An Evolutionary Systems Biology View on Metabolic System Structure and Dynamics

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Abstract Cellular metabolism consists of many interconnected reactions that present feedbacks through cyclic reaction motifs and through metabolite regulation of enzyme kinetics. In addition, metabolism is interlinked with gene regulation and other cellular, energy-driven processes such as division and motility. While many important insights have been gained on metabolism in the last decades, we are still far from a complete, predictive understanding of it. This is reflected in our current, limited ability to pinpoint the drivers of metabolic system dynamics and devising ways to engineer it.

In this review paper, we argue that the study of metabolism through the lens of evolutionary biology can provide further insights into its structure and dynamics. By structure, we mean the composing reactions of a metabolic system, and how these reactions are connected with each other through shared metabolites, while by dynamics, we mean the temporal behaviour and responses of the resulting metabolic system. Following an introductory section, we summarise the key findings on the structure and dynamics of cellular metabolism within an evolutionary systems

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perspective in Sects. 2 and 3. In doing so, we highlight two key ways of thinking about metabolic systems, one based on considering metabolism optimised for biomass production, and another one based on considering metabolism as a self-regulating emergent system for maintaining nonequilibrium metabolic fluxes. From this second consideration, we then expand to discuss the possible biophysical drivers that could have played a key role in shaping metabolic systems in Sect. 4. Finally, in Sect. 5, we call for an evolutionary perspective on metabolism that takes into account both of the above considerations. We conclude by highlighting key areas of future research where this combined view can provide valuable insights.

1 Introduction

Metabolism is the collective biochemical reaction set through which cells convert available chemicals from the environment into energy and cell components. Cells cannot and could not have existed without metabolism, thus, metabolic systems must have been very early inventions in the evolution of life, possibly emerging before cells. Such an ancient character of metabolism is evident in the fact that many metabolic reactions and systems are conserved across a range of uni- and multicellular organisms (Yamada et al., 2006). These highly conserved aspects of metabolism, as well as variations across organisms may provide a clue back to the chemical conditions during early origins of life and the current environments of organisms.

Discussions regarding the origin of life usually emphasise the need for self-replication and information carrier molecules. The emergence of such molecules must have been linked tightly with the emergence of early metabolic systems that provided the building blocks needed for their synthesis. Such systems might have had an abiotic nature initially (Branscomb & Russell, 2013; Keller et al., 2014, 2017; Messner et al., 2017), but it is possible that their structure and tight linkage with reproduction came to be embedded into the fabric of current-day biological systems. Indeed, it is difficult to separate metabolism from the other aspects of cell physiology, including membrane potential (Merrins et al., 2016), gene expression and regulation (Chubukov et al., 2014; You et al., 2013), motility (Egbert et al., 2010), and cell division (Papagiannakis et al., 2017). It is this linkage that makes an understanding of cell metabolism a prerequisite for a complete understanding of cell physiology. Besides its importance as a fundamental research topic, metabolism and its connections to cell physiology relate to biotechnological applications such as bioproduction in microbial or mammalian cells, and to medicine, including possible cancer treatment (Jain et al., 2012; Locasale, 2013).

Evolutionary considerations in the study of metabolism are not rare (Nam et al., 2011; Papp et al., 2009) but do commonly take an adaptive stance that views metabolism as optimised for biomass production. While there have been cases where laboratory evolution experiments resulted in metabolic adaptations matching such optimality predictions (Ibarra et al., 2002; Fong & Palsson, 2004), there are

also many instances where solely adaptive explanations cannot capture observations from metabolic systems (Papp et al., 2009; Schuster et al., 2008). In this review, we advocate the importance of considering also the possibility of metabolic system features emerging through nonadaptive mechanisms, as by-products of biophysical and biochemical requirements on maintaining metabolic fluxes out of equilibrium. In particular, the first emergence of metabolic systems and their early evolution must have been driven by achieving nonequilibrium flux across system boundaries, and the drivers for achieving such a state must have influenced system structure and dynamics in ways that is still visible in current-day metabolism. We argue that such an extended, evolutionary view on metabolism can provide better insights on '*why the metabolism is the way it is*', and in turn, lead to new ways of understanding and engineering current metabolic systems.

In the following two sections, we first review the current knowledge on the structure (i.e. composition and connectedness) and dynamics (i.e. temporal behaviour of metabolic fluxes) of metabolic systems and discuss them from the perspective of evolutionary systems biology. Rather than providing an exhaustive summary of the vast literature on metabolism, we highlight findings that we believe are representative or of key importance to our current thinking and understanding of metabolism and its evolution. In Sect. 4, we consider the possible key biological and biophysical factors that might drive and constrain the evolution of metabolic systems. Finally, in Sect. 5, we provide a future outlook that evaluates how taking an evolutionary systems biology approach to metabolism can open up new ways of understanding and engineering metabolic systems.

2 Structural Features of Metabolic Systems

The elucidation of the structure of metabolic systems starts with the pioneering biochemical studies of early 1900s. These Nobel-winning studies mapped key metabolic conversions within cells onto specific enzymes and organised these into so-called 'pathways' (Gottschalk, 1986). Known today mostly through the names of their discoverers, these include the Entner–Doudoroff (ET), Embden–Meyerhof–Parnas (EMP) and pentose-phosphate (PP) pathways involved in glucose uptake and conversion into pyruvate, and the Krebs pathway (a.k.a. tricarboxylic acid cycle) involved in the conversion of pyruvate into biomass precursors (Neidhardt et al., 1990). As biochemical studies continue to define new enzymatic reactions and pathways, organised databases of enzyme function (e.g. KEGG: Kanehisa, 2013; BRENDA: Jeske et al., 2019) allow us to increasingly achieve biochemical annotation through evolutionary relatedness among enzymes. This, together with increasing sequencing ability allows obtaining the lists of enzymatic reactions from genome sequences and derive insights on how cellular metabolism is organised in different organisms. It must be noted, however, that any analysis of reaction maps will be limited by the accuracy of such maps, which will relate to the quality of enzyme function annotations (derived biochemically or from genomic sequences by

homology). Setting aside such limitations, the study of metabolic reaction maps has flourished in the last few decades with the application of graph theory.

2.1 Metabolic Maps as Graphs (Networks)

Conceptually, it is easy to make the transition from a biochemist's drawing of a metabolic reaction pathway to a mathematically well-defined graph or network representation (see Fig. 1). Indeed, metabolites can readily be envisioned as nodes in a network connected through enzymatic reactions. The actual practice of mapping a biochemical reaction set into a network, however, can be done in several different ways that preserve or lose different types of information (Sandefur et al., 2012; Montañez et al., 2010; Zhou & Nakhleh, 2011; Arita, 2004; Beber et al., 2012) (Fig. 1).

In one approach, networks are defined such that there are two sets of nodes in the network; the molecular species in one set and the reactions themselves in the other set. This approach defines a bipartite graph, where the edges connect nodes from elements of one set (species nodes) to elements of the other set (reaction nodes) only (Fig. 1b). To make this network representation simpler, a logical first step is to combine the set of reaction nodes with their corresponding edges across the network. This results in a unipartite graph representation with only one type of node, which corresponds to the metabolites. Edges in this representation correspond to reactions that involve (or connect) the associated nodes (metabolites) (Fig. 1c). While some information on reaction mechanisms is lost in the unipartite graph representation, it is commonly used in databases and many graph theoretical network analyses. The latter aspect is important, because the representation of a metabolic system as a uni- or bipartite graph can have direct impact on the results of common network analyses such as degree distribution and modularity (Montañez et al., 2010; Zhou & Nakhleh, 2011; Arita, 2004; Beber et al., 2012) (see legend of Fig. 1).

2.2 Connectivity Within Metabolic Networks

Notwithstanding the importance of choices associated with the abstraction of a metabolic system as a network, the analyses of the resulting networks using graph theory can provide insights into their large-scale properties. An early, key finding in this regard was that the connectivity distributions of metabolic systems are more aligned with a scale-free distribution. For this distribution the probability of finding a node with connectivity k, scales with $k^{-\gamma}$, with γ being a constant degree exponent. This contrasts with a Poisson distribution expected from a random network (Jeong et al., 2000). The scale-free like distribution indicates the presence

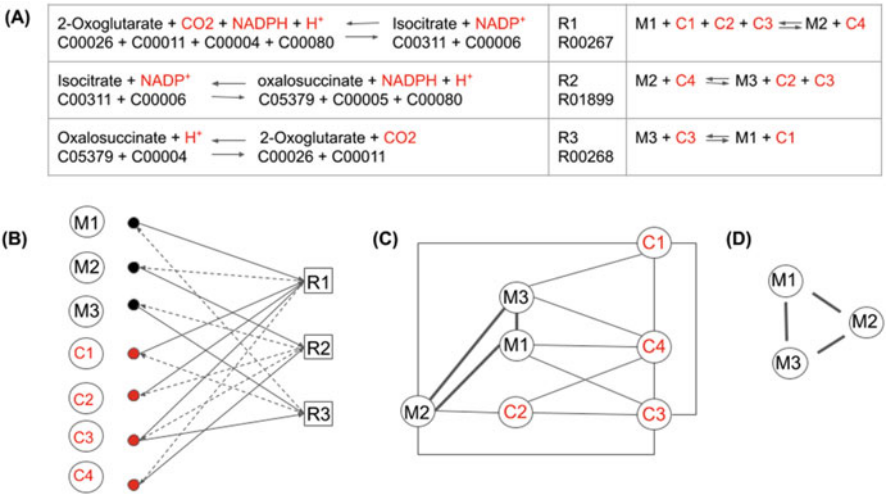

Fig. 1 Cartoon diagram showing a selection of approaches to capture biochemical reaction information as a graph, and possible discrepancies that can arise from this choice. We consider a small section of the central metabolism as an illustrative example. (**a**). The system contains three enzymatic reactions labelled as R1, R2 and R3, as well as with their KEGG reaction IDs (R00267, R01899, and R00268). The main metabolites {M1, M2 and M3} and additional ones {C1, C2, C3 and C4} are highlighted in black and red font respectively. (**b**) A directed bipartite graph corresponding to the reaction system given in (**a**). The enzyme information can be encoded within the reaction class (rectangular boxes), while substrates and products are represented by a metabolite class (circles). Solid and dashed lines represent involvement of substrates and products respectively in each reaction. (**c**). An undirected unipartite graph representing the same system. A reduced graphical complexity has been obtained by omitting the enzymatic information (i.e. the reaction class). Note that this results in the loss of the specificity of products and substrates towards reactions. (**d**) Another alternative graph representation with further reduction of complexity. Additional metabolites C1, C2, C3 and C4 have been absorbed into the graph. A comparison between these different graphs shows differences in graph-based statistics such as centrality and node degree; for example metabolites M1, M2 and M3 have different degrees {4, 5, 4}, in (**c**), but the same degree of 2 in (**d**)

of highly connected nodes, so-called hubs, in the system. It has been argued that these highly connected metabolites, that include the well-known energy carriers (such as ATP and GTP) and reductive equivalents (such as NADH and NADPH), represent evolutionarily ancient parts of the system (Wagner & Fell, 2001).

In order to better understand if any specific connectivity distribution could infer a functional (or evolutionary) benefit to a network, it is necessary to consider possible mechanisms that can generate such distribution. A general, empirical model for network expansion, which can lead to a scale-free connectivity distribution has been proposed and involves the preferential attachment of new nodes onto existing ones based on their connectivity (Ravasz et al., 2002). While the general nature of this model is useful to construct random networks with scale-free connectivity distribution, it has been shown that alternative, and biochemically more realistic

network expansion schemes can also lead to networks that display such connectivity distributions (Keller, 2005; Salathé et al., 2005; Takemoto & Akutsu, 2008; Pastor-Satorras et al., 2003). Additionally, *in silico* evolution of toy metabolic systems of enzymatic reactions, under selection for supporting growth, has shown to lead to the emergence of networks with scale-free connectivity distribution and hubs (Pfeiffer et al., 2005; Hintze & Adami, 2008). In one such study, the initial system that was composed of broad-specificity enzymes evolved, under selection for faster growth, into one that is composed of enzymes with high specificity, some of which highly connected, i.e. enzyme specificity and hubs have emerged (Pfeiffer et al., 2005). However, whether hubs emerged or not was dependent on the presence/absence of group transfer reactions in the toy metabolism, suggesting that the observed connectivity distributions in metabolic networks might be a by-product of the nature of biochemical reactions involved. An alternative theory suggests that the observed connectivity distributions (and in particular the presence of hubs) have emerged from selection for increased robustness of metabolic systems against loss of enzymes, since presence of hubs can infer to the system such robustness (Jeong et al., 2000). This argument, however, is not fully supported by subsequent analyses. In particular, it is indicated that the robustness of metabolic systems to enzyme loss is *apparent*; many of the enzymes that can be seemingly dispensable under a metabolically rich environment (suggesting high robustness to enzyme loss) are actually required in some other, metabolically limited environment (Papp et al., 2004). This kind of apparent robustness has emerged under *in silico* evolution experiments, where toy metabolic models were evolved under selection for growth under fluctuating environments (Soyer & Pfeiffer, 2010).

In summary, these findings show that the general connectivity distribution of metabolic networks is different from random ones, but might not have been directly selected for during evolution and rather resulted as a by-product of other evolutionary forces acting on metabolic systems. More broadly, they highlight the need for any adaptive arguments proposed for the possible evolutionary origins and significance of any structural network features to be evaluated carefully against simpler and possibly nonadaptive explanations (Papp et al., 2009; Zhou & Nakhleh, 2011; Basler et al., 2012).

2.3 Modules in Metabolic Networks

Another structural feature observed in graph representation of metabolic networks is the presence of clusters, or modules, where member nodes in a module present higher connectivity among themselves compared to the rest of the network (Ravasz et al., 2002; Guimerà & Amaral, 2005). It is shown that many of these modules correspond to known pathways that are described based on biochemical studies (Guimerà & Amaral, 2005). As with connectivity distributions, several evolutionary mechanisms have been proposed that can lead to the emergence of modular networks, including selection for increased enzyme specialisation (Soyer, 2007;

Espinosa-Soto & Wagner, 2010) or robustness to gene loss (Ravasz et al., 2002), or growth under static (Takemoto, 2012), randomly fluctuating (Hintze & Adami, 2008), or structured environments (Lipson et al., 2002; Kashtan & Alon, 2005). Modularity being the result of selection for robustness, is not supported by a subsequent study, which found no relation between robustness against genetic and metabolic perturbations and modularity in computationally generated toy models of metabolic systems (Holme, 2011). Modularity emerging from selection for growth under fluctuating or modular environments had a mixed support from subsequent analyses. Analysis of network modularity in different organisms that were grouped according to the environmental variability that they experience suggested some correlation between the two (Parter et al., 2007). Analysis of randomly generated metabolic networks also suggested a relation between modularity (based on flux distributions) and ability of a metabolic system to sustain growth in different simulated environments (Samal et al., 2011). Several other analyses found distinct levels of modularity for organisms living in different habitats or living different lifestyles (e.g. auto- vs. heterotrophic) (Kreimer et al., 2008; Takemoto & Borjigin, 2011; Mazurie et al., 2010), however there was no simple correlation between possible indicators of environmental variability (for example number of transport enzymes) and network modularity (Kreimer et al., 2008). Few studies found instead a strong correlation between habitat temperature and the level of metabolic modularity (Takemoto et al., 2007; Takemoto & Akutsu, 2008). *In silico* evolution of a toy metabolic system under fluctuating environments has found no positive relation between the level of modularity emerging over an evolutionary period and the level of environmental fluctuation over that time (Hintze & Adami, 2008). Taken together, these findings leave it inconclusive at the moment if the nature and frequency of environmental fluctuations had a strong influence on the selection of the level of network modularity.

It must be noted that any analysis of modularity in a network will depend on the definition of modularity and its quantification, as well as the choice of system abstraction used for the network representation. Methods that are alternative to those based on simple graph representation of metabolic systems have been attempted to define and measure modules (Kanehisa, 2013; Yamada et al., 2006; Muto et al., 2013; Sorokina et al., 2015). Perhaps the most straightforward of these is to define metabolic modules based on sets of reactions that are associated with each other according to the information in the literature. The KEGG database for example employs this strategy to define 'metabolic modules', which contain reactions resulting from literature-based pathway definitions, or are associated to a set of enzymes that are shown to either form a larger complex or are organised together on the genome (Kanehisa, 2013). The latter aspect is further developed into a 'phylogenetically' motivated metabolic module description, where modules are identified from the phylogenetic distribution profiles of individual enzymes and their 'connectedness' within metabolic reaction maps (Yamada et al., 2006). This approach indicated that enzymes that are closely connected (in terms of how many reactions separate their substrates) share more similar phylogenetic distributions across sequenced genomes. While plausible, this finding needs to be reevaluated in

light of phylogenetic distributions of modules corrected for phylogenetic relatedness of the organisms that they are found in, since such a correction is shown to impact the level of 'true' modularity in all biological networks analysed (Snel & Huynen, 2004).

Another alternative approach is to define modules based on reaction similarities. In this case, all known reactions are grouped into categories based on the similarity of the atomic conversions that they enable and then are used to analyse existing, known metabolic systems for common reaction patterns. This approach has allowed identification of so-called reaction modules, which represent patterns of specific reaction groups reoccurring in different parts of metabolism (Muto et al., 2013; Sorokina et al., 2015). While some of these reaction modules correspond to aforementioned, enzyme-based metabolic modules, some of them represent unique modules (Kanehisa, 2013). The existence of such modules suggests that despite the differences in the enzymes employed, different parts of metabolism employ similar reactions and that metabolic system evolution might be driven by adaptation of existing reactions to 'work' on new metabolites (Schmidt et al., 2003). Supporting this suggestion, simulating the evolution of metabolic systems through a reaction-based expansion algorithm, where new reactions are added to a network when their corresponding metabolites are made available by previous reactions or by the environment, showed that the resulting expanded networks display similar properties as those observed in nature (Ebenhoh et al., 2004; Ebenhöh et al., 2005; Raymond & Segrè, 2006). These findings can be interpreted as metabolic systems having emerged as a core set of chemical reactions, which are then reused in slightly different biochemical contexts when that network expanded to accommodate new molecules (Raymond & Segrè, 2006; Muto et al., 2013). This reaction-based view of metabolic evolution would be in agreement with an enzyme-based evolutionary scenario that considers early enzymes of low specificity subsequently diverging and specialising on different substrates, while maintaining a core set of reaction types (Jensen, 1976; Schmidt et al., 2003; Pfeiffer et al., 2005). It is important to note, in this context, that many of the current-day enzymes display promiscuous (low specificity) functions that are shown to facilitate the evolution of new pathways within the metabolic system (Kim et al., 2010; Soo et al., 2011).

2.4 Network Motifs

An alternative structural analysis of metabolic systems abstracted as networks, is to search for small interaction patterns within them that are overrepresented in the original network compared to randomised networks serving as null models. These patterns, or so-called motifs, were first identified in signalling and transcription networks (Milo et al., 2002). These two types of networks are found to display different motif prevalence, suggestive of a link between network function and type of motifs present (Milo et al., 2004). Indeed, subsequent studies have shown

that several of the motifs found in signalling and transcription networks can embed specific functions in a dynamical context, including noise filtering in signal transduction, and decoupling of the expression speed and level, in gene regulation (Goentoro et al., 2009; Mangan & Alon, 2003; Mangan et al., 2006; Alon, 2007; Lipshtat et al., 2008).

These findings, where overrepresented motifs from specific networks display specific dynamical and functional properties that are relevant to the overall function of those networks, suggest that motifs could provide the link between structural and dynamical (or functional) analyses of networks. This possibility, however, is called into question both in terms of statistical significance of overrepresented motifs and in terms of how specific their functional and dynamical properties are. The identification of overrepresented motifs is directly influenced by the choice of null models that the original, analysed networks are compared to (Artzy-Randrup, 2004). This raises the issue of identifying a suitable null model for the network being analysed, with alternative null models giving rise to different motif significance results (Konagurthu & Lesk, 2008; Avetisov et al., 2010). Even if overrepresented motifs are correctly identified, their functional significance is difficult to assess. For example, the bi-fan motif, identified in gene regulatory motifs and suggested to display specific dynamical properties, displays a range of dynamics under different parameter sets and modelling choices (Ingram et al., 2006). Similarly, an analysis of all three-node signalling network motifs indicated dependence of their response dynamics on the specifics of biochemical implementation choices in the model used (Soyer et al., 2006).

Identification and analysis of network motifs in metabolic systems are subject to these same issues as well. While specific metabolic network motifs were identified as significant (Eom et al., 2006), it was subsequently shown that this result is dependent both on the original network representation used and the randomised networks used for comparison (Beber et al., 2012).

3 Dynamics of Metabolic Systems

Cell metabolism is a dynamical process that converts an initial set of environmentally available metabolites into a set of end products that are released into the environment or incorporated into biomass (Fig. 2). While many reactions take part in this process, an overall chemical reaction can be written to describe the full conversion from substrates to end products. This overall reaction takes the form of a redox reaction, indicating that cell metabolism enables the flux of electrons across many reactions and between an initial electron donor and a final electron acceptor (Gottschalk, 1986) (Fig. 2a). It is shown that these intercoupled reactions, the involvement of conserved moieties in many of them, and metabolite-mediated allosteric regulation of enzymes (Fig. 2b) can all lead to rich temporal dynamics including oscillations and multi-stability (Reich & Sel'kov, 1981). These

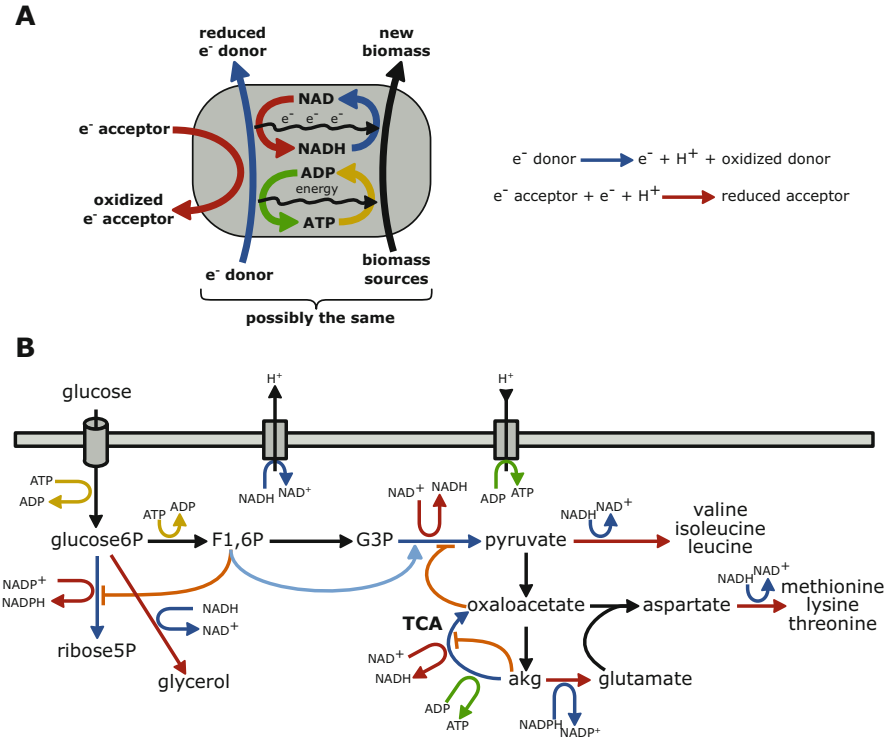

Fig. 2 Schematic representation of the chemical transformations carried out by a cell. (**a**) Representation of metabolic reactions at cell scale, considering the cell itself as a black box. From this standpoint, the phenomenon of cell growth appears as the combination of a catabolic and an anabolic reaction, as well as energy extraction from redox reactions that transfer electrons from an initial electron donor to a final electron acceptor. (**b**) More detailed representation of metabolic reactions, where the focus is shifted from the cell to its metabolic network, and highlighting both the many redox reactions and other types of chemical conversions. The metabolites are linked by chemical reactions (black arrows), sometimes involving the conversion of conserved moieties that are involved in redox (e.g. NAD/NADH, yellow for reduction and green for oxidation) and energy (e.g. ADP/ATP, red for energy investment and blue for energy extraction) balances. The upkeep of conserved moieties' balances can involve interaction with protein complexes embedded in the membrane (shown in grey). Some of the enzymes catalysing the reactions of the metabolic network are regulated by the concentration of metabolites, through allosteric regulations (shown as light blue and orange arrows)

nonlinear dynamics, on their own, or in combination with gene regulation, can then give rise to dynamic cellular behaviours. One of the key, open challenges in metabolic research is to decipher these higher-level metabolic behaviours and pinpoint structural features in the network that can be used as their explanatory and predictive indicators.

3.1 Overflow Metabolism and the Respiration-Fermentation Switch

Perhaps one of the earliest dynamical observation on higher-level behaviour of metabolism is a shift from pure respiration into fermentation or respiro-fermentation with changing conditions. This shift, known as contre-effet Pasteur, Warburg, or, Crabtree effect, is described initially in yeast and mammalian cells (De Deken, 1966). In a respiratory mode, metabolism utilises a strong electron acceptor such as oxygen and the associated, membrane bound electron transport chain. In fermentative mode, instead, metabolism utilises weak organic acids as electron acceptors and associated pathways, which provide a stoichiometric balance between reactions oxidising and reducing the key electron carriers (such as NAD^+ and NADH) (Gottschalk, 1986). Cells are found to display a shift from respiration to fermentation with changing availability of electron acceptors or carbon source, and with increasing growth rate (De Deken, 1966; Christen & Sauer, 2011; Meyer et al., 1984; Nanchen et al., 2006; Schulze & Lipe, 1964; Valgepea et al., 2010; Luli & Strohl, 1990; Postma et al., 1989; Rieger et al., 1983; Dauner et al., 2001). While a shift into fermentative pathways due to lack of strong electron acceptors can be intuitively understood as the only route to sustain electron flow, a similar shift due to increased carbon availability or growth rate are nonintuitive as they occur under the continued presence of strong electron acceptors such as oxygen and despite higher energy efficiency of respiration.

It has been suggested that a switch into fermentative pathways in presence of oxygen, happens due to limitations on the respiratory chain and associated pathways (Postma et al., 1989; Rieger et al., 1983). In this argument, the increasing carbon flow cannot be sustained by respiration alone, and any overflow needs to be directed into fermentative pathways to maintain stoichiometric balances and an assumed optimal growth (Majewski & Domach, 1990; Varma et al., 1993). This 'limitation-based' view, is extended by recent studies, which have argued for cellular space (Szenk et al., 2017; Zhuang et al., 2011) or protein content being key limiting factors that can favour fermentation over respiration, because fermentation is more efficient with regards to these features compared to respiration (i.e. higher energy produced per protein or space investment) (Molenaar et al., 2009; Basan et al., 2015; Schuster et al., 2011; Goelzer & Fromion, 2017; Wortel et al., 2018). The idea that overall protein amounts can be limiting is linked to the observation that increasing growth rates, where a switch to fermentation happens, results in an increased investment from the shared proteome pool into ribosomes (Klumpp et al., 2009) and that this can cause a shift into the more protein efficient fermentation (Basan et al., 2015; Schuster et al., 2011). Recent temporal measurements on proteome allocation, however, do not necessarily show a shift in the expression of the enzymes involved in central metabolism vs. respiration when cells undergo a respiration-to-fermentation switch (Goel et al., 2015; Metzl-Raz et al., 2017). Moreover, the fact that not all yeast species exhibit the Crabtree effect (De Deken, 1966) indicates that

the extent and dynamics of the Crabtree effects can be tuned, instead of being the result of an insurmountable limitation arising from resource allocation.

A plausible alternative explanation for the onset of a respiration-fermentation shift is the dynamics of the oxidised and reduced forms of key redox carriers within metabolism. These forms are involved in many of the reactions of the central metabolism, including glycolysis, TCA cycle, and pathways branching from these, as well as the respiratory chain (Fig. 2b). Thus, it is possible that shifts in the NAD^+/NADH balance can directly affect the flux distribution across these different pathways (Hatakeyama & Furusawa, 2017; Reich & Sel'kov, 1981). It is observed, both in yeast and *E. coli*, that altering NADH/NAD^+ dynamics with synthetically incorporated oxidases alters the critical level of glucose, at which the respiration-to-fermentation switch happens (Vemuri et al., 2006, 2007). Additionally, it is found that changes in the activity of pyruvate kinase, a key enzyme implicated in cancer cells' increased capacity for the respiration-fermentation switch (Diaz-Ruiz et al., 2009), can cause a feedback onto the dynamics of NADPH, which acts both as an electron carrier and a neutraliser of reactive oxygen species through redox reactions (Grüning et al., 2011). It is also possible to envision similar limitations, and impacts on metabolic fluxes, arising from ATP/ADP balances. Indeed, ATP balance is implicated to affect metabolic fluxes under different conditions in *B. subtilis*, including conditions favouring a respiration-fermentation switch (Dauner et al., 2001). That limitations arising from ATP/ADP and NAD^+/NADH balances can cause changes in overall metabolic fluxes, and lead to metabolic switches and overflows, is an attractive mechanistic explanation that could explain several additional metabolic overflows such as of amino acids and vitamins (Dauner et al., 2001; Jiang et al., 2018; Ponomarova et al., 2017).

The proposition that metabolic shifts are due to certain cellular limitations is an interesting concept to consider through the lens of evolution. It can be argued that any limitations on cellular resources can be overcome under appropriate selective pressures. If such limitations have not been overcome, then this is suggestive that they are either linked to physical hard bounds that evolution cannot surpass, or relate to trade-offs between different selective pressures. One such trade-off is proposed between growth rate and yield (Novak et al., 2006; Schuster et al., 2011; Bachmann et al., 2013; Wortel et al., 2018). For example, increasing the number of ATP producing reactions in a linear pathway would slow its overall flux rate, presenting a simple mechanism of a rate-yield trade-off (Pfeiffer et al., 2001; Heinrich et al., 1991). Such a thermodynamic basis for a rate-yield trade-off, combined with the higher energy yield of respiration, is used to argue for it driving the respiration-to-fermentation shift (Pfeiffer et al., 2001).

The respiration-to-fermentation shift, or overflow metabolism, results in the excretion of organic acids from cells, such as acetate and lactate. These organics can be used as a carbon source by other cells and result in a so-called cross-feeding interaction. It has been theoretically shown that trade-offs among uptake efficiencies of different carbon sources can lead to cells evolve into specialists on one of such carbon sources, creating the basis for the emergence of cross-feeding even within a single population (Doebeli, 2002). Indeed, long-term evolution experiments with

Escherichia coli resulted in the emergence of different clones that are shown to interact metabolically through cross-feeding (Le Gac et al., 2008; Rozen & Lenski, 2000; Grosskopf & Soyer, 2016).

3.2 Carbon Preference and Catabolic Pathway Switching

Another phenomenon observed with overall metabolic dynamics is the 'carbon preference' or 'carbon catabolic repression'. It was found that when microbes are cultivated in the presence of a mix of different substrates that they can catabolise, consumption follows a sequential pattern (Görke & Stülke, 2008; Monod, 1949), with an order of carbon sources specific to the microbial species (Collier et al., 1996; Van Den Bogaard et al., 2000; Parche et al., 2006). While it is shown that such carbon source switching can involve genetic regulatory networks with possible bistable dynamics (Ozbudak et al., 2004; Solopova et al., 2014; van Hoek & Hogeweg, 2006), it is less clear how the initial sensing of different carbon sources is achieved and conveyed to the regulatory level. One simplistic explanation is that carbon sources are directly sensed to trigger activation and inhibition of the associated downstream catabolic pathways to enforce the carbon preference hierarchy. This mechanism has been shown to be implemented in a few bacteria, but only for a small subset of their possible substrates (Aidelberg et al., 2014). Recent studies suggest that the adaptation of the microbial metabolic networks is not modulated by direct detection of the concentration of the entry-point substrates, but rather by the temporal dynamics of the concentration of key metabolites inside the network. It is proposed that there could be sensing of the metabolic fluxes through these key metabolites, which can react with transcription factors and thus affect the expression of catabolic enzymes to create feedback systems (Kotte et al., 2010; Görke & Stülke, 2008; Aidelberg et al., 2014).

A kinetic model of metabolism and gene regulation featuring central carbon pathways and several regulatory interactions between specific metabolites and transcription factors has allowed successful simulation of the carbon preference hierarchy (Kotte et al., 2010). Analysis of this model suggested that a given metabolite can become a 'flux-reporting', key metabolite, either because it is only produced under specific environmental conditions, or because it sits between two low-energy reactions, where its concentration can act as a reporter of the flux direction at this point in the metabolic system (Kotte et al., 2010). For example, in the *E. coli* metabolic system, fructose-1,6-bisphosphate, an intermediate of glycolysis, and its interaction with the transcription factor Cra is implicated as a flux-sensing regulatory system (Kochanowski et al., 2013).

Allosteric regulation of enzymes, that is regulation of enzyme activity through binding of substrates and products or additional metabolites, is another possible route to altering metabolic fluxes. This is possibly an evolutionary more ancient route to regulating metabolic systems, as it would not require gene regulation and the intermediary role of transcription factors. Additionally, allosteric regulation could allow for a quicker response to changing environmental conditions. Information on

the allosteric regulation of many enzymes is available and catalogued in databases (Jeske et al., 2019; Huang et al., 2011), and its regulatory role is implicated in several computational and experimental studies. The dynamic variations of many fluxes in the metabolic network of *B. subtilis* were not correlated to the expression level of its enzymes, suggesting that other means of regulation were at play, including allosteric regulation (Chubukov et al., 2013). Recent experimental work shows that allosteric regulation is required in addition to transcriptional control to explain the observed flux dynamics during catabolic repression and co-utilisation in *B. subtilis* (Buffing et al., 2018). Similarly, in *E. coli*, explaining metabolic flux shifts in response to changes in the nature of the carbon source required multiple regulatory layers including allosteric regulation (Link et al., 2013; Gerosa et al., 2015). A metabolic network model implementing such candidate allosteric regulations was able to predict flux dynamics under changing substrate availability (Machado et al., 2015).

3.3 Oscillations and Bistability

The high-level observations of pathway switching and catabolic hierarchy indicate that metabolic system fluxes can be abruptly altered upon changes in conditions. Such dynamics are suggestive of multistable, nonlinear dynamics, which could be expected from any system that displays high interconnectedness as seen in metabolic systems; many metabolites are acted upon by many different enzymes, individual enzymes can form dimers and heteromers that can bind multiple substrates and additional, nonsubstrate metabolites, and multiple reactions can connect through their metabolites to form cyclic or feedback reaction systems. These features provide a significant potential for metabolic systems to implement nonlinear dynamics such as bistability, oscillation, and homeostasis (Reich & Sel'kov, 1981).

Among these, bistability refers to a dynamical system that can attain two different steady states depending on initial conditions. Changes from one of these steady states to the other can be caused through perturbations in parameters or concentrations of system components. In the context of metabolic systems, two steady states would manifest themselves as different flux rates across reactions and perturbations can arise from changes in enzyme or metabolite concentrations, or through changes in catalytic rates of enzymes (induced for example through allosteric regulation). Bistability in metabolic system dynamics has been implicated in the context of respiration-to-fermentation switch (Lei et al., 2003), and when carbon metabolism is initiated on glucose (van Heerden et al., 2014) or switches from glucose to other carbon sources (Kotte et al., 2014; Şimşek & Kim, 2018; Ozbudak et al., 2004; Solopova et al., 2014). In particular, the latter studies found subpopulations, within isogenic populations, that show different metabolic behaviours not caused by mutations. In glucose-shift experiments, additional experiments with isotope labelled carbon indicated that these subpopulations emerged at the time of the shift, i.e. in response to changing conditions, and in a manner dependent on the concentrations of the different carbon source (Kotte et al., 2014). This suggests

that the metabolic system implements bistable dynamics, such that changes in external glucose concentrations can lead some cells to shift to a new metabolic steady-state flux distribution. Indeed, mathematical models implementing bistability are proposed to explain these experimental observations, in some cases involving transcriptional feedback in addition to metabolic dynamics (Kotte et al., 2014; Ozbudak et al., 2004; Solopova et al., 2014), and in other cases just the metabolic dynamics (Planqué et al., 2014).

In terms of bistability arising solely from metabolic system dynamics, there have been many theoretical studies indicating the possibility of bistability within simple enzymatic reaction systems (Fig. 3a). For example, bistability is shown to

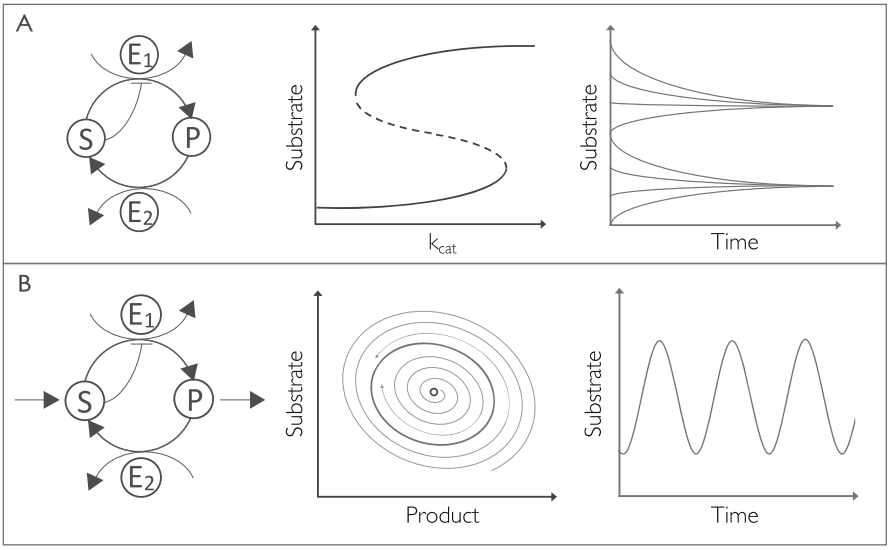

Fig. 3 Two cyclic reaction motifs and their corresponding dynamics. Note that such cyclic reaction motifs are readily found in natural metabolic systems, and in particular for reactions involving NAD^+/NADH-linked dehyrogenases (e.g. isocitrate and dihydrolipoyl dehydrogenases) and kinase-phosphatase pairs (e.g. phosphofructokinase—fructosebiphosphate pair). Reaction motifs are shown as cartoons with labelled circles representing substrate (S), product (P) and enzymes (E_1 and E_2). (**a**) Substrate (S) is converted into product (P) and then back again by two different enzymes (E_1 and E_2). These two enzymatic reactions can involve additional and different substrates and products (not shown) such that thermodynamic feasibility of the cycle is ensured. The activity of E_1 is substrate inhibited, that is, high concentrations of substrate reduce the rate of substrate to product conversion. Such a motif can display bistability under certain parameter conditions as shown in the subsequent panels. The middle panel shows the steady-state concentration of the substrate against the catalytic constant for E_1 (k_{cat}), while the last panel shows the time evolution of the substrate concentration, simulated from different starting conditions. (**b**) The same reaction motif as in (**a**), with the addition of flux into substrate from an external reservoir or upstream reaction (not shown) and flux out from the product into a downstream reaction (not shown). In this case, substrate concentration may oscillate within certain parameter regimes as shown in the subsequent panels. The first panel shows the steady-state concentration of product against the steady-state concentration of substrate, while the second panel shows the evolution of substrate concentration over time

be possible in systems of few coupled enzymatic reactions (Edelstein, 1971; Reich & Sel'kov, 1981). A particular 'reaction motif' that has been studied extensively is a two-enzyme cyclic reaction system, where a substrate is converted into a product and then back again, with both forward and backward reactions usually involving different co-substrates. It is common that the enzyme catalysing the forward reaction is regulated through substrate inhibition or substrate inhibition coupled with product activation (Hervagault & Canu, 1987; Cimino & Hervagault, 1990; Simonet et al., 1996; Guidi & Goldbeter, 1998; Mulukutla et al., 2014). This motif is found in several locations within metabolism, particularly around dehydrogeneases such as lactate dehydrogenase (Simonet et al., 1996), and kinase/phosphatase pairs such as those involved around fructose-6-phosphate (Mulukutla et al., 2014). These reactions convert different metabolites back and forth, using the NAD^+/NADH or ADP/ATP pairs as reaction partners. The theoretical findings from such cyclic reaction models were further supported by several in vitro re-constitution experiments that confirmed bistability experimentally and that were performed with different pyruvate kinase, lactate dehydrogenase, and isocitrate dehydrogenase enzymes and their corresponding partners in creating cyclic reaction schemes (Cimino & Hervagault, 1990; Simonet et al., 1996; Guidi et al., 1998).

In addition to bistability, threshold dynamics known as ultrasensitivity can arise from metabolic branching points (LaPorte et al., 1984), and can also lead to heterogeneities in metabolic phenotypes. Both ultrasensitivity and bistability are manifested by nonlinear 'input–output' relations, where the output of a system can change its steady-state value abruptly at a threshold value of a specific parameter of the system (Fig. 3a). Thus, if these dynamics are coupled with intrinsic or external noise in a relevant parameter across a population of cells, heterogenous metabolic outputs and cellular phenotypes can be observed. In this context, it is notable that significant level of noise or variance is seen in several metabolic parameters, including sugar uptake (Nikolic et al., 2013, 2017), ATP levels (Yaginuma et al., 2014), and expression levels of the enzymes involved in glycolysis and the TCA cycle (Rosenthal et al., 2018).

The same basic models that show bistable behaviour (as discussed above) can readily be extended with in- and out-fluxes of involved metabolites, to display oscillations (Fig. 3b) (Higgins, 1964; Sel'kov, 1968; Guidi & Goldbeter, 1998, 2000; Goldbeter & Guilmot, 1996). While these theoretical demonstrations of specific enzymatic schemes leading to oscillations have not been explored in detail in vitro, metabolic oscillations are readily observed both in vivo (Satroutdinov et al., 1992; Richard et al., 1993, 1996; Keulers et al., 1996; Sohn et al., 2000; Wittmann et al., 2005; Dodd & Kralj, 2017; Papagiannakis et al., 2017) and in situ, with cell extracts (Boiteux et al., 1975; Frenkel, 1968; Chance et al., 1964). In the latter case, both damped and sustained oscillations are observed, usually with a phase ranging from few to tens of minutes. It is possible that these oscillations relate to artificial changes in ATP dynamics arising from cell extract preparations (Frenkel, 1968), however, the fact that oscillations could be entrained by controlled glucose additions (Boiteux et al., 1975), show that there is an inherent ability for oscillatory dynamics in the underpinning enzymatic system. This ability is suggested to be linked

to the enzyme phosphofructokinase (PFK), which catalyses the phosphorylation of fructose-6-phosphate into fructose-diphosphate (Laurent et al., 1979). Several mathematical models of this enzymatic reaction, and incorporating the observed allosteric regulation of PFK both by its substrates and products, confirm the possibility of sustained oscillations (Higgins, 1964; Sel'kov, 1968; Goldbeter & Lefever, 1972).

In the case of intact cells, oscillatory dynamics are observed to occur within the central carbon pathways and displaying a phase of tens of minutes (Satroutdinov et al., 1992) up to several hours (Wittmann et al., 2005; Papagiannakis et al., 2017). Metabolic oscillations were demonstrated at single-cell level and are found to be autonomous but coupled with cell cycle oscillations (Papagiannakis et al., 2017). Additional studies across cell populations found that cells can synchronise metabolic oscillations under some conditions (Satroutdinov et al., 1992; Richard et al., 1993), and proposed several possible mediators including acetaldehyde, hydrogen sulphide, carbon dioxide, and pH (Richard et al., 1996; Keulers et al., 1996; Sohn et al., 2000; Dodd & Kralj, 2017). Models, involving some of these proposed synchronisation molecules, were also developed (Wolf & Heinrich, 2000; Wolf et al., 2001) and could reproduce experimental findings.

4 Evolutionary and Physical Drivers (and Constraints) on Metabolic Systems

We have highlighted, so far, a diverse range of structural and dynamical features of metabolism. We would argue that despite this accumulated wealth of information, we still lack a predictive understanding of metabolism at a systems level. For example, for many of the observed dynamics, it is not clear what their causative mechanisms are and how they could be influenced with external and internal perturbations. Additionally, for many of the observed structural features, we do not know what their functional significance are. Answering these open questions, as well as better conceptualising metabolic systems and devising new means to influencing their behaviour can benefit from identification of evolutionary drivers and constraints. While we have alluded to specific evolutionary arguments and studies in the above sections on observed properties, here we would like to summarise additional evolutionary and biophysical drivers and constraints relating to metabolism.

4.1 Thermodynamics

As collections of chemical reactions, metabolic systems must obey the laws of thermodynamics (Alberty, 2005). An active metabolic system remains away from

thermodynamic equilibrium through interactions of cells with external energy sources including molecular, thermal or pH gradients, and photo radiation, and as such, metabolism and its link to cellular growth can be described through the formalisms of nonequilibrium thermodynamics (Cannon & Baker, 2017; Westerhoff et al., 1983; Hellingwerf et al., 1982; Desmond-Le Quéméner & Bouchez, 2014; Goldbeter, 2018). Thus, considering thermodynamics-based ideas and criteria can provide insights into the evolution and present organisation of metabolic systems. For example, it has been shown that the utilisation of low-energy reactions that are more prone to thermodynamic inhibition due to product accumulation can allow relaxation of the ecological competitive-exclusion principle, and lead to co-existence of diverse species implementing different metabolic conversions, starting from a single substrate (Grosskopf & Soyer, 2016). Such low-energy reactions are present within cellular metabolism itself (Fig. 4), suggesting that metabolic pathway diversification could have evolved as a way to overcome thermodynamic bottlenecks. Other studies made similar suggestions for thermodynamics playing a key role in determining the overall organisation of cellular metabolism (Bar-Even et al., 2012a, b) and metabolically interacting multiple species (Vallino, 2010). In the latter case, computational simulations showed that applying the theory of 'entropy maximisation' in a way such that entropy production is maximised in a toy metabolic model over a time span and across a varying environment, results in a system behaviour similar to that observed from experimental microbial microcosms (Vallino, 2010). It remains to be seen if this type of optimisation can also explain the organisation of cellular metabolism or not.

Besides its possible role in shaping metabolic system organisation, thermodynamic constraints could also be directly influencing their temporal dynamics. Evidence for this possibility comes from the observation that many reactions within central carbon metabolism have free energies of reaction close to zero (Miller & Smith-Magowan, 1990; see also Fig. 4 legend). These reactions can become thermodynamic bottlenecks or reverse pathway flux direction under certain conditions (González-Cabaleiro et al., 2013; Dauner et al., 2001), thereby becoming influencing points for metabolic system dynamics. Thus, a combination of measuring metabolic concentrations and assessing reaction thermodynamics can allow an understanding of metabolic fluxes within a system, or conditions for enabling a certain flux distribution (Kümmel et al., 2006; Bennett et al., 2009; Noor et al., 2014). It is also possible that thermodynamic limitations under certain conditions can serve a regulatory or feedback role. For example, it is indicated that some proportion of observed flux shifts with changing carbon sources are explained by changes in reaction thermodynamics (Gerosa et al., 2015) and the excretion or consumption of acetate can be thermodynamically controlled by external acetate concentration (Enjalbert et al., 2017).

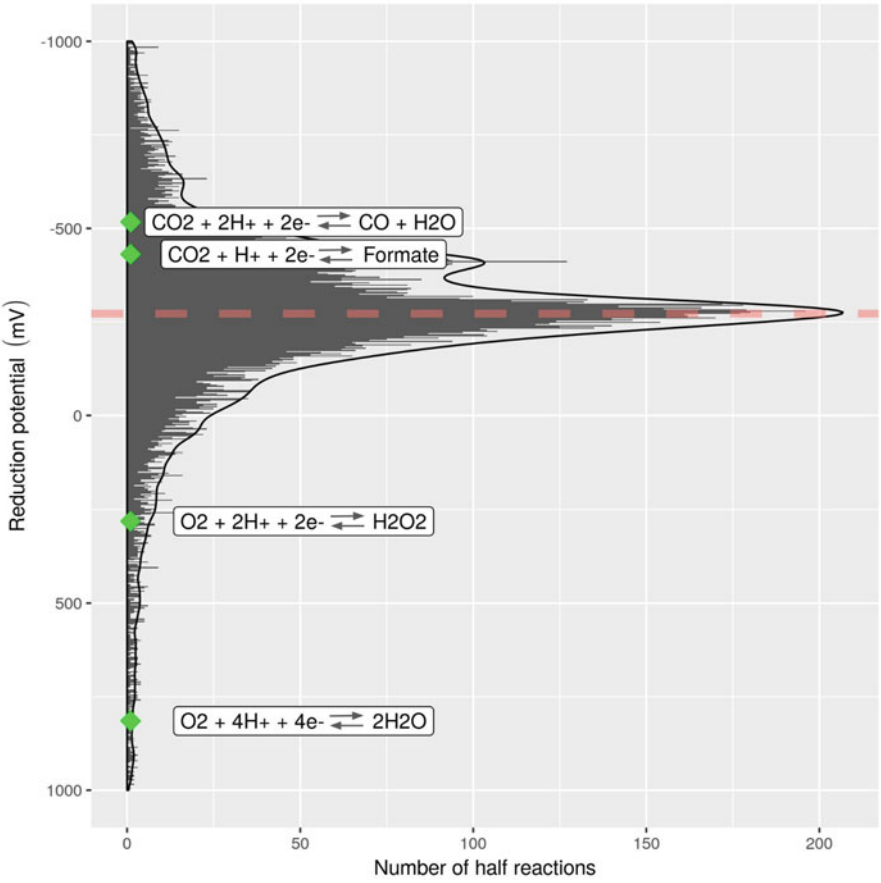

Fig. 4 Frequency histogram showing the standard reduction potentials (at pH = 7) of a set of metabolic redox half-reactions among a select set of 140 metabolites. This set of metabolites was compiled by Thauer (1977) along with their standard free energies of formation (at pH = 7). Using this set, we have computationally generated all possible atom balanced reduction half-reactions among the 140 metabolites (a total of 14,563 reactions) and computed the standard reduction potential for each reaction from the standard free energies of formation of the constituting metabolites and the number of electrons involved. The mean reduction potential of these half-reactions was found to be −272.03 mV (red dashed line). While the presented results contain both biologically realised and unrealised reactions, it is interesting to note that the mean of the distribution is close to the reduction potential of the NAD^+/NADH pair at −320 mV. Four examples of biologically observed reactions are indicated on the distribution, at their corresponding standard reduction potentials; calculated values according to the presented approach are {−517, −431, 281, 815 mV}, while corresponding values from the literature are {−500, −420, 300, 820 mV} (Voet et al., 2013). Note that higher (more positive) values of standard reduction potentials indicate metabolites affinity for electrons, i.e. their tendency to be reduced in a redox reaction. Thus, half-reactions with more negative (positive) reduction potentials have a tendency to run in the oxidation (reduction) direction

4.2 Biomass and Energy Production

The connection between metabolism and the production of cell constituents (i.e. biomass) is clear and well-formulated (Neidhardt et al., 1990). This clear connection has led to a dominant evolutionary view on metabolism that puts it as a servant to the cell and argues that selection for cells' proliferation dominates the evolution of metabolism. This view has led to the development of the commonly used stoichiometric modelling through flux balance analysis (FBA), which uses optimisation for biomass yield (on a given substrate) as its basis (Price et al., 2004). Alternative optimality criteria for FBA has also been formulated, but these also assume links to biomass formation; maximisation of ATP yield (Schuster et al., 2008; Schuetz et al., 2007) and minimisation of enzyme investment (i.e. total flux in the FBA context) along with biomass maximisation (Holzhütter, 2004). The latter idea has recently given rise to the Resource Balance Analysis (RBA), where the focus is still the optimisation of biomass production but considering not only metabolic flux constraints but also constraints arising from protein allocation to different cellular processes including transcription and house-keeping (Goelzer & Fromion, 2017).

Optimality ideas are also used to explore the biochemically feasible space of reactions using both kinetic simulations and graph theoretical approaches and to see if observed metabolic pathways are superior to alternatives in terms of supporting cellular growth. Both kinetic studies using optimality analysis and the simulation of feasible possible pathways indicated that glycolysis represents an optimal solution for maximising ATP flux (Heinrich et al., 1997; Court et al., 2015). Graph theoretical approaches suggested that the central carbon pathways represent enzymatically minimal routes among the different metabolites that act as precursors to biomass (Noor et al., 2010), and that the pentose-phosphate pathway represent the most enzyme-efficient solution to the sugar conversion it implements (Meléndez-Hevia & Isidoro, 1985). Enzyme cost minimisation with biomass optimisation, as used in the RBA approach, is also used to explain the presence of enzymatically different, but seemingly functionally redundant pathways in central metabolism, in particular, the glycolytic Embden–Meyerhoff–Parnass and Entner–Doudoroff pathways (Flamholz et al., 2013). These pathways are shown to require different levels of enzyme investment for achieving the same flux, a point that is used to argue that their evolution was driven for a requirement to sustain efficient biomass formation under environmental conditions that can support different levels of protein production (Flamholz et al., 2013). Following on from these findings, it was shown that when a metabolic system is simultaneously optimised for maximum flux and minimal enzyme investment, the resulting flux distributions correspond to so-called elementary flux modes (Wortel et al., 2014; Müller et al., 2014), which are paths through the metabolic system that have minimal enzymatic steps and that can sustain the required metabolic conversion under steady-state conditions (Schuster et al., 2000). In a recent study, these elementary flux modes are enumerated and analysed for their biomass yield and the growth rate that they can sustain when assuming minimal enzyme investment (Wortel et al., 2018).

4.3 Maintenance of Metabolic Gradients and Physicochemical Constraints

Selection alone for more or faster biomass formation could not have been a key driver of early metabolic systems that must have predated cells (or at least cells as we know them today). Instead, these systems must have been directly born out of nonequilibrium thermodynamics emerging from chemical gradients (Branscomb & Russell, 2013). Thus, the presence and maintenance of these gradients, as well as the chemicophysical properties of associated metabolites can still be relevant and informative for the current-day metabolic systems.

Stabilisation of mechanisms that can generate extracellular entropic gradients on which metabolism can operate is considered an important prerequisite for the emergence of metabolism (Branscomb & Russell, 2013). Extracellular spatial organisations that can allow metabolic systems to operate on chemical gradients are argued not to readily form in a well-mixed 'primordial soups' based on considerations of diffusion rates (Barge et al., 2017; Branscomb & Russell, 2013). A possible solution to this problem is mounds of hydrothermal vents that can sustain metabolic gradients (Branscomb & Russell, 2013; Martin & Russell, 2007). There is also a possible role for self-forming coacervates, a type of phase separation driven by charged molecules, that could maintain short-scale metabolic gradients due to different diffusion rates through the bulk and coacervate phases (Oparin, 1965). In this context, it is interesting to note that synthetic coacervates are shown to be able to harbour enzymatic processes (Nakashima et al., 2018) and coacervate-like phase separations are observed in current-day cells (Nott et al., 2016).

Adaptation of metabolites into these early cell-like formations (or liquid phases) and ultimately into cellular metabolism could have been driven by their physicochemical properties. For example, metabolites that are more readily involved in the formation of coacervates or that are readily trapped in them, might end up being locked-in into later metabolic systems. From this perspective, it is interesting to note that the analysis of physicochemical properties of current-day metabolites indicate some trends in terms of reactivity, solubility, and diffusion across membranes (Morowitz et al., 2002; Srinivasan & Morowitz, 2009; Bar-Even et al., 2012a, b), there seems to be relations between metabolites' connectivity in graph representations of metabolism and their polarity (Zhu et al., 2011), and that simple physicochemical pruning rules applied on the available chemical space can lead to biologically relevant subsets of molecules (Morowitz et al., 2002).

If the early evolution of metabolic systems involved a phase-separated, cell-like environment, than there must have been mechanisms to ensure maintaining a metabolic gradient across such an environment. It has been suggested that maintaining such a gradient in light of fluctuating external metabolite concentrations can be ensured by cyclic reaction systems (Hatakeyama & Furusawa, 2017; Reich & Sel'kov, 1981). Such cyclic systems are highly prevalent in current-day metabolism in form many coupled reactions that use the same conserved moieties such as NAD/NADH and ADP/ATP in opposing directions (see Fig. 3).

5 Conclusion and Future Outlook

The extensive biochemical and genetic study of metabolism provided us with detailed information on metabolic systems. While this information can and has been condensed to graphical representations, graph theoretical analyses of metabolic systems have not necessarily yielded clear insights to structure–function relations and predictive capabilities. It is indeed possible that many of the structural features of metabolic networks are by-products of biochemical and biophysical drivers. Indeed, when using biochemically appropriate representation and randomisation schemes, the significance of the gross structural properties of metabolic networks are highly dependent on the exact null model utilised (Basler et al., 2012; Zhou & Nakhleh, 2011). When compared to similarly complex abiotic chemical systems, many structural features of metabolism are found not to be unique (Holme et al., 2011), suggesting that there is not necessarily any functional (or evolutionary) significance to these properties.

In terms of dynamics, most focus has been on modelling pathway dynamics, while considering them as isolated entities. This divide-and-conquer type approach might be justified due to the curse of dimensionality and lack of detailed kinetic parameters associated with large-scale metabolic models, yet, we argue that it will eventually be limited because metabolism is so highly interconnected. Thus, we advocate a push for analysis of models that can take into account the connected nature of metabolism, especially through conserved moieties such as redox and energy carriers. It is possible that simulations with tractable toy models (e.g. combining metabolism with other cellular processes; Molenaar et al., 2009; Weisse et al., 2015), or new approaches, such as statistical thermodynamics applied to metabolic dynamics (Cannon, 2014; Thomas et al., 2014), can provide headways in this direction.

It has been a common practice to conceptualise metabolism within an adaptive evolutionary framework and see it as a servant to achieving optimal biomass production. Within this view, it is proposed that pathway dynamics can be understood through supply–demand type relations (van Heerden et al., 2015; Hofmeyr & Cornish-Bowden, 2000), where cell growth determines demand for biomass precursors (and ATP), which is then delivered through pathways such as glycolysis. This adaptive view has also given rise to the development of whole-genome scale stoichiometric models of metabolism and their study through FBA and biomass optimisation. In our view, and as noted also by others (Schuetz et al., 2007; Schuster et al., 2008), this strong reliance on an adaptive evolutionary argument and ad hoc constraints limits the FBA approach. Efforts are now being made to improve FBA's predictive power with the development of flux rate constraints that are based on biophysical arguments (Mori et al., 2016), and with the development of approaches that enable sampling of larger number of possible flux distributions rather than using linear optimisation on a single objective to obtain a single flux distribution (Binns et al., 2015). The former point is also being addressed with the recently developed RBA, which assumes metabolism to be simultaneously optimised for maximum

biomass production at minimum enzyme investment (Wortel et al., 2018; Goelzer & Fromion, 2017; Flamholz et al., 2013; Schuster et al., 2011; Molenaar et al., 2009; Holzhütter, 2004).

It remains to be verified if selection for fast or high-yield biomass production is, or was, a dominant force on the evolution of metabolism and if the issue of enzyme allocation is a widespread and evolutionarily relevant limitation. For example, while unicellular organisms are shown to evolve rapidly under selection for faster growth, selection for individual cell proliferation is certainly not the dominant factor in the case of multicellular organisms, where cell collectives have to limit their growth to achieve developmental constraints and requirements. Even in the case of unicellular organisms, many environments only support extremely slow growth (Jørgensen & Marshall, 2015) and selection for fast growth might never become relevant. Similarly, the argument that minimisation of enzyme investment being a strong factor shaping metabolic system evolution needs to be considered carefully. While it is likely that cellular protein content has an upper bound, it is less clear if cells are close to this upper bound to the extent that limits on enzyme amount would directly influence metabolic fluxes, or if such effects would operate equally for every enzyme or under different conditions (Brown, 1991). While some studies indicate control architectures for transcriptional regulation of enzyme levels to confer to optimality criteria (Chubukov et al., 2012), these were confined to linear, biosynthesis pathways within metabolism. In contrast, global analyses of metabolic systems suggest that enzyme levels are not necessarily tightly regulated as metabolic fluxes change with changing conditions. For example, proteomics studies have found that levels of different enzymes do not change as metabolic fluxes shift (Goel et al., 2015; Metzl-Raz et al., 2017) and several experimental analysis pointed to the importance of allosteric and thermodynamic regulation (Rossell et al., 2006; Chubukov et al., 2013; Link et al., 2013; Gerosa et al., 2015; Machado et al., 2015; Buffing et al., 2018) rather than transcriptional regulation of enzyme levels.

It has also been shown that the commonly pre-assumed tight linkage between energy harvesting through catabolic pathways and biomass formation through anabolic pathways is not necessarily observed and there are many instances of significant ‘energy spilling’ (Russell & Cook, 1995; Dauner et al., 2001). In its simplest form, such spilling can happen, and some of the harvested energy is ‘lost’ by the cell to drive chemical reactions. It has been largely documented that the amount of harvested energy not converted into biomass is correlated to some properties of the anabolic pathways, across many taxa and growth conditions (Heijnen et al., 1992; von Stockar et al., 2006; Roden & Jin, 2011; Smeaton & Van Cappellen, 2018) and this observed link has been used to couple the stoichiometry of energy harvest and biomass synthesis, and successfully predict population growth yields (González-Cabaleiro et al., 2015). However, a mechanistic understanding of this correlation remains elusive.

Considering links to the pre-cellular metabolic systems, an alternative evolutionary view can be formulated that considers metabolic evolution as primarily being shaped and constrained by thermodynamics and physicochemical factors (Vallino, 2010; Branscomb & Russell, 2013). Thus, these factors need to be included in

formulating both evolutionary ideas and dynamical models of metabolism. While steady-state, stoichiometric models tried to include reaction thermodynamics as additional constraints in their optimisation formulations (Flamholz et al., 2013; Henry et al., 2007; Hoppe et al., 2007), dynamical simulations incorporating thermodynamics are only recently being started to be explored in the context of metabolism (Cannon, 2014; Cannon & Baker, 2017; Cannon et al., 2018; González-Cabaleiro et al., 2013, 2015; Thomas et al., 2014).

We argue that it will be productive to reconcile the two evolutionary views on metabolism; metabolism as a highly regulated system optimised solely for cell growth vs. a self-organising system governed by thermodynamics and biophysical factors. It is possible, for example, to consider that thermodynamically driven and self-organising, early metabolic systems could have been stabilised by cell-like structures, which could have then helped stabilise and perpetuate those metabolic systems. There could be remnants of such feedbacks still evident in current-day metabolic systems, where features that have emerged (and maintained) solely due to thermodynamic and other physicochemical drivers are intertwined with those that resulted from selection for increased biomass production rate or yield. To this end, we note that considering metabolism as composed of interlinked catabolic and anabolic pathways within a thermodynamic framework allows a successful empirical description of cell growth and biomass yield (von Stockar et al., 2006), and in certain cases, growth dynamics (González-Cabaleiro et al., 2015). Similarly, explaining metabolic regulation seems to benefit from the synthesis of regulatory mechanisms based solely on metabolite-driven effects, and those based on transcriptional control of enzyme levels (Chubukov et al., 2014).

While many ideas and studies about metabolism concerns steady-state fluxes, it is clear that metabolic dynamics are highly nonlinear and can readily give rise to bistability and non-steady-state dynamics such as oscillations. Both of these dynamical features are linked to higher functionalities, with bistability implicated in dynamic switching of metabolic fluxes and oscillations linked to the regulation of cell cycle (Lloyd et al., 2003; Papagiannakis et al., 2017), management of superoxide generation during growth (Murray et al., 2007), and resilience and communication in multicellular structures such as biofilms (Liu et al., 2015). Thus, further study of metabolic dynamics, and their molecular driving mechanisms, can provide important insights on how higher-level cellular and multicellular behaviours arise and are maintained through metabolism. While it is possible that the emergence of oscillations is intertwined with bistability (Martinez-Corral et al., 2018), one can already note that the same metabolic 'motif' that can mediate bistability can also readily be extended with additional features to mediate oscillations (as discussed above). Interestingly, and as a side note, the association of simple metabolic motifs with potentially complex nonlinear dynamics led to the suggestion that engineering of enzymatic dynamics could be an ideal route for implementing specific dynamics with biological systems (Arkin & Ross, 1994). Subsequent focus in the then emerging field of synthetic biology, however, focused on engineering of transcriptional regulation. It would be useful, in our view, to now reconsider enzymatic systems from an engineering perspective, to use them to implement

specific system dynamics (such as bistability). This would be facilitated by new mathematical and experimental tools (such as ability to create multidomain proteins) and by further exploring the biochemical and kinetic determinants of enzyme reaction motifs that enable in them specific dynamics. In this context, identifying allosteric interactions, as being done with emerging proteomic approaches (Li et al., 2010; Piazza et al., 2018), and considering the resulting reaction motifs from these interactions (Reich & Sel'kov, 1981) can allow us to better understand the role of allosteric regulation in metabolic system evolution, either as a positive factor or as something to be avoided (Alam et al., 2017). Finally, an electrochemical view on metabolism (Berry, 1981; Zerfass et al., 2018) can allow interfacing and controlling metabolism through such reaction motifs, for example, by controlling redox states of conserved moieties to drive the dynamics of interlinked reaction cycles.

Experimental analysis of metabolic systems is constantly benefiting from the application of omics and biophysical techniques, including metabolomics and imaging. We argue that, as these new techniques develop, old ideas need also be revisited with new tools. For example, thermodynamic information on many reactions within metabolism are not available, and measurement of key physiological parameters such as pH, respiration rate, or ATP/ADP ratio are still mostly lacking at the single-cell level. Such single-cell measurements can be essential to develop better understanding of cellular metabolism, which is implicated to show heterogeneities within clonal populations (Nikolic et al., 2013, 2017; Yaginuma et al., 2014; Rosenthal et al., 2018) and discover the key trade-offs arising from metabolic system structure and dynamics. Single-cell analyses can also allow identifying metabolic interactions within populations (Rosenthal et al., 2018), especially in populations with an inherent structure, such as biofilms and tissue. This, in turn, can allow us to make connections between metabolic dynamics and emergence of division of labour and multicellularity (Liu et al., 2015). Within spatially organised systems, the role of diffusion of metabolites, especially charged ones, across or within membranes needs to be considered, as they can give rise to the formulation of new modes of communication such as metabolite-driven electrochemical signalling (Prindle et al., 2015). Finally, the analysis of metabolic systems under conditions of no growth, but sustained viability, is another under-studied area, which can give better insights into the connections between metabolism and other physiological processes, and in particular membrane potential and cell division.

Evolutionary thinking can provide a canvas on which to evaluate findings from metabolic systems and draw up new experiments. More specifically, the use of evolutionary thinking and experiments for the identification of selective trade-offs, physicochemical constraints, and ecology-evolutionary feedbacks can provide insights into current-day metabolic systems. For example, the consideration of possible feedbacks between ecological and evolutionary dynamics can help us better understand the emergence of metabolic interactions within microbial communities (Grosskopf et al., 2016). The consideration of trade-offs between different selectable traits, on the other hand, can allow proposition of multistable metabolic behaviours that might become embedded in the metabolism of different

cell types in multicellular organisms. The advancement of an evolutionary thinking in metabolic research can thus bear important insights into the future.

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