The regulation of gene expression in eukaryotes: bistability and oscillations in repressilator models

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The regulation of gene expression in eukaryotes: bistability and oscillations in repressilator models

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Abstract

To model the regulation of gene expression in eukaryotes by transcriptional activators and repressors, we introduce delays in conjugation with the mass action law. Delays are associated with the time gap between the mRNA transcription in the nucleoplasm and the protein synthesis in the cytoplasm. After re-parameterisation of the m-repressilator model with the Hill cooperative parameter n, for $n = 1$, the m-repressilator is deductible from the mass action law and, in the limit $n \to \infty$, it is a Boolean type model. With this embedding and with delays, if m is odd and $n > 1$, we show that there is always a choice of parameters for which the m-repressilator model has sustained oscillations (limit cycles), implying that the 1-repressilator is the simplest genetic mechanism leading to sustained oscillations in eukaryotes. If m is even and $n > 1$, there is always a choice of parameters for which the m-repressilator model has bistability.

Keywords: regulation of gene expression in eukaryotes, delays, m-repressilator, limit cycles, bistability

1. Introduction

In the cell, protein synthesis begins with the transcription of mRNA from its DNA template. Then, the mRNA is translated into protein in the ribosomes. This is the central dogma of molecular biology, as discussed in Crick

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(1967) and Crick (1970). This mechanism of protein synthesis is common to both prokaryote and eukaryote organisms.

As prokaryote organisms lack a nuclear membrane, both DNA molecules and ribosomes are located in the cytoplasm. In eukaryotes, the DNA molecules are in the nucleoplasm and the ribosomes are in the cytoplasm. This different localisation of the DNA implies that, in eukaryotes, for protein translation to occur, the mRNA molecules have to migrate through the nucleoplasm, crossing the nuclear membrane.

The transcription of mRNA is done by a catalytic enzymatic process involving the RNA polymerase enzyme that is continuously produced in the cell. In this process, the DNA is the catalyst. The RNA polymerase binds to the promoter regions of the DNA and translation begins. Some genes have control regions where transcription activators and repressors can bind, enabling the activation or inhibition of the mRNA transcription, Alberts *et al.* (2008). At the molecular spatial scale and during short time scales, it is assumed that RNA polymerase molecules move randomly in the cytoplasm (prokaryotes) or in the nucleoplasm (eukaryotes). In models, it is implicitly assumed that the mRNA motion is intrinsically stochastic, Larson *et al.* (2009).

A general mathematical framework to describe quantitatively the transcription, translation and protein synthesis in prokaryotes, based on the mass action law, has been proposed in Alves & Dilão (2005). This class of models is built with genes and proteins. The mass action law assumes that reaction kinetics result from the random motion of the molecules present in the media, Gillespie (1977, 1992); Gardiner (1997); van Kampen (2007), and all the model parameters have a biochemical meaning. However, as in eukaryotes ribosomes are separated from the DNA template by the nuclear wall, the mobility of mRNA is constrained. The transit from inside to outside the nucleus is done through porous in the nuclear membrane, and no active transport process is known. The nuclear wall has the effect of delaying both the entry of proteins into the nucleus and the transport of mRNA chains to ribosomes. Mathematical modelling of single cell gene expression with stochastic techniques shows the existence of delays between transcription and translation in eukaryotes, Larson *et al.* (2009). Experiments inducing protein synthesis in *Drosophila* have quantified that the temporal delay between the mRNA transcription and the protein synthesis can be in the range $8 - 12$ min, O'Brien & Lis (1993).

To model the regulation of gene expression in eukaryotes by transcrip-

tional activators and repressors, we propose here to introduce delays in conjugation with the mass action law. Several authors have introduced delays in systems biology models, Allwright (1977), Mackay & Glass (1977), Murray (1989), Fall (2002), Chen & Aihara (2002), Chen *et al.* (2004), Smith (2011), among others. These models have been used to describe the intrinsic delays between the production of a substance and its action on a specific target. The stability analysis of specific low dimensional linear models evolving delay effects between mRNA transcription and protein production have been analysed by Monk (2003) and Mincheva & Roussel (2007). In these examples, delays were important to explain the oscillations or even chaos in the observed phenomena. On the other hand, in some of these models, the functional response of the systems was described by Hill type functional forms, which have neither a direct correspondence nor a theoretical justification within the microscopic dynamical mechanisms associated with the mass action law.

It is known that some biochemical systems described by the mass action law have a stable steady state in phase space, but when modified to a Hill type functional description they show oscillatory behaviour. The best well-known example is the (3-)repressilator model, Elowitz & Leibler (2000). The (3-)repressilator model has a steady state for the corresponding mass action description, but, in the framework of the Hill type response functions, it shows oscillatory behaviour with limit cycles in phase space, Buse *et al.* (2010); Kuznetsov & Afraimovich (2012). Due to the intrinsic difficulty in calibrating models of protein synthesis with experimental or observational data, it is not known if the oscillations emerging from the Hill specific parameterisation have a biological meaning or if they are a mathematical artefact. In the enzyme kinetics literature, the Hill approach is generally criticised as non realistic, (Bisswanger, 2008, pp. 32-43).

One of goals here is to have a biologically meaningful and physically consistent framework for the modelling of the regulation of gene expression in eukaryotes. To be consistent with the mechanism of molecular interactions and dynamics, we follow a strategy where only bi-molecular kinetic mechanism based on the mass action law are considered. Then, delays are introduced whenever we consider both the action of transcription activators and repressors on genes and the corresponding protein synthesis. Within this approach, we analyse mechanisms that can eventually lead to biological oscillations (damped or persistent) and are described with a family of parameterised models containing the mass action law, the Hill type response

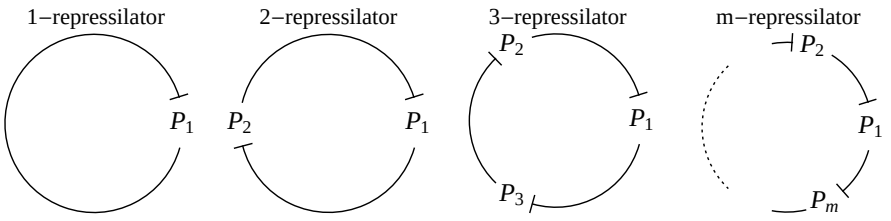

Figure 1: Graphs describing the different types of repressilator interactions. P_i represent proteins and the end symbol $\dashv$ indicates that the interactions are inhibitory.

functions and the Boolean formalism. The repressilator model is one of the models that best adapts to these conditions.

This paper is organised as follows. In section 2, using the mass action law, we derive the 1-repressilator model. In this model, a protein is produced and, as its concentration increases, it represses its own production, figure 1. Re-parameterising this model with the Hill extra parameter n, for $n = 1$, the model is deductible from the mass action law. For $n > 1$, we have a Hill type empirical description, and, in the limit $n \to \infty$, we obtain a Boolean type model. Then, for these different cases with and without delays, the asymptotic solutions of the family of parameterised models have been analysed. In section 3, we analyse the general m-repressilator model, figure 1, and we narrow this analysis to the 2- and 3-repressilator models, with and without delays. In section 4, we analyse the general solutions of the Boolean m-repressilator models for $m \geq 2$. In this case, we show that all the asymptotic solutions are bistable or oscillatory stable of limit cycle type, depending if m is even or odd, respectively. Finally, in section 5, we summarise and discuss the main conclusions of the paper. To simplify the flow of this exposition, all the formal proofs were moved to the Appendix A.

2. The 1-repressilator model

The 1-repressilator model describes the production of a protein that represses itself by binding to some operator site in its template DNA, figure 1. Following the mathematical formalism developed in Alves & Dilão (2005) and the discussion therein, in prokaryote organisms, the kinetic diagrams

describing the 1-repressilator protein production model are,

$$\begin{array}{l} \mathrm{G} \xrightarrow{k_1} \mathrm{G} + \mathrm{P} \\ \mathrm{P} + \mathrm{G} \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} \mathrm{G_P} \\ \mathrm{P} \xrightarrow{k_3} \end{array} \tag{1}$$

where P, G and $\mathrm{G_P}$ represent, respectively, the protein, its gene template and the gene with the protein bound to the DNA repressor site. The first diagram in (1) describes the protein free production, and the last diagram represents protein degradation. The second diagram represents the inactivation of the gene by the protein. The constants k_i are the rates at which production, inhibition and degradation occur.

From (1) and the mass action law, Alves & Dilão (2005); Dilão & Muraro (2010), the time evolution of the protein concentrations is described by the system of ordinary differential equations,

$$\begin{cases} \dot{\mathrm{G}} = k_{-2}\mathrm{G_P} - k_2 \mathrm{G.P} \\ \dot{\mathrm{G}}_\mathrm{P} = -k_{-2}\mathrm{G_P} + k_2 \mathrm{G.P} \\ \dot{\mathrm{P}} = k_{-2}\mathrm{G_P} - k_2 \mathrm{G.P} + k_1 \mathrm{G} - k_3 \mathrm{P} \end{cases} \tag{2}$$

with the conservation law,

$$\mathrm{G}(t) + \mathrm{G_P}(t) = \mathrm{G}(0) \tag{3}$$

and G(0) is the initial concentration of the gene G. As usual, the small dots represent derivatives in order to time t. Introducing (3) into (2), in order to eliminate the equation for $\mathrm{G_P}$ and applying the steady state simplification $\dot{\mathrm{G}} = 0$ to eliminate G, we obtain the equation for the 1-repressilator model,

$$\dot{\mathrm{P}} = \frac{k_1 \mathrm{G}(0)}{1 + \frac{k_2}{k_{-2}} \mathrm{P}} - k_3 \mathrm{P}\,. \tag{4}$$

Due to the conservation law (3) and the steady state assumption, the concentrations of $\mathrm{G_P}$ and of G are given by, $\mathrm{G_P} = k_1 \mathrm{G}(0)\mathrm{P}(t)/(k_{-2} + k_2 \mathrm{P})$ and $\mathrm{G} = k_1 \mathrm{G}(0)/(k_{-2} + k_2 \mathrm{P})$. Equation (4) describes the production of the self inhibiting or self repressing protein P.

To adimensionalize equation (4), we introduce the new variables, $x = \mathrm{P}k_3/(k_1 \mathrm{G}(0))$, $\tau = t/k_3$ and the new constant, $\gamma = k_1 k_2 \mathrm{G}(0)/(k_{-2} k_3) > 0$.

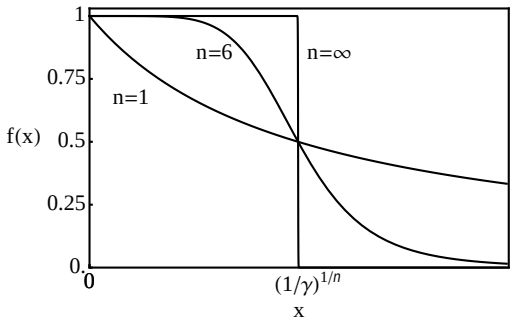

Figure 2: Graphs of the family of regulatory functions $f(x)$ defined in (6), describing the self-repressive behaviour of a regulatory protein. For $n = 1$, the regulatory function $f(x)$ is derived from the mass action law. For $n > 1$, the regulatory function has the Hill or inverted sigmoidal form and, for $n = \infty$, $f(x)$ is a Boolean or step function.

Substitution of these variables into (4) leads to the 1-repressilator adimensional model equation,

$$\frac{dx}{d\tau} = \frac{1}{1+\gamma x} - x\,. \tag{5}$$

In order to include the Hill type functional form in the 1-repressilator model, we introduce the Hill parameter n into (5), obtaining,

$$\frac{dx}{d\tau} = \frac{1}{1+\gamma x^n} - x = f(x) - x\,. \tag{6}$$

Some authors call n the cooperative parameter, Kuznetsov & Afraimovich (2012). In this way, the 1-repressilator model is embedded in a larger class of models.

Equation (6) describes a mechanism of protein production with self-repression in a prokaryote organism, and, for $n = 1$, it is derivable from the mass action law. If $n > 1$, the physical mechanisms associated with the mass action law and leading to (6) are no longer true. In figure 2, we show, for several values of n, the graph of the regulatory function $f(x)$. For $n > 1$, the function $f(x)$ has the inverted sigmoidal Hill form and, for $n \to \infty$, $f(x)$ converges to a Boolean or step function. Due to the particular form of the function $f(x)$, for large values of n, it is sometimes argued that $f(x)$ describes a threshold mechanism. This threshold occurs for $x_{th} = (1/\gamma)^{1/n}$, and $f(x_{th}) = 1/2$.

By a simple analysis, it follows that the equation (6) has a unique stable fixed point or steady state. For any value of $n \geq 1$, and as $\tau \to \infty$, the protein concentration converges to a steady state. For $n = 1$, the fixed point is $x^* = (-1 + \sqrt{1 + 4\gamma})/(2\gamma)$ and, in the limit $n \to \infty$, $x^* \to 1$.

Let us assume now that the protein x is produced in an eukaryote organism. In this case, there exists a delay between the protein synthesis and the effective transcriptional inhibition. To model this effect, the equation (6) is transformed into the new equation,

$$\frac{dx}{d\tau} = \frac{1}{1 + \gamma x^n(\tau - s)} - x(\tau) \tag{7}$$

which is an ordinary differential equation with delays. In this case, the delay is represented by s, being s the mean (renormalised) time that the protein takes to reach the interior of the nucleus of the cell of the eukaryote organism. To be more precise, in this cases, we must represent the delayed contribution for the kinetics by the diagram $\mathrm{P} + \mathrm{G} \xrightarrow{k_2}_r \mathrm{G_P}$, explicitly indicating that the delay $r = sk_3$ is associated with the reaction ratio k_2.

From the mathematical point of view, equation (7) defines an infinite dimensional dynamical system. In fact, the solutions of equation (7) depend on the choice of continuous real functions $\phi(t) : [-s, 0] \to \mathbb{R}_{\geq 0}$, where $x(t) = \phi(t)$, for $t \in [-s, 0]$. The infinite dimensionality of the dynamical system comes from the infinite dimensionality of the set of admissible ϕ-functions. In the following, we will consider solutions of delay equations with initial conditions $\phi(t) \equiv 0$, for $t \in [-s, 0]$.

For sufficient small values of the delay s, the asymptotic solutions of equations (6) and (7) coincide, Smith (2011), and the steady state solution x^* is stable. For larger delays, to determine the stability of the steady state x^*, we have to linearise equation (7) around x^*.

Let x^* be the unique fixed point of the delay equation (7). The point with coordinate $x^* \equiv x^*(\gamma, n)$ is the unique positive real root of the equation $1/(1 + \gamma x^n) - x = 0$. The delay equation obtained by linearising (7) around the steady state x^* is,

$$\frac{dx}{d\tau} = -\gamma n (x^*)^{n+1} \left(x(\tau - s) - x^*\right) - \left(x(\tau) - x^*\right) . \tag{8}$$

With $y = x - x^*$ and the new parameter $\beta = \gamma n (x^*)^{n+1} = n(1 - x^*(\gamma, n)) > 0$, equation (8) becomes,

$$\frac{dy}{d\tau} = -\beta y(\tau - s) - y(\tau) . \tag{9}$$

The stability of the steady state x^* of equation (7) is determined by the stability of the zero steady state of equation (9). Using standard techniques of stability analysis for delay equations, Hale (1977) and Smith (2011), we now construct the characteristic equation associated with (9). Assuming that the solution of equation (9) has the form $y(\tau) = e^{\lambda\tau}$ and after substitution of this solution into equation (9), we obtain the characteristic equation,

$$p(\lambda) = \lambda + 1 + \beta e^{-\lambda s}\,. \tag{10}$$

Lemma 1. *Let $p(\lambda)$ be the characteristic equation of the linear delay equation (9), with $\beta > 0$ and $s > 0$, and let δ_1 be the unique solutions of the equation, $-\tan(s\delta) = \delta$, with $\delta \in]\pi/(2s), \pi/s[$. Let $\beta_0 = e^{-s-1}/s$ and $\beta_1 = -1/\cos(\delta_1 s) > 0$. Then, the roots of the characteristic equation (10) are:*

a) If $\beta < \beta_0$, $p(\lambda)$ has two real negative roots.
b) If $\beta = \beta_0$, $p(\lambda)$ has one real negative root.
c) If $\beta_0 < \beta < \beta_1$, $p(\lambda)$ has two complex conjugate roots with $Real(\lambda) < 0$.
d) If $\beta = \beta_1$, $p(\lambda)$ has two complex conjugate roots with $Real(\lambda) = 0$.
e) If $\beta > \beta_1$, $p(\lambda)$ has two complex conjugate roots with $Real(\lambda) > 0$.

The above lemma establishes the conditions of applicability of the Hopf bifurcation theorem to the adimensional non-linear delay equation (7).

Theorem 2. *Consider the delay equation (7), with $\gamma > 0$, $n \geq 1$, $s > 0$, and the positive parameter $\beta = n(1-x^*(\gamma, n))$, where $x^*(\gamma, n)$ is the coordinate of the unique positive fixed point of (7). Let $\beta_0 > 0$ and $\beta_1 > 0$ be the constants as defined in Lemma 1, and let $x(t) = \phi(t) = 0$, for $t \in [-s, 0]$, be the initial data. Then, we have,*

a) If $\beta \leq \beta_0$, then the fixed point x^* is asymptotically stable.
b) If $\beta_0 < \beta < \beta_1$, then the fixed point x^* is (oscillatory) asymptotically stable.
c) If $\beta = \beta_1$, then the fixed point x^* has a supercritical Hopf bifurcation and x^* is asymptotically stable.
d) If $\beta > \beta_1$, then for initial conditions away from the unstable fixed point, the delay equation (7) has an asymptotically stable periodic solution.

As the conditions on β of theorem 2 encapsulate the parametric information about the coordinates of the fixed point $x^*(\gamma, n)$, we must be more specific.

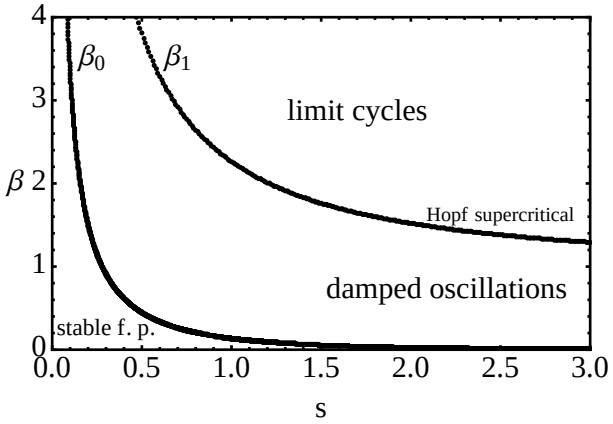

Figure 3: Bifurcation diagram for the delay equation (7), for solutions reached from the initial condition $x(t) = \phi(t) = 0$, for $t \in [-s, 0]$. The functions $\beta_0(s)$ and $\beta_1(s)$ are defined in lemma 1. In the region marked "damped oscillations", the asymptotic solutions of the delay equation converge to the stable fixed point (f.p.) x^*. Limit cycle solutions are asymptotically stable oscillatory solutions of the delay equation.

Corollary 3. *The delay equation (7) with the choice $n = 1$ and initial data $x(t) = \phi(t) = 0$, for $t \in [-s, 0]$, has no asymptotically stable periodic solutions but, for sufficiently large values of s, it can have damped oscillations, asymptotically converging to the fixed point $x^*(\gamma, 1)$. If $n > 1$, for sufficiently small γ and sufficiently large delays s, the equation (7) with initial data $x(t) = \phi(t) = 0$, for $t \in [-s, 0]$, has an asymptotically stable periodic solution (limit cycle), and the fixed point $x^*(\gamma, n)$ is unstable.*

Corollary 4. *In the limit $n \to \infty$, the solutions of the delay equation (7), with initial data, $x(t) = \phi(t) = 0$, for $t \in [-s, 0]$, converge monotonically to the steady state $x^* = 1$.*

From theorem 2, we can construct the bifurcation diagram of the solutions of the delay equation (7) as a function of the delay s and of the parameter $\beta = n(1-x^*(\gamma, n))$, where $x^*(\gamma, n)$ is the coordinate of the unique fixed point of equation (7), figure 3. From corollary 3, it follows that, for $n = 1$, the delay equation (7) has no asymptotically stable oscillatory solutions. However, choosing a Hill parameter $n > 1$, it is always possible to choose values of n such that $\beta = n(1 - x^*(\gamma, n)) > \beta_1(s)$, where $\beta_1(s)$ is the parameter that determines a supercritical Hopf bifurcation. In this case, the delay equa-

tion (7) has asymptotically stable oscillatory solutions (limit cycle solutions), figure 3.

In Table 1, we summarise the type of asymptotic solutions of the model equation (7), with and without delay. Both the mass action law and the Boolean based models don't have asymptotically oscillatory stable solutions, with or without delays. However, for the same model, but with the Hill parameter $n > 1$ and delay $s > 0$, for a suitable choice of the parameters γ and n, it is always possible to have stable oscillations in the protein concentration. In the limit $n \to \infty$, corollary 4 applies.

In figure 4, we show an oscillatory solution of equation (7), for the parameter values $\gamma = 1.0$, $n = 10.0$ and $s = 20.0$. In this case, all the solutions of equation (7) oscillate for delays $s > 2.0$. In the same figure, we also show a damped oscillatory solution obtained with $n = 1$.

	$s = 0$	$s > 0$
$n = 1$ (mass action)	steady state no oscillations	steady state damped oscillations
$n > 1$ (Hill form)	steady state no oscillations	steady state damped and stable oscillations Hopf bifurcation
$n = \infty$ (Boolean form)	steady state no oscillations	steady state no oscillations

Table 1: Asymptotic solutions of the 1-repressilator model equation (7) as a function of the delay s and for different values of the Hill parameter n.

Assuming that the Hill functional form describes genetic transcriptional regulation, we conclude that the 1-repressilator model is the prototype of the simplest genetic mechanism being able to produce oscillations in eukaryote organisms. On the other hand, if this self repressing mechanism of protein synthesis does not show persistent oscillations, two situations can occur. Or we are in a particular region of parameters for which natural delays are not enough to induce oscillations, or the Hill functional form does not accurately describe the mechanism of protein synthesis. These facts stress the importance of the calibration and validation of the mathematical models for the mechanisms associated with the central dogma of molecular biology.

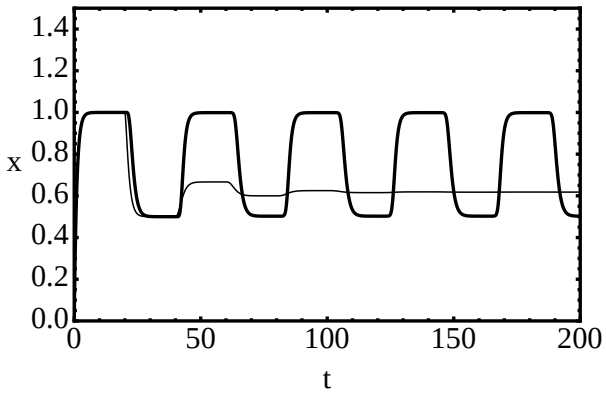

Figure 4: Solution of the delay equation (7), for the parameter values $\gamma = 1.0$, $n = 10.0$, $s = 20.0$ (thick line) and initial condition $x(t) = \phi(t) = 0$, for $t \in [-s, 0]$. For this choice of γ and n, the solution of equation (7) oscillates for delays $s > 2.0$. For the case $n = 1$ and in agreement with corolary 3, the delay equation (7) shows dumped oscillations (thin line).

3. The *m*-repressilator model

We consider m proteins P_i, $i = 1, \ldots, m$, continuously produced by its gene template G_i but repressed by protein P_{i+1}. This sequential process can be viewed as a circular chain of repressor proteins where P_m is repressed by P_1, figure 1. The kinetic diagrams for this process are,

$$\begin{array}{l} \mathrm{G}_i \xrightarrow{k_{3i-2}} \mathrm{G}_i + \mathrm{P}_i \\ \mathrm{P}_{i+1} + \mathrm{G}_i \xrightarrow{k_{3i-1}}_{r} \mathrm{GP}_i \\ \mathrm{P}_{i+1} + \mathrm{G}_i \xleftarrow{k_{-3i+1}} \mathrm{GP}_i \\ \mathrm{P}_i \xrightarrow{k_{3i}} \end{array} \qquad i = 1, \ldots, m \tag{11}$$

and the subscript r in the second diagram indicates that this mechanism is delayed. As we have discussed in the introduction, the delay is due to the re-entry of the protein into the nucleus.

Following the same approach as in the previous section, after substitution of conservation laws and making the steady state simplification, the equations for the time evolution of the proteins are,

$$\dot{\mathrm{P}}_i = \frac{k_{3i-2}\mathrm{G}_i(0)}{1 + \frac{k_{3i-1}}{k_{-3i+1}}\mathrm{P}_{i+1}} - k_{3i}\mathrm{P}_i\,, \qquad i = 1, \ldots, m \tag{12}$$

where $\mathrm{P}_{m+1} = \mathrm{P}_1$. Equations (12) describe the time evolution of proteins involved in the m-repressilator model, figure 1, and as been derived from the mass action law.

With the new variables, $x_i = \mathrm{P}_i/(k_{3i-2}\mathrm{G}_i(0))$, and the new constants $\gamma_i = k_{3i-1}k_{3i-2}\mathrm{G}_i(0)/k_{-3i+1}$ and $\mu_i = k_{3i}$, with $i = 1, \ldots, m$, the system of equations (12) reduce to,

$$\dot{x}_i = \frac{1}{1+\gamma_i x_{i+1}} - \mu_i x_i \,, \qquad i = 1, \ldots, m \,. \tag{13}$$

To include the Hill parameter $n \geq 1$ and the delays associated with the repressing proteins, we transform (13) into the new delay equation,

$$\dot{x}_i = \frac{1}{1+\gamma_i x_{i+1}^n(t-r)} - \mu_i x_i(t) \,, \qquad i = 1, \ldots, m \tag{14}$$

where $x_{m+1} = x_1$ and $r \geq 0$, and the delay term has been introduced only in the terms involving the rate constant k_{3i-1}, as indicated in (11). The systems of equations (14) is the reparameterized m-repressilator model with delays.

Lemma 5. *Consider the delay equation (14) with $\mu_i > 0$ and $\gamma_i > 0$. Then, we have:*

a) If m is odd, equation (14) has a unique fixed point with positive coordinates.

b) If m is even, equation (14) has at least one fixed point with positive coordinates.

By lemma 5, the systems of equations (14) has at least one fixed point with positive coordinates. We denote the generic fixed point by $x^* = (x_1^*, \ldots, x_m^*)$. Linearizing the systems of equations (14) around the fixed point x^*, we obtain,

$$\dot{y} = By(t-r) + Ay(t) \tag{15}$$

where $y(t) = (x(t) - x^*)$,

$$B = \begin{pmatrix} 0 & -\alpha_1 & 0 & \ldots & 0 \\ 0 & 0 & -\alpha_2 & \ldots & 0 \\ \vdots & \vdots & \vdots & \ldots & \vdots \\ 0 & 0 & 0 & \ldots & -\alpha_{m-1} \\ -\alpha_m & 0 & 0 & \ldots & 0 \end{pmatrix}, \quad A = \begin{pmatrix} -\mu_1 & 0 & \ldots & 0 \\ 0 & -\mu_2 & \ldots & 0 \\ \vdots & \vdots & \vdots & \vdots \\ 0 & 0 & \ldots & -\mu_m \end{pmatrix} \tag{16}$$

and $\alpha_i = n\gamma_i(x^*_{i+1})^{n-1}/(1+\gamma_i(x^*_{i+1})^n)^2 = n\gamma_i\mu_i^2(x^*_{i+1})^{n-1}(x^*_i)^2$. The coordinates of the fixed point x^* are also functions of the constants μ_i, γ_i and n.

The characteristic equation of the linear delay equation (15) is easily calculated and we obtain (see Smith (2011) for details),

$$\begin{aligned} p(\lambda) &= \det(\lambda I - A - e^{-\lambda r}B) \\ &= \prod_{i=1}^{m}(\lambda+\mu_i) - (-1)^m e^{-\lambda m r}\prod_{i=1}^{m}\alpha_i\,. \end{aligned} \tag{17}$$

The stability of the fixed points of the delay equations (14) and (15) are determined through the analysis of the roots of the characteristic equation (17). As, by lemma 5, there are differences between m-repressilator models, eventually affecting the stability of asymptotic solutions, we now analyse separately the cases $m = 2$ and $m = 3$.

3.1. The 2-repressilator model

We first consider the case of the 2-repressilator model, figure 1, without delays, $r = 0$. For the case $m = 2$, the characteristic equation (17) reduces to,

$$p(\lambda) = (\lambda+\mu_1)(\lambda+\mu_2) - \alpha_1\alpha_2 e^{-\lambda 2r}\,. \tag{18}$$

With $r = 0$, $\mu_i > 0$ and $\alpha_i > 0$, a simple geometric analysis shows that the characteristic polynomial (18) has two real roots, both negative or one negative and the other positive. In the first case, the fixed points of equations (14) are of stable node type and, in the second case, the fixed points are of saddle type and are unstable.

If one of the steady states (fixed points) of equations (14), for $m = 2$ and $r = 0$, is of saddle type, then this fixed cannot be the unique fixed point of equation (14). Otherwise, the flow on the unstable manifold of the saddle point would go to infinity. This is not possible since it would be incompatible with the direction of the vector field taken along a circular path in phase space with sufficiently large radius.

If $m = 2$, $r = 0$ and $n = 1$ in equations (14), due to the convexity and monotonicity of the vector field components, equations (14) have a unique fixed point of stable node type, figure 5a).

In the extreme case of $n \to \infty$, equations (14), for $m = 2$ and $r = 0$, reduce to,

$$\begin{cases} \dot{x}_1 &= g(x_2) - \mu_1 x_1 \\ \dot{x}_2 &= g(x_1) - \mu_2 x_2 \end{cases} \tag{19}$$

where $g(x) = 1$, for $x \leq 1$, and $g(x) = 0$, otherwise.

If $\mu_1 \geq 1$ or $\mu_2 \geq 1$, the vector field (19) has one fixed point. If $\mu_1 \geq 1$ and $\mu_2 \geq 1$, the coordinates of the fixed point are $(1/\mu_1, 1/\mu_2)$. If $\mu_1 \geq 1$ and $\mu_2 < 1$, the coordinates of the fixed point are $(0, 1/\mu_2)$. If $\mu_1 < 1$ and $\mu_2 \geq 1$, the coordinates of the fixed point are $(1/\mu_1, 0)$. In any of these cases, the fixed point is of stable node type.

If $\mu_1 < 1$ and $\mu_2 < 1$, the vector field (19) has two fixed points with coordinates, $x^*_{n1} = (1/\mu_1, 0)$ and $x^*_{n2} = (0, 1/\mu_2)$. These fixed points are of stable node type. Therefore, for $\mu_1 < 1$ and $\mu_2 < 1$, the dynamics of the system of equations (19) is bistable.

In the bistable case, due to the integrability of equation (19), the boundary of the basin of attraction of the two fixed points can be easily found. For $\mu_1 < 1$ and $\mu_2 < 1$, we can define the set,

$$\begin{aligned} W^s &= \{(x_0, y_0) \in \mathbb{R}^2_{\geq 0} : x_0 e^{-\mu_1 t} = 1, y_0 e^{-\mu_2 t} = 1, \text{ for some } t \geq 0\} \\ &\cup \{(x_0, y_0) \in \mathbb{R}^2_{\geq 0} : \tfrac{1}{\mu_1} - (\tfrac{1}{\mu_1} - x_0) e^{-\mu_1 t} = 1, \tfrac{1}{\mu_2} - (\tfrac{1}{\mu_2} - y_0) e^{-\mu_2 t} = 1, \\ &\quad \text{for some } t \geq 0\} . \end{aligned} \tag{20}$$

Geometrically, the set W^s behaves as the stable manifold of the point $x_s = (1, 1)$. The point in phase space with coordinates $x_s = (1, 1)$ is not a fixed point, and, as is easily derived from (20), is attained in finite time.

Analogously, we can define the set of points that converge backwards in time to x_s, analogously defining a "kind" of the unstable manifold W^u of x_s. In figure 5b) we show $x_s = (1, 1)$, W^s and W^s. The two branches of W^u end up at x^*_{n1} and x^*_{n2}. The set W^s partition the positive quadrant of phase space in two invariant regions, marked A and B in figure 5b). Initial conditions $(x_1(t), x_2(t)) = (\phi_1(t), \phi_2(t))$ in A or B, for $t \in [-r, 0]$, lead to solutions of equations (19) with asymptotic limits x^*_{n1} or x^*_{n2}, respectively.

Thus, if $\mu_1 < 1$ and $\mu_2 < 1$, for some Hill parameter value $n > 1$, there exists a bifurcation from a system with one stable steady state to a system with two stable steady states — bistable system.

To analyse the transition to the bistable system as the Hill parameter n is varied, we analyse the coordinates of the stable fixed points of equations (14). By a simple manipulation, the fixed points of equations (14) with $m = 2$ are

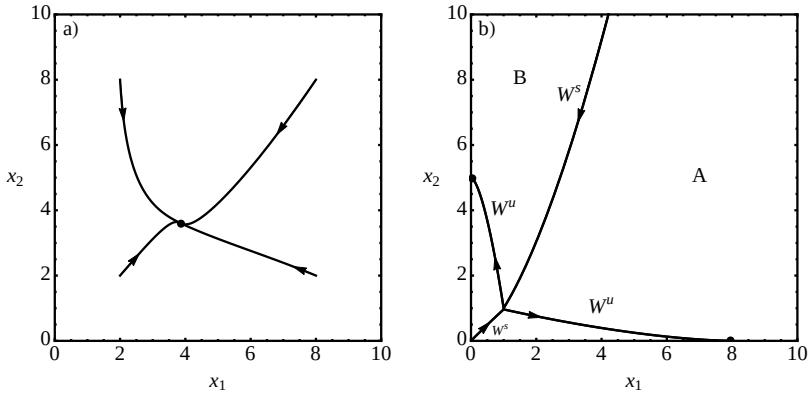

Figure 5: Solutions in phase space of equations (14) and (19), for $m = 2$, $r = 0$, and kinetic parameters, $\mu_1 = 1/8$, $\mu_2 = 1/5$, $\gamma_1 = 0.3$ and $\gamma_2 = 0.1$. Bullets represent fixed points. In a), $n = 1$, we show four solutions converging to the stable steady state. In b), $n = \infty$, we show the stable (W^s) and the unstable (W^u) manifolds of the point $x_s = (1, 1)$. In this case, the set W^s, defined in (20), splits the phase space in two invariant regions denoted by A and B. In both cases a) and b), $\mu_1 < 1$ and $\mu_2 < 1$.

solutions of the equations,

$$\begin{cases} x_1 &= \dfrac{1}{\mu_1} \dfrac{1}{1+\gamma_1 \left(\frac{1}{\mu_2} \frac{1}{(1+\gamma_2 x_1^n)} \right)^n} = F(x_1) \\ x_2 &= \dfrac{1}{\mu_2} \dfrac{1}{(1+\gamma_2 x_1^n)} \end{cases} \tag{21}$$

(see the proof of lemma 5 in Appendix A). From the first equation in (21), it follows that, for any $n \geq 1$, $F(0) > 0$ and $F(1/\mu_1) < 1/\mu_1$. Therefore, for any $n \geq 1$, the equation $x = F(x)$ has at least one solution in the interval $[0, 1/\mu_1]$. If $\mu_1 < 1$ and $\mu_2 < 1$, in the limit $n \to \infty$ and in the same interval, the same equation has three solutions. To analyse the change in the number of solution of the first equation in (21), we proceed numerically. In figure 6, we show the solutions of the first equation in (21) as a function of the Hill parameter n and for two different choices of the kinetic parameters μ_i and γ_i. The solutions of the first equation in (21) give the first coordinates of the fixed points of equations (14), for $m = 2$. From the numerical analysis shown in figure 6, it follows that, for this choice of parameters and when the Hill parameter n is varied, the system of equations (14), with $m = 2$ and

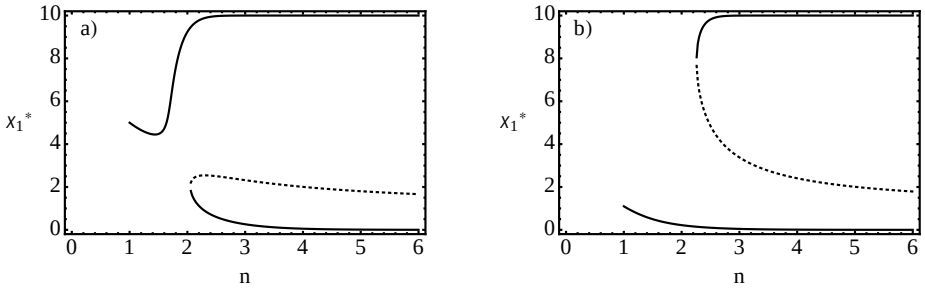

Figure 6: Bifurcation diagram of the fixed points of equations (14), for $m = 2$, $r = 0$, $\mu_1 < 1$ and $\mu_2 < 1$. We represent the first coordinate of the fixed points of equations (14) as a function of the Hill parameter n. The dotted lines represent unstable fixed points, and full lines represent stable fixed points. In a), the kinetic parameters are, $\mu_1 = 1/8$, $\mu_2 = 1/5$, $\gamma_1 = 0.3$ and $\gamma_2 = 0.1$, and the saddle-node bifurcation occurs for $n \simeq 2.04$. In b), the kinetic parameters are, $\mu_1 = 1/8$, $\mu_2 = 1/5$, $\gamma_1 = 1.8$ and $\gamma_2 = 0.1$, and the saddle-node bifurcation occurs for $n \simeq 2.24$. For values of n above the saddle-node bifurcation, equation (14), for $m = 2$ and $r = 0$, has one fixed point of saddle node type and two fixed points of stable node type.

$r = 0$, has a saddle-node bifurcation. The value of the parameter n where the bifurcation occurs, depends on all the kinetic parameters.

Increasing n for values above the saddle-node bifurcation values, equation (14), for $m = 2$ and $r = 0$, has one fixed point of saddle node type and two fixed points of stable node type. The fixed point of saddle type has a stable and an unstable manifold similar to the ones constructed previously in the $n \to \infty$ case. The stable manifold partition the first quadrant of phase space into two invariant regions and initial conditions on these separated regions lead to solutions with different asymptotic limits. In this case, the topology of the phase space orbits are similar to the ones found in the $n \to \infty$ case.

All the analysis we have done so far is for the case without delays in equations (14). With delays, $r > 0$, the number of fixed points of equations (14) are the same. Also, from a straightforward geometric analysis and as $\alpha_1\alpha_2 > 0$, the exponential term in the characteristic polynomial (18) does not affect the stability results previously obtained. Therefore, the introduction of delays in the 2-repressilator model do not lead to significative qualitative differences between the asymptotic solutions of the differential equation (14), for $m = 2$, provided $(x_1(t), x_2(t)) = (\phi_1(t), \phi_2(t)) = (0, 0)$, for $t \in [-s, 0]$.

In Table 2, we summarise the type of asymptotic solutions of the model

equation (14), for $m = 2$. In all the cases, all the solutions converge asymptotically in time to a steady state solution. As we have seen in the bifurcation analysis, if the Hill parameter n is large enough and if, $\mu_1 < 1$ and $\mu_2 < 1$, it is possible to have bistable behaviour and the choice of the asymptotic state depends on the initial conditions.

	$r = 0$	$r > 0$
$n = 1$ (mass action)	steady state	steady state
$n > 1$ (Hill form)	steady state bistable saddle-node bifurcation	steady state bistable saddle-node bifurcation
$n = \infty$ (Boolean form)	steady state bistable	steady state bistable

Table 2: Asymptotically stable solutions of the 2-repressilator equation (14) as a function of the delay r and for different values of the Hill parameter n.

3.2. The 3-repressilator model

By lemma 5, the system of equations (14), for $m = 3$, has only one fixed point, and, by (17), the characteristic equation associated to the fixed point is,

$$p(\lambda) = (\lambda + \mu_1)(\lambda + \mu_2)(\lambda + \mu_3) + \alpha_1\alpha_2\alpha_3 e^{-\lambda 3r} . \tag{22}$$

As, $\alpha_1\alpha_2\alpha_3 > 0$ and with $r = 0$, a simple geometric analysis shows that the characteristic equation (22) has three roots. These three roots can be real and negative or, one real and negative and the two others complex conjugate.

Lemma 6. *Consider the system of equations (14), with $m = 3$, $r = 0$, $\mu_i > 0$ and $\gamma_i > 0$, for $i = 1, 2, 3$. Let $\beta_1 = (\mu_1 + \mu_2)(\mu_1 + \mu_3)(\mu_2 + \mu_3)$. For $\alpha_1\alpha_2\alpha_3 = \beta_1$, the system of equations has a supercritical Hopf bifurcation. If, $\alpha_1\alpha_2\alpha_3 > \beta_1$, then the system of equations has an asymptotically stable periodic solution in phase space (limit cycle). There exists a constant β_0, with $0 \leq \beta_0 < \beta_1$, such that, if $\beta_0 < \alpha_1\alpha_2\alpha_3 < \beta_1$, the solutions of the system equation are oscillatory asymptotically stable. If, $0 < \alpha_1\alpha_2\alpha_3 \leq \beta_0$, then the solutions of the system equation are monotonically asymptotically stable.*

We have tested numerically the results of lemma 6. For the choice of parameters, $\mu_1 = 0.1$, $\mu_2 = 0.2$, $\mu_3 = 0.3$, $\gamma_1 = 0.3$, $\gamma_2 = 0.1$ and $\gamma_3 = 0.5$, we have obtained $\beta_0 = 0.00039$ and $\beta_1 = 0.06$. In this case, the 3-repressilator model has damped oscillations for $1.04 \lessapprox n \lessapprox 2.8$ and has a Hopf bifurcation for the Hill parameter $n \simeq 2.8$. For the same kinetic parameters, if $n > 2.8$, the asymptotic solutions of the 3-repressilator model show stable oscillations (limit cycles).

The introduction of delays in the 3-repressilator model does not change these results significantly, as it is easily seen from a simple geometric analysis of the roots of the characteristic equation (22). For $n = 1$ and sufficiently large r (> 2), it is possible to have damped oscillations, but a Hopf bifurcation is never reached.

For $n \to \infty$, the 3-repressilator model has the Boolean form,

$$\left\{ \begin{array}{rcl} \dot{x}_1 & = & g(x_2) - \mu_1 x_1 \\ \dot{x}_2 & = & g(x_3) - \mu_2 x_2 \\ \dot{x}_3 & = & g(x_1) - \mu_3 x_3 \end{array} \right. \tag{23}$$

where $g(x) = 1$, for $x \leq 1$, and $g(x) = 0$, otherwise. If $\mu_1 < 1$, $\mu_2 < 1$ and $\mu_3 < 1$, the system of equations (23) has no fixed points. Systematic numerical analysis of the geometry of the orbits in phase space suggest that the asymptotic solution of the system of equations (23) converges always to a function periodic in time, a limit cycle type solution. In figure 7, we show such an asymptotically periodic solution of the system of equations (23). In the next section, these cases will be analysed in more detail.

For $n \to \infty$, but $\mu_1 \geq 1$, $\mu_2 \geq 1$ and $\mu_3 \geq 1$, there exists one fixed point of stable node type, with coordinates $(1/\mu_1, 1/\mu_2, 1/\mu_3)$. As in the case $m = 2$, delays do not introduce qualitative changes in the dynamics of the 3-repressilator model.

In Table 3, we summarise the type of asymptotic solutions of the 3-repressilator model.

4. Boolean m-repressilator model for $m \geq 2$

In the previous sections, we have studied Boolean m-repressilator models for $m \leq 3$. We have shown that the 1-repressilator model with positive delays can have limit cycle solutions. The 2-repressilator model with $\mu_1 < 1$ and $\mu_2 < 1$ has bistability, with or without delays, and has no limit cycle solutions. The 3-repressilator model, with $\mu_i < 1$, for $i = 1, 2, 3$, and sufficiently

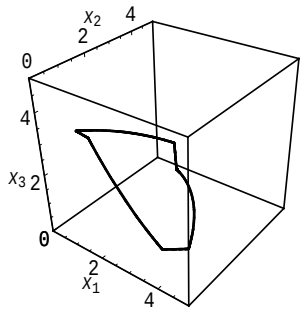

Figure 7: Limit cycle solution of the 3-repressilator model equations (23), for the parameter values, $\mu_1 = 0.1$, $\mu_2 = 0.2$, $\mu_3 = 0.3$.

	$r = 0$	$r > 0$
$n = 1$ (mass action)	steady state	steady state damped oscillations
$n > 1$ (Hill form)	steady state damped and stable oscillations Hopf bifurcation	steady state damped and stable oscillations Hopf bifurcation
$n = \infty$ (Boolean form)	steady state stable oscillations	steady state stable oscillations

Table 3: Asymptotically stable solutions of the 3-repressilator model as a function of the delay r and for different values of the Hill parameter n.

large Hill parameter n, has asymptotically stable periodic solutions. This shows that for $m = 2$ and $m = 3$, the asymptotic solutions of the system of equations (14) in the limiting case $n \to \infty$ have the same phase space topology as the solutions of the same family of equations for finite but large values of the Hill parameter n.

We analyse now the general properties of the asymptotic solutions of the m-repressilator model in limit $n \to \infty$ and for $m \geq 2$. In this case, by (14), the Boolean m-repressilator model has the form,

$$\dot{x}_i = g(x_{i+1}(t - r)) - \mu_i x_i(t)\,, \qquad i = 1, \ldots, m \tag{24}$$

where $x_{m+1} = x_1$, $g(x) = 1$, for $x \leq 1$, and $g(x) = 0$, otherwise.

Theorem 7. *Consider the system of equations (24) with* $r = 0$ *and* $\mu_i > 0$, *for* $i = 1, \ldots, m$. *Then, we have:*

a) If $m \geq 2$ *and if there exists at least one integer* p, *with* $1 \leq p \leq m$, *such that* $\mu_p \geq 1$, *then the system of equations (24) has a unique fixed point with positive coordinates, and this fixed point is of stable node type.*

b) If $m \geq 2$ *and* m *is even, and if* $\mu_i < 1$, *for every* $i = 1, \ldots, m$, *then the system of equations (24) has two fixed points with coordinates,*

$$(1/\mu_1, 0, 1/\mu_3, \ldots, 0)$$
$$(0, 1/\mu_2, 0, \ldots, 1/\mu_m)\,.$$

These fixed point are of stable node type.

c) If $m \geq 3$ *and* m *is odd, and if* $\mu_i < 1$, *for every* $i = 1, \ldots, m$, *then the system of equations (24) has no fixed points.*

For the particular case where $\mu_i \geq 1$, for every $i = 1, \ldots, m$, the coordinates of the fixed point are, $x_i = 1/\mu_i$, for $i = 1, \ldots, m$. For the other possible cases in theorem 7a), in the proof of the theorem, we give a combinatorial algorithm in order to calculate the coordinates of the unique fixed point of equations (24).

For the case b) in theorem 7, m is even, and the solutions of the piecewise linear system of equations (24) show bistability. For the case c), the numerical solutions of the piecewise linear system of equations (24) converge to an asymptotically stable periodic function of time or limit cycle. However, this system has no fixed points in phase space.

In this Boolean case and for the initial condition $(x_1(t), x_2(t), \ldots, x_m(t)) = (0, \ldots, 0)$, for $t \in [r, 0]$, delays do not introduce topological modifications on the solutions of the piecewise linear system of equations (24).

5. Conclusions

We have shown that the m-repressilator model is one of the simplest genetic mechanisms showing bistability ($m = 2$) and stable oscillations ($m = 1$ and $m = 3$). In general, in have shown that for $m \geq 2$, there exists always a choice of the Hill parameter n such that:

a) If m is even, the asymptotic solutions of the m-repressilator model are bistable, and do not show damped or persistent oscillations.

b) If m is odd, the asymptotic solutions of the m-repressilator model are periodic of limit cycle type.

In case a), upon variation of the Hill parameter n, bistability is obtained through a saddle-node bifurcation. In case b), limit cycle solutions are obtained through a Hopf Bifurcation.

The 1-repressilator model with delay is the simplest mechanism that can originate oscillations in eukaryote organisms or in organisms where there exists a delay between mRNA transcription and protein synthesis. As the 1-repressilator model involves the synthesis of only one protein, due to its simplicity, it may be important in synthetic biology applications. The importance of the 2-repressilator model relies on the fact that bistability is a switch mechanism tuned by initial conditions.

Some of the results obtained in this paper depend on the parameterisation obtained through the Hill cooperative parameter n. If $n = 1$, neither oscillations nor bistability exist. On the contrary, for $n > 1$ and with delays, these phenomena do appear. Synthetic biology models have been used to validate qualitatively the Hill embedding of the 3-repressilator model, Elowitz & Leibler (2000). For other models involving activation and repression of genes, it is not straightforward to discover how to do similar embeddings. Confrontation of the literature on enzyme kinetics with the systems biology approach, leads to the conclusion that experiments leading to the quantitative calibration of models of protein synthesis are indeed needed. Synthetic biology gives a good experimental framework to calibrate and validate experimentally m-repressilator models.

Appendix A.

Proof. Lemma 1. Let $g(\lambda) = -1 - \beta e^{-\lambda s}$. As $g(\lambda) < 0$ for every $\lambda \in \mathbb{R}$, the characteristic equation (10) has one real negative root λ^* only if, $\lambda^* < 0$ and $g'(\lambda^*) = 1$. These conditions imply that $\lambda^* = \log(\beta s)/s$, with $\beta s < 1$ and $s > 0$. With the characteristic equation condition $\lambda^* = g(\lambda^*)$, we obtain,

$$\begin{cases} \beta = \dfrac{1}{s} e^{-1-s} \\ \lambda^* = \dfrac{1}{s} \log(\beta s) = -\dfrac{s+1}{s} \end{cases}$$

and b) is proved. If $\beta < e^{-1-s}/s$, the characteristic equation has two negative real roots and a) is also proved.

For $\beta > e^{-1-s}/s$, the characteristic equation has two imaginary root. This is straightforwardly proved by expanding the characteristic equation in series around $\lambda = \lambda^*$. In this case, if $(\beta - e^{-1-s}/s)$ is positive and sufficiently small, these complex roots have negative real parts. Let us show now that, if β increases, these two characteristic roots cross the imaginary axis of the complex plane. For that, we assume that there exists a solution of the characteristic equation of the form, $\lambda = i\delta$, with $\delta > 0$. So, the real and imaginary parts of the characteristic equation obey to,

$$\begin{cases} 1 + \beta \cos(\delta s) = 0 \\ \beta \sin(\delta s) = \delta \, . \end{cases}$$

Solving the above equations for β and δ, for every $s > 0$, there exists a countable number of solutions in δ and β. With $\delta > 0$ and $\beta > 0$, the solutions in δ are the unique roots of the equations, $-\tan(s\delta_q) = \delta_m q$, where $\delta_q \in]-\pi/(2s) + q\pi/s, q\pi/s[$ with $q = 1, 3, \ldots$ (odd), and $\beta_q = -1/\cos(\delta_q s)$. For $q = 1$, c) and d) are proved. If $q > 1$, the other roots can not be reached by a continuous pass in the complex plane and so β_1 is the unique value of the parameter β for which the eigenvalues of the characteristic polynomial cross the imaginary axis. This proves e). □

Proof. Theorem 2. The proofs of a) and b) follow from Lemma 1 and the analysis of the roots of the characteristic polynomial of the linearised delay equation (9). c) and d) are a result of the Hopf bifurcation theorem for delay equations, Hale (1977); Smith (2011). To show that the Hopf bifurcations is indeed supercritical, we calculate now the derivatives at bifurcation, $\beta = \beta_1$. Let $\lambda = \alpha + i\delta$ be a root of the characteristic equation $p(\lambda)$. The real part of λ is a root of the function $h(\alpha) = 1 + \alpha + \beta e^{-s\alpha} \cos s\delta$, and $\alpha \equiv \alpha(\beta)$. As we have seen in the proof of lemma 1, $h(\alpha) = 0$ has the solution $\alpha(\beta) = 0$. So developing $h(\alpha)$ in Taylor series around $\alpha = 0$ and keeping only the first order terms, $h(\alpha) = 1 + \beta \cos s\delta + (1 - \beta s \cos s\delta)\alpha$, the local behaviour of $\alpha(\beta)$ near 0 is,

$$\alpha(\beta) = -\frac{1 + \beta \cos(\delta s)}{1 - s\beta \cos(\delta s)}$$

and,

$$\frac{\partial \alpha}{\partial \beta} = -\frac{(1+s)\cos(\delta s)}{(1 - s\beta \cos(\delta s))^2} \, .$$

For $\alpha = 0$, $(\cos(\delta s))_{\alpha=0} = \cos(\delta_1 s) = -1/\beta_1$, we obtain,

$$\left(\frac{\partial \alpha}{\partial \beta}\right)_{\alpha=0} = \frac{1}{\beta_1(1+s)} > 0\,.$$

By the Hopf bifurcation theorem, this last condition guarantees that the Hopf bifurcation at $\beta = \beta_1$ is supercritical, proving c) and d). □

Proof. Corollary 3. By theorem 2 and lemma 1, the delay equation (7) has a stable oscillatory solution if it is possible to find a value for the parameter δ_1 such that, $s\delta_1 \in]\pi/2, \pi[$. As $\beta_1 = -1/\cos(s\delta_1)$, this implies that $\beta_1 \in]1, +\infty[$. As, for $n = 1$, $\beta = (1 - x^*(\gamma, 1)) = 1 + 1/(2\gamma) - \sqrt{1 + 1/(4\gamma^2)} < 1$, for every $\gamma > 0$, no solutions with $\beta > \beta_1 > 1$ exist. As $\beta > 0$, it is always possible to find sufficiently large values of s such that, $e^{-s-1}/s < \beta < \beta_1$. Therefore, by lemma 1c), damped oscillatory solutions exist. This proves the first part of the corollary.

To prove the second part, we first note that $(1 - x^*(\gamma, n)) \leq 1$, since x^* is a solution of $1/(1 + \gamma(x)^n) = x$. So, as $\beta(\gamma, n) = n(1 - x^*(\gamma, n)) \leq n$, in the limit $\gamma \to 0$, $\beta(\gamma, n) = n$. On the other hand, with $-\tan(s\delta_1) = \delta_1$ and $u = s\delta_1$, we have $-\tan(u) = \delta_1/s$, and in the limit $s \to \infty$, the solution of $-\tan(s\delta_1) = \delta_1$ converges to $s\delta_1 = \pi$, and, in the same limit, $\beta_1 \to 1$. Therefore, we have proved that, for any $n > 1$, there exists always a sufficiently small γ and a sufficiently large delay s, such that the delay equation (7) has an asymptotically stable periodic solution (limit cycle). □

Proof. Corollary 4. In the limit $n \to \infty$, the delay equation (7) reduces to $\dot{x} = g(x) - x$, where $g(x) = 1$ for $x \leq 1$, and $g(x) = 0$, otherwise. For the initial condition, $x(t) = \phi(t) = 0$, with $t \in [-s, 0]$, the solution is $x(t) = (1 - e^{-t})$, for $t \geq 0$, converging monotonically to the steady state $x^* = 1$. □

Proof. Lemma 5. For $m = 1$, the lemma is straightforward. Let us prove it in general for any finite $m \geq 1$. The coordinates of the fixed points of equation (14), if they exist, are solutions of the equations,

$$x_i = \frac{1}{\mu_i} \frac{1}{(1 + \gamma_i x_{i+1}^n)} := f_{i+1}(x_{i+1})\,, \quad i = 1, \ldots, m$$

where $x_{m+1} = x_1$, $\mu_i > 0$ and $\gamma_i > 0$, for $i = 1, \ldots, m$. The functions $f_{i+1}(x) : \mathbb{R}^+ \to]0, 1/\mu_i]$ are strictly decreasing, positive and smooth and

$f_{i+1}(0) = 1/\mu_i$. Then, the first coordinate of a fixed point of equation (14) is a root of the equation,

$$x_1 = (f_2 \circ f_3 \circ \cdots f_m \circ f_{m+1})(x_1) := F(x_1)$$

where $F(x_1) : \mathbb{R}^+ \rightarrow]0, 1/\mu_1]$ and $F(0) > 0$.

If m is odd, $F(x_1)$ is strictly decreasing, positive and smooth. As $F(F(0)) = F(0) + F(0)F'(\xi)$, where, $F'(\xi) < 0$ and $\xi \in [0, F(0)]$, then, we have, $F(F(0)) - F(0) < 0$ and $F(F(0)) < F(0)$. So, by the continuity of $F(x_1)$, there exists a unique $x_1^* \in [F(0), F(F(0))]$, such that $F(x_1^*) = x_1^*$. This solution is the first coordinate of the unique fixed point of equation (14). The other coordinates are the unique solutions of, $x_i = f_{i+1}(x_{i+1})$, with $i = 1, \ldots, m$, where $x_{m+1} = x_1$.

If m is even, $F(x_1)$ is strictly increasing, positive and smooth. But as $F(x_1)$ has values in the interval $]0, 1/\mu_1]$, we have $F(1/\mu_1) \leq 1/\mu_1$. As $F(0) > 0$, then, in the interval $[0, 1/\mu_1]$, there exists at least one solution of the equation $F(x_1) = x_1$. The other coordinates are determined as previously. □

Proof. Lemma 6. Under the conditions of the lemma, the system of equations has a Hopf bifurcation if the characteristic polynomial (22) has one negative real root $-\lambda_1$ and two pure imaginary roots, $\lambda_{2,3} = \pm i\delta$. In this case, $p(\lambda) = p^*(\lambda) = \lambda^3 + \lambda_1\lambda^2 + \delta^2\lambda + \delta^2\lambda_1$. Comparing the characteristic polynomial (22) with $p^*(\lambda)$, and solving for δ and λ_1, we obtain, $\alpha_1\alpha_2\alpha_3 = \beta_1$, where $\beta_1 = (\mu_1 + \mu_2)(\mu_1 + \mu_3)(\mu_2 + \mu_3)$. For $\alpha_1\alpha_2\alpha_3 \geq \beta_1$, the Hopf bifurcation theorem applies. The second part of the lemma follows from the fact that for $\alpha_1\alpha_2\alpha_3 = 0$, the characteristic polynomial (22) has three real negative roots. □

Proof. Theorem 7. To calculate the coordinates of the fixed points of the system of equations (24), the right hand sides of equations (24) are equated to zero. For each pair of parameter values (μ_i, μ_{i+1}), with $i = 1, \ldots, m$ and

$\mu_{m+1} = \mu_1$, there exist at most two possible solutions,

$$\begin{aligned}
(\mu_i \geq 1, \mu_{i+1} \geq 1) &\implies (x_i = \frac{1}{\mu_i}, x_{i+1} = \frac{1}{\mu_{i+1}}) \\
(\mu_i \geq 1, \mu_{i+1} < 1) &\implies (x_i = \frac{1}{\mu_i}, x_{i+1} = 0) \\
&\qquad\quad (x_i = 0, x_{i+1} = \frac{1}{\mu_{i+1}}) \\
(\mu_i < 1, \mu_{i+1} \geq 1) &\implies (x_i = \frac{1}{\mu_i}, x_{i+1} = \frac{1}{\mu_{i+1}}) \\
&\qquad\quad (x_i = \frac{1}{\mu_i}, x_{i+1} = 0) \\
(\mu_i < 1, \mu_{i+1} < 1) &\implies (x_i = \frac{1}{\mu_i}, x_{i+1} = 0) \\
&\qquad\quad (x_i = 0, x_{i+1} = \frac{1}{\mu_{i+1}}) \,.
\end{aligned}$$

Let $(\mu_p, \ldots, \mu_{p+m-1})$, where $\mu_{p+m} = \mu_p$, be the sequence of parameter values such that p, with $1 \leq p \leq m$, is the first index for which $\mu_p \geq 1$. From the correspondence table between ordered pairs of parameters and ordered pairs of fixed point coordinates just derived, to the sequence of parameter values $(\mu_p, \ldots \mu_{p+m-1})$ correspond at most two sequences of coordinates,

$$\begin{aligned}
&(x_p = \frac{1}{\mu_p}, x_{p+1} = \cdots, \ldots, x_{p+m-1} = \cdots) \\
&(x_p = 0, x_{p+1} = \cdots, \ldots, x_{p+m-1} = \cdots)
\end{aligned}$$

where $x_{p+m} = x_p$. As $g(x_{m+1})) - \mu_m x_m = g(x_1)) - \mu_m x_m = 0$, we mast have, $x_{p+m} = x_p$, and, by the correspondence table, only one of the two possible solutions corresponds to the coordinates of the fixed point of the system of equation (24). Therefore, we have proved that, in the conditions in a), there exists a unique fixed point of equation (24). The stability of this fixed point follows from the eigenvalues of the Jacobian matrix of the system of equations (24), calculated at the fixed point. As $\mu_i > 0$, for $i = 1, \ldots, m$, this Jacobian matrix has only diagonal terms and they are all negative. This proves a).

To prove b), we use the correspondence table above. If $\mu_i < 1$, for every $i = 1, \ldots, m$, we have two possible fixed point solutions,

$$\begin{aligned}
&(x_1 = \frac{1}{\mu_1}, x_2 = 0, x_3 = \frac{1}{\mu_3}, \ldots) \\
&(x_1 = 0, x_2 = \frac{1}{\mu_2}, x_3 = 0, \ldots) \,.
\end{aligned}$$

If m is even, both solutions are compatible with the parameter signs in the correspondence table. Therefore, the system of equations (24) has two fixed points. Both fixed points are of stable node type. This proves b) for $m \geq 2$.

If m is odd, the solutions for $x_{m+1} = x_1$ are not compatible with the parameter signs in the correspondence table, and the system of equations (24) has no fixed points. □

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