Control-theoretic methods for biological networks

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***Abstract*— Feedback is both a pillar of control theory and a pervasive principle of nature. For this reason, control-theoretic methods are powerful to analyse the dynamic behaviour of biological systems and mathematically explain their properties, as well as to engineer biological systems so that they perform a specific task by design. This paper illustrates the relevance of control-theoretic methods for biological systems. The first part gives an overview of biological control and of the versatile ways in which cells use feedback. By employing control-theoretic methods, the complexity of interlaced feedback loops in the cell can be revealed and explained, and layered feedback loops can be designed in the cell to induce the desired behaviours, such as oscillations, multi-stability and activity regulation. The second part is mainly devoted to modelling uncertainty in biology and understanding the robustness of biological phenomena due to their inherent structure. Important control-theoretic tools used in systems biology are surveyed. The third part is focused on tools for model discrimination in systems biology. A deeper understanding of molecular pathways and feedback loops, as well as qualitative information on biological networks, can be achieved by studying the "dynamic response phenotypes" that appear in temporal responses. Several applications to the analysis of biological systems are showcased.**

I. Introduction

System-theory and control-theory are particularly well suited for crossing borders among disciplines: practically any phenomenon can be represented as a dynamical system and this allows us to put mathematics into action in the real world, to understand it (explain and unravel the essence of natural behaviours) and to improve it (by designing controllers that suitably govern behaviours). In particular, many tools from control theory that have been developed to tackle crucial problems in engineering can be employed, if suitably adapted, to address problems in the life sciences and in biology. Indeed, the mathematical exactness of control-theoretic tools can not only streamline technological progress, but also help us gain more insight into the complex, fascinating and apparently haphazard phenomena occurring in biology. With the increase of complexity, the greater availability of huge amounts of data, and the growing attention for the contributions that can be achieved thanks to interdisciplinary approaches, new disciplines are arising that tackle fundamental and complex problems in biology and in the life sciences with a system-level approach [1], [65], [93]. In particular, systems biology aims at analysing the behaviour of natural systems to unravel the design principles underlying their complex dynamics and to mathematically explain their peculiar properties. Conversely, synthetic biology aims at engineering artificial biological systems *de novo*, so that they exhibit the desired dynamic behaviour.

The control community has shown a growing interest in this area and has proven that, by using control-theoretic tools, it is possible to streamline both the analysis and the synthesis of biological systems. Why are control-theoretic methods so effective? Because the fundamental concept of feedback is at the core of control theory and, at the same time, is one of the most pervasive principles of nature. Living beings rely for their survival on a huge amount of interlaced feedback loops that regulate biological functions. Hence, control-theoretic tools are naturally well tailored to analyse and design feedback loops, also in biology.

Here we give a broad overview of interesting developments in the study of biological networks enabled by control-theoretic methods. Although control-theoretic approaches have led to significant insight, the complexity of biological systems is such that new tools need to be developed or refined to be able to successfully address many open problems; future challenges are also outlined, to stimulate novel research ideas. The paper is organised in three parts, summarised in the next subsections, which focus on diverse and complementary aspects to propose a well-rounded illustration of control-theoretic methods for biological systems.

A. The versatile ways in which cells use feedback loops

In 1939, Walter Cannon wrote in his book *The wisdom of the Body*: "The living being is an agency of such sort that each disturbing influence induces by itself the calling forth of compensatory activity to neutralize or repair the disturbance". Since this remarkable statement that postulates the use of feedback control to support life, we have come to appreciate that the use of feedback loops is ubiquitous at every level of biological organization, from the gene to the ecosystem. Section II, by Hana El-Samad, focuses on examples that demonstrate the versatile roles and functions that feedback loops play in cells, and also discusses the need for tools, technologies and mathematical frameworks for studying biological feedback control. First, we discuss examples of the use for layered feedback loops to produce oscillations and biological rhythms. We describe the use of mathematical tools that led to establishing and analyzing

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these phenomena. We also discuss the use of feedback in producing multi-stability, with examples illustrating biological switches. Then, we discuss more nuanced use of feedback to modulate quantitatively the activity of biological pathways. We present examples that include the use of feedback to dynamically shift the dose response of a pathway or to modulate the variability of pathway activity to induce different distributions of behaviors across a population. Finally, we discuss available tools for studying and measuring feedback activity in cellular pathways, and illustrate the difficulty inherent in these endeavors. We motivate the need for new tools, both experimental and computational, to study biological feedback. As a closing statement, we pose the nascent challenge of designing feedback control systems using biomolecules for many biotechnological applications.

B. Biological phenomena, mathematical explanations

The control community has developed many mathematical tools that are tailored to face important problems in engineering, but can also be successfully adopted to address problems in systems biology, since feedback is both a fundamental concept at the core of control theory and an ubiquitous feature in biology: no living being could survive without the myriad of entangled feedback loops that rule its biological functions. When adopting control-theoretic tools for the study of biological problems, it is crucial to deal with the huge complexity of biological systems by means of simplifications that allow us to nicely capture the essence of the system and describe it in a framework that allows for a significant mathematical analysis; and crucial to convince biologists that simplifications are worth adopting because, together with non-trivial mathematical tools, they enable a deeper qualitative and quantitative understanding of biological phenomena. Section III, by Franco Blanchini and Giulia Giordano, discusses the use of the mathematical language to address, formulate and solve problems in biology, which needs to be always supported by an effective communication between mathematicians and biologists. We investigate the role of models well-suited to capture the inherent uncertainty in biological systems, focusing on the concept of robust and structural properties. We consider techniques for system simplification based on time-scale separation and on lumping subsystems with special properties (monotonicity, or positivity of the impulse response) into *aggregate elements*. Then, we overview mathematical tools and notions that have been shown to be useful in systems biology, ranging from graph theory, differential equations, BDC–decomposition to frequency methods, from degree theory to Lyapunov and parametric robustness tools. We show how the presented methods have been actually applied to the analysis of biological systems, including the structural stability of biochemical networks and the structural steady-state influence.

C. Dynamic response phenotypes as tools for model discrimination in systems biology

Understanding the roles of signal transduction pathways and feedback loops is fundamental in both systems and synthetic biology. Section IV, by Eduardo D. Sontag, discusses certain types of network qualitative information that can be gleaned from "dynamic phenotypes", which encompass both the transient characteristics of temporal responses and the use of rich classes of probing signals beyond step inputs. We focus on three examples: fold-change detection, non-monotonic responses, and subharmonic oscillations. An ubiquitous property of sensory systems is "adaptation": a step increase in stimulus triggers an initial change in a biochemical or physiological response, followed by a more gradual relaxation toward a basal, pre-stimulus level. Adaptation helps maintain essential variables within acceptable bounds and allows organisms to readjust themselves to an optimum and non-saturating sensitivity range when faced with a prolonged change in their environment. Certain adapting systems, ranging from bacterial chemotaxis pathways to signal transduction mechanisms in eukaryotes, enjoy a remarkable additional feature: scale invariance or "fold change detection", meaning that the initial, transient behavior remains approximately the same even when the background signal level is scaled ("log sensing"). We review the biological phenomenon, and formulate a theoretical framework leading to a general theorem characterizing scale invariant behavior by equivariant actions on sets of vector fields that satisfy appropriate Lie-algebraic nondegeneracy conditions. The theorem allows one to make experimentally testable predictions, and we discuss the validation of these predictions using genetically engineered bacteria and microfluidic devices, as well their use as a "dynamical phenotype" for model invalidation. Systems described by order-preserving dynamics are called "monotone systems". Such systems can be shown to have monotone response properties when starting from steady states: a nondecreasing input can never give rise to a biphasic response, for example. We briefly review some of this theory and show as an example how this tool can be used to invalidate a published model of *M. tuberculosis* stress response (hypoxic induction pathway). One challenging question in systems biology is that of comparing different architectures for perfect adaptation. For example both incoherent feedforward loops (IFFLs) and integral feedback systems give rise to perfect adaptation and, in some configurations, scale invariance. Recent work has proposed the use of periodic signals to discriminate between these models. We review a theoretical result showing that feedforward loops and monotone systems both lead to entrainment, but nonlinear feedback architectures (such as nonlinear integral feedback) may lead to period doubling bifurcations and even chaos. This result is illustrated through experimental work with *C. elegans* AIA interneurons, in which odor-evoked intracellular Ca2+ response signatures, to periodic on-off pulses of diacetyl, display subharmonic behavior at high forcing frequencies.

II. Biological control: The versatile ways in which cells use feedback loops

The idea that feedback loops are at the fundamental core of life is not new. In fact, researchers of physiology and

anatomy, as well as medical doctors, have long explored and endorsed the idea of feedback in their attempts to understand phenomena such as homeostasis. Concepts of feedback also percolated into the thinking of molecular biologists since the dawn of that field. For example, a review article, titled "Biological Feedback Control at the Molecular Level" [15] highlights what perhaps are the first two established examples of metabolic feedback regulation at the molecular level. Since then, hundreds (if not thousands) of feedback loops have been identified in cellular networks, and have been shown to play critical role of virtually every aspect of biological function, from the response to stress conditions [59], [51], to developmental pathways [21], pathogens and disease [85], symbiotic relationships of organisms to reside within their eukaryotic hosts [98], and for the synthesis of [83], resistance to [33], and persistence in the presence of [17] antibacterial agents.

More specifically, the role of negative feedback to establish robust and malleable operation has been explored in a number of biological systems. Additionally, many examples of feedback strategies that are recognizable by control practitioners have been documented in cellular regulation, including integral control. For example, integral feedback was demonstrated to be at work in the *E. coli* chemotaxis circuit, where the percentage of active CheY proteins that are responsible for regulating the bacteria's tumbling frequency adapts perfectly to step-changes in chemoattractant concentration, maintaining the system's sensitivity to new concentration changes [20], [18], [3], [100]. This was termed *Biochemical Perfect Adaptation*. Other examples of such behavior include the perfect adaptation of the nuclear enrichment of the *S. cerevisiae* MAPK Hog1 after step changes in osmolarity [73], and the control of blood calcium concentration in mammals [42]. In both cases, integral control was shown to be the structural underpinning of this adaptation and many ideas from classical feedback theory, such as the internal model principle [47], were productively exploited to accelerate our understanding of these systems. In addition, many studies explored the role of negative feedback loops as a fundamental requirement for oscillatory behaviors and biological rhythms [74], [45], and positive feedback loops to produce multi-stable systems that can accommodate processes such as differentiation [46]. Here, we argue that these biological functions of feedback, while crucially important, cover only a small portion of a long list. We suspect that there is still a large and largely unexplored canon of roles that biological feedback loops may play. We discuss an example, and then pose three questions for the field. The examples are intently not described in great mathematical details, and the questions are posed in simple form. We hope that this accessible description will be read and understood by a wide slice of the control community, perhaps tempting them to embark on the study of biological feedback.

A. Positive feedback and switches in differentiation programs

The maturation of Xenopus frog oocytes has long been used as a prototype model for cell fate induction [99], with the immature oocyte representing the default fate and the mature oocyte representing the induced fate. Transition from immature to mature (maturation) is a progesterone-induced resumption of meiosis I. This transition is irreversible with a well-defined threshold: Oocytes treated with a sufficient concentration of progesterone undergo maturation, whereas those treated with a lower concentration do not. At the biochemical level, a large progesterone stimulus up-regulates the translation and gradual accumulation of the protein kinase Mos through a largely unidentified pathway. Mos is the essential initiator of meiosis; it signals through the mitogen-activated protein kinase (MAPK) cascade (composed of Mos, the MAPK kinase MEK1, and p42 MAPK) and promotes the activation of the cyclin-dependent kinase 1(Cdk1) cyclin B complex. Numerous positive feedback loops have been identified in the p42 MAPK/Cdc2 network of an oocyte. For example, Mos activates p42 MAPK through the intermediacy of MEK, and active p42 MAPK feeds back to promote the accumulation of Mos (Fig. 1). This positive feedback was the subject of many studies, which established that it is the engine for the sharp and irreversible switching behavior of this maturation system. Analyses that spanned a decade established both computationally and experimentally the conditions under which this feedback loop can generate a bistable system of this sort [99], [46]. Specific nonlinearities, such as a so-called Hill function, were implicated in the feedback to produce bistability, and the plausibility of their existence at work in this system was also experimentally probed. This (now) textbook material was an influential result, and a generation of biologists were trained with the idea that positive feedback loop is necessary for producing multistability, looked for it and found it in many developmental systems. Irrespective of the details, the computational model that conveyed the concept and ushered its experimental investigation was a simple one, capturing the essence of the phenomenon (shown in Fig. 1(c)).

B. Layers upon layers of feedback

Subsequent investigations found many other feedback loops wrapped around the same Oocyte maturation positive feedback loop switch. For example, we discovered that glycogen synthase kinase $GSK-3\beta$, which prevents progesterone-induced translation of Mos, is itself inhibited by M-phase feedback (Fig. 1) [63]. This discovery brought to the forefront the presence of another positive feedback loop, formed by the aggregation of two negative interactions. Disabling this feedback loop revealed a remarkable phenotype–the Oocyte still maturated in all or none fashion, but at a much lower concentration of progesterone stimulus (Fig. 2). We concluded through simple analyses of an updated phenomenological model (Fig. 1(c)), supported by careful experimentation, that this stratification of feedbacks also led to a stratification of their functions. The original positive feedback established the switch, and the discovered positive feedback involving $GSK-3\beta$ established its flipping threshold (Fig. 2), moving the dose response without changing its shape. By impinging on this threshold-setting

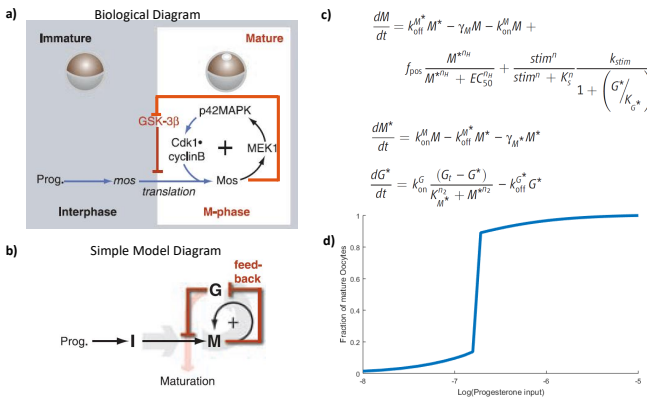

Fig. 1: Positive Feedback loops in the Oocyte maturation switch. (a) Biological diagram showing the canonical P42-MAPK positive feedback loop (black) and the double-negative feedback loop implemented through $GSK-3\beta$ (orange). (b) A diagram with a simplified set of interactions that are modeled. (c) Model equations for diagram in (b). M is newly synthesized Mos; M^* is an aggregate of stabilized Mos and all Mos-dependent signaling; G^* is active $GSK-3\beta$; k_{on} designates activation terms; k_{off} designates inactivation terms; γ designates destruction; n terms are theoretical Hill coefficients. (d) Fraction of mature Oocytes (fraction of M^* to total M) as a function of stimulus (progesterone). Results adapted from [63].

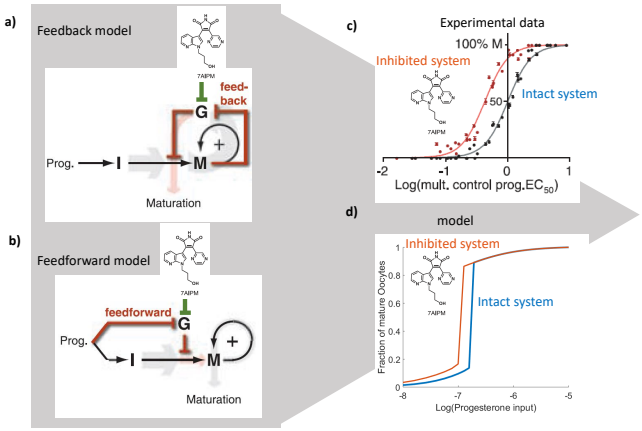

Fig. 2: Inhibition of double-negative $GSK-3\beta$ feedback loop shifts the dose response of maturation as a function of the progesterone stimulus, making the switch occur at lower level of stimulus. (a) Feedback model block diagram. $GSK-3\beta$ double negative feedback loop can be disabled by addition of the inhibitor 7AIPM. (b) A plausible feedforward model involving $GSK-3\beta$. (c) Fraction of mature Oocytes as a function of progesterone stimulus determined experimentally in wild type and following inhibition of $GSK-3\beta$ with $7AIPM$ to disable the double negative $GSK-3\beta$ feedback loop. (d) Fraction of mature Oocytes as a function of stimulus (progesterone) for models following inhibition of G. Both feedback and feedforward loop architectures can produce a similar shift of the dose response observed in the data. Results adapted from [63].

loop, environmental variables can move the tipping point of the switch accordingly, therefore tuning the maturation of the Oocytes according to their surroundings and allowing them to integrate information from different inputs such as the availability and abundance of amino acids [63]. This picture is almost certainly partial. Many other feedback loops decorate this system, and to date, their function remains obscure.

C. Some (hopefully) thought-provoking questions

1) Why feedback? Is it the only way to achieve this function?: The use of feedback in technological systems is mainly studied and understood in terms of the ability of feedback regulation to ensure robust stable operation in the presence of disturbances, tracking references, and shaping the dynamic response of a system. Therefore, it is perhaps self-evident why negative feedback would be abundantly used to achieve robust operation, including disturbance rejection and setpoint tracking, in biological systems. However, in the absence of abundant technological examples, it is far from clear why feedback loops would be used to achieve purposes such as sensitization or desensitization of a pathway based on its environment (e.g. shifting the dose response discussed above). Is feedback in this capacity superior to a plethora of other solutions that can be imagined or designed to fulfill this function? If so, in what respect? If not, why did it preferentially evolve? In fact, we show in Fig. 2 that a feedforward architecture involving $GSK-3\beta$ can achieve modulation of the dose response of the maturation system, in the same way that a feedback loop does. Understanding the properties, constraints, and tradeoffs involved in the use of feedback for implementing different non-canonical functions will enable more predictive and compelling understanding of biology, as well as the extension of the feedback control field in new and exciting dimensions.

2) How many feedback functionalities in biology?: The biological literature is full of examples of feedback loops, most of which remain unstudied and their function largely unassigned. It is therefore natural to ask whether there is a finite set of functionalities that feedback loops have evolved to fulfill in biological networks and explore how this list can be rigorously compiled and vetted. For every functionality, is there a particular physical implementation that preferentially evolved? If so, what are its general properties? Even if it is impossible to build such an exhaustive list, it might be interesting to clearly state the broader and most abundant categories, facilitating the way for their careful examination.

3) Are existing tools in feedback and dynamical systems theories sufficient to capture and analyze the biological use of feedback?: There are probably thousands of papers in the control literature prescribing analysis methods or design strategies for, or proving theorems about, important properties such as stability of a controlled system. There are no such papers or mathematical results for how, for example, to design a biological system with a prescribed dose response, devise a controller that can achieve this dose response, or ascertain any of its properties. While arguably not of great relevance to technological systems, such a property is essential for a biological system. Perhaps, dose-response characteristics can be re-cast and embedded in existing theories, or maybe we need new investigations and results. It is interesting to consider a few such properties that are biologically relevant, and attempt to extend existing theories to study them in their own right, as valid and important mathematical objects.

III. Understanding biological phenomena with control-theoretic tools

Understanding the design principles that rule the behaviour of biological systems is one of the most fascinating research challenges that we are currently facing.

A fundamental question is whether these principles can be described in mathematical terms, or at least to which extent mathematics is a proper language to describe them [1]. Mathematics is a beautiful language to explain biology from the point of view of mathematicians, physicists and engineers, who traditionally use this language to formulate and solve problems in their disciplines. Yet, is mathematics equally useful for biologist to solve *problems in their discipline*? The interactions between mathematics and biology have recently become deep, leading to the new field of systems biology, characterised by an interdisciplinary approach [1], [65], [93]. Yet there are some risks to avoid: on the one hand, mathematical biology might be seen as an excuse to study wonderful mathematical problems, claiming that they have relevant applications, without contributing much to the solution of actual biological problems; on the other hand, the mathematical language might be seen as not worth adopting by biologists, while the standard statistical approaches for data analysis or extensive numerical simulations [37] might be deemed sufficient to get biological insight.

Assuming that all parties have agreed on the benefit of dealing with problems arising in the biological world using a mathematical language, thanks to the many successful examples in the literature [6], [35], [39], [43], [68], [62], [76], the adventure can begin. Clearly, a mathematical approach requires models [34], [41], which can be either based on physical-chemical laws and principles, or phenomenological (based on macroscopic empirical relationships, mathematically described based on experimental data). In both cases, the models we build must be valid, effective and detailed enough to make the analysis meaningful, but simple enough to enable a mathematical analysis:

- to be a realistic representation of a biological phenomenon, a model cannot be too simple;
- to be useful to reveal the essential principles on which the phenomenon relies, a model cannot be too complex.

Is then the common investigation space empty? We believe that it is not. Of course, in the trade-off between essential and detailed models, the choice depends on what we mean by "explaining/understanding the design principles".

A very complicated model can provide some answers: for instance, resorting to numerical simulations, systems of many (partial) differential equations can be solved to check whether the numerically-determined solutions fit the experimental data; this allows the validation of the model, which can then be adopted to make predictions, as in the case of Insulin models (see [75] for a survey). However, this is not the main focus of this section. Our aim and hope is to show that mathematics can reveal and explain simple, essential and "universal" laws – such as the fact that peculiar types of *motifs* in biological systems generate a particular dynamic behaviour, regardless of the value of the parameters [1], [2] – when suitably tailored tools and approaches are adopted.

Fig. 3: Positive (left) and negative (right) loops of two elements: hammerhead arrows represent inhibition, while pointed arrows represent activation.

A. Ask the right questions – and find their answers

Let us start with an example. Assume that a certain system $\mathcal{S}(p)$ of differential equations, depending on the parameter vector p, can represent a genetic regulatory network, and denote as $\hat{p}$ a particular choice of the parameters, fitted based on experimental data.

Q1) Can we prove that the system $\mathcal{S}(\hat{p})$ is stable?

Is question Q1 of any interest? The answer from a biological perspective is often no (unless we are considering special problems such as biological oscillators or bistable systems), because most of the times, if the biological entity exists and survives, it must be stable. Is then any stability analysis useless? Let us change our question as:

Q1 – revised) Why is the system $\mathcal{S}(p)$ stable even under huge variations of p?

This question is definitely more interesting. For instance, in negative feedback loops time-scale separation is a fundamental property to ensure stability [1]; this is a general principle and does not depend on a specific choice of the parameter values. Investigating the astounding robustness of biological systems [4], [18], [66], [67], [86] by means of a *structural analysis* [22], [48] is often deeply interesting.

Definition 1: Let $\mathcal{C}$ be a class of systems and $\mathcal{P}$ be a property pertaining to such a class. Given a family $\mathcal{F} \subset \mathcal{C}$ the property $\mathcal{P}$ is *robustly verified* by $\mathcal{F}$, in short *robust*, if it is satisfied by each element of $\mathcal{F}$; $\mathcal{P}$ is *structurally verified* by $\mathcal{F}$, in short *structural*, if the family $\mathcal{F}$ is not specified resorting to quantitative parameter bounds.

Example 1: Consider the two matrices

$$A_1 = \begin{bmatrix} -a_1 & a_2 \\ a_3 & -a_4 \end{bmatrix} \quad \text{and} \quad A_2 = \begin{bmatrix} -a_1 & -a_2 \\ a_3 & -a_4 \end{bmatrix},$$

where a_i, $i = 1, \ldots, 4$, are unknown *positive* coefficients. Matrix A_1 is robustly Hurwitz for proper numerical bounds $a_i^- \leq a_i \leq a_i^+$. Matrix A_2 is structurally Hurwitz: no bounds are required, as long as the parameters are positive. This corresponds to a neat biological law: the positive feedback loop of two self-degrading species activating each other (as in A_1) can be unstable, while the negative feedback loop of two self-degrading species, an inhibitor and an activator (as in A_2), is always (structurally) stable (cf. the graph representation in Fig. 3).

A massive literature has proposed many different types of models for systems biology. We will here consider models represented by Ordinary Differential Equations of the form

$$\dot{x}(t) = Sg(x(t)) + g_0, \tag{1}$$

where $x \in \mathbb{R}^n$ is a vector of species concentrations, $S \in \mathbb{Z}^{n\times m}$ is a matrix representing an *interconnection structure* (e.g., in chemical reaction networks [7], the reactions stoichiometry), $g : \mathbb{R}^n \to \mathbb{R}^m$ is a vector function whose components are monotonic in each argument (in chemical reaction networks, the vector of reaction rates) and $g_0 \in \mathbb{R}^n$ is an external input.

Example 2: Consider the chemical reaction network

$$\emptyset \xrightarrow{a_0} A, \quad \emptyset \xrightarrow{b_0} B, \quad B \xrightarrow{g_b} \emptyset$$
$$A + B \xrightarrow{g_{ab}} C \xrightarrow{g_c} D, \quad A + D \xrightarrow{g_{ad}} \emptyset$$

Species A and B are supplied with flow a_0 and b_0, respectively, and combine at rate g_{ab} to produce C, which converts into D at rate g_c. B has a self-degradation rate g_b and finally D combines with A, thus repressing its own production, at rate g_{ad}. Species concentrations are denoted with the corresponding lowercase letters and evolve according to the equations

$$\begin{bmatrix} \dot{a} \\ \dot{b} \\ \dot{c} \\ \dot{d} \end{bmatrix} = \begin{bmatrix} -1 & 0 & 0 & -1 \\ -1 & -1 & 0 & 0 \\ 1 & 0 & -1 & 0 \\ 0 & 0 & 1 & -1 \end{bmatrix} \begin{bmatrix} g_{ab}(a,b) \\ g_b(b) \\ g_c(c) \\ g_{ad}(a,d) \end{bmatrix} + \begin{bmatrix} a_0 \\ b_0 \\ 0 \\ 0 \end{bmatrix},$$

which can be cast in the framework of (1), where the stoichiometric matrix S represents the system structure and all the reaction rate functions are monotonically increasing in their arguments.

For reasons that will become clear later on, we notice that the Jacobian of a system of the form (1), evaluated at *any* point x, admits the BDC-decomposition [23], [49], [48]

$$J(x) = B\Delta(x)C,$$

where $\Delta(x)$ is a diagonal matrix carrying on the diagonal the absolute value of the partial derivatives of the vector function g, while B and C are constant matrices that capture the system interconnection structure. In Example 2,

$$B = \begin{bmatrix} -1 & -1 & 0 & 0 & -1 & -1 \\ -1 & -1 & -1 & 0 & 0 & 0 \\ 1 & 1 & 0 & -1 & 0 & 0 \\ 0 & 0 & 0 & 1 & -1 & -1 \end{bmatrix}, \; C = \begin{bmatrix} 1 & 0 & 0 & 0 \\ 0 & 1 & 0 & 0 \\ 0 & 1 & 0 & 0 \\ 0 & 0 & 1 & 0 \\ 1 & 0 & 0 & 0 \\ 0 & 0 & 0 & 1 \end{bmatrix}$$

and $\Delta = \operatorname{diag}\left\{\frac{\partial g_{ab}}{\partial a}, \frac{\partial g_{ab}}{\partial b}, \frac{\partial g_b}{\partial b}, \frac{\partial g_c}{\partial c}, \frac{\partial g_{ad}}{\partial a}, \frac{\partial g_{ad}}{\partial d}\right\} \succ 0$. Hence, the system linearised around any equilibrium point $\bar{x}$, such that $0 = Sg(\bar{x}) + g_0$, has the form

$$\dot{z}(t) = B\Delta(\bar{x})Cz(t) \tag{2}$$

(*local BDC-decomposition*), where $z(t) = x(t) - \bar{x}$ is the shifted variable. The nonlinear system can also be equivalently rewritten, according to the *global BDC-decomposition* [23], [24], [48], as

$$\dot{z}(t) = BD(z(t))Cz(t), \tag{3}$$

where $D(z)$ is a diagonal matrix with strictly positive functions on the diagonal. The shifted system (3) is obtained by exploiting the formula [64]

$$g(x) - g(\bar{x}) = \left(\int_0^1 \frac{\partial g}{\partial x}[\sigma(x-\bar{x}) + \bar{x}]d\sigma\right)(x - \bar{x}),$$

and can be studied in a differential inclusion framework [5], [12], [23].

B. Boundedness, stability, instability, bistability, oscillations

When analysing a system, a fundamental property is the boundedness of the solutions. Unbounded behaviours in nature are typically due to unbounded (exponential) growth, such as that of bacteria. To assess boundedness, the theory of invariant sets and of Lyapunov functions can be adopted [5], [23], [25]. To investigate the boundedness of biochemical systems, we can exploit their positivity: if $x(0) \geq 0$ (component-wise), then $x(t) \geq 0$ for all $t > 0$.

Theorem 1: System $\dot{x} = f(x)$, with f regular enough, is positive iff, for all k, $f_k(x_1, \ldots, x_{k-i}, 0, x_{k+i}, \ldots, x_n) \geq 0$ for all $x_i \geq 0$, if $i \neq k$.

Once boundedness of the solutions has been established, it has a fundamental consequence: the existence of an equilibrium [79], [95].

Theorem 2: Given the system $\dot{x} = f(x)$, with f regular enough, assume that all its solution are ultimately bounded in a convex and compact set $\mathcal{C}$ with a non-empty interior. Then, the system admits an equilibrium point in $\mathcal{C}$.

In some cases, we are interested in finding conditions on the parameters that ensure the existence of an equilibrium.

Consider again Example 2. The equilibrium values $\bar{b}$ and $\bar{c}$ are fixed by $g_c(\bar{c}) = a_0/2$ and $g_b(\bar{b}) = b_0 - a_0/2$. Then $\bar{a}$ is deduced from the equation $g_{ab}(a, \bar{b}) = b_0 - g_b(\bar{b})$ and $\bar{d}$ is derived from $g_{ad}(\bar{a}, d) = g_c(\bar{c}) = a_0/2$. Under proper assumption (unboundedness and monotonicity of the functions in g), the equilibrium exists, but we must have $b_0 - a_0/2 > 0$, hence $2b_0 > a_0$, because $g_b(\bar{b})$ must be positive. If this condition fails, the solution is unbounded.

Once the existence of equilibria is ensured, then the issues are their number and their stability.

Assume for brevity that the system admits a positively invariant convex and compact set $\mathcal{C}$ with non-empty interior. Hence, there exists an equilibrium in $\mathcal{C}$ and we assume that there are none on the boundary. We call the equilibrium $\bar{x} \in \text{int}(C)$. Is it unique? An answer can come from the *topological degree theory* [58], [71].

Theorem 3: Given the system $\dot{x} = f(x)$, with f regular enough, assume that it admits m equilibria $\bar{x}^{(k)}$, $k = 1, \ldots, m$, which are all in the interior of $\mathcal{C}$, and that the Jacobian is non-singular in all of them. Then

$$\sum_{k=1}^{m} \operatorname{sign}\left[\det(-J(\bar{x}^{(k)}))\right] = 1.$$

This theorem has interesting consequences. If an equilibrium $\bar{x}$ is unstable due to a single real positive eigenvalue (while all the other eigenvalues have negative real part), then the characteristic polynomial has a negative known term. On the other hand, the known term is $\det(-J)$. In view of Theorem 3, this equilibrium cannot be unique and at least

other two must exists; under suitable assumptions, they are exactly two and stable. This is a typical situation when a local stability analysis can reveal a global property such as bistability. Conversely, if the equilibrium is unstable due to precisely two complex eigenvalues with positive real part, then $\det(-J)$ is positive and this situation is compatible with the uniqueness of the equilibrium. This type of instability is typically associated with an oscillatory behaviour.

In general, it is possible to discriminate the type of instability associated with either real or complex non-negative roots as follows. Consider a system of the form

$$\dot{x}(t) = f(x(t), \theta),$$

where $\theta \in \Theta$ is a parameter vector. Assume that an equilibrium $\bar{x}_\theta$ exists for all $\theta \in \Theta$ and that, for some nominal value θ^*, the Jacobian computed at the equilibrium is Hurwitz. What type of transition to instability can occur when θ is changed? [26] If the transition is due to a single real root that crosses 0 to become positive, at some critical value θ_{cr}, then the Jacobian determinant must be 0. So, the critical values can be found by analyzing $\det[J(\theta)]$. Conversely, if a pair of complex roots crosses the imaginary axis, then $\det[-J] > 0$. Finding critical values is harder: we must typically consider the characteristic polynomial $\det[\lambda I - J(\theta)]$ and, e.g., compute the Routh-Hurwitz table.

Uniqueness of the equilibrium is guaranteed if the Jacobian is non-singular for all x in the invariant set $\mathcal{C}$ [101]. An algorithm to check structural non-singularity for Jacobian matrices that admit the BDC-decomposition will be discussed later on.

Concerning structural stability, the Zero-Deficiency Theorem [44] is the most famous result. For a network of the form $\dot{x} = Sg(x)$, the *stoichiometric compatibility class* [7] is the positively invariant set

$$SCC(x(0)) = x(0) + \mathrm{Im}\,[S]\,.$$

Assume that the rate functions follow the law of mass action [57], hence the reaction rates are monomials: a reaction of the form $mA + nB \rightharpoonup C$ has rate $g_{ab}(a, b) = ka^m b^n$ (constant terms can be accommodated by assuming ka^0 for the input flow $\emptyset \to A$). If the reaction network has zero deficiency (a quantity that depends on the network interconnection topology) and is weakly reversible, then it admits the entropy as a Lyapunov function [44], [53], hence it admits in each stoichiometric compatibility class a single equilibrium point that is locally stable. The conditions to be tested are structural, independent of parameter values.

Other stability criteria have been suggested based on special classes of Lyapunov functions: polyhedral [23], [50], [80] and piecewise-linear-in-rate [5]. When the network admits a polyhedral Lyapunov function (as in the Example 2) and its Jacobian is structurally non-singular, no matter how the smooth and increasing function g is taken, the equilibrium point, if it exists, is globally stable [24].

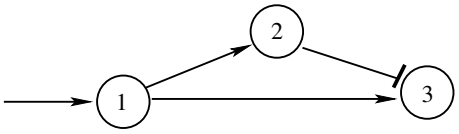

Fig. 4: The incoherent feed-forward loop motif (IFFL).

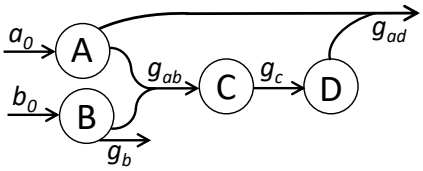

Fig. 5: The flow graph corresponding to the chemical reaction network in Example 2.

C. Graph representation

The graph representation of biochemical systems is a fundamental communication tool to enable mutual understanding between biologists and mathematicians [22], [77]. As an example, consider the incoherent feed-forward loop motif (IFFL) [1], [2]. Looking at Fig. 4, a biologist immediately understands the mechanism: node 1 has a positive (activating) effect on node 3, which is compensated by the negative (inhibitory) signal passing through node 2 (with a belated effect if the reactions have comparable rates, since two steps are required for the inhibitory signal to reach its target). Anyone used to the mathematical language immediately understands that the graph can be associated with the equations

$$\begin{cases} \dot{x}_1 = g_u(u) - k_1 x_1 \\ \dot{x}_2 = g_{21}(x_1) - k_2 x_2 \\ \dot{x}_3 = g_{31}(x_1) + g_{32}(x_2) - k_3 x_3 \end{cases}$$

where g_{21} and g_{31} are increasing functions, while g_{32} is decreasing. Typical choices are the Hill functions

$$g_{inc}(x) = \frac{qx^m}{p + x^m}, \qquad g_{dec}(x) = \frac{q}{p + x^m}.$$

To preserve this crucial communication channel, any graph description should be easy to translate in equations and vice-versa. There are mainly two types of graph representations:

- flow or reaction graph (where the arcs are flows);
- signal graph (where the arcs are signals).

Fig. 4 shows a *signal graph*, while the *flow graph* associated with the chemical reaction network in Example 2 is in Fig. 5. Graph analysis of biological networks is a very well investigated subject [40], [72], [77]. As an example, a bistable behaviour is typically associated with the presence of a positive cycle in the network graph, while an oscillatory behavior requires the presence of a negative cycle (see [26], [28] for a more general classification for systems having both positive and negative cycles).

D. Influence and adaptation

Identifying *structural influences* can be very useful to analyse systems and invalidate models. An input u has a

structurally positive (negative, zero) influence if, no matter how the parameters are chosen, the variation in the steady-state value of the output y due to a step input u is positive (negative, zero); the influence is indeterminate if it does depend on the parameter values. For systems admitting a BDC-decomposition, under stability assumptions, we can assess steady-state influences by looking at the steady-state output of the system

$$\dot{z} = B\Delta C z + Eu, \quad y = Hx,$$

where $u > 0$ is constant, and checking if the sign of

$$\phi(\Delta) = \det \begin{bmatrix} -B\Delta C & -E \\ H & 0 \end{bmatrix}$$

is the same for all $\Delta = \text{diag}\{\Delta_1, \ldots, \Delta_m\}$ with $\Delta_i > 0$. In fact, the steady-state output variation is $\delta\bar{y} = [\phi(\Delta)/\psi(\Delta)]\,\delta\bar{u}$, where $\psi(\Delta) = \det(-B\Delta C) > 0$, in view of the assumed stability, and the input variation is $\delta u > 0$.

Theorem 4: [49] The function $\phi(\Delta_1, \Delta_2, \ldots, \Delta_m)$ is positive (negative) for all $\Delta_k > 0$ if and only if $\phi(1, 1, \ldots, 1) > 0$ (< 0) and

$$\phi(\hat{\Delta}_1, \hat{\Delta}_2, \ldots, \hat{\Delta}_m) \geq 0 \quad (\leq 0)$$

for all possible choices $\hat{\Delta}_k \in \{0, 1\}$, while it is zero if and only if it is zero for all choices. It is undetermined otherwise.

The result relies on the fact that $\phi(\Delta_1, \Delta_2, \ldots, \Delta_m)$ is a multi-affine function of the Δ_k, and a multi-affine function defined on a hyper-rectangle reaches its minimum and maximum on the vertices. A different approach for this type of investigation has been proposed in [92].

An interesting outcome is the influence matrix [49], whose (i, j) entry is the signed influence resulting from applying an additive input to the equation of species j and taking species i as output. The structural influence matrix for Example 2 is

$$\Sigma = \begin{bmatrix} 1 & -1 & -1 & -1 \\ -1 & 1 & 1 & 1 \\ 1 & 0 & 1 & -1 \\ ? & 1 & 1 & 1 \end{bmatrix}. \tag{4}$$

A zero steady-state influence, as in the case of Σ_{32}, is associated with perfect adaptation, a remarkable feature of some living systems [1], [31], [100]. The only indeterminate sign, which depends on the choice of Δ, is Σ_{41}.

Remark 1: To assess the structural non-singularity of the matrix $B\Delta C$, the same vertex-type result can be exploited. Indeed, also $\psi(\Delta_1, \ldots, \Delta_m) = \det[-B\Delta C]$ is multi-affine. Hence it is positive (negative) iff it is nonnegative (nonpositive) on all vertices and $\psi(1, \ldots, 1) > 0$ (< 0). In view of multi-affinity, to check Hurwitz stability of $B\Delta C$, given bounds of the form $\Delta^- \leq \Delta \leq \Delta^+$, we can adopt value-set techniques, well established in the control literature [19], and in particular the Mapping Theorem. These techniques extend in a remarkable way to uncertain systems well established frequency-domain methods for the stability and harmonic analysis, in particular those based on the Nyquist plot.

Structural influences provide a remarkable tool to invalidate biological models: given a model, if we assess the presence of a structurally signed influence and the predicted sign is contradicted even by a single experimental observation, then the model is invalidated. The importance of model invalidation will be stressed also in Section IV.

E. Time-scale separation

Time-scale separation is a fundamental ingredient in the analysis of biological networks. In general, chemical reactions in the same network may have completely different rates, which allows for approximations: the most famous one is due to Michaelis and Menten. Consider the reaction

$$E + S \underset{\beta}{\overset{\alpha}{\rightleftharpoons}} X \overset{\gamma}{\rightharpoonup} E + P$$

where $X = [ES]$ is an intermediate species, E an enzyme, S the substrate, P the product. We can apply a singular perturbation [64] argument: we assume that the reversible reaction is much faster than the other (hence $\gamma \approx 0$) and consider the steady state relation $\alpha es = \beta x$. Then, since $e + x = e_0 = \text{const}$, we have $\alpha e_0 s = \beta x + \alpha x s$ and we can derive the approximate production speed of p

$$\dot{p} = \gamma x = \frac{\gamma e_0 s}{\beta/\alpha + s} = \frac{V_{max} s}{K_D + s}.$$

A similar argument can be used to investigate multi-molecular reactions. The reaction $2A + B \overset{k}{\rightharpoonup} C$ in practice occurs in two steps, because it is unlikely that the three molecules (A, A, B) meet at the same instant. Hence

$$A + B \underset{\beta}{\overset{\alpha}{\rightleftharpoons}} X, \quad X + A \overset{\gamma}{\rightharpoonup} C.$$

However, if the reversible reaction is faster, then $\alpha ab = \beta x$ and the production of C is

$$\dot{c} = \gamma x a = \frac{\gamma\alpha}{\beta} a^2 b = k_{ab} a^2 b,$$

which is the accepted formula for trimolecular reactions.

Time-scale separation is important in many other contexts, for instance to explain the robust stability of negative loops [1, Section 6.5].

F. Complexity and aggregation

Given the huge complexity of biological networks, it is crucial to find systematic methods for simplification. A possible strategy is to identify subsystems of nodes that can be considered as a single *aggregate element*, because they are characterised by a strong property, such as being monotone or near-monotone [8], [10], [90], [94]. A system

$$\dot{x}(t) = f(x(t), u(t)), \quad y(t) = g(x(t))$$

is input-output monotone if, given $x_A(0) \geq x_B(0)$ and $u_A(t) \geq u_B(t)$, the corresponding state and output solutions satisfy $x_A(t) \geq x_B(t)$ and $y_A(t) \geq y_B(t)$ (all componentwise). As further discussed in Section IV, in monotone (or cooperative) systems, all the variables tend to the same ordered behaviour. Exploiting this fact, all the "cooperating

variables" can often be seen as a single entity. A recent endeavor to generalise this concept considers systems with a positive impulse response [28].

G. Other systems-and-control challenges in biology

Many other fundamental aspects in systems biology can attract people from the systems and control community.

Modularity. In electrical engineering we know how to generate a cascade of systems, each influencing the downstream one without being influenced by it, and the properties of these cascades are well studied. How to achieve the same goal in biological systems is not always clear, due to the *retroactivity* issues considered in [38], [39].

Self-organising systems. How do complex living systems organise themselves? This is a wide chapter in biology [32], including morphogenesis, pattern formation, and tissue differentiation. The current standard methods in control theory are perhaps not powerful enough to investigate problems of such a complexity; still it is a nice challenge to employ control tools in at least some particular problems [13], [56].

Optimality. This is a crucial aspect in nature, in view of competitive evolutionary selection, but has received relatively scarce attention. Optimality in evolution is a knife-edge battle: a very small increment of the survival chances can produce a huge evolutionary advantage in the long run [1]. Formalising this concept is not easy at all. Typically, if two species A and B share the same environment, one is more likely to dominate but several factors need to be considered to predict which one. Even the simple "chance to survive" can change completely based on the environment conditions.

Biologically inspired mechanism. Nature can inspire us and suggest engineering solutions (for synchronization [82], swarming [30], decentralized control [16], [27], [29], [60], [61]) that mimic those in biological systems, whose efficiency is proven by natural selection.

We finally stress that, as thoroughly discussed in [1], mathematical tools are precious to explain why biological systems can preserve their function in spite of enormous variations of the parameters and the external conditions.

This problem often has to be faced case by case. Importantly, assessing structural stability of a certain class of systems does not necessarily explain where the robustness originates from. For instance, for the network in Example 2, as long as an equilibrium exists, it is globally stable, as ensured by a piecewise–linear Lyapunov function that certifies structural stability, but that cannot provide any explanation for it. Conversely, in matrix Σ in (4), the fact that $\Sigma_{32} = 0$ can be explained by looking at the steady state computed before and noting that $g_c(\bar{c}) = a_0/2$, hence constant perturbations on b_0 ($b_0 \to b_0 + \delta b_0$) cannot alter the value of $\bar{c}$. The computation of the steady state requires less sophisticated mathematical notions than Lyapunov theory. Therefore, sometimes the simpler is the mathematics involved, the more useful is the biological explanation achieved.

IV. Transient behaviors as signatures of biological motifs, and their use as tools for model invalidation

One of the central questions in systems and synthetic biology is that of understanding the roles of signal transduction pathways and feedback loops, from the elucidation of such pathways in natural systems to the engineering design of networks that exhibit a desired behavior. This part of the tutorial discusses certain types of network qualitative information that can be gleaned from "dynamic phenotypes", a term that we take as encompassing both the transient characteristics of temporal responses and the use of rich classes of probing signals beyond step inputs. We focus on three examples: fold-change detection or scale-invariance, non-monotonic responses, and subharmonic oscillations, sketching both mathematical theory and biological applications.

We consider dynamical systems with inputs and outputs in the standard sense of control systems theory [91],

$$\dot{x} = f(x,u)\,, \qquad y = h(x,u)\,. \tag{5}$$

The functions $f = (f_1, \ldots, f_n)^T$, h describe respectively the dynamics and the read-out map. Here, $u = u(t)$ is a generally time-dependent input (stimulus, excitation) function, $x(t) = (x_1(t), \ldots, x_n(t))$ is an n-dimensional vector of state variables, and $y(t)$ is the output (response, reporter) variable. In order to describe positivity of variables as well as other constraints, we introduce the following additional notations. States, inputs, and outputs are constrained to lie in particular subsets $\mathbb{X}$, $\mathbb{U}$, and $\mathbb{Y}$ respectively, of Euclidean spaces $\mathbb{R}^n, \mathbb{R}^m, \mathbb{R}^q$, and the functions f and h are continuously differentiable. We assume that for each piecewise-continuous input $u : [0,\infty) \to \mathbb{U}$, and each initial state $\xi \in \mathbb{X}$, there is a unique solution $x : [0,\infty) \to \mathbb{X}$ of (5) with initial condition $x(0) = \xi$, which we write as $\varphi(t,\xi,u)$, and we denote the corresponding output $y : [0,\infty) \to \mathbb{Y}$, given by $h(\varphi(t,\xi,u),u(t))$, as $\psi(t,\xi,u)$.

A. Scale-invariance

Biological sensory systems are often "perfectly adapted" to constant inputs. This means that a step change in an input triggers an initial change in a response (which may be biochemical, such as activation of a pathway or expression of a particular gene, or physiological), and there is a consequent relaxation toward a pre-stimulus basal level. Perfect adaptation has a role in maintaining important variables within acceptable bounds, and allows organisms to readjust themselves to an optimum and non-saturating dynamic range when faced with a long-lasting change in their environment. It has been recently observed that some adapting systems, which play a role in bacterial chemotaxis pathways or in signal transduction mechanisms in eukaryotes, have in addition a stronger feature, called scale invariance or "fold change detection" ("FCD"). This means that the initial (transient) behavior remains approximately the same even when the background signal level is scaled ("log sensing"). This property, related to Weber's law in psychophysics, can

be interpreted as robustness to scale uncertainty, and plays an important role in key signaling transduction mechanisms in eukaryotes, including the ERK and Wnt pathways, as well as in *Escherichia coli* and possibly other prokaryotic chemotaxis pathways. Theoretical predictions about FCD behavior made in [88] were subsequently experimentally verified in [70] for bacterial chemotaxis.

We next discuss the formulation of the FCD property, present a "certificate" for its validity (equivariances), and explain how this property can be used as a "dynamical phenotype" for model invalidation. The main references for the material here are [88] and [87]. The general setup is that of invariance under the action of a more general set of symmetries in inputs, and in that context FCD is the special case where the symmetries are given by the action of the multiplicative group of positive real numbers.

We will assume that for each constant input $u(t) \equiv \bar{u}$, there is a unique solution $\bar{x} = \sigma(\bar{u})$ of the algebraic equation $f(\bar{x}, \bar{u}) = 0$. Often one also assumes in this context that this steady state is globally asymptotically stable (GAS): it is Lyapunov stable and globally attracting for the system when the input is $u(t) \equiv \bar{u}$: $\lim_{t\to\infty} \varphi(t, \xi, u) = \sigma(\bar{u})$ for every initial condition $\xi \in \mathbb{X}$. The GAS property is not required for the results to follow, however.

If $\mathbb{X}$ is an open set, or the closure of an open set, in $\mathbb{R}^n$, we say that the system (5) is *analytic* if f and h are real-analytic (can be expanded into locally convergent power series around each point) with respect to x, and it is *irreducible* if it is accessible and observable. By an accessible system we mean one for which the accessibility rank condition holds: $\mathcal{F}_{\mathrm{LA}}(x_0) = \mathbb{R}^n$ for every $x_0 \in \mathbb{X}$, where $\mathcal{F}_{\mathrm{LA}}$ is the accessibility Lie algebra of the system. Intuitively, this means that no conservation laws restrict motions to proper submanifolds. For analytic systems, accessibility is equivalent to the property that the set of points reachable from any given state x has a nonempty interior; see a proof and more details in the textbook [91]. An observable system is one for which $\psi(t, x_0, u) = \psi(t, \widetilde{x_0}, u)$ for all u, t implies $x_0 = \widetilde{x_0}$. Intuitively, observability means that no pairs of distinct states can give rise to an identical temporal response to all possible inputs. For analytic input-affine systems, observability is equivalent to the property that any distinct two states can be separated by the observation space; see [91], Remark 6.4.2 for a proof and discussion. In the context of applications to biomolecular systems, analyticity and irreducibility are weak technical assumptions, often satisfied.

We say that the system (5) *perfectly adapts to constant inputs* if the steady-state output $h(\sigma(\bar{u}), \bar{u})$ equals some fixed $y_0 \in \mathbb{Y}$, independently of the particular input value $\bar{u} \in \mathbb{U}$. That is, the steady-state output value is independent of the actual value of the input, provided that the input is a constant (a step function).

Invariance will be defined relative to a set $\mathcal{P}$ of continuous and onto input transformations $\pi : \mathbb{U} \to \mathbb{U}$. For each input $u(t)$ and $\pi \in \mathcal{P}$, we denote by "πu" (even when π is nonlinear) the function of time that equals $\pi(u(t))$ at time t. (The continuity assumption is only made in order to ensure that πu is a piecewise continuous function of time if u is. The ontoness assumption, that is, $\pi\mathbb{U} = \mathbb{U}$, can be weakened considerably: it is only used in the main theorem in order to prove that a system $\dot{x} = f(x, \pi u)$, $y = h(x, \pi u)$ is irreducible if the original system is irreducible, but far less than ontoness is usually required for that. An example is *scale invariance*, in which $\mathbb{U} = \mathbb{R}_{>0}$ and $\mathcal{P} = \{u \mapsto pu, p \geq 0\}$. Scale invariance is sometimes called "fold-change detection" (FCD), because the only changes that can be detected in a response are due to different fold-changes in inputs. We say that the system (5) has *response invariance to symmetries in* $\mathcal{P}$ or, for short, that it is $\mathcal{P}$*-invariant* if

$$\psi(t, \sigma(\bar{u}), u) \;=\; \psi(t, \sigma(\pi\bar{u}), \pi u) \tag{6}$$

holds for all $t \geq 0$, all inputs $u = u(t)$, all constants $\bar{u}$, and all transformations $\pi \in \mathcal{P}$.

Under the assumption that the action of $\mathcal{P}$ is transitive, i.e., for any two $\bar{u}, \bar{v} \in \mathbb{U}$, there is some π such that $\bar{v} = \pi\bar{u}$, $\mathcal{P}$-invariance implies perfect adaptation, because the outputs in (6) must coincide at time zero, and any two inputs can be mapped to each other.

Given a system (5), we will say that a set of input transformations $\mathcal{P}$, a parametrized set of differentiable mappings $\{\rho_\pi : \mathbb{X} \to \mathbb{X}\}_{\pi\in\mathcal{P}}$ is a $\mathcal{P}$*-equivariance family* if

$$f(\rho_\pi(x), \pi u) = (\rho_\pi)_*(x) f(x, u),\ h(\rho_\pi(x), \pi u) = h(x, u)$$

for each π, and for all $x \in \mathbb{X}$ and $u \in \mathbb{U}$, where $(\rho_\pi)_*$ denotes the Jacobian matrix of ρ_π. If this property holds, the system is said to be ρ_π-equivariant under the input transformation π.

The first equality is a first order quasilinear partial differential equation on the n components of the vector function ρ_π, for each $u \in \mathbb{U}$, and one may solve such equations, in principle, using the method of characteristics. The second equality is an additional algebraic constraint on these components. Observe that the verification of equivariance does not require the computation of solutions $\psi(t, \sigma(\pi\bar{u}), \pi u)$. We omit the subscript π when clear from the context.

The main result in [87] is a necessary and sufficient "certificate" for scale invariance, as follows.

Theorem. An analytic and irreducible system is $\mathcal{P}$-invariant if and only if there exists a $\mathcal{P}$-equivariance family.

An application to model invalidation: The paper [97] studied the adaptation kinetics of a eukaryotic chemotaxis signaling pathway, employing a microfluidic device to expose *Dictyostelium discoideum* to changes in chemoeffector cyclic adenosine monophosphate (cAMP). The work focused on the dynamics of activated Ras (Ras-GTP), which was in turn reported by RBD-GFP (the Ras binding domain of fluorescently tagged human Raf1), and showed almost perfect adaptation of previously unstimulated cells to cAMP concentrations ranging from 10^{-2} nM to 1 μM. Furthermore, the authors compared alternative models for adaptation and concluded that the best fit was obtained by using an incoherent feedforward structure. The model that they identified

was given by a system of 6 differential equations:

$$\begin{aligned}
dR_1/dt &= k_{R_1}(v+r_1)(R_1^{\text{tot}} - R_1) - k_{-R_1}R_1\\
dR_2/dt &= k_{R_2}(v+r_2)(R_2^{\text{tot}} - R_2) - k_{-R_2}R_2\\
dGEF/dt &= k_{GEF}\,u - k_{-GEF}GEF\\
dGAP/dt &= k_{GAP}\,u - k_{-GAP}GAP\\
dRas^{GTP}/dt &= k_{RAS}\,GEF\,(RAS^{\text{tot}} - Ras^{GTP})\\
&\quad -k_{-RAS}\,GAP\,Ras^{GTP}\\
dRBD^{\text{cyt}}/dt &= k_{RBD}^{\text{off}}\,(RBD^{\text{tot}} - RBD^{\text{cyt}})\\
&\quad -k_{RBD}^{\text{on}}\,Ras^{GTP}\,RBD^{\text{cyt}}
\end{aligned}$$

where $u := R_1 + R_2$. The symbol v stands for the chemoeffector cAMP, and the authors assumed the existence of two different receptor populations (R_1 and R_2, with very different K_d's) which when bound pool their signals to downstream components (through u). The constants r_1 and r_2 represent levels of constitutive activation. The variables GEF and GAP represent activation and deactivation of RasGEF and RasGAP, Ras^{GTP} represents the activated Ras, and RBD^{cyt} describes the cytosolic reporter molecule RBD-GFP. The best-fit parameters were obtained as: $R_1^{\text{tot}} = 0.1$, $R_2^{\text{tot}} = 0.9$, $r_1 = 0.012$nM, $r_2 = 0.115$nM, $k_{R_1} = 0.00267\text{nM}^{-1}\text{sec}^{-1}$, $k_{-R_1} = 0.16\text{sec}^{-1}$, $k_{R_2} = 0.00244\text{nM}^{-1}\text{sec}^{-1}$, $k_{-R_2} = 1.1\text{sec}^{-1}$, $k_{GEF} = 0.04\text{sec}^{-1}$, $k_{-GEF} = 0.4\text{sec}^{-1}$, $k_{GAP} = 0.01\text{sec}^{-1}$, $k_{-GAP} = 0.1\text{sec}^{-1}$, $RAS^{\text{tot}} = 1$, $k_{RAS} = 390\text{sec}^{-1}$, $k_{-RAS} = 3126\text{sec}^{-1}$, $RBD^{\text{tot}} = 1$, $k_{RBD}^{\text{off}} = 0.53\text{sec}^{-1}$, $k_{RBD}^{\text{on}} = 1.0\text{sec}^{-1}$. We now show how, for certain input regimes, this system satisfies a scale-invariance property. For more details, see [89].

With these parameters, and cAMP concentrations which are small yet also satisfy $r_1 \ll v(t)$ and $r_2 \ll v(t)$, it follows that $\dot{R}_1 \approx k_{R_1}R_1^{\text{tot}}v - k_{-R_1}R_1$ and $\dot{R}_2 \approx k_{R_2}R_2^{\text{tot}}v - k_{-R_2}R_2$, so we may view $u(t)$ as an input (linearly dependent on the external $v(t)$) to the three-variable system described by GEF, GAP, Ras^{GTP}. Since RBD^{cyt} depends only on Ras^{GTP}, we may view the latter as the output. As a final simplification, observe that with the given parameters there is a significant time-scale separation, with Ras^{GTP} being a fast variable compared to GEF and GAP ($k_{GEF}, k_{-GEF}, k_{GAP}, k_{-GAP}$ are in the range $0.01 - 0.4$, while $k_{RAS} = 390, k_{-RAS} = 3126$). So this three-dimensional system naturally reduces (at the slower time scale) to:

$$\begin{aligned}
\dot{x}_1 &= -a_1x_1 + b_1u\\
\dot{x}_2 &= -a_2x_1 + b_2u\\
y &= \frac{Kb_3x_1}{a_3x_2 + b_3x_1}
\end{aligned}$$

where $x_1 = GEF$, $x_2 = GAP$, and y is the quasi-steady state value of Ras^{GTP} as a function of x_1 and x_2. The constants are $a_1 = k_{-GEF}$, $b_1 = k_{GEF}$, $a_2 = k_{-GAP}$, $b_2 = k_{GAP}$, $a_3 = k_{-RAS}$, $b_3 = k_{RAS}$, and $K = RAS^{\text{tot}}$. This system is equivariant under $x_i \mapsto px_i$, so it is scale-invariant. Indeed, Fig. 6 shows a simulation of the entire six-dimensional system (not merely of our two-dimensional reduction) when using a step from 1 to 2 nM of cAMP, confirming that essentially the same response is obtained when stepping from 2 to 4 nM.

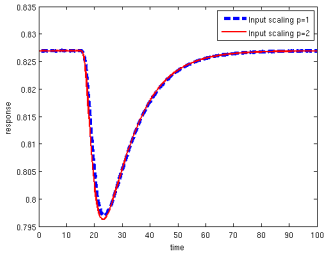

Fig. 6: Scale-invariance for model from [97]: responses to steps 1→2 and 2→4 coincide.

This prediction of scale-invariant behavior was found to contradict experimental observations (personal communication from authors of [97]): testing the system with double the input, at least in the given ranges, resulted in responses that were far from similar.

What did we learn from this example? For one thing, that the published model (at very the least, the fitted parameters) is not quite right. But more conceptually, it shows the power of scale-invariance as a "dynamic phenotype" to invalidate models: a very high-level experiment, simply testing if outputs are approximately the same when inputs are doubled, serves to obtain important information about the model.

B. Monotonicity

Systems described by order-preserving dynamics are called "monotone systems". Such systems can be shown to have monotone response properties when starting from steady states: a nondecreasing input can never give rise to a biphasic response, for example. We briefly review some of this theory and show as an example how this tool can be used to invalidate a published model of *M. tuberculosis* stress response (hypoxic induction pathway).

We assume here that $\mathbb{X}$ and $\mathbb{U}$ are an open subsets of $\mathbb{R}^n$ and $\mathbb{R}$ (we restrict to scaler inputs, $m = 1$, only for notational simplicity, but results easily generalize). We assume that the partial derivatives

$$\frac{\partial f_j}{\partial x_i}(x,u) \quad \text{and} \quad \frac{\partial f_j}{\partial u}(x,u)$$

have the same sign (either ≥ 0 or ≤ 0) for all $(x,u) \in \mathbb{X}\times\mathbb{U}$. For those derivatives that are not identically zero, we write φ_{ij} and γ_i for their signs (± 1):

$$\varphi_{ij} := \text{sign}\frac{\partial f_j}{\partial x_i}(x,u) \quad \text{and} \quad \gamma_i := \text{sign}\frac{\partial f_i}{\partial u}(x,u)$$

and let $\varphi_{ij} = 0$ or $\gamma_i = 0$ if the corresponding derivative is identically zero.

A *(graph) path* π from the input u to a node x_j means, by definition, a sequence of k indices

$$\ell_1, \ell_2, \ldots, \ell_k = j$$

such that $\gamma_{\ell_1} \neq 0$ and

$$\varphi_{\ell_i,\ell_{i+1}} \neq 0$$

for all $i = 1, \ldots, k-1$. We denote by $s(\pi)$ the sign of the path, defined as the product

$$s(\pi) := \gamma_{\ell_1}\, \varphi_{\ell_1\ell_2}\, \varphi_{\ell_2\ell_3}\, \cdots\, \varphi_{\ell_{k-1}\ell_k}\,.$$

Similarly, a path from a node x_i to a node x_j means, by definition, a sequence of k indices

$$\ell_1, \ell_2, \ldots, \ell_k = j$$

such that

$$\varphi_{i,\ell_1} \neq 0$$

and

$$\varphi_{\ell_i,\ell_{i+1}} \neq 0$$

for all $i = 1, \ldots, k-1$. We denote by $s(\pi)$ the sign of the path, defined as the product

$$s(\pi) := \varphi_{i\ell_1}\, \varphi_{\ell_1\ell_2}\, \varphi_{\ell_2\ell_3}\, \cdots\, \varphi_{\ell_{k-1}\ell_k}\,.$$

If there is a path from the input u to a node x_j, we say that x_j is *(graph) reachable*. If there is a path from a node x_i to the output node x_n, we say that the node x_i is *(graph) observable*.

The following Theorem and proof are from [14], and heavily rely upon an earlier version of the result given in [11].

Theorem. If the system is initially in steady state, the response of the output $x_n(t)$ will monotonically increase or decrease in time in response to changes in the input $u(t)$ if $u(t)$ is monotonically increasing or decreasing in time and all the directed paths from input node $u(t)$ to the output node $x_n(t)$ have the same parity. Furthermore, monotonically increasing (decreasing) $u(t)$ will trigger monotonic increase (respectively, decrease) of $x_n(t)$ if parity is positive or will trigger monotonic decrease (respectively, increase) if parity is negative.

In other words, a non-monotonic (biphasic, bell-shaped) response *requires* a negative feedback and/or and incoherent feedforward loop, see Fig. 7.

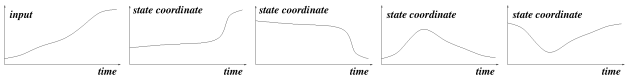

Fig. 7: Left: monotonic input. Middle: two possible state behaviors. Right: two impossible behaviors, as the response is biphasic.

A sketch of the proof is as follows. We start by "pruning" those state variables x_j which do not lie in any path from the input node to the output node x_n. We now formalize this construction, which is analogous to the "Kalman decomposition" reduction to minimal systems in linear control theory [91]. We start by splitting the set of variables X into four disjoint subsets of variables $x = (x, y, z, w)$, as follows:

1) the output node x_n is a component of the vector x,
2) the components of x are reachable and observable,
3) the components of y are observable but not reachable,
4) the components of z are reachable but not observable, and
5) the components of w are neither reachable nor observable.

We assume without loss of generality that the output node x_n is in the first set of variables, x, since otherwise there would be no path from the input to the output, and the output is then constant when starting from a steady state. It is clear that, with this partition, the equations look as follows:

$$\begin{aligned} \dot{x} &= f(x, y, u) \\ \dot{y} &= g(y) \\ \dot{z} &= h(x, y, z, w, u) \\ \dot{w} &= k(y, w) \end{aligned}$$

(for example, there cannot be a z nor w dependence in f and in g, since otherwise the z and/or w variables would be observable).

To prove the Theorem, we need to show, for the original system $\dot{x} = f(x, u)$, that if we start from a steady state $f(x_0, u_0) = 0$ and if $u(t)$ is monotonic in time, with $u(0) = u_0$, then x_n will be also monotonic in time (with the same, or opposite, monotonic behavior depending on parity). Write $x_0 = (x_0, y_0, z_0, w_0)$, so $f(x_0, u_0) = 0$ means that $f(x_0, y_0, u_0) = g(y_0) = h(x_0, y_0, z_0, w_0, u_0) = k(y_0, w_0) = 0$.

The assumption that all directed paths from the input node u to the output node x_n have the same parity applies also to the subsystem given by the variables in x in which the y variables are set to y_0:

$$\dot{x} = \hat{f}(x, u) = f(x, y_0, u) \tag{7}$$

with initial state $x(0) = x_0$, because partial derivatives of $\hat{f}$ with respect to x and u are also partial derivatives of the original f.

Suppose that we have already proved the theorem for this subsystem in which all variables are reachable and observable. We claim next that the same is then true for the original system. Consider the solution $x(t)$ of (7) with input $u = u(t)$ and $x(0) = x_0$. Consider also the solution of the full system $\dot{x} = f(x, u)$ with $x(0) = x_0$ and the same input u, and write it in the corresponding block form

$$x(t) = (\xi(t), \psi(t), \zeta(t), \omega(t)).$$

We want to prove that $\xi(t) = x(t)$ for all $t \geq 0$, from which the claim will follow. But this just follows because $g(y_0) = 0$ implies that $y(t) \equiv y_0$. (Note that the variables $\zeta(t)$ and $\omega(t)$ do not affect the output variable, which is a component of $\xi(t)$.)

We now prove the theorem for the x-subsystem, for which all variables are reachable and observable. For ease of notation, we will write $\hat{f}$ simply as f, use n for the size of x, and assume that the output node is x_n. Pick any index $i \in \{1, \ldots, n\}$. By reachability, there is at least one path π from the input to x_i and, if $i < n$, then by observability there is at least one path θ from x_i to the output node x_n.

We claim that every other path π' from the input to x_i has the same parity as π. Suppose without loss of generality that the parity of π is $+1$. We need to see that every other path π' from the input to x_i also has parity $+1$. If $i = n$, this is true by assumption (all paths from input to output have the same parity). So assume $i < n$. Suppose that π' has parity -1. Then, the path $\pi\theta$ obtained by first following π and then following θ has parity $(+1) * \rho = \rho$, where ρ is the parity of θ, and the path $\pi'\theta$ obtained by first following π' and then following θ has parity $(-1) * \rho = -\rho$. So we have two paths from input to output with different parity, which contradicts the assumption of the Theorem. In conclusion, every two paths from the input to any given node have the same parity.

We assign a label with values "$+1$ or -1" σ_u and σ_i, $i = 1, \ldots, n$, to the nodes u and each node $x_1, \ldots, x_n$ respectively, as follows: $\sigma_u := +1$, $\sigma_i :=$ sign of any path from u to x_i. A key observation is that, if $\varphi_{ij} = +1$ then $\sigma_i = \sigma_j$, and if $\gamma_i = +1$ then $\sigma_u = \sigma_i$. Indeed, if we have a path π from the input to x_i, then a path π' can be obtained, from the input to x_j, by simply adjoining the edge from i to j, which has parity equal to the parity of π. Since σ_j is the sign of any path from the input to x_j, it follows that $\sigma_i = \sigma_j$, as claimed. The statement for $\gamma_i = +1$ is simply (since we defined $\sigma_u := +1$) that $\sigma_i = +1$ if the one-step path from the input to node x_i has parity 1, which means that all paths have this parity. Similarly, if $\varphi_{ij} = -1$ then $\sigma_i = -\sigma_j$, and if $\gamma_i = -1$ then $\sigma_u = -\sigma_i$.

Now make the change of variables $x_i \mapsto \sigma_i x_i$ (i.e., reverse the sign of variables with a "-1" label). Writing the system in the new variables, we have now that

$$\frac{\partial f_i}{\partial u}(x, u) \geq 0 \quad \text{and} \quad \frac{\partial f_j}{\partial x_i}(x, u) \geq 0$$

for all $i = 1, \ldots, n$ and all $i, j = 1, \ldots, n$ respectively. Thus in the new variables we have what is called a *cooperative system* [90].

We must prove that, if $u = u(t)$ is a monotonically increasing input for a cooperative system, and if $x(0) = x_0$ is a steady state $f(x_0, u_0) = 0$, then every coordinate $x_i(t)$ of $x(t)$ (and, in particular, the output node) is monotonically increasing as well. (In the original coordinates, before sign reversals, $x_i(t)$ will decrease if $\sigma_i = -1$.) Similarly if $u = u(t)$ is a monotonically decreasing input for a cooperative system, and if $x(0) = x_0$ is a steady state $f(x_0, u_0) = 0$, then every coordinate $x_i(t)$ of $x(t)$ (and, in particular, the output node) is monotonically decreasing as well. We prove the increasing statement, since the second statement is proved analogously. From now on, for any two vectors $a, b \in \mathbb{R}^n$, we write simply $a \leq b$ to mean that $a_i \leq b_i$ for each $i = 1, \ldots, n$.

We let $\varphi(t, x_0, v)$ denote the solution of $\dot{x} = f(x, u)$ at time $t > 0$ with initial condition $x(0) = x_0$ and input signal $v = v(t)$. *Kamke's Comparison Theorem* (see [90] for systems without inputs, and [8] for an extension to systems with inputs), asserts as follows: Let $y(t)$ and $z(t)$ be two solutions of the system $\dot{x} = f(x, u)$ corresponding, respectively, to an input $v(t)$ and an input $w(t)$. Suppose that $y(0) \leq z(0)$ and that $v(t) \leq w(t)$ for all $t \geq 0$. Then, $y(t) \leq z(t)$ for all $t \geq 0$.

Now pick an input v that is non-decreasing in time and an initial state x_0 that is a steady state with respect to $v_0 = v(0)$, that is, $f(x_0, v_0) = 0$. Since $v(t)$ is non-decreasing, we have that $v(t) \geq v(0)$ so that, by comparison with the input that is identically equal to $v(0)$, we know that

$$\varphi(h, x_0, v) \geq \varphi(h, x_0, v_0)$$

for all $h \geq 0$, where, by a slight abuse of notation, "v_0" is the function that has the constant value v_0. We used the comparison theorem with respect to inputs and with the same initial state. The assumption that the system starts at a steady state gives that $\varphi(h, x_0, v_0) = x_0$ for all $h \geq 0$. Therefore:

$$x(h) \ \geq \ x(0) \qquad \text{for all } h \geq 0\,. \tag{8}$$

Next, we consider any two times $t \leq t+h$. We wish to show that $x(t) \leq x(t+h)$. Using (8) and the comparison theorem now applied with respect to initial states and the same input, we have that:

$$x(t+h) \ = \ \varphi(t, x(h), v_h) \ \geq \ \varphi(t, x(0), v_h)\,,$$

where v_h is the "tail" of v, defined by: $v_h(s) = v(s+h)$. On the other hand, since the function v is non-decreasing, it holds that $v_h \leq v$, in the sense that the inputs are ordered: $v_h(t) \leq v(t)$ for all $t \geq 0$. Therefore, using once again the comparison theorem with respect to inputs and with the same initial state, we have that

$$\varphi(t, x(0), v_h) \ \geq \ \varphi(t, x(0), v) = x(t)$$

and thus we proved that $x(t+h) \geq x(t)$. So x is a non-decreasing function. This concludes the proof.

Sometimes we only care about conditional monotonicity, depending on monotonic behavior of a particular node, even if the input is not monotonic. The following theorem from [14] is useful in that context.

Theorem. If the system is initially in steady state, the response of the output $x_n(t)$ will monotonically increase or decrease in time in response to changes in the input $u(t)$ if all the directed paths from the input nodes to the output node pass through an internal node $x_i(t)$ with monotonically increasing or decreasing dynamics and all the directed paths from input node $x_i(t)$ to the output node $x_n(t)$ have the same parity. Furthermore, monotonically increasing (decreasing) $x_i(t)$ will trigger monotonic increase (respectively, decrease) of $x_n(t)$ if parity is positive or will trigger monotonic decrease (respectively, increase) if parity is negative.

A proof is as follows. The assumption that all directed paths from the input node u to the output node x_n must pass through the internal node x_i can be formalized by splitting the set of nodes x into three subsets, $x = (x, y, z)$, where

1) the components of x are those nodes x_j, $j \neq i$, for which there is at least one path from the input node u to x_j which does not pass through node x_i,
2) $y = x_i$, and
3) the components of z are all remaining nodes, including

x_n.

For this partition, the equations look as follows:

$$\begin{aligned}\dot{x} &= f(x,y,z,u)\\ \dot{y} &= g(x,y,z,u)\\ \dot{z} &= h(z,y)\end{aligned}$$

because, if there were any dependence of h on some coordinate x_j, then there would be a path from the input to some component of z (follow a path to x_j and concatenate it with an edge from x_j to this component).

The condition that all the directed paths from $y = x_i$ to the output node x_n have the same parity means that in the system

$$\dot{z} = h(z,v)$$

(where we now view $y(t)$ as an input, which we write as "$v(t)$" to avoid confusion) all paths from the input to the output have the same parity, as in the hypothesis of the Theorem. Suppose that we consider an input u, starting from a steady state (x_0, y_0, z_0). Think of $v(t) = y(t)$ as an input. Since we started from a steady state, we know that $h(v(0), z_0) = 0$. Thus, if $v(t)$ is monotonic, the previous theorem gives us that the output is monotonic, increasing or decreasing depending on parity and on the increasing or decreasing character of the input.

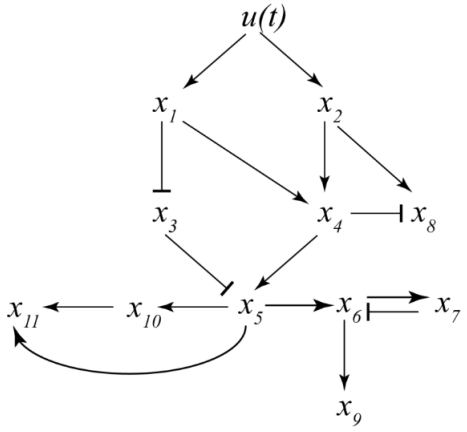

Fig. 8: Example to illustrate monotone dependence results, from [14].

For example, on the system shown in Fig. 8, the first theorem allows us to conclude that monotonically increasing input $u(t)$ will ensure monotonic increase of $x_1, x_2, x_4, x_5, x_{10}, x_{11}$ (since all directed paths from u to the respective node have positive parity), and monotonic decrease is ensured for x_3, but monotonicity cannot be guaranteed for x_6, x_7, x_8, x_9. On the other hand, if we do not know whether input signal $u(t)$ is monotonic or in case an additional negative path in the network from $u(t)$ to x_5 is added, we may still use the second formulation to conclude that if $x_5(t)$ is monotonic so will be x_{10} and x_{11}. Indeed, all the paths to x_{10} and x_{11} from input $u(t)$ pass through x_5 and all the paths from x_5 to x_{10} and x_{11} have positive parity. The argument does not work for x_9 due to a negative feedback loop between x_6 and x_7 (a directed path that goes around this loop will have the opposite parity from the path that does not). These results play a useful role in ruling out putative biological pathways, as illustrated next (see [14] for more details).

Regulation by the sigma-factor σ^E affects the hypoxic stress response pathway in *M. tuberculosis*, and specifically the expression of two critical central metabolism genes, *icl1* (Rv0467, glyoxylate shunt) and *gltA1* (Rv1131, methylcitrate cycle), which play a role in persistence of tubercle bacilli in infections. Transcription of *icl1* requires both σ^B, which is transcribed under σ^E control, and the σ^B-regulated transcription factor *lrpI* (Rv0465c, local regulatory protein of icl1). The resulting circuit (see [36]) is a feedforward coherent loop, as illustrated in the left panel of Fig. 9. This circuit has no feedback loops nor IFFL's, and hence monotone activation of σ^E should result in monotone gene expression. However, experiments in which oxygen is depleted over a three-day course, with concomittant monotone σ^E activation, lead to a biphasic, not monotone, activation of the target gene, as shown in the right panel of Fig. 9 (from [84]). This model invalidation motivated the search for new regulatory architectures in [14].

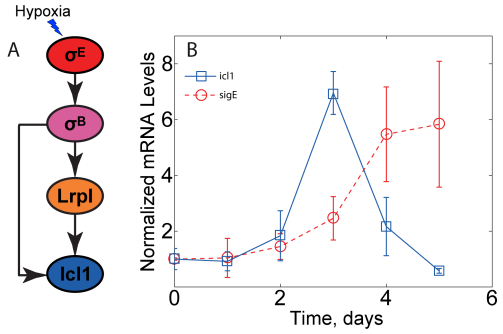

Fig. 9: Left: network from literature. Right: experimental σ^E and gene expression dynamics, inconsistent with network.

C. Distinguishing between adaptation topologies: Response to periodic inputs

One challenging question in systems biology is that of comparing different architectures for perfect adaptation. For example both incoherent feedforward loops (IFFL's) and integral feedback systems give rise to perfect adaptation and, in some configurations, scale invariance. Recent work has proposed the use of periodic signals to discriminate between these models. We review a theoretical result showing that feedforward loops and monotone systems both lead to entrainment, but nonlinear feedback architectures (such as nonlinear integral feedback) may lead to period doubling bifurcations and even chaos. This result is illustrated through experimental work with *C. elegans* interneurons, in which odor-evoked intracellular Ca^{2+} response signatures, to periodic on-off pulses of diacetyl, display subharmonic behavior at high forcing frequencies.

Perfect adaptation means that, for constant (step) inputs, outputs are not identically zero, but, after a transient, recover

asymptotically to their steady state value. In particular, such systems exhibit nonmonotone responses to step inputs (which are monotonic), and it follows therefore that such systems must include an IFFL or a negative feedback loop. How to distinguish between them using i/o data? One answer is provided in [78], and says that feedforward systems (including IFFL's) entrain to periodic inputs. That paper also shows that monotone systems (under some mild conditions) also entrain to periodic inputs. Thus non-entrainment to inputs is a "signature" of a negative feedback loop in the system. We outline some details next. In this section, we assume that inputs, states, and outputs all take nonnegative values.

Feedforward systems: Consider first purely feedforward systems, meaning that the state variables $\{x_1, \ldots, x_n\}$ satisfy the property that if x_i influences x_j, then x_j does not influence x_i. One can always re-label variables in such a way that no element x_i influences any element x_j with $j < i$. In this form the system has a cascade structure, that is to say the Jacobian $(J_{ij} = \partial f_i/\partial x_j)$ is lower triangular. Let us make the following mild (and reasonable for biological systems) assumptions on trajectories, given an input u: (i) solutions are bounded ($x_i(t) \leq c_i$ for some $c_i > 0$) for all t; (ii) the diagonal elements of J are negative ($\partial f_i/\partial x_i < 0$), which means biologically that every species is degraded, typically in a concentration-dependent manner such as a linear degradation term like $-k_i x_i$ or a Michaelis-Menten term like $-k_i x_i^M/(1+x_i^M/x_{i0}^M)$, where M is a Hill coefficient; (iii) the off-diagonal elements of J are bounded, i.e., all $|J_{ij}| \leq p_{\max}$ for some $p_{\max} > 0$.

Suppose from now on that the input u is T-periodic. Then the system has a unique periodic solution with period T (same as stimulus), to which every other solution converges. The proof consists of choosing a diagonal matrix P with $P_{ii} = 1/p^i$, so as to make the off-diagonal elements of PJP^{-1} arbitrarily close to zero, the larger that $p \gg p_{\max}$ is. Then, the matrix measures μ_1, μ_2, or μ_∞, associated with the L^1, L^2, or L^∞-norms, respectively, of PJP^{-1} are all approximately equal to the largest (i.e. least negative) diagonal element. Thus, the system is infinitesimally contracting and by Theorem 2 in [81] all $x_i(t)$ are T-periodic, as claimed.

Cooperative systems: For systems that are not feedforward, there are entrainment results as well, as long as all loops are positive. We now sketch the case of cooperative systems, meaning (we allow now vector inputs) that

$$\frac{\partial f_i}{\partial u_j}(x,u) \geq 0 \quad \forall i,j \quad \text{and} \quad \frac{\partial f_i}{\partial x_j}(x,u) \geq 0 \quad \forall i \neq j$$

hold for all states x and input values u. This discussion is also from [78]. Such systems are particular cases of monotone systems with inputs as defined in [9], meaning that they satisfy:

$$x^{(1)}(t_0) \geq x^{(2)}(t_0), u^{(1)}(t) \geq u^{(2)}(t) \quad \forall t \geq t_0$$
$$\Rightarrow \quad x^{(1)}(t) \geq x^{(2)}(t) \quad \forall t \geq t_0$$

where we abbreviate the component-wise inequality $x_i \geq y_i$ for all vector components i by $x \geq y$. An important result for periodically forced monotone systems $\dot{x} = f(x(t), u(t))$ is given as Theorem 5.26 in [54], which credits the unpublished 1997 Ph.D. thesis by I. Těšćak. This result applies to systems that are irreducible, meaning that all its Jacobian matrices are irreducible (that is, every variable can indirectly affect every other variable, possibly through an arbitrary number of intermediates; see also [55]). The result states that $x(t)$ converges to a solution with period kT, where $k \geq 1$ is an integer, for almost all initial conditions if the stimulus $u(t)$ is periodic with period T ($u(t) = u(t+T)$). It is important to note that, generally, there may be stable periodic solutions with period kT and $k > 1$, as shown in [96]. Thus, if we are interested in entrainment (global convergence to period-T trajectories), we need to find additional conditions which rule out $k > 1$.

Special case: two-dimensional systems with no negative loops. First, we show that a monotone system which only contains two dynamical elements and which is stimulated with period T, if it has a solution with period kT, where k is an integer, then k must equal 1. A related result exists in the literature, namely that for two-dimensional periodically forced irreducible cooperative systems, solutions approach a T-periodic solution [52]. We present the following results to make the exposition self-contained and build towards the results in the subsequent case, and also because we do not need to assume irreducibility.

Lemma. Consider a two-dimensional dynamical system, driven by an input of period T ($u(t) = u(t+T)$), and suppose that $x(t_0)$ is a periodic point with some period kT, where k is a positive integer. Then there is some time t_1 so that $x(t_1) \leq x(t_1+T)$ or some time t_2 so that $x(t_2) \geq x(t_2+T)$.

A proof is as follows. Suppose without loss of generality that $t_0 = 0$ (otherwise we are done, with $t_1 = 0$) and that $x_1(0) > x_1(T)$ and $x_2(0) < x_2(T)$ (if the opposite inequalities hold, the reasoning is analogous). There is some integer $s > 1$ so that $x_1((s-1)T) \leq x_1(sT)$ since, otherwise, $x_1((s-1)T) > x_1(sT)$ for all s, and therefore $x_1(0) = x_1(kT) < x_1((k-1)T) < \ldots < x_1(0)$, which is a contradiction. Now pick any such s and let $S = (s-1)T$. Then, the continuous function $p(t) := x_1(t) - x_1(t+T)$ has $p(0) > 0$ and $p(S) \leq 0$, so there is some minimal t_1 so that $p(t_1) = 0$. Similarly, consider $q(t) := x_2(t) - x_2(t+T)$, which has $q(0) < 0$, and conclude that there is some minimal t_2 so that $q(t_2) = 0$. Suppose that $\min\{t_1, t_2\} = t_1$. Then $x_1(t_1) = x_1(t_1+T)$ and $x_2(t_1) \leq x_2(t_1+T)$. If instead $\min\{t_1, t_2\} = t_2$, then the other inequality holds. This completes the proof of the Lemma.

Assuming the input has period T, we introduce the notation $F(x(s)) = x(s+T)$ for the solution to the differential equation $\dot{x}(t) = f(x(t), u(t))$ at time $s+T$ when starting with initial condition $x(s)$ at time s. Furthermore, we denote by $F^k = F \circ \ldots \circ F$ (k times) the k-fold iteration of F. We next show by induction that $F^k(x(s)) = x(s+kT)$ for all k. For $k = 1$, this follows from the definition. Next, assuming that $F^n(x(s)) = x(s+nT)$ holds, we define

$z(t) = x(t+nT)$ and note that $\dot{z}(t) = \dot{x}(t+nT) = f(x(t+nT), u(t+nT)) = f(z(t), u(t))$, where the last equality follows from the definition of z and the periodicity of u. So, like $x(s)$ before, we can map $z(s)$ forward in time by one period $z(s+T) = F(z(s)) = F(F^n(x(s))) = F^{n+1}(x(s))$, and, also, $z(s+T) = x(s+nT+T) = x(s+(n+1)T)$. Thus, we have $F^{n+1}(x(s)) = x(s+(n+1)T)$, as claimed.

Corollary: Consider a two-dimensional monotone system, with a periodic input, and consider a solution of period kT, $x(t) = x(t+kT)$. Then $x(t+T) = x(t)$ for all t, i.e., the orbit of x has period T.

To prove this, observe that the mapping F is monotone by assumption. We pick the time t_1 as in the Lemma (the proof would be analogous with t_2), so $x(t_1) \leq F(x(t_1))$. By monotonicity, the inequality is preserved under repeated mappings, $F^n(x(t_1)) \leq F^{n+1}(x(t_1))$. So, iterating, $x(t_1) \leq x(t_1+T) \leq x(t_1+2T) ... \leq x(t+kT) = x(t)$, where the last equality comes from the assumption that $x(t)$ has period kT. Since $x(t_1) = x(t_1+T)$, we have $x(t) = x(t+T)$ for all times t.

Positive feedback loops stimulated from rest: Next, we consider cooperative systems of arbitrary dimension, not just two, but instead take the special case where the initial state is an equilibrium $\bar{x}$ corresponding to the zero input, that is, $f(\bar{x}, 0) = 0$. We again follow [78].

Any (nonnegative) input starting from $\bar{x}$ results in a trajectory that is (coordinatewise) larger than $\bar{x}$. Indeed, it is a general fact that $x^{(1)}(t) \leq x^{(2)}(t)$ for all $t \geq 0$ if the systems starts from the same initial state $x(0)$ and if the inputs satisfy $u^{(1)}(t) \leq u^{(2)}(t)$ for all $t \geq 0$. So, if we compare the steady state (i.e. with input $u^{(1)}(t) = 0$) to the system after the onset of stimulation, i.e., $u^{(2)}(t) \geq 0$ for all $t \geq 0$, and $x(0) = \bar{x}$, it follows that $\bar{x} = x^{(1)}(t) \leq x^{(2)}(t)$ for all $t \geq 0$. In particular, $\bar{x} \leq F^\ell(\bar{x})$ for all positive integers ℓ.

Suppose that, as in the conclusion of Těšćak's Theorem cited earlier, we know that a trajectory $x(t)$ converges to a periodic orbit of period kT, for some positive integer k. We want to show that if $x(0) = \bar{x}$ then in fact $k = 1$. Call the periodic orbit Γ. Since $x(t) \to \Gamma$ as $t \to \infty$, in particular it holds that $x(\ell T) \to \Gamma$ for integers ℓ as $\ell \to \infty$. As Γ is compact, the sequence $x(\ell T)$ stays in a compact set and thus has a converging subsequence $F^{\ell_j}(\bar{x}) = x(\ell_j T) \to \widetilde{x}$ as $j \to \infty$, with $\ell_1 < \ell_2 < \ldots \to \infty$. Necessarily $\widetilde{x} \in \Gamma$, so solutions starting from $\widetilde{x}$ are periodic of period k, i.e. $F^k(\widetilde{x}) = \widetilde{x}$. Moreover, $\bar{x} \leq F(\bar{x})$ and monotonicity of F imply

$$F^{\ell_j}(\bar{x}) \leq F^{\ell_j}(F(\bar{x})) = F(F^{\ell_j}(\bar{x})) \to F(\widetilde{x})$$

as $j \to \infty$, and coupled with $\bar{x} \leq F^{\ell_j}(\bar{x})$ for all j, this gives, by passing to the limit, $\bar{x} \leq F(\widetilde{x})$.

Theorem. Let F (introduced above) be a monotone mapping. Suppose that these properties hold for two fixed states $\bar{x}$ and $\widetilde{x}$:

(1) $\bar{x} \leq F(\widetilde{x})$

(2) $F^k(\widetilde{x}) = \widetilde{x}$ for some integer $k \geq 1$

(3) $F^{\ell_j}(\bar{x}) \to \widetilde{x}$ as $j \to \infty$, with $\ell_1 < \ell_2 < \ldots \to \infty$.

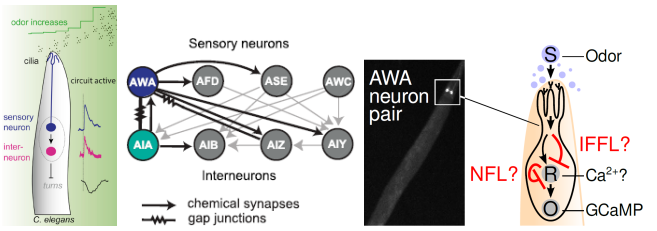

Fig. 10: Odor sensing in *C. elegans* and sensory/interneurons. Two possible circuits for adaptation, a negative feedback loop and an IFFL. Figures from [69] and [78].

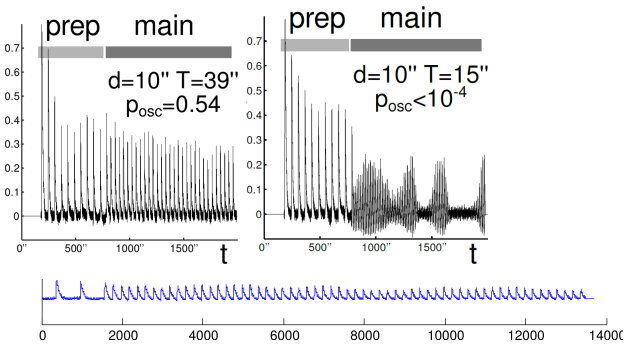

Fig. 11: Top: Testing for odor-evoked intracellular Ca^{2+} response signature via periodic on-off pulses of diacetyl, population measurements. Entrainment at high periodic inputs ($T = 39s$) and subharmonic behvior at lower period ($T = 15s$). Bottom: single-neuron recording $T = 20$ gives response of period about ~ 200, indicating that IFFL's (or positive feedback systems) cannot be the reason for behavior.

Then $F(\widetilde{x}) = \widetilde{x}$.

To prove this, first pick an arbitrary integer $n \geq 0$. Now observe that for any j, and letting for simplicity $r := \ell_j$:

$$F^n(\widetilde{x}) = F^{rk+n}(\widetilde{x}) = F^{rk+n}(\widetilde{x}) - F^{rk+n}(\bar{x}) + F^{rk+n}(\bar{x}) \leq q_j + F^{rk+n+1}(\widetilde{x}) = q_j + F^{n+1}(\widetilde{x})$$

where $q_j := F^{\ell_j k+n}(\widetilde{x}) - F^{\ell_j k+n}(\bar{x})$ and where we used (2) to obtain $F^n(\widetilde{x}) = F^{rk+n}(\widetilde{x})$, then (1) to get $F^{rk+n}(\bar{x}) \leq F^{rk+n}(F(\widetilde{x})) = F^{rk+n+1}(\widetilde{x})$ and finally again (2) to get $F^{rk+n+1}(\widetilde{x}) = F^{n+1}(\widetilde{x})$. Observe that $F^k(\widetilde{x}) = \widetilde{x}$ implies that $F^{\ell_j k+n}(\widetilde{x}) = F^n(F^{\ell_j k}(\widetilde{x})) = F^n(\widetilde{x})$. So $F^{\ell_j k}(\bar{x}) \to \widetilde{x}$ implies that $F^{\ell_j k+n}(\bar{x}) = F^n(F^{\ell_j k}(\bar{x})) \to F^n(\widetilde{x}) = F^{\ell_j k+n}(\widetilde{x})$, or $F^{\ell_j k+n}(\widetilde{x}) - F^{\ell_j k+n}(\bar{x}) \to 0$. Thus, $q_j \to 0$ as $j \to \infty$, so we conclude that $F^n(\widetilde{x}) \leq F^{n+1}(\widetilde{x})$ for all $n \geq 0$.

From this,

$$\widetilde{x} \leq F(\widetilde{x}) \leq F^2(\widetilde{x}) \leq \ldots \leq F^k(\widetilde{x}) = \widetilde{x},$$

The worm *C. elegans* can locate odor sources across a 100,000-fold concentration range, and various sensory and interneurons participate in the recognition pathway, see Fig. 10. The paper [78] discussed how the above theorems can be used to rule out IFFL's as responsible for adaptation in a local circuit controlling the AWA sensory neuron, leading to the postulation of a negative feedback model. This is because at high frequencies of inputs, one does not obtain entrainment, see Fig. 11.

V. Conclusions

We have surveyed successful applications of control-theoretic approaches to the study of problems in biology. Studying structures based on feedback and layered feedback loops in biological systems enables a deeper understanding of their function (Section II). Assessing structural properties, which rely on the topology of the system interconnections and do not depend on specific parameter values, helps us discover the source of the extraordinary robustness of biological systems and can be also used for model invalidation (Section III). The mathematical analysis of dynamic phenotypes can provide fundamental qualitative insight into phenomena such as fold-change detection, non-monotonic responses, and subharmonic oscillations, giving us powerful tools to invalidate biological models (Section IV). All the sections testify the importance of simple phenomenological models, able to provide significant explanations, and the fundamental role of *structures* and *qualitative behaviors*, given the inherent uncertainties affecting the parameters of biological systems. Sections II and III end with open questions and problems, hoping to make more control-theorists eager to apply their tools (or develop novel tools) to gain a better understanding of the complex and fascinating world of biology.

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