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# Robustness and autonomy in biological systems

# How regulatory mechanisms enable functional integration, complexity and minimal cognition through the action of second-order control constraints.

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**Abstract** Living systems employ several mechanisms and behaviors to achieve robustness and maintain themselves under changing internal and external conditions. Regulation stands out from them as a specific form of higher-order control, exerted over the basic regime responsible for the production and maintenance of the organism, and provides the system with the capacity to act on its own constitutive dynamics. It consists in the capability to selectively shift between different available regimes of self-production and self-maintenance in response to specific signals and perturbations, due to the action of a dedicated subsystem which is operationally distinct from the regulated ones. The role of regulation, however, is not exhausted by its contribution to maintain a living system's viability. While enhancing robustness, regulatory mechanisms play a fundamental role in the realization of an autonomous biological organization. Specifically, they are at the basis of the remarkable integration of biological systems, insofar as they coordinate and modulate the activity of distinct functional subsystems. Moreover, by implementing complex and hierarchically organized control architectures, they allow for an increase in structural and organizational complexity while minimizing fragility. Finally, they endow living systems, from their most basic unicellular instances, with the capability to control their own internal dynamics to adaptively respond to specific features of their interaction with the environment, thus providing the basis for the emergence of minimal forms of cognition.

**Keywords** Regulation; Control; Functional integration; Organization; Autonomy; Cognition.

## *1. Introduction*

One of the characteristics that differentiate living systems from physicochemical ones is the capability to self-produce and self-maintain by means of continuous exchanges of matter and energy with the environment. While doing so, organisms exhibit a remarkable robustness in how they respond to external perturbations and manage internal variations in such a way as to

maintain their viability. They do not just produce, modify and maintain their components in order to persist: another distinctive feature of life ─ often included in definitions employed in origins of life and synthetic biology (e.g. Ruiz-Mirazo et al. 2004; Damiano and Luisi 2010; Bich and Damiano 2012a) ─ is the adaptive capability to constantly oppose the thermodynamic tendency towards disintegration, and to counteract potentially destabilizing interactions with the environment.

From this perspective, robustness is a crucial and ubiquitous biological property, that allows living systems to adaptively cope with variation. It is generally regarded as the capability of a system to maintain its functions and performances despite perturbations, or under uncertainty in general (Kitano 2004; Stelling et al. 2004; Chen 2008). It is often associated with degeneracy in the realisation of functions, and characterized in terms of flexibility of behaviours or of steady maintenance of some property (Mitchell 2009).

Physiological mechanisms and behavioral strategies by which organisms achieve robustness and maintain their viability as integrated wholes, are implemented at all levels of biological organization, and they can range from distributed network properties to more complex mechanisms, which rely on different degrees of modularity. An open question, which requires careful theoretical scrutiny, is whether mechanisms responsible for achieving robustness do not just assure the survival of living systems under changing internal and external conditions, but also play a fundamental, inherent, role already in the realization of biological organizations and of relevant biological properties. A closely related issue concerns whether complex forms of robustness – relying on hierarchical or modular mechanisms – are already necessary at the level of minimal life or, instead, they are later additions that ensured the survival of living systems in more variable environments than the ones where life might have originated. This chapter tackles the open question ─ and aims to provide conceptual tools to address the issue related to minimal life ─ from an organizational standpoint. In doing so, it regards robustness as a system property, and analyzes it in relation to the whole organization of the organism and the environmental conditions in which it operates[1].

A candidate theoretical framework to address these issues is that of *biological autonomy*. Historically inspired by the work of Immanuel Kant (Kant 1790) and Claude Bernard (Bernard 1865), among others, on the internally self-determined organization of living systems, this perspective has been developed in Systems Theory, Cybernetics and Theoretical Biology starting from the 1960s (Bich and Damiano, 2008; Bich and Arnellos, 2012; Mossio and Bich, 2017). The main contributions to this line of research include: Jean Piaget's work on organizational closure and thermodynamic openness (Piaget 1967); Robert Rosen's theory of M/R-Systems and his formal model of minimal biological organization (Rosen 1972; Rosen 1991; Letelier et al. 2006); Humberto Maturana and Francisco Varela's theory of autopoiesis (Varela et al. 1974; Maturana and Varela 1980) and its applications to origins of life and synthetic biology (Luisi, 2006); Tibor Ganti's Chemoton Theory (Ganti 1975; Ganti 2003a; Ganti 2003b); Stuart Kauffman's auto-catalytic sets and his theory of autonomous systems (Kauffman 1986;; Kauffman 2000); and, more recently, the notion of basic autonomy and the

[1] An alternative way to address biological robustness, closer to engineering approaches, would be to focus on individual behaviours and mechanisms, and on the maintenance of specific functions or performances.

organizational approach developed by Alvaro Moreno and collaborators (Ruiz-Mirazo and Moreno 2004; Moreno and Mossio 2015).

The notion of biological autonomy is grounded in the idea that living systems are metabolic self-producing systems able to self-maintain and keep their network organization invariant through the continuous exchange of matter and energy with the environment. According to this perspective, living systems are "endogenously active" (Bechtel 2008), due to their distinctive thermodynamic nature. They realize a specific kind of internal organization ─ defined by the notion of *organizational closure* (Varela 1979; Rosen 1991; Bich and Damiano 2008; Mossio and Moreno 2010) ─ where not only the very existence and activity of the constituents depend on the network of processes of transformation that they realize but, in addition, they collectively promote the conditions of their own existence through their interaction with the environment. The thermodynamic nature of biological organization, which combines at its core endogenous activity with essential interaction with changing environments, implies also that the system is required to harbor an internal dynamical variability to enable different viable responses to a variety of environmental perturbations. This is one of the reasons why robustness plays a crucial role in the characterization of living systems from their most basic instances.

By adopting this perspective, this chapter advocates the view that complex mechanisms implemented by living systems to achieve robustness are essential for an understanding of life and of some of its distinctive features. Specifically, by leaning on a theoretical characterization of the concepts of biological control and signal (Section 2), it focuses on the organization and role of those specialized regulatory mechanisms that contribute to the robustness of living systems by coordinating compensatory responses (Section 3). Regulatory mechanisms are characterized as forms of higher-order control, exerted over the basic regime responsible for the production and maintenance of the organism, in response to specific signals and perturbations (see also Bich et al. 2016). The thesis defended in Section 4 is that while enhancing robustness, regulatory mechanisms play also a fundamental role in the realization of an autonomous biological organization. By enabling the integration and coordination of the basic biological functions, they contribute the construction of biological identity. They also allow living systems to overcome bottlenecks of complexity in the transitions from basic self-maintaining (bio)chemical networks to increasingly more sophisticated organizations. Moreover (Section 4.2), regulatory mechanisms lay the basis for the emergence of minimal forms of cognition already at the unicellular level, when a system becomes capable to internally generate operational meanings ─ expressed through self-regulatory loops ─ associated with environmental variations.

### *2. Basic concepts: stability, control and signal in autonomous systems*

Robustness, as applied to biology, is a general and flexible concept that includes diversified properties and phenomena, related to how organisms or their parts cope with variation. It admits different approaches depending on level of description and goals: the engineering approaches, centered on the maintenance of individual functions and performances; those approaches interested in local properties such as the persistence and stability of specific molecules or classes of molecules (see for example Pascal & Pross, 2016); and system-oriented approaches that address robustness as a property related to (and emerging from) collective self-production. This chapter, which aims to contribute to an understanding of the fundamental

adaptive properties common to living organisms, takes the system-oriented path, and addresses robustness in the context of biological autonomous organizations[2].

In this scenario, robustness is usually characterized as a network property, mostly related to forms of stability (Kitano 2007), and described dynamically in terms of properties of attractors or of the capability to shift between attractors. When mechanistic aspects are taken into account, robustness is often understood in terms of feedback loops, and of their capability to make attractors more stable or to facilitate the establishing of new steady states when the systems' dynamics are displaced from the initial ones[3].

These properties are very general and widespread, and not specifically biological: they can be exhibited by many types of natural and artificial networks. A case of special interest for its proximity to biology is that of out-of-equilibrium self-maintaining chemical systems: *dissipative structures* emerging and maintaining themselves under specific external boundary conditions, like Benard cells, tornados, whirlpools etc. (Nicolis and Prigogine 1977). They share some properties with living systems, such as stability and out-of-equilibrium self-maintenance but, importantly, they lack the capability of self-production, and the internal organization and functional differentiation that are distinctive of biological systems.

Let us consider the most basic instances of robustness in biological systems. It is possible to think of prebiotic systems and the of earliest (and simplest) forms of life as distributed biochemical networks of processes of production capable of generating and maintaining stable dynamic regimes, encapsulated by a compartment of their own making. An example is the basic scenario centered on metabolic self-production provided by the theory of autopoiesis (Varela et al. 1974; Maturana and Varela 1980), among similar approaches. The emphasis is on the network capability of the system as a whole to achieve a stable regime and employ distributed compensations for perturbations in such a way as to maintain the global organization of the system stable. In such a scenario robustness could be achieved, for example, through the capability to recover after the loss of most of its catalysts (Piedrafita et al. 2010).

A more detailed model of minimal biological organization is represented by Tibor Ganti's Chemoton (Ganti 1979; Ganti 2003b), a system characterized by an internal differentiation between three coupled chemical subsystems ─ a metabolic cycle, a template subsystem and a compartment ─ working like the cogwheels of a clock[4]. This model describes a non-hierarchical and very fragile basic biological network, exhibiting a certain (although low) degree of functional differentiation, and a minimal robustness that allows it to be viable under

[2] As argued by Cornish-Bowden (2006), among others, most engineering approaches "often seem to imply little more than reductionist biology applied on a large scale" while a "systemic approach to biology ought to put the emphasis on the entire system". A somehow similar categorization is proposed by Dupré and O'Malley (2005), who distinguish between 'pragmatic systems biologists' – who find it useful to refer to some systems properties in terms of interactions and of integration of data – and 'systems-theoretic biologists' – who focus on the investigation of general systems principles. An interesting case is that of Robert Rosen, whose contributions include both engineering (Rosen, 1967; 1970) and system-oriented approaches (Rosen, 1972; 1985; 1991), and who saw the former as inadequate to capture the distinctive features of living organisms *as systems* (Rosen, 1991).

[3] For different ways to achieve biological robustness through stability see for example Rosen (1970), a classic textbook in dynamical systems theory.

[4] The coupling between these subsystems is realised by means of supply and demand of metabolites necessary for the production of the components in the different subsystems (*metabolic complementarity*). The subsystems provide the necessary substrates for the internal processes of production taking place in the others and, in turn, consume the metabolites supplied by the others.

a limited range of environmental conditions (Bechtel, 2007), and to cope with stochastic variation (Segbroeck et al. 2009).

In this scenario, a minimal form of biological robustness is achieved. It can be understood in terms of *dynamic stability*[5] of the self-producing and self-maintaining distributed network organization that puts together the basic living system. This organization realizes highly distributed endogenous patterns of compensations that respond to variation in such a way that the system remains within its viability region. The system simply ''absorbs'' the effects of perturbations or internal variations as a network, by compensating them through internal reciprocal adjustments between tightly coupled subsystems, while the whole dynamics is maintained in the initial attractor—or shifts to a new one[6].

Implicit in these accounts is the idea that prebiotic and early living systems were characterized by a distributed organization capable to achieve a minimal form of robustness as stability under very specific (almost invariant) and favorable environmental conditions[7]. Only later, an increase in the scope and efficiency of mechanisms related to robustness would have allowed living systems to survive in more variable environments. *Vice versa*, according to this view, today when the environments of current living systems are particularly favorable and almost invariant, many regulatory mechanisms would not be needed anymore. This is the case of many intracellular endosymbionts, which live under very stable conditions within the cellular internal environment of the host organism. They tend to undergo a reduction of their organizational complexity and genome size, and they lose several constitutive and compensatory mechanisms. An example is *Buchnera aphidicola BCc* ─ an endosymbiont of the cedar aphid *Cinara cedri* ─ which lost some of its regulatory mechanisms and also the capability to realize the full pathway for the synthesis of the amino acid tryptophan and the co-enzyme riboflavin necessary for its own functioning and that of the host. The production of these components is achieved only by sharing biosynthetic pathways with other endosymbionts in the same host (Lamelas et al. 2011).

Dynamic stability is an important property to understand and model some aspects of life and its robustness, but it is not an exclusively biological property. It is also crucial to explain the emergence of several relevant phenomena in chemistry and at the edge of biology, such as oscillatory behaviors in reaction networks (Semenov et al. 2016). The paradigmatic case of dynamic stability in these infra-biological domains is represented by dissipative structures, where a high number of microscopic elements spontaneously self-organize and realize a stable global, macroscopic ordered configuration in the presence of a specific flow of energy and matter in far from thermodynamic equilibrium conditions (Nicolis and Prigogine 1977). These

[5] Dynamic stability is the capability to counterbalance the displacement of the system from a certain initial state, provoked by a perturbation, and end up in the same final state (Rosen 1970). It is a widespread property in the natural world, instantiated by any system whose dynamic behavior is characterized by the presence of at least one stable attractor. When realized collectively, dynamic stability is a distributed property of a whole network of reactions—it cannot be attributed to any single transformation, or to a partial subset of transformations.

[6] See Bich et al. (2016) for a discussion of dynamic stability in models of minimal living organizations.

[7] These favorable conditions might also include the presence of most of the necessary building blocks for early living systems, as claimed for example by the heterotrophic models of the origin of life (see for example Mansy et al. 2008).

dynamic structures show the capacity of reacting conservatively to a certain range of perturbations, due solely to distributed network properties.

Yet, unlike these cases at the edge of biology, dynamic stability is not the only way actual living systems can achieve robustness. As pointed out by Hiroaki Kitano, the study of biological robustness should not be limited to dynamic stability, considered as a distributed property. Robustness should be understood, instead, as a more general phenomenon.

> "Whereas homeostasis and stability are somewhat related concepts, robustness is a more general concept according to which a system is robust as long as it maintains functionality, even if it transits through a new steady state or if instability actually helps the system to cope with perturbations. […] Examples of extreme robustness under harsh stress conditions show that organisms can attain an impressive degree of robustness by switching from one steady state to the other, rather than trying to maintain a given state. […] Robustness is also not identical to stability. Some species gain robustness by increasing instability in a part of its system" (Kitano 2007, 1–2).

A theoretical account of biological robustness needs to take into consideration not only stability, but also more complex ways of achieving robustness. In particular, it should take into consideration modularity and hierarchy – two aspects which, according to Kitano, still require a proper formal characterization (Kitano 2004; Kitano 2007)[8]. Modularity may enable functional differentiation and, therefore, different contributions of subsystems to the maintenance of the more comprehensive system that harbors them. Hierarchy allows to modulate and coordinate such contributions in an efficient and precise way, and to specify in each circumstance how to ensure the viability of the whole system.

As a matter of fact, biological systems can locally produce distributed network responses. However, one of their distinctive features is that they are internally organized in such a way that different subsystems contribute in different ways to the maintenance of the system (Montévil and Mossio 2015; Moreno and Mossio 2015; Mossio et al. 2016). These features are absent in infra-biological thermodynamically open systems (see also Moreno and Ruiz-Mirazo 2009; Mossio et al. 2009; Arnellos and Moreno 2012).

One of the consequences of being internally organized and functionally differentiated is that the interactions between the components of a system can take place in ways that are qualitatively different from one another, and they can play distinct causal roles within the system. This differentiation might be invisible to a network characterization, focused on patterns of interconnectivity and numbers of connections, rather than types of interactions. As a consequence, alternative and more complex way to achieve achieving robustness that are distinctively biological might be passed over[9]. This is a serious conceptual issue if we consider

[8] It does not imply that different forms of robustness, based respectively on dynamic stability, and modular and hierarchical control mechanisms cannot coexist in the same system or subsystem.

[9] This issue is closely related to the debate on the relationship between mechanistic and network descriptions. Whereas mechanisms describe distinguishable parts which play different specific tasks, network descriptions focus on global properties and patterns of connectivity (Moreno et al., 2011). Finding ways to bring together network and mechanistic descriptions, apparently irreducible to each other, is on the of the challenges faced by complex systems theory. An attempt to develop an heuristic to move between the two descriptive approaches has been proposed by Bechtel (2017a; 2017b). The basic idea is that clusters of interconnections in a network description are possible candidates for the parts of the mechanistic description of the same system. The limit of

that in all known instances of living systems, biological robustness is not achieved exclusively through reciprocal adjustments between the coupled subsystems of a distributed network, and indeed all living systems employ also other forms of direct control of the basic dynamics that imply a more active role of the system organization in handling variation (Bich and Damiano 2012b).

With the aim to tackle this issue, the following sections will not dwell upon robustness as a property of stable networks, but on mechanisms that are more demanding in terms of organizational requirements, such as hierarchical regulatory mechanisms. However, this approach needs to face some conceptual difficulties related to the specific object of study. Basic living systems, such as bacteria, are characterized by fluid biochemical machineries with low internal structural differentiation, realized by highly distributed interactions where each component interacts with ─ or is indirectly influenced by ─ many others. These features favor network descriptions and seem to make it particularly difficult to provide a characterization of robustness in terms different functional contributions to the viability of the system. This scenario, therefore, requires the introduction of theoretical tools to operationally distinguish between distinct kinds of interactions in the systems. These tools can then allow us to address issues related to complex biological robustness such control and regulation, to operationally identify which components or subsystems act as controllers or regulators, and to distinguish them from those other subsystems that are the targets of their activities.

### *2.1. Control and signal*

Let us proceed stepwise by first clarifying some basic concepts such as control and signal in the framework of autonomy.

Autonomous biological systems constantly produce, transform, repair and replace their own components, and maintain themselves through exchanges of matter and energy with the environment. Unlike dissipative structures, which are mostly and largely determined by external boundary conditions, they do not emerge spontaneously under appropriate environmental conditions, but they contribute to determine their own conditions of existence (Mossio and Bich, 2017). In order to maintain themselves in far-from-equilibrium conditions, they need to exert some *control* over their underlying thermodynamic processes which would otherwise proceed toward equilibrium.

Control is generally defined as the capability to modify the dynamics of a system toward a certain state (e.g. parameters acting upon variables, enzyme upon concentrations of metabolites, etc.[10]). It is an *asymmetric interaction*. In biological systems control is exerted by some molecules or supra-molecular structures, generated and maintained by the system itself,

this approach is that patterns and network configurations derived from numbers of connections do not provide the same kind of information on the system as the identification of different types of contributions (e.g. in metabolism a complex hierarchy involving, metabolites, enzymes acting on metabolites, energy currencies, kinases acting on enzymes, etc.), but a complementary one.

[10] See for example Rosen 1970; Hofmeyr and Cornish-Bowden 1991.

which act as *constraints*[11] on thermodynamic (matter/energy) flows[12]. A biological system is capable of generating some of the (internal) constraints that control its dynamics in such a way that they collectively achieve self-maintenance. These constraints are involved in at least three main kinds of control mechanisms. One is *kinetic control* (e.g., catalysis), which specifies the rates of diverse synthetic pathways: e.g. an enzyme that harnesses (catalyzes) a chemical reaction (without being directly affected by it). A second one is *spatial control*, which defines the spatial scale of the system, the selective passage of molecules, and thereby, the concentrations of its components. Examples are selectively permeable boundaries and diffusion barriers, which avoid the dilution of certain key compounds and keep their concentration above critical threshold values. A third kind is *template control* (e.g. DNA, mRNA[13]), responsible for constraining the sequences of amino acids in proteins.

The distinctive feature of biological systems is that the constraints which exert these basic types of control are organized in such a way that they are mutually dependent for their production and maintenance, and collectively contribute to maintain the conditions at which the whole network can persist (Moreno and Mossio 2015). This continuous operational integration puts together the *constitutive regime* (C) responsible for the basic self-production and self-maintenance of a living system[14]. This basic regime involves at least two different kinds of interactions ─ *processes* and *constraints* acting on processes ─ and it is characterized by the strict *stoichiometric coupling* between the subsystems involved. Responses to variation are essentially governed by changes in concentrations (both of the substrates that take part in the processes, and of the molecular structures that carry out control tasks on those metabolites). In this context, robustness is achieved as a network property: variations in concentrations affecting a given process or subsystem can propagate throughout the system, producing the change of one or several other processes and control subsystems which, in turn, compensate for the initial one. As a result, the system can be regarded as stable.

The activity of control constraints can be affected in different ways[15]. In the context of the constitutive regime alone, the most basic ways to affect the activity and rates of control subsystems are through direct molecular interactions, changes in the concentrations of the substrates and products of the processes upon which the constraints act[16] and, finally, through variations in the processes responsible for the synthesis of the control components themselves, resulting in changes in the concentrations of the control components in the system.

[11] Given a particular thermodynamics process P, a molecular configuration C acts as a constraint upon P if: (1) at a time scale characteristic of P, C is locally unaffected by P; (2) at this time scale C exerts a causal role on P, i.e. there is some observable difference between free P, and P under the influence of C (Mossio et al. 2013, 164. A more detailed characterisation can be found in Montévil and Mossio 2015).

[12] See Pattee 1972; Bich et al. 2016; Winning and Bechtel 2018, for a discussion of control in biological systems. See Arnellos et al. 2014 and Veloso 2017, for a discussion of inter-cellular control mechanisms in development.

[13] See for example Mossio et al. 2016 for a characterisation of the role of mRNA as a constraint.

[14] See Nghe et al. (2015) and Ruiz-Mirazo and Moreno (2004) for a discussion of the some of the key elements for the origin of these self-producing and self-maintaining networks in prebiotic conditions.

[15] The requirement for a structure to be a constraint is to be locally unaffected by the process they are harnessing. But a constraint can be affected by other interactions in the system.

[16] For example, the effects described by the law of mass action.

All these are constitutive interactions governed by stoichiometry. However, there are additional ways in which the activity of control subsystems can be affected. These additional modalities open new spaces of possibilities for the action of control subsystems (new degrees of freedom) and are responsible for the complexification of biological organizations. It is the case of *signal molecules*. In living systems they play a fundamental role in the coordination and integration between subsystems in response to both internal variation and interactions with the environment. The distinctive features of signals in general, as argued by Haven Wiley, are the capability to trigger a response in the targets without providing the energy for it, and the fact that their causal power is insufficient for determining the response. The response, therefore, depends to a large degree on the properties of the receiver (Wiley 2013).

In the framework of autonomy described above, signals are not part of constitutive processes. They are usually by-products of biosynthetic processes and, more importantly, their role *as signals* does not consist in participating as metabolites in production processes. They *do not act as* constraints either, but they usually *interact with* control constraints by giving rise to activated or inhibited molecular complexes. A paradigmatic case is constituted by cyclic AMP, a ubiquitous signal molecule in cells, which forms molecular complexes with regulatory proteins like kinases ─ responsible for the activation or inhibition of enzymes ─ and also with other regulatory proteins such as CAP (catabolic activation protein), which interacts with the promoters of the genes coding for the enzymes involved in the metabolism of glucose.

### *3. Biological Regulation*

Another way control constraints can be affected or modulated beyond constitutive interactions is hierarchically, by the direct action of other specialized constraints in the system. These internally produced, second-order control subsystems provide a living system with the capability to act on its own constitutive dynamics. They can be functionally recruited into regulatory mechanisms (R) that modulate constitutive control constraints on the basis of internal and external signals in such a way as to maintain the overall viability of the system[17]. These higher-order architectures open a new space of possible control operations. They enable the realization of more complex mechanisms that contribute to enhancing the robustness of the system, *beyond and on top* of the constitutive ones, which instead are ruled by changes in concentrations and embedded in the basic self-maintaining network.

In this new organizational architecture, the functional role of a regulatory subsystem is to modulate the basic constitutive network, by shifting between distinct metabolic regimes available to the system in relation to changes in environmental conditions. It does so in such a way that the new metabolic/constitutive regimes brought forth by regulatory shifts should be capable of coping with the new environmental conditions and internal variation, thus extending the range of perturbations or stimuli to which the system may respond in a rapid and efficient way, as well as enriching the sphere of dynamic functional behaviors available.

One of the difficulties that arise when addressing control and regulation, is to find an operational/naturalized way to distinguish between the regulator and what is regulated. Regulatory control cannot be regarded as a straightforward extension of the collective control that enables the dynamical stability of the constitutive regime. It is not the consequence of

[17] For a more detailed discussion of biological regulation as second-order control see Bich et al. (2016).

either a different way to wire constraints and processes, or of the introduction of additional functional nodes in the basic self-maintaining network. In these cases, the result would still be a constitutive network.

A more complex organizational architecture is required in order to realize regulation. Its distinctiveness and the difficulty in characterizing it, stem from the fact that a regulatory subsystem (R) is part of the living system, and like the other parts is produced and maintained by the processes integrating the constitutive regime (C). Yet, to act as a second order controller of C, R needs to work according to a different logic than the one characteristic of C, which is based on the strict (stoichiometric) coupling between control subsystems[18]. R needs to be able to exhibit some independence ─ i.e. a *dynamical decoupling* ─ from what takes place in C, in order to freely and asymmetrically modulate the activity of the control constraints in C on the basis of signals. This capability can be achieved when R exhibits additional degrees of freedom with respect to the controllers in C. Some of these new degrees of freedom make R sensitive to activation and inhibition signals, while not being directly dependent on the state of C. Others endow R with the effector capability to modulate the activity of the controllers in C (Bich et al. 2016).

The decoupling required in order for R to be operationally distinct from C, and capable to independently modulate the latter, is realized when the operations of the regulatory subsystem R are neither specified nor directly determined by the metabolic activity of C: i.e. they are 'stoichiometrically free' from the latter (Griesemer and Szathmáry 2009). More specifically, the activation and operation of a regulatory subsystem R is not directly dependent on its concentration (or variation of concentration) ─ that is, on its production by C ─ even though C guarantees its presence in the system (Bich et al. 2016). Instead, the activation of R is triggered by signals, and its operations depend on its internal organization and on the structure of its functional parts (in particular molecular geometries that are complementary to those of the controlled constraints in C). In such a way the activity of R is operationally distinct from C, and R can act as a dedicated regulatory controller of C. The functional regulatory loop realized by a control architecture so built, takes place in three steps: (1) a signal or perturbation activates R, which (2) in turn modulates C and brings forth a new constitutive regime. Finally, (3) the modification of C enables the system to cope with the specific variation which triggered the regulatory response.

This general theoretical model applies to autonomous systems at different levels of organization. Let us now consider two examples of how living systems ─ basic ones such as bacteria and multicellular systems like mammals ─ rely on mechanisms of regulation to adaptively respond to the composition (or variation in the composition) of their internal and external environments.

Some of the most basic mechanisms of regulation are present at the very core of biological machinery, such as protein synthesis. Organisms need to be able to determine which proteins/enzymes to produce on the basis of their internal state, of the availability of specific

[18] As argued by William Bechtel, "Although stoichiometric linkages between reactions are effective for insuring linkages between operations, they do not provide a means for varying the reactions independently. Such independent control can only be achieved by a property not directly linked to the critical stoichiometry of the system" (Bechtel 2007, 229).

amino acids, and of the characteristics of the environment. To do so they need to activate, inhibit and modulate the synthesis of specific proteins according to their needs. The regulation of protein synthesis can take place at many different steps of this process: at the level of DNA through the control of transcription, which involves longer time scales; at the level of RNA through RNA processing control, RNA transport and localization control, translational control, mRNA degradation control, etc.; and at the post-translational level by modulating, at shorter time scales, the folding and activity of proteins ─ e.g. through forms of allosteric control, phosphorylation, etc.

Let us consider for example how bacteria modulate the metabolism of tryptophan through a regulatory control of the transcription step. Bacteria are able to produce this amino acid when it is not available in the environment. The genes responsible for the five enzymes which contribute to its synthesis are grouped together into one operon. A repressor protein exerts a regulatory control upon the promoter of the operon, by repressing it in presence of tryptophan in the cell. Two molecules of this amino acid act as the signals that allosterically activate the repressor protein. When tryptophan is present in the environment, the repressor protein is activated and blocks the endogenous production of this amino acid by repressing the synthesis of the enzymes responsible for it. In absence of tryptophan, instead, the repressor protein is in an inhibited state, and the cell can start synthesizing the enzymes responsible for the production of the amino acid. In this example the regulator R (the repressor protein) is dynamically decoupled from the constitutive regime C, i.e. from the enzyme-coding operon and the metabolism of tryptophan. The activity of the repressor protein does not depend on variation in its own concentration ─ which is determined by the rates of C[19] ─ but, rather, on its (stoichiometrically free) structural affinities with the signal molecules and with the promoter sequence.

The second example concerns the regulation of glucose concentrations in mammals through glycogen synthesis during fast and food uptake[20]. The metabolic pathway that in mammal cells connects glucose to G6P (through the GT/HK subsystem) and then to glycogen (through the GSase subsystem) is characterized by homeostatic capabilities, that in the fasting state of low glucose level can compensate slight variations in glucose through negative feedbacks and other distributed responses (Schafer et al. 2005).[21] Yet, the unregulated metabolic pathway alone is not able to cope through a network response with the strong increase in glucose that takes places during food uptake.

Therefore, additional mechanisms such as the release of insulin are implemented to prevent glucose from reaching dangerously high levels, by activating and increasing glycogen synthesis sufficiently and quickly enough to cope with the rise in plasma concentration of glucose.

[19] The concentration of repressor proteins is usually low (proportional to the number of copies of the promoter sequence it regulates) and does not undergo variation in concentrations to bring forth its regulatory effect. The *lac*-operon system, a more complex example of genetic regulation, which coordinates of the metabolism of two sugars (lactose and glucose) through the second-order control of the transcription step, follows the same logic (see Bich et al. 2016 for an analysis of regulatory decoupling in this latter case).

[20] Specifically, the example relies on the analysis of glycogen synthesis in the rat gastrocnemius muscle provided in Schafer et al. (2005).

[21] See also J. S. Hofmeyr and Cornish-Bowden (2000) for an analysis of this type of stoichiometrically dependent responses.

"Larger-scale flux changes operating on a more intermediate time scale will involve an external detector/effector (e.g. pancreas/insulin) that stimulates both up- and downstream subsystems, thereby maintaining excellent internal and external homeostasis despite increased flux" (Schafer et al. 2005, 69).

The release of insulin by the pancreas is triggered by signals in presence of high concentrations of glucose, and it leads to the "coordinate activation of glucose transport, hexokinase and glycogen synthase" (Schafer et al. 2005, 67)[22] which allows metabolizing the exceeding sugar. In virtue of the action of the pancreas/insulin regulatory subsystem, dynamically decoupled from the intracellular metabolism of muscular cells, the system as a whole can reach a new regime that is able to cope with the perturbing variation ─ the absorption of glucose through food.

This example shows how distributed network properties (dynamic stability) and regulatory mechanisms coexist and interact to enhance the organism's robustness, and what is the crucial role played by regulation in integrating and coordinating some core metabolic functions to maintain the viability of the whole system. The main point is that, whereas intracellular stability network mechanisms that compensate for slight variations in glucose concentrations are always at work during fast, they do not guarantee the viability of the system in response to a strong increase of glucose that occur during food uptake. During food uptake, regulatory mechanisms intervene by releasing insulin, and they bring forth a coordinate response of the organism's metabolism to transform glucose into glycogen. This case also shows that regulation does not depend solely on genetic control ─ which requires more time to exert its effects. When quick responses are needed, they can be achieved by the coordinated control of the activation state of several enzymes and subsystems in a pathway.

### *4. Regulation at the crossroads between identity, complexity, and cognition*

A possible approach to the study of the nature and evolution of biological robustness is to consider dynamic stability as the default property of self-producing and self-maintaining prebiotic and minimal living systems. According to this view, additional and more complex regulatory mechanisms were later developed beyond the constitutive regime C. These mechanisms act on top of C, and they allow the system to cope with a wider range of variations and to improve the specificity of its responses to perturbations. Regulation, therefore, contributes to extend the viability space of the already dynamically stable biological system[23].

In this view, the requirements for the emergence of biological regulation would also roughly coincide with fundamental transitions in the process of evolution of robustness, leading from basic stability to full-fledged adaptive regulation. They would include the emergence of: (a) organizational self-production and self-maintenance; (b) functional differentiation; (c) network stability; (d) multistability; and (e) sequence dependent components that enable stoichiometric freedom by means of interactions based on complementary molecular geometry.

[22] One of the effects of the release of insulin is the rapid change of the phosphorylation state of the enzyme glycogen synthase (GSase), which alters its kinetics. It is a clear case of second order control interaction, in which a regulatory subsystem acts on a first order control constraints (GSase) by activating (or inhibiting) it.

[23] Another way to express the idea is that systems can be alive under very special stable conditions, without regulatory mechanisms, by relying only on the constitutive network. Regulation would then become necessary only when the system is immersed in changing environments and develops a higher internal differentiation.

In particular, steps (d) and (e) would be crucial for the transition towards regulation. Multistability enables the realization of distinct constitutive regimes which, in turn, may allow the system to remain viable under different conditions. As it has been recently shown, bistability and related properties such as oscillations, can arise already in simple autocatalytic networks of relevant organic reactions such as those involved in origins of life (Semenov et al. 2016). The problem that arises at this step is how to govern multiple stability precisely and efficiently: a distributed system characterized by multiple possible stable regimes can be more and more fragile the higher the number of attractors. Yet, the existence of more than one viable regime is the basis upon which specialized second-order control subsystems can functionally act and bring forth transitions in C, compatibly with internal and external conditions. One way to look at multistability is to regard it as a starting point, capable to generate a new *adjacent possible* (Kauffman 2000) in the evolution of robustness. The additional requirement for the development of regulatory capabilities is then the presence in the system of geometry-dependent molecular complexes that can be recruited by the system to operate as dynamically decoupled switches that control the shifts between attractors in C.

It is therefore reasonable to think that regulation is dependent on the existence of a full-fledged constitutive network already capable to exhibit stability (and multistability) and, consequently, that it is both logically and historically secondary to more basic forms of biological robustness. A possible weakness of this idea it that the stoichiometric couplings that characterize the constitutive regime are very fragile: anything disturbing the delicate balance of metabolites or creating a new branching in the metabolic pathways would easily break the system apart. Moreover, some further considerations that will be discussed in Sect. 6.4.1. might make us question this idea and admit the possibility that mechanisms responsible for complex form of robustness might have already been necessary for the realization of a constitutive regime and for the emergence of some of the fundamental properties of biological systems.

### *4.1. Functional integration and organizational complexity*

*Functional integration* can be characterized as the degree of interdependence between the (functional) subsystems that are necessary to realize and maintain the system that harbors them. As argued in Section 2.1, the integration into one coherent system of several types of kinetic, spatial and template control constraints is the basis for the realization of a self-maintaining and self-producing biochemical machinery. The way these subsystems are put together constitutes the specific organization of the constitutive regime of the system. This organization is what determines its *identity* as a living system, characterized by functional autonomy, cohesion, and asymmetry with respect to the environment. There are different ways in which the subsystems of a basic autonomous organization can operate in a functionally integrated fashion (Bich 2016). The simplest ones are constituted by (1) metabolic complementarities ─ in which the products of one subsystem become the substrates for the processes controlled by another, like in the case of the chemoton ─ or (2) forms of cross control ─ in which control constraints are the direct products of the process harnessed by other constraints in the network (e.g. cross-catalysis).

Yet, the integration of subsystems into a basic constitutive network cannot be achieved by simply coupling pre-existing molecular or supramolecular complexes and recruiting them as control constraints. As pointed out by Shirt-Ediss, “the first systems with the ability to robustly

maintain themselves far-from-equilibrium […] could only have existed as such if they were encapsulated and their internal (proto-metabolic) organization and compartment were tightly integrated'' (Shirt-Ediss 2016, 85). This tight integration requires a matching between the features of the subsystems involved: for instance, the composition of the membrane and the position of its molecular machineries to meet the demands of metabolism; the synthesis of the right components by metabolism to be used in compartments to achieve the required permeability. As it has been recently argued, "the encapsulation of a self-maintaining chemical system has far-reaching organizational implications since its viability imposes significant changes on both parts (compartments and metabolic networks) in order to enable a functional coupling between them" (Moreno 2016, 10).

A minimal constitutive network might not be enough to realize a living autonomous system, and surely not to achieve more complex forms of biological organization, not only because of its overall fragility. The basic functional subsystems, in fact, need not only to be matched. Also their activity and rates need to be functionally coordinated in order to achieve integration, to avoid conflict, and to realize robustness at the system level under changing internal and external conditions[24]. The functional coordination necessary to realize and maintain this integrated organization might already require control hierarchies such as those realized by regulatory mechanisms.

As a matter of fact, all current living systems employ forms of hierarchical control to modulate the relations between their constitutive subsystems in such a way that they are capable to coordinate their basic functions and achieve integration. In bacteria, for example, membrane channels are not only activated directly by concentration gradients, but also by protein phosphorylation triggered by specific signals from the environment and the metabolism (Karpen 2004; Kulasekara and Miller 2007). In turn, signals from the membrane can trigger the regulation of gene expression, which can affect metabolism in a way that is compatible with the state of the membrane (Stock et al. 1989). The coordination between genome and metabolism is exemplified by genetic regulatory mechanisms such as the tryptophan operon, described above, which modulates metabolism through protein synthesis, compatibly with the composition of the internal and external environments of the living system. The *lac*-operon provides an even more interesting case: the regulatory mechanisms that govern the diauxic shift between glucose and lactose metabolisms, coordinate two different functional regimes within metabolism itself according to the state of the internal and external environments (Jacob and Monod 1961; Bich et al. 2016). Bacterial chemotaxis is another example in which the coordination between two functional subsystems ─ metabolism and the flagellum responsible for movement ─ is achieved, in different ways, through the activity of a regulatory mechanism (Eisenbach 2004; van Duijn et al. 2006; Eisenbach 2007; Alexandre 2010; Bich and Moreno 2016). These examples show that regulation plays a role in achieving the functional integration

[24] Different subsystems might present different internal norms of operation, and need to be coordinated to ensure both their compatibility and their joint functional contribution to the maintenance of the system. Another relevant feature of biological cells is that they cannot synthesise all the possible molecules at the same time, due to energetic and spatial limits. Therefore, they have to exert some control over biosynthetic processes in order to produce the necessary components at the right times. Other types of functional resources need coordination as well. Let us think of the interplay between metabolic regimes relying on different carbon sources, such as lactose and glucose. Without the proper regulation (realised for example by the *lac*-operon subsystem) these regimes would compete for basic catalytic resources in the cell.

necessary for the continuous realization of the constitutive regime of biological autonomous systems, starting from their simplest instances in the prokaryotic world.

A further remark concerns organizational complexity. As argued above, a basic constitutive regime already exhibits a relevant internal complexity that allows it to harbor the functional differentiation necessary for its production and maintenance. Such degree of complexity cannot be supported reliably by means of network properties alone in presence of variations, insofar as generating a compensatory effect depends on propagating changes through many local interactions. As argued by Wayne Christensen (2007), in this context achieving reliability and specificity of responses becomes an issue. The time required for the responses depends on both the size of the system and the degree of complexity found in it, and, as argued above, the internal complexity required for a constitutive network is already high. Additional problems derive from the difficulties in generating multiple differentiated global states and in reaching the appropriate one for a given perturbation. The lack of specificity in the responses is responsible for the increased fragility of the systems when its internal complexity rises, unless additional switch mechanisms devoted to the selective modulation of the basic dynamics are in place. What regulation does is precisely to make it possible to overcome this bottleneck of complexity, by endowing the system with the capability to induce the appropriate collective pattern of behavior in a more rapid and efficacious way. The responses so produced are specific, and they are not negatively affected by the size of the system.

Moreover, evolutionarily speaking, the adaptation of constitutive networks to new environmental perturbations would require each time a modification of the organization of the core constitutive network of the system, which would more likely drive the system to disruption than to the complex responses. A modification of those subsystems dedicated to handling internal changes, such as switches, instead, provides more reliable solutions (see Kirschner and Gerhart 2005), that do not only provide further viability, but also enable the increase of complexity.

The individual and evolutionary capability to maintain or enhance robustness at the system level ─ by extending the range of internal and external variations the system is capable to cope with, and by enabling internal differentiation without loss of viability ─ are the results of a trade-off between stability and complexity. Dynamically stable distributed systems are fragile under qualitatively different conditions, and unspecific in their responses. To implement those differential and specific responses necessary to maintain a complex organization and to respond to a variety of changing conditions, internal variability (increased degrees of freedom) under the coordination of regulatory mechanisms is fundamental, and it is realized at the expenses of the intrinsic stability of the constitutive regime and of its subsystems[25].

In sum, the increase of robustness at the system level goes hand in hand with increases of complexity and of internal variability, and it can take place only through the action of regulatory mechanisms. Novel and higher levels of regulation need to be invented to ensure the maintenance of an increasingly complex constitutive (or low-level) part of the system. As

[25] The idea of improving adaptivity and robustness by increasing the degrees of freedom available to the systems' dynamics at the expense of distributed stability of the network can be found already in Piaget's school (Meyer 1967). For a recent discussion of the interplay between organisation and variation in biology see instead Montévil et al. (2016).

the complexity of the system increases, the corresponding regulatory subsystem is bound to become increasingly necessary for the continuous maintenance of the basic organization, from minimal living systems to more complex forms of life.

### *4.2. Minimal cognition*

The capability of living systems to adaptively cope with their interactions with the environment by means of regulatory mechanisms is also closely related to the question of the origin and characterization of minimal cognition[26]. Traditionally, the authors that first developed the framework of autonomy ─ in particular Piaget (1967) and Maturana and Varela (1980) ─ defended the view according to which cognition consists basically in the viable interactions that the organism can enter with the environment without losing its identity, and the internal modifications it undergoes in this process. This approach considers cognition in its minimal form as coextensive with life or coinciding with the interactive dimension of life. The general idea underlying this thesis is that what for physical systems would be just external influence, in living system is adaptively integrated and transformed into a "meaningful interpretation" (Heschl 1990, 13). Another way to formulate it, is that since autonomous systems ─ and, therefore, all living beings ─ are capable of "enacting a meaningful world", they would also *ipso facto* be cognitive agents, at least in a minimal sense (Maturana and Varela 1980; Varela et al. 1991; Bitbol and Luisi 2004; Bourgine and Stewart 2004). According to this perspective, then, the adaptive behavior of minimal organisms such as bacteria is already a cognitive phenomenon.

A different view is supported by those who argue that even though some of the properties exhibited by minimal living systems are important aspects of cognition, they are not sufficient to capture cognition. One of the weaknesses of the thesis that cognition emerges already with unicellular life, it has been argued, is that that by dissolving cognition in broader biological phenomena, then it would become difficult to understand the nature, function, and evolutionary history of cognition as a specific phenomenon (Moreno et al. 1997). According to these latter approaches, it is increased *behavioral capacities* (Christensen and Hooker 2000) or a higher degree of organizational complexity ─ namely, a nervous system decoupled from metabolism, and with its own *distinctive norms* (Barandiaran and Moreno 2006) ─ which are the primary discriminating dimensions of cognition.

Bechtel, among others, sustains a position closer to the former one, and provides a theoretical and heuristic justification for addressing cognition already at the level of bacteria: "Evolution is a highly conserved process, and the mechanisms developed in our common ancestors with these species provide the foundation for many of our cognitive activities. Since these organisms lack some of the complications that have evolved in us, research on them can help reveal key features of our cognitive mechanisms" (Bechtel 2014, 158). Other supporters of minimal cognition at the unicellular level argue that the decoupling between metabolism and

[26] See Bich and Moreno (2016) for a more detailed discussion of minimal cognition in relation to biological regulation.

cognition takes place already in bacterial chemotaxis, and propose an account of minimal cognition based on sensory-motor activity (van Duijn et al. 2006).[27]

The account of regulation proposed here provides a generalized theoretical model to understand the most basic decoupling responsible for minimal forms of cognition: the decoupling between adaptive regulation and constitutive metabolism. The adaptive capability provided by regulation is not specific of sensory-motor activity, but rather exhibits a more fundamental logic common to all regulatory mechanisms dedicated to the interaction with the environment, such as for example the tryptophan and *lac* operons (Bich et al. 2016). When applied to cognition, therefore, the notion of decoupling from constitutive metabolism needs to be addressed in this more encompassing context, which transcends sensory-motor activity to include other forms of (*sensory effector*) adaptivity. The central idea is that one of the essential aspects of cognition that can be analyzed at the basic level of biological organization, is that cognitive agents should be able to distinguish between some specific features of their interaction with the environment and to act accordingly, in such a way as to maintain their viability. And this fundamental requirement for cognition can be met only in presence of regulatory mechanisms (Bich and Moreno 2016).

When a mechanism of regulation is at work, the environment is not only a source of indistinguishable perturbations, but also of specific and recognizable ones. The crucial point is that the regulated system reacts in a distinctive way: it does things according to what it distinguishes (what activates the regulatory subsystem) in its interactions with the environment. In the presence of regulatory mechanisms perturbations do not directly drive the response of the system. It is the regulatory subsystem, activated by specific perturbations, which modulates the constitutive one. It does so in such a way that the system as a whole becomes able to cope with the environmental perturbations which triggered the regulatory response: the organism eats a new source of food, or secrets chemicals to neutralize a lethal substance, etc.

In this context, an environmental perturbation becomes a specific and recognizable interaction because of the nature of the relation it holds with the regulatory subsystem. The response of the system is the result of the evaluation operated by the regulatory subsystem (activation-plus-action). The regulatory subsystem establishes "classes of equivalence" (Rosen 1978) in the environment according to how the variation activates it and triggers the regulatory action, so that such categories are actually employed by the system to modify its own internal dynamics in a viable way. Therefore perturbations achieve an endogenous, operational, significance for the system: the interactions with the environment become more than just a source of indistinct noise, but are converted into a world of endogenously generated (naturalized) significances.

This approach still leaves open the question whether these cognitive relevant properties are sufficient for cognition ─ and bacteria should be considered as fully-fledged cognitive ─ or a new source of normativity needs to emerge on top of the one related to the metabolic viability of the cell. This latter view is supported for example by the advocates of the emergence of cognition with the nervous system (Barandiaran and Moreno 2006).

[27] See Godfrey-Smith (2016) for a critical discussion of this account of minimal cognition focused on sensory-motor activity.

A related question is whether a theory of minimal cognition should account for the simplest instantiation of those features we ascribe to human or higher animal cognition ─ and depict its evolution as continuous from the appearance of the nervous system ─ or consider minimal cognition as a distinct category in itself, specified by a first decoupling from the constitutive regime and capable to generate behavioral capabilities analogous to those found in multicellular systems. In the latter case the evolution of cognition would be understood as a discontinuous process characterized by the emergence of several decouplings. However, whichever position is adopted, the adaptive regulatory mechanisms that confer robustness to the system, by realizing different types of decoupling, play a relevant, if not crucial, role in the origin of cognition.

### *5. Final Remarks*

What is the relationship between robustness and autonomy in living systems? An answer can be found by looking at biologically distinctive ways to achieve robustness beyond and on top of general network properties. The advantages of pursuing an organizational approach to this question, is that it allows us to identify and analyze robustness mechanisms by focusing on different types of contributions to the realization and maintenance of a biological system: for instance, processes, constraints, control, signals and regulation. This approach also provides an understanding of robustness, functional differentiation, modularity and integration in the encompassing context of a living cell or of a multicellular organism, rather than in relation to individual properties or performances of a system, or of parts of it. Moreover, the thermodynamic nature of the notion of constraint, as used in this approach,[28] can be useful in the attempt to lay a bridge between robustness and thermodynamics, one of the open issues in the field of robustness (Kitano 2007).

From this theoretical standpoint, this paper has advocated the view according to which mechanisms related to robustness ─ and, specifically, regulatory ones ─ play a fundamental role at the core of biological organization. They contribute not only to enhancing the viability of the system under changing conditions. They also make it possible to coordinate and integrate the basic constitutive functions of a living system and to overcome bottlenecks of complexity. Moreover, they are the basic requirements for the emergence of minimal forms of cognition or of cognitively relevant properties. Some questions remain open, among which: whether or not such mechanisms played a role in the origin of life, and whether a viable minimal biological system integrating metabolism, compartment and template can be actually realized without regulatory mechanisms, although in very special or controlled environmental conditions. A negative response to the latter question would then require a revision of our theoretical models of minimal life.

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[28] See Pattee (1972; 1973), Kauffman (2000), Umerez and Mossio (2013), Moreno and Mossio (2015), Winning and Bechtel (2018).

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