Pareto Optimality in Organelle Energy Metabolism Analysis

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Abstract—In low and high eukaryotes, energy is collected or transformed in compartments, the organelles. The rich variety of size, characteristics and density of the organelles makes it difficult to build a general picture. In this paper we make use of the Pareto front analysis to investigate the optimisation of energy metabolism in mitochondria and chloroplasts. Using the Pareto optimality principle, we compare models of organelle metabolism on the basis of single- and multi-objective optimisation, approximation techniques (the Bayesian Automatic Relevance Determination), robustness and pathway sensitivity analysis. Finally, we report the first analysis of the metabolic model for the hydrogenosome of Trichomonas vaginalis, which is found in several protozoan parasites. Our analysis has shown the importance of the Pareto optimality for such comparison and for insights into the evolution of the metabolism from cytoplasmic to organelle bound, involving a model order reduction. We report that Pareto fronts represent an asymptotic analysis useful to describe the metabolism of an organism aimed at maximising concurrently two or more metabolite concentrations.

Index Terms—Mitochondrion, Chloroplast, Hydrogenosome, Sensitivity analysis, Multi-objective optimisation, Robustness analysis.

1 INTRODUCTION

In recent years, the fields of mitochondria, chloroplasts and other mitochondrion-like organelles (MLO), e.g. hydrogenosome and mitosome, have witnessed an extraordinary expansion. This is mainly due to the identification of the pivotal role that mitochondria play in human disease and ageing [1], to the synergy shown by chloroplasts and mitochondria in energy output [2], to the development of new organelle proteomics tools [3], and to the discovery of novel factors involved in organelle division, movement, signalling and adaptation to varying environmental conditions [4]. Remarkably, no examples of examined eukaryotes lacking a mitochondrion-related organelle exist [5].

It is now widely accepted that all extant eukaryotes are descended from an ancestor that had a mitochondrion. In anaerobic eukaryotes, this organelle has been modified into either a hydrogenosome, which continues to generate energy for the host cell, or a mitosome, which does not. Therefore, the evolutionary history of chloroplasts and mitochondria are intertwined, and a better knowledge of the pathways in both organelles and vestigial organelles (e.g. apicoplast [6] and hydrogenosome [7]) may lead to drug discovery. Interestingly, when a mitochondrion and other organelles coexist in the same cell, their volume and activity can be strongly related with one another. In these cells, mitochondria exhibit behaviours that are absent in other eukaryotic cells [8].

Chloroplasts, mitochondria, hydrogenosomes and other MLO share many common features. For instance, the hypothesis of Co-location for Redox Regulation (CoRR) predicts that the regulatory coupling operated continuously before, during, and after the transition from prokaryote to eukaryotic organelle [9]. According to the CoRR hypothesis, these organelles would lose their genomes in case of loss of the redox and proton-motive machinery of oxidative phosphorylation. Moreover, they are “smart” energy-transducing devices and make decisions on the basis of environmental changes affecting redox poise. CoRR operates today and will continue to operate in living cells, being a necessary condition for the compatibility of energy conversion with genome function. Finally, these organelles exploit a common pathway to harness energy for biological purposes. This process is called chemiosmotic coupling, since it involves both the chemical bond-forming reactions that generate ATP (chemi) and the membrane-transport processes (osmotic).

Although mitochondria and chloroplasts are two different organelles with different functions, it is useful to compare the electron-transport processes in mitochondria, which convert energy from chemical fuels, with those in chloroplasts, which convert energy from sunlight. Chloroplasts produce glucose from sunlight energy. This glucose then transfers to the mitochondrion for aerobic respiration. The function of chloroplasts is basically to make food, by trapping light energy to convert water and carbon dioxide to form oxygen and glucose (this process is called photosynthesis). It is noteworthy that the plant produces most of its ATP from aerobic respiration, not from photosynthesis. Conversely, the function of mitochondria is to provide energy for the cell’s use. Mitochondria extract the energy from the glu-
cose molecules, and store it in ATP molecules; the ATP molecules will then diffuse out of the mitochondrion for the cell’s use.

We aim at investigating and comparing the complexity of these organelles through a common framework that includes single- and multi-objective optimisation, robustness analysis and sensitivity analysis. In addition to the possible goals and methods reviewed by Handl et al. [10], the optimisation of biological networks has been recently performed using mixed integer approaches [11]. The multi-objective optimisation in organelles such as the mitochondrion may be related to the different tasks of maximising the ATP or the heat, or intermediate compounds of the Krebs in order to provide input for biosynthetic pathways (e.g. the amino acids synthesis). Therefore, we propose the investigation of the evolution from the ancestral bacterium in light of their multi-objective optimisation. Specifically, Pareto fronts are related to the sensitivity and robustness of an organism, and represent a key tool for the metabolic analysis.

The ODE models we take into account are the chloroplast model by Zhu et al. [12] and the mitochondrion model by Bazil et al. [13]. First, we perform a Sensitivity Analysis to identify the most important components of both organelles. Second, we optimise the ability of these organelles to produce CO₂ and ATP respectively. The maximisation of these metabolites allows the optimisation of both metabolic networks. Finally, we assess the fragileness of the multi-optimised metabolic networks using the Robustness Analysis [14]. Our framework focuses on the fundamental properties of these organelles, and their integration into broader physiological processes. The paper will emphasise the commonalities and the differences in the energy production in mitochondria, chloroplasts and hydrogenosomes, in light of their evolution from bacterial endosymbionts. A comprehensive analysis of these organelles through a multi-objective optimisation approach highlights engineered endosymbiotic relationships between different species and therefore represents a valuable tool for synthetic biology [15].

Finally, we propose a model for the hydrogenosomes in *Trichomonas vaginalis*, with the aim of understanding similarities and differences relative to other organelles (e.g. which pathways are retained from mitochondria, and which ones are lost). The hydrogenosome was first noted in *Trichomonas foetus* as a component that produced hydrogen and ATP. The origin of this organelle remains a matter of debate, and an extensive analysis on its energy production could give new hints in understanding its ancestral relationship to mitochondria. Thus, using our model, we carry out a flux balance analysis [16] taking into account the ATP production as one of the objective functions to optimise.

## 2 A Common Optimisation Framework for Organelles

In Figure 1 we propose the steps of a common pipeline to analyse organelles. Although not reported in the figure, it is noteworthy that organelles are not closed systems. Indeed, they receive input signals from the nuclear genome, thus several proteins of the cell are transported to them. In every parameterised dynamical system with a given initial condition, each value assumed by the vector of parameters in the parameter space corresponds to a trajectory of the system in the state space. The system converges to the steady state through a dynamics, thus the steady state acts as an attractor [18]. In the ODE models described in this paper, the parameter and state spaces are Euclidian spaces. As regards the size of the state space, we expect the following inequalities to hold: (i) ancestral bacterium > mitochondrion, and (ii) chloroplast > hydrogenosome > apicoplast.

In order to apply the sensitivity analysis in the whole parameter space, we build an organelle metamodel using SUMO toolbox [19]. In this paper we apply it to the mitochondrial model proposed by Bazil et al. [13] to obtain a less accurate model that can be deeply analysed even if the number of inputs is very large. In our case, we are able to investigate the combined effect of 42 input parameters on the ATP yield and to perform a sensitivity analysis by means of the Bayesian Automatic Relevance Determination (ARD). Since the parameter space is 42-dimensional, through a metamodel we avoid the computational cost of running the whole mitochondrial model in the parameter space. The parameters of the meta-model belong to a neighbourhood of the parameter values of the original model. Since the meta-model is an approximation of the real model, given the same initial condition, the trajectories in the state meta-space differ from those in the original space. Thanks to the adaptive sampling of the original model, the attractor (i.e. the steady state point) is the same as that of the original model. Therefore, we can easily investigate the sensitivity of the parameters and obtain a rank that indicates to what extent a change in each parameter affects the behaviour of the system.

The multi-objective optimisation exploits the concept of Pareto optimality to maximise two or more desired metabolites in the organelle, thus obtaining new artificial strains. The choice of the target metabolites depends on the organelle under investigation. For instance, in mitochondria we will analyse ATP and NADH, the energy molecules in the cell, while in chloroplasts we will focus on the CO₂ uptake, which drives the photosynthesis. They are the most important products in these organelles and, when maximised, allow them to work at the optimal level. In particular, the multi-objective optimisation turns out to be very useful when we need to maximise simultaneously two or more metabolites responsible for the energy production.

Finally, the robustness analysis assesses the fragility of a strain obtained through the optimisation when it undergoes small perturbations, both external (changes in the nutrients) and internal (changes in the metabolism). One can define the robustness region, in which a change in the initial condition does not prevent the algorithm to
Fig. 1: The common framework to analyse organelles. We start from the complete model of the organelle, whose state space reflects its metabolism. We reduce the organelle model (A) to a meta-model (B) in which we can analyse its high-dimensional parameter space and evaluate the sensitivity of all its parameters by perturbing them in a neighbourhood of the original values. Then, we perform a multi-objective optimisation (C) on the organelle metabolism, in order to find the Pareto-optimal front involving two or more metabolites of interest (e.g. ATP and NADH); the genetic algorithm underlying the optimisation allows us to reach the optimal Pareto front, i.e. to move the front towards the optimal point (e.g. maximum ATP and NADH), which is unfeasible if the two objective are negatively correlated with one another. Finally, we evaluate the robustness (D) of the Pareto-optimal solutions. Kitano [17] has remarked on the need for a general theory of biological robustness. According to him, a system is robust if it maintains its functionality, even if it transits through a new steady state or if it is unstable (a). According to Gunawardena [18], the robustness to change of initial conditions is called dynamical stability; for instance, one can evaluate the differences in the dynamics of the system (b). According to Stracquadanio and Nicosia [14], the robustness of a system is the number of robust trials over the total number of trials; a perturbation trial is said to be robust when the perturbation is in the robust neighbourhood (c) such that the output remains in a given interval.

We apply this methodology to mitochondria and chloroplasts not only to provide meaningful insights into the production or uptake of their key metabolites, but also to obtain a comparison based on Pareto fronts between these two organelles.

### 3 Comparison Between Mitochondria and Chloroplasts Optimisation Models

In this section and in the following ones, we compare the behaviour, the sensitivity, the optimisation and robustness of the mitochondrion and chloroplast models.

In mitochondria, electrons from a carbohydrate food molecule degrading to CO$_2$ are transferred through the membrane by a chain of electron carriers, and finally reduce oxygen gas (O$_2$) to form water. Since they flow down this path from a high-energy state to a low-energy state, electrons release free energy, which allows to drive a series of three H+ pumps in the inner mitochondrial membrane. The third H+ pump catalyses the transfer of the electrons to O$_2$. The mechanism of electron transport between two sites is carried out by diffusible molecules that can pick up electrons at one location and deliver them to another [20]. In mitochondria, the first electron carrier, NAD+, is able to take up two electrons (plus an H+) to become NADH, a water-soluble small molecule that ferries electrons from the sites where food molecules are degraded to the inner mitochondrial membrane. All the proteins in the membrane and all the small molecules involved in the electron transfers form an electron-transport chain.

In chloroplasts, the transfer of electrons is driven by photosystems. In these membrane components, a pigment called chlorophyll captures the energy from the light and, through the C3 cycle, produces the energy-storage molecules ATP and NADH. This energy allows to transfer electrons. Remarkably, while the mitochondrion consumes O$_2$, the chloroplast generates it. This is due to the fact that the direction of transfer in chloroplasts is opposite to that in mitochondria. Indeed, electrons are taken from water to produce O$_2$, and they are donated to CO$_2$ to synthesise carbohydrate (the donation occurs through NADPH, which is closely related to NADH).

The mitochondrial model [13] consists of 73 DAEs (Differential Algebraic Equations), each of which represents either a constraint or the rate of variation...
of a metabolite involved in bioenergetic reactions of mitochondria. The state variables were initialised to achieve the fully oxidised state [13]. In this work, we calculated the metabolite content that leads to maximising the matrix ATP and NADH, maintaining constant oxidised cytochrome c, reduced cytochrome c, ubiquinone, ubiquinol, NAD_{mtx}, NADH_{mtx}, GTP_{mtx}, GDP_{mtx} (mtx = matrix), the mitochondrial membrane potential (1 mV), the matrix O_{2} (0.0652 mmol/mg), the total CO_{2} (21.4762 nmol/mg) and Ca^{2+}. We initialised Ca^{2+} with five different values to evaluate the behaviour of mitochondria. First we used Ca^{2+} = 10^{-5}, i.e. the standard condition, and then 10^{-4}, 10^{-6}, 10^{-5}×1.5 and 10^{-5}/1.5 nmol/mg.

As regards chloroplasts, in the model by Zhu et al. [12] the photosynthetic metabolism is modelled through equations for conserved quantities (e.g. total leaf nitrogen) and a set of 31 linked ODEs, each of which describes the rate of change in a metabolite. The C3 metabolism takes place in four compartments: thylakoid membranes, thylakoid lumen, chloroplast stroma and cytosol. Photoreactions and electron carriers are embedded in the membrane, while protons accumulate in the lumen. Carbon reduction and starch synthesis pathways are located in the stroma with sucrose synthesis in the cytosol. The phosphate translocator transports metabolites through the chloroplast membrane. The molar concentrations of metabolites required in the kinetic equations are the state variables. If necessary, the total amount of a specific compound per unit leaf area is expressed by multiplying its concentration by the volume of the compartment per unit leaf area.

4 Sensitivity Analysis

It is extremely important to focus the in silico design on a set of restricted significant parameters, in order to decrease the complexity of a future biological implementation. In this research work, we perform sensitivity analysis both on mitochondria and on chloroplast. We infer that the most sensitive enzymes govern the key reactions in the metabolism, and they are directly linked with the energy production.

4.1 Pathway Sensitivity Analysis in a Chloroplast Model

The representation of the chloroplast as a set of linked ODEs gives a mathematical description of the chemical process and, successively, the Morris analysis gives useful insights on linear and non-linear contribution of enzymes to the Carbon metabolism. In the set of ODEs of the model by Zhu et al. [12], we have evaluated the most sensitive components through a one-factor-at-a-time (OAT) method proposed by Morris [21]. According to the OAT method, only one input is perturbed while the others are kept at their nominal value. We consider our pathway as a black-box with certain inputs and certain outputs. Each step-variation computed for each input is an elementary effect calculated as $u_i = (P(x_1, ..., x_i + \Delta_i, ..., x_k) - P(x_1, ..., x_i, ..., x_k))/\Delta_i$, where $P$ represents the pathway, $(x_1, ..., x_i, ..., x_k)$ is the nominal vector and $\Delta_i$ is the perturbation affecting the $i$th input. For each factor, we collect an ensemble of elementary effects and compute their mean $\mu_i$ and the standard deviation $\sigma_i$. We expect a high global influence from those inputs with a high $\mu_i$, and a highly non-linear or counterintuitive behaviour from those inputs with a high $\sigma_i$.

For each enzyme (i.e. input) we use the concentration reported in Table SI1 as nominal value [22], computing twenty different factor levels, each of which is altered ten times. We perturb each nominal value by a factor of 10 (both up and down). The sensitivity analysis shows that eleven (out of 23) enzymes are extremely sensitive [23], namely RubisCO, PGA kinase, GAP dehydrogenase, FBP aldolase, FBPase, SBP aldolase, SBPase, Phosphoribulose kinase, ADPGPP, Phosphoglycolate phosphatase, and GDC. These enzymes showed indeed high values of $\sigma_i$ (i.e. $1 < \sigma_i < 15$), when compared to all of the others (i.e. $10^{-4} < \sigma_i < 1$).

In order to validate these results, we take into account the interaction map defined by the photosynthesis pathway. In particular, we expect that the sensitive enzymes represent the hubs of the pathway. Indeed, the Roswall’s community detection method [24] confirmed our assumptions, detecting RubisCO and GAP dehydrogenase as the most strongly regulated enzymes of the C3 cycle (both enzymes are light regulated). Another key enzyme is Transketolase, because it uses as substrates Fructose-6-P (which otherwise would exit from the cycle towards the starch biosynthetic pathway) and 3-P-Glyceraldehyde (produced by GAP dehydrogenase). These enzymes correspond to the main nodes of the C3 cycle leading to the other biosynthetic pathways [22].

4.2 Sensitivity Analysis in a Mitochondrial Model

As in the sensitivity analysis carried out on the chloroplast model, the representation of the mitochondrion as 73 DAEs (Differential Algebraic Equations) and, successively, the Morris analysis gives useful insights into linear and non-linear contribution of metabolite concentrations involved in the bioenergetic reactions of mitochondria. For each metabolite (i.e. input) we use the fully oxidised state as nominal values, computing twenty different factor levels, each of which is altered ten times. We perturb each nominal value by a factor of 10 (both up and down). The sensitivity analysis results are shown in Supplementary Information (Table SI2, third and fourth column). The metabolites are sorted from the most sensitive to the least sensitive. Only the total Adenosine monophosphate (AMP) content and the total fumarate (FUM) content can be considered insensitive.
variables as they have values of $\mu$ and $\sigma$ near zero. The other variables are sensitive or extremely sensitive. Notably, the most sensitive variable is the free potassium (K$^+$) content.

### 4.3 Bayesian ARD Sensitivity on Mitochondrial Parameters

After the sensitivity analysis performed on the initial concentrations, our idea is to investigate the model by Bazil et al. [13] by evaluating how the choice of the adjustable parameters influences the output. The right value of the parameters is hard to determine experimentally. After the publication of the mitochondrial model, the authors proposed an update of the vast majority of the model parameters after the modifications outlined by Wu et al. [25] due to the change to the function used to compute the ionic strength. These corrections witness the fact that both the estimation and the sensitivity of parameters deserve a closer look.

Currently, the most commonly used estimation procedures rely only on nonlinear least squares or on Monte Carlo techniques based on optimisation algorithms (both have been used by Bazil et al. in the mitochondrial model). These approaches are computationally expensive and often poorly suited for statistical inference. Alternative methods that use the P-spline theory to estimate the state functions and the ODE parameters were proposed by Ramsay et al. [26]. When the number of parameters is high, the sensitivity analysis can detect the most important ones. Therefore, one can put more effort in estimating the key parameters, rather than using the same technique for all the parameters. Nevertheless, because of the high-dimensional parameter space of the mitochondrial model, typical approaches (e.g. the OAT method adopted for the chloroplast) may not accurately assess the sensitivity of the system; furthermore, the simulation code needed to evaluate the output in the whole parameter space is computationally expensive. Using a surrogate model in the way described in this paper, we are able to globally investigate a high-dimensional parameter space, in order to identify all the sensitive and uncertain points.

Using the SUMO toolbox [19], we build a surrogate mathematical model that mimics the behaviour of the mitochondrion over the complete parameter space. The metamodel adaptively selects as few data points as possible in the parameter space, and reproduces the output as accurately as possible. This way, we are able to explore the design space and investigate how the combined effect of all the parameters influences the output of the model. We use a least squares support vector machine (LS-SVM) in order to build a continuous model from the discrete ATP output in the 42-dimensional parameter space. LS-SVM is a learning method that recognises patterns from given data points [27]. Support Vector Machines (SVM), introduced within statistical learning theory, is a powerful methodology for solving problems such as function estimation, which has also led to kernel based learning methods.

Given the parameters $p_i, i = 1, ..., 42$, proposed by Bazil et al. we consider the parameter space $\prod_i [0, 2p_i]$. LS-SVM adaptively samples the parameter space in order to build a continuous model of the output, by minimising the least squares error between the model and the output evaluated on the points already sampled in the parameter space. The regression is carried out by weighting the inputs so as to obtain the most probable model for the given data points. The first 20 points are selected randomly in the parameter space so as to start the training of the model. Then, the parameter space is sampled dynamically, thus the surrogate model is built and refined as more points are available. The procedure is halted when the least squares error becomes smaller than a fixed tolerance.

Starting from the surrogate model, we perform a sensitivity analysis by means of the Bayesian Automatic Relevance Determination (ARD) [28]. This allows to investigate the sensitivity of the parameters and understand how a change in a parameter affects the behaviour of the whole system. ARD is used to determine the subset of the most relevant inputs for the proposed model. We assign a different weighting parameter to each dimension in the kernel inference. Each input is assigned a value $\sigma^2$. In each step, LS-SVM performs a backward selection by removing the input with the largest optimal $\sigma^2$. For every step, the Bayesian cost criterion is computed, based on the singular value decomposition (SVD) of the kernel matrix.

In order to rank the parameters, the effect of each parameter must be compared to the effect of all the others. To this end, in each step of the ARD algorithm a parameter is removed according to a minimal cost criterion. Although at each step a parameter is removed with the smallest cost at that step, it may happen that at a step after the smallest cost is larger. This way, we obtain a list of inputs in decreasing order of relevance (see Table SI3).

According to the Bayesian ARD, the Hexokinase max rate and the $F_1F_0$ ATP synthase activity are the parameters having the largest influence on the production of ATP in mitochondria. In order to analyse more thoroughly the role of these two key players, in Figure 2 we plot the concentration of ATP over the total concentration of adenine nucleotides, according to our metamodel. The minus sign indicates a production of ATP. Since the mitochondrial species for adenine nucleotides are conserved in the model, the following algebraic equations of conservation holds: $[ATP] + [ADP] = A_{tot}$. On the z axis we plot $10^6 ([ATP] / A_{tot}) = 10^6 (1 - [ADP] / A_{tot})$.

In Figure 2b we show the metamodel curve along with the 185 points sampled by SUMO to create the metamodel. The plot confirms that $F_1F_0$ ATP synthase activity is a highly sensitive parameter. In particular, there are some regions of the parameter space of $F_1F_0$ ATP synthase activity, and specifically the neighbour-
hood of $1 \times 10^9$, in which SUMO finds points considered as outliers that cause a fast decrease of the ATP production.

In order to have a closer look at the model behaviour as function of the parameter values, we build a second-order and a third-order polynomial surrogate models using 250 and 1028 samples (respectively) in the parameter space. Let us denote by $p$ the vector of the parameter values. For the second-order approximation, the ATP is given by

$$ a_0 + c^\top p + p^\top A p, \tag{1} $$

where $a_0 = -5.98$, while $c$, $p$ and $A$ are defined in Figure SI1 and Figure SI3 in Supplementary Information, and the $\tau$ symbol denotes the transpose operation. The heat map of $A$ is shown in Figure SI2 in Supplementary Information. For the sake of completeness, in Figure SI4 we report the curve for the third-order polynomial metamodel.

The sensitivity applied to the mitochondrial model allows to draw the same conclusion as that applied to the chloroplast model. In both cases, the sensitive enzymes are the key players in the pathway of these organelles, i.e. the enzymes directly associated with the production of energy.

5 Multi-objective Optimisation

The methodology we propose in this section allows to optimise simultaneously several biotechnological targets, i.e. the output of the computation carried out by organelles. This approach highlights the complex behaviour that may arise in metabolic networks, and hints at future investigation of the concept of optimality in organelles (see Section in Supplementary Information). A multi-objective optimisation is needed when the system performs multiple tasks, and a given phenotype cannot be optimal at all of them (e.g. when two tasks are in contrast with each other).

Let us assume to have $r$ objective functions $f_1, \ldots, f_r$ to optimise. The problem of multi-objective optimisation can be formalised as follows:

$$ \max_x (f_1(x), f_2(x), \ldots, f_r(x))^\top, \tag{2} $$

where $x$ is the variable in the search space. Without loss of generality, we have assumed that all the functions have to be maximised; indeed, minimising a function $f_i$ can be thought of as maximising $-f_i$.

The solution of a multi-objective problem is a potentially infinite set of points, called Pareto optimal solutions or Pareto front. A point $y^*$ in the solution space is said to be Pareto optimal if there does not exist a point $y$ such that $f(y)$ dominates $f(y^*)$, i.e. $f_i(y) > f_i(y^*), \forall i = 1, \ldots, r$, where $f$ is the vector of $r$ objective functions to optimise in the objective space. We use the Pareto-front concept to find the set of designs that represent the best trade-off between two or more requirements. The Pareto front can also show interesting trade-off knee points, considered by Higuera et al. as preferred solutions in metabolic networks [29]. The Pareto front is the set of all the phenotypes that remain after eliminating all the feasible phenotypes dominated on all tasks [30].

5.1 Optimisation of the Chloroplast Model

Once the eleven sensitive enzymes have been identified through the sensitivity analysis, we employ the multi-objective optimisation algorithm on the “sensitive domain” made up of the eleven most sensitive enzymes ($x \in \mathbb{R}^{11}$). We use Parallel Multi-objective Optimisation (PMO2) [31] to find all those sensitive enzyme concentration vectors $\hat{x} = (c_1, c_2, \ldots, c_{11})$ such that when $\hat{x}$ has the other twelve enzyme values are kept at their nominal value, the resulting CO\textsubscript{2} uptake function is maximised and the nitrogen consumption is minimised:

$$ \max_{\hat{x} \in \mathbb{R}^{11}} (f_1(\hat{x}), -f_2(\hat{x}))^\top, \tag{3} $$

where $f_1$ represents the CO\textsubscript{2} uptake, while $f_2 = \left(\sum_{i=1}^{11} \frac{2c_i}{M_i} K_i \right)$ represents the nitrogen consumption, $K_i$ is the catalytic number (or turnover number), and $M_i$ the molecular weight of the $i$th enzyme. Gaining higher CO\textsubscript{2} uptake rates while employing less nitrogen means absorbing more CO\textsubscript{2} while consuming less “leaf-fuel”; this renders the metabolism cycle more efficient. Hence, our search for $\hat{x}$ must take into account a trade-off between maximal CO\textsubscript{2} uptake rate and minimal nitrogen employment. Figure 3 shows the Pareto front made up of the solutions that simultaneously maximise $f_1$ and minimise $f_2$. In the simulation we adopt the actual CO\textsubscript{2} atmospheric concentration, i.e. 270 $\mu$mol mol\textsuperscript{-1}.

5.2 Optimisation of the Mitochondrial Model

In order to optimise multiple objectives, we used the Non-dominated Sorting Genetic Algorithm II, also known as NSGA-II [32], that exploits evolution among
a population of individuals in the search space in order to obtain the best offspring.

Here we optimise the concentration of of Adenosine triphosphate (ATP) and Nicotinamide adenine dinucleotide (NADH) in the matrix compartment in the model of the mitochondrial bioenergetics [13]. Since NADH and ATP represent the most important energy molecules, by means of our computational framework we seek and investigate their trade-off and the conditions (basal concentrations of metabolites in mitochondria) such that ATP and NADH are maximised.

Mitochondria convert free energy in substrates (carbohydrate and fatty acid derived) into the free energy of the ATP hydrolysis reaction. The Gibbs free energy of ATP hydrolysis reaction (ATP ⇔ ADP + Pi) is equal to \(\Delta G_0 = -RT\ln\left(\frac{[ATP]}{[ADP][Pi]}\right)\), and is negative, therefore the ATP hydrolysis is an exergonic reaction, i.e., the energy content of the products is less than that of reactants. During the reaction there is a release of energy. In this work, we investigate the ATP concentration in matrix compartment of the mitochondria since this molecule has an high potential to transfer the phosphoric group. Therefore, optimising ATP concentration in the matrix compartment would mean optimise the free energy transduction.

The model we adopt consists of 73 differential-algebraic equations (DAEs) to represent the mitochondrial bioenergetics [13]. In particular, the model accounts for 35 biochemical reactions, including the oxidative phosphorylation, the electron transport system, the tricarboxylic acid cycle and related reactions, the Na\(^+\)/Ca\(^{2+}\) cycle and the K\(^+\)-cycle.

The variable space is defined as the space of the feasible initial concentrations of metabolites. We first initialise the population and compute the fitness score, based on the output functions we aim at optimising. The individuals of the initial population can be initialised in different ways, e.g. randomly or assigning specific values to all the concentrations. In our analysis, we assign specific values – the fully oxidised state reported in the original work [13] – from which we start searching optimal points. We maintain fixed all the other variables and constants of the model (such as temperature to 25°C and the water volume in buffering, intermembrane and matrix space and thermodynamics constraints), varying only the initial concentration of metabolites when solving the DAEs system.

An individual is a feasible vector of concentrations of metabolites. Once a vector of concentrations of metabolites is fixed, the objective functions are calculated by solving the DAEs system. For each ATP and NADH curve, we evaluate its integral, considering a single measure. Each individual is assigned a rank, and between two solutions with different non-domination ranks we prefer the one with the lower rank. After sorting the individuals according to the level of non-domination, the fitness score of each individual is computed by evaluating the objective functions associated with it.

Successively, we carry out three steps in a loop: (i) in a binary tournament selection process, two individuals are selected at random, their fitness is compared and the individual with the best fitness is selected as a parent for the next population; (ii) the algorithm selects a number of parents (i.e. the best individuals) equal to the half of the population, and then mutates them using a mutation operator and a crossover operator; (iii) a novel population of the same size of the initial population is formed selecting the best individuals from the parents and the offspring. Each individual of the final population will be a point of the Pareto front in the objective space.

Before the optimisation, at the fully oxidised state we obtain NADH = \(1.5987 \times 10^{-10}\) nmol/mg (formation) and ATP = \(-0.0014\) nmol/mg (consumption). Then, each metabolite involved in the metabolic network varies in
concentration depending on the reactions. The metabolite undergoes several processes, i.e. its formation and degradation, transport, and cellular utilisation. For every metabolite, a mass balance equation depends on the fluxes in input and output in the compartment. The variation of the metabolite concentration depends on the amount of mass formed and consumed. Therefore, when we maximise the concentration of ATP in the matrix compartment, we can obtain positive values (indicating a formation in the compartment of that molecule) or negative values (indicating that that molecule is being transported out of the system). After the optimisation, we obtain the Pareto-optimal points shown in Figure 4. Pareto fronts and the reported values of NADH and ATP represent an asymptotic analysis. They describe how the mitochondrial metabolism evolves when it needs to optimise simultaneously the concentrations of ATP and NADH. The Pareto curve can be useful to analyse the trend of the mitochondrial processes that have to satisfy specific constraints.

Furthermore, we analyse more thoroughly two particular Pareto-optimal solutions, i.e. the point with maximum ATP synthesis (and lower NADH formation) and the point with maximum NADH formation (and lower ATP synthesis). After setting Ca$_{2+}$=$10^{-5}$ nmol/mg, the first solution provides NADH = $6.17 \times 10^{-15}$ nmol/mg (that can be considered null) and ATP = $2027.34$ nmol/mg, with overproduction of SUC$_{mtx}$, SCoA$_{mtx}$, CoASH$_{mtx}$, H$_{mtx}^+$ and ATP$_{ims}$ (ims=intermembrane space, mtx=matrix) and underproduction of ISOC$_{mtx}$, aKG$_{mtx}$, MAL$_{ims}$, CIT$_{ims}$, ISOC$_{ims}$, aKG$_{ims}$, SUC$_{ims}$, MAL$_{ims}$ and GLU$_{cyt}$, ASP$_{cyt}$ (cyt=cytosolic space).

The second solution provides NADH = $6.07 \times 10^{-6}$ nmol/mg and ATP = $-3734.6$ nmol/mg (consumption), underproducing the following metabolites: H$_{mtx}^+$, ISOC$_{mtx}$, SCoA$_{mtx}$ and ATP$_{ims}$ whereas CIT$_{mtx}$, MAL$_{ims}$ and AMF$_{ims}$, PYR$_{ims}$, GLU$_{ims,cyt}$ and aKG$_{ims}$ are totally consumed. These results indicate that the mitochondrion is requesting an amount of ATP that is not available in the matrix. With respect to the state where no ATP is available in the matrix, a negative value of concentration in the model can be thought of as the change of ATP concentration needed to produce the corresponding NADH. Both the extreme solutions are shown in Figure 5 (top).

Increasing the matrix calcium content from $10^{-5}$ to $10^{-4}$ nmol/mg causes ATP synthesis and NADH formation to stop, and both molecules are consumed by the metabolism (see Figure 4, red signs). As in the previous case, we analyse the two extreme Pareto-optimal points in Figure 5 (bottom). This achievement can demonstrate that a perturbation in mitochondrial Ca$_{2+}$ homeostasis has major implications for cell function at the level of ATP synthesis and NADH generation.

If Ca$_{2+}$ increases by a little step, i.e. it is fixed at $1.5 \times 10^{-5}$ nmol/mg, we obtain an increase in NADH formation, while ATP remains constant (see Figure 4, green signs). If Ca$_{2+}$ drastically decreases to $10^{-6}$ nmol/mg, there is a lower ATP synthesis (see Figure 4, blue circles). Conversely, with Ca$_{2+}$ = $1.5 \times 10^{-5}$ nmol/mg, both objectives are maximised.

6 Robustness Analysis

The concept of robustness is frequent in nature, and it seems to be one of the driving forces of evolution [14]. The ability of a system to preserve its behaviour despite internal or external perturbations is a crucial design principle for any biological and synthetic system [17], [18]. The basic principle of the robustness analysis consists of defining the perturbation as a function $\gamma(\Psi, \sigma)$, where $\gamma$ applies a stochastic noise $\sigma$ to the system $\Psi$ and generates a trial sample $\tau$. We assume that the noise is defined by a random distribution and we generate a set $T$ of trial samples $\tau = \gamma(\Psi, \sigma)$. Let us define:

$$\rho(\Psi, \tau, \phi, \epsilon) = \begin{cases} 1 & \text{if } |\phi(\Psi) - \phi(\tau)| \leq \epsilon \\ 0 & \text{otherwise} \end{cases}$$

where $\Psi$ is the reference system, $\phi$ is a metric (or property), $\tau$ is a trial sample of the set $T$, and $\epsilon$ is a robustness threshold. The definition of this condition makes no assumptions about the function $\phi$, which is not necessarily related to properties or characteristics of the system; however, it is implicitly assumed that it is quantifiable. Each element $\tau \in T$ is said to be robust to the perturbation, due to stochastic noise $\sigma$, for a given property (or metric) $\phi$, if the condition $\rho(\Psi, \tau, \phi, \epsilon) = 1$ holds.

The robustness of a system $\Psi$ is defined as the number of robust trials $\tau \in T$, with respect to the property $\phi$, over the total number of trials $|T|$. Formally, the robustness of a system is a dimensionless quantity defined as

$$\Gamma(\Psi, T, \phi, \epsilon) = \frac{\sum_{\tau \in T} \rho(\Psi, \tau, \phi, \epsilon)}{|T|}.$$
of enzyme perturbations at which the system property of enzyme level perturbation. Applying the concept of which the achieved CO$_2$ concentration of each enzyme of the C3 cycle at a given time.jected to noise, making it difficult to ensure a certain con-

tant is Ca$^{2+}$, maximum NADH formation, in green. The calcium con-

tentions for maximum ATP synthesis, in red, and

![Graph](image1.png)

![Graph](image2.png)

Fig. 5: Ratio of metabolite concentrations optimised by the multi-objective algorithm compared to the initial concentrations for maximum ATP synthesis, in red, and maximum NADH formation, in green. The calcium content is Ca$^{2+}$ = 10$^{-5}$ nmol/mg (top) and Ca$^{2+}$ = 10$^{-4}$ nmol/mg (bottom) respectively. In the inset, we focus on the [0, 3] y-axis range.

We note that $\Gamma$ is a function of $\epsilon$, so the choice of this parameter is crucial and a reasonable value is often chosen after conducting several computational experiment.

### 6.1 Robustness in the Chloroplast Model

During the “in vitro” implementation, our chloroplast must be robust with respect to possible experimental errors or unpredicted unknowns. Although it is possible to find maximal values for the CO$_2$ uptake, efficiency of transcription promoters cannot be easily foreseen. Moreover, transcription, translation and enzyme efficiency can vary depending on many factors, even of environmental origin. In other words, the enzyme concentration is subjected to noise, making it difficult to ensure a certain concentration of each enzyme of the C3 cycle at a given time. For this reason, it is fundamental to know the extent to which the achieved CO$_2$ uptake will be preserved in case of enzyme level perturbation. Applying the concept of robustness to the C3 cycle allows to calculate the limits of enzyme perturbations at which the system property of interest (a given level of CO$_2$ uptake) is maintained. Among read-out-equivalent designs, one should prefer the one with the most CO$_2$ uptake read-out preserved when the enzyme input noise increases.

Let $\bar{x} \in \mathbb{R}^{23}$ be an enzyme partitioning and $f : \mathbb{R}^{23} \rightarrow \mathbb{R}$ a function computing the expected CO$_2$ uptake rate value of $\bar{x}$. Given an enzyme partition $\bar{x}$, obtained by perturbing $\bar{x}$, the condition $\rho$ for the robustness of enzymes partitions is defined by adapting the general definition (4) as

$$
\rho(\bar{x}, \bar{x}^*, f, \epsilon) = \begin{cases} 
1 & \text{if } | f(\bar{x}) - f(\bar{x}^*) | \leq \epsilon \\
0 & \text{otherwise},
\end{cases}
$$

where the robustness threshold $\epsilon$ denotes the maximum percentage of variation allowed from the nominal CO$_2$ uptake value.

The ensemble $T$ has been generated using a Monte-Carlo algorithm. We consider both mutations occurring on all the enzymes (global robustness analysis) and mutations on one enzyme at time (local robustness analysis) [14]. We adopt a maximum perturbation of 10% for each enzyme concentration. We generate an ensemble of 5·10$^3$ trials for the global robustness analysis, and 200 trials, for each enzyme, for the local robustness analysis. In all the experiments we fix $\epsilon = 5\%$ of the nominal uptake rate value. In Figure 6 we show the robustness landscape of each enzyme. Remarkably, most enzymes do not change significantly the uptake rate value, despite the presence of noise. This analysis seems to confirm that the C3 cycle lies in a robust configuration.

The results of our methodology, in terms of new enzyme concentrations for maximal CO$_2$ uptake, are presented in Table SI1. Our optimisation results take into account enzyme kinetic parameters, largely obtained as an average of different plant values, as done in [12]. Such results consider hence an ideal plant. In a future research effort, we plan to consider the preparation of specific optimisation models for a given C3 crop, as rice, soybean, wheat or barley by taking into account the specific enzyme kinetic parameters in these plants, and the relative condition of temperature and water availability typical of their areal distribution. Moreover, the study and the use in optimisation models of enzymes kinetic parameters of plants adapted to specific climatic conditions would be a starting point for further biotechnological targets, e.g. to improve photosynthetic efficiency of crops in specific environmental conditions. As a matter of fact, the C4 photosynthesis type represents itself an adaptation to lower CO$_2$ atmospheric concentration. The possibility of redesigning the energy circuits of the cells, such as those in the chloroplast and in the mitochondrion, will make it possible to obtai

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Fig. 6: Robustness analysis. Robustness of the individual enzymes for the natural chloroplast. The enzymes are shown in the same order as in Table SI1. For each enzyme, as mentioned in section 6.1, we create 200 trials by perturbing the corresponding enzyme concentration, and we show the distribution of the CO\textsubscript{2} uptake rate values obtained. The y values are in percentage (\%), that is, the percentage (out of 200 trials) that obtains the corresponding value (±0.1) on the x axis. The chloroplast is robust to perturbations of the enzyme concentration if the values of CO\textsubscript{2} uptake rate are close to the nominal value (15.48 \(\mu\text{mol mg}^{-1} \text{fwt}\)) in the majority of the perturbation trials. For example, it is more robust with respect to perturbation of GAP dehydrogenase concentration and it is less robust with respect to perturbation of RuBisCO concentration.

### 6.2 Robustness in the Mitochondrial Model

Also in the case of “in vitro” implementation of mitochondrial models, the parameters must be robust with respect to possible experimental errors or unpredicted unknowns. Consequently, we apply the concept of robustness also to the optimised mitochondria. In this case, among read-out-equivalent designs, one should prefer the one that ensures the largest NADH formation and ATP consumption read-out preserved when the noise in the input concentrations of metabolites increases.

As in the previous section, the problem can be formalised as follows. Let \(\bar{x} \in \mathbb{R}^{50}\) be the array of the concentrations of metabolites and \(f : \mathbb{R}^{50} \rightarrow \mathbb{R}^{2}\) a function computing the expected NADH formation and ATP consumption values corresponding to \(\bar{x}\). Given a metabolite partition \(\bar{x}\), obtained by perturbing \(\bar{x}\), the condition \(\rho\) for the robustness of metabolite partitions is defined by adapting the general definition (4) as made in the equation (6), where the robustness threshold \(\epsilon\) denotes the maximum percentage of variation allowed from the nominal NADH formation and ATP consumption values. The remarks on the ensemble \(T\), the muta-
tions, the perturbation and the threshold in chloroplasts apply also to mitochondria. The results of our methodology, in terms of new metabolite concentrations for maximal NADH formation and ATP consumption, are presented in Table SI2. Our results highlight that the natural mitochondrion (C1) is the most robust, since it features a global value of 26.94\% and a local value of 9.00\% (referred to the concentration of free potassium). The C2 and the C3 mitochondria are less robust than the natural mitochondrion, both globally and locally.

Remarkably, we need to find a trade-off between the performance of the optimisation and the robustness of the solution. Since the multi-objective optimisation returned thousands of solutions (the number can decrease if the number of objectives increases) and the robustness analysis is computationally expensive, the choice of the most robust and optimal solution can require a high computational time. A good trade-off workaround could be of thinking the robustness index as another objective of the optimisation.
In addition to this, we conducted an additional robustness analysis on natural mitochondrion perturbing the 42 parameters listed in Table SI3. The formulation of the problem is identical, where the vector $\bar{x}$ in the equation (6) represents the parameters. The results are presented in the last column of Table SI3.

7 A MODEL OF THE HYDROGENOSOME

In several unicellular eukaryotes, including ciliates, fungi, and trichomonads, instead of the traditionally studied mitochondria, there is an alternate organelle called hydrogenosome. Hydrogenosomes are anaerobically functioning ATP-producing organelles of mitochondrial origin that represent a particular adaptation of mitochondrial metabolism, and possess the ability of producing molecular hydrogen by using protons as electron acceptor [7]. One of the best studied hydrogenosomes, and thus the one that we consider in our analysis, is that of the sexually transmitted human parasite *Trichomonas vaginalis*.

In order to investigate the hydrogenosome with the same techniques introduced before, we propose a model of the hydrogenosome metabolism in *T. vaginalis* and we evaluate the ATP flux, comparing it with that of mitochondria. The model we propose is a FBA model [16] that contains all the main reactions occurring in the organelle [33], [34] (see Table SI4), as well as reactions dealing with the import of serine, glycine, pyruvate and malate into the hydrogenosome. Although our model already shows interesting behaviours, we plan to extend it by adding new reactions. To our knowledge, no hydrogenosome models are present in literature, thus we believe our model will have great value as the best description that we have to date of the hydrogenosome, and will provide a foundation upon which more accurate models can be arrived at. A complete model of the hydrogenosomal metabolism would be of great biological relevance in the design of new antiparasitic drugs [35].

We specify the reaction network of the hydrogenosome through the LIM package of R, which allows to generate the mass balance for each component. Moreover, we estimate the optimal reaction rates in the flux balance analysis (FBA) approach [16]. (See Supplementary Information for details on the modelling technique for the hydrogenosome. The source code of the model and all the required libraries are available from the authors at request.) In Figure 7 we show the multi-objective optimisation carried out in the flux balance analysis framework.

In order to obtain the optimal solution in all the feasible two-objective optimisations, we randomly sample the solution space. In Figure 8 we display the pairs plot and mark the optimal solution with a red dot. Most reactions are coupled together; it happens frequently that a reaction can occur only when the substrates, which are products of another reaction, are available. For instance, the ATP production depends on the Acetate production, since ATP requires SuccinylCoA, which is a product of the same reaction that produces Acetate. It is noteworthy that a few reactions are in conflict with all the other reactions. For instance, the NaFe reaction, which produces NADH, is in conflict with Hydro, which produces H$_2$. Indeed, the last row of the pairs plot shows that the NADH production is in conflict with the H$_2$ production. The presence of a feasible point in this plot does not imply that the hydrogenosome metabolism, even if optimised, is able to reach that point. Notably, the CO$_2$-NADH plot shows that the hydrogenosome is versatile and can produce both, but it cannot specialise in producing only one metabolite, since there are few points near the axes and the Pareto front exhibits a higher curvature than the other fronts.

8 CONCLUSIONS

Both mitochondria and chloroplasts are energy-converting organelles in the cytoplasm of eukaryotic cells; while chloroplasts capture and convert energy of sunlight in plants, mitochondria synthesise energy (ATP). The genetic and the energy-converting networks of mitochondria and chloroplasts are descended, with little modification, from those of their ancestor bacteria. In this regard, we can explore how the Pareto front analysis related to the energy metabolism can provide interesting insights into the evolutionary dynamics leading to the formation of organelle structures in the single- and multi-celled life. Pareto fronts combined with sensitivity and robustness are useful tools to understand
Fig. 7: hydrogenosome. We report the reactions of its metabolism and their fluxes (mmol h\(^{-1}\) gdW\(^{-1}\)) on the x axis. A line next to a reaction represents all the feasible fluxes for that reaction, while a point represents the value of the specific solution proposed in this configuration of the hydrogenosome. The maximisation of the ATP yield (a) implies the consumption of all the H\(_2\), CO\(_2\) and NADH in the organelle. The maximisation of CO\(_2\) (b) implies also the maximisation of H\(_2\), but requires the maximum import of malate, pyruvate and glycine. Moreover, it impairs the NaFe reaction, thus keeping the NADH at the initial value. The two-objective optimisation of ATP and NADH (c) causes the impairment of the Hydro reaction, thus the hydrogenosome cannot produce H\(_2\). It is thus evident the need for a Pareto-optimal trade-off between energy and H\(_2\) production.

the steps of the cellular evolution and the engulfments and specialisation of organelles. In Figure 9 we show the putative evolution through the Pareto front analysis. The whole figure should be seen in an evolutionary perspective, where the passage from the engulfment to the full functionality of the organelle has required a number of adaptive evolutionary steps and a similar less optimised system working outside the organelle. During the optimisation, i.e. the maximisation of a metabolite, the Pareto front of an organism undergoes both expansion and contraction phases. These steps can be numerically assessed using the hypervolume [36], in order to quantify the evolution of the Pareto front. We intend to make use of machine learning algorithms to predict whether a protein enters a membrane according to the transient peptide, and measures on Pareto fronts (e.g. hypervolume [36]) can avail or disprove these predictions.

We also discussed the concept of robustness and parameter sensitivity for the models of organelles, and in general for compartmentalised structures in the cell. While cross comparing the biological systems, we showed also a methodological variety to analyse them. We are delighted to report that the modelling of metabolic processes, which is a thriving field of research, has two immediate and important benefits: (i) a comprehensive insight into the energy balance in the cell, and (ii) the improved understanding of the processes that shape health and diseases, thus providing the possibility to design new drugs.

Following our framework and extending it, in the near future we plan to design, analyse and optimise the metabolism of systems composed of different species living and interacting in the same organism, as introduced by Cottret et al. [37] in the case of two endosymbiotic bacteria. In this paper we also analysed the metabolite production of organelles and identified genetic interventions needed to overproduce metabolites of interest. We performed an in silico design that can also explore the reaction network and seek in the search space the solutions that optimise two or more objectives simultaneously. Our technique can be extended using global and local sensitivity measures based on partial derivatives [38], which allow to obtain rankings of parameters according to their influence. This kind of analysis could easily highlight the complementarity of different metabolic networks. For instance, mitochondria and chloroplasts are (usually) both found in plants, and are part of the same functional pipeline: starting from CO\(_2\), the photosynthesis in the chloroplast creates glucose that enters the mitochondrion to create ATP.

Although in this paper we have considered the single organelle, in cells there are usually many organelles that could differ for activity depending on their location. Therefore, we expect that applications of compartmentalisation in synthetic biology (e.g. the use of organelles to optimise a cell) will increase in the near future.

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Fig. 9: Putative Pareto front evolution associated with the engulfments in cell evolution. The double-membrane organelle is a bacterium that has been engulfed by a single-membrane eukaryote (A). After an engulfment (B), the guest organelle shows a concave Pareto front that expands along one axis, since it specialises in the production of a metabolite, thus losing versatility and decreasing the area under the front. Conversely, the hosting cytoplasm specialises in the production of all the other metabolites, thus it shows a convex Pareto front. The process is repeated for the second and third engulfment (C), after which the organelles are specialised in the production of a metabolite each. Since a slight reduction of metabolite 1 dramatically increases the production of metabolite 2, the organelle 1 can further specialise (see C) in the next evolutionary step. Organelle 3 is the most specialised, and therefore it could be thought of as an apicoplast, with Metabolite 5 representing the IPP. The histogram (D) highlights the increase of specificity, gradient and signalling cost in the evolutionary steps.

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