A remark on the number of steady states in a multiple futile cycle

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Abstract

This note studies the number of positive steady states in biomolecular reactions consisting of activation/deactivation futile cycles, such as those arising from phosphorylations and dephosphorylations at each level of a MAPK cascade. It is shown 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 $2n$ 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

1 Introduction

A motif of great interest in current systems biology research is that of a "futile cycle" in which a substrate, 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 Figure 1.

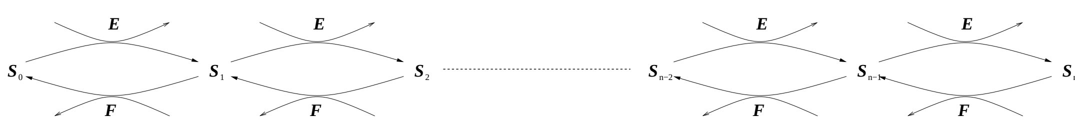

Figure 1: Futile cycle

The chemical reactions are as follows:

$$S_0 + E \underset{k_{\text{off}_0}}{\overset{k_{\text{on}_0}}{\rightleftarrows}} ES_0 \overset{k_{\text{cat}_0}}{\rightarrow} S_1 + E$$

$$\vdots$$

$$S_{n-1} + E \underset{k_{\text{off}_{n-1}}}{\overset{k_{\text{on}_{n-1}}}{\rightleftarrows}} ES_0 \overset{k_{\text{cat}_{n-1}}}{\rightarrow} S_n + E$$

$$S_1 + F \underset{l_{\text{off}_0}}{\overset{l_{\text{on}_0}}{\rightleftarrows}} FS_1 \overset{l_{\text{cat}_0}}{\to} S_0 + F$$

$$\vdots$$

$$S_n + F \underset{l_{\text{off}_{n-1}}}{\overset{l_{\text{on}_{n-1}}}{\rightleftarrows}} FS_n \overset{l_{\text{cat}_{n-1}}}{\to} S_{n-1} + F$$

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. A mass-action kinetics ODE model, described later, is used.

Futile cycles (with any number of intermediate steps, and also called substrate cycles, enzymatic cycles, or enzymatic inter-conversions, see [1]) underlie signaling processes such as GTPase cycles [2], bacterial two-component systems and phosphorelays [3, 4] actin treadmilling [5]), and glucose mobilization [6], as well as metabolic control [7] and cell division and apoptosis [8] and cell-cycle checkpoint control [9]. One very important instance is that of Mitogen-Activated Protein Kinase ("MAPK") cascades, which regulate primary cellular activities such as proliferation, differentiation, and apoptosis [10–13] in eukaryotes from yeast to humans. MAPK cascades usually consist of three tiers of similar structures with multiple feedbacks [14–16]. An individual level of a MAPK cascade is often modeled as a futile cycle as depicted in Figure 1 with $n = 2$.

Numerical analysis of the model with $n = 2$ to $n = 5$ indicates that the system may be monostable or bistable (subject to conservation relations), depending on parameter values [17–19]. Bistable parameter regimes allow possible switch-like behavior and memory, which are ubiquitous in cellular pathways [20–23].

In either case, simulations under meaningful biological parameters show convergence, not other dynamical properties such as periodic behavior or even chaotic behavior. Analytical studies done for the quasi-steady-state version of the model (slow dynamics), which is a monotone system, indicate that the reduced system is indeed monostable or bistable, see [24]. Our paper [25] (see also [26] for preliminary results) established mathematically, for $n = 2$ and based upon the theory of singular perturbations of monotone systems, that, at least in certain parameter ranges (as required by singular perturbation theory), the full system indeed inherits convergence properties from the reduced system. Other related theoretical work is [27], where the general case $n = 2$ (any parameter values, not necessarily near quasi-steady state) is studied and a persistence result is shown, as well as [28], where, for $n = 1$, it is shown, also for all parameter values, that global convergence to steady states (subject to stoichiometric constraints) holds.

In this note, we study a complementary problem, namely the question of analyzing *how many* positive steady states are possible. (Hereafter, when we say "steady state" we always mean a *positive* steady state. Observe that there always exists at least one nonnegative steady state, because the dynamics evolve in a compact convex set.)

Our main results are informally summarized as follows (precise statements are given later):

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 $2n$ steady states.

3. For parameter ranges near the standard Michaelis-Menten quasi-steady state conditions, there are at most $n + 1$ steady states.

4. For parameter ranges very far from the standard Michaelis-Menten quasi-steady state conditions, there is at most one steady state.

Our results make heavy use of beautiful observations made in [18], that allow one to reduce the search for positive steady states to the study of algebraic equations involving the ratio $u = e/f$, where e and f are the concentrations of E and F respectively. This construction was also used in [29] in order to show that there are, for some parameters sets, n (or $n+1$) steady states for a limiting quasi-steady state reduction of the same system, as well as numerical evidence for the existence of $n+1$ states for the full system.

Acknowledgment

We thank Jeremy Gunawardena for very helpful discussions.

2 Statements and Proofs of Results

We model the futile cycle described in the introduction by means of a system of $3n+3$ differential-algebraic equations, consisting of the ordinary differential equations:

$$
\begin{aligned}
\frac{ds_0}{dt} &= -k_{\mathrm{on}_0} s_0 e + k_{\mathrm{off}_0} c_0 + l_{\mathrm{cat}_0} d_1 \\
\frac{ds_i}{dt} &= -k_{\mathrm{on}_i} s_i e + k_{\mathrm{off}_i} c_i + k_{\mathrm{cat}_{i-1}} c_{i-1} - l_{\mathrm{on}_{i-1}} s_i f + l_{\mathrm{off}_{i-1}} d_i + l_{\mathrm{cat}_i} d_{i+1}, \quad i = 1, \ldots, n-1 \\
\frac{dc_j}{dt} &= k_{\mathrm{on}_j} s_j e - (k_{\mathrm{off}_j} + k_{\mathrm{cat}_j}) c_j, \quad j = 0, \ldots, n-1 \\
\frac{dd_k}{dt} &= l_{\mathrm{on}_{k-1}} s_k f - (l_{\mathrm{off}_{k-1}} + l_{\mathrm{cat}_{k-1}}) d_k, \quad k = 1, \ldots, n,
\end{aligned}
\tag{1}
$$

together with the algebraic "conservation equations":

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

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

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

respectively. For each positive vector

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

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

We introduce a mapping

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

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

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

and let $\Phi(x, \kappa, \mathcal{C})$ have components $\Phi_1, \ldots, \Phi_{3n+3}$ where the first $3n$ components are

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

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

$$e + \sum_{0}^{n-1} c_i - E_{\mathrm{tot}},$$

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

For each $\kappa, \mathcal{C}$, let

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

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 $x \in \mathcal{Z}(\kappa, \mathcal{C})$.

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

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

where

$$\lambda_i = \frac{k_{\mathrm{cat}_i} L_{M_i}}{K_{M_i} l_{\mathrm{cat}_i}}, \quad K_{M_i} = \frac{k_{\mathrm{cat}_i} + k_{\mathrm{off}_i}}{k_{\mathrm{on}_i}}, \quad L_{M_i} = \frac{l_{\mathrm{cat}_i} + l_{\mathrm{off}_i}}{l_{\mathrm{on}_i}}, \quad i = 0, \ldots, n-1. \tag{3}$$

For each $\kappa, \mathcal{C}$, define $\tilde{\mathcal{Z}}(\kappa, \mathcal{C})$ as the set of $x \in \mathbb{R}_+^{3n+3}$ such that:

$$s_{i+1} = \lambda_i (e/f) s_i, \tag{4}$$

$$c_i = \frac{e s_i}{K_{M_i}} \tag{5}$$

$$d_{i+1} = \frac{f s_{i+1}}{L_{M_i}}, \tag{6}$$

$$e = \frac{E_{\mathrm{tot}}}{1 + s_0 \varphi_1^\kappa(e/f)} \tag{7}$$

for $i = 0, \ldots, n-1$, and

$$s_0 \left(\frac{e}{f} \varphi_1^\kappa(\frac{e}{f}) - \frac{E_{\mathrm{tot}}}{F_{\mathrm{tot}}} \varphi_2^\kappa(\frac{e}{f}) \right) = \frac{E_{\mathrm{tot}}}{F_{\mathrm{tot}}} - \frac{e}{f} \tag{8}$$

$$G^{\kappa,\mathcal{C}}(s_0, e/f) = 0, \tag{9}$$

where $G^{\kappa,\mathcal{C}} : \mathbb{R}_+^2 \longrightarrow \mathbb{R}$ is given by

$$G^{\kappa,\mathcal{C}}(s_0, u) = \varphi_0^\kappa(u)\varphi_2^\kappa(u) s_0^2 + \left(\varphi_0^\kappa(u) - S_{\mathrm{tot}}\varphi_2^\kappa(u) + F_{\mathrm{tot}} u \varphi_1^\kappa(u) + F_{\mathrm{tot}}\varphi_2^\kappa(u)\right) s_0 - S_{\mathrm{tot}}.$$

Lemma 1 For each $\kappa, \mathcal{C}$, we have $\mathcal{Z}(\kappa, \mathcal{C}) = \tilde{\mathcal{Z}}(\kappa, \mathcal{C})$.

Proof. We pick an arbitrary $x \in \mathcal{Z}(\kappa, \mathcal{C})$. Since $\Phi_{n+1}(x, \kappa, \mathcal{C}), \ldots, \Phi_{3n}(x, \kappa, \mathcal{C})$ are all zero, equations (5) and (6) follow easily. We next prove that

$$\frac{d_{i+1}}{c_i} = \frac{k_{\mathrm{cat}_i}}{l_{\mathrm{cat}_i}} \tag{10}$$

for all $i = 0, \ldots, n-1$ by induction.

When $i = 0$, from $\Phi_1(x, \kappa, \mathcal{C}) + \Phi_{n+1}(x, \kappa, \mathcal{C}) = 0$, we get

$$\frac{d_1}{c_0} = \frac{k_{\mathrm{cat}_0}}{l_{\mathrm{cat}_0}}.$$

Suppose that (10) holds for $i = k$, then for $i = k+1$, we have

$$\begin{aligned} 0 &= \Phi_{k+2}(x, \kappa, \mathcal{C}) + \Phi_{n+k+2}(x, \kappa, \mathcal{C}) + \Phi_{2n+k+1}(x, \kappa, \mathcal{C}) \\ &= k_{\mathrm{cat}_k} c_k + l_{\mathrm{cat}_{k+1}} d_{k+2} - k_{\mathrm{cat}_{k+1}} c_{k+1} - l_{\mathrm{cat}_k} d_{k+1} \\ &= l_{\mathrm{cat}_{k+1}} d_{k+2} - k_{\mathrm{cat}_{k+1}} c_{k+1}, \end{aligned}$$

which is (10) when $i = k+2$. Therefore, by induction, (10) holds for all $i = 0, \ldots, n-1$. Together with equations (5) and (6), we can obtain (4). Using (4)-(6), we may now express $\sum_0^n s_i$, $\sum_0^{n-1} c_i$ and $\sum_1^n d_i$ in terms of κ, e and f:

$$\begin{aligned} \sum_0^n s_i &= s_0 \left(1 + \lambda_0 \left(\frac{e}{f}\right) + \lambda_0\lambda_1 \left(\frac{e}{f}\right)^2 + \cdots + \lambda_0 \cdots \lambda_{n-1} \left(\frac{e}{f}\right)^n\right) = s_0 \varphi_0^\kappa \left(\frac{e}{f}\right), \\ \sum_0^{n-1} c_i &= e s_0 \left(\frac{1}{K_{M_0}} + \frac{\lambda_0}{K_{M_1}} \left(\frac{e}{f}\right) + \cdots + \frac{\lambda_0 \cdots \lambda_{n-2}}{K_{M_{n-1}}} \left(\frac{e}{f}\right)^{n-1}\right) = e s_0 \varphi_1^\kappa \left(\frac{e}{f}\right), \\ \sum_1^n d_i &= f s_0 \left(\frac{\lambda_0}{L_{M_0}} \left(\frac{e}{f}\right) + \frac{\lambda_0\lambda_1}{L_{M_1}} \left(\frac{e}{f}\right)^2 + \cdots + \frac{\lambda_0 \cdots \lambda_{n-1}}{L_{M_{n-1}}} \left(\frac{e}{f}\right)^n\right) = f s_0 \varphi_2^\kappa \left(\frac{e}{f}\right). \end{aligned} \tag{11}$$

From $\Phi_{3n+2}(x, \kappa, \mathcal{C}) = 0$, we have

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

and thus (7) holds. To check (8), we use $\Phi_{3n+2}(x, \kappa, \mathcal{C}) = 0$ and $\Phi_{3n+3}(x, \kappa, \mathcal{C}) = 0$ to get:

$$\frac{E_{\mathrm{tot}}}{F_{\mathrm{tot}}} = \frac{e(1 + s_0 \varphi_1^\kappa (e/f))}{f(1 + s_0 \varphi_2^\kappa (e/f))}, \tag{12}$$

which is (8) after multiplying by $1 + s_0 \varphi_2^\kappa (e/f)$ and rearranging terms. From $\Phi_{3n+3}(x, \kappa, \mathcal{C}) = 0$, we get

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

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

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

that is, $G^{\kappa,\mathcal{C}}(s_0, e/f) = 0$. Now we have checked that x satisfies (4)-(9), so $x \in \tilde{\mathcal{Z}}(\kappa, \mathcal{C})$.

Conversely, for any $x \in \tilde{\mathcal{Z}}(\kappa, \mathcal{C})$, because of (5) and (6), we know that $\Phi_{n+1}(x, \kappa, \mathcal{C}), \ldots, \Phi_{3n}(x, \kappa, \mathcal{C})$ are all zero. For each $i = 0, \ldots, n-1$, because of (4), we have

$$\frac{d_{i+1}}{c_i} = \frac{k_{\text{cat}_i}}{l_{\text{cat}_i}},$$

and thus

$$\Phi_1(x, \kappa, \mathcal{C}) + \Phi_{n+1}(x, \kappa, \mathcal{C}) = 0, \text{ when } i = 0,$$

and

$$\Phi_{i+1}(x, \kappa, \mathcal{C}) + \Phi_{n+i+1}(x, \kappa, \mathcal{C}) + \Phi_{2n+i}(x, \kappa, \mathcal{C}) = 0, \text{ otherwise.}$$

We already know that $\Phi_{n+1}(x, \kappa, \mathcal{C}), \ldots, \Phi_{3n}(x, \kappa, \mathcal{C})$ are zero, so $\Phi_1(x, \kappa, \mathcal{C}), \ldots, \Phi_n(x, \kappa, \mathcal{C})$ are zero too. We next check $\Phi_{3n+1}(x, \kappa, \mathcal{C}) = 0, \ldots, \Phi_{3n+3}(x, \kappa, \mathcal{C}) = 0$. By (4)-(6), (11) holds. Because of (7), we have that $\Phi_{3n+1}(x, \kappa, \mathcal{C}) = 0$ since:

$$e + \sum_0^{n-1} c_i = e\left(1 + s_0\varphi_1^\kappa(\frac{e}{f})\right) = E_{\text{tot}}.$$

From (8), we can derive (12), and therefore $\Phi_{3n+2}(x, \kappa, \mathcal{C}) = 0$:

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

Since $1 + s_0\varphi_2^\kappa(e/f) > 0$, we have

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

which implies

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

Therefore, $x \in \tilde{\mathcal{Z}}(\kappa, \mathcal{C})$. ∎

For each $\kappa, \mathcal{C}$, let us define two functions $H^{\kappa,\mathcal{C}}, F^{\kappa,\mathcal{C}} : \mathbb{R}_+ \longrightarrow \mathbb{R}$ as:

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

Lemma 2 For each $\kappa, \mathcal{C}$, there is a bijection between the set $\tilde{\mathcal{Z}}(\kappa, \mathcal{C})$ and the set of solutions of $F^{\kappa,\mathcal{C}}(u) = 0$.

Proof. We will show that the mapping $\theta(x) := u = e/f$ is a bijection.

First, we show that θ maps $\tilde{\mathcal{Z}}$ into the set of solutions of $F^{\kappa,\mathcal{C}}(u) = 0$. Any $x \in \tilde{\mathcal{Z}}(\kappa, \mathcal{C})$ is a solution of (9), i.e. $G^{\kappa,\mathcal{C}}(s_0, e/f) = 0$. Regarding $G^{\kappa,\mathcal{C}}(s_0, e/f)$ as a quadratic polynomial in s_0 with $G^{\kappa,\mathcal{C}}(0, e/f) < 0$, it has a unique positive root, namely

$$s_0 = \frac{-H^{\kappa,\mathcal{C}}(e/f) + \sqrt{H^{\kappa,\mathcal{C}}(e/f)^2 + 4S_{\text{tot}}\varphi_0^{\kappa}(e/f)\varphi_2^{\kappa}(e/f)}}{2\varphi_0^{\kappa}(e/f)\varphi_2^{\kappa}(e/f)}. \tag{13}$$

Plugging into (8) and multiplying by $\varphi_0^{\kappa}(e/f)$, we get $F^{\kappa,\mathcal{C}}(e/f) = 0$.

Next, we show that θ is onto. Pick any solution u of $F^{\kappa,\mathcal{C}}(u) = 0$. We can construct a solution of (4)-(9), i.e. an element of $\tilde{\mathcal{Z}}(\kappa, \mathcal{C})$, as follows.

Let

$$s_0 = \frac{-H^{\kappa,\mathcal{C}}(u) + \sqrt{H^{\kappa,\mathcal{C}}(u)^2 + 4S_{\text{tot}}\varphi_0^{\kappa}(u)\varphi_2^{\kappa}(u)}}{2\varphi_0^{\kappa}(u)\varphi_2^{\kappa}(u)}, \tag{14}$$

so that $G^{\kappa,\mathcal{C}}(s_0, u) = 0$. Substituting (14) into $F^{\kappa,\mathcal{C}}(u) = 0$ and dividing by $\varphi_0^{\kappa}(u)$, we get

$$s_0 \left(u\varphi_1^{\kappa}(u) - \frac{E_{\text{tot}}}{F_{\text{tot}}}\varphi_2^{\kappa}(u) \right) = \frac{E_{\text{tot}}}{F_{\text{tot}}} - u. \tag{15}$$

If we define

$$\begin{aligned}
s_{i+1} &= \lambda_i u s_i, \\
e &= \frac{E_{\text{tot}}}{1 + s_0\varphi_1^{\kappa}(u)} \\
f &= \frac{e}{u} \\
c_i &= \frac{e s_i}{K_{M_i}} \\
d_{i+1} &= \frac{f s_{i+1}}{L_{M_i}}
\end{aligned} \tag{16}$$

for $i = 0, \ldots, n-1$, then the vector $(s_0, \ldots, s_n, c_0, \ldots, c_{n-1}, d_1, \ldots, d_n, e, f)$ is an element of $\tilde{\mathcal{Z}}(\kappa, \mathcal{C})$ such that $\theta(x) = u$.

Finally, we show that θ is one to one. Suppose that $\theta(x_1) = \theta(x_2)$. The formula (13) shows that s_0 is the same for x_1 and x_2. Equation (7) gives that

$$e_1 = \frac{E_{\text{tot}}}{1 + s_0^1\varphi_1^{\kappa}(\theta(x_1))} = \frac{E_{\text{tot}}}{1 + s_0^2\varphi_1^{\kappa}(\theta(x_2))} = e_2.$$

So also $f_1 = f_2$. Now equations (4)-(6) show that $x_1 = x_2$. ∎

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

$$F_{tot} = E_{tot}/\gamma < \varepsilon_0 S_{tot}/\gamma; \tag{17}$$

then the system $\Sigma(\kappa, \mathcal{C})$ with $\mathcal{C} = (E_{tot}, F_{tot}, S_{tot})$ 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 $\mathbb{R}_+ \times \mathbb{R}_+ \longrightarrow \mathbb{R}$ as follows:

$$\begin{aligned}\tilde{H}^{\kappa,\gamma,S_{\text{tot}}}(\varepsilon, u) &= H^{\kappa,(\varepsilon S_{\text{tot}},\varepsilon S_{\text{tot}}/\gamma,S_{\text{tot}})}(u) \\ &= \varphi_0^\kappa(u) - S_{\text{tot}}\varphi_2^\kappa(u) + \varepsilon\frac{S_{\text{tot}}}{\gamma}u\varphi_1^\kappa(u) + \varepsilon\frac{S_{\text{tot}}}{\gamma}\varphi_2^\kappa(u),\end{aligned} \tag{18}$$

and

$$\begin{aligned}\tilde{F}^{\kappa,\gamma,S_{\text{tot}}}(\varepsilon, u) &= F^{\kappa,(\varepsilon S_{\text{tot}},\varepsilon S_{\text{tot}}/\gamma,S_{\text{tot}})}(u) \\ &= u\varphi_0^\kappa(u) + \frac{-\tilde{H}^{\kappa,\gamma,S_{\text{tot}}}(\varepsilon,u) + \sqrt{\tilde{H}^{\kappa,\gamma,S_{\text{tot}}}(\varepsilon,u)^2 + 4S_{\text{tot}}\varphi_0^\kappa(u)\varphi_2^\kappa(u)}}{2\varphi_2^\kappa(u)}\left(u\varphi_1^\kappa(u) - \gamma\varphi_2^\kappa(u)\right) - \gamma\varphi_0^\kappa(u).\end{aligned} \tag{19}$$

By Lemma 1 and Lemma 2, it is enough to show that there exist $\varepsilon_0 > 0$ and $\kappa \in \mathbb{R}_+^{6n-6}$ 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_{tot}, γ, E_{tot}, and F_{tot} satisfying (17), we let $\varepsilon = E_{\text{tot}}/S_{\text{tot}} < \varepsilon_0$, and apply the result.)

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

$$\begin{aligned}\tilde{F}^{\kappa,\gamma,S_{\text{tot}}}(0, u) &= u\varphi_0^\kappa(u) + S_{\text{tot}}\left(u\varphi_1^\kappa(u) - \gamma\varphi_2^\kappa(u)\right) - \gamma\varphi_0^\kappa(u) \\ &= \lambda_0\cdots\lambda_{n-1}u^{n+1} + \lambda_0\cdots\lambda_{n-2}\left(1 + \frac{S_{\text{tot}}}{K_{M_{n-1}}}(1-\gamma\beta_{n-1}) - \gamma\lambda_{n-1}\right)u^n \\ &+ \cdots + \lambda_0\cdots\lambda_{i-2}\left(1 + \frac{S_{\text{tot}}}{K_{M_{i-1}}}(1-\gamma\beta_{i-1}) - \gamma\lambda_{i-1}\right)u^i + \cdots \\ &+ \left(1 + \frac{S_{\text{tot}}}{K_{M_0}}(1-\gamma\beta_0) - \gamma\lambda_0\right)u - \gamma,\end{aligned} \tag{20}$$

where the λ_i's and K_{M_i}'s are defined as in (3), 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

$$(u - u_1)\cdots(u - u_{n+1}) = u^{n+1} + a_n u^n + \cdots + a_1 u + a_0, \tag{21}$$

where $a_0 = -\gamma < 0$. Our goal is to find a vector $\kappa \in \mathbb{R}_+^{6n-6}$ such that (20) and (21) are the same. We pick

$$\lambda_0 = \cdots = \lambda_{n-1} = 1.$$

For each $i = 0, \ldots, n-1$, comparing the coefficients of u^{i+1} in (20) and (21), we have:

$$\frac{S_{\text{tot}}}{K_{M_i}}(1 + a_0\beta_i) = a_{i+1} - a_0 - 1. \tag{22}$$

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 (22). From the given

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

we will find a vector

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

such that $\beta_i = k_{\text{cat}_i}/l_{\text{cat}_i}, i = 0, \ldots, n-1$, and (3) 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 is as follows. For each $i = 0, \ldots, n-1$, we start by defining:

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

consistently with the definitions in (3). 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 $\beta_i = k_{\text{cat}_i}/l_{\text{cat}_i}, i = 0, \ldots, n-1$, and (3).

In order to apply the Implicit Function Theorem, we now view the functions defined by formulas in (18) and (19) as defined also for $\varepsilon \leq 0$, i.e. as functions $\mathbb{R} \times \mathbb{R}_+ \longrightarrow \mathbb{R}$. 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 $i = 1, \ldots, n+1$, there exist open intervals E_i containing 0 and U_i containing u_i, and a differentiable function

$$\alpha_i : E_i \to U_i$$

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

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

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

Remark 3 For an arbitrary vector κ, we do not expect the derivative at each positive root to be non zero. Here is an example to show that more conditions are needed: with

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

we have that

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

has a double root at $u = 1$.

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

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

$$S_{tot}\left|\frac{1-\gamma\beta_j}{K_{M_j}}\right| \leq \frac{1}{n} \tag{23}$$

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

Proof. Recall that (dropping the u's in $\varphi_i^\kappa, i = 0,1,2$)

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

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,

$$
\begin{aligned}
\frac{\partial \tilde{F}^{\kappa,\gamma,S_{\text{tot}}}}{\partial u}(0,\bar{u}) &= \varphi_0^\kappa + S_{\text{tot}}(u\varphi_1^\kappa - \gamma\varphi_2^\kappa)' - \frac{S_{\text{tot}}(\bar{u}\varphi_1^\kappa - \gamma\varphi_2^\kappa)}{\varphi_0^\kappa}(\varphi_0^\kappa)' \\
&= \varphi_0^\kappa + \frac{S_{\text{tot}}}{\varphi_0^\kappa}\left(\varphi_0^\kappa(u\varphi_1^\kappa - \gamma\varphi_2^\kappa)' - (\bar{u}\varphi_1^\kappa - \gamma\varphi_2^\kappa)(\varphi_0^\kappa)'\right) \\
&= \varphi_0^\kappa + \frac{S_{\text{tot}}}{\varphi_0^\kappa}((1+\lambda_0\bar{u}+\lambda_0\lambda_1\bar{u}^2+\cdots+\lambda_0\cdots\lambda_{n-1}\bar{u}^n)\times \\
&\left(\frac{1}{K_{M_0}}(1-\gamma\beta_0)+2\frac{\lambda_0}{K_{M_1}}(1-\gamma\beta_1)\bar{u}+\cdots+n\frac{\lambda_0\cdots\lambda_{n-2}}{K_{M_{n-1}}}(1-\gamma\beta_{n-1})\bar{u}^{n-1}\right) \\
&-\left(\lambda_0+2\lambda_0\lambda_1\bar{u}+\cdots+n\lambda_0\cdots\lambda_{n-1}\bar{u}^{n-1}\right)\times \\
&\left(\frac{1}{K_{M_0}}(1-\gamma\beta_0)\bar{u}+\frac{\lambda_0}{K_{M_1}}(1-\gamma\beta_1)\bar{u}^2+\cdots+\frac{\lambda_0\cdots\lambda_{n-2}}{K_{M_{n-1}}}(1-\gamma\beta_{n-1})\bar{u}^n\right)) \\
&= \varphi_0^\kappa + \frac{S_{\text{tot}}}{\varphi_0^\kappa}\sum_{i=0}^{n}\lambda_0\cdots\lambda_{i-1}\bar{u}^i\left(\sum_{j=0}^{n-1}(j+1-i)\frac{\lambda_0\cdots\lambda_{j-1}}{K_{M_j}}(1-\gamma\beta_j)\bar{u}^j\right) \\
&= \frac{1}{\varphi_0^\kappa}\sum_{i=0}^{n}\lambda_0\cdots\lambda_{i-1}\bar{u}^i\sum_{j=0}^{n}\lambda_0\cdots\lambda_{j-1}\bar{u}^j \\
&+ S_{\text{tot}}\sum_{i=0}^{n}\lambda_0\cdots\lambda_{i-1}\bar{u}^i\left(\sum_{j=0}^{n-1}(j+1-i)\frac{\lambda_0\cdots\lambda_{j-1}}{K_{M_j}}(1-\gamma\beta_j)\bar{u}^j)\right) \\
&= \frac{1}{\varphi_0^\kappa}\sum_{i=0}^{n}\lambda_0\cdots\lambda_{i-1}\bar{u}^i\left(\lambda_0\cdots\lambda_{n-1}\bar{u}^n+\sum_{j=0}^{n-1}\lambda_0\cdots\lambda_{j-1}\bar{u}^j\left(1+S_{\text{tot}}(j+1-i)\frac{1-\gamma\beta_j}{K_{M_j}}\right)\right),
\end{aligned}
$$

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

Because of (23),

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

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

Corollary 4 For each positive numbers S_{tot}, γ, and vector $\kappa \in \mathbb{R}_+^{6n-6}$ satisfying condition (23), there exists $\varepsilon_0 > 0$ such that for any $F_{\text{tot}}, E_{\text{tot}}$ satisfying $F_{\text{tot}} = E_{\text{tot}}/\gamma < \varepsilon_0 S_{\text{tot}}/\gamma$, the number of positive steady states of system $\Sigma(\kappa, \mathcal{C})$ is greater or equal to the number of (positive) roots of $\tilde{F}^{\kappa,\gamma,S_{\text{tot}}}(0,u)$.

Proof. Follows directly from Lemmas 1, 2, the Implicit Function Theorem and Theorem 2. ∎

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

Proof. Let us define a polynomial $P^{\kappa,\mathcal{C}} : \mathbb{C} \longrightarrow \mathbb{C}$ as:

$$\varphi_0^\kappa\varphi_2^\kappa\left(\frac{E_{\text{tot}}}{F_{\text{tot}}}-u\right)^2+(\varphi_0^\kappa - S_{\text{tot}}\varphi_2^\kappa + F_{\text{tot}}u\varphi_1^\kappa + F_{\text{tot}}\varphi_2^\kappa)\left(\frac{E_{\text{tot}}}{F_{\text{tot}}}-u\right)\left(u\varphi_1^\kappa - \frac{E_{\text{tot}}}{F_{\text{tot}}}\varphi_2^\kappa\right) - S_{\text{tot}}\left(u\varphi_1^\kappa - \frac{E_{\text{tot}}}{F_{\text{tot}}}\varphi_2^\kappa\right)^2.$$

We claim that the number of positive steady states of $\Sigma(\kappa, \mathcal{C})$ is less or equal to the number of positive roots of $P^{\kappa,\mathcal{C}}(u)$. Recall that $\mathcal{Z} = \tilde{\mathcal{Z}}$ and the map θ is a bijection between the set $\tilde{\mathcal{Z}}$ and the set of (positive) roots of $F^{\kappa,\mathcal{C}}(u)$. Then it is enough to show that the set of (positive) roots of $F^{\kappa,\mathcal{C}}(u)$ is a subset of the set of positive real roots of $P^{\kappa,\mathcal{C}}(u)$.

In the proof of Lemma 2, we have showed that for any root $u > 0$ of $F^{\kappa,\mathcal{C}}(u)$, s_0 defined as in (14) satisfies $G^{\kappa,\mathcal{C}}(s_0, u) = 0$ and (15). Multiplying the equation $G^{\kappa,\mathcal{C}}(s_0, u) = 0$ by $(u\varphi_1^\kappa(u) - E_{\text{tot}}/F_{\text{tot}}\varphi_2^\kappa(u))^2$ and substituting (15) into it, we get $P^{\kappa,\mathcal{C}}(u) = 0$, that is, u is a positive root of $P^{\kappa,\mathcal{C}}(u)$.

Next we will show that the polynomial $P^{\kappa,\mathcal{C}}(u)$ of degree $2n+2$ has at most $2n$ positive real roots, and thus $\Sigma(\kappa, \mathcal{C})$ has at most $2n$ positive steady states.

It is easy to see that $P^{\kappa,\mathcal{C}}(u)$ is divisible by u; therefore, if we can find one more non positive root, we are done. Consider the polynomial $P^{\kappa,\mathcal{C}}(u)/u$ of degree $2n + 1$. 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 $P^{\kappa,\mathcal{C}}(u)/u$ has at least one negative root, and so does $P^{\kappa,\mathcal{C}}(u)$. ∎

Remark 5 This upper bound holds for every κ and $\mathcal{C}$.

The following is a standard result on continuity of roots; see for instance Lemma A.4.1 in [30]:

Lemma 6 Let $g(z) = z^n + a_1 z^{n-1} + \cdots + a_n$ be a polynomial of degree n and complex coefficients having distinct roots

$$\lambda_1, \ldots, \lambda_q,$$

with multiplicities

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

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

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

then h has precisely n_i roots in $B_\delta(\lambda_i)$ for each $i = 1, \ldots, q$.

Theorem 4 *For each $\gamma > 0$ and $\kappa \in \mathbb{R}_+^{6n-6}$ such that $\varphi_1^\kappa(\gamma) \neq \varphi_2^\kappa(\gamma)$, and each $S_{tot} > 0$, there exists $\varepsilon_1 > 0$ such that for all positive numbers F_{tot}, E_{tot} satisfying $F_{tot} = E_{tot}/\gamma < \varepsilon_1 S_{tot}/\gamma$, the system $\Sigma(\kappa, \mathcal{C})$ has at most $n+1$ positive steady states.*

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

$$\begin{aligned}
\tilde{P}^{\kappa,\gamma,S_{\text{tot}}}(\varepsilon, u) &= P^{\kappa,(\varepsilon S_{\text{tot}}, \varepsilon S_{\text{tot}}/\gamma, S_{\text{tot}})}(u) \\
&= \varphi_0^\kappa \varphi_2^\kappa (\gamma - u)^2 + \left(\varphi_0^\kappa - S_{\text{tot}}\varphi_2^\kappa + \varepsilon\frac{S_{\text{tot}}}{\gamma}u\varphi_1^\kappa + \varepsilon\frac{S_{\text{tot}}}{\gamma}\varphi_2^\kappa\right)(u\varphi_1^\kappa - \gamma\varphi_2^\kappa)(\gamma - u) \\
&\quad - S_{\text{tot}}(u\varphi_1^\kappa - \gamma\varphi_2^\kappa)^2.
\end{aligned}$$

As shown in the proof of Theorem 3, there is a bijection, given by the map θ, between $\tilde{\mathcal{Z}}(\kappa, (\varepsilon S_{\text{tot}}, \varepsilon S_{\text{tot}}/\gamma, S_{\text{tot}}))$ (denoted as $\tilde{\mathcal{Z}}$ for the rest of the proof) and a subset of the real positive roots of $\tilde{P}^{\kappa,\gamma,S_{\text{tot}}}(\varepsilon, u)$. Since $\tilde{P}^{\kappa,\gamma,S_{\text{tot}}}(\varepsilon, u)$ is a polynomial of degree $2n+2$, if we can show that there exists $\varepsilon_1 > 0$ such that for any $\varepsilon \in (0, \varepsilon_1)$, $\tilde{P}^{\kappa,\gamma,S_{\text{tot}}}(\varepsilon, u)$ has at least $n+1$ roots that are not in $\theta(\tilde{\mathcal{Z}})$, that is, do not have pre-images in $\tilde{\mathcal{Z}}$, then we are done.

First of all, any root of $\tilde{P}^{\kappa,\gamma,S_{\text{tot}}}(\varepsilon, u)$ that is not real or positive does not have a pre-image in $\tilde{\mathcal{Z}}$ under the map θ. Since $\tilde{P}^{\kappa,\gamma,S_{\text{tot}}}(\varepsilon, u)$ is divisible by u, $u = 0$ is a root of $\tilde{P}^{\kappa,\gamma,S_{\text{tot}}}(\varepsilon, u)$ that is not in $\theta(\tilde{\mathcal{Z}})$.

In order to apply Lemma 6, we regard the function $\tilde{P}^{\kappa,\gamma,S_{\text{tot}}}$ as defined on $\mathbb{R} \times \mathbb{C}$. At $\varepsilon = 0$:

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

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

$$u_1, \ldots, u_q,$$

with multiplicities

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

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

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

with multiplicities

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

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

1. $u_i > \gamma$. We have
$$u_i\varphi_1^\kappa(u_i) - \gamma\varphi_2^\kappa(u_i) > \gamma(\varphi_1^\kappa(u_i) - \varphi_2^\kappa(u_i)) = 0.$$

2. $u_i < \gamma$. We have
$$u_i\varphi_1^\kappa(u_i) - \gamma\varphi_2^\kappa(u_i) < \gamma(\varphi_1^\kappa(u_i) - \varphi_2^\kappa(u_i)) = 0.$$

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

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

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

1. For all $i = 1, \ldots, p$, if u_i is not real, then $B_\delta(u_i)$ has no intersection with the real axis.

2. For all $i = 1, \ldots, p$, if u_i is real, then for any real $u \in B_\delta(u_i)$, the following inequality holds:
$$(u\varphi_1^\kappa(u) - \gamma\varphi_2^\kappa(u))(\gamma - u) < 0. \tag{24}$$

3. $B_\delta(u_j)\bigcap B_\delta(u_k)=\emptyset$ for all $j\neq k=1,\dots,q$.

By Lemma 6, there exists $\varepsilon_1>0$ such that for all $\varepsilon\in(0,\varepsilon_1)$, the polynomial $\tilde{P}^{\kappa,\gamma,S_{\text{tot}}}(\varepsilon,u)/u$ 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 $B_\delta(u_i), i=1,\dots,p$ has pre-image in $\tilde{\mathcal{Z}}$. If so, we are done, since there are $\sum_1^p n_i\geq\sum_1^p m_i=n$ such roots of them, together with the root $u=0$, there are at least $n+1$ roots of $\tilde{P}^{\kappa,\gamma,S_{\text{tot}}}(\varepsilon,u)$ that do not have pre-images in $\tilde{\mathcal{Z}}$.

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 all $k=1,\dots,n_i$, and thus is not in $\theta(\tilde{\mathcal{Z}})$.
2. u_i is real. Suppose one of the roots $u_i^k(\varepsilon)$ has pre-image in $\tilde{\mathcal{Z}}$, i.e. $\theta(x)=u_i^k(\varepsilon)$ for some $x\in\tilde{\mathcal{Z}}$ and $k=1,\dots,n_i$. Since x is constructed as in the proof of Lemma 2, the s_0 coordinate of x satisfies (15), i.e.
$$s_0\big(u_i^k(\varepsilon)\varphi_1^\kappa(u_i^k(\varepsilon))-\gamma\varphi_2^\kappa(u_i^k(\varepsilon))\big)=\gamma-u_i^k(\varepsilon).$$
But condition 2 says that $u_i^k(\varepsilon)\varphi_1^\kappa(u_i^k(\varepsilon))-\gamma\varphi_2^\kappa(u_i^k(\varepsilon))$ and $\gamma-u_i^k(\varepsilon)$ have opposite signs, so s_0 has to be negative, which contradicts the fact that $x\in\mathbb{R}_+^{3n+3}$.

∎

Theorem 5 *For each $\gamma>0,\kappa\in\mathbb{R}_+^{6n-6}$ such that $\varphi_1^\kappa(\gamma)\neq\varphi_2^\kappa(\gamma)$, and each $E_{tot}>0$, there exists $\varepsilon_2>0$ such that for all positive numbers F_{tot}, S_{tot} satisfying $F_{tot}=E_{tot}/\gamma>S_{tot}/(\varepsilon_2\gamma)$, the system $\Sigma(\kappa,\mathcal{C})$ has at most one positive steady state.*

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

$$\begin{aligned}\bar{P}^{\kappa,\gamma,E_{\text{tot}}}(\varepsilon,u)&=P^{\kappa,(E_{\text{tot}},E_{\text{tot}}/\gamma,\varepsilon E_{\text{tot}})}(u)\\&=(\gamma-u)^2\varphi_0^\kappa\varphi_2^\kappa+\left(\varphi_0^\kappa-\varepsilon E_{\text{tot}}\varphi_2^\kappa+\frac{E_{\text{tot}}}{\gamma}u\varphi_1^\kappa+\frac{E_{\text{tot}}}{\gamma}\varphi_2^\kappa\right)(\gamma-u)\left(u\varphi_1^\kappa-\gamma\varphi_2^\kappa\right)\\&-\varepsilon E_{\text{tot}}\left(u\varphi_1^\kappa-\gamma\varphi_2^\kappa\right)^2.\end{aligned}$$

By the same argument as in the proof of Theorem 4, it is enough to show that there exists $\varepsilon_2>0$ such that for any $\varepsilon\in(0,\varepsilon_2)$ there is at most one positive root of $\bar{P}^{\kappa,\gamma,E_{\text{tot}}}(\varepsilon,u)$ that has pre-image in $\tilde{\mathcal{Z}}(\kappa,(E_{\text{tot}},E_{\text{tot}}/\gamma,\varepsilon E_{\text{tot}}))$ (denoted as $\tilde{\mathcal{Z}}$ for the rest of the proof).

In order to apply Lemma 6, we now view the function $\bar{P}^{\kappa,\gamma,E_{\text{tot}}}$ as defined on $\mathbb{R}\times\mathbb{C}$. At $\varepsilon=0$:

$$\bar{P}^{\kappa,\gamma,E_{\text{tot}}}(0,u):=(\gamma-u)\left((\gamma-u)\varphi_0^\kappa\varphi_2^\kappa+\left(\varphi_0^\kappa+\frac{E_{\text{tot}}}{\gamma}u\varphi_1^\kappa+\frac{E_{\text{tot}}}{\gamma}\varphi_2^\kappa\right)\left(u\varphi_1^\kappa-\gamma\varphi_2^\kappa\right)\right):=(\gamma-u)\,Q^{\kappa,\gamma,E_{\text{tot}}}(u).$$

Let us denote the distinct roots of $\bar{P}^{\kappa,\gamma,E_{\text{tot}}}(0,u)/u$ ($\bar{P}^{\kappa,\gamma,E_{\text{tot}}}(\varepsilon,u)$ is divisible by u) as

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

with multiplicities

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

and $u_2, \ldots, u_q$ are the roots of $Q^{\kappa,\gamma,E}\mathrm{tot}(u)/u$ other than γ.

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

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

$$(\gamma - u_i)\,\varphi_0^\kappa(u_i)\varphi_2^\kappa(u_i) = -\left(\varphi_0^\kappa(u_i) + \frac{E_{\mathrm{tot}}}{\gamma} u_i \varphi_1^\kappa(u_i) + \frac{E_{\mathrm{tot}}}{\gamma}\varphi_2^\kappa(u_i)\right)\left(u_i\varphi_1^\kappa(u_i) - \gamma\varphi_2^\kappa(u_i)\right).$$

If $u_i > 0$, then $\varphi_0^\kappa(u_i)\varphi_2^\kappa(u_i)$ and $\varphi_0^\kappa(u_i) + \frac{E_{\mathrm{tot}}}{\gamma} u_i \varphi_1^\kappa(u_i) + \frac{E_{\mathrm{tot}}}{\gamma}\varphi_2^\kappa(u_i)$ 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

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

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

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:
$$(u\varphi_1^\kappa(u) - \gamma\varphi_2^\kappa(u))(\gamma - u) < 0. \tag{25}$$
3. If u_i is real and negative, then $B_\delta(u_i)$ has no intersection with the imaginary axis.
4. $B_\delta(u_j) \bigcap B_\delta(u_k) = \emptyset$ for all $i \neq k = 2, \ldots, q$.

By Lemma 6, there exists $\varepsilon_2 > 0$ such that for all $\varepsilon \in (0, \varepsilon_2)$, the polynomial $\bar{P}^{\kappa,\gamma,E}\mathrm{tot}(\varepsilon, u)$ has exactly n_j roots in each $B_\delta(u_j), j = 1, \ldots, q$, denoted by $u_j^k(\varepsilon), k = 1, \ldots, n_j$.

We pick one such ε, and if we can show that none of the roots in $B_\delta(u_i), i = 2, \ldots, q$ has pre-images in $\tilde{\mathcal{Z}}$, then we are done, since the only roots that may have pre-images in $\tilde{\mathcal{Z}}$ are the roots in $B_\delta(u_1)$, and there is $n_1 = 1$ root in $B_\delta(u_1)$.

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

1. u_i is not real. Then condition 1 guarantees that $u_i^k(\varepsilon)$ is not real for all $k = 1, \ldots, n_i$, and thus is not in $\theta(\tilde{\mathcal{Z}})$.
2. u_i is real and negative. By conditions 1 and 3, $u_i^k(\varepsilon)$ is not in $\theta(\tilde{\mathcal{Z}})$ neither.
3. u_i is real and positive. Suppose one of the roots $u_i^k(\varepsilon)$ has pre-image in $\tilde{\mathcal{Z}}$, i.e. $\theta(x) = u_i^k(\varepsilon)$ for some $x \in \tilde{\mathcal{Z}}$ and $k = 1, \ldots, n_i$. Since x is constructed as in the proof of Lemma 2, the s_0 coordinate of x satisfies (15), i.e.
$$s_0\big(u_i^k(\varepsilon)\varphi_1^\kappa(u_i^k(\varepsilon)) - \gamma\varphi_2^\kappa(u_i^k(\varepsilon))\big) = \gamma - u_i^k(\varepsilon).$$
But condition 2 says that $u_i^k(\varepsilon)\varphi_1^\kappa(u_i^k(\varepsilon)) - \gamma\varphi_2^\kappa(u_i^k(\varepsilon))$ and $\gamma - u_i^k(\varepsilon)$ have opposite signs, so s_0 has to be negative, which contradicts the fact that $x \in \mathbb{R}_+^{3n+3}$.

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