

On the number of steady states in a multiple futile cycle

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Abstract The multisite phosphorylation-dephosphorylation cycle is a motif repeatedly used in cell signaling. This motif itself can generate a variety of dynamic behaviors like bistability and ultrasensitivity without direct positive feedbacks. In this paper, we study the number of positive steady states of a general multisite phosphorylation–dephosphorylation cycle, and how the number of positive steady states varies by changing the biological parameters. We show analytically that (1) for some parameter ranges, there are at least $n + 1$ (if n is even) or n (if n is odd) steady states; (2) there never are more than $2n - 1$ steady states (in particular, this implies that for $n = 2$, including single levels of MAPK cascades, there are at most three steady states); (3) for parameters near the standard Michaelis–Menten quasi-steady state conditions, there are at most $n + 1$ steady states; and (4) for parameters far from the standard Michaelis–Menten quasi-steady state conditions, there is at most one steady state.

Keywords Futile cycles · Bistability · Signaling pathways · Biomolecular networks · Steady states

Mathematics Subject Classification (2000) 92C45

1 Introduction

A promising approach to handling the complexity of cell signaling pathways is to decompose pathways into small motifs, and analyze the individual motifs. One particular motif that has attracted much attention in recent years is the cycle formed by two

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Fig. 1 A futile cycle of size n

or more inter-convertible forms of one protein. The protein, denoted here by S_0 , is ultimately converted into a product, denoted here by S_n , through a cascade of “activation” reactions triggered or facilitated by an enzyme E ; conversely, S_n is transformed back (or “deactivated”) into the original S_0 , helped on by the action of a second enzyme F . See Fig. 1.

Such structures, often called “futile cycles” (also called substrate cycles, enzymatic cycles, or enzymatic inter-conversions, see [27]), serve as basic blocks in cellular signaling pathways and have pivotal impact on the signaling dynamics. Futile cycles underlie signaling processes such as GTPase cycles [10], bacterial two-component systems and phosphorelays [5, 15] actin treadmilling [8], and glucose mobilization [19], as well as metabolic control [25] and cell division and apoptosis [26] and cell-cycle checkpoint control [20]. One very important instance is that of mitogen-activated protein kinase (“MAPK”) cascades, which regulate primary cellular activities such as proliferation, differentiation, and apoptosis [2, 7, 18, 32] in eukaryotes from yeast to humans.

Mitogen-activated protein kinase cascades usually consist of three tiers of similar structures with multiple feedbacks [6, 13, 33]. Each individual level of the MAPK cascades is a futile cycle as depicted in Fig. 1 with $n = 2$. Markevich et al.’s paper [21] was the first to demonstrate the possibility of multistationarity at a single cascade level, and motivated the need for analytical studies of the number of steady states. Conradi et al. studied the existence of multistationarity in their paper [9], employing algorithms based on Feinberg’s chemical reaction network theory (CRNT). (For more details on CRNT, see [11, 12].) The CRNT algorithm confirms multistationarity in a single level of MAPK cascades, and provides a set of kinetic constants which can give rise to multistationarity. However, the CRNT algorithm only tests for the existence of multiple steady states, and does not provide information regarding the precise number of steady states. See [3, 4, 17, 22, 23, 28, 30, 31] for other work on multistationarity of biological systems.

In [16], Gunawardena proposed a novel approach to the study of steady states of futile cycles. His approach, which was focused in the question of determining the proportion of maximally phosphorylated substrate, was developed under the simplifying quasi-steady state assumption that substrate is in excess. Nonetheless, our study of multistationarity uses in a key manner the basic formalism in [16], even for the case when substrate is not in excess.

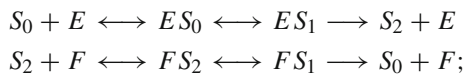
In Sect. 2, we state our basic assumptions regarding the model. The basic formalism and background for the approach is provided in Sect. 3. The main focus of this paper is on Sect. 4, where we derive various bounds on the number of steady states of futile cycles of size n . The first result is a lower bound for the number of steady states. Currently available results on lower bounds, as in [29], can only handle the case when quasi-steady state assumptions are valid; we substantially extend these results to the fully general case by means of a perturbation argument which allows one to

get around these restricted assumptions. Another novel feature of our results in this paper is the derivation of an upper bound of $2n - 1$, valid for all kinetic constants. Models in molecular cell biology are characterized by a high degree of uncertainty in parameters, hence such results valid over the entire parameter space are of special significance. However, when more information on the parameters is available, sharper upper bounds can be obtained, see Theorems 4 and 5. We finally conclude our paper in Sect. 5 with a conjecture of an $n + 1$ upper bound.

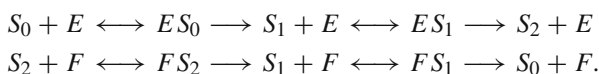
We remark that the results here do not address the stability of the steady states. However, we see from simulations that the stable and unstable steady states tend to alternate if ranked by the ratio of their steady state concentrations of the kinase and the phosphatase. Complementary work dealing with the *dynamical* behavior of futile cycles of size two is studied in [4, 31]. In [31], we showed that the model exhibits generic convergence to steady states but no more complicated behavior, at least within restricted parameter ranges, while [4] showed a persistence property (no species tends to be eliminated) for any possible parameter values. See [1] for a global convergence result in the single-phosphorylation case.

2 Model assumptions

Before presenting mathematical details, let us first discuss the basic biochemical assumptions that go into the model. In general, phosphorylation and dephosphorylation can follow either a distributive or a processive mechanism. In the processive mechanism, the kinase (phosphatase) facilitates two or more phosphorylations (dephosphorylations) before the final product is released, whereas in the distributive mechanism, the kinase (phosphatase) facilitates at most one phosphorylation (dephosphorylation) in each molecular encounter. In the case of $n = 2$, a futile cycle that follows the processive mechanism can be represented by reactions as follows:



and the distributive mechanism can be represented by reactions:



Biological experiments have demonstrated that both dual phosphorylation and dephosphorylation in MAPK are distributive, see [6, 13, 33]. In their paper [9], Conradi et al. showed mathematically that if either phosphorylation or dephosphorylation follows a processive mechanism, the steady state will be unique, which, it is argued in [9], contradicts experimental observations. We therefore assume that both phosphorylations and dephosphorylations in the futile cycles follow the distributive mechanism.

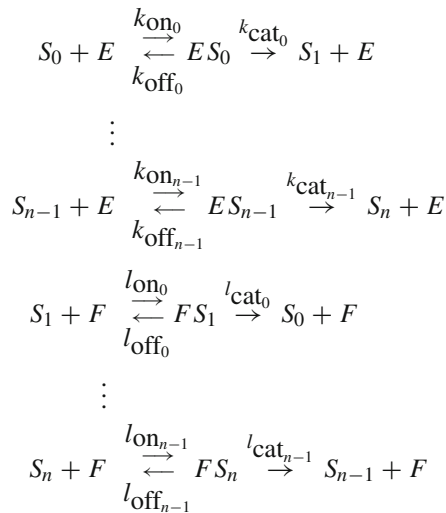
Our structure of futile cycles in Fig. 1 also implicitly assumes a sequential instead of a random mechanism. By a sequential mechanism, we mean that the kinase phosphorylates the substrates in a specific order, and the phosphatase works in the reversed order.

A few kinases are known to be sequential, for example, the auto-phosphorylation of FGF-receptor-1 kinase [14]. This assumption dramatically reduces the number of different phospho-forms and simplifies our analysis. In a special case when the kinetic constants of each phosphorylation are the same and the kinetic constants of each dephosphorylation are the same, the random mechanism can be easily included in the sequential case.

To model the reactions, we assume mass action kinetics, which is standard in mathematical modeling of molecular events in biology.

3 Mathematical formalism

In this section, we set up a mathematical framework for studying the steady states of futile cycles. Let us first write down all the elementary chemical reactions in Fig. 1:



where k_{on_0} , etc., are kinetic parameters for binding and unbinding, ES_0 denotes the complex consisting of the enzyme E and the substrate S_0 , and so forth. These reactions can be modeled by $3n + 3$ differential-algebraic equations according to mass action kinetics:

$$\begin{aligned}
 \frac{ds_0}{dt} &= -k_{\text{on}_0}s_0e + k_{\text{off}_0}c_0 + l_{\text{cat}_0}d_1, \\
 \frac{ds_i}{dt} &= -k_{\text{on}_i}s_i e + k_{\text{off}_i}c_i + k_{\text{cat}_{i-1}}c_{i-1} - l_{\text{on}_{i-1}}s_i f \\
 &\quad + l_{\text{off}_{i-1}}d_i + l_{\text{cat}_i}d_{i+1}, \quad i = 1, \dots, n-1, \\
 \frac{dc_j}{dt} &= k_{\text{on}_j}s_j e - (k_{\text{off}_j} + k_{\text{cat}_j})c_j, \quad j = 0, \dots, n-1, \\
 \frac{dd_k}{dt} &= l_{\text{on}_{k-1}}s_k f - (l_{\text{off}_{k-1}} + l_{\text{cat}_{k-1}})d_k, \quad k = 1, \dots, n,
 \end{aligned} \tag{1}$$

together with the algebraic “conservation equations”:

$$\begin{aligned}
 E_{\text{tot}} &= e + \sum_0^{n-1} c_i, \\
 F_{\text{tot}} &= f + \sum_1^n d_i, \\
 S_{\text{tot}} &= \sum_0^n s_i + \sum_0^{n-1} c_i + \sum_1^n d_i.
 \end{aligned}
 \tag{2}$$

The variables $s_0, \dots, s_n, c_0, \dots, c_{n-1}, d_1, \dots, d_n, e, f$ stand for the concentrations of

$$S_0, \dots, S_n, ES_0, \dots, ES_{n-1}, FS_1, \dots, FS_n, E, F$$

respectively. For each positive vector

$$\begin{aligned}
 \kappa &= (k_{\text{on}_0}, \dots, k_{\text{on}_{n-1}}, k_{\text{off}_0}, \dots, k_{\text{off}_{n-1}}, k_{\text{cat}_0}, \dots, k_{\text{cat}_{n-1}}, \\
 & \quad l_{\text{on}_0}, \dots, l_{\text{on}_{n-1}}, l_{\text{off}_0}, \dots, l_{\text{off}_{n-1}}, l_{\text{cat}_0}, \dots, l_{\text{cat}_{n-1}}) \in \mathbb{R}_+^{6n}
 \end{aligned}$$

(of “kinetic constants”) and each positive triple $\mathcal{C} = (E_{\text{tot}}, F_{\text{tot}}, S_{\text{tot}})$, we have a different system $\Sigma(\kappa, \mathcal{C})$.

Let us write the coordinates of a vector $x \in \mathbb{R}_+^{3n+3}$ as:

$$x = (s_0, \dots, s_n, c_0, \dots, c_{n-1}, d_1, \dots, d_n, e, f),$$

and define a mapping

$$\Phi : \mathbb{R}_+^{3n+3} \times \mathbb{R}_+^{6n} \times \mathbb{R}_+^3 \longrightarrow \mathbb{R}^{3n+3}$$

with components $\Phi_1, \dots, \Phi_{3n+3}$ where the first $3n$ components are

$$\Phi_1(x, \kappa, \mathcal{C}) = -k_{\text{on}_0} s_0 e + k_{\text{off}_0} c_0 + l_{\text{cat}_0} d_1,$$

and so forth, listing the right hand sides of the Eq. (1), Φ_{3n+1} is

$$e + \sum_0^{n-1} c_i - E_{\text{tot}},$$

and similarly for Φ_{3n+2} and Φ_{3n+3} , we use the remaining equations in (2).

For each κ, \mathcal{C} , let us define a set

$$\mathcal{Z}(\kappa, \mathcal{C}) = \{x \mid \Phi(x, \kappa, \mathcal{C}) = 0\}.$$

Observe that, by definition, given $x \in \mathbb{R}_+^{3n+3}$, x is a positive steady state of $\Sigma(\kappa, \mathcal{C})$ if and only if $x \in \mathcal{Z}(\kappa, \mathcal{C})$. So, the mathematical statement of the central problem in this paper is to count the number of elements in $\mathcal{Z}(\kappa, \mathcal{C})$. Our analysis will be greatly simplified by the following preprocessing. Let us introduce a function

$$\Psi : \mathbb{R}_+^{3n+3} \times \mathbb{R}_+^{6n} \times \mathbb{R}_+^3 \longrightarrow \mathbb{R}^{3n+3}$$

with components $\Psi_1, \dots, \Psi_{3n+3}$ defined as

$$\begin{aligned}\Psi_1 &= \Phi_1 + \Phi_{n+1}, \\ \Psi_i &= \Phi_i + \Phi_{n+i} + \Phi_{2n+i-1} + \Psi_{i-1}, \quad i = 2, \dots, n, \\ \Psi_j &= \Phi_j, \quad j = n+1, \dots, 3n+3.\end{aligned}$$

It is easy to see that

$$\mathcal{Z}(\kappa, \mathcal{C}) = \{x \mid \Psi(x, \kappa, \mathcal{C}) = 0\},$$

but now the first $3n$ equations are:

$$\begin{aligned}\Psi_i &= l_{\text{cat}_{i-1}} d_i - k_{\text{cat}_{i-1}} c_{i-1} = 0, \quad i = 1, \dots, n, \\ \Psi_{n+1+j} &= k_{\text{on}_j} s_j e - (k_{\text{off}_j} + k_{\text{cat}_j}) c_j = 0, \quad j = 0, \dots, n-1, \\ \Psi_{2n+k} &= l_{\text{on}_{k-1}} s_k f - (l_{\text{off}_{k-1}} + l_{\text{cat}_{k-1}}) d_k = 0, \quad k = 1, \dots, n,\end{aligned}$$

and can be easily solved as:

$$s_{i+1} = \lambda_i (e/f) s_i, \quad (3)$$

$$c_i = \frac{e s_i}{K_{M_i}}, \quad (4)$$

$$d_{i+1} = \frac{f s_{i+1}}{L_{M_i}}, \quad (5)$$

where

$$\lambda_i = \frac{k_{\text{cat}_i} L_{M_i}}{K_{M_i} l_{\text{cat}_i}}, \quad K_{M_i} = \frac{k_{\text{cat}_i} + k_{\text{off}_i}}{k_{\text{on}_i}}, \quad L_{M_i} = \frac{l_{\text{cat}_i} + l_{\text{off}_i}}{l_{\text{on}_i}}, \quad i = 0, \dots, n-1. \quad (6)$$

For each κ , we introduce three functions $\varphi_0^\kappa, \varphi_1^\kappa, \varphi_2^\kappa : \mathbb{R}_+ \longrightarrow \mathbb{R}_+$ as follows:

$$\begin{aligned}\varphi_0^\kappa(u) &= 1 + \lambda_0 u + \lambda_0 \lambda_1 u^2 + \dots + \lambda_0 \dots \lambda_{n-1} u^n, \\ \varphi_1^\kappa(u) &= \frac{1}{K_{M_0}} + \frac{\lambda_0}{K_{M_1}} u + \dots + \frac{\lambda_0 \dots \lambda_{n-2}}{K_{M_{n-1}}} u^{n-1}, \\ \varphi_2^\kappa(u) &= \frac{\lambda_0}{L_{M_0}} u + \frac{\lambda_0 \lambda_1}{L_{M_1}} u^2 + \dots + \frac{\lambda_0 \dots \lambda_{n-1}}{L_{M_{n-1}}} u^n.\end{aligned}$$

We may now write

$$\begin{aligned}
 \sum_0^n s_i &= s_0 \left(1 + \lambda_0 \left(\frac{e}{f} \right) + \lambda_0 \lambda_1 \left(\frac{e}{f} \right)^2 + \dots + \lambda_0 \dots \lambda_{n-1} \left(\frac{e}{f} \right)^n \right) \\
 &= s_0 \varphi_0^\kappa \left(\frac{e}{f} \right), \\
 \sum_0^{n-1} c_i &= e s_0 \left(\frac{1}{K_{M_0}} + \frac{\lambda_0}{K_{M_1}} \left(\frac{e}{f} \right) + \dots + \frac{\lambda_0 \dots \lambda_{n-2}}{K_{M_{n-1}}} \left(\frac{e}{f} \right)^{n-1} \right) \\
 &= e s_0 \varphi_1^\kappa \left(\frac{e}{f} \right), \\
 \sum_1^n d_i &= f s_0 \left(\frac{\lambda_0}{L_{M_0}} \left(\frac{e}{f} \right) + \frac{\lambda_0 \lambda_1}{L_{M_1}} \left(\frac{e}{f} \right)^2 + \dots + \frac{\lambda_0 \dots \lambda_{n-1}}{L_{M_{n-1}}} \left(\frac{e}{f} \right)^n \right) \\
 &= f s_0 \varphi_2^\kappa \left(\frac{e}{f} \right).
 \end{aligned}
 \tag{7}$$

Although the equation $\Psi = 0$ represents $3n + 3$ equations with $3n + 3$ unknowns, next we will show that it can be reduced to two equations with two unknowns, which have the same number of positive solutions as $\Psi = 0$. Let us first define a set

$$\mathcal{S}(\kappa, \mathcal{C}) = \{(u, v) \in \mathbb{R}_+ \times \mathbb{R}_+ \mid G_1^{\kappa, \mathcal{C}}(u, v) = 0, G_2^{\kappa, \mathcal{C}}(u, v) = 0\},$$

where $G_1^{\kappa, \mathcal{C}}, G_2^{\kappa, \mathcal{C}} : \mathbb{R}_+^2 \rightarrow \mathbb{R}$ are given by

$$\begin{aligned}
 G_1^{\kappa, \mathcal{C}}(u, v) &= v(u\varphi_1^\kappa(u) - \varphi_2^\kappa(u)E_{\text{tot}}/F_{\text{tot}}) - E_{\text{tot}}/F_{\text{tot}} + u, \\
 G_2^{\kappa, \mathcal{C}}(u, v) &= \varphi_0^\kappa(u)\varphi_2^\kappa(u)v^2 + (\varphi_0^\kappa(u) - S_{\text{tot}}\varphi_2^\kappa(u) + F_{\text{tot}}u\varphi_1^\kappa(u) \\
 &\quad + F_{\text{tot}}\varphi_2^\kappa(u))v - S_{\text{tot}}.
 \end{aligned}$$

The precise statement is as follows:

Lemma 1 *There exists a mapping $\delta : \mathbb{R}^{3n+3} \rightarrow \mathbb{R}^2$ such that, for each κ, \mathcal{C} , the map δ restricted to $\mathcal{Z}(\kappa, \mathcal{C})$ is a bijection between the sets $\mathcal{Z}(\kappa, \mathcal{C})$ and $\mathcal{S}(\kappa, \mathcal{C})$.*

Proof Let us define the mapping $\delta : \mathbb{R}^{3n+3} \rightarrow \mathbb{R}^2$ as $\delta(x) = (e/f, s_0)$, where

$$x = (s_0, \dots, s_n, c_0, \dots, c_{n-1}, d_1, \dots, d_n, e, f).$$

If we can show that δ induces a bijection between $\mathcal{Z}(\kappa, \mathcal{C})$ and $\mathcal{S}(\kappa, \mathcal{C})$, we are done.

First, we claim that $\delta(\mathcal{Z}(\kappa, \mathcal{C})) \subseteq \mathcal{S}(\kappa, \mathcal{C})$. Pick any $x \in \mathcal{Z}(\kappa, \mathcal{C})$, we have that x satisfies (3)–(5). Moreover, $\Phi_{3n+2}(x, \kappa, \mathcal{C}) = 0$ yields

$$E_{\text{tot}} = e + e s_0 \varphi_1^\kappa \left(\frac{e}{f} \right),$$

and thus

$$e = \frac{E_{\text{tot}}}{1 + s_0\varphi_1^\kappa(e/f)}. \tag{8}$$

Using $\Phi_{3n+1}(x, \kappa, C) = 0$ and $\Phi_{3n+2}(x, \kappa, C) = 0$, we get:

$$\frac{E_{\text{tot}}}{F_{\text{tot}}} = \frac{e(1 + s_0\varphi_1^\kappa(e/f))}{f(1 + s_0\varphi_2^\kappa(e/f))}, \tag{9}$$

which is $G_1^{\kappa,C}(e/f, s_0) = 0$ after multiplying by $1 + s_0\varphi_2^\kappa(e/f)$ and rearranging terms.

To check that $G_2^{\kappa,C}(e/f, s_0) = 0$, we start with $\Phi_{3n+3}(x, \kappa, C) = 0$, i.e.

$$S_{\text{tot}} = \sum_0^n s_i + \sum_0^{n-1} c_i + \sum_1^n d_i.$$

Using (7) and (8), this expression becomes

$$\begin{aligned} S_{\text{tot}} &= s_0\varphi_0^\kappa\left(\frac{e}{f}\right) + \frac{E_{\text{tot}}s_0\varphi_1^\kappa(e/f)}{1 + s_0\varphi_1^\kappa(e/f)} + \frac{F_{\text{tot}}s_0\varphi_2^\kappa(e/f)}{1 + s_0\varphi_2^\kappa(e/f)} \\ &= s_0\varphi_0^\kappa\left(\frac{e}{f}\right) + \frac{eF_{\text{tot}}s_0\varphi_1^\kappa(e/f)}{f(1 + s_0\varphi_2^\kappa(e/f))} + \frac{F_{\text{tot}}s_0\varphi_2^\kappa(e/f)}{1 + s_0\varphi_2^\kappa(e/f)}, \end{aligned}$$

where the last equality comes from (9).

After multiplying by $1 + s_0\varphi_2^\kappa(e/f)$, and simplifying, we get

$$\begin{aligned} &\varphi_0^\kappa\left(\frac{e}{f}\right)\varphi_2^\kappa\left(\frac{e}{f}\right)s_0^2 + \left(\varphi_0^\kappa\left(\frac{e}{f}\right) - S_{\text{tot}}\varphi_2^\kappa\left(\frac{e}{f}\right)\right) \\ &+ \frac{e}{f}F_{\text{tot}}\varphi_1^\kappa\left(\frac{e}{f}\right) + F_{\text{tot}}\varphi_2^\kappa(u)s_0 - S_{\text{tot}} = 0, \end{aligned}$$

that is, $G_2^{\kappa,C}(e/f, s_0) = 0$. since both $G_1^{\kappa,C}(e/f, s_0)$ and $G_2^{\kappa,C}(e/f, s_0)$ are zero, $\delta(x) \in \mathcal{S}(\kappa, C)$.

Next, we will show that $\mathcal{S}(\kappa, C) \subseteq \delta(\mathcal{Z}(\kappa, C))$. For any $y = (u, v) \in \mathcal{S}(\kappa, C)$, let the coordinates of x be defined as:

$$s_0 = v, \quad s_{i+1} = \lambda_i u s_i, \quad e = \frac{E_{\text{tot}}}{1 + s_0\varphi_1^\kappa(u)}, \quad f = \frac{e}{u}, \quad c_i = \frac{e s_i}{K_{M_i}}, \quad d_{i+1} = \frac{f s_{i+1}}{L_{M_i}},$$

for $i = 0, \dots, n - 1$. It is easy to see that the vector $x = (s_0, \dots, s_n, c_0, \dots, c_{n-1}, d_1, \dots, d_n, e, f)$ satisfies $\Phi_1(x, \kappa, C) = 0, \dots, \Phi_{3n+1}(x, \kappa, C) = 0$. If $\Phi_{3n+2}(x, \kappa, C)$ and $\Phi_{3n+3}(x, \kappa, C)$ are also zero, then x is an element of $\mathcal{Z}(\kappa, C)$ with $\delta(x) = y$. Given the condition that $G_i^{\kappa,C}(u, v) = 0$ ($i = 1, 2$) and $u = e/f, v = s_0$, we have $G_1^{\kappa,C}(e/f, s_0) = 0$, and therefore (9) holds. Since

$$e = \frac{E_{\text{tot}}}{1 + s_0\varphi_1^\kappa(e/f)}$$

in our construction, we have

$$F_{\text{tot}} = f(1 + s_0\varphi_2^\kappa(e/f)) = f + \sum_1^n d_i.$$

To check $\Phi_{3n+3}(x, \kappa, C) = 0$, we use

$$\frac{G_2^{\kappa, C}(e/f, s_0)}{1 + s_0\varphi_2^\kappa(e/f)} = 0,$$

as $G_2^{\kappa, C}(e/f, s_0) = 0$ and $1 + s_0\varphi_2^\kappa(e/f) > 0$. Applying (7)–(9), we have

$$\begin{aligned} & \sum_0^n s_i + \sum_0^{n-1} c_i + \sum_1^n d_i \\ &= s_0\varphi_0^\kappa(e/f) + \frac{eF_{\text{tot}}s_0\varphi_1^\kappa(e/f)}{f(1 + s_0\varphi_2^\kappa(e/f))} + \frac{F_{\text{tot}}s_0\varphi_2^\kappa(e/f)}{1 + s_0\varphi_2^\kappa(e/f)} = S_{\text{tot}}. \end{aligned}$$

It remains for us to show that the map δ is one to one on $\mathcal{Z}(\kappa, C)$. Suppose that $\delta(x^1) = \delta(x^2) = (u, v)$, where

$$x^i = (s_0^i, \dots, s_n^i, c_0^i, \dots, c_{n-1}^i, d_1^i, \dots, d_n^i, e^i, f^i), \quad i = 1, 2.$$

By the definition of δ , we know that $s_0^1 = s_0^2$ and $e^1/f^1 = e^2/f^2$. Therefore, $s_i^1 = s_i^2$ for $i = 0, \dots, n$. Equation (8) gives

$$e^1 = \frac{E_{\text{tot}}}{1 + v\varphi_1^\kappa(u)} = e^2.$$

Thus, $f^1 = f^2$, and $c_i^1 = c_i^2, d_{i+1}^1 = d_{i+1}^2$ for $i = 0, \dots, n - 1$ because of (3)–(5). Therefore, $x^1 = x^2$, and δ is one to one. □

The above lemma ensures that the two sets $\mathcal{Z}(\kappa, C)$ and $\mathcal{S}(\kappa, C)$ have the same number of elements. From now on, we will focus on $\mathcal{S}(\kappa, C)$, the set of positive solutions of equations $G_1^{\kappa, C}(u, v) = 0, G_2^{\kappa, C}(u, v) = 0$, i.e.

$$G_1^{\kappa, C}(u, v) = v(u\varphi_1^\kappa(u) - \varphi_2^\kappa(u)E_{\text{tot}}/F_{\text{tot}}) - E_{\text{tot}}/F_{\text{tot}} + u = 0, \tag{10}$$

$$\begin{aligned} G_2^{\kappa, C}(u, v) &= \varphi_0^\kappa(u)\varphi_2^\kappa(u)v^2 + (\varphi_0^\kappa(u) - S_{\text{tot}}\varphi_2^\kappa(u) + F_{\text{tot}}u\varphi_1^\kappa(u) \\ &+ F_{\text{tot}}\varphi_2^\kappa(u))v - S_{\text{tot}} = 0. \end{aligned} \tag{11}$$

4 Number of positive steady states

4.1 Lower bound on the number of positive steady states

One approach to solving (10) and (11) is to view $G_2^{\kappa, \mathcal{C}}(u, v)$ as a quadratic polynomial in v . Since $G_2^{\kappa, \mathcal{C}}(u, 0) < 0$, Eq. (11) has a unique positive root, namely

$$v = \frac{-H^{\kappa, \mathcal{C}}(u) + \sqrt{H^{\kappa, \mathcal{C}}(u)^2 + 4S_{\text{tot}}\varphi_0^\kappa(u)\varphi_2^\kappa(u)}}{2\varphi_0^\kappa(u)\varphi_2^\kappa(u)}, \tag{12}$$

where

$$H^{\kappa, \mathcal{C}}(u) = \varphi_0^\kappa(u) - S_{\text{tot}}\varphi_2^\kappa(u) + F_{\text{tot}}u\varphi_1^\kappa(u) + F_{\text{tot}}\varphi_2^\kappa(u). \tag{13}$$

Substituting this expression for v into (10), and multiplying by $\varphi_0^\kappa(u)$, we get

$$F^{\kappa, \mathcal{C}}(u) := \frac{-\tilde{H}^{\kappa, \mathcal{C}}(u) + \sqrt{\tilde{H}^{\kappa, \mathcal{C}}(u)^2 + 4S_{\text{tot}}\varphi_0^\kappa(u)\varphi_2^\kappa(u)}}{2\varphi_2^\kappa(u)} \left(u\varphi_1^\kappa(u) - \frac{E_{\text{tot}}}{F_{\text{tot}}}\varphi_2^\kappa(u) \right) - \frac{E_{\text{tot}}}{F_{\text{tot}}}\varphi_0^\kappa(u) + u\varphi_0^\kappa(u) = 0. \tag{14}$$

So, any $(u, v) \in \mathcal{S}(\kappa, \mathcal{C})$ should satisfy (12) and (14). On the other hand, any positive solution u of (14) (notice that $\varphi_0^\kappa(u) > 0$) and v given by (12) (always positive) provide a positive a solution of (10) and (11), that is, (u, v) is an element in $\mathcal{S}(\kappa, \mathcal{C})$. Therefore, the number of positive solutions of (10) and (11) is the same as the number of positive solutions of (12) and (14). But v is uniquely determined by u in (12), which further simplifies the problem to one equation (14) with one unknown u . Based on this observation, we have:

Theorem 1 *For each positive numbers S_{tot}, γ , there exist $\varepsilon_0 > 0$ and $\kappa \in \mathbb{R}_+^{6n}$ such that the following property holds. Pick any $E_{\text{tot}}, F_{\text{tot}}$ such that*

$$F_{\text{tot}} = E_{\text{tot}}/\gamma < \varepsilon_0 S_{\text{tot}}/\gamma, \tag{15}$$

then the system $\Sigma(\kappa, \mathcal{C})$ with $\mathcal{C} = (E_{\text{tot}}, F_{\text{tot}}, S_{\text{tot}})$ has at least $n + 1$ (n) positive steady states when n is even (odd).

Proof For each $\kappa, \gamma, S_{\text{tot}}$, let us define two functions $\mathbb{R}_+ \times \mathbb{R}_+ \rightarrow \mathbb{R}$ as follows:

$$\begin{aligned} \tilde{H}^{\kappa, \gamma, S_{\text{tot}}}(\varepsilon, u) &= H^{\kappa, (\varepsilon S_{\text{tot}}, \varepsilon S_{\text{tot}}/\gamma, S_{\text{tot}})}(u) \\ &= \varphi_0^\kappa(u) - S_{\text{tot}}\varphi_2^\kappa(u) + \varepsilon \frac{S_{\text{tot}}}{\gamma} u\varphi_1^\kappa(u) + \varepsilon \frac{S_{\text{tot}}}{\gamma} \varphi_2^\kappa(u), \end{aligned} \tag{16}$$

and

$$\begin{aligned} \tilde{F}^{\kappa, \gamma, S_{\text{tot}}}(\varepsilon, u) &= F^{\kappa, (\varepsilon S_{\text{tot}}, \varepsilon S_{\text{tot}}/\gamma, S_{\text{tot}})}(u) \\ &= \frac{-\tilde{H}^{\kappa, \gamma, S_{\text{tot}}}(\varepsilon, u) + \sqrt{\tilde{H}^{\kappa, \gamma, S_{\text{tot}}}(\varepsilon, u)^2 + 4S_{\text{tot}}\varphi_0^\kappa(u)\varphi_2^\kappa(u)}}{2\varphi_2^\kappa(u)} \\ &\quad \times (u\varphi_1^\kappa(u) - \gamma\varphi_2^\kappa(u)) - \gamma\varphi_0^\kappa(u) + u\varphi_0^\kappa(u). \end{aligned} \tag{17}$$

By Lemma 1 and the argument before this theorem, it is enough to show that there exist $\varepsilon_0 > 0$ and $\kappa \in \mathbb{R}_+^{6n}$ such that for all $\varepsilon \in (0, \varepsilon_0)$, the equation $\tilde{F}^{\kappa, \gamma, S_{\text{tot}}}(\varepsilon, u) = 0$ has at least $n + 1$ (n) positive solutions when n is even (odd). (Then, given $S_{\text{tot}}, \gamma, E_{\text{tot}}$, and F_{tot} satisfying (15), we let $\varepsilon = E_{\text{tot}}/S_{\text{tot}} < \varepsilon_0$, and apply the result.)

A straightforward computation shows that when $\varepsilon = 0$,

$$\begin{aligned} \tilde{F}^{\kappa, \gamma, S_{\text{tot}}}(0, u) &= S_{\text{tot}}(u\varphi_1^\kappa(u) - \gamma\varphi_2^\kappa(u)) - \gamma\varphi_0^\kappa(u) + u\varphi_0^\kappa(u) \\ &= \lambda_0 \cdots \lambda_{n-1} u^{n+1} + \lambda_0 \cdots \lambda_{n-2} \\ &\quad \times \left(1 + \frac{S_{\text{tot}}}{K_{M_{n-1}}}(1 - \gamma\beta_{n-1}) - \gamma\lambda_{n-1}\right) u^n \\ &\quad + \cdots + \lambda_0 \cdots \lambda_{i-2} \left(1 + \frac{S_{\text{tot}}}{K_{M_{i-1}}}(1 - \gamma\beta_{i-1}) - \gamma\lambda_{i-1}\right) u^i \\ &\quad + \cdots + \left(1 + \frac{S_{\text{tot}}}{K_{M_0}}(1 - \gamma\beta_0) - \gamma\lambda_0\right) u - \gamma, \end{aligned} \tag{18}$$

where the λ_i 's and K_{M_i} 's are defined as in (6), and $\beta_i = k_{\text{cat}_i}/l_{\text{cat}_i}$. The polynomial $\tilde{F}^{\kappa, \gamma, S_{\text{tot}}}(0, u)$ is of degree $n + 1$, so there are at most $n + 1$ positive roots. Notice that $u = 0$ is not a root because $\tilde{F}^{\kappa, \gamma, S_{\text{tot}}}(0, u) = -\gamma < 0$, which also implies that when n is odd, there can not be $n + 1$ positive roots. Now fix any S_{tot} and γ . We will construct a vector κ such that $\tilde{F}^{\kappa, \gamma, S_{\text{tot}}}(0, u)$ has $n + 1$ distinct positive roots when n is even.

Let us pick any $n + 1$ positive real numbers $u_1 < \cdots < u_{n+1}$, such that their product is γ , and assume that

$$(u - u_1) \cdots (u - u_{n+1}) = u^{n+1} + a_n u^n + \cdots + a_1 u + a_0, \tag{19}$$

where $a_0 = -\gamma < 0$ keeping in mind that a_i 's are given. Our goal is to find a vector $\kappa \in \mathbb{R}_+^{6n}$ such that (18) and (19) are the same. For each $i = 0, \dots, n - 1$, we pick $\lambda_i = 1$. Comparing the coefficients of u^{i+1} in (18) and (19), we have:

$$\frac{S_{\text{tot}}}{K_{M_i}}(1 + a_0\beta_i) = a_{i+1} - a_0 - 1. \tag{20}$$

Let us pick $K_{M_i} > 0$ such that $\frac{K_{M_i}}{s_{\text{tot}}}(a_{i+1} - a_0 - 1) - 1 < 0$, then take

$$\beta_i = \frac{\frac{K_{M_i}}{s_{\text{tot}}}(a_{i+1} - a_0 - 1) - 1}{a_0} > 0$$

in order to satisfy (20). From the given

$$\lambda_0, \dots, \lambda_{n-1}, K_{M_0}, \dots, K_{M_{n-1}}, \beta_0, \dots, \beta_{n-1},$$

we will find a vector

$$\kappa = (k_{\text{on}_0}, \dots, k_{\text{on}_{n-1}}, k_{\text{off}_0}, \dots, k_{\text{off}_{n-1}}, k_{\text{cat}_0}, \dots, k_{\text{cat}_{n-1}}, l_{\text{on}_0}, \dots, l_{\text{on}_{n-1}}, l_{\text{off}_0}, \dots, l_{\text{off}_{n-1}}, l_{\text{cat}_0}, \dots, l_{\text{cat}_{n-1}}) \in \mathbb{R}_+^{6n}$$

such that $\beta_i = k_{\text{cat}_i}/l_{\text{cat}_i}$, $i = 0, \dots, n - 1$, and (6) holds. This vector κ will guarantee that $\tilde{F}^{\kappa, \gamma, S_{\text{tot}}}(0, u)$ has $n + 1$ positive distinct roots. When n is odd, a similar construction will give a vector κ such that $\tilde{F}^{\kappa, \gamma, S_{\text{tot}}}(0, u)$ has n positive roots and one negative root.

One construction of κ (given $\lambda_i, K_{M_i}, \beta_i, i = 0, \dots, n - 1$) is as follows. For each $i = 0, \dots, n - 1$, we start by defining:

$$L_{M_i} = \frac{\lambda_i K_{M_i}}{\beta_i},$$

consistently with the definitions in (6). Then, we take

$$k_{\text{on}_i} = 1, \quad l_{\text{on}_i} = 1,$$

and

$$k_{\text{off}_i} = \alpha_i K_{M_i}, \quad k_{\text{cat}_i} = (1 - \alpha_i) K_{M_i}, \quad l_{\text{cat}_i} = \frac{1 - \alpha_i}{\beta_i} K_{M_i}, \quad l_{\text{off}_i} = L_{M_i} - l_{\text{cat}_i},$$

where $\alpha_i \in (0, 1)$ is chosen such that

$$l_{\text{off}_i} = L_{M_i} - \frac{1 - \alpha_i}{\beta_i} K_{M_i} > 0.$$

This κ satisfies $\beta_i = k_{\text{cat}_i}/l_{\text{cat}_i}$, $i = 0, \dots, n - 1$, and (6).

In order to apply the Implicit Function Theorem, we now view the functions defined by formulas in (16) and (17) as defined also for $\varepsilon \leq 0$, i.e. as functions $\mathbb{R} \times \mathbb{R}_+ \rightarrow \mathbb{R}$. It is easy to see that $\tilde{F}^{\kappa, \gamma, S_{\text{tot}}}(\varepsilon, u)$ is C^1 on $\mathbb{R} \times \mathbb{R}_+$ because the polynomial under the square root sign in $\tilde{F}^{\kappa, \gamma, S_{\text{tot}}}(\varepsilon, u)$ is never zero. On the other hand, since $\tilde{F}^{\kappa, \gamma, S_{\text{tot}}}(0, u)$ is a polynomial in u with distinct roots, $\frac{\partial \tilde{F}^{\kappa, \gamma, S_{\text{tot}}}}{\partial u}(0, u_i) \neq 0$. By the

Implicit Function Theorem, for each $i = 1, \dots, n + 1$, there exist open intervals E_i containing 0, open intervals U_i containing u_i , and a differentiable function

$$\alpha_i : E_i \rightarrow U_i$$

such that $\alpha_i(0) = u_i$, $\tilde{F}^{\kappa, \gamma, S_{tot}}(\varepsilon, \alpha_i(\varepsilon)) = 0$ for all $\varepsilon \in E_i$, and the images $\alpha_i(E_i)$'s are non-overlapping. If we take

$$(0, \varepsilon_0) := \bigcap_1^{n+1} E_i \cap (0, +\infty),$$

then for any $\varepsilon \in (0, \varepsilon_0)$, we have $\{\alpha_i(\varepsilon)\}$ as $n+1$ distinct positive roots of $\tilde{F}^{\kappa, \gamma, S_{tot}}(\varepsilon, u)$. The case when n is odd can be proved similarly. \square

The above theorem shows that when E_{tot}/S_{tot} is sufficiently small, it is always possible for the futile cycle to have $n + 1$ (n) steady states when n is even (odd), by choosing appropriate kinetic constants κ . We should notice that for arbitrary κ , the derivative of \tilde{F} at each positive root may become zero, which breaks down the perturbation argument. Here is an example to show that more conditions are needed: with

$$n=2, \quad \lambda_0=1, \quad \lambda_1=3, \quad \gamma=6, \quad \beta_0=\beta_1=1/12, \quad K_0=1/8, \quad K_1=1/2, \quad S_{tot} = 5,$$

we have that

$$\tilde{F}^{\kappa, \gamma, S_{tot}}(0, u) = 3u^3 - 12u^2 + 15u - 6 = 3(u - 1)^2(u - 2)$$

has a double root at $u = 1$. In this case, even for $\varepsilon = 0.01$, there is only one positive root of $\tilde{F}^{\kappa, \gamma, S_{tot}}(\varepsilon, u)$, see Fig. 2.

However, the following lemma provides a sufficient condition for $\frac{\partial F^{\kappa, \gamma, S_{tot}}}{\partial u}(0, \bar{u}) \neq 0$, for any positive \bar{u} such that $\tilde{F}^{\kappa, \gamma, S_{tot}}(0, \bar{u}) = 0$.

Lemma 2 For each positive numbers S_{tot} , γ , and vector $\kappa \in \mathbb{R}_+^{6n}$, if

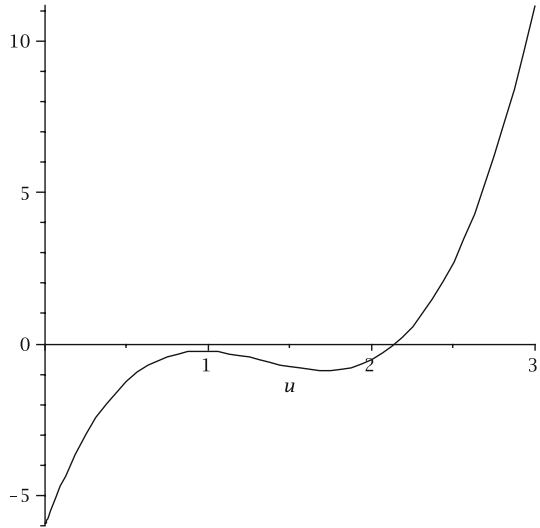
$$S_{tot} \left| \frac{1 - \gamma\beta_j}{K_{M_j}} \right| \leq \frac{1}{n} \tag{21}$$

holds for all $j = 1, \dots, n - 1$, then $\frac{\partial \tilde{F}^{\kappa, \gamma, S_{tot}}}{\partial u}(0, \bar{u}) \neq 0$.

See Appendix for the proof.

Theorem 2 For each positive numbers S_{tot} , γ , and vector $\kappa \in \mathbb{R}_+^{6n}$ satisfying condition (21), there exists $\varepsilon_1 > 0$ such that for any F_{tot}, E_{tot} satisfying $F_{tot} = E_{tot}/\gamma < \varepsilon_1 S_{tot}/\gamma$, the number of positive steady states of system $\Sigma(\kappa, \mathcal{C})$ is greater or equal to the number of (positive) roots of $\tilde{F}^{\kappa, \gamma, S_{tot}}(0, u)$.

Fig. 2 The plot of the function $\tilde{F}^{\kappa, \gamma, S_{\text{tot}}}(0.01, u)$ on $[0, 3]$. There is a unique positive real solution around $u = 2.14$, the double root $u = 1$ of $\tilde{F}^{\kappa, \gamma, S_{\text{tot}}}(0, u)$ bifurcates to two complex roots with non-zero imaginary parts



Proof Suppose that $\tilde{F}^{\kappa, \gamma, S_{\text{tot}}}(0, u)$ has m roots: $\bar{u}_1, \dots, \bar{u}_m$. Applying Lemma 2, we have

$$\frac{\partial \tilde{F}^{\kappa, \gamma, S_{\text{tot}}}}{\partial u}(0, \bar{u}_k) \neq 0, k = 1, \dots, m.$$

By the perturbation arguments as in Theorem 1, we have that there exists $\varepsilon_1 > 0$ such that $\tilde{F}^{\kappa, \gamma, S_{\text{tot}}}(\varepsilon, u)$ has at least m roots for all $0 < \varepsilon < \varepsilon_1$. □

The above result depends heavily on a perturbation argument, which only works when $E_{\text{tot}}/S_{\text{tot}}$ is sufficiently small. In the next section, we will give an upper bound of the number of steady states with no restrictions on $E_{\text{tot}}/S_{\text{tot}}$, and independent of κ and \mathcal{C} .

4.2 Upper bound on the number of steady states

Theorem 3 For each κ, \mathcal{C} , the system $\Sigma(\kappa, \mathcal{C})$ has at most $2n - 1$ positive steady states.

Proof An alternative approach to solving (10) and (11) is to first eliminate v from (10) instead of from (11), i.e.

$$v = \frac{E_{\text{tot}}/F_{\text{tot}} - u}{u\phi_1^\kappa(u) - (E_{\text{tot}}/F_{\text{tot}})\phi_2^\kappa(u)} := \frac{A(u)}{B(u)}, \tag{22}$$

when $u\varphi_1^\kappa(u) - (E_{\text{tot}}/F_{\text{tot}})\varphi_2^\kappa(u) \neq 0$. Then, we substitute (22) into (11), and multiply by $(u\varphi_1^\kappa(u) - (E_{\text{tot}}/F_{\text{tot}})\varphi_2^\kappa(u))^2$ to get:

$$P^{\kappa,C}(u) := \varphi_0^\kappa \varphi_2^\kappa \left(\frac{E_{\text{tot}}}{F_{\text{tot}}} - u \right)^2 + (\varphi_0^\kappa - S_{\text{tot}}\varphi_2^\kappa + F_{\text{tot}}u\varphi_1^\kappa + F_{\text{tot}}\varphi_2^\kappa) \times \left(\frac{E_{\text{tot}}}{F_{\text{tot}}} - u \right) \left(u\varphi_1^\kappa - \frac{E_{\text{tot}}}{F_{\text{tot}}}\varphi_2^\kappa \right) - S_{\text{tot}} \left(u\varphi_1^\kappa - \frac{E_{\text{tot}}}{F_{\text{tot}}}\varphi_2^\kappa \right)^2 = 0. \tag{23}$$

Therefore, if $u\varphi_1^\kappa(u) - (E_{\text{tot}}/F_{\text{tot}})\varphi_2^\kappa(u) \neq 0$, the number of positive solutions of (10) and (11) is no greater than the number of positive roots of $P^{\kappa,C}(u)$.

In the special case when $u\varphi_1^\kappa(u) - (E_{\text{tot}}/F_{\text{tot}})\varphi_2^\kappa(u) = 0$, by (10), we must have $u = E_{\text{tot}}/F_{\text{tot}}$, and thus $\varphi_1^\kappa(E_{\text{tot}}/F_{\text{tot}}) = \varphi_2^\kappa(E_{\text{tot}}/F_{\text{tot}})$. Substituting into (11), we get a unique v defined as in (12) with $u = E_{\text{tot}}/F_{\text{tot}}$. Since $u = E_{\text{tot}}/F_{\text{tot}}$ is a root of $P^{\kappa,C}(u)$, also in this case the number of positive solutions to (10) and (11) is no greater than the number of positive roots of $P^{\kappa,C}(u)$.

It is easy to see that $P^{\kappa,C}(u)$ is divisible by u . Consider the polynomial $Q^{\kappa,C}(u) := P^{\kappa,C}(u)/u$ of degree $2n + 1$. We will first show that $Q^{\kappa,C}(u)$ has no more than $2n$ positive roots, then we will prove by contradiction that $2n$ distinct positive roots can not be achieved.

It is easy to see that in the polynomial $Q^{\kappa,C}(u)$ the coefficient of u^{2n+1} is

$$\frac{(\lambda_0 \cdots \lambda_{n-1})^2}{L_{M_{n-1}}} > 0,$$

and the constant term is

$$\frac{E_{\text{tot}}}{F_{\text{tot}}K_{M_0}} > 0.$$

So the polynomial $Q^{\kappa,C}(u)$ has at least one negative root, and thus has no more than $2n$ positive roots.

Suppose that $\mathcal{S}(\kappa, C)$ has cardinality $2n$, then $Q^{\kappa,C}(u)$ must have $2n$ distinct positive roots, and each of them has multiplicity one. Let us denote the roots of $Q^{\kappa,C}(u)$ as u_1, \dots, u_{2n} in ascending order, and the corresponding v 's given by (22) as v_1, \dots, v_{2n} . We claim that none of them equals $E_{\text{tot}}/F_{\text{tot}}$. If so, we would have $\varphi_1^\kappa(E_{\text{tot}}/F_{\text{tot}}) = \varphi_2^\kappa(E_{\text{tot}}/F_{\text{tot}})$, and $E_{\text{tot}}/F_{\text{tot}}$ would be a double root of $Q^{\kappa,C}(u)$, contradiction.

Since $Q^{\kappa,C}(0) > 0$, $Q^{\kappa,C}(u)$ is positive on intervals

$$I_0 = (0, u_1), \quad I_1 = (u_2, u_3), \dots, I_{n-1} = (u_{2n-2}, u_{2n-1}), \quad I_n = (u_{2n}, \infty),$$

and negative on intervals

$$J_1 = (u_1, u_2), \dots, J_n = (u_{2n-1}, u_{2n}).$$

As remarked earlier, $\varphi_1^\kappa(E_{\text{tot}}/F_{\text{tot}}) \neq \varphi_2^\kappa(E_{\text{tot}}/F_{\text{tot}})$, the polynomial $Q^{\kappa, \mathcal{C}}(u)$ evaluated at $E_{\text{tot}}/F_{\text{tot}}$ is negative, and therefore, $E_{\text{tot}}/F_{\text{tot}}$ belongs to one of the J intervals, say $J_s = (u_{2s-1}, u_{2s})$, for some $s \in \{1, \dots, n\}$.

On the other hand, the denominator of v in (22), denoted as $B(u)$, is a polynomial of degree n and divisible by u . If $B(u)$ has no positive root, then it does not change sign on the positive axis of u . But v changes sign when u passes $E_{\text{tot}}/F_{\text{tot}}$, thus v_{2s-1} and v_{2s} have opposite signs, and one of (u_{2s-1}, v_{2s-1}) and (u_{2s}, v_{2s}) is not a solution to (10) and (11), which contradicts the fact that both are in $\mathcal{S}(\kappa, \mathcal{C})$.

Otherwise, there exists a positive root \bar{u} of $B(u)$ such that there is no other positive root of $B(u)$ between \bar{u} and $E_{\text{tot}}/F_{\text{tot}}$. Plugging \bar{u} into $Q^{\kappa, \mathcal{C}}(u)$, we see that $Q^{\kappa, \mathcal{C}}(\bar{u})$ is always positive, therefore, \bar{u} belongs to one of the I intervals, say $I_t = (u_{2t}, u_{2t+1})$ for some $t \in \{0, \dots, n\}$. There are two cases:

1. $E_{\text{tot}}/F_{\text{tot}} < \bar{u}$. We have

$$u_{2s-1} < E_{\text{tot}}/F_{\text{tot}} < u_{2t} < \bar{u}.$$

Notice that v changes sign when u passes $E_{\text{tot}}/F_{\text{tot}}$, so the corresponding v_{2s-1} and v_{2t} have different signs, and either $(u_{2s-1}, v_{2s-1}) \notin \mathcal{S}(\kappa, \mathcal{C})$ or $(u_{2t}, v_{2t}) \notin \mathcal{S}(\kappa, \mathcal{C})$, contradiction.

2. $E_{\text{tot}}/F_{\text{tot}} > \bar{u}$. We have

$$\bar{u} < u_{2t+1} < E_{\text{tot}}/F_{\text{tot}} < u_{2s}.$$

Since v changes sign when u passes $E_{\text{tot}}/F_{\text{tot}}$, so the corresponding v_{2t+1} and v_{2s} have different signs, and either $(u_{2t+1}, v_{2t+1}) \notin \mathcal{S}(\kappa, \mathcal{C})$ or $(u_{2s}, v_{2s}) \notin \mathcal{S}(\kappa, \mathcal{C})$, contradiction.

Therefore, $\Sigma(\kappa, \mathcal{C})$ has at most $2n - 1$ steady states. □

4.3 Fine-tuned upper bounds

In the previous section, we have seen that any $(u, v) \in \mathcal{S}(\kappa, \mathcal{C})$, $u \neq E_{\text{tot}}/F_{\text{tot}}$ must satisfy (22)–(23), but not all solutions of (22)–(23) are elements in $\mathcal{S}(\kappa, \mathcal{C})$. Suppose that (u, v) is a solution of (22)–(23), it is in $\mathcal{S}(\kappa, \mathcal{C})$ if and only if $u, v > 0$. In some special cases, for example, when the enzyme is in excess, or the substrate is in excess, we could count the number of solutions of (22)–(23) which are not in $\mathcal{S}(\kappa, \mathcal{C})$ to get a better upper bound.

The following is a standard result on continuity of roots; see for instance Lemma A.4.1 in [24]:

Lemma 3 *Let $g(z) = z^n + a_1 z^{n-1} + \dots + a_n$ be a polynomial of degree n and complex coefficients having distinct roots*

$$\lambda_1, \dots, \lambda_q,$$

with multiplicities

$$n_1 + \dots + n_q = n,$$

respectively. Given any small enough $\delta > 0$ there exists a $\varepsilon > 0$ so that, if

$$h(z) = z^n + b_1 z^{n-1} + \dots + b_n, \quad |a_i - b_i| < \varepsilon \quad \text{for } i = 1, \dots, n,$$

then h has precisely n_i roots in $B_\delta(\lambda_i)$ for each $i = 1, \dots, q$, where $B_\delta(\lambda_i)$ is the open ball in \mathbb{C} centered at λ_i with radius δ .

Theorem 4 For each $\gamma > 0$ and $\kappa \in \mathbb{R}_+^{6n}$ such that $\varphi_1^\kappa(\gamma) \neq \varphi_2^\kappa(\gamma)$, and each $S_{tot} > 0$, there exists $\varepsilon_2 > 0$ such that for all positive numbers E_{tot}, F_{tot} satisfying $F_{tot} = E_{tot}/\gamma < \varepsilon_2 S_{tot}/\gamma$, the system $\Sigma(\kappa, C)$ has at most $n + 1$ positive steady states.

Proof Let us define a function $\mathbb{R}_+ \times \mathbb{C} \rightarrow \mathbb{C}$ as follows:

$$\tilde{Q}^{\kappa, \gamma, S_{tot}}(\varepsilon, u) = Q^{\kappa, (\varepsilon S_{tot}, \varepsilon S_{tot}/\gamma, S_{tot})}(u),$$

and a set $\mathcal{B}^{\kappa, \gamma, S_{tot}}(\varepsilon)$ consisting of the roots of $\tilde{Q}^{\kappa, \gamma, S_{tot}}(\varepsilon, u)$ which are not positive or the corresponding v 's determined by u 's as in (22) are not positive. Since $\tilde{Q}^{\kappa, \gamma, S_{tot}}(\varepsilon, u)$ is a polynomial of degree $2n + 1$, if we can show that there exists $\varepsilon_2 > 0$ such that for any $\varepsilon \in (0, \varepsilon_2)$, $\tilde{Q}^{\kappa, \gamma, S_{tot}}(\varepsilon, u)$ has at least n roots counting multiplicities that are in $\mathcal{B}^{\kappa, \gamma, S_{tot}}(\varepsilon)$, then we are done.

In order to apply Lemma 3, we regard the function $\tilde{Q}^{\kappa, \gamma, S_{tot}}$ as defined on $\mathbb{R} \times \mathbb{C}$. At $\varepsilon = 0$:

$$\begin{aligned} &\tilde{Q}^{\kappa, \gamma, S_{tot}}(0, u) \\ &= \left[\varphi_0^\kappa \varphi_2^\kappa (\gamma - u)^2 + (\varphi_0^\kappa - S_{tot} \varphi_2^\kappa)(u \varphi_1^\kappa - \gamma \varphi_2^\kappa)(\gamma - u) - S_{tot}(u \varphi_1^\kappa - \gamma \varphi_2^\kappa)^2 \right] / u \\ &= \left[\varphi_0^\kappa (\gamma - u)u(\varphi_1^\kappa - \varphi_2^\kappa) + S_{tot}u(u \varphi_1^\kappa - \gamma \varphi_2^\kappa)(\varphi_2^\kappa - \varphi_1^\kappa) \right] / u \\ &= (\varphi_2^\kappa - \varphi_1^\kappa)(u \varphi_0^\kappa + S_{tot}(u \varphi_1^\kappa - \gamma \varphi_2^\kappa) - \gamma \varphi_0^\kappa) \\ &= (\varphi_2^\kappa - \varphi_1^\kappa) \tilde{F}^{\kappa, \gamma, S_{tot}}(0, u) \end{aligned}$$

Let us denote the distinct roots of $\tilde{Q}^{\kappa, \gamma, S_{tot}}(0, u)$ as

$$u_1, \dots, u_q,$$

with multiplicities

$$n_1 + \dots + n_q = 2n + 1,$$

and the roots of $\varphi_1^\kappa - \varphi_2^\kappa$ as

$$u_1, \dots, u_p, \quad p \leq q,$$

with multiplicities

$$m_1 + \dots + m_p = n, \quad n_i \geq m_i, \quad \text{for } i = 1, \dots, p.$$

For each $i = 1, \dots, p$, if u_i is real and positive, then there are two cases ($u_i \neq \gamma$ as $\varphi_1^k(\gamma) \neq \varphi_2^k(\gamma)$):

1. $u_i > \gamma$. We have

$$u_i \varphi_1^k(u_i) - \gamma \varphi_2^k(u_i) > \gamma(\varphi_1^k(u_i) - \varphi_2^k(u_i)) = 0.$$

2. $u_i < \gamma$. We have

$$u_i \varphi_1^k(u_i) - \gamma \varphi_2^k(u_i) < \gamma(\varphi_1^k(u_i) - \varphi_2^k(u_i)) = 0.$$

In both cases, $u_i \varphi_1^k(u_i) - \gamma \varphi_2^k(u_i)$ and $\gamma - u_i$ have opposite signs, i.e.

$$(u_i \varphi_1^k(u_i) - \gamma \varphi_2^k(u_i))(\gamma - u_i) < 0.$$

Let us pick $\delta > 0$ small enough such that the following conditions hold:

1. For all $i = 1, \dots, p$, if u_i is not real, then $B_\delta(u_i)$ has no intersection with the real axis.
2. For all $i = 1, \dots, p$, if u_i is real and positive, the following inequality holds for any real $u \in B_\delta(u_i)$:

$$(u \varphi_1^k(u) - \gamma \varphi_2^k(u))(\gamma - u) < 0. \tag{24}$$

3. For all $i = 1, \dots, p$, if u_i is real and negative, then $B_\delta(u_i)$ has no intersection with the imaginary axis.
4. $B_\delta(u_j) \cap B_\delta(u_k) = \emptyset$ for all $j \neq k = 1, \dots, q$.

By Lemma 3, there exists $\varepsilon_2 > 0$ such that for all $\varepsilon \in (0, \varepsilon_2)$, the polynomial $\tilde{Q}^{\kappa, \gamma, S_{\text{tot}}}(\varepsilon, u)$ has exactly n_j roots in each $B_\delta(u_j)$, $j = 1, \dots, q$, denoted by $u_j^k(\varepsilon)$, $k = 1, \dots, n_j$.

We pick one such ε , and we claim that none of the roots in $B_\delta(u_i)$, $i = 1, \dots, p$ with the v defined as in (22) will be an element in \mathcal{S} . If so, we are done, since there are $\sum_1^p n_i \geq \sum_1^p m_i = n$ such roots of $\tilde{Q}^{\kappa, \gamma, S_{\text{tot}}}(\varepsilon, u)$ which are in $\mathcal{B}^{\kappa, \gamma, S_{\text{tot}}}(\varepsilon)$.

For each $i = 1, \dots, p$, there are two cases:

1. u_i is not real. Then condition 1 guarantees that $u_i^k(\varepsilon)$ is not real for each $k = 1, \dots, n_i$, and thus is in $\mathcal{B}^{\kappa, \gamma, S_{\text{tot}}}(\varepsilon)$.
2. u_i is real and positive. Pick any root $u_i^k(\varepsilon) \in B_\delta(u_i)$, $k = 1, \dots, n_i$, the corresponding $v_i^k(\varepsilon)$ equals

$$\frac{\gamma - u_i^k(\varepsilon)}{u_i^k(\varepsilon) \varphi_1^k(u_i^k(\varepsilon)) - \gamma \varphi_2^k(u_i^k(\varepsilon))} < 0$$

followed from (24). So $(u_i^k(\varepsilon), v_i^k(\varepsilon)) \notin \mathcal{S}(\kappa, \mathcal{C})$, and $u_i^k(\varepsilon) \in \mathcal{B}^{\kappa, \gamma, S_{\text{tot}}}(\varepsilon)$.

3. u_i is real and negative. By condition 3, $u_i^k(\varepsilon)$ is not positive for all $k = 1, \dots, n_i$. □

The next theorem considers the case when enzyme is in excess:

Theorem 5 For each $\gamma > 0, \kappa \in \mathbb{R}_+^{6n}$ such that $\varphi_1^\kappa(\gamma) \neq \varphi_2^\kappa(\gamma)$, and each $E_{tot} > 0$, there exists $\varepsilon_3 > 0$ such that for all positive numbers F_{tot}, S_{tot} satisfying $F_{tot} = E_{tot}/\gamma > S_{tot}/(\varepsilon_3\gamma)$, the system $\Sigma(\kappa, C)$ has at most one positive steady state.

Proof For each $\gamma > 0, \kappa \in \mathbb{R}_+^{6n}$ such that $\varphi_1^\kappa(\gamma) \neq \varphi_2^\kappa(\gamma)$, and each $E_{tot} > 0$, we define a function $\mathbb{R}_+ \times \mathbb{C} \rightarrow \mathbb{C}$ as follows:

$$\bar{Q}^{\kappa, \gamma, E_{tot}}(\varepsilon, u) = Q^{\kappa, (E_{tot}, E_{tot}/\gamma, \varepsilon E_{tot})}(u).$$

Let us define the set $C^{\kappa, \gamma, E_{tot}}(\varepsilon)$ as the set of roots of $\bar{Q}^{\kappa, \gamma, E_{tot}}(\varepsilon, u)$ which are not positive or the corresponding v 's determined by u 's as in (22) are not positive. If we can show that there exists $\varepsilon_3 > 0$ such that for any $\varepsilon \in (0, \varepsilon_3)$ there is at most one positive root of $\bar{Q}^{\kappa, \gamma, E_{tot}}(\varepsilon, u)$ that is not in $C^{\kappa, \gamma, E_{tot}}(\varepsilon)$, we are done.

In order to apply Lemma 3, we now view the function $\bar{Q}^{\kappa, \gamma, E_{tot}}$ as defined on $\mathbb{R} \times \mathbb{C}$. At $\varepsilon = 0$:

$$\begin{aligned} \bar{Q}^{\kappa, \gamma, E_{tot}}(0, u) &= (\gamma - u) \left((\gamma - u) \varphi_0^\kappa \varphi_2^\kappa + \left(\varphi_0^\kappa + \frac{E_{tot}}{\gamma} u \varphi_1^\kappa + \frac{E_{tot}}{\gamma} \varphi_2^\kappa \right) \right. \\ &\quad \left. \times (u \varphi_1^\kappa - \gamma \varphi_2^\kappa) \right) / u \\ &:= (\gamma - u) R^{\kappa, \gamma, E_{tot}}(u). \end{aligned}$$

Let us denote the distinct roots of $\bar{Q}^{\kappa, \gamma, E_{tot}}(0, u)$ as

$$u_1(= \gamma), u_2, \dots, u_q,$$

with multiplicities

$$n_1 + \dots + n_q = 2n + 1,$$

and u_2, \dots, u_q are the roots of $R^{\kappa, \gamma, E_{tot}}(u)$ other than γ .

Since $\varphi_1^\kappa(\gamma) \neq \varphi_2^\kappa(\gamma)$, $R^{\kappa, \gamma, E_{tot}}(u)$ is not divisible by $u - \gamma$, and thus $n_1 = 1$.

For each $i = 2, \dots, q$, we have

$$\begin{aligned} (\gamma - u_i) \varphi_0^\kappa(u_i) \varphi_2^\kappa(u_i) &= - \left(\varphi_0^\kappa(u_i) + \frac{E_{tot}}{\gamma} u_i \varphi_1^\kappa(u_i) + \frac{E_{tot}}{\gamma} \varphi_2^\kappa(u_i) \right) \\ &\quad \times (u_i \varphi_1^\kappa(u_i) - \gamma \varphi_2^\kappa(u_i)). \end{aligned}$$

If $u_i > 0$, then $\varphi_0^\kappa(u_i) \varphi_2^\kappa(u_i)$ and $\varphi_0^\kappa(u_i) + \frac{E_{tot}}{\gamma} u_i \varphi_1^\kappa(u_i) + \frac{E_{tot}}{\gamma} \varphi_2^\kappa(u_i)$ are both positive. Since $u_i \varphi_1^\kappa(u_i) - \gamma \varphi_2^\kappa(u_i)$ and $\gamma - u_i$ are non zero, $u_i \varphi_1^\kappa(u_i) - \gamma \varphi_2^\kappa(u_i)$ and

$\gamma - u_i$ must have opposite signs, that is

$$(u_i \varphi_1^k(u_i) - \gamma \varphi_2^k(u_i))(\gamma - u_i) < 0.$$

Let us pick $\delta > 0$ small enough such that the following conditions hold for all $i = 2, \dots, q$:

1. If u_i is not real, then $B_\delta(u_i)$ has no intersection with the real axis.
2. If u_i is real and positive, then for any real $u \in B_\delta(u_i)$, the following inequality holds:

$$(u \varphi_1^k(u) - \gamma \varphi_2^k(u))(\gamma - u) < 0. \tag{25}$$

3. If u_i is real and negative, then $B_\delta(u_i)$ has no intersection with the imaginary axis.
4. $B_\delta(u_j) \cap B_\delta(u_k) = \emptyset$ for all $i \neq k = 2, \dots, q$.

By Lemma 3, there exists $\varepsilon_3 > 0$ such that for all $\varepsilon \in (0, \varepsilon_3)$, the polynomial $\bar{Q}^{\kappa, \gamma, E_{\text{tot}}}(\varepsilon, u)$ has exactly n_j roots in each $B_\delta(u_j)$, $j = 1, \dots, q$, denoted by $u_j^k(\varepsilon)$, $k = 1, \dots, n_j$.

We pick one such ε , and if we can show that all of the roots in $B_\delta(u_i)$, $i = 2, \dots, q$ are in $C^{\kappa, \gamma, E_{\text{tot}}}(\varepsilon)$, then we are done, since the only roots that may not be in $C^{\kappa, \gamma, E_{\text{tot}}}(\varepsilon)$ are the roots in $B_\delta(u_1)$, and there is one root in $B_\delta(u_1)$.

For each $i = 2, \dots, p$, there are three cases:

1. u_i is not real. Then condition 1 guarantees that $u_i^k(\varepsilon)$ is not real for all $k = 1, \dots, n_i$.
2. u_i is real and positive. Pick any root $u_i^k(\varepsilon)$, $k = 1, \dots, n_i$, the corresponding $v_i^k(\varepsilon)$ equals

$$\frac{\gamma - u_i^k(\varepsilon)}{u_i^k(\varepsilon) \varphi_1^k(u_i^k(\varepsilon)) - \gamma \varphi_2^k(u_i^k(\varepsilon))} < 0.$$

So, $u_i^k(\varepsilon)$ is in $C^{\kappa, \gamma, E_{\text{tot}}}(\varepsilon)$.

3. u_i is real and negative. By condition 3, $u_i^k(\varepsilon)$ is not positive for all $k = 1, \dots, n_i$. □

5 Conclusions and discussion

Here we have set up a mathematical model for multisite phosphorylation-dephosphorylation cycles of size n , and studied the number of positive steady states based on this model. We reformulated the question of number of positive steady states to question of the number of positive roots of certain polynomials, through which we also applied perturbation techniques. Our theoretical results depend on the assumption of mass action kinetics and distributive sequential mechanism, which are customary in the study of multisite phosphorylation and dephosphorylation.

An upper bound of $2n - 1$ steady states is obtained for arbitrary parameter combinations. Biologically, when the substrate concentration greatly exceeds that of the enzyme, there are at most $n + 1$ (n) steady states if n is even (odd). And this

upper bound can be achieved under proper kinetic conditions, see Theorem 1 for the construction. On the other extreme, when the enzyme is in excess, there is a unique steady state.

As a special case of $n = 2$, which can be applied to a single level of MAPK cascades. Our results guarantees that there are no more than three steady states, consistent with numerical simulations in [21].

We notice that there is an apparent gap between the upper bound $2n - 1$ and the upper bound of $n + 1$ (n) if n is even (odd) when the substrate is in excess. If we think the ratio $E_{\text{tot}}/F_{\text{tot}}$ as a parameter ε , then when $\varepsilon \ll 1$, there are at most $n + 1$ (n) steady states when n is even (odd), which coincides with the largest possible lower bound. When $\varepsilon \gg 1$, there is a unique steady state. If the number of steady states changes “continuously” with respect to ε , then we do not expect the number of steady states to exceed $n + 1$ (n) if n is even (odd). So a natural conjecture would be that the number of steady states never exceed $n + 1$ under any condition.

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Appendix

Proof of Lemma 2: Recall that (dropping the u ’s in $\varphi_i^k, i = 0, 1, 2$)

$$\tilde{F}^{\kappa,\gamma,S_{\text{tot}}}(0, u) = u\varphi_0^\kappa + S_{\text{tot}}(u\varphi_1^\kappa - \gamma\varphi_2^\kappa) - \gamma\varphi_0^\kappa.$$

So

$$\frac{\partial \tilde{F}^{\kappa,\gamma,S_{\text{tot}}}}{\partial u}(0, u) = \varphi_0^\kappa + S_{\text{tot}}(u\varphi_1^\kappa - \gamma\varphi_2^\kappa)' - (\gamma - u)(\varphi_0^\kappa)'$$

Since $\tilde{F}^{\kappa,\gamma,S_{\text{tot}}}(0, \bar{u}) = 0$,

$$S_{\text{tot}}(\bar{u}\varphi_1^\kappa - \gamma\varphi_2^\kappa) = (\gamma - \bar{u})\varphi_0^\kappa,$$

that is,

$$\gamma - \bar{u} = \frac{S_{\text{tot}}(\bar{u}\varphi_1^\kappa - \gamma\varphi_2^\kappa)}{\varphi_0^\kappa}.$$

Therefore,

$$\begin{aligned} \frac{\partial \tilde{F}^{\kappa,\gamma,S_{\text{tot}}}}{\partial u}(0, \bar{u}) &= \varphi_0^\kappa + S_{\text{tot}}(u\varphi_1^\kappa - \gamma\varphi_2^\kappa)' - \frac{S_{\text{tot}}(\bar{u}\varphi_1^\kappa - \gamma\varphi_2^\kappa)}{\varphi_0^\kappa}(\varphi_0^\kappa)' \\ &= \varphi_0^\kappa + \frac{S_{\text{tot}}}{\varphi_0^\kappa} (\varphi_0^\kappa(u\varphi_1^\kappa - \gamma\varphi_2^\kappa)' - (\bar{u}\varphi_1^\kappa - \gamma\varphi_2^\kappa)(\varphi_0^\kappa)') \end{aligned}$$

$$\begin{aligned}
&= \varphi_0^\kappa + \frac{S_{\text{tot}}}{\varphi_0^\kappa} ((1 + \lambda_0 \bar{u} + \lambda_0 \lambda_1 \bar{u}^2 + \cdots + \lambda_0 \cdots \lambda_{n-1} \bar{u}^n) \\
&\quad \times \left(\frac{1}{K_{M_0}} (1 - \gamma \beta_0) + 2 \frac{\lambda_0}{K_{M_1}} (1 - \gamma \beta_1) \bar{u} + \cdots \right. \\
&\quad \quad \left. + n \frac{\lambda_0 \cdots \lambda_{n-2}}{K_{M_{n-1}}} (1 - \gamma \beta_{n-1}) \bar{u}^{n-1} \right) \\
&\quad - (\lambda_0 + 2\lambda_0 \lambda_1 \bar{u} + \cdots + n\lambda_0 \cdots \lambda_{n-1} \bar{u}^{n-1}) \\
&\quad \times \left(\frac{1}{K_{M_0}} (1 - \gamma \beta_0) \bar{u} + \frac{\lambda_0}{K_{M_1}} (1 - \gamma \beta_1) \bar{u}^2 + \cdots \right. \\
&\quad \quad \left. + \frac{\lambda_0 \cdots \lambda_{n-2}}{K_{M_{n-1}}} (1 - \gamma \beta_{n-1}) \bar{u}^n \right) \\
&= \varphi_0^\kappa + \frac{S_{\text{tot}}}{\varphi_0^\kappa} \sum_{i=0}^n \lambda_0 \cdots \lambda_{i-1} \bar{u}^i \\
&\quad \times \left(\sum_{j=0}^{n-1} (j+1-i) \frac{\lambda_0 \cdots \lambda_{j-1}}{K_{M_j}} (1 - \gamma \beta_j) \bar{u}^j \right) \\
&= \frac{1}{\varphi_0^\kappa} \sum_{i=0}^n \lambda_0 \cdots \lambda_{i-1} \bar{u}^i \sum_{j=0}^n \lambda_0 \cdots \lambda_{j-1} \bar{u}^j \\
&\quad + S_{\text{tot}} \sum_{i=0}^n \lambda_0 \cdots \lambda_{i-1} \bar{u}^i \\
&\quad \times \left(\sum_{j=0}^{n-1} (j+1-i) \frac{\lambda_0 \cdots \lambda_{j-1}}{K_{M_j}} (1 - \gamma \beta_j) \bar{u}^j \right) \\
&= \frac{1}{\varphi_0^\kappa} \sum_{i=0}^n \lambda_0 \cdots \lambda_{i-1} \bar{u}^i \left(\lambda_0 \cdots \lambda_{n-1} \bar{u}^n + \sum_{j=0}^{n-1} \lambda_0 \cdots \lambda_{j-1} \bar{u}^j \right) \\
&\quad \times \left(1 + S_{\text{tot}} (j+1-i) \frac{1 - \gamma \beta_j}{K_{M_j}} \right),
\end{aligned}$$

where the product $\lambda_0 \cdots \lambda_{n-1}$ is defined to be 1 for the convenience of notation.

Because of (21),

$$S_{\text{tot}} \left| (j+1-i) \frac{1 - \gamma \beta_j}{K_{M_j}} \right| \leq 1,$$

so we have $\frac{\partial \tilde{F}^{\kappa, \gamma, S_{\text{tot}}}}{\partial u}(0, \bar{u}) > 0$. □

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