Modular analysis of two cyclic biological circuits: Technical report

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Abstract

We conduct a formal analysis of two biological network circuits, that come about from the cyclic interconnection of motifs that commonly recur in biology. Specifically, we first analyze a generalized version of the gene regulatory network known as the "repressilator", which is the interconnection of an arbitrary number of transcriptional regulators connected in feedback. We further analyze an enzymatic covalent modification network, which consists of an arbitrary number of enzyme-substrate motifs connected in feedback. A key technical tool used to obtain these results is the representation of these motifs as modules that are functionally isolated from each other, so that the behavior of the composite network of two or more modules can be predicted from the input-output characteristics of each individual module. We demonstrate that the stable parameter regions of the repressilator tend to shrink as we add more transcriptional regulators. We further demonstrate that enzyme and substrate concentrations in covalent modification networks will always completely degrade, regardless of the network parameters and its size.

I. INTRODUCTION

Network science and control systems theory have played a significant role in the advancement of systems and synthetic biology in recent years. Tools from graph theory and system identification have been used to understand the basic subunits or "motifs" in biological networks [1], [2], and to identify the existence (or non-existence) of pathways in large networks [3], [4], [5]. With a deeper understanding of mechanisms that exist in biology, synthetic biologists have used

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bottom-up construction techniques to engineer new biological networks, and modify or optimize the behavior of existing networks [6], [7], with impressive results [8], [9], [10].

Some interesting classes of biological networks that are commonly studied are gene regulatory networks and enzymatic networks, both of which play important roles in many vital life processes [11] and are also well-characterized mathematically [12], [13]. A class of gene regulatory networks that has been the subject of many theoretical studies is known as the repressilator [9], which was the first known synthetic oscillator to be built [14]. Repressilators are topologically equivalent to a class of cyclic negative gain networks [14], and numerical analysis of the model revealed the parameter regions for which the network would oscillate and when it would converge to a stable steady-state [9]. Since then, there has been a plethora of work on theoretically establishing oscillatory regions of the network, for varying degrees of generalizations of the network model [14], [15], [16], [17]. While the results of these studies provide deep insights about when the repressilator oscillates, these results are often complex and require the satisfaction of multiple assumptions.

The Goldbeter-Koshland model for covalent modification of proteins [18] is another enzymatic network that has been the subject of many studies [19], [20]. In this network, there exists a protein X_1 that is modified to another form X_2 through an interaction with an enzyme E_1. X_2 is also modified back to X_1 through an interaction with an enzyme E_2. The dynamical model describing this behavior is simple, and yet reveals interesting results about sensitivity of the protein concentrations to changes in enzyme concentration. Typically, these covalent modification reactions happen in signaling cascades [21], and the enzymes and substrates are subject to degradation.

In this paper, we study a generalized repressilator which consists of a cyclic interconnection of an arbitrary number of transcriptional activators and repressors. For the symmetric case, where all parameters across all the modules are equivalent (which is typical in an experimental setting [9]), we provide results for when the network will converge to a stable steady-state, both in the local and global sense. Our results clearly show how the stable parameter region for the repressilator tends to become smaller as more transcriptional regulators are added to the network. These results were inspired by studies by Arcak and Sontag [22], [23]. We further study a generalized covalent modification network, consisting of a cascade of an arbitrary number of enzymatic reactions connected in feedback. We show that regardless of the parameters chosen for each

enzyme-substrate interaction, the substrates will necessarily degrade away for almost all initial conditions, in spite of being connected in positive feedback. These results were inspired by the work on Monotone Systems Theory by Angeli and Sontag [24], [25], [26].

A key technical tool that we used to obtain these results is the conception of the biological networks as a collection of functionally isolated interacting components, or *modules*. Hartwell *et al.* were among the first to suggest that this idea could reduce the complexity of analyzing these networks [27]. For example, properties like stability and robustness can be predicted just from properties of each individual component in the network and knowledge of the interconnection structure. From a computational perspective, computing network parameters such as its equilibrium point(s) can be greatly simplified, since the computations can be done over a set of components as opposed to over the entire network.

We require that a biological module should admit both *dynamic modularity* and *parametric modularity*. The former implies that the properties of each module do not change upon interconnection with other modules, and the latter implies that the network parameters within a module appear in no other module. In this sense, the model of a synthetic component that undergoes loading effects upon interconnection with other components does *not* exhibit dynamic modularity [28], [29], and a component whose internal dynamics depend on parameters that also affect other components does *not* exhibit parametric modularity. In many studies, biological networks were often partitioned into modules that could result in parameters appearing across multiple modules [30], [31].

The remainder of this paper is organized as follows. In Section II, we introduce the transcriptional regulation module and the covalent modification module that appear in biological networks, and describe their properties. In Section III, we show how the interconnection of an arbitrary number of transcriptional regulation modules leads to the generalized repressilator network structure, and derive a result that relates the stability region to the number of interconnected modules. Finally in Section IV, we build the generalized covalent modification network through an interconnection of covalent modification modules, and show how this network always converges to the origin regardless of the parameters in each module or the number of modules.

II. COMMON BIOLOGICAL MODULES

In this section, we consider a few key biological modules that arise in gene regulatory networks and enzymatic networks. We characterize these modules in terms of system theoretic properties that can be used to establish properties of complex interconnections involving these modules. Before introducing the biological modules of interest, we briefly recall some of these system theoretic properties.

A. Module properties

Consider a generic input-output module, expressed by an ODE of the form

$$\dot{x} = A(x,u), \qquad y = B(x,u), \qquad x \in \mathbb{R}^n, u \in \mathbb{R}^k, y \in \mathbb{R}^m, \tag{1}$$

where $x(t)$ denotes the n-vector state of the module, $u(t)$ the k-vector input to the module, and $y(t)$ the m-vector output from the module.

The module described by (1) is *positive* if the entries of its state vector $x(t)$ and output vector $y(t)$ never take negative values, as long as all the entries of the initial condition $x(0)$ and of the input vector $u(t)$, $\forall t \geqslant 0$ never take negative values. All the modules described in this section are positive.

We say that the module described by (1) is *cooperative* (also known as *monotone with respect to the positive orthant*) if for all initial conditions $x_0, \bar{x}_0 \in \mathbb{R}^n$ and inputs $u(t), \bar{u}(t) \in \mathbb{R}^k$, $\forall t \geqslant 0$, we have that

$$\begin{aligned} & x_0 \gg \bar{x}_0 \quad \& \quad u(t) \succeq \bar{u}(t),\ \forall t \geqslant 0 \\ \Rightarrow \quad & x(t; x_0, u) \gg x(t; \bar{x}_0, \bar{u}),\ \forall t > 0 \end{aligned}$$

where $x(t; x_0, u)$ denotes the solution to (1) at time t, starting from the initial condition $x(0) = x_0$ and with the input u. Given two vectors $v, \bar{v}$, we write $v \gg \bar{v}$ if every entry of v is strictly larger than the corresponding entry of $\bar{v}$ and we write $v \succeq \bar{v}$ if every entry of v is larger than or equal to the corresponding entry of $\bar{v}$. The reader is referred to [24], [26], [25] for a more comprehensive treatment of monotone dynamical systems, including simple conditions to test for monotonicity and results that allow one to infer monotonicity of a complex network from the monotonicity of its constituent parts. Several modules described in this section are cooperative.

The *Input-to-State Static Characteristic Function (ISSCF)* $g(u^*)$ *of* (1) specifies how a constant input $u(t) = u^*$, $\forall t \geqslant 0$ to the module maps to the corresponding equilibrium value of the state $x(t) = x^*$, $\forall t \geqslant 0$. In terms of (1), the value of $g(u^*)$ is the (unique) solution x^* to the steady-state equation $A(x^*, u^*) = 0$. When this equation has multiple solutions x^*, the ISSCF is not well defined.

For modules with a well-defined ISSCF, the *Input-to-Output Static Characteristic Function (IOSCF)* $f(u^*)$ *of* (1) specifies how a constant input $u(t) = u^*$, $\forall t \geqslant 0$ to the module maps to the corresponding equilibrium value of the output $y(t) = y^*$, $\forall t \geqslant 0$. In terms of (1), the value of $f(u^*)$ is given by $B(g(u^*), u^*)$. The equilibrium point of a network can be obtained obtained from the interconnection of several input-output modules like (1), from the IOSCFs and the ISSCFs of the constituent modules.

For systems with a well-defined ISSCF, the *Linearized Transfer Function (LTF)* $H(s)$ *of* (1) *around an equilibrium defined by the input* u^* determines how a small perturbation $\delta u(t) \coloneqq u(t) - u^*$ of the input $u(t)$ around the constant input $u(t) = u^*$, $\forall t \geqslant 0$ leads to a perturbation $\delta y(t) \coloneqq y(t) - y^*$ of the output $y(t)$ around the constant equilibrium output $y(t) = y^* \coloneqq f(u^*)$, $\forall t \geqslant 0$.

B. Transcriptional regulation (TR) module

A gene regulatory network consists of a collection of transcription factor proteins, each involved in the regulation of other proteins in the network. Such a network can be decomposed into *transcriptional regulation (TR) modules*, each containing a transcription factor $\mathcal{S}_0$, the promoter regions of a set of genes $\mathcal{G}_1, \mathcal{G}_2, \ldots, \mathcal{G}_F$ that $\mathcal{S}_0$ up-regulates or down-regulates, and the corresponding mRNA molecules $mRNA_1, mRNA_2, \ldots, mRNA_F$ transcribed. The case $F > 1$ is referred to in the literature as *fan-out* [32].

The input u to a TR module is the rate of production of $\mathcal{S}_0$ due to exogenous processes such as regulation from other TR modules, and can be associated with a generic reaction of the form

$$\varnothing \xrightarrow{u} \mathcal{S}_0.$$

A number $q_j \geqslant 1$ of molecules of the transcription factor $\mathcal{S}_0$ can bind to the promoter region P_j

of the gene $\mathcal{G}_j$, which is represented by the reaction

$$q_j\mathcal{S}_0 + P_j \underset{k_j^{\text{off}}[\mathcal{S}_0:P_j]}{\overset{k_j^{\text{on}}[\mathcal{S}_0]^{q_j}[P_j]}{\rightleftharpoons}} \mathcal{S}_0{:}P_j, \qquad \forall j \in \{1, 2, \ldots, m\}.$$

The total concentration of promoter regions $P_j^{\text{tot}} = [P_j] + [\mathcal{S}_0{:}P_j]$ for the gene $\mathcal{G}_j$ (bound and unbound to the transcription factor) is assumed to remain constant. When $\mathcal{S}_0$ *activates* the gene $\mathcal{G}_j$, the bound complex $\mathcal{S}_0{:}P_j$ gives rise to transcription, which is expressed by a reaction of the form

$$\mathcal{S}_0{:}P_j \xrightarrow{\alpha_j[\mathcal{S}_0:P_j]} \mathcal{S}_0{:}P_j + mRNA_j.$$

Alternatively, when $\mathcal{S}_0$ *represses* the gene $\mathcal{G}_j$, it is the unbounded promoter P_j that gives rise to transcription, which is expressed by a reaction of the form

$$P_j \xrightarrow{\alpha_j[P_j]} P_j + mRNA_j.$$

Additional reactions in the module include the translation of $mRNA_j$ to $\mathcal{S}_j$

$$mRNA_j \xrightarrow{\beta_j[mRNA_j]} mRNA_j + \mathcal{S}_j,$$

and the protein and mRNA degradation reactions

$$\mathcal{S}_0 \xrightarrow{\bar{\beta}[\mathcal{S}_0]} \varnothing,$$
$$mRNA_j \xrightarrow{\gamma_j[mRNA_j]} \varnothing$$

The TR module has F outputs $y_1, y_2, \ldots, y_F$ that are equal to the rates of translations of the proteins $\mathcal{S}_1, \mathcal{S}_2, \ldots, \mathcal{S}_F$, respectively. In particular,

$$y_j = \beta_j\,[mRNA_j]$$

When $F = 1$, we refer to each module simply as a TR activator or TR repressor module and the subscript j's can be omitted, as is done in the Repressilator example in Section III. Figure 1 shows a biological representation of a TR module.

Using the Law of Mass Action Kinetics (LMAK), which assumes that the species are well-mixed and their copy numbers are sufficiently large, we obtain the dynamics of a TR module

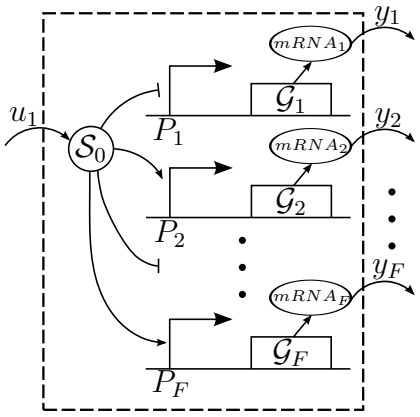

Fig. 1. Biological representation of a TR module.

to be

$$\begin{aligned}
[\dot{\mathcal{S}}_0] &= u - \bar{\beta}\,[\mathcal{S}_0] + \\
&\qquad \sum_{j=1}^{m} q\big(k^{\text{off}}\,[\mathcal{S}_0{:}P_j] - k^{\text{on}}\,[\mathcal{S}_0]^q\,(P^{\text{tot}} - [\mathcal{S}_0{:}P_j])\big) \\
[\mathcal{S}_0\dot{:}P_j] &= -k^{\text{off}}\,[\mathcal{S}_0{:}P_j] + k^{\text{on}}\,[\mathcal{S}_0]^q\,(P^{\text{tot}} - [\mathcal{S}_0{:}P_j]) \\
[m\dot{R}NA_j] &= \alpha_j\,[\mathcal{S}_0{:}P_j] - \gamma_j\,[mRNA_j] \ \text{(activating)} \\
[m\dot{R}NA_j] &= \alpha_j(P^{\text{tot}} - [\mathcal{S}_0{:}P_j]) - \gamma_j\,[mRNA_j] \ \text{(repressing)},
\end{aligned} \tag{2}$$

where we assume that the association and dissociation constants, the total promoter concentration, and the stoichiometric coefficients are the same for every gene for simplicity of presentation, i.e., that $k_j^{\text{off}} = k^{\text{off}}$, $k_j^{\text{on}} = k^{\text{on}}$, $P_j^{\text{tot}} = P^{\text{tot}}$ and $q_j = q$, $\forall j \in \{1, 2, \ldots, F\}$. The IOSCFs of each module are given by

$$y_j^* = \alpha_j\beta_j P^{\text{tot}}/\gamma_j\Big(1 + \frac{K}{(\theta u^*)^q}\Big) \tag{3a}$$

for every activating output and

$$y_j^* = \alpha_j\beta_j P^{\text{tot}}/\gamma_j\Big(1 + \frac{(\theta u^*)^q}{K}\Big) \tag{3b}$$

for every repressing output, where $K = \frac{k^{\text{off}}}{k^{\text{on}}}$.

To simplify the LTF and to make use of results from the literature subsequently [22], [23], we make the following simplifying assumptions:

Assumption 1 (Parameters in TR module)**.** The following assumptions on the parameter values are considered:

1) The binding-unbinding reactions are on timescales much faster than those of the transcription, translation, and decay reactions, i.e., $k^{\text{off}}, k^{\text{on}} \gg \gamma,\ \beta_j,\ \bar{\beta}$ and $u(t)\ \forall t \geqslant 0$ [28].
2) The dissociation constant $K = \frac{k^{\text{off}}}{k^{\text{on}}}$ is much higher than the total promoter concentration i.e $K \gg P^{\text{tot}}$, implying that the affinity of each binding site is small. □

The LTF to every activating output can then be simplified to

$$H_j(s) = \frac{qKP^{\text{tot}}\alpha_j\beta_j(\theta u^*)^{q-1}}{(K + (\theta u^*)^q)^2(s+\gamma_j)(s+\bar{\beta})}, \tag{4}$$

where $K \coloneqq \frac{k^{\text{off}}}{k^{\text{on}}}$ and $\theta =\coloneqq \frac{1}{\bar{\beta}}$. The LTF to every repressing output is simply the negation of (4). The TR module is positive and the equilibrium defined by any constant input u^* is locally asymptotically stable, under Assumption 1. When all outputs are activating, the TR module is also cooperative. The reader may verify that the module satisfies dynamic modularity conditions in that its state contains the concentrations of all the chemical species associated with the module, and parametric modularity conditions in that the parameters of all the chemical reactions associated with the module are not needed outside this module.

C. Covalent Modification (CM) module

The *covalent modification (CM) module* represents the process by which a substrate protein $\mathcal{S}_0$ is covalently modified by an enzyme E into an alternative form $\mathcal{S}_1$. The chemical species associated with this module are the substrate protein $\mathcal{S}_0$, the enzyme E, and the complex $\mathcal{S}_0{:}E$ formed by the enzyme-substrate binding. The input u to the CM module is the rate of production of the substrate $\mathcal{S}_0$ due to an exogenous process (e.g., a TR or another CM module) and can be associated with the generic reaction

$$\varnothing \xrightarrow{u} \mathcal{S}_0.$$

The additional reactions associated with the CM module include the $\mathcal{S}_0$ degradation reaction

$$\mathcal{S}_0 \xrightarrow{\gamma[\mathcal{S}_0]} \varnothing,$$

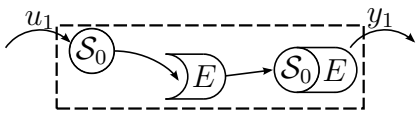

Fig. 2. Biological representation of a CM module.

and the reactions involved in the Michaeles-Menten model for the enzyme-substrate interaction:

$$\mathcal{S}_0 + E \underset{k^{\mathrm{r}}[\mathcal{S}_0{:}E]}{\overset{k^{\mathrm{f}}[\mathcal{S}_0][E]}{\rightleftharpoons}} \mathcal{S}_0{:}E \xrightarrow{k^{\mathrm{cat}}[\mathcal{S}_0:E]} \mathcal{S}_1 + E,$$

where the total concentration of enzyme $E^{\mathrm{tot}} = [E] + [\mathcal{S}_0{:}E]$ is assumed to remain constant. The output y of the CM module is the rate of production of the modified substrate $\mathcal{S}_1$, given by

$$y = k^{\mathrm{cat}} [\mathcal{S}_0{:}E] .$$

For simplicity, instead of presenting the exact dynamics of the module (which are straightforward to derive using MAK), we present a common approximation to the Michaeles-Menten model:

Assumption 2 (Equilibrium Approximation [13])**.** The reversible reaction is in thermodynamic equilibrium (i.e., $k^{\mathrm{f}} [\mathcal{S}_0] [E] = k^{\mathrm{r}} [\mathcal{S}_0{:}E]$), which is valid when $k^{\mathrm{r}} \gg k^{\mathrm{cat}}$. □

Under this assumption, the dynamics of the module simplify to be

$$\begin{aligned} [\dot{\mathcal{S}}_0] &= \frac{u - \gamma [\mathcal{S}_0] - \frac{k^{\mathrm{cat}} E^{\mathrm{tot}} [\mathcal{S}_0]}{K^{\mathrm{d}} + [\mathcal{S}_0]}}{1 + \frac{K^{\mathrm{d}} E^{\mathrm{tot}}}{(K^{\mathrm{d}} + [\mathcal{S}_0]^2)}} \\ y &= \frac{k^{\mathrm{cat}} E^{\mathrm{tot}} [\mathcal{S}_0]}{\frac{k^{\mathrm{r}}}{k^{\mathrm{f}}} + [\mathcal{S}_0]} \end{aligned} \tag{5}$$

with corresponding IOSCF

$$y^* = \frac{1}{2}(2u^* + K^{\mathrm{e}} - \sqrt{(K^{\mathrm{e}})^2 + 4K^{\mathrm{d}} u^* \gamma}), \tag{6}$$

where $K^{\mathrm{e}} \coloneqq -u^* + k^{\mathrm{cat}} E^{\mathrm{tot}} + K^{\mathrm{d}} \gamma$. The LTF is given by the form $H(s) = \dfrac{K^{\mathrm{p}}(u^*)}{s + K^{\mathrm{q}}(u^*)}$, where we omit further details for brevity. It is worth noting that the Quasi Steady-State Approximation [33] would also lead to quantitatively similar values for the LTF and IOSCF. This module is positive,

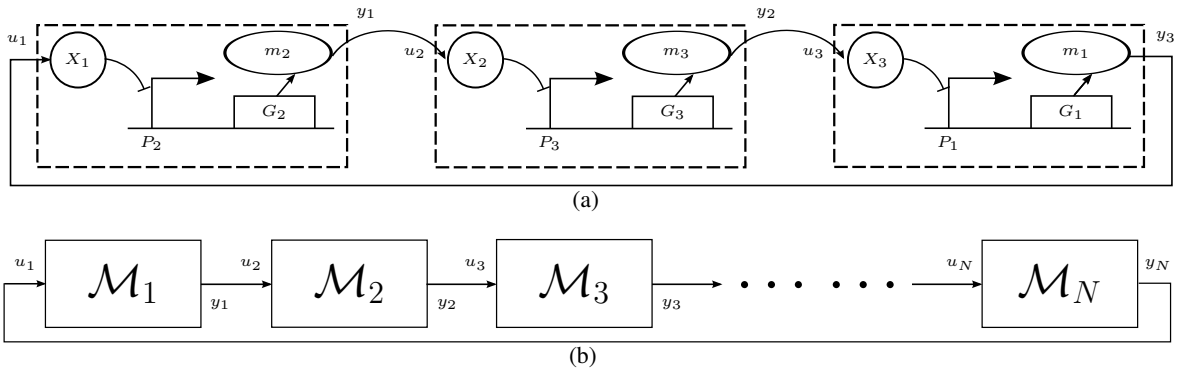

Fig. 3. (a) Biological realization of a three gene Repressilator network. In experiment, this was done with three naturally-occurring repressor proteins $LacI$, $tetR$ and cI, each corresponding to a TR repressor module. (b) Cascade of N TR repressor modules connected in feedback, where each module is denoted by $\mathcal{M}_i$, $i \in \{1, \cdots, N\}$.

cooperative, and the equilibrium defined by any constant input u^* is globally asymptotically stable under both assumptions. Figure 2 illustrates a biological representation of this module.

III. GENERALIZED REPRESSILATOR NETWORK

The Repressilator is a synthetic network designed to gain insight into the behavior of biological oscillators [9]. In its generalized form, this network consists of an odd number N of repressor proteins $\mathcal{S}_1, \cdots, \mathcal{S}_N$, where $\mathcal{S}_i$ represses the gene $\mathcal{G}_{i+1}$, for $i \in \{1, \cdots, N-1\}$ and $\mathcal{S}_N$ represses the gene $\mathcal{G}_1$. These networks can be decomposed into a cascade of N single-gene TR repressor modules connected in a (negative) feedback loop with no exogenous input. The biological realization of this decomposition with $N = 3$ (as in [9]) is shown in Figure 3(a), with the corresponding block diagram representation depicted in Figure 3(b).

The equilibrium point of the network must satisfy the equation

$$f_N\big(\cdots f_2\big(f_1(u_1^*)\big)\cdots\big) = u_1^*, \tag{7}$$

where $f_i(\cdot)$ denotes the IOSCF of the ith TR module (see (3a)). Since each $f_i(\cdot)$ is monotone decreasing, the composition of the N (odd) functions is also monotone decreasing and we have a feedback interconnection with a unique solution u_1^* to (7) *for all values of the parameters.* For simplicity, in the remainder of this section we assume that the parameters of the chemical reactions within each TR repressor module are exactly the same, implying that each module is identical and the network is *symmetric*. However, it is straightforward to modify the results

below for networks consisting of non-identical modules.

For this example, we follow two alternative approaches to determine whether or not the concentrations of the species converge to the unique equilibrium. The first approach is based on the *Nyquist Stability Criterion* [34] and will allow us to determine whether trajectories that start close to the equilibrium eventually converge to it.

Lemma 1. *Consider the feedback interconnection of a cascade of N modules, all with the same LTF $H_1(s)$ around a given equilibrium point of the feedback interconnection. Then the LTF of the feedback connection is BIBO stable if and only if*

$$\#OUP = -\frac{1}{N}\sum_{\ell=1}^{N} \#END[e^{j\frac{2\pi\ell}{N}}],$$

where $\#OUP$ represents the number of (open-loop) unstable poles of $H_1(s)$ and $\#END[e^{j\frac{2\pi\ell}{N}}]$ denotes the number of clockwise encirclements of the Nyquist contour of $H_1(j\omega)$, $\omega \in \mathbb{R}$ around the point $e^{j\frac{2\pi\ell}{N}}$ on the complex plane.[1] □

Proof of Lemma 1. To investigate the BIBO stability of the LTF of the network, we consider the characteristic equation of the feedback loop: $1 - H_1(s)^N = 0$. The number of unstable poles is thus given by the unstable solutions to the equation:

$$1 - H_1(s)^N = 0 \quad \Leftrightarrow \quad \exists i \in \{1, 2, \cdots, n\},\ H_1(s) = z_i,$$

where $z_\ell \coloneqq e^{j\frac{2\pi\ell}{N}}$ are the N roots to the equation $z^N = 1$.
To count the number of unstable poles of the network, we must then add the number of unstable poles of each of the N equations

$$H_1(s) = z_\ell, \quad \ell \in \{1, 2, \cdots, N\},$$

which can be done using Cauchy's argument principle by counting the number of clockwise encirclements of the point $z_\ell \in \mathbb{C}$ for the Nyquist contour of $H_1(j\omega)$, $\omega \in \mathbb{R}$. ■

[1] We assume here that $H_1(s)$ has no poles on the imaginary axis. If this were the case, the standard "trick" of considering an infinitesimally perturbed system with the poles moved off the axis can be applied [34].

Theorem 1. *Consider a Repressilator network, that consists of an odd number N of equal single-gene TR repressor modules ($F = 1$) connected in feedback as in Figure 3(b). The network has a unique equilibrium point that is locally asymptotically stable if and only if*

$$\sum_{\ell=1}^{N} \#END[e^{j\frac{2\pi\ell}{N}}] = 0, \tag{8}$$

where $\#END[e^{j\frac{2\pi\ell}{N}}]$ denotes the number of clockwise encirclements of the Nyquist plot of the LTF of a single TR repressor module given by (4) *around the point $e^{j\frac{2\pi\ell}{N}}$.* □

Proof of Theorem 1. We assume that Assumption 1 is satisfied for simplicity, although it is straightforward to extend the proof for the case when it is not. Theorem 1 follows from Lemma 1 by recognizing that a single-gene repressor TR module can be represented by the LTF in (4) under Assumption 1. Moreover, since the network input $u(t) = 0\ \forall t \geqslant 0$ and all modules have identical parameters, the values of the inputs and outputs of each module at equilibrium will be the same, each given by the unique solution u^* to (10). Therefore all modules have equal LTFs, and this enables us to use Theorem 1 to analyze when the LTF of the Repressilator is BIBO stable.

It is then straightforward to show that the he realization of the linearized network is minimal, since both the controllability and observability matrices of this system have full rank. This then implies that the LTF of the Repressilator network is BIBO stable, and hence the equilibrium point is locally asymptotically stable. ■

The symmetric repressilator we analyze has a convenient property that the equilibrium point of the network, and hence the Nyquist plot of the LTF of a TR repressor module, remains the same regardless of the number of modules added to the network. Because of this, the greater the number of TR repressor modules in the network, the larger the parameter range over which the repressilator will be unstable. This is demonstrated in Figure 4, which shows the Nyquist plot for $\beta = 10^{-1.5}$ to be stable when there are only three TR repressor modules, but becomes unstable when there are five TR repressor modules in the network. As more TR repressor modules are added to a network, there will be a wider parameter region for which the Nyquist plot which encircles the points $e^{j\frac{2\pi l}{N}}\ \forall l \in \{1, 2, \cdots, N\}$.

An alternative approach that can be used to determine global convergence to the equilibrium

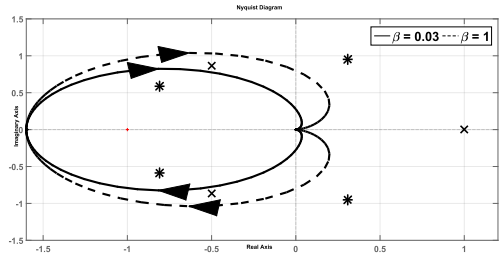

Fig. 4. Nyquist plots of the LTF of a TR repressor module which satisfies Assumption 1 for two different sets of parameters: $\beta = \bar{\beta} = 10^{-1.5}$ (solid) and $\beta = \bar{\beta} = 1$ (dashed). The remaining parameters are the same for both plots: $Ptot = 1$, $K = 100$, $\gamma = 1$, $q = 2$, $\alpha = 100$. The points $e^{j\frac{2\pi\ell}{3}}$, $j \in \{1, 2, 3\}$ that appear in the criterion (8) are marked with "X", and the points $e^{j\frac{2\pi\ell}{5}}$, $j \in \{1, \cdots, 5\}$ are marked with "*". For the network with $N = 3$, the solid plot does not encircle any of the three points so we have BIBO stability for the feedback LTF, whereas for the dashed Nyquist plot this is not the case. When N becomes 5, the repressilator represented by the solid plot becomes unstable

of a negative feedback interconnection is based on the *Secant Criterion* [35], [36] and some of its more recent variations [22].

Theorem 2. *Consider a Repressilator network, that consists of an odd number N of equal single-gene TR repressor modules ($F = 1$) connected in feedback as in Figure 3(b), with $q = 2$. Under Assumption 1 for the TR repressor modules, the network has a unique equilibrium point that is LAS if*

$$\frac{2P^{\text{tot}}\alpha\beta\frac{u^*}{\bar{\beta}}}{\bar{\beta}K\gamma\left(1+\frac{1}{K}(\frac{u^*}{\bar{\beta}})^2\right)^2} < \sec\left(\frac{\pi}{2N}\right)^2, \tag{9}$$

where u^* is the unique solution to

$$\frac{\alpha\beta P^{\text{tot}}}{\gamma\left(1+\frac{(\frac{u^*}{\bar{\beta}})^2}{K}\right)} = u^*, \tag{10}$$

and is GAS if

$$\frac{3P^{\text{tot}}\alpha\beta}{8\bar{\beta}\gamma}\sqrt{\frac{3}{K}} < \sec\left(\frac{\pi}{2N}\right)^2. \tag{11}$$

Both of these conditions are sufficient but not necessary. (11) in particular provides a condition which guarantees global convergence of the repressilator to its steady-state. This result again clearly shows for a symmetric repressilator that the stability regions of the network shrink as N grows, since the equilibrium point of the network does not change with N. Figure 5 shows the stability regions for a 3-gene Repressilator as we vary two of the TR module parameters.

To prove Theorem 2, we first prove the following result based on the Secant Criterion [35], [36] and some of its more recent variations [22], by choosing an appropriate coordinate transformation.

Lemma 2. *Consider the feedback interconnection depicted in Figure 3(b), with each of the N modules $\mathcal{M}_i$ of the form*

$$[\dot{\mathcal{S}}_i] = u_i - c_i([\mathcal{S}_i]), \qquad y_i = d_i([\mathcal{S}_i]), \qquad i \in \{1, 2, \ldots, N\}, \tag{12}$$

where

1) *the $c_i(\cdot)$ are continuous and monotone strictly increasing;*
2) *an odd number $M \leqslant N$ of the $d_i(\cdot)$ are continuous and monotone strictly decreasing, while the remaining $N - M$ of the $d_i(\cdot)$ are continuous and monotone strictly increasing.*

Then the IOSCF of the cascade is monotone strictly decreasing and the feedback interconnection has a unique equilibrium. This equilibrium is locally asymptotically stable provided that

$$\prod_{i=1}^{N} \left| \frac{\frac{\partial d_i(s_i)}{\partial s_i}\Big|_{s_i=[\mathcal{S}_i]^*}}{\frac{\partial c_i(s_i)}{\partial s_i}\Big|_{s_i=[\mathcal{S}_i]^*}} \right| < \sec\left(\frac{\pi}{N}\right)^N, \tag{13}$$

where $[\mathcal{S}_i]^*$ denotes the value of $[\mathcal{S}_i]$ at the equilibrium; and it is globally asymptotically stable if there exist constants $\phi_i > 0$, $i \in \{1, 2, \ldots, N\}$ for which

$$\left| \frac{d_i(z_i) - d_i([\mathcal{S}_i]^*)}{c_i(z_i) - c_i([\mathcal{S}_i]^*)} \right| \leqslant \phi_i \quad \forall z_i \neq [\mathcal{S}_i]^* \tag{14}$$

and

$$\prod_{i=1}^{N} \phi_i < \sec\left(\frac{\pi}{N}\right)^N. \qquad \square$$

An alternative condition to (28) which is simpler to verify is given by

$$\left| \frac{\partial d_i(z_i)}{\partial z_i} \right| \leqslant \phi_i \frac{\partial c_i(z_i)}{\partial z_i}, \quad \forall z_i \neq [\mathcal{S}_i]^*.$$

Proof of Lemma 2. The proof of this result relies on making a coordinate transformation to our original system to take it into a form that allows us to use the secant criterion [35], [36], [22] and results in [22].

The dynamics of the feedback interconnection under consideration can be written as

$$[\dot{\mathcal{S}}_1] = -c_1([\mathcal{S}_1]) + d_N([\mathcal{S}_N]), \tag{15a}$$

$$[\dot{\mathcal{S}}_2] = -c_2([\mathcal{S}_2]) + d_1([\mathcal{S}_1]) \tag{15b}$$

$$\vdots \tag{15c}$$

$$[\dot{\mathcal{S}}_N] = -c_N([\mathcal{S}_N]) + d_{N-1}([\mathcal{S}_{N-1}]). \tag{15d}$$

with an equilibrium state defined by concentrations $[\mathcal{S}_i]^*$ for which

$$c_1([\mathcal{S}_1]^*) = d_N([\mathcal{S}_N]^*), \tag{16a}$$

$$c_2([\mathcal{S}_2]^*) = d_1([\mathcal{S}_1]^*), \tag{16b}$$

$$\vdots \tag{16c}$$

$$c_N([\mathcal{S}_N]^*) = d_{N-1}([\mathcal{S}_{N-1}]^*). \tag{16d}$$

To verify that such an equilibrium exists and is unique, note that we have a feedback interconnection of a cascade of N systems, each with an IOSCF given by

$$f_i(u_i^*) = d_i\big(c_i^{-1}(u_i^*)\big), \quad \forall u_1^* \in \mathbb{R},$$

where c_i^{-1} denotes the inverse function of c_i, which is invertible and monotone strictly increasing since c_i is monotone strictly increasing. Therefore, $f_i(u_i^*)$ has the same (strict) monotonicity as d_i. Since an odd number M of the d_i are monotone strictly decreasing, an odd number of the f_i are also monotone strictly decreasing and therefore the composition of all the f_i is monotone strictly decreasing. This shows that we have a negative feedback interconnection and thus a unique equilibrium.

Using (16), we can re-write (15) as

$$\begin{aligned}
[\dot{\mathcal{S}}_1] &= -c_1([\mathcal{S}_1]) + c_1([\mathcal{S}_1]^*) + d_N([\mathcal{S}_N]) - d_N([\mathcal{S}_N]^*),\\
[\dot{\mathcal{S}}_2] &= -c_2([\mathcal{S}_2]) + c_2([\mathcal{S}_2]^*) + d_1([\mathcal{S}_1]) - d_1([\mathcal{S}_1]^*),\\
&\vdots\\
[\dot{\mathcal{S}}_N] &= -c_N([\mathcal{S}_N]) + c_N([\mathcal{S}_N]^*) + d_{N-1}([\mathcal{S}_{N-1}]) - d_{N-1}([\mathcal{S}_{N-1}]^*).
\end{aligned}$$

Since we have an odd number $M \geqslant 1$ of functions d_i that are monotone strictly decreasing and there is perfect symmetry in the cycle (15), we shall assume without loss of generality that d_N is monotone strictly decreasing; if that were not the case we could simply shift the numbering of the modules appropriately.

We consider a coordinate transformation with

$$x_1 = [\mathcal{S}_1] - [\mathcal{S}_1]^*\,, \qquad\qquad x_N = [\mathcal{S}_N] - [\mathcal{S}_N]^*\,, \tag{17}$$

and the remaining x_i, $i \in \{2, 3, \ldots, N-1\}$ either given by

$$x_i = [\mathcal{S}_i] - [\mathcal{S}_i]^* \tag{18}$$

or given by

$$x_i = -\,[\mathcal{S}_i] + [\mathcal{S}_i]^*\,; \tag{19}$$

each to be determined shortly. The coordinate transformation (17) leads to

$$\begin{aligned}
\dot{x}_1 &= -c_1([\mathcal{S}_1]) + c_1([\mathcal{S}_1]^*) + d_N([\mathcal{S}_N]) - d_N([\mathcal{S}_N]^*) \\
&= -c_1([\mathcal{S}_1]^* + x_1) + c_1([\mathcal{S}_1]^*) + d_N([\mathcal{S}_N]^* + x_N) - d_N([\mathcal{S}_N]^*) \\
&= -a_1(x_1) - b_N(x_N)
\end{aligned}$$

with

$$a_1(x_1) \coloneqq c_1([\mathcal{S}_1]^* + x_1) - c_1([\mathcal{S}_1]^*), \qquad b_N(x_N) \coloneqq -d_N([\mathcal{S}_N]^* + x_N) + d_N([\mathcal{S}_N]^*).$$

Note that because c_1 is monotone strictly increasing and d_N is monotone strictly decreasing, we have that

$$a_1(x_1) \quad \begin{cases} > 0 & x_1 > 0 \\ = 0 & x_1 = 0 \\ < 0 & x_1 < 0. \end{cases}, \qquad\qquad b_N(x_N) \quad \begin{cases} > 0 & x_N > 0 \\ = 0 & x_N = 0 \\ < 0 & x_N < 0. \end{cases} \tag{20}$$

For the remaining variables x_i, $i \in \{2, 3, \dots, N\}$, the coordinate transformation leads to

$$\dot{x}_i = \begin{cases} c_i([\mathcal{S}_i]^*) - c_i([\mathcal{S}_i]) + d_{i-1}([\mathcal{S}_{i-1}]) - d_{i-1}([\mathcal{S}_{i-1}]^*) & \text{if } x_i = [\mathcal{S}_i] - [\mathcal{S}_i]^*,\ x_{i-1} = [\mathcal{S}_{i-1}] - [\mathcal{S}_{i-1}]^* \\ & \text{or } x_i = [\mathcal{S}_i] - [\mathcal{S}_i]^*,\ x_{i-1} = [\mathcal{S}_{i-1}]^* - [\mathcal{S}_{i-1}] \\ c_i([\mathcal{S}_i]) - c_i([\mathcal{S}_i]^*) - d_{i-1}([\mathcal{S}_{i-1}]) + d_{i-1}([\mathcal{S}_{i-1}]^*) & \text{if } x_i = [\mathcal{S}_i]^* - [\mathcal{S}_i],\ x_{i-1} = [\mathcal{S}_{i-1}] - [\mathcal{S}_{i-1}]^* \\ & \text{or } x_i = [\mathcal{S}_i]^* - [\mathcal{S}_i],\ x_{i-1} = [\mathcal{S}_{i-1}]^* - [\mathcal{S}_{i-1}] \end{cases}$$
$$= -a_i(x_i) + b_{i-1}(x_{i-1})$$

where, for every $i \in \{2, 3, \dots, N\}$,

$$a_i(x_i) \coloneqq \begin{cases} c_i([\mathcal{S}_i]^* + x_i) - c_i([\mathcal{S}_i]^*) & x_i = [\mathcal{S}_i] - [\mathcal{S}_i]^* \\ -c_i([\mathcal{S}_i]^* - x_i) + c_i([\mathcal{S}_i]^*) & x_i = [\mathcal{S}_i]^* - [\mathcal{S}_i] \end{cases}$$

$$b_{i-1}(x_{i-1}) \coloneqq \begin{cases} d_{i-1}([\mathcal{S}_{i-1}]^* + x_{i-1}) - d_{i-1}([\mathcal{S}_{i-1}]^*) & \text{if } x_i = [\mathcal{S}_i] - [\mathcal{S}_i]^*,\ x_{i-1} = [\mathcal{S}_{i-1}] - [\mathcal{S}_{i-1}]^* \\ d_{i-1}([\mathcal{S}_{i-1}]^* - x_{i-1}) - d_{i-1}([\mathcal{S}_{i-1}]^*) & \text{if } x_i = [\mathcal{S}_i] - [\mathcal{S}_i]^*,\ x_{i-1} = [\mathcal{S}_{i-1}]^* - [\mathcal{S}_{i-1}] \\ -d_{i-1}([\mathcal{S}_{i-1}]^* + x_{i-1}) + d_{i-1}([\mathcal{S}_{i-1}]^*) & \text{if } x_i = [\mathcal{S}_i]^* - [\mathcal{S}_i],\ x_{i-1} = [\mathcal{S}_{i-1}] - [\mathcal{S}_{i-1}]^* \\ -d_{i-1}([\mathcal{S}_{i-1}]^* - x_{i-1}) + d_{i-1}([\mathcal{S}_{i-1}]^*) & \text{if } x_i = [\mathcal{S}_i]^* - [\mathcal{S}_i],\ x_{i-1} = [\mathcal{S}_{i-1}]^* - [\mathcal{S}_{i-1}]. \end{cases}$$

Since all the c_i are monotone strictly increasing, we have that

$$a_i(x_i) \quad \begin{cases} > 0 & x_i > 0 \\ = 0 & x_i = 0 \\ < 0 & x_i < 0. \end{cases}, \qquad \forall i \in \{2, 3, \dots, N\}.$$

We have already selected x_1 and x_N according to (17) to obtain (20). Our goal is now to select

the remaining x_i, $i \in \{2, 3, \ldots, N-1\}$ according to (18) or (19) so that we also have

$$b_{i-1}(x_{i-1}) \quad \begin{cases} > 0 & x_{i-1} > 0 \\ = 0 & x_{i-1} = 0 \\ < 0 & x_{i-1} < 0, \end{cases} \qquad \forall i \in \{2, 3, \ldots, N\}.$$

which would require us to have $\forall i \in \{2, 3, \ldots, N\}$

$$\begin{cases} d_{i-1} \text{ monotone strictly increasing} & \text{if } x_i = [\mathcal{S}_i] - [\mathcal{S}_i]^*,\ x_{i-1} = [\mathcal{S}_{i-1}] - [\mathcal{S}_{i-1}]^* \\ & \text{or } x_i = [\mathcal{S}_i]^* - [\mathcal{S}_i],\ x_{i-1} = [\mathcal{S}_{i-1}]^* - [\mathcal{S}_{i-1}] \\ d_{i-1} \text{ monotone strictly decreasing} & \text{if } x_i = [\mathcal{S}_i] - [\mathcal{S}_i]^*,\ x_{i-1} = [\mathcal{S}_{i-1}]^* - [\mathcal{S}_{i-1}] \\ & \text{or } x_i = [\mathcal{S}_i]^* - [\mathcal{S}_i],\ x_{i-1} = [\mathcal{S}_{i-1}] - [\mathcal{S}_{i-1}]^* \end{cases}$$

It turns out that this is always possible because there is an even number of the d_{i-1} with $i \in \{2, 3, \ldots, N\}$ are monotone strictly decreasing (recall that d_N is monotone strictly decreasing and there are in total an odd number of d_i that are monotone strictly decreasing). All we need to do is to start with x_1 as in (17) and alternate between (18) and (19) each time d_{i-1} is monotone strictly decreasing. Since there is an even number of the d_{i-1} with $i \in \{2, 3, \ldots, N\}$, we will end up with x_N as in (17).

The coordinate transformation constructed above, leads us to a system of the following form

$$\dot{x}_1 = -a_1(x_1) - b_N(x_N) \tag{21a}$$

$$\dot{x}_2 = -a_2(x_2) + b_1(x_1) \tag{21b}$$

$$\vdots \tag{21c}$$

$$\dot{x}_N = -a_N(x_N) + b_{N-1}(x_{N-1}) \tag{21d}$$

To prove the local stability result, we apply the secant criterion [35], [36], [22] to the local linearization of this system around the equilibrium $x_i = 0$, $\forall i$, which has a Jacobian matrix of

the form

$$\begin{bmatrix} -\frac{\partial a_1(x_1)}{\partial x_1}\Big|_{x_1=0} & 0 & \cdots & 0 & -\frac{\partial b_N(x_N)}{\partial x_N}\Big|_{x_N=0} \\ \frac{\partial b_1(x_1)}{\partial x_1}\Big|_{x_1=0} & -\frac{\partial a_2(x_2)}{\partial x_2}\Big|_{x_2=0} & \ddots & & 0 \\ 0 & \frac{\partial b_2(x_2)}{\partial x_2}\Big|_{x_2=0} & -\frac{\partial a_3(x_3)}{\partial x_3}\Big|_{x_3=0} & \ddots & \vdots \\ \vdots & \ddots & \ddots & \ddots & 0 \\ 0 & \cdots & 0 & \frac{\partial b_{N-1}(x_{N-1})}{\partial x_{N-1}}\Big|_{x_{N-1}=0} & -\frac{\partial a_N(x_N)}{\partial x_N}\Big|_{x_N=0} \end{bmatrix}, \tag{22}$$

where

$$\frac{\partial a_i(x_i)}{\partial x_i}\Big|_{x_i=0} = \frac{\partial c_i(s_i)}{\partial s_i}\Big|_{s_i=[\mathcal{S}_i]^*} > 0$$

$$\frac{\partial b_i(x_i)}{\partial x_i}\Big|_{x_i=0} = \begin{cases} \frac{\partial d_i(s_i)}{\partial s_i}\Big|_{s_i=[\mathcal{S}_i]^*} > 0 & \text{if } d_i \text{ monotone increasing} \\ -\frac{\partial d_i(s_i)}{\partial s_i}\Big|_{s_i=[\mathcal{S}_i]^*} > 0 & \text{if } d_i \text{ monotone decreasing,} \end{cases} \qquad \forall i \in \{1, 2, \ldots, N\}.$$

This matrix matches precisely the one considered in the secant criteria, which states that the Jacobian matrix (22) is Hurwitz if (13) holds.

For the global asymptotic stability result we use [22, Corollary 3], which applies precisely to systems of the form (21) with

$$x_i a_i(x_i) > 0, \qquad x_i b_i(x_i) > 0, \qquad \forall x_i \neq 0, i \in \{1, 2, \ldots, N\}.$$

Two additional conditions are needed by [22, Corollary 3]:

$$\lim_{|x_i| \to \infty} \int_0^{x_i} b_i(\sigma) d\sigma = \infty. \tag{23}$$

and there must exist $\phi_i > 0$, $\forall i \in \{1, 2, \ldots, N\}$ for which

$$\frac{b_i(x_i)}{a_i(x_i)} \leqslant \phi_i, \quad \forall i, \ x_i \neq 0, \tag{24a}$$

$$\prod_{i=1}^{N} \phi_i < \sec\left(\frac{\pi}{N}\right)^N. \tag{24b}$$

The first condition (23) holds because our functions b_i are all zero at zero and monotone strictly increasing.

We then prove two conditions to be sufficient for (24a) to be satisfied. Before proceeding, we

make the following observations:

$$\frac{b_i(x_i)}{a_i(x_i)} \coloneqq \begin{cases} \frac{d_i([\mathcal{S}_i]^*+x_i)-d_i([\mathcal{S}_i]^*)}{c_i([\mathcal{S}_i]^*+x_i)-c_i([\mathcal{S}_i]^*)} \quad \text{or} \quad \frac{-d_i([\mathcal{S}_i]^*+x_i)+d_i([\mathcal{S}_i]^*)}{c_i([\mathcal{S}_i]^*+x_i)-c_i([\mathcal{S}_i]^*)} & \text{if } x_i = [\mathcal{S}_i] - [\mathcal{S}_i]^* \\ \\ \frac{d_i([\mathcal{S}_i]^*-x_i)-d_i([\mathcal{S}_i]^*)}{-c_i([\mathcal{S}_i]^*-x_i)+c_i([\mathcal{S}_i]^*)} \quad \text{or} \quad \frac{-d_i([\mathcal{S}_i]^*-x_i)+d_i([\mathcal{S}_i]^*)}{-c_i([\mathcal{S}_i]^*-x_i)+c_i([\mathcal{S}_i]^*)} & \text{if } x_i = [\mathcal{S}_i]^* - [\mathcal{S}_i]\,. \end{cases} \tag{25}$$

$$\frac{\partial a_i(x_i)}{\partial x_i} \coloneqq \begin{cases} \frac{\partial}{\partial x_i} c_i([\mathcal{S}_i]^* + x_i) & \text{if } x_i = [\mathcal{S}_i] - [\mathcal{S}_i]^* \\ -\frac{\partial}{\partial x_i} c_i([\mathcal{S}_i]^* - x_i) & \text{if } x_i = [\mathcal{S}_i]^* - [\mathcal{S}_i]\,. \end{cases} \tag{26}$$

$$\frac{\partial b_i(x_i)}{\partial x_i} \coloneqq \begin{cases} \frac{\partial}{\partial x_i} d_i([\mathcal{S}_i]^* + x_i) \quad \text{or} \quad -\frac{\partial}{\partial x_i} d_i([\mathcal{S}_i]^* + x_i) & \text{if } x_i = [\mathcal{S}_i] - [\mathcal{S}_i]^* \\ \frac{\partial}{\partial x_i} d_i([\mathcal{S}_i]^* - x_i) \quad \text{or} \quad -\frac{\partial}{\partial x_i} d_i([\mathcal{S}_i]^* - x_i) & \text{if } x_i = [\mathcal{S}_i]^* - [\mathcal{S}_i]\,. \end{cases} \tag{27}$$

First, we prove that the condition

$$\Big|\frac{d_i(z_i) - d_i([\mathcal{S}_i]^*)}{c_i(z_i) - c_i([\mathcal{S}_i]^*)}\Big| \leqslant \phi_i \quad \forall z_i \neq [\mathcal{S}_i]^*\,. \tag{28}$$

implies (24a). We see that (28) implies that

$$\frac{d_i(z_i) - d_i([\mathcal{S}_i]^*)}{c_i(z_i) - c_i([\mathcal{S}_i]^*)} \leqslant \phi_i \quad \text{and} \quad -\frac{d_i(z_i) - d_i([\mathcal{S}_i]^*)}{c_i(z_i) - c_i([\mathcal{S}_i]^*)} \leqslant \phi_i \qquad \forall z_i \neq [\mathcal{S}_i]^*\,. \tag{29}$$

With the change of co-ordinates $x_i = -[\mathcal{S}_i]^* + z_i$ and $x_i = [\mathcal{S}_i]^* - z_i$, we see that (29) implies that

$$\begin{aligned} &\frac{d_i([\mathcal{S}_i]^* + x_i) - d_i([\mathcal{S}_i]^*)}{c_i([\mathcal{S}_i]^* + x_i) - c_i([\mathcal{S}_i]^*)} \leqslant \phi_i\,, \quad &&\frac{-d_i([\mathcal{S}_i]^* + x_i) + d_i([\mathcal{S}_i]^*)}{c_i([\mathcal{S}_i]^* + x_i) - c_i([\mathcal{S}_i]^*)} \leqslant \phi_i \\ &\frac{d_i([\mathcal{S}_i]^* - x_i) - d_i([\mathcal{S}_i]^*)}{-c_i([\mathcal{S}_i]^* - x_i) + c_i([\mathcal{S}_i]^*)} \leqslant \phi_i\,, \quad &&\frac{-d_i([\mathcal{S}_i]^* - x_i) + d_i([\mathcal{S}_i]^*)}{-c_i([\mathcal{S}_i]^* - x_i) + c_i([\mathcal{S}_i]^*)} \leqslant \phi_i \qquad \forall x_i \neq 0. \end{aligned} \tag{30}$$

From (25), we conclude that (30) implies (24a).

We then prove that the condition

$$\Big|\frac{\partial d_i(z_i)}{\partial z_i}\Big| \leqslant \phi_i \frac{\partial c_i(z_i)}{\partial z_i}, \quad z_i \neq [\mathcal{S}_i]^* \tag{31}$$

implies (24a). We see that (31) implies that

$$\frac{\partial d_i(z_i)}{\partial z_i} \leqslant \phi_i \frac{\partial c_i(z_i)}{\partial z_i} \quad \text{and} \quad -\frac{\partial d_i(z_i)}{\partial z_i} \leqslant \phi_i \frac{\partial c_i(z_i)}{\partial z_i} \qquad \forall z_i \neq [\mathcal{S}_i]^* \tag{32}$$

With the change of co-ordinates $x_i = -[\mathcal{S}_i]^* + z_i$ and $x_i = [\mathcal{S}_i]^* - z_i$, (32) can be seen to imply that

$$\begin{aligned}
&\frac{\partial}{\partial x_i} d_i([\mathcal{S}_i]^* + x_i) \leqslant \phi_i \frac{\partial}{\partial x_i} c_i([\mathcal{S}_i]^* + x_i)\,, \quad -\frac{\partial}{\partial x_i} d_i([\mathcal{S}_i]^* + x_i) \leqslant \phi_i \frac{\partial}{\partial x_i} c_i([\mathcal{S}_i]^* + x_i) \\
&\frac{\partial}{\partial x_i} d_i([\mathcal{S}_i]^* - x_i) \leqslant -\phi_i \frac{\partial}{\partial x_i} c_i([\mathcal{S}_i]^* - x_i)\,, \quad -\frac{\partial}{\partial x_i} d_i([\mathcal{S}_i]^* - x_i) \leqslant -\phi_i \frac{\partial}{\partial x_i} c_i([\mathcal{S}_i]^* - x_i) \quad \forall x_i \neq 0
\end{aligned} \tag{33}$$

From (26)–(27), we can observe that (33) implies that

$$\frac{\partial b_i(x_i)}{\partial x_i} < \phi_i \frac{\partial a_i(x_i)}{\partial x_i} \quad \forall x_i \neq 0 \tag{34}$$

Let $h_i(x_i) \coloneqq b_i(x_i) - \phi_i a_i(x_i)$. Then, (34) implies that

$$\frac{\partial h_i(x_i)}{\partial x_i} \leqslant 0 \quad \forall x_i \neq 0. \tag{35}$$

Since $a_i(0) = 0$ and $b_i(0) = 0$, we know that $h_i(0) = 0$. Therefore (35) implies that

$$h_i(x_i) \begin{cases} \leqslant 0 & \forall x_i > 0 \\ \geqslant 0 & \forall x_i < 0, \end{cases}$$

which further implies that

$$b_i(x_i) \begin{cases} \leqslant \phi_i a_i(x_i) & \forall x_i > 0 \\ \geqslant \phi_i a_i(x_i) & \forall x_i < 0 \end{cases} \tag{36}$$

Since

$$a_i(x_i) \quad \begin{cases} > 0 & x_i > 0 \\ < 0 & x_i < 0 \end{cases}$$

(36) implies (24a), hence completing our proof. ■

We can then apply Lemma 2 to prove Theorem 2.

Proof of Theorem 2. Each SISO repressor module can be further decomposed into two modules that can be described by the equations

$$[\dot{\mathcal{S}}_0] = u_1 - \bar{\beta}\,[\mathcal{S}_0]$$
$$y_1 \coloneqq h([\mathcal{S}_0]) = \begin{cases} \frac{\alpha P^{\text{tot}}}{1+\frac{1}{K}[\mathcal{S}_0]^2} & \text{if } [\mathcal{S}_0] \geqslant 0 \\ \alpha P^{\text{tot}} \frac{1+\frac{2}{K}[\mathcal{S}_0]^2}{1+\frac{1}{K}[\mathcal{S}_0]^2} & \text{if } [\mathcal{S}_0] < 0. \end{cases}$$

and

$$[\dot{mRNA_1}] = u_2 - \gamma\,[mRNA_1]$$
$$y_2 = \beta\,[mRNA_1]$$

respectively. It can be seen that a small modification has been made to y_1, the repressing output from the TR repressor module. Since our network is positive, this modification has no effect on the network behavior. However, this change makes it more straightforward to apply Lemma 2 to analyze this network, since the theorem relied on each output function being monotone strictly increasing or monotone strictly decreasing $\forall\,[\mathcal{S}_0]$.

From the (13) in Lemma 2, (9)–(10) guarantee that the equilibrium point of the Repressilator network will be LAS.

For GAS, we first need to pick ϕ_1 and ϕ_2 to satisfy

$$\left|\frac{\partial h(z)}{\partial z}\right| \leqslant \phi_1 \bar{\beta} \quad z \neq [\mathcal{S}_0]^*$$
$$\beta \leqslant \phi_2 \gamma.$$

It is straightforward to show that

$$\max_{z \neq [\mathcal{S}_0]^*} \left|\frac{\partial h(z)}{\partial z}\right| = \frac{3\alpha P^{\text{tot}}}{8}\sqrt{\frac{3}{K}},$$

so we can pick

$$\phi_1 = \frac{3\alpha P^{\text{tot}}}{8\bar{\beta}}\sqrt{\frac{3}{K}}, \quad \phi_2 = \frac{\beta}{\gamma}.$$

From the proof of Lemma 2 [37], we can infer that (11) guarantees that the equilibrium point of the Repressilator network will be GAS. □

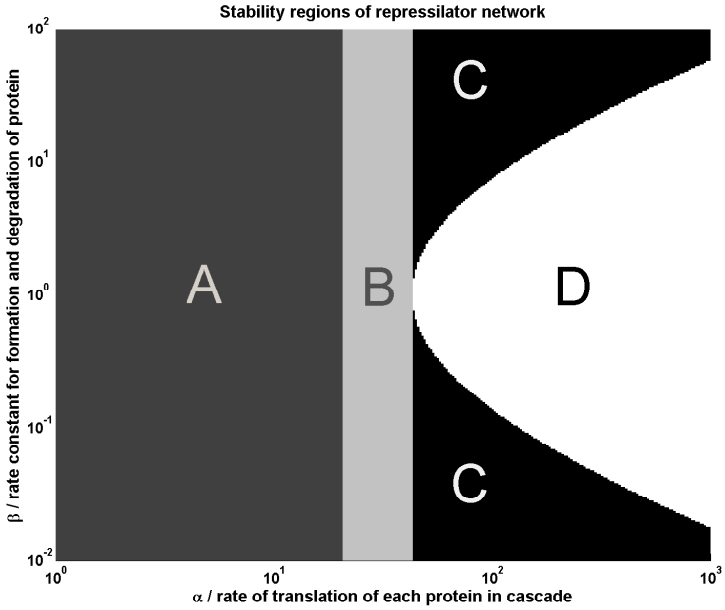

Fig. 5. Stability regions for the 3-gene Repressilator, as a function of the parameters α and $\beta = \bar{\beta}$, under Assumption 1. The (sufficient) condition (11) allows us to conclude that the equilibrium is GAS in region "A;" the (sufficient) condition (9) allows us to conclude that the equilibrium is LAS in regions "A" and "B;" and the (necessary and sufficient) condition (8) allows us to conclude that the equilibrium is LAS in regions "A," "B" and "C;" and also that it is unstable in region "D.".

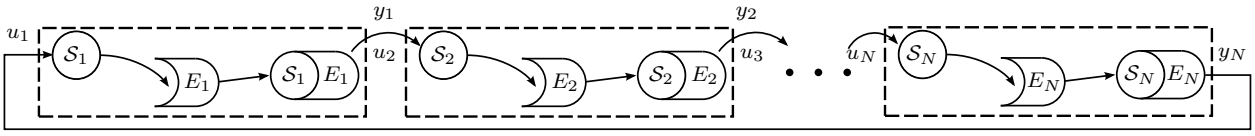

Fig. 6. Biological representation of a Covalent Modification network decomposition.

IV. GENERALIZED COVALENT MODIFICATION NETWORK

We derive a result for the covalent modification network shown in Figure 6. From a biological perspective, this result implies that regardless of the parameters chosen, substrates that are covalently modified in this cyclic manner will almost certainly completely degrade. Despite the fact that each of the substrates is essentially "activating" the next substrate in the cascade, the degradation reactions dominate in this case.

Theorem 3. *Consider the covalent modification network shown in Figure 6, which consists of a cascade of N CM modules connected in (positive) feedback. The substrate concentrations in each module converges to 0 as $t \to \infty$.* □

To prove Theorem 3, we use the following result which is adapted from [25, Theorems 2-3], and provides conditions that can be used to establish the stability of the equilibrium points for positive feedback networks. To express the result, we need the following definitions which are closely related to that of cooperativity: We say that the system

$$\dot{x} = A(x,u), \qquad y = B(x), \qquad x \in \mathbb{R}^n, u \in \mathbb{R}^k, y \in \mathbb{R}^m, \tag{37}$$

is *excitable (with respect to the positive orthant)* if for every initial condition $x_0 \in \mathbb{R}^n$ and all inputs $u(t), \bar{u}(t) \in \mathbb{R}^k$, $\forall t \geqslant 0$, we have that

$$u(t) \succ \bar{u}(t),\ \forall t \geqslant 0 \quad \Rightarrow \quad x(t;x_0,u) \gg x(t;x_0,\bar{u}),\ \forall t > 0,$$

and it is *transparent (with respect to the positive orthant)* if for all initial conditions $x_0, \bar{x}_0 \in \mathbb{R}^n$ and every input $u(t) \in \mathbb{R}^k$, $\forall t \geqslant 0$, we have that

$$x_0 \succ \bar{x}_0 \quad \Rightarrow \quad x(t;x_0,u) \gg x(t;\bar{x}_0,u),\ \forall t > 0.$$

Given two vectors $v, \bar{v}$, we write $v \succ \bar{v}$ if every entry of v is larger than or equal to the corresponding entry of $\bar{v}$ and $v \neq \bar{v}$.

Lemma 3. *Consider the feedback interconnection of a SISO module $\mathcal{M}$ of the form* (37)*, whose output y is fed back to its input u. Assume that*

1) *$\mathcal{M}$ is excitable, transparent, and cooperative with a well-defined ISSCF and IOSCF;*
2) *for every constant input $u(t) = u^*$, $\forall t \geqslant 0$ to $\mathcal{M}$, the Jacobian matrix $\frac{\partial A(x,u)}{\partial x}$ is nonsingular at the corresponding equilibrium, which is globally asymptotically stable;*
3) *the IOSCF $f(u^*)$ of $\mathcal{M}$ has fixed points $\bar{u}^*$ for which $f_1(\bar{u}^*) = \bar{u}^*$ and $\left.\frac{\partial f_1(u^*)}{\partial u^*}\right|_{u^*=\bar{u}^*} \neq 1$;*
4) *all trajectories of the feedback interconnection are bounded.*

Then, for almost all initial conditions, the solutions converge to the set of points for which $f(u^*) = u^*$ and $\frac{\partial f(u^*)}{\partial u^*} < 1$. □

We then use Lemma 3 to prove Theorem 3.

Proof of Theorem 3. Every CM module $\mathcal{M}_i$ for $i \in \{1, \cdots, N\}$ is given by

$$\boldsymbol{\mathcal{M}_i}: [\dot{\mathcal{S}}_i] = A_i([\mathcal{S}_i], u_i), \qquad y_i = B_i([\mathcal{S}_i])$$

where A_i and B_i can be deduced from (5). We first show that the cascade of two CM modules satisfies the properties in item 1 from Lemma 3. For the cascade network with $y_1 = u_2$, we have

$$\frac{\partial A_1([\mathcal{S}_1], u_1)}{\partial u_1} = 1 \qquad \frac{\partial A_2([\mathcal{S}_2], u_2)}{\partial [\mathcal{S}_1]} > 0 \qquad \frac{\partial y_2}{\partial [\mathcal{S}_2]} > 0$$
$$\frac{\partial A_1([\mathcal{S}_1], u_1)}{\partial [\mathcal{S}_2]} = 0 \qquad \frac{\partial A_2([\mathcal{S}_2], u_2)}{\partial u_1} = 0 \qquad \frac{\partial y_2}{\partial [\mathcal{S}_1]} = 0,$$

from which we can infer several properties: From [26, Propositon 1], we conclude that the cascade is cooperative, from [25, Theorem 4] that it is excitable, and from [25, Theorem 5] that it is transparent. The IOSCF of the cascade is well-defined and given by $f_2(f_1(u_1^*))$, where u_1^* denotes a constant input to the cascade and $f_i(u_i^*)$ can be obtained from (6). The ISSCF of the cascade is also well-defined, and therefore, the cascade of two CM modules satisfies the properties in item 1 of Lemma 3.

We now show that the cascade of two CM modules satisfies the properties in item 2 from Lemma 3. For some constant input $u_1^* \geqslant 0$, the Jacobian matrix of the interconnection is given by the 2×2 lower triangular matrix

$$J = \begin{bmatrix} J_{11} & 0 \\ J_{21} & J_{22}, \end{bmatrix}$$

which is non-singular because $J_{11} > 0$ and $J_{22} > 0$, under the implicit assumption that all parameters within each module are positive.

Each of the modules $\mathcal{M}_1$ and $\mathcal{M}_2$ has an equilibrium point that is globally asymptotically stable for some constant input u_i^* into each module. This can be verified by doing the coordinate transformation $x_i = [\mathcal{S}_i] - [\mathcal{S}_i]^*$ and observing that the Lyapunov function $V(x_i) = x_i^2$ is zero-at-zero, locally positive definite and $\dot{V}(x_i) < 0$, $\forall x_i$, $i \in \{1, 2\}$. The cascade of both the modules also has a globally asymptotically stable equilibrium point, as can be seen from the argument in [38].

To verify that the cascade of the CM modules satisfies the property in item 3 from Lemma 3, we will show that $f_2(f_1(u_1^*)) = u_1^*$ has a unique solution at $u_1^* = 0$, and also that

$$\left.\frac{\partial f_2(f_1(u_1^*))}{\partial u_1^*}\right|_{u_1^*=0} < 1.$$

To show that $f_2(f_1(u_1^*)) = u_1^*$ has a unique solution at $u_1^* = 0$, we first observe that the IOSCFs of $\mathcal{M}_1$ and $\mathcal{M}_2$ are each monotone increasing and strictly concave, since $f_i'(u_i^*) > 0$ and $f_i''(u_i^*) < 0$, $\forall i \in \{1, 2\}$. Then from

$$\frac{\partial f_2\left(f_1\left(u_1^*\right)\right)}{\partial u_1^*} = f_1'(u_1^*) f_2'\left(f_1\left(u_1^*\right)\right) \tag{38}$$

$$\frac{\partial^2 f_2\left(f_1\left(u_1^*\right)\right)}{\partial (u_1^*)^2} = f_1''(u_1^*) f_2'\left(f_1\left(u_1^*\right)\right) + \left(f_1'\left(u_1^*\right)\right)^2 f_2''\left(f_1\left(u_1^*\right)\right), \tag{39}$$

it can be seen that the cascade of $\mathcal{M}_1$ and $\mathcal{M}_2$ is also monotone increasing and strictly concave because

1) $f_1'(u_1^*) > 0$ and $f_2'(u_2^*) > 0 \quad \forall u_1^*, u_2^* > 0 \implies \frac{\partial f_2\left(f_1\left(u_1^*\right)\right)}{\partial u_1^*} > 0$ from (38).
2) $f_1''(u_1^*) < 0$ and $f_2''(u_2^*) < 0 \quad \forall u_1^*, u_2^* > 0 \implies \frac{\partial^2 f_2\left(f_1\left(u_1^*\right)\right)}{\partial (u_1^*)^2} < 0$ from (39).

Therefore, there can be no other solution to $f_2(f_1(u_1^*)) = u_1^*$ other than $u_1^* = 0$. In addition, the derivative of the IOSCF of the cascade at this equilibrium is given by

$$\left.\frac{\partial f_2(f_1(u_1^*))}{\partial u_1^*}\right|_{u_1^*=0} = \frac{E_1^{\text{tot}} E_2^{\text{tot}} k_1^{\text{cat}} k_2^{\text{cat}}}{(E_1^{\text{tot}} k_1^{\text{cat}} + K_1^d \gamma_1)(E_2^{\text{tot}} k_2^{\text{cat}} + K_2^d \gamma_2)},$$

which is always less than 1 since all parameters are positive by definition.

The boundedness property in item 4 of Lemma 3 follows from techniques used to analyze MAK ODEs from [39, Main Technical Lemma], which can be applied to the covalent modification network.

This argument can be extended over each of the N modules to complete the proof. ■

V. CONCLUSIONS AND FUTURE WORK

In this paper, we studied two important classes of biological networks. An important technical tool that permitted the use of many powerful results from control theory was the conception of these networks as a set of functionally isolated but interconnected modules. Using this technique, we can extend these results to include more complex behavior such as delayed networks and stochastic gene regulation.

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