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Modelling Biological Rhythms

Review

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With our growing awareness of the complexity underlying biological phenomena, our need for computational models becomes increasingly apparent. Due to their properties, biological clocks have always lent themselves to computational modelling. Their capacity to oscillate without dampening — even when deprived of all rhythmic environmental information — required the hypothesis of an endogenous oscillator. The notion of a 'clock' provided a conceptual model of this system well before the dynamics of circadian oscillators were probed by computational modelling. With growing insight into the molecular basis of circadian rhythmicity, computational models became more concrete and quantitative. Here, we review the history of modelling circadian oscillators and establish a taxonomy of the modelling world to put the large body of circadian modelling literature into context. Finally, we assess the predictive power of circadian modelling and its success in creating new hypotheses.

Introduction

Some biological experimentalists claim that they can get by without modelling, and furthermore, that modelling is often unhelpful — even counterproductive, rarely contributing to the understanding of a scientific problem. The aim of this review is to constructively contradict this prejudice by demonstrating that modelling is more than solving differential equations on a computer and adapting them to fit the observed results. Biological oscillators have always lent themselves to modelling and their dynamic behaviour — as single oscillators or interacting with others — has been modelled mathematically [1] well before biologists started to scrutinise the mechanisms underlying biological rhythms. In this review, we focus on circadian rhythms but most of what we present could just as well concern the modelling of other biological oscillators with both shorter (ultradian) or longer (infradian) periods.

The world of modelling is rich in methods and approaches, and since 1960, more than 600 modelling papers have been published alone in the circadian field. Models can be as simple as a diagram or as complex as hundreds of equations that can only be solved by computers. In this review, we will try to make the modelling landscape more accessible by creating a taxonomy of the different approaches. Within this taxonomy, some modelling methods are applied frequently while other modelling approaches await further testing.

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A Taxonomy of Modelling Approaches

Every experiment is based on a question, and a hypothesis that should be verified or falsified. Hypotheses are to some extent already implicit models of the investigated phenomena. Yet, implicit modelling already starts at an even lower level, specifically in the language that we use to describe a phenomenon, to formulate a scientific question, or to explain methods and results. Implicit models and concepts are therefore prevalent in all research, continually influencing its outcome. The fact that the circadian system was labelled a 'clock' already in the 18th century [2] is an example of a concept of the circadian system which has profoundly influenced subsequent research.

Explicit, non-computational modelling is also part of most scientific endeavours, for instance when scientists summarise their findings in diagrams. Compared to the frequent use of diagrammatic models, explicit computational modelling is much rarer. The first bifurcation of the taxonomy for explicit modelling distinguishes between 'conceptual' and 'contextual' (Figure 1). The former deals with concepts without placing the respective model into a defined context (as the latter does). The mentioned concept of a 'clock' belongs to an abstract class of conceptual models. It invokes different images in different individuals and in different eras. Some people see a grandfather's clock, others a wristwatch, some think of a mechanical clockwork and others of a timer based on counting atomic events. All of these different clock images have common denominators: The hands of clocks move with the same pace over the course of the day and their task is to represent time as reliably as possible. Clocks are reference devices for the passing of time that can be consulted in order to take an appropriate action at the appropriate time. All these notions were evoked with the introduction of the word 'clock' and have strongly influenced our concepts, experimental approaches and interpretations of results in circadian research. This is especially apparent when researchers investigate phenomena that require 'reading' the correct time, such as in the dance language of bees [3], the orientation of migrating birds [4,5] or the timing of night length in seasonal measurements [6]. Temperature independence was important for accuracy in early mechanical clocks and was accordingly defined as one of the basic properties in their circadian counterparts [7]. Modelling so-called 'temperature compensation' has been an aim of clock models early on (e.g., [8–15]).

While the concept of a 'clock' is abstract, explicit models always have some concrete basis. Conceptual models follow a top-down approach, starting with a phenotype they try to simulate as accurately as possible. Components are introduced that interact with each other to simulate the observed phenomenon without specifically aiming for a given context, such as a molecular circadian machinery of a cell or a network of neurons. It is irrelevant how many components are introduced and how they interact, as long as they simulate the observed properties. Thus, circadian models should show the following set of properties [16]: first, they have to

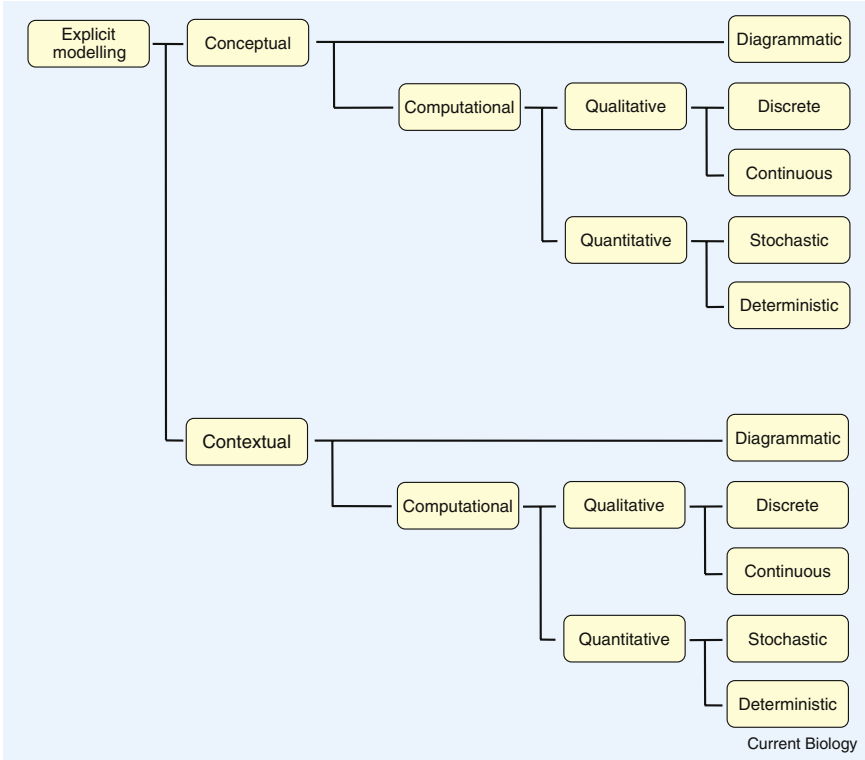

Figure 1. Taxonomy of the modelling world.
Explicit models can either be conceptual (e.g., pertaining to the circadian clock in general, irrespective of the organism) or contextual (i.e., focussing on the specificities of a given organism, tissue or cell). Both can be simply described by a diagram (diagrammatic) or are implemented with a set of equations which can be solved by a computer programme, giving rise to computational models which can be sub-divided into further sub-classes (see text for details).

be rhythmic; second, they have to show a circadian period (although time is relative in computational models as it doesn't take 24 hours to simulate one day); third, the rhythm's amplitude should be strong enough to drive outputs; fourth, the rhythm's amplitude should not damp in constant conditions; fifth, the rhythm's period should be temperature-compensated; and sixth, the rhythm's period should entrain to external rhythms (*zeitgeber*, German for 'time giver').

In contrast to conceptual models, contextual models aim at specific implementations of circadian rhythms in a defined context — a given organism, tissue or cell. The sub-divisions of both conceptual and contextual models are identical (Figure 1). Both can produce non-computational models, i.e., diagrams. In most circadian papers, from the molecular to the anatomical level, results are summarised in a *diagrammatic* model, depicting the presumed components and their interactions — negative, positive, feed-forward, feedback, etc. The step from a *diagrammatic* to a *computational* model is both small and big. Translation of the diagrammatic flow into equations is a small, though laborious step. The big step is that the model brings time to the diagram. Diagrammatic models hypothesise components, their connections and their relationships. In computational models, their dynamic changes (states, rates, concentrations, etc.) can be 'monitored', and thereby the diagram's predictions can be tested, and sometimes new predictions emerge.

Computational models can either be quantitative or qualitative. Quantitative (or dynamic) models consider time as one of their most important qualities and are therefore very common in circadian research. If a component's waxing and waning concentrations are computed, the model is *deterministic*. In this case, the fate of all the molecules of a component (e.g., all the proteins expressed from a single (clock) gene) is presumed to be identical. Stochastic models are typically used for systems where events occur probabilistically and randomness is present. In stochastic models, variable states are described by probabilistic distributions rather than by unique values. In circadian research, they would, for example, consider each clock molecule (e.g., each mammalian PER protein) or each neuron in the mammalian master clock, the suprachiasmatic nucleus (SCN) — even if they are identical — as an individual entity interacting with others under given probabilities and/or given noise levels. Until recently, most models in circadian research have been deterministic; stochastic models are, however, more realistic [17].

Qualitative models are less concerned with time than with whether and how the components of a system are interacting. Cellular expression systems, which have been very successful in determining the action of and the interaction between clock proteins [18], can be regarded as experimental implementation of a qualitative model. If a component's concentration changes continuously, the model is *continuous* while it is *discrete* if components change from one state to another, e.g., if they are activated by phosphorylation or move from the cytosol to the nucleus.

As always, the distinction between classes can blur as components of a model sometimes can be described in more than one category. Some elements may be continuous (concentrations), others discrete (phosphorylation or nuclear entry). Some, predominantly deterministic models may include stochastic processes and/or noise. Models which combine different methods are usually called 'hybrid' in the modelling community and are quite popular in engineering.

Modelling Clocks

Modelling clock mechanisms and circadian behaviour has been an active part of rhythms research since early on and excellent publications have reviewed the circadian modelling landscape [17,19–22]. The proceedings of the famous and influential Cold Spring Harbor conference in 1960 already contained a section dealing with concepts and models [23–29], including Pittendrigh's milestone paper summarising the "*empirical generalizations about circadian rhythms*". His paper also introduces the concept of two interacting oscillators, prompted by the observation that the free-running eclosion rhythm in *Drosophila* shows very different transients after perturbations by either light or temperature [30].

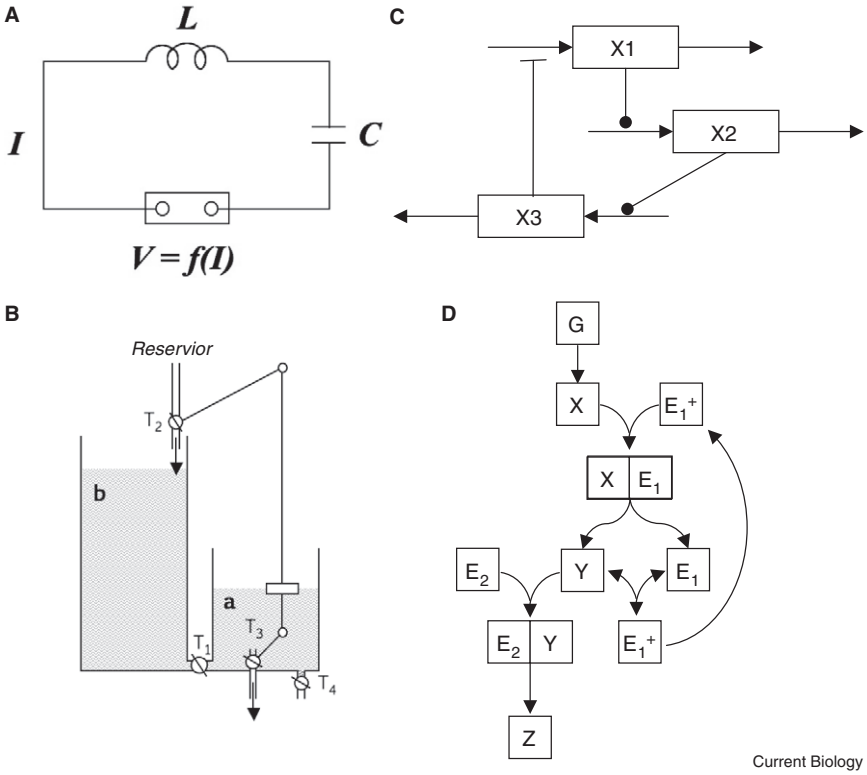

Figure 2. Four examples of oscillator models.
(A) In 1920, van der Pol constructed an oscillator based on an electronic circuit consisting a capacitor (C), an inductor (L), a DC voltage supply (E_0) and a vacuum tube containing a tetrode [1] (redrawn with permission from [106]). (B) A hydraulic oscillator was suggested by Kalmus and Wiggleworth at the Cold Spring Harbor meeting in 1960 as a metaphor for the circadian clock [24]. Water from a reservoir flows into chamber *b* and then via an adjustable (T_1) connection into chamber *a* and out through another adjustable tap (T_4). The water level of chamber *a* is connected to the taps T_2 and T_3, with opposite effects: rising levels in chamber *a* will close the inflow and open the outflow forming a negative feedback loop (redrawn with permission from [24], Copyright Cold Spring Harbor Laboratory Press). (C) The first biochemical, negative-feedback oscillator was constructed by Goodwin in 1965 [40] involving three components. The concentration of each component is controlled by a production and a degradation rate. The production of X2 is positively influenced by the concentration of X1 as is the production of X3 by X2, while the production of X1 is inhibited by X3 (redrawn with permission from [40]). (D) A more complex biochemical feedback oscillator was described by Pavlidis in 1969 [41] involving a series of enzymatic reactions whereby X is a substrate and Y an activator. E_1 and E_2 are enzymes, of which the former exists in an active (E_1^+) and an inactive state (E_1). The first reaction converts X to Y and the active form of E_1 to its inactive form which can be reactivated by Y. Y is 'flushed' out of the system either by being converted to Z by the enzyme E_2 or by reactivation of E_1 to E_1^+. This set of reactions has also been used to describe the glycolytic oscillator, whereby G corresponds to glucose, X to fructose-diphosphate, E_1 to phosphofructokinase, E_2 to adolase, and Y to ADP [42,43] (redrawn with permission from [41]).

A Genealogy of Clock Models

Sets of equations, put together to simulate oscillations, are often associated with a realistic metaphor or a device that one could build to function as an oscillator. Figure 2 shows four examples of basic oscillator models. The van der Pol oscillator — the oldest among them — is based on an electronic circuit (Figure 2A) [1], and it is the least intuitive oscillator model for biologists. Unlike other models, its interactions, functions and parameters have no correspondence to biological substrates. The van der Pol oscillator does, however, produce rhythms with all the typical qualities of circadian clocks, including their limited range of entrainment [31] and the fact that they can entrain to *zeitgeber* cycles twice as fast as their endogenous frequency by skipping every other cycle, a phenomenon called 'frequency demultiplication' [32]. In spite of its rather technical origin, this model has been widely used to simulate circadian behaviour.

Most of the modelling work by Kronauer and coworkers [33–36] probing the human circadian clock is based on one or several van der Pol oscillators. These have also been employed to model the concerted action of SCN neurons in the creation of a robust circadian output [37] as well as the circadian behaviour in different mutants of the *frequency* gene in the *Neurospora* clock [38]. Because the van der Pol oscillator has no biological correlates, its models must be viewed as primarily conceptual even if they simulate clocks in humans or *Neurospora*. The only contextual van der Pol models are those which explore oscillator networks by using this oscillator as a 'black box' to represent, for example, the oscillation of individual SCN neurons (e.g., [37]).

A hydraulic model was suggested by Kalmus and Wiggleworth at the Cold Spring Harbor meeting in 1960 (Figure 2B) [24]. Although it has — unlike the other three models shown in Figure 2 — not been used in subsequent endeavours of circadian modelling, we include this model because it is highly intuitive. It is a simple negative feedback loop. The chambers *b* and *a* could represent RNA and protein of a molecular negative feedback loop, which do not necessarily have to oscillate with a circadian period [39]. The higher the levels in *a* (for instance, concentration of the clock protein), the more it shuts down the inflow into chamber *b* (for instance, transcription of the clock RNA). Its basic principle of flow, connectivity and feedback is inherent also in the two biochemical models, the Goodwin oscillator (Figure 2C) [40], which connects three components (with specific production and degradation rates) into a negative feedback loop, and the Pavlidis oscillator (Figure 2D) [41], which consists of a series of 'enzymatic' reactions (its equations have also been used to simulate short period mitochondrial oscillations [42,43]).

The Goodwin oscillator (or its derivatives) (Figure 2C) is by far the most widely used in circadian modelling, in both single and multiple oscillator models. Its deterministic equations are commonly used in clock models, both conceptual and contextual, that try to simulate feedback loops on the molecular level (e.g., [44–47]). The Pavlidis (Figure 2D) oscillator was mainly applied, by Pavlidis himself, to model formal aspects of the circadian system (e.g., [41]). More recently, two Pavlidis oscillators were used to simulate the presumed Morning and Evening oscillators in the *tau* mutant hamster

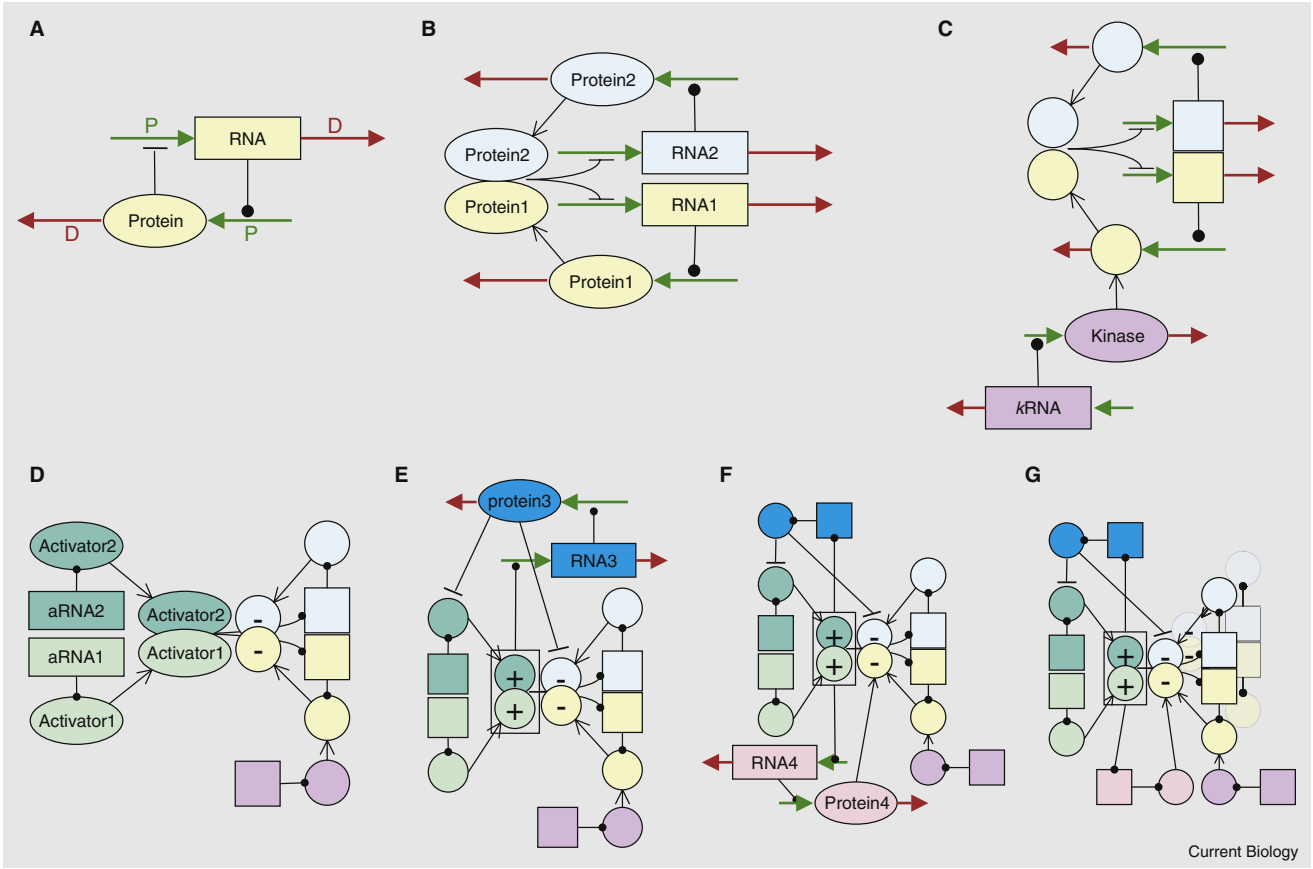

Figure 3. The history of diagrammatic clock models.

RNAs are represented in rectangles or squares, proteins in ovals or circles. With growing complexity of the models, details such as production and degradation of the individual components are omitted. (A) A simple version of the historic feedback in *Drosophila* (the original one incorporated a lag and putative intermediates [55]). (B) Inhibiting clock proteins act as a heterodimer (e.g., *per* and *tim* or *per* and *cry*). (C) At least one of them is modified by a kinase (e.g., Double-time or casein kinase 1ε). (D) The activators (e.g., Clock and Cycle or Clock and Bmal1) also function as a heterodimer. (E,F) additional loops are discovered in the mammalian system (e.g. Vrille, Rev-Erbα or Dec). (G) The negative components come in pairs (e.g., Per1/Per2 and Cry1/Cry2).

[48]. Pittendrigh, who was for some time Pavlidis' colleague at Princeton, combined two of the basic oscillators shown in Figure 2 to simulate a system that was very successful in describing the systematic differences of *Drosophila*'s eclosion rhythm at various latitudes [27,30,49–52]. The Pavlidis oscillator represented the pacemaker (with a self-sustained, non-damped oscillation) and the van der Pol oscillator simulated the 'slave oscillator', which would be damped without input from the pacemaker.

When translated into equations, the oscillators shown in Figure 2 produce deterministic models. Experimental biologists are sometimes alienated by the multitude of maths in model papers, yet all they represent is a detailed translation of all the model's inherent steps into differential equations which describe the changes in all factors over time.

A Diagrammatic Explosion

The true driving force of circadian modelling at the molecular level is diagrammatic (Figure 3). The discovery of the first clock genes, in *Drosophila* and *Neurospora*, respectively, [53,54] and the experimental results describing a time lag between the rhythms of RNA and protein expression led to the model of a transcriptional-translational negative feedback loop being responsible for generating the circadian oscillation at the cellular level [55] (Figure 3A). This initial model was basically a two-component Goodwin oscillator (Figure 2C), which does not mean that Goodwin could have predicted the transcriptional-translational feedback with his model — too little was known about genes and their regulation in the 1960s. Over the years, many components were added to the initial feedback loop in *Drosophila*. Similar feedback loops were found in very different organisms, from cyanobacteria and fungi to birds and mammals. The number of components in the single feedback loop doubled (Figure 3B), kinases were added (Figure 3C), and the loop's activators were discovered (Figure 3D). With more components being identified, the diagrammatic models started to introduce additional loops (Figure 3E,F). Finally, the gene duplications in mammals gave rise to a series of orthologues and thus doubled or even tripled the potential loop components (Figure 3G). Until recently, diagrammatic models depicted the regulation of multiple clock components often as if they were transcribed as a single operon. Meanwhile many researchers see the circadian molecular machinery as a complex network of feedback loops [56,57].

The diagrammatic explosion generated formidable substrates for computational deterministic models, which have

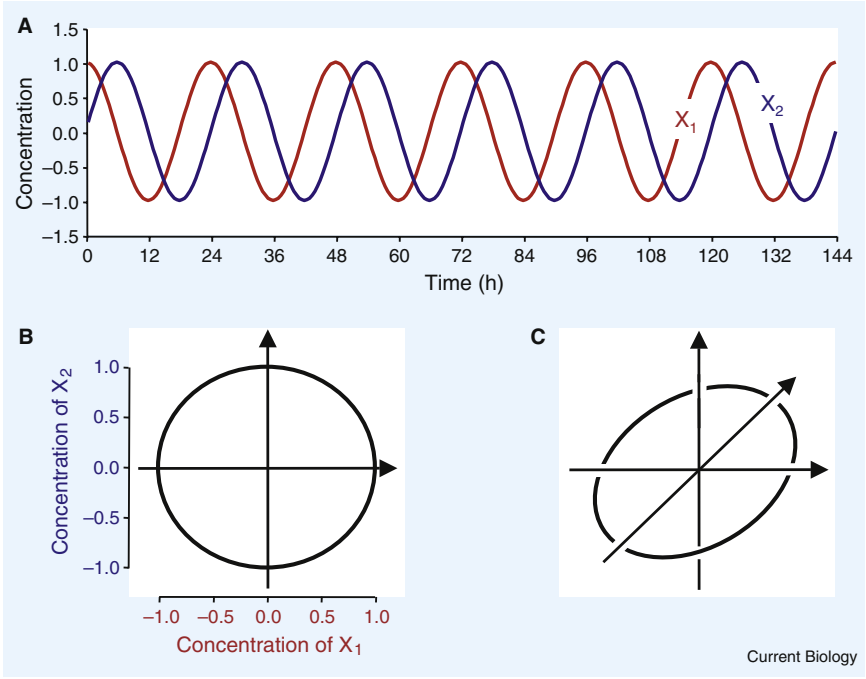

Figure 4. State variables and limit cycles.

(A) The two state variables of a given oscillator (e.g., mRNA and protein) often oscillate with a time lag. (B) If their respective concentrations are plotted against each other, they form a 'limit cycle' in which each phase of the oscillator is represented by a point on the cycle representing the current concentrations. Limit cycle representations of oscillators with more than two state variables still form circles in a multi-dimensional space. (C) Three state variables, e.g., mRNA, protein and its phosphorylation state.

become more quantitative as they try to predict the underlying biochemical reactions in the concrete context of the presumed components. When translating the diagrammatic models into differential equations for the computation of deterministic models, eventually all the diagram's reactions should be considered. Thus, deterministic computer models that investigate the inherent possibilities and constraints have to deal with a colossal parameter space, and long before such models can be fully explored, new components are added to the system by experimentalists. While new components are discovered for the circadian molecular network in animals, plants and fungi, the recent finding that circadian rhythmicity can be generated by surprisingly few molecular components in a test tube [10,58] provides a clearly defined system, which is ideal for quantitative modelling [59–61] (see below). Yet, even in the cyanobacterial system, the true challenge for modelling is still to come, namely when trying to couple the 'test-tube-oscillator' to the transcriptional feedback loops formerly presumed responsible for generating circadian rhythmicity [62].

Clock Formalisms

Practically all formalisms of circadian clocks are based on oscillator theory [63]. Oscillators with at least two components (state variables), e.g., a clock gene RNA and its protein, show so-called 'limit cycle' behaviour: Depending on the time-lag in their accumulation, the two components can oscillate out of phase, similar to a sine and a cosine function (Figure 4A). When their respective 'concentrations' at different times of the cycle (phases) are plotted against each other, they form a circle (Figure 4B), a so-called 'limit cycle'. Depending on waveform and lag of the oscillations, limit cycles can adopt many shapes, ranging from a perfect circle, an almost straight line — if the two state variables oscillate in synchrony or 180° out of phase — to a square, e.g., if they switch between discrete states. If rhythms dampen, their phase specific concentrations leave the stable limit cycles and spiral in; if amplitudes increase, they spiral out. If they oscillate around different levels and/or adopt different amplitudes (e.g., in constant darkness compared to constant light), their limit cycle moves to a different location and/or its radius changes.

The behaviour of circadian clocks based on their limit cycles has been extensively modelled by Arthur Winfree, summarised in a highly recommended book [64]. Figure 4A,B shows a simple limit cycle based on a two component oscillator, which is inadequate to describe the complex network of molecular components shown in Figure 3. A limit cycle in a three-component-system would still form a circle which lies in a 3-D coordinate system (Figure 4C). Thus, limit cycle behaviour can be described diagrammatically for up to three components, while predictions for more complex limit cycle oscillators can only be derived by computational modelling.

Modelling Entrainment

While free-running rhythms are the most conspicuous property of circadian clocks, they cannot have been the property under selection in the evolution of clocks [56] — with very few exceptions, organisms live in a cyclic environment. Free-running rhythms, therefore, reveal how clocks have evolved to adaptively entrain to their natural *zeitgebers*. Thus, modelling the interactions of clock components without 'external' input only indicates how the system is put together, without exploring the system's adaptive evolutionary advantages. The true test for clock models is, therefore, adequately simulating entrainment, including its complex qualities such as transients, history-dependence and after-effects, frequency demultiplication, or its temperature dependency. It is noteworthy that, while circadian rhythms are temperature compensated under constant conditions, their entrainment can be temperature-dependent (e.g., at different latitudes [51]). Relatively few modelling papers have been specifically dedicated to entrainment, be it at the contextual levels of molecules [65–68] and cells [69,70] or at the conceptual level [11,71–76]. Recently, modellers have begun to explore the complexity of entrainment beyond the simple synchronisation to a *zeitgeber* (e.g., [77]).

Resetting the circadian clock is thought of as a result of perturbing the free-running rhythm of a clock component (Figure 5A), leading to a change in its concentration and thereby resetting the phase of the rhythm. Regular perturbations can thus result in a stable entrainment of the rhythm to a *zeitgeber*. All oscillators, even a simple swing (Figure 5B),

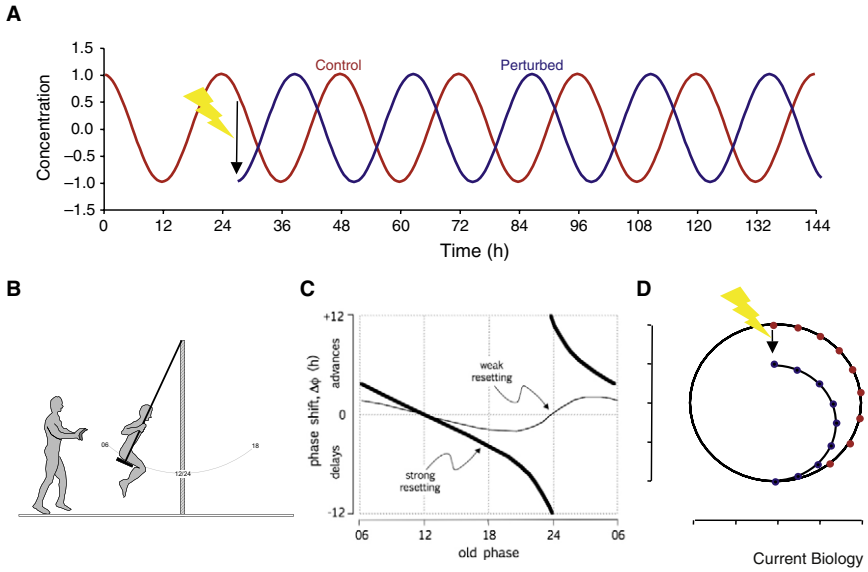

Figure 5. Resetting the clock as a basis for entrainment.

(A) The concentration of a cycling clock component is light-labile, such as Tim in the *Drosophila* clock. Its phase can, therefore, be reset by a light pulse and direction and amount of response will depend on the phase when the light pulse is administered. (B) For all oscillators, even a simple swing, the response to a stimulus will depend on their phase which gives rise to a phase response curve (PRC). (C) Depending on the strength of a stimulus, oscillators show weak or strong phase response curves. (D) Responses to perturbations and the resulting phase shift can also be described with the help of limit cycles (see Figure 4). The stimulus drives the state variable away from its attracting limit cycle to which it spirals back, thereby taking 'short-cuts' resulting in advances or 'detours' resulting in delays. A stimulus that drives the limit cycle exactly to its centre produces arrhythmicity.

respond differently to the same perturbation depending on when the stimulus is given. For example, an identical push will change the progression of the swing differently depending on whether it swings towards or away from the pusher [63]. The systematic responses of an oscillator to perturbations, e.g., light-pulses, can be described by a phase-response curve (Figure 5C). The power of the phase response curve in explaining phase resetting or entrainment and even circadian behaviour in constant conditions is remarkable. It can predict, for example, an oscillator's periodicity in constant light of different intensities. This circadian quality, called 'Aschoff's rule', has already been modelled 40 years ago using the van der Pol oscillator [41]. The phase response curve also predicts the clock's phase relationship to a *zeitgeber* and the range of *zeitgeber* periods it can entrain to. Under normal conditions, all organisms on earth would entrain to a 24 hour cycle, but the theory of the phase response curve has even been applied to answer the question of whether the human clock would be able to entrain to the 24.65 h day on Mars [78]. Phase response curves are formal, mathematical representations of an oscillator's resetting capacity and can be established experimentally for circadian behaviour at all levels of the organism. Phase resetting of an oscillator can also be modelled on the basis of its limit cycle (Figure 5D). As the phase-response curve represents the system's capacity to entrain, it is a fundamental feature that all models should be tested for [41], similar to their capacity to free-run in constant conditions.

Circadian Models — Test Tracks, Predictors or Crystal Balls?

Fortune telling can be very successful. Not that the future can actually be seen in a crystal ball, but if the fortune teller finds the right words they will strike a chord in the client. Similarly, modelling risks simply echoing what the modeller expects in the first place, i.e., it behaves in its basic features as would be expected of a circadian system. If a model can produce free-running rhythms, and has an appropriate phase response curve, enabling entrainment, then it merely echoes that the equations produce an oscillator adequate for the purpose. Once this has been established, models can be used as test tracks to ensure all the intuitive predictions applied to a diagrammatic model are feasible. The increasing complexity of the clock's diagrammatic models (Figure 3) invites computational testing. We are incapable of intuitively grasping the results obtained by modern chip technology — the same can be said of understanding and predicting the dynamics of the highly complex networks. Thus, computational modelling serves an important purpose in handling and understanding the complexity revealed by experimenters. Beyond their role as test tracks, models should be predictive, to uncover unforeseen features of the system.

The Predictive Power of Clock Models

The true predictive power of circadian modelling is hard to judge, simply because it takes an experimenter to pick up the prediction and test it experimentally. In most cases, this has been achieved when the modeller and the experimenter were the same person or part of the same team. Here, we review only a few examples for the predictive power of circadian modelling. Pittendrigh's work (for references, see [52]) is a good example of the power of this personal union. His ability of using formalisms and models to make sense of his many experimental observations was exceptional, starting with the concept that two oscillators are necessary to account for certain circadian observations [30] and ending with a comprehensive model that explained latitude-dependent variations of photoperiodic responses in different *Drosophila* species [49]. He summarised the enormous value of moving between experimentation, modelling and verifying the models' predictions in further experiments in his highly recommended book '*Reflections of a Darwinian Clock-Watcher*' [52].

While Pittendrigh was an experimenter-modeller, Arthur Winfree was a modeller-experimenter. His approach to describe circadian clocks as limit cycles (Figures 4 and 5) made clear predictions that a critical pulse, given at just the right phase and with just the right strength, would collapse the limit cycle to its centre (point of singularity), rendering the system arrhythmic; an example for the effect of a non-critical pulse on a limit cycle can be seen in Figure 5D. This prediction has been verified experimentally for many

systems ranging from unicells [79] to humans [35]. Winfree's models also predicted that once an oscillator is driven into singularity, a second pulse could re-initiate rhythmicity, which was also simulated by a contextual deterministic computer model [80]. Winfree was critical enough to recognise that a flat rhythm and its restoration — when found in clocks of real organisms — could either represent true singularities in each individual clock of the system (i.e., those in a population of unicells or SCN neurons) or could result from a desynchronised ensemble of oscillators — he actually favoured the latter explanation for experimental observations [64]. Only recently, this issue has been revisited [81], showing how long-term predictions can affect scientific discovery.

Another example of the predictive power of modelling was the discovery of a hitherto unknown molecular clock component in plants. Andrew Millar and his colleagues found a behaviour in their model of the *Arabidopsis* clock demanding an additional component [82] which they identified experimentally to be the gene *GIGANTEA* [83].

A conceptual deterministic model of the circadian system showed that the observed consequences of clock mutants can also be explained if the respective genes were part of an input pathway loop rather than of the actual rhythm generator [16]. Notably, as in Pittendrigh's 'pacemaker and slave' model, only both elements together create a functional circadian clock. The model predicted that if the clock mutant was acting as part of a light-input pathway, temperature cycles should still be able to reveal an underlying circadian oscillator. This prediction was experimentally validated in *Neurospora* [74,84] and led to a series of experiments that supported the existence of an oscillator outside of the transcriptional-translational feedback loop involving *Neurospora*'s *frequency* gene [85]. The extended role of the clock's input, proposed in this model, led to the *zeitnehmer* (German for 'time taker') concept whereby the circadian programme actively influences its own reception of the external *zeitgeber* signals. The existence of *zeitnehmer* mechanisms have subsequently been verified experimentally [86].

Another conceptual, top-down modelling paper [87] picked up a loose end in a study combining *in vitro* experimentation and *in silico* modelling concerning the effects of phosphorylation on human PER2 [88] and its effects on human chronotype. They found that the hPer2 mutation leads to an accelerated degradation of the protein but that it would remain longer in the nucleus. Gallego and colleagues [87] showed in their model that decreased nuclear clearance alone would lengthen the circadian period. Yet, subjects affected by this mutation are extreme early chronotypes, which is traditionally associated with a short period. Therefore, the authors predicted that the accelerated degradation must be causal for the phenotype. The importance of degradation of clock components on period length had already been predicted by models based on the Goodwin oscillator [89].

A purely conceptual diagrammatic model by Daan and colleagues [90] proposed that each member of the gene pairs of the *period* and the *cryptochrome* in mammals (*Per1*/*Per2* and *Cry1*/*Cry2*) are associated with the morning and the evening oscillator, respectively. Twenty-five years earlier, Pittendrigh and Daan [91] had suggested the concerted action of two oscillators, one that locks to dawn and one that locks to dusk to explain circadian behaviour, indicating that the clock has a history-dependent memory, specifically in the context of seasons. The new model made an attempt to find substrates for the two oscillators in clock genes. Based on their expression patterns in light–dark cycles the model predicted Per1/Cry1 to be part of the morning oscillator and Per2/Cry2 to be components of the evening oscillator. Although the model was criticised for making too big a leap from known experimental results to a new concept that was not readily predictable from the published data, it did make very distinct and testable predictions which subsequently led to a series of experiments proving several, but not all, of the predictions to be correct [92,93].

Within the eukaryotic field, transcriptional feedback loops are still favoured models for oscillator mechanisms. However, in prokaryotes, a circadian rhythm in phosphorylation of one of the clock proteins (KaiC) of the cyanobacterial clock shows circadian clock properties even though the experiment is done in a test tube containing nothing but three proteins (KaiA, KaiB, and KaiC) together with ATP. This finding obviously demands scrutiny in terms of models as they show how the interactions of the components could generate and stabilise the observed oscillation. A stochastic computational model of an hourglass mechanism predicted that the rhythm would be stabilised by a collective assembly or disassembly of KaiC proteins [94] which was verified experimentally [95]. Another model predicted that the monomer exchange maintains synchrony among the KaiC hexamers, sustaining a high amplitude oscillation [96]. Again the predictions could be confirmed by experimentation [97].

New Alleys — Promising Niches

Circadian modelling has traditionally followed the deterministic track that simulates only average behaviour of the components involved. This may be sufficient if very many molecules contribute to the phenomenon but may cause problems when there are only few — as has been shown for the regulation of gene expression and specifically for the circadian negative feedback loop [98]. This limitation can be overcome by stochastic modelling which is becoming more prevalent in circadian research (see [17]). Models that incorporate stochastic aspects, such as intrinsic and extrinsic noise, are more realistic than their deterministic counterparts when it comes to describing molecular events, such as the individual binding of an estimated number of proteins to an estimated number of promoters. In the stochastic version of a model simulating the mammalian molecular clock [99], for example, PER2 shows its expected rhythmicity while it did not show it in the deterministic version [100]. Stochastic models can also adequately address the important question of how robust deterministic simulations perform when the realistic scenario of molecular noise is introduced [101].

Beyond using differential equations — both deterministic and stochastic — other modelling methods have so far rarely been used for computer simulations of the circadian clock. The pi-calculus, for example, method was originally developed to simulate concurrent processes and interactions in networks, for example, those created by mobile phones [102]. A stochastic version of pi-calculus [103] can be used to model biochemical systems which form networks of thousands of mobile elements involving concurrent processes and has recently also been introduced to simulate the circadian clock [104].

The proportion of modelling in biology increases exponentially which may partly reflect the fact that systems biology approaches are increasingly used in the attempt to

understand biological functions. The necessity to analyse thousands of interacting variables will foster the development of alternative modelling methods and/or the use of hybrid models (mixing different methods and approaches), which will have to prove their usefulness in simulating biological mechanisms, such as the intra- and/or intercellular processes underlying circadian rhythmicity and temporal adaptivity.

Going beyond the Clock Paradigm

As discussed at the beginning of this review, the most persuasive model ever in circadian biology was the abstract concept of a 'clock'. Providing an internal time frame is surely one of the functions of the circadian programme but its adaptation to seasons and the fact that its rhythms in constant conditions can show history-dependent aftereffects highlight that this programme also has a memory; notably, it was initially called *zeitgedächtnis* (German for 'time memory') [3,4]. Entrainment shows that this temporal programme is also a perception system. Such systems are rarely only passive responders. Our visual perception, for example, is predominantly updating an endogenous spatial representation or template. In analogy, the circadian system could be viewed as an endogenous representation of a temporal space, namely the 24-hour day with all its reoccurring features such as light and darkness or warm and cold [105]. As in spatial perception, the endogenous *template* of a day is continually updated by external and internal information that results in what we call entrainment. To move away from the clock metaphor towards a perception system may enable us to reconcile the timing and memory functions of the system. Prolonged regular external time structures, such as certain photoperiods or non 24-hour *zeitgeber* cycles, would continuously shape the endogenous template that represents a *zeitgedächtnis.* Such a process would enable seasonal adaptation or could produce aftereffects in constant conditions. As in other perception processes, the underlying mechanisms are not simple linear pathways — neither at the tissue nor the molecular level — but form complex networks with some form of memory. Logically, constant conditions cannot exist if a system influences its own inputs depending on the current endogenous template, which would explain why we can record sustained free-running rhythms, in some cases for years, without evolution ever having to select for them [56,57].

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