# High order epistasis, Complexity and mutation-selection-drift balance: insights from a mechanistic approach

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**Abstract** — Epistasis is a pervasive phenomenon tightly linked with the complexity of biological systems that both influence trajectories followed by Evolution and its steady-state. However, the specific role played by complex high dimensional epistatic relationships in the establishment of evolutionary steady-state has mostly remained a conundrum up until now. Here, we put forward a new kind of epistasis - namely complementary epistasis - and shows how this improvement based on mechanistic considerations may change some predictions about mutation-selection-drift balance, and, in turn, help interpret genomic data. We argue that this idea seems well-suited to be studied analytically within a theoretical population genetics framework that remains to be developed in that sense, which would be the first part of this project. We then discuss how this idea can be further explored and how it could broaden our theoretical knowledge on both general and more specific questions in evolutionary and molecular biology. To illustrate this, we then focus on the phenotypic evolution of proteins, especially enzymes, to demonstrate that it cannot be fully understood without this new framework due to their embedding in metabolic pathways. Such a study would be the second part of the project. Finally, we argue that combined with other biological considerations, enriching the framework could also help make predictions about molecular evolution, which would be one of the several possible extension to this project.

***Key words***— Complementary epistasis, high-order epistasis, mutation-selection-drift balance, complexity, population genetics, emerging fitness, neutral evolution, pathway evolution, molecular evolution

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# 1 Introduction

"*The limits of my language are the limits of my world*".

(Wittgenstein, 1922)

Evolution is the ongoing process that gave birth to the wide zoo of organisms that have ever lived and shall ever live (Darwin, 1859; Wallace, 1858). It is, as it were, the language of Nature when it deals with mutable self-replicating entities. If this language has been fruitful, it still sets the limits of what can exist and how this space of possibilities is explored, as pinpointed by Wittgenstein (1922). Melting many mechanisms acting at different levels of space and time, these limits are yet to be fully understood. It has long been known indeed that the power of Natural Selection is limited by genetic drift (Wright, 1930) such that organisms are the best of the possible ones under the conflicting mutational and selective pressures (Kimura, 1962; Ohta, 1992), but how Evolution combines with the complex genetics underlying traits remains, to say the least, inchoate (Hansen, 2013; Blanquart and Bataillon, 2016; Barton, 2017; Harpak and Przeworski, 2021).

Epistasis[1] is a ubiquitous phenomenon in which the effect of a mutation differs depending on the genetic background in which it occurs (Bateson, 1909; Phillips, 2008). As Weinreich et al. (2013) pointed out, it is a measure of our "surprise" insofar as we *a priori* expect mutational effects to be additive (Phillips, 2008). Epistasis has long been known to occur between pairwise mutations where the combined effect between them results in a phenotype or a fitness that is not the sum of that they would have in isolation from one another: if Adaptation can still occur through few genetic changes (Orr, 2005), such epistatic interactions in general comes with several consequences, narrowing the paths towards adaptation (Poelwijk et al., 2007) or influencing genetic architecture (Hermisson et al., 2003) for instance, and could also create fitness valleys under certain circumstances called reciprocal sign epistasis (Weinreich et al., 2005; Poelwijk et al., 2011).

Because of the process of genetic drift (Wright, 1930; Kimura, 1958; Ohta, 1992) that entails a mutational load (Haldane, 1937; Muller, 1950; Agrawal and Whitlock, 2012), it was thus supposed that small effective population sizes could help escape from a local fitness peak by facilitating fixation of intermediate deleterious mutations (Wright, 1930, 1932); therefore, subdivision in small populations should be better at finding the highest peak though they are less efficient to climb up to this peak. However, introducing polymorphism and recombination disproved this conclusion (Weinreich and Chao, 2005) since combined mutations can either be found through stochastic tunneling (Iwasa et al., 2004) or be brought together after having emerged in different lineages, and it was later shown that considering the width – *i.e.* the number of loci making up the valley – of valleys would even reverse Wright's initial intuition with higher populations more prone to cross large fitness valleys, especially if these valleys are much alike plateaus (Weissman et al., 2009).

Known as higher-order epistasis, this phenomenon involving many loci usually gives rise to rugged

[1] see Glossary at the end of this document for further details on the different kind of genetic interactions with a specific focus on epistasis ones.

fitness landscapes (Kauffman and Levin, 1987; Kauffman and Weinberger, 1989; Weinreich et al., 2013) where peaks and valleys quickly follow each other in both the phenotype and the genotype spaces. Based on the NK-model, Kauffman and Levin (1987) shown that complexity – in terms of complex epistasis relationships which may exist between nucleotides, genes, pathways, or even organisms subunits such as organs – comes at a great cost for fitness since more ruggedness in turn increases the probability of being trapped on a local optimum (Kauffman and Levin, 1987; Geard et al., 2002), a finding which was further confirmed when accounting for the possibility of neutral mutations, through NKq and NKp models (Barnett, 1998; Newman and Engelhardt, 1998; Geard et al., 2002). Under certain assumptions, this model makes possible to estimate analytically the average expected mutation-selection-drift balance (Weinberger, 1991), which can be contrasted to other such predictive frameworks.

Indeed, the influence of complexity on evolutionary trajectories adn outcomes has in parallel thoroughly been addressed through the lens of Fisher's geometrical model (Fisher, 1930; Orr, 1998, 2000; Poon and Otto, 2000; Martin and Lenormand, 2006; Tenaillon et al., 2007; Tenaillon, 2014) where epistasis builds up from the mathematical underpinnings on which the framework relies (Hartl and Taubes, 1996; Orr, 2000; Tenaillon, 2014) and the assumption of pleiotropy preexistence (Tenaillon, 2014) - see (Stearns, 2010) for a review on pleiotropy. Besides enabling to study the distribution of fitness effects (Martin and Lenormand, 2006; Lourenço et al., 2011), it is in principle possible through this framework to approach how complexity – in a broad sense covering independent phenotypic traits, epistasis, pleiotropy among other (and more organismic) features – emerges and evolves (Orr, 1998; Martin et al., 2007; Gros et al., 2009; Le Nagard et al., 2011), what it really portrays and even to try and derive that of an organism from the strength of drift an organism experiences (Tenaillon et al., 2007). This is because epistasis and pleiotropy are intrinsic features to the multidimensional formulation of the model (Tenaillon, 2014) such that complexity needs not be defined in terms of the unknown explicit genotypic-phenotypic relationships, but can on the contrary be understood as the factor limiting the strength of selection when adopting a top-down approach (Le Nagard et al., 2011).

Though fascinating, both these frameworks lack a mechanistic basis as highlighted recently by Martin (2014) and Yi and Dean (2019) – even if Fisher's geometrical model was specifically revived for this purpose by Hartl and Taubes (1996) and subsequently led to several insights (Poon and Otto, 2000; Martin and Lenormand, 2006; Martin et al., 2007) – and, at least, deserves to be informed by the very biological processes giving rise to epistasis and pleiotropy if we are to succesfully achieve a functional synthesis (Dean and Thornton, 2007) in an integrative approach (Gudelj et al., 2010) that enables one to disentangle causes from effects in Evolution. For instance, it has been shown that the distribution of fitness effects can be captured using such models (Martin and Lenormand, 2006; Huber et al., 2017), but how alternate and potentially more parsimonious explanations could account for similar patterns is largely unknown (Lourenço et al., 2011), a case that can also be made for fitness landscapes (Blanquart and Bataillon, 2016). In the same vein, determining how features such as modularity (Wagner et al., 2007; Segrè et al.,

2005; Hartwell et al., 1999) changes the landscape and whether it is a specific kind of intrinsic interactions or the evolutionary product that these interactions favours or even made necessary requires to understand how they all dynamically behave together, as was argued for molecular networks (Alexander et al., 2009).

This is especially significant - and, noticeably, a major challenge for human biological understanding (Young et al., 2019) - inasmuch as the genotype-phenotype-fitness map stems from the intertwining between interacting genes - susceptible to evolve (Gros et al., 2009) during the course of Adaptation - with their basic and emerging physiochemical properties (Bershtein et al., 2017). Yi and Dean (2019) proposed that, contrary to Dobzhansky (1973)'s view, "nothing in Evolution makes sense except in the light of Biology", which rightly questioned the original statement and rejuvenated the debate on the influence of mechanisms and constraints (Gould and Lewontin, 1979; Pigliucci and Kaplan, 2000), but failed to avoid circular reasoning. In fact, since Biology is the product of the joint Evolution of physical entities whose combined properties are explored in a (non-random) particular way (Monod, 1971, 1974; Wagner, 2012) during the process, it seems more likely that Dobzhansky (1973)'s statement holds if and only if we acknowledge that nothing in Evolution makes sense without accounting for physics and chemistry, which raises the need for more investigation merging these fields (Dean and Thornton, 2007; Serohijos and Shakhnovich, 2014). Lately, shy first steps to fill in this theoretical gap have adopted the paradigm of statistical physics in an interesting attempt to derive the isotropic instance of Fisher's geometrical model from first principles (Martin, 2014), drawing inspiration from results of systems biology such as those of the FBA (Flux Balance Analysis) (Orth et al., 2010), which, for several reasons[2] should however not be the appropriate framework to study Evolution - see for example Schuster et al. (2008).

To our knowledge, it has not yet been determined how the combination of complementary epistasis – where a phenotypic trait can only be competitive if each and any of its underlying loci are (see Figure 1 for more details) – with global epistasis (Otwinowski et al., 2018) could specifically change population genetics predictions arising from the Neutral Theory of Evolution and its extensions (Kimura, 1968; Ohta, 1973, 1992) while this process seems to be supported by mechanistic underpinnings (Kacser and Burns, 1973; Hartl et al., 1985; Yi and Dean, 2019; Taverna and Goldstein, 2002; Bloom et al., 2005; Labourel and Rajon, 2021). This is what we propose to do in this project; to justify the interest of such an investigation, we start with the presentation of a toy model based on simplistic assumptions from global epistasis. We then show how this framework may have a profound impact on our understanding of Evolution at different levels of biological organization and put forward the key components that a more complete instance of the model should include. Finally, we introduce how it can be tested and applied by contrasting its results to those obtained when simulating enzyme evolution along one and/or multiple pathways, and conclude by discussing possible further developments, among which the link with protein stability and evolutionary rates are of particular interest as well as the influence of stochastic environments on the actual coefficient of selection.

[2]First, FBA relies on an assumption of optimality to solve the systems of equations describing the process as well as on the existence of fixed molecule contents. Yet more importantly, because this framework does not deal with real mechanisms but only describes a complex system that has already evolved, it cannot explain why nor how it evolved the way it did.

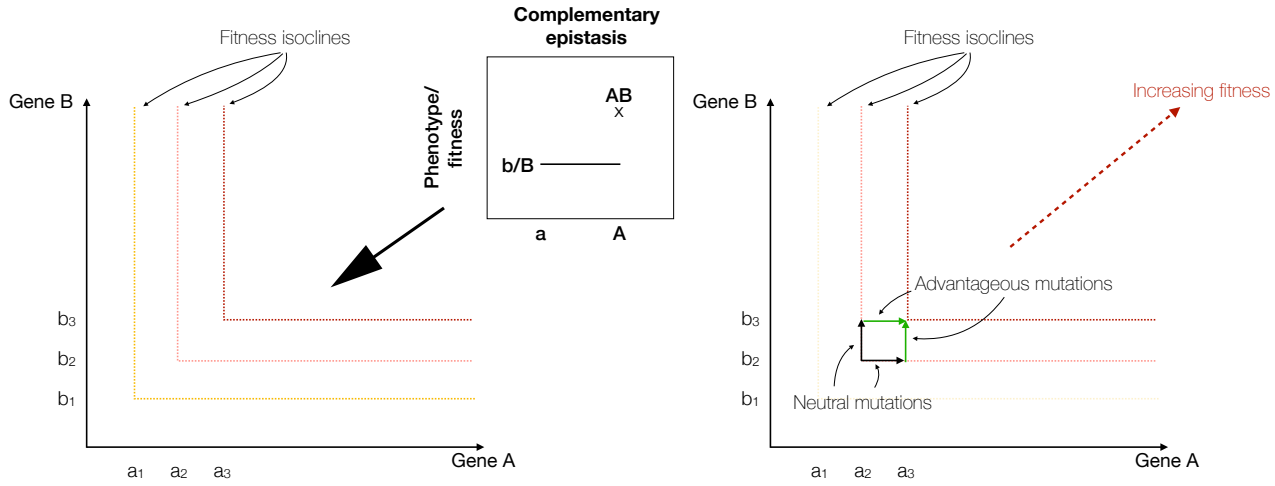


Figure 1: Description of complementary epistasis: on the left panel, its effect in the classical a/A-b/B case is shown in the short window, with the need for both A and B mutations to experience any gain of fitness, while the larger plot represents how phenotype/fitness isoclines can be mapped in the genotype space when more mutations are considered. On the right panel is shown the mutational path that allows to gain an extra fitness: first, a potentially advantageous but actually perfectly neutral mutation needs - at least - to exist on either one of the locus before the advantageous mutation can occur and give an actual extra push of fitness. This phenomenon can involve higher order interactions leading to the need for the segregation of numerous neutral mutations which may be subject to mutational biases.

# 2 A first approach

## 2.1 Prediction

We expect that complementary epistasis should influence both the speed of adaptation, as was observed for evolutionary escape (Weinreich and Chao, 2005; Weissman et al., 2009) and the mutation-selection-drift balance (see introduction for previous approaches on this idea), and focus on this latter phenomenon. More particularly, we assume that fitness should decrease with the number of genes/loci involved and that the mutational bias should play a large part in limiting adaptation because it influences the preexistence of potentially complementary beneficial mutations (at loci which are drifting because there differ from the worst one). Nevertheless, it should be possible to derive a prediction from the underlying premises of such a phenomenon.

Based on results of the neutral theory of Evolution (Kimura, 1962; Ohta, 1973), we indeed know that Natural Selection cannot filter mutations whose selective effect $|s|$ is below $1/N_e$. If fitness is limited by a maximum value, this means that deleterious mutations with effects $|s| \approx 1/N_e$ evolve through genetic drift and that fitness at the mutation-selection-drift balance establishes around $\widetilde{f_1} \approx 1-1/N_e$ when mutations are mostly deleterious and one locus is considered (Kimura, 1958). Let us say that an organism starts with the maximum possible fitness. Through drift on the first gene, $f$ should decrease on average to approximately $1-1/N_e$. Drift on the second gene should again push fitness downwards since the maximum fitness relatively to which drift occurs is now set to $1-1/N_e$, such that $\widetilde{f_2} \approx (1-1/N_e)\times(1-1/N_e) = (1-1/N_e)^2$. Therefore, considering $n$ complementary genes yield the following prediction $\widetilde{f_n} \approx (1-1/N_e)^n$. If $N_e >> 1$, this can be summarized by $\widetilde{f_n} \approx 1-n/N_e$. This rough estimate is very similar to that from the Fisher's geometrical

model in a N-dimensional space (Hartl and Taubes, 1996; Poon and Otto, 2000) that considers the influence of complexity on the evolutionary equilibrium.

### 2.2 Toy model

In this toy model, we consider a fitness landscape subject to saturation due to diminishing returns epistasis (Tokuriki et al., 2012; Kaltenbach and Tokuriki, 2014) as has widely been documented for proteins in the case of stability (Taverna and Goldstein, 2002; Bloom et al., 2005, 2006; Kaltenbach and Tokuriki, 2014) and catalytic efficiency (Dykhuizen et al., 1987; Hartl et al., 1985; Yi and Dean, 2019; Labourel and Rajon, 2021). It has also been recently shown that global diminishing returns epistasis (Kryazhimskiy et al., 2014; Bahcall, 2014; Otwinowski et al., 2018) should arise for a complex trait as a by-product of the distribution of fitness effects (Reddy and Desai, 2020). Such a fitness landscape is typically describe through a S-shaped function whose shape is given by the following equation (similar to that of Michaelis Menten):

$$f(x) = \frac{x}{x + K_X} \tag{1}$$

The evolutionary process is simulated using the probability of fixation (McCandlish and Stoltzfus, 2014), which is a classical result in population genetics (Haldane, 1927; Kimura, 1962; Wright, 1931). Under this assumption, the probability that a mutation occurring in a haploid population is eventually fixed is given by:

$$P_{\text{fix}}(s, N_e) = \frac{1 - e^{-2s}}{1 - e^{-2N_e s}} (\approx \frac{2s}{1 - e^{-2N_e s}}, \text{ when } s << 1) \tag{2}$$

Let us say that $\boldsymbol{X}$ is the vector representing a state of the pool of complementary genes[3] where $X_i$ denotes the phenotypic value of any gene $(i)$, and that $s'_m = \frac{f(X'_m) - f(X_m)}{f(X_m)}$ denotes the potential selective value of a mutation with fitness $f(X'_m)$ occurring on the gene $(m)$. The fitness function detailed above determines the maximum fitness a gene can potentially induce (*e.g.* the maximum catalytic flux an enzyme may be able to sustain). Owing to the specific process of complementary epistasis, the fate of a mutation is decided by the genomic background in which it occurs since it changes the selective effect it provides to its carrier. Two cases have to be distinguished, as we detailed below.

First, if the mutation affects the less efficient gene of a pool of complementary genes – *i.e.* $X_m = \min_{i \in S_g} X_i$ such that $f_{\boldsymbol{X}} = f(X_m)$, with $S_g$ the set of genes involved in the phenotypic set $\boldsymbol{X}$ – the probability of fixation of the mutation $X'_m$ rises only up to the threshold where it is no longer the worst gene of the pool. It yields:

$$P_{\text{fix},X'_m} = \begin{cases} P_{\text{fix}}(s'_m, N_e), \text{ when } s'_m \leq \Delta s_{max,m} \\ P_{\text{fix}}(\Delta s_{max,m}, N_e), \text{ otherwise,} \end{cases} \tag{3}$$

[3]For convenience, we only mention genes, but as stated previously, it may also apply to loci or organismic units, for instance.

where $\Delta s_{max,m} = \min_{\substack{i \in S_g \\ i \neq m}} \frac{f(X_i)}{f(X_m)} - 1$ (with $\Delta s_{max,m} \geq 0$).

Conversely, if the mutation influences the phenotypic value of any other gene – *i.e.* $X_m > \min_{i \in S_g} X_i$, with $S_g$ the set of genes involved in the phenotype $\boldsymbol{X}$ – its probability of fixation is that of a perfectly neutral mutation as long as the phenotypic value for this gene remains above the minimum of the set while it is that of a disadvantageous mutation – relatively to this threshold – when it falls under it, such that:

$$P_{\text{fix},X'_m} = \begin{cases} 1/N_e, \text{ when } s'_m \geq \Delta s_{min,m} \\ P_{\text{fix}}(s_{act,m'}, N_e), \text{ otherwise,} \end{cases} \tag{4}$$

where $\Delta s_{min,m} = \min_{i \in S_g} \frac{f(X_i)}{f(X_m)} - 1$ (with $\Delta s_{min,m} \leq 0$) and $s_{act,m'} = \frac{f(X'_m)}{\min_{i \in S_g} f(X_i)} - 1$.

Within such a framework, the actual selective advantage of a mutation is largely dependent on the worst loci in the set; as a corollary, it does not depend on the phenotypic value of the focal locus. One should notice that neither clonal interference nor double/multiple mutants are considered, meaning that the fixation process concerns only one mutation (on one gene) at a time.

Finally, we tested the influence of different distribution of fitness effects ranging from cases with no mutational biases ($b = 0$) to some with high ones ($|b| = \lambda X_m$, where $\lambda >> 0$). To comply with estimates on biological phenotypic traits be they catalytic constants (Carlin et al., 2016) or gene expression (Metzger et al., 2016; Hodgins-Davis et al., 2019), the distribution of mutational phenotypic effects is modelled through a Gaussian distribution whose mean depends on the present value of the trait $X_m$ at the loci $m$, a mutational bias $b$ pushing the trait value downwards and a variance $\sigma_X^2$ of the fitness effects of mutants. Both because it seems more realistic (Carlin et al., 2016) and because the optimization process would otherwise be far longer, mutations are drawn for the $log_{10}$ value of the trait such that mutations affecting higher phenotypic values have a proportionally higher variability and are more biased (when the bias is not null). This can be summed up by the following mutational distribution, in which $X'_m$ is drawn:

$$log_{10}(X'_m) \sim \mathcal{N}(log_{10}(X_m) - b, \sigma) \tag{5}$$

Note that there exists a broad scientific literature on the distribution of fitness effects of mutations (Keightley and Eyre-Walker, 2007; Orr, 2003; Gillespie, 1984) and some previous theoretical hypothesis for it (see for instance Martin and Lenormand (2006) and Rice et al. (2015)), but we are here interested in the making of these effects from underlying causes and cannot, as a consequence, use distributions that result from the phenotype-fitness map. We shall later discuss this point as a direct perspective of the present project.

### 2.3 Simulation and results

In order to demonstrate the relevance of studying this phenomenon, we tested as a premise the influence of complementary epistasis on the mutation-selection-drift balance. To do so, we set $K_X = 1$ in equation

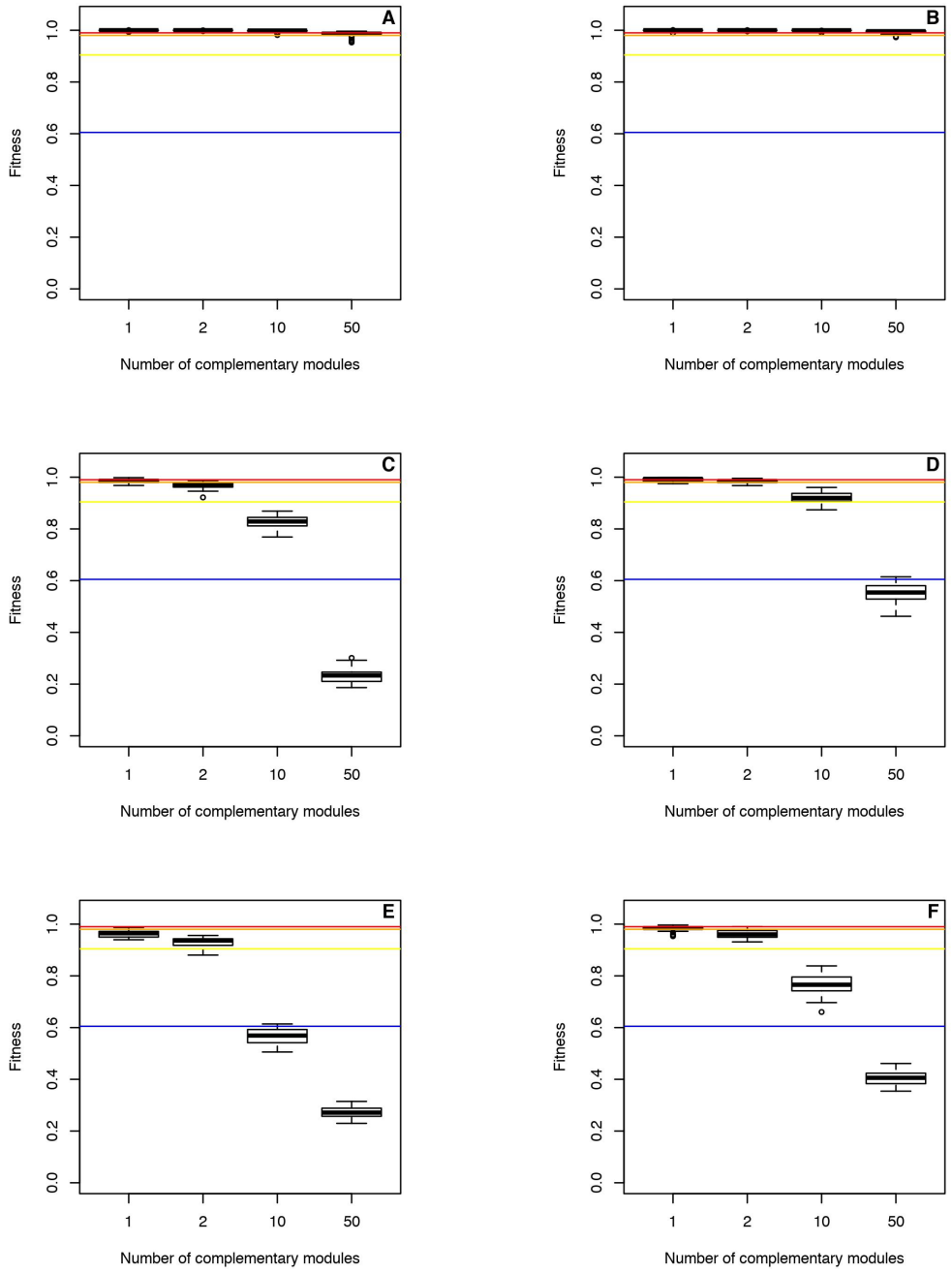


Figure 2: Simulation outcomes for the mutation-selection-drift balance with $N_e = 10^2$ when the interplay of complementary epistasis and diminishing returns epistasis (through the fitness landscape of traits) are accounted for. The number of complementary modules (*eg.* genes) varies from 1 to 50. Each line corresponds to a level of mutational bias: null in (A) and (B), low in (C) and (D), high in (E) and (F) - see text for details; each column represents a level of mutational variability, with (A), (C) and (E) having low variability while (B), (D) and (F) display a moderately high variability.

(1). Three mutational biases were considered $(0; -\sigma_X; -2\sigma_X)$ ranging from no bias to a high one through which only 1 mutations out of 40, approximately, is advantageous. We also studied two different levels of phenotypic variability among mutants for the $log_{10}$ of $X'_m$ ($\sigma_X = 0.25$ and $\sigma_X = 0.5$), with the highest one producing advantageous mutations with greater selective effects while decreasing the relative pool of

slightly deleterious ones. These sets of parameters are in line with the aforementioned estimates for the distribution of phenotypic effects of mutations (Carlin et al., 2016; Metzger et al., 2016). The initial value of each complementary phenotypic trait was set to $X_0 = 1 - 1/N_e$ to start from the null hypothesis of nearly-neutral Evolution. Finally, the probability of fixation was computed through either equation (3) or equation (4) depending on the locus which was affected and the simulations were ran for an average of 1000 mutation events per gene.

We simulated the evolutionary process for $N_e = 10$ (see APPENDIX section at the end) and $N_e = 100$ and present results obtained for the mutation-selection-drift balance in this latter case. In line with expectations, we observe that the number of complementary genes (denoted under the generic term of modules in the figure) severely impairs the strength of Natural Selection, with a decrease more or less of the order of $N_{mod} \times 1/N_e$ when mutational biases are considered. However, this is not the case when no bias is considered with a decrease being far more limited that does not jeopardise the optimisation process albeit marginally - see (A) and (B) on Figure (2). Because deviations from the expected equilibrium can accumulate, the balance is also more sensitive to the variability of mutational effects, with lower variability coming with a predictable decreased fitness - due to an increased amount of slightly deleterious mutations and a decreased amount of largely advantageous mutations - for the same level of mutational bias (compare (C) and (E) with (D) and (F) on Figure (2)). Owing to the very low effective population sizes, drift overwhelms Natural Selection and pushes the balance towards very low values, a process already documented in models based on universal pleiotropy such as (Hartl and Taubes, 1996) and (Poon and Otto, 2000). But we show here using a toy model that such complexity-selection trade-off may be readily observed without any preexisting pleiotropic relationship (and without accounting for the decrease of effective population size usually associated with complexity), and lends credit to the idea of an intrinsic cost to complexity.

### 2.4 Consequences and perspectives for this project

Obviously, there is a need to study higher and more realistic population sizes in order to draw robust conclusions. But, because the aim of this new framework is to try and understand the influence of epistasis from physical and chemical first principles, it definitely also needs include other relevant features that should influence evolutionary outcomes. First, it is known that mutations occurring on coding sequences, even being synonymous, are rarely exactly neutral, reflecting in particular the cost arising from codon usage bias (Ikemura, 1985; Galtier et al., 2018; LaBella et al., 2019), a feature that definitely influences fitness landscapes (Fragata et al., 2018). In parallel, not all kind of phenotypic trait undergo deleterious mutational biases: this is especially true for levels of expression[4], which result from changes in gene networks features such as the level of trans-regulatory elements, their specificity and a complex pleitropic set of relations with cis-regulatory elements involving the whole genome (Hodgkin, 1998; Chesmore et al., 2016). Last but not least, if biological systems may be thought to be under directional selection to maximize growth and

[4] And organic shapes, albeit for different reasons.

biomolecules production, the intertwining of pathways and biological reactions is more likely to result in a slightly different picture than that displayed by a simple positive complementary epistasis : genes that are too efficient may at some point yield collateral damages either because they monopolize resources in vain or induce toxicity (*eg.* producing too many metabolites) (Lilja and Johnson, 2017; Niehaus and Hillmann, 2020; Labourel and Rajon, 2021). Consequently, it seems more realistic that each of the non-worst genes is under more or less relaxed stabilizing selection towards an optimum, coinciding with the worst gene - that can only change when the worst gene of the set improves to a higher value - which means that even when no mutational bias exists, most genes involved in complementary epistasis should be pulled towards the worst one, for complementary epistasis becomes negative in those cases. Likewise, it seems judicious to study whether the effect of such epistasis differs when it occurs within entities part of a linear system or between parallel entities (which could of course be made by lower-level entities themselves) like two pathways comprised of proteins, which would also be philosophically interesting for its shared similarity with electric or heat systems - as recently outlined by Yi and Dean (2019) - where parallel and series circuit were shown to work differently a long while ago.

Being directly based on functional insights, such a framework seems very relevant to start tackling the challenges set out by the joint evolution between basic functional epistasis – and more broadly, genetic interactions – and Adaptation from a population genetics theoretical perspective insofar as it may allow to draw very general and testable conclusions. In spite of its apparent specificity, it can indeed describe both intra-level and multilevel evolution: it is appropriate to describe the joint evolution of organs/appendages, as well as enzymes along a pathway, or organs/appendages and enzymes all together, provided that one knows how the genotype-phenoype-fitness map builds up. A first step of the project would therefore aim to deal analytically with simple instances of this model[5], an approach which could then be further refine through simulations to study cases where analytic tools fail to yield predictions. This step should be the main subject of a first research article and comes with many perspectives.

One natural sequel would of course be to refine its components and account for the existence of compensatory mutations entailed by the multidimensional phenotypic redundancy of some biological features: higher enzyme concentrations can buffer lower kinetic enzymes - though it comes with the cost of a protein burden (Koch, 1983; Dill et al., 2011; Kafri et al., 2016); villi can theoretically relax the selective pressure acting on enzymes for the absorption of nutrients; a longer calf can compensate for a smaller thigh or vice-versa, etc. To expand further in this area, it would also be relevant to see what happens when genes undergoing true stabilizing selection come into play, as they are also widespread[6], and whether the joint evolution with the fitness effects of mutations - whose distribution both impacts the course and the outcome of Evolution while inescapably being subject to It - could overturn expectations. However, it would seem premature to further investigate these specific building blocks more than others while we know

[5]Fisher's geometric model or Kaufman's NK complexity model seem well suited to feed into the thinking, but at this stage, it is not possible to state if they can properly translate the model assumptions.

[6]This is noticeably interesting for such fitness landscapes - including stabilizing selection - have been empirically documented in the case of drug resistance in microorganisms (Ford et al., 2020).

that there exists plenty other kind of genetic interactions (*eg.* biological modularity (Wagner, 1996)), that each deserves to be accounted for on a mechanistic basis: for instance, pleiotropy is, like epistasis and the distribution of fitness effects, both a result of Evolution and an intrinsic biological phenomenon (Wagner, 1996; Chesmore et al., 2016) depending on its underpinnings, which are numerous. This means that understanding its joint evolution with epistasis requires first to inform which part is intrinsic to biological systems[7], when and how it can be alleviated, and how it finally impacts the genotype-phenotype-fitness map before these systems are studied using a complete framework, which undeniably sketches a rather more distant objective.

Instead, it seems more appropriate to adopt a step-by-step approach where population genetics and first principle fitness landscapes are built in parallel, as was done in the past to understand the evolution of stability (Taverna and Goldstein, 2002; Bloom et al., 2004) and its evolutionary (Drummond et al., 2005; Bloom et al., 2006; Drummond and Wilke, 2008; Tokuriki and Tawfik, 2009; Serohijos et al., 2012; Dasmeh et al., 2014; Echave and Wilke, 2017) and functional consequences (Bloom et al., 2007; Geiler-Samerotte et al., 2011; Dasmeh and Serohijos, 2018), and to derive the models of genetic interactions and constraints from these latter ones rather than taking them for granted because they currently exist after billion years of evolutionary history. This would be the purpose of the second part of this project.

## 3 Testing the framework and improving functional hypothesis through the evolution of enzymes embedded in pathways

Thereafter, the second step of the project could aim at studying how cellular fitness emerges as the intertwining of metabolic fluxes within a cell and how this relationship in turn influences enzyme evolution. Such a task would therefore intend to see how basic theoretical predictions match those explicitly modelling mechanisms, and contribute to draw conclusions about higher level constraints that population genetics approaches must take into account. It could build on insights from a previous approach that was recently built to prove that an enzyme's selective pressure is driven by several biochemical and ecological factors (Labourel and Rajon, 2021), which explain why their enzyme kinetic features resemble a zoo (Bar-Even et al., 2011; Davidi et al., 2018) and seem far off physical optima if not sloppy (Bar-Even et al., 2015). Noticeably, an enzyme's selective pressure partly relies on the efficiency of more upstream enzymes and the other way around, which fully legitimates to study theoretically the influence of complementary epistasis (as also do other models and experiments we already mentioned). Perhaps more importantly yet, because fitness relied on specific parts of pathways, our conclusions point to the need of looking deeper into how epistasis builds up in a pathway (series epistasis[8]) and between them (parallel epistasis[8]), as well as it provides a framework to test ideas about the emergence and the further evolution of metabolic pathways.

As stated in the introduction, some authors have already raised the need to address the consequences of high order epistasis - see (Weinreich et al., 2013) for instance - in the context of molecular evolution. In

[7] In order to avoid introducing constraints which may be the result of Evolution, be it adaptive or not.
[8] In terms of electric/heat analogy.

fact, Heckmann et al. (2018) have lately tried to do so precisely in the case of enzyme kinetic parameters (focusing on turn-over numbers $k_{cat}$s). In their model relying on FBA[9], the fitness of *E.coli* cells results from the complex combination of thousands of enzymes whose $k_{cat}$s can undergo mutations. Mutation fixation of one variant is computed by a random draw from a binomial distribution with Kimura (1962)'s formula for fixation probability. Through that framework, they did observe that Evolution fails to produce optimal enzymes but this is mainly due to the premise that some enzymes are constrained under an efficiency ceiling, so that their study, though promising, does not provide a reliable answer to the influence of epistasis on Adaptation in the case of enzymes. This is even truer since FBA is already the fruit of a long evolutionary process and should therefore not be considered the appropriate framework to understand the joint evolution between epistasis and Adaptation.

Previously, the Evolution of enzymes and pathways had usually been studied theoretically using the Flux Control Theory (Kacser and Burns, 1973; Heinrich and Rapoport, 1974; Hartl et al., 1985; Dean et al., 1986), where diminishing returns effects on flux are the result of complementary epistasis (named differently) because of the flux summation theorem (Kacser and Burns, 1973; Kacser et al., 1995; Kaltenbach and Tokuriki, 2014): this latter states that flux control has to be spread between all enzymes of a pathway. Though it is only valid under certain circumstances (Bagheri-Chaichian et al., 2003), this theory has met some empirical success (Dykhuizen and Dean, 1990; Fell, 1992) but came short of explaining why the system does not improve further its observed state since some enzymes - especially transporters among empirically documented cases (Kacser and Burns, 1981; Hartl et al., 1985; Yi and Dean, 2019) - necessarily have a large control and should therefore lead to a step-by-step increase through which large control coefficients continuously travel from one enzyme to another. Besides, the fact that transporters play a specific role is also puzzling and needs a careful examination for it can reflect many distinct causes (physical constraints limiting their efficiency through trade-offs (Gudelj et al., 2010; Bosdriesz et al., 2018), cellular constraints acting on metabolite and enzymatic content that, along with organisms evolving under stabilizing selection for the efficiency of reactions, favour upstream control (Wright and Rausher, 2010) are explanations that have been proposed in the past; but they are, in one way or another, *ad hoc* assumptions).

Surely, enzymes - and more generally proteins - face physical constraints at a point that prevents them from being more efficient, but this does not explain why the same enzyme can be more efficient – both *in vitro* and *in vivo* (Bar-Even et al., 2011; Davidi et al., 2016, 2018) – in another species by many orders of magnitude and the fact that their kinetic activity can also be improved through readily evolvable levels of expression seems to contradict this argument and lends credence to initiatives trying to map fitness from first principles (Bershtein et al., 2017). And even when these constraints are relevant, they have to be modelled in order to assess their influence. What we propose to do here is to build a model where fitness results from the flux sustained by different pathways and is initiated by nutrients for which cells are in competition alike it occurs in Nature. As a first approach relevant for theoretical purposes, a metabolic pathway can be modelled as a succession of more or less reversible Michaelis Menten reactions initiated

[9]Flux Balance Analysis.

by a transport process (Labourel and Rajon, 2021), according to the following scheme comprised of one initiating transport step followed by $n$ reversible reactions:

$$S_{j,env} + T_j \rightleftharpoons S_j + E_{j,1} \rightleftharpoons P_{j,1}+E_{j,2} \rightleftharpoons (...) \rightleftharpoons P_{j,n-1}+E_{j,n} \rightleftharpoons \mathrm{P}_{j,f} \ , \quad (6)$$

with $j$ denoting the $j^{ieth}$ nutrient, $S_{j,env}$ and $S_j$ corresponding to the environmental and the cellular substrate ($j$), $T_j$ and $E_{j,i}$ representing respectively the transporter protein and the $i_{eth}$ enzyme of the pathway involved in processing substrate $S_j$, while $P_{j,f}$ represents the final product of this pathway (*eg.* energy).

The first step of the model would intend to determine the evolutionary outcomes at mutation-selection-drift balance, how fitness builds up from the different enzymes and whether there is space for evolutionary contingency or not. To do so, one should model the joint evolution of enzymes embedded in one such pathway, where fitness is represented by the final point: the efficiency of enzymes derives from its kinetic parameters and its concentration, while the need for an efficient enzyme (*i.e.* the fitness landscape on which it evolves) is driven by the extra flux it provides. It needs to account for possible loss of fitness due to excessive concentrations of metabolite (for instance, because it creates an imbalance with ubiquitous promiscuous reactions(Khersonsky and Tawfik, 2010; Peracchi, 2018; Tawfik, 2020; Niehaus and Hillmann, 2020)), and that contingent to the protein burden related to the cost of expression, and, to a lesser point, cellular and membrane molecular crowding (Chou et al., 2014; Labourel and Rajon, 2021). It also needs to include noise in gene expression as noisiness was shown to harbour a potential for very deleterious effects on such systems(Wang and Zhang, 2011) - not to mention that some concentrations are biochemically unachievable - that should be contingent to cell sizes (Labourel and Rajon, 2021) in such a way that evolutionary outcomes must be sensitive to this latter parameter.

The follow-up step of this project would be to unveil what occurs when there are multiple pathways that contribute to a cell's fitness under diverse circumstances (pathways could be parallel or branched, fitness could be determined only by the last step of processes, like for phospholipid synthesis, or result from multiple additions that add along the pathway, like for energy). With these latter realistic components, there would emerge a need to synchronize pathways in order for cells to be efficient and ensure them, in particular, to avoid some adverse effects of over-competitiveness: the intrinsic constraint of epistasis would therefore concern two different levels of interactions. Interestingly, if we also explicitly introduce substrate competition, we may see that some cells sacrifice part of their functions in order to maximize their fitness. This should be especially true when the environment is considered dynamically and can be depleted by cells, because cells may rather feed on nutrients than produce them *de novo*: determining when it happens and which cellular constraints (membrane crowding, protein burden) drive the process would open up avenues about the evolutionary trajectories that followed from the advent of the eukaryotic cell or transitions towards multicellularity.

Concurrently, it has been established that the ability of a protein to fold and adopt its active conformation is a prerequisite to an enzyme's function (Taverna and Goldstein, 2002; Bloom et al., 2004; Echave and Wilke, 2017), not to mention that misfolded states conceals the potential to be harmful (Bucciantini et al., 2002; Geiler-Samerotte et al., 2011). Accounting for the fact that enzymes cannot sacrifice too much stability (Taverna and Goldstein, 2002) subsequently enabled the community to better understand many processes in molecular evolution, such as the distribution of evolutionary rates or the effects of mutations (Zeldovich et al., 2007; Tokuriki et al., 2007; Lobkovsky et al., 2010; Tokuriki and Tawfik, 2009; Echave and Wilke, 2017; Bershtein et al., 2017), and led to insights about the evolvability of new functions (Bloom et al., 2004, 2006; Tokuriki et al., 2008). Meanwhile, it has been documented that mutations are on average destabilizing (Tokuriki et al., 2007, 2008) so that it gives rise to an apparent function-stability trade-off (Shoichet et al., 1995; DePristo et al., 2005; Weinreich et al., 2006; Lunzer et al., 2010), which can preclude many enzymes to be highly efficient (Tomala et al., 2019). It has thus become increasingly apparent that understanding enzyme (and pathway) evolution crucially requires to integrate both these fitness components. At this stage however and in spite of commendable first proposals (Bloom et al., 2004), combining mechanistically the effects of residues on stability and catalysis - through their contribution to the respective $\Delta G$ - within a general framework remains a major challenge (Echave and Wilke, 2017; Bershtein et al., 2017) because the intensity of the trade-off and its existence itself[10] seem to be largely enzyme-dependent (Schreiber et al., 1994; van den Burg and Eijsink, 2002; Knies et al., 2017; Miller, 2017; Tomala et al., 2019), and it is also anything but obvious to draw a two-dimensional phenotype-fitness map involving both these quantities (see (Echave, 2019) for one of the most recent and significant efforts relying on the ansatz of neutral threshold in the fitness landscapes). Thence, this issue of bringing together stability and catalysis still calls for more in-depth reflection to pick the right way to capture its founding principles, and, in turn, the arising evolutionary consequences for the question at hand.

Because there are many dimensions that contribute to enzymes, the project may have to be split into two different research articles, the first one focusing on the effect of epistasis and competition, while the second would seek to bring together enzyme activity and stability within a single evolutionary framework. Such a model could be used later to test ideas not only about the evolution of the lower level entities embodied by proteins but also for the upper level entities constituted by pathways, as well as this could be a starting point to tackle the influence of the same mechanism when it concerns cells, another kind of essentially fungible entities (Grosberg and Strathmann, 2007). It should also be useful to understand some patterns in molecular evolution because it has been shown that rates of Evolution are determined by a complex functional-stability relationship (Marcos et al., 2015; Echave et al., 2016; Jack et al., 2016; Jimenez et al., 2018), and also that highly expressed enzymes evolve more slowly (Drummond et al., 2005; Drummond and Wilke, 2008; Serohijos et al., 2012). Understanding what in the first place governs an enzyme's level of expression would therefore be useful to predict evolutionary rates - at least in the case of enzymes - and, coupled with the activity-stability gradient (Echave, 2019), it may shed light on questions

[10] As many residues act specifically on stability, they can even buffer some of the deleterious effects of mutations affecting both (Tokuriki and Tawfik, 2009; Storz, 2018).

that are contingent to these estimates.

## 4 Further perspectives

We have designed here a project that intends to draw theoretical evolutionary predictions from first principles and to foster the dialog between functional and evolutionary approaches in order to better understand why organisms work the way they do. Because further developments on both ends of the project are contingent to their results, it is not possible to anticipate precisely the next steps, but we have already put forward how some of these natural steps will emerge from the project. Two other concomitant perspectives deserve to be considered at this stage.

First, in the present outline of the project, the environment is considered to be constant such that fitness landscapes always look exactly the same, an unreasonable assumption for *in vivo* conditions. Acknowledging that ecological conditions impact Adaptation dates all the way back to the very beginning of Evolutionary biology insofar as it is the tenet of Natural Selection identified by Darwin (1859) and Wallace (1858): different environments can be seen as different fitness landscapes (Wright, 1931) where the optima are not located at the same place. Conversely, this means that species evolving under different environment are not subject to the same selective pressure, which therefore contribute to define evolutionary outcomes such as the mutation-selection-drift balance. This is all the more true since environment are always subject to fluctuations - albeit with different magnitude, temporality and stochasticity - a phenomenon which has largely been proven to determine optimal phenotypic strategies such as predictive plasticity (Gotthard and Nylin, 1995; Ghalambor et al., 2007), bet-hedging (Cohen, 1966; Slatkin, 1974; Cooper and Kaplan, 1982; Philippi and Seger, 1989) or polymorphism (Wittmann et al., 2017), be it among macro- (Menu and Debouzie, 1993; Philippi, 1993; Harpak and Przeworski, 2021) and micro-organisms (Ratcliff and Denison, 2010; Solopova et al., 2014) or in between (Martínez-García and Tarnita, 2017).Wilke et al. (2001) and Mustonen and Lässig (2008) compellingly unraveled how it should impact the long-term selective coefficient $s$, but it was not until recently that these ecological pressures were shown to impair severely the strength of Natural Selection under certain circumstances (Cvijović et al., 2015). Introducing ecological factors and other phenomenon likely to harm the potential of Natural Selection (Graves and Weinreich, 2017) cannot thus be overlooked when one is willing to make accurate predictions about *in vivo* Evolution and should be added for further developments on either part of this project.

Simultaneously, it could also become relevant to attempt to detect complementary epistasis in genomic data using well identified and characterized genes and pathways (*eg.* genes involved in glycolysis) among closely related species - sharing similar ecological niches - or even within populations, whose genetic divergence concerns few loci. Identifying genes and/or loci of the same pathway that face distinct selective pressures depending on the genomic background to which they belong may yield testable predictions about the actual metabolic effects of mutations even when they depart from *a priori* (for example, a mutation improving catalytic properties of an enzyme is supposed to increase the metabolic flux and may well do so - or not - in different organisms but still have few or no effect at all on the fitness in some but not all of them,

because in those where it does not, it is not the fitness limiting step) and to reconcile expectations about fitness effects of mutations with their actual counterpart. Reciprocally, investigating why some mutations increasing fluxes[11] display similar fitness effects than synonymous mutations would help appreciate how existing phenotypes and their underlying genotypes are translated into fitness while it should also feeds the community with information about mechanisms responsible for adaptation in functional sites such as the catalytic site of an enzyme.

Then, using species with more or less different life histories and phylogenetic relatedness, it may become practicable to test these ideas on a wider scale as some pathways are largely conserved, and to see whether it influences the inference of population features (such as $N_e$). Obviously, there are many contributions that can equally account for similar selective signatures and the objective being to disentangle these manifold contributions, data analysis needs either be restricted to typical cases where flux, fitness and genomic index of adaptations can readily be determined, or to be seen from a broader perspective. This suggests another long-term line of research, where the combination of such a framework with the knowledge of other mechanisms involved in molecular fitness - namely protein stability (Dasmeh et al., 2014; Dasmeh and Serohijos, 2018; Echave and Wilke, 2017; Echave, 2019) - would aim at explaining part of the variability observed when characterizing the fitness of specific sites or codons along and across phylogenies (Rodrigue et al., 2010; Rodrigue and Lartillot, 2017; Parto and Lartillot, 2017; Jones et al., 2020) and may help to better grasp adaptation signals as was shown in (Dasmeh et al., 2014), and also to identify what governs the adaptive process, a yet contentious issue (Harpak and Przeworski, 2021). Along with many other lines of research, for instance on convergent Adaptation (Stoltzfus and McCandlish, 2017), such an effort would also help uncover which part of the evolutionary process is contingent and which one is necessary (Monod, 1971; Ben-Menahem, 1997), for a similar strength of selection may yield pathways whose weakest links are spread differently, and, eventually, lead to variability in levels of evolvability.

[11] In principle, it could also be done with organs for instance, or symbiotic relationships or any complementary interaction, but the link with fitness for a seemingly improved parameter is yet more complex and makes it all but attainable at this stage.

**Glossary**

Complex epistasis arises from the interaction between two or more mutations in which these mutations do not contribute additively on any underlying trait they code.

Complementary epistasis arises when two mutations or more are necessary to produce a new phenotype. Positive complementary epistasis describes the redundancy of a system/trait and can be quantified by the robustness of this system/trait to mutations. Conversely, negative complementary epistasis means that any gain in the value of a phenotype needs that two or more positive mutations occur to produce a more effective phenotype. This is true for example when a specific color results from the involvement of several pigments.

Diminishing-returns epistasis stands for any mutational interaction in which the effect of combined advantageous mutations is less than the sum of their isolated effect.

Genetic redundancy describes a state where a function is processed by several redundant genes, which may be the result of subfunctionnalizaiton for instance.

Global epistasis describes the interaction between two or more mutations combine additively on an underlying unobserved trait which influences an observed trait or phenotype (including fitness) through a non-linear relationship.

Higher-order epistasis is a phenomenon where epistasis involves many different genes/residues and is used, for instance, to describe the influence of genetic background on epistasis involving two or few specific mutations.

Modularity defines the existence of relatively independent modules (*eg.* the arm and the leg) - in that they contribute independently to the phenotype - that are made of tightly interacting parts.

Modular epistasis extends the concept of epistasis to interacting modules: in this case, each part of a module interact displays similar epistasis relationships with another module.

Multidimensional epistasis is the general case where the interaction can involve any number of genes/residues.

Phenotypic redundancy describes the existence of multiple ways - generally involving sub-phenotypic traits - to optimize a phenotypic trait (*eg.* concentration, affinity and catalytic rates for enzyme efficiency).

Pleiotropy represents a process in which a specific gene/binding motif (or even single residue) influences several traits at once and may therefore be involved in a trade-off.

# 5 Project outline

## 5.1 Proposed timeline

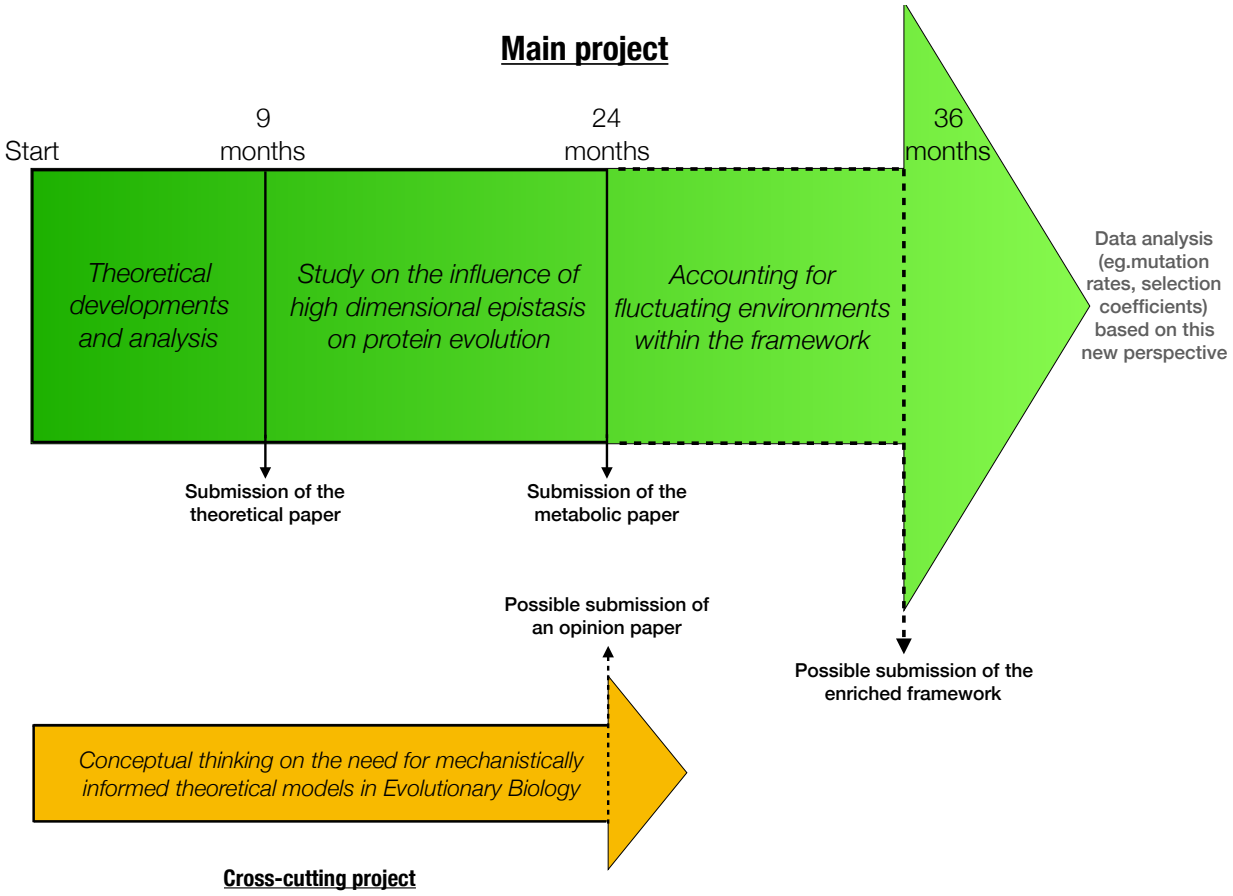


Figure 3: Proposed schedule for the project: the green arrow details a possible roadmap for a research project funded for 24 to 36 months while the gold arrow introduces the possibility of an opinion paper making the case for the development of theoretical population genetics and evolutionary models with more biologically realistic underlyings.

## 5.2 Possible host laboratories

Among several others, three laboratories match with the needs and aspirations of this cross-sectional line of research and could possibly be interested by it:

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# APPENDIX: Simulation outcomes for $N_e = 10$

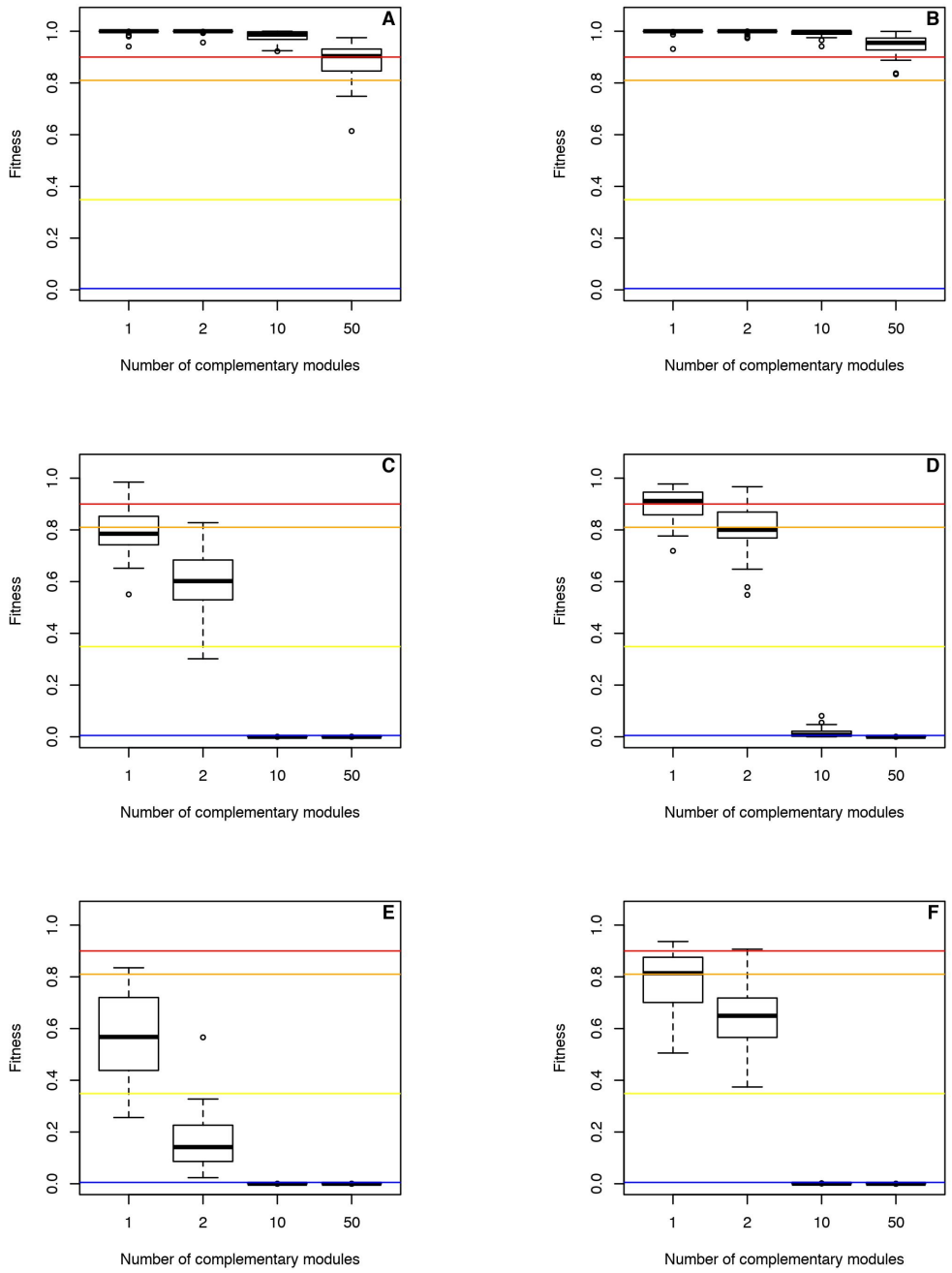


Figure 4: Simulation outcomes for the mutation-election-drift balance with $N_e = 10$ when the interplay of complementary epistasis and diminishing returns epistasis (through the fitness landscape of traits) are accounted for. The number of complementary modules varies from 1 to 50. Each line corresponds to a level of mutational bias: null in (A) and (B), low in (C) and (D), high in (E) and (F) - see text for details; each column represents a level of mutational variability, with (A), (C) and (E) having low variability while (B), (D) and (F) display a moderate to high variability.