Design of a molecular bistable system with RNA-mediated regulation

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Abstract—we design a new biomolecular circuit with the potential for bistable behavior. We study this candidate toggle switch and find sufficient conditions on key parameters that guarantee bistability. The circuit structure is based on a positive feedback loop created by the mutual repression of two synthetic genes. Repression is generated not by direct inactivation of the promoter region, but by inactivation of the enzymes performing transcription. Inactivation is experimentally possible by designing the RNA outputs of the two genes to be inhibitory aptamers for the enzymes.

I. INTRODUCTION

In vitro synthetic molecular circuits are attractive because they allow us to build new biological functions in a simplified context [1]. Circuits that rely on the rational design of nucleic acids are particularly powerful, because a variety of reaction primitives are available [2]. Using RNA and DNA as regulatory molecules, in this and a companion paper [3] we design two canonical dynamic networks in biology: a bistable switch (toggle switch) and an oscillator. We use synthetic genes whose RNA outputs create regulatory loops by modulating the activity of the enzymes producing them.

In this paper, we design and analyze the model of a toggle switch where two synthetic genes produce two different RNA transcripts relying on different enzyme species. Each RNA species is designed to inhibit the transcribing enzyme of the other RNA species, overall creating a positive feedback loop (Fig. 1). This type of regulation is experimentally achievable using recently published RNA sequences [4], [5], and our laboratory is actively pursuing the construction of this circuit.

Here we consider a simple model capturing the essential interactions among molecular species. This model is not exhaustive, but is useful to provide intuition on what are the key reaction rates to achieve bistability. We focus on sufficient conditions for bistability that relate the RNA transcription rates and the enzyme activation rates in the system. We strive to derive analytical conditions that make a limited number of assumptions on the parameters [6], [7], [8]. Transcription rates are often not controllable in the system, because they depend on the enzyme properties, which are difficult to tune. Mechanisms to achieve enzyme reactivation are currently under study in our laboratory, thus it is important for us to understand what is their role in the system, relative to parameters that are not under our control such as transcription rates.

In the past decade several bistable molecular circuits have been designed and built [9], [10], with recent in vitro implementations [1], [11], [12]. All these circuits are based on the creation of an overall positive feedback loop. Our circuit is based on transcription reactions as in [1], [12], however regulation is not achieved by modulating the activity of the genes’ promoter region, but by modulating the activity of the transcribing enzymes. Because it relies on reactions naturally occurring in the cellular environment, this circuit may function in vivo, in contrast with other recently proposed in vitro designs [1], [11], [12].

![Fig. 1. A: Structure of our two node transcriptional toggle switch; G1 and G2 are synthetic genes. B: Proposed experimental implementation of our RNA-based switch, where the transcription enzymes are inhibited by RNA aptamers.](image)

II. CIRCUIT DESCRIPTION AND MODELING

Biomolecular bistable switches are generally built based on the principle that a positive feedback loop is required; the presence of a positive feedback loop is a well known necessary conditions set forth by Thomas [13], [14]. We propose to design a positive loop using in vitro transcription reactions, designing RNA inputs to regulate the ability of synthetic genes to generate RNA outputs. We will then discuss sufficient conditions on the reaction parameters to achieve bistability. Regulation of transcription is achieved by...
modulating the activity of RNA polymerases (RNAP), rather than the activity of promoters. Using specific RNA aptamers (short, noncoding RNA sequences that bind to a desired target [15, 16]) we can inhibit the activity of two well characterized and commercially available bacteriophage RNAP species [4, 5].

Fig. 1 shows our circuit structure: two synthetic genes produce RNA outputs $R_1$ and $R_2$. The RNA outputs are transcribed by two different enzymes $E_1$ and $E_2$; for example, one could use bacteriophage SP6 and T7 RNAPs, and the corresponding promoter sequences (purple and blue domains on the genes in Fig. 1). We assume that enzymes can either be in an active or inactive state, and their mass is conserved: $[E_i^{tot}] = [E_i] + [E_i^*]$, $i = 1, 2$. Each enzyme $E_i$ is inhibited by RNA species $R_j$ (through a direct aptamer binding pathway), and we assume it reverts to its active state at a given rate which could be driven by a strand displacement reaction [17] (i.e. another RNA or DNA specie binds to $R_2$ and releases the enzyme).

The designed reactions are:

$$E_i + g_i \xrightarrow{k_i} E_i + R_i + g_i \quad (1)$$
$$R_i \xrightarrow{\delta_i} 0 \quad (2)$$
$$E_i + R_j \xrightarrow{\gamma} E_i^* \quad \gamma \quad \beta E_i^* \xrightarrow{\beta_i} E_i \quad (4)$$

for $i = 1, 2$ and $j = 2, 1$, where $E_i$ are active enzymes, $E_i^*$ are inactive enzymes, $R_i$ are RNA species, $g_i$ are genes (constant). From now on, for simplicity we assume that both subcircuits have the same reaction rates, i.e. $k_1 = k_2 = k$, $\delta_1 = \delta_2 = \delta$ etc. (This simplifying assumption may be violated in experimental implementations of the circuit.)

Using mass action laws, we can derive the ODEs describing the dynamics of the system:

$$\dot{[R_i]} = k[E_i][g_i] - \delta_i[R_i] - \gamma_i[E_j][R_i] \quad (5)$$
$$\dot{[E_i]} = \beta_i([E_i^{tot}] - [E_i]) - \gamma_i[E_j][R_i] \quad (6)$$

We now switch to a more compact notation, and define $x_1 = [R_1]$, $x_2 = [E_1]$, $x_3 = [R_2]$, and $x_4 = [E_2]$. Because the concentration of genes is constant, we define $\kappa_1 = k g_1$ and $\kappa_2 = k g_2$. The system of ODEs modeling our system can be rewritten as:

$$\begin{align*}
\dot{x}_1 &= \kappa_1 x_2 - \delta x_1 - \gamma_1 x_2 x_1 \\
\dot{x}_2 &= \beta_1 x_2^{tot} - \beta x_2 - \gamma_2 x_3 \\
\dot{x}_3 &= \kappa_2 x_4 - \delta_1 x_3 - \gamma_2 x_3 x_4 \\
\dot{x}_4 &= \beta_2 x_4^{tot} - \beta x_4 - \gamma_4 x_1 x_4
\end{align*} \quad (7)$$

In the next sections, we investigate analytically the potential for bistability of model (7), supporting our findings with simple numerical simulations. Under some simplifying assumptions on the parameters, we will find conditions on RNA production rates and enzyme recovery rates that can guarantee bistability.

III. STABILITY ANALYSIS

We begin by deriving equilibrium conditions. We set each ODE in model (7) equal to zero for $i = 1, \ldots, 4$, and derive the equations below which describe the equilibrium relationship between the enzyme concentrations $x_2$ and $x_4$:

$$\begin{align*}
\beta_1 \gamma_1 x_2^3 + (\gamma \kappa_1 x_2 - \beta_1 \gamma_2 x_2^{tot} + \beta \delta) x_4 - \beta_1 \delta x_4^{tot} &= 0, \quad (8) \\
\beta_2 \gamma_2 x_2^3 + (\gamma \kappa_2 x_4 - \beta_2 \gamma_2 x_2^{tot} + \beta \delta) x_2 - \beta_2 \delta x_4^{tot} &= 0. \quad (9)
\end{align*}$$

It is easy to verify that each of these second order equations has a unique positive solution; thus from the first equation we can find an expression of $x_4$ as a monotonic strictly decreasing function of $x_2$, and from the second equation we can find $x_2$ as a monotonic strictly decreasing function of $x_4$. Expressions are reported in Appendix A. We numerically plot these equilibrium conditions in Fig. 2; for illustrative purposes, we choose the following parameters: $\beta = 4 \cdot 10^{-3}$ /s, $\gamma = 5 \cdot 10^5$ /M/s, $\delta = 3 \cdot 10^{-3}$ /s, $\kappa_1 = \kappa_2 = 1 \cdot 10^{-2}$ /s, $x_4^{tot} = x_2^{tot} = 100$ nM. These parameters are close to realistic values for in vitro circuits [18], however our assumed inhibition rate $\gamma$ may be relatively high for aptamer systems. The equilibrium values $\bar{x}_2$ and $\bar{x}_4$ satisfying the equations above can be used to find the equilibria of $x_1$ and $x_3$, using again our model (7).

$$\bar{x}_1 = \beta_1 \frac{x_4^{tot} - \bar{x}_4}{\gamma_1}, \quad \bar{x}_3 = \beta_2 \frac{x_2^{tot} - \bar{x}_2}{\gamma_2}. \quad (10)$$

Fig. 2. Equilibrium conditions (8) (orange) and (9) (cyan), and overlapped selection of trajectories (magenta).
To investigate the local stable or unstable nature of each equilibrium, we look at the linearized dynamics. The Jacobian is:

\[
J = \begin{bmatrix}
-\gamma \bar{x}_4 - \delta & \kappa_1 & 0 & * \\
0 & -\beta - \gamma \bar{x}_3 & -\gamma \bar{x}_2 & 0 \\
0 & -\gamma \bar{x}_3 & -\gamma \bar{x}_2 - \delta & \kappa_2 \\
-\gamma \bar{x}_4 & 0 & 0 & -\beta - \gamma \bar{x}_1 \\
\end{bmatrix}
\]  

(11)

By examining the Jacobian and its characteristic polynomial, we find that instability of the linearized system can only occur through transition of the real dominant eigenvalue to the positive half plane.

**Lemma 1** Any equilibrium of system (7) is unstable if and only if the constant term of the characteristic polynomial is negative. Instability can only be driven by a simple, real (positive) eigenvalue.

**Proof** The Jacobian is similar to a Metzler matrix through a transformation \( T = \text{diag}(1, 1, -1, -1) \):

\[
T^{-1}JT = \begin{bmatrix}
-\gamma \bar{x}_4 - \delta & \kappa_1 & 0 & * \\
0 & -\beta - \gamma \bar{x}_3 & -\gamma \bar{x}_2 & 0 \\
0 & -\gamma \bar{x}_3 & -\gamma \bar{x}_2 - \delta & \kappa_2 \\
-\gamma \bar{x}_4 & 0 & 0 & -\beta - \gamma \bar{x}_1 \\
\end{bmatrix}
\]

Therefore, the Jacobian always has a real dominant eigenvalue, i.e. \( \lambda_{\text{max}} > \Re \epsilon(\lambda_i), \forall \lambda_i \in J \) [19].

The characteristic polynomial of the Jacobian (11) is computed in Appendix B, expression (32). All its coefficients are real and are positive with the exception of the constant term, which can be positive or negative.

Assume the constant term of the characteristic polynomial \( p_2(s) \) is negative. Then, we know that \( p_2(0) < 0 \) and it is real. Take \( s \to \infty, p_2(s) > 0 \), because all other coefficients are positive. Thus, there must be at least one point in the right half plane that is a root of \( p_2 \). Thus, the system is unstable. Because the matrix is Metzler, the largest root must be real.

If the system is unstable, then the characteristic polynomial must have at least one root with positive real part. *Ab absurdo*, suppose the constant term is positive. Then instability can only occur with a pair of complex conjugate eigenvalues with positive real part. This is impossible because the Jacobian is a Metzler matrix and the dominant eigenvalue must be real. Then, the constant term of the characteristic polynomial must be negative.

**IV. SUFFICIENT CONDITIONS FOR BISTABILITY**

We now seek any conditions on the transcription and recovery reaction rates that can guarantee bistability of the system. We begin by making two additional simplifying assumptions:

1) The total amount of enzyme is identical for the two subcircuits: \( E_1^{\text{tot}} = E_2^{\text{tot}} = E^{\text{tot}}, \) i.e. \( x_2^{\text{tot}} = x_4^{\text{tot}} = E^{\text{tot}} \).

2) The rates of transcription of the RNA species are identical (see reaction (1)): \( \kappa_1 = \kappa_2 = \kappa \).

Bistability requires the presence of two stable and one unstable equilibrium. From our equilibrium conditions (8) and (9), we establish that the system presents three equilibria when \( \kappa > 2\beta \). Calculations are shown in Appendix C.

We then derive some additional conditions on the equilibria. First, by subtracting the equations (8) and (9) (equilibrium conditions), we get:

\[
\beta \gamma (x_4^2 - x_2^2) + (\beta \delta - \beta \gamma E^{\text{tot}})(x_4 - x_2) = 0. 
\]

(12)

This equation is satisfied when either of these two equations holds:

\[
x_4 = x_2, 
\]

(13)

\[
x_4 = -x_2 + \left( E^{\text{tot}} - \frac{\delta}{\gamma} \right). 
\]

(14)

Note that \( x_4 + x_2 \) must be a positive quantity, thus \( (E^{\text{tot}} - \frac{\delta}{\gamma}) \) must be positive for our problem to be physically meaningful. We plot equations (13) and (14) overlapped to the equilibrium conditions (8) and (9) in Fig. 3. If we pick \( \kappa > 2\beta \), we find three intersection points A, B, and C which define the equilibria of the system.

![Equation plots](image)

Fig. 3. Equilibrium conditions (13), (14) and their intersections with conditions (8) and (9).

Based on Lemma 1, we know that stability of any equilibrium depends on the constant term \( a_0 \) of the characteristic polynomial (31):

\[
a_0 = \left( \beta \gamma \bar{x}_4 + \beta \delta + \gamma \bar{x}_1 \right) \left( \beta \gamma \bar{x}_2 + \beta \delta + \delta \bar{x}_3 \right) - \gamma^2 \bar{x}_2 \bar{x}_4. 
\]

(15)

If \( a_0 > 0 \), then the equilibrium is stable; if \( a_0 < 0 \), then the equilibrium is unstable. We further manipulate
expression (15), after multiplying it by the positive factor ∆x₄. The modified constant term is now \( P₀ = a₀\bar{x}_2\bar{x}_4 \).

\[
P₀ = \left( \beta \gamma \bar{x}_2^3 + \beta \delta \bar{x}_4 + \delta \gamma \bar{x}_4 \right) \left( \beta \gamma \bar{x}_2^3 + \beta \delta \bar{x}_2 + \delta \gamma \bar{x}_3 \bar{x}_2 \right)
- \gamma^2 \kappa^2 \bar{x}_3^2 \bar{x}_4^2
\]

(16)

We finally substitute the equilibrium expressions for \( \bar{x}_3 \) and \( \bar{x}_4 \) found in equation (10), thus equation (16) becomes:

\[
P₀ = \left( \beta \gamma \bar{x}_2^3 + \beta \delta E^{tot} \right) \left( \beta \gamma \bar{x}_2^3 + \beta \delta E^{tot} \right) - \gamma^2 \kappa^2 \bar{x}_3^2 \bar{x}_4^2
\]

(17)

**Unstable node (Point B)** For bistability, we require that point B is unstable. This occurs when \( P₀ < 0 \). Point B is the only intersection of equation (13) and the equilibrium conditions (8) and (9). We substitute \( x_2 = \bar{x}_4 = \bar{z} \) in equation (18), and we find a fourth order polynomial in \( \bar{x}_4 \):

\[
P₀^δ(\bar{z}) = \left( \beta \gamma^2 + \beta \delta E^{tot} \right)^2 - \gamma^2 \kappa^2 \bar{z}^4
\]

(18)

Keeping in mind that \( z > 0 \), we can rewrite \( P₀^δ(\bar{z}) \) as \( P₀^δ(t) = -at^2 + bt + c \), where \( t = \bar{z}^2 \) and \( a, b, c > 0 \). Due to the negative sign in front of \( a \), this polynomial is negative for \( t \) larger than its biggest root. Thus, \( P₀^δ(t) < 0 \) when:

\[
t = \bar{z}^2 = \bar{x}_4 > \frac{\beta \delta E^{tot}}{\gamma} \frac{1}{\kappa - \beta}
\]

(19)

We also know, from (14), that

\[
\bar{x}_4 < \left( E^{tot} - \frac{\delta}{\gamma} \right)
\]

(20)

Combining these bounds (19) and (20), we find the following sufficient condition that guarantees \( P₀^δ < 0 \) and thus instability of point B:

\[
\kappa > \beta (1 + \Theta), \quad \Theta = \frac{\gamma \delta E^{tot}}{(\gamma E^{tot} - \delta)^2}
\]

(21)

**Stable node (Points A and C)** Stable nodes result from the intersection of equilibrium conditions (8) and (9) with equation (14). As done for the unstable node, we use these conditions on the equilibria within the expression for term \( P₀ \), in order to find the relationship between the transcription and recovery parameters that guarantee stability of points A and C.

We begin by summing equations (8) and (9). It is convenient to rewrite their sum as:

\[
\beta \gamma (x_2 + x_4)^2 + (\beta \delta - \beta \gamma E^{tot}) (x_2 + x_4)
+ 2\gamma (\kappa - \beta) x_2 x_4 - 2\beta \delta E^{tot} = 0
\]

(22)

Now, we can substitute term \( x_2 + x_4 \) with the equivalent parametric value that can be found from equation (14).

Thus, we find a parametric expression for the product \( x_2 x_4 \):

\[
x_2 x_4 = \frac{\beta \delta E^{tot}}{\gamma (\kappa - \beta)}
\]

(23)

We then rewrite (17) as:

\[
P₀ = (\beta \delta E^{tot}) (\beta \gamma (x_2^2 + x_4^2) + \gamma^2 (\beta^2 - \kappa^2) x_2^2 x_4^2 + (\beta \delta E^{tot})^2)
\]

(24)

and as done before we substitute the parametric expressions for \( x_2 x_4 \) and \( x_2 x_4 \) found above, and we impose that the resulting new expression for \( P₀ \) be positive. We find:

\[
(\beta \gamma) (\beta \delta E^{tot}) \left( E^{tot} - \frac{\delta}{\gamma} \right)^2 - 4\beta \kappa^2 \gamma \delta E^{tot} > 0
\]

(25)

We find the following sufficient condition relating \( \kappa \) and \( \beta \),

\[
\kappa > \beta (1 + \Theta), \quad \Theta = \frac{\gamma \delta E^{tot}}{(\gamma E^{tot} - \delta)^2}
\]

(26)

where \( \Theta \) was defined at condition (21). By imposing condition (26) we guarantee that the system is bistable.

V. NUMERICAL ANALYSIS

We ran numerical simulations using MATLAB, checking the bistability region for the system in a range of values for \( \beta \) and \( \kappa \). First, we compute equilibria using our equilibrium conditions described in the previous sections, discarding negative solutions which are not physically acceptable. For each equilibrium point, we find the eigenvalues of the corresponding Jacobian. A combination of parameters is classified as yielding bistability if we find two stable and one unstable equilibrium between them. Our results are shown in Fig. 4, and they are compared with the analytical bounds for \( \kappa \) and \( \beta \) derived at the previous sections. While we coarsely vary \( \kappa \) and \( \beta \) between 0.001 and 0.9, all other parameters are held constant as: \( \gamma = 5 \cdot 10^5 \text{ M/s} \), \( \delta = 3 \cdot 10^{-3} \text{ s} \), \( x_2^{tot} = x_4^{tot} = 100 \text{ nM} \) (these are the same parameters used to plot Figs. 2 and 3).

Referring to Fig. 4, light green dots correspond to the bistability region. Dark blue dots correspond to a region where the system is not bistable. The orange line plots condition (26) for our specific choice of parameters, while the red line marks the \( \kappa > 2\beta \) conservative condition that guarantees the presence of three equilibria. We remark that three equilibria may occur also for \( \beta < \kappa < 2\beta \), as highlighted by our numerical results; unfortunately, we were not able to analytically find a condition less conservative than \( \kappa > 2\beta \).

VI. CONCLUSIONS

We have described a design idea for a new molecular bistable system, which relies on the use of RNA aptamers for regulation. A simple model for this switch is thoroughly analyzed and its potential for bistability is demonstrated. In particular we derive sufficient conditions for bistability that focus on RNA transcription and enzyme reactivation rates, which we consider key experimental parameters. Our laboratory is actively pursuing
the construction of this circuit. We expect that a more exhaustive model will be required for the purpose of data fitting; however analysis of this simple model provided significant intuition on the potential for bistability of this circuit.

**APPENDIX**

A. Monotonicity of equilibrium conditions (8) and (9)

From equation (8) we derive an expression for \( x_4 \) as a function of \( x_2 \):

\[
x_4 = \frac{\beta \delta E_{x4} - \gamma E_{x4}^2 x_2 - \gamma x_2^2}{\gamma k x_2}.
\]

(27)

Define:

\[
p_1 = \beta \gamma, \quad p_2 = \left( E_{x2}^2 - \frac{\delta}{\gamma} \right),
\]

\[
p_3 = \beta \delta E_{x2}, \quad p_1 = \gamma k.
\]

(28)

Note that \( p_i > 0, \ i = 1, ..., 4 \). We can rewrite (27) as:

\[
x_4 = \frac{p_3 + p_1 p_2 x_2 - p_1 x_2^2}{p_4 x_2} = \frac{p_3}{p_4 x_2} + \frac{p_1 p_2}{p_4} - \frac{p_1 x_2}{p_4},
\]

(29)

where \( x_2 \neq 0 \). We now take the derivative of \( x_4 \) with respect to \( x_2 \):

\[
\frac{dx_4}{dx_2} = -\frac{p_3 p_4}{p_1 x_2^2} + 0 - \frac{p_1}{p_4} = -\frac{p_3}{p_4 x_2} - \frac{p_1}{p_4}
\]

\[
= \frac{1}{p_4} \left( \frac{p_1}{x_2^2} + p_1 \right).
\]

(30)

The above derivative is strictly negative if all parameters are positive, thus equilibrium condition (27) is a monotonic strictly decreasing function. The same procedure can be repeated for the equilibrium condition of \( x_2 \) as a function of \( x_4 \).

B. Analysis of the characteristic polynomial

We compute the characteristic polynomial of the Jacobian matrix (11), reported in equation (32). We find that all the coefficients of the polynomial are positive, with the exception of the constant term. To explore the existence of any parameter-independent, we first rewrite the polynomial with a set of aggregate coefficients, then use the Routh-Hurwitz criterion to derive stability/instability conditions. We define the positive coefficients:

\[
P : = \beta + \delta + \gamma (\bar{x}_2 + \bar{x}_3), \quad Q : = \beta + \delta + \gamma (\bar{x}_1 + \bar{x}_4),
\]

\[
W : = \beta + \delta + \gamma \bar{x}_2 + \delta \bar{x}_3, \quad Z : = \beta \delta + \delta \bar{x}_1 + \beta \gamma \bar{x}_4.
\]

Then, the characteristic polynomial (32) can be rewritten as:

\[
p(s) = s^4 + s^3 (P + Q) + s^2 (W + Z + PQ) + s (QW + PZ) + WZ - \gamma^2 k_1 k_2 \bar{x}_2 \bar{x}_4
\]

\[
= s^4 + a_2 s^3 + a_3 s^2 + a_4 s + a_0
\]

(31)

All coefficients of the polynomial are real; all coefficients are positive, with the exception of the constant term \( a_0 \) which may be positive or negative.

C. Admissible number of equilibria

We rewrite the equilibrium condition (8) as:

\[
a_1 x_4^2 + b_1 x_4 + c_1 = 0,
\]

(33)

where \( a_1 = \beta \gamma, b_1 = \gamma k x_2, c_1 = -\beta \delta E_{x4} \). This equation has a single positive root \( x_4 = \frac{-b_1 + \sqrt{b_1^2 - 4a_1c_1}}{2a_1} \).

We can equate the expression above with expression (27) derived at Appendix A. We thus find a polynomial in variable \( x_2 \). After several computations we find:

\[
A_2 x_2^4 + B_2 x_2^3 + C_2 x_2^2 + D_2 x_2 + E_2 = 0,
\]

(34)

where:

\[
A_2 = A_1^2 - (\gamma k)^2 f_{12},
\]

(35)

\[
B_2 = 2A_1 B_1 - 2 (\gamma k)^2 f_{11} f_{12},
\]

(36)

\[
C_2 = B_1^2 + 2A_1 C_1 - (\gamma k)^2 (f_{11}^2 - 4a_1 c_1),
\]

(37)

\[
D_2 = 2B_1 C_1
\]

(38)

\[
E_2 = C_1^2
\]

(39)

\[
A_1 = -2a_1 \beta \gamma + (\gamma k) f_{12},
\]

(40)

\[
B_1 = 2a_1 \beta \gamma E_{x4} - 2a_1 \beta \delta + (\gamma k) f_{11},
\]

(41)

\[
C_1 = 2a_1 \beta \delta E_{x4}
\]

(42)

\[
f_{11} = \beta \delta - \beta \gamma E_{x4}, \quad f_{12} = \gamma k.
\]

(43)

The Routh table for polynomial (34) is:

| row 0: | \( R_2 \) | \( R_{12} \) | \( E_2 \) |
| row 1: | \( R_2 = \frac{R_1 (D_2 - B E_2)}{R_1} \) | \( E_2 \) |
| row 2: | \( R_2 = \frac{R_1 (B_2 - A_1 D_2)}{R_1} \) | \( E_2 \) |
| row 3: | \( B_2 = \frac{B_1 + C_1 - A_1 D_2}{R_1} \) | \( E_2 \) |
| row 4: | \( A_2 = \frac{A_1 B_1 - A_1 D_2}{R_1} \) | \( C_2 \) | \( E_2 \) |
\[
\det(sI - J) = s^3 + \left(2\beta + 2\delta + \gamma(\bar{x}_1 + \bar{x}_2 + \bar{x}_3 + \bar{x}_4)\right) s^2 \\
+ \left(\beta \gamma(\bar{x}_2 + \bar{x}_4) + 2\beta \delta + \delta \gamma(\bar{x}_1 + \bar{x}_3) + \left(\beta + \gamma(\bar{x}_1 + \bar{x}_4) + \delta\right) \left(\beta + \gamma(\bar{x}_2 + \bar{x}_3) + \delta\right)\right) s \\
+ \left(\beta \gamma(\bar{x}_1 + \bar{x}_4) + \delta\right) \left(\beta \gamma \bar{x}_2 + \delta \gamma \bar{x}_3 + \beta \delta\right) + \left(\beta \gamma \bar{x}_4 + \beta \delta + \delta \gamma \bar{x}_1\right) \left(\beta + \gamma(\bar{x}_2 + \bar{x}_3) + \delta\right) s \\
+ \left(\beta \gamma \bar{x}_4 + \beta \delta + \delta \gamma \bar{x}_1\right) \left(\beta \gamma \bar{x}_2 + \beta \delta + \delta \gamma \bar{x}_3\right) - \gamma^2 \kappa_1 \kappa_2 \bar{x}_2 \bar{x}_4 = 0.
\]

(32)

As previously noted, \(E_{\text{tot}} - \frac{\delta}{\gamma}\) must be positive; then we can show that the possible sign changes in the first row of the table are:

row 4: \(\pm\)
row 3: \(+\)
row 2: \(R_1: \pm\)
row 1: \(R_2: \pm\)
row 0: \(+\)

The first row of the table has at most 3 sign changes. We conclude that the polynomial which describes our equilibrium conditions has at most three positive roots, thus system (7) has at most three positive equilibria. To have at least one positive root, then \(A_2\) must be negative.

We examine \(A_2\) and rewrite it as:

\[
A_2 = A_1^2 - (\kappa \gamma)^2 f_1^2 = A_1^2 - (\kappa \gamma)^4 \\
= ((\kappa \gamma)^2 - 2(\beta \gamma)^2)^2 - (\kappa \gamma)^2 \\
= (\kappa \gamma)^4 - 4(\kappa \gamma)^2(\beta \gamma)^2 + 4(\kappa \gamma)^4 - (\beta \gamma)^4 \\
= 4(\beta \gamma)^2((\beta \gamma)^2 - (\kappa \gamma)^2) \\
= 4a_1^2(\gamma^2 - (\kappa \gamma)^2).
\]

If \(\kappa > \beta\), then \(A_2 < 0\) and we have at least one positive equilibrium. If \(R_1\) or \(R_2\), or both \(R_1\) and \(R_2\) are negative, then we have three positive roots. Analytical computations are long and tedious for this case. However, we are able to show that if \(k > 2 \beta\), then \(R_1 < 0\). This is a conservative condition. We may find three equilibria also for \(\beta < \kappa < 2 \beta\), as highlighted in the numerical simulation in Fig. (4).

First, take \(\alpha = \left(E_{\text{tot}} - \frac{\delta}{\gamma}\right) > 0\). If we substitute this term in \(B_2\), we find that \(B_2\) is always positive:

\[
B_2 = 4a_1^2(a_1 \gamma \kappa - 2a_1^2 + (\gamma \kappa)^2) = 4a_1^2(\gamma^2 + \beta \kappa - 2\beta^2).
\]

If \(\kappa > \beta\), then \(B_2 > 0\).

We can also simplify term \(E_2\) and we find that it is always positive:

\[
E_2 = 4a_1^2(\beta \delta E_{\text{tot}}) = 4(\beta \gamma)^2(\beta \delta E_{\text{tot}}) > 0
\]

The sign of \(R_1\) now depends on the sign of \(C_2\) and \(D_2\). Before discussing those elements, we derive simplified expressions for \(B_1\) and \(f_{11}\). First, a simplified expression for \(B_1\) is:

\[
B_1 = 2a_1 \beta \gamma E_{\text{tot}} - 2a_1 \beta \delta + (\kappa \gamma)(\beta \delta - \beta \gamma E_{\text{tot}}) \\
= 2a_1 \beta \gamma E_{\text{tot}} - 2a_1 \beta \delta + \kappa(\beta \gamma)(\delta - \gamma E_{\text{tot}}) \\
= a_1 \left(2\beta \gamma E_{\text{tot}} - 2\beta \delta + \kappa(\delta - \gamma E_{\text{tot}})\right) \\
= a_1 \left(2\beta \gamma \left(\frac{E_{\text{tot}} - \frac{\delta}{\gamma}}{\delta - \gamma E_{\text{tot}}}\right) - (\kappa \gamma) \left(\frac{E_{\text{tot}} - \frac{\delta}{\gamma}}{\delta - \gamma E_{\text{tot}}}\right)\right) \\
= a_1 \gamma \left(E_{\text{tot}} - \frac{\delta}{\gamma}\right)(2\beta - \kappa)
\]

We now simplify the expression of \(f_{11}\):

\[
f_{11}^2 = (\beta \delta - \beta \gamma E_{\text{tot}})^2 \\
4a_1 c_1 = -4(\beta \gamma)(\beta \delta E_{\text{tot}}) \\
E_{\text{tot}} - 4a_1 c_1 = (\beta \delta + \beta \gamma E_{\text{tot}})^2
\]

Finally, we discuss the sign of \(C_2\) (for brevity we omit a few steps):

\[
C_2 = B_1^2 + 2a_1 C_1 - (\kappa \gamma)^2 f_1^2 = 4a_1^2 \gamma^2 \left(E_{\text{tot}} - \frac{\delta}{\gamma}\right)^2 (2\beta - \kappa)^2 + \\
+ 4a_1 \beta \delta E_{\text{tot}}(-2a_1^2 + (\gamma \kappa)^2) - (\kappa \gamma)^2(\beta \delta + \beta \gamma E_{\text{tot}})^2 \\
= \cdots \\
= 4a_1^2 \beta \gamma \left(E_{\text{tot}} - \frac{\delta}{\gamma}\right)^2 (\beta - \kappa - 2\delta a_1 E_{\text{tot}})
\]

If \(\kappa > \beta\), then \(C_2 < 0\).

Finally, we inspect \(D_2\):

\[
D_2 = 2B_1 C_1 \\
= 2 \left(a_1 \gamma \left(E_{\text{tot}} - \frac{\delta}{\gamma}\right)(2\beta - \kappa)\right) (2a_1 \beta \delta E_{\text{tot}}) \\
= 4a_1^2 \delta \gamma \beta E_{\text{tot}} \left(E_{\text{tot}} - \frac{\delta}{\gamma}\right)(2\beta - \kappa)
\]
We finally find that:

\[
\begin{cases}
    D_2 > 0 & \text{if } \kappa < 2\beta \\
    D_2 < 0 & \text{if } \kappa > 2\beta
\end{cases}
\]

Thus, to guarantee that \( R_1 < 0 \), and that there are three sign positive roots, we ask \( \kappa > 2\beta \).

We consider this bound to be conservative. A thorough analysis of the sign of \( R_2 \) may provide a less conservative condition; we were not able to find analytically any such condition.

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