Linear systems approach to analysis of complex dynamic behaviours in biochemical networks

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Abstract: Central functions in the cell are often linked to complex dynamic behaviours, such as sustained oscillations and multistability, in a biochemical reaction network. Determination of the specific mechanisms underlying such behaviours is important, e.g. to determine sensitivity, robustness, and modelling requirements of given cell functions. In this work we adopt a systems approach to the analysis of complex behaviours in intracellular reaction networks, described by ordinary differential equations with known kinetic parameters. We propose to decompose the overall system into a number of low complexity subsystems, and consider the importance of interactions between these in generating specific behaviours. Rather than analysing the network in a state corresponding to the complex non-linear behaviour, we move the system to the underlying unstable steady state, and focus on the mechanisms causing destabilisation of this steady state. This is motivated by the fact that all complex behaviours in unforced systems can be traced to destabilisation (bifurcation) of some steady state, and hence enables us to use tools from linear system theory to qualitatively analyse the sources of given network behaviours. One important objective of the present study is to see how far one can come with a relatively simple approach to the analysis of highly complex biochemical networks. The proposed method is demonstrated by application to a model of mitotic control in *Xenopus* frog eggs, and to a model of circadian oscillations in *Drosophila*. In both examples we are able to identify the subsystems, and the related interactions, which are instrumental in generating the observed complex non-linear behaviours.

1 Introduction

The link between information encoded in the genome and specific cell functions is created by complex interactions between DNA, mRNA, proteins and metabolites in large-scale intracellular biochemical networks. Unravelling the workings of these networks calls for systematic approaches based on mathematical modelling and analysis. So far, significant efforts have been invested in modelling and analysis of steady state properties of biochemical networks, e.g. [1–6]. An important objective in much of this work has been network modularisation, i.e. dividing a network into smaller subnetworks (modules) with functions that are essentially separable from other parts of the network [7, 8].

While steady state modelling and analysis can provide important information about the links between components and network properties, transient responses and dynamic behaviours are inherent parts of many cell functions [9–11]. In particular, a number of vital functions in the cell, such as cell cycle control [12–15], and circadian rhythms [16], are directly related to complex dynamic behaviours involving limit cycles and multistability. Such behaviours are in general caused by some form of feedback interactions between network components. Previous work in this area has largely focused on modelling the subnetworks generating the phenomena, and analysis of these models based on simulation and bifurcation analysis. In [14] bifurcation analysis is used to analyse the complex behaviour of a system, involved in cell cycle control, on a global level. The same system is studied in [17] where the authors use bifurcation analysis to analyse the robustness of the behaviour with respect to parameter variations.

Experimental results regarding the bifurcation behaviour of biological systems have also been obtained. For instance, [18] were able to experimentally determine a Hopf bifurcation, corresponding to circadian oscillations in *Drosophila*. In [15, 19], experimental evidence is provided for the irreversibility of the cell cycle transitions due to a bistable switch, caused by a positive feedback loop.

Bifurcation analysis, using tools such as AUTO [20], is a powerful tool for analysing the possible behaviours, and determining the sensitivity and robustness to parameter variations, of biochemical networks. However, even for relatively small networks the number of model parameters quickly grows large and mapping of the behaviour for all possible parameter combinations easily becomes impractical. Furthermore, the use of bifurcation analysis is limited to the determination of the existence of complex behaviours, and does not provide any insight into the mechanisms generating these behaviours. To obtain such insight, [14] employ bifurcation analysis of various simplifications of the full network structure for the cell cycle control in frog eggs. Based on this approach the authors are able to determine the most important subsystems generating specific bifurcations in the overall network. However, this approach is based on hypothesis postulation and testing, which requires significant system insight, and furthermore, substantial computational efforts for larger networks.

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In [21, 22] methods are presented that allow for the classification of reaction networks depending on their capacity of admitting multiple steady state solutions, corresponding to saddle-node bifurcations. In [23] a method is proposed based on 'opening' an identified feedback loop and analysing the non-linear characteristics of the resulting open-loop system. Sufficient conditions for the ability of the loop to create bistability are then determined based on the open-loop characteristics. However, as for bifurcation analysis, all these methods are limited to the determination of specific behaviours for a given system and provide little or no insight into what parts, and corresponding mechanisms, of a network are instrumental in generating these behaviours.

In this paper our aim is to identify the subsystem of a biochemical reaction network, described by a set of ordinary differential equations, that is instrumental in creating a certain complex dynamic behaviour, such as sustained oscillations or multiple steady states. The fundamental assumption made is that the source of complex behaviours can be determined by analysing the feedback mechanisms destabilising the underlying steady state. We first present a framework for representing a system of non-linear ordinary differential equations as a network of feedback interconnected subsystems. Representing networked systems as interconnected subsystems is a key ingredient to understand the inner workings of cell processes [10]. The resulting structured feedback system is then linearised around the unstable steady state, and by removing all feedback loops we obtain a linear non-interactive, or open-loop, system. Using results from linear feedback control theory we are then in a position to analyse the impact of interactions imposed by feedback and thereby gain insight into the source of a certain behaviour in the non-linear system. Using this approach, we can determine the feedback mechanisms that are instrumental in generating complex behaviours in a given system, for instance, the feedback loops that need to exist for a biochemical network to oscillate. In a similar way, the source of bi- or multistability can also be determined.

The motivation behind the use of tools from linear feedback control theory to analyse strongly non-linear complex behaviour, is the fact that the main purpose here is to qualitatively understand the mechanisms giving rise to a given behaviour of the system, and not to globally assess the non-linear behaviour of the system. We stress that the aim here is limited to the determination of important subsystems in a qualitative sense, and that we do not seek to determine quantitative properties such as parametric sensitivities or robustness of the network. However, the determination of the most important subsystems will prove valuable in guiding the search for the most important parameters and structural perturbations with respect to sensitivity and robustness of given cell functions. Also, this approach is in line with the idea of modularising intracellular biochemical networks [7].

2 Cell cycle control model

We consider a model of the reaction network controlling the cell cycle in *Xenopus* frog eggs, developed in [24] based on a combination of hypothesised molecular interactions and experimental data. The model consists of a set of nine non-linear ordinary differential equations, with a total of 26 parameters.

$$
\begin{aligned}
\dot{x}_1 &= k_1 - k_2 x_1 - k_3 x_1 \\
\dot{x}_2 &= k_{pp} x_5 - (k_{wee} + k_{cak} + k_2) x_2 + k_{25} x_3 + k_3 x_1 \\
\dot{x}_3 &= k_{wee} x_2 - (k_{25} + k_{cak} + k_2) x_3 + k_{pp} x_4 \\
\dot{x}_4 &= k_{wee} x_5 - (k_{pp} + k_{25} + k_2) x_4 + k_{cak} x_3 \\
\dot{x}_5 &= k_{cak} x_2 - (k_{pp} + k_{wee} + k_2) x_5 + k_{25} x_4 \\
\dot{x}_6 &= \frac{k_a x_5 (1 - x_6)}{1 + K_a - x_6} - \frac{k_b x_6}{K_b + x_6} \\
\dot{x}_7 &= \frac{k_e x_5 (1 - x_7)}{1 + K_e - x_7} - \frac{k_f x_7}{K_f + x_7} \\
\dot{x}_8 &= \frac{k_g x_5 (1 - x_8)}{1 + K_g - x_8} - \frac{k_h x_8}{K_h + x_8} \\
\dot{x}_9 &= \frac{k_c x_8 (1 - x_9)}{1 + K_c - x_9} - \frac{k_d x_9}{K_d + x_9}
\end{aligned}
\tag{1}
$$

where

$$
\begin{aligned}
k_2 &= V_2' + x_9 (V_2'' - V_2') \\
k_{wee} &= V_{wee}'' + x_7 (V_{wee}' - V_{wee}'') \\
k_{25} &= V_{25}' + x_6 (V_{25}'' - V_{25}')
\end{aligned}
$$

Here, $x_i, (i = 1 \ldots n)$ represent the concentrations, or activities, of free cyclin (x_1), the four phosphorylation states of the cyclin-Cdc2 dimer ($x_{2,3,4,5}$), and the four regulatory enzymes Cdc25, Wee1, IE and APC ($x_{6,7,8,9}$). Of particular interest is the variable x_5, representing the concentration of the M-phase promoting factor (MPF). The level of MPF triggers the transition between the different phases during the cell cycle. For instance, a high concentration of MPF triggers the cell to divide into two daughter cells.

For the nominal parameters in [25] the system displays sustained oscillations in all variables x_i. Physiologically, these sustained oscillations can be interpreted as the driving force behind the first 12 synchronous cell divisions occurring in the early embryonic state after fertilisation of the arrested egg [14]. Figure 1 shows the simulated temporal

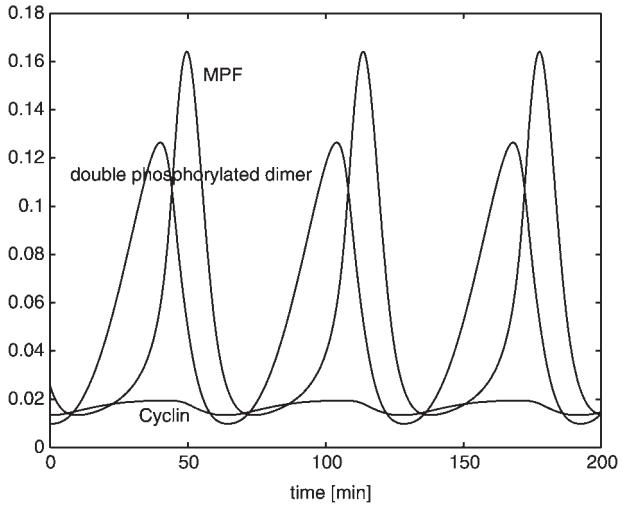

Fig. 1 *Numerical solution of the cell cycle model (1) displaying sustained oscillations in all concentrations. Here only three of the nine concentrations are shown. Parameter values [25]:* $K_a = 0.1$, $K_b = 1.0$, $K_c = 0.01$, $K_d = 1.0$, $K_e = 0.1$, $K_f = 1.0$, $K_g = 0.01$, $K_h = 0.01$, $k_1 = 0.01$, $k_3 = 0.5$, $V_2' = 0.005$, $V_2'' = 0.25$, $V_{25}' = 0.017$, $V_{25}'' = 0.17$, $V_{wee}' = 0.01$, $V_{wee}'' = 1.0$, $k_{cak} = 0.64$, $k_{pp} = 0.004$, $k_a = 2.0$, $k_b = 0.1$, $k_c = 0.13$, $k_d = 0.13$, $k_e = 2.0$, $k_f = 0.1$, $k_g = 2.0$, $k_h = 0.15$

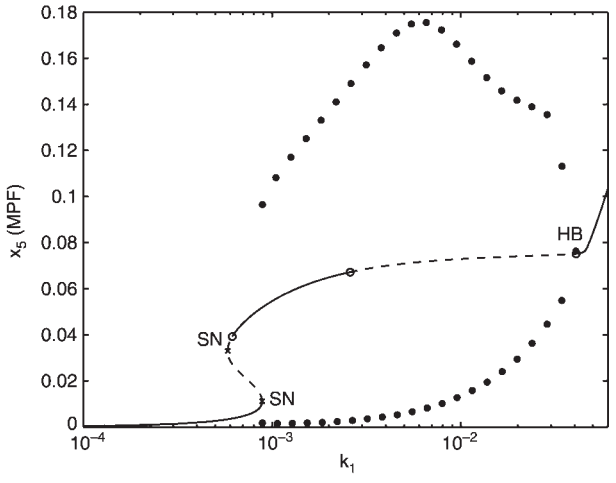

Fig. 2 *Bifurcation diagram of the cell cycle model. The Figure shows MPF solutions as function of parameter k_1, the cyclin synthesis rate. Stable steady states are indicated by full lines, unstable steady states by dashed lines and the maximum and minimum values of the oscillations by filled circles*

behaviour of the concentrations of cyclin, MPF, and the doubly-phosphorylated dimer PYTP (x_4).

Apart from sustained oscillations, the considered cell cycle model also displays bistable behaviour, as can be seen from the bifurcation diagram in Fig. 2. Here, the rate constant for cyclin synthesis k_1 has been used as the bifurcation parameter. The cyclin synthesis rate is related to the actual size of the cell, and thus the bistable behaviour of the cell cycle reaction network can be interpreted as serving as a control mechanism, allowing switching between different phases of the cell cycle, depending on the growth state of the cell [15, 19]. This control mechanism is active at a later stage of the embryonic growth, i.e. after the initial 12 rapid synchronous mitotic cycles.

3 Analysis method

In this Section we propose a method for the analysis of biochemical reaction networks, with the aim of identifying the subnetworks that cause complex behaviours, such as oscillations and steady state multiplicity.

3.1 Networks of interacting subsystems

In the following, we consider a general biochemical reaction network, described by a set of ordinary differential equations (ODEs)

$$\dot{\mathbf{x}} = \mathbf{f}(\mathbf{x}, \mathbf{p}), \quad \mathbf{x} = [x_1, \ldots, x_n]^T \tag{2}$$

where $\mathbf{x}$ and $\mathbf{p}$ are vectors, $\mathbf{x}$ representing the concentrations, or activities, of the components taking part in the reactions, and $\mathbf{p}$ the system parameters, including the reaction rate constants. The vector-valued function $\mathbf{f}$ determines the dynamics of the system. In cases of small molecular concentrations and/or small diffusivity, partial differential or stochastic equations may be required but this is outside the scope of this paper.

While each single ODE in (2) by itself displays trivial first order dynamic behaviour, complex behaviours can be created by feedback type interactions between the system equations. Such feedback mechanisms can be viewed as combinations of pairwise interactions between the individual components of the system. To obtain insight into the source of a certain complex behaviour of the overall network (2) we thus study the impact of the interactions between the different components within the biochemical reaction network.

We start by decomposing the overall network into smaller subsystems Γ_i, here representing the concentration of a single component only, i.e.

$$\Gamma_i: \quad \dot{x}_i = f_i(x_i, \mathbf{u_i}, \mathbf{p})$$

Following this definition, a subsystem has only one state and one output x_i. The inputs $\mathbf{u_i}$ consist of the outputs $x_j, j \neq i$ of all subsystems but the ith subsystem. The decomposition of the cell cycle reaction network (1) is illustrated in Fig. 3.

Although we here limit ourselves to consider single components as modules, the method presented below is applicable also to larger modules involving several components. However, how these should be chosen based on a systematic model analysis is largely an open problem. In specific problems, however, biological insight may guide the division into proper modules.

3.2 Analysis of destabilising interactions

Consider a single parameter p_i of (2). Depending on the value of this parameter the system can have different stability properties, and display qualitatively different kinds of asymptotic dynamic behaviours. For example, for some values it can have a single stable steady state, for others it can have multiple (stable and unstable) steady states, sustained oscillations, and also more complex behaviours not being addressed in this paper. A point in the parameter-state space at which the system switches its stability properties, and at which a branch of asymptotic solutions also starts or ends, is called a bifurcation point. In Fig. 2 two types of *codimension one* bifurcation points are depicted. Codimension one refers to the fact that the bifurcation points appear when varying a single parameter only, in this case k_1. The two types of bifurcation points in Fig. 2 are a Hopf bifurcation point (HB), from which a stable limit cycle and an unstable steady state emanate, and saddle-node bifurcation points (SN) from which one stable and one unstable steady state emanate. For unforced systems, i.e. systems with constant inputs, any complex phenomena can eventually be traced to a steady state bifurcation point for some value of the system parameters.

We here limit ourselves to consider behaviours which stem from local codimension one bifurcations, and consider in particular Hopf and saddle-node bifurcations.

From Lyapunov's indirect method (e.g. [26]) it follows that the local steady state stability of a general non-linear system can be determined from the linearisation of the

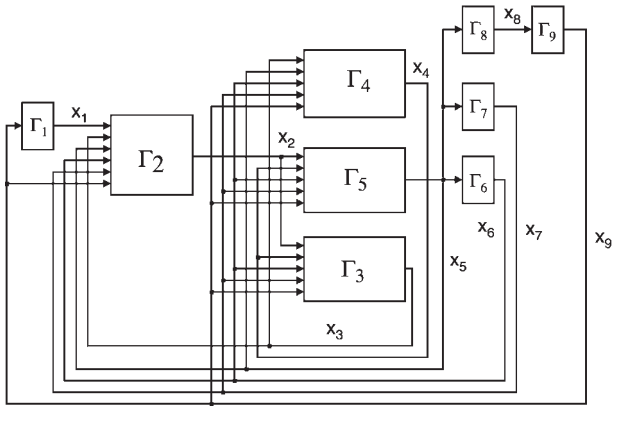

Fig. 3 *The cell cycle reaction network (1) represented as a network of interacting subsystems. Each subsystem Γ_i corresponds to the ith ordinary differential equation in the model (1)*

system around the steady state. Thus, the non-linear system is locally unstable at a certain steady state if the linearised model at this state has a Jacobian with some eigenvalues in the open complex right-half plane, and stable if all eigenvalues are in the open left-half plane. Since complex behaviours can be traced to destabilisation of an underlying steady state, linear systems theory can in principle be used to identify the mechanisms causing the behaviour by analysing the destabilising mechanisms of the underlying steady state. Thus, linear systems analysis can be used to determine the mechanisms generating the controls in the cell cycle model (1), or similar phenomena in any other biochemical reaction network that can be represented in the form of (2).

In the following we assume that for the values of the parameters $\mathbf{p}$, for which the model of the reaction network displays a certain complex behaviour, there also exists an underlying unstable steady state. This will always hold in the case of saddle-node bifurcations, giving rise to multiple steady states and bistability. For the case of Hopf bifurcations, it holds provided the bifurcation is supercritical and there are no additional bifurcations between the original bifurcation point and the considered parameter value on either of the two solution branches emanating from the bifurcation point. If the latter does not hold, one can simply use any model parameter to move the system closer to the Hopf bifurcation point so as to obtain a solution on the branch of unstable steady states emanating from the Hopf point.

For the early embryonic cell cycle in *Xenopus* frog eggs, [25] considered the limit cycle corresponding to a cyclin production rate constant $k_1 = 0.01$. From the bifurcation diagram in Fig. 2 it can be seen that there are no additional bifurcations on any of the solution branches emanating from the Hopf point and the solutions for $k_1 = 0.01$, and hence we have an unstable steady state underlying the limit cycle. Thus, we propose that the source of the observed limit cycle can be analysed by determining the source of the destabilisation of the steady state at $k_1 = 0.01$ using linearisation of the model at this point. The source of the bistable behaviour for the cell cycle model, at low rates of cyclin synthesis, can be analysed in the same way. The linearisation of system (2) around a steady state $\mathbf{x_0}$ is given by

$$\Delta\dot{\mathbf{x}}(t) = \mathbf{A}\Delta\mathbf{x}(t), \quad \mathbf{A} = \frac{\partial \mathbf{f}(\mathbf{x}(t), \mathbf{p})}{\partial \mathbf{x}}\Big|_{\mathbf{x_0}} \tag{3}$$

where $\Delta\mathbf{x}(t) = \mathbf{x}(t) - \mathbf{x_0}$ denote deviations of the component concentrations, or activities, from their steady state values.

The decomposition of the linearised system (3) into linear one-component subsystems, corresponding to the subsystems Γ_i, is now given by

$$\Delta\dot{\mathbf{x}}(t) = \tilde{\mathbf{A}}\Delta\mathbf{x}(t) + (\mathbf{A} - \tilde{\mathbf{A}})\Delta\mathbf{u}(t) \tag{4}$$

where $\tilde{\mathbf{A}}$ is a diagonal matrix containing the diagonal elements of $\mathbf{A}$. The interactions between the single linear subsystems are given by the feedback

$$\Delta\mathbf{u} = \Delta\mathbf{x} \tag{5}$$

In this framework, the biochemical reaction network can be seen as being composed of an interaction-free open-loop system (4) and the feedback imposed by the pairwise interactions (5) between the proteins, metabolites, and genes of the network. We are now in a position to use standard tools from feedback control theory to analyse the destabilisation of the underlying steady state by considering the properties of the open-loop system.

The idea of analysing a feedback-free system to derive properties of the system with feedback is standard within the area of feedback control theory, e.g. [27]. In [28] a similar concept is used in steady state sensitivity, or metabolic control, analysis of biochemical networks. In [23], the non-linear characteristic of an open-loop system is used to derive sufficient conditions on when feedback generates bistability in biochemical systems.

Following Laplace transformation, the open-loop system (4) can be written as

$$\Delta\mathbf{x}(s) = \mathbf{L}(s)\Delta\mathbf{u}(s) \tag{6}$$

where $\mathbf{L}(s) = (s\mathbf{I} - \tilde{\mathbf{A}})^{-1}(\mathbf{A} - \tilde{\mathbf{A}})$. The element $L_{ij}(s)$ corresponds to the transfer function from the activity, or concentration, of component j to the activity of component i in the absence of any feedback effects.

The poles of the closed-loop system, corresponding to the eigenvalues of the network Jacobian A, are the solutions s_i to

$$\det(\mathbf{I} - \mathbf{L}(s))_{|s_i} = 0 \tag{7}$$

In the case of a limit cycle, the closed-loop system will have at least one pair of conjugate complex poles in the right-half plane, while in the case of multiple steady states the closed-loop system will have at least one real pole in the right-half plane.

In the following we will assume that the open-loop system $\mathbf{L}(s)$ is stable. Extension to unstable open-loop systems, or modules, is trivial, but we assume stability here in order to keep the exposition simple. The open-loop system $\mathbf{L}(s)$ is stable if none of the biochemical reactions, in the overall system (2), are autocatalytic.

3.3 Linear stability analysis

For stability analysis of linear feedback systems, a standard approach in control theory is to consider the feedback signals Δx_i as composed of sinusoidals with different frequencies ω in the range from 0 to ∞. For an open-loop systems $L(s)$ having only one input and one output, corresponding to a single feedback loop, results from feedback control theory can be used to determine the stability properties of the closed-loop system based on such *frequency responses*. For instance, for a scalar stable open-loop system, the Nyquist stability criterion states that the closed-loop system is unstable under positive feedback if the frequency response $L(j\omega), \omega = -\infty \ldots + \infty$ encircles the critical point $(1, 0)$ in the complex plane, corresponding to the loop gain being real and exceeding $+1$ for some frequency ω_{crit}. Essentially, a sinusoidal with this frequency will get amplified every time it passes around the loop, and thus grow out of bounds with time. An important property of the Nyquist criterion is that it can be used to determine the stability margin of the closed-loop system, i.e. the factor by which the open-loop system has to be scaled to bring the closed-loop system onto the stability limit.

As an example of the use of the Nyquist stability criterion consider the following stable linear open-loop system

$$\Delta\dot{x}(t) = -\Delta x(t) + a\Delta u(t) \tag{8}$$

where a is a parameter, Δx and Δu are scalar variables. We want to determine the stability properties of the resulting closed-loop system, when the feedback is given by $\Delta u = \Delta x$.

The stable open-loop transfer function $L(s)$ is obtained by Laplace transformation of (8), and given by

$$L(s) = \frac{\Delta x(s)}{\Delta u(s)} = \frac{a}{s+1}$$

The frequency response of the open-loop system is then obtained by replacing s by the imaginary number $j\omega$ in $L(s)$

$$L(j\omega) = \frac{a}{j\omega + 1}$$

Plotting the Nyquist curve, i.e. the frequency response $L(j\omega)$ in the complex plane for all ω, with the parameter value $a = 1.1$ results in the curve shown in Fig. 4. The curve is encircling the critical point (1,0), marked with **x** on the positive real axis, and the curve crosses the real axis at the value 1.1. Thus, according to the Nyquist stability criterion the closed-loop system is unstable for this choice of the parameter. It is straightforward to see that the closed-loop system can be brought onto the stability limit by scaling the open-loop system $L(j\omega)$ with the stability margin $\gamma = 1/1.1$, since then the Nyquist curve would exactly cross the critical point $+1$. This leads to the conclusion that the closed-loop system is stable if the parameter a is chosen such that $a < 1.1 \times \gamma = 1$.

Clearly, this is a trivial example, and the same result could easily have been obtained by simply considering the closed-loop system equation, obtained by combining the open-loop system (8) with the feedback $\Delta u = \Delta x$

$$\Delta\dot{x}(t) = -\Delta x(t) + a\Delta x(t) = (a-1)\Delta x(t)$$

showing directly that the closed-loop system is stable if $(a-1) < 0$ and unstable if $(a-1) > 0$. However, the purpose of the example was to clarify the use of the Nyquist stability criterion, which is applicable in exactly the same way for linear systems of any complexity. Furthermore, the concept of stability margin, which is hard to define based on the Jacobian of the differential equations, is useful to analyse the robustness of feedback systems with respect to model perturbations, as considered below.

For multivariable systems, the eigenloci play the role of gains when considering stability. The eigenloci are the eigenvalues $\lambda_i(j\omega)$ of the frequency response of the open-loop system $L(j\omega)$, and can be interpreted as multivariable loop gains. Applying the generalised Nyquist stability criterion (see, e.g. [29]) we obtain the result that, given a stable open-loop system $L(s)$, the closed-loop system is unstable, if there exists a frequency ω_{crit}, for which at least one of the eigenloci has a real value larger than $+1$, and thus encircles the critical point (1, 0). Note that we here consider positive feedback structures, i.e. signals as added rather than subtracted, and that the critical point $(-1, 0)$ typically considered in control theory is due to the use of negative feedback structures. We will label the eigenlocus encircling (1, 0) the *critical eigenlocus* λ_{crit}. If $\omega_{crit} = 0$, the critical eigenlocus indicates a static instability, while if $\omega_{crit} > 0$, then the critical eigenlocus indicates a dynamic instability, resulting in an unstable conjugate complex pole pair in the closed-loop system.

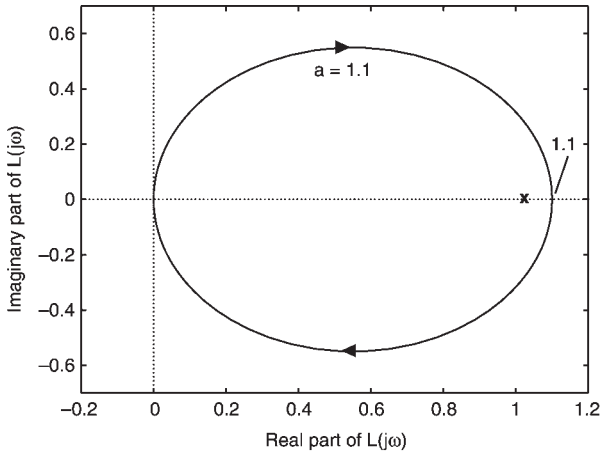

Fig. 4 *Nyquist frequency response curve for the positive feedback example system with parameter $a = 1.1$. The curve encircles the critical point (1, 0) and thus the closed-loop system is unstable for this choice of a*

3.4 The effect of individual proteins and metabolites

In order to determine the role of individual proteins or metabolites in the creation of the observed complex network behaviour, one may simply consider disconnecting the feedback from one component at the time, by letting $\Delta u_i(t) = 0$ for some i in (5). Biologically, this corresponds to holding the concentration of the i-th component in the reaction network fixed at its steady state value. While this is not feasible in *in vivo* or *in vitro* experiments, it can be useful as a model analysis tool to obtain insight into the impact of the various proteins in generating complex network behaviour.

A somewhat more general approach than outlined above, however, is to add a real perturbation Δ_i to the feedback of the concentration Δx_i of component i, as illustrated in Fig. 5, and determine the smallest $|\Delta_i|$ that will stabilise the closed-loop system, i.e. the considered steady state of the reaction network.

The transfer function $T_i(s)$ from Δu_i to Δx_i, incorporating all feedback from the different components but from the ith component, is given by

$$T_i(s) = [(\mathbf{I} - \mathbf{L_{i0}}(s))^{-1}[\mathbf{L}(s)]_i]_i \qquad (9)$$

where $\mathbf{L_{i0}}(s)$ corresponds to $\mathbf{L}(s)$ with all entries of the ith row set to zero, and $[\mathbf{L}(s)]_i$ corresponds to the ith column of $\mathbf{L}(s)$. The smallest perturbation $|\Delta_i|$ that will stabilise the considered steady state can now easily be determined from the Nyquist criterion based on T_i.

The relative size of the required perturbations $|\Delta_i|$ will indicate the role of the various feedback connections in destabilising the steady state, and hence in creating the complex behaviour of the biochemical reaction network (2). Small required perturbations indicate that the stability of the system is sensitive to the feedback of the corresponding component concentration, and that the dynamic behaviour of this concentration is hence instrumental in the creation of the considered behaviour.

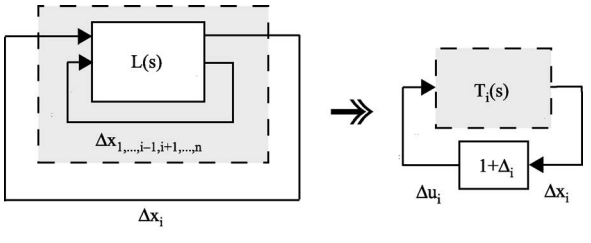

Fig. 5 *Reformulation of the multivariable feedback system as a scalar feedback loop. The aim is, by applying the Nyquist criterion, to determine the required perturbation Δ_i for each single feedback variable Δx_i that will stabilise the closed-loop system corresponding to the unstable steady state of the network*

3.5 The effect of pairwise interactions

As a complement to the method outlined above, in which the role of single components in the destabilisation of the considered steady state was investigated, we here aim at determining the specific pairwise interactions between network components, corresponding to the elements $L_{ij}(s)$ of the open-loop system, that are most instrumental in causing steady state instability, and thus the complex behaviour under consideration.

The stability of the considered steady state is reflected in the poles of the closed-loop system, corresponding to the solutions $s_i = \sigma_i \pm j\omega_i$ of (7). In the case of an unstable steady state the closed-loop system has some poles s^* with $\sigma^* > 0$, i.e. in the open complex right-half plane. If the steady state underlies bistability we assume a static instability, implying that the single right-half plane pole has a zero imaginary part ($\omega^* = 0$), while if the steady state underlies sustained oscillations we assume a dynamic instability, implying that there exists a pair of conjugate complex poles with $\omega^* > 0$. To obtain insight into which pairwise interactions are most important for causing these right-half plane poles, we seek the smallest relative perturbations in the pairwise component interactions $L_{ij}(s)$, that will stabilise the closed-loop system by moving the right-half plane poles onto the imaginary axis at $s^* = \pm j\omega_{crit}$, corresponding to moving the critical eigenlocus λ_{crit} onto the point $(1, 0)$ at the frequency ω_{crit}. Note that the perturbation obtained at ω_{crit} may not be the smallest perturbation required to move the poles to the imaginary axis, since the frequency is kept fixed, but is a sufficient perturbation which furthermore will be close to the minimum perturbation for those elements of $L_{ij}(s)$ that require relatively small perturbations for stabilisation.

The relative perturbation of a single element in $\mathbf{L}(s)$ that will move some poles of the closed-loop system to $s^* = \pm j\omega_{crit}$ solves

$$\det(\mathbf{I} - \mathbf{L_p}(j\omega_{crit})) = 0$$

where $\mathbf{L_p}(s)$ is equal to $\mathbf{L}(s)$ with one element $L_{p,ij}$ perturbed to $L_{p,ij} = L_{ij}(1 + \Delta_{ij})$, and where Δ_{ij} in general is a complex valued perturbation.

The required perturbations Δ_{ij} can be computed from the relative gain array (RGA) [30]. The RGA of a square complex matrix $\mathbf{M}$ is given by

$$\mathbf{RGA}(\mathbf{M}) = \mathbf{M} \times (\mathbf{M}^{-1})^T$$

where $\times$ denotes element-by-element multiplication (Hadamard or Schur product). One important property of the elements of the RGA is that they provide the relative change needed in the corresponding element of $\mathbf{M}$ to make $\mathbf{M}$ singular. In particular, if the single element M_{ij} is changed to $M_{p,ij} = M_{ij}(1 - 1/[RGA]_{ij})$ then the perturbed matrix $\mathbf{M_p}$ becomes singular [31].

The relative perturbations Δ_{ij} of element $L_{ij}(s)$ required to move some poles of the closed-loop system to the imaginary axis, are hence given by

$$\Delta_{ij}(\omega_{crit}) = -\frac{1}{[\mathbf{RGA}(\mathbf{I} - \mathbf{L}(j\omega_{crit}))]_{ij}} \qquad (10)$$

Note that the perturbation only ensures that the perturbed closed-loop system has poles at $\pm j\omega_{crit}$ on the imaginary axis, and that it is not guaranteed that it actually is the right-half plane poles which have been moved onto the imaginary axis. The perturbation may in principle move some stable poles to the imaginary axis instead, in which case the perturbation does not stabilise the steady state. This can easily be checked by applying the perturbation computed from (10) to the matrix $\mathbf{L}(j\omega_{crit})$ to verify whether the critical eigenlocus is moved to $(1, 0)$. If this is not the case, then there will not exist any perturbation of the considered element at the frequency ω_{crit} that will stabilise the system. This follows from the fact that the element perturbation making the matrix singular is unique.

In summary, based on the analysis outlined above, we can rank-order the importance of feedback effects from individual components, such as metabolites or enzymes, and pairwise interactions between components, respectively, in destabilising the steady state of the network and thereby creating the corresponding complex behaviour related to a specific physiological function in the cell. This information is relevant for identifying the mechanisms underlying specific functions of the cell, and also for guiding the modelling of these functions.

4 Examples

Here, we apply the proposed analysis methods to two examples, the cell division control in *Xenopus* frog eggs, and circadian oscillations in *Drosophila*.

We stress that our intention is not to interpret the results with respect to their biological implications, but rather to demonstrate that a relatively simple, but systematic, analysis can provide insight into the mechanisms generating the complex behaviours observed in these example reaction networks.

4.1 Analysis of the cell cycle model

We consider the reaction network, described by (1), involved in the cell division cycle of frog eggs. With the parameter values given in Fig. 1, the system displays sustained oscillations in all proteins. Applying the analysis method proposed above we aim at determining which proteins of the reaction network, and corresponding interactions, are most instrumental in generating the periodic oscillations.

The first step is to determine the unstable steady state solution $\mathbf{x_0}$ underlying the complex behaviour in question. This is trivially determined by solving $\dot{\mathbf{x}} = \mathbf{f}(\mathbf{x_0}, \mathbf{p}) = 0$, for the given parameters $\mathbf{p}$. However, since a system can have multiple steady states, the steady state underlying the considered behaviour should in general be determined using continuation from a bifurcation point, for some parameter p_i, at which the complex behaviour is born.

From the linearisation around the underlying steady state we find that the closed-loop system has a complex conjugate pair of poles at $s^* = 0.041 \pm j0.153$. For $\omega = \omega_{crit} = 0.104$ rad/min the critical eigenlocus $\lambda_{crit}(L(j\omega))$ crosses the real axis to the right of of the critical point (1,0). Decomposition of the linear model according to (4) reveals that the open-loop system is stable, implying that no autocatalytic reactions are involved in the cell cycle reaction network (1).

We first determine the smallest real perturbations Δ_i in the feedback of the individual proteins that will stabilise the closed-loop system. The results are given in Fig. 6*a*. As can be seen from the Figure, only three proteins require perturbations of a magnitude less than unity to stabilise the system. These correspond to MPF (x_5), the anaphase-promoting complex APC (x_9), and the enzyme IEP (x_8). Indeed, we find that the linear subsystem containing only these three components has an oscillating instability. Also, keeping all other components at their steady state values and simulating the non-linear model with the three differential equations for x_5, x_8, and x_9 yields MPF oscillations, as can

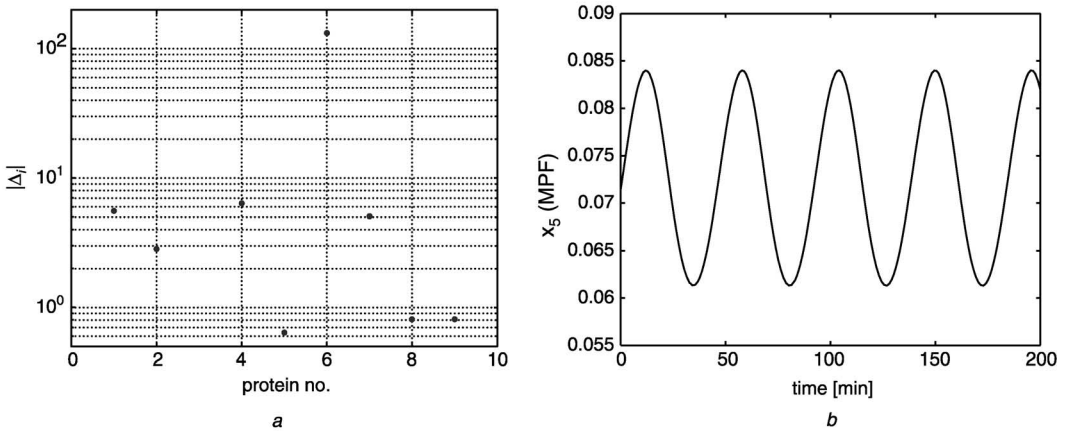

Fig. 6 *Analysis of the cell cycle control model*

a Magnitude of the relative real perturbation Δ_i in the feedback of component i required to stabilise the steady state of the cell cycle network at $k_1 = 0.01$
b MPF oscillations within the reduced reaction network, where only the differential equations for x_5, x_8, and x_9 were included, keeping all other concentrations x_i at their steady state values

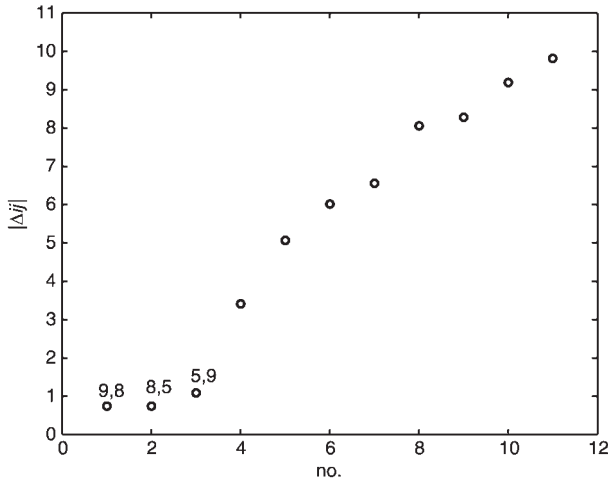

Fig. 7 *Analysis of the cell cycle model. The magnitude of the relative perturbations Δ_{ij} required in element L_{ij} of the open-loop model, i.e. the effect of component j on component i in absence of feedback interactions, to stabilise the closed-loop system*

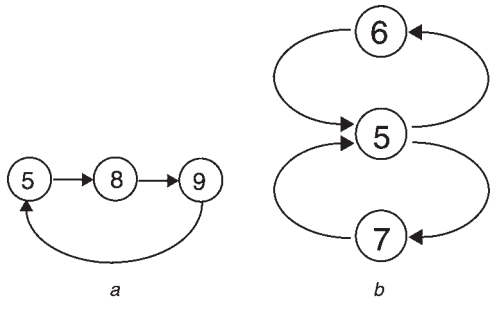

Fig. 8 *Identified interactions behind control mechanisms in the cell cycle model*

a Component interactions underlying sustained oscillations and
b Component interactions underlying bistability in the cell cycle reaction network
The numbers i refer to the concentration x_i of the ith component

be seen in Fig. 6*b*. Thus, the result provides a strong indication that it is the subnetwork consisting of these three components that is the source of the oscillations observed in the cell cycle control network.

The above result is supported by the computed magnitudes of the required relative changes in the single elements of the open-loop system $\mathbf{L}(s)$ that will stabilise the steady state. Figure 7 shows these values for the non-zero elements in $\mathbf{L}(s)$, evaluated at the frequency $\omega_{crit} = 0.104$ rad/min. From the Figure it can be seen that the most important pairwise interactions correspond to the elements $L_{9,8}$, $L_{8,5}$, and $L_{5,9}$, i.e. involving the same components x_5, x_8, and x_9, as found to be most important above. This result furthermore suggests that there is a scalar feedback loop $5 \rightarrow 8 \rightarrow 9 \rightarrow 5$, see Fig. 8*a*, which creates the oscillatory behaviour of the network.

The results above confirm previous results found by Tyson *et al.* through hypothesis postulation and testing. The main purpose of our study, however, is to show that the central mechanisms underlying specific behaviours and functions in biochemical networks of the cell can be determined in a simple and highly systematic fashion. Furthermore, the method is equally applicable to networks of essentially any complexity.

To complete the study of the cell cycle model (1) we consider determination of the mechanism causing bistability in the reaction network. To analyse this mechanism we linearise the model around the unstable steady state for $k_1 = 0.0007$, corresponding to the middle of the unstable branch between the two saddle-node bifurcations (see Fig. 2). Figure 9*a* shows the smallest real perturbations Δ_i that will stabilise this steady state. From the Figure it can be seen that the components corresponding to x_5, x_6, and x_7 appear to be most instrumental for the static instability of the closed-loop system. This is confirmed by the plot of the element-wise perturbations Δ_{ij}. There are in fact only two elements of the open-loop system $\mathbf{L}(s)$ that can be perturbed to render the network stable. Both correspond to interactions involving proteins number 5, 6, and 7. Indeed, we find that retaining only the differential equations for the components 5, 6, and 7 in the model (1), while keeping all other concentrations x_i at their steady state values, yields a static instability at the considered steady state. The bifurcation diagram, with the steady state of component 4 ($x_{4,ss}$) as the bifurcation parameter, for the corresponding three-state model is shown in Fig. 10, showing that bistability is obtained with all concentrations except x_5, x_6 and x_7 kept constant. The reason for using $x_{4,ss}$ as the bifurcation parameter is that k_1 is not influencing the differential equations for x_5, x_6, and x_7 directly, but rather via the steady state concentration of x_4.

In conclusion, through a systematic model analysis of the biochemical reaction network controlling the cell cycle in

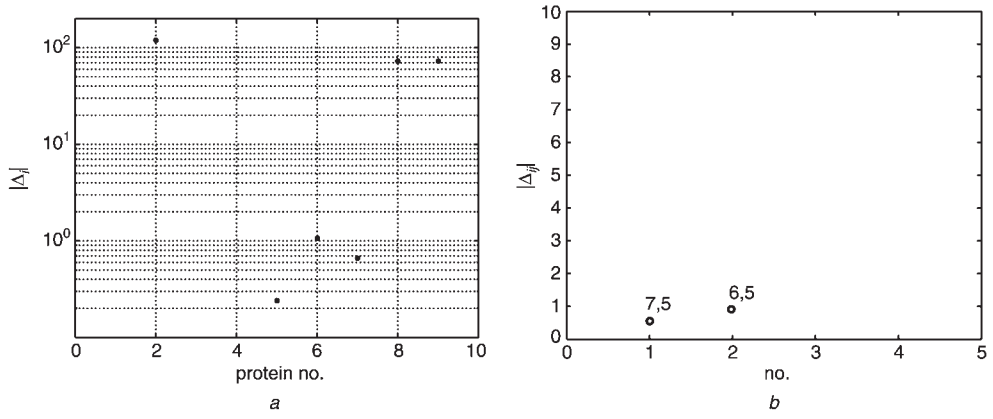

Fig. 9 *Analysis of the cell cycle model. The magnitudes of the required relative perturbations stabilising the underlying steady state at* $k_1 = 0.0007$

a Δ_i
b Δ_{ij}

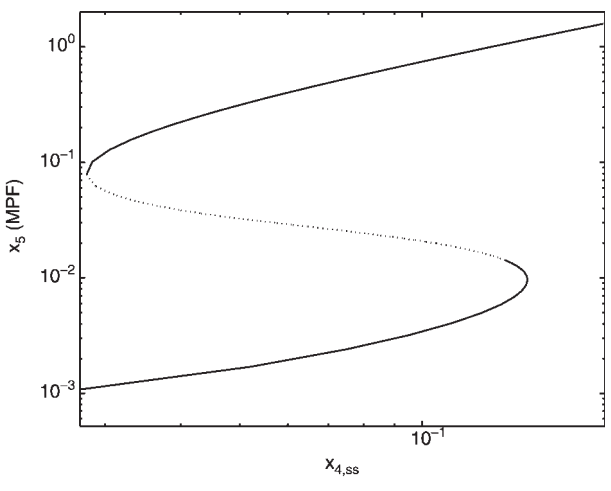

Fig. 10 *Bifurcation diagram of the three-state cell cycle model with states* x_5, x_6 *and* x_7. *The Figure shows MPF solutions as function of the steady state concentration of component 4. Stable steady states are indicated by full lines, unstable steady states by dotted lines*

frog eggs, we have been able to determine the component interactions that are causing the sustained oscillation at high rates of cyclin synthesis, as well as those causing the bistable switch at low cyclin synthesis rates.

4.2 Analysis of a model for circadian oscillations

As a second example we consider a model for circadian rhythms in *Drosophila*, proposed by [16]. Circadian rhythms are limit-cycles with a period of about 24 hours, corresponding to regular changes in physiological functions like, e.g. sleep, body temperature, etc. The considered reaction network contains ten components and the model is given by

$$
\begin{aligned}
\dot{x}_1 &= v_{sP}\frac{K_{IP}^n}{K_{IP}^n + x_{10}^n} - v_{mP}\frac{x_1}{K_{mP} + x_1} - k_d x_1 \\
\dot{x}_2 &= k_{sP}x_1 - V_{1P}\frac{x_2}{K_{1P} + x_2} + V_{2P}\frac{x_3}{K_{2P} + x_3} - k_d x_2 \\
\dot{x}_3 &= V_{1P}\frac{x_2}{K_{1P} + x_2} - V_{2P}\frac{x_3}{K_{2P} + x_3} \\
&\quad - V_{3P}\frac{x_3}{K_{3P} + x_3} + V_{4P}\frac{x_4}{K_{4P} + x_4} - k_d x_3 \\
\dot{x}_4 &= V_{3P}\frac{x_3}{K_{3P} + x_3} - V_{4P}\frac{x_4}{K_{4P} + x_4} \\
&\quad - k_3 x_4 x_8 + k_4 x_9 - v_{dP}\frac{x_4}{K_{dP} + x_4} - k_d x_4 \\
\dot{x}_5 &= v_{sT}\frac{K_{IT}^n}{K_{IT}^n + x_{10}^n} - v_{mT}\frac{x_5}{K_{mT} + x_5} - k_d x_5 \\
\dot{x}_6 &= k_{sT}x_5 - V_{1T}\frac{x_6}{K_{1T} + x_6} + V_{2T}\frac{x_7}{K_{2T} + x_7} - k_d x_6 \\
\dot{x}_7 &= V_{1T}\frac{x_6}{K_{1T} + x_6} - V_{2T}\frac{x_7}{K_{2T} + x_7} \\
&\quad - V_{3T}\frac{x_7}{K_{3T} + x_7} + V_{4T}\frac{x_8}{K_{4T} + x_8} - k_d x_7 \\
\dot{x}_8 &= V_{3T}\frac{x_7}{K_{3T} + x_7} - V_{4T}\frac{x_8}{K_{4T} + x_8} \\
&\quad - k_3 x_4 x_8 + k_4 x_9 - v_{dT}\frac{x_8}{K_{dT} + x_8} - k_d x_8 \\
\dot{x}_9 &= k_3 x_4 x_8 - k_4 x_9 - k_1 x_9 + k_2 x_{10} - k_{dC} x_9 \\
\dot{x}_{10} &= k_1 x_9 - k_2 x_{10} - k_{dN} x_{10}
\end{aligned}
\tag{11}
$$

where x_i corresponds to the concentration, or activity, of the ith component. The key component of the reaction network is the nucleic PER-TIM complex C_N (component 10) that inhibits the transcription of the *per* mRNA, M_P (component 1) and of the *tim* mRNA, M_T (component 5), which again synthesise the PER P_0 and the TIM T_0 proteins (components 2 and 6). After two reversible phosphorylations of PER $P_{1,2}$ (components 3 and 4) and TIM $T_{1,2}$ (components 7 and 8), the doubly phosphorylated PER and TIM proteins combine reversibly to the cytosolic PER-TIM complex C (component 9). This complex is then reversibly transported into the nucleus. Several degradation reactions are included in the model, for which the degradation of the doubly phosphorylated TIM protein, described by the rate constant v_{dT}, is sensitive to light.

This reaction network has been modelled as two identical loops, coupled via components 9 and 10. By identical we here imply that the dynamics for the PER and TIM proteins, and the corresponding mRNAs, are modelled identically and that we furthermore consider identical kinetic parameters. The very construction of the model implies that the source of the oscillations are more or less given. However, the aim here is to test the ability of the proposed method, which is intended for general biochemical networks, to identify the key mechanisms generating specific behaviours.

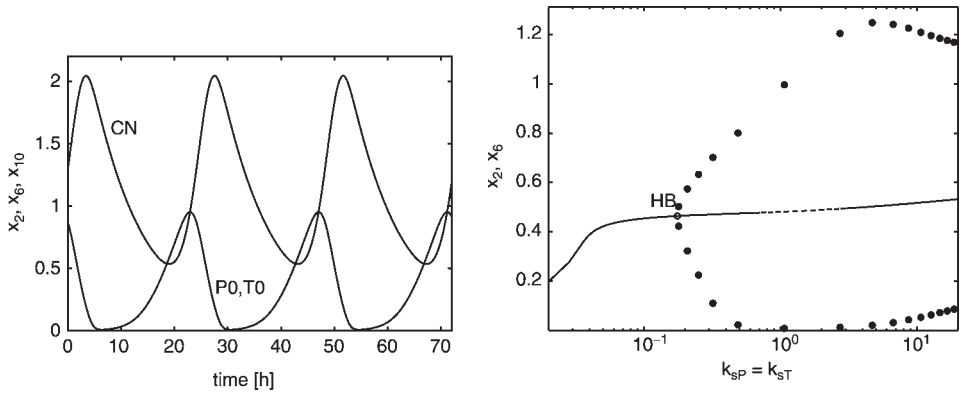

Fig. 11

a Oscillations within the biochemical reaction network (11) for circadian rhythms. Parameters: $v_{sP} = v_{sT} = 1$, $v_{dP} = v_{dT} = 2$, $k_1 = 0.6$, $k_3 = 1.2$, $K_{mP} = K_{mT} = K_{dP} = K_{dT} = 0.2$, $V_{1P} = V_{1T} = 8$, $V_{2P} = V_{2T} = 1$, $V_{3P} = V_{3T} = 8$, $V_{4P} = V_{4T} = 1$, $v_{mP} = v_{mT} = 0.7$, $k_{sP} = k_{sT} = 0.9$, $k_2 = 0.2$, $k_4 = 0.6$, $K_{1P} = K_{1T} = K_{2P} = 2$, $K_{3P} = K_{3T} = K_{4P} = K_{4T} = 2$, $K_{IP} = K_{IT} = 1$, $k_d = k_{dC} = k_{dN} = 0.01$, $n = 4$
b Bifurcation diagram showing the PER (x_2) and TIM (x_6) steady state solutions for different values of the synthesis rates for these proteins. Note that both synthesis rates are chosen to have the same values

Thus, it is of interest to see if the proposed systematic method is able to identify the 'correct' mechanism in the circadian rhythm model, as proposed in [16], solely by considering the unstructured models equations (11).

The parameters we consider here have been determined to lead to a period close to 24 hours under conditions corresponding to constant darkness [16]. Figure 11*a* shows the oscillatory behaviour of the concentrations of the nucleic PER-TIM complex C_N (x_{10}), and the unphosphorylated PER and TIM proteins P_0, T_0 (x_2, x_6). Note that all concentrations x_i within (11) oscillate with the same period. The corresponding bifurcation diagram, with the parameters $k_{sP} = k_{sT}$ as bifurcation parameters, is shown in Fig. 11*b*. We here change the two parameters simultaneously, as we consider only the case where the coupled oscillators are parameterised identically. As can be seen, for the nominal values of $k_{sP} = k_{sT} = 0.9$ the system has only one unstable steady state surrounded by a stable limit cycle. Following the approach outlined above, we linearise the non-linear model around the unstable steady state and analyse the component interactions inducing instability of this steady state.

The system linearised around the steady state for $k_{sP} = k_{sT} = 0.9$ has a complex pair of unstable poles $s = 0.102 \pm j0.38$. Based on the decomposed linear model, we compute the perturbations of pairwise interactions Δ_{ij} required to stabilise the steady state of the network, as computed from the RGA at $\omega_{crit} = 0.269$ rad/h, see Fig. 12. As can be seen from the Figure, the most instrumental interactions for the dynamic instability underlying the circadian oscillations involve the impact from component 9 on component 10, i.e. $L_{10,9}$. Successively adding the second most instrumental interactions until the resulting system is becoming unstable, which happens after adding $L_{8,7}$, leads to the interaction diagram in Fig. 13. The Figure shows one closed-loop, corresponding to the TIM oscillator and one not completely closed-loop corresponding to the PER oscillator, where the interaction corresponding to $L_{4,3}$ is missing. This result shows that around the considered steady state the loop consisting of the interactions $5 \to 6 \to 7 \to 8 \to 9 \to 10 \to 5$ is sufficient to induce oscillations. This means that a minimum of six of the 20 non-zero elements in the open-loop system $L(s)$ are needed to create the oscillating behaviour around the considered steady state. The diagram in Fig. 13 and the plot in Fig. 12 suggests also to test the case where $L_{4,3}$ is considered instead of $L_{8,7}$, resulting in a closed-loop for the PER oscillator and an opened loop for the TIM oscillator. The additional result is that also the loop consisting only of the interactions $1 \to 2 \to 3 \to 4 \to 9 \to 10 \to 1$ is inducing oscillations, which is not surprising as the two oscillators are identical. This also explains why the interaction analysis is unable to discriminate between the elements of the two loops. Note, however, that the analysis reveals that a number of pairwise dynamic interactions present in the model are unimportant for the creation of oscillations. In fact, we find that only 11 out of a total of

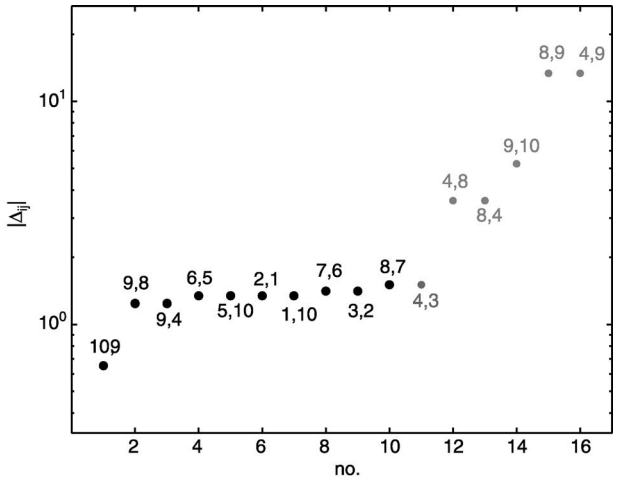

Fig. 12 *Magnitudes of the required relative perturbations Δ_{ij}, stabilising the steady state underlying the circadian rhythm in Drosophila at $k_{sP} = k_{sT} = 0.9$*

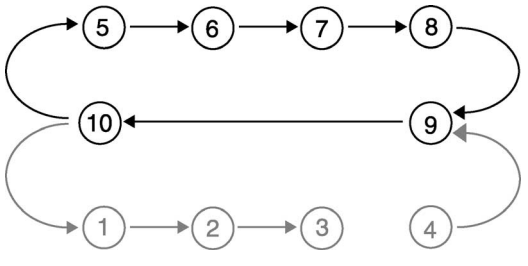

Fig. 13 *Component interactions generating sustained oscillations in the circadian rhythm network. The numbers i refer to the component i*

20 pairwise protein interactions are required to create the double-loop oscillator. For instance, as can be seen from the results in Fig. 12 the direct interactions between proteins 4 and 8 only have a small influence on the stability of the steady state and are not part of the feedback loops creating the double oscillator.

In summary, based on a linear systems analysis, we find directly from the model equations that the feedback mechanism creating the circadian rhythm in Drosophila consists of a double feedback loop, connected via the PER-TIM complexes x_9 and x_{10}. However, the results also reveal that only one out of the two loops in the model of [16] are required to create the circadian oscillations. The latter result supports the simpler single-loop model proposed earlier in [32]. This result also suggests that the existence of a double-loop is for the purpose of robustness of the oscillatory behaviour.

5 Summary and conclusions

The work in this paper has been based on the assumption that complex behaviours, such as multiple steady states and limit cycles, in biochemical reaction networks, have their origin in feedback mechanisms, destabilising a steady state that underlies the considered behaviour. Based on this we proposed a method for determining subnetworks being the source of a given behaviour, corresponding to a specific cell function. The method is based on a decomposition of the system model, in the form of a set of ordinary differential equations with known kinetic parameters, into a network of feedback interconnected subsystems, each being of significantly lower complexity than the overall system. The impact of perturbations of single feedback loops and pairwise component interactions, in destabilising the steady state, can then be determined and rank ordered.

As an example of a moderately complex biological system we considered the model of the cell cycle control in frog eggs. Using results from linear feedback control and linear systems theory we determined the feedback mechanisms which are the main sources of the limit cycle and bistable behaviour present in the network, respectively. The results obtained are consistent with results previously obtained from non-linear simulation and bifurcation analysis, combined with biological insight. Furthermore, we used the proposed method to analyse a model of circadian oscillations.

One aim of the presented work was to consider how far one can come with relatively simple tools in a systematic analysis of complex biochemical networks. The strength of the proposed method is that highly complex systems are greatly simplified, allowing consideration of simple linear subsystems. By employing powerful tools from feedback control theory one can gain insight into where in a complex biochemical reaction system the source of a certain behaviour is located. This information can, for example, be used to guide the modeller as to which parts of the network to focus on when modelling specific functions.

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