Retroactivity attenuation through signal transduction cascades

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Abstract—This paper considers the problem of attenuating retroactivity, that is, the effect of loads in biological networks and demonstrates that signal transduction cascades incorporating phosphotransfer modules have remarkable retroactivity attenuation ability. Uncovering the biological mechanisms for retroactivity attenuation is relevant in synthetic biology to enable bottom-up modular composition of complex circuits. It is also important in systems biology for deepening our current understanding of natural principles of modular organization. In this paper, we perform a combined theoretical and computational study of a cascade system comprising two phosphotransfer modules, ubiquitous in eukaryotic signal transduction, when subject to load from downstream targets. Employing singular perturbation on the finite time interval, we demonstrate that this system implements retroactivity attenuation when the input signal is sufficiently slow. Employing trajectory sensitivity analysis about nominal parameters that we have identified from in vivo data, we further demonstrate that the key parameters for retroactivity attenuation are those controlling the timescale of the system.

I. INTRODUCTION

The description of biomolecular circuits through functional modules [1] relates cellular biology to synthetic disciplines such as computer science and engineering. Modularity is the property that allows the assembly of larger system through the use of units that perform independently (modules) as building blocks [2]. Yet, the predictable and functional composition of complex systems from simpler modules still remains one of the challenges in synthetic biology [3][4]. One problem lies in the presence of an impedance-like effect known as retroactivity, which creates context dependencies that change the internal state of the individual modules upon interconnection [5][6][7].

It has been demonstrated in vivo that retroactivity can affect the level of MAPK (mitogen-activated protein kinase) phosphorylation depending on substrate concentration [8]. It has also been reported that retroactivity can change the response time of a uridylyltransferase/uridylyl-removing enzyme (UTase/UR)–PII system [9], and induce time delays in gene transcription networks [10], thus affecting the dynamic behavior of biomolecular systems. To attenuate retroactivity effects, which can disrupt a module functionality, the implementation of insulation devices was proposed [6][11]. The behavior of an insulation device is similar to that of a non-inverting amplifier, which buffers the dynamics of the upstream circuit from the impedance effects due to downstream load. Similarly, the insulation device allows for the preservation of the temporal behavior of the interconnected modules by mitigating retroactivity effects upon interconnection.

A formal mathematical treatment of systems with the retroactivity attenuation property employing the principle of timescale separation is provided in [11]. This principle states that if the insulation device internal dynamics are on a much faster timescale compared to the input timescale, the output presents no tracking error even in the presence of high load. The main result, based on Tikhonov’s theorem of singular perturbation on the finite time interval [12], suggests that the fast internal dynamics of the insulation device allows the system trajectories to immediately approach a (asymptotically stable) slow manifold. This manifold, in turn, can be made independent from the load when key regulatory elements are in sufficiently large amounts.

This work presents the analysis of an insulation device realization based on the natural YPD1/SKN7 phosphotransfer cascade in Saccharomyces cerevisiae. The YPD1/SKN7 pathway is part of a phosphorelay circuit of the osmotic stress response mechanism in yeast [13]. It consists of a series of phosphotransfer reactions with timescale in the sub seconds range, ideal to provide the fast internal dynamics of the insulation device. It is also reported that both YPD1/SKN7 and the YPD1/SSK1 pathway of the osmotic stress response mechanism are responsible for regulating multiple downstream clients, with SKN7 specifically being a transcription factors of many genes [14]. Thus, nature utilizes intermediate phosphotransfer cascades in order to ensure high response time to changes in osmotic stress, independent of the amount of downstream clients. Based on this, it is our goal to show that the fast nature of the phosphotransfer reactions indeed allow for the successful operation of the insulation device based on timescale separation.

This paper is organized as follows. Section II mathematically characterizes a class of systems that have the retroactivity attenuation property through timescale separation. In Section III, the mathematical model of a specific YPD1/SKN7 pathway is presented. It also shows how the YPD1/SKN7 system fits this class of systems described in Section II, thus possessing the retroactivity attenuation property. A parameter sensitivity analysis on the insulation device output error is provided in Section IV, while Section V describes the dependence of the output error on phosphorylation timescale and total concentration of key regulatory elements.

II. RETROACTIVITY ATTENUATION

An interconnection diagram of two general biomolecular systems is presented in Fig. 1. Here, the arrows traveling
in the rightward direction model the information carrier signals, while arrows traveling in the leftward direction model retroactivity signals, while arrows traveling in the leftward direction...internal to the insulation device evolve in a significantly faster timescale than its upstream and downstream system.

![Diagram](image)

Fig. 1: System Σ transmits input signal u to the output y while its internal state x is subjected to retroactivity to the output s. System Ω with internal state v has y as an input and applies the retroactivity to the output signal s to Σ.

We can claim system Σ is an insulation device if it possesses the following properties.

**Definition 1.** System Σ has small retroactivity to the input if the retroactivity r is close to zero. System Σ further has the retroactivity to the output attenuation property if the effect of retroactivity s on the internal state x is attenuated. This means \( \|x(t, s) - x(t, 0)\| \approx 0 \)

**Definition 2.** System Σ is called an insulation device if it has both small retroactivity to the input and the retroactivity to the output attenuation property.

The following proposition introduces a specific class of systems Σ that employ the principle of timescale separation for retroactivity to the output attenuation.

**Proposition 1.** Consider system Σ

\[
\begin{align*}
\dot{x} &= g(t, u), \\
\dot{y} &= f(u, x, \epsilon_1 \psi(v)) + \epsilon_1 (x) + \epsilon M s(y, v), \\
y &= C x, \\
\dot{v} &= B s(y, v) + h(y, v),
\end{align*}
\]

where \( \|\psi(v)\| \leq a, C, B \) and M are constant matrices, 0 < \( \epsilon \ll 1 \) is a singular perturbation parameter and 0 < \( \epsilon_1 \) is a constant parameter. Let the following assumptions hold for \( t \in [0, t_1] \):

1. For \( f(u, x, \epsilon_1 \psi(v)) = 0 \) there exists a unique global mapping \( x = \gamma(u, \epsilon_1 \psi(v)) \) and for \( f(u, x_0, 0) = 0 \) there is a unique global mapping \( x_0 = \gamma_0(u) \).
2. Functions \( g, f, \lambda, C, C, B \) are smooth on all parameters.
3. The slow system \( \dot{u} = g(t, u), \dot{v} = B s(C \gamma(u, \epsilon_1 \psi(v)), v) + h(C \gamma(u, \epsilon_1 \psi(v)), v) \) has a unique solution \( (u(t), \psi(t, \epsilon_1)) \).
4. System \( \frac{dx}{dt} = f(u, x, \epsilon_1 \psi(v)) \), where \( \tau = t/\epsilon \) and \( u \) and \( v \) are frozen in time, has a locally exponentially stable equilibrium point \( \bar{x} \) uniformly in \( (u, \epsilon_1 \psi(v)) \), with \( x(t_0) \) inside the region of attraction of \( \bar{x} \).

Then, the solution \( x(t, \epsilon, \epsilon_1) \) of system (1)-(4) can be approximated by \( \gamma_0(u(t)) \), with an error given by

\[
\|x(t, \epsilon, \epsilon_1) - \gamma_0(u(t))\| = O(\epsilon) + O(\epsilon_1). \tag{5}
\]

**Proof.** Assumptions 1) - 4) allow the application of the singular perturbation theorem (Theorem 11 [12]) which implies that there exists a constant \( \epsilon^* \) such that for any initial condition inside the region of attraction of the equilibrium \( \bar{x} \) of system \( \frac{dx}{dt} = f(u, x, \epsilon_1 \psi(v)) \) for a time \( t_b > t_0 \) and \( 0 \leq \epsilon \leq \epsilon^* \) the singular perturbation problem (1) - (4) has a unique solution \( (u(t), x(t, \epsilon, \epsilon_1), v(t, \epsilon, \epsilon_1)) \)

\[
\begin{align*}
v(t, \epsilon, \epsilon_1) &= v(0, \epsilon_1) + \epsilon f(y(t, \epsilon, \epsilon_1), s(t, \epsilon, \epsilon_1), v(t, \epsilon, \epsilon_1)) \\
\|x(t, \epsilon, \epsilon_1) - v(t, \epsilon_1)\| &= O(\epsilon) \tag{6} \\
x(t, \epsilon, \epsilon_1) &= \gamma_0(u(t), \epsilon_1) \tag{7}
\end{align*}
\]

Furthermore, since \( f \) is at least of class \( C^1 \), it follows from the Implicit Function Theorem [15] that the implicit function \( \gamma(u, \epsilon_1 \psi(v)) \), such that \( f(u, \gamma(u, \epsilon_1 \psi(v))) = 0 \), is also of class \( C^1 \). From the Mean Value Theorem [15] we now have that there exists a \( K > 0 \) such that \( |\gamma(u, \epsilon_1 \psi(v)) - \gamma(u, 0)| \leq \epsilon K \psi(v) \) for all \( v \) making

\[
|\gamma(u, \epsilon_1 \psi(v)) - \gamma_0(u)| \leq K \epsilon_1 a. \tag{8}
\]

Now, using the triangular inequality we have \( \|x(t, \epsilon, \epsilon_1) - \gamma(u, \epsilon_1 \psi(v))\| + \|\gamma(u, \epsilon_1 \psi(v)) - \gamma(u, 0)\| \leq \|x(t, \epsilon, \epsilon_1) - \gamma(u, \epsilon_1 \psi(v))\| + \|\gamma(u, \epsilon_1 \psi(v)) - \gamma(u, 0)\| \). From the definition of \( \gamma_0(u) \) in Assumption 1 and using results (7)-(8) yields (5).

**Remark 1.** The specific class of system Σ introduced in Proposition 1 is a special instance of an insulation device in which the retroactivity to the input \( r = 0 \) if \( \epsilon, \epsilon_1 \ll 1 \). This can be seen from Definition 2 since \( r = 0 \) and parameters \( \epsilon, \epsilon_1 \ll 1 \) make \( \|x(t, s) - x(t, 0)\| \) arbitrarily small.

**Remark 2.** Having \( \epsilon \ll 1 \) implies that the insulation device dynamics are much faster than both upstream and downstream systems, thus the system employs timescale separation as a mechanism for retroactivity attenuation. This result is an extension of [11] and it allows us to consider models of existing natural signal transduction cascades as shown in the next section.

**III. PROBLEM FORMULATION AND SYSTEM MODEL**

We will demonstrate that the natural phosphotransfer cascade realized by the YPD1/SKN7 pathway [13] can function as an insulation device attenuating the retroactivity effects due to loading. Specifically, we will show that the YPD1/SKN7 system satisfies Proposition 1, thus implementing the principle of timescale separation for retroactivity attenuation. Then we will identify the key biochemical parameters controlling its retroactivity attenuation property. Although, the analysis will focus on checking the assumptions of Proposition 1 for a set of parameters inside the allowable physical range, this result applies to a much broader set of parameters as long as the kinetic rates internal to the insulation device evolve in a significantly faster timescale than its upstream and downstream system.
A. YPD1/SKN7 system reactions and model

Using the YPD1/SKN7 pathway, we consider a candidate phosphorylation device as follows. In this description the asterisk notation (\(*\) ) represents phosphorylation and for a species \(Y\) its italic \(Y\) represents its concentration. The notation considered for the YPD1/SKN7 system species is: 

\[ Z \text{ represents a phosphate donor for YPD1, } W \text{ represents protein YPD1 and } X \text{ represents protein SKN7.} \]

A diagram of the YPD1/SKN7 system is given in Fig. 2, which describes the SKN7 activation pathway. It should be noted that both, \(X^*\) and \(X^{**}\) can bind to the downstream system as in Fig. 2. This downstream system consists of DNA binding sites \(p\), but only the bound \(X^{**}\) leads to transcriptional activation. Complexes \(C^*\) and \(C^{**}\) denote the bound form of \(X^*\) and \(X^{**}\), respectively, which can also be phosphorylated by \(W^*\) and dephosphorylated by \(W\). The decay of both \(Z\) and \(Z^*\) as well as the spontaneous dephosphorylation of all phosphorylated species was considered.

\[
\begin{align*}
X^* \rightarrow & x_1 (X^* - X^* - X^{**} - C^* - C^{**}) W^* \\
& + (k_3 X^*(p_T - C^* - C^{**}) + k_4 C^*) (W_T - W^*)
\end{align*}
\]

with the conservation laws: \(X_T = X + X^* + X^{**} + C^* + C^{**}\), \(W_T = W + W^*\), \(p_T = p + C^* + C^{**}\), and all rate parameters as defined in the corresponding reactions. This model fits the box description presented in Fig. 1 by setting \(u = Z_T\), the insulation device state vector \(x = (Z^*, W^*, X^*, X^{**})^T\) and the output vector \(y = (W^*, X^*, X^{**})^T\). The internal dynamics of system \(\Sigma\) are given by equations (10)-(13) while the downstream system \(\Omega\) with state vector \(v = (C^*, C^{**})^T\) has internal dynamics given by equations (14)-(15). The retroactivity to the input \(r\) is zero since \(Z_T\) is independent of all downstream processes, and the retroactivity to the output \(s\) is given by the terms over braces \(s_i\).

B. YPD1/SKN7 retroactivity attenuation property

To see how system (9)-(15) fits Proposition 1, let us write a nondimensional version of the system and make the separation of timescale explicit by including a singular perturbation parameter \(\epsilon\). All concentrations in (9)-(15) can be normalized by their maximum value. We can define these non-dimensional concentrations as \(x^* := X^*/X^0\), \(x^{**} := X^{**}/X^0\), \(w^* := W^*/W^0\), \(z_T := Z_T/Z_T^0\), \(z^* := Z^*/Z^0\), \(c^* := C^*/C^0\), \(c^{**} := C^{**}/C^{**0}\), where \(Z_T^0\) is the maximum concentration \(Z_T\) can reach and it is given by \(Z_T^0 := \max\{ Z(t)/\delta \}\). This makes the range of all states \([0, 1]\) for all time. From this non-dimensional model we can see that the timescale of the \(z_T\) differential equation is given by the decay rate \(\delta \in [0.004, 0.01]\) min\(^{-1}\) [16], but the remaining differential equations evolve in a faster timescale determined by phosphotransfer reactions. We consider, specifically, that all reactions involving kinetic rates \(k_{pi}, W_Tk_1, W_Tk_2, W_Tk_3, W_Tk_4, k_6\) evolve in a fast timescale characterized by the phosphotransfer rate \(k_1 W_T \in [1,600, 1,600] \text{ min}^{-1}\) [13]. The other phosphotransfer reactions evolve in a slower time scale since \(k_{p1/0}, k_{5/0}\) in \([0.004, 1]\) min\(^{-1}\) [13], and \(k_8, k_9 p_T, k_{10} p_T, k_{11}, k_{12} p_T \leq 0.02 \text{ min}^{-1}\) [18]. Also, the binding/unbinding reactions of \(x^*\) and \(x^{**}\) with DNA load promoter sites occurs at a maximum rate \(k_{pd} = 0.13 \text{ min}^{-1}\) [19]. Thus, we can justify the application of singular perturbation theory with small parameter \(\epsilon = \delta/(k_1 W_T)\). In particular, we define constants not depending on \(\epsilon\): \(c_1 := k_1/k_4, c_2 := k_2/k_4, c_3 := k_3/k_4, c_5 := k_5/\delta, c_6 := k_6/k_4 W_T, c_7 := k_7/\delta, c_8 := k_8/\delta, c_9 := k_9 /k_1 p_T/\delta, c_{10} := k_{10} p_T/\delta, c_{11} := k_{11}/\delta, c_{12} := k_{12} p_T/\delta, k_0 := k_0/p_T/k_4 W_T, k_p := k_p/\delta, k_0 := k_0/k_4 W_T, k_p := k_p/\delta, \alpha := X_T/W_T, k_{con} := k_{con}/p_T/X_T/\delta, k_{init} := k_{init}/p_T/X_T/\delta, \text{ and } \epsilon_1 = p_T/X_T\). Defining \(k(t) := k(t)/Z_T^0\), system (9)-(15)
becomes:
\[
\dot{z}_T = \bar{k}(t) - \delta z_T \tag{16}
\]

\[
\epsilon \dot{z}^* = c_1 \delta z^*(1 - w^*) + c_2 \delta w^*(z_T - z^*) + \kappa_p \delta (z_T - z^*)
- \epsilon \kappa_\rho \delta z^* - \epsilon \delta z^* \tag{17}
\]

\[
\epsilon \dot{w}^* = c_1 \delta Z_0 W_T z^*(1 - w^*) - c_2 \delta Z_0 \epsilon w^*(z_T - z^*)
- c_3 \alpha (1 - \epsilon c^* - \epsilon c_{**}) w^*
+ \alpha \delta x^*(1 - w^*) + \alpha \delta x^{**}(1 - w^*) - \epsilon \kappa_7 \delta w^*
+ c_9 \delta c \epsilon w^* + c_{10} \delta c^{**}(1 - w^*) + \epsilon c_{12} \delta c(1 - w^*) \tag{18}
\]

\[
\epsilon \dot{c}^* = c_9 \delta X_T x^*(1 - c^* - c_{**}) - \kappa_a \delta X_T c + c_8 \delta c^{**} - c_{11} \delta c^*
- c_{12} \delta W_T (1 - w^*)c \tag{19}
\]

\[
\dot{c}^{**} = c_9 \delta X_T c^{**}(1 - c^* - c_{**}) - \kappa_a \delta X_T c^{**} - c_8 \delta c^{**} + c_9 \delta c^{**} + c_{10} \delta W_T c^{**} - c_{11} \delta c^* \tag{20}
\]

System (16)-(22) fits the structure of (1)-(4) with \( u = z_T \), \( x = (z^*, w^*, x^*, x^{**})^T \), \( v = (c^*, c_{**})^T \). The dynamics of \( u(t) \) are given by (16), with abuse of notation we define the vector \( s = (s_1, s_2, s_3)^T \) using the functions indicated by braces in (18)-(20). For example, let \( \psi(v) = c^* + c^{**} \) which is bounded since both \( c^* \) and \( c_{**} \) are inside \([0, 1]\). Functions \( f(u, x, \epsilon \psi(v)), l(x), h(y, v) \) and matrices \( C, M \) and \( B \) can be defined by inspection. We will now prove Proposition 4 and show that Assumption 4 holds, which is the crucial assumption, and assume all smoothness and uniqueness conditions are met.

Assumption 4: To prove that the slow manifold is locally exponentially stable, we verified that the Jacobian of the fast system with \( \epsilon = 0 \) is negative semi-definite, where \( u(t) \) and \( v(t) \) are frozen in time and \( \tau = t/\epsilon \) has eigenvalues with strictly negative real parts uniformly in \( z_T \) when (\( z^*, w^*, x^*, x^{**} \)) belong to the slow manifold (\( \epsilon = 0 \) in equations (17)-(20)). We numerically calculated the Jacobian of the system as a function of \( z_T \) and showed that the eigenvalues have strictly negative real part, as seen in Fig. 3, with the largest eigenvalue bounded above by -0.025.

Hence, by Proposition 1 we have that system (16)-(22) works as an insulation device employing by virtue of timescale separation. The insulation device retroactivity attenuation dependence on fast internal dynamics is depicted in Fig. 4. Here, we see that the retroactivity attenuation is decreased as the parameters controlling the internal timescale are decreased. This is consistent with the requirement of a small \( \epsilon = \delta/(k_4 W_T) \).

**IV. Sensitivity Analysis**

To further explore how the retroactivity attenuation property depends on the internal parameters of the system, we performed a sensitivity analysis on the insulation device output. Specifically, we expect that uniform decrease in the parameters \{\(W_T k_1, W_T k_2, W_T k_3, W_T k_4, k_6\)\} controlling the insulation device’s timescale will worsen retroactivity attenuation.

For a general state equation \( x = f(t, \lambda) \), continuous in \((t, x, \lambda)\) and with continuous first partial derivatives on the set of internal parameters \( \lambda \), we can define the sensitivity of a state trajectory \( x(t, \lambda) \) to parameters \( \lambda \) by taking the...
partial derivative with respect to $\lambda$ [12]. This is given by:

$$S_\lambda(t,\lambda) = \frac{\partial}{\partial \lambda} x(t,\lambda),$$

which is a matrix in $\mathbb{R}^{nxp}$, where $n$ is the number of states and $p$ is the number of parameters in $\lambda$. By defining the Jacobian matrices $J_1(t,\lambda) := \frac{\partial f(t,x,\lambda)}{\partial x}$ and $J_2(t,\lambda) := \frac{\partial f(t,x,\lambda)}{\partial \lambda}$, we can write the differential equation for the sensitivity matrix as $\dot{S}_\lambda = J_1(t,\lambda)S_\lambda(t) + J_2(t,\lambda)$, where matrices $J_1$ and $J_2$ are evaluated at the nominal trajectory $x(t,\lambda_0)$ and parameter values $\lambda_0$. In our case we have $x = (Z^*, W^*, X^*, X^{**}, C^*, C^{**})^T$ and $\lambda = (\delta, k_p, k_p^*, k_1, k_2, k_3, k_4, k_5, k_6, X_T, W_T)^T$ with nominal parameter values $\lambda_0$ as given in Fig. 3. Defining the diagonal matrix $D_\lambda := \text{diag}\{\lambda_1, \lambda_2, \ldots, \lambda_n\}$, we can obtain the normalized sensitivity $S_\lambda = S_\lambda D_\lambda$. The normalized sensitivity can be seen as the concentration change of species $x_i$ due to a percentile change in the individual parameter $\lambda_j$, which allows for sensitivity comparison between parameters of different nature.

We define the output error as the squared difference between the unloaded output trajectory $X^{**}$ and loaded output trajectory $X^{**}_L$, $e_\lambda(t,\lambda) := (X^{**}(t,\lambda) - X^{**}_L(t,\lambda))^2$, where the unloaded system has $p_T = 0$ in (12)-(15) and the loaded system has $p_T \neq 0$. This allows to assess the sensitivity of the retroactivity attenuation property to parameters $\lambda$, since we are directly comparing the behavior of the insulation device in isolation and to that when connected. This sensitivity is given by:

$$\frac{\partial e_\lambda(t,\lambda)}{\partial \lambda} = 2[X^{**}(t,\lambda) - X^{**}_L(t,\lambda)][\dot{S}_\lambda(t,\lambda) - \dot{S}^*_\lambda(t,\lambda)],$$

(23)

where $\dot{S}_\lambda$ and $\dot{S}^*_\lambda$ denote the normalized unloaded and loaded output sensitivities respectively. The simulation results of the normalized output error sensitivity are given in Fig. 5 for periodic square wave input with a fixed on-time of 50 minutes and period of 500 minutes.

An increase in the squared error is given by positive error sensitivity while a decrease in the squared error is given by negative error sensitivity. From Fig. 5 (c), we can see that the output error performance is highly sensitive to the total phosphatase concentration $W_T$ and the $X^{**}$ dephosphorylation rate $k_6$. Increasing these parameters leads to an improvement in the insulation device’s performance, consistent with having $\epsilon \ll 1$ in Proposition 1. We can also see from Fig. 5 (a) that the output error is sensitive to parameter $k_p$ which has positive sensitivity. The $Z^*$ phosphorylation rate $k_p$ leads to an increase in the insulation device signal. This, in turn, increases the formation of $C^*$ and $C^{**}$, worsening the approximation of $x$ with $\gamma_0(t)$ from Proposition 1 (since it is no longer true that $\epsilon_1 \psi(\nu) \ll 1$).

V. RETROACTIVITY ATTENUATION PERFORMANCE

The timescale and sensitivity analysis presented thus far motivates further study of the insulation devices output error dependence on both internal dynamic timescale and individual tunable parameters $X_T$ and $W_T$. This was explored by simulating the unloaded and loaded insulation device using the same input signal with a fixed on-time of 50 minutes and period of 500 minutes, and calculating the mean squared output error given by (24) for changes in phosphorylation timescale and protein concentrations $X_T$, $W_T$:

$$E = \frac{1}{T} \int_0^T \left[ \frac{X^{**}(t) - X^{**}_L(t)}{\max[X^{**}(t)]} \right]^2 dt.$$  

(24)

Changes in timescale illustrate how employing timescale separation allows for retroactivity attenuation. Furthermore, concentrations $X_T$ and $W_T$ are the physical tunable parameters in the system since reaction timescale cannot be easily modulated experimentally. Thus their relation to the retroactivity attenuation property is important for the insulation device’s practical implementation.

The insulation device performance dependence on timescale was evaluated by simulating the normalized system (16)-(22) after modulating the parameter $1/\epsilon$ in (17)-(20). Increasing $1/\epsilon$ leads to faster timescale while decreasing this parameter leads to a slow down in the insulation devices timescale. The output error performance was assessed by calculating the mean squared error in (24) across a range of promoter sites $p_T$, which controls the amount of load placed on the insulation device. The parameter ratio $\epsilon_1 = p_T/X_T$ was kept constant across all simulations by increasing $X_T$ proportionally to $p_T$. This prevents from changes in the slow manifold of system (16)-(22).

We can see from the simulation results shown in Fig. 6 (a)-(b) that the error increases with the amount of promoter sites for a constant $1/\epsilon$. This is expected since an increase in $p_T$ leads to a higher effect of the retroactivity fluxes $s_1$, $s_2$, $s_3$ on the (16)-(22) system dynamics. By contrast, the mean squared error is decreased for an increase in parameter $1/\epsilon$. This implies that the insulation device is able to attenuate higher retroactivity effects as the timescale of the internal processes becomes faster.

The insulation device dependence YPD1 and SKN7 concentration was evaluated by simulating system (9)-(15) assuming $x = X_T/W_T$ is kept constant. By increasing proportionally concentrations $X_T$ and $W_T$, the error due to an increase in the $p_T$ promoter sites concentration was assessed
using expression (24). As before, we see that the error increases as the amount of promoter sites $p_T^*$ is increased, which is shown in Fig. 6 (c)-(d). Also, the error is reduced as the concentrations $X_T^*$ and $W_T^*$ are increased for all values of load, since they are directly related to the retroactivity attenuation property of the insulation device as described in Section III. Having high $X_T^*$ prevents from changes in steady state of system (9)-(15) due to the load by making $\epsilon_1 \ll 1$. Furthermore, in the case where the reactions with timescale $k_1 W_T^*, k_3 W_T^*, k_5 W_T^*$, and $k_6 W_T^*$ are rate limiting (or slowest), increasing $W_T^*$ improves the approximation $\epsilon \approx 0$, as it follows from the definition of $\epsilon$ given in Section III.

VI. CONCLUSIONS AND FUTURE WORK

This work demonstrates that the natural phosphotransfer YPD1/SKN7 cascade can function as an insulation device by employing timescale separation for retroactivity attenuation. This was validated through a singular perturbation analysis, which showed that the fast nature of phosphotransfer reactions allows for the insulation device’s dynamics to be decoupled from the load, in the case the cycle substrate is in sufficient amount. This finding was further validated through a sensitivity analysis in which it was shown that increasing the concentration $W_T^*$ and the spontaneous dephosphorylation rate $k_6$ leads to a reduction in the insulation device output error. It was demonstrated through simulation that reducing the phosphotransfer reaction speed diminishes the retroactivity attenuation ability, and that the physical tunable parameters of the system (concentrations $X_T^*$ and $W_T^*$) could be modulated to enhance retroactivity attenuation. Our results highlight a new role that cascades of fast phosphotransfer reactions may play in natural networks and suggest that they can be employed as insulation devices in synthetic biology to enable modular composition.

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