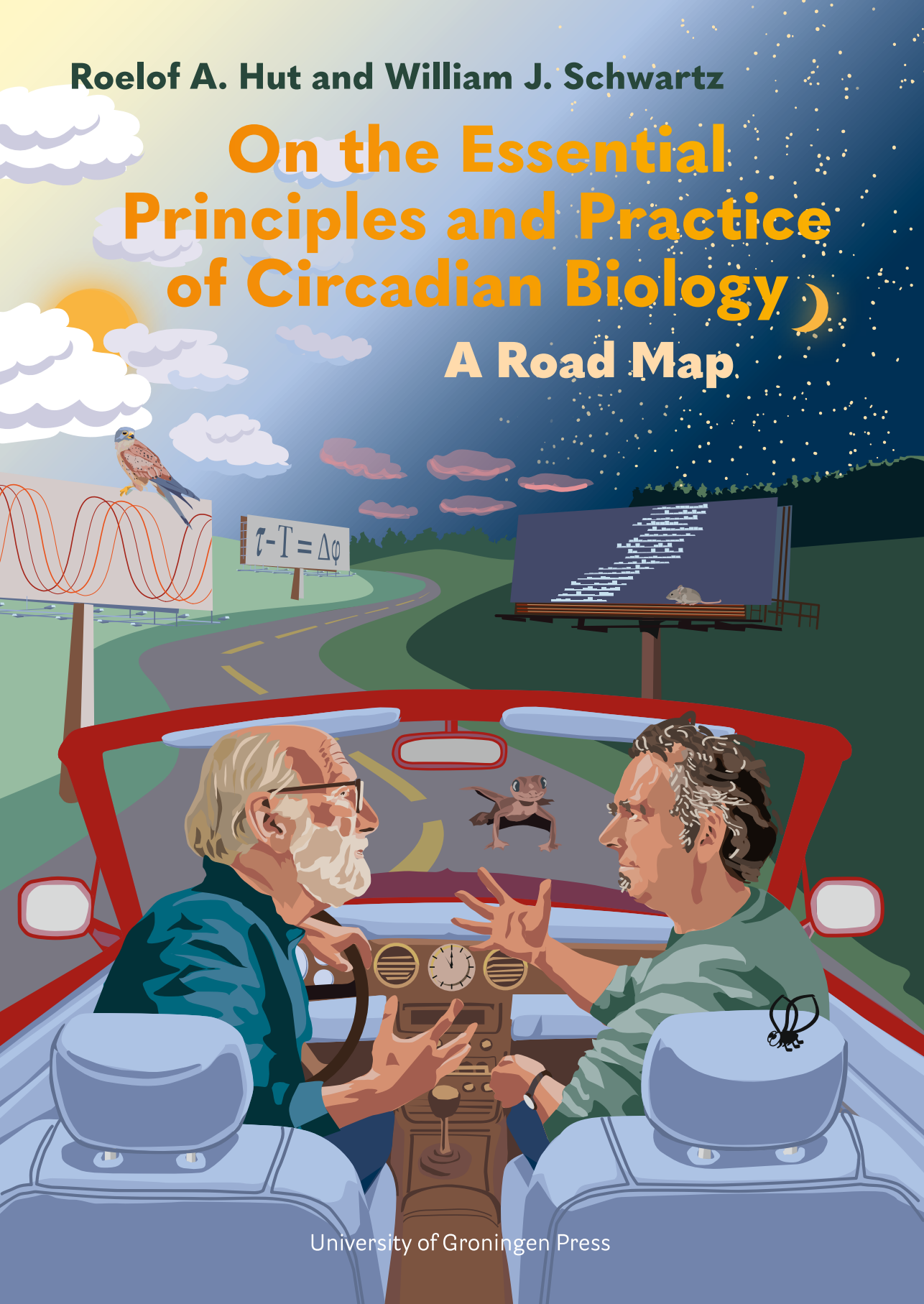
Roelof A. Hut and William J. Schwartz

On the Essential Principles and Practice of Circadian Biology

A Road Map



University of Groningen Press

On the Essential Principles and Practice of Circadian Biology

On the Essential Principles and Practice of Circadian Biology: A Road Map

by Roelof A. Hut and William J. Schwartz

University of Groningen Press

Published by University of Groningen Press
Broerstraat 4 9712 CP
Groningen The Netherlands

This book has been published open access thanks to the financial support of the Open Access Book Fund of the University of Groningen.

Cover design: Riëtte van Zwol
Typesetting and production support : LINE UP boek en media bv

Open textbook edition https://opentextbooks.rug.nl/circadianbiology/

DOI: https://doi.org/10.21827/68629ef93c8d0

This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License.

CONTENTS

PREFACE: Read This First! **7**

PROFILE: The Circadian System in 920 Words **9**

THE ROAD MAP **13**

Chapter 1. OBSERVATION: Daily Rhythmicity **13**

How do I know if variable data collected at different times represent a daily rhythm? 14

Why display rhythmic time series as "actograms"? 15

What do I do if it is not possible to make repeated measurements over multiple cycles? 17

Terminology check-in 19

Chapter 2. INVESTIGATION: What is Your Question? **21**

Question 1: What are the mechanisms for generation & expression of daily rhythms? 21

Question 2: What is the functional utility of a circadian clock? 29

Question 3: What is the systems physiology of circadian networks? 39

Terminology check-in 48

Chapter 3. APPLICATION: Consulting the Clock **51**

Adapting to other consequential geophysical cycles 51

Wayfinding on a rotating world 57

A final word – On opting out 58

POINTS OF INTEREST **59**

ABOUT THE AUTHORS **130**

PREFACE: Read This First!

We, the authors, have borne witness to the extraordinary transformation of a scientific field, which we will refer to here as *circadian biology*. It's the biology of adapting to life on our rotating world, to the Earth's 24-hour rhythm of day and night. Circadian biology is not really a sub-discipline of biology – like zoology or immunology or genetics – but instead spans the whole of the biomedical sciences and across biological kingdoms. Not surprisingly, the field has attracted considerable popular interest as well as new immigrants seeking further study or applications – students, investigators, and collaborators – ethologists, physiologists, oncologists, urban architects, agriculturists, wildlife conservators, and more. This new diversity is exciting and invigorating!

But there's a problem, at least as we see it. Formal courses in biological time and timekeeping are still uncommon in undergraduate, graduate, and medical curricula. So, newcomers may have little or no foundational knowledge on the essential principles and practice of circadian biology, no framework upon which to incorporate their disciplinary experience and expertise. Our language, data visualization, and concepts might seem as daunting as the science itself. If this is you – whether your aim is to conduct research, collaborate and/or communicate with others, or understand advanced experimental literature – then this book is meant for you. It is not intended to be a popular book (of which there are already several); or as an up-to-date manual of experimental methods and discipline-specific approaches (about which you are likely already expert in your area); or as a comprehensive textbook (although a new one is certainly past due). Instead, our intention is to provide you with a *Road Map* – consider it a launching pad, jump start, gateway, or primer – as a first (and hopefully engaging and helpful) step to enter the field and as an entrée to its now voluminous literature.

We strongly recommend you read the book in order, from beginning to end (you know, just like an old-fashioned book!); or perhaps it could be used as a template underpinning a series of didactic sessions for laboratory members and students. Our *Road Map* is really not that long, at around 13,000 words, with the three chapters building on one another in succession. Make sure to read the figure legends as well. Along the *Road*, you will find links to Points of Interest, denoted by ★1.1; these indicate supplementary (bonus) material meant to clarify, expand, comment, or illustrate the main text without interrupting its

flow. They are self-contained (you will not be dropped directly onto the internet). Consider the Points of Interest as informative footnotes and selected (rather than complete) references intended to serve as portals for further learning.

We must apologize in advance that our presentation leans heavily on mammals and mammalian circadian timekeeping; we do include some material on insects (especially *Drosophila*) and birds, as well as in the Points of Interest, but in general we admit that bacteria, unicellular eukaryotes, fungi, and plants really do get short shrift. And to keep the main text short and readable, we were forced to decide – from all the incredible work in this burgeoning field – what we felt was crucial for you to know as part of the *Road Map*. For that, we admit relying on our subjective views, based on our combined 70 person-years in the field, and for which we take full responsibility.

Lastly, we wish to thank each other for many profitable hours of animated and fruitful discussions, none of which resulted in (significant) bodily injuries to either of us. We greatly appreciate the insightful comments of Danny Forger, Mary Harrington, Renske Lok, Laura van Rosmalen, Paul Taghert, and Dave Weaver on an early version of the manuscript. We are grateful to Riëtte van Zoll for her expert transformation of the *Road Map* to an original cover design and Nicole Mangum for administrative support. And we are indebted to the Groninger University Fund's WETEX grant and the University of Groningen Press for partial funding of our project, and to Margreet Nieborg and Pieter van der Veen of the Press for their expert and invaluable advice and support, without which this *Road Map* would not have seen the light of day.

Roelof and Bill.

PROFILE: The Circadian System in 920 Words

Circadian rhythms are biological rhythms cycling once every 24 hours, expressing a wide array of individual shapes and peak times with respect to day and night. A true circadian rhythm persists in an environment free of any recurring external timing cues ("constant conditions," usually requiring a laboratory) with a near 24-hour cycle length that remains relatively stable even under ambient temperatures that may vary within the natural range ("temperature compensation"). Such features point to the existence of an innate, self-sustained oscillatory mechanism ("circadian clock") underlying the rhythms, a mechanism that both recognizes the local time of day and measures the passage of time. Under natural conditions, the clock's near 24-hour cycle length is reset (entrained) to match the Earth's 24-hour axial rotation, establishing a stable relationship between them. A key, but not exclusive, timing cue for this process is the 24-hour alternation of light and darkness. The selective advantages of such a timekeeping system are myriad – including the regulation of the temporal organization of biological processes, anticipation of periodic daily events, adaptation to the seasons, and spatial orientation and navigation by the sun.

The mechanisms responsible for generating overt circadian rhythms bridge multiple levels of biological organization. The core oscillatory mechanism lies within individual cells, utilizing a common design principle that involves a suite of clock genes functioning within transcription-translation negative feedback loops, with delayed repression of their mRNAs by their own protein products. In eukaryotes, this regulatory mechanism is largely conserved, although the identities of the genes may differ between organisms (however, in a prokaryote [a cyanobacterium] the core mechanism instead involves three interacting proteins and rhythmic phosphorylation). In the mammalian case, the positive (activator) limb of the loop is represented by the binding of the transcription factors BMAL1 and CLOCK as a heterodimeric complex to E (enhancer) box response elements on the DNA of clock-controlled, genome-wide target genes. Among the genes activated by BMAL1/CLOCK are *period* (*per1*, *per2*, *per3*) and *cryptochrome* (*cry1*, *cry2*), which represent the negative (repressor) limb of the loop, as PER and CRY protein products are translocated to the nucleus and assemble as parts of large complexes that inhibit BMAL1/CLOCK activity and thus their own transcription. Cycle length is set by the repressors; by their post-translational accumulation and phosphorylation via kinases such as casein kinases 1δ and 1ε and by their degradation through the ubiquitin-proteosome pathway. Also identified are two additional loops that feedback on BMAL1/CLOCK tran-

scriptional activity. The first involves E-box mediated transcription of genes encoding nuclear hormone receptors REV-ERBα/β and RORγ that bind to RORE (retinoic acid-related orphan receptor) response elements on the *bmal1* and *clock* promoters; the second involves bZIP transcription factors DBP and NFIL3 that regulate the expression of RORα/β. Beyond the loops, it is the combinatorial actions of these elements on clock-controlled output genes that contribute to the regulation of cellular-, tissue-, and time of day-specific transcriptional rhythms within organisms.

In metazoan animals, in addition to the presence of widespread intracellular clocks, the brain harbors specialized, heterogeneous populations of neurons and glia that function as centralized circadian pacemakers to coordinate rhythmicity at the supra-cellular level. In mammals, there are about 20,000 neurons in the hypothalamic suprachiasmatic nucleus (SCN); and in *Drosophila*, about 150 neurons in the head. Neurotransmitters and neuropeptides in the SCN – such as γ-amino-butyric acid (GABA), vasoactive intestinal polypeptide (VIP), and arginine vasopressin (AVP) – and in *Drosophila* – such as pigment-dispersing factor (PDF) – play key roles in building dynamic neural circuits through synaptic and other signaling mechanisms. Such circuitry enables multicellular pacemaker precision (from sloppy cellular oscillations), robustness (against incidental perturbations), plasticity (as with changes in daylength), and the capacity for multiphasic oscillatory outputs. In mammals, entrainment of the circadian system to the light-dark cycle is via a monosynaptic retinal input to the SCN ("retino-hypothalamic tract"), involving the activity of melanopsin-expressing photoreceptive ganglion cells, which are especially sensitive to short wavelength light (blue, around 479 nm). Light-induced neuronal depolarization in the SCN activates second-messenger signal transduction pathways and the induction of *per1* and *per2* to affect the phase of the molecular feedback loop. If the circadian oscillation in the SCN is inactivated, either by physical lesion or genetic mutation, some downstream rhythms will be rendered arrhythmic (for example, resulting in continuous locomotor activity and absent pineal melatonin secretion) while others will persist but de-couple from the light-dark cycle (for example, in some but not all peripheral organs and tissues). In *Drosophila*, in addition to circadian light input from the compound eye (and perhaps the ocelli), light resets the molecular loop by the rapid degradation of the clock protein TIM, mediated by the blue-light sensitive protein CRY in pacemaker neurons and other cells in the fly body (the *Drosophila* CRY is distinct from the mammalian CRYs that are part of the transcription-translation negative feedback loop and are not photo-receptive).

At the organismal level, the phase alignments of cell, tissue, and organ rhythms with each other and the day and night (the latter largely dependent on the SCN) is determined by the action of a panoply of coupling modes, including behavioral (such as feeding and sleeping), thermal, hormonal, autonomic, and metabolic. Misalignments – between solar time, internal (circadian) time, sleep time, and/or social (societal) time – contribute to the symptoms of jet lag and the health risks of shiftwork and possibly of chronic or repeated stress. Much current research is now focused on the significance and impact of circadian dysrhythmias in humans, such as mistimed food and medication intake, nighttime light pollution, aging, and vulnerability to diseases like metabolic syndrome, cancer, and neurodegeneration.

THE ROAD MAP

Chapter 1. OBSERVATION: Daily Rhythmicity

Millennia ago, our ancestors had already recognized rhythms of plant and animal behavior that appeared to cycle every 24 h, seemingly to match the length of time it takes for the Earth to complete one rotation about its own axis. We will discuss terminology at the end of this chapter, but here we reserve the term "daily rhythm" for a biological rhythm expressing a 24-h cycle length in the presence of a repeating 24-h external timing event (for example, external events such as the sun rising every morning, an alarm clock ringing at 6:00 AM daily, artificial lights turning on in an animal facility at 8:00 AM or a bolus of food appearing at 6:00 PM daily).

There is not one single "daily rhythm," but a universe of them ★1.1. Daily rhythmicity is a feature of biological processes (in biochemistry, metabolism, physiology, and behavior) and is now studied across organisms (including photosynthetic bacteria, unicellular eukaryotes, fungi, plants, and animals), within organisms (in organelles, cells, tissues, organs, and physiological systems), and between organisms (flowering plants and their pollinators, eusocial insects, conspecific group living, parasitism and mutualism, pathogens and their vectors and hosts, and human societies). They are wide-ranging, from rhythms of overt activity to rhythms of sensitivity and responsiveness only revealed by an imposed challenge or perturbation. They express a variety of shapes (*waveforms*), from near-sinusoidal to bimodal, or even more complex; as well as differential timing (*phases*; for example, the timing of a rhythm's onset, peak, offset, or other marker) that can occur at any point throughout the entire 24-h cycle.

Now let us suppose that by serendipity, sloppiness, or design, you have repeated the same measurement or observation of a biological process at multiple times around the clock, and the values appear to be different! Perhaps inaccurate technique is the culprit, but is a process that undergoes a predictable daily rhythm in its level or activity another explanation for this difference?

Or let us suppose that your transcriptomic profiling of normal and diseased tissues collected at a specified time point reveals strong differential expression of several canonical "clock" genes! Perhaps the disease state is indeed associated with altered absolute levels of gene expression, but is another explanation

that the transcripts are similarly expressed in both conditions but as daily rhythms with their peaks at different phases?

Interesting! So, what do you do now?

How do I know if variable data collected at different times represent a daily rhythm?

Even if a 24-h rhythm in the data appears obvious by visual inspection, you'll want to apply statistical analyses to confidently establish and describe its features. For sure, the nuances of time series analyses are complex and the subject of an extensive literature, so you may eventually wish to consult or even collaborate with a knowledgeable statistician. However, there is no substitute for initiating your analysis by first plotting the raw data points before submitting them to automated processing. You will have the opportunity to identify possible outliers, perhaps interesting or unexpected features, and potential trends across the record.

Choosing the most appropriate analysis for a time series ★1.2 is guided in large part by the characteristics of the dataset: these include what is being measured, the number of cycles and the number of time points recorded per cycle, whether the data points are equally or unequally spaced and normally or non-normally distributed, the complexity of the waveform, and the presence of zeros (as for example, in a behavioral activity rhythm with long intervals of absent activity). Typically, and for practical purposes, 6 time points over 24 h – that is, sampling every 4 h – would be considered adequate for the most basic description of a daily rhythm. Obviously, more sample points would allow for more precise description of waveform ★1.3. However, confidence in the rhythmicity itself and in the estimation of its parameters is generally improved more by increasing the number of cycles in the series than by increasing the sampling rate.

Key rhythm parameters (Fig. 1) are:
period, ***phase***, ***amplitude***, and ***mean level***.

• Period is the interval of time for the rhythm to complete one cycle; in the case of a daily rhythm as we have defined, the period is thus 24 h. By the way, *period* and *frequency* are related; frequency is the reciprocal of period, in this case as the number of cycles per hour. When there is one cycle per 24-h day, the period is 24 h, but this can also be expressed as a frequency of 1/24 = 0.04166... h^{-1} (cycles/h or in International System (SI) Units as 0.000015741 Hz), but thankfully no one does that.

• Phase is a defined reference point within the cycle, representing the instantaneous state of the oscillation. The time interval between two identical phases in successive cycles – for example, onset of activity – is equal to the period of the rhythm. For analytical purposes, the choice of a phase marker(s) is important, non-trivial, and will be noted in Chapter 2.

• Amplitude is the difference between the maximum or minimum value of a rhythm and its mean level (not the difference between the maximum and the minimum values, which is the range of the oscillation).

• Mean level is the value around which the rhythm oscillates, corresponding to the arithmetic mean if the rhythm waveform is symmetrical. Of course, the mean level can change with or without a change in amplitude.

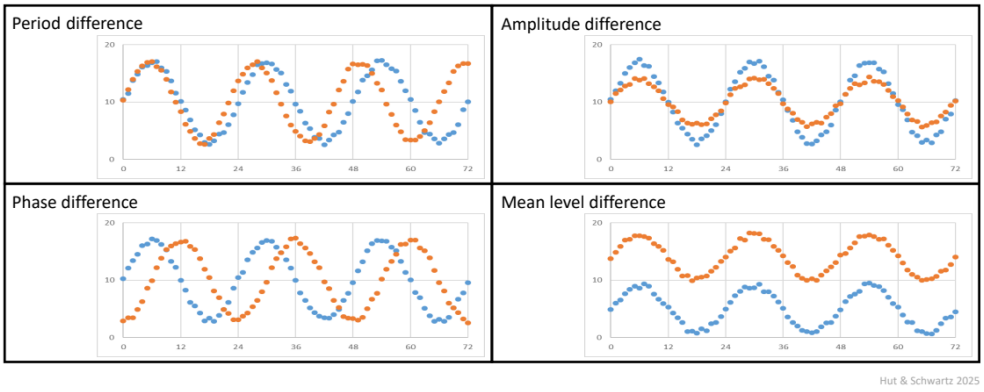


Figure 1. Key rhythm parameters (see text)

Note that a difference in period cannot be distinguished from a difference in steady-state phase if the rhythms are only recorded over a single cycle.

Why display rhythmic time series as "actograms"?

Ordinarily time series are graphed with the independent variable (time) plotted horizontally from left to right on the x-axis, while the dependent variable (say, measured value) plotted vertically on the y-axis. Changes in amplitude and mean level are easily visualized in this way, but subtle variations in period and phase may be less apparent. The "secret handshake" of the circadian biologist

is an alternative display known as the actogram (but also useful for plotting variables other than activity), with one 24-h period plotted horizontally and successive calendar days vertically from top to bottom. In this way, any disruption in the vertical alignment of rhythm phases over succeeding cycles is usually clear (Fig. 2).

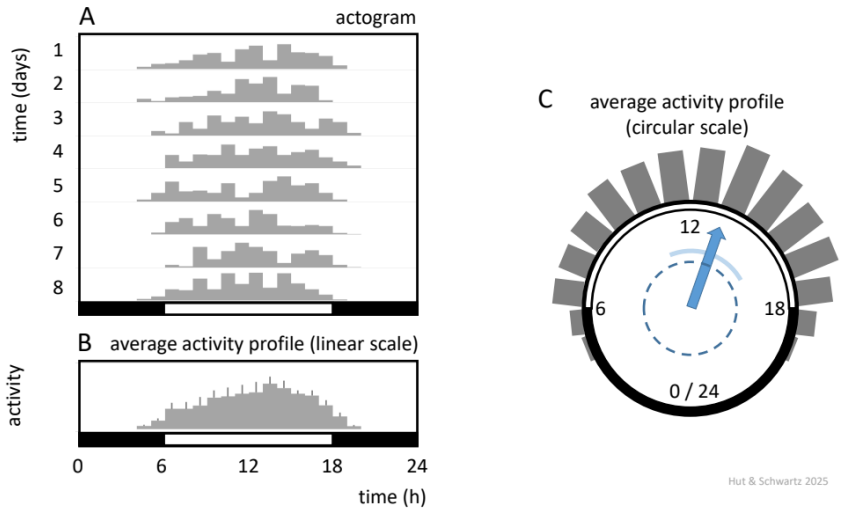


Figure 2. Graphical displays of a daily activity rhythm

(A) Actogram, in which the daily variation of activity patterns in a diurnal animal is shown over 8 consecutive 24-h days, as a bar graph showing total activity per hour. At bottom: white bar, lights-on; black bar, lights-off.
(B) Average Activity Profile from (A) plotted on a linear scale, constructed by averaging activity levels for each hour over 24 hours; error bars indicate standard errors based on variation per hour between days.
(C) Average Activity Profile from (B) plotted on a circular scale, allowing determination of the center of gravity (CoG, blue vector) by goniometric approach (using the average of the activity-weighted sines and cosines to calculate the direction and length of the CoG vector by the arcTan function). The width of the circular distribution (the spread around the circle) is represented by the circular standard deviation (light blue arc). Rhythmicity in the original data can be determined when the Raleigh test identifies significant directionality (difference from an equally spread circular distribution), when the CoG vector length is greater than the radius of the dashed circle.

Actograms were initially popularized especially for displaying the behavioral rhythms of rodents (hence the name, "activity-gram"). It was at a time that rhythm amplitude was not readily measurable; the methods used to record events involved pens on white paper or needles on smoked paper and did not have the temporal resolution to accurately measure the degree (intensity) of activity. Such actograms highlighted period and phase, which was a chief aim being pursued by investigators at the time ★1.4.

What do I do if it is not possible to make repeated measurements over multiple cycles?

There are certainly circumstances when multi-cycle, longitudinal measurements from an individual organism or preparation cannot be made (for example, if obtaining tissue for biochemical, immunohistochemical, or transcriptomic analyses). The practical solution to this situation has been to sample multiple groups of individuals at pre-determined time points – say, every 4 h – and to test for a statistically significant variation between the groups as a function of time over the 24-h cycle. Obviously and importantly, the fidelity of any educed "rhythm" determined in such cross-sectional fashion will critically depend on the confidence of randomization across the groups and the number of sampling times (as well as, for example, such details as the care in processing samples from all groups together). While ANOVA is commonly employed to show differences across groups over time, it is important to remember that significant non-uniformity of groups by ANOVA does not demonstrate periodicity. Also, a reminder that temporal data on phase are circular (periodic), not linear; that is, a 24-h clock time of, say, 23 h 55 min is closer in time to 0 h 5 min than it is to 23 h 40 min on a circular scale, as on a clock face. If computing an average daily phase across individuals, one cannot simply perform an arithmetic average of the clock times as placed on a linear scale. The most common statistical procedure to distinguish whether circular data are uniformly distributed or with a preferred (unimodal) direction is the Rayleigh test, which calculates the position of the circular average by goniometry (Fig. 2).

And here is one final note of caution. Biological variability of a measure within a group at a specified time point (say, the intensity of an immunohistochemical signal) could arise in two ways if the measure is indeed rhythmic (Fig. 3). One is by variability in individual *amplitudes* (typically acknowledged by error bars at that time point), and the other is by variability in individual *phases* (which cannot be known by a measurement at a single time point); and these two sources of variation are not mutually exclusive. Thus, the parameters of a

"rhythm" calculated from multiple groups of individuals sampled over a 24-h interval may not mirror those of any one rhythmic individual.

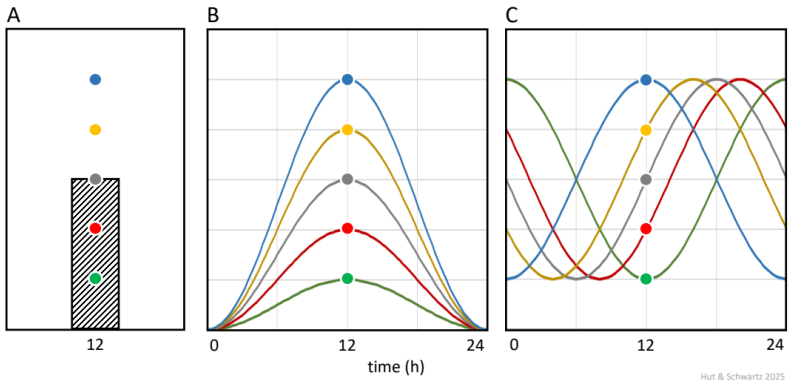


Figure 3. Interpreting biological variability within a group at a specified time point

Biological variation across samples at a specified time point (A) may arise from variation in rhythm amplitude (B) or phase (C), or both, and cannot be distinguished.

In the extreme case, absence of 24-h rhythmicity might be concluded from group data even if there actually were a rhythm but with widely distributed phases between the individuals (Fig. 4).

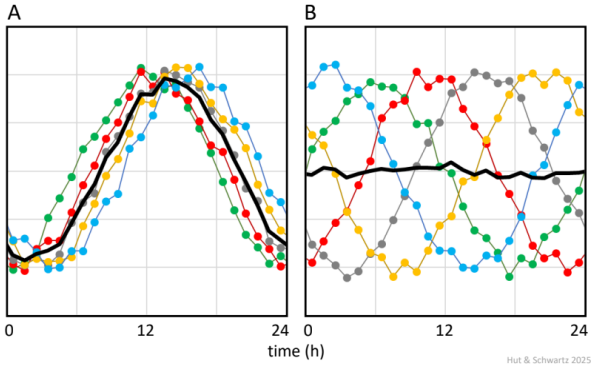


Figure 4. Educed rhythms from group data

Average rhythms extracted from group data (in black) may show reduced amplitudes when the phase difference between individual patterns increases (compare panel A with B).

Note that for a stable *daily* rhythm inferred by the timed sampling of multiple groups, extending the experiment from 24 h to 48 h or longer does not provide a longitudinal measurement, although it can serve to produce independent biological replicates of samples at similar time points over the 24 h cycle (importantly, however, see ★1.5).

Terminology check-in

We wish to earnestly assure you that we are not overly fastidious people, but we do believe that the terminology we use in our field merits special attention and care. Vague or inconsistent use of terms can lead to miscommunication, propagation of errors, and faulty design and interpretation of experiments. In this regard, we are aware that daily rhythms are sometimes referred to as "diurnal rhythms," which we feel is confusing. A "nocturnal" rhythm is understood to be one that is night-active (peaking during the dark interval); so, we recommend that the term "diurnal" rhythm be restricted to one that is day-active (peaking during the light interval). In this way, both diurnal and nocturnal rhythms are daily rhythms but with (roughly) opposite phases.

We favor "daily" as the adjective for a rhythmic pattern that occurs every 24 h in the presence of a repeating 24-h external timing event, but you may come across "diel" or "nycthemeral" (or spelled "nychthemeral"), apparently used synonymously. Actually, nycthemeral is arguably a more specific term than daily; according to the Oxford English Dictionary, it is derived from nycthemeron, defined as a period of 24 h that consists of a day and a night.

Incidentally, we've also seen that the word "day," in isolation, can be ambiguous! Does it refer to the light interval of a light-dark cycle (that is, as in day and night)? Or is it the Earth's 24-h "day" (in distinction, for example, to Mars' 24.62 h "day")? Or is it that part of the 24-h cycle occupied by the waking hours (as in, "he filled his day with participation in outdoor sports"). For these reasons, we try to avoid "day" except if put in context (as in "24-h day," "day and night," or "daytime and nighttime," or "subjective day and subjective night" [see Chapter 2 for a description of the term "subjective"]).

For an accessible and reasonably complete glossary of terms in circadian biology, consider
https://www.circadian.org/dictionary.html

Chapter 2. INVESTIGATION: What is Your Question?

We imagine that you may not be entirely satisfied with solely describing, however rigorously, the daily rhythm you've now demonstrated (see previous chapter). Like other great discoveries, yours should also generate more questions than answers. But the issue is:

What is your question?

There are numerous questions that could be tackled, for sure, but based on budgetary time and effort, available person-power, and your own lifespan, we recommend first choosing wisely and focusing on one of them to get your experiments underway. In this chapter we put forward and discuss three broad questions; procedurally they are somewhat separate but conceptually quite interdependent.

Question 1: What are the mechanisms for generation & expression of daily rhythms?

This kind of question is of the type that seeks to understand how a biological process occurs ("proximate" causation), nowadays most often by employing reductionistic approaches. We begin with the assumption that the parameters of the daily rhythm of interest – its period, phase, amplitude, and mean level – are shaped by multiple sources to form the final, composite, overt rhythmic waveform; generally speaking, some influences will arise externally outside the organism (exogenous), while other factors will be generated by the organism itself (endogenous). Over a couple of hundred years, the existence of an internal timekeeping component – popularly known as the "circadian clock," the subject of this book – was proven, and here we discuss three experimental strategies for revealing the presence and influence of circadian clocks through the dissection of daily rhythms into their exogenous and endogenous components.

a. Rhythm dissection in an environment without external time cues

Considerable effort has been expended in the laboratory to remove rhythmic external timing cues that might be driving daily biological rhythms or impacting their shapes, especially the alternation of light/darkness and high/low temperatures over 24 h (but including other conceivable cues as well) ★2.1. Such direct effects on rhythm expression are referred to as "masking" – because they mask

(hide) the presence or action of any endogenous timing element from the investigator's view – and will be discussed in additional detail later in this chapter. In any case, the experimental elimination of such masking effects leads to a stunning result: (a) the persistence of rhythmicity for many daily rhythms under such constant conditions, and (b) an expressed period that is relatively precise but with a value that may deviate considerably from 24 h ★2.2. The period is thus *circadian* ("about a day" or "around a day"), referred to as "*free-running*" (that is, free of 24-h external synchronization), and its value denoted by the lowercase Greek letter tau (τ).

Under these conditions, the passage of internal biological time ("circadian time") no longer matches that of local time, as defined by the light-dark cycle. This situation calls for a special nomenclature (Fig. 5).

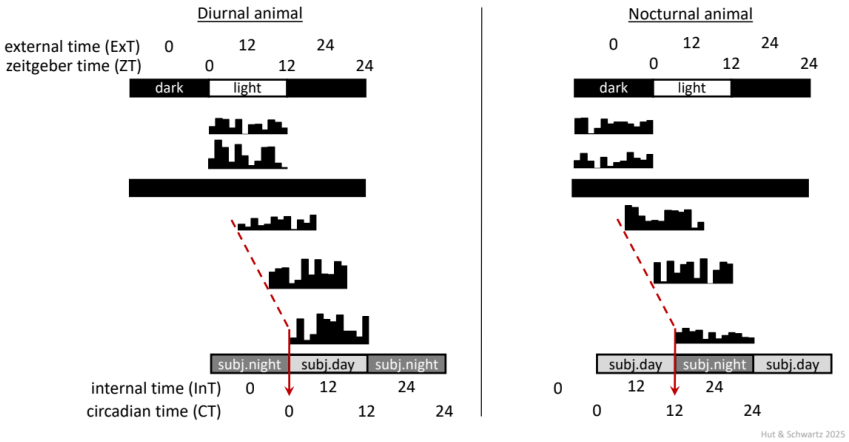


Figure 5. Time scales: external and internal time; zeitgeber and circadian time (see text)

Actograms of a diurnally-active (left) and nocturnally-active (right) animal, initially maintained in a 12 h:12 h light-dark cycle and then free-running in constant darkness. Dotted red line indicates activity onset as a phase marker during constant darkness. In the cases shown here, the value of τ is greater (longer) than 24 h, or put another way, the rhythm runs slower than 24 h.

At least for a daily rhythm expressed in the presence of a light-dark (often abbreviated as LD) cycle, the simplest convention would seem to be the one that is already familiar to us: setting local time point "0" to the middle of the dark phase and partitioning the entire cycle into 24 equal hourly segments (this is

akin to our concept of "midnight," even though our local 12:00 AM does not always occur at mid-dark phase). This solution has been designated "External Time" (ExT), with the number of hours elapsed since "0," as in "lights-on at ExT 10 and off at ExT 22" (or other combinations that might not be 12 h of light and 12 h of dark).

You have undoubtedly encountered the term "*zeitgeber*" Time (ZT). *Zeitgeber* is a German word (capitalized as a noun) for "timer" or "timegiver." Like ExT, ZT also denotes the passage of local time in the presence of a repeating 24-h timing cue, and is likewise divided into 24 equal hours, but ZT 0 is assigned as the time point of lights-on. Traditionally, this scheme has been applied to the nomenclature of free-running rhythms in constant conditions, where "Circadian Time" 0 (CT 0) represents a projection over time of the previous ZT 0. Using a single phase marker per circadian cycle allows partitioning the cycle into 24 equal parts, now as circadian hours, with one circadian hour = τ/24 h (of note, for one circadian hour to have the same duration across all phases of the cycle, the speed (velocity) of the oscillation is considered to be constant across all phases of the cycle). For a nocturnal animal like a mouse or hamster, the clear activity onset may serve as a convenient phase marker for each circadian cycle. Since activity onset during a light-dark cycle occurs at the onset of darkness (ZT 12), activity onset under free-running conditions may serve as the phase marker for CT 12. So, for a mouse with a circadian period length around 23.7 h in constant darkness, under these conditions one circadian hour = 23.7/24 = 0.9875 h.

The terms "subjective day" and "subjective night" are used to represent projections of the preceding light and dark phases, respectively, over circadian time; this definition is thus irrespective of the timing of an organism's usual activity and rest phases (thus, CT 0 is identical for a diurnally active human and a nocturnally active mouse). Of course, the ExT terminology can be analogously extended in constant conditions to "Internal Time" (InT). A significant advantage of the ExT/InT over ZT/CT configuration becomes evident when comparing rhythms under different photoperiods (light phase durations); with the changing seasons in nature, both dawn and dusk shift to different relative phases of the cycle rather than one of them (dawn, ZT 0) remaining anchored and immovable by definition.

An actogram is a very effective graphical display for visualizing τ and for its visual estimation via an eye fit line through activity onsets (Fig. 6).

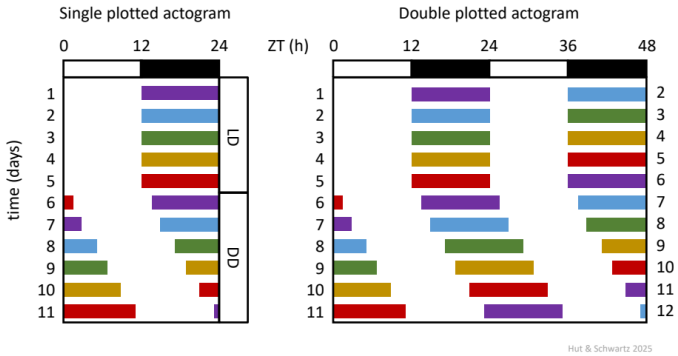


Figure 6. Single- and double-plotted actograms

Stylized actograms of a nocturnally-active animal under a 12 h: 12 h light-dark cycle (LD) released into constant darkness (DD). The double-plotted actogram repeats the format for 48 h, where day n is followed by day n + 1 horizontally, succeeded by day n + 1 and n + 2 on the next line, then by day n + 2 and n + 3, and so on. The double plot design allows for the visualization of an unbroken free run.

In practice, most of the attention to constant conditions in the laboratory has focused on lighting, and the classical procedure has been to employ constant darkness (DD). Although the use of constant light (LL) was key to the early discovery and analysis of circadian rhythms in plants ★2.3, its effects tend to be complicated, especially for animals that exhibit rhythmic sleep-wake cycles with the eyes alternately closed and open ★2.4. In studies of humans in temporal isolation, whose behavior can be directed, a "constant routine" protocol seeks to unmask the innermost kernel of the circadian timekeeping machinery underlying physiological rhythms, especially for the body temperature rhythm. In addition to the elimination of external light and temperature cycles, the protocol also minimizes willful rest-activity patterns such as physical activity and posture, intermittent meal timing, and sleep. In this way, estimation of the relative contribution of the "central" internal timekeeper to the ultimate, integrated waveform of the overt natural rhythm might be feasible. A similar approach has been attempted to dissect other daily physiological rhythms, such as blood pressure. Constant routine protocols are understandably short in duration given the intensive monitoring involved. Moreover, a significant confound of the protocol is an associated and inevitable element of sleep deprivation, and perhaps also the continued presence of ambient dim light.

In 1979, a very simple linear diagram of an archetypical circadian system was published, inspired by work on the experimental dissection of daily rhythms, now commonly referred to as the "Eskinogram" (Fig. 7) [★2.5].

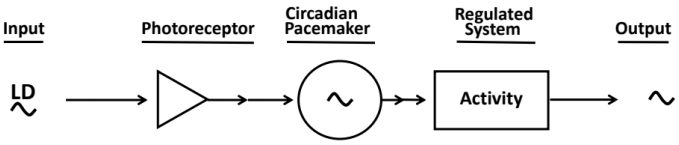


Fig. 7. The original "Eskinogram" (1979) composed by Arnold Eskin

Drawn from: Eskin A. Identification and physiology of circadian pacemakers. Fed Proc 38:2570-2572, 1979.

A critical feature of the model is the non-identity of the central timekeeper ("circadian pacemaker") from the observable rhythm that it either drives, regulates, or generates ("output" via a separate "regulated system"). This sequential segregation means that an output rhythm can be eliminated or altered somehow downstream of the pacemaker – become dissociated from it – without interrupting or affecting the continued timekeeping by the pacemaker itself [★2.6]. An "output" rhythm is not necessarily a faithful, passive "read out" of the state of the upstream pacemaker, as might be implicit in the diagram. As we have discussed above, the "regulated system" must be integrating the circadian signal along with a number of non-circadian ones to mold an output rhythm with phase, amplitude, and waveform that might not replicate the pacemaker's parameters. Furthermore, and importantly, how we as investigators choose to assay output rhythms may alter properties and performance of the system itself. For example, locomotor activity in fruit flies and rodents can be measured using a variety of apparatuses, most popularly with *Drosophila* activity monitoring (DAM) systems and rodent running wheels, respectively, but our methods may have an impact on features of circadian timekeeping *per se* [★2.7]. In humans, one of the attractive features of using the nocturnal rhythm of pineal melatonin levels to monitor circadian timing is its relative stability and tight regulation in the face of physiological, pharmacological, and behavioral variables (although production of the hormone is suppressed by bright light).

By the way, we tend to habitually and casually refer to "*the* free running circadian period" as though it were a fixed value for an individual, strain, or species.

However, it is a dynamic value, influenced not only by the running wheel but also by the intensity of constant light ★2.8, preceding lighting conditions (so-called after-effects), age, sex, and other factors. The statistical approaches traditionally employed in biological rhythm research require that the data are *stationary* over time (that is, of unchanging period, amplitude, and waveform from the 1st to the *n*th cycle). For rhythms that are *non-stationary*, a different analytical toolkit applies ★2.9.

b. Rhythm dissection by genetic and/or physical lesions

In a sense, this strategy is the opposite of the one that removes exogenous timing cues. Instead, here the aim is to modify or remove suspected endogenous timing elements, seeking telltale changes in the free-running period or even frank arrhythmicity (in the circadian range) as evidence that an endogenous timer has been impacted.

As is well documented, thanks to this strategy and to the power of molecular genetics and neurobiology, our knowledge of circadian systems made spectacular progress in the latter part of the 20th century. A genetic substrate underlying circadian rhythmicity had already been surmised in the 1930's in studies of plant hybrids and populations of fruit flies maintained in constant light over multiple generations. In the early 1970's, mutations at single genetic loci in *Neurospora* (*frequency* [*frq*]) and *Drosophila* (*period* [*per*]) were generated that led to abnormally fast, slow, or absent rhythms; and by the 1990's, a framework for the architecture of the core oscillatory machinery emerged, based on intracellular transcriptional-translational negative feedback loop(s) with built-in adjustable time delays ★2.10. Meanwhile, in the 1960's, destructive lesions of multiple brain areas and organs in rats focused specific attention on a critical role for the hypothalamus in generating circadian locomotor activity rhythms. By the 1980's, the suprachiasmatic nucleus (SCN) had been shown to exhibit properties expected of the central circadian pacemaker envisioned in the Eskinogram; and at the end of that decade came the impressive rescue of arrhythmic SCN-lesioned hamsters by fetal SCN transplants, with the values of τ (of wheel-running activity) restored in the hosts dependent on donor genotype ★2.11. Continued work using these and increasingly sophisticated techniques has elegantly mapped circadian systems at the molecular, cellular, tissue, and whole organismal levels of biological organization.

It is customary to assess the phenotypes of lesioned organisms and preparations under constant conditions, because in some cases rhythmicity can appear normal if a recurrent 24-h exogenous time cue is present (that is, masking;

for example, locomotor activity in a light-dark cycle). There are other confounds inherent in genetic and physical lesion-making that are well known. Identical mutations on different genetic backgrounds may have dramatically different phenotypic effects ★2.12, and there are remarkable examples of organisms' capacity for functionally compensating for lost parts, especially but not exclusively during development ★2.13. For this reason, it is prudent to keep in mind that some lesion phenotypes might actually reflect more about how an organism successfully (or unsuccessfully) adapts to the loss of specific genetic and anatomical elements than about how these elements function under normal conditions. Also interesting to consider are cases labeled as "no phenotype," since latent vulnerabilities of such systems might be concealed under favorable laboratory environments, only to be revealed by challenges such as sickness, stress, aging, or natural conditions.

A plethora of abnormal metabolic, physiological, and behavioral phenotypes has been associated with circadian arrhythmicity following the experimental elimination of some essential "clock" genes (for example, *bmal1*), raising exciting hypotheses about the possible roles of circadian dysfunction in the etiology, pathogenesis, and symptomatology of disease. Here it is key to remember that "clock" gene activity is pleiotropic and not restricted to the core transcriptional-translational timing loop; *bmal1*, for instance, encodes a multifunctional transcription factor that regulates expression of a large portion of the genome. Thus, inactivation of the core circadian timing loop by knocking out a critical "clock" gene might also lead to a state of persistently high or persistently low expression of many circadian-controlled, downstream transcripts. Resulting pathological mechanisms, or even the presence or absence of pathology *per se*, may therefore depend on how circadian arrhythmicity is being induced, whether by interruption of the positive or the negative limbs of the molecular oscillator's feedback loop ★2.14.

c. Rhythm dissection by "forced desynchronization"

In humans, the "forced desynchrony" strategy is a non-invasive behavioral manipulation that aims to disentangle rhythms of physical activity, meals, and sleep from the core circadian timing signal. However, rather than eliminating these behavioral rhythms through a "constant routine," forced desynchrony schedules participants to follow relatively normal daily routines but with sleep opportunities and meal times cycling with abnormally short or long periods of 20 or 28 hours, usually in a dim, very low amplitude light-dark regimen. Circa-

dian body temperature and melatonin rhythms cannot synchronize to these extreme periods (see *limits of entrainment*, discussed in Question 2 of this chapter) and instead oscillate with a period believed to approximate τ (Fig. 8).

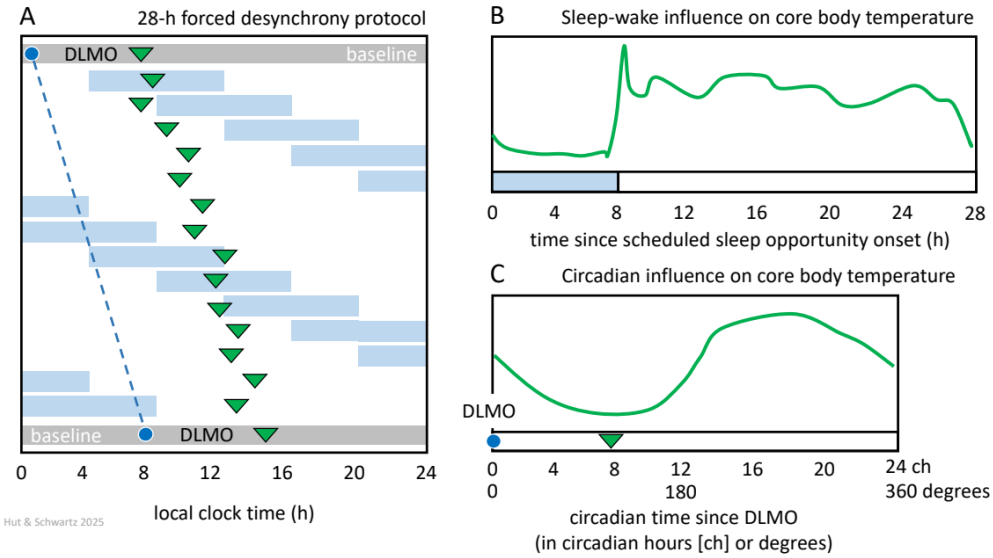


Figure 8. Human forced desynchrony protocol

Shown is a stylized forced desynchrony (FD) protocol with a scheduled sleep-wake cycle of 28 h with an 8-h scheduled sleep opportunity (A; blue bars). The protocol begins after an initial baseline day in which dim light melatonin onset is measured (DLMO, dark blue dot). DLMO is measured again after the FD protocol. The 28-h FD protocol lasts for two beat cycles, meaning that the 12 sleep-wake cycles cover two times all phases of a full daily cycle (12x28 h=14x24 h=336 h) and approximately two times all phases of the yet to be established circadian cycle. The period of the circadian cycle can be estimated post-hoc using the final DLMO measurement after the FD schedule. In this example, initial DLMO=02:00 h, final DLMO=09:30 h, thus free-running period = 24 h + (7.5 h over 15 d)=24.5 h (indicated by the dashed dark blue line). Core body temperature is measured continuously, and for each circadian cycle the timing of core body temperature minimum is indicated (green triangles), which reveals the nature of its free-running circadian regulation. Measurements like body temperature obtained during the protocol can now be binned and plotted on a sleep-wake-related time scale (in hours after sleep onset; B) and on a circadian time scale (in hours after interpolated DLMO; C).

Thus, over the course of this imposed desynchrony, as the dissociated behavioral and circadian rhythms cycle past each other, a series of changing phase relationships is generated between them; typically, these protocols should be continued long enough for all possible phase combinations to be expressed (a "beat cycle"). Then by averaging the data over either the circadian or sleep/wake time axes, the relative contributions of behavioral and circadian rhythms on each other's profiles can be estimated.

This method has been especially useful in humans ★2.15 for assessing, for example, intrinsic circadian periodicity as well as the complex regulation of wakefulness, sleep efficiency, sleep stages, and other variables. Thus, the rhythm of wakefulness is seen to be impacted by both circadian- and sleep-dependent processes: it is lowest (and sleep latency the shortest) around the minimum of the body temperature rhythm, while wakefulness within sleep episodes increases as a function of elapsed time within the episode. An additional finding is that the circadian contribution to the regulation of rapid eye movement and slow-wave (delta) sleep stages differs significantly, with an influence on the former considerably greater than the latter. Notably, an inherent feature of the forced desynchrony protocol – that is, the varying alignments expressed between central circadian (presumably SCN) cycles and behavioral (fasting/feeding and sleep/wake) cycles over time – reveals that such temporal disruption can result in physiological and metabolic consequences that are likely to be clinically relevant.

Question 2: What is the functional utility of a circadian clock?

This kind of question is of the type that seeks to better understand the evolution of a biological process and how it contributes to enhanced adaptation to the environment (fitness), eventually leading to increased lifetime reproduction ("ultimate" causation). Raising this question about the adaptive functions of a system can in turn guide the focus of investigations at the proximate level.

It is of course a truism that most all organisms on earth are faced with a host of 24-h cycling stimuli, presenting both challenges and opportunities for synchronization. We use the term *synchronization* here in its most restrictive sense, courtesy of the Oxford English Dictionary, as "coincidence or agreement in point of time;" that is, *simultaneity*, as in synchronized swimming. It is certainly possible for organisms to synchronize some of their functions and behaviors to the day or to the night without the need of a circadian clock. One such synchronizing mechanism is the phenomenon we referred to as "masking" in Question 1 of this chapter, as may be observed in animals with genetic and/or physical

lesions of their circadian pacemakers that express apparently normal locomotor activity rhythms when they are maintained in the presence of a light-dark cycle. Importantly, a clock-less state is not a necessary condition for masking; recall our discussion of the human "constant routine" protocol, in which certain behavioral patterns are suppressed with the aim to "unmask" the innermost circadian timing signal underlying the body temperature rhythm.

Although a sound physiological mechanism, masking is ultimately a reactive one ★2.16. Without an internal sense of time, there is no possibility for anticipating a recurring external event (for example, morning lights-on or periodic food availability) and preparing for it. ★2.17. Such an internal sense allows not only for anticipation but also for the dynamic regulation of optimal rhythm phases – synchronizing compatible processes, separating incompatible ones, properly sequencing others, and tuning temporal organization in response to environmental circumstances. An adaptable timekeeping mechanism should be favored by evolution and key to an organism's selective advantage in nature. Indeed, in the relatively few species that have been studied both in nature and the laboratory, the phases and waveforms of some daily rhythms are plastic and can differ dramatically between the two conditions ★2.18. Across biological kingdoms, the ubiquity of internal timekeeping systems that enable such adaptable phase control is a testament to the benefits of a clock mechanism that essentially represents the external day-night cycle within organisms rather than having them simply react to it. Such a mechanism not only ensures the organism's external alignment to daily environment cycles but also the internal alignment of its metabolic, physiological, and behavioral rhythms to each other.

Obviously, this function demands that the circadian oscillator adopts a stable phase relationship to the day-night cycle. Entrainment is the process by which two oscillating systems assume the same period – in our case, 24 h – generally with one driving (entraining) the other, thereby establishing a stable phase relationship between them over succeeding cycles. Their entrained phases may be synchronous (that is, with a "phase difference" or "phase angle difference" of 0°) or not (for example, in antiphase, at 180°). Note that in our case, with the entrainment of two qualitatively different oscillating systems (rhythms of living matter to the day-night cycle), we can define the phase of entrainment between them as we wish by our choice of a phase reference point (e.g., onset, offset, peak, or center of gravity of rhythmic behavioral activity). Obviously, the reference point(s) become a practically important decision for real-world rhythms that may not be sinusoidal or symmetrical.

a. Circadian entrainment by the light-dark cycle & discrete light pulses

Theoretically, there must be a multitude of candidate entrainment cues (referred collectively to as *Zeitgebers*), but the most prominent ones (and the most intensively studied) are light and temperature, alone or in combination ★2.19. The role of a *Zeitgeber* in circadian entrainment can be demonstrated using a "jet lag" protocol, especially popular in laboratory studies of nocturnal rodents (although, admittedly, jet lag is not a condition represented in their evolutionary history). By the way, to our knowledge, this experimental approach was first reported and presented in actogram format in 1926 ★2.20, well before there were jet airplanes or jet lag.

We have found that the descriptive vocabulary of jet lag can be confusing for students or newcomers to the field, so let us begin with the actual problem that most of us may have experienced (Fig. 9). Imagine a non-stop flight from Boston's Logan Airport (BOS) to Amsterdam's Schiphol Airport (AMS) (Fig. 9A); they are 6 time zones apart, with local AMS time *ahead – later than* – local BOS time (since the Earth rotates from west to east, the sun rises at AMS 6 hours earlier than at BOS). Now, with a favorable tailwind, flight duration from BOS to AMS will be about 6 hours. A BOS departure at 19:00 local time + 6 hours in flight results in an AMS arrival at 01:00 the next day from the traveler's perspective, whose subjective (internal) time is still set to local BOS time. Of course, the problem is that local AMS time is actually 07:00, so re-entrainment to the new local time requires that our traveler's rhythm must *advance* or *speed up* towards it; another way of expressing this is that external AMS phase reference points – say, the morning (lights-on) or a dinner reservation – will now come *earlier* than expected from a BOS point of view. The same logic can be applied to the return trip from AMS to BOS (Fig. 9B), with local BOS time *behind – earlier than* – local AMS time. Now on this leg there is likely a headwind, so flight duration from AMS to BOS will be about 8 hours. An AMS departure at 12:00 local time + 8 hours in flight results in a BOS arrival at 20:00 the same day from the traveler's perspective, whose internal time is still set to local AMS time. Of course, the problem is that local BOS time is actually 14:00, so re-entrainment to the new local time requires that our traveler's rhythm must *delay* or *slow down* towards it; another way of expressing this is that an external BOS phase reference point – say, the dinner reservation or bedtime (lights-off) – will now come *later* than expected from an AMS point of view. Note that in this example the BOS to AMS traveler flies in the direction of the Earth's rotation while the AMS to BOS traveler flies against it; at these roughly realistic departure times, the former compresses the duration of their night, while the latter expands the duration of their day.

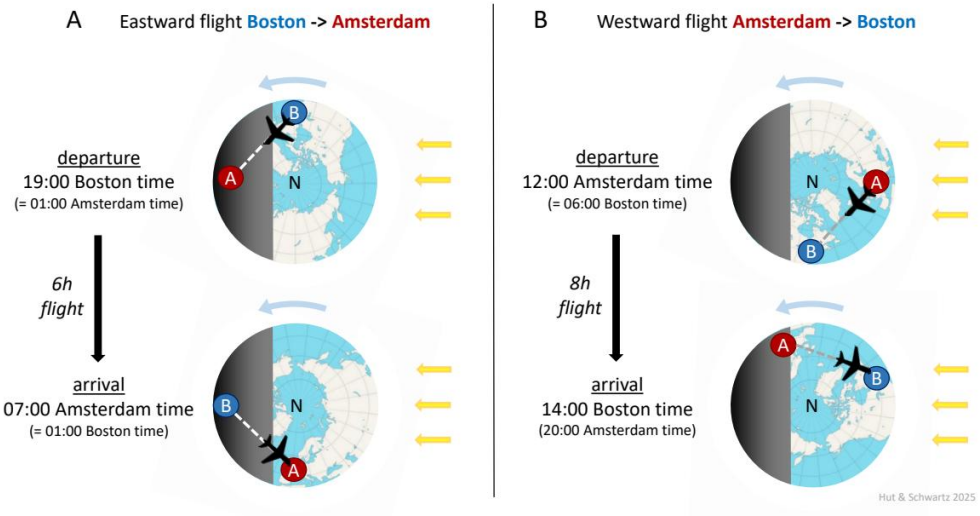


Figure 9. Visualizing jet lag flying west to east (A) and east to west (B) (see text)

Polar views of the Earth (N, north pole) rotating from west to east (blue arrow) with the sun illuminating daytime in the northern hemisphere during summer (yellow arrows). Local time in Amsterdam (red circle A) is 6 hours ahead (later than) local time in Boston (blue circle B).

In the typical laboratory experiment (Fig. 10), the animal (usually a mouse, hamster, or rat) is initially maintained on a 24-h light-dark cycle (on the order of weeks, generally as a 12 h: 12 h light-dark schedule) and locomotor activity monitored (by running wheel or infrared beam sensor); ordinarily, daily activity onset will occur around lights-off. Then, the timing of the light-dark cycle is abruptly shifted; for example, with lights-on and lights-off now occurring 6 h earlier the next day (Fig. 10A). Over several days, animal activity onset gradually advances each 24-h cycle until it is re-aligned with the new lights-off. The shifted light-dark cycle has re-entrained the circadian pacemaker that drives the locomotor activity rhythm, and the process is referred to as a *phase advance* of the pacemaker (or the rhythm). The opposite re-entrainment maneuver – with lights-on and lights-off occurring 6 h later the next day (Fig. 10B) – is referred to as a *phase delay*.

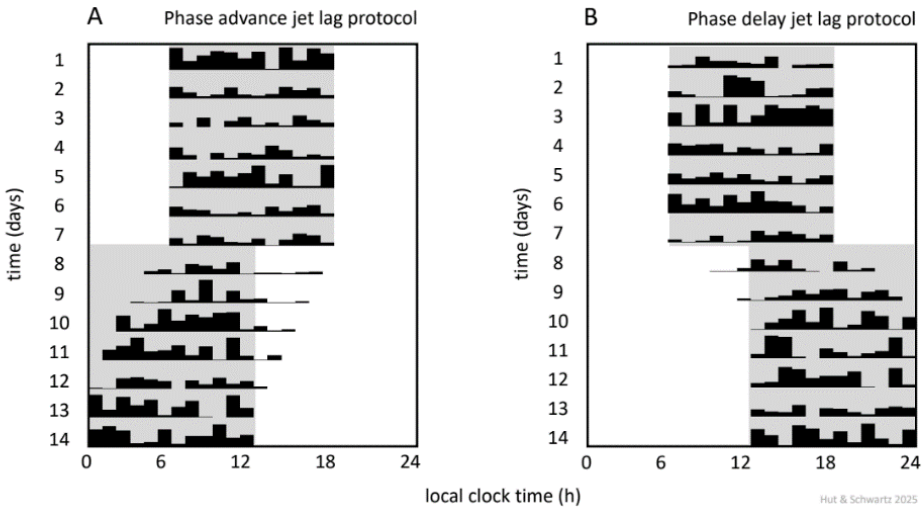


Figure 10. Modeling rodent jet lag in the laboratory (see text)

Actograms of two nocturnally-active animals, initially maintained in a 12 h: 12 h light-dark cycle and then subjected to a 6-h advance (A) or delay (B) of the cycle. Shading represents darkness. Note the difference between the advance and delay shifts in the speed of re-alignment of activity onset.

But wait! Is synchronization after the shift in the light-dark cycle due to entrainment of the circadian pacemaker or to "masking" of the locomotor activity rhythm? The answer in the illustrated case depends on the marker being used to track rhythm phase and the direction of the induced shift. If the marker is activity onset – as it often is, since onset is usually easily and reproducibly identified in nocturnal rodents – its slow movement over several days (referred to as *transients*) during the phase advance (Fig. 10A) argues for entrainment as a mechanism. On the other hand, activity onset during the phase delay appears to shift immediately and completely (Fig. 10B). This asymmetry in the speed of re-entrainment hints at a possible role for masking in the delay direction, because the shifted light phase in that direction is initially coincident with the animal's activity phase (and the masking effect of light is known to suppress the expression of locomotor activity in nocturnal rodents). In the ideal design to distinguish entrainment from masking, the jet lag protocol should be preceded and followed by measurement of the animal's free-running rhythm in constant darkness. If entrainment has occurred, the phase of activity onset after jet lag should clearly differ from that predicted by extrapolation of the pre-protocol free run (Fig. 11).

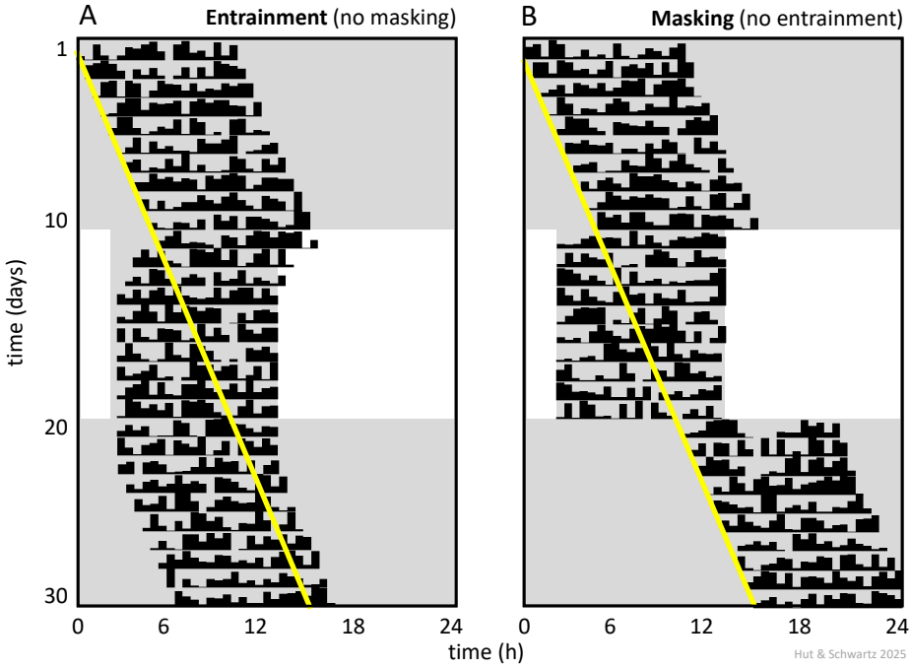


Figure 11. Entrainment vs. masking

Shown is the "classical" experimental protocol to determine if synchronization of an activity rhythm to a light-dark cycle represents entrainment or masking (in this case, to a 12 h: 12 h light-dark cycle during days 11 – 20). Shading represents darkness. The key is to record free-running period in constant conditions before and after the synchronization interval. With entrainment (A), the phase of the rhythm after synchronization (in this case, activity onset on day 21) is predicted by its phase just before release from synchronization. With masking (B), the phase is instead predicted by extrapolation of the pre-protocol free run (yellow line), indicating that the underlying oscillator has continued to run unperturbed through the experiment, with synchronization of the activity rhythm due to a direct action of the light-dark cycle. Note that the argument assumes an invariant value for the free-running period through the entire procedure. Also shown in the figure are two additional characteristic features of entrainment: synchronization achieved gradually over several cycles (in this case, with 4 days of advancing "transients") and the presence of an after-effect that reflects the preceding entrained period (in this case, the 2 initial days in constant darkness that express a period equal to 24 h). Neither of these features is seen with masking.

The light-dark cycles we have been discussing can be described as *complete photoperiods* (in the case of Fig. 10, it is the 12 h duration of the light phase), and it is "complete" in the sense that light is continuous for the full 12 h interval. Notably, stable entrainment and re-entrainment with jet lag can also be achieved under certain *skeleton photoperiods*, characterized by two short, discrete light pulses (say, 15 min to 1 h in length) that define the onset and offset of the light phase. Interestingly, the differential speed of re-entrainment in the jet lag protocol – with delays completed quickly but advances only slowly after a series of transients – is also a feature of the protocol under a skeleton photoperiod, so differential masking cannot be the only explanation for the asymmetry in the shifts ★2.21.

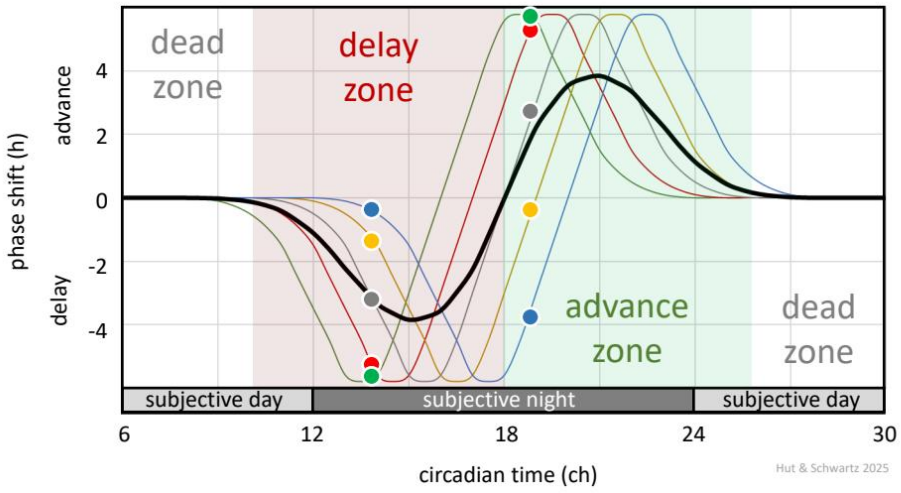


Figure 12. The photic phase response curve (PRC)

The canonical PRC to brief light pulses (black waveform), with delay phase shifts after light administered during the late subjective day / early subjective night, advance phase shifts during the late subjective night / early subjective day, and little or no phase shifts during most of the subjective day. In practice, PRCs are experimentally derived from populations of animals, likely with inter-individual variation in their phases (see Fig. 3); here we show how such variation in individual data points from 5 animals might appear after light pulses around circadian times 13 – 14 and 19. Note that the blue animal would actually exhibit a phase delay after a light pulse administered during the early part of the average "advance zone."

Entrainment by discrete light pulses indicates that the circadian pacemaker shifts differentially to light applied at different phases across the circadian cycle, and a graph of such shifts as a function of the phase of light pulse administration can be plotted as a "phase response curve" (PRC) (Fig. 12). The general configuration of the PRC is similar for all organisms, whether their behavior is diurnal, nocturnal, or something else; it is thus a property of the pacemaker itself. Light pulses administered during the late subjective day / early subjective night (that is, around subjective lights-off [CT 12]) result in a phase delay (the pacemaker and rhythm move to a later phase); light pulses administered during the late subjective night / early subjective day (that is, around subjective lights-on [CT 0]) result in a phase advance (the pacemaker and rhythm move to an earlier phase); and light pulses administered during most of the subjective day have little effect.

The shape of the PRC suggests a provisional blueprint for natural entrainment of the circadian pacemaker to 24 h by daily phase shifts to light around dawn and dusk. If the pacemaker's period is too long – oscillating slower than once every 24 h – exposure to daily morning light will advance its phase; if the period is too short – oscillating faster than once every 24 h – exposure to daily evening light will delay its phase. Quantitatively, with entrainment, the light induces phase shifts that exactly compensate for the period mismatch between the light-dark cycle and the free running pacemaker (Fig. 13). In this way, the shape of the PRC and the difference between τ and 24 h determine a rhythm phase that is stable upon entrainment to the light-dark cycle.

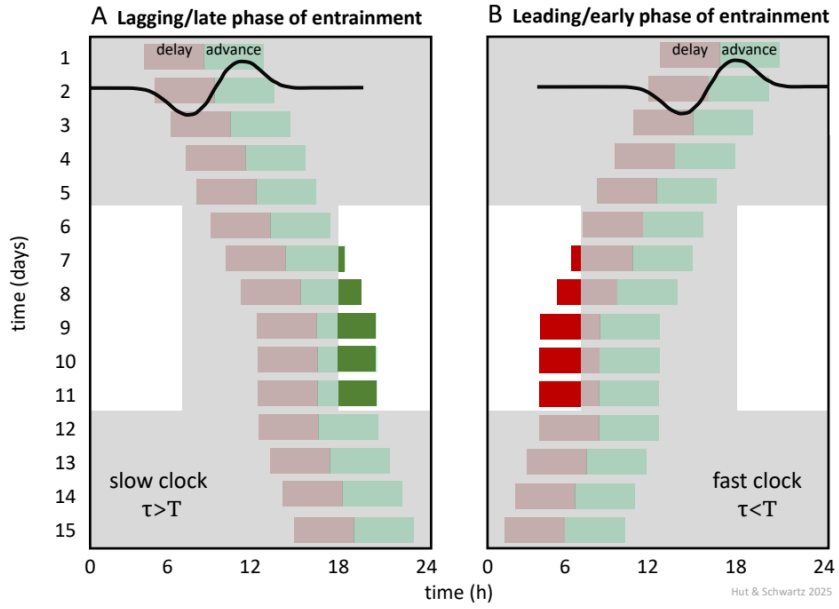


Figure 13. Phase of entrainment of slow and fast clocks explained by the PRC

The free-running rhythms shown here represent the PRCs of two circadian oscillators with periods either longer (A) or shorter (B) than 24 h. Shading represents darkness; advance (green) and delay (red) zones of the PRCs are as denoted in Fig. 12. Both oscillators can be stably entrained to the 12 h: 12 h light-dark cycle but do so assuming different phases of entrainment. When the circadian period is too long, exposure to daily morning light will advance oscillator phase; when the period is too short, exposure to daily evening light will delay oscillator phase. Entrainment on day 9 occurs when the magnitude of the light-induced phase shift compensates for the difference between the periods of the free-running oscillator and the entraining cycle, expressed as $\tau - T = \Delta\phi$, where τ is the circadian period, T is the entraining period, and $\Delta\phi$ is the magnitude of the phase shift.

The discrete (non-parametric) model of entrainment outlined above demonstrates considerable explanatory power and has been widely adopted. And yet, it assumes that phase shifts are instantaneous, complete photoperiods are irrelevant, and τ is fixed (even though we know that the value of τ is altered by light; refer to ★2.8 on the shortening or lengthening of τ under constant light conditions). So an alternative – the continuous (parametric) model – posits that light decelerates (slows down) or accelerates (speeds up) the pacemaker's oscillation in a phase-dependent fashion, based on a velocity response curve qualitatively comparable to light's non-parametric effects in constant darkness. This model may help to resolve the conundrum of photic entrainment in animals that do not see dawn or dusk ★2.22. Most likely, the truth involves aspects of both entrainment models.

b. τ, light sensitivity, and phase of entrainment

Regulating the phase of entrainment in nature – for example, as an "early bird" or a "night owl" – is surely critical for adapting to dynamic environmental challenges; so, from an evolutionary point of view, entrained phase would seem to be the pre-eminent circadian parameter undergoing natural selection ★2.23. Mathematical analyses and simulations as well as experimental data have suggested that organisms could adjust their phase of entrainment to a 24-h *Zeitgeber* by regulating the pacemaker 's velocity (that is, the value of τ) and/or its sensitivity to the entraining signal.

Experimentally, we don't yet have a reliable method for systematically varying and clamping the pacemaker's velocity in any one individual living organism.

However, we can vary the mismatch between τ and the entraining cycle (referred to as **T)** either by exploiting a range of short and long period mutants or by altering **T** across a range of non-24 h periods (**T** cycles) [★2.24]. The results suggest that entrainment phase is a monotonic function of the degree of τ - **T** mismatch, as well as defining the range (limits) of entrainment to the shortest and longest **T** cycles, beyond which synchronization is not possible (recall the "forced desynchrony" protocol introduced earlier in this chapter). Particular examples using golden hamsters show that these τ and **T** cycle influences on the phase of entrainment can be quite dramatic even in mammals [★2.25].

With regard to the role of the pacemaker's sensitivity to the entraining signal, light is of course the most widespread and powerful 24-h *Zeitgeber*, and in the last decades spectacular progress has been made in our understanding of the photic entrainment pathway to the SCN. These include its strictly ocular localization; the key role of the photopigment melanopsin (OPN4) expressed by multiple classes of intrinsically photosensitive retinal ganglion cells interacting with rods and cones; the responsible neurotransmitters, receptors, and second-messenger signal transduction pathways in the SCN itself; and ultimately in immediate-early and *per* gene transcription [★2.26]. Nevertheless, it remains experimentally difficult to systematically vary the photic sensitivity of circadian systems (as examples, altered sensitivity due to retinal adaptation or pupillary size), so the empirical approach has primarily focused on varying stimulus strength (irradiance, duration, and wavelength). Increasing the photic "dose" will change the shape of the PRC, augmenting delays and/or advances by a few hours (a "type 1 PRC", as in Fig. 12), and, in humans, increasing the degree of suppression of the nocturnal level of melatonin (light exposure via the SCN inhibits pineal melatonin synthesis and release; this suppression is thus used as a proxy to measure light sensitivity [★2.27]). In "lower" organisms (like fungi, insects) as well as some rodent genetic mutants, very large phase shifts around 12 h can be generated – so large that it may be impossible to classify them as advances or delays (a "type 0 PRC") [★2.28]. As a practical matter, most routine laboratory experiments employ saturating light intensities (a couple of hundred lux for rodents) that may not be standardized across laboratories; "lux" is a measure based on human, not rodent, perception of brightness, and equal illuminance in lux is often not equivalent in the distribution of wavelengths and their intensities. Importantly, measures of light exposure that account for species differences in wavelength sensitivities have been proposed recently [★2.29].

So the bottom line, generally speaking, is that phase of entrainment can be related to the magnitude of the τ - **T** mismatch and degree of *Zeitgeber* strength.

As a common rule: if $\tau < 24$ h, and the greater the mismatch or the less the *Zeitgeber* strength, then the earlier the entrainment phase; if $\tau > 24$ h, then the later the entrainment phase ★2.30. But it is fair to say that quantitative prediction of the phase of any one individual remains a work in progress. Modeling suggests additional oscillator properties need to be included – properties that may not be easily or unambiguously obtained in the laboratory – such as oscillator amplitude (the effective strength of the *Zeitgeber* is related to oscillator amplitude, increasing as amplitude decreases) and relaxation rate (for systems that switch between states, as with feedback loops of activators and repressors). Additionally, and perhaps most inconveniently for simple models, populations of coupled oscillators – not only in the SCN and the *Drosophila* head but throughout the body (see Question 3 in this chapter) – can behave quite differently from the autonomous behavior of one oscillator alone ★2.31. And ultimately, we must confront the complexity of entrainment in the natural world: where variance of light irradiance and spectral composition at actual dawn and dusk is not mimicked by our usual laboratory "lights-on" and "lights-off;" where τ is not a fixed unchanging value; where an array of non-photic biotic stimuli (with non-photic PRCs) appear irregularly; where age and sex are factors; and where "noise" in the photic entrainment signal is unavoidable.

Question 3: What is the systems physiology of circadian networks?

The cloning of circadian clock genes made possible the development of transgenic luciferase reporters of real-time circadian gene expression – and the subsequent discovery that the core molecular circadian timing loop oscillates in cells, tissues, and organs throughout the body (Fig. 14) (although already in the late 1950's and early 60's there had been claims that isolated rodent organs could express circadian rhythms ★2.32). We have since learned that cell-autonomous circadian rhythmicity is a ubiquitous feature of nearly all living matter – not only in certain bacteria, unicellular eukaryotes, and cell cultures & cell lines, but also nearly everywhere in multicellular organisms: in the cells of tissues, organs, brain regions, and cancers; endocrine, hematopoietic, and immune systems; and embodied parasites and symbionts.

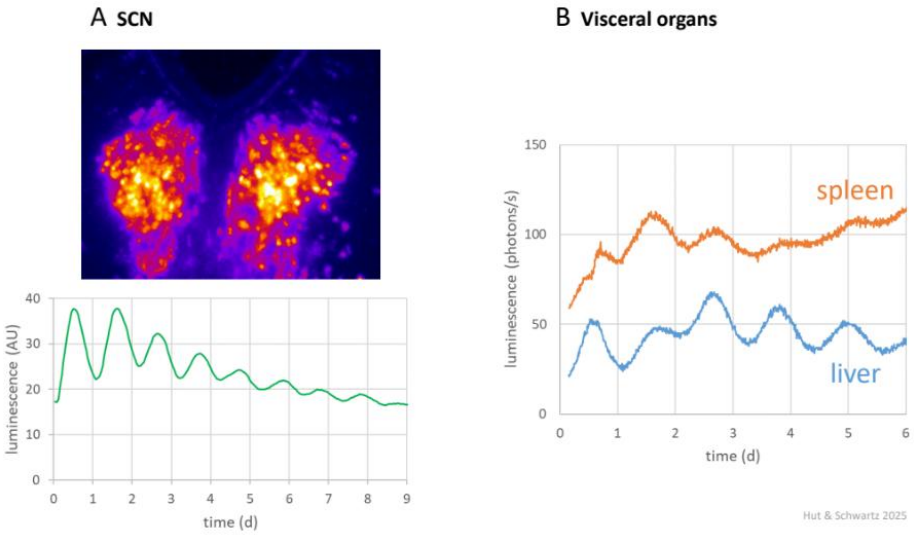


Figure 14. Real time recordings of circadian gene expression ex vivo

SCN (A) and visceral organs (B, spleen and liver) were obtained from mPer2^{Luc} knockin mice (Jackson Laboratory, Bar Harbor, Maine) expressing a mPER2::LUC fusion protein; the mice were previously entrained to a 12 h: 12 h light-dark cycle. The culture medium in the sealed culture dish includes the substrate luciferin, which emits light when metabolized by luciferase in the tissues, allowing real-time quantitative reporting of the amount of fusion protein. Upper panel in (A), 9-day video recording of ~8.5 circadian cycles of the transgenic SCN ex vivo.

So now we find ourselves beyond the simple linear Eskinogram; deciphering the design principles of the timing of bodily functions will require a deep understanding of the dynamics of complex oscillator networks, coupled both within and across biological levels (from molecules to metabolic, physiological, and behavioral systems) and between anatomical regions. For sure, this is a very hard problem!

a. Conceptualizing the temporal architecture of circadian timekeeping

The current conception of mammalian circadian organization is usually described as hierarchical. Typically, the SCN appears alone at the top, as the repository of the internal representation of the ambient light-dark cycle. External time (or circadian time, in constant conditions) is conveyed by the SCN to the brain and body via a panoply of neural, humoral, thermal, and behavioral signals ★2.33 that drive, gate, or entrain ★2.34 daily or circadian rhythmicity in downstream targets. In a cell-, tissue-, and organ-specific manner, combinations of

these signals in concert with cellular energetic status engage the intracellular circadian timing loop to regulate circadian rhythms of gene expression. This regulation is mediated by mechanisms at different levels – including epigenetic regulation, transcription, RNA splicing, mRNA stability and translation, and post-translational modification ★2.35 – ultimately leading to the flexible timing of biological functions. Customarily, the connectivity in this schema has been portrayed with multiple arrows projecting out from the SCN to its targets. The special position of the SCN at the pinnacle of the hierarchy is justified not only for its role in photic entrainment but also for its strong intercellular coupling – conferring precision and robustness to its output rhythms as well as resistance to the actions of its own downstream signaling modes (like feeding-, glucocorticoid-, and temperature-induced changes in phase) ★2.36. Of note, temperature *entrainment* should not be confused with temperature *compensation*; the latter refers to a requisite property of a free-running rhythm with its period relatively resistant to differences in ambient temperature ★2.37.

A schematic diagram like the popular one described above is important for advancing scientific concepts because it helps to shape our mental models and the design of our experiments. So any re-working with additional data should reflect conceptual advances and not merely artistic refinements, and we do have some suggestions. First of all, the exclusive position of the SCN as the pre-eminent circadian pacemaker in mammals is not conserved across other vertebrate classes; in some fish, reptiles, and birds, that function lies along an axis that includes not only an SCN but circadian pacemakers that are directly photo-entrainable in retina and pineal gland ★2.38. And second, that the isolated SCN continues to oscillate *ex vivo* does not mean that its cells are unresponsive to bodily influences *in vivo*. Indeed, the amplitude of its electrophysiological rhythm is altered by locomotor activity, and the nucleus receives many mono- and poly-synaptic neural afferents, bears receptors for molecules expressing rhythms that it controls, and responds to systemic cytokines ★2.39. We need to learn much more about the SCN's fine anatomy and compartmental organization, but these examples argue for a revised schematic that also includes a host of arrows projecting backwards, into the nucleus from the brain and body (Fig. 15). It is tempting to imagine that the SCN and its extensive web of efferent signaling pathways has been co-opted by evolution to also meet unexpected homeostatic challenges to the internal milieu – such as starvation, dehydration, stress, inflammation, and activity state – that demand highly integrated metabolic, physiological, and behavioral responses ★2.40.

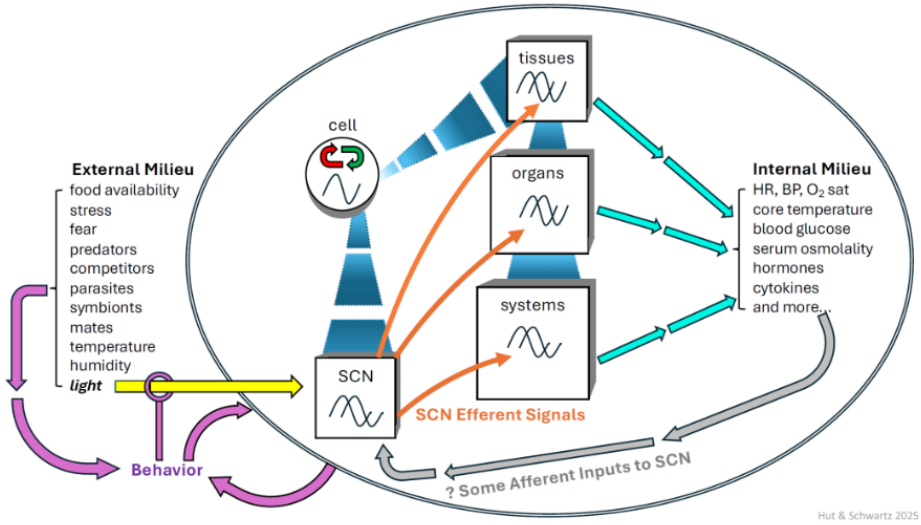


Figure 15. A working schematic of the SCN within the circadian timing network

Within an organism (the oval bordered by a compound double line), the underlying oscillatory mechanism is within the cell, and cells form tissues, tissues build organs, and organs function together as physiological and metabolic systems. In this schematic, the SCN is a specialized neural tissue embedded within the network. On one hand, photic entrainment of its oscillation (yellow arrow) provides time-of-day information to tissues, organs, and systems via a variety of SCN efferent signaling mechanisms (orange arrows); on the other hand, the state of the internal milieu (green arrows) also appears to influence aspects of SCN rhythmicity (gray arrows), perhaps by modulating its electrophysiological activity and thus leading to possible consequences in efferent signaling. Also shown are additional rhythmic features of the external milieu, some of which act through changes in behavior of the organism itself (purple arrows); and these altered behaviors, in turn, may affect photic entrainment and masking. Not included here are signaling mechanisms between organs (see ★2.41) nor tissue- and organ-specific Zeitgebers independent of the SCN (for example, local photic entrainment pathways [see ★2.42]). HR, heart rate; BP, blood pressure; O_2 sat, oxygen saturation.

b. Phase alignment, realignment, and misalignment

Sophisticated experimental techniques have been employed – including trans-

genic reporters for real-time tracking of rhythmicity in cells and tissues, optogenetics and chemogenetics, transcriptome profiling, and organ-specific conditional mutants – to discern how the architecture and dynamic connectivity of the circadian network leads to homeostasis at the level of the whole organism. The emerging picture appears extraordinarily complex. Circadian gene expression in cells, tissues, and organs is not only determined by systemic signals and behaviors set by the SCN but also by the autonomous oscillators within the cells, tissues, and organs themselves; the resulting phase map is complicated by the fact that these local oscillators are not uniformly beholden to the SCN. For metabolically active organs, the behavioral feeding-fasting cycle is a dominant *Zeitgeber* and remains so even when the behavior is uncoupled from SCN control (for example, by artificially displacing and restricting feeding to the light phase in a nocturnal animal); for skeletal muscle and lung, scheduled exercise has an analogous effect. A host of candidate metabolites, hormones, neurotransmitters, and paracrine factors acting via specific receptor and second-messenger signal pathways underlies communication and coordination of rhythms and/or their local oscillators ★2.41. Some of these oscillators – including in retina, cornea, and exposed skin in mammals – appear to be independently photoentrainable via activation of the photopigment neuropsin (OPN5) ★2.42.

It is through the proper alignment of rhythmic network components – for example, liver, pancreas, skeletal muscle, adipose tissue, intestine – that regulated blood glucose levels can be achieved even in the face of strong rhythms of feeding and fasting. Crucially, alignments are malleable and adaptive; they are neither fixed internally within the network nor externally to geophysical time ★2.43. The mechanisms underlying these kinds of readjustments of entrainment phase are not fully known, but from our discussion in this chapter (see Question 2, above), we can guess that such control might be mediated, at least in part, by changes in downstream oscillator $\boldsymbol{\tau}$ and/or in the strength of oscillator coupling to resetting signals. The principles for how cell, tissue, and organ oscillators integrate multiple incoming signals, both in their quantity and quality, are not understood.

Establishing the daily temporal organization of biological systems requires coordination among multiple time axes: not only between external day-night (solar) time and internal body (circadian) time, but also behavioral (sleep) time and social or societal (clock) time, which in humans undergoes a yearly 1-h scheduled phase advance in spring and phase delay in autumn ★2.44. Now given our appreciation of the complexity of the circadian timekeeping network, and the significance of optimal (and adaptable) alignment of its oscillating parts, a

practical problem regarding circadian time becomes the definition and assessment of its phase with respect to the other axes, especially over repeated cycles in living animals. To this end, several measures have been promoted as indicators of mammalian phase of entrainment to the solar cycle. "Chronotype" is a word coined in 1974 to mean "temporal phenotype," originally envisioned as a 24-h landscape of the distribution of peak phases of an array of rhythms in behavior, physiology, hormonal and biochemical levels, mitosis, circulating white blood cells, pharmacological sensitivities, and more ★2.45. Since then, the term has been re-purposed as sleep phase, yielding practical insights on the variation of sleep timing relative to solar, circadian, or clock times, depending on lighting, age, sex, genetics, and even east-west position within a time zone. There are clinical consequences for individuals with extreme sleep phases, especially those that are delayed relative to modern societal school and work hours (Fig. 16).

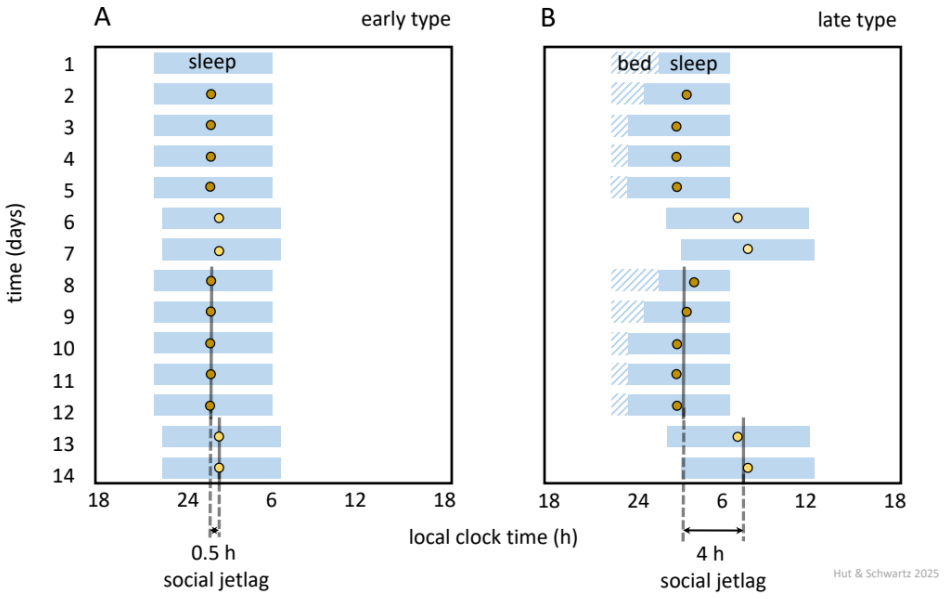


Figure 16. Chronotype and social jet lag

Stylized actograms of sleep duration and timing (blue bars) of two exemplary human chronotypes, early (A) and late (B), over 2 weeks of recording, with days 6/7 and 13/14 representing "free days" (weekends) unencumbered by alarm clocks or other social or societal demands. Here, with both individuals required to awaken at 06:00 on "work days" (weekdays), the late type's attempts to initiate

sleep earlier than their natural late phase leads to sleep-onset insomnia ("bed," hatched bars) and a shortened sleep duration. This sleep deprivation during weekdays is compounded by "social jet lag" during weekends, when innately later sleep onset and wake up times generate phase delays and advances on Friday and Sunday evenings, respectively. Measured as the difference in mid-sleep phase between weekdays and weekends (colored dots), the degree of social jet lag has been associated with cognitive, somatic, metabolic, and behavioral pathologies.

We have previously referred to the nocturnal rhythm of pineal melatonin synthesis and secretion as a reliable assay of circadian time, or at least as a gauge of "central SCN" time, with its phase in humans usually measured as rhythm onset under dim lighting conditions (dim light melatonin onset, or DLMO). The rhythm is driven by the SCN via a well-described polysynaptic neural pathway, and, like the SCN, its phase is similar in diurnally- and nocturnally-active animals (for melatonin, high during nighttime in the dark, so phylogenetically it is a "darkness" hormone rather than a "sleep" hormone). The key regulatory step in melatonin synthesis is in the activity of the enzyme aryl alkylamine *N*-acetyltransferase (AANAT); interestingly, the mechanism involves changes in AANAT gene transcription in rodents but not in ungulates or primates, reminding us that natural selection selects for outcomes, not necessarily for mechanisms. On a discouraging practical note, however, most inbred laboratory mouse strains do not synthesize melatonin (including popular ones like C57BL/6 and BALB/c, but not including CBA and C3H), and even in humans multiple blood samples and specified lighting conditions are required to calculate DLMO. Recently, there has been exciting work on developing novel assays of circadian time that may supplant DLMO, using one or two single blood samples for metabolomics or gene expression profiles of circulating blood cells ★2.46.

Equally exciting has been the increasing interest – among investigators and the lay public alike – in the causes and consequences of *improper* alignment of the circadian network to the time axes, as recognized in jet lag and shift work, and by several terms (disruption, desynchrony, misalignment) that attempt to acknowledge the myriad circumstances that could lead to circadian trouble. These include, as examples, desynchrony due to differing periods between rhythms; misalignment due to anomalous phase relationships between rhythms; internal disruption within or between network levels; and external disruption between the network and the environment. Such conditions have been associated with symptoms and signs of disease and health outcomes ★2.47, but interpretation of many of these associations will be a daunting task. Which misalignments are adaptive (a physiological compensation to a challenge), which

are pathogenic, and which are consequential rather than causal? Which maladies result from associated disturbances of sleep rather than circadian misalignment *per se*? Which misalignments are actually due to natural variation? And what about the known confounds of most pre-clinical studies: laboratory mice, unlike most humans, are inbred, nocturnal, and as small rodents expend much of their energy defending body temperature. On this point, typical animal facility temperatures lie below the lower critical temperature that defines the murine thermoneutral zone, so the alignment of the circadian network in our key model species during such "baseline" conditions actually represents a configuration under a metabolically stressful environment for them.

c. Elaborating the clinical physiology of disturbed circadian function

Our field has now developed the conceptual framework, investigative tools, and powerful analytics to further our understanding of the pathophysiology of circadian dysrhythmias. As the circadian system becomes a therapeutic target for pharmacological and behavioral interventions, humans are obviously the favored model, not only for their clinical relevance but also for the long tradition of applying holistic, integrative approaches to their study (including the recording of multiple rhythms from individual subjects). We have emphasized from the beginning of this chapter that the expressed patterns of most daily rhythms are shaped by both endogenous and exogenous factors beyond the timing of clock genes. So now an important task will be work directed to understanding natural rhythm waveforms, aiming to estimate the relative contributions and weights of circadian and non-circadian influences.

Consider the case of body temperature, with (a) a *value* adaptively regulated ("tuned") for optimal physiological performance via changes in heat production and/or heat loss, and (b) a *rhythm*, at least one function of which is acting as an internal 24-h *Zeitgeber* for the entrainment of some cellular and organ rhythms. In adult humans, parameters of the overt temperature rhythm (mean level, range of oscillation, phase, waveform) can be altered by multiple factors, including ambient temperature, physical activity, sleep, meals, age, and the menstrual cycle. Clearly, discovery of a disturbed temperature rhythm as part of a pathological state will require comprehensive studies to determine mechanistic connections and the arrow of causality, given the bidirectional relationship between circadian disruption and disease.

The daily rhythm of sleep and wakefulness provides another case study of an overt rhythm shaped by circadian and non-circadian influences. Two key oscillatory processes, conceptually distinct but physiologically interconnected, interact to shape the nominal 8 h-16 h waveform of the human sleep-wake

rhythm. One is circadian, based on internal time; the other relates to elapsed time, with the likelihood of sleep (known as sleep propensity or sleep pressure) increasing as a function of time spent awake and dissipating as a function of time spent asleep ★2.48 (a homeostatic regulation of sleep). Forced desynchrony and other protocols have suggested how the combination of circadian and homeostatic influences might act to create the human consolidated but malleable sleep-wake rhythm (Fig. 17).

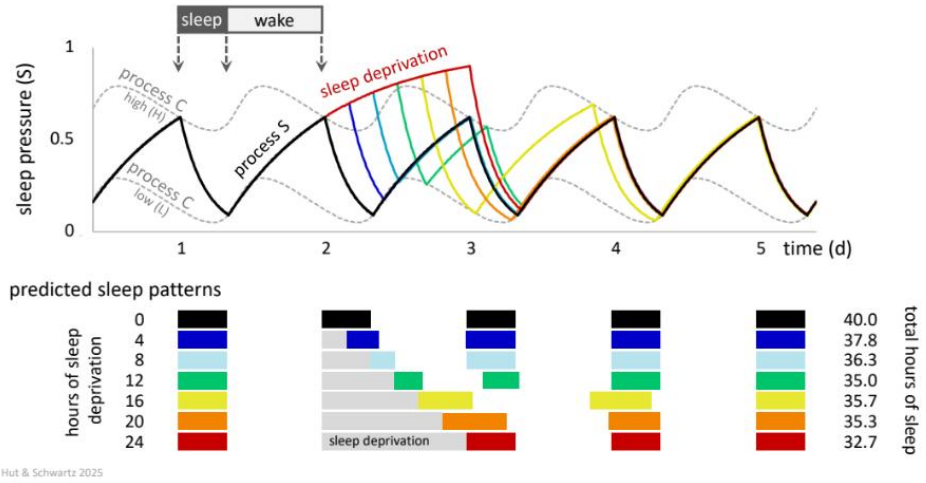


Figure 17. Circadian and homeostatic regulation of the human sleep-wake rhythm

The two-process model was developed to account for the circadian influence on the timing of sleep and the response to sleep deprivation. During wakefulness, sleep pressure (homeostatic process S, black curve) builds up by an inverse exponential increase that approaches an asymptote at 1; then during sleep, the pressure dissipates by an exponential decrease that approaches an asymptote at 0. The switch from wakefulness to sleep, and from sleep to wakefulness, occurs when S reaches high and low threshold levels, respectively. These levels fluctuate over 24 h following a skewed sines wave (circadian process C, high [H] and low [L] gray dashed curves). The model (see references in ★2.48) has been instrumental, among other insights, in predicting the duration of sleep after prolonged deprivation (4 h, blue; 8 h, turquoise; 12 h, green; 16 h, yellow; 20 h, orange; 24 h, red), explaining the counterintuitive finding that sleep duration after 4 – 16 h of sleep deprivation is generally shorter than normal.

Note that an alternative schematic (Edgar, 1996; Dijk and Edgar, 1999) has focused on a circadian "alerting signal" that opposes a rising "sleep load" over the day, resulting in relatively stable wakefulness until the circadian signal declines in the evening. Both models may not be very different, since the value of S – H might be a good measure of fatigue, and therefore H – S could be interpreted as an indicator of the circadian alerting signal.

Edgar DM. Circadian control of sleep/wakefulness: Implications in shiftwork and therapeutic strategies. In: Physiological Basis of Occupational Health: Stressful Environments (Shiraki K, Sagawa S, Yousef MK, eds), Academic Publishing, Amsterdam, 1996, pp. 253-265.

Dijk DJ, Edgar DM. Circadian and homeostatic control of wakefulness and sleep. In: Regulation of Sleep and Circadian Rhythms (Turek FW, Zee PC, eds), Marcel Dekker, New York, 1999, pp. 111-147.

Circadian and homeostatic regulation also appears to be a defining feature of sleep in other animals, probably including those with relatively simple nervous systems, but the outcome is not necessarily a monophasic pattern. For example, nocturnally-active inbred mice and rats do spend more time asleep in the light phase than the dark phase, but in the range of 60 – 70% of their time during the light and 30 – 40% during the dark, and with much more frequent alternations between sleep and wake in each of the phases (influenced, not unexpectedly, by the availability of a running wheel ★2.49).

The circadian/homeostatic construct is useful for considering sources of natural variation or the pathophysiology of sleep disorders as well as therapeutic approaches to their treatment. As one example, the delayed sleep phase syndrome, with the natural sleep interval occurring relatively late with respect to the environment, may present as a disorder with complaints of sleep onset insomnia, morning sleep inertia, and excessive daytime somnolence. It is considered a circadian rhythm sleep disorder, and two recognized subtypes are (a) a misalignment of sleep time with circadian time (with delayed sleep but normal DLMO) or (b) a misalignment of internal time with the environment (with both sleep time and DLMO delayed but aligned with each other), with likely practical implications for approaches to therapy. But also plausible might be subtypes with alterations in homeostatic regulation (that is, in the rate of daytime rise and/or nighttime fall of sleep propensity) or in the amplitude of the circadian signal. And, of course, such variations in the homeostatic and circadian systems are not mutually exclusive.

Terminology check-in

Here we wish to reflect, with all due respect, on some of the labels adopted by practitioners in the field – among whom we are counted – for concepts that have been critical to fueling advances in our understanding of circadian systems.

These labels have been so effective and used for so long that they are, as a practical matter, irreplaceable. Nevertheless, we offer some impressions on how they may have – and probably still are – biasing our views. A good example is the term "masking," referring to the direct action of an environmental feature on rhythmicity, which can obscure altered phase or even loss of rhythmicity in our experiments. While it is certainly a valuable concept, we feel that the label is unnecessarily pejorative, as though it is a contaminant or nuisance that needs to be expunged, rather than a natural mechanism that can make a significant impact on shaping the overt waveforms of daily rhythms ★2.16.

"Output" is another interesting word choice. The usual meaning of output is the amount or quantity of something that is produced by a process, a machine, or even by the body ("urinary output"). So it is easy to imagine how statements such as "...an overt output of the circadian clock is the sleep-wake cycle..." can be misconstrued. The clock does not produce sleep/wake like the kidney produces urine. Yes, we know, it's an innocent sentence construction (meant to describe a measurable rhythm from which we can infer properties of the underlying circadian system), but it tends to depreciate the concept that the parameters of the sleep-wake rhythm are shaped by multiple sources to form the final, composite rhythmic waveform. Sleep is not driven by the clock in the way that the melatonin rhythm is driven by the SCN. Perhaps "sleep is gated by" might better reflect our current understanding.

Naming the network components of the mammalian circadian system remains a real problem. The SCN as "master" over cell, tissue, and organ "slaves" is, at the very least, physiologically incorrect. "Central" and "peripheral" oscillators are a convenient partitioning but also perhaps not the best. It seems that "central" is usually referring to the SCN, but it's not always clear. Among endocrinologists and neurologists, "central" generally refers to the central nervous system (that is, dysfunction in hypothalamus rather than endocrine gland, or pathology in spinal cord rather than peripheral nerve). Are extra-SCN brain oscillators central? What about glia or migrating microglia into the SCN? The other difficulty is that "peripheral" oscillators that are lumped indiscriminately together perpetuate a strict top-down network, a view that seems at odds with accumulating experimental data on non-uniform tissue responses to internal *Zeitgebers*.

And finally, of course, we come to the biological or circadian "clock," as well as terms such as "oscillator" and "pacemaker." An oscillator is an entity that moves or swings rhythmically, a pacemaker imposes its rhythmicity on other entities, and a clock is a device for measuring time. The clock metaphor ★2.50

really has powered the field forward in the search for a self-sustained, resettable, and reliable timekeeping mechanism, but it has also constrained our thinking into a binary choice of assigning network elements as either clock or non-clock. If the "clock" is the entirety of the timing network, with multiple nodes and links between and across levels of circadian oscillators, is "masking" part of the clock or not? To avoid confusion, we have tried to be judicious in using "clock," primarily in chapter titles and subtitles and in referring to clock genes. For now, we feel that it is best for everyone to take care to communicate explicitly what they mean, on what level or part of the network they are focused, whether "molecular clock," "SCN clock," "clock gene," and so on. We continue our discussion of the "clock" concept in Chapter 3 of our *Road Map*.

Chapter 3. APPLICATION: Consulting the Clock

Looking back on our *Road Map*, you will see that we have deconstructed, reconstructed, and peered into the adjustable moving parts of the circadian timing system. It functions as an innate ★3.1, inherited, and adaptable timekeeping mechanism that coordinates rhythmic biological processes with each other and with predictable 24-h environmental changes.

But is it a clock?

For this, we return one last time to our Oxford English Dictionary: *clock* (Middle English, likely mid-14th century) is "a device for the measurement and indication of the passage of time." So, do organisms consult their circadian clocks as reference tools for marking the local time and measuring the passage of time, in addition to their role in temporal organization? The answer is a resounding yes.

Adapting to other consequential geophysical cycles

Of course, as the Earth is rotating daily on its axis it is also orbiting annually around the sun. Because the Earth's axis remains tilted in a fixed direction (at 23.44°) throughout its entire orbit, the northern and southern hemispheres are alternately directed towards and away from the sun for half a year each, resulting in the seasons (Fig. 18).



Figure 18. Sun, Earth, and Moon

All axial rotation and orbital periods are as viewed from Earth (synodic), to suit our biological interests. Corresponding periods relative to the "fixed" position of the stars are indicated in brackets (sidereal). Synodic and sidereal periods differ because the Earth orbits the sun (precession effect). The Earth rotates around

its axis in 24 h (that is, the average time it takes for the sun to reach its highest position in the sky again the next day; the synodic or solar day). The Moon orbits the Earth, and it takes 29.53 d from one full moon till the next (synodic month). Due to tidal friction, the Moon's axial rotation became phase locked with its orbit around Earth, resulting in a synodic rotation period also of 29.53 d, causing the Moon to face the Earth always with the same side. Because the sun is not a solid body but a plasma, its sidereal rotation decreases with latitude (24.47 d at the equator and 34.19 d at the poles). Sunspots observed from Earth rotate with an average synodic period of ~27.28 d. The Earth takes 365.24 d to complete one orbit around the sun (synodic, tropical or solar year). Because of the Earth's 23.44° axis tilt, the northern hemisphere is tilted towards the sun around June 21st (summer solstice, longest day length), while the southern hemisphere is tilted away from the sun (winter solstice, shortest day length). On December 21st, the situation is reversed (southern hemisphere: summer solstice, northern hemisphere: winter solstice). The progression of the seasons for the northern hemisphere is therefore in anti-phase with the southern hemisphere, as indicated respectively above and below the dashed orbital path of Earth.

Source: Hiesinger H, Jaumann J, in Encyclopedia of the Solar System (Third Edition), 2014. https://en.wikipedia.org/wiki/Rotation_period_(astronomy)

And seasonal changes in photoperiod (often referred to as "daylength," that is, the length of the light phase) lead to large seasonal differences in ambient temperature, precipitation, and nutrient availability – all of which challenge anticipation and adaptation for life in temperate latitudes. These are met with dramatic annual cycles in reproduction, growth, body weight and composition, thermogenesis, morphology, and behavior. For some small nocturnal mammals, winter adaptation may even include switching to ultradian rhythmicity or being active during the diurnal temporal niche, since activity shifted to the higher ambient temperatures of the light phase should reduce overall energy expenditure ★3.2; others may minimize their energy demands during winter through states of suspended animation (torpor and hibernation). The cycling of the seasons presents an additional critical problem: optimizing survival of the next generation, which is best if birth is timed to the spring, with plentiful food for growth and development of the young. This means that the anticipated seasonal window for mating will differ depending on the length of gestation. For small mammals (as in hamsters) with a short gestation lasting weeks (and also for birds like Japanese quail), breeding occurs in early spring as the light phase lengthens (and the dark phase shortens), and they are referred to as "long day" (LD) (or better, long photoperiod [LP]) breeders; for large mammals (as in sheep, deer) with a long gestation lasting months, breeding occurs in autumn as the light phase shortens (and the dark phase lengthens), and they are referred to as "short day" (SD) (or better, short photoperiod [SP]) breeders. Reproductive

competence is triggered when the change of photoperiod reaches a critical duration, depending on species, latitude, temperature, food intake, and, most importantly, prior photoperiodic history ★3.3.

This kind of anticipatory phenotypic switch hints at the possible existence of an endogenous circa-annual oscillator. The search for this putative oscillator has been a challenging enterprise: demonstrating free-running circannual rhythms requires measurements over several cycles (years) in constant conditions, and many of our favorite model species are short-lived and their breeding is completed after one season ★3.4. Nevertheless, circannual rhythms in a variety of long-lived species (maintained under a fixed photoperiod, usually a 12 h: 12 h light-dark cycle) have been clearly documented, including rhythms in body weight, reproduction, hibernation, and molting (Fig. 19). Generally, free-running circannual periods are in the range of 10 – 11 months.

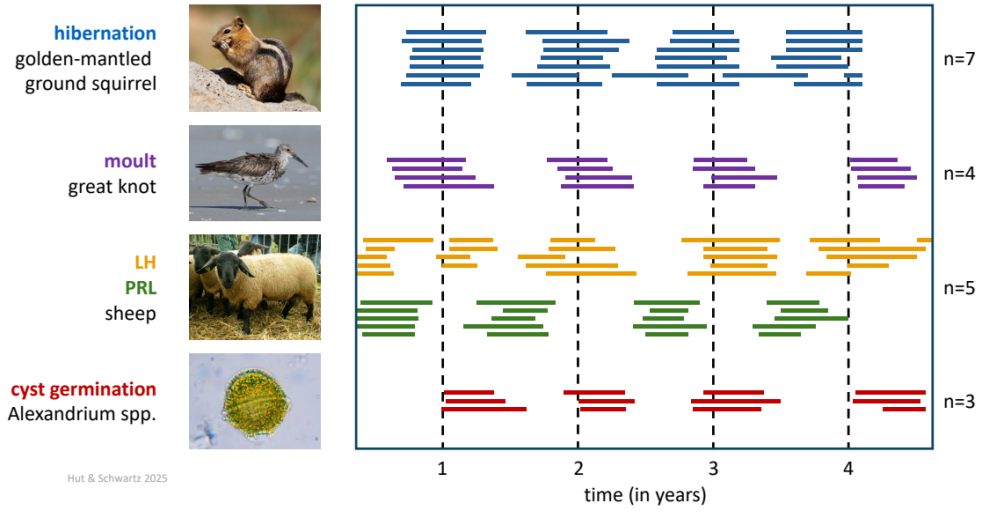


Figure 19. Examples of free-running circannual rhythms

Data collected over four years; vertical dotted lines represent January 1 of each year.

Stacked blue bars: *timing of hibernation in seven individual golden mantled ground squirrels (Callospermophilus lateralis) under constant conditions (12h: 12h light-dark cycle; temperature 3°C).*

***Stacked purple bars**: timing of moult in four individual great knots (Calidris tenuirostris) under constant conditions (12h: 12h light-dark cycle; temperature 15°C).*
***Stacked orange and green bars:** timing of high serum luteinizing hormone (LH) and prolactin (PRL) concentrations, respectively, in five individual sheep (Suffolk ewes) under constant lighting conditions (8h :16h light-dark cycle, temperature not regulated).*
***Stacked red bars:** timing of cyst germination in three populations of dinoflagellate algae (Alexandrium spp.) under constant conditions (constant darkness; temperature 2°C).*

References and photographic acknowledgements on page 128.

Of all the potential *Zeitgebers* for the accurate yearly entrainment of circannual clocks, the most powerful and predictive would seem to be the seasonal change in photoperiod. It was in the 1930's that the circadian clock was proposed as the underlying mechanism for photoperiodic time measurement; over the next few decades it was experimentally implicated through stepwise manipulation of dark phase duration or by interruption of the dark phase with light pulses applied at different times after dark onset ★3.5. You may have come across the terms "external" and "internal" coincidence. These were ideas for how the changing photoperiod might be detected by the circadian oscillator: the former suggested photoperiodic variation of illumination on a light-sensitive phase of the clock's oscillation, while the latter posited variation of the phase relationship between two coupled circadian oscillators (or sets of oscillators) individually entrained to dawn and dusk. At least for mammals, we now know that the SCN encodes photoperiod by a reconfiguration of the phases of individual oscillating SCN cells (perhaps as part of an "internal" coincidence concept), adopting wide and narrow distributions across long and short photoperiods, respectively ★3.6. In this way, the duration of the dark phase (or of the subjective night in constant darkness) is accurately reflected in the duration of elevated nocturnal pineal melatonin levels – both in diurnal and nocturnal animals and in long- and short-photoperiod breeders – creating a faithful photo-neuro-endocrine signal transduction pathway (Fig. 20).

Great progress is now being made in dissecting the anatomical, biochemical, and molecular substrates downstream of the melatonin rhythm that underlie seasonal physiological responses. Attention has been directed especially to the pars tuberalis as a key element in birds and mammals, in large part because it is the only nervous system structure that consistently expresses melatonin receptors across a wide range of long- and short-photoperiod breeders.

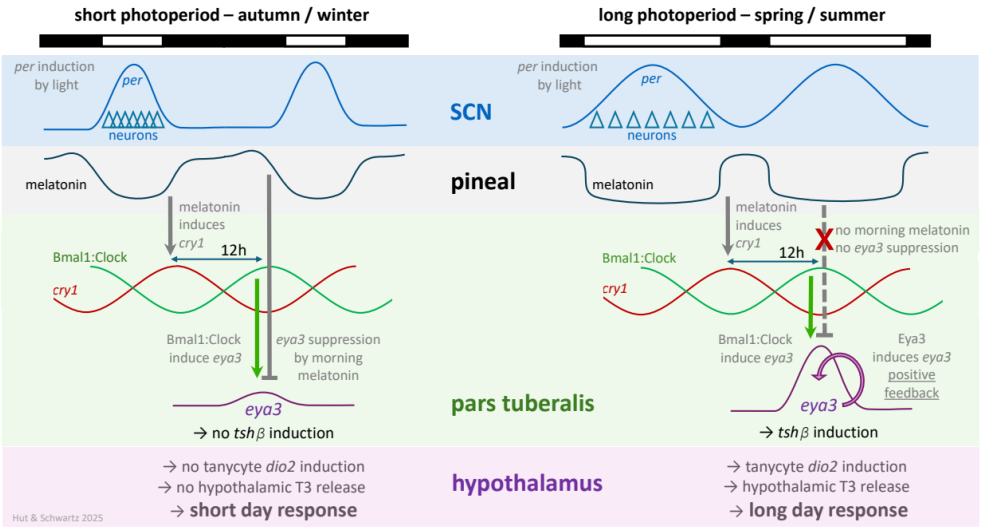


Figure 20. Encoding the seasons

Photic induction of SCN per genes leads to the entrainment of the rhythm of SCN electrical activity, with compressed (left) and expanded (right) durations of high activity in short and long photoperiods, respectively (see ★3.6); as a result, the duration of high pineal melatonin levels during the dark phase is long in the short photoperiod and short in the long photoperiod. Under both photoperiods, plasma melatonin onset induces cry1 in pars tuberalis cells, entraining their circadian clocks to evening lights-off; 12 h later, Bmal1:Clock heterodimers induce eya3 (a transcriptional co-activator; see ★3.7). Under the short photoperiod, the presence of melatonin in the morning suppresses further eya3 transcription; but under the long photoperiod (with night lengths shorter than 12 h), the absence of melatonin in the morning allows positive feedback of the Eya3 protein to reinforce its own transcription. Once photoperiod length has surpassed a critical duration (critical photoperiod), augmented Eya3 expression subsequently activates TSHβ transcription. The ensuing cascade involves the stimulation of ependymal tanycytes to upregulate deiodinase type 2 (dio2) and downregulate dio3, thereby activating thyroid hormone conversion from T4 → T3 in the hypothalamus, and ultimately a long day response phenotype.

As a tubular structure that wraps around the pituitary stalk, the pars tuberalis is not only in direct contact with the portal vessels of the median eminence but also lies adjacent to the anterior pituitary gland (pars distalis). Both the pars tuberalis and the pars distalis contain cells that release thyroid stimulating hor-

mone (TSH), but their regulation is distinctly different. Pars tuberalis thyrotropes do not express triiodothyronine (T3) or thyroid releasing hormone receptors and are thus not a part of the classic hypothalamo-pars distalis-thyroid feedback axis; instead, TSHβ secretion in the pars tuberalis is regulated by a melatonin-dependent network of circadian clock and clock-controlled genes (the emerging details of which are perhaps akin to an "external" coincidence concept ★3.7) (Fig. 20). TSH-receptor-bearing ependymal tanycytes lining the third ventricle then control the levels of the bioactive thyroid hormone T3 in the mediobasal hypothalamus through enzymatic processing by deiodinases. The distal mechanisms responsible for species-specific differential responses, and for integrating the reproductive, behavioral, and metabolic components of the seasonal phenotype, are under active investigation ★3.8, as are the identit(ies) of hypothesized paracrine factor(s) from the pars tuberalis that regulate circannual prolactin secretion by pars distalis lactotrophs ★3.9.

By the way, there is an additional geophysical force affecting life on Earth – the Moon's orbit around the planet, which gives rise to multiple astronomical cycles and obvious environmental consequences that are exceedingly complex. The *lunar* cycle of 29.53 days (the "synodic" month; Fig. 18) is the interval of time for the Moon to orbit the Earth, progressing through phases of moonlight intensity from maximum (full moon) to minimum (new moon) defined by illumination from the sun. The *lunidian* cycle of 24.8 h (the lunar "day") is the interval of time between consecutive moonrises as viewed on Earth, the gravitational consequences of which help shape the ocean tides (the 12.4 h *tidal* cycle, with two high and two low tides per lunar day). The lunar cycle also contributes to the periodicity of the tides: twice a synodic month (that is, the *semilunar* cycle), the sun, Earth, and Moon are all aligned at the full and new moons, creating an increased gravitational force that generates a higher high tide and lower low tide than normal (spring tides) (this in contrast to the decreased gravitational force at the first and last quarter moons [neap tides]). Note that there are additional levels of complexity shaping tidal phenomena, due to the Moon's elliptical orbit and its declination relative to the plane of the Earth's equator.

While monthly reproductive rhythms and intertidal behavioral rhythms have been appreciated since antiquity, only recently has there been a resurgence of interest in the possible role of endogenous circa-oscillators entrained by rhythms in the intensity of moonlight or the rise and fall of the tides. Research has been fueled by links to circadian clock gene homologs and rhythms in diverse marine organisms, transcriptome analyses, and longitudinal studies of human physiology and behavior (the latter, for example, on menstrual cycles and bipolar disorder) ★3.10.

Wayfinding on a rotating world

There exists another remarkable strategy for adapting to the seasons. Several species of birds (and some insects and marine mammals) undertake round-trip migrations every spring and autumn to and from their breeding grounds; some involve long distance routes to destinations thousands of miles and months away, with future local conditions at the target sites unknown at the time of departure. Such a mobile lifestyle relies on both circannual and circadian temporal programs. For timing of migration, anticipatory physiological and morphological preparations for the journey are timed by a photoperiod-entrained circannual clock, culminating in a departure time that can be studied in captive birds by the onset of near constant 24-h locomotor activity (termed *Zugunruhe*, or "migratory restlessness"). For orientation during migration, birds likely exploit multiple navigational cues while in flight, but one is based on the position of the sun. Early in the 1950's, studies of captive European starlings demonstrated the existence of an innate sun compass necessarily adjusted for the passage of time by a circadian mechanism ★3.11. Notably, outside the laboratory, such a time-compensated sun-compass mechanism might face some interesting challenges; as an example, for trans-meridian flight paths at very high latitudes, is there a mechanism for rapid, repeated resetting of the circadian system to local solar time (a jet-lagged sun compass is surely not a functional device)? Eventually, along the way, flexible re-calibration of the itinerary is needed in response to homeostatic demands (for example, in the timing of flight segments and stopovers based on energy reserves) and environmental cues (for example, in setting arrival and breeding times independently based on local conditions). And eventually there is the final challenge of the return trip, now using the compass to plot a reversed path. At least in monarch butterflies, ambient temperature plays a key role in the directional switch. These insects complete a trans-generational migration between North America and their overwintering grounds in Mexico, and winter cold exposure appears to be required for northward reorientation in the spring ★3.12.

Lastly, on a spatial scale quite different from wayfinding around the globe – but equally critical to survival in the wild – is wayfinding around the neighborhood. On a rotating world, resources may become available in particular locations at specific times of the day and night – as well as the presence of predators – and learning to visit or avoid such sites at the right times (time-place learning) is believed to be a strategy to optimize energy expended in the search, minimizing futile or dangerous forays. This ability has been described in a number of animals – in certain species of insects, birds, and mammals – and at least in some

cases a role for the circadian system in time measurement has been demonstrated ★3.13.

A final word – On opting out

Although we (the authors) are clearly unabashed champions of living in harmony with one's circadian clock, even we must admit that there could be circumstances when going off the clock might be the right thing to do. This is apparently so, as indicated by fascinating natural examples of animals that exhibit plastic locomotor activity patterns ★3.14. We have already mentioned the switch from diurnal to near-constant activity during seasonal avian migrations, a change also reported in a polygynous Arctic shorebird (pectoral sandpiper) for a few weeks during the continuous light of the summer breeding season. In eusocial insects like ants, honeybees, and bumblebees, workers adopt locomotor regimens congruent with their assigned tasks, uninterrupted if caring ("nursing") the brood in the nest but strongly diurnal if foraging outside. Bumblebee queens without and with brood alternate between circadian and continuous activity levels, respectively, and the switch can even anticipate the arrival of brood.

Essentially unknown are the mechanisms responsible for shifting activity levels from rhythmic to constant over 24 h, but they probably do not involve a complete arrest of the entire circadian system; some network components may remain operational ★3.15. Svalbard reindeer and ptarmigan (a member of the grouse family) are herbivorous arctic residents during the continuous light and dark of summer and winter, respectively. In reindeer, circadian regulation of melatonin rhythmicity is lost under these conditions, whereas locomotor activity remains weakly rhythmic; in ptarmigan, locomotor rhythmicity is lost, while circadian-based photoperiodic timing persists.

With new and developing technologies that allow for remote, multi-modal, real-time rhythm tracking as well as the expansion of our armamentarium of "model" species, the significance of cases like these will not be as zoological curiosities but as exciting opportunities for better understanding the diversity and dynamic architectures of animal circadian clockworks and their role in sustaining the diversity of life.

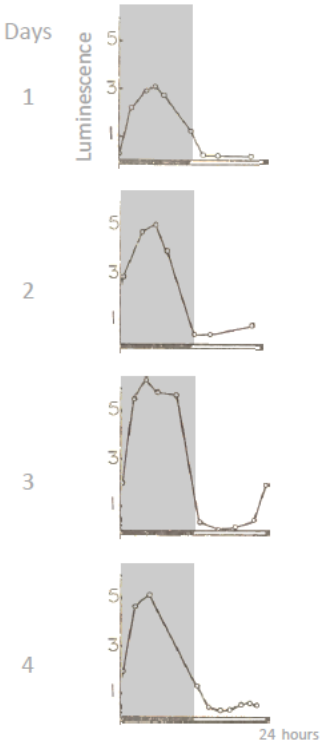
POINTS OF INTEREST

★1.1 On a Cornucopia of Daily Rhythms

Daily rhythmicity is a widespread attribute of living entities, spanning biological kingdoms and functions, as illustrated by just a few examples here.

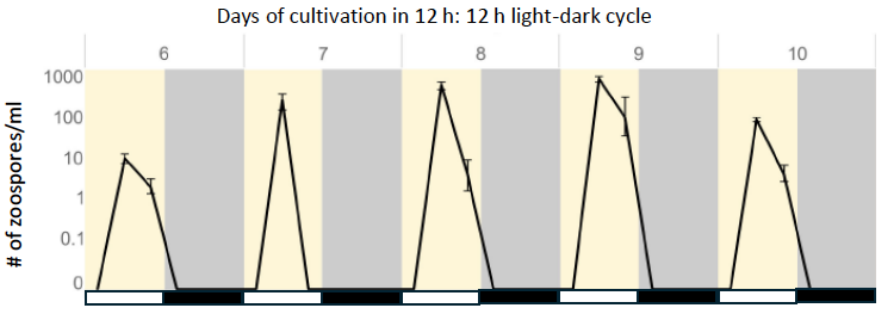
In unicellular eukaryotes

The marine dinoflagellate *Gonyaulax polyedra* (now *Lingulodinium polyedra*) expresses a 24-h rhythm of luminescence in culture, with a high level during the dark phase of a 12 h: 12 h light-dark cycle. Black bars and gray shading represent the dark phase, white bars represent the light phase.



Re-drawn from Fig. 2 in: Hastings JH, Sweeney BM. A persistent diurnal rhythm of luminescence in Gonyaulax polyedra. Biol Bull 115:440-458, 1958 [doi.org/10.2307/1539108].

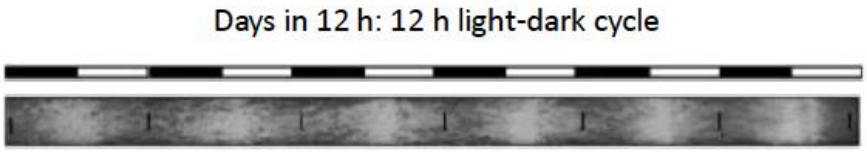
The complex alga *Chromera velia* exhibits a 24-h rhythm in the formation of its motile stage, zoospores, with a high level during the light phase of a 12 h: 12 h light-dark cycle.



Modified from Fig. 1 in: Richtová J, Bazalová O, Horák A, Tomčala A, Gonepogu VG, Oborník, Doležel D. Front Plant Sci 12:1226027, 2023 [doi.org/10.3389/fpls.2023.1226027]; under Creative Commons Attributions License 4.0.

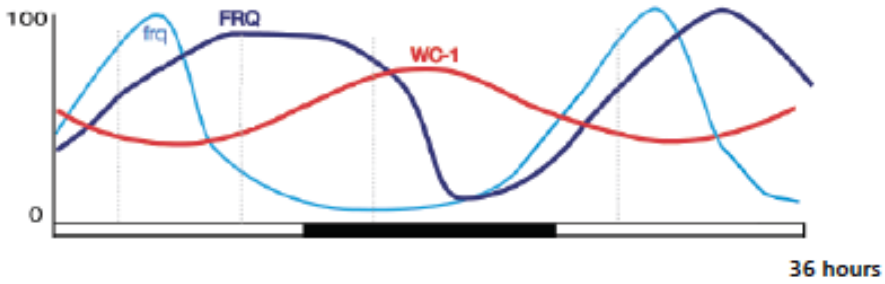
In fungi

The filamentous fungus *Neurospora crassa* reproduces asexually from spores (conidiation) with a 24-h rhythm when grown in long tubes ("race tubes") in a 12 h: 12 h light-dark cycle.



Modified from Fig. 1 in: Ma H, Li L, Yan J, Zhang Y, Ma X, Li Y, Yuan Y, Yang X, Yang L, Guo J. J Fungi 8:27, 2022 [doi.org/10.3390/jof8010027]; under Creative Commons Attributions License 4.0.

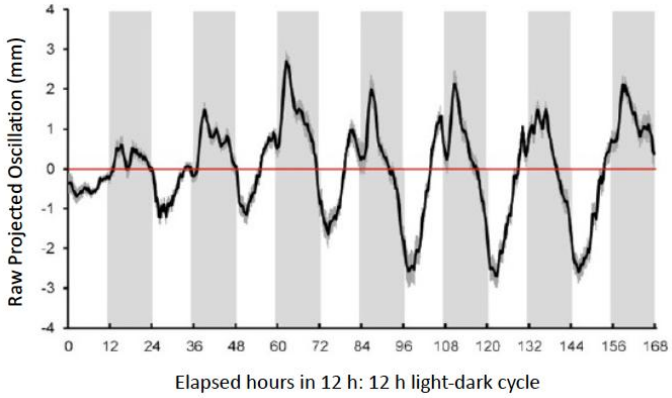
The levels of circadian clock RNAs and proteins involved in the *Neurospora* core transcription-translation feedback loop oscillate with 24-h rhythms in a 12 h: 12 h light-dark cycle. Shown are frq, frequency RNA; FRQ, FREQUENCY protein; WC-1, WHITE COLLAR-1 protein.



Modified from Fig. 3 in: Dunlap JC, Loros JJ. Making time: Conservation of biological clocks from fungi to animals. Microbiol Spectrum 5(3): FUNK-0039-2016, 2017 [doi.org/10.1128/microbiolspec.FUNK-0039-2016]; under Creative Commons Attributions License 4.0.

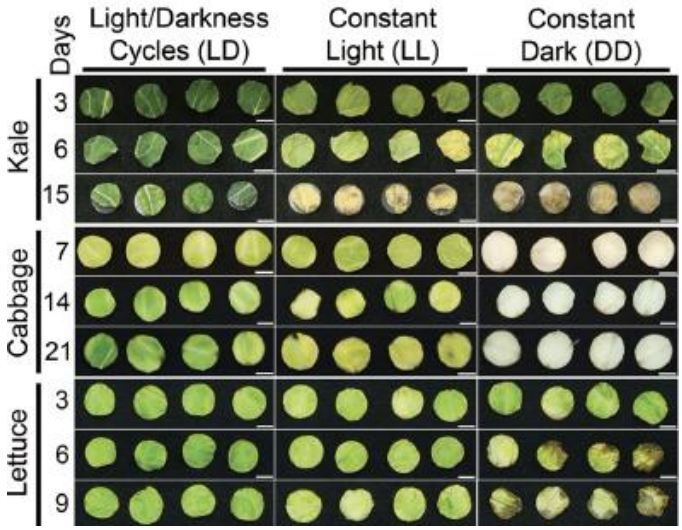
In plants

Infrared time-lapse imaging of mature *Arabidopsis thaliana* plants quantifies a 24-h rhythm of leaf movement in a 12 h: 12 h light-dark cycle (in the detrended plot below, the maximum upright leaf position during the light phase is inverted).



Modified from Fig. 1 in: Bours R, Muthuraman M, Bouwmeester H, van der Krol A. OSCILLATOR: A system for analysis of diurnal leaf growth using infrared photography combined with wavelet transformation. Plant Methods 8:29, 2012 [doi.org/10.1186/1746-4811-8-29]; under Creative Commons Attribution License 2.0.

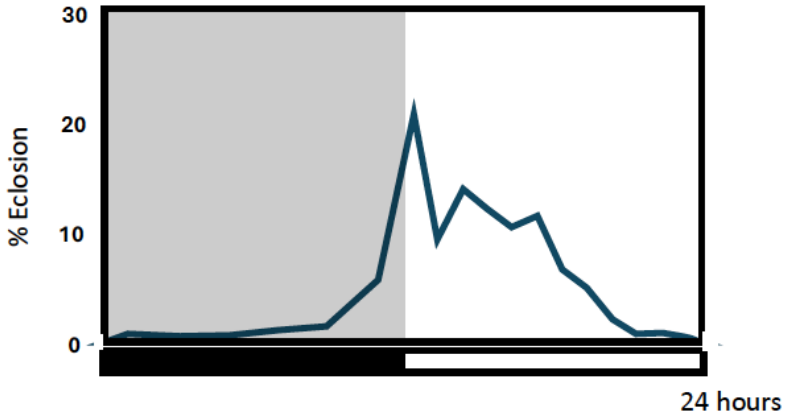
Leaf discs from green vegetables stored after harvest in a 12 h: 12 h light-dark cycle are better preserved than those maintained in constant light or darkness.



Modified from Fig. 1 in: Liu JD, Goodspeed D, Sheng Z, Li B, Yang Y, Kliebenstein DJ, Braam J. Keeping the rhythm: light/dark cycles during postharvest storage preserve the tissue integrity and nutritional content of leafy plants. BMC Plant Biology 15:92, 2015 [doi.org/10.1186/s12870-015-0474-9]; under Creative Commons Attributions License 4.0.

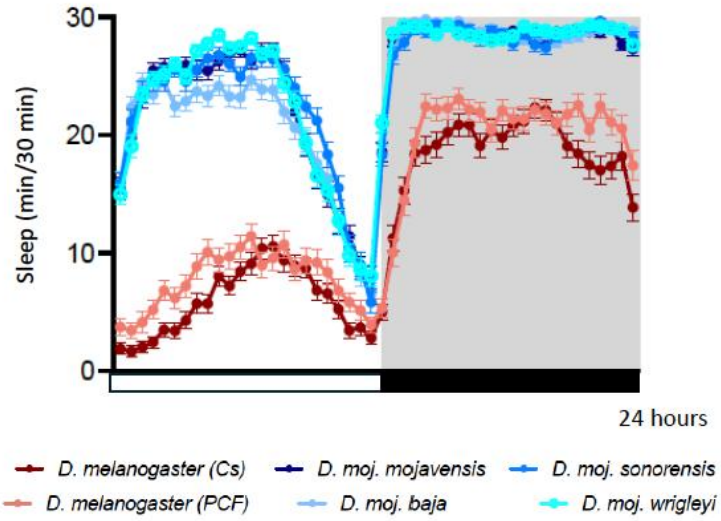
In fruit flies

Pupal eclosion in *Drosophila melanogaster* occurs mostly during the first half of the light phase in a 12 h: 12 h light-dark cycle, with some anticipation of lights-on.



Re-drawn from Fig. 1 in: Qiu J, Hardin P. Developmental state and the circadian clock interact to influence the timing of eclosion in Drosophila melanogaster. J Biol Rhythms 11:75-86, 1996 [doi.org/10.1177/074873049601100108].

Sleep in stock *Drosophila melanogaster* and desert-adapted *Drosophila mojanvensis* strains exhibit 24-h rhythms under laboratory conditions in a 12 h: 12 h light-dark cycle. *Cs*, Canton-S; *PCF*, Population Cage Flies.

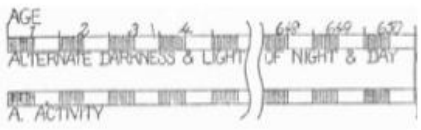


Modified from Fig. 1 in: Yano J, Nave C, Larratt K, Honey P, Roberts M, Jingco C, Fung ML, Trotter D, He X, Elezi G, Whitelegge JP, Wasserman S, Donlea JM. Elevated sleep quota in a stress-resilient Drosophila species. Curr Biol 34:2487-

2501, 2024 [doi.org/10.1016/j.cub.2024.04.060]; under Creative Commons Attributions License 4.0.

In rats

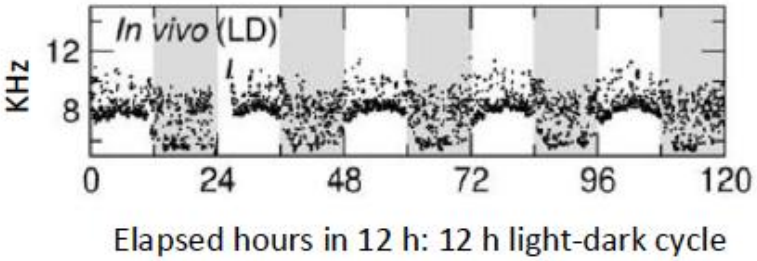
Laboratory rats (*Rattus norvegicus*) singly housed inside a "revolving drum" (an early version of a running wheel) are behaviorally active with a 24-h rhythm, with high levels of activity during the dark phase of a 12 h: 12 h light-dark cycle.





Modified from Figs. 13 & 19 in: Richter CP. A behavioristic study of the activity of the rat. Comparative Psychology Monographs 1:1-54, 1922 [doi.org/10.5962/bhl.title.151527].

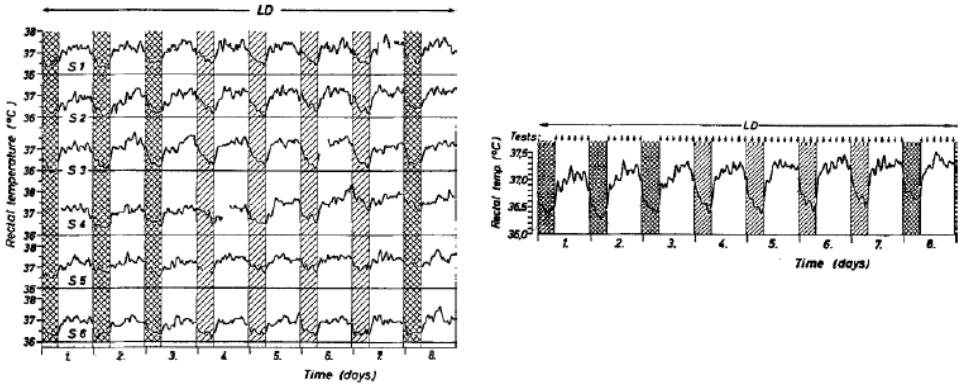
Multi-unit (electrophysiological) activity of the suprachiasmatic nucleus in albino rats (Wistar) oscillates with a 24-h rhythm *in vivo*, with higher levels during the light phase of a 12 h: 12 h light-dark cycle.



Modified from Fig. 1 in: Hu K, Meijer JH, Shea SA, vanderLeest HT, Pittman-Polletta B, Houben T, van Oosterhout F, Deboer T, Scheer FAJL. Fractal patterns of neural activity exist within the suprachiasmatic nucleus and require extrinsic network interactions. PLoS ONE 7:e48927, 2012 [doi.org/10.1371/journal.pone.0048927]; under Creative Commons Attributions License 4.0.

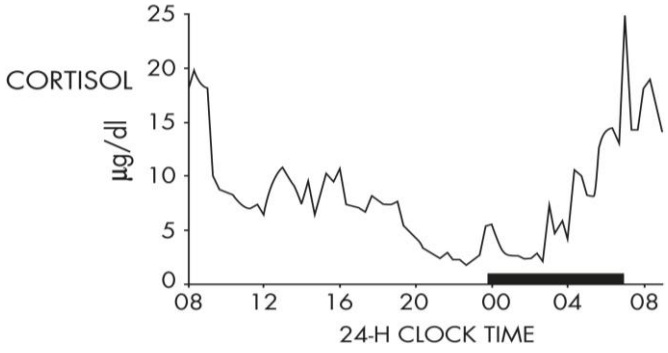
In humans

Rectal temperatures in six male subjects housed in groups of two express a 24-h rhythm when recorded in an isolation facility under a 16 h: 8 h light-dark cycle (individual records, left; mean of six, right).



By permission, modified from Figs. 1 & 2 in: Aschoff J, Fatranska M, Gerecke U, Giedke H. Twenty-four-hour rhythms of rectal temperature in humans: Effects of sleep-interruptions and of test-sessions. Pflügers Arch 346:215-222, 1974 [doi.org/10.1007/BF00595708].

Plasma cortisol levels in a healthy young man under non-restrictive conditions show a 24-h rhythm with an early morning maximum (black bar in this plot is scheduled sleep time).



Modified from Fig. 5 in: Oster H, Challet E, Ott V, Arvat E, de Kloet ER, Dijk D-J, Lightman S, Vgontzas A, Van Cauter E. The functional and clinical significance of the 24-hour rhythm of circulating glucocorticoids. Endocrine Rev 38:3-45,

2017 [doi.org/10.1210/er.2015-1080]; under Creative Commons Attributions License 4.0.

★1.2 On Methods for the Statistical Detection of Daily Rhythms

We acknowledge the large interdisciplinary literature on the mathematics and implementation of diverse algorithms for analyzing data collected over time. While we cannot comprehensively tackle the subject here, we can offer a few points to help you get started. To begin, we recommend a set of two papers as a balanced, practical, and very readable introduction to the use of statistics in biological rhythm research:

Bianchi MT, Phillips AJK, Wang W, Klerman EB. Statistics for sleep and biological rhythms research: From distributions and displays to correlation and causation. J Biol Rhythms 32:7-17, 2017 [doi.org/10.1177/0748730416670050].

Klerman EB, Wang W, Phillips AJK, Bianchi MT. Statistics for sleep and biological rhythms research: Longitudinal analysis of biological rhythms data. J Biol Rhythms 32:18-25, 2017 [doi.org/10.1177/0748730416670051].

Also available from the Society for Research on Biological Rhythms is a helpful list of publicly available tools for time series analysis, at https://srbr.org/education-outreach/research-tools/.

The goal of these kinds of tools is to (1) determine the period that best explains the time-dependent variation in a data series, (2) confirm that the rhythmicity detected in the data is statistically greater than attributable to random noise, and (3) characterize other rhythm parameters like phase and waveform. Notably, the approaches traditionally employed in biological rhythm research require that the data are *stationary* over time (that is, of constant period, amplitude, and waveform from the 1st to the n^{th} cycle), and a few examples of such methods will be mentioned here (for a consideration of *non-stationary* datasets, see ★2.9 in Chapter 2).

For data collected over only one or two cycles

Cosinor is a least squares method for analyzing relatively sinusoidal waves by fitting one or more cosine curves to the data, seeking to minimize the residuals between the actual measurement and the fitted model. Assigning a best-fit period allows calculation of rhythm parameters such as amplitude and

phase. More complex analyses add a Fourier extension to the single cosine function by incorporating sine and cosine components as harmonics.

For large genome-scale data sets, such as microarrays or RNA-seq, there are special challenges in experimental design and statistical analysis, the solutions to which are still evolving. A consensus statement authored by a who's-who of 93 prominent circadian biologists lays out the critical issues (*Hughes ME, et al., Guidelines for genome-scale analysis of biological rhythms. J Biol Rhythms 32:380-393, 2017 [doi.org/10.1177/0748730417728663]*).

For data collected over multiple cycles

The chi-square (Sokolove-Bushell) periodogram is a popular method for computing period and its statistical significance, as long as the dataset is composed of equally spaced time points over several cycles (say, 10 at the least). It is an adaptation of the Enright periodogram, based on segmenting the rhythm into equal lengths corresponding to a range of periods, and then overlaying ("folding") the sections to determine the period at which the amplitude of the resulting waveform is the highest (i.e., when the overlaid peaks and troughs coincide). The Lomb-Scargle periodogram, a Fourier-based method originally applied to astronomical observations, has an added advantage of handling datasets with unequally spaced or even missing time points.

For a discussion of additional methods and their comparative efficacies for the estimation of periodicity, you might consider beginning your reading with:

Sokolove PG, Bushell WN. The chi square periodogram: its utility for analysis of circadian rhythms. J Theor Biol 72:131-160, 1978 [doi.org/10.1016/0022-5193(78)90022-x].

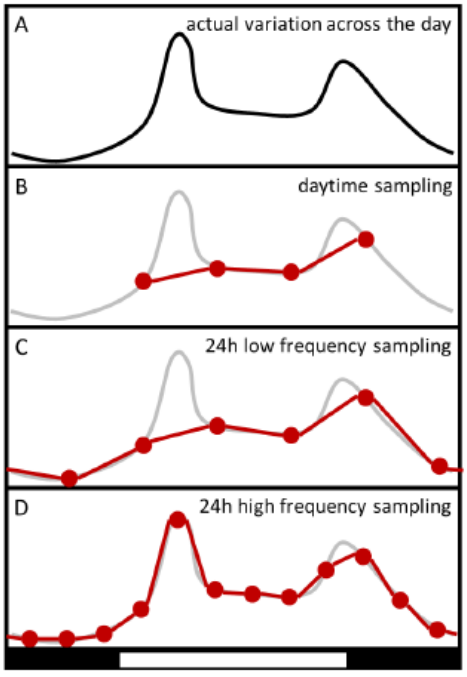
Ruf T. The Lomb-Scargle Periodogram in Biological Rhythm Research: Analysis of Incomplete and Unequally Spaced Time-Series. Biol Rhythm Res 30:178-201, 2010 [doi.org/10.1076/brhm.30.2.178.1422].

Refinetti R, Cornélissen G, Halberg F. Procedures for numerical analysis of circadian rhythms. Biol Rhythms Res 38:275-325, 2007 [doi.org/10.1080/09291010600903692].

Zielinski T, Moore AM, Troup E, Halliday KJ, Millar AJ. Strengths and limitations of period estimation methods for circadian data. PLoS ONE 9:e96462, 2014 [doi.org/10.1371/journal.pone.0096462].

★1.3 On the Influence of Sampling Frequency on Rhythm Detection & Waveform

Increasing sampling frequency increases the probability of detecting a rhythm and characterizing its waveform. When only two points per 24-h cycle are sampled, a rhythmic process might not be detected; even when equally spaced, the sampling points may catch the rhythm at its rising and falling phases and thus describe the average value of an otherwise dramatic wave shape. The cartoon below shows how actual rhythm waveform (A) might be mis-interpreted if sampling only takes place during half of the cycle (for example, in the lab during daytime [B]). Sampling across the entire cycle with six equidistant sample points (once every 4 hours) improves the description of the underlying rhythmic shape, but peaks with a width less than the distance between sampling points might be completely missed (C). This leads to the wrong conclusion that the peak of the rhythm is in the early dark phase, whereas increasing the sampling rate to once every two hours (12 sampling points) allows for the interpretation that the peak is actually in the early light phase (D).

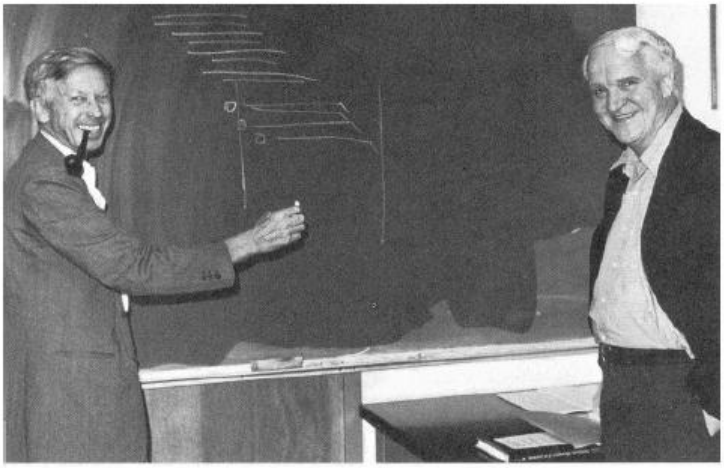


Hut & Schwartz 2024

★1.4 On Actograms: Period and Phase but No Amplitude

In addition to the technical limitations of pen and paper recording instruments 50 years ago (normal paper speed of the Esterline Angus multichannel event recorder was ¾ inch/h), investigators at the time were generally less interested in rhythm amplitude than in period and phase. In large part, this was because the search was on to identify the biological timing mechanism for rhythm generation and its possible localization; even if an experimental manipulation could be found that altered the amplitude of an overt rhythm, it would not be possible to distinguish whether such an effect was acting on the output "hands" downstream of the timer or on its actual "gears."

And here are two of the acknowledged founders of modern circadian biology engaged in friendly debate, aided by an actogram with period and phase but no amplitude.



Jürgen Aschoff
1913 – 1998

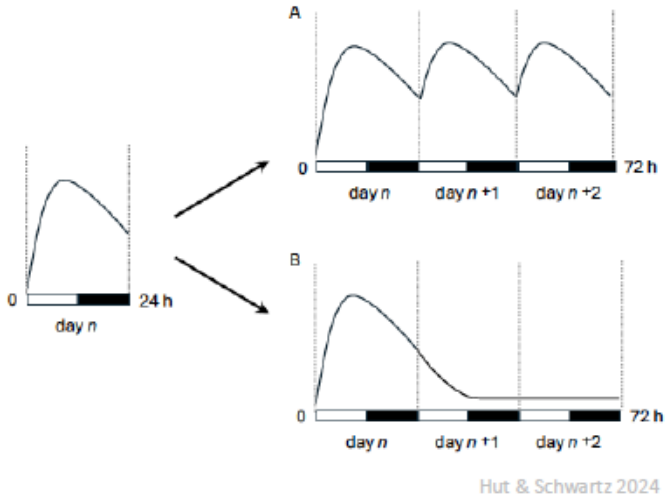
Colin Pittendrigh
1918 – 1996

Aschoff and Pittendrigh, ca. 1970, captured by author Ritchie R. Ward in the library of the Max Planck Institut für Verhaltensphysiologie in Erling-Andechs, Germany. Photograph in Aschoff's estate, and also on p. 375 of Ward RR. The Living Clocks, 1st edition. Alfred A. Knopf, New York, 1971.

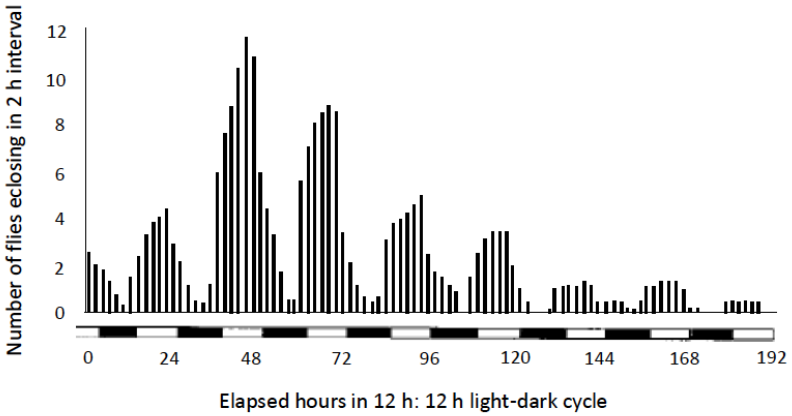
★1.5 On the Need for Extended Sampling Beyond 24 h for Daily Rhythms

There is an important exception to our statement that the timed sampling of multiple groups beyond 24 h – extending the experiment to 48 h or 72 h or beyond – is *not* needed to infer daily rhythmicity. The obvious situation is if the population of subjects does not remain in the same biological state over successive 24 h cycles; and a clear case in point would be during development, when a developmental window might extend over several daily cycles.

The following cartoon illustrates the problem. At left, suppose there is variation over the day-night cycle in a measure across multiple groups undergoing development, as denoted by the drawn curve through their mean values. Only by extending the collection could one distinguish between the presence of a daily rhythm on one hand (as in A) or some kind of singular developmental checkpoint on the other (as in B).



One famous daily rhythm during development is that of *Drosophila* (example below), when the emergence of adult flies from their pupal cases (eclosion) is timed by an interaction between daily rhythmicity and developmental state. Eclosion is a once-in-a-lifetime event for each individual, but there is a rhythm of the population that extends over several 24-h cycles, gating emergence to the early part of the light phase with some anticipation of lights-on.



Re-drawn from Fig. 3 in: Paranjpe DA, Anitha D, Kumar S, Kumar D, Verkhedkar K, Chandrashekaran MK, Joshi A, Sharma VK. Entrainment of eclosion rhythm in Drosophila melanogaster populations reared for more than 700 generations in constant light environment. Chronobiol Int 20:977-987, 2003 [doi.org/10.1081=CBI-120025247].

★2.1 On Excluding an Exogenous but Obscure 24-h Timing Cue

For many years investigators searched for a role for rhythmic 24-h geophysical or cosmic timing cues that might serve as alternatives to the existence of an internal circadian clock. Even as late as the second half of the 20th century, such experiments continued – near the South Pole and in geocentric orbit 157 miles above the surface of the Earth – but failed to show such an exogenous factor.

Hamner KC, Finn JC Jr, Sirohi GS, Hoshizaki T, Carpenter BH. The biological clock at the South Pole. Nature 195:476-480, 1962 [doi.org/10.1038/195476a0].

Sulzman FM, Ellman D, Fuller CA, Moore-Ede MC, Wassmer G. Neurospora circadian rhythms in space: a reexamination of the endogenous-exogenous question. Science 225:232-234, 1984 [doi.org/10.1126/science/11540800].

But what about a more prosaic problem: when circadian periods measured in a laboratory with constant light, temperature, humidity, and *ad libitum* food and water are so close to 24 h that an unrecognized daily cue remains a concern (e.g., an animal technician running the vacuum cleaner in a neighboring room at the same time every morning). Recording the phase of free-running

rhythmicity after housing in a reversed light-dark cycle may be one useful test. Another is based on the continuous distribution of free-running periods among individuals; eventually in constant conditions individual rhythms will diverge over time and express a range of differing phases (as long as recording is done individually and independently, avoiding possible social interactions).

★2.2 On the *Circa*-24 h Nature of Free-Running Period

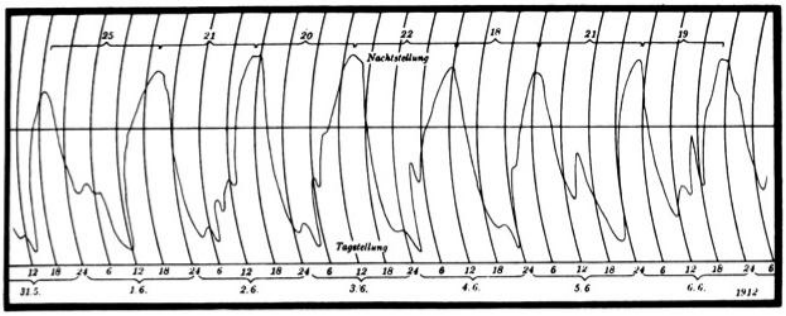
The sources of variation impacting the value of τ are legion: within an individual, at different life stages and circumstances; between individuals within a species, of different sexes and at different latitudes; and across species. For the most part, τ is revealed under special laboratory conditions; even the extreme cases of constant darkness and constant light in nature do not imply the absence of some kind of *Zeitgeber*. From an evolutionary point of view, the control of phase, not period, must be the pre-eminent circadian parameter that is under natural selection, because circadian period is not revealed under entrained conditions.

Just a comment here on two other "circa" terms that you will likely see. The term *ultradian* refers to rhythms with short periods, of several minutes to a few hours; it does not refer to a rhythm with a period less than 24 h but in the circadian range ("circadian range" depends on the organism, light intensity, and other factors, but typically is just a few hours away from 24; see *limits of entrainment* in Question 2 of this chapter). *Ultradian* also does not refer to bimodal or more complex 24-h rhythm waveforms driven by the circadian clock, e.g., crepuscular rhythms with activity around dawn and dusk. The term *infradian* refers to rhythms with long periods, typically monthly or annually; it does not refer to a rhythm with a period greater than 24 h but in the circadian range.

★2.3 On Nyctinasty

The origins of circadian biology lie in studies of the daily (nyctinastic) movements of leaves and petals. Although such movements had been known for millennia, Jean-Jacques d'Ortous de Mairan (1678 – 1771) is given credit for reporting (in 1729) that the daytime opening and nighttime closing of *Mimosa* leaves continued when the plant was placed in a dark place, "sensing" the sun without seeing it. His observation was published as a short News and Views-like communication (in 1731) that was actually not written by him. It was the Swiss botanist Augustin Pyramus de Candolle (1778 – 1841) who later focused his investigations on light: by reversing the light-dark cycle he inverted

the leaf movement rhythm, and, by placing the plants in constant light, he reported that a persistent rhythm was shorter than 24 h and varied from plant to plant. He concluded that the movements "…are linked to a disposition to periodic movement inherent to plants…" (in 1832). Nearly 100 years of debate and experimentation had to pass, including perfection of technique and instrumentation, before a skeptical Wilhelm Friedrich Philipp Pfeffer (1845 – 1920), the pre-eminent plant physiologist of his time, clearly demonstrated persistent, endogenous, free-running rhythms of leaf movements in bean plants housed in constant light (in 1915).



Circadian rhythm of leaf movement in Phaseolus vitellinus in constant light ("4 tungsten lamps with 50 candles each"). Fig. 21b from: Pfeffer W. Beiträge zur Kenntnis der Entstehung der Schlafbewegungen. Abh Math-Physiol Kl K Sächs Ges Wiss 34:1-154, 1915.

For a scholarly and enjoyable review of the early botanical origins of circadian biology, see:

Emery P, Klarsfeld A, Stanewsky R, Shafer OT. Sensitive timing: A reappraisal of chronobiology's foundational texts. J Biol Rhythms 38:245-258, 2023 [doi.org/10.1177/07487304231169080].

★2.4 On the Complex Effects of Constant Light in Laboratory Rodents

Changing the intensity of constant light may increase or decrease τ, depending on the organism (Daan and Pittendrigh, 1976; and see ★2.8, below), but it generally also destabilizes rhythmicity in mammals, especially at higher intensities. The result may be frank arrhythmicity or a phenomenon known as "splitting," when an animal's normally consolidated activity/rest interval separates into two activity bouts that free-run with different periods for a number of

cycles and then stably re-synchronize with a common period but 180° in antiphase (12 circadian hours apart). These phenomena appear to be due to aberrant intercellular coupling within the SCN, either by desynchronizing cellular rhythms from each other (resulting in behavioral arrhythmicity) or by reorganizing the left and right sides of the SCN to oscillate in antiphase (resulting in splitting) (de la Iglesia et al., 2000; Ohta et al., 2005; Yan et al., 2005). Given the SCN's central role in driving, gating, or synchronizing a host of brain and body rhythms, constant light's disruptive effects on timing can be multi-dimensional (including the real-world effects of artificial light at night).

Daan S, Pittendrigh CS. A functional analysis of circadian pacemakers in nocturnal rodents. III. Heavy water and constant light: homeostasis of frequency? J Comp Physiol A 106:267-290, 1976 [doi.org/10.1007/BF01417858].

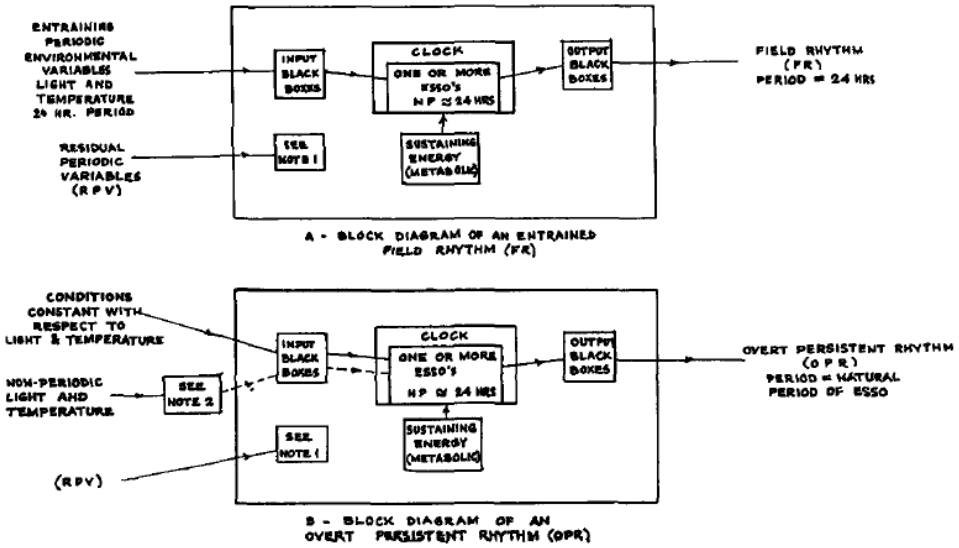
de la Iglesia HO, Meyer J, Carpino A Jr, Schwartz WJ. Antiphase oscillation of the left and right suprachiasmatic nuclei. Science 290:799-801, 2000 [doi.org/10.1126/science.290.5492.799].

Ohta H, Yamazaki S, McMahon DG. Constant light desynchronizes mammalian clock neurons. Nat Neurosci 8:267-269, 2005 [doi.org/10.1038/nn1395].

Yan L, Foley NC, Bobula JM, Kriegsfeld LJ, Silver R. Two antiphase oscillations occur in each suprachiasmatic nucleus of behaviorally split hamsters. J Neurosci 25:9017-9026, 2005 [doi.org/10.1523/JNEUROSCI.2538-05.2005].

★2.5 On the Origin of the "Eskinogram"

Named after Arnold Eskin (1940 – 2019), who acknowledged in his 1979 paper that his diagram was inspired by an earlier 1957 schematic by Pittendrigh and Victor Bruce that was published in the proceedings of a symposium.



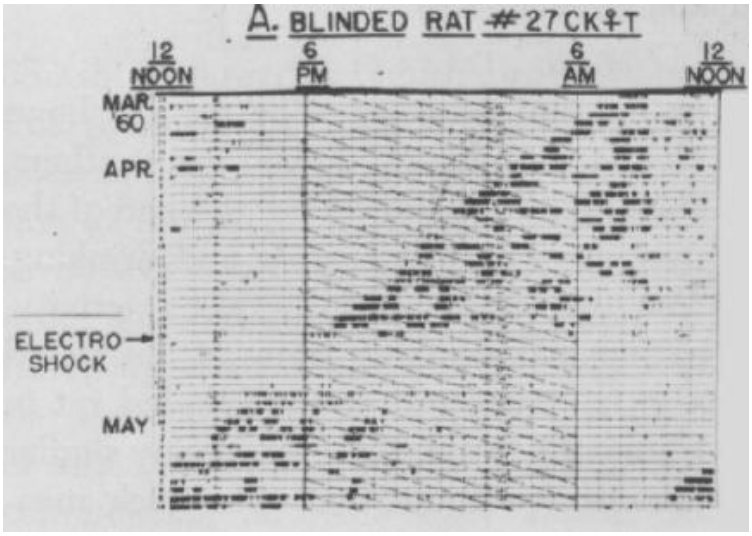
By permission, from Fig. 1 in: Pittendrigh CS, Bruce VG. An oscillator model for biological clocks. In: Rhythmic and Synthetic Processes in Growth (ed D Rudnick, from the Fifteenth Symposium for the Study of Development and Growth), 75-109, 1957.

Notes 1 and 2 were defined in the text of the article:

Note 1: All periodically fluctuating environmental variables other than light and temperature are called residual periodic variables (RPV). They are assumed to be never coupled to the oscillator.
Note 2: Light shocks, temperature shocks, and non-periodic treatment with light and temperature may affect the phase of OPR.

★2.6 On Distinguishing the Pacemaker's Oscillation from its Rhythmic Output

One example of the non-identity of the pacemaker and a rhythm that it drives is shown in an experiment by Curt Richter (1894 – 1998) below, with the reversible inactivation of locomotor (running wheel) activity for several days after an experimental perturbation (electric shock). Upon recovery, it is clear that the pacemaker has continued to run unperturbed but unseen through the shock, which apparently affected only the pacemaker's "hands" (output) but spared its "gears" (internal timekeeping mechanism). Richter obtained a similar result using 6 h of nitrous oxide inhalation as the experimental perturbation.

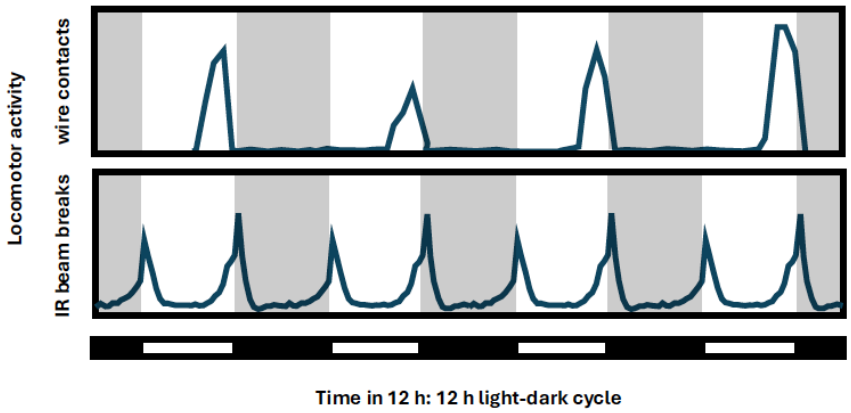


By permission, from Fig. 21A in: Richter C. Biological Clocks in Medicine and Psychiatry, Charles C. Thomas: Springfield, IL, 1965.

★2.7 On the Impact of Our Methods for Assaying Locomotor Activity Rhythms

In reality, the Eskinogram arrow from the pacemaker to the output rhythm is not unidirectional; as an example, how we assay the locomotor activity rhythm can impact the expression of the rhythm itself, including the daily rhythm's shape and the free-running rhythm's period.

To our knowledge, the first report of the *Drosophila* locomotor activity rhythm was in 1956 (de F Roberts, 1956); in that report, 6 male and 6 female *D. robusta* were housed in a common chamber with a grid of fine wires, revealing a daily population rhythm with activity restricted to a few hours before lights-off (upper panel below, of four consecutive 24-h days in a 12 h: 12 h light-dark cycle). Now *Drosophila* locomotor activity is usually monitored using the *Drosophila* Activity Monitoring (DAM) System (TriKinetics, Waltham, Massachusetts), wherein individual flies are housed in glass tubes and activity is recorded over days via infrared beam breaks, yielding instead bimodal peaks around lights-on and lights-off that anticipate the lighting transitions (lower panel below, quadruple plotted for comparison).

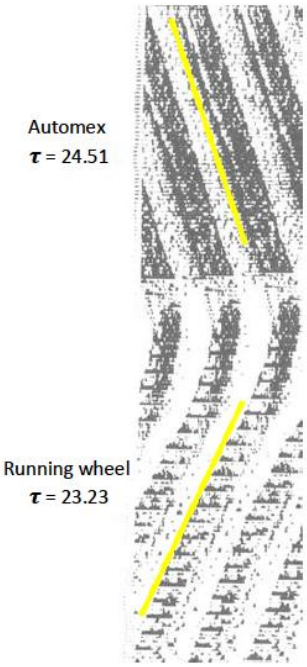


Upper panel, re-drawn from Fig. 1 in: de F Roberts SK. "Clock" controlled activity rhythms in the fruit fly. Science 124:172, 1956 [doi.org/10.1126/science.124.3213.172.a].

Lower panel, re-drawn from Fig. 1 in: Allada R, Chung B. Circadian organization of behavior and physiology in Drosophila. Ann Rev Physiol 72:605-624, 2010 [doi.org/10.1146/annurev-physiol-021909-135815].

And in 6-week old rats, blinded at birth by bilateral ocular enucleation, the value of $\boldsymbol{\tau}$ is shorter for rats with a running wheel than that for rats without a wheel (the latter with their locomotor activity recorded via an electromagnetic animal activity monitor [Automex system, Columbus Instruments, Columbus, Ohio]). In the figure below, the yellow stripes are added for visualizing $\boldsymbol{\tau}$ via eye fit lines through activity onsets. The greater the wheel revolutions/day, the shorter the $\boldsymbol{\tau}$ (Shiori et al., 1991).

Shioiri T, Takahashi K, Yamada N, Takahashi S. Motor activity correlates negatively with free-running period, while positively with serotonin contents in SCN in free-running rats. Physiol Behav 49:779-786, 1991 [doi.org/10.1016/0031-9384(91)90318-I].



By permission, modified from Fig. 3 in: Yamada N, Shimoda K, Ohi K, Takahashi S, Takahashi K. Free-access to a running wheel shortens the period of free-running rhythm in blinded rats. Physiol Behav 42:87-91, 1988 [doi.org/10.1016/0031-9384(88)90265-X].

★2.8 On the Intensity of Constant Light and τ ("Aschoff's Rule")

Coined by Pittendrigh, "Aschoff's Rule" is based on Aschoff's empirical observations of the relationship between the intensity of constant light and the value of τ in diurnally- and nocturnally-active animals. For diurnal animals, increasing light intensity tends to shorten τ of their free-running behavioral rhythm, while it is the opposite for nocturnal animals, with increasing intensity lengthening τ. Although there are exceptions, the rule is part of the evidence for a theory of photic entrainment based on tonic (parametric) effects of light (see discussion in Question 2 of this chapter).

★2.9 On the Analysis of Non-stationary Time Series

A helpful introduction to the analysis of data sets with variable periods and amplitudes, rhythms at multiple time scales, noise, and trend over time is:

Leise TL. Analysis of nonstationary time series for biological rhythms research. J Biol Rhythms 32:187-194, 2017 [doi.org/10.1177/0748730417709105].

Of particular interest is the utility of wavelet transforms, which do not rely on fixed sines and cosines for the decomposition of time series. Discrete transforms can extract a circadian component in the presence of discontinuities, noise, and trend; and continuous transforms can quantify variability in period and amplitude over time. Some starting references for circadian applications of wavelets include:

Leise TL, Harrington ME. Wavelet-based time series analysis of circadian rhythms. J Biol Rhythms 26:454-463, 2011 [doi.org/10.1177/0748730411416330].

Leise TL, Indic P, Paul MJ, Schwartz WJ. Wavelet meets actogram. J Biol Rhythms 28:62-68, 2013 [doi.org/10.1177/0748730412468693].

Leise TL. Wavelet-based analysis of circadian behavioral rhythms. Methods in Enzymology 551:95-119, 2015 [doi.org/10.1016/bs.mie.2014.10.011].

★2.10 On Some Suggested Reviews: Molecular and Genetic Analyses

Here are a few reviews as starting points to enter the literature on molecular and genetic analyses of circadian clocks in model organisms:

Takahashi JS. Transcriptional architecture of the mammalian circadian clock. Nat Rev Genetics 18:164-179, 2017 [doi.org/10.1038/nrg.2016.150].

Swann JA, Golden SS, LiWang A, Partch CL. Structure, function, and mechanism of the core circadian clock in cyanobacteria. J Biol Chem 293:5026-5034, 2018 [doi.org/10.1074/jbc.TM117.001433].

Loros, JJ. Principles of the animal molecular clock learned from Neurospora. Eur J Neurosci 51:19-33, 2020 [doi.org/10.1111/ejn.14354].

Nakamichi N. The transcriptional network in the Arabidopsis circadian clock system. Genes 11:1284, 2020 [doi.org/10.3390/genes11111284].

Patke A, Young MW, Axelrod S. Molecular mechanisms and physiological importance of circadian rhythms. Nat Rev Molec Cell Biol 21:67-84, 2020 [doi.org/10.1038/s41580-019-0179-2].

★2.11 On Some Suggested Reviews: Suprachiasmatic Nucleus

Here are a few reviews as starting points to enter the literature on the suprachiasmatic nucleus:

Weaver DR. The suprachiasmatic nucleus: a 25-year retrospective. J Biol Rhythms *13:100-112, 1998 [doi.org/10.1177/074873098128999952].*

Welsh DK, Takahashi JS, Kay SA. Suprachiasmatic nucleus: cell autonomy and network properties. Annu Rev Physiol 72:551-577, 2010 [doi.org/10.1146/annurev-physiol-021909-135919].

Ono D, Weaver DR, Hastings MH, Honma K-I, Honma S, Silver R. The suprachiasmatic nucleus at 50: Looking back, then looking forward. J Biol Rhythms 39:135-165, 2024 [doi.org/10.1177/07487304231225706].

★2.12 On the Influence of Genetic Background

The circadian phenotypes of mutated mice are strongly influenced by genetic background, as a few examples can illustrate. While mouse *per2* knockouts congenic with the 129sv strain lead to arrhythmicity (Zheng et al., 1999; Bae et al., 2001), knockouts congenic with C57BL/6J do not (Xu et al., 2007; Pendergast et al., 2010). And the pathologically lengthened free-running period of the $clock^{\Delta 19}$ mutation, initially induced on a C57BL/6J background, is suppressed by crosses with BALB/cJ or C3H/HeJ strains (Shimomura et al., 2010, 2013). The translational implications of such background effects are consequential: while $clock^{\Delta 19/\Delta 19}$ on C57BL/6J develops high fat diet-induced obesity, the mutation on ICR mice does not (in fact, the mice lose weight; Oishi et al., 2006).

Zheng B, Larkin DW, Albrecht U, Sun ZS, Sage M, Eichele G, Lee CC, Bradley A. The mPer2 gene encodes a functional component of the mammalian circadian clock. Nature 400:169-173, 1999 [doi.org/10.1038/22118].

Bae K, Jin X, Maywood ES, Hastings MH, Reppert SM, Weaver DR. Differential functions of mPer1, mPer2, and mPer3 in the SCN circadian clock. Neuron 30:525-536, 2001 [doi.org/10.1016/s0896-6273(01)00302-6].

Xu Y, Toh KL, Jones CR, Shin J-Y, Fu Y-H, Ptáček LJ. Modeling of a human circadian mutation yields insights into clock regulation by PER2. Cell 128:59-70, 2007 [doi.org/10.1016/j.cell.2006.11.043].

Pendergast JS, Friday RC, Yamazaki S. Distinct functions of Period2 and Period3 in the mouse circadian system revealed by in vitro analysis. PLoS ONE 5:e8552, 2010 [doi.org/10.1371/journal.pone.0008552].

Shimomura K, Lowrey PL, Vitaterna MH, Buhr ED, Kumar V, Hanna P, Omura C, Izumo M, Low SS, Barrett RK, LaRue SI, Green CB, Takahashi JS. Genetic suppression of the circadian Clock mutation by the melatonin biosynthesis pathway. Proc Natl Acad Sci USA 107:8399-8403, 2010 [doi.org/10.1073/pnas.1004368107].

Shimomura K, Kumar V, Koike N, Kim T-K, Chong J, Buhr ED, Whiteley AR, Low SS, Omura C, Fenner D, Owens JR, Richards M, Yoo S-H, Hong H-K, Vitaterna MH, Bass J, Pletcher MT, Wiltshire T, Hogenesch J, Lowrey PL, Takahashi JS. Usf1, a suppressor of the circadian Clock mutant, reveals the nature of the DNA-binding of the CLOCK:BMAL1 complex in mice. eLife 2:e00426, 2013 [doi.org/10.7554/eLife.00426].

Oishi K, Atsumi G-I, Sugiyama S, Kodomari I, Kasamatsu M, Machida K, Ishida N. Disrupted fat absorption attenuates obesity induced by a high-fat diet in Clock mutant mice. FEBS Lett 580:127-130, 2006 [doi.org/10.1016/j.febslet.2005.11.063].

★2.13 On Functional Compensation After Genetic Lesions

Manifestly normal circadian function may belie underlying genetic disruption. Examples of such compensation can occur at multiple biological levels, including the up-regulation of gene paralogs (Baggs et al., 2009) and the presence of intercellular coupling within neural circuits (Liu et al., 2007).

Baggs JE, Price TS, DiTacchio L, Panda S, FitzGerald GA, Hogenesch JB. Network features of the mammalian circadian clock. PLoS Biology 7:e1000052, 2009 [doi.org/10.1371/journal.pbio.1000052].

Liu AC, Welsh DK, Ko CH, Tran HG, Zhang EE, Priest AA, Buhr ED, Singer O, Meeker K, Verma IM, Doyle FJ III, Takahashi JS, Kay SA. Intercellular coupling confers robustness against mutations in the SCN circadian clock network. Cell 129:605-616, 2007 [doi.org/10.1016/j.cell.2007.02.047].

★2.14 On Breaking the Positive vs. the Negative Limbs of the Molecular Feedback Loop

Pathological mechanisms and even pathological states ascribed to the experimental inactivation of the circadian molecular feedback loop may depend on how the loop is broken, whether by disrupting genes in the activating (*clock*, *bmal1*) vs. the repressing (*per*, *cry*) limbs of the loop. For example, either *bmal1* or *per* loss is associated with pathological defects in murine wound healing, but the underlying mechanisms are different: the *bmal1* knockout results in insufficient proliferation of fibroblasts or keratinocytes, while *per* loss leads to hyperproliferation with an excess of fibroblasts and polymorphonuclear cells (Kowalska et al., 2013). Even more dramatic are the results of *bmal1* or *per* loss on brain pathology, with signs of neural degeneration in the former but not in the latter (Musiek et al., 2013). As a counter-example, abnormal glucose homeostasis follows either *bmal1* or *per* loss, suggesting that this pathology is indeed a consequence of disrupted circadian rhythmicity *per se* (Lamia et al., 2008).

Kowalska E, Ripperger JA, Hoegger DC, Bruegger P, Buch T, Birchler T, Mueller A, Albrecht U, Contaldo C, Brown SA. NONO couples the circadian clock to the cell cycle. Proc Natl Acad Sci USA 110: 1592-1599, 2013 [doi.org/10.1073/pnas.1213317110].

Musiek ES, Lim MM, Yang G, Bauer AQ, Qi L, Lee Y, Roh JH, Ortiz-Gonzalez X, Dearborn JT, Culver JP, Herzog ED, Hogenesch JB, Wozniak DF, Dikranian K, Glasson BI, Weaver DR, Holtzman DM, FitzGerald GA. Circadian clock proteins regulate neuronal redox homeostasis and neurodegeneration. J Clin Invest 123:5389-5400, 2013 [doi.org/10.1172/JCI70317].

Lamia KA, Storch K-F, Weitz CJ. Physiological significance of a peripheral tissue circadian clock. Proc Natl Acad Sci USA 105:15172-15177, 2008 [doi.org/10.1073/pnas.0806717105].

★2.15 On Some Suggested Further Reading: Human Forced Desynchrony Protocol

Scheer FAJL, Hilton MF, Mantzoros CS, Shea SA. Adverse metabolic and cardiovascular consequences of circadian misalignment. Proc Natl Acad Sci USA 106:4453-4458, 2009 [doi.org/10.1073/pnas.0808180106].

Wang W, Yuan RK, Mitchell JF, Zitting K-M, St. Hilaire MA, Wyatt J, Scheer FAJL, Wright KP Jr, Brown EN, Ronda JM, Klerman EB, Duffy JF, Dijk D-J, Czeisler CA. Desynchronizing the sleep-wake cycle from circadian timing to assess their separate contributions to physiology and behaviour and to estimate intrinsic circadian period. Nature Protocols 18:579-603, 2023 [doi.org/10.1038/s41596-022-00746-y].

★2.16 On Masking

Masking is characterized as either positive (enhancing) or negative (inhibiting), although such categorization depends on the measure chosen. For example, light is known to inhibit the expression of locomotor activity in nocturnal rodents (negative) while simultaneously enhancing features of sleep (positive). Different neural pathways may underlie the locomotor-suppressing vs. sleep-inducing effects of the masking light pulse.

For a helpful review on the nature of masking, see:

Mrosovsky N. Masking: history, definitions, and measurement. Chronobiol Int 16:415-429, 1999 [doi.org/10.3109/07420529908998717].

★2.17 On Anticipation of the Morning or of a Meal

One adaptive benefit of an internal timekeeping mechanism is the capacity to anticipate a recurring, periodic event, like sunrise. As one example, plants like *Arabidopsis* increase their expression of photosynthesis-related genes well before the daily dark to light transition occurs. When plants are rendered arrhythmic by constitutive overexpression of the clock gene *CCA1*, the transgenic mutant remains responsive to light, but it fails to anticipate its periodic re-occurrence and appears less fit than wild type when tested under certain laboratory conditions (Green et al., 2002). And young heliotropic sunflower plants track the sun as it moves from east to west and then reorient overnight by a circadian mechanism to face the east in anticipation of the coming dawn (Atamian et al., 2016); warming of the flowers by the rising sun appears to increase pollinator visits in the early morning hours.

Also, a wide array of animals can anticipate periodic mealtimes, and research on rodents indicates that such “food anticipatory activity” represents the action of a circadian oscillator (“food entrainable oscillator” [FEO]) that is not the SCN (Stephan 2002); its identity remains under active investigation (Crosby et al., 2019; Petersen et al., 2022; Ehichioya et al., 2023), as well as

its relationship to a “methamphetamine sensitive circadian oscillator” (Taufique et al., 2022).

Green RM, Tingay S, Wang Z-Y, Tobin EM. Circadian rhythms confer a higher level of fitness to Arabidopsis plants. Plant Physiol 129:576-584, 2002 [doi.org/10.1104/pp.004374].

Atamian HS, Creux NM, Brown RI, Garner AG, Blackman BK, Harmer SL. Circadian regulation of sunflower heliotropism, floral orientation, and pollinator visits. Science 353:587-590, 2016 [doi.org/10.1126/science.aaf9793].

Stephan FK. The “other” circadian system: food as a zeitgeber. J Biol Rhythms 17:284-292, 2002 [doi.org/10.1177/074873040201700402].

Crosby P, Hamnett R, Putker M, Hoyle NP, Reed M, Karam CJ, Maywood ES, Stangherlin A, Chesham JE, Hayter EA, Rosenbrier-Ribeiro L, Newham P, Clevers H, Bechtold DA, O’Neill JS. Insulin/IGF-1 drives PERIOD synthesis to entrain circadian rhythms with feeding time. Cell 177:896-909, 2009 [doi.org/10.1016/j.cell.2019.02.017].

Petersen CC, Cao F, Stinchcombe AR, Mistlberger RE. Multiple entrained oscillator model of food anticipatory rhythms. Sci Rep 12:9306, 2022 [doi.org/10.1038/s41598-022-13242-w].

Ehichioya DE, Taufique SKT, Farah S, Yamazaki S. A time memory engram embedded in a light-entrainable circadian clock. Curr Biol 33:5233-5239, 2023 [doi.org10.1016/j.cub.2023.10.027].

Taufique SKT, Ehichioya DE, Pendergast JS, Yamazaki S. Genetics and functional significance of the understudied methamphetamine sensitive circadian oscillator (MASCO). F1000 Research 11:1018, 2022. [doi.org/10.12688/f1000research.125432.2]

★2.18 **On Lab vs. Nature**

Perhaps not unexpectedly, there are significant, qualitative differences in locomotor activity rhythms expressed by animals when they are moved from the laboratory to natural conditions of light and temperature outdoors. *Drosophila melanogaster* under such “semi-natural” environments (they remain housed in glass tubes) show differences in the timing of their morning and evening activity bouts, and their diurnal siesta in the lab is replaced by a

prominent afternoon activity bout outdoors (Vanin et al., 2012; Menegazzi et al., 2012; Green et al., 2015).

Vanin S, Bhutani S, Montelli S, Menegazzi P, Green EW, Pegoraro M, Sandrelli F, Costa R, Kyriacou CP. Unexpected features of Drosophila circadian behavioural rhythms under natural conditions. Nature 484:371-375, 2012 [doi.org/10.1038/nature10991].

Menegazzi P, Yoshii T, Helfrich-Förster C Laboratory versus Nature: the two sides of the Drosophila circadian clock. J Biol Rhythms 27:433-442, 2012 [doi.org/10.1177/0748730412463181].

Green EW, O'Callaghan EK, Hansen CN, Bastianello S, Bhutani S, Vanin S, Armstrong JD, Costa R, Kyriacou CP. Drosophila circadian rhythms in seminatural environments: Summer afternoon component is not an artifact and requires TrpA1 channels. Proc Natl Acad Sci USA 112:8702-8707, 2015 [doi.org/10.1073/pnas.1506093112].

Notably, our dependable nocturnal laboratory rodents may not be so reliably nocturnal outdoors. Golden spiny mice (*Acomys russatus*) are nocturnal when housed in the laboratory but become diurnal within a day after transfer to 1000 m^2 enclosures in the Judean desert (Levy et al., 2007). Their immediate transition to diurnality in the field suggests a masking mechanism downstream of the circadian pacemaker (presumably the pacemaker in the SCN). Male and female laboratory mice housed together in outdoor experimental pens in Western Russia for two years – where they were exposed to natural weather conditions & aerial predation and competed among themselves for food, water, shelter, and mating – exhibited variable feeding rhythms that were not restricted to the night (Daan et al., 2011). And even the golden hamster (*Mesocricetus auratus*), the archetypical nocturnal rodent species in the laboratory, is not really nocturnal in its natural habitat in southern Turkey, as it assumes a more crepuscular-like pattern (Gattermann et al., 2008).

Levy O, Dayan T, Kronfeld-Schor N. The relationship between the golden spiny mouse circadian system and its diurnal activity: an experimental field enclosures and laboratory study. Chronobiol Int 24:599-613, 2007 [doi.org/10.1080/07420520701534640].

Daan S, Spoelstra K, Albrecht U, Schmutz I, Daan M, Daan B, Rienks F, Poletaeva I, Dell'Omo G, Vyssotski A, Lipp H-P. Lab mice in the field: unorthodox

daily activity and effects of a dysfunctional circadian clock allele. J Biol Rhythms 26:118-129, 2011 [doi.org/10.1177/0748730410397645].

Gattermann R, Johnston RE, Yigit N, Fritzsche P, Laarimer S, Özkurt S, Neumann K, Song Z, Colak E, Johnston J, McPhee ME. Golden hamsters are nocturnal in captivity but diurnal in nature. Biol Lett 4:253-255, 2008 [doi.org/10.1098/rsbl.2008.0066].

★2.19 On the Primacy of Light in Mammals

Unlike in non-mammalian vertebrates, 24-h entrainment of the circadian locomotor activity rhythm in adult mammals depends on intact ocular photoreception; the rhythm free runs after bilateral orbital enucleation (but see ★2.42). Moreover, when eight rodent species were blinded and housed outdoors at the University of California Berkeley Field Station, exposure to other 24-h cues – temperature, humidity, noise, and so on – failed as effective *Zeitgebers*. However, the natural environment is admittedly complex, and certain biotic factors may well act as effective non-photic *Zeitgebers* (Bussi et al., 2024).

Nelson RJ, Zucker I. Absence of extraocular photoreception in diurnal and nocturnal rodents exposed to direct sunlight. Comp Biochem Physiol 69A:145-148, 1981 [doi.org/10.1016/0300-9629(81)90651-4].

Bussi IL, Ben-Hamo M, Salazar Leon LE, Casiraghi LP, Zhang VY, Neitz AF, Lee J, Takahashi JS, Kim JJ, de la Iglesia HO. The circadian molecular clock in the suprachiasmatic nucleus is necessary but not sufficient for fear entrainment. Proc Natl Acad Sci USA 121:e2316841121, 2024 [doi.org/10.1073/pnas.2316841121].

★2.20 On Maynard Stickney Johnson

To the best of our knowledge, the first "modern" actogram was conceived by Maynard S. Johnson (1900 – 1981). Johnson graduated from Bates College, Lewiston, Maine, U.S.A. in 1921 with a Bachelor of Arts in Zoology. The 1921 Bates yearbook described him as "A Progeny of Learning" and also detailed in part "*Maynard is not avaricious but he does love lady scientists' fudge. He is ready to try anything or anyone once, from a moonlight waltz to a sermon….*" (incidentally, we have failed in our attempts to identify "fudge" as a 1920's-era slang term for anything other than a chocolate confection).

He was awarded a Ph.D. in Zoology from the University of Illinois in 1925, and a paper was published in 1926 (Johnson, 1926). In it he described the rhythmic locomotor activity of forest deer mice (*Peromyscus leucopus*). He housed each mouse in a cage suspended by rubber bands, and an indicator attached to the cage traced cage movement on a paper disk rotated by an alarm clock over 12 h. When the mouse was quiet, the record was smooth; when active, the record was irregular (Johnson's Figure 1 below shows such a tracing).

Then, without any explanation for how he decided on the design of his plots, he wrote "*There was fairly constant activity at night, and nearly continuous quiet during the day. The time when activity began and ceased agreed very closely with the time of arrival of darkness and light, as may be seen from figure 2. In this figure, periods of activity are shown by blocks of black, and periods of quiet by single lines. The heavy vertical line shows time of sunset. A study of the figure shows a relation between the seasons and time of activity. In the winter, when the days are short and the nights are long, the daily period of quiet is shorter and of activity is longer*." His Figure 2 (below) represents the first *bona fide* actograms.

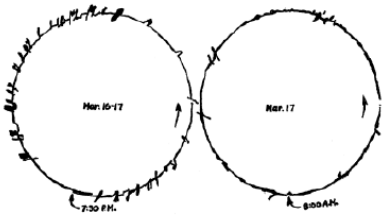


FIG. 1. TRACING OF MOUSE ACTIVITY

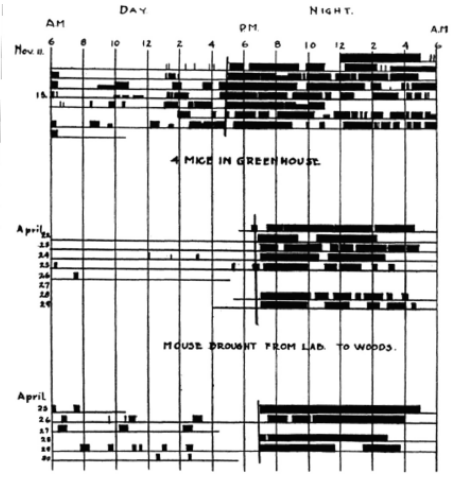
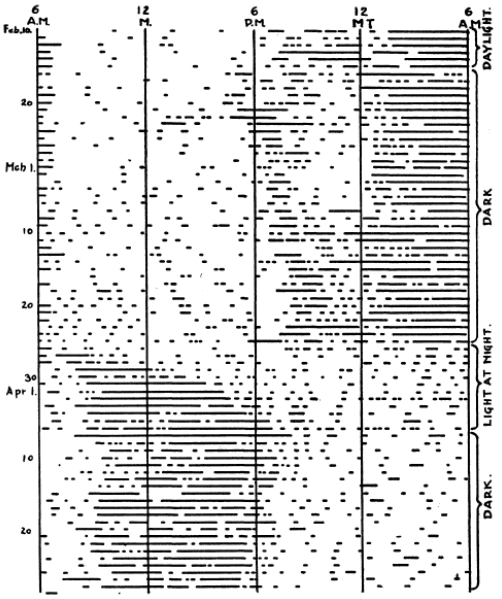


FIG. 2. NORMAL ACTIVITY OF FOREST DEER MICE, SHOWING NOCTURNAL ACTIVITY AND CHANGES WITH SEASONS. UPRIGHT LINE MARKS TIME OF SUNSET

In the same paper, he presented an actogram showing persistence of the rhythm in constant darkness, then rhythm re-entrainment with reversed lighting, followed again by constant darkness.



Figures modified from: Johnson MS. Activity and distribution of certain wild mice in relation to biotic communities. J Mammol 7:245-277, 1926 [doi.org/10.2307/1373575].

In 1939 a second laboratory-based paper appeared (Johnson, 1939), this time from the Biological Laboratories of Harvard University. His official position at Harvard is unclear and lasted for only two years (in his paper, he thanked Drs. H.W. Rand and T.J.B. Stier for laboratory space and equipment; Stier had published a paper on the thermoregulatory function of mouse locomotor activity in 1933). Johnson tested the effects of different intensities of constant light on free-running period, demonstrating what eventually would become part of Aschoff's Rule (recall ★2.8), and Johnson concluded that "...*this animal has an exceptionally substantial and durable self-winding and self-regulating physiological clock, the mechanism of which remains to be worked out*..." He soon left academia for a post with the newly created U.S. Fish and Wildlife Service, and he later served in World War II and afterwards throughout Asia working on pest control.

Johnson MS. Effect of continuous light on periodic spontaneous activity of white-footed mice (Peromyscus). J Exp Zool 82:315-328, 1939 [doi.org/10.1002/jez.1400820209].

★2.21 On the Asymmetry of Advancing and Delaying Transients

It's interesting that the differential speed of photic re-entrainment – with advancing phase shifts proceeding more slowly than delaying ones – seems to be a general feature of circadian systems. Pittendrigh et al. in 1958 noted (p. 972), "*Finally, we note that there is strong evidence in hamsters, cockroaches, and birds that advancing transients persist for many more cycles than do delaying transients; in other words, the whole system is more easily reset by a "dawn" that comes later than by one coming earlier.*" And we can even extend this list from unicellular eukaryotes (Dunlap et al., 1980) to humans (Aschoff et al., 1975), including contributions to the performance of transmeridian-traveling athletes (Heller et al., 2024). The phenomenon is irrespective of whether τ is less than or greater than 24 h. One idea is that delays are completed more quickly because they are self-enhancing (Taylor et al., 2021), in the sense that delays expand the time within the circadian cycle for further delay. Intriguingly however, re-entrainment to advancing and delaying *temperature* cycles in *Drosophila* appears to be the opposite, with advance shifts proceeding more quickly than delay ones (Abhilash et al., 2020).

Pittendrigh C, Bruce V, Kaus P. On the significance of transients in daily rhythms. Proc Natl Acad Sci USA 44:966-973, 1958 [doi.org/10.1073/pnas.44.9.965].

Dunlap JC, Taylor W, Hastings JW. The effects of protein synthesis inhibitors on the Gonyaulax clock. I. Phase-shifting effects of cycloheximide. J Comp Physiol B 138:1-8, 1980 [doi.org/10.1007/BF00688728].

Aschoff J, Hoffman K, Pohl H, Wever R. Re-entrainment of circadian rhythms after phase-shifts of the zeitgeber. Chronobiologia 2:23-78, 1975 [PMID: 1192905].

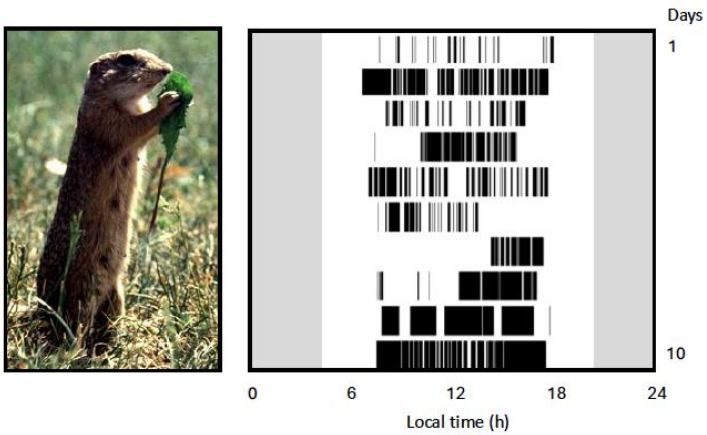
Heller HC, Herzog E, Brager A, Poe G, Allada R, Scheer F, Carskadon M, de la Iglesia HO, Jang R, Montero A, Wright K, Mouraine P, Walker MP, Goel N, Hogenesch J, Van Gelder RN, Kriegsfeld L, Mah C, Colwell C, Zeitzer J, Grandner M, Jackson CL, Prichard JR, Kay SA, Paul K. The negative effects of travel on student athletes through sleep and circadian disruption. J Biol Rhythms 39:5-19, 2024 [doi.org/10.1177/07487304231207330].

Taylor S. Delays are self-enhancing: an explanation of the east-west asymmetry in recovery from jetlag. J Biol Rhythms 36:127-136, 2021 [doi.org/10.1177/0748730421990482].

Abhilash L, Kalliyil A, Sheeba V. Responses of activity rhythms to temperature cues evolve in Drosophila populations selected for divergent timing of eclosion. J Exp Biol 223:jeb222414, 2020 [doi.org/10.1242/jeb.222414].

★2.22 On the Photic Entrainment of a Diurnal Animal That Does Not See Dawn or Dusk

European ground squirrels (*Spermophilus citellus*) are diurnal, burrow-dwelling rodents that do not see solar light-dark transitions; observational and telemetry data show that the squirrels first emerge from their dark burrows to perceive natural light 3 - 4 h after dawn and retreat back below ground 2 - 3 h before dusk (Hut et al. 1999, and representative actogram below). Nevertheless, their above-ground activity rhythms remain entrained, maintaining a relatively constant phase relationship of activity offset to the changing photoperiod. A plausible entrainment mechanism might involve phase-dependent changes of $\boldsymbol{\tau}$ in response to varying irradiance levels over the duration of the light phase, likely averaged or integrated across several days (Beersma et al., 1999).



Actogram (right) of light-sensitive telemetry recording of a European ground squirrel (left) in Bugac Puszta, Hungary; vertical marks indicate when the animal was above ground in the light, and shaded areas indicate night phases bordered by civil twilight times (Hut, van Oort, Daan, unpublished).

Hut RA, van Oort BEH, Daan S. Natural entrainment without dawn and dusk: the case of the European ground squirrel (Spermophilus citellus). J Biol Rhythms 14:290-299, 1999 [doi.org/10.1177/074873099129000704].

Beersma DGM, Daan S, Hut RA. Accuracy of circadian entrainment under fluctuating light conditions: contributions of phase and period responses. J Biol Rhythms 14:320-329, 1999 [doi.org/10.1177/074873099129000740].

★2.23 On Circadian "Resonance"

It is believed that circadian systems have evolved to enhance fitness, at least in part, by optimizing the timing of internal biological processes to the external day-night cycle. A series of studies has been conducted in cyanobacteria (*Synechococcus*; Ouyang et al., 1998; Woelfle et al., 2004) and plants (*Arabidopsis;* Dodd et al., 2005) to address this concept, based on the dependence of entrainment phase on the value of τ and the period of the entraining light-dark cycle. When short and long τ mutant bacteria and plants are grown across a range of light-dark cycle lengths, and when short τ mutant mice are maintained in a semi-natural environment (Spoelstra et al., 2016), those with τ's similar to the environmental period (referred to as "circadian resonance" by the authors) enjoy competitive advantages over those grown when the two periodicities are mismatched.

Ouyang Y, Andersson CR, Kondo T, Golden SS, Johnson CH. Resonating circadian clocks enhance fitness in cyanobacteria. Proc Natl Acad Sci USA 95:8660-8664, 1998 [doi.org/10.1073/pnas.95.15.8660].

Woelfle MA, Ouyang Y, Phanvijhitsiri K, Johnson CH. The adaptive value of circadian clocks: an experimental assessment in cyanobacteria. Curr Biol 14:1481-1486, 2004 [doi.org/10.1016/j.cub.2004.08.023].

Dodd AN, Salathia N, Hall A, Kévei E, Tóth R, Nagy F, Hibberd JM, Millar AJ, Webb AAR. Plant circadian clocks increase photosynthesis, growth, survival, and competitive advantage. Science 309:630-633, 2005 [doi.org/10.1126/science.1115581].

Spoelstra K, Wikelski M, Daan S, Hau M. Natural selection against a circadian clock gene mutation in mice. Proc Natl Acad Sci USA 113:686-691, 2016 [doi.org/10.1073/pnas.1516442113].

★2.24 On Entrainment Phase and τ - T Mismatch

In addition to the references in ★2.23 above, see also:

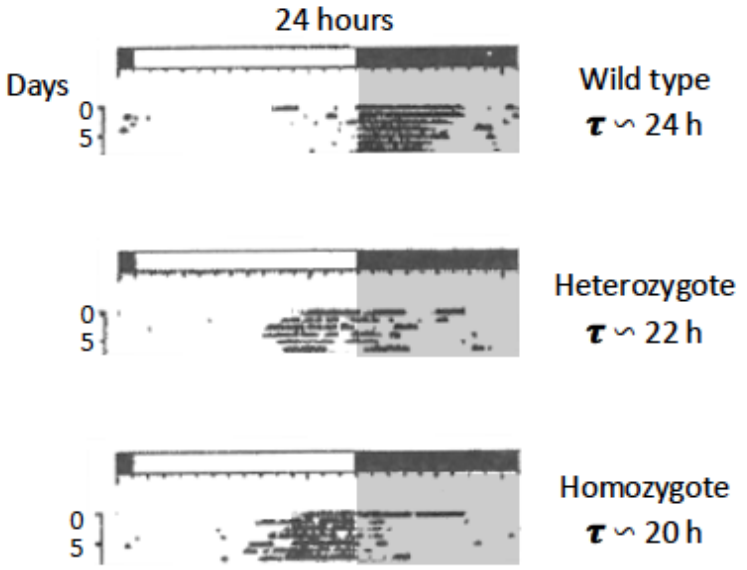
Aschoff J, Pohl H. Phase relations between a circadian rhythm and its Zeitgeber within the range of entrainment. Naturwissenschaften 65:80-84, 1978 [doi.org/10.1007/BF00440545].

Merrow M, Brunner M, Roenneberg T. Assignment of circadian function for the Neurospora clock gene frequency. Nature 399:584-586, 1999 [doi.org/10.1038/21190].

Duffy JF, Rimmer DW, Czeisler CA. Association of intrinsic circadian period with morningness–eveningness, usual wake time, and circadian phase. Behav Neurosci 115:895-899, 2001 [doi.org/10.1037//0735-7044.115.4.895].

★2.25 On τ, T, and Phase of Entrainment in Golden Hamsters

The wheel-running rhythm of golden hamsters offers striking examples to illustrate the large variations of entrainment phase that arise from altered values of τ or **T**. The hamster *tau* mutant is a semi-dominant point mutation in the catalytic region of casein kinase one epsilon (CK1ε); the wild-type-typical τ in constant darkness of about 24 h is shortened to ∽22 h in heterozygotes and ∽20 h in homozygotes. Shown below, the phase of entrained activity onset in a 14 h: 10 h LD cycle occurs normally at the light to dark transition in the wild type (top panel); the heterozygote begins running earlier, during the light phase, several hours before dark onset (middle panel); and the homozygote has essentially become a diurnal rodent (bottom panel).



By permission, modified from Fig. 1b in: Lowrey PL, Shimomura K, Antoch MP, Yamazaki S, Zemenides PD, Ralph MR, Menaker M, Takahashi JS. Positional syntenic cloning and functional characterization of the mammalian circadian mutation tau. Science 288:483-492, 2000 [doi.org/10.1126/science.288.5465.483].

On the other hand, wild type hamsters can stably entrain their wheel-running rhythms to a 1 h light pulse with a pulse period as short as 23.33 h and as long as 24.67 h, but they align with diametrically opposite phases. In the 23.33 h case, the animal entrains by advances, resulting in its active phase preceding the light pulse (the pulse acts as subjective dawn); while in the 24.67 h case, the animal entrains by delays, resulting in its active phase following the light pulse (the pulse acts as subjective dusk).

Pittendrigh CS, Daan S. A functional analysis of circadian pacemakers in nocturnal rodents. I. The stability and lability of spontaneous frequency. J Comp Physiol 106:223-252, 1976 [doi.org/10.1007/BF01417856].

Schwartz WJ, Tavakoli-Nezhad M, Lambert CM, Weaver DR, de la Iglesia HO. Distinct patterns of Period gene expression in the suprachiasmatic nucleus underlie circadian clock photoentrainment by advances or delays. Proc Natl Acad Sci USA 108:17219-17224, 2011 [doi.org/10.1073/pnas.1107848108].

★2.26 On Some Suggested Reviews: Photic Entrainment of the Suprachiasmatic Nucleus

Here are a few reviews as starting points to enter the literature on photic entrainment of the SCN:

Golombeck DA, Rosenstein RE. Physiology of circadian entrainment. Physiol Rev 90:1063-1102, 2010 [doi.org/10.1152/physrev.00009.2009].

Do MT, Yau KW. Intrinsically photosensitive retinal ganglion cells. Physiol Rev 90:1547-1581, 2010 [doi.org/10.1152/physrev.00013.2010].

Foster RG, Hughes S, Peirson SN. Circadian photoentrainment in mice and humans. Biology 9:180, 2020 [doi.org/10.3390/biology9070180].

Ashton A, Foster RG, Jagannath A. Photic entrainment of the circadian system. Int J Mol Sci 23:729, 2022 [doi.org/10.3390/ijms23020729].

★2.27 On Interindividual Variation in Photic Sensitivity of the Human Circadian System

The sensitivity of the human circadian system to light shows wide variation, including across individuals (Phillips et al., 2019), between children and adults (Higuchi et al., 2014), and by eye pigmentation and/or ethnicity (Higuchi et al., 2007).

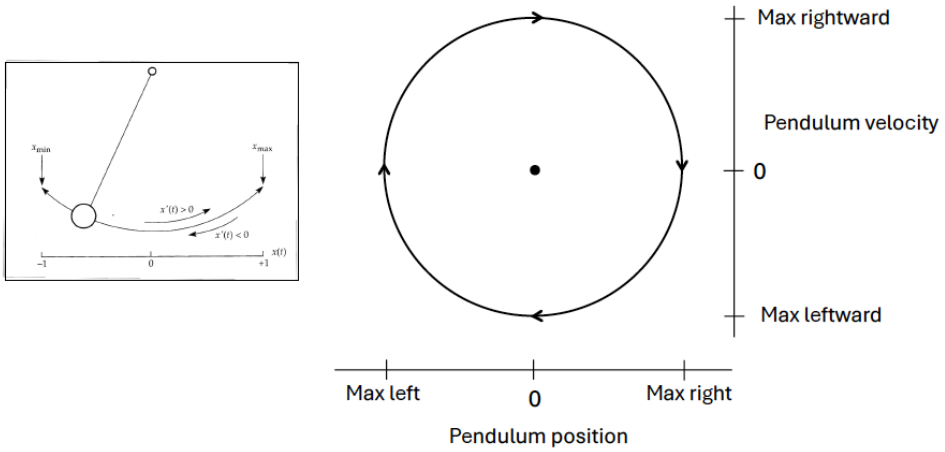
Phillips AJK, Vidafar P, Burns AC, McGlashan EM, Anderson C, Rajaratnam SMW, Lockley SW, Cain SW. High sensitivity and interindividual variability in the response of the human circadian system to evening light. Proc Natl Acad Sci USA 116:12019-12024, 2019 [doi.org/10.1073/pnas.1901824116].

Higuchi S, Nagafuchi Y, Lee S, Harada T. Influence of light at night on melatonin suppression in children. J Clin Endocrinol Metab 99:3298-3303, 2014 [doi.org/10.1210/jc.2014-1629].

Higuchi S, Motohashi Y, Ishibashi K, Maeda T. Influence of eye colors of Caucasians and Asians on suppression of melatonin secretion by light. Am J Physiol 292:R2352-R2356, 2007 [doi.org/10.1152/ajpregu.00355.2006].

★2.28 On Stable Limit Cycles, Phase Response Curves, and Phase Transition Curves

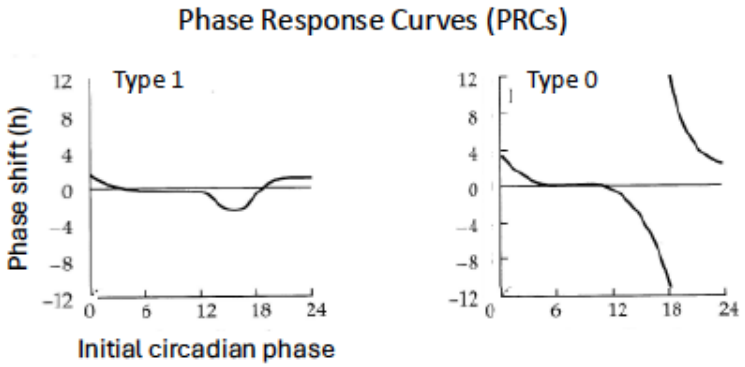
As a graduate student of Pittendrigh, Arthur Winfree (1942 – 2002) pioneered a theoretical model of the self-sustained circadian oscillator as a stable, attractive limit cycle; that is, a closed trajectory in which the state variables of the system exhibit oscillating values. This approach has helped to predict how phase-specific perturbations (like light pulses) affect the oscillator's behavior. A clock pendulum is often cited as a simple limit cycle defined by two dynamic state variables as the pendulum swings back and forth: its position in space and its velocity. In the phase plane diagram below, you can trace the oscillation as the pendulum moves left and right, with the black dot in the center representing the motionless pendulum, hanging vertically at rest. This equilibrium point is often referred to as a "singularity" or "singular region."



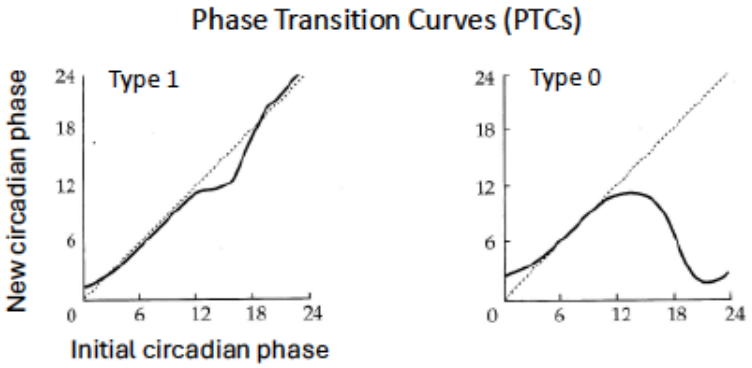
A clock pendulum

Perturbing this system at different phases of its oscillation can shift the state variables to other areas of the phase plane. If the strength of the perturbation is weak, such that the shifted system remains on the near side of the singularity, a phase response curve is generated with a continuous transition between small delay and advance phase shifts (Type 1 PRC). On the other hand, if the strength of the perturbation is strong (or the diameter [amplitude] of the limit cycle is small), the shifted system may move to the far side of the singularity, to the opposite side of the limit cycle. This generates a phase response curve with large phase shifts and a discontinuity at the transition between the delays and advances (Type 0 PRC). Illustrated below are generic shapes for Type 1 (left) and Type 0 (right) PRCs to light pulses for a circadian system; the phases

of pulse onset (initial circadian phase) are plotted in circadian time and the resulting phase shifts are measured in hours.



There is another way to display these data, as (PTCs); instead of the phase shifts (in hours) on the y-axis, PTCs plot the new circadian phases (in circadian time) that result from the shifts. Illustrated below are the shapes for Type 1 (left) and Type 0 (right) PTCs that correspond to the PRCs above.



Limit cycle, PRC, and PTC graphics re-drawn or modified from Figs. 3.7 & 3.22 in: Dunlap JC, Loros JJ, DeCoursey PJ (eds). Chronobiology; Biological Timekeeping, Sinauer, 2004.

The Type 1 / Type 0 designations for PRCs and PTCs refer to the average slopes of the PTCs, with an average slope of 1 for Type 1 and 0 for Type 0 (dotted lines show a slope of 1). While PRCs have been widely adopted by the circadian field (Johnson, 1999), several topological considerations favor the use of PTCs (Winfree, 2001). Moreover, there is a perceptual advantage to using PTCs ra-

ther than PRCs for displaying Type 0 resetting: PTCs avoid the apparent discontinuity at the transition between delays and advances on PRCs. Discontinuous PRCs give the impression that there might be mechanistic differences between the shifts in opposite directions, when in fact the discontinuity is most often an artifact of defining a delay or an advance as no greater than 12 h (one half cycle). Plotting PRCs monotonically – with all phase shifts labeled as delays from 0 h to 24 h – is another approach that can address this confound.

Johnson CH. Forty years of PRCs – what have we learned? Chronobiol Int 16:711-743, 1999 [doi.org/10.3109/07420529909016940].

Winfree AT. The Geometry of Biological Time, second edition, Springer, 2001.

Here we note an interesting notion raised by Winfree's limit cycle model. Could a light pulse of exactly the right magnitude, delivered at exactly the right phase, lead to circadian arrhythmicity by propelling the oscillator to its singularity, rendering it motionless and phaseless (see arguments in Jewett et al., 1991)? Indeed, attempts to do so with rhythmic cell cultures have led to a loss of rhythmicity, but – given even slight differences in phase between cells or instability of the singular region – these results seem most likely due to desynchronization rather than abolition of individual cellular rhythms (Miwa et al., 1987; see also Nagoshi et al., 2004 and Welsh et al., 2004 for examples of population arrhythmicity due to cellular desynchrony). It's also important to emphasize that multi-dimensional limit cycles need not be perfect circles with singularities at their centers. For example, a two-dimensional mathematical representation of the Forger/Peskin model oscillator (composed of 73 differential equations) reveals a narrow, elongated oval trajectory with the singularity close to one of its ends (Indic et al., 2006).

Jewett ME, Kronauer RE, Czeisler CA. Light-induced suppression of endogenous circadian amplitude in humans. Nature 350:59-62, 1991 [doi.org/10.1038/350059a0].

Miwa I, Nagatoshi H, Horie T. Circadian rhythmicity within single cells of Paramecium bursaria. J Biol Rhythms 2:57-64, 1987 [doi.org/10.1177/074873048700200105].

Nagoshi E, Saini C, Bauer C, Laroche T, Naef F, Schibler U. Circadian gene expression in individual fibroblasts: cell-autonomous and self-sustained oscillators pass time to daughter cells. Cell 119:693-705, 2004 [doi.org/10.1016/j.cell.2004.11.015].

Welsh DK, Yoo S-H, Liu AC, Takahashi JS, Kay SA. Bioluminescence imaging of individual fibroblasts reveals persistent, independently phased circadian rhythms of clock gene expression. Curr Biol 14:2289-2295, 2004 [doi.org/10.1016/j.cub.2004.11.057].

Indic P, Gurdziel K, Kronauer RE, Klerman EB. Development of a two-dimension manifold to represent hiogh dimension mathematical models of the intracellular mammalian circadian clock. J Biol Rhythms 21:222-232, 2006 [doi.org/10.1177/0748730406287357].

★2.29 On Measuring and Reporting Light Exposure Across Species

See references:

Spitschan M, Stefani O, Blattner P, Gronfier C, Lockley SW, Lucas RJ. How to report light exposure in human chronobiology and sleep research experiments. Clocks Sleep 1:280-289, 2019 [doi.org/10.3390/clockssleep1030024].

Lucas RJ, Allen AE, Brainard GC, Brown TM, Dauchy RT, Didikoglu A, Do MTH, Gaskill BN, Hattar S, Hawkins P, Hut RA, McDowell RJ, Nelson RJ, Prins J-B, Schmidt TM, Takahashi JS, Verma V, Voikar V, Wells S, Peirson SN. Recommendations for measuring and standardizing light for laboratory mammals to improve welfare and reproducibility in animal research, PLoS Biology 22:e3002535, 2024 [doi.org/10.1371/journal.pbio.3002535].

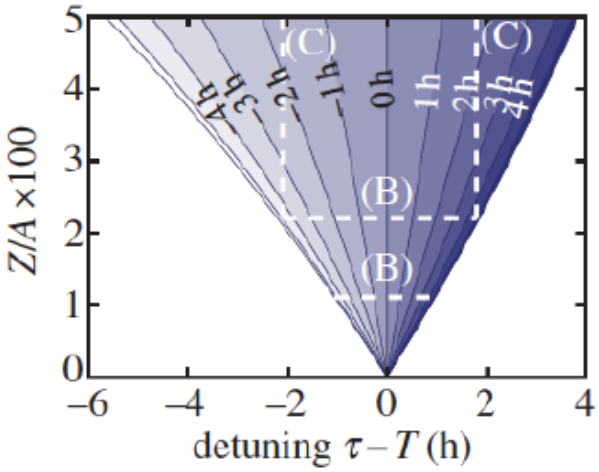
Lucas RJ, Peirson SN. Practical advice on measuring and applying light for laboratory animals. J Biol Rhythms 39:323-330, 2024 [doi.org/10.1177/07487304241259514].

McDowell RJ, Didikoglu A, Woelders T, Gatt MJ, Moffatt F, Notash S, Hut RA, Brown TM, Lucas RJ. Beyond Lux: Methods for species and photoreceptor-specific quantification of ambient light for mammals. BMC Biology 22:257, 2024 [doi.org/10.1186/s12915-024-02038-1].

★2.30 On a Generic Mathematical Model for the Phase and Range of Entrainment

Granada et al. (2013) and Bordyugov et al. (2015) have sought a systematic understanding of how entrainment phase relates to $\boldsymbol{\tau}$ - **T** and *Zeitgeber*

strength using theoretical models and numerical simulations. Their core result is illustrated below.



By permission, from Fig. 1a in: Bordyugov G, Abraham U, Granada A, Rose P, Imkeller K, Kramer A, Herzel H. Tuning the phase of circadian entrainment. J R Soc Interface 12:20150282, 2015 [doi.org/10.1098/rsif.2015.0282].

Note that as the ratio of *Zeitgeber* strength to oscillator amplitude (Z/A) increases (horizontal lines B), the range of entrainment also increases for greater mismatches of $\boldsymbol{\tau}$ - **T** (within the colored wedge-shaped region termed "Arnold tongue" [after the Russian mathematician V.I. Arnold]). Within the tongue, as the $\boldsymbol{\tau}$ - **T** mismatch becomes more negative or more positive, the entrainment phase becomes earlier or later, respectively. Vertical lines C show that for a given negative or positive $\boldsymbol{\tau}$ – **T** mismatch, decreasing *Zeitgeber* strength leads to an earlier or later entrainment phase, respectively.

Granada AE, Bordyugov G, Kramer A, Herzel H. Human chronotypes from a theoretical perspective. PLoS ONE 8:e59464, 2013 [doi.org/10.1371/journal.pone.0059464].

★2.31 On Coupled Limit Cycle Oscillators

Winfree realized that the behavior of a population of weakly-coupled limit cycle oscillators would likely be substantially different from that of a single oscillator alone. And he surmised, quite presciently, that intercellular synchronization might determine as much about circadian clock dynamics as does its autonomous cellular mechanism. Indeed, theoretical and experimental studies suggest that the coupling and phase distribution of SCN neurons significantly

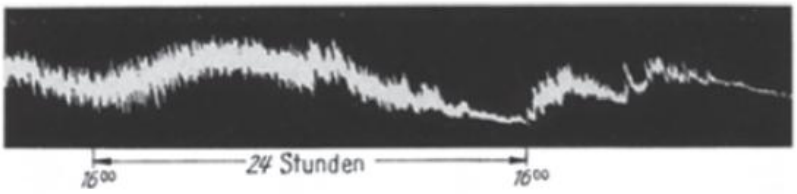
impact entrainment properties and phase-shifting capacities of the nucleus as a whole (Van der Leest et al., 2009; Abraham et al., 2010).

Van der Leest HT, Rohling JHT, Michel S, Meijer JH. Phase shifting capacity of the circadian pacemaker determined by the SCN neuronal network organization. PLoS ONE 4:e4976, 2009 [doi.org/10.1371/journal.pone.0004976].

Abraham U, Granada AE, Westermark PO, Heine M, Kramer A, Herzel H. Coupling governs entrainment range of circadian clocks. Molec Systems Biol 6:438, 2010 [doi.org/10.1038/msb.2010.92].

★2.32 On Early Claims for the Existence of Multiple Body Clocks

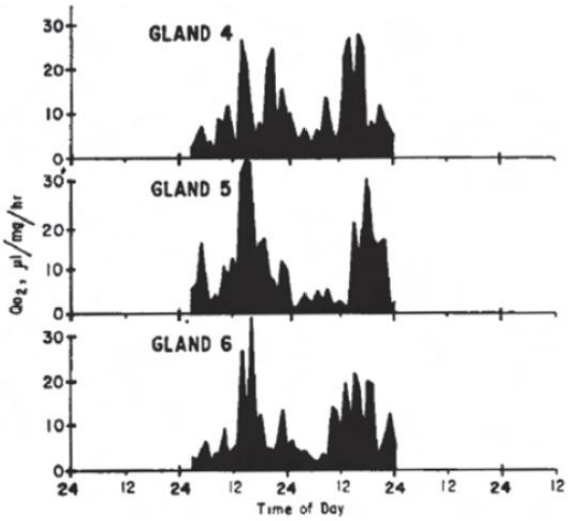
Erwin Bünning (1906 – 1990) (along with Jürgen Aschoff and Colin Pittendrigh, considered one of the three founders of modern circadian biology) noted that some physiological rhythms in vertebrates appear to resynchronize with different speeds after a shift in the light-dark cycle; so inspired, he recorded the peristaltic movements of isolated hamster intestine in a tissue bath over 2-3 days and reported that gut tension expressed a circadian rhythm, with stronger contraction during the projected night. Inspection of his figure (below) suggests why the existence of an intestinal clock was not universally accepted at the time.



By permission, from Fig. 1 in: Bünning E. Das Weiterlaufen der "physiologischen Uhr" im Säugerdarm ohne zentrale Steuerung. Naturwissenschaften 45:68, 1958 [doi.org/10.1007/BF00638628].

In the 1960's, Richard Andrews and G. Edgar Folk, Jr. cultured hamster adrenal glands and measured oxygen consumption and steroid secretion over a few days. They made a case for a circadian metabolic rhythm in culture, with low activity during the subjective night, and also described that (a) the phase of the rhythm was not influenced by the time of sacrifice of the donors; (b) the period of the rhythm was relatively independent of flask temperature; and (c)

the pattern of the rhythm appeared 12 h out of phase if donor animals were maintained in a reversed light-dark cycle.



Oxygen consumption of three adrenal glands from donor hamsters entrained to a 12 h: 12 h light-dark cycle, lights on at 0800 h. By permission, from Fig. 1b in: Andrews RV, Folk GE Jr. Circadian metabolic patterns in cultured hamster adrenal glands. Comp Biochem Physiol 11:393-409, 1964 [doi.org/10.1016/0010-406x(64)90006-4].

And 1 year later, Gerald Tharp and Folk reported a circadian rhythm of heart rate in isolated rat hearts, hypothesizing that "*the circadian rhythms of resting heart rate are controlled by a 'clock' located within the heart cell*."

Tharp GD, Folk GE Jr. Rhythmic changes in rate of the mammalian heart and heart cells during prolonged isolation. Comp Biochem Physiol 14:255-273, 1965 [doi.org/10.1016/0010-406X(65)90202-1].

★2.33 On the Diversity of Downstream Efferent Pathways

The SCN's efferent pathways are impressively multimodal, translating its electrophysiological rhythm widely through (a) first-order neural projections primarily to nearby hypothalamic areas and then trans-synaptically, including to the autonomic nervous system (with innervation of the pineal gland); and (b) secretion of diffusible substances, with transmission via the ventricular

system and a recently-described venous portal system to the organum vasculosum lamina terminalis (OVLT) (Yao et al., 2021). In turn, some of the resulting physiological and behavioral rhythms – of hormone secretion, body temperature, and activity/rest (and consequently, feeding/fasting) – convey external time (or circadian time, in constant conditions) throughout the organism to act as internal *Zeitgebers* for phase-setting of downstream targets.

Yao Y, Taub AB, LeSauter J, Silver R. Identification of the suprachiasmatic nucleus venous portal system in the mammalian brain. Nat Commun 12:5643, 2021 [doi.org/10.1038/s41467-021-25793-z].

★2.34 On the Diversity of Downstream Phase-setting Mechanisms

The phase-setting of cell, tissue, and organ rhythms by the SCN involves a multitude of target-specific signaling pathways (for two reviews as starting points, consider Dibner et al., 2010 and Mohawk et al., 2012). The regulation of circadian gene transcription by the signals is primarily, but not exclusively (Kornmann et al., 2007), via the entrainment of local oscillators.

Dibner C, Schibler U, Albrecht U. The mammalian circadian timing system: Organization and coordination of central and peripheral clocks. Annu Rev Physiol 72:517-549, 2010 [doi.org/10.1146/annurev-physiol-021909-135821].

Mohawk JA, Green CB, Takahashi JS. Central and peripheral circadian clocks in mammals. Annu Rev Neurosci 35:445-462, 2012 [doi.org/10.1146/annurev-neuro-060909-153128].

Kornmann B, Schaad O, Bujard H, Takahashi JS, Schibler U. System-driven and oscillator-dependent circadian transcription in mice with a conditionally active liver clock. PLoS Biol 5:e34, 2007 [doi.org/10.1371/journal.pbio.0050034].

On the other hand, the nocturnal rhythm of pineal melatonin synthesis is directly driven via a multi-synaptic neural pathway from the SCN. During the light phase (or subjective day), SCN GABAergic inhibition of the paraventricular nucleus (Kalsbeek et al., 2000) suppresses sympathetic activation of the rate-limiting enzyme for melatonin synthesis, aryl alkylamine *N*-acetyltransferase (AANAT). During the dark phase (or subjective night), the inhibition is released, and along with a stimulatory signal from a small group of glutamatergic SCN neurons (Perreau-Lenz et al., 2004), melatonin synthesis can proceed. Incidentally, in addition to its other roles (see Chapter 3), the melatonin

rhythm acts as an internal *Zeitgeber* during pregnancy; it is sufficient (although not necessary) as a transplacental entraining signal of the fetal circadian system (Davis and Mannion, 1988).

Kalsbeek A, Garidou ML, Palm IF, Van Der Vliet J, Simonneaux V, Pévet P, Buijs RM. Melatonin sees the light: blocking GABA-ergic transmission in the paraventricular nucleus induces daytime secretion of melatonin. Eur J Neurosci 12:3146-3154, 2000 [dio.org/10.1046/j.1460-9568.2000.00202.x].

Perreau-Lenz, Kalsbeek A, Pévet P, Buijs RM. Glutamatergic clock output stimulates melatonin synthesis at night. Eur J Neurosci 19:318-324, 2004 [doi.org/10.1111/j.0953-816x.2003.03132.x].

Davis FC, Mannion J. Entrainment of hamster pup circadian rhythms by prenatal melatonin injections to the mother. Am J Physiol 255:R439-448, 1988 [doi.org/10.1152/ajpregu.1988.255.3.R439].

Yet another mechanism appears responsible for the SCN's control of the locomotor activity rhythm in nocturnal rodents. Rather than entraining or driving the rhythm, the SCN acts to "gate" locomotor activity by allowing expression during the dark phase (or subjective night) and suppressing it during the light phase (or subjective day), when the rhythm of overall SCN electrical activity is low or high, respectively. The width of this permissive window is defined by SCN activity falling below or rising above a 50% level (Houben et al., 2009; see also Davis and Menaker, 1980). Note that this tight relationship can be altered under certain circumstances (see ★2.43 in this chapter and ★3.2 in Chapter 3).

Houben T, Deboer T, van Oosterhout F, Meijer JH. Correlation with behavioral activity and rest implies circadian regulation by SCN neuronal activity levels. J Biol Rhythms 24:477–487, 2009 [doi.org/10.1177/0748730409349895].

Davis FC, Menaker M. Hamsters through time's window: temporal structure of hamster locomotor rhythmicity. Am J Physiol 239:R149-R155, 1980 [doi.org/10.1152/ajpregu.1980.239.1.R149].

★2.35 On the Production and Degradation Rates of Clock-controlled mRNAs and Proteins

With the advent and advances in genome-wide transcriptomic and proteomic studies came the realization that not all rhythmic transcripts lead to protein

rhythms and not all protein rhythms are encoded by rhythmic transcripts. Of course, rates of both production and degradation determine the levels of mRNA and protein abundance; models suggest that, depending on the mean half-lives of the molecules, rhythmic degradation itself can shape the amplitudes and peak phases of the molecular rhythms (Lück et al., 2014; Wang et al., 2018). In any case, a practical lesson is that the presence or absence of rhythmic transcripts alone (or their amplitudes and phases) cannot define the functional rhythmicity of circadian systems.

Lück S, Thurley K, Thaben PF, Westermark PO. Rhythmic degradation explains and unifies circadian transcriptome and proteome data. Cell Rep 9:741-751, 2014 [dio.org/10.1016/j.celrep.2014.09.021].

Wang J, Symul L, Yeung J, Gobet C, Sobel J, Lück S, Westermark PO, Molina N, Naef F. Circadian clock-dependent and -independent posttranscriptional regulation underlies temporal mRNA accumulation in mouse liver. Proc Natl Acad Sci USA 115:E1916-E1925, 2018 [doi.org/10.1073/pnas.1715225115].

★2.36 On the SCN's Intrinsic Inter-cellular and Inter-compartmental Circuitry

In addition to critical roles in photic entrainment (see ★2.31 in this chapter) and seasonal encoding (see Chapter 3), cellular communication within the SCN – established by complex mechanisms of intercellular coupling and topographical compartmentalization – is key for building an SCN pacemaker that is precise (Herzog et al., 2004), robust (Liu et al., 2007), and resistant to entrainment by the body temperature rhythm (Buhr et al., 2010). For reviews of candidate mechanisms, see Evans and Gorman, 2016 and Herzog et al., 2017.

Herzog ED, Aton SJ, Numano R, Sakaki Y, Tei H. Temporal precision in the mammalian circadian system: a reliable clock from less reliable neurons. J Biol Rhythms 19:35-46, 2004 [doi.org/10.1177/0748730403260776].

Liu AC, Welsh DK, Ko CH, Tran HG, Zhang EE, Priest AA, Buhr ED, Singer O, Meeker K, Verma IM, Doyle FJ III, Takahashi JS, Kay SA. Intercellular coupling confers robustness against mutations in the SCN circadian clock network. Cell 129:605-616, 2007 [doi.org/10.1016/j.cell.2007.02.047].

Buhr ED, Yoo S-H, Takahashi JS. Temperature as a universal resetting cue for mammalian circadian oscillators. Science 330:379-385, 2010 [doi.org/10.1126/science.1195262].

Evans JA, Gorman MR. In synch but not in step: circadian clock circuits regulating plasticity in daily rhythms. Neuroscience 320:259-280, 2016 [doi.org/10.1016/j.neuroscience.2016.01.072].

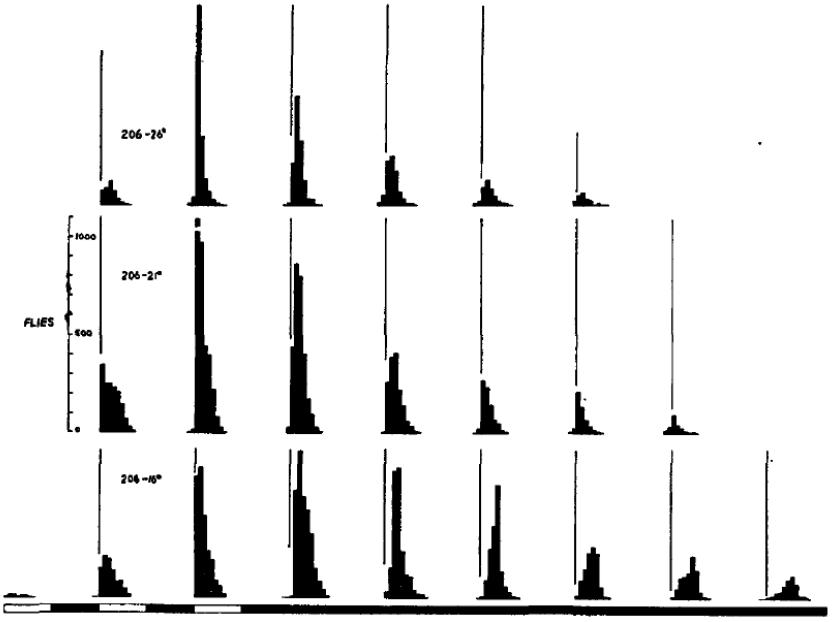
Herzog ED, Hermanstyne T, Smyllie NJ, Hastings MH. Regulating the suprachiasmatic nucleus (SCN) circadian clockwork: interplay between cell-autonomous and circuit-level mechanisms. Cold Spring Harb Perspect Biol 9:a027706, 2017 [doi.org/10.1101/cshperspect.a027706].

★2.37 On Temperature Compensation

Originally described as temperature independence, Pittendrigh argued that such a property must be required for a physiological clock to function as a reliable timekeeper and not as a thermometer (Pittendrigh, 1960). He made the case with a classical set of experiments on the timing of *Drosophila* eclosion under different ambient temperatures (Pitttendrigh, 1954), also making a point that the internal circadian timekeeping mechanism is separate from the downstream behaviors it regulates (see also ★2.6 in this chapter).

Pittendrigh CS. Circadian rhythms and the circadian organization of living systems. Cold Spring Harb Symp Quant Biol 25:159-184, 1960 [doi.org/10.1101/sqb.1960.025.01.015].

Pittendrigh CS. On temperature independence in the clock system controlling emergence time in Drosophila. Proc Natl Acad Sci USA 40:1018-1029, 1954 [doi.org/10.1073/pnas.40.10.1018].



Relative temperature compensation of the Drosophila eclosion rhythm in constant darkness, at 26° (upper), 21° (middle), and 16°C (lower). Vertical lines represent 24-h intervals. By permission, from Fig. 2 in: Pittendrigh CS, Bruce VG. An oscillator model for biological clocks. In: Rhythmic and Synthetic Processes in Growth (ed D Rudnick, from the Fifteenth Symposium for the Study of Development and Growth), 75-109, 1957.

★2.38 On Vertebrate Circadian Organization

The relative roles of the retina, pineal, and SCN in the circadian systems of fish, reptiles, birds, and mammals roughly correspond along phylogenetic lines (but important details are variable even within a vertebrate class). Differing roles for the structures might also reflect evolutionary adaptation to different photic niches and also changing requirements of developmental life stages (Menaker et al., 1997). Now thanks to the availability of modern genome editing technologies, a revitalized comparative approach incorporating novel animal models can take advantage of the strengths of additional species.

Menaker M, Moreira LF, Tosini G. Evolution of circadian organization in vertebrates. Braz J Med Biol Res 30:305-313, 1997 [doi.org/10.1590/S0100-879X1997000300003].

★2.39 On SCN Inputs

We have already pointed out that the rodent running wheel is not only an assay of the locomotor activity rhythm but also a modulator of it (see ★2.7). The mechanisms appear complex – involving effects related to exercise and central reward pathways – but also include "feedback" on the electrophysiological activity of the SCN itself, enhancing the amplitude of its circadian electrical rhythm (Van Oosterhout et al., 2012; Houben et al., 2014). In this regard, it's interesting that provision of a running wheel to the common vole (*Microtus arvalis*) enhances the circadian component of its activity rhythm but suppresses the ultradian component of its activity/rest and feeding/fasting rhythms, especially during the light phase. Although electrical activity has not been recorded in the vole SCN, clock gene expression in the vole nucleus remains circadian in the presence or absence of the wheel (Van der Veen et al., 2006).

Much of the past work on SCN neural inputs initially focused on innervation by the retina (retinohypothalamic tract), intergeniculate leaflet (geniculohypothalamic tract), and median raphe nucleus. But the SCN receives on the order of hundreds of monosynaptic and polysynaptic non-photic inputs from interoceptive neural systems, including olfactory, limbic, circumventricular, hypothalamic, visceral, and nociceptive sites (Krout et al., 2002). And the SCN bears high-affinity receptors for melatonin (Dubocovich, 2007) and sex hormones (Karatsoreos et al., 2007; Hatcher et al., 2020) and responds to humoral factors that regulate energy metabolism (see Bass, 2013) and inflammation (Cermakian et al., 2014).

Van Oosterhout F, Lucassen EA, Houben T, vanderLeest HT, Antle MC, Meijer JH. Amplitude of the SCN clock enhanced by the behavioral activity rhythm. PLoS ONE 7:e39693, 2012 [doi.org/10.1371/journal.pone.0039693].

Houben T, Coomans CP, Meijer JH. Regulation of circadian and acute activity levels by the murine suprachiasmatic nuclei. PLoS ONE 9:e110172, 2014 [doi.org/10.1371/journal.pone.0110172].

Van der Veen FR, Le Minh N, Gos P, Americ M, Gerkema MP, Schibler U. Impact of behavior on central and peripheral circadian clocks in the common vole Microtus arvalis, a mammal with ultradian rhythms. Proc Natl Acad Sci USA 103:3393-3398, 2006 [doi.org/10.1073/pnas.0507825103].

Krout KE, Kawano J, Mettenleiter TC, Loewy AD. CNS inputs to the suprachiasmatic nucleus of the rat. Neuroscience 110:73-92, 2002 [doi.org/10.1016/s0306-4522(01)00551-6].

Dubocovich ML. Melatonin receptors: role on sleep and circadian rhythm regulation. Sleep Med 8:S34-S42, 2007 [doi.org/10.1016/j.sleep.2007.10.007].

Karatsoreos IN, Wang A, Sasanian J, Silver R. A role for androgens in regulating circadian behavior and the suprachiasmatic nucleus. Endocrinology 148:5487-5495, 2007 [doi.org/10.1210/en.2007-0775].

Hatcher KM, Royston SE, Mahoney MM. Modulation of circadian rhythms through estrogen receptor signaling. Eur J Neurosci 51:217-228, 2020 [doi.org/10.1111/ejn.14184].

Bass J. Forever (FGF) 21. Nat Med 19:1090-1092, 2013 [doi.org/10.1038/nm.3334].

Cermakian N, Westfall S, Kiessling S. Circadian clocks and inflammation: reciprocal regulation and shared mediators. Arch Immunol Ther Exp 62:303-318, 2014 [doi.org/10.1007/s00005-014-0286-x].

★2.40 On the Circadian and Homeostatic Regulation of Thirst

A prime example of the SCN's position at the nexus of a circadian and homeostatic pathway is in the neural regulation of thirst. The murine SCN drives *anticipatory* thirst, secretion of anti-diuretic hormone, and a reduction in body temperature a few hours before sleep to protect against dehydration. Projections from SCN vasopressin neurons to the OVLT are necessary and sufficient to drive circadian thirst (Gizowski and Bourque, 2018). But the *homeostatic* response to an unanticipated rise in serum osmolality (hypertonic saline injection, ZT 19) is also mediated by SCN vasopressin neurons, driven by an excitatory GABA-ergic input from osmo-sodium-sensing neurons in the OVLT (Gizowski and Bourque, 2020).

Gizowski C, Bourque CW. The neural basis of homeostatic and anticipatory thirst. Nat Rev Nephrol 14:11-25, 2018 [doi.org/10.1038/nrneph.2017.149].

Gizowski C, Bourque CW. Sodium regulates clock time and output via an excitatory GABAergic pathway. Nature 583:421-424, 2020 [doi.org/10.1038/s41586-020-2471-x].

★2.41 On the Temporal Coherence of Tissue and Organ Oscillators

A rapidly expanding list of communicating signals, cellular mediators, and clock gene targets are being identified with local actions that bridge oscillatory functions across neighboring and distant tissues and organs. For two reviews as starting points:

Sinturel F, Petrenko V, Dibner C. Circadian clocks make metabolism run. J Molec Biol 432:3680-3699, 2020 [doi.org/10.1016/j.jmb.2020.01.018].

Koronowski KB, Sassone-Corsi P. Communicating clocks shape circadian homeostasis. Science 371:eabd0951, 2021 [doi.org/10.1126/science.abd0951].

★2.42 On Local Photic Entrainment of Circadian Timing in Retina, Cornea, and Skin

Local oscillators regulate aspects of circadian gene expression in cornea and retina, but their entrainment to the light-dark cycle does not require the SCN, melanopsin (OPN4), or rod or cone opsins. Instead, neuropsin (OPN5) – already known as a deep brain photopigment in birds – has been identified as the responsible opsin, expressed in a small subset of retinal ganglion cells (Buhr et al., 2015). Neuropsin is also present in murine melanocytes – as in some invertebrates and non-mammalian vertebrates – and can mediate photic entrainment of local epidermal oscillators (Buhr et al., 2019). Additional possible SCN-independent actions of light on the coherence of circadian network elements are not fully understood (Husse et al., 2014; Koronowski et al., 2019; Welz et al., 2019).

Buhr ED, Yue WWS, Ren X, Jiang Z, Liao H-WR, Mei X, Vemaraju S, Nguyen M-T, Reed RR, Lang RA, Yau K-W, Van Gelder RN. Neuropsin (OPN5)-mediated photoentrainment of local circadian oscillators in mammalian retina and cornea. Proc Natl Acad Sci USA 112:13093-13098, 2015 [doi.org/10.1073/pnas.1516259112].

Buhr ED, Vemaraju S, Diaz N, Lang RA, Van Gelder RN. Neuropsin (OPN5) mediates local light-dependent induction of circadian clock genes and circadian photoentrainment in exposed murine skin. Curr Biol 29:3478-3487, 2019 [doi.org/10.1016/j.cub.2019.08.063].

Husse J, Leliavski A, Tsang AH, Oster H, Eichele G. The light-dark cycle controls peripheral rhythmicity in mice with a genetically ablated suprachiasmatic nucleus clock. FASEB J 28:4950-4960, 2014 [doi.org/10.1096/fj.14-256594].

Koronowski KB, Kinouchi K, Welz P-S, Smith JG, Zinna VM, Shi J, Samad M, Chen S, Magnan CN, Kinchen JM, Li W, Baldi P, Benitah SA, Sassone-Corsi P. Defining the independence of the liver circadian clock. Cell 177:1448-1462, 2019 [doi.org/10.1016/j.cell.2019.04.025].

Welz P-S, Zinna VM, Symeonidi A, Koronowski KB, Kinouchi K, Smith JG, Guillén IM, Castellanos A, Furrow S, Aragón F, Crainiciuc G, Prats N, Caballero JM, Hidalgo A, Sassone-Corsi P, Benitah SA. BMAL1-drive tissue clocks respond independently to light to maintain homeostasis. Cell 177:1436-1447, 2019 [doi.org/10.1016/j.cell.2019.05.009].

★2.43 On Developmental and Adaptive Phase Realignments of the Circadian Network

An example of a developmental realignment of oscillating network components is in developing rats, which show a nearly 12-h shift of the liver and thyroid oscillators' phase with respect to that of the SCN around post-natal days 20 – 22, corresponding to the switch from suckling during mother's rest phase (light phase) to independent feeding during the pups' own active phase (dark phase) (Yamazaki et al., 2009). And in laboratory mice, increasing the energetic costs of feeding – rather than displacing and restricting its timing – or lowering ambient temperature can functionally reconfigure the network phenotype from nocturnal to essentially diurnal (Van der Vinne et al., 2014; we will consider the likely adaptive significance of such "temporal niche" switching in Chapter 3).

Yamazaki S, Yoshikawa T, Biscoe EW, Numano R, Gallaspy LM, Soulsby S, Papadimas E, Pezuk P, Doyle SE, Tei H, Sakaki Y, Block GD, Menaker M. Ontogeny of circadian organization in the rat. J Biol Rhythms 24:55-63, 2009 [doi.org/10.1177/0748730408328438].

Van der Vinne V, Riede SJ, Gorter JA, Eijer WG, Sellix MT, Menaker M, Daan S, Pilorz V, Hut RA. Cold and hunger induce diurnality in a nocturnal mammal. Proc Natl Acad Sci USA 111:15256-15260, 2014 [doi.org/10.1073/pnas.1413135111].

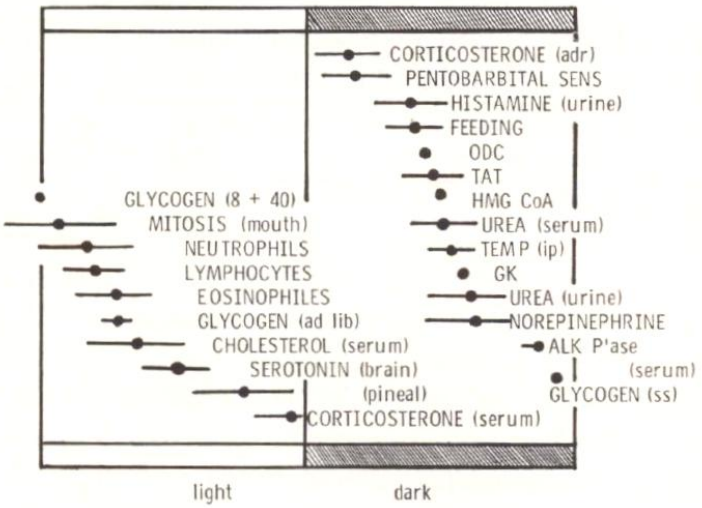
★2.44 On New Ecological Challenges in the Anthropocene

Increasingly (and well beyond our forced switches between standard and daylight savings times), human activity is affecting the daily timing of other species, either directly by invasion of their ecosystems or indirectly through climate change and light pollution. The consequences – both adaptive and maladaptive – have only begun to be explored; for a review and research agenda see:

Gilbert NA, McGinn KA, Nunes LA, Shipley AA, Bernath-Plaisted J, Clare JDJ, Murphy PW, Keyser SR, Thompson KL, Maresh Nelson SB, Cohen JM, Widick IV, Bartel SL, Orrock JL, Zuckerberg B. Daily activity timing in the Anthropocene. Trends Ecol Evol 38:324-336, 2023 [doi.org/10.1016/j.tree.2022.10.008].

★2.45 On the Original Definition of Chronotype

Charles Ehret (1923 – 2007) coined the term *chronotype* as the overall temporal phenotype of an organism; for example, he illustrated the *circadian chronotype* of the rat in a 12 h: 12 h light-dark cycle from a review of the literature:



The horizontal bars represent 95% confidence intervals around the mean for the peak phase of each measure as calculated by the cosinor method. By permission, from Fig. 16 in: Ehret CF. The sense of time: evidence for its molecular basis in the eukaryotic gene-action system. Adv Biol Med Phys 15:47-77, 1974 [dio.org/10.1016/b978-0-12-005215-8.50009-7].

Presciently, in the paper cited above, Ehret quoted a 1972 student paper in "circadian cybernetics" (T. Hefty, University of Wisconsin, Madison) that "*even if the molecular circadian clock is someday well defined, working out the complexities of the interactions of these 'little clocks' to form larger systems will still be a monumental task*."

★2.46 On Assaying Human Circadian Time

See references:

Kasukawa T, Sugimoto M, Hida A, Minami Y, Mori M, Honma S, Honma K, Mishima K, Soga T, Ueda HR. Human blood metabolite timetable indicates internal body time. Proc Natl Acad Sci USA 109:15036–15041, 2012 [doi.org/10.1073/pnas.1207768109].

Hughey JJ. Machine learning identifies a compact gene set for monitoring the circadian clock in human blood. Genome Med 9:19, 2017 [doi.org/10.1186/s13073-017-0406-4].

Laing EE, Möller-Levet CS, Poh N, Santhi N, Archer SN, Dijk DJ. Blood transcriptome based biomarkers for human circadian phase. Elife 6:e20214, 2017 [doi.org/10.7554/eLife.20214].

Wittenbrink N, Ananthasubramaniam B, Münch M, Koller B, Maier B, Weschke C, Bes F, de Zeeuw J, Nowozin C, Wahnschaffe A, Wisniewski S, Zaleska M, Bartok O, Ashwal-Fluss R, Lammert H, Herzel H, Hummel M, Kadener S, Kunz D, Kramer A. High-accuracy determination of internal circadian time from a single blood sample. J Clin Invest 128:3826-3839, 2018 [doi.org/10.1172/JCI120874]

Woelders T, Revell VL, Middelton B, Ackermann K, Kayser M, Raynaud FI, Skene DJ, Hut RA. Machine learning estimation of human body time using metabolomic profiling. Proc Natl Acad Sci USA 120:e2212658120, 2023 [doi.org/10.1073/pnas.2212685120]

Huang Y, Braun R. Platform-independent estimation of human physiological time from single blood samples. Proc Natl Acad Sci USA 121:e2308114120, 2024 [doi.org/10.1073/pnas.2308114120].

★2.47 On Some Suggested Reviews: Circadian Disruption and Disease

Here are a few reviews as starting points to enter the burgeoning literature on the association of circadian dysrhythmias and the possible etiopathogenesis of neuropsychiatric, cardiovascular, metabolic, neoplastic, and immune disorders:

Fishbein AB, Knutson KL, Zee PC. Circadian disruption and human health. J Clin Invest 131:e148286, 2021 [doi.org/10.1172/JCI148286].

Colwell CS. Defining circadian disruption in neurodegenerative disorders. J Clin Invest 131:e148288, 2021 [doi.org/10.1172/JCI148288].

Fekry B, Eckel-Mahan K. The circadian clock and cancer: links between circadian disruption and disease pathology. J Biochem 171:477-486, 2022 [doi.org/10.1093/jb/mvac017].

Schrader LA, Ronnekleiv-Kelly SM, Hogenesch JB, Bradfield CA, Malecki KMC. Circadian disruption, clock genes, and metabolic health. J Clin Invest 134:e170998, 2024 [doi.org/10.1172/JCI170998].

★2.48 On Slow Wave Activity as a Marker of Sleep Homeostasis

Electroencephalographic slow wave activity (SWA, oscillations in the delta band 0.5 – 4 Hz) is believed to reflect the homeostatic sleep regulatory process ("sleep need"), as SWA increases with the duration of wakefulness and decreases with daytime naps and over the course of sleep (Borbély, 1982; Daan et al., 1984; Dijk et al., 1990, 1995; Werth et al., 1996). The rise and fall of the process during episodes of sleep deprivation has been mathematically modeled (see Fig. 17); advanced analyses are complicated by variation in local SWA time courses across cortical regions and with chronic sleep restriction over multiple nights.

For a review of the two process (circadian + homeostatic) model of sleep regulation, see:
Borbély A. The two-process model of sleep regulation: Beginnings and outlook. J Sleep Res 31:e13598, 2022 [doi.org/10.1111/jsr.13598].

Borbély AA. A two process model of sleep regulation. Human Neurobiol 1:195-204, 1982 [PMID: 7185792].

Daan S, Beersma DG, Borbély A. Timing of human sleep: recovery process gated by a circadian pacemaker. Am J Physiol 246:R161-183, 1984 [doi.org/10.1152/ajpregu.1984.246.2.R161].

Dijk D-J, Brunner DP, Borbély A. Time course of EEG power density during long sleep in humans. Am J Physiol 258:R650-661, 1990 [doi.org/10.1152/ajpregu.1990.258.3.R650].

Dijk D-J. EEG slow waves and sleep spindles: windows on the sleeping brain. Behav Brain Res 69:109-116, 1995 [doi.org/10.1016/0166-4328(95)00007-g].

Werth E, Dijk D-J, Achermann P, Borbély AA. Dynamics of the sleep EEG after an early evening nap: experimental data and simulations. Am J Physiol 271:R501-510, 1996 [doi.org/10.1152/ajpregu.1996.271.3.R501].

★2.49 On the Influence of a Running Wheel on Murine Sleep

The sleep-wake circadian rhythm of C57BL/6 male mice is altered by the provision of a running wheel; with the wheel locked, total wake time decreases and non-REM and REM sleep time increases by about 11%, without a change in the amplitude of the drinking rhythm. However, the τ-shortening effect of running in the wheel (see ★2.7 in this chapter) impacts both sleep-wake and drinking rhythms equally.

Edgar DM, Kilduff TS, Martin CE, Dement WC. Influence of running wheel activity on free-running sleep/wake and drinking circadian rhythms in mice. Physiol Behav 50:373-378, 1991 [doi.org/10.1016/0031-9384(91)90080-8].

★2.50 On the Value and Limits of the Circadian "Clock" Metaphor

See:

Daan S, Hut RA. Circadian clock, program, oscillator, pacemaker, synchroniser?: On the language of biological rhythms research. In: Circadian Clocks (Honma K, Honma S, eds), Hokkaido University Press, 2016, pp. 21-32.

Aviram R, Manella G. A metaphor that keeps on ticking: the "clock" as a driving force in the history of chronobiology research. Philos Theor Pract Biol 12:7, 2020 [doi.org/10.3998/ptpbio.16039257.0012.006].

★ 3.1 On the Innateness of the Circadian Timekeeping Mechanism

Even before the hereditary basis for the circadian clock was demonstrated, its inborn nature had already been realized; and, of course, the study of leaf movements led the way. Richard Semon (1859 – 1918), who went on to consider the nature of memories (he coined the term "engram"), published data on young beansprouts (*Phaseolus*) that had never seen any light, were then exposed to light-dark cycles of 12, 24 or 48 h, and afterwards returned to constant conditions. The plants expressed only a circa 24-h period (Semon, 1905). And in 1928, the Dutch botanist Anthonia Kleinhoonte (1887 – 1960) published her work on jack bean plants (*Canavalia ensiformis*), demonstrating that leaf movements exhibited a circa-24 h rhythm in constant darkness even if the seedlings were first maintained in an 8 h: 8 h light-dark cycle (Kleinhoonte, 1928). She also reported that brief light pulses – as short as one minute in duration – could reset rhythm phase depending on the phase of administration of the light!

Semon R. Über die Erblichkeit der Tagesperiode. Biologisches Centralblatt 25:241-252, 1905.

Kleinhoonte A. De door het licht geregelde autonome bewegingen der Canavalia-bladeren. Meinema, Delft, 142 pp, 1928.

★ 3.2 On Switching the Temporal Niche

Some microtine rodents are nocturnal in summer and diurnal in winter, and this shift can be induced in the laboratory by changing photoperiod alone (for example, in the montane vole [*Microtus montanus*]; Rowsemitt et al., 1982). Such temporal niche switching has been proposed as a seasonal strategy for adapting to energetic challenges (e.g., cold, hunger), and the mechanism appears to lie downstream of the SCN, as assayed by the unchanging phase of SCN clock gene expression (Van der Vinne et al., 2015). Conservation of SCN phase is also a feature of animals that are naturally diurnal or nocturnal in their locomotor activity rhythms or that switch their rhythm phase when provided with a running wheel (for review, see Smale et al., 2003).

Rowsemitt CN, Petterborg LJ, Claypool LE, Hoppensteadt FC, Negus NC, Berge PJ. Photoperiodic induction of diurnal locomotor activity in Microtus montanus, the montane vole. Can J Zool 60:2798-2803, 1982 [doi.org/10.1139/z82-358].

Van der Vinne V, Riede SJ, Gorter JA, Eijer WG, Sellix MT, Menaker M, Daan S, Pilorz V, Hut RA. Cold and hunger induce diurnality in a nocturnal mammal. Proc Natl Acad Sci USA 111:15256-15260, 2014 [doi.org/10.1073/pnas.1413135111].

Smale L, Lee T, Nunez AA. Mammalian diurnality: some facts and gaps. J Biol Rhythms 18:356-366, 2003 [doi.org/10.1177/0748730403256651].

★ 3.3 On Variation of the Critical Photoperiod

The critical photoperiod is the light phase duration required for a biological response to occur (e.g., flowering in plants, reproductive status in animals) and is influenced by a number of factors. For example, horses are certainly large mammals, but breeding occurs mostly in spring rather than autumn, given their very long gestation time of about 11 months (Bos and Van der Mey, 1980). Differences in the steepness of a population's photoperiodic response curve – the slope at the inflection point – may reflect individual variation in photoperiodic sensitivity. A shallower curve suggests that there may be a role for other environmental features that are less predictable from year to year, like food availability and ambient temperature, to shape the photoperiodic response to optimally time seasonal responses (Van Rosmalen and Hut, 2021). Of note, annual rhythms of both light and temperature vary with differences in latitude, and critical photoperiod within a species is known to vary with distance from the equator (for review, see Hut et al., 2013). Importantly, the critical photoperiod must also depend on photoperiodic history. In nature, each daylength (except at the solstices) occurs twice per year and thus could signal either upcoming winter or upcoming summer, depending on the trajectory of preceding daylengths (Gorman and Zucker, 1995; Butler et al., 2007).

Bos H, Van der Mey GJW. Length of gestation periods of horses and ponies belonging to different breeds. Livestock Prod Sci 7:181-187, 1980 [doi.org/10.1016/0301-6226(80)90105-0].

Van Rosmalen L, Hut RA. Food and temperature change photoperiodic responses in two vole species. J Exp Biol 224:jeb243030, 2021 [doi.org/10.1242/jeb.243030].

Hut RA, Paolucci S, Dor R, Kyriacou CP, Daan S. Latitudinal clines: an evolutionary view on biological rhythms. Proc Biol Sci 280:20130433, 2013 [doi.org/10.1098/rspb.2013.0433].

Gorman MR, Zucker I. Seasonal adaptations of Siberian hamsters. II. Pattern of change in daylength controls annual testicular and body weight rhythms. Biol Reprod 53:116-125, 1995 [doi.org/10.1095/biolreprod53.1.116].

Butler MP, Turner KW, Park JH, Butler JP, Trumbull JJ, Dunn SP, Villa P, Zucker I. Simulated natural day lengths synchronize seasonal rhythms of asynchronously born male Siberian hamsters. Am J Physiol 293:R402-R412, 2007 [doi.org/10.1152/ajpregu.00146.2007].

★ 3.4 On Photoperiodic and Circannual Timers

Short-lived seasonal species (for example, the Siberian [Djungarian] hamster [*Phodopus sungorus*]) generally don't survive for more than one breeding season, so laboratory research has focused on their reproductive activation with the lengthening of the photoperiod that naturally occurs from winter to spring. However, even if the animals are maintained on the short (simulated winter) photoperiod, reproductive activation will still occur after an interval of weeks to months (that is, the animals become refractory to the short-photoperiod inhibitory condition). Once they become reproductively active, though, they do not undergo any further changes in their physiological state. On the other hand, long-lived animals that breed repeatedly over two or more years will undergo continued circannual cycles when maintained on a daily light-dark cycle of a fixed photoperiod.

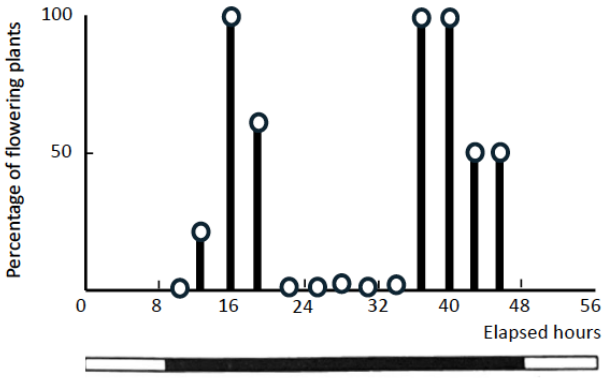
For reviews of the properties of photoperiodic and circannual timing schemes, see:

Paul MJ, Zucker I, Schwartz WJ. Tracking the seasons: the internal calendars of vertebrates. Phil Trans R Soc B 363:341-361, 2008 [doi.org/10.1098/rstb.2007.2143].

Lincoln G. A brief history of circannual time. J Neuroendocrinol 31:e12694, 2019 [doi.org/10.1111/jne.12694].

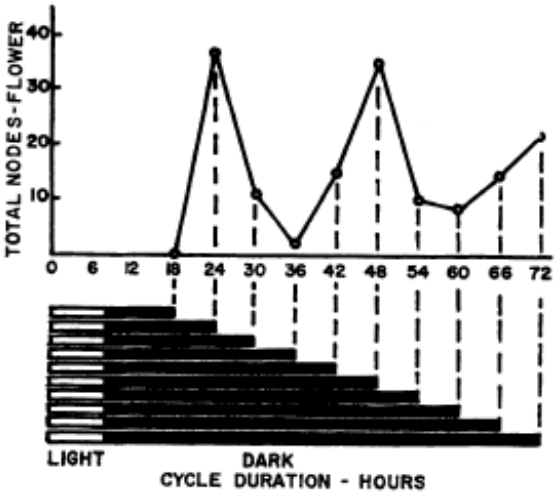
★ 3.5 On the Bünsow and Hamner (Nanda-Hamner) Protocols

It was Bünning (1936) who put forward the hypothesis that photoperiodic (seasonal) time measurement might involve an underlying circadian mechanism. Over the next decades supportive evidence was gathered, based largely on two experimental protocols that were initially tested in seasonally-flowering plants. Using a short photoperiod with an extended dark phase that was interrupted by brief light pulses delivered at different times, Robert C. Bünsow (1953) and others could inhibit or stimulate the flowering of short-day or long-day plants, respectively, if the light pulses were delivered 24 h apart.



Black henbane (Hyoscyamus niger), a long-day flowering plant, is induced to flower if exposed to 2 h light pulses (timing indicated by open circles) across the extended dark phase of a 9 h: 39 h light-dark cycle; controls not exposed to light pulses do not flower. Sensitive phases of maximum flowering are 24 h apart.
Drawn from the original data in: Claes H, Lang A. Die Blütenbildung von Hyoscyamus niger in 48-stündigen Licht-Dunkel-Zyklen und in Zyklen mit aufgeteilten Lichtphasen. Z Naturforsch 2b:56–63, 1947 [doi.org/10.1515/znb-1947-1-214]; under Creative Commons Attribution License 3.0. See the data also graphed as Fig. 100 in: Bünning E. Die Physiologische Uhr. Springer-Verlag, Berlin, 1958.

Karl C. Hamner and his students, most prolifically with K.K. Nanda (Nanda and Hamner, 1958), designed a "resonance" protocol, with light-dark cycles ranging from 18 to 72 h, all typically with an 8 h light phase but extended dark phases. Under these conditions, flowering of a short-day plant could be stimulated if the period of the light-dark cycle (**T**) was a multiple of 24 h (see example below).



Biloxi soybean (Glycine max), a short-day flowering plant, is induced to flower under light-dark cycles with periods of 24, 48, and 72 h, all with an 8 h light phase but extended dark phases. By permission, from Fig. 1 in: Hamner KC. Photoperiodism and circadian rhythms. Cold Spring Harb Symp Quant Biol 25:269-277, 1960 [doi.org/10.1101/SQB.1960.025.01.029].

Following these early studies, Bünsow and Nanda-Hamner protocols have since been applied to assess the circadian contribution to seasonal adaptation in a number of animals, especially for the regulation of winter diapause in insects (see Meuti and Denlinger, 2013).

Bünning E. Die endonome Tagershythmik als Grundlage der photoperiodischen Reaktion. Ber Deut Bot Ges 54:590-607, 1936 [doi.org/10.1111/j.1438-8677.1937.tb01941.x].

Bünsow RC. Über Tages- und Jahresrhythmische Anderugen der Photoperiodischen Lichteropfindlischkeit bie Kalanchoe blossfeldiana und ihre Beziehugen zuer endogogen Tagesrhythmik. Z Bot 41:257-276, 1953.

Nanda KK, Hamner KC. Studies on the nature of the endogenous rhythm affecting photoperiodic response of Biloxi soybean. Bot Gaz 120:14-25, 1958 [doi.org/10.1086/335992].

Meuti ME, Denlinger DL. Evolutionary links between circadian clocks and photoperiodic diapause in insects. Integr Comp Biol 53:131-143, 2013 [doi.org/10.1093/icb/ict023].

★ 3.6 On the Photoperiodic Clockwork in the SCN (and *Drosophila* too)

The intrinsic SCN oscillation that is most obviously altered by ambient photoperiod is its rhythmic waveform. Under long photoperiods, the light phase and subjective day are characterized by expanded durations of elevated electrical activity; under short photoperiods, the durations are compressed. The underlying basis for the plasticity in overall waveform has been clarified by analyses of murine SCN rhythms at the cellular level, by recording electrical activity and imaging bioluminescent clock gene reporter expression. The data reveal that photoperiod is encoded by a spatiotemporal reconfiguration of the phases of individual oscillating SCN cells – dispersed dorsoventrally or rostrocaudally during long photoperiods and more narrowly during short photoperiods – rather than by an expansion or compression of the rhythm duration of individual cells (for discussion and references, see Evans and Schwartz, 2024). In *Drosophila* too, discrete groups of circadian pacemaker neurons generate Ca^{2+} rhythms with altered phases under long and short photoperiods (Liang et al., 2016).

Evans JA, Schwartz WJ. On the origin and evolution of the dual oscillator model underlying the photoperiodic clockwork in the suprachiasmatic nucleus. J Comp Physiol A 210: 503-511, 2024 [doi.org/10.1007/s00359-023-01659-1].

Liang X, Holy TE, Taghert PH. Synchronous Drosophila circadian pacemakers display non-synchronous Ca^{2+} rhythms in vivo. Science 351:976-981, 2016 [doi.org/10.1126/science.aad3997].

★ 3.7 On the Photoperiodic Molecular Switch Regulating Pars Tuberalis TSHβ Expression

In Soay sheep (Dardente et al., 2010) and melatonin-proficient CBA/N mice (Masumoto et al., 2010), a molecular switch governing pars tuberalis *TSHβ* transcription has been described. Under long photoperiods, the melatonin signal engages the local circadian oscillator in the pars tuberalis, inducing the expression of coactivator eyes absent 3 (*eya3*); Eya3 interacts with the circadian transcription factor thyrotroph embryonic factor (TEF) on the D box response element of the *TSH*β promoter to activate *TSH*β transcription in pars tuberalis cells. Notably, Eya3 forms a positive feedback by reinforcing its own transcription, thereby creating a molecular switch that augments transcrip-

tion with every circadian cycle (under long photoperiods, there is no suppression of *eya3* transcription, given the absence of melatonin in the morning [see Fig. 20]).

Dardente H, Wyse CA, Birnie MJ, Dupré SM, Loudon ASI, Lincoln GA, Hazlerigg DG. A molecular switch for photoperiod responsiveness in mammals. Curr Biol 20:2193-2198, 2010 [doi.org/10.1016/j.cub.2010.10.048].

Masumoto K, Ukai-Tadenuma M, Kasukawa T, Nagano M, Uno KD, Tsujino K, Horikawa K, Shigeyoshi Y, Ueda HR. Acute induction of Eya3 by late-night light stimulation triggers TSHβ expression in photoperiodism. Curr Biol 20:2199-2206, 2010 [doi.org/10.1016/j.cub.2010.11.038].

★ 3.8 On the Generation of Seasonal Reproductive Phenotypes

With regard to the seasonal regulation of reproduction, T3 does not act directly on gonadotropin-releasing hormone neurons but instead through distinct populations of neurons that express RFamide peptides (kisspeptins and RF-amide related peptides). Notably, these are expressed in hypothalamic nuclei involved in a range of metabolically-relevant functions, including food intake, fat mass regulation, and body temperature.

For review, see:

Henningsen JB, Gauer F, Simonneaux V. RFRP neurons – the doorway to understanding seasonal reproduction in mammals. Front Endocrinol 7:36, 2016 [doi.org/10.3389/fendo.2016.00036].

★ 3.9 On Hypothalamo-Pituitary Disconnected Sheep

Soay sheep have served as a key large-animal model for elaborating the preeminent role of the pars tuberalis in seasonal timekeeping. They can be bred under controlled lighting regimens, and their size allows for precise surgical procedures around the hypothalamus and pituitary gland and for multiple blood sampling, including from portal capillaries of the median eminence. In hypothalamo-pituitary disconnected Soay rams – in which the pituitary gland is surgically isolated from all hypothalamic neural inputs – a direct action of melatonin on the pars tuberalis could be demonstrated, as assayed by cyclic prolactin secretion from adjacent pars distalis lactotrophs and by the timing of the pelage (hair) molt (Lincoln et al., 2006).

Lincoln GA, Clarke IJ, Hut RA, Hazlerigg DG. Characterizing a mammalian circannual pacemaker. Science 134:1941-1944, 2006 [doi.org/10.1126/science.1132009].

★ 3.10 On Timing by the Moon

Some of the recent excitement about circalunar and circatidal clocks can be appreciated in recent reviews (Rock et al. 2022; Häfker et al., 2023; Ritter and Tessmar-Raible, 2024), a role for *bmal1* in circatidal rhythms of crustacean model organisms (Kwiatkowski et al., 2023; Zhang et al., 2023), and natural observations in humans (Casiraghi et al., 2021; Helfrich-Förster et al., 2021; Wehr and Helfrich-Förster, 2021).

Rock A, Wilcockson D, Last KS. Towards an understanding of circatidal clocks. Front Physiol 13:830107, 2022 [doi.org/10.3389/fphys.2022.830107].

Häfker NS, Andreatta G, Manzotti A, Falciatore A, Raible F, Tessmar-Raible K. Rhythms and clocks in marine organisms. Ann Rev Mar Sci 15:509-538, 2023 [doi.org/10.1146/annurev-marine-030422-113038].

Ritter A, Tessmar-Raible T. Time me by the moon: The evolution and function of lunar timing systems. EMBO reports 25:3169-3176, 2024 [doi.org/10.1038/s44319-024-00196-5].

Kwiatkoski ER, Schnytzer Y, Rosenthal JJC, Emery P. Behavioral circatidal rhythms require Bmal1 in Parhyale hawaiensis. Curr Biol 33:1867-1882, 2023 [doi.org/10.1016/j.cub.2023.03.015].

Zhang L, Green EW, Webster SG, Hastings MH, Wilcockson DC, Kyriacou CP. The circadian clock gene bmal1 is necessary for co-ordinated circatidal rhythms in the marine isopod Eurydice pulchra (Leach). PLoS Genet 19: e1011011, 2023 [doi.org/10.1371/journal.pgen.1011011].

Casiraghi L, Spiousas I, Dunster GP, McGlothlen K, Fernández-Duque E, Valeggia C, de la Iglesia HO. Moonstruck sleep: Synchronization of human sleep with the moon cycle under field conditions. Sci Adv 7:eabe0465, 2021 [doi.org/10.1126/sciadv.abe0465].

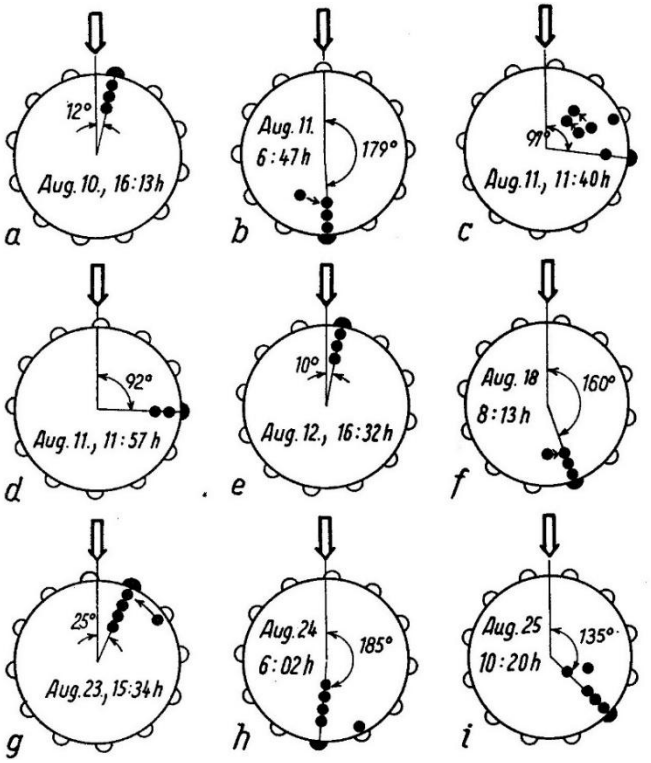
Helfrich-Förster C, Monecke S, Spiousas I, Hovestadt T, Mitesser O, Wehr TA. Women temporarily synchronize their menstrual cycles with the luminance

and gravimetric cycles of the moon. Sci Adv 7:eabe1358, 2021 [doi.org/10.1126/sciadv.abe1358].

Wehr TA, Helfrich-Förster C. Longitudinal observations call into question the scientific consensus that humans are unaffected by lunar cycles. BioEssays 43:2100054, 2021 [doi.org/10.1002/bies.202100054].

★ 3.11 On Directional Orientation by a Time-Compensated Sun Compass

Gustav Kramer (1910 – 1959) observed that European starlings (*Sturnus vulgaris*) housed in an outdoor aviary became increasingly active ("restless") at the time of their usual spring migration, orienting themselves in a direction appropriate to the season. So inspired, he devised a circular cage and, by the ingenious use of mirrors that deflected the apparent position of the sun in the sky, re-directed the birds' orientation (Kramer, 1950). The obvious fact that the sun changes its position over time means that the birds must be able to compensate for that motion by an endogenous sense of the time of day. Further experiments by Kramer and others (Kramer, 1952; see Hoffmann, 1960) confirmed that the azimuth component of the sun's position was the relevant daytime visual clue and also that the innate sun compass was being compensated for the sun's predicted movement over time by an endogenous circadian mechanism. Thus, a shift of the light-dark cycle by 6 h re-oriented the birds' direction by 90°; while illumination of the cage by a stationary (fixed) artificial "sun" – but interpreted by the birds as a moving sun – rotated their directional orientation by about 15° every hour.



Tests with a starling trained to fly west under the natural sun show a changed directional orientation over time with reference to an artificial sun (open arrow). On the circumference of each circle are 12 feeders, with the feeder the bird was expected to select drawn solid. Each dot represents one choice; dots are joined by an arrow if the bird corrected its choice. By permission, from Fig. 2 in: Hoffmann K. Experimental manipulation of the orientational clock in birds. Cold Spring Harb Symp Quant Biol 25:379-387, 1960 [doi.org/10.1101/SQB.1960.025.01.040].

Kramer G. Weitere Analyse der Faktoren, welche die Zugaktivität des gekäfigten Vogels orlentieren. Naturwiss 37:377-378, 1950 [doi.org/10.1007/BF00626007].

Kramer G. Experiments on bird orientation. Ibis 94:265-285, 1952 [doi.org/10.1111/j.1474-919X.1952.tb01817.x].

★ 3.12 On Some Suggested Reviews: Monarch Migration

For reviews, see:

Merlin C, Iiams SE, Lugena AB. Monarch butterfly migration moving into the genetic era. Trends Genetics 36:689-701, 2020 [doi.org/10.1016/j.tig.2020.06.011].

Freedman MG, Kronforst MR. Migration genetics take flight: genetic and genomic insights into monarch butterfly migration. Curr Opin Insect Sci 59:101079, 2023 [doi.org/10.1016/j.cois.2023.101079].

★ 3.13 On Circadian Time-Place Learning

Time-place learning, also known as time-place discrimination or time-place association, serves to link pivotal events with their specific spatial locations and times of day. Mechanistic analyses have been complicated, because in addition to circadian-based memory "time stamps," there are other possible non-circadian mechanisms that can underlie this ability (such as recognizing contextual cues, memorizing sequences of events, or tracking short intervals of time) (for review, see Mulder et al., 2013). The first, and perhaps most famous, example of circadian time-place learning is the "time memory" (*Zeitgedächtnis*) in the foraging behavior of honeybees (Beling, 1929; Renner, 1960; Bloch et al., 2017) that involves a time-compensated sun compass (von Frisch and Lindauer, 1956). While circadian mechanisms are also involved in examples of mammalian time-place learning, the neurobiological basis remains uncertain (Mulder et al., 2014; Cole et al., 2016)

Mulder CK, Gerkema MP, Van der Zee EA. Circadian clocks and memory: time-place learning. Front Molec Neurosci 6:article 8, 2013 [doi.org/10.3389/fnmol.2013.00008].

Beling I. Über das Zeitgedächtnis der Bienen. Z Vergl Physiol 9:259-338, 1929 [doi.org/10.1007/BF00340159].

Renner M. The contribution of the honey bee to the study of time-sense and astronomical orientation. Cold Spring Harb Symp Quant Biol 25:361-367, 1960 [doi.org/10.1101/SQB.1960.025.01.037].

Bloch G, Bar-Shai N, Cytter Y, Green R. Time is honey: circadian clocks of bees and flowers and how their interactions may influence ecological communities. Phil Trans R Soc B 372:20160256, 2017 [doi.org/10.1098/rstb.2016.0256].

von Frisch K, Lindauer M. The "language" and orientation of the honey bee. Annu Rev Entomol 1:45–58, 1956 [doi.org/10.1146/annurev.en.01.010156.000401].

Mulder CK, Papantoniou C, Gerkema MP, Van der Zee EA. Neither the SCN nor the adrenals are required for circadian time-place learning in mice. Chronobiol Int 31:1075-1092, 2014 [doi.org/10.3109/07420528.2014.944975].

Cole E, Mistlberger RE, Merza D, Trigiani LJ, Madularu D, Simundic A, Mumby DG. Circadian time-place (or time-route) learning in rats with hippocampal lesions. Neurobiol Learning Memory 136:236-243, 2016 [doi.org/10.1016/j.nlm.2016.09.004].

★ 3.14 On Activity Around the Clock

For review, see:

Bloch G, Barnes BM, Gerkema MP, Helm B. Animal activity around the clock with no overt circadian rhythms: patterns, mechanisms and adaptive value. Proc R Soc B 280:20130019, 2013 [doi.org/10.1098/rspb.2013.0019].

★ 3.15 On the Adaptation of Svalbard Reindeer and Ptarmigan to the Arctic

See references:

Lu W, Meng Q-J, Tyler NJC, Stokkan K-A, Loudon ASI. A circadian clock is not required in an Arctic mammal. Curr Biol 20:533-537, 2010 [doi.org/10.1016/j.cub.2010.01.042].

Arnold W, Ruf T, Loe LE, Irvine RJ, Ropstad E, Veiberg V, Albon SD. Circadian rhythmicity persists through the polar night and midnight sun in Svalbard reindeer. Sci Rep 8:14466, 2018 [doi.org/10.1038/s41598-018-32778-4].

Reierth E, Stokkan K-A. Activity rhythm in high Arctic Svalbard ptarmigan (Lagopus mutus hyperboreus). Can J Zool 76:2031-2039, 1998 [doi.org/10.1139/z98-173].

Appenroth D, Wagner GC, Hazlerigg DG, West AC. Evidence for circadian-based photoperiodic timekeeping in Svalbard ptarmigan, the northernmost resident bird. Curr Biol 31:2720-2727, 2021 [doi.org/10.1016/j.cub.2021.04.009].

Figure 19: References and photographic acknowledgements

Data from:
Squirrel: Pengelley ET, Asmundson SJ, Barnes B, Aloia RC. Relationship of light intensity and photoperiod to circannual rhythmicity in the hibernating ground squirrel, *Citellus lateralis*. *Comp Biochem Physiol* 53:273-277, 1976 *[doi.org/10.1016/S0300-9629(76)80035-7]*.
Great knot: Piersma T, Brugge M, Spaans B, Battley PF. Endogenous circannual rhythmicity in body mass, molt, and plumage of great knots *(Calidris tenuirostris)*. *The Auk* 125:140-148, 2008 *[doi.org/10.1525/auk.2008.125.1.140]*.
Sheep: Karsch FJ, Robinson JE, Woodfill CJI, Brown MB. Circannual cycles of luteinizing hormone and prolactin secretion in ewes during prolonged exposure to a fixed photoperiod: Evidence for an endogenous reproductive rhythm. *Biol Reprod* 41:1034-1046, 1989 *[doi.org/10.1095/biolreprod41.6.1034]*.
***Alexandrium spp.*:** Matrai P, Thompson B, Keller M. Circannual excystment of resting cysts of *Alexandrium spp.* from eastern Gulf of Mexico populations. *Deep Sea Res* 52:2560-2568, 2005 *[doi.org/10.1016/j.dsr2.2005.06.013]*.

Photo credits:
Squirrel: © Frank Schulenburg / CC BY-SA 4.0, https://commons.wikimedia.org/wiki/File:*Callospermophilus_lateralis*_near_Lake_Almanor.jpg.
Great knot: Sunphlo, South Australia - *Calidris tenuirostris* / Great Knot, CC BY-SA 2.0, https://commons.wikimedia.org/w/index.php?curid=36492740.
Sheep: Saruman - Own work, Public Domain, https://commons.wikimedia.org/w/index.php?curid=2839618.
Alexandrium: A. minutum by Philippe Garcelon, https://www.flickr.com/photos/78178083@N05/51841087009/, CC BY 2.0, https://commons.wikimedia.org/w/index.php?curid=139183910

ABOUT THE AUTHORS



Roelof A. Hut (left) is Professor of Chronobiology at the University of Groningen. He was trained as a chronobiologist and received his Ph.D. (2001) in the laboratory of Serge Daan at the University of Groningen. Post-doctoral fellowships were conducted in the laboratories of Howard Cooper (Lyon), Michael Menaker (Charlottesville, Virginia), and in Groningen. His scientific work has covered many aspects of chronobiology, including circadian light responses, suprachiasmatic nucleus physiology, evolutionary ecology of circadian organization, photoreception, thermoregulation, sleep, and photoperiodism, using species like insects, various nocturnal and diurnal rodent species, and humans. He is also a founding member and current chair of the International Hibernation Society, where his early fascination with hibernation is combined with his interest in physiology and biological rhythms. Being trained by prominent early scientists in chronobiology, who were themselves trained by the "founding fathers" of the field, has afforded Dr. Hut a wide perspective on all aspects of chronobiology, with a focus to the future and a respect for the past.

William J. Schwartz (right) is a Distinguished Teaching Professor of The University of Texas System, in the Department of Neurology, Dell Medical School, The University of Texas at Austin; and a Visiting Professor of Chronobiology at the University of Groningen. He received his M.D. (1974) and training in clinical neurology at the University of California, San Francisco and post-doctoral work in neurophysiology and cerebral metabolism at the National Institute of Mental Health (Bethesda, Maryland). For over 40 years, his scientific work has focused on the neural regulation of mammalian circadian rhythms. He was elected Chair of the Gordon Research Conference on Chronobiology (1993) and President of the Society for Research on Biological Rhythms (SRBR) (2004-2006) and served a 6-year term as Editor-in-Chief of the *Journal of Biological Rhythms* (2014-2019). Honors include Boerhaave Professor (Leiden, 2005), Baerends Visiting Chair (Groningen, 2008), 9th Pittendrigh-Aschoff Lecturer (SRBR, 2014), Clark Way Harrison Visiting Professor (Washington Univ. St. Louis, 2016), and 5th Johannes Ariëns Kappers Lecturer (European Biological Rhythms Society, 2017), among other distinctions.

Our increasing knowledge of circadian clocks and rhythms is fueling exciting applications in agriculture, wildlife conservation, architecture and lighting design, and human lifestyles and treatments for disease. And yet, newcomers to the field may not be familiar with its basic concepts, technical language, and data visualization. To fill this gap, Roelof A. Hut and William J. Schwartz have composed an introductory Road Map, as a first and engaging step towards understanding the essential principles and practice of circadian biology. Three expanded chapters, along with 20 original figures, build on one another, and supplementary (bonus) material is included to support the main text without interrupting its flow. The result is an accessible launching pad for those wishing to acquire a basic and practical knowledge of the field and its expanding literature.



University of Groningen Press