Model reduction of genetic-metabolic networks via time scale separation

Juan Kuntz, Diego Oyarzún and Guy-Bart Stan

Abstract Model reduction techniques often prove indispensable in the analysis of physical and biological phenomena. A succesful reduction technique can substantially simplify a model while retaining all of its pertinent features. In metabolic networks, metabolites evolve on much shorter time scales than the catalytic enzymes. In this chapter, we exploit this discrepancy to justify the reduction via time scale separation of a class of models of metabolic networks under genetic regulation. We formalise the concept of a metabolic network and employ Tikhonov's Theorem for singularly perturbed systems. We demonstrate the applicability of our result by using it to address a problem in metabolic engineering: the genetic control of branched metabolic pathways. We conclude by providing guidelines on how to generalise our result to larger classes of networks.

1 Introduction

Biological systems often display large discrepancies in the speed at which different processes occur. In such cases, time scale separation is frequently employed to reduce ordinary differential equation (ODE) models of biological phenomena. A classical example is found in enzyme kinetics (Segel and Slemrod, 1989), whereby the difference between the speed of substrate-enzyme binding and product formation is explicitly used to derive the classic Michaelis-Menten equation.

Another discrepancy is found in genetic-metabolic systems prominent in the field of Metabolic Engineering. These systems describe networks of enzymatic reactions where the concentrations of the catalytic enzymes are dynamically regulated by gene expression. Metabolic reactions occur at rates in the order of seconds or less, while gene expression usually takes between minutes and hours to complete (Madigan

Juan Kuntz, Diego Oyarzún and Guy-Bart Stan
Centre for Synthetic Biology and Innovation, Department of Bioengineering, Imperial College London, SW7 2AZ, UK, e-mail: {juan.kuntz08, d.oyarzun, g.stan}@imperial.ac.uk

et al, 2011). For this reason, the reduction of models of metabolic networks under genetic control by time scale separation is sometimes used as a stepping stone in the analysis of such models (e.g., (Oyarzún et al, 2012; Baldazzi et al, 2012)). However, the justification behind these reductions is typically limited to qualitative arguments discussing the discrepancy in speed between metabolic and genetic processes. Unfortunately, these arguments sometimes are not sufficient and the reduced model generated does not behave at all like the original (e.g., see (Flach and Schnell, 2006) for a discussion regarding several models of metabolic networks for which the reduction fails).

In this chapter, we provide sufficient conditions under which reduction via time scale separation of models of metabolic networks under genetic control can confidently be carried out. In Section 2 we introduce some notation to describe a general class of ODE models of metabolic networks under genetic regulation. In addition, we make certain assumptions on the shape of the dynamics of the metabolites. In Section 3 we, first, introduce the main ideas behind time scale separation and we consider networks in which the enzyme concentrations are fixed. Then, we present our results regarding the validity of time scale separation as a model reduction tool for metabolic networks. In Section 4 we conclude the chapter by a discussing the plausibility of the assumptions we made throughout the text and the applicability of our results. We illustrate the concepts discussed in the chapter by applying them to the Metabolic Engineering problem presented in Box 1.

Box 1: Genetic control of a branched metabolic network

The control of metabolic activity of microbes is a long standing problem of the field of Metabolic Engineering. It encompasses the genetic modification of a host organism and its metabolism to optimise or even artificially induce the organism's production of a chemical compound that is of commercial value, e.g., pharmaceuticals, fuels, commodity chemicals, etc., see (Zhang and Keasling, 2011) and references therein. Often, this consists of two steps. First, the selection of a well studied microbial organism as a host (e.g., *E. coli* and *S. cerevisae*) with some native metabolite that is a precursor to the chemical of interest. Second, the genetic modification of the microbe so that it expresses the enzymes that catalyse the reactions which convert the precursor into the desired molecule (Nielsen and Keasling, 2011).

We study a simple instance of the above scenario. Consider the native metabolic pathway in Fig. 1A, which converts metabolite 1 into metabolite 3. Suppose that metabolite 2 is a precursor to a chemical of interest, metabolite 4. Suppose that we can design a plasmid that contains the gene coding for enzyme e, which catalyses the reaction that converts metabolite 2 into metabolite 4 which diffuses across the cell membrane. Since the host requires metabolite 3 to live and grow, we would like to maximise the production of metabolite 4, without greatly disrupting that of metabolite 3. The question

now becomes when and how should e be expressed so that these goals are met.

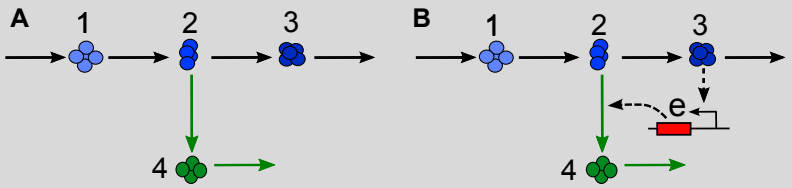

Figure 1: Control of a branched metabolic pathway. (**A**) The native pathway (black) converts metabolite 1 into metabolite 3. The synthetic 'branch' (green) converts the native intermediate, metabolite 2, into a valuable chemical, metabolite 4, and exports it outside the cell. (**B**) It is possible to implement positive feedback from metabolite 3 to the reaction that converts metabolite 2 into 4 by designing a plasmid coding for the enzyme e, who's expression is activated by high concentrations of metabolite 3.

Consider implementing the controller architecture in Fig. 1B. Roughly, if there is an excess of 3, indicating that it is safe to divert resources to the production of 4, then the controller activates the expression of e, which leads to an increase in the rate of the branch reaction. The branch reaction consumes 2 and, by lowering the concentration of 2, causes a decrease in the production of 3. This drop in production contributes to driving the concentration of 3 back to normal levels. If, on the contrary, the concentration of 3 is initially low, then expression of e drops and the branch shuts off. In this fashion, 2 is exclusively converted into 3, which hopefully restores the concentration of 3 to normal levels.

One could describe the above scenario using a model consisting of five ODEs, one of them describing the dynamics of the enzyme concentration and the other four describing the dynamics of the metabolite concentrations. Coarsely, model reductions employing time scale separation consist of grouping model variables into 'slow' variables and 'fast' variables and then neglecting the dynamics of the fast ones. In our case this grouping would naturally be the four metabolites as the 'fast' variables and the enzyme as the 'slow' variables. Thus, if applicable, the reduction would permit us to draw conclusions on the behaviour of the network by studying a 1 dimensional model instead of a 5 dimensional model. This would be highly desirable given that the analysis of a 1 dimensional model is straightforward while that 5 dimensional model can be exceedingly complicated (Khalil, 2002).

2 Models for metabolic reactions under genetic control

Suppose we have a network of n metabolites and m irreversible enzymatic reactions each of which converts a single metabolite into another. Consider the model for the

network under genetic regulation

$$\dot{s}(t) = f(s(t), e(t)), \qquad s(0) = s_0, \tag{1a}$$
$$\dot{e}(t) = g(s(t), e(t)), \qquad e(0) = e_0, \tag{1b}$$

where s denotes the vector of concentrations of the metabolites and e denotes the vector of concentrations of the enzymes catalysing the m reactions in the network. The metabolite dynamics, $f(\cdot)$, are defined by the rate at which the reactions consume and produce the different metabolites. The enzyme dynamics, $g(\cdot)$, model all the processes involved in enzyme synthesis and degradation.

In this section, we discuss what model (1) represents and make certain assumptions about it. We begin by discussing the kinetics of individual enzymatic reactions. Next, we construct the metabolite dynamics (1a) from first principles. We conclude by briefly discussing the enzyme dynamics (1b).

2.1 Enzyme kinetics

We consider irreversible enzymatic reactions like the one shown in Fig. 2. The reaction converts a single reactant A into a single product B. The rate at which the reaction occurs, $v(s_A, e)$, depends exclusively on the concentration of the reactant, s_A, and the concentration of the catalysing enzyme, e.

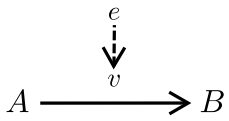

Figure 2: An irreversible, enzymatic reaction. The reaction converts metabolite A into metabolite B at a rate $v(s_A, e)$ which depends exclusively on the concentration of the reactant, s_A, and that of the catalysing enzyme, e.

Assumption 1. *The reaction rate, $v(s_A, e)$, is smooth and globally Lipschitz continuous. For any given constant enzyme concentration $e > 0$, we assume that $v(\cdot, e)$ is bounded, that*

$$\frac{\partial v(s_A, e)}{\partial s_A} > 0, \qquad \forall s_A \neq 0,$$

and that $v(\cdot, e)$ is positive definite*, that is,*

$$v(0, e) = 0, \qquad v(s_A, e) > 0, \qquad \forall s_A > 0.$$

We denote its least upper bound with

$$\lim_{s_A \to +\infty} v(s_A, e) = \hat{v}(e).$$

At a network level we need to distinguish between different reactions. To do this, we write $v_{A\to B}$ and $e_{A\to B}$ to refer to the rate and the concentration of the catalysing enzyme of the reaction with reactant A and product B.

Our assumptions on the kinetics are satisfied by a wide range enzyme kinetics proposed in the literature (Cornish-Bowden, 2004) (e.g., Michaelis-Menten and Hill type kinetics). Essentially, they state that:

- (Positive definite) If there are no reactant molecules present, the reaction rate is zero. If there are some reactant and some enzyme molecules present, the reaction rate is non-zero.
- (Strictly increasing) If there are some enzyme molecules present, then the more reactant molecules present, the faster the reaction rate.
- (Bounded) Enzymes have a limited number of active sites to which the reactants attach to react. Thus, given a fixed number of enzyme molecules, the reaction rate cannot exceed the maximum rate achieved when all the enzymes' active sites are bound by the reactants.

Implicit in our definition of the reaction rates is the assumption that they are time invariant. It is well known that the rate of a reaction depends on the temperature and pressure of the medium in which the reaction is taking place. Hence, assuming time invariance of the reaction kinetics is equivalent to assuming that the cytoplasm can be approximated to be isobaric and isothermal. This is a common assumption in the literature on ODE models of biochemical reactions (Heinrich and Schuster, 1996; Cornish-Bowden, 2004).

2.2 Metabolic model

Assuming that the cytoplasm may be approximated to be an isovolumetric and spatially homogeneous medium (Heinrich and Schuster, 1996), the law of mass balance applied to the concentration of metabolite number i yields

$$\dot{s}_i(t) = P_i(t) - C_i(t) + I_i(t) - E_i(t), \tag{2}$$

where P_i denotes the rate at which s_i is produced by the considered genetic-metabolic network, C_i the rate at which s_i is consumed by the network, I_i the rate at which s_i enters the network from outside and E_i the rate at which s_i leaves the network. From now on, we use the convention $v_{i\to j} \equiv 0$ if there is no reaction that converts metabolite i into j.

A metabolite is produced (consumed) by the reactions of which it is the product (reactant). We limit our attention to networks whose metabolites can be ordered in such a way that the following condition is satisfied.

Condition 1. *For any i, if $j > i$ then $v_{j\to i} \equiv 0$. In other words, metabolite i is not the product of any reaction whose reactant is metabolite $i+1, i+2, \ldots, n$.*

Condition 1 has a simple graphical interpretation. Consider the directed graph whose vertices represent the metabolites and whose edges represent the transfer of mass (via reactions) from one metabolite to another. Condition 1 is equivalent to the graph being **acyclic**, that is, starting at any given vertex one cannot return to that same vertex by following the edges, see Fig. 3. Examples of such networks can be found in the amino-acid biosynthesis pathways of *E. coli* (Zaslaver et al, 2004).

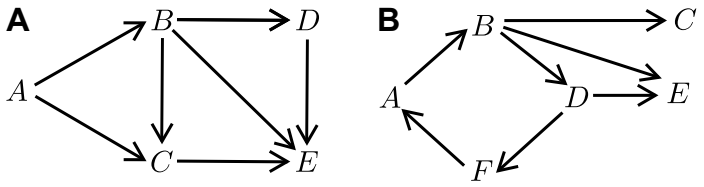

Figure 3: Acyclicity in networks. (**A**) The network is acyclic. (**B**) The network is not acyclic; A, B, D, F form a cycle.

Let $N_{i,i\to j}$ denote the *stoichiometric coefficient* of i in reaction $i \to j$, that is, the number of molecules of i involved in reaction $i \to j$. If Condition 1 holds, we can write the rates of production as

$$P_1(t) := 0, \qquad P_i(t) := \sum_{j=1}^{i-1} N_{i,j\to i} v_{j\to i}(s_j, e_{j\to i}), \quad i = 2,3,\dots,n, \tag{3}$$

and the rates of consumption as

$$C_n(t) := 0, \qquad C_i(t) := \sum_{j=i+1}^{n} N_{i,i\to j} v_{i\to j}(s_i, e_{i\to j}), \quad i = 1,2,\dots,n-1. \tag{4}$$

Consider the import and export rates I_i and E_i, respectively, in (2).

Assumption 2. *The import rates are constant, $I_i(t) := I_i \geq 0 \,\forall\, i$. The export rate of a metabolite i, if it exists, is a smooth, globally Lipschitz continuous, positive definite, bounded function of its concentration such that*

$$\frac{\partial E_i(s_i)}{\partial s_i} > 0.$$

We denote its least upper bound with

$$\lim_{s_i\to+\infty} E_i(s_i) = \hat{E}_i.$$

One can use the import and export rates to model a variety of phenomena. For instance, they may represent the rates at which the metabolites flow in and out of the cell. Or the rates at which the metabolites are consumed/produced by other metabolic pathways inside the cell. Additionally, one may use the export rates to circumvent the isovolumetric assumption and model dilution. Regardless, in any of

these cases the physical interpretations of Assumption 2 are similar to those we made regarding the assumptions on the enzyme kinetics (Assumption 1). In addition, assumptions of the type of Assumption 2 are common in the systems biology literature (for example, see (Craciun et al, 2011; Radde et al, 2010)) and for this reason we shall not discuss them any further.

We can now rewrite the metabolite dynamics, $f(s,e)$, in the model (1) as

$$\begin{aligned}
\dot{s}_1 &= I_1 - E_1(s_1) - \sum_{j=2}^{n} N_{1,1\to j} v_{1j}(s_1, e_{1\to j}), \\
\dot{s}_i &= I_i + \sum_{j=1}^{i-1} N_{i,j\to i} v_{j\to i}(s_j, e_{j\to i}) - E_i(s_i) - \sum_{j=i+1}^{n} N_{i,i\to j} v_{i\to j}(s_i, e_{i\to j}), \quad i = 2,3,\ldots,n-1, \\
\dot{s}_n &= I_n + \sum_{j=1}^{n-1} N_{n,j\to n} v_{j\to n}(s_j, e_{j\to n}) - E_n(s_n).
\end{aligned} \tag{5}$$

Box 2: Metabolic model

In our example network we assume that all reactions follow Michaelis Menten kinetics

$$v_{1\to 2} := \frac{k_{cat1} s_1}{K_{M1} + s_1} e_{1\to 2}, \quad v_{2\to 3} := \frac{k_{cat2} s_2}{K_{M2} + s_2} e_{2\to 3}, \quad v_{2\to 4} := \frac{k_{cat3} s_2}{K_{M3} + s_2} e_{2\to 4}.$$

It is straightforward to verify that Michaelis-Menten kinetics satisfy Assumption 1, see Fig. 4.

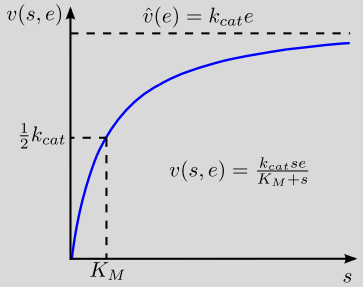

Figure 4: Michaelis Menten kinetics. The kinetics are strictly increasing, positive definite and bounded.

The network in Fig. 1 has a single import rate, I_1 and two export rates, E_3 and E_4. We assume that the export rates may also be described by Michaelis-Menten functions

$$E_3 := \frac{\hat{E}_3 s_3}{K_{O3} + s_3}, \quad E_4 := \frac{\hat{E}_4 s_4}{K_{O4} + s_4}.$$

Hence, we get the metabolite dynamics

$$\dot{s}_1 = I_1 - \frac{k_{cat1} s_1}{K_{M1} + s_1} e_{1\to 2}, \tag{6a}$$

$$\dot{s}_2 = \frac{k_{cat1} s_1}{K_{M1} + s_1} e_{1\to 2} - \frac{k_{cat2} s_2}{K_{M2} + s_2} e_{2\to 3} - \frac{k_{cat3} s_2}{K_{M3} + s_2} e_{2\to 4}, \tag{6b}$$

$$\dot{s}_3 = \frac{k_{cat2} s_2}{K_{M2} + s_2} e_{2\to 3} - \frac{\hat{E}_3 s_3}{K_{O3} + s_3}, \tag{6c}$$

$$\dot{s}_4 = \frac{k_{cat3} s_2}{K_{M3} + s_2} e_{2\to 4} - \frac{\hat{E}_4 s_4}{K_{O4} + s_4}. \tag{6d}$$

From Fig. 1A it is easy to see that our network satisfies Condition 1, i.e., it is acyclic. To simplify future computations, we choose $k_{cati} = k_{cat} = 32\text{s}^{-1}$, $K_{Mi} = K_M = 4.7\mu\text{Ms}^{-1}$ $\forall i$ and $e_{1\to 2} = e_{2\to 3} = e_N = 200\text{nM}$. These values are representative of reactions in the tryptophan pathway (extracted from the BRENDA database (Scheer et al, 2011), EC number 5.3.1.24). We also assume that $\hat{E}_3 = \hat{E}_4 = k_{cat} e_N$, $K_{O3} = K_{O4} = K_M$ and use the shorthand $e := e_{2\to 4}$.

2.3 Enzymatic model

The enzyme dynamics, $g(\cdot)$, are a lumped representation of all the processes involved in enzyme synthesis and destruction. Synthesis encompasses the transcription of genes encoding the enzymes by RNA polymerases into mRNA strands and the translation of these by ribosomes into polypeptides that later fold into the actual enzyme proteins. Most enzyme-enzyme and metabolite-enzyme interactions occur in synthesis, specifically in transcription. In particular, metabolites often act as, or bind to, *transcription factors* (TFs) that inhibit or activate the transcription of genes coding for other enzymes. Destruction, typically, includes enzyme degradation by the cell and dilution due to cell growth.

To keep this exposition general, we shall not define the function $g(\cdot)$ explicitly. We will only make the following minimal assumptions.

Assumption 3. *The enzymes dynamics $g(\cdot)$ are smooth and globally Lipschitz continuous.*

Enzyme degradation and dilution are typically modelled as linear functions of the enzyme concentration (Alon, 2006). Synthesis is usually modelled as the sum of a constant (or basal) expression rate a set of sigmoids (e.g., Hill functions) representing the activating or repressing effects of the TF on the enzyme expression (Oyarzún and Stan, 2013; Baldazzi et al, 2012; Oyarzún and Stan, 2012). These are all smooth and globally Lipschitz continuous functions. The enzyme dynamics, $g(\cdot)$, are a linear combination of these and, thus, are also a smooth and globally Lip-

schitz continuous. For this reason, Assumption 3 holds for a significant portion of the models presented in the literature.

Box 3: Enzymatic model

Consider the controller for the branched metabolic pathway previously discussed in Box 1. Implementing such a controller can be achieved, for example, by designing the promoter of the gene coding for e such that 3 binds to some TFs that activates the transcription of e, see Fig. 5A. We model the expression of the branch enzyme e as

$$\dot{e} = k_0 + k_1\sigma(s_3) - \gamma e, \quad \sigma(x) := \frac{x^h}{\theta^h + x^h}. \tag{7}$$

This model comes from the balance between protein synthesis and degradation. We consider a first order destruction process with kinetic constant γ, which accounts for the aggregate effect of degradation and dilution by cell growth (Alon, 2006). The synthesis term, $k_0 + k_1\sigma(s_3)$, describes both transcription and translation of e. The parameter k_0 represents the leaky, or constitutive, expression of the enzyme that occurs regardless whether the gene is activated or repressed, while k_1 represents the compound effect of transcription and translation when the gene is fully expressed. The function $\sigma(\cdot)$ takes values in $[0,1)$ and depends on the specific molecular mechanisms underlying the interactions both between metabolite 3 and the TF and those between the TF and the promoter of the gene coding for the enzyme. Typically, these types of interactions are modelled as sigmoidal (or Hill) functions (Oyarzún and Stan, 2013; Baldazzi et al, 2012), see Fig. 5B.

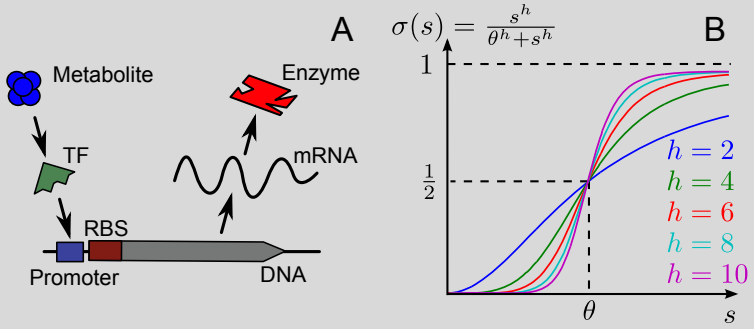

Figure 5: The implementation of feedback via promoter design. (**A**) Metabolite 3 induces a conformation change on the transcription factor, which then binds to the promoter of the gene coding for e and activates its expression. (**B**) Hill functions with different Hill coefficients, note that their range is $[0,1)$, hence $k_0 + k_1$ represent the maximum expression of e.

We chose the parameter values $k_0 = 0.03n\text{M}$, $k_1 = 100k_0$, $\gamma = 2\times 10^{-4}s^{-1}$, $\theta = 0.2\mu\text{M}$ and $h = 2$ which are representative of enzyme expression in the Tryptophan Pathway (Oyarzún and Stan, 2013).

3 Model reduction via time scale separation

In this section we present our results regarding time scale separation in genetic-metabolic systems. We first consider the behaviour of metabolic networks when the enzyme concentrations are kept fixed in time. There are two reasons behind this. First, it is a prerequisite to the time scale separation results regarding networks with varying enzyme concentrations. Second, the study itself is instructive with regards to understanding the behaviour of the networks. After this, we introduce abstractly the main ideas of time scale separation and give our results justifying the time scale separation based reduction of the networks.

3.1 Metabolic networks with constant enzyme concentrations

Suppose that the enzyme concentrations are positive constants, i.e., $e(t) \equiv e \in \mathbb{R}^m_{>0}$. We find it convenient to rewrite (5) as

$$\dot{s}_i = g_i(s_1,\dots,s_{i-1},e) - h_i(s_i,e), \qquad i = 1,2,\dots,n, \tag{8}$$

where $g_1 := I_1$,

$$g_i(s_1,\dots,s_{i-1},e) := I_i + \sum_{j=1}^{i-1} N_{i,j\to i} v_{j\to i}(s_j, e_{j\to i}), \qquad i = 2,3,\dots,n,$$

and $h_n(s_n) := E_n(s_n)$,

$$h_i(s_i,e) := E_i(s_i) + \sum_{j=i+1}^{n} N_{i,i\to j} v_{i\to j}(s_i, e_{i\to j}), \qquad i = 1,2,\dots,n-1.$$

The function $g_i(\cdot) \geq 0$ represents the total rate of increase (via both import and production) of the concentration of metabolite i. Similarly, the function $h_i(\cdot) \geq 0$ represents the total rate of decrease (via both export and consumption) of the concentration of metabolite i. To avoid pathological scenarios in which the concentration of a given metabolite keeps on rising because there is no process that removes the metabolite, we impose the following condition.

Condition 2. *Every metabolite has at least one reaction that consumes it, or it has a non-trivial export term. In other words, for all i, $E_i \not\equiv 0$ or there exists a j such that $v_{i\to j} \not\equiv 0$. Thus, $h_i \not\equiv 0$ for all i.*

The non-zero E_i and $v_{i\to j}$ functions (if they exist, and Condition 2 ensures at least one does exists for all $i = 1,2,\ldots,n$) are bounded, strictly increasing, positive definite functions of s_i. Hence, h_i, which is a sum of these functions, is also positive definite and strictly increasing with s_i and it maps from $\mathbb{R}_{\geq 0}$ to $[0,\hat{h}_i(e))$, where

$$\hat{h}_i(e) := \hat{E}_i + \sum_{j=i+1}^{n} N_{i,i\to j}\hat{v}_{i\to j}(e_{i\to j}).$$

We now examine the conditions under which system (8) has an equilibrium. By definition, $g_1 \equiv I_1 \geq 0$, thus $\dot{s}_1 = 0$ implies

$$h_1(\bar{s}_1, e) = I_1.$$

This algebraic or transcendental equation has a solution if and only if the I_1 is in the range of the function $h_1(\bar{s}_1, e)$. In other words, we require $\hat{h}_1(e) > I_1$. In addition, $h_1(\bar{s}_1, e)$ is a strictly increasing function of $\bar{s}_1$, thus if a solution exists it is unique. Now, if we assume that $\bar{s}_1,\ldots,\bar{s}_{i-1}$ exist, then $\dot{s}_i = 0$ implies

$$h_i(\bar{s}_i, e) = g_i(\bar{s}_1,\ldots,\bar{s}_{i-1}, e).$$

Similarly as before, the equation has a solution if and only if the constant $g_i(\bar{s}_1,\ldots,\bar{s}_{i-1}, e)$ is in the range of the function $h_i(\bar{s}_i, e)$, i.e., if $\hat{h}_i(e) > g_i(\bar{s}_1, \bar{s}_2,\ldots,\bar{s}_{i-1}, e)$. In addition, $h_i(\bar{s}_i, e)$ is a strictly increasing function of $\bar{s}_i$, Hence if a solution exists it is unique.

Thus, by induction, an equilibrium exists if and only if the following condition is satisfied.

Condition 3. *The vector of constant enzymes e is such that $\hat{h}_i(e) > g_i(\bar{s}_1,\ldots,\bar{s}_{i-1}, e)$ $\forall i = 1,2,\ldots,n$.*

Furthermore, by monotonicity, if the equilibrium exists it is unique.

Condition 3 is important and has an intuitive interpretation. Regard the metabolites in the network as large water tanks, their concentrations as the water level in the tanks, the reactions as pipes connecting the tanks and the reaction rates as the rate of flow of water through the pipes. In this context, the enzymes may be regarded as valves whose concentrations modulate the resistance to flow through them. Then $g_i(\cdot)$ may be interpreted as the rate at which water enters the i^{th} tank through the incoming pipes and $h_i(\cdot)$ as the rate at which it leaves through the outgoing pipes. The monotonicity of h_i can be interpreted as 'the more volume of water in the tank, the greater the water pressure and thus the bigger the rate at which the water is pushed out of the tank through the outgoing pipes'. Condition 3 simply ensures that the outgoing pipes are 'sufficiently large' in the sense that the maximum rate at which water can escape the tank is higher than the equilibrium rate at which water enters.

Condition 1, that the network is acyclic, implies that there is no chain of reactions that convert metabolite i into metabolites $1,2,\ldots,i-1$. Thus, if metabolites $1,2,\ldots,i-1$ are at their equilibrium concentrations, they will remain there forever irrespective of what is happening to the concentrations of metabolites $i,i+1,\ldots,n$. So, if Condition 3 does not hold for a given metabolite i and metabolites $1,2,\ldots,i-1$ are at their equilibrium concentrations, then metabolite i will simply accumulate and its concentration will tend to infinity.

Box 4: Network fluxes

Consider Condition 3 applied to the network in Fig. 1A

$$\hat{v}_{1\to2}(e_N) > I_1, \quad \hat{v}_{2\to3}(e_N) + \hat{v}_{2\to4}(e) > v_{1\to2}(\bar{s}_1, e_N),$$

$$\hat{E}_3 > v_{2\to3}(\bar{s}_2, e_N), \quad \hat{E}_4 > v_{2\to4}(\bar{s}_2, e).$$

By definition, all the reaction rates are non-negative, so $\hat{v}_{2\to3}(e_N)+\hat{v}_{2\to4}(e) \geq \hat{v}_{2\to3}(e_N)$. Also note that because $\bar{s}$ is an equilibrium

$$I_1 = v_{1\to2}(\bar{s}_1, e_N) = v_{2\to3}(\bar{s}_2, e_N) + v_{2\to4}(\bar{s}_1, e).$$

In Box 2 we assumed that

$$\hat{v}_{1\to2}(e_N) = \hat{v}_{2\to3}(e_N) = \hat{E}_3 = \hat{E}_4 = k_{cat} e_N.$$

So, Condition 3 is satisfied for any postive enzyme concentration, that is $e \in (0,+\infty)$, if and only if $k_{cat} e_N > I_1$.

It can be shown that the fulfilment of Condition 3 does not just imply that the network has a unique equilibrium, it also implies that the equilibrium is stable.

Lemma 1. *Assume that the metabolic network is such that Conditions 1 and 2 are satisfied and Assumptions 1 and 2 hold. If the enzyme concentrations are fixed in time at some value such that Condition 3 is satisfied, then* (8) *has a unique equilibrium which is globally asymptotically stable.*

The proof of the above lemma can be found in Appendix 2.

3.2 Time scale separation

Time scale separation is applicable to systems that may be written as

$$\varepsilon \dot{z} = f(x,z), \qquad z(0) = z_0 \tag{9a}$$

$$\dot{x} = g(x,z), \qquad x(0) = x_0 \tag{9b}$$

where the components of $f : \mathbb{R}^n \times \mathbb{R}^m \to \mathbb{R}^n$, $g : \mathbb{R}^n \times \mathbb{R}^m \to \mathbb{R}^m$ are in the same order of magnitude for all $(x,z) \in \mathbb{R}^{n+m}$ and $0 < \varepsilon \ll 1$ is a small positive real number. The characterising feature of these systems is that the dynamics of some of the state variables (z) are multiple orders of magnitude faster than those the other state variables (x), i.e., $\dot{z} = f(x,z)/\varepsilon \gg g(x,z) = \dot{x}$. Suppose that during a small interval of time within which the value of the slow variables (x) remain approximately constant, the fast variables (z), which are evolving hundreds/thousands times faster, reach some steady state or *quasi-steady state*. If we assume that the dynamics of the variables z reach this steady-state very quickly (almost instantaneously at the time scale of the slow variables x), then we can assume that, at the time scale of the slow variables x, $\dot{z} = 0$ or, equivalently, that

$$f(x,z) = 0.$$

Suppose that the above has a unique root $z = \phi(x)$, i.e., $f(x,\phi(x)) = 0$ for all x. Then, at the time scale of the slow variables x, one can focus on studying the *reduced* dynamical system

$$\dot{\bar{x}} = g(\bar{x},\phi(\bar{x})), \qquad \bar{x}(0) = x_0 \tag{10a}$$

$$\bar{z} = \phi(\bar{x}), \tag{10b}$$

instead of the original system (9).

Notice that in contrast with the fast variable z of the original system (9), which starts at time 0 from a given z_0, the fast variable $\bar{z}$ of the reduced system (10) is not free to start from z_0 and there may be a large discrepancy between its initial value, $\phi(x_0)$, and z_0. Thus, there must at least be a short period of time where the behaviour of reduced system does not approximate well that of the complete system.

Before carrying out the above reduction, we need to address a number of outstanding issues. For instance, does a quasi-steady state even exist? Is it unique? If it is not, which quasi-steady state should be used in the reduction? Do the fast variables of the complete system always tend to their quasi-steady state?

Theorem 2, known as *Tikhonov's Theorem*, partly answers these questions by providing sufficiency conditions under which the behaviour of the original system (9) is well approximated by that of the reduced system (10). More specifically, if its assumptions are satisfied, Tikhonov's Theorem ensures that after some period of time of order $\varepsilon\ln(1/\varepsilon)$, i.e., $O(\varepsilon\ln(1/\varepsilon))$, during which the initial discrepancy between z and $\bar{z}$ dies out, the norm difference between the trajectory of the complete system (9) and that of the reduced system (10) remains of order ε and no more.

3.3 Sufficiency conditions for time scale separation

To be able to state our results regarding time scale separation in genetic-metabolic systems, we must first re-write the network model (1) in the same form as (9). Usu-

ally, this involves some, possibly complicated, change of variables. However, in the case of genetic-metabolic networks this is not necessary; the 'fast' variables are the metabolite concentrations while the 'slow' variables are the enzyme concentrations. Thus all that must be done is to scale the variables so that the new metabolite dynamics, $f(\cdot)$, and the enzyme dynamics, $g(\cdot)$, are of the same order of magnitude and all the normalising constants are grouped into a parameter ε multiplying $\dot{s}$. A systematic way to do this is to *non-dimensionalise* the network model (1), which consists of performing a set of variable substitutions such that the new variables have no physical dimensions associated with them (Lin and Segel, 1988).

Box 5: Non-dimensionlisation

Consider substituting the variables of our network model (equations (6) in Box 2 and (7) in Box 3) with

$$z := \frac{s}{K_M}, \qquad x := \frac{e}{\hat{e}}, \qquad \tau := \gamma t, \qquad \hat{e} := \frac{k_0 + k_1}{\gamma}. \tag{11}$$

Notice that the new variables (x,z) have no physical units associated with them. After re-arranging we get

$$\varepsilon \frac{dz_1}{d\tau} = \tilde{I} - \frac{z_1}{1+z_1} \tag{12a}$$

$$\varepsilon \frac{dz_2}{d\tau} = \frac{z_1}{1+z_1} - \frac{z_2}{1+z_2} - \frac{\hat{e}}{e_N}\frac{z_2 x}{1+z_2} \tag{12b}$$

$$\varepsilon \frac{dz_3}{d\tau} = \frac{z_2}{1+z_2} - \frac{z_3}{1+z_3} \tag{12c}$$

$$\varepsilon \frac{dz_4}{d\tau} = \frac{\hat{e}}{e_N}\frac{z_2 x}{1+z_2} - \frac{z_4}{1+z_4} \tag{12d}$$

$$\frac{dx}{d\tau} = \frac{k_0}{k_0+k_1} + \frac{k_1}{k_0+k_1}\sigma^*(z_3) - x \tag{12e}$$

where $\tilde{I} = \frac{I_1}{k_{cat} e_N}$, $\sigma^*(z_3) := \sigma(K_M z_3)$ and $\varepsilon = \frac{K_M \gamma}{k_{cat} e_N} \approx 1.5 \times 10^{-4}$.

We can now state our results regarding time scale separation in metabolic networks under genetic regulation. The proofs for the following lemma and theorem may be found in Appendix 2.

Lemma 2. *Suppose that* (1) *is such that Conditions 1, 2 and Assumptions 1 to 3 hold. Consider a non-dimensionalised version of* (1)

$$\varepsilon \dot{s}(t) = f(s(t), e(t)), \qquad s(0) = s_0 \tag{13a}$$

$$\dot{e}(t) = g(s(t), e(t)), \qquad e(0) = e_0 \tag{13b}$$

Then, the unique solution of (13), $\left[s(t)\ e(t)\right]^T$, *exists for all* $t \geq 0$. *In addition, let* A *denote the subset of* $\mathbb{R}^m_{>0}$ *whose elements are such that Condition 3 holds. There exists a unique function* $\phi : A \to \mathbb{R}^n$ *such that* $f(\phi(e), e) = 0$ *for all* $e \in A$. *In addition,* $\phi(\cdot)$ *is continuously differentiable. Consider the reduced system*

$$\dot{\bar{e}}(t) = g(\phi(\bar{e}(t)), \bar{e}(t)), \qquad \bar{e}(0) = e_0. \tag{14}$$

Suppose that there exists a compact set $B \subseteq A$ *that is forward invariant with respect to* (14). *Then, if* $e_0 \in B$, (14) *has a unique solution* $\bar{e}(t) \in B$ *for all* $t \geq 0$.

Theorem 1. *Suppose that the assumptions of Lemma 2 are satisfied and that* $e_0 \in B$. *Then, for any finite time* $T \geq 0$

$$e(t) = \bar{e}(t) + O(\varepsilon) \tag{15}$$

holds for all $t \in [0, T]$ *and there exists a time* $t_1 \geq 0$, $O(\varepsilon \ln(1/\varepsilon))$, *such that*

$$s(t) = \bar{s}(t) + O(\varepsilon), \tag{16}$$

where $\bar{s}(t) := \phi(\bar{e}(t))$, *holds for all* $t \in [t_1, T]$.

Box 6: Model Reduction

As discussed in Box 4, Condition 3 is satisfied for all values of $e \in (0, +\infty)$, or equivalently $x \in (0, +\infty)$, if and only if $\tilde{I} < 1$. Suppose that this is so and define $A := (0, +\infty)$. Then, for any $x \in A$, the non-dimensionalised model (12) has the unique root

$$\phi_1(x) = \frac{\tilde{I}}{1-\tilde{I}}, \quad \phi_2(x) = \phi_3(x) = \frac{\tilde{I}}{\frac{\hat{e}}{e_N}x + 1 - \tilde{I}}, \quad \phi_4(x) = \frac{\tilde{I}}{\frac{e_N}{\hat{e}}\frac{1}{x} + 1 - \tilde{I}}.$$

Thus, the reduced model is given by

$$\dot{\bar{x}} = \frac{k_0}{k_0 + k_1} + \frac{k_1}{k_0 + k_1}\sigma^*(\phi_3(\bar{x})) - \bar{x}, \qquad \bar{z} = \phi(\bar{x}). \tag{17}$$

To satisfy the premise of Theorem 1, and thus justify the reduction, all that remains to be done is to find a compact subset of A that is forward invariant with respect to (17). Given that $\sigma(x)^* \in [0, 1)$ for all $x \in [0, +\infty)$ we have that

$$\frac{k_0}{k_0 + k_1} - \bar{x} \leq \dot{\bar{x}} \leq 1 - \bar{x}. \tag{18}$$

From the above it is straightforward to see that $[\frac{k_0}{k_0+k_1}, 1]$ is a compact subset of A that is forward invariant with respect to (17). Suppose that $x_0 \in [\frac{k_0}{k_0+k_1}, 1]$, or, equivalently, $e_0 \in [\frac{k_0}{\gamma}, \frac{k_0+k_1}{\gamma}]$. Then, using the substitutions in (11), Theorem 1

implies that the norm of the difference between the enzyme trajectory of the our original model (6), (7) and that of the reduced model (17) will be of order $0.037n$M and that, after a short period of time (of order $1.3ms$), the norm of the difference between metabolite trajectory of both models will be of order $0.69n$M, see Fig. 6.

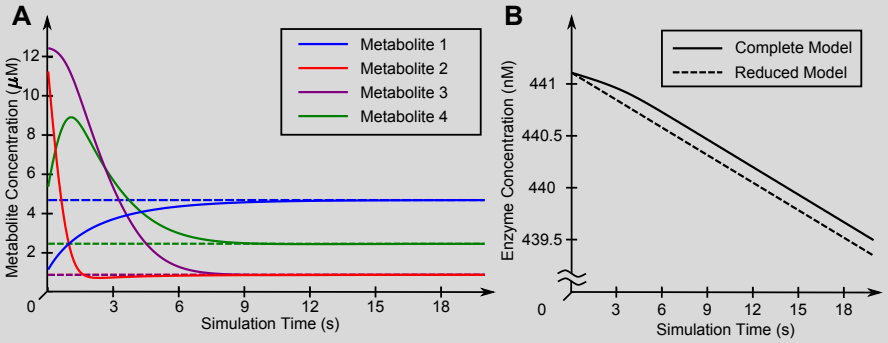

Figure 6: Model reduction. The plots were generated using Matlab and show the first few seconds of a simulation of a single trajectory of both the original and reduced models, (17) and (12), respectively. These were generated using $I_1 = \frac{1}{2}k_{cat}e_N$ (thus $\tilde{I}_1 = \frac{I}{k_{cat}e_N} = 1/2 < 1$). (**A**) The trajectory of the metabolites of the complete model (solid lines) converges rapidly to that of the reduced model (dashed lines). (**B**) The trajectory of enzyme of the complete model remains a fraction of a nano molar away from that of the reduced model.

The main benefit of carrying out this reduction, is that it can often be considerably easier to extract analytical results from the lower dimensional reduced model than from the higher dimensional original model. This is particularly obvious in our example given that in Box 6 we reduced a 5 dimensional model to a 1 dimensional model.

Box 7: Global stability of the reduced model

The dynamics of the reduced system (17), $g(\bar{x},\phi(\bar{x}))$, is a strictly decreasing function of $\bar{x}$. This follows from the fact that ϕ_3 is a decreasing function of its argument while σ^* is an increasing function of its argument. So $\sigma^*(\phi_3(x))$ is a decreasing function of x. In addition, due to inequality (18), $g(0,\phi(0)) \geq \frac{k_0}{k_0+k_1} > 0$ and $g(1,\phi(1)) \leq 0$. This, together with the fact that $g(\phi(x),x)$ is a continuous function of x implies that the reduced model has a unique equilibrium $\bar{x}_{eq} \in [0,1]$. Lastly, the reduced model is a 1 dimensional system, hence, the fact that $g(\bar{x},\phi(\bar{x}))$ is strictly decreasing in $\bar{x}$, implies that the unique equilibrium is globally asymptotically stable, see Fig. 7.

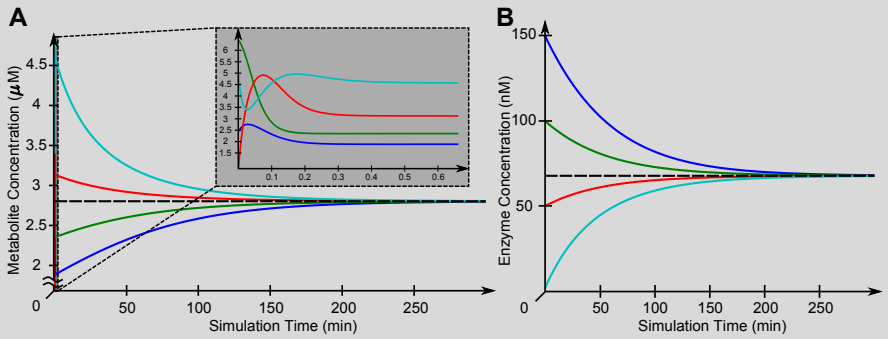

Figure 7: Global stability. (**A**) Concentration of metabolite 3 versus time. (**A Inset**) After a rapid transient, the initial metabolite concentrations become irrelevant; the metabolites quickly reach their quasi-steady state that depends exclusively on the value of the enzyme concentrations. (**B**) Concentration of the branch enzyme versus time. Four trajectories with different initial conditions are plotted alongside the equilibrium (black, dashed). All trajectories converge to the equilibrium. If the initial enzyme concentration is higher than its equilibrium value (as it is the case for the dark blue and green trajectories), the branch drains resources away from the native pathway depleting the concentration of metabolite 2 and, as a consequence, that of metabolite 3 too. The drop in concentration of 3 is detected by the genetic controller and the expression of the branch enzyme is repressed. This causes the enzyme concentration to return back to its equilibrium level, and that of metabolites 2 and 3 to return back to theirs.

In conclusion, such a controller architecture ensures that the network has a unique steady state to which the concentrations of the metabolites and of the enzyme always tend to, regardless of initial their values. In addition, by modifying the promoter parameters (in particular, the basal expression k_0 and promoter strength k_1) one can move the steady state to a more desirable location (e.g., maximise the steady state concentrations of metabolite 4 while keeping that of metabolite 3 above a prescribed minimum value). It is also worth mentioning, that one can replicate the above analysis to design a controller for branched metabolic networks with arbitrarily long main pathway and branch.

4 Discussion

In this chapter, we exploited the discrepancy in the speeds at which metabolic reactions and gene expression occur to justify the reduction of genetic-metabolic networks via time scale separation. If applicable, time scale separation reduces a model with n 'fast' variables and m 'slow' variables to one with just the m 'slow' variables. Such a model reduction can have strong benefits with regards to obtaining analytical results on the model (e.g., see (Oyarzún et al, 2012; Baldazzi et al, 2012)).

The framework we use to describe genetic-metabolic systems is flexible. The assumptions made on the enzyme kinetics are minimal and are satisfied by a wide collection of kinetics models employed in the literature (Cornish-Bowden, 2004). Furthermore, we make few assumptions regarding the ODEs describing the enzyme dynamics. Thus, we allow for just about any model of enzyme dynamics presented in the literature, with the notable exception of switch like models occasionally employed, e.g., (Oyarzún et al, 2012). However, our framework has some important drawbacks that can seriously limit the applicability of Theorem 1.

First, we deal only with enzymatic reactions, i.e., reactions catalysed by an enzyme. Although many reactions involved in cellular metabolism are enzymatic reactions (Cornish-Bowden, 2004), there are also some that are not. This is a not a serious issue, one may simply not define an enzyme for the non-enzymatic reactions. Once this is done, one can follow the same approach as the one presented in this chapter, to obtain similar results regarding the validity of time scale separation for the metabolic networks with both enzymatic and non-enzymatic reactions.

Implicit in our framework is the assumption that each reaction has a single reactant. One could potentially include reactions with multiple reactants by following the example set by Jackson, Horn and Feinberg and in their work on chemical reaction network theory (CNRT) (Feinberg, 1987). They introduce the idea of chemical complexes, separate from chemical species (what we refer to as 'metabolites'). For example, if one has the reaction $A + 2B \to C$, A, B and C are the chemical species involved in the reaction and $A + 2B$ and C are the chemical complexes.

Another subtle but important issue is that the enzyme kinetics our framework is aimed for (e.g., Michaelis Menten, Hill type functions, etc.) are, themselves, the outcome of a previous reduction involving a quasi-steady state approximation. Key to these reductions is the assumption that the enzyme concentrations are constant. Although this is not the case in the type of models we are examining, where the enzyme concentrations are modelled as dynamic variables, there has recently been some headway in showing that these reductions are also valid if the enzyme concentrations vary, see (Kumar and Josić, 2011).

The applicability of our results to the class of genetic-metabolic systems we consider has two main limitations. The first is that to carry out the reduction, one must show that the premise of Theorem 1 is satisfied. The second is that our results are only applicable to acyclic networks, i.e., networks that satisfy Condition 1. The former is not as much of a hindrance as one expects it to be; the enzyme dynamics, often, are such that the premise of Theorem 1 is not hard to satisfy. The later is more serious, in particular because it rules out networks with reversible reactions. However, one can build on our current result to construct a more general one for the case of certain non-acyclic networks, e.g., ones that include reversible reactions.

To apply our result, one must first be able to find a compact subset of the set of all enzyme concentrations such that Condition 3 is satisfied, that is forward invariant with respect to the reduced model (14). Often, in models for enzyme dynamics, the differential equation describing the evolution of an individual enzyme is coupled to the metabolites and other enzymes via saturable functions (Oyarzún and Stan, 2013, 2012; Baldazzi et al, 2012). Hence, one can often extract certain differential inequal-

ities regarding the time evolution of individual enzymes that are decoupled from the other metabolites and enzymes. These can then be used to find the desired forward invariant regions. Indeed, this is exactly what we did in our example network, see Box 6.

The requirement that the network must be acyclic, i.e., that it satisfies Condition (1), is a limitation. This is especially true because it rules out networks with reversible reactions. However, if one is willing to impose some more conservative inequalities than those in Condition 3, it is straightforward to extend the result to a significantly more general class of networks.

Our proof for the acyclic case consists of showing that the metabolite dynamics, $\dot{s} = f(s,e)$, are such that the premise of Tikhonov's Theorem (Theorem 2) is satisfied. In particular, we show that for any fixed enzyme vector $e \in \mathbb{R}^m_{>0}$ the system $\dot{s} = f(s,e)$ has globally asymptotically stable equilibrium. To do this, we use the fact that the network is acyclic to decompose the system $\dot{s} = f(s,e)$ into a series of interconnected 1 dimensional subsystems, or blocks, such that the input the i^{th} subsystem comes only from the previous $i-1$ systems. We then prove certain properties about these subsystems (essentially that they are *converging input converging state* (CICS)) and use these to establish properties about the complete system required to satisfy the theorem's premise. However, there is no reason why to only use 1 dimensional subsystems other than that it is easier to show that they are CICS. If one can show that larger blocks, e.g., a 2 dimensional block representing a reversible reaction, are also CICS then the result would be almost immediate for 'block-acyclic' networks containing a mixture of 1 dimensional irreversible reaction blocks and larger blocks. Indeed, by imposing stronger inequalities than those in Condition 3, it is straightforward to show that much more general blocks are CICS, e.g., chains of reversible reactions and loops of irreversible reactions. However, to simplify this exposition we limit ourselves to the acyclic case. Strictly speaking, to satisfy the premise of Tikhonov's Theorem, one must also show that the eigenvalues of the Jacobian of $\dot{s} = f(s,e)$ all have negative real parts. This can be done easily because the network being acyclic implies that the Jacobian is triangular. If one considers a block acyclic network then the Jacobian will be block triangular. All that one needs to show in this case is that the eigenvalues of the Jacobian of each of the blocks have negative real parts.

An interesting alternative would be to attempt to use the existing CNRT machinery, specifically the Deficiency Zero Theorem (Feinberg, 1987), to re-derive and potentially extend our results, at least to networks with mass-action kinetics.

Acknowledgements We thank Aivar Sootla for very useful discussions about various topics described in this chapter and Alexandros Houssein and Keshava Murthy for their valuable advice regarding how to improve this script.

Appendices

In the appendices we assume that the reader has some familiarity with non-linear systems theory. Specifically, we assume that the reader is comfortable with the various notions of stability of equilibria, Lyapunov functions and the existence and uniqueness results. If not, we refer the reader to the excellent text (Khalil, 2002).

We begin by presenting Tikhonov's Theorem over finite time intervals and some related results. Next, we discuss the notion of converging input converging state systems. Lastly, we employ the previous two to prove Lemmas 1 and 2 and Theorem 1.

Throughout the appendices we use $||\cdot||$ to denote any vector norm.

A: Tikhonov's Theorem

As discussed in the main text, a method for dimensionality reduction of non-linear systems is time scale separation. This is applicable in systems whose state variables exhibit large differences in the 'speed' of their time responses. Core to time scale separation is the following result first proved by Tikhonov 60 years ago (Tikhonov, 1948), (Tikhonov, 1952). The version of it presented here is not the original version by Tikhonov, but instead the version published by Vasil'eva in 1963, which we find easier to work with.

Theorem 2 ((Vasil'eva, 1963; Kokotovic et al, 1986)). *Let $f : \mathbb{R}^n \times \mathbb{R}^m \times \to \mathbb{R}^n$ and $g : \mathbb{R}^n \times \mathbb{R}^m \to \mathbb{R}^m$ both be smooth functions. Consider the system*

$$\varepsilon \dot{z}(t) = f(x(t), z(t)), \quad z(0) = z_0, \quad z \in \mathbb{R}^n, \tag{19a}$$

$$\dot{x}(t) = g(x(t), z(t)), \quad x(0) = x_0, \quad x \in \mathbb{R}^m, \tag{19b}$$

where $\varepsilon > 0$. Assume for all $t \in [0,T]$ where $T \in \mathbb{R}_{\geq 0}$ that (19) *has the unique solutions $x(t), z(t)$. Consider the following conditions:*

1. *There exists a unique function $\phi(\cdot)$ such that $g(\bar{x}(t), \phi(\bar{x}(t))) = 0$ for all $t \in [0,T]$ where $\bar{x}(t)$ denotes the unique solution over $[0,T]$ of the* reduced system $\dot{\bar{x}} = g(\bar{x}, \phi(\bar{x})), \quad x(0) = x_0$.
2. *Consider the 'boundary layer' system*

$$\frac{d\hat{z}}{d\tau}(\tau) = f(x_0, \hat{z}(\tau) + \phi(x_0)). \tag{20}$$

   Assume that the equilibrium $\hat{z} = 0$ of (20) *is globally asymptotically stable, uniformly in x_0.*
3. *The eigenvalues of $\left[\frac{\partial f}{\partial z}(\cdot)\right]$ evaluated along $\bar{x}(t)$, $\bar{z}(t)$, have real parts smaller than a fixed negative number, i.e.,*

$$Re\left(\lambda_i\left(\left[\frac{\partial f}{\partial z}\right](\bar{x}(t), \bar{z}(t))\right)\right) \leq -c, \quad c \in \mathbb{R}_{>0}, \quad \forall i, \quad \forall t \geq 0.$$

where $Re(a)$ denotes the real part of $a \in \mathbb{C}$ and $\lambda_i(A)$ denotes the i^{th} eigenvalue of $A \in \mathbb{R}^{n\times n}$.

If the three conditions above are satisfied, then relations (21) *and* (22) *hold for all $t \in [0,T]$ and there exists a time $t_1 \geq 0$, $O(\varepsilon \ln(1/\varepsilon))$, such that* (23) *holds for all $t \in [t_1,T]$.*

$$x(t) = \bar{x}(t) + O(\varepsilon). \tag{21}$$

$$z(t) = \bar{z}(t) + \hat{z}(t) + O(\varepsilon). \tag{22}$$

$$z(t) = \bar{z}(t) + O(\varepsilon). \tag{23}$$

The Theorem's first condition ensures that there exists a well defined reduced model. The second condition verifies that, initially, the trajectory of the complete system rapidly converges to the one of the reduced system. The third condition guarantees that after the initial transient dies out the trajectory of the complete system remains close to the that of the reduced system. It is worth mentioning, that the above is Tikhonov's theorem restricted to the special case when the systems are time invariant and (19a) has a unique root. For an excellent treatment of Tikhonov's theorem (including its most general form) and its applications in control theory see (Kokotovic et al, 1986).

In verifying the theorem's last two conditions the following two lemmas will be useful.

Lemma 3 ((Tikhonov, 1952)). *Consider the boundary system* (20). *Assume that f and the root ϕ are continuous functions and that $x_0 \in \mathscr{P}$ where $\mathscr{P}$ is a compact subset of $\mathbb{R}^m$. Suppose that for all $x_0 \in \mathscr{P}$, the origin of* (20) *is globally asymptotically stable. Then the origin of* (20) *is globally asymptotically stable, uniformly in x_0.*

Lemma 4. *Consider $f(\cdot)$ in* (19). *Let A be a compact subset of $\mathbb{R}^{n+m}$ and suppose that*

$$Re\left(\lambda_i\left(\left[\frac{\partial f}{\partial z}\right](x,z)\right)\right) < 0, \quad \forall i, \quad \forall \left[x,z\right]^T \in A.$$

Then, there exists a $c \in \mathbb{R}_{>0}$ such that

$$Re\left(\lambda_i\left(\left[\frac{\partial f}{\partial z}\right](x,z)\right)\right) \leq -c, \quad \forall \begin{bmatrix} x \\ z \end{bmatrix}^T \in A.$$

Proof. First, we show that

$$\lambda^*(x,z) := \max_i \left(\lambda_i\left(\left[\frac{\partial f}{\partial z}\right](x,z)\right)\right), \tag{24}$$

that is, the maximum real part of the eigenvalues of the Jacobian, is a continuous function of x and z.

The eigenvalues are the roots of the characteristic polynomial of the Jacobian (i.e., the solutions to $\det\left(\lambda I - \left[\frac{\partial f}{\partial z}\right](x,z)\right) = 0$ where $\lambda \in \mathbb{C}$). The roots of a polynomial depend continuously on the coefficients of a polynomial. The coefficients of the characteristic polynomial of the Jacobian above depend continuously of the entries of the Jacobian. The entries of the Jacobian are continuous functions of x and z. The composition of two continuous functions is also a continuous function. Thus, the eigenvalues of the Jacobian are continuous functions of x and z. Thus, (24) is a continuous function of x and z.

The supremum of a continuous function over a compact set is achieved by an element in the set. This fact and the lemma's premise imply that $\sup_{[x,z]^T \in A} \lambda^*(x,z) < 0$ which completes the proof. □

B: Converging input converging state systems

In Appendix C, we need to prove that the unique equilibrium of the network with the enzyme concentrations fixed in time (system (8)) is globally asymptotically stable (GAS). To accomplish this we exploit the acyclycity of the network to break system (8) down into n one dimensional subsystems and study how they interact. To this end, we introduce the notions of *converging input bounded state* (CIBS) and *converging input converging state* (CICS) systems. These were original presented in (Sontag, 1989) and relate to other more well known concepts such as *input to state stable* (ISS) systems.

Definition 1. We say that $u(\cdot)$ is an *input* if it is a continuous function that maps from $\mathbb{R}_{\geq 0}$ to $\mathbb{R}^m$.

Now, consider the non-autonomous system

$$\dot{x}(t) = f(x(t), u(t)), \tag{25}$$

where $f(\cdot)$ is continuous, $x \in \mathbb{R}^n$ and $u(\cdot)$ is an input. In addition, consider the same system with 'zero input'

$$\dot{x}(t) = f(x(t), 0). \tag{26}$$

Definition 2. System (25) is said to be converging input bounded state (CIBS) if for any input $u(\cdot)$ such that $u(t) \to 0$ as $t \to +\infty$ and for any initial conditions $x_0 \in \mathbb{R}^n$, the solution exists for all $t \geq 0$ and is bounded.

Definition 3. System (25) is said to be converging input converging state (CICS) if for any input $u(\cdot)$ such that $u(t) \to 0$ as $t \to +\infty$ and for any initial conditions $x_0 \in \mathbb{R}^n$, the solution exists for all $t \geq 0$ and converges to 0.

Lemma 5. *Assume that for any input, $x(t)$ exists for all $t \geq 0$. Let $V : \mathbb{R}^n \to \mathbb{R}$ be $\mathscr{C}^1$, bounded from below and radially unbounded (i.e., $||x|| \to +\infty \Rightarrow V(x) \to +\infty$). If there exists constants $\alpha > 0$ and $\beta > 0$ such that*

$$\dot{V}(x) = \frac{\partial V}{\partial x} f(x,u) \leq 0 \quad \forall (x,u) \in \mathbb{R}^{n+m} : \quad ||x|| \geq \beta, \quad ||u|| \leq \alpha,$$

then system (25) *is CIBS.*

Proof. We prove by contradiction. Assume that the premise of the Lemma is satisfied, i.e., that $||u(t)|| \to 0$ as $t \to +\infty$ and that $x(t)$ is unbounded. By our premise, $x(t)$ is defined for all $t \geq 0$. Thus, there does not exist a finite escape time, i.e., there does not exists a time $T \geq 0$ such that $||x(t)|| \to +\infty$ as $t \to T$. Thus, the fact that $x(t)$ is unbounded implies that $||x(t)|| \to +\infty$ as $t \to +\infty$.

Now, $||u(t)|| \to 0$ as $t \to +\infty$ implies that there exists a $t_1 \geq 0$ such that $\forall t \geq t_1$, $||u(t)|| \leq \alpha$. In addition, $||x(t)|| \to +\infty$ as $t \to +\infty$ implies that there exists a $t_2 \geq 0$ such that $\forall t \geq t_2$, $||x(t)|| \geq \beta$. Let $t_3 := \max\{t_1, t_2\}$. Thus, $\forall t \geq t_3$, $\dot{V}(x(t)) \leq 0$ which implies that $\forall t \geq t_3$, $V(x(t)) \leq V(x(t_3))$. This implies that $\forall t \geq t_3$, $||x(t)|| \leq ||x(t_3)|| < +\infty$. Hence, $||x(t)||$ does not tend to $+\infty$ as $t \to +\infty$. We have reached a contradiction. □

Theorem 3 ((Sontag, 1989)). *If* 0 *is a GAS equilibrium of* (26) *then CIBS and CICS are equivalent for* (25)*.*

Theorem 4 ((Sontag, 1989)). *Consider the cascade formed by system* (25) *and the autonomous system* $\dot{y} = g(y)$*,*

$$\dot{x} = f(x,y), \tag{27a}$$
$$\dot{y} = g(y), \tag{27b}$$

where g is continuous, $y \in \mathbb{R}^m$*. Assume the origin of* (27b) *is GAS and that* (25) *is CICS. Then the origin of* (27) *is GAS.*

C: Proof of the main results

We begin by demonstrating a series of results regarding the metabolic model when enzymes are kept at a fixed value. In other words, up to and including the proof of Lemma 1 we neglect the enzyme dynamics (1b) and assume $e(t) \equiv e$, where $e \in \mathbb{R}^m_{>0}$ is a constant such that Conditions 1-3 hold. In Section 3.1, we argued that if Conditions 1-3 are satisfied, the metabolic network (8) has a unique equilibrium $\bar{s}$.

We now establish global asymptotic stability of the equilibrium. To do this, instead of studying the behaviour of the whole network in one go, we examine the behaviour of individual metabolites, or individual *subsystems* first, and then using these we establish the stability property for the whole network. We call

$$\dot{x}(t) = f_1(x(t), e), \qquad x(0) = x_0 \in \mathbb{R}_{\geq 0}$$

the 1^{st} *subsystem* where f_1 is defined as in (8). Similarly, we call

$$\dot{x}(t) = f_i\left(w(t), x(t), e\right), \qquad x(0) = x_0 \in \mathbb{R}^{i-1}_{\geq 0}$$

the i^{th} *subsystem*[1] where $w : \mathbb{R}_{\geq 0} \to \mathbb{R}^{i-1}_{\geq 0}$ plays the role o an input and f_i is defined as in (8) for $i = 2, \ldots, n$. Note that, given that the domain of f_i, with $i = 2, \ldots, n$, is $\mathbb{R}^{i-1}_{\geq 0} \times \mathbb{R}_{\geq 0} \times \mathbb{R}_{\geq 0}$ (the reaction rates, $v_{j \to i}$ are only defined for non-negative arguments, i.e., the metabolite concentrations must be non-negative), it is important that the range of w is $\mathbb{R}^{i-1}_{\geq 0}$ instead of $\mathbb{R}^{i-1}$. For this reason, if we want to employ the CICS machinery introduced in Appendix 2, we first must alter slightly our definition of an input $u(\cdot)$ (Definition 1, Appendix B).

Definition 4. We say that $u(\cdot)$ is an input to the system $\dot{x} = f(x,u)$, $f : A \times B \to \mathbb{R}^n$ where $A \times B \subset \mathbb{R}^n \times \mathbb{R}^m$, if it is a continuous function that maps from $\mathbb{R}_{\geq 0}$ to B.

It can be shown, in a similar manner as in Appendix B and (Sontag, 1989), that Lemma 5 and Theorems 3 and 4 hold if one replaces the original definition of an input (Definition 1) with the one above (Definition 4) and $x_0 \in \mathbb{R}^n$ with $x_0 \in A$.

Returning to our original problem, it is convenient to introduce the change of coordinates $z := x - \bar{s}$ and $u(\cdot) := w(\cdot) - \bar{s}^i$ where $\bar{s}^i := \begin{bmatrix} \bar{s}_1 & \ldots & \bar{s}_{i-1} \end{bmatrix}^T$ for $i = 2, \ldots, n$. Then, we can re-write the 1^{st} subsystem as

$$\dot{z}(t) = f_1\left(z(t) + \bar{s}_1, e\right), \qquad z(0) = z_0 \in [-\bar{s}_1, +\infty).$$

and the i^{th} subsystem

$$\dot{z}(t) = f_i\left(u(t) + \bar{s}^i, z(t) + \bar{s}_i, e\right), \qquad z(0) = z_0 \in [-\bar{s}_i, +\infty). \tag{28}$$

for $i = 2, \ldots, n$. In addition, from now onwards we will say an input $u(\cdot)$ meaning an input to the i^{th} subsystem (28) in the sense of Definition 4.

Proposition 1. *For any input given $u(\cdot)$, then the i^{th} subsystem has a unique, continuous solution $z(t) \in [-s_i, +\infty)$ for all $t \geq 0$.*

Proof. Each component of $f(\cdot)$ is a linear combination of globally Lipschitz continuous functions (Assumptions 1 and 2), hence $f(\cdot)$ is globally Lipschitz continuous as well. This and the definition of $u(\cdot)$ (which implies that it is a continuous function of t), ensure that the i^{th} subsystem, $\dot{z} = f_i\left(u(t) + \bar{s}^i, z(t) + \bar{s}_i, e\right)$, satisfies the usual conditions for global existence of solutions of time varying systems. Hence the i^{th} subsystem has a unique, continuous solution $z(t)$ that exists for all $t \geq 0$. Then, due to the positive definiteness of the $g_i s$ and $h_i s$

$$z = -\bar{s}_1 \Rightarrow \dot{z} = f_1\left(0, e\right) = I_1 - h_1(0, e) = I_1 \geq 0$$

which proves that $z(t) \in [-s_1, +\infty)$ for all $t \geq 0$ were $z(t)$ is the solution of the 1^{st} subsystem, and

[1] Here we are abusing slightly our notation by writing the first $i-1$ scalar arguments of f_i as a single $i-1$ dimensional vector argument.

$$z = -\bar{s}_i \Rightarrow \dot{z} = f_i\left(u(t)+\vec{s}^i, 0, e\right) = g_i\left(u(t)+\vec{s}^i, e\right) - h_i(0,e) = g_i\left(u(t)+\vec{s}^i, e\right) \geq 0$$

which proves that $z(t) \in [-s_i, +\infty)$ for all $t \geq 0$ were $z(t)$ is the solution of the i^{th} subsystem, $i = 2, \ldots, n$. □

Proposition 1 is important for two reasons. First, it allows us to regard the state space of i^{th} subsystem, (28), to be $[-\bar{s}_i, +\infty)$ instead of $\mathbb{R}$. This makes sense, we are only interested in non-negative concentrations of the metabolites. Second, it shows that the vector containing the state of the first $i-1$ subsystems is input to the i^{th} subsystem, in the sense of Definition 4.

Proposition 2. *The i^{th} subsystem is CIBS, for any $i = 2, \ldots, n$.*

Proof. Let $V : \mathbb{R}_{\geq 0} \to \mathbb{R}_{\geq 0}$ be defined as

$$V(z) := \frac{1}{2}z^2 \Rightarrow \dot{V}(z) = \frac{\partial V}{\partial z}\dot{z} = z\dot{z} = z\left(g_i\left(u+\vec{s}^i, e\right) - h_i\left(z+\bar{s}_i, e\right)\right).$$

By Condition 3, $\hat{h}_i(e) > g_i(\vec{s}^i, e)$ thus $\hat{h}_i(e) \geq g_i(\vec{s}^i, e) + \delta_1$, for some $\delta_1 > 0$. In addition, by continuity and monotonicity of g_i (monotonicity in each of its arguments), there exists a sufficiently small $\alpha > 0$ such that

$$g_i\left(\alpha\mathbb{1}+\vec{s}^i, e\right) - g_i\left(\vec{s}^i, e\right) \leq \frac{\delta_1}{2},$$

where $\mathbb{1} := \begin{bmatrix}1 & \ldots & 1\end{bmatrix}^T$. In addition,

$$||u||_\infty \leq \alpha \Rightarrow g_i\left(u+\vec{s}^i, e\right) \leq g_i\left(\alpha\mathbb{1}+\vec{s}^i, e\right) \leq g_i\left(\vec{s}^i, e\right) + \frac{\delta_1}{2} \leq \hat{h}_i(e) - \frac{\delta_1}{2}.$$

Hence, we have

$$||u||_\infty \leq \alpha \Rightarrow g_i\left(u+\vec{s}^i, e\right) - h_i\left(z+\bar{s}_i, e\right) \leq \hat{h}_i(e) - \frac{\delta_1}{2} - h_i(z+\bar{s}_i, e).$$

Because $h_i(z+\bar{s}_i, e) \to \hat{h}_i(e)$ from below as $z \to +\infty$ we can always find a β_1 such that $z \geq \beta_1 \Rightarrow \hat{h}_i(e) - h_i(z+\bar{s}_i, e) \leq \delta_2$ for any $\delta_2 > 0$. In addition, because $z \in [-\bar{s}_i, +\infty)$, $||z|| > \bar{s}_i$ implies $||z|| = z$. Hence, choosing $\delta_2 \leq \frac{\delta_1}{2}$ and defining $\beta := \max\{\beta_1, \bar{s}_i + \varepsilon\}$, where $\varepsilon > 0$, we have

$$u, z : ||u||_\infty \leq \alpha, ||z|| \geq \beta \Rightarrow \dot{V}(z) \leq z(\hat{h}_i(e) - h(\hat{s}_i, e) - \frac{\delta_1}{2}) \leq z(\delta_2 - \frac{\delta_1}{2}) \leq 0$$

Then, applying Lemma 5 completes the proof. □

Proposition 3. *The origin of i^{th} subsystem with zero input (i.e., $u(t) \equiv 0$) is a globally asymptotically stable equilibrium, for any $i = 1, \ldots, n$.*

Proof. We use the Lyapunov function

$$V(z) := \frac{1}{2}z^2 \Rightarrow \dot{V}(z) = \frac{\partial V}{\partial z}\dot{z} = z f_i(u(t)+\bar{s}^i, z(t)+\bar{s}_i, e) = z(g_i(\bar{s}_1,\ldots,\bar{s}_{i-1},e) - h_i(z+\bar{s}_i,e)).$$

By the definition of $\bar{s}$, we have that $g_i(\bar{s}_1,\ldots,\bar{s}_{i-1},e) = h_i(\bar{s}_i,e)$. So

$$\dot{V}(z) = z(h_i(\bar{s}_i,e) - h_i(z+\bar{s}_i,e)).$$

Due to the strict monotonicity of h_i, z and $(h_i(\bar{s}_i,e) - h_i(z+\bar{s}_i,e))$ have opposite signs and are both equal to zero if and only if $z = 0$. Hence, applying Lyapunov's Direct Method completes the proof. □

Proposition 4. *The i^{th} subsystem is CICS, for any $i = 1,\ldots,n$.*

Proof. This follows directly from Propositions 2 and 3 and Theorem 3. □

With these preliminary results in mind, we are now ready to prove Lemma 1.

Proof (Lemma 1). As previously pointed out, the solution to the first subsystem is an input to the second subsystem, in the sense of Definition 4. Consider the cascade obtained by setting the input of the 2^{nd} subsystem to the state of the 1^{st} subsystem,

$$\dot{z}_1(t) = f_1\left(z_1(t)+\bar{s}_1, e\right),$$

$$\dot{z}_2(t) = f_2\left(z_1(t)+\bar{s}_1, z_2(t)+\bar{s}_2, e\right).$$

Propositions 3 (i.e., the origin of the 1^{st} subsystem is a GAS equilibrium) and 4 (i.e., the 2^{nd} subsystem is CICS) and Theorem 4 (i.e., that the origin of the interconnection of an autonomous system which has a GAS equilibrium at the origin and a CICS system is a GAS equilibrium) imply that the origin of the above cascade is GAS. Then, by induction, we see that the origin of the system obtained by iteratively cascading the i^{th} subsystem with the cascade formed by the previous $i-1$ subsystems is a GAS equilibrium. In other words, the origin of

$$\dot{z} = f(z+\bar{s}, e)$$

is a GAS equilibrium, which completes the proof. □

Proposition 5. *Let A denote the subset of $\mathbb{R}^m_{>0}$ whose elements are such that Condition 3 holds. There exists a unique function $\phi : A \to \mathbb{R}^n_{>0}$ such that $f(\phi(e),e) = 0$ for all $e \in A$. Furthermore, this function is continuously differentiable and globally Lipschitz continuous.*

Proof. Existence and uniqueness of ϕ follows from our discussion in Section 3.1 of the main text regarding the existence and uniqueness of an equilibrium if the enzymes are constant. Each component of $f(\cdot)$ is a linear combination of continuously differentiable and globally Lipschitz continuous functions (Assumptions 1 and 2). Thus, $f(\cdot)$ is continuously differentiable and globally Lipschitz continuous or, equivalently its partial derivatives exist everywhere and are bounded. The fact that $f(\phi(e),e) = 0$ for all $e \in A$ implies that the total derivative of $f(\cdot)$ along

$\left[\phi(e)\ e\right]^T$ is also equal to zero, i.e., $f'(\phi(e),e)=0$ for all $e\in A$. The total derivative of a function exists and is continuous if and only if the partial derivatives of the function exist and are continuous. Hence,

$$\frac{\partial f}{\partial \phi}(\phi(e),e)\frac{\partial \phi}{\partial e}(e)+\frac{\partial f}{\partial e}(\phi(e),e)=0$$

which implies that

$$\frac{\partial \phi}{\partial e}(e)=-\left(\frac{\partial f}{\partial \phi}(\phi(e),e)\right)^{-1}\frac{\partial f}{\partial e}(\phi(e),e).$$

By Condition 1, $v_{j\to i}\equiv 0$ if $i<j$. Hence, $i<j\Rightarrow \frac{\partial f_i}{\partial \phi_j}(\phi(e),e)=\frac{\partial v_{j\to i}}{\partial \phi_j}(\phi_j(e),e)=0$. Thus, $\frac{\partial f}{\partial \phi}(\phi(e),e)$ is lower triangular. Furthermore, by Condition 2, h_i is strictly increasing, hence

$$\frac{\partial f_i}{\partial \phi_i}(\phi(e),e)=-\frac{\partial h_i}{\partial \phi_i}(\phi_i(e),e)<0.$$

Thus, $\left(\frac{\partial f}{\partial \phi}(\phi(e),e)\right)^{-1}$ exists for all $e\in A$. Hence, $\frac{\partial \phi}{\partial e}(e)$ exists for all $e\in A$. Furthermore, $\frac{\partial \phi}{\partial e}(e)$ is continuous and bounded which shows that ϕ is continuously differentiable and globally Lipschitz continuous. □

We are now in a position to prove Lemma 2 and Theorem 1.

Proof (Lemma 2). The existence and uniqueness of $s(t)$ and $e(t)$ follow from our assumption that $f(\cdot)$ and $g(\cdot)$ are smooth and globally Lipschitz continuous (Assumptions 1 - 3). The existence and uniqueness of $\phi(\cdot)$ is proven in Proposition 5. The domain of $\phi(\cdot)$ is A. Thus, (14) is well-defined if and only if $\bar{e}(t)$ remains in A. This is ensured by the premise, $B\subseteq A$ is forward invariant with respect to (14) and $e_0\in B$. In addition, $g(\cdot)$ and $\phi(\cdot)$ are globally Lipschitz continuous (Assumption 3, Proposition 5, respectively), which implies that (14) satisfies the usual conditions for global existence and uniqueness solutions. □

Proof (Theorem 1). The proof is an application of Tikhonov's Theorem on finite time intervals (Theorem 2). The existence and uniqueness of $\phi(\cdot)$ satisfies the first condition in the premise of Theorem 2 which requires that the metabolite dynamics, $f(s,e)$, has a unique root.

The second condition of Tikhonov's Theorem is that $z=\phi(e_0)$ is a globally asymptotically stable equilibrium, uniformly in e_0, of the boundary layer system $\dot{z}=f(z,e_0)$. Lemma 1 shows that for any given $e_0\in B\subseteq A$, $\phi(e_0)$ is a globally asymptotically stable equilibrium of $\dot{z}=f(z,e_0)$. The fact that B is compact combined with the previous statement form the premise of Lemma 3. Then, Lemma 3 establishes the desired result, i.e., that the equilibrium $z=\phi(e_0)$ is a globally asymptotically stable, uniformly in e_0.

Proposition 5 shows that $\phi(e)$ is continuous with respect to e. Because $\bar{e}(t)\in B$ for all time, and B is a compact set, $\bar{s}(t)=\phi(\bar{e}(t))$ must also be confined to some

compact set. In the proof of 5 we established that for any given $e \in B \subseteq A$, the eigenvalues of the Jacobian of the boundary layer system evaluated at $[\phi(e), e]^T$, $\frac{\partial f}{\partial \phi}(\phi(e), e)$, have negative real parts. The previous two statements form the premise of Lemma 4 which shows that the eigenvalues of the Jacobian of the boundary layer system, evaluated along $\left[\phi(\bar{e}(t))\ \bar{e}(t)\right]^T$ have real parts smaller than a certain negative real number, i.e., that the third condition of Tikhonov's theorem is satisfied. □

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