Propagation of Fluctuations in Biochemical Systems, I: Linear SSC Networks

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Abstract

We investigate the propagation of random fluctuations through biochemical networks in which the concentrations of species are large enough so that the unperturbed problem is well-described by ordinary differential equation. We characterize the behavior of variance as fluctuations propagate down chains, study the effect of side chains and feedback loops, and investigate the asymptotic behavior as one rate constant gets large. We also describe how the ideas can be applied to the study of methionine metabolism.

1 Introduction.

There are two different natural contexts in which stochastic dynamics arises in the study of biochemical reaction networks. In the first, the stochastic chemical dynamics arises from the randomness inherent in the formation and breaking of chemical bonds. This "intrinsic stochasticity" is particularly relevant when the numbers of molecules are small such as in gene transcription and small gene regulatory networks where the mean concentrations no longer faithfully model the chemical dynamics. There is a large literature in this field beginning with [4], including [12], [8], and recently exemplified by [7][13]. In this setting, one typically assumes that the reaction system is described by a Poisson process that models individual discrete chemical reactions. One then derives a partial differential equation for the time evolution of concentration densities. As all species have their own intrinsic stochasticity, this partial differential equation is parabolic with a uniformly elliptic generator.

In the second context, which is our focus here, one wants to investigate the response of a large biochemical system to external excitation. It is natural and theoretically useful to consider stochastic excitations and to study the emergent properties of the network as the random fluctuations propagate through the system. Here the randomness is a tool used to study the out-of-equilibrium dynamics of the biochemical system. In this setting, we assume that the concentrations are large enough so that the unperturbed dynamics is faithfully modeled by ordinary differential equations. Typically, one in interested in perturbing a single (or small number of) input(s) with white noise. Hence, the perturbed problem becomes a stochastic differential equation with a hypoelliptic generator.

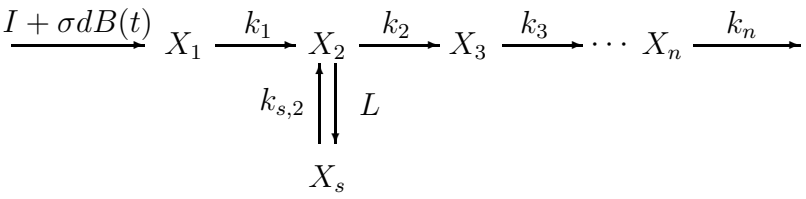
The central biological goal driving our work is to understand the behavior of biochemical systems in cells, which *in vivo* are exceptionally large and complicated. A metabolite can be the substrate for many different enzymes and participate in apparently unrelated reactions. Individual reactions usually have nonlinear kinetics catalyzed by enzymes that are themselves inhibited or excited by products or distant substrates in the network. Cells and tissues differ because the genes that code for certain enzymes have tissue specific expression patterns and biochemical substrates themselves also influence gene expression. Further, each cell's environment, its inputs and outputs, and its internal state (e.g. stage of cell cycle) are not constant but vary in time. This continual variation affects both the concentrations of substrates and the expression of genes that catalyze particular reactions. Thus, the gene-biochemical network should not be viewed as a fixed object but as one that is continuously changing.

For each signal, either external or internal, that causes a particular cell to dramatically change its operation, there are two natural questions. First, how does the gene-biochemical network respond to accomplish the change? Second, how does the network enable the cell to maintain homeostasis in all its other operations despite the change? One would like to understand the structural and kinetic principles that allow the network to accomplish both tasks simultaneously. We take two distinct approaches to this biological goal. First, we study how fluctuations propagate through relatively simple systems. We are interested in discovering how different network geometries magnify or suppress fluctuations since this may give clues to why biochemical networks look the way they do. Secondly, we apply fluctuations to *in silico* representations of specific biological networks. By observing how fluctuations propagate we can identify reactions or subsystems that are

buffered against such fluctuations, i.e. are homeostatic. Then, through *in silico* experimentation (e.g. removing particular reactions), we can take the system apart piece by piece to discover the regulatory mechanisms that give rise to the homeostasis.

In this paper, we develop fluctuation theory for chemical reaction systems for which each complex (in Feinberg's terminology, [5]) consists of a single chemical species and the kinetics are mass action. Thus the corresponding differential equations are linear, so we refer to such networks as linear SSC (single species complexes) networks. Because of the linearity, the technical difficulties involved in studying the associated stochastic processes are minimized. Thus, linear SSC systems are an excellent arena for investigating the effects of network geometry on the propagation, magnification, and suppression of fluctuations. The principles discovered then become the natural goal for generalization to nonlinear settings [2].

To see the kinds of questions we want to ask, consider a simple chain with a side branch. The chemical species are $X_1, \ldots, X_n, X_s$; the corresponding concentrations are denoted by $x_1, \ldots, x_n, x_s$.



The chain has a constant input I, which is perturbed by some random process, in this case, white noise. If the input is fluctuating, then each of the concentrations will fluctuate as will the fluxes, $k_i x_i$. Suppose the side chain is absent. Then, will the variations of the fluxes increase, decrease, or stay the same as we move down the chain? Does the answer depend on the rate constants k_i? If the side chain is present, does it affect the variances of the fluxes on the chain? If so, what is the effect of the size of L.

The chemical reaction diagram corresponds to set of differential equations for the concentrations and, similarly, the diagram with stochastic forcing corresponds to a system of stochastic differential equations (SDEs):

$$\begin{aligned} dx_1 &= (I - k_1 x_1)dt + \sigma dB(t) \\ \dot{x}_2 &= k_1 x_1 - L x_2 - k_2 x_2 + k_{s,2} x_s \\ \dot{x}_3 &= k_2 x_2 - k_3 x_3 \\ &\vdots \end{aligned}$$

These SDEs in turn give rise to a stochastic process on the state space $\mathbb{R}^{n+1}$. We prove that this stochastic process has a unique stationary measure. Intuitively, this means that at large times the joint distribution of values of the concentrations becomes independent of the initial condition and independent of time. That is, the statistics converge to an equilibrium distribution. The variances of the concentrations referred to above are the variances of the marginal distributions of this measure. We prove the existence of the stationary measure for linear SSC systems in Section 2.2. In Section 3 we study the propagation of fluctuations in chains. In Section 4 we study the effects of side reaction

systems, and feedback loops. In Section 5 we ask what happens to variances in the asymptotic limit as one of the rate constants goes to ∞, corresponding to a very fast reaction. In Section 6 we show how to use the fluctuation theory ideas to investigate methionine metabolism.

It is important to note that our, goals, methods and results are different from those in classical biochemical control theory [11],[3],[9],[21]. In that theory one takes a system at a fixed steady state, makes a small perturbation in a parameter (perhaps an input), and allows the system to relax to a new steady state. By comparing the new value of a variable (a concentration or flux) to the old value, one computes the percentage change of the variable per unit percentage change in the parameter. Technically, one is computing a partial derivative. This kind of sensitivity analysis gives good information about local, linearized behavior near the initial steady state. By contrast, we are concerned with responses to large scale fluctuations in inputs. Technically, this means computing properties of the distribution of each concentration or flux from properties of the stationary measure.

It is true that the classical biochemical control theory can be made "stochastic" in the following way. Suppose that the system has input I and is at steady-state. Consider the same system with input $I + \eta$, where η is a random variable drawn from some density. For each η we let the system relax to steady state and measure the value, v, of some concentration or flux. v is a random variable and comparing it's variance to the variance of η gives information about how much **the steady state value of** v changes as η changes. However, this modified biochemical control theory often gives completely different answers from the fluctuation theory that we are developing and the differences are biologically significant. Consider the chain (without the side chain) in the example above. If the input is $I + \eta$, then, at steady state, the flux $k_n x_n$ must equal $I + \eta$, so $Var(k_n x_n) = Var(\eta)$; thus the variance remains constant down the chain. By contrast, we will see below that in our fluctuation theory, under a variety of reasonable assumptions, that the variances of the fluxes *decrease* as one proceeds down the chain. This result is interesting from a biological point of view because it says that one way to stabilize the flux out of a chain (i.e. small variance) is to have many intervening biochemical steps between the input and the output.

2 SSC networks and the stationary measure

In this section we introduce the class of chemical reaction systems that we will study and prove the existence of a stationary measure.

2.1 SSC systems with mass actions kinetics

Throughout we use the terminology introduced by Horn, Jackson, and Feinberg [10][5]. Let m be the number of chemical species. We shall study chemical reaction systems such that each complex contains a single chemical species and refer to such systems as *SSC networks*. In the sequel, we use only the statements in Lemma 2.3.

Lemma 2.1 (Deficiency of SSC networks). *An SSC network has deficiency zero.*

Proof. Suppose the network has a single linkage class and let S denote the stoichiometric subspace. Choose any reaction in the network, $X_i \to X_j$. Here we have two complexes and one reaction vector in S. Thus, if there are no other complexes, we are done. Because the diagram has one linkage class, if there are other complexes, then there must be one, call it X_k, with an arrow to or from either X_i or X_j. This adds one complex and one dimension to S since X_k is not a linear combination of X_i and X_j. Continuing in this manner until we have exhausted all the complexes, we see that the number of complexes is one greater than $dim\{S\}$. Since there is one linkage class the deficiency of the network is zero. The case where there is more than one linkage class follows easily because the reaction vectors in different linkage classes are orthogonal. □

We will concentrate on SSC networks containing the zero complex that have one linkage class.

Lemma 2.2 (Dimension of S). *In an SSC system containing the zero complex with one linkage class, $dim\{S\} = m$.*

Proof. Since the network contains the zero complex, the number of complexes, n, is one greater than the number of species, m. If $s = dim\{S\}$, then, by Lemma 2.1, $0 = n - s - 1 = m - s$, so $s = m$. □

We assume mass action kinetics so the differential equations governing the system are linear:

$$\dot{x}(t) = Ax(t) + I, \tag{1}$$

where $A \in \mathbb{R}^{m\times m}$ and $x(t), I \in \mathbb{R}^m$. The matrix A is the matrix of rate constants for the system and the vector I represents any constant flow into the species of the system from the zero complex. Thus the components of I are non-negative. We denote the open positive orthant and its closure by $\mathbb{R}^m_{>0}$ and $\mathbb{R}^m_{\geq 0}$, respectively.

Lemma 2.3. *If a linear SSC system is weakly reversible and contains the zero complex, then*

(a) The differential equations (1) *have a unique equilibrium which is globally asymptotically stable and contained in $\mathbb{R}^m_{>0}$.*

(b) The eigenvalues of the matrix of rate constants, A, have strictly negative real parts.

(c) For all vectors $v \in \mathbb{R}^m_{\geq 0}$, we have $e^{At}v \cdot e_j \geq 0$.

Proof. Part (a) is a special case of the zero deficiency theorem [5]. Since A is the Jacobian at the equilibrium point, (b) follows from (a) and linearity. (c) holds because $\mathbb{R}^m_{\geq 0}$ is invariant under the flow of the differential equation. □

2.2 The Stationary Measure.

Consider the following weakly reversible SSC system with mass action kinetics, input vector I, and matrix of rate constants A perturbed by a mean zero, finite variance stationary stochastic process $\xi(t)$:

$$\begin{cases} \dot{x}(t) = Ax(t) + I \ + \ \xi(t) \ , \\ x(0) = x_0 \ . \end{cases} \tag{2}$$

From this definition and the stationarity of $\xi(t)$ one easily sees that (2) generates a time-homogeneous Markov process.

Theorem 2.4. *The process $x^*(t) = x^*(t, \xi)$ defined by*

$$x^*(t, \xi) = \int_{-\infty}^{t} e^{A(t-s)} I \ ds + \int_{-\infty}^{t} e^{A(t-s)} \xi_s \ ds \tag{3}$$

is a stationary solution to (2)*. Furthermore given any initial condition x_0, if $x(t, x_0, \xi)$ is a solution to equation* (2) *then $x(t, x_0, \xi)$ converges to $x^*(t, \xi)$ as $t \to \infty$ in that*

$$\mathbb{E}|x(t, x_0, \xi) - x^*(t, \xi)|^2 \to 0 \quad \textit{as} \quad t \to \infty \ .$$

Proof. Observe that for any $t, \tau \in \mathbb{R}$,

$$\begin{aligned} x^*(t+\tau) &= \int_{-\infty}^{t+\tau} e^{A(t+\tau-s)} I \ ds + \int_{-\infty}^{t+\tau} e^{A(t+\tau-s)} \xi(s) \ ds \\ &= \int_{-\infty}^{t} e^{A(t-s)} I \ ds + \int_{-\infty}^{t} e^{A(t-s)} \xi(s+\tau) \ ds \ . \end{aligned}$$

This can be written succinctly as

$$(\theta_\tau x^*)(t, \xi) = x^*(t, \theta_\tau \xi) \tag{4}$$

where the shift θ_t is defined by $(\theta_t f)(s) = f(t+s)$ for all $s, t \in \mathbb{R}$ and functions f on $\mathbb{R}$. Hence for any $t_1 \leq \cdots \leq t_n$,

$$\big(x^*(\tau + t_1, \xi), \cdots, x^*(\tau + t_n, \xi)\big) = \big(x^*(t_1, \theta_\tau \xi), \cdots, x^*(t_n, \theta_\tau \xi)\big) \ .$$

Since ξ is a stationary process, the distribution of the right hand side is independent of τ which proves that x^* is stationary. Clearly, $x^*(t, \xi)$ is a solution in that $x(t, x^*(0, \xi), \xi) = x^*(t, \xi)$.

We now turn to convergence. It follows from Lemma 2.3(b) that there are constants $\alpha, M > 0$ such that $\|e^{At}\| < Me^{-\alpha t}$ for all $t > 0$. Subtracting the solution of (2),

$$x(t, x_0, \xi) = e^{At} x_0 + \int_0^t e^{A(t-s)} I \ ds + \int_0^t e^{A(t-s)} \xi_s \ ds, \tag{5}$$

from $x^*(t)$, squaring, and taking expected values gives,

$$\begin{aligned}\mathbb{E}|x(t,x_0,\xi)-x^*(t,\xi)|^2 \leq & 3\|e^{At}\|^2|x_0|^2 + 3\mathbb{E}\left|\int_{-\infty}^0 e^{A(t-s)}Ids\right|^2 + 3\mathbb{E}\left|\int_{-\infty}^0 e^{A(t-s)}\xi_s ds\right|^2 \\ \leq & 3M^2|x_0|^2e^{-2\alpha t} + \frac{3M^2|I|^2}{\alpha^2}e^{-2\alpha t} \\ & + 3\mathbb{E}\left(\int_{-\infty}^0 \|e^{A(t-s)}\|ds\right)\left(\int_{-\infty}^0 \|e^{A(t-s)}\||\xi_s|^2 ds\right) \\ \leq & 3M^2|x_0|^2e^{-2\alpha t} + \frac{3M^2|I|^2}{\alpha^2}e^{-2\alpha t} + \frac{3M^2}{\alpha^2}e^{-2\alpha t}Var(\xi).\end{aligned}$$

Thus, $\mathbb{E}|x(t,x_0,\xi)-x^*(t,\xi)|^2 \to 0 \quad$ as $\quad t\to\infty$. □

Remark. If one takes expectations on both sides of equation (5), one sees immediately that the model is consistent in the mean, that is, the mean of the perturbed problem is equal to the solution of the unperturbed problem.

If instead of random perturbations given by the vector ξ_t we had allowed the system to be perturbed by independent white noise processes, we arrive at the following system of Itô stochastic differential equations:

$$\begin{cases} dx(t) = (Ax(t)+I)\,dt \;+\; \Sigma dB(t)\;, \\ x(0) = x_0\;, \end{cases} \tag{6}$$

where $\Sigma \in \mathbb{R}^{m\times p}$ and $B(t)$ is standard p-dimensional Brownian motion. The following theorem is proved in the same manner as Theorem 2.4.

Theorem 2.5. *The process $x^*(t) = x^*(t,B)$ defined by*

$$x^*(t,B) = \int_{-\infty}^t e^{A(t-s)}I\;ds + \int_{-\infty}^t e^{A(t-s)}\Sigma dB(s) \tag{7}$$

is a stationary solution to (6)*. Furthermore given any x_0, if $x(t,x_0,B)$ is a solution to equation* (6) *then $x(t,x_0,B)$ converges to $x^*(t,B)$ as $t\to\infty$ in that*

$$\mathbb{E}|x(t,x_0,B)-x^*(t,B)|^2 \to 0 \quad \textit{as} \quad t\to\infty\;.$$

Proof. The proof is identical to that of Theorem 2.4, except that the Itô Isometry is used to control the expected value of the square of the Itô integral term. □

Since $x^*(t)$ is stationary, the distribution of $x^*(t)$ is independent of t and invariant under the dynamics of (2) (or (6)). More precisely, defining the measure $\mu(A) = \mathbb{P}(x^*(0)\in A)$ for all measurable $A\subset\mathbb{R}^m$, we see that

$$\mu(A) = \int \mathbb{P}(x(t,y,\xi)\in A)\mu(dy)\;.$$

Furthermore, μ characterizes the longtime behavior of the solution in that the distribution of $x(t, x_0, \xi)$ converges to μ as $t \to \infty$. This follows from $\mathbb{E}|x(t, x_0, \xi) - x^*(t, \xi)|^2 \to 0$ and the fact that $\mu(A) = \mathbb{P}(x^*(t) \in A)$ for all t.

Thus μ contains information about the average, long-term behavior of fluxes and concentrations. It will be μ, therefore, which we shall probe in order to gain an understanding of how different graphical structures and asymptotic limits of biochemical reaction systems increase, decrease, and otherwise modify the exogenous fluctuations of biochemical reaction systems. Throughout the rest of this paper, it is understood that each mean or variance is computed with respect to this stationary measure.

2.3 A General Bound

We can now prove a simple general bound for the variance of the concentration of any species in an SSC system in terms of the variance of the input fluctuations. We assume that the fluctuations, ξ_t, are one-dimensional, stationary, mean zero, and finite variance. By taking the expected value in equation (3) and using that ξ_t has mean zero one sees that

$$m_i = I \int_{-\infty}^{t} e^{A(t-s)} e_1 \cdot e_i \, ds \tag{8}$$

is the mean of the i^{th} species.

Theorem 2.6. *Let $x^*(t)$ be the stationary solution of an SSC system with one input, I, to a single species, X_1, that is perturbed by a stationary stochastic process, ξ_t, with finite variance and mean zero. Then for each i,*

$$Var(x_i^*) < \left(\frac{m_i}{I}\right)^2 Var(\xi).$$

Proof. Using Lemma 2.3(c) and the Cauchy-Schwarz inequality gives

$$\begin{aligned}
Var(x_i^*(t)) &= \mathbb{E}\left(\int_{-\infty}^{t} \xi_s e^{A(t-s)} e_1 \cdot e_i \, ds\right)^2 \\
&= \mathbb{E}\left(\int_{-\infty}^{t} \xi_s \left(e^{A(t-s)} e_1 \cdot e_i\right)^{1/2} \left(e^{A(t-s)} e_1 \cdot e_i\right)^{1/2} \, ds\right)^2 \\
&< \mathbb{E}\left(\int_{-\infty}^{t} \xi_s^2 e^{A(t-s)} e_1 \cdot e_i \, ds\right)\left(\int_{-\infty}^{t} e^{A(t-s)} e_1 \cdot e_i \, ds\right) \\
&= Var(\xi)\left(\int_{-\infty}^{t} e^{A(t-s)} e_1 \cdot e_i \, ds\right)^2 \\
&= \left(\frac{m_i}{I}\right)^2 Var(\xi).
\end{aligned}$$

The strictness of the inequality follows because ξ_t is not a constant. □

This simple result is all that we need in this paper. An analogous proof works in the more general case where there are inputs to more than one species and any number of the inputs undergo independent fluctuations.

3 Chains

In this section we consider non-reversible chains with mass action kinetics:

$$0 \overset{I}{\longrightarrow} X_1 \overset{k_1}{\longrightarrow} X_2 \overset{k_2}{\longrightarrow} \cdots \overset{k_{m-2}}{\longrightarrow} X_{m-1} \overset{k_{m-1}}{\longrightarrow} X_m \overset{k_m}{\longrightarrow} 0. \tag{9}$$

Theorem 2.6 allows us to see that variances of the fluxes of the stationary solution decrease as one proceeds down the chain.

Theorem 3.1. *Let the input, I, of a non-reversible chain with mass action kinetics be perturbed by a stationary stochastic process, ξ_t, with finite variance and mean zero. Let $x^*(t)$ denote the stationary solution for the chain. Then, for all i, $Var(k_i x_i^*) < Var(\xi)$ and*

$$Var(k_{i+1}x_{i+1}^*) \;<\; Var(k_i x_i^*). \tag{10}$$

Proof. From the remark following Theorem 2.4, we know that the mean, m_i, of $x_i^*(t)$ is the equilibrium value of x_i for the unperturbed problem. For the chain this implies that $m_i = \frac{I}{k_i}$, so the bound $Var(k_i x_i^*) < Var(\xi)$ follows immediately from Theorem 2.6. To prove (10) note that the input to X_2 is

$$k_1 x_1^*(t) \;=\; I \;+\; (k_1 x_1^*(t) - I)$$

and $k_1 x_1^*(t) - I$ is a stationary stochastic process of mean zero and finite variance. Thus, by Theorem 2.6,

$$Var(k_2 x_2^*) \;<\; Var(k_1 x_1^* - I) \;=\; Var(k_1 x_1^*)\,.$$

The input to X_3 is $k_2 x_2^*(t)$, so repeating this argument down the chain proves (10). □

Note that the variances of the fluxes are strictly decreasing as one moves down the chain even though the means of the fluxes remain unchanged (i.e., equal to I). The next natural question is how much do the variances decrease down the chain? This cannot be answered without more detailed information about ξ_t. To investigate it, we will perturb the input I by white noise, $\sigma dB(t)$, which will allow us to use the Itô calculus.

Theorem 3.2. *Let $x^*(t)$ be the stationary solution of the linear chain* (9) *where the input is perturbed by white noise. We assume that the rate constants, k_i, are distinct. Then*

$$Var(x_i^*) = \sigma^2 \sum_{j=1}^{i} \sum_{r=1}^{i} p_{ij} p_{ir} \frac{1}{k_j + k_r}, \tag{11}$$

where

$$p_{ij} = \begin{cases} \left(\prod_{n=1}^{i-1} k_n\right) \Big/ \left(\prod_{n=1, l\neq j}^{i} (k_n - k_j)\right) & i \geq j \\ 0 & i < j \end{cases}. \tag{12}$$

Proof. The matrix of rate constants, A, is given by

$$A = \begin{bmatrix} -k_1 & 0 & \dots & 0 \\ k_1 & -k_2 & \dots & 0 \\ \vdots & \vdots & \ddots & \vdots \\ 0 & \dots & k_{m-1} & -k_m \end{bmatrix}.$$

Let $P = \{p_{ij}\}$. A straightforward calculation shows that the jth column of P is the eigenvector of A corresponding to eigenvalue $-k_j$. Thus, $D = P^{-1}AP$ is diagonal. In addition, P takes the vector $(1, 1, \cdots, 1)^T$ to the vector $(1, 0, \cdots, 0)^T$. Using these facts, the formula (7) for $x^*(t)$, and the Itô Isometry,

$$\begin{aligned} Var(x_i^*) &= \sigma^2 \mathbb{E}\left(\int_{-\infty}^{t} e^{A(t-s)} e_1 \cdot e_i dB_s\right)^2 \\ &= \sigma^2 \int_{-\infty}^{t} \left(P e^{D(t-s)} P^{-1} e_1 \cdot e_i\right)^2 ds \\ &= \sigma^2 \int_{-\infty}^{t} \left(P e^{D(t-s)} \begin{bmatrix} 1 \\ \vdots \\ 1 \end{bmatrix} \cdot e_i\right)^2 ds \\ &= \sigma^2 \int_{-\infty}^{t} \left(P \begin{bmatrix} e^{-k_1(t-s)} \\ \vdots \\ e^{-k_m(t-s)} \end{bmatrix} \cdot e_i\right)^2 ds \\ &= \sigma^2 \int_{-\infty}^{t} \left(\sum_{j=1}^{i} p_{ij} e^{-k_j(t-s)}\right)^2 ds \\ &= \sigma^2 \sum_{j=1}^{i} \sum_{r=1}^{i} p_{ij} p_{ir} \frac{1}{k_j + k_r}. \end{aligned}$$

□

We assumed that the k_i's were distinct so that the explicit formulas above make sense. It can be shown that the variances of the concentrations are continuous functions of the rate constants. This fact, together with the bound given by (10) allows us to conclude that formula (11) has finite limits as various subsets of the k_i's become identical.

We can use the explicit formula (11) to answer several natural questions:

Example 3.3. (Magnitude of decrease) Theorem 3.1 shows that variances of fluxes are strictly decreasing as one moves down a chain. To investigate how much they decrease, consider the chain (9) where $m = 2$ and the input is perturbed by white noise. Using (11) we see that $Var(k_1 x_1^*) = \frac{\sigma^2 k_1}{2}$ and $Var(k_2 x_2^*) = \frac{\sigma^2 k_1 k_2}{2(k_1+k_2)}$. Thus,

$$\frac{Var(k_2 x_2^*)}{Var(k_1 x_1^*)} = \frac{k_2}{k_1 + k_2} \ .$$

This simple example shows that the ratio of successive variances can be any number between zero and one.

Example 3.4. (Long chains) Assume that $k_i = k$ for some fixed $k > 0$ and all i. Taking the limit of (11) is difficult. Instead, since all the k_i are equal, an induction proof shows that

$$x_i^*(t) = \frac{I}{k} + \sigma \frac{k^{i-1}}{(i-1)!} \int_{-\infty}^{t} (t-s)^{i-1} e^{-k(t-s)} dB(s).$$

Using the Itô Isometry, it follows that

$$Var_\infty(x_i^*) \ = \ \sigma^2 \frac{2(2i-2)!}{4^i (i-1)!^2} \frac{1}{k} \ ,$$

and using Stirling's formula

$$Var(k x_i^*) \ \sim \ \sigma^2 \frac{k}{2\sqrt{\pi}} \frac{1}{\sqrt{i}} + O(i^{-3/2}) \ .$$

Thus the variances decrease to zero in a regular fashion if all of the rate constants are the same.

Example 3.5. (A small rate constant) Suppose that one rate constant, k_i, in a chain is very small. Using the explicit formula (11), one can easily compute that

$$Var(k_i x_i^*) \sim \sigma^2 \frac{1}{2} k_i + O(k_i^2), \quad \text{as } k_i \to 0,$$
$$Var(k_j x_j^*) \sim \sigma^2 \frac{1}{2} k_i + O(k_i^2), \quad \text{as } k_i \to 0, \text{ for } j > i.$$

Notice that the small rate constant has the effect of significantly decreasing the variances of the ith and all subsequent fluxes while the means of the fluxes remain unchanged. Therefore a small rate constant is not "rate limiting" but instead is "variance limiting."

Example 3.6. (A large rate constant) Suppose that one rate constant, k_i, in a chain is very large. Again, using (11), one can compute that

$$Var(k_i x_i^*) \to Var(k_{i-1} x_{i-1}^*), \quad \text{as } k_i \to \infty.$$

Furthermore, for all $j > i$,

$$Var(k_j x_j^*) \to Var(k_j \tilde{x}_j^*), \quad \text{as } k_i \to \infty,$$

$$\xrightarrow{I+\sigma dB(t)} \tilde{X}_1 \xrightarrow{k_1} \tilde{X}_2 \xrightarrow{k_2} \cdots \tilde{X}_{i-1} \xrightarrow{k_{i-1}} \tilde{X}_{i+1} \xrightarrow{k_{i+1}} \cdots$$

where $\tilde{x}_j$ is from the process arising from the following system:

This shows that in the asymptotic limit where $k_i \to \infty$ one can replace the original chain by the chain with the substrate X_i removed. Here we implicitly use the fact that since the kinetics are linear and hence the concentrations are Gaussian the statistics are determined by the means and variances.

4 Side Reaction Systems and Feedback Loops.

A *side reaction system* on a chain is any SSC system that gets its input from a species on the chain and has output that flows back into the same species; see Figure 4.1 below.

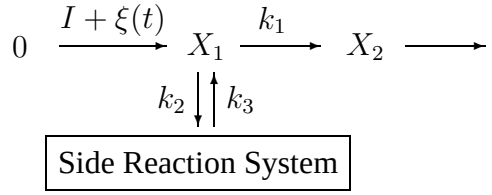


Figure 4.1: A side reaction on a linear chain

Note that there must be a species within the side reaction system whose output flows to X_1 with some rate constant, k_3. Define Y to be that species. The SDE governing the behavior of $x_1(t)$ is then given by

$$\frac{d}{dt}x_1(t) = I - k_1x_1(t) - k_2x_1(t) + k_3y(t) + \xi(t). \tag{13}$$

If $\tilde{x}_1$ is the solution to the above system when there is no side reaction system (i.e. $k_2 = k_3 = 0$), then

$$\frac{d}{dt}\tilde{x}_1(t) = I - k_1\tilde{x}_1(t) + \xi(t). \tag{14}$$

Theorem 4.1 (Side reactions lower variance). *Let x_1^* and $\tilde{x}_1^*$ be the first components of the stationary solutions to* (13) *and* (14)*, respectively, where $\xi(t)$ is a finite variance, mean zero, random process or white noise. Then,*

$$Var(k_1x_1^*) < Var(k_1\tilde{x}_1^*).$$

Proof. We give the proof in the case where $\xi(t) = \sigma dB(t)$ is white noise; the proof in the general case is similar but more complicated [1]. Let $z(t) = \mathbb{E}(k_1x_1(t) - I)^2$ and $\tilde{z}(t) = \mathbb{E}(k_1\tilde{x}_1(t) - I)^2$, where $x_1(t)$ and $\tilde{x}_1(t)$ are solutions of (13) and (14). By theorem 2.5 $z(t)$ and $\tilde{z}(t)$ converge to

$Var(k_1x_1^*)$ and $Var(k_1\tilde{x}_1^*)$, respectively. We will prove the theorem by comparing the differential equations satisfied by $z(t)$ and $\tilde{z}(t)$.

By using Kolmogorov's backward equation [18], we see that $\tilde{z}(t)$ satisfies

$$\tilde{z}'(t) = -2k_1\tilde{z}(t) + k_1^2\sigma^2. \tag{15}$$

Therefore, $Var(k_1\tilde{x}_1^*) = k_1\sigma^2/2$ because $Var(k_1\tilde{x}_1^*)$ is the equilibrium value of (15). Similarly, $z(t)$ satisfies

$$z'(t) = -2k_1z(t) + k_1^2\sigma^2 + 2k_1\mathbb{E}(k_1x_1(t) - I)(k_3y(t) - k_2x_1(t)),$$

and so, by Theorem 2.5,

$$Var(k_1x_1^*) = \frac{k_1\sigma^2}{2} + \frac{1}{2}\mathbb{E}(k_1x_1^* - I)(k_3y^* - k_2x_1^*).$$

Thus, to complete the proof we need only show that $\mathbb{E}(k_1x_1^* - I)(k_3y^* - k_2x_1^*) < 0$. The remark following Theorem 2.4 implies that $\mathbb{E}x_1^* = I/k_1$ and $\mathbb{E}k_3y^* = \mathbb{E}k_2x_1^*$. Therefore, $\mathbb{E}k_3y^* = \frac{k_2I}{k_1}$, and

$$\mathbb{E}(k_1x_1^* - I)(k_3y^* - k_2x_1^*) = \frac{k_1}{k_2}\mathbb{E}\left(k_2x_1^*k_3y^* - k_2^2x_1^{*2}\right).$$

By Theorem 2.6 $\mathbb{E}\left(k_3y^*\right)^2 < \mathbb{E}\left(k_2x_1^*\right)^2$, so

$$\begin{aligned}|\mathbb{E}\left(k_2x_1^*k_3y^*\right)| &\leq \left(\mathbb{E}\ k_2^2x_1^{*2}\right)^{\frac{1}{2}}\left(\mathbb{E}\ k_3^2y^{*2}\right)^{\frac{1}{2}} \\ &< \mathbb{E}\ k_2^2x_1^{*2}.\end{aligned}$$

Thus, $\mathbb{E}(k_1x_1^* - I)(k_3y^* - k_2x_1^*) < 0$, as desired. □

A *feedback loop* on a chain is an SSC system together with an input from one species on the chain, X_n, and an output to an earlier species, X_1; see Figure 4.2.

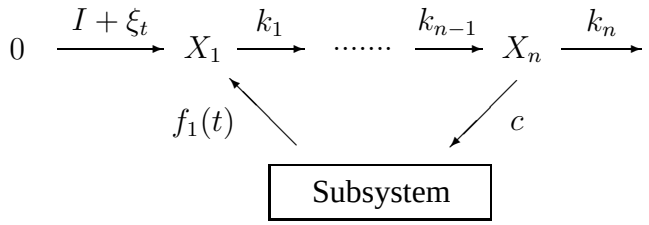


Figure 4.2: A chain with a feedback loop

Theorem 4.2. *Let $\tilde{x}(t)$ be the vector of species concentrations for the chain* (9) *and let $x(t)$ be the vector of species concentrations for the chain with feedback loop (Figure 4.2), where $\xi(t)$ is a finite variance, mean zero, random process or white noise. Then,*

$$Var(k_nx_n^*) < Var(k_n\tilde{x}_n^*).$$

Proof. Let $\{V_i\}$ be the substrates and B be the matrix of rate constants of the SSC subsystem in Figure 4.2. We suppose that V_j is the species which gives input to X_1 with rate constant α. Then the input to X_1 from the feedback loop is

$$f_1(t) = \alpha e^{Bt} v(0) \cdot e_j + \alpha c \int_0^t e^{B(t-s)} x_n(s) \cdot e_j \, ds,$$

which depends explicitly only on x_n. If we let $R(t) = k_{n-1} x_{n-1}(t)$ then the differential equation for $x_n(t)$ is $\dot{x}_n(t) = R(t) - c x_n(t) - k_n x_n(t)$.

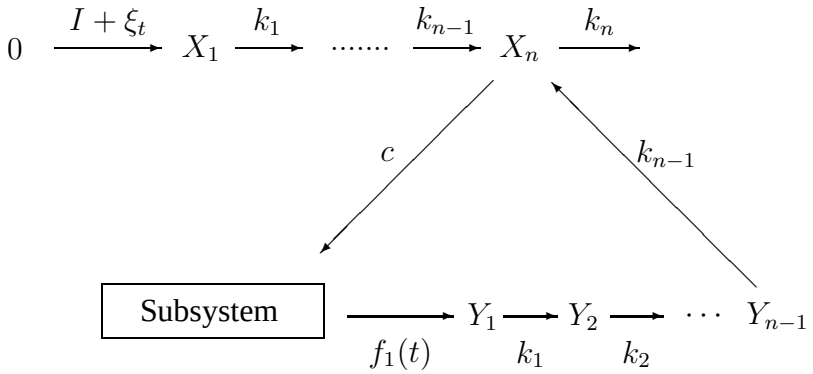


Figure 4.3: A chain with a side reaction system

Consider the chain with side reaction system given in Figure 4.3 where the subsystem is the same as in Figure 4.2 and the flux to Y_1 comes from V_j with rate constant α. Let $Q(t) = k_{n-1} x_{n-1}(t)$ and $P(t) = k_{n-1} y_{n-1}(t)$ be the inputs to X_n in Figure 4.3. Since the input to the Y-chain is $f_1(t)$ and the rate constants for the two chains are the same, $R(t) = Q(t) + P(t)$ because the differential equations are linear. Thus, the differential equation governing $x_n(t)$ in Figure 4.2 is the same as the differential equation governing $x_n(t)$ in Figure 4.3. Since the system in Figure 4.3 is a chain with a side reaction system, the result follows from Theorem 4.1. □

5 One large rate constant in a general SSC system

We now consider a general weakly reversible SSC system with input and characterize the effect of a large rate constant.

Theorem 5.1. *Suppose that independent white noise processes perturb the inputs to a weakly reversible SSC system with m substrates. Let X_a be a particular substrate and suppose that the rate constant L for one flux out of X_a to another complex (possibly the zero complex) is large. Then,*

$$Var(x_a^*) \sim O\left(\frac{1}{L}\right) \quad \text{as } L \to \infty. \tag{16}$$

Proof. We will assume that one of the perturbed inputs goes directly to X_a. The proof of the general case is similar. The stochastic differential equation governing $x_a(t)$ is given by

$$dx_a(t) = \left(C + \sum_{i=1}^{m} c_i x_i(t) - (L+K)x_a(t) \right) dt + \sigma dB(t) \ , \tag{17}$$

where $L + K > 0$ is equal to the sum of all the rate constants for reactions leaving X_a, $C > 0$ is the input flow to X_a from the zero complex, $\sigma > 0$, and $c_i \geq 0$ is the rate constant associated with the reaction $X_i \to X_a$. Solving (17) for x_a^* in terms of the x_i^* and using the Itô Isometry, one can easily bound $Var(x_a^*)$,

$$Var(x_a^*) \ \leq \ \frac{\beta}{2(L+K)} + \beta \sum_{i=1}^{m} \frac{c_i^2 Var(x_i^*)}{(L+K)^2},$$

for some constant β. To complete the proof we will show that $Var(x_i^*) \leq O(L)$.

Let A be the matrix of rate constants for the SSC system. Using the formula (7) for the stationary solution and the Ito Isometry, one easily calculates:

$$Var(x_i^*) = \sigma^2 \int_{-\infty}^{t} (e^{A(t-s)} e \cdot e_i)^2 \, ds, \tag{18}$$

for some vector e. By Lemma 2.3(b) we know that the real parts of the eigenvalues of A, $\{\lambda_i\}$, are strictly negative; let $\lambda = \inf \{|\lambda_i|\}$. There exist positive constants c and M so that for all $t - s > 0$, we have $\|e^{A(t-s)}\| \leq c e^{-M\lambda(t-s)}$. Using this inequality in (18), we have

$$Var(x_i^*) \leq \frac{\sigma^2 c^2 |e|}{2M} \frac{1}{\lambda}.$$

In Appendix A we prove that $\lambda \geq O(1/L)$, so $Var(x_i^*) \leq O(L)$, which concludes the proof. □

Example 6.1 (A side chain with a large rate constant) To illustrate the theorem, we consider the linear chain with a side reaction given in the diagram in the Introduction. As the rate constant L becomes large, Theorem 5.1 tells us that $Var(x_2^*) \leq O(1/L)$. Therefore the flux out of X_2 down the chain has variance $Var(k_2 x_2^*) \leq O(1/L)$. By Theorem 2.6,

$$Var(k_i x_i^*) \leq Var(k_2 x_2^*) \leq O(1/L) \quad \text{ for all } i \geq 2.$$

Thus, for all $i \geq 2$, the means of the fluxes remain equal to I, while the variances of the fluxes go to zero as $L \to \infty$.

6 Application to Methionine Metabolism.

The actual biochemical systems involved in cell metabolism are much more complicated and more difficult to analyze than the single species systems considered in the previous sections. Consider,

for example the diagram in Figure 6.1 that shows the methionine cycle and part of the folate cycle. Firstly, most reactions have two or more substrates and many enzymes are inhibited by the products of the reactions they catalyze. Thus, the kinetics will be highly nonlinear. Secondly, many reactions are catalyzed by two different enzymes that have very different properties. Thirdly, some substrates inhibit or activate distant enzymes in the reaction diagram (red arrows in the diagram). These long-range interactions make it virtually impossible to intuit the emergent properties of the network by tracing influences from point to point.

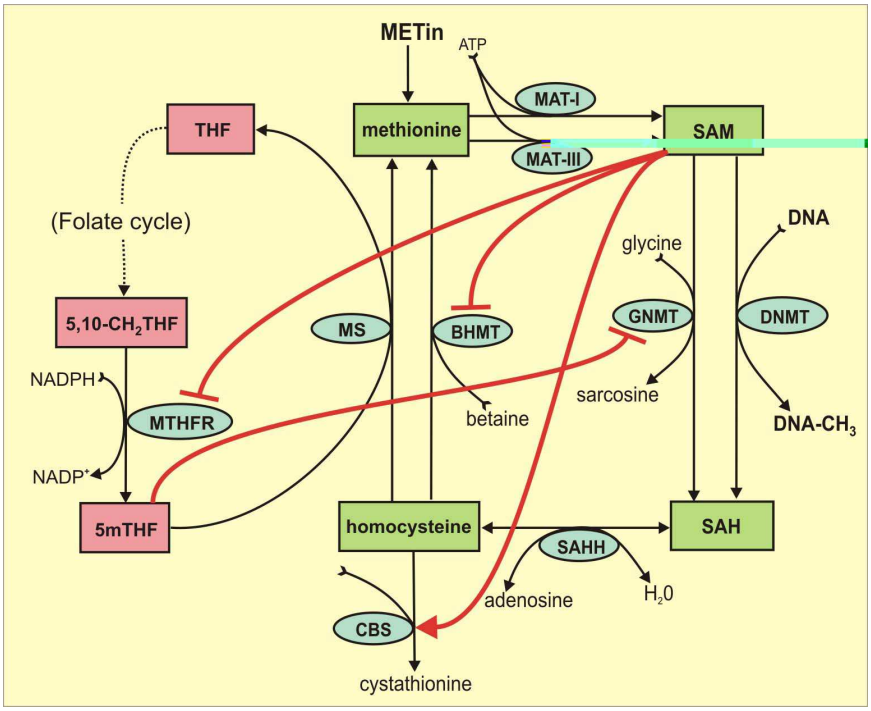


Figure 6.1. Methionine Metabolism. Substrates of the methionine cycle and (part of) the folate cycle are shown in green and red rectangles, respectively. Enzyme acronyms are in ellipses. Long-range interactions are shown by red curves with the arrow indicating activation and the bars indicating inhibition. SAM, s-adenosyl-methionine, activates CBS and inhibits BHMT and MTHFR, while 5mTHF, 5-methyltetrahydrofolate, inhibits GNMT.

Epidemiological evidence correlates changes in folate and methionine metabolism to serious human health consequences (cancer, heart disease, depression) and there are several important public health issues involved in folate supplementation as currently practiced in the United States and Canada. Thus, this part of cell metabolism has been the object of numerous experimental studies and several modeling studies [14][20][16][19][17]. Our purpose here is simply to illustrate how fluctuation analysis can be used to understand such a complex system.

The velocities of the individual reactions in the methionine cycle [17] are typically highly nonlinear functions that depend on the concentrations of several substrates. For example, the velocity of the GNMT reaction, V_{GNMT}, depends on SAM, on SAH because of product inhibition, and on $5mTHF$ because of a long-range interaction. Because of the complexity and the nonlinearities, a rigorous mathematical analysis of this system is beyond current mathematical techniques. Even

proving the existence and uniqueness of a stationary measure is a delicate issue. Nevertheless, we have investigated this question by numerical computation in the case where the methionine input ($METin$) is an Ornstein-Uhlenbeck process with mean 100 μM/hr and standard deviation 30 (variance = 900). We found that the joint distribution of the substrates does indeed stabilize as time gets large, and thus for each concentration or flux, X, we can compute the ratio

$$r \;=\; \frac{\text{Variance}(X)}{\text{Variance}(METin)},$$

which tells us how much X varies compared to the variance of the input. Table 1 shows the values of r for two substrates and two fluxes in the case where all the long-range interactions are present (regulated) and the case where the long-range interaction are absent (unregulated). Methionine is quite stable in both cases but is more stable in the regulated case. SAM is much less stable than methionine, which agrees with what is seen experimentally. Notice that in the unregulated case, the variances of V_{GNMT} and V_{DNMT} are similar, but in the regulated case the variance of V_{GNMT} doubles and the variance of V_{DNMT} becomes exceptionally small. There are good biological reasons why one would want the DNA methylation rate to be stabilized against fluctuations in methionine input. Thus, fluctuation analysis shows that this stabilization is achieved by the long-range interactions. We have also computed the values or r in all the intermediate cases where some but not all of the long-range interaction are present and this has enabled us to quantify each of their effects and propose an evolutionary scenario [17].

Table 1. Values of r

Methionine	r
regulated	.064
unregulated	.082
SAM	r
regulated	.22
unregulated	1.23
V_{GNMT}	r
regulated	.15
unregulated	0.079
V_{DNMT}	r
regulated	0.007
unregulated	0.09

In liver cells the reaction from methionine to SAM is catalyzed by two isoforms of the same enzyme, MAT–I and MAT–III, that have very different properties [15]. MAT–I is inhibited by SAM and MAT–III is activated by SAM, and it has been proposed that it is this unusual combination that stabilizes the methionine concentration. To test this, we recomputed r after eliminating the $MAT{-}III$ reaction and raising the V_{max} of the $MAT{-}I$ reaction so that it carried the same flux previously carried by both. The values in Table 2 show conclusively that, indeed, the presence of the $MAT{-}III$ reaction somewhat destabilizes SAM but greatly increases the stability of the methionine concentration.

Table 2. Values of r

Methionine	r
with $MAT\text{–}III$	0.06
no $MAT\text{–}III$	0.16
SAM	r
with $MAT\text{–}III$	0.22
no $MAT\text{–}III$	0.17

7 Discussion.

In Sections 2-5, we developed the theory of propagation of fluctuations for the special case of linear SSC networks and proved theorems relating variances to network structure. Variances decrease down a chain and the presence of side reactions and feedback loops always lowers the variances further down the chain. These results are very general in that they hold independent of the choice of rate constants. It is tempting to speculate that biochemical systems evolved to be as complicated as they are partly because of the homeostasis of exit fluxes achieved by having many intermediate steps. We also showed how the large size of a single rate constant affects variances. It is known that most of these results generalize to non-linear SSC networks with restrictions on the nature of the nonlinearity [2]. It remains to be seen whether they generalize to networks in which complexes contain more than one species. In these highly non-linear contexts, a fundamental mathematical issue is the proof of the existence of a stationary measure.

A reasonable concern with the idealized models in Sections 2-5 is that, under the influence of the fluctuations, the concentrations can become negative. By modifying the forcing processes appropriately this could have been avoided. However, this would complicate the analysis and prevent us from obtaining explicit formulae and straightforward bounds. Since our goal with these idealized models is to build intuition and develop general principles, we have purposely avoided complicating the analysis.

In Section 6 we showed how the ideas of fluctuation theory could be used to investigate a network of biological interest, the methionine cycle. It is reasonable to ask whether methionine input actually fluctuates randomly and if so what are the properties of the fluctuations. There are really two answers. The input to the methionine pool in liver cells is certainly continually varying. There are large deviations on the time scale of hours depending on the times and content of meals. Methionine is always being used for protein synthesis and is being made available by protein catabolism, two processes that are themselves variable and not always in balance. The methionine available for input to the methionine cycle is also affected by the use of methionine in other metabolic reactions. Finally, all these processes are affected by the time-varying regulation of the genes that code for the various enzymes. Thus, the first answer is that we don't know how methionine input varies but it certainly fluctuates with standard deviations of the order of 30-50 μM/hr on the time scale of hours and with smaller standard deviations on the time scales of minutes and seconds. The second answer is that it doesn't matter. We are using the fluctuations in methionine input as a probe of the dynamical properties of the system away from equilibrium. Of

course, we need to be sure that the properties we find do not depend on the detailed properties of the noise.

For simplicity of exposition, we have discussed the special case where a single input to a biochemical system is varied. The same ideas can be used to introduce fluctuations in a concentration, a flux, or in several places, and then study how the fluctuations propagate throughout the system. Understanding the consequences of fluctuations in kinetic parameters is also important because kinetic parameters depend on enzyme concentrations and other properties that are variable and themselves dependent on time-varying genetic regulation. Analyzing this case requires some technical extensions of this work.

In the Introduction we referred to "intrinsic stochasticity" in contrast to the external stochastic forcing that we consider. It would be interesting to consider models with both forms of stochasticity, and indeed both surely arise in gene networks. In gene networks that are coupled to biochemical networks, the intrinsic stochasticity at the gene and gene regulation level can be viewed as external stochastic forcing to the biochemical level. Therefore, both types of questions and analyses will be necessary to gain full understanding of real biological networks.

Appendix A

We derive the bound used in Theorem 5.1. There are two cases which need consideration:

1. The flux out of X_a with rate constant L goes to another species. This case is handled in Theorem A.1 below.

2. The flux out of X_a with rate constant L leaves the system. The proof of the result in this case is similar to the proof of the theorem below and so the details are omitted.

Theorem A.1 *Let $A = \{a_{ij}\}$ be an $n \times n$ matrix with the following properties:*

(1) For each i, $a_{ii} < 0$ and $|a_{ii}| \geq \sum_{j \neq i}^{n} |a_{ji}|$.

(2) $a_{11} = -L + \alpha_{11}$ and $a_{21} = L + \alpha_{21}$ for some $\alpha_{11} < 0$ and $\alpha_{21} \in \mathbb{R}$.

(3) For every $L > 0$, the real parts of the eigenvalues of A are all strictly negative.

Denote the eigenvalues of A by $\{\lambda_i\}$ and let $\lambda = \inf \{|Re(\lambda_i)|\}$. Then

$$\lambda \geq O(1/L), \text{ as } L \to \infty.$$

Proof. Let $B = \frac{1}{L}A$. The eigenvalues of B are $\{\frac{1}{L}e_i : e_i \text{ is an eigenvalue of } A\}$. We will use the characteristic polynomials of A and B to show that the magnitude of the real parts of the eigenvalues of B are no smaller than $O(1/L^2)$, which implies our result.

Because L only appears in the first column of A, all $O(1)$ terms of B occur in the first column. Expanding the determinant of B by cofactor expansion along the first column then shows that

$det(B)$ must be of order $O(1/L^n)$ or $O(1/L^{n-1})$. Similarly, the cofactors of B must be of order $O(1/L^{n-1})$ or $O(1/L^{n-2})$. Therefore, computing the inverse of B (which exists by assumption (3) above) by cofactors, we see that the possible order of the entries of B^{-1} are 1, L, and L^2. Therefore, $\|B^{-1}\| \leq O(L^2)$.

One may view B as a $1/L$ matrix perturbation of the matrix $C = \{c_{ij}\}$, where $c_{11} = -1$, $c_{21} = 1$, and $c_{ij} = 0$ for all other entries. Therefore, each eigenvalue, ρ, of B is an analytic functions of $1/L$:

$$\rho = \rho_0 + \frac{1}{L}\rho_1 + \frac{1}{L^2}\rho_2 + O\left(\frac{1}{L^3}\right), \tag{19}$$

where ρ_0 is -1 or 0. If $\rho_0 = -1$ there is nothing to prove; so we assume $\rho_0 = 0$. If $\rho_1 = \rho_2 = 0$ then $\rho = O(1/L^3)$. However, this would imply that $O(1/\rho) = O(L^3)$. Since $1/\rho$ is an eigenvalue of B^{-1}, this would contradict the norm bound for B^{-1}, above. Thus ρ_1 and ρ_2 can not both be zero. It remains to be shown that the leading order term in equation (19) can not be purely imaginary. We will do this through asymptotic matching.

Consider two different formulations for the characteristic polynomial of A, $p_A(x)$:

$$\begin{aligned} p_A(x) &= det(xI_n - A) && (20)\\ &= x^n + Lu(x) + v(x) && (21)\\ &= x^n + c_{1,n-1}Lx^{n-1} + c_{0,n-1}x^{n-1} + \cdots + c_{1,2}Lx^2 + c_{0,2}x^2 \\ &\quad + c_{1,1}Lx + c_{0,1}x + c_{1,0}L + c_{0,0}, && (22)\end{aligned}$$

where $u(x)$ and $v(x)$ are polynomials of degree $n-1$ that are independent of L, and $c_{i,j} \in \mathbb{R}$ for $i = 1, 2$ and $j = 1, ..n-1$ (i gives the power of L and j gives the power of x for the term $c_{ij}L^i x^j$). We note that we can not have $c_{1,0} = c_{0,0} = 0$, for then there would be a zero eigenvalue, which would contradict assumption (3).

To show that the leading order term in equation (19) is not purely imaginary we will consider two cases: $\rho_1 = 0$ and $\rho_1 \neq 0$. We begin by supposing $\rho_1 = 0$ and $\rho_2 \neq 0$. Then $\rho = O(1/L^2)$ and there is a solution to (22) which is $O(1/L)$. Putting $x = \rho_2/L$ into (22) and setting the equation equal to zero gives us:

$$O\left(\frac{1}{L^3}\right) + \frac{c_{1,2}\rho_2^2}{L} + \frac{c_{0,2}\rho_2^2}{L^2} + c_{1,1}\rho_2 + \frac{c_{0,1}\rho_2}{L} + c_{1,0}L + c_{0,0} = 0.$$

Matching like terms in L tells us that $c_{1,0} = 0$, $c_{0,0} \neq 0$, and $c_{1,1} \neq 0$. Solving for ρ_2 gives us $\rho_2 = -c_{0,0}/c_{1,1} \in \mathbb{R}$. Therefore, ρ_2 has a nonzero real part.

We now suppose that $\rho_1 \neq 0$. Because finding an $O(1/L)$ solution to equation (19) is equivalent to finding an $O(1)$ solution to (21), ρ_1 must satisfy $u(\rho_1) = 0$. Let $D(x) = xI_n - A$. Then $u(x) = D(x)_{11} + D(x)_{21}$, where $D(x)_{ij}$ is the i, j^{th} cofactor of $D(x)$. $D(x)_{11}$ and $D(x)_{21}$ differ only in the first row, so we may combine the determinants by adding the first two rows. We

conclude that

$$u(x) = \begin{vmatrix} -a_{22} - a_{12} + x & -a_{13} - a_{23} & -a_{14} - a_{24} & \cdots & -a_{1n} - a_{2n} \\ -a_{32} & -a_{33} + x & -a_{34} & \cdots & -a_{3n} \\ -a_{42} & -a_{43} & -a_{44} + x & \cdots & -a_{4n} \\ \vdots & \vdots & & \ddots & \vdots \\ -a_{n2} & -a_{n3} & -a_{n4} & \cdots & -a_{nn} + x \end{vmatrix}.$$

Solving $u(x) = 0$ for non-zero solutions is therefore equivalent to finding the non-zero eigenvalues of the matrix

$$\tilde{A} = \begin{bmatrix} a_{22} + a_{12} & a_{13} + a_{23} & a_{14} + a_{24} & \cdots & a_{1n} + a_{2n} \\ a_{32} & a_{33} & a_{34} & \cdots & a_{3n} \\ a_{42} & a_{43} & a_{44} & \cdots & a_{4n} \\ \vdots & \vdots & & \ddots & \vdots \\ a_{n2} & a_{n3} & a_{n4} & \cdots & a_{nn} \end{bmatrix}.$$

By assumption (1), the diagonal entries of $\tilde{A}$ are non-positive and have magnitudes that are greater than or equal to the sums of the magnitudes of all the other terms in that column. Therefore, Gershgorin's Theorem says that the non-zero eigenvalues of $\tilde{A}$, and hence the non-zero solutions of $u(x) = 0$, have strictly negative real part. Thus, $Re(\rho_1) \neq 0$. This completes the proof. □

If the flux out of X_a with rate constant L leaves the system, the only change in the statement of the above theorem is that a_{21} is independent of L. The proof is identical except that $u(x) = D(x)_{11}$ and so we no longer have to add two determinants together to simplify $u(x)$.

Acknowledgements

This research was supported by NIH grant R01-CA105437, NSF grants DMS0109872, DMS0449910, and the Alfred P. Sloan Foundation (Mattingly).

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