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Temperature-Compensation in Biological Clocks: Models and Experiments

Peter Ruoff

School of Science and Technology, Stavanger University College,
P. O. Box 8002 Ullandhaug, N-4068 Stavanger, Norway

1. Introduction

In order to function as physiological clocks, circadian (and certain ultradian) rhythms contain homeostatic mechanisms which compensate for external temperature variations and other environmental influences. In this paper a theory for temperature-compensation and general homeostasis for physiological clocks is presented and compared with experimental findings. Results obtained from different organisms indicate that circadian pacemakers are based on one or several negative feedback loops where protein products of clock genes act as inhibitors of their own transcription. We have simulated the occurrence of temperature-compensation by using a simple reaction-kinetic model (the so-called Goodwin oscillator) which mimicks the negative feedback loop of a circadian pacemaker. The comparison between simulation calculations and experiments of *Neurospora* and *Drosophila* clock mutants shows that both period length and temperature-compensation appear closely connected through the stability/degradation rate of clock proteins.

2. Compensation Mechanisms in Biological Clocks

Circadian clocks [1–3] play important roles in the adaptation of organisms to the daily changes in their environments. They are found in eukaryotic single-cell and multicellular organisms and even in certain prokaryotes. A central task of biological clocks involve the timing of physiological events both on a daily and seasonal basis. Examples for daily adaptations include the regulation of sleep or activity periods in humans and higher organisms, conidiation in fungi, fruit fly eclosion or leaf movements in plants. Studies on honey bees show that spatial orientation to the sun is closely related to the ability of the insects to determine the time of day [4]. Seasonal timing events that are directed by circadian rhythms and daylength are reproduction, hibernation or migration of organisms as well as flower induction. As described by *Van't Hoff's rule*, temperature has a profound effect on most chemical and biochemical processes with Q_{10} values of about 2 or higher [5]. Although biochemical (enzyme-catalyzed) reactions are quite dependent on temperature [6], the period lengths of circadian rhythms remain practically

unaffected by changing the environmental temperature. As a result, Q_{10} values of circadian rhythms are generally close to 1. This indicates that circadian rhythms function as true physiological clocks, and therefore must contain compensation mechanisms against fluctuations of temperature or other environmental influences [7,8].

Although little is known about how these compensation mechanisms work, it is possible to make some general statements when temperature-compensation (or period-homeostasis) occurs. Here I will present a theory for temperature-compensation, analyse the implications of models for properties of circadian rhythmicity and show that both the circadian period length and temperature-compensation appear to be closely related through the stability of certain clock proteins. Some of these theoretical predictions have experimentally been confirmed for the model organisms *Neurospora crassa* and *Drosophila.*

2.1. Modelling Complex Reaction Networks

It is now generally believed that all chemical change, no matter how complex, is the consequence of a number of *elementary processes* [10]. An elementary process takes place in a single step (it cannot be broken down into smaller steps) and involves at most three reactant and three product molecules. The kinetic orders of an elementary process are identical with the stoichiometric coefficients, and the net direction of an elementary process is determined from the free energies of formation and the concentrations of the participiating species. These constraints on kinetic order and reaction direction make the consideration of elementary reactions a powerful tool in the modelling of complex reaction systems: the temporal behavior of any reacting system can be described by a simultaneous set of nonlinear differential equations, where the number of equations is equal to the number of elementary processes. If all rate constants are known, computer programs [12] can perform numerical simulations of the development for any system of interest, where the computations can be compared with experimental observations. Although the constraints of elementary reactions do in principle not apply to component stoichiometric processes (for example taking the process of transcription), we still can try to approximate them by elementary reactions and refer to them as *pseudo-elementary processes.* The usefulness in looking at elementary or pseudo-elementary reactions in the description of complex reaction systems has clearly been demonstrated in the study of oscillating reactions [11]. Oscillating reactions are probably the most complicated (nonliving) chemical processes that have been elucidated in detail. Although a living cell is considerably more complex than any of the chemical oscillators considered so far, the principles outlined above together with the use of high-speed computers provide the language by which the dynamics of various cell processes can (quantitatively) be described.

2.2. General Criteria for Period-Homeostasis

2.2.1. Temperature-compensation To understand how temperature-compensation can be achieved in circadian oscillators (or any other reaction kinetic oscillator) I consider *all* (elementary or pseudo-elementary) processes R_i that may occur in a single cell. Index i identifies the process R_i and its rate constant k_i. N is the total number of processes. The influence of temperature T on R_i is described by the Arrhenius equation

$$k_i = A_i e^{-E_i/RT} \tag{1}$$

where A_i is the (here assumed temperature-independent) pre-exponential factor, E_i is the activation energy, and R is the gass constant. The period length P of the rhythm will be a complicated function f of the $k_i's$:

$$P = f(k_1, k_2, k_3, \ldots, k_N) \tag{2}$$

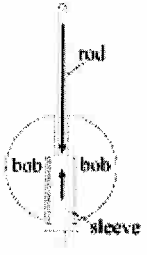
By applying the chain rule and expressing $\partial k_i/\partial T$ by means of Eq. (1), the condition for temperature-compensation can be written as

$$\frac{\partial P}{\partial T} = \sum_i \left(\frac{\partial f}{\partial k_i}\right)\left(\frac{\partial k_i}{\partial T}\right) = 0 \Rightarrow \sum_i \left(\frac{\partial lnf}{\partial lnk_i}\right) \times E_j = 0 \tag{3}$$

Each process i will either positively or negatively (or not) contribute to the overall $\partial P/\partial T$. Thus, by rearranging the processes into period-increasing or period-decreasing contributions (when T is increased), we can describe temperature-compensation as a balance between these two *opposing* sets of reactions:

$$\sum_{i,P-increasing} \left(\frac{\partial lnf}{\partial lnk_i}\right) \times E_i = - \sum_{j,P-decreasing} \left(\frac{\partial lnf}{\partial lnk_j}\right) \times E_j \tag{4}$$

Thus, temperature-compensation is expected to occur whenever the E_i-weighted sensitivity coefficients $\partial lnf/\partial lnk_j$ of period increasing processes balance with the contributions of the period-decreasing processes. In fact, for a given reaction network an *infinite* (!) number of E_i-combinations is possible that will satisfy Eq. (4) and lead to temperature-compensation. The experimental task therefore is to find those (during evolution developed) reactions together with their E_i-combinations which are responsible for temperature-compensation of a certain rhythm.



Invar Pendulum

The above concept of opposing reactions has a nice analogy in the construction of a temperature-compensated mechanical pendulum. In the mechanical compensation pendulum a variety of approaches exist which differ in the type of arrangement to keep the bob of the pendulum at approximately the same length [15]. In the *Invar-pendulum* (figure to the left) the increase of the

pendulum-rod is compensated by a sleeve that expands in opposite direction to the expansion of the rod. Hastings and Sweeney [14] were the first to explain temperature-compensation in circadian rhythms by the concept of opposing reactions. However, while the Hastings and Sweeney approach puts the control mechanism outside of the oscillator, there is evidence that the molecular mechansims of temperature-compensation ("the opposing mechanism") are an integral part of the oscillator. According to Franck [16] both positive and negative (i.e., "opposing") elements ("feedbacks") are necessary to build a chemical (or physiological) oscillator. Thus, any reaction kinetic oscillator has, in principle, all elements available to become temperature-compensated [19].

2.2.2. General homeostasis of the circadian period It is now well established that temperature-compensation is only one aspect of a more general homeostatic mechanism, which compensates circadian rhythms against various environmental influences such as pH, food supply, salinity, etc [7]. Theoretically, *simultaneous* homeostasis conditions may be formulated for each influencing physico-chemical property ξ, i.e.,

$$\frac{\partial P}{\partial \xi} = \sum_i \left(\frac{\partial f}{\partial k_i}\right)\left(\frac{\partial k_i}{\partial \xi}\right) = 0 \tag{5}$$

that lead to an overall compensation of the circadian period [21].

3. Models of Circadian Rhythmicity

3.1. Basic Mechanisms

During recent years considerable progress has been made to unravel the mechanisms of circadian rhythmicity [2]. The use of clock mutants has made it possible to study the influence of point mutations on period length, phase resetting, as well as temperature- or pH-compensation. A considerable amount of evidence suggests that negative feedback loops play important roles not only in the generation of circadian rhythmicity, but also for the compensation mechanisms of circadian oscillators. Fig. 1A shows the basic mechanism of the circadian clock in *Neurospora crassa* together with the putative light input pathpays. The *Neurospora* clock rhythm is normally studied in growth tubes or on petri-plates by means of a sporulation assay where the period is determined as the time interval between rhythmic sporulation peaks (Fig. 1B), [9, 17]. The *frequency* (*frq*) gene plays an important role as it may be considered as the pacemaker of the rhythm: after transcription of *frq*, *frq*-mRNA is transported into the cytosol and there translated into the FRQ-protein. FRQ is transported back into the nucleus where it inhibits its own transcription. Thus, transcription, translation and inhibition of *frq* generates a negative feedback loop. A correct timing between active transcription (the positive element) and inhibition of transcription (the negative element) is necessary to generate oscillations [16]. Phosphorylation of FRQ-protein influences

FRQ-stability, and, as we will see below, is an important factor in determining period length, temperature-compensation and resetting behavior of the rhythm. The white-collar proteins WC-1 and WC-2 form a heterodimer (white-collar complex, WCC) which serves as a transcription factor in the generation of *frq*-mRNA. Constitutively elevated expression of *frq* results in the loss of rhythmicity (because the timed interplay between positive and negative elements causing the oscillations is disturbed), while step changes in the level of *frq*-mRNA resets the phase of the clock [13]. Light and temperature, two very important environmental Zeitgeber signals, reset the *Neurospora* clock by changing the levels of *frq*-mRNA and FRQ-protein [9]. By phosphorylating the WCC, light increases the transcription rate of *frq* while Z (the transcription inhibition factor which is probably a phosphorylated form of FRQ, Fig. 1A) is no longer able to inhibit *frq* transcription. Temperature changes, on the other hand, are expected to influence all processes dependent on their activation energies as described in the Arrhenius-equation (1).

3.2. The Goodwin Model

Although the reaction scheme in Fig. 1A is clearly a simplified caricature of the biochemistry of the *Neurospora* clock, I think it is of importance to consider mechanisms in form of *minimal models*. Minimal models contain a minimum amount of information, and their study can provide valuable predictions or suggestions for further experimental work. Interestingly, more than 30 years ago Brian Goodwin [18] proposed the possibility of physiological oscillations for the same three-dimensional negative feedback loop as described in Fig. 1A. In the Goodwin model, the time-dependence of the variables X (*frq*-mRNA), Y (FRQ-protein) and Z (transcription inhibition factor) is described by a set of three coupled differentiel equations

$$\frac{dX}{dt} = \frac{k_1}{1+Z^9} - k_4 X \tag{6}$$

$$\frac{dY}{dt} = k_2 X - k_5 Y \tag{7}$$

$$\frac{dZ}{dt} = k_3 Y - k_6 Z \tag{8}$$

where each rate constant k_i is related to reaction R_i (Fig. 1A). The transcriptional inhibition term $1/(1+Z^9)$ is often criticized due to its large and unrealistic cooperativity exponent (Hill-constant). This criticism appears unjustified, because the large cooperativity is the result of the small number of intermediates: as soon as the number of intermediates in the feedback loop are increased (or the reaction order in the kinetic equations is increased [25]) the cooperativity decreases.

The oscillations generated by the Goodwin equations can be viewed as repeated relaxations to "high" and "low" steady states dependent on whether transcription process R_1 (Fig. 1A) is either "on" or "off". The relaxation time τ, which describes how fast these steady states are approached determines the period. Intermediates Ξ ($\Xi = X, Y, or Z$) are synthesized and degraded by the rates r_s and

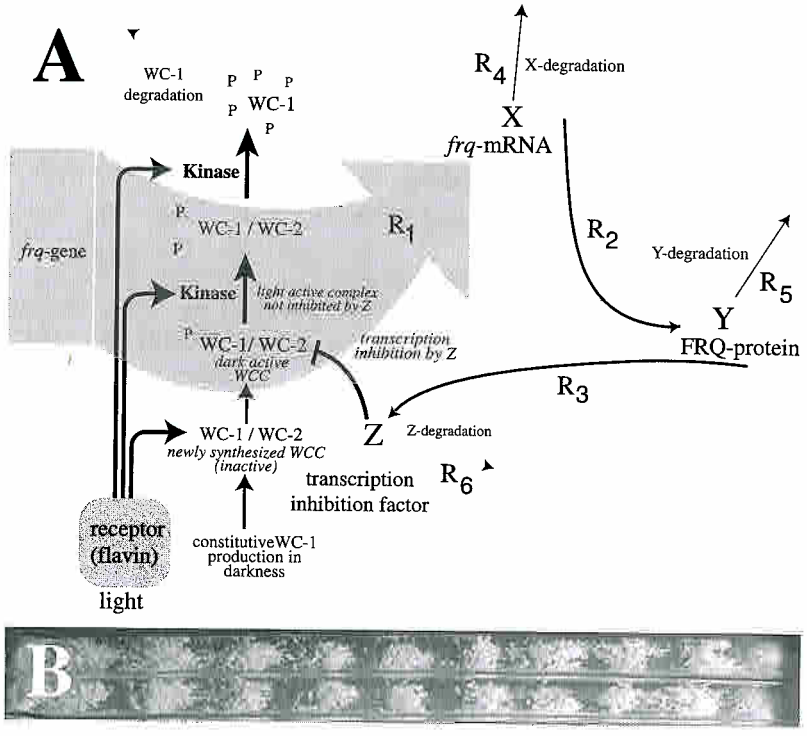


FIGURE 1. (**A**) Basic mechanism of the circadian clock in *Neurospora crassa* (for details see text, replotted from [24]. (**B**) Rhythmic bands of conidial spores are generally used as an assay for the circadian clock in *N.crassa*. The picture shows the rhythmic sporulation observed in two growth tubes for a strain with an average period of approximately 21 h.

$r_d(= k_d\Xi)$, and the relaxation time τ to the (low or high) steady states, Ξ_{SS}, is only dependent on the degradation rate constant k_d (with $\tau = k_d^{-1}$ and $\Xi_0 =$ Ξ-concentration at t=0):

$$\xrightarrow{r_s} \Xi \xrightarrow{k_d} \quad \Rightarrow \quad \frac{d\Xi}{dt} = r_s - k_d\Xi \quad \Rightarrow \quad \Xi = \Xi_{SS} + (\Xi_0 - \Xi_{SS})e^{-k_d t} \tag{9}$$

Although the simple structure of the Goodwin equations may overemphasize the importance of the degradation rate constants in the feedback loop, the "$\rightarrow \Xi \rightarrow$" feature of the model makes the prediction that a decreased degradation of any intermediate should result in an increased τ and period length: as k_d (i.e., k_4, k_5, or k_6) decreases, τ and the period length will increase. Because the long and short period *frq* clock mutants differ by point mutations (a single amino acid exchange) in the FRQ-protein [27], we proposed [26] that the *frq* clock mutants

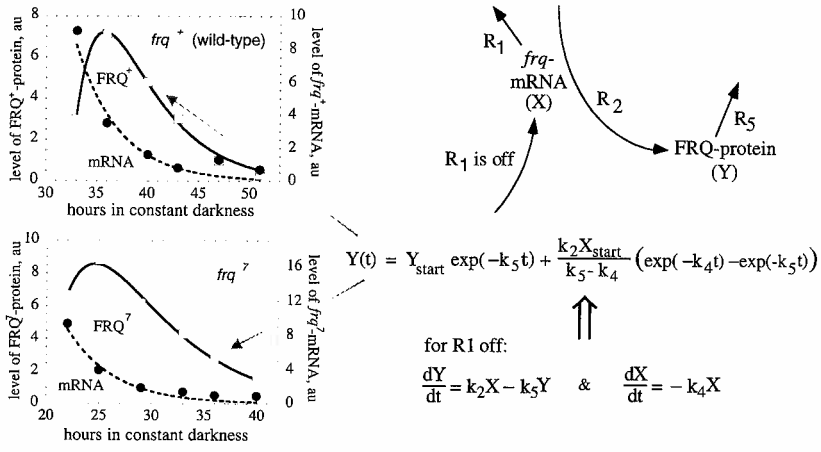


FIGURE 2. Experimental data [28] showing wild-type frq^+-mRNA and FRQ^+-protein levels, as well as levels for frq^7-mRNA and FRQ^7-protein (left). By using the determined value of k_4, the analytical solution of the FRQ-protein (Y) concentration from the Goodwin model (no transcription occurs) can be fitted to the experimental data by using k_2 and k_5 as adjustable parameters.

differ in the stabilities of their respective FRQ-proteins. For example, the long period length in the frq^7-mutant is the result of a more stable FRQ-protein. By use of experimental data from Garceau et al. [28] showing how *frq*-mRNA and FRQ-protein cycles in both wild-type and the frq^7-mutant, it became possible to estimate the FRQ-degradation rate constant R_5 (Fig. 2). To do this, we assumed that during a first-order *frq*-mRNA decrease the transcription reaction R_1 is off ($k_1 = 0$). First, the degradation rate constant k_4 for *frq*-mRNA (X) was determined; then the analytical solution of the FRQ-protein concentration was fitted to the experimental data by using k_2 and k_5 as adjustable parameters [29]. The fits and the derived FRQ half-lifes are show in Fig. 2 and Table 1, respectively. The table clearly shows that the FRQ^7-protein is more stable than wild-type FRQ.

Recently, Liu et al. [30] confirmed more directly the relationship between clock-protein stability and circadian period as predicted by the Goodwin model. In one experiment the phosphorylation of FRQ-protein was blocked by 6-DMAP (6-dimethylaminopurine), and a decrease in the FRQ-protein degradation rate was observed together with a corresponding lengthening of *Neurospora's* clock period. In another set of elegant experiments certain phosphorylation sites in FRQ were blocked by site-directed mutagenesis. Especially the replacement of Ser-513 by isoleucine or aspartic acid resulted in a significantly slower FRQ degradation rate and a very long circadian period length [30]. Interestingly, for *Drosophila* similar observations were made in the study of the clock gene *double-time* (*dbt*) [31].

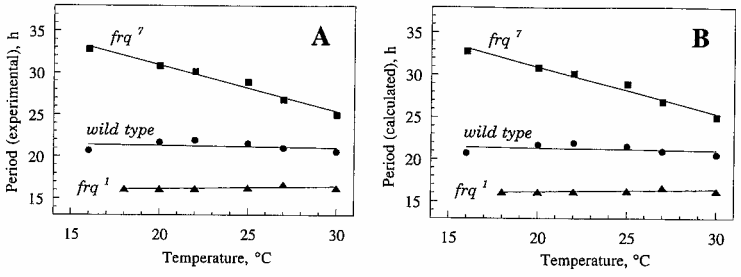


FIGURE 3. (**A**) Experimental period lengths as a function of temperature in wild-type and in the short period mutant frq^1 and in the long period mutant frq^7. Note the loss of temperature compensation in the frq^7 mutant. (**B**) Calculated temperature behavior for the same system as in A by use of the Goodwin model.

Double-time expresses a mammalian casein kinase Iε homolog [32], which regulates the clock (PER) protein phosphorylation and its stability (turnover), which in *Drosophila* plays a similar role as FRQ does in *Neurospora*. It has been found that long period and short period mutants of *dbt*, dbt^L and dbt^S, respectively, appear to be related to more stable or less stable PER proteins.

3.3. Modelling Temperature-Compensation

Fig. 3A shows the temperature dependence of period length of *Neurospora crassa* wild-type (frq^+) together with the long and short period mutants frq^7 and frq^1, respectively. Both wild-type (frq^+) and frq^1 have temperature compensated periods, while the rhythm of the long period mutant has lost temperature-compensation. The period of frq^7 decreases with increasing temperature, very much like what is observed in chemical oscillating reactions [33]. This behavior can be understood by determining the $\partial ln f/\partial ln k_j$ sensitivities of the Goodwin model, i.e., determine the contributions for the period increasing the reactions R_1, R_2 and R_3 and the period decreasing reactions R_4, R_5, R_6 according to Eq. (4) [20]:

$$0.0023(E_1 + E_2 + E_3) = 0.3583(E_4 + E_5) + 0.3416E_6 \tag{10}$$

TABLE 1. frq^+-mRNA AND FRQ HALF-LIFES [29]

Species	*Determined Half-Live, h*
frq^+ *(wild-type)*-mRNA	3.39 ± 0.42
frq^7-mRNA	2.86 ± 0.31
FRQ^+ (wild-type)	2.9
FRQ^7	6.2

The relatively large numbers in front of the activation energies E_4 - E_6 (Eq. (10)) indicate the high sensitivity of the period length to the degradation rate constants k_4 - k_6 as already mentioned (Eq. (9)). The loss of temperature-compensation by a change in FRQ-protein leading to a long-period mutant can be explained as the result of a point-mutation that leads to a more stable FRQ-protein whose rate of degradation (i.e., k_5) is reduced. This can be accomplished by an increase in activation E_5 and a decrease in the pre-exponential factor A_5 Eq. (1). Both will lead to a decrease in k_5, but only an increase in E_5 will result in an increased temperature-dependence of the period. The larger the increase in E_5 gets, the larger becomes the temperature-dependence of the rhythm. In this way the loss of temperature-compensation in *frq*^{7} can be explained due to a more stable FRQ-protein (Fig. 3B). The presence of temperature-compensation in the short period *frq*^{1} mutant indicates a less stable FRQ-protein (due to a increased A_5-factor), but with the temperature balance (Eq. (10)) still intact.

3.4. Ockham's Razor: An Outlook

We have seen that the relatively simple Goodwin model can describe temperature-compensation in the *Neurospora* clock. Apart from this, the Goodwin model is also able to model experimental observations of phase resetting by pulses of temperature, heat-shock, light, or cycloheximide [20, 22, 24]. Unfortunately, space limitations do not allow to describe these experiments and model calculations in further detail. Today, computers make it possible to integrate hundreds of elementary reaction with the possibility to model highly complex reaction networks. So why bother with minimal models? The problem is that for a large model many of the rate constants and activation energies are not known. A model with many adjustable parameters may describe the time evolution of a complex system with high accuracy. However, the model's capability to make realistic predictions is generally blurred by the different sets of "acceptable" parameter values which can be obtained in the fitting process.

To develop consistent models one can use Ockham's razor [23], i.e., avoid unnecessary assumptions and complexities. Then, by gradually increasing the complexity of a model in accordance with the availability of new experimental findings, it should be possible to arrive at models which consistently represent an experimental system. In this respect, it will be interesting to see how our understanding of circadian rhythms and their models develop in the future.

Acknowledgment

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[5] The Q_{10} is defined as the ratio between reaction velocities v at $(T + 10)$ and T Kelvin, i.e, $Q_{10} = v(T + 10)/v(T)$. Van't Hoff's rule states that most chemical reactions increase by a factor of two or more when the temperature is increased by 10 centrigrades.

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