial value for males was around 100 revs/pellet.

Sex difference in rate of body weight loss and modulation of activity rhythms

After starting to increase the workloads we noticed that males started to decrease in bodyweight and gradually phase advance their rhythms into the light-phase relatively early in the protocol (when workloads were between 120 and 160 revolutions per 45mg food pellet), whereas females remained stable in their phase at increasing workload and appeared to increase their wheel running efforts to secure a higher amount of food (Fig. 1a,b). In line with this, females maintained their initial body-weight longer than males. As we continued to increases workloads, females did eventually decrease in bodyweight and simultaneously advanced their rest-activity rhythm (Fig. 1b). For the records of all individual animals please refer to the supplementary materials (Fig. S1-S3).

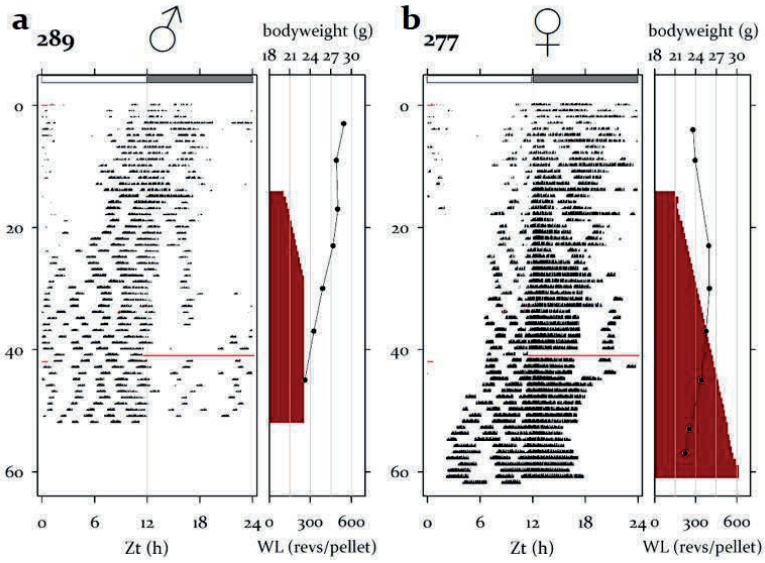

Figure 1. Both male and female mice become increasingly day-active under a progressive workload protocol*. Representative actogram of a male and female mouse with the corresponding changes in bodyweight (●) and workload (WL; red horizontal bar plot). ZT = Zeitgeber Time (h), with lights on between ZT0 and ZT12. WL = workload. For an overview of all actograms in this study please refer to supplemental figures S1 (males), S2 (females) and S3 (naïve second cohort, both sexes). Horizontal red line in actogram denotes missing data.*

Sex differences in the workloads that enhance diurnal activity

Our WFF-protocol directly links the amount of daily wheel running activity with the amount of food obtained. Differences in the activity levels between mice thus have strong implications for the amount of food obtained, and the rate of caloric restriction experienced. We therefore expressed the percentage of activity in the light phase (the Diurnality Index) as a function of workload for both genders (Fig. 2). In line with the actograms shown, we see that male mice show a gradual increase in diurnal wheel running activity as soon as workloads started to increase (Fig. 2a). In contrast, for females the percentage of diurnal remained low for a wider range of workloads (up to 300 revs/pellet) that would correspond with very high diurnality scores in male mice. However, exceeding these workloads ultimately resulted into weight loss and increased diurnal wheel running activity in female mice as well (Fig 2b). Note that differences between males appear much smaller, than those between female mice.

Differences in basal and maximum activity levels between the sexes

Female mice on average where almost twice as active in their wheels as males during *ad libitum* feeding with dispenser pellets (Fi.g 3a). This difference in activity level remained under high workloads, while both sexes increased their individual activity

levels (Fig. 3a). Female mice were able to withstand workloads that far exceeded the maximal amount of (workload induced) food restriction we previously utilized in our WFF protocols. In order to withstand these severe restrictions, females initially compensated the low food-return by greatly increasing the amount of daily wheel revolutions. We therefore quantified the amounts of wheel running activity under *ad libitum* (low WL, during which more pellets were obtained than consumed but with the functional association between running and food obtainment being established) versus the seven days most wheel running activity (max act) and the last seven days under high workloads (high WL; Fig. 3b). Males tended to become more active in their wheels, but quickly reached their maximal limit and started to lose weight and become diurnal, whilst maintaining this the higher level of activity. Females, despite being on average already twice as active under low WL, increased their daily wheel running tremendously as an initial response, with some individuals managing over 60.000 revolution (corresponding to distances of close to 30 km a

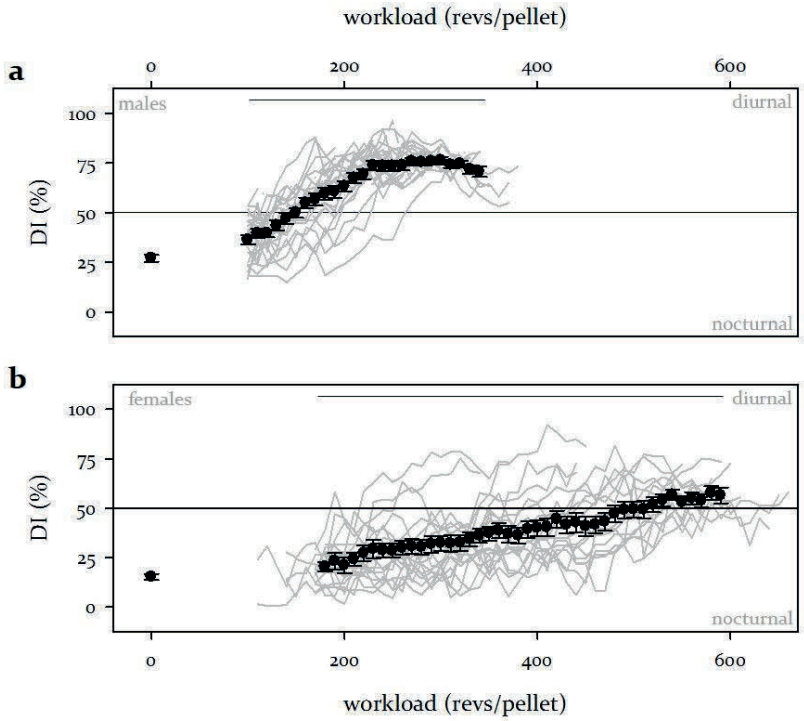

Figure 2. Diurnality in female mice is induced at higher workloads than in male mice. *a) Male mice (n=25) gradually increase day time activity when facing increasing workloads to obtain their food. Individuals are shown as grey lines, symbols denote mean with SE and are only shown for workloads experienced by at least half of the mice in that group. b) Same plot for female mice (n=20). Horizontal bars denote a significant change from AL baseline diurnality index, indicating that as soon as running wheel efforts are linked to obtaining food, the amount of daytime activity starts to increase in both sexes, but females are much more reluctant than males to show diurnal activity at higher workloads.*

day), after which they too started to lose weight and became increasingly diurnal, stabilizing at an activity level still well above their starting levels, but below their maximum intensity (Fig. 3b).

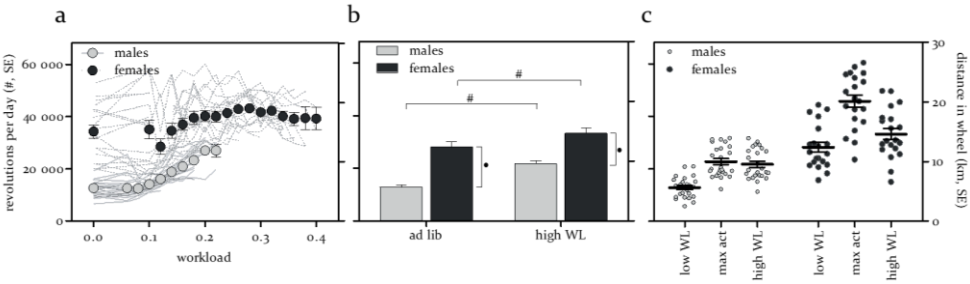

Figure 3. Quantification of activity levels of both sexes for low (ad libitum) and high workloads*. a) male mice show significantly lower levels of activity than female mice, both during ad libitum feeding - prior to starting the WFF phase - as during the final week of high workload (t-test,]● p<0.001). In addition, both sexes had higher activity levels during their last week on high workload (WL) compared with their ad lib. baseline level (paired t-test, # p<0.001). b) Activity levels (7d average) of the individual mice during al libitum/low workload feeding, their 7 most active days throughout the protocol, and the final days under high WL.*

Workload induced weight loss produces changes in phase markers of activity

As the Diurnality Index is a relatively crude measure for the changes in activity rhythm (it does not take the timing within the light or dark phase into account) we therefore also compared the changes in the onset and offset of activity (Fig 4). Under *ad libitum* fed conditions, males had an earlier onset and offset than females (males = ZT 8.74 ±SD 1.02 vs females = 10.40 ±SD 1.07; p<0.0001 unpaired t-test with Welch's correction). Offsets under ad-libitum conditions were also significantly earlier in males (males = 18.08 ±SD 0.95 vs females = 21.26 ±SD 1.82; p<0.0001 unpaired t-test with Welch's correction). The onset of males advanced with the increase in workload, their mean activity onset at high workload (HWL) being around ZT 4.15 ±SD 2.06; a mean advance of 4.58 hours. Likewise, offsets advanced in males by an average of 3.90 hours (Fig. 4). Also females advanced in both of their activity phase markers: onset from ZT 10.40±1.07 to 5.15±1.29, offset from 21.26±1.82 to 15.51±1.38, *ad libitum* versus high workload respectively. All advances were highly significant (p<0.0001), as tested by paired t-test. The ultimate onsets under HWL were not significantly different for males versus females (p=0.051) but the offset of females remained significantly later than that of males (p=0.008, unpaired t-test with Welch's correction). Therefore, a bigger portion of their activity remained expressed during the early hours of the dark phase, explaining the lower DI scores found in the previous analyses.

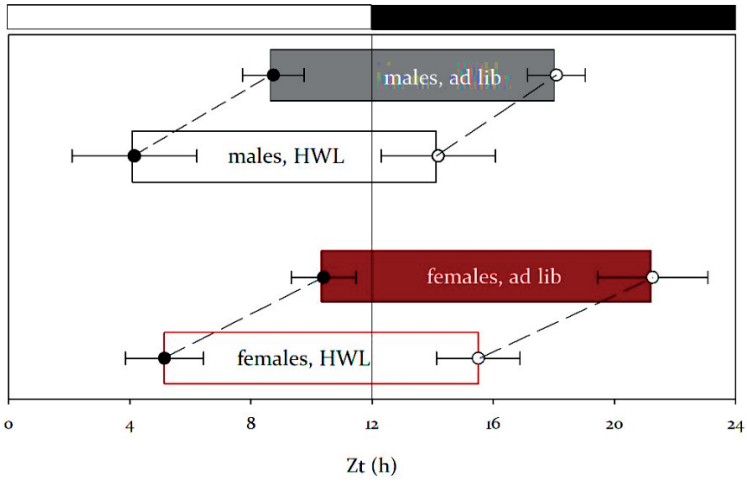

Figure 4. Advance of activity phase of male and females by high workloads. *Both experimental batches were combined as there were no significant differences found between batch 1 (offspring) and 2 (naïve). Included are the last 7 days of ad-libitum feeding, prior to the start of the work for food protocol, and the final 7 days of each individual at high workload. For males, the onset phase-advanced by 4.58 ±1.87 hours, the offset by 3.9 ±1.99 hours. Female onsets and offsets advanced on average by 5.25 ±1.67 and 5.75 ±2.01 hours respectively.*

Weight loss induces diurnality

Rather than expressing diurnality as a function of workload, the combined data from male and female mice suggest the increase in diurnality is influenced by the ability of mice to maintain energy balance. We therefore plotted changes in BW in relation to the experienced workload (Fig. 5a). Under the WFF protocol, weight loss is induced rapidly in males, whereas females maintain a relatively stable bodyweight, even at relatively high workloads. However, females will also decrease in body weight if workloads are further increased (Fig. 5a). Female mice show higher levels of (foraging) activity as well as more plasticity in the ability to increase their daily foraging activity. As they can cover larger distances, they are capable to secure more food, and thereby prevent negative energy balance and thus maintain their bodyweight for longer (Fig. 5a). Resultantly, they also maintain their nocturnal phenotype longer than males. The workloads at which females start to experience negative energy balance and loose bodyweight correlated with the moment in time at which they started to display phase advances (Fig. 5b).

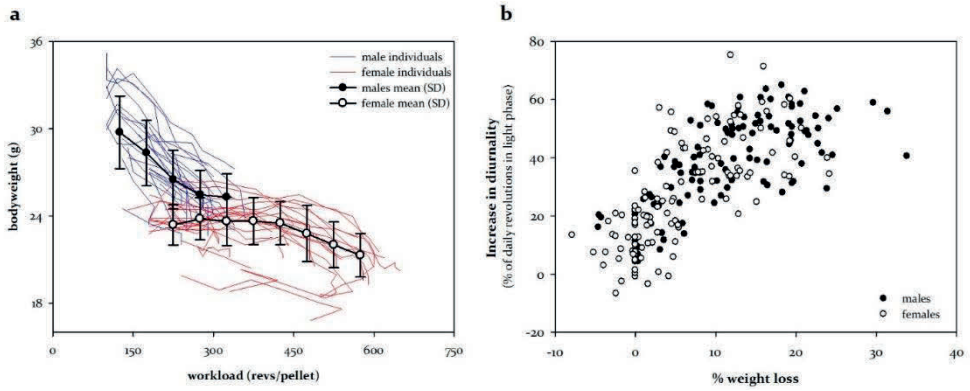

Figure 5. Changes in body mass with progressive workload versus changes in diurnal running with decreasing bodyweight*. A) Males show a decrease in bodyweight soon after the start of the work for food protocol, which increases with mounting workloads, whereas female mice maintain their body mass longer. B) The amount of weight loss in turn shows a clear correlation with increases in diurnality (7day running mean), which holds true for both sexes. Negative X-values indicate the animal was in a weight gaining phase (e.g. when increasing from 95% to 100% of the maximum weight being expressed as -5%). Changes in diurnality were expressed against each individuals mean percentage of light phase activity under AL feeding conditions prior to the WFF phase.*

Discussion

Our main finding is that female mice, like males, show increased diurnal activity when energetically challenged under the WFF protocol. The substantial plasticity in activity levels observed in female mice, plays a major role in explaining a lack of this diurnal response found in previous studies. Workloads that tend to be sufficient to make male mice become predominantly diurnal, can be easily tolerated by females that increase their wheel running efforts, hence securing sufficient food to maintain energy balance and thus maintaining a nocturnal circadian niche. When the workload is increased further ('food density' decreases), females do to start to display phase advances as soon as they decline in bodyweight and in a similar relationship as male mice (Fig.5b). This observation matches well with the results found by Perrigo et al. (1987) in pregnant female mice, where a high workload in combination with lactation (an additional energetic challenge) produced diurnal behaviour, whereas the same workload during different parts of the gestation period did not (Perrigo, 1987).

It is interesting to speculate why female mice have a stronger ability to enhance activity levels and foraging behaviour than males. Potentially, it may be explained because their ability to harvest energy from their environment has a more direct effect on their reproduce success, which therefore may result in a sex specific selection for this feature. Similar sex differences in time allocation can be found in

other species like fish, where males spend less time foraging and more on social interactions and territorial maintenance (Sano, 1993). However, it remains unclear if this plasticity in foraging behaviour is relevant for all mouse strains. The major increase in wheel running activity we found in our female CBA/CaJ mice contrasts with the behaviour observed in female CD-1 mice; where females in response to high foraging costs decreased food intake without significant effects on total daily activity level, but rather compensated by suppression of energy allocated to reproduction, immune function (Schubert et al., 2008), and thermogenesis (Schubert et al., 2010). This may be an unexplained result of strain differences between CBA/CaJ and CD-1 females, but also the presence of members of the opposite sex in the same experimental room might have altered behavioural responses.

An alternative, not mutually exclusive explanation for the sex difference might relate to the perceived risk of diurnal activity, where risk is meant here as the chance of predation times the fitness costs of predation. The light phase might be associated with enhanced risk of diurnal predation in natural habitats, and differences in perceived risk can contribute to both sex- and strain differences in the readiness to increase diurnal activity. Similar sex differences in relation to risk avoidance are reported for rats, in which animals were housed in a safe home cage but needed to forage in an adjacent arena with a grid floor they gave unpredictable foot shocks. Males in this protocol responded by ingesting larger meals with reduced frequency, reducing their foraging time whereas female rats reduced metabolic needs to avoid food shocks (Pellman et al., 2017). Data obtained from female CBA/CaJ mice in outdoor enclosures likewise showed that reducing the perceived predation risk, providing runway-cover to a feeder platform, resulted in more daytime feeder visits in females (Vinne et al., 2019).

The underlying evolutionary mechanism may include that females have longer periods of critical time investment during reproduction than males by including gestation (three weeks) and lactation (three weeks). Predation in females will therefore often result in loss of the complete litter during a period of at least six additional weeks per reproductive attempt. In males, however, predation would only result in loss of a reproductive attempt up to the time of mating. A first approximation shows that, after weaning, it might take about four weeks until adulthood when mice may experience their first mating. However, only the female would need to survive for another six weeks to complete gestation and lactation. This means that the fitness consequences of predation after weaning, or the accumulated risk of predation during independence, may be more than twice as high for females (10/10=1) as for males (4/10=0.4). This may result in a considerably higher selective advantage for prudent behaviour in females than in males, which could result in

riskier behaviour in males specifically. In short; there is no genetic contribution of females that fail to become less than 10 weeks old, whereas there might be some contribution of males that last less than 10 (but more than 4) weeks. In stable biological populations the median number of offspring reaching full maturity would be around 2, as prolonged deviations would lead to exponential growth or population collapse, likely attributing substantial weight to the success of the first litter(s) of mice populations in their natural habitat.

An underlying physiological mechanism for the sex differences in "willingness" to become diurnal might be underpinned by the sex differences in the ability to reduce energetic needs. Implanted temperature loggers in both male and female CBA/CaJ mice housed in outdoor enclosures revealed that males show a larger capacity to reduce their resting body temperature than female mice (Van der Vinne et al. 2019). This larger amplitude between body-temperature in the active and resting phase would make the energetic benefit of becoming diurnal larger for males (van der Vinne et al., 2015c; Vinne et al., 2019). As the energetic benefit of diurnality would be smaller in females they might first respond with other coping strategies such as increasing foraging efforts or decreasing the energy attributed to reproduction or immune function, before partaking with the more risky behaviour of aligning the active phase with the warmer, but potentially more predation prone light phase of the day.

Interestingly, deer mice in the previously mentioned study by Perrigo (1987) did not increase diurnality, even when losing weight during the combination of both lactation and high foraging demands. Instead they even opted to reduce their energetic burden by committing infanticide. However, deer mice can produce many litters per year and have more complex social structures than house mice, they might valuate survival of themselves versus survival of the offspring in a different ratio (potentially being more hesitant to expose themselves to diurnal predators). It remains unclear why *Peromyscus* in Perrigo's study failed to take advantage of adjusting their chronotype to preserve energy. Potentially, deer mice are more directly sensitive of the (lack of) ambient temperature rhythms in their habitat, whereas house-mice may expect such patterns to exist in their natural context. Whether or not the presence of ambient temperature cycles can induce diurnal activity during energetic challenge in *Peromyscus* remains an interesting question for future experiments.

We did not observe significant differences in the circadian behavioural response to high workloads between offspring of parents without history of encountering simulated food scarcity (naïve) and the offspring of males that did experience the WFF prior to producing offspring. Whereas this confirms the validity of previously

generated data, regarding the robust behavioural response of CBA/CaJ mice (increasing diurnal activity during high workload) as well as that the relative early chronotype during *ad libitum* feeding is common in this strain, the data from this study do not support the hypothesis that males influence the behavioural responsiveness and chronotype enhance the ability of their offspring to cope with (simulated) food scarcity. It might be that such adaptations that make a strain or population increase circadian flexibility occur by standard genetic diversity and natural selection, or that any cross-generational effects on energy homeostasis and circadian phenotype occur during gestation or maternal care, and therefore are not present in the presented study. It remains worthwhile to test if the offspring of WFF- or food shortage experienced mothers is more prone to show the diurnal phenotype in future experiments.

In summary, we showed considerable sex differences in the response to deal with increasing workloads. Although negative energy balance ultimately induces increased diurnal (foraging) behaviour in both sexes, females are able to work a lot harder to secure food while remaining nocturnal for a longer period of time, whereas males more readily shifted their behavioural activity into the day, potentially resulting in higher energy savings but also increased predation risk in a natural context.

Supplemental figures

Supplementary figure 1: actograms of all males (n=18) for experiment 1*. One of the 18 males returned to a nocturnal phenotype and high running intensity (ID#300). All others obtained a stable diurnal activity rhythms. For axis and clarification see larger figure 1 and associated caption in main text.*

Supplemental Figure 1: all actograms of female mice (n=12) in experiment 1*. Even though there is some heterogeneity in both the magnitude of the shift (e.g. ±4h in ID#276 and ±12h in ID#299) as well as in the severity of the workload at which these shifts were induced (up to over 600 revolutions per 45mg pellet), the general picture is that like their male counterparts, also female mice revert to a more diurnal phenotype when they are exposed to prolonged and/or severe simulated food shortage. For axis and clarification see larger figure 2 and associated caption in main text*

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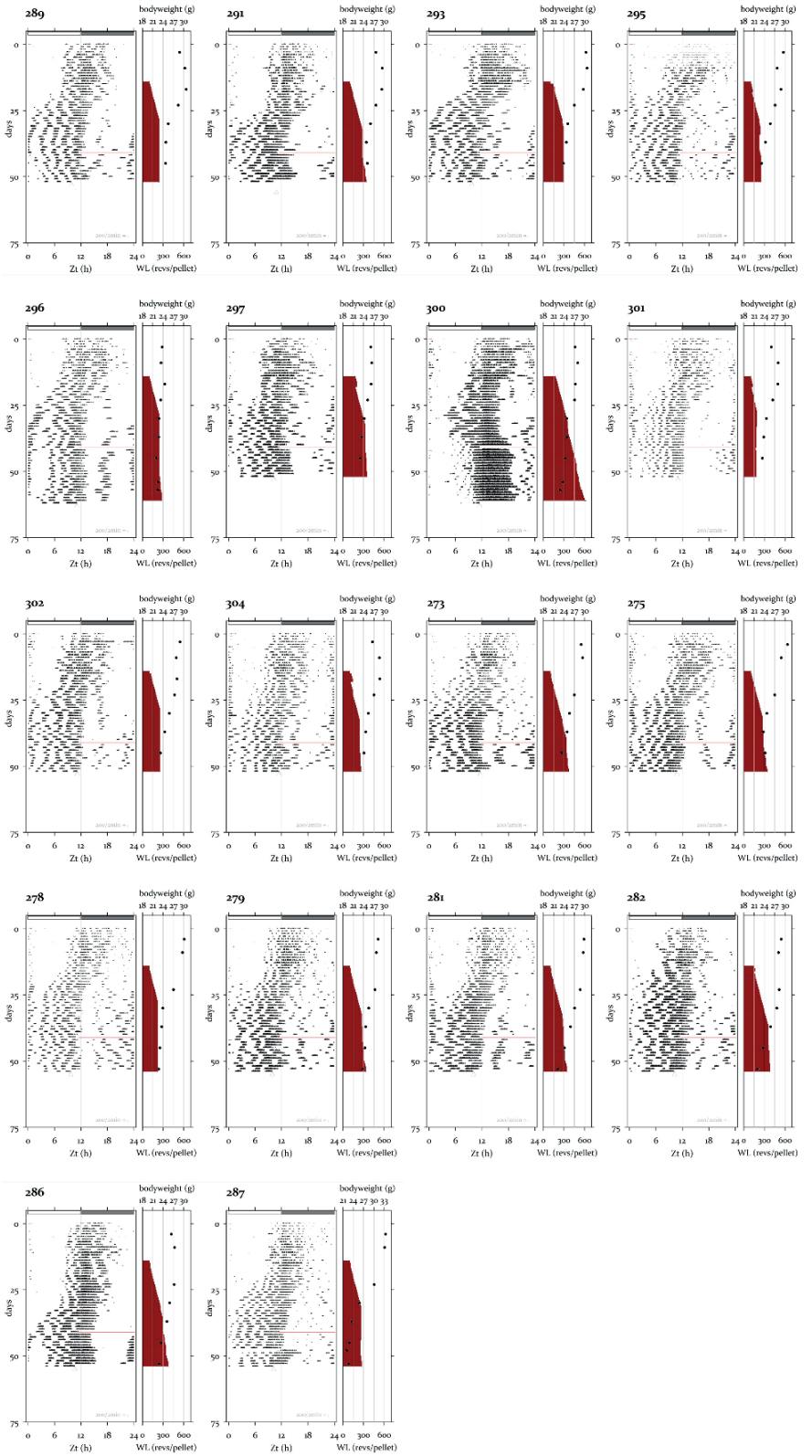
Supplemental Figure 2: Individual quantitative actograms from all mice in experiment 2. *Males (n=8, top rows) showed a more pronounced phase advance which occurred at lower workloads than females (lower rows). For more detailed description of the plots see the preceding figures.*

Supplemental Figure 3: Onset and offsets of both genders and both experimental groups over the progression of workload. *Plotted are the mean onset/offset with SD. Note that the progressive advance of the phase-markers to earlier times of day was consistent between the two male groups (blue and black symbols) as well as between the two female cohorts (red and red-open symbols). Upward triangles denote onset, downward triangles denote offset.*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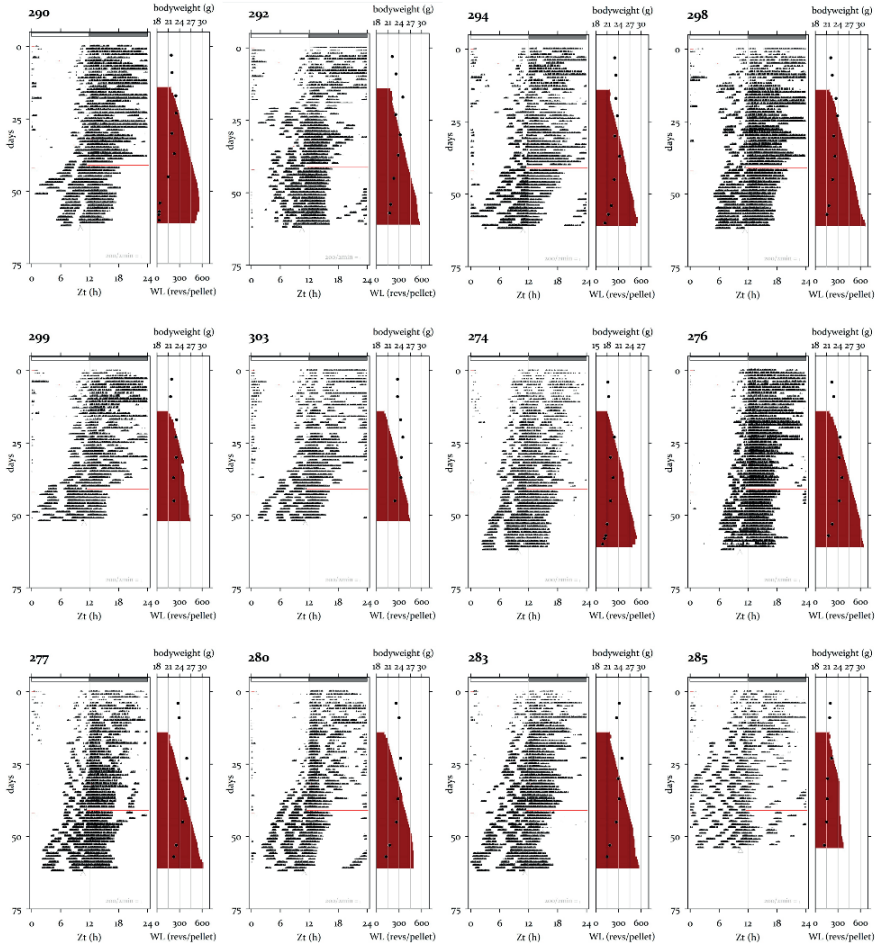
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Fig. S 2

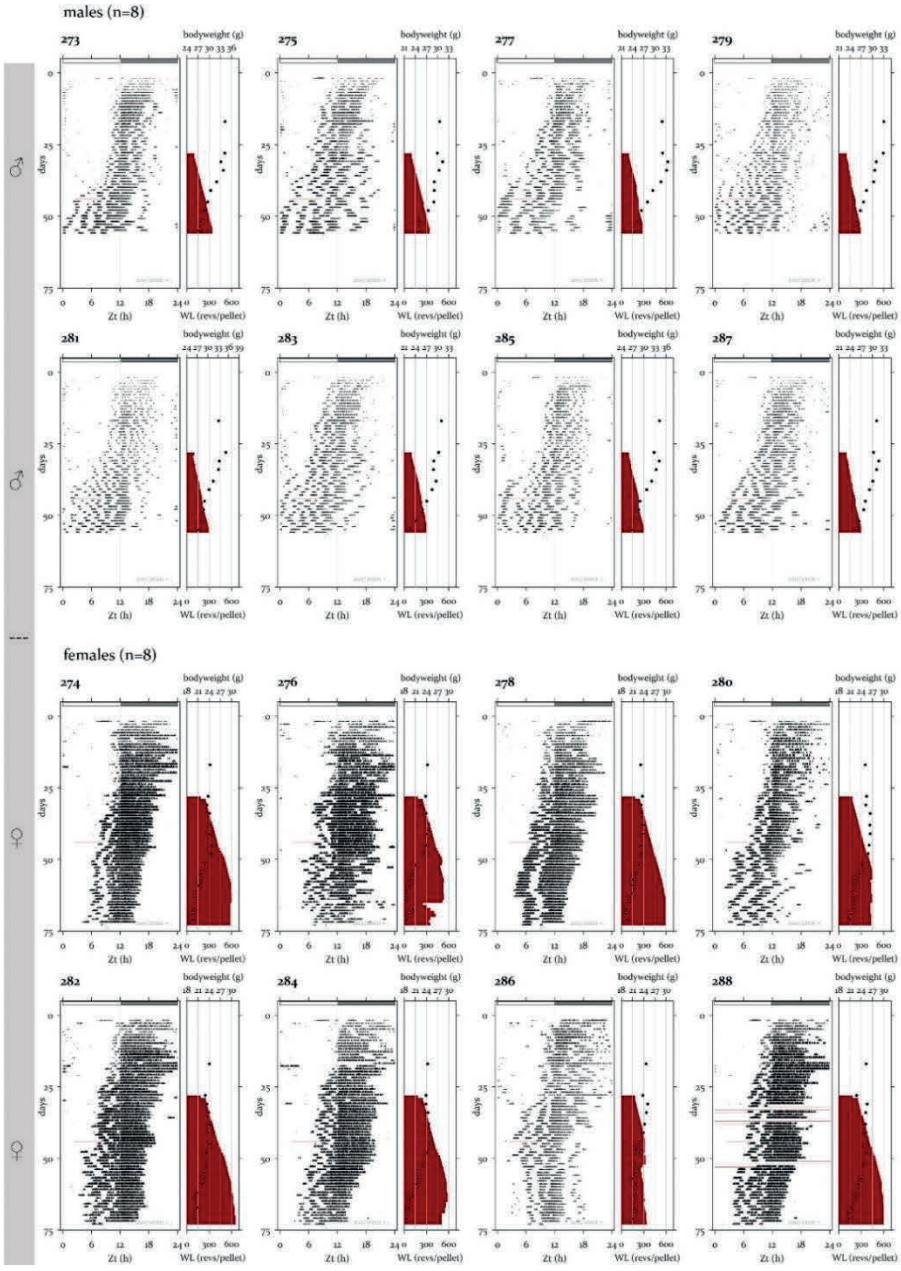
males (n=8)
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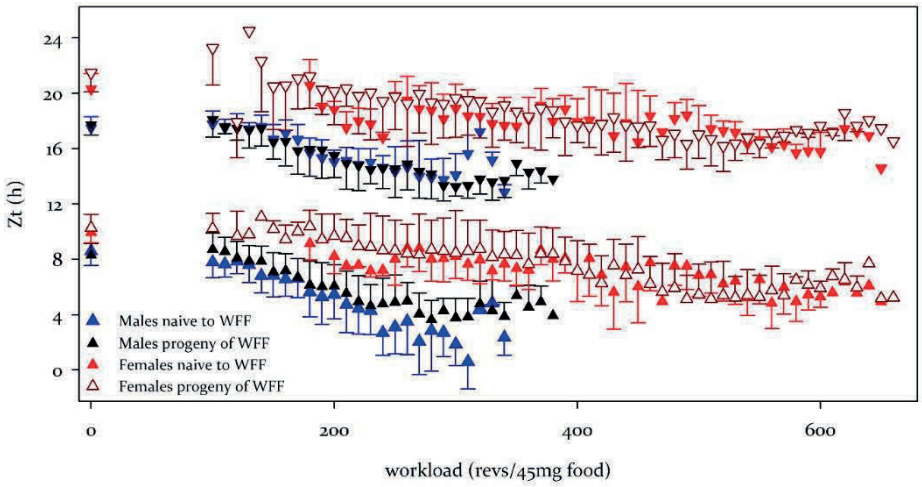
Fig. S 3.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workload (revs/45mg food)
Males naive to WFF
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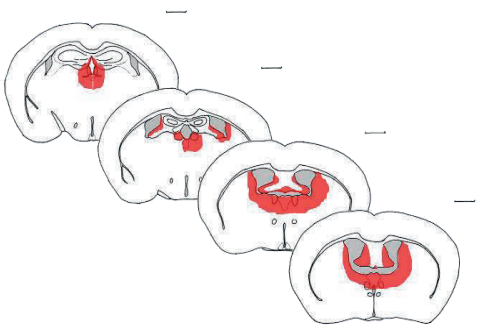
Females naive to WFF
Females progeny of WFF

Fig. S 4.



Chapter 6

Mechanisms of WFF induced diurnality

DATA CHAPTER

To test the mechanisms underlying the response to display more diurnal activity during negative energy balance, we performed a series of lesion-experiments described in this chapter. Specifically, we addressed the potential involvement of the adrenal glands, the role of the supra-chiasmatic nucleus, performed a neuronal activation screening of multiple brain regions by FOS-immunocytochemistry and lesioned the paraventricular nucleus of the thalamus.

Chapter 6

Negative energy balance induced diurnal activity in mice: the role of the suprachiasmatic nucleus, thalamic paraventricular nucleus and adrenal glands

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Chapter is a compilation of 4 extensive experiments and might be edited for future submission

Abstract

Negative energy balance was previously shown to induce diurnal behaviour in generally nocturnal euthermic, burrowing, rodents like mice and rats. Whereas the functional explanation of this phase advance in daily activity patterns appears to relate to reducing the thermogenesis costs, the neurobiological, endocrine and physiological mechanisms that facilitate this shift remain elusive. In previous work we found that the phase of clock gene (*Per2*) expression in the suprachiasmatic nuclei remains stably locked to the LD-cycle, whereas clock gene rhythms in the adrenal glands show phase advances in line with the shifted behavioural patterns. In the current chapter we tested if the adrenal glands and the SCN are necessary for the diurnal phenotype emerging during negative energy balance by subjecting adrenalectomized and SCN-lesioned mice to the work-for-food protocol. Adrenalectomy failed to prevent a shift to diurnality, whereas in SCN-lesioned mice, negative energy balance was unable to induce diurnal circadian rhythms. Third, we compared neuronal activation patterns by FOS immunocytochemistry staining in the brains of mice sacrificed across the 24h LD-cycle under high workload (diurnal) versus ad-libitum feeding conditions (nocturnal controls). Elevated and altered phase of FOS-expression was observed in the anterior portions of the paraventricular thalamic nucleus (aPVT), a structure involved with balancing risk-perception and reward and guiding motivated behaviours. Despite the area being a potential candidate for rearranging the phase of activity patterns during high workload, lesions aimed at the aPVT failed to prevent a shift towards diurnality in our WFF-paradigm. In conclusion, an intact SCN in mice is required to change chronotype from nocturnal to diurnal. Although adrenal products, such as plasma corticosterone, might alter phase angles in metabolic tissues the adrenals are not required to adopt diurnal behavioural rhythms, nor is the aPVT. An intact SCN appears to be required in order to provide the time-of-day framework to downstream modulators.

Introduction

Importance of circadian rhythms

Timing of behaviour and physiology relative to rhythmic variation occurring in the habitat influences fitness in a complex manner. By capitalizing on opportunities and reducing the risks to which an organism is exposed, daily (circadian) rhythms provide extrinsic benefits (van der Veen et al., 2017; Vaze and Sharma, 2013). In addition, partitioning behaviour and physiology over time of day helps to separate conflicting biological processes (e.g. catabolic and anabolic pathways), improves efficient use of available nutrients and can optimize how well different tissues cooperate, collectively referred to as the intrinsic benefits of circadian rhythms

(Sharma, 2003). Circadian rhythms are considered highly advantageous when they predict future conditions, allowing the body to anticipate and prepare for upcoming events and thereby aiding homeostasis (Riede et al., 2017). The extrinsic fitness benefit provided by circadian organisation depends strongly on the rhythmic variation in the environment.

Why circadian rhythms are adaptive

Even in a single location, temporal dynamics of natural environments can vary over time, in some cases changing which circadian niche is best suited for an individual's survival and its long-term fitness. One extreme example is the variation in daylength over the seasons, with circadian rhythmicity in arctic reindeer dissolving during the middle of summer (when continuous daylight allows round-the-clock opportunities for efficient grazing and migration (Bloch et al., 2013). Another example can be shifts in diets of the individual, camouflaged caterpillars might feeding on the always available leaves throughout day and night, whereas in their adult stages the flowers they visit for nourishment are selectively accessed during the day (butterflies) or night (moths) (Niepoth et al., 2018). Similarly circadian patterns of feeding change in fish that prioritize quick growth and maturation to leave their spawning grounds versus individuals that extend they stay for next year's migration (that remain more nocturnal) (Metcalfe et al., 1999; Orpwood et al., 2006). Besides dietary changes or growth demands, also changes in circadian predation pressures might affect the circadian niche. Studying a population of rabbits, Bakker et al. showed that circadian foraging behaviour patterns become more diurnal when they find nocturnal predator dropping, and likewise more nocturnal when faeces of a diurnal predator was introduced to their enclosure (Bakker et al., 2005). A desert animal might prioritize minimizing water loss by avoiding diurnal activity when water is scarce, but adopt a nocturnal predator avoiding life-style when food and water are found aplenty, while such decisions may change when temperatures drop during winter and energy preservation becomes more important for survival (Tachinardi et al., 2017; Tomotani et al., 2012). Observing animals in their natural habitats can provide interesting examples of variation in temporal niche and highlights that flexibility in circadian organisation of daily rhythms is adaptive (reviewed in Hut et al., 2012).

Relevance of adaptive circadian organisation to society

Whereas we become increasingly aware and appreciative of the importance of adaptive flexibility in circadian rhythms, we remain largely at a loss to which mechanisms allow such flexibility. A better understanding could provide important insights that are quite relevant to our own health and performance as well (Dyar et al., 2018; Karatsoreos et al., 2011; Plano et al., 2017). As the current 24h society has a substantial proportion of the workforce working irregular or night-shifts we might

be able to facilitate our biology to cope with such shifts, with clear health and socioeconomic benefits (Chellappa et al., 2018; Zimmet et al., 2019). To gain such insights, we require reproducible methods of inducing adaptive circadian rhythmicity under relatively controlled conditions, where we can monitor and study the internal changes that trigger and modify adaptive circadian rhythmicity.

Simulated food shortage induces adaptive circadian (re)organization under controlled laboratory conditions

In previous experiments we have identified foraging efforts as a potent modulator of the circadian niche in mice (Hut et al., 2011; Riede et al., 2017; van der Vinne et al., 2014b; van der Vinne et al., 2015c). When individually housed mice must obtain food by wheel running effort, we see a substantial phase advance of the rest-activity cycle with increased expression of day-time activity when high wheel-running efforts are required to secure food. This contrasts with the predominant nocturnal phenotype observed in the same mice under standard housing conditions, with low running efforts of freely available food. In the current set of experiments, we utilize this work-for-food paradigm in combination with a set of targeted anatomical lesions in the hope to gain insights which structures are critically involved in adjusting the temporal niche of mice, shifting their daily pattern of rest-activity relative to the light-dark cycle.

Research Questions

In this manuscript we seek to answer the following research questions: 1) whether the adrenal glands are necessary to shift activity rhythms under negative energy balance, 2) whether the SCN is necessary to display diurnal activity rhythms under negative energy balance, 3) which brain structure(s) show rhythmicity changes that correspond to the shifted behavioural activity pattern and 4) are those brain structures necessary to induce shifted behavioural patterns.

Adrenal glands as the hormonal arm of the clock

In our previous studies we established that mice that become increasingly diurnal in the WFF-paradigm have a markedly elevated and phase advanced pattern of their plasma corticosterone concentrations (van der Vinne et al., 2014b). Clock gene rhythms in the adrenal gland, as established by the expression of the Period2 gene, likewise are phase advanced in in mice with a diurnal activity pattern (van der Vinne et al., 2014b). As glucocorticoids in turn are considered an important Zeitgeber-signal for peripheral tissue clocks (Asher and Schibler, 2011; Dibner et al., 2010; Sujino et al., 2012) we speculate that adrenal output might be necessary to reorganize circadian rhythms towards diurnality under simulated food scarcity. Therefore, we

subjected adrenalectomized mice, along with their sham-controls, to the WFF-protocol in experiment 1.

The dependency of circadian rhythms on the suprachiasmatic nucleus

The mammalian SCN has widely been established as one of the most important structures in regulating circadian rhythms in behaviour and physiology (Belle, 2015; Blum et al., 2012; Davidson et al., 2003; Sujino et al., 2003). Experiments in various mammalian species, diurnal and nocturnal alike, have shown that many overt rhythms are lost in constant/arrhythmic conditions when this structure is damaged or impaired by genetic deficits, and become increasingly irregular or dampened in rhythmic environments (Kaufman and Menaker, 1993; Stephan and Zucker, 1972; Zhang et al., 2004). However, it is known that several circadian patterns can re-emerge or be restored along SCN-independent routes, for instance by having scheduled meals, activity or rewards provided in daily recurrent cycles (Jansen et al., 2012; Mistlberger, 2011; Mrosovsky, 1996). Whereas the anatomical components and mechanisms behind such SCN-independent circadian oscillators remains largely elusive, we could imagine that the diurnal patterns emerging in mice under negative energy balance might be driven by such a non-SCN circadian pacemaker (Pezuk et al., 2010). Indicative data that the phase of the SCN remains unchanged in phase relation to the LD-cycle during WFF in our mice (van der Vinne et al., 2014b) and retains a stable LD-relation regardless of whether the behavioural phenotype is predominantly nocturnal or diurnal in other species showing temporal niche switching (Cohen et al., 2010b), makes the option of a non-SCN oscillator responsible for governing activity-rhythms under energetic challenge a distinct possibility. To answer the question whether the SCN is a necessary component of the emerging diurnal behaviour under WFF protocol, we tested the behavioural response of SCN-lesioned mice to the high workload of a WFF paradigm along with that of sham-lesioned controls in experiment 2. In addition to performing the WFF protocol under LD, LL and DD settings, and evaluating circadian periodicity under HWL in LD, LL and DD, we also addressed if induction/restoration of circadian rhythmicity by food entrainment (using timed feeding at mid-day) could be maintained in SCN_X mice by a high workload.

CNS-based modulation of circadian rhythms; evaluation of FOS dynamics in the brain

As many components of behaviour and physiology are regulated or initiated at the level of the central nervous system, we raised the question as to which parts of the brain show circadian phase-advances in neuronal activity patterns in line with the advanced behavioural phenotype under simulated food scarcity. To address this, and raise new hypothesis as to which structures are involved in circadian reorganization,

we evaluated the patterns of FOS expression using a c-fos antibody on sections of various brain regions. Brain sections of 36 male mice with induced diurnality due to high workload were obtained in 2h intervals spanning the circadian cycle and compared with sections from *ad libitum* fed (nocturnal) controls. Our procedures and results are summarized in this chapter as experiment 3.

Dependence of diurnality on the paraventricular thalamic nucleus

Based on the data from our c-Fos evaluation described above, we hypothesized the anterior portion of the paraventricular thalamic nucleus (aPVT) would be a potential candidate to rearrange circadian patterns in foraging and feeding behaviour under an energetic challenge. The biological role attributed to this nucleus appears to connect motivated behaviours, reward-systems and the circadian clock (Cheng et al., 2018; Choi and McNally, 2017; Colavito et al., 2015; Van der Werf et al., 2002). In addition, the aPVT has been suggested to receive photic information from the environment, either by direct input from melanopsin positive retinal ganglion cells or through projections that pass the adjacent habenula and the more distant SCN to which it is bidirectionally connected (Alamilla and Aguilar-Roblero, 2010; Hattar et al., 2006). A pilot study to address whether the aPVT is critically involved in circadian flexibility was therefore performed by subjecting a group of aPVT-lesioned mice to negative energy balance, comparing their behavioural response with that of sham lesioned controls in experiment 4.

Framework of this study

How the phase of behavioural rhythms is organized remains complex and might depend heavily on the context, involving modulation and integration of multiple brain regions that in turn might respond to complex feedback of peripheral (cyclic) signals. Self-sustaining patterns of neuronal activity in the SCN appear to have a rather constant phase-relation with the environmental LD-cycle, with a higher firing rates during the light phase in both diurnal and nocturnal species (Brown and Piggins, 2007; Dibner et al., 2010; Rose et al., 1999; Welsh et al., 2010) and keeping the same phase when behavioural circadian niche changes within a species (Cohen et al., 2010b; van der Vinne et al., 2014b). Rather than being merely enslaved, behavioural patterns are governed by downstream structures that besides rhythmic input from the SCN, might integrate many more cues to entrain and fine-tune their own rhythms, likely including and interconnected network of brain structures. How this incompletely identified network that determines the timing of rest-activity patterns can be modulated by energy balance is a daunting question to solve. Conceptually, the simplest interpretation might be that within or downstream from the SCN there is a switch that can be operated by negative energy balance cues, for example the coupling with either GABAergic or glutamatergic interneurons could

'flip a switch' and invert the signal, altering nocturnal to diurnal behavioural patterns (Fig 1a). More complex, there might be a dedicated structure downstream of the SCN, that drives the behavioural patterns, that itself acts like an oscillator. The structure might be capable of maintaining rhythmicity alone as an SCN-independent clock, or require rhythmic input from the LD-cycle, or the SCN, to be able to distinguish between diurnal and nocturnal phasing. Such an activity-timing coordinating oscillator (ACO) might itself change phase during negative balance or might only become involved during negative energy balance (Fig 1b). As we generally observe transient phase advances during the WFF-protocol, our interpretation is that the behaviour might be more likely governed by some activity oscillator mechanism, that a switch like inversion. By the experiments in this chapter, we hope to gain more insight which components might be constituting this ACO.

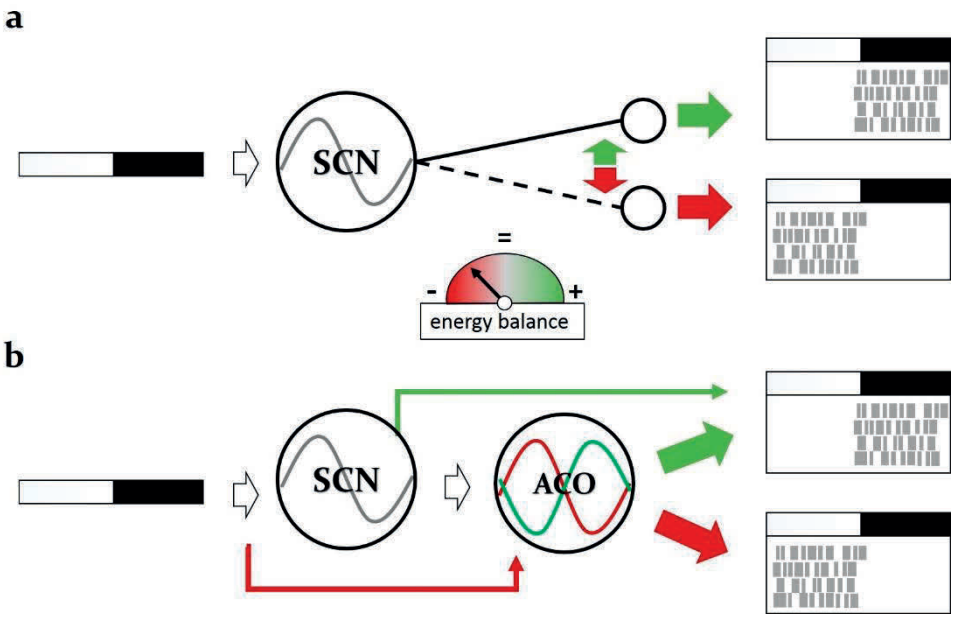


Figure 1. Theoretical framework of how the phase of behavioural rhythms might be modified by energy balance*. A) Provides a simple switch-like control, in which a rhythmic signal might change directionality. B) The more complex alternative is that a rhythmically oscillating structure that is downstream of the SCN, changes phase angle in relation to either this SCN or the LD-cycle during negative energy balance, allowing more transient shifts between a nocturnal and diurnal phenotype.*

Methods

Subjects

Male CBA/CaJ mice, born and raised the University of Groningen GELIFES-institute were used in a series of four experiments. In experiment 1, we compared circadian

activity patterns in wheel running behaviour of 8 adrenalectomized (AD_X) and 8 sham-controls (AD_{SHAM}). In experiment 2 the final sample size was 12 SCN-lesioned (SCN_X) versus 8 sham-lesioned controls (SCN_{SHAM}), with one-mislesioned individual excluded from the final analysis. In experiment 3, brains were obtained from 36 male mice on high workload (HWL) across different phases of the LD-cycle and compared with the brains from mice sacrificed at the same time points that were fed *ad libitum* (AL), after which these brains were processed and analysed after immunocytochemical staining for the immediate early gene C-Fos. Finally, in experiment 4 we compared behavioural responses in the WFF paradigm of 3 mice that received a double midline lesion aimed at the coordinates of the anterior paraventricular nucleus of the thalamus ($aPVT_X$) and 3 sham lesioned controls ($aPVT_{SHAM}$).

Housing and WFF system

In all four experiments, mice were individually housed in transparent running wheel cages (w14 x l40 x h21 cm), wheel diameter 14cm. Drinking water was present *ad libitum*. The food consisted of dust free rodent 45mg Precision Pellets® (#F0021, Bio-Serv, Flemington NJ, USA) that where either supplied in excess of daily intake (*ad libitum*) or had to be earned by completing wheel running revolutions (WFF-phase) operating an automated food dispenser system (#ENV-203-45IR, Med Associates Inc, Fairfax VT, USA). A standardized 12h:12h light-dark (LD_{24}) cycle was present for most of the experiments, during which two ceiling mounted fluorescent bright-polychromatic white light tubes provided 200-300 lux at cage level. In addition, we used dim red light during a 2-week period of "constant darkness" in experiment 2 (SCNx) to evaluate the success of the SCN-lesions and a period of continuous lights-on to determine whether self-sustained circadian rhythms were detectable during a high workload. The work-for-food paradigm was matched to individual activity levels, in which starting low workloads were determined by dividing the spontaneous number of daily wheel revolutions by the estimated spontaneous pellet intake. After habitation to the dispenser pellets and forming the association that wheel running would trigger the supply of food, excess pellets were removed and the workload (i.e., the number of revolutions needed per pellet) was increased with daily increments. Workloads used are discussed in more detail within the results section for the separate experiments. Body weights were measured multiple times a week, either at irregular and unpredictable times of day, or during times in which the animals were already active in their wheels (late light phase for example). The daily amount of wheel revolutions and the corresponding number of pellets supplied could be read-out and reported remotely from outside the experimental room, thus minimizing external stimuli that could modulate the behavioural patterns. Light and temperature were controlled automatically in our climate controlled experimental

rooms, which provided an environment with constant temperature (21-22°C) and stable humidity (55-65% RH) and minimal sound interference from other parts of the facility. Experiments 1 and 4 (adrenal and aPVT) were conducted with both the experimental and control group being housed interspersed in a single room. In the two experiments with larger sample size, 2 (SCNx) and 3 (cFOS) we used two adjacent climate rooms with identical set-up, dividing treatment and control groups across both locations.

Experiment 1: Adrenalectomized mice and WFF

In the first experiment we compared the behavioural response of adrenalectomized mice to high workloads in the WFF-paradigm with sham lesioned controls to evaluate if the adrenal glands play a pivotal role within the emerging diurnal patterns of activity during negative energy balance. We selected 16 young male CBA/CaJ mice for the experiment that were randomly assigned to the adrenalectomized (AD_X) or sham control group (AD_{SHAM}). Surgery procedures followed general guidelines and are described in more detail in the supplemental information. Final group sizes were 6 AD_{SHAM} and 7 AD_X respectively. Adrenalectomized were supplied with 1% saline as drinking water supplement in order to compensate for the impairments in renal salt retention following the procedure. Plasma corticosterone was determined in blood collected at the last light phase (ZT10-12) at the end of the study, after mice were returned to *ad lib.* feeding; AD_X = 36.5 ±23.3 (SD) ng/ml (min=5.5, max=61.0) versus AD_{SHAM} = 113.7 ± 33.8 ng/ml (min=66.5, max=170.0). No general differences in activity levels were noted and both groups received the standard WFF-protocol to evaluate circadian flexibility.

Experiment 2: SCN-lesioned mice and WFF

In the second experiment we tested if the observed diurnal circadian activity patterns that emerge under high workload conditions in intact mice are dependent on their suprachiasmatic nucleus (SCN) to (re)organize their behavioural rhythms. In total 24 male CBA/CaJ mice entered the study, 16 receiving bilateral thermos-electric lesions aimed at the coordinates of the SCN (SCN_X) and 8 serving a sham controls (SCN_{SHAM}). After recovery and screening for residual circadian activity in AL-fed mice under constant darkness (DD, 14 days), final group sizes were 13 SCN_X and 8 SCN_{SHAM} controls. Workloads in the WFF paradigm were adapted to start at lower than usual workloads, as the surgery caused a severe reduction in the spontaneous daily running-wheel activity levels, tailored as [average revolutions per day/120] and ranged from 20-40 for SCN_X and 70-100 for SCN_{SHAM}. The timeline of the SCN-lesioned WFF-experiment was complex, as we tested many different combinations of workload and light-dark environment, including WFF in LD, HWL in LL, LD and

DD, as well as the ability to maintain circadian rhythms following timed feeding. More detailed description of the surgery procedure, as well as the timeline of the experiment is provided in the supplemental information.

Experiment 3: CNS-screening for candidate regions involved in altered circadian rhythmicity under HWL

To gain additional insight which brain regions would be a candidate-proper for circadian flexibility in response to induced negative energy balance, we obtained the brains of diurnal mice sacrificed during HWL and those of ad-lib fed nocturnal controls in 2h intervals across their circadian cycle. 72 mice were randomly assigned to either the diurnal (HWL, n=36) or the nocturnal (AL-fed, n=36) group. The WFF protocol (in a 12:12 LD-cycle) lasted about five weeks for the WFF group: 1 week habituation and training, 3 weeks of increasing workloads ramping from ~100 revs/pellet to ~250 revs/pellet and one final week of stable high workload during which bodyweight remained stable. The AL group were fed the same dispenser pellets during the same duration. Mice were than sacrificed in their home cage by CO2-overdose immediately followed by transcardial perfusion and fixation. Post-fixation, storage, dehydration and sectioning details are provided in the supplemental information. Brains were prepared for the cryotome and cut into 25µm coronal sections. Sections containing our structures of interest spanned the whole hypothalamus (VLPO, SCN, DMH, VMH, ARC, LH, PVT), thalamic regions (habenula, PVT) and the more caudally located locus *coeruleus* (LC) which were microscopically identified and stored in 0.1M PBS/0.01% Azide (PH7.2) at 4 degrees prior to staining. Sections were stained by rabbit anti-c-FOS-Ab in 1:4000 as primary (Calbiochem, Ab-5, 4-14 Cat# PC38, Lot#D00134698) and biotinylated goat-anti rabbit as secondary antibody. After washing sections were further developed by a standardized DAB-staining procedure. Sections were mounted and analysed on microscope (Olympus Bh-2 at 4x magnification) and captured using a Leica DFC320 camera system. Images were processed using optical density for the regions of interest, subtracting their bordering areas as background using ImageJ software by an experimenter (MV) blind to both the time point and experimental condition of the image. For each experimental group, data from the same time point (n=3) were subsequently averaged, log-transformed and analysed for rhythmicity using CircWave software (Hut et al, unpublished) which applied forward linear harmonic regression to the data.

Experiment 4: aPVT-lesioned mice and WFF

As one of the areas of interest in experiment 3 we found a significant alteration in FOS-rhythmicity in the anterior portion of the thalamic paraventricular nucleus. As this structure is attributed a key-function in motivated behaviours (such as foraging

activity during energy deprivation) we speculated the aPVT might be a key candidate for the circadian behavioural flexibility. We therefore conducted experiment 4 as a pilot study to evaluate if this region is an essential coordinator of the diurnal activity that emerges under energetic challenge. 3 male mice received two thermoelectric lesions aimed at the aPVT-coordinates ($aPVT_X$) and 3 served as Sham lesioned controls ($aPVT_{SHAM}$). Both groups were subjected to a standard WFF-paradigm of about five weeks in length, in a regular 12:12 LD cycle, and the circadian characteristics of their wheel running activity were assessed.

Results

Experiment 1: Response of adrenalectomized mice to WFF

To determine if the adrenal glands are an integral and required component in circadian plasticity in dealing with induced negative energy balance by simulated food shortage, we applied a 6-week WFF protocol to AD_X and AD_{SHAM} controls in a standard 12:12 LD cycle. Following surgery and recovery, mice were housed individually in running wheel cages that registered the timing of wheel running. After being habituated to their wheels and gaining additional weight (24+ grams) we started to gradually increase the amount of wheel revolutions required to obtain a 45mg food pellet. After an initial increase in activity, the growth of the animals stagnated as they became food deprived at higher workloads. Once increasing workloads and the resulting food shortage induced weight loss, mice showed a phase advance in their circadian patterns of wheel running activity (Fig. 2) as previously reported (Hut et al., 2011; van der Vinne et al., 2014b). We did not observe notable differences between the adrenalectomized group and the control mice, which indicated the adrenal glands are not required in driving this adaptive change in circadian rhythms under negative energy balance. Follow up of the diurnality-inducing high workload with a low workload (leading to weight gains and with mice obtaining surplus daily pellets), both groups showed a transient return to a more nocturnal activity pattern (Fig 2). After 7 days, mice were sacrificed at the end of the light phase (ZT 9-12) and blood samples were obtained for plasma corticosterone analysis. AD_X mice had significantly lower levels of circulating corticosterone compared too Sham-controls; 36.5± 10 and 113.7± 12 ng/ml, mean and SEM, $p<0.0007$ by two tailed t-test with Welsch-correction (Fig S1).

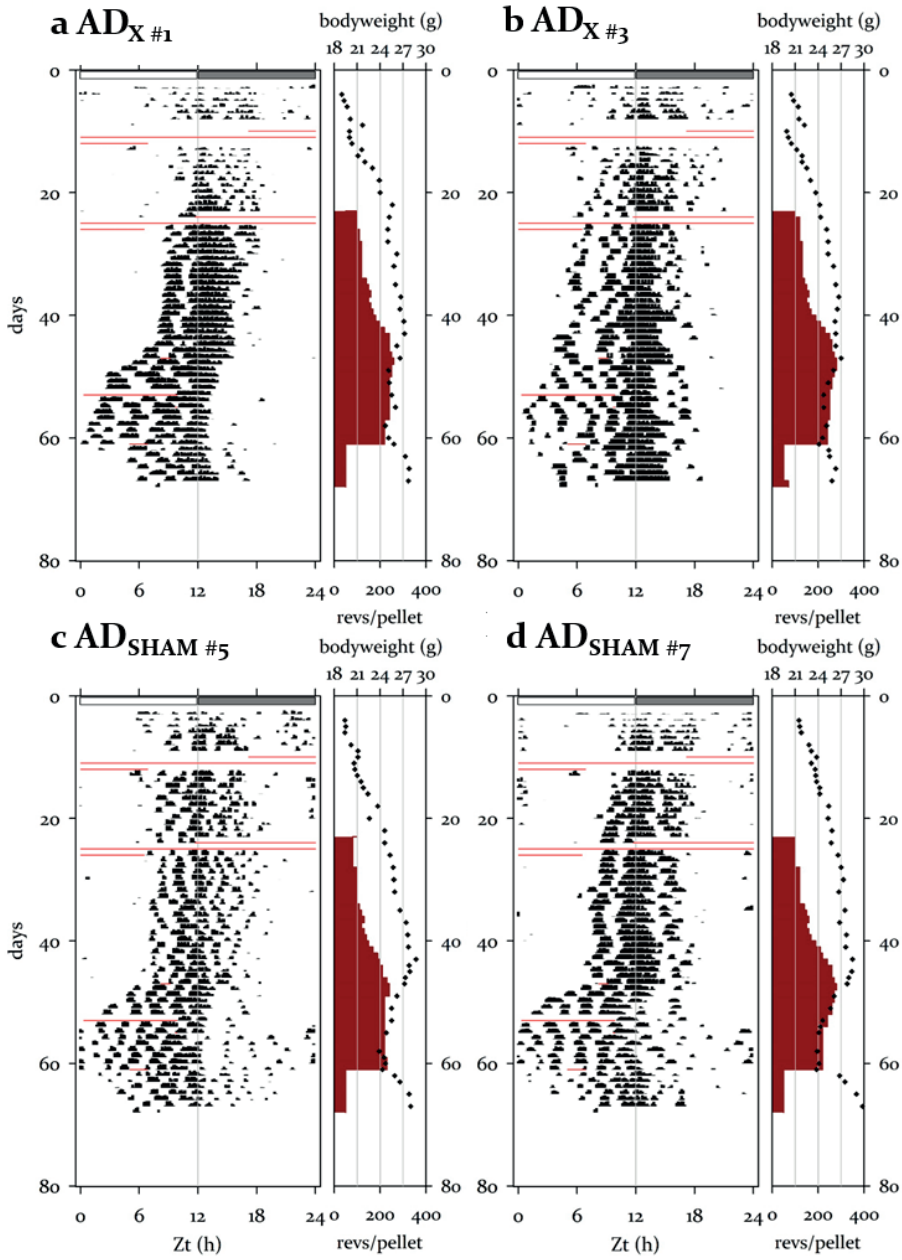


Figure 26. Behavioural response of adrenalectomized mice (a,b) and sham controls (c,d) to the work-for-food protocol. *Panels next to each actogram display change in BW (top axis) and workload (horizontal bars, bottom axis) respectively. Horizontal red-lines in the actogram denote missing data, due to registration-system malfunction. During high workload induced negative energy balance, as evidenced by weight loss, both AD_X and AD_{SHAM} controls showed phase advances and increased diurnality.*

Experiment 2: Response of SCN-lesioned mice to WFF

By subjection SCN_X mice to the WFF-paradigm we wanted to establish if the SCN is required for the changes in behavioural activity phasing during negative energy balance. First, we checked if SCN-lesions were successful in disrupting circadian behaviour by housing the mice in continues dim light whilst fed *ad libitum*. Activity patterns over 14 consecutive days were analysed by Lomb-Scargle (LS) periodogram analysis (Ruf, 1999) to determine the dominant periods in wheel running patterns, using the Chronoshop software package (Spoelstra, 2015). Periodogram analysis is the behavioural equivalent of spectrograms for light-wave composition, aligning underlying frequencies to find the dominant wavelength(s) or period(s), respectively. Strong circadian impairments and near eradication of circadian behavioural patterns after lesions were clearly evident, and supported by actigraphy inspection (Fig. 3, S2). In addition, histological evaluation at the end of the study revealed no clearly discernible SCN-structure in SCN_X and no signs of SCN damage in SCN_{SHAM}.

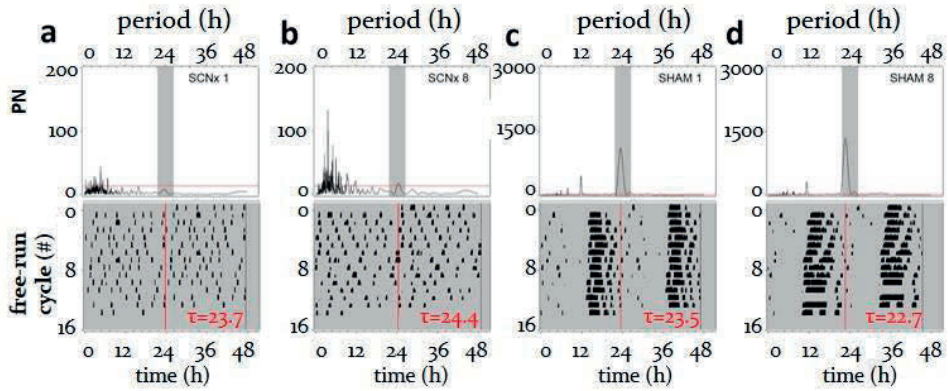


Figure 3. After surgery and recovery animals were housed in constant dim red light to assess the impact of SCN-targeted bilateral lesions in circadian rhythmicity. *Data of two representative SCN_X (a,b) and SCN_{SHAM} controls (c,d) are shown. Lomb-Scargle periodogram analysis of the wheel running data revealed near absent periodicity in the circadian domain of lesioned individuals and clear and dominant circadian rhythmicity in the sham controls (top panels). Actograms in the lower panels are double-plotted based on each individual's maximal circadian peak as indicated by the red vertical line.*

Following lesion evaluation, both groups were exposed to the WFF-paradigm under a standard LD_{24} cycle (Fig 4). Grouped activity profiles for high and low workloads revealed that SCN_X mice in general had slightly higher activity during the day (Fig 5a) although it was chaotically scattered in little bouts (Fig 4a, b). Sham controls, in agreement with previous findings, showed a gradual phase advance and increased

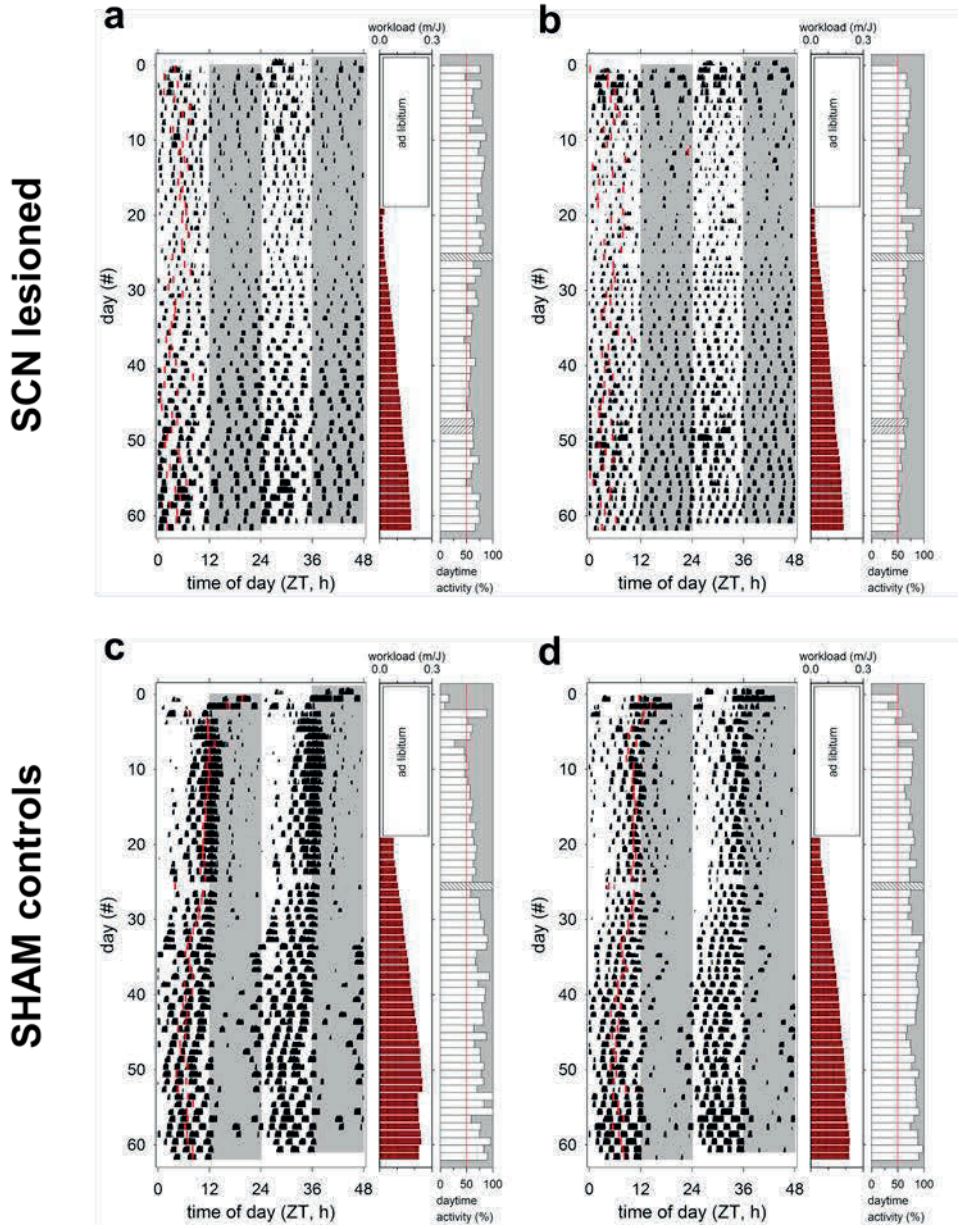


Figure 4. Behavioural response of SCN-lesioned mice (a,b) and sham controls (c,d) to the work-for-food protocol. *Panels denote workload and the percentage of daily activity shown during the light phase. Red marker indicates CoG in left half of the actograms. Double plots are used to improve readability of the actigraphy.*

the proportion of their daily wheel running during the light phase (Fig 4c, d). Mean activity profiles for SHAM group conformed a clear phase advance into the light

phase (Fig 5b). After reaching the high workloads under an LD-cycle, we subsequently tested how the two treatment-groups organized their behaviour under constant light (LL) with HWL (Fig 6). SCNx mice did not display any phase or regularity in their behaviour under LL when experiencing HWL. In Sham mice, the combination of LL and HWL disrupted circadian rhythmicity in some (Fig 6c) and lengthened the circadian period in others (Fig 6d). Within external rhythmic input (LL) and without SCN, high workload was thus not able to induce rhythmicity in the wheel running behaviour of lesioned mice.

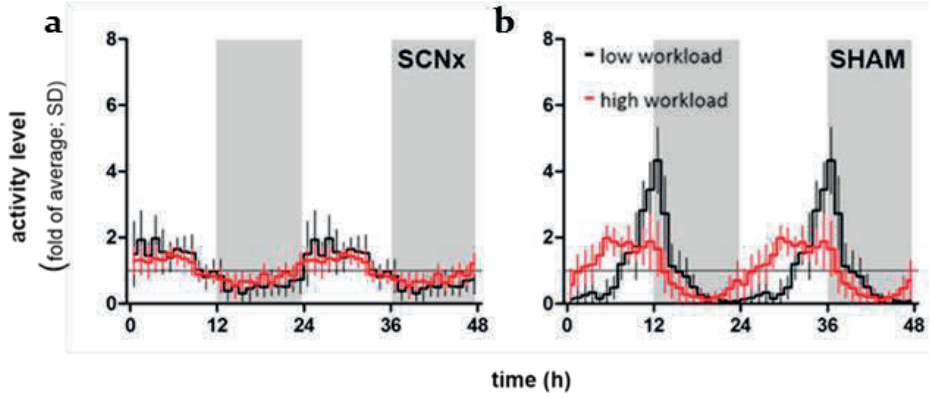


Figure 5. Mean activity profiles from 7-days of activity from low and high workload conditions for SCN_X (a) and Sham controls (b). *Due to highly variable activity levels, both between and within treatments, data was normalized to fold of average (daily counts/24) and plotted in hourly bins. Sham mice showed a phase-advance under high workload whereas the weak overall circadian profile for lesioned mice remained similar in phase.*

Next, we tested if inducing diurnal circadian rhythmicity by timed feeding could induce more condensed circadian rhythms in SCNx mice, and if subsequent HWL-LD release was able to maintain this diurnal organisation of behaviour when an LD-cycle was present. Furthermore, we also repeated timed feeding followed by a HWL

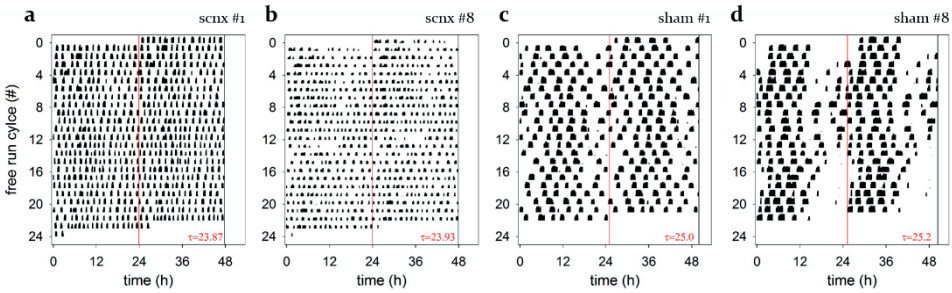


Figure 6. Double-plotted actigraphy based on dominant circadian peak of two SCN_X (a,b) and sham controls (c,d) of 21 days in LL+HWL. *In most animals, LL increased arrhythmicity and remaining circadian patterns were increased in period.*

release in constant darkness to address if endogenous circadian rhythms engaged with timed feeding (attributable to the FEO) could be maintained under negative energy balance. In our interpretation, TF induced clear food anticipatory activity which was maintained in phase for prolonged periods of time (>2 weeks) in the LD-HWL release for SCN_X and sham controls (Fig 7a-d). Without an external LD-cycle, HWL release in constant light led to a transient loss of the FEO-organised rhythmicity in SCN_X mice, suggesting part of the FEO might be capable to incorporate LD-input. HWL-LL release after timed feeding in Sham controls lead to

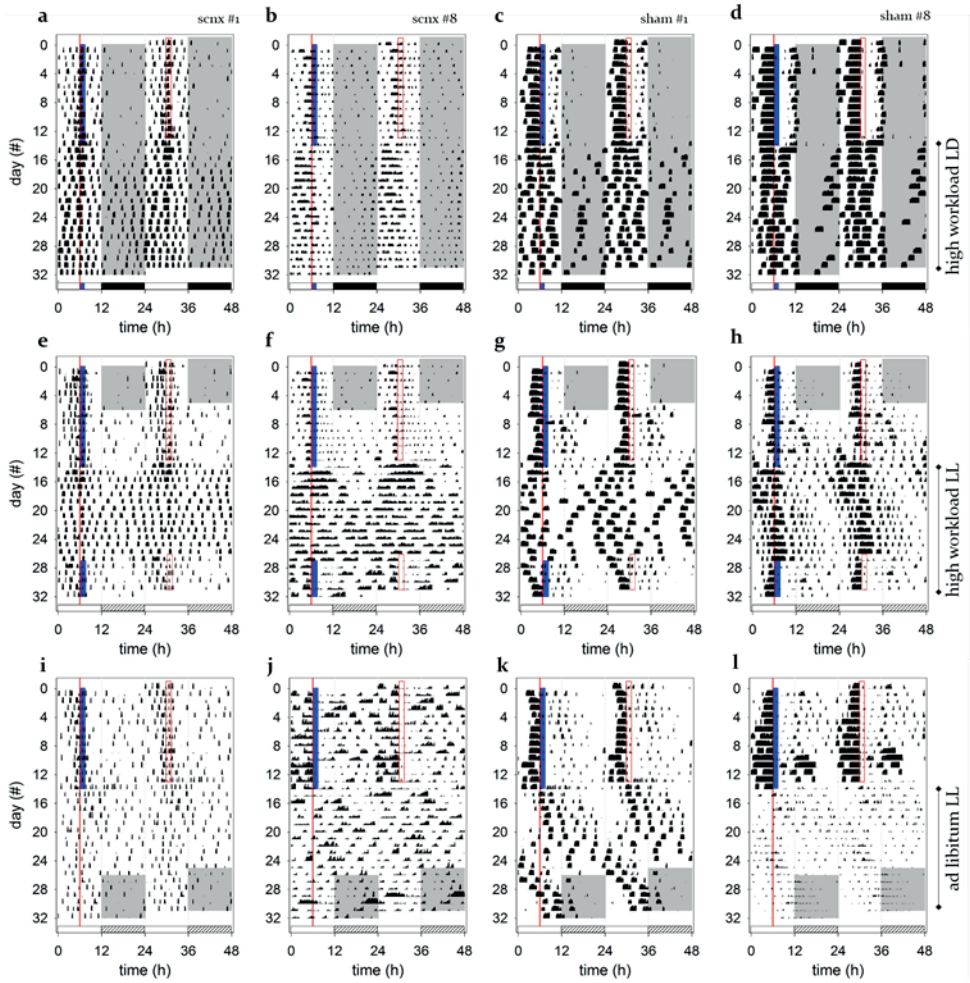


Figure 77. Induction of FEO-driven circadian rhythms by timed feeding in SCNx (a,b,e,f,i,j) and sham controls (c,d,g,h,k,l), *with subsequent releases with HWL+LD cycle (a-d), HWL in constant light (e-h) and release with AL feed availability in LL (i-l). Blue bars denote timed feeding of ~75 % of AL intake (comparable with HWL intake).*

mixed phenotypes, with some individuals drifting in phase and dispersing their rhythm cohesion (Fig 7g) and others maintaining their phase as induced during TF quite well (Fig 7h). Release of lesioned mice in LL with AL food led to immediate arhythmicity (Fig 7i, j) and free running patterns with long tau (as expected under LL) with onsets departing from the previous feeding time (fig 7k,l). A complete overview of the consecutive stages and behavioural patterns across the experiment is provided in figure 8.

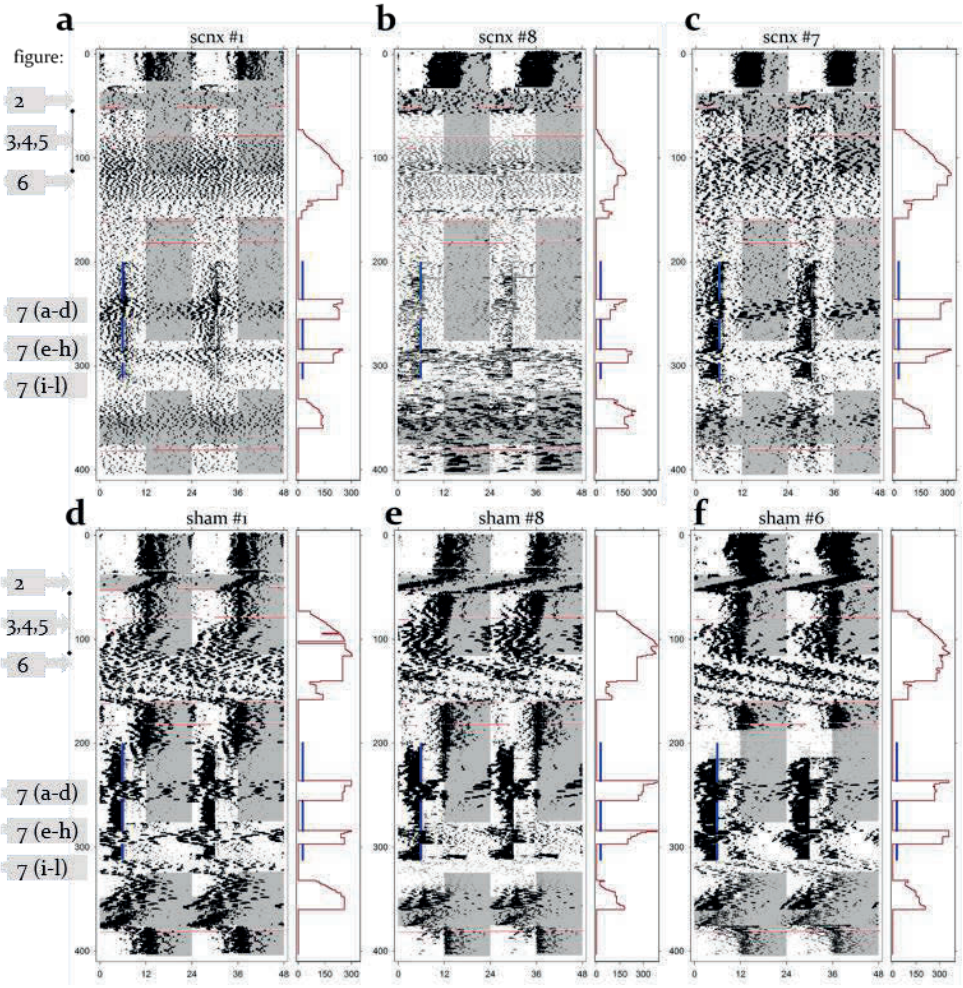


Figure 8. Actigraphy across the full duration of the study, indicating general behavioural patterns for SCN$_X$ (a-c) and Sham controls (d-f) across the various stages of the experiment. *Closer discussion of relevant responses is provided in the preceding figures (indexed in the left margin).*

After completing our behavioural analysis based on the visual evaluation of actigraphy, we also mathematically assessed the changes in circadian organization of wheel running patterns across the various stages of these experiments, applying the Lomb-Scargle periodogram analysis over ten-day intervals of our dataset (Fig 9) hoping to provide some insights into the relative effects of workload, light-dark cycles and the FEO on circadian organization. For each interval, we determined each individual's dominant circadian period (tau) and the dominance of the period in the overall wheel running patterns (normalized power = PN).

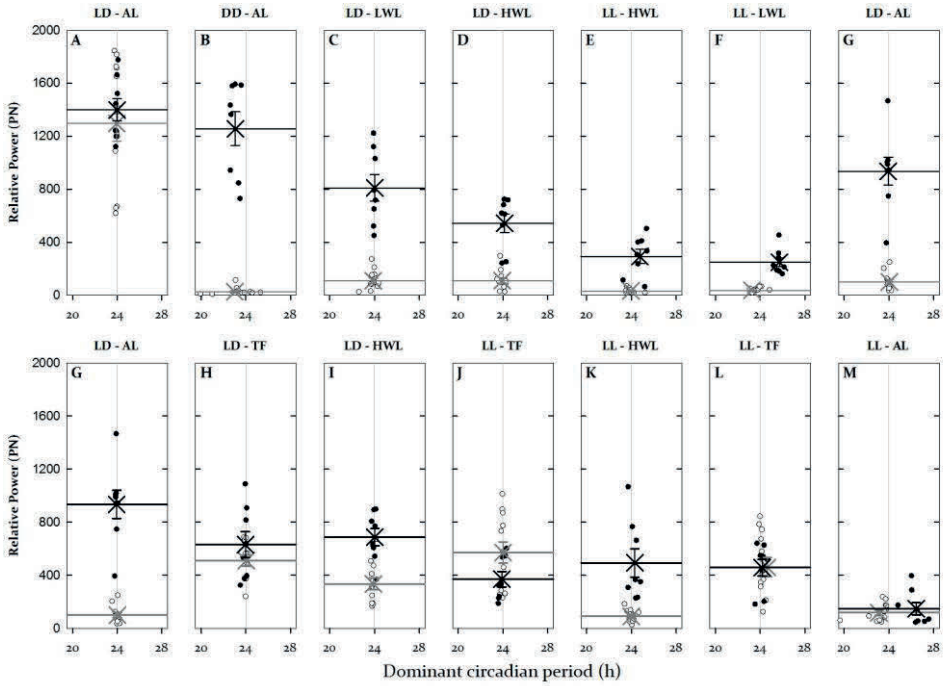


Figure 9. Lomb-Scargle periodogram analysis showing the contributions of the SCN, the FEO, ambient light conditions and energy balance to the circadian organisation of wheel running behaviour. *Dominant circadian period (tau) of each individual is plotted against the dominance of the period within the overall wheel running pattern (PN). Groups are indicated by the symbols (open for SCN_X, black for sham-lesioned controls) and their mean and SD are shown in grey and black respectively.*

Experiment 3: Identification of CNS candidate regions involved in circadian flexibility

Behavioural responses of the 36 mice in the HWL group matched our previous observations that exposure to a high workload (~250 revs/pellet) causes a phase advance in the activity-rest patterns of male mice. However, we observed a large variation in phases between individuals in both groups, with the average COG of HWL mice being only 111 minutes phase advanced. Of the regions of interest, we

found a significant circadian rhythm in the optical densities of c-FOS-stained sections for the suprachiasmatic nuclei (SCN; AL=0.001/WFF=0.022), supramammilary nucleus (SUM; AL=0.001/WFF=0.006) and the anterior region of the thalamic paraventricular nucleus (aPVT; AL=0.166 /WFF=0.018). Differences in phase and amplitude of these rhythms were small for the SCN and SUM when comparing HWL and AL controls, meaning circadian patterns in activation were not significantly altered by the work-for-food condition (SCN; p=0.83 / SUM; p=0.78). For the aPVT however, the weak circadian oscillation observed under AL (p=0.17) was clearly more rhythmic in the high-workload group (p=0.018), with high FOS expression occurring during the hours of food consumption (ZT 0-10) (Fig 10). Of note, whereas we did a significant single harmonic fit (i.e., a convincing 24-hour pattern to the FOS levels) for brain regions like the arcuate nuclei (ARC), dorsomedial hypothalamic nucleus (DMH) and ventromedial hypothalamic nucleus (VMH), structures involved with feeding and energy balance, we failed to find evidence for either significant changes in amplitude or phase between HWL and AL group, although the approach of using optical density in c-FOS/DAB contrasted sections is a relatively crude measure, despite our best efforts to perform a strong circadian test by choosing a high frequency 2-hour interval between our time points.

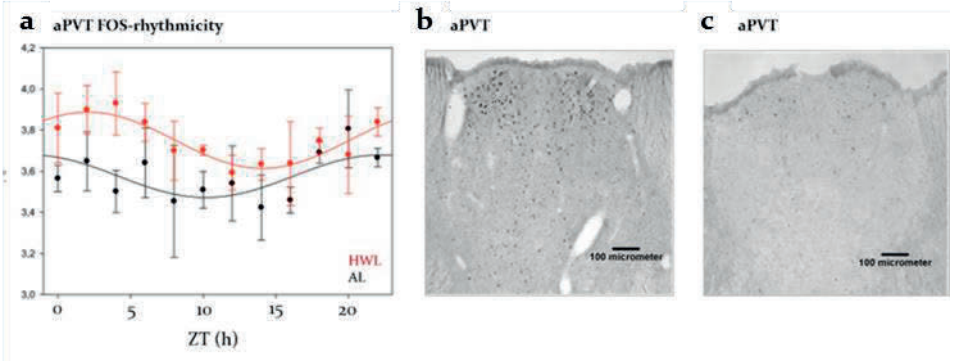


Figure 108. Shifted FOS rhythmicity in the aPVT of mice under HWL. *A) Fitted circadian patterns in optical density of the aPVT after FOS-staining. Fits consist of a single harmonic wave (indicating a 24-hour pattern) that was ns. in AL-fed (black line) but became significant in HWL mice (red line). B) Example of FOS immunoreactivity in the aPVT of a diurnal HWL mouse at ZT2. C) Example of FOS immunoreactivity in the aPVT of a HWL mouse at ZT 12.*

Experiment 4: aPVT-lesioned mice retain adaptive circadian flexibility in response to high workload

Lesions aimed at the coordinates of the aPVT were unable to prevent the adaptive circadian advance of behaviour in the work-for-food protocol. Lesion dimensions were assessed by histology and converted to standardized coronal sections (Fig. 11).

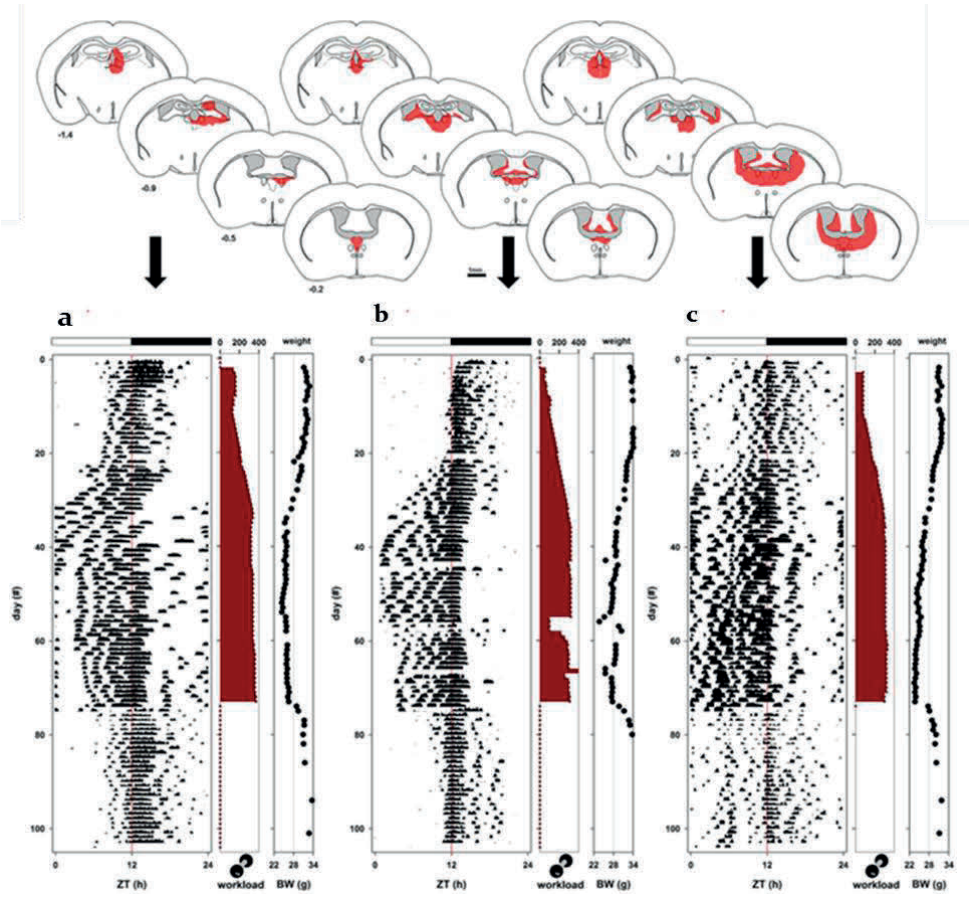


Figure 91. Behavioural response to the work-for-food protocol of aPVT lesioned mice. *Despite varying degrees of damage the aPVT following surgery (destroyed tissue marked in red) all animals retained a pronounced diurnality shift during high workload. Release to AL-feeding furthermore appeared to induced a normal direct return to the nocturnal phenotype, with some possible irregularities in individual c.*

The behavioural response to the work-for-food protocol, with diurnality increasing during high workloads, was not altered by damage to the aPVT and remained fully intact even in the individuals with most extensive damage (Fig. 11c).

Discussion

Adrenal glands are not required for increased diurnality during WFF

We showed that adrenalectomy, which greatly reduced circulating levels of plasma corticosterone (S1), did not prevent the behavioural response of increased diurnal wheel running activity under negative energy balance induced during the WFF

protocol (Fig 2). Following up the high workload with a very low workload, resulting in surplus food acquisition resulting and weight gain, led to a transient return to a more nocturnal phenotype. In previous experiments, and in experiments 2 and 4 this return to the nocturnal niche seems to occur more abruptly (Hut et al., 2011) but the protocols differed in that in those instance the mice were provided *ad libitum* chow, uncoupling the relation between wheel-running and food acquisition. It is however conceivable that the adrenals, or the hormones produced, play a facilitating or stabilizing function of physiological rhythms, reducing the rate in which physiology can change phase. Most importantly however, adrenalectomized and sham control mice did differ in their gradual behavioural phase-advance during increasing workloads, leading to the interpretation that the adrenal glands are not critically required in the adaptive circadian phenotype of behavioural rhythms induced by the WFF paradigm.

The SCN is required for circadian organisation under HWL

Without a functional SCN, behavioural organisation is largely arrhythmic under constant conditions irrespective of the metabolic status (fed *ad lib.*/low workload/high workload). When a LD-cycle was present, our SCN-lesioned animals tend to show slightly more activity in the light phase, but this was not dependent on their energy balance (Fig. 5a). Negative energy balance by itself is not capable of triggering diurnal behavioural activity patterns in the absence of the SCN, and these experiments thus do not provide proof for a self-sustaining extra-SCN circadian activity oscillator with a diurnal phase preference (ACO, Fig.1).

FEO might provide circadian input or overlap with the circuitry coordinating diurnal activity under negative energy balance.

The FEO was identified to alter circadian light entrained activity patterns by food timing. It thus may provide overlap with a putative non-SCN circadian pacemaker that drives WFF induced diurnality. Timed feeding indeed reinstated circadian patterns in the wheel running behaviour of SCN-lesioned mice (Fig 7), as many others showed before (Dibner et al., 2010; Mistlberger, 2011; Stephan et al., 1979). Subsequent release with untimed but reduced food availability (i.e., high workload) was capable to maintain this circadian organisation of behaviour in LD (Fig 7a-d), but much less in combination with constant light (Fig 7e-h). To our knowledge, this is the first time that circadian rhythmicity that likely results from the FEO, can be observed as an endogenously driven circadian oscillation for extended periods of time, as *ad lib.* feeding leads to instant abolishment (Fig 7 i-l). Difficulties faced when studying the FEO are that food restriction generally provides external timing cues, and complete food deprivation cannot be maintained for long periods in small mammals with high metabolic rates due to starvation. The combination of TRF and

HWL in a WFF paradigm thus might also been an interesting experimental tool to utilize in research on the underlying mechanisms of food entrainment, as it can apply varying levels of food restriction without providing a timing signal (the food-reward per rotation is the same irrespective of time of day). The strong contrast between HWL-LD and HWL-LL release of SCN_X mice seems to suggest that the underlying circuitry that drives these circadian rhythms is capable of integrating and/or phase-locking with the LD-cycle. Combinations of TRF at different circadian phases followed by prolonged food restriction using the WFF-paradigm might offer a new approach to gain more insights into the FEO-circuitry and its properties, including questions such as *if* and *to what extend* the FEO and our proposed ACO (Fig. 1) are (parts of) the same functional circuitry.

High diurnality in SCN_{SHAM} controls prior to WFF

As to our SCN_{SHAM} mice, in Fig 4, we showed the behavioural response of SCN_X and SCN_{SHAM} mice during the WFF paradigm. Although there is a clear phase advance in the phase of entrainment in the controls, there is already high diurnal activity (up or even above 50%) preceding the increases in workload. A possible explanation might be that this batch of animals had some difficulties with accepting the food dispenser pellets as their new diet, thus losing bodyweight (encountering negative energy balance) prior to starting the workloads. We tried to counter this by providing a mixture of grinded dispenser pellets mixed with standard chow and eventually replaced dispenser pellets with newly ordered food-dispenser pellets (even though the bags were within their expiration date, newly opened for the experiment and stored at -20°C prior to use). Indeed, the phase of entrainment when fed *ad lib.* chow (prior to the surgeries, see Fig 8, day 0-15) appeared later and more nocturnal when no such potential challenge was present.

Circadian rhythm periods and strength (PN scores during various phases)

In an attempt to infer the relative impacts of workload (energy balance), the food-entrainable oscillator (FEO) and ambient light conditions we scored the changes in tau and normalized circadian power across the experiment in figure 9. Although this analysis remains incomplete to draw solid conclusions and express the contributions numerically, some interesting aspects should be discussed. Starting with periodicity in the data from the SCN_{SHAM} controls, we observed that mice remained entrained (their dominant period matching 24h) in all LD-conditions (Fig 9 A,C,D,G,H,I) and in the timed feeding intervals (Fig 9 H,J,L); as would be expected since a Zeitgeber with a 24h period is provided. Notably, this period remains closer to 24h when mice are released from timed feeding on high workload in LL (Fig. 9k), but not when fed *ad lib.* in LL (Fig. 9m) when the shams free-run with periods much longer than 24h. We interpret that due to the HWL, mice maintain a feedback cycle of obtaining a

large meal at the time when they anticipated the previously provided meal, as the anticipatory activity itself yields food rewards and food intake follows immediately upon acquisition. In terms of the dominance of the circadian rhythms in their activity patterns, Sham mice show a reduction of the circadian power when they have to work for their food versus when they are fed AL (Fig. 9 G>C>D). This makes sense, as we tend to see that activity patterns become more fragmented under WFF, with smaller bouts of high intensity wheel running (to reach the threshold to obtain a food pellet) followed by breaks. This fragmented nature amplifies the relative contribution of ultradian frequencies in the overall rhythm and as they are not occurring at the identical time of day on subsequent days will reduce the circadian power. Constant light furthermore is known to decrease circadian organisation in wild-type rodents, which is partly due to effects on constant light on SCN-amplitude (Hughes et al., 2015) as well as potential direct effects of light suppressing wheel running activity (Alves-Simoes et al., 2016; Daan and Pittendrigh, 1976). The finding that circadian power is lower in intact mice during timed feeding also makes sense; in these animals both the SCN and the FEO have an impact on the behavioural patterns, which can have divergent phases (the timed meal was provided mid-day) – and under LL can in addition have divergent periods (Fig 9 H>J,L). Noteworthy, release from TF with high workload in LL (Fig 9K) preserved circadian patterns better than release with AL in LL (Fig 9M) for the SCN_{SHAM} controls.

For the SCN-lesioned mice, the circadian organisation of their wheel running patterns was greatly reduced following surgery, as was intended. In terms of the remaining dominant period, all conditions were close to 24h, suggesting the period modulating effects of constant light or darkness might invoke changes to period solely due effects on the SCN. The relative circadian power was low overall but was fortified when an LD-cycle was present, possibly due residual masking activity (Gall et al., 2016; Redlin and Mrosovsky, 1999). Modulating energy balance by the WFF-paradigm, despite having large effects on total daily activity levels, did not alter the relative circadian power (Fig 9 C=D=G). This suggest that the WFF paradigm itself does not "engage" an extra-SCN circadian oscillator. Timed feeding was effective to induce robust circadian rhythmicity in lesioned mice, which appeared unaffected by light conditions (Fig. 9 H=J=L). Release from timed feeding under high workload was able to preserve circadian organisation only when a rhythmic LD-cycle was provided (Fig. 9 I>K). It is an interesting thought that the mechanism involved in food entrainment might also be capable of utilizing light-dark information. The WFF protocol might be an interesting tool to study the "free running" and self-sustaining properties of the FEO in future experiments.

Concluding remarks

In this chapter, we show the results of a series of experiments aimed at gaining a better understanding of which structures might play a crucial role in making mice adopt a more diurnal phase of entrainment during negative energy balance. The results reveal a clear role for the SCN in the temporal niche switch between nocturnal and diurnal behaviour, while the adrenal glands and the aPVT do not play a critical role. In addition, we found modest indications that the FEO and the temporal niche switching mechanism may share common regulatory elements.

Supplemental Methods

General surgery procedures in the lesion-experiments: Surgery, aimed to bilaterally ablate the SCN, ablate the PVT or remove the adrenals respectively, was performed when mice were between three and four months of age. Surgery was performed in our in-house surgery theatre that is exclusively used for surgery in small rodents (mice and rats). Gas anaesthesia was provided in the form of isoflurane (1-1.5%) and oxygen-enriched air (2%) supplied over a fitted nose cone. Measures to retain body temperature were taken during each surgery procedure. Eyes were lubricated with salve to prevent dehydration, eye infection or damage. Long-acting pain relief was provided prior to the surgery, as well as 24h post-op. Skin and muscle incisions were preceded by topical analgesics (lidocaine). Body weights were checked once or twice a day for the week following surgery to ensure animal welfare. Adrenalectomy procedures were performed by our most experienced veterinarian technician (C. Reitzema) whereas assistance and advice for SCNX was provided by training in Charlottesville VA (J. Mohawk) and experienced researchers in our own department (C. Mulder), the aPVT surgeries being performed almost identical apart from different lesion coordinates. Detailed descriptions of the individual surgery protocols used is provided in the sections below, and supplemental information. Mice were habituated to the experimental room and solitary housed in wheel running cages for a minimum of 1 week prior to surgery and allowed ample time to recover, including pain management and thermal support by heating pads during and days post-surgery. Body weights were checked daily in the week following surgery. Recovery and recuperation periods were around 10-14 days, before starting the subsequent experimental conditions. In the ADX group, drinking water was replaced after surgery by 1% saline solution to compensate the renal salt-recovery impairment inflicted by this procedure. Experiments were ethically approved by the University of Groningen (DEC#6545) and followed European guidelines on animal studies.

Experiment 1 – Adrenalectomy procedure: as survival following adrenalectomy is reportedly highest in young animals, a cohort of n=16 male CBA-CaJ mice 7-8 weeks of age (~20 grams) were selected to participate in this experiment aimed to establish the involvement of the adrenals in WFF-induced circadian realignment. From the mice that underwent surgery, 6 out of 8 sham-controls (AD_X-sham) and 7 out of 8 adrenalectomized mice fully recovered after surgeries and completed the study successfully. Animals received gas anaesthesia and injection analgesia during surgery and after recovery were provided with 1% saline in their drinking water, compensating the impaired salt-retention following adrenal ablation. Adrenals were reached and exposed through a small (1.5-2cm) dorsolateral incisions in their flank

and the adrenal structures externalized and removed by cauterization (AD_X) or merely inspected and returned to their original location (SHAM) for the two treatment groups respectively. Following recovery - which was rather quick with animals seemingly returning to normal active and explorative behaviour on the first day post-surgery – wheels were returned after 2-3 days. A stable period of 10 days of wheel running behaviour was obtained for each animal after which the WFF paradigm was started. Weights were monitored daily, and high workloads and the associated increased diurnality phenotype was reached within 1.5 to 2 weeks. A 10 day period of stable high workloads was analysed for circadian characteristics followed by a two week wash-out period with mice returned to low workload.

Experiment 2 – SCN-surgery procedures: to evaluate if the SCN is required for the diurnal wheel running rhythms that appear during simulated food shortage, we subjected SCN lesioned mice and appropriate sham-surgery controls to the work for food paradigm. Animals were sedated by isoflurane gas inhalation (3% Isoflurane and 5 units of oxygen). After the animal was fully sedated, as checked by toe pinch reflex, animals were secured in a stereotact using ear bars and tooth bar and levelled horizontally. After placement, isoflurane was turned back to 1.5% to maintain anaesthetized state within the stereotactic device. Regular breathing and hart rate were monitored during the full procedure. Animals were shaved using small animal clippers and their scalp was disinfected three sets of alternating swaps with iodine (betadine) and 70% ethanol. Topical analgesia (lidocaine) was applied to the scalp prior to making a midline incision, starting behind the eyes (just before Bregma) and continuing until lambda was visible. A single small midline hole (⌀ 1 mm) was drilled 0.3mm posterior to Bregma using a dental drill bit. Bilateral thermoelectric lesions were placed to the coordinates of the SCN (0.32 ⌀0.1 mm posterior to Bregma, 0.25 mm lateral to midline, 5.32 ⌀ 0.1 mm deep, adjusted to skull size by measurement of Bregma-Lambda distance) by inserting a coated tungsten-wire electrode with a 0.2mm exposed tip into the hypothalamus and running at 110 volts, 1.1 mA current for 20 seconds. No current was run and the electrode inserted 0.3 mm less deep for SCN_{SHAM} controls to avoid damage to their SCN. After running the currents, 30 seconds of cool down were applied prior to retreating the electrode. After both sides were lesioned, the scalp was closed and fixed using surgical wound glue. All procedures were carried out under clean and where possible sterile conditions. Prior to recovery, a 0.8 ml injection of warmed (37 °C) physiological saline with 2.5% glucose was given subcutaneously, as well as 0.2 cc of Finadyne (1 mg/ml) for pain management and its anti-inflammatory properties. During both surgery and recovery normothermia was supported, by 50ml warm-water tubes flanking the mouse in the stereotact and by heating pads under the cage respectively.

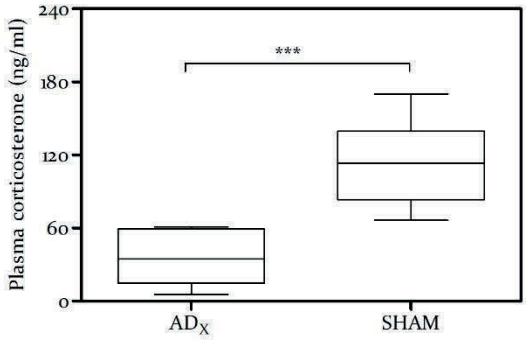
Experiment 3 – evaluating CNS- neuronal activation rhythms by c-Fos expression: In order to screen for potentially relevant structures involved with the circadian reorganization observed under high workload in the WFF-paradigm we obtained brains from 36 (diurnal) mice from a high workload group (~250 revs/pellet) and 36 nocturnal controls (fed *ad libitum*) at 2h intervals. Mice were sacrificed by CO_2 overdose in their home cage under their standard LD-cycle, immediately followed by transcardial perfusion with 0.1 M phosphate buffered saline (PBS, pH 7.2) and subsequently fixated by paraformaldehyde (4% PFA/0.1 M PBS). Brains dissected and post-fixed in (4% PFA/0.1 M PBS) for 48 hours at 4°C after which they were transferred to 0.1 M PBS/0.01% azide (pH 7.2). Brains were dehydrated in a 30% sucrose/0.1 M PBS (pH 7.2) solution for 24h prior to freezing and sectioning into 25µm slices on a cryotome. Sections were collected and stored in in 0.1 M phosphate buffered saline/0.01% azide (pH 7.2) until processing. Sections were stained in three separate series (series 1: SCN, PVN, PVT, series 2: VMH, DMH, ARC, SUM, series 3: LC). Sections were washed (3x 10-min., 0.01 M PBS, pH 7.2) and endogenous peroxidase activity was removed (1x 60-min., 0.01 M PBS/0.75% H_2O_2 /50% alcohol). Incubation of brain sections took place (1% normal goat serum (NGS)/0.01 M PBS/0.5% Triton X/0.01% Azide) for 60-min. Sections were placed back on 0.01 M PBS (pH 7.2). Rabbit polyclonal antisera for FOS (Ab-5, 4-14, Cat# pc38, Lot#D00134698, Calbiochem, 1:4000) was added to NGS solution. The second incubation lasted 72 hours (1% NGS/0.01 M PBS/0.5% Triton X/0.01% Azide/1:4000 FOS) at 5°C. Following incubation sections were washed (2x 10-min., 0.01 M PBS/0.5% Triton X, pH 7.2) and incubated with the secondary antibody (1% normal goat serum/0.01 M PBS/0.5% Triton X/0.01% Azide/1:300 2nd AB (biotinylated goat – anti rabbit, code# 111-066-047, lot# 70903, Jackson Immunoresearch, Westgrove, PA)). Subsequently sections were washed (2x 10-min., 0.01 M PBS, pH 7.2) and processed using avidin-biotin-peroxidase (Vectastain Elite ABC-kit, PK6100, Vector Laboratories, Burlingame, CA). Sections were washed (2x 10-min., 0.01 M PBS/0.5% Triton X, pH 7.2) and pre-incubated with TRIS-HCL (2x 10-min, 0.05M TRIS, pH 7.6). Sections were developed with DAB (20 mg. DAB/0.05 gr. Nickel(II)sulfate/100 ml 0.05 M TRIS-HCL) which was activated with 333 µl H_2O_2 (0.6% H_2O_2/0.01 M PBS/0.5% Triton X) in 1 liter DAB. Staining was definitively stopped by washing with PBS (3x 10-min., 0.01 M PBS, pH 7.2) followed by a standard rinsing and mounting procedure.

Experiment 4: Lesions of the aPVT.

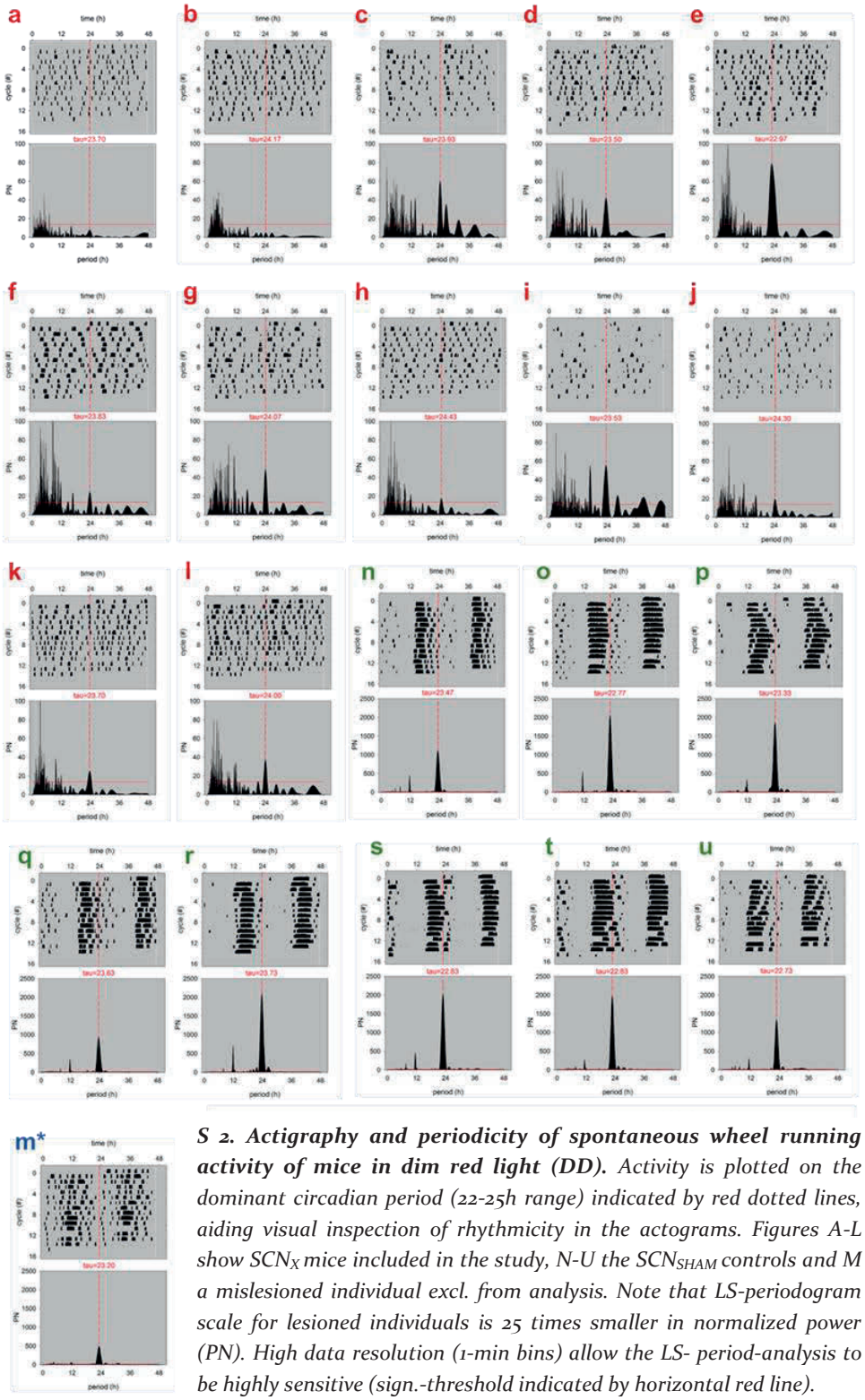
Experimental procedures in experiment 4 were identical to those for SCN-lesions as described under experiment 2, apart from the placement of the lesions. To lesion the aPVT two lesions were made in the midline; 0.87 and 1.63 mm posterior to Bregma.

The electrode was lowered to a depth of 3.0 mm. Following the experiment from which behavioural actigraphy was collected, mice were sacrificed, and brains were histologically assessed to evaluate dimensions of the resulting lesions, as shown in the main text. Due to the much closer succession between surgery and the end of the study, this evaluation of damage and lesion dimensions was much more feasible than in experiment 2 (where lesions and sacrifice were separated by more than 12 months).

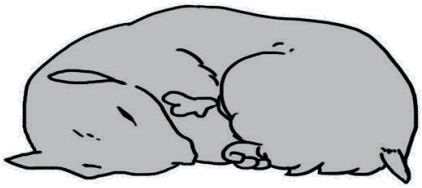
Supplemental Figures



S 1: Circulating corticosterone levels at the end of the experiment 1 *(obtained during low workloads and resulting weight gain and return towards a more nocturnal niche). T=4.87, 10, P<0.0007 by two tailed t-test with Welch correction. Wiskers denote minimal and maximal values within each treatment group.*



S 2. Actigraphy and periodicity of spontaneous wheel running activity of mice in dim red light (DD). *Activity is plotted on the dominant circadian period (22-25h range) indicated by red dotted lines, aiding visual inspection of rhythmicity in the actograms. Figures A-L show SCN_X mice included in the study, N-U the SCN_{SHAM} controls and M a mislesioned individual excl. from analysis. Note that LS-periodogram scale for lesioned individuals is 25 times smaller in normalized power (PN). High data resolution (1-min bins) allow the LS- period-analysis to be highly sensitive (sign.-threshold indicated by horizontal red line).*



Chapter 7

How does a hibernator know when to stop hibernating

DISPATCH PAPER

This chapter was published as an invited dispatch paper to accompany and highlight the importance of the findings of the paper "*A Circannual Clock Drives Expression of Genes Central for Seasonal Reproduction*" by Sáenz de Miera et al., Current Biology 2014.

Chapter 7

How does a hibernator know when to stop hibernating

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Chapter has been published as a dispatch paper
In *Current Biology* (2014); doi: 10.1016/j.cub.2014.05.061

Deep hibernators that spend winter in a hypothermic coma below ground can still emerge and reproduce in spring at the right moment. A recent study shows that specific cells of the pituitary may harbor the internal calendar responsible for this.

Many species in seasonal environments enter a state of dormancy in winter to avoid unfavourable conditions, such as low temperatures and reduced energy resources. This behaviour can be found in micro-organisms, plants, invertebrates and vertebrates. An example of a unicellular organism that enters winter dormancy is the dinoflagellate *Alexandrium (Gonyaulaceae*). It drops to the sea floor to enter a state of winter quiescence when days get short and light availability is low. A similar behaviour occurs in deep hibernators like ground squirrels and several hamster species: they retreat in their burrows, seal the entrance and stay below ground for 6–8 months in a state of deep hibernation with body temperatures slightly above ambient temperature (~5–10°C) (Hut et al., 2002). Although winter dormancy may increase survival, it also introduces a problem: both the algae in the mud of the sea floor and the hibernators in their burrows are overwintering under stable conditions in the absence of light. Since perception of day length — photoperiod — is critical for seasonal timing, how can they possibly 'know' when spring has come? These organisms thus need a seasonal timing mechanism that works like a calendar: an internal representation of the annual cycle. Such a circannual clock was indeed first described in ground squirrels (reviewed in Helm et al., 2013) and later also in *Alexandrium* (Andersen and Keafer, 1987). The advantage of having a circannual clock is particularly clear in hibernators, but it is certainly not limited to this group of vertebrates and circannual clocks have been well characterized in sheep, rodents and birds (reviewed in Helm et al., 2013). Although the site of the circannual pacemaker has long remained elusive, evidence in sheep indicated that an autonomous circannual timing mechanism may reside in a small layer of endocrine cells surrounding the pituitary stalk, the pars tuberalis (Lincoln et al., 2006; Sáenz de Miera et al., 2013). Now, as reported in this issue of Current Biology, Sáenz de Miera et al. (Sáenz de Miera et al., 2014) provide further evidence using a deep hibernator species, the European hamster, that the pars tuberalis could be the site of the circannual pacemaker.

The internal calendar in hibernators regulates the timing of hibernation, body mass and the activity of the reproductive system. The latter two parameters are essential for hibernation to occur: sex steroids inhibit hibernation and body fat reserves are required to enter (and survive) hibernation. It therefore seems impossible to use hibernation as a winter survival strategy without a functional circannual clock. This

clock is usually self-sustained and keeps oscillating under a limited set of species-specific conditions, but it requires at least part of the annual photoperiodic cycle for seasonal synchronisation. Whereas *Alexandrium* can carry these essentials in a single cell, the annual timing system in vertebrates is much more complicated (Figure 1) (Hut, 2011).

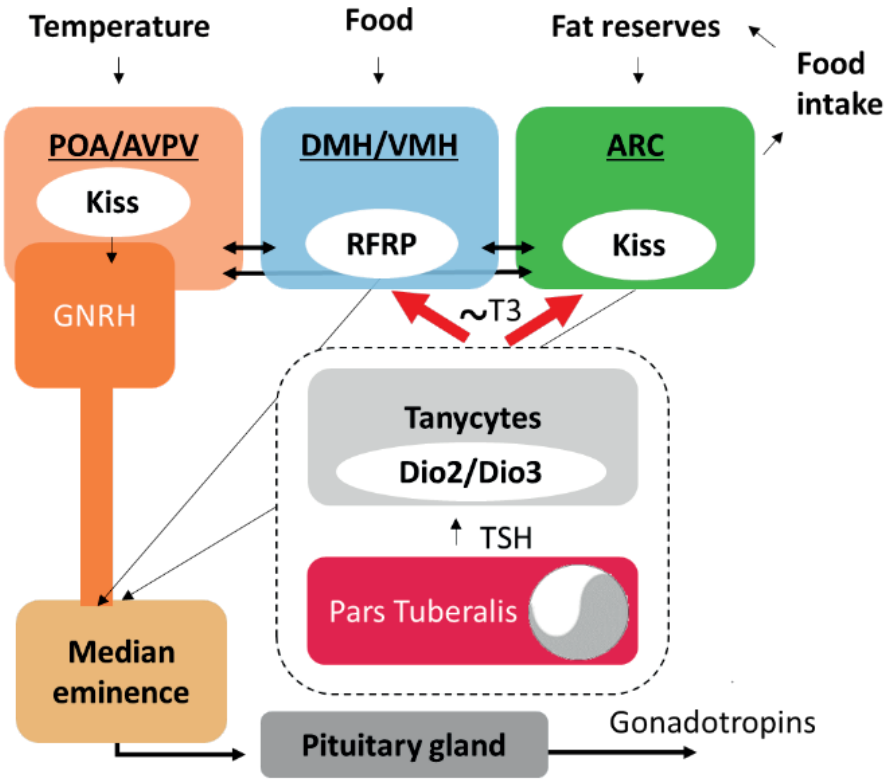


Figure 1. Current view of the circannual system driving reproduction. *A mammalian circannual pace-maker in the pars tuberalis drives conserved seasonal changes in thyroid hormone metabolism (dashed box). How this signal converges on the regulation of reproductive state remains partly speculative (indicated by the red arrows). The RF-amide neuropeptides Kiss-peptin and RFRP may play an important role to mediate and modify the pars tuberalis-driven T3 signal. Interestingly, they are localized in areas (arcuate nucleus, ARC; dorso- and ventromedial hypothalamus, DMH/VMH; pre-optic area, POA; anteroventral periventricular nucleus, AVPV) involved in fat mass regulation, food intake, body temperature, and sexual behaviour, respectively. A simplified representation shows that the annual T3 signal may be mediated by go/no-go signals from Kiss and RFRP neurons in these areas that drive seasonal breeding when the state of the animal and its habitat would allow for successful reproduction.*

The synchronising photoperiodic induction pathway has been well characterized over the past decade (Figure 2). In mammals, melatonin constitutes the internal representation of night and therefore carries day length information to target tissues.

Among these, the pars tuberalis is central. The photoperiod-decoding mechanism within the pars tuberalis is complex and involves an interaction between the melatonin signal and a local circadian clock, which results in the long-day induction of the transcription factor EYA3. The latter allows the production of thyroid-stimulating hormone (TSH) to increase dramatically as animals experience lengthening days in spring (Dardente et al., 2010). TSH triggers tanycytes around the third ventricle to produce deiodinase type-2 (DIO_2), which converts the thyroid hormone Thyroxine (T_4) into the biologically active form T_3. Hypothalamic T_3 levels eventually affect gonadotropin releasing hormone (GnRH) neurons in the preoptic area (POA), which govern gonadal development (Figure 1). This chain of events constitutes the missing link between photoperiod and the long-recognized role of thyroid hormone in seasonal reproduction (Dardente et al., 2014). Even though the exact mechanisms are not understood, there is broad consensus that T_3 does not impact GnRH neurons directly but rather acts through distinct hypothalamic populations of neurons, which express two different RF-amide peptides, namely kisspeptins (Kiss) and RF-amide related peptide (GnIH in birds, RFRP in mammals) (Parhar et al., 2012). Recent data from Klosen et al. (Klosen et al., 2013) have considerably substantiated this working model for the photoperiodic control of reproduction. Now Sáenz de Miera et al. take another step forward and demonstrate that expression of TSH in the pars tuberalis and RF-amides in the hypothalamus exhibit a spontaneous change when hamsters are kept under the same photoperiod for an extended period of time. These data strongly suggest that the pars tuberalis possesses a circannual clock responsible for ending hibernation and starting reproduction in early spring.

The photoperiodic induction pathway, from light perception to heightened T_3 production under long days, presents some important similarities in mammals and birds (Hazlerigg, 2012; Nakao et al., 2008). However, contrary to small mammals that develop gonads under long photoperiods (long day breeders), larger mammals with long gestation times (like sheep) develop gonads under short days (short day breeders); yet all these species show the same photoperiodic control of T_3 (Figure 2). Furthermore, the photoperiodic response may be modified by food availability and temperature (Caro et al., 2013; Visser et al., 2009). Indeed, some species (e.g., mice, rats, and voles) even display a largely opportunistic behaviour and their gonadal activation seems to depend mainly on food availability and temperature rather than T_3 signalling (Caro et al., 2013; Król et al., 2012). The action of T_3 on gonadotropin production thus differs between species and conditions. The underlying mechanism for this variation is largely unknown, but some clues exist.

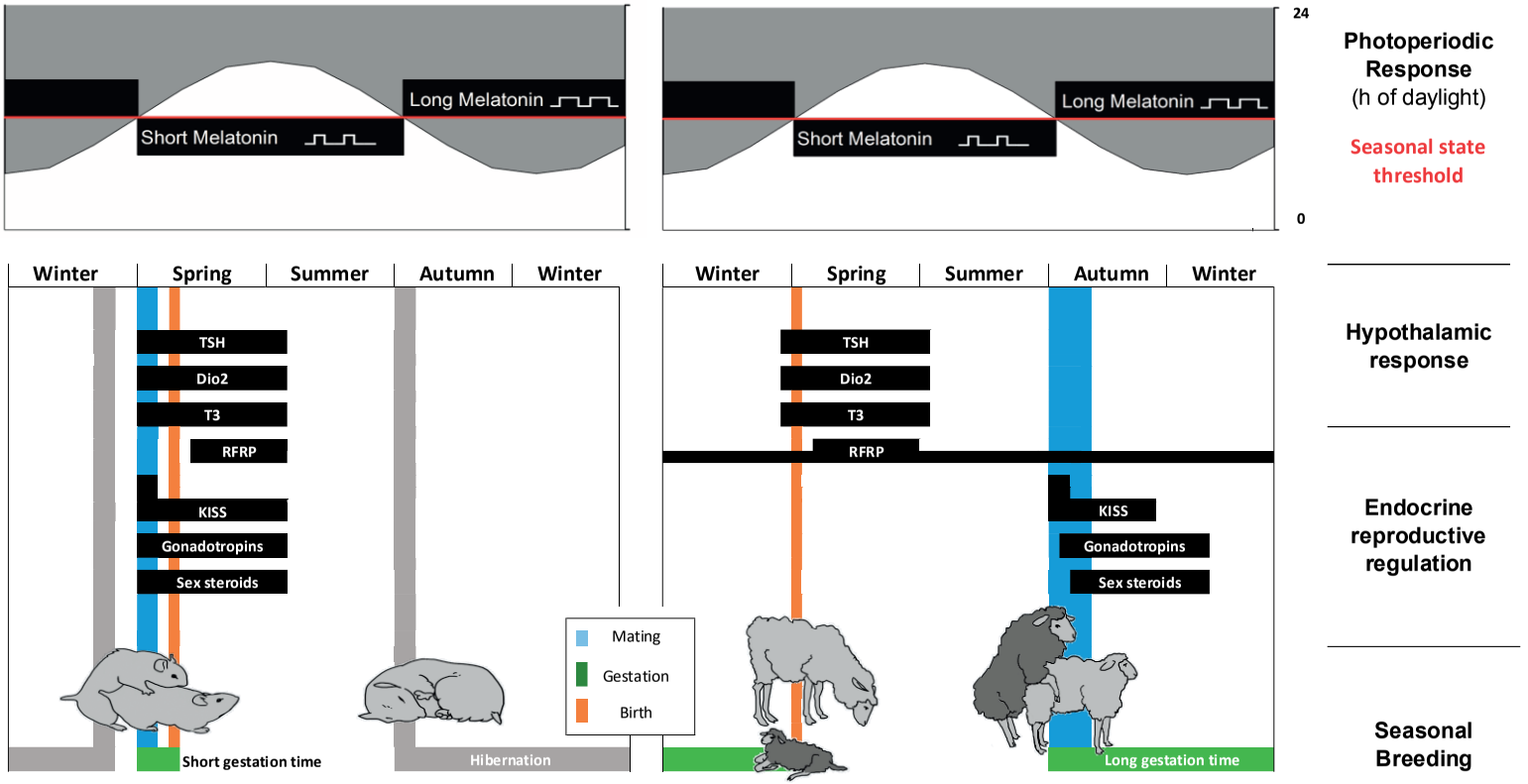

A. Long-day breeders (e.g . hamster); short gestation time
B. Short-day breeders (e.g . sheep); long gestation time
Long Melatonin
Short Melatonin
Long Melatonin
Short Melatonin
24
0
Photoperiodic Response
(h of daylight)
Seasonal state threshold
Winter
Spring
Summer
Autumn
Winter
TSH
Dio2
T3
RFRP
KISS
Gonadotropins
Sex steroids
Mating
Gestation
Birth
Short gestation time
Hibernation
Long gestation time
Hypothalamic response
Endocrine reproductive regulation
Seasonal Breeding

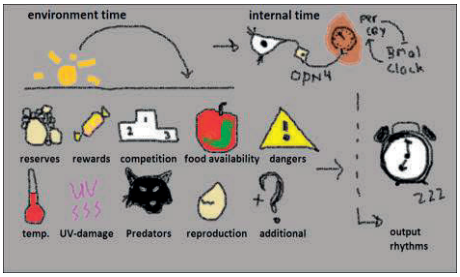
Figure 2. Simplified annual timeline of a short day breeder (European hamster) and long day breeder (sheep). *Changes in day length (photoperiod) shape the pattern of melatonin synthesis by the pineal gland (top panels). A conserved hypothalamic response (equal in short and long day breeders), driven by the melatonin-sensitive pars tuberalis, relays this seasonal information to the production of T3. From there, species show considerable differences. Kisspeptin marks the onset of reproduction in both short and long day breeders (but is repressed by testosterone feedback). The role and phase of RFRP expression is less clear, but suggested here to be expressed at higher levels during the summer for both species, possibly indicating opposing modes of action.*

Compared to mammals, birds seem to lack (parts of) the Kiss signalling system (Pasquier et al., 2014) and have different types and anatomical locations of the gonadotropin inhibitory hormone receptors (the avian RFRP homolog) (Ikemoto and Park, 2005). This may relate mechanistically to the observation that all non-tropical birds are long day breeders (the emperor penguin is an interesting exception Groscolas et al., 1986). A long day breeding strategy combines with fast embryonic development, which minimizes the duration of the vulnerable incubation period in the egg. Such differences between the avian and the mammalian RFamide systems may accommodate the variation in mammalian reproductive strategies (Figure 2). These strategies involve various levels of interaction between photoperiodic, nutritional and thermal cues. The RFamide peptides appear well placed to integrate these and direct differential responses of the gonadal axis. Therefore, understanding how these cues impact the RFamide system and how and where they interact is crucial (Figure 1).

One site of interaction might be the preoptic area, which is involved in temperature regulation. GnRH (and Kiss) neurons are located in the preoptic area (among other regions) and environmental temperature responses of the annual cycle might be mediated here (Figure 1; reviewed in Caro et al., 2013). Additionally, Kiss neurons in the Arcuate nucleus (Arc) may be involved in mediating energy balance to the reproductive system. Arc-Kiss neurons have excitatory synapses to POMC neurons which may serve as a conditional relay station allowing reproduction only when sufficient food is present (Fu and van den Pol, 2010). Mammalian Arc-Kiss neurons also express leptin receptors, suggesting that the Kiss system incorporates signals regarding the amount of fat reserves. Additionally, RFRP neurons are located in the dorsal and ventral hypothalamic nuclei, which are regions regulating feeding behaviour and energy balance (Figure 1).

Overall, the hypothalamic RFamide systems (Kiss, GnIH, RFRP) are excellent candidates for modifying the proximate cue of photoperiod towards species-specific seasonal strategies and integrating energy balance information (food, fat, temperature) to the reproductive system. Describing second messenger pathways and mapping the neuroanatomical network of hypothalamic RFamide signalling will

be the challenge for the near future. Comparison of mammal and bird species with different reproductive strategies will most certainly help to solve this intriguing puzzle of neuroanatomical mechanisms underlying optimal timing of hibernation and reproduction.



Chapter 8

Discussion

DISCUSSION

Circadian rest-activity rhythms (and circannual rhythms) are tailored by environmental influences to adapt rhythmic output patterns, making them display the right behaviours at the right time to optimize their fitness. In this discussion we summarize the main findings and statements provided in this thesis.

Chapter 8
Discussion

The interaction between energy balance and circadian niche in small mammals

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Discussion

Daily changes in the complex natural environment may offer opportunities for animals to maximize survival and fitness by optimizing behavioural timing and maintaining energy balance (**Chapter 2**; van der Veen et al., 2017; Vinne et al., 2019). In balancing the (changes in) different needs, it may therefore be optimal for animals to change from nocturnality to diurnality or *vice versa* (temporal niche switching). Specifically, flexibility in circadian organization has been shown to occur when food scarcity is simulated in the lab using the work-for-food protocol (Hut et al., 2011; van der Vinne et al., 2014b), as well as in enclosures under semi-natural conditions (Vinne et al., 2019). According to the previously postulated Circadian Thermo-Energetics hypothesis (Hut et al., 2012), the shift from nocturnal to diurnal activity patterns allows small euthermic animals to conserve energy, mainly because insulation and heterothermia during the rest phase saves more energy when aligned with the night when ambient temperatures are colder (**Chapter 3**; Riede et al., 2017).

Light is known to have direct effects on behaviour, such that it stimulates activity and arousal in diurnal species, while it suppresses activity and stimulates sleep in nocturnal species (Altimus et al., 2008; Fernandez et al., 2016; Mrosovsky, 1994; Pilorz et al., 2016; Redlin, 2001). One prerequisite for nocturnal animals to exploit the diurnal niche, is therefore that the activity suppressing effect of light become weaker or reversed to an activity stimulatory effect under negative energy balance. When nocturnal mice become diurnal under the work-for-food protocol, the modest activity suppressing effect of light is indeed reduced during the subjective night, while light seemed to increase activity levels during the subjective day (**Chapter 4**).

In contrast to expectations based on previous work (Mrosovsky, 1994) we found very small effects of light exposure suppressing activity levels in *ad lib.*-fed control mice during their (subjective) night. We can only speculate why this is the case. One explanation might be that suppression of non-goal directed activity (spontaneous wheel-running activity) is different from the effect of light on foraging activity (running in a wheel to obtain food), although this would only account for the HWL treatment. Second, the light levels used in our experiment (~200-300 lux at cage level) might be less potent in modulating activity levels than intensities of light used previous studies that did show larger activity suppression by light during the subjective night (Thompson et al., 2008). Furthermore, the melatonin proficient CBA/CaJ mice might display lower light masking than other strains, such as for example like the C57bl/6J background. As the circadian niche of animals determine the strength and direction of light masking, the relatively early phenotype in our AL-fed mice could also reduce the magnitude of their negative light masking (Redlin, 2001). It would be interesting to test light-masking and WFF-responses in different strains in future experiments. We have identified previously that male mice from the C57bl6/Per2::Luc strain also show phase-advances to diurnality during HWL (Master

thesis S.J. Riede), and *ad lib*. fed c57bl/6J mice have a strong nocturnal preference and robust behavioural masking response, making the newly generated melatonin-proficient C57bl/6J line (Zhang et al., 2018) and the standard (melatonin-deficient) C57bl/6J lines an interesting comparison for future masking evaluation both without and during energetic challenge.

The change in circadian niche, displaying more diurnal activity during challenging energetic conditions, was previously explored using male mice in the work for food paradigm. Whether or not female mice show similar adaptive circadian responses during (simulated) food scarcity was uncertain. Previous work to energetically challenge mice without external timing cues is limited. Instead reported strategies utilize daily torpor to reduce thermogenesis costs and reduced reproductive capacity as primary coping mechanisms (Perrigo and Bronson, 1985; Schubert et al., 2008; Schubert et al., 2010), albeit tested under different experimental settings. We now show that female mice also show a change in their circadian organization in the work-for-food paradigm when subjected to increasing workloads, but they have considerable more flexibility in their amount of daily wheel-running or foraging efforts, thereby delaying the moment they start to encounter weight loss. Once weight loss occurs, activity patterns start to phase advance like in males, leading to more diurnal wheel running activity (**Chapter 5**).

Thermogenic differences due to body size, metabolic rate or reproductive physiology might make females less inclined to alter their circadian organization towards a diurnal phenotype. First, males in many species have more variable reproductive success than females (Bateman, 1948). Leaner and smaller males might be expected to be less likely to acquire social dominance or maintain a territory reducing their chances of generating offspring which could arguable make them weight risks and benefits different from females (Jolles et al., 2015). As the survival of the offspring depends strongly on maternal survival until they reach independence, females might show a higher level of predator/risk avoidance. As the metabolic demands on females are very high during lactation, females might have more capacity to increase foraging efforts and time-allocation to obtaining foraging than males (Dias et al., 2011). In addition, it has been postulated that selection pressures in females might have been oriented towards heat-loss rather than preserving energy according to the heat dissipation limitation theory (Speakman and Król, 2010). Collectively all these arguments point that selection pressures might favor females to be more resilient in maintain a nocturnal phenotype during decreasing food availability compared to males.

In humans, cortisol is sometimes referred to as the internal alarm clock. It rises during the night, prior to waking up and shows an additional 'cortisol awakening response' peak (Clow et al., 2010; Jones and Gwenin, 2021). This suggests that cortisol, or the rodent corticosterone equivalent, is crucial for driving the onset of activity. Indeed when mice become diurnal under WFF, their corticosterone-rhythms are

markedly phase advanced (van der Vinne et al., 2014b). To test the involvement of corticosterone in driving diurnality in mice, we tested adrenalectomized mice in the WFF protocol (**Chapter 6**). We found that adrenalectomized mice retained the capacity to become diurnal. This shows that the adrenals and their secreted hormones (including adrenal corticosterone, aldosterone, adrenalin and noradrenalin) are not crucially involved in temporal niche switching in mice. Cautionary, these hormones can also be produced to lesser extends by other tissues and organs. Furthermore, the adrenal glands can potentially show re-growth after ectomisation. However, it is important to note that the most successful ADx operated mice, which having very low levels of plasma corticosterone, still readily became diurnal under high workload conditions (**Chapter 6**).

In both nocturnal and diurnal mammals, the SCN is crucial for driving normal daily rhythms in sleep and activity. The neuronal activity and clock gene expression in the SCN is entrained by the light-dark cycle and therefore does not differ between nocturnal and diurnal mammals. Nocturnality and diurnality is therefore expected to be determined by neuronal (or even peripheral) structures downstream of the SCN (Hut et al., 2012). It has been hypothesized that this downstream structure could either be characterized as a 'switch' or perhaps as an alternative circadian (slave?) oscillator. Such an alternative oscillator is known to exist in circadian food entrainment and is called the food entrainable oscillator (FEO; see Blum et al., 2012; Mistlberger, 2011; Pezuk et al., 2010). Under temporal food restriction protocols, where ~70% of the *ad lib*. daily food intake is offered at the same time of day, the food entrained FEO can drive anticipatory daily activity rhythms independent of the SCN (Mistlberger, 2009; Pendergast and Yamazaki, 2014). To test whether the WFF diurnality is also independent of the SCN, we subjected SCN lesioned mice to high workloads (HWL). Rhythmic behaviour was virtually absent in SCNx mice under HWL LD, and completely disappeared under DD and LL (**Chapter 6**). This indicates that HWL, which also reduced food intake to ~70%, is by itself insufficient to induce an alternative circadian oscillator driving diurnal activity. This does not exclude the possibility that the FEO is essential for driving the diurnal phenotype. We tested for this possibility by kick-starting the FEO under food restriction, immediately followed by HWL. This indeed seems to induce modest levels of circadian rhythmicity under LL (and LD) in SCNx mice, suggesting that there is a fair possibility that the alternative oscillator, driving temporal niche switching in mice, is indeed independent and downstream from the SCN and potentially shares similar network structures with the FEO (**Chapter 6**).

Testing patterns of neuronal-activation in several brain regions involved with energy homeostasis by immune-histological analysis we identified that FOS-levels appeared to be elevated and in a different phase in brains of mice sacrificed during high workload versus nocturnal *ad lib.* fed controls in the anterior portion of the paraventricular nucleus of the thalamus (**Chapter 6**). As this brain region has been

implied with regulation of (food)motivated behaviours and modulated circadian behaviours (Do-Monte et al., 2017; Salazar-Juárez et al., 2002) it made an interesting target to assess potential involvement in negative energy balance induced circadian adaptive behaviours. aPVT-lesions however did not prevent a shift towards diurnality indicating this brain region is not crucially involved in the diurnality response to high workload (**Chapter 6**).

The possibility that nocturnal and diurnal activity patterns are driven by an extra-SCN oscillator, poses the question what the importance of the SCN is under natural conditions. Out of a spectrum of potential answers, we chose to highlight the essential importance of the SCN for annual rhythmicity and photoperiodic response (**Chapter 7**). The primary role of the SCN therefore seems to be to produce an internal representation of the phase and ratio of the external light-dark cycle, which is most important for burrowing mammals that exclude themselves regularly from this environmental information.

Taken together, the chapters in this thesis show consistently that the circadian system is capable of considerable flexibility in that sleep-wake rhythms can adaptively change their phase angle with the external light-dark cycle. The core circadian pacemaker in the SCN is essential for this temporal niche switching behaviour, but maintains its light-dark driven phase angle of entrainment and thereby maintains a truthful internal representation of the external light-dark cycle. This stable SCN entrainment is functional because it satisfies its important role in the photoperiodism endocrine system that drives timing of reproduction. The mechanism responsible for temporal niche switching is likely a slave oscillator downstream of the SCN that requires its input from the SCN but may also share mechanisms or structures with the food entrainable oscillator.

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Acknowledgements

The road to completing the research and this final booklet, has been far from easy. It has certainly not been the direct path I envisioned when it all started, back in 2013 when I received the initial email by the (than) BCN-graduate school director **Prof. Jaap Koolhaas** that he was delighted to inform me my research proposal was awarded with a 2+3 Ubbo Emmius award. Even writing down this acknowledgement section poses the daunting task to remember all the people that have guided, taught, cooperated, supported and helped me in any shape or form to reach this moment. But some major words of gratitude are certainly warranted as I could never have reached this milestone, nor gain the valuable experience without the help of countless friendly, patient, clever and enthusiastic people providing me with their help, time and input at the right moments in time.

First and foremost I am very grateful for the input, help, support, patience and guidance of **Roelof Hut.** You have really made all the difference in helping me get to where I am today. It was your style of supervision during the bachelor course that really showed me how much fun it is to do science; putting down an open assignment (design an animal-model for human shiftwork) and going through all the steps of experimental design. Instead of merely teaching or directing research projects you are a true master in triggering people to think scientifically for themselves, develop idea's and become capable to discuss them with peers and experts and design the right exoerperimental methods to test and validate them. You welcome diversity in inputs and angles, which really strengthens the quality of the whole team. I am very lucky to have had you as my supervisor, mentor and friend! The believe you have put in me has been astounding, from arranging a temporal position for me with help of **Luc Schlangen** (Phillips) for a project on twilight effects on entrainment, sending me as a first year BCN-research master student abroad to **Mike Sellix** and **Prof. Menaker** in Charlottesville (VA) to run WFF-experiments (thank you to **Jenifer, Asli, Pinar, the three Mike's** -including mini-mike -**, Denise, Ron, Dan & Erin** for the great time I had with you) right up until the last couple of years when at times I was struggling to keep the work on this thesis going. Also outside of office hours you were always there for me, and I carry many fond memories including whiskey Fridays in Haren with you and Peter, the guitar evening on our C'ville porch, the darts matches (which strengthened my academic Excel skills as a bonus), the sailing at Zuidlaardermeer and the visits to Onnen.

Besides Roelof there was a whole team of people that were essential to both the science and the joy I found in my PhD project. **Domien, Menno, Peter** and **Serge** were great in our weekly meetings to discuss all the different projects ongoing

within the group, providing key questions, sharing crucial experiences and anecdotes that helped to solve the complex puzzles, identify gaps in knowledge or flaws in reason. But the atmosphere created by them also made that all the people in the group, from PhD-students to master and bachelor students felt invited to ask their questions, cooperate and provide inputs. This truly is one of Groningen's major strengths!

In this regard I of course owe many thanks to **Vincent!** I think we were a great team despite in some facets being polar opposites. I am still hugely impressed by your drive and attitude when it comes to science and together we had a great run on doing the many WFF-experiments. I am greatly in debt to the massive amounts of work you poured into the projects we did and very thankful to have co-authored so many excellent papers with you. Hopefully some of the chapters in this thesis will also be submitted at some point to repay the favour. You are a great scientist and most of all a great personality and I hope our paths will cross again in the future.

Kees. Wat een geweldige tijd hebben we gehad in Groningen. Ik kan me nog goed herinneren hoe wel op een avondje uitgaan (met Herman-Jan, Lutzen en Wandert geloof ik) toevallig jou tegen het lijf liepen. Jij had net te horen gekregen dat je huisgenoot ging samenwonen, had het super druk met je bestuurs-jaar, je bijbaan en en je studie LS&T en toen ik tussen neus en lippen liet blijken een kamer te zoeken was een afspraak om langs te komen in Beijum snel gemaakt. Naast dat jij een belangrijke factor was in mijn keuze om na een jaar architectuur voor een studie LS&T te kiezen heb je mijn project erg geholpen. Het door jou geprogrammeerde Actoview template, de hulp bij de SCN-lesies, het letterlijk en figuurlijk meeliften naar een plekje in Haren en binnen GLV-idun en de hulp en adviezen bij zowel de onderzoeksprojecten als het doen van een PhD hebben me ontzettend veel gebracht. Daarnaast is onze vriendschap ontzettend belangrijk voor me geworden. Onze **Eva**, ons darten, de Batavieren race, ons thuis-biljard, het "*best-of-seven* UT'en" om de huishoudelijke taken te verdelen, zoveel mooie herinneringen. Ook **Anniek** met wie (+ **Kay**) je nu een gezin vormt bleek een onschatbare steun voor mij tijdens mijn PhD tijd, ik kon altijd bij jullie terecht voor advies en voor vriendschap en ontspanning! En Nieky, jou brommertje maakte het controleren van de muizen in de tijd dat het biologisch centrum nog in Haren stond een flink stuk dragelijker natuurlijk! Enorm veel dank aan jullie beide voor al jullie steun.

Wandert. Ook een van mijn grote voorbeelden en een grote positieve invloed bij het zorgen dat ik uiteindelijk biologie ben gaan studeren en op de juiste plaats belandde. Jou enthousiasme over de vakken en het onderzoek wisten deze vonk

ook op mij te doen overslaan. Naast onze vriendschap en de vele gezellige avonden heb je me ook veel geleerd toen ik tijdens mijn master met jou als en **Bart** als supervisors de epigentica heb leren kennen. Daarnaast ben jij een geweldig voorbeeld dat je bovenal je hart moet blijven volgen in het leven, wat jou van een PhD in de immunologie tot het opbouwen van een wijngaard, wijnmakerij en gezinnetje onder de Spaanse zon heeft gebracht! Alle liefs aan **Laura & Leo!** Ik ben blij dat we nog altijd contact hebben en het word hoog tijd weer eens face-2-face te kunnen afspreken, in Spanje of Duitsland!

Tijdens mijn tijd als PhD heb ik veel kunnen sparren met, en waardevolle input gekregen van de gehele Chronobiologie groep. De enorme diversiteit van onze projecten hebben echt een grote rol gespeeld in mijn brede interesses en er mede voor gezorgd dat ik nu een leuke baan heb gevonden bij Chrono@Work met **Marijke Gordijn.** Hierom een welgemeend dankwoord aan al mijn vrienden en collega's van de afgelopen jaren; **Giullia**, **Jamey**, **Jenke**, **Jildert**, **Kevin**, **Laura, Lauren, Maan, Meghan, Moniek, Michelle, Patricia, Renske, Simone, Sjoerd, Theresa, Tom** en de vele anderen die voor korte of langere tijd in onze Chronogroep hebben meegedraaid! Ook van de studenten die ik heb mogen begeleiden heb ik zelf veel geleerd.

Naast de *peers* en professoren wil ik ook nog mijn dank uitspreken voor al het ondersteunde personeel dat het doen van onderzoek mogelijk maakt. **Marlies, Pleunie, Diana, Martijn, Miriam, Roelie, Gerard, Wanda, Christa, Diane, Willem, Roel** en de vele anderen die voor en achter de schermen ervoor zorgen dat het doen van onderzoek überhaupt mogelijk is, van administratie tot dierverzorging, van het onderhoud tot het bestellen van materialen, van de operaties tot het stroomlijnen van meetings en conferenties. Ook jullie hulp was onmisbaar!

I also would like to thank all the colleagues from other Universities that have provided inputs and help in shaping our research be discussing our data and designs over the years. I hold very fond memories especially to all those that attended the Wild clocks meeting on Texel, where it was great to have so many people together that were interested in uniting field and laboratory findings and matching mechanisms with their ecological importance. Especially the co-authors on the Flexible clock paper (Chapter 4) as a product of this conference were a joy to work with!

Thanks go out as well to my German friends and colleagues. After moving to Germany in the end of 2017, I have had a great opportunity to do some highly interesting research projects at the University of Leipzig. **Wiebke**, **Nadine**, **Ute**, **Julia, Jiesie, Anja, Katha, Eileen** and **Elly;** you were a great group of people to

work with. When I struggled to remain productive and tackle my personal problems you have been very patient and supportive!

Besides the many years that spanned the research phase of my PhD, I owe an important word of thanks to those that are here to perform the final evaluation and judgement. Thank you to **Gertjan van Dijk** as my secondary promotor and all the people from the reading committee; **Martien Kas, Andries Kalsbeek** and **Henrik Oster** as well as all those present during the final ceremony on April 12th. I am looking forward to discuss and present this work to all of you.

It is only befitting to end this section with a final thanks to my family and friends, both in Germany and those back "home" in the Netherlands, for their interest, support and patience. But there is no person more important to mention than **Nele**! I am blessed to have you at my side. You're the most wonderful person I know and your support and understanding mean the world to me. Let's hope your thesis is next; you can do it too!

Curriculum Vitae

About me:

Born in Emmen on the 4th of July 1986 in Emmen I grew up living with my parents and two younger brothers in Nieuw-Weerdinge, a small agricultural village in Drenthe. Around the age of nine, our family moved to the Frisian capital of Leeuwarden, where after completing my primary education I attended CSG Comenius where I obtained my "VWO" degree, with the profile mathematics and natural sciences. Upon completion in 2005, I started to study architecture in Groningen (Hanzehogeschool) but was disappointed with the program and instead became more and more interested in biology and life sciences. In 2006 I started the bachelor Life Science and technology which I finished in 2009 and followed up by doing the behavioral and Cognitive Neuroscience research master, - both at the University of Groningen - which I finished *cum Laude* in 2011.

Within my masters I had the opportunity to perform a five-month research project at the University of Virginia, Charlottesville (USA) – in the lab of Professor Menaker - which later proved to become the foundation of my PhD-project. After doing a 6 month research project funded by Philips in Groningen – on the effects of twilight on entrainment and circadian stability – I obtained funding from the Ubbo Emmius Fund allowing me to start my own PhD-project in the lab of Roelof Hut, at the University of Groningen in 2013.

At the end of 2017 I moved to the Germany to work full time as junior Scientist on the topic of obesity prevention by intermittent fasting in the group of Dr. Med. Fenske where I was employed until December 2020. While I originally intended to complete the thesis during my spare time, the combination of a full time research position and remaining work required for the PhD-thesis proved to be more than I could manage. Afterwards, while looking for new career opportunities (slightly hampered by the coronavirus outbreak), I managed to make good progress on resuming the work on the PhD thesis in 2021. December 2021 I started my current project at Chrono@Work, based in Groningen, translating chronobiological principles in to health advices for employees and patients. I am pleased to defend the PhD-work and complete my thesis in April 2022.

Skills and strengths

During my work as junior scientist and my PhD-project I acquired a diverse array of valuable skills, including a good feel for data visualization, a good sense of experimental design, critical and logical reasoning, working with large datasets, circadian and statistical analysis, rodent behavior and physiology, neurobiology

and anatomy, minor rodent surgery, tissue culturing, molecular techniques like qPCR, western blots, histology and immunocytochemistry, microscopy, project management and especially enjoyed to present and communicate findings both written and verbally to audiences from students to conference attendees and enjoyed the teaching and supervision of students.

Conferences and presentations

- European biological rhythms Society (Munchen, 2013, Poster)
- Society of Research on biological rhythms (2014, Big Sky–Montana, Presenter)
- SCNI summerschool (Oxford, 2014, Participant)
- European Biological rhythms society (2015, Manchester, Poster)
- Wild Clocks Meeting (2015, Texel, volunteer organization and Poster)
- Dutch Neuroscience Conference (2017, Lunteren, Presenter)
- European Society for Endocrinology (2019, St Odile, Poster)
- Versuchstierkund. Kolloquim (2020; Leipzig, Lecturer)

Published work

RA Hut, H Dardente, SJ Riede (2014). Seasonal timing: how does a hibernator know when to stop hibernating? Current Biology 24 (13) R602-R605. https://doi.org/10.1016/j.cub.2014.05.061. (Hut et al., 2014)

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SM Ota, RA Hut, SJ Riede, P Crosby, D Suchecki and P Meerlo. Social stress and glucocorticoids alter PERIOD2 rhythmicity in the liver, but not in the suprachiasmatic nucleus. Hormones and behavior 120, 104683 (Ota et al., 2020).

Balancing Expectations

Adaptive flexibility of mammalian circadian organisation