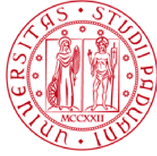

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CYCLE: XXX

EFFECTS OF PHENOTYPIC ROBUSTNESS ON ADAPTIVE EVOLUTIONARY DYNAMICS

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0.1 Summary

This work is mainly the results of a theoretical approach to the origin and evolution of phenotypic robustness in living systems. The work is structured as a paper collection of four articles. These reflect the logic of the different methods adopted to address the main question and they actually constitute the parts of a single argument. In particular we started from an experiment to verify theoretical predictions on the role of phenotype robustness on adaptive dynamics (1), then we raised new question answered by mathematical (2) and computational modelling (3), and finally we tested the new predictions on genomic data (4).

In the Introduction we showed how the main topic of investigation is framed under the umbrella of the "extended evolutionary synthesis". We presented the main aspect and concepts regarding the recent advances in the understanding of phenotypic robustness including its modelling through the genotype networks theory. In particular we focused on the effects of phenotypic robustness on evolvability and innovability, remarking the lack of evolutionary experimental evidence on whole living organisms and stressing the fact that the mechanisms and evolutionary forces by which robustness might be established during evolution are far from clear and overall little explored.

In Chapter I, we introduced the concept of cryptic genetic variation (CGV), that is a direct consequence of the existence of phenotypic robustness. We experimentally tested some theoretical expectations on the role of CGV in adaptive dynamics for the first time in vivo on an evolving populations of whole organisms (the bacterium *E. coli*). We found that according to the theoretical expectations, indeed CGV promotes a faster adaptation to novel environments even in the absence of significant phenotypic variation. This is a very counter intuitive results, since we expect the

adaptation rate to be proportional to the phenotypic variation and actually not to the neutral genetic variation to which selection is apparently blind.

In Chapter II we presented part of the theoretical work through the mathematical modelling. In particular we worked on the questions raised in the introduction about the origin and evolution of phenotypic robustness. In the introduction to the article, we highlighted the main questions under studying and illustrated the main models used or assumed in our theoretical analysis. In particular we introduce to the Quasi-species model, the Price equation, the Fisher infinitesimal model and the concept of universal pleiotropy. All these broadly accepted models constitutes the working framework of our mathematical modelling. We found that phenotypic robustness can affects the evolutionary adaptive dynamic itself. Elaborating on two independent evolutionary models, we found that, counterintuitively, a critical level of phenotypic robustness is a necessary condition for adaptation to occur even in the case of positive selection coefficients and infinite population sizes. Indeed ,this resulted in an example of how a feature of the genotype-phenotype map (robustness) can directly influence evolutionary outcomes. We argued that this could be the explanation for the widespread high levels of phenotypic robustness observed among organisms.

The aim of Chapter III is to test for stochastic effects on the results derived from the deterministic theoretical work of the Article 1. In the introduction to Article 3 we explained why randomness is important to be taken into account in evolutionary modelling, and why we adopted a computational approach for this task, rather than a mathematical stochastic model. For instance, we explained the limits of the diffusion approximation theory in modelling our problem, and explained the appropriateness of the simulation approach. We introduced also some few essential concepts and overview on simulations and computational models. The work of Article 3 resulted

in a substantial consistency with the theoretical results of the deterministic models. Even in more realistic stochastic scenarios and with finite population sizes, phenotypic robustness appears to keep its role of a necessary condition for adaptation to occur. However, we highlighted the need to explore the role of more complex environmental effects on the minimum level of robustness required.

Finally in Chapter IV we test some theoretical expectations on real biological data adopting a phylogenetic comparative approach (PCM). In the introduction to Article 4 we briefly introduce to the main concepts behind the PCMs, we illustrate the PGLS analysis and the evolutionary models adopted in our work. We tested two main predictions on genomic data on a sample of 210 eukaryotic taxa, deriving from the theoretical expectation that more complex organisms require higher levels of phenotypic robustness in order to adapt to environments. In particular since we argued that the proportion of genomic neutral DNA and the splicing levels are likely to be robustness proxies, we tested if they can explain at least a part of the organismal complexity, calculated as the proteome size (number of different proteins expressed by a particular genome). We found that, even accounting for the phylogenetic relationships, the relations actually hold with a very good explanation power. We argue that these results should be taken into account in exploring the problem of the genome size evolution, however we also argue that more, different and more accurate proxies of robustness and complexity are required to expand this prelaminar analysis.

0.2 Introduction

Despite the great achievements of the Modern Evolutionary Synthesis theoretical efforts, in recent times a growing body of opinion has suggested that the study of heritable variation (one of the pillars of the evolutionary theory), its origin and its structure, has been substantially neglected (Pigliucci, 2007). For nearly 150 years after the publication of the Origin of species by Charles Darwin (Darwin, 1859), evolutionary biology focused primarily on the role of selection, chance and inheritance in the adaptation process and on the dynamics of molecular and morphological evolution (Gould, 2002). Actually, not only evolution influences variation, but the latter can also play an "instructive" role, rather than merely a "permissive" one, in determining evolutionary outcomes (Fusco et al., 2012; Gould, 2002). This heterogeneous collection of studies are grouped, among other, under the umbrella term of "extended evolutionary synthesis" (Huxley et al., 2010), which includes for instance the interdisciplinary field of studies known as evolutionary developmental biology (or, evo-devo). General concepts deriving from the evo-devo tradition, like the influence of the genotype-phenotype map structure on evolution, also apply to living systems. In fact while evo-devo claims that development can bias the production of phenotypic variation, it is not true that the structure of variation, its instructive role, must come from development exclusively. Indeed, development is only a segment of an organisms' life cycle (Minelli and Fusco, 2010) and there are biological processes other than development that can be source of anisotropic phenotypic variation. These are for example standard mutation and recombination through the constrains imposed by standing genetic architecture (e.g.,Hansen (2006); Rajon and Plotkin (2013)), epigenetic effects (e.g., Richards et al. (2012); Mesoudi et al. (2013)), different forms of biased transmission (Dalton and Carroll, 2013), and not

fully appreciated effects of several kind of stochastic events (e.g.,Lenormand et al. (2009); Vogt (2015)). Thus in the need of a more comprehensive ”theory of variation”, moving forward from the limited concept of developmentally biased variation (Fusco, 2015), this work is an exploration of the origin and evolution of a specific feature of the genotype-phenotype map, namely phenotypic robustness, or mutational robustness, (also known as genetic canalization (Gibson and Wagner, 2000). Phenotype mutational robustness, from now simply ”phenotypic robustness”, is generally referred to as the ability of a phenotype to resist to mutational perturbations at the genetic level, stemming from the fact that multiple genotypes can encode the same phenotype. This corresponds to some extent to Kimura' s (Kimura et al., 1968) claim that much of genotypic change in evolution is selectively neutral (mutations responsible for an effect on fitness are only a small minority). Empirically, the observation that RNA and protein structures are more conserved during evolution than their sequences indicates that most point mutations are neutral. In other words, only a minority of sites is conserved in sequences evolved from a single ancestor, indicating a high level of degeneracy in genotype-phenotype maps. Such mutational robustness has been observed in biological RNA structures (Huynen et al., 1993), simulations of the evolution of RNA secondary structure (Huynen and Hogeweg, 1994), ribozymes and living organisms (Rigato and Fusco, 2016).

0.2.1 Genotype networks

A phenotype may be realized by a number of different genotypes, which are said to form a *neutral network* (Fontana et al., 1993; Kauffman, 1993; Schuster et al., 1994; Grüner et al., 1996; Fontana, 2002), also called a genotype network (Wagner, 2011; Payne et al., 2014). Genotype networks resides in the genotype space. A geno-

type space comprises all sequences of a given length L. This is astronomically large, comprising 20^L protein genotypes (for amino acid sequences) or 4^L RNA or DNA genotypes (for nucleotide sequences). Evolutionary change takes place in populations of organisms, and each member of a population is considered having a single genotype. It is thus useful to think of a population as a collection of genotypes in the genotype space. The members of this population "explore" this space through mutations. An especially important class of mutations is that of point mutations, which transform a genotype into one of its neighbours, the set of genotypes that differ only for one amino acid or one nucleotide from the original genotype. Typical genotype networks i) are vast (count very many genotypes), ii) extend widely across the genotype space (genotypes of the neutral network can differ significantly in their sequences), but iii) occupy a vanishing small volume of the genotype space (the ratio between the size of the genotype network and the size on the genotype space). Thus, a network can be traversed through many small mutational steps with little or no phenotypic change. Existence of these vast genotype networks was first suggested by computational models of phenotypic formation (Lipman and Wilbur, 1991; Schuster et al., 1994), but they had been also observed in real macromolecules (Babajide et al., 1997). It is important to remark that the definition of neutral genotype network adopted here is derived from a broader definition of neutrality (Wagner, 2012). In most cases mutations arising in a genotype are not strictly neutral, i.e. they can actually slightly affect the phenotype and thus fitness. For example, weakly deleterious mutations are more abundant than neutral mutations in most macromolecules, but they are often accompanied by compensatory genotypic changes that allow a preservation of the phenotype. The simultaneous occurrence of multiple mutations can help a population "tunnel" through a low fitness region in the genotype space, and thus help to preserve a phenotype (Sawyer et al., 2007; Eyre-Walker et al., 2002).

In other words, a combination of non-strictly neutral mutations can lead to an effective neutral phenotype due to their epistatic interaction. Another central feature of the genotype space is the genotype' s neighbourhood, that is the collection of those genotypes that can be reached from a given genotype through one or few mutations. Neighbourhoods are important from both a qualitative and a quantitative point of view. This is because the set of different phenotypes in a neighbourhood is easily accessible by mutation. The size of this set is thus a simple measure of how phenotypically variable a genotype is in response to mutations (Wagner, 2008). Genotype networks allow individuals in a population to preserve their phenotypes while changing their genotypes by many small mutational steps. This could lead to an increase in the population genetic variation even in the presence of a high selective pressure. This neutral genetic variation is cryptic since is not manifested at the phenotypic level, however, as neighbourhoods of different genotypes typically contain different novel phenotypes (Wagner, 2005b), a population of different genotypes on a genotype network can access a more vast sets of different novel phenotypes (Espinosa-Soto et al., 2011).

0.2.2 Phenotypic robustness and evolvability

Phenotypic robustness has the appearances of an attribute of the genotype-phenotype map that should oppose the adaptation process of populations. This is because there is a tension between the need of biological complex systems to evolve in a changing environment, and the need to preserved their complex phenotypes from mutations (Draghi et al., 2010). Consider a particular genotype of a genotype network. We can define the mutational robustness of its associated phenotype as the proportion of neutral genotype neighbours at one or more mutational step of distance. This

proportion of neutral genotype neighbours can be different for different genotypes in the neutral network. In fact, computational studies (Wilke et al., 2001; Wagner, 2005a) show that in general neutral networks in genotype space can be arranged as "the galaxies in our universe". This means that there are zones of high interconnection (high neutrality) and zones with lower connectivity. However, if we consider a population of individuals distributed in a neutral network, we can define a mean phenotype robustness, which is the mean of proportion of neutral neighbours of each genotype of a given population. This scenario highlights some very important properties of robustness for adaptive dynamics. Adaptive dynamics (and ultimately evolution) are population phenomena but can be influenced by phenotypic robustness, an individual property, in several ways. Firstly, as saw before, highly robust phenotypes allow populations to access greater phenotypic variability, namely the total of neighbours with new different phenotypes. Secondly, phenotype robustness allows the accumulation of cryptic genetic variation that could be exapted (Gould, 2002) to new mutational or environmental perturbations (Wagner, 2008; Hayden and Wagner, 2012). Thirdly, phenotypic robustness allows a faster cryptic exploration of the genotype space, increasing the probability to find a new superior phenotype even in the absence of substantial phenotypic variation. All these properties, deriving from the structures of neutral network, have been highlighted computationally or experimentally for molecules such as RNAs (Lipman and Wilbur, 1991; Grüner et al., 1996), rybozymes (Stelling et al., 2004; Tanner et al., 1996) and proteins (Lipman and Wilbur, 1991; Rost, 1997), while for whole organisms some evidence are provided in this work (see Chapter 1).

0.2.3 Origin and evolution of phenotypic robustness

The present work is structured as a paper collection articulated in four chapters, one for each article. Three out of four articles are in advance stage of preparation and have not been submitted yet. Each chapter contains an introduction to the corresponding article, to allow the reader to familiarize with the most important concepts and theoretical or technical tools specific of the work, and to logically connect one article to the others. As highlighted above, most of the preceding studies aimed at exploring the long-term effects of phenotypic robustness on adaptation and evolvability. However, the lack of in vivo experimental evolution evidence, lead us to design an experiment to test the above-mentioned effects on evolvability in a real, organism level living system (Article I). In addition, except for some few studies, the mechanisms by which robustness might be established during evolution are far from clear and overall little explored (Masel and Siegal, 2009; Rigato and Fusco, 2016). Given that phenotypic robustness seems a quality that would oppose the adaptation process, how such a feature of living systems can be maintained throughout generations without, apparently, any short-term benefits and in a continuously changing environment? Is robustness an adaptation in historical sense, i.e. a feature that has been shaped by natural selection? Or is it simply a by-product of evolution? Long term beneficial effects on evolvability cannot explain why high levels of phenotypic robustness are preserved in complex living system. A more complete treatment of adaptation within evolutionary theory should try to include phenotypic robustness as an evolvable parameter, rather than to treat it as a given. Accordingly, we tried to explore the possible causes of the origin and widespread persistence of phenotypic robustness in evolving complex living systems. We started adopting a theoretical approach with the aim of exploring the relation between phenotypic robustness,

mutation, selection, adaptation and complexity either through deterministic models (Article II) and simulations accounting for stochasticity (Article III). Finally, we tried to test predictions of the theoretical model and to find evidences from empirical data supporting theoretical results (Article IV).

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0.3 Chapter I

0.3.1 Introduction to Article I

A consequence of phenotypic mutational robustness at the population level is the accumulation of neutral genetic variation (Hayden et al., 2011), also known as cryptic genetic variation (CGV). This represents an unexpressed, bottled-up genetic potential (Gibson and Reed, 2008). Although not normally observable, CGV can be expressed under new or unusual conditions such as in a new environment or in a different genetic background. Phenotypic robustness allows the accumulation of cryptic genetic variation that could be exapted (Gould, 2002) to new mutational or environmental perturbations (Wagner, 2008; Hayden and Wagner, 2012). In a sense, the expressed component of genetic variation is just the tip of an "iceberg of genetic possibilities" that are hidden below the visible surface. For example, *Antennapedia* is a mutation in *Drosophila melanogaster* that transforms the antennae into legs. When this mutation is placed in a dozen different wild-type genetic backgrounds, each strain will show a different phenotype, ranging from almost perfect antennae to almost perfect legs where the antennae should be (Gibson et al., 1999). This is the effect of cryptic genetic variation, which modifies the mutant phenotype, even though it is unobservable in normal flies. Through CVG, phenotypic robustness has also an intimate relationship with the phenomenon of genetic canalization. Canalization refers to the evolution of phenotypic robustness that occurs under conditions of long-term stabilizing selection. It leads to suppression of the effects of genetic variation under normal, unperturbed circumstances (Gibson and Reed, 2008). Phenotypic robustness allows cryptic genetic variation to accumulate in a population, that can be "released" as observable phenotypic variation when the environment

changes or a new mutation appears, or when novel genotypes are introduced as in hybrid zones. Thus, cryptic genetic variation might have a key role in the kinetic of adaptation. Since evolutionary adaptation by natural selection requires phenotypic variation, the fraction of variation emerging from cryptic genetic variation could enhance evolvability and innovability (sensu Wagner, 2011). This property was demonstrated computationally for biological systems at different level of organization, and experimentally for molecular systems such as RNAs (Lipman and Wilbur, 1991), rybozymes (Stelling et al., 2004; Tanner et al., 1996) and proteins (Lipman and Wilbur, 1991; Martinez-Pastor et al., 1996; Rost, 1997). However, the effects of phenotypic robustness on adaptation had not been investigated in more complex evolving systems, as are whole organisms, where adaptation depends on several, generally little-known parameters of the genotype-phenotype map, such as the amount of epistasis, pleiotropy and neutrality. We designed a first evolutionary experiment to investigate the effects of robustness on adaptation in a biological system at the organism level. Laboratory evolution has recently grown into a standard tool for the study of the evolutionary process in a controlled manner in microbial communities (Helling et al., 1987; Nakatsu et al., 1998; Lenski et al., 1998; Papadopoulos et al., 1999; Massey et al., 1999; Cooper et al., 2001), and we choose the well-studied bacterium *Escherichia coli* to test the role of CGV on adaptive dynamics. The trait selected for in this study is a component of fitness: the population growth rate in a given environment, and the only environmental variables used in this experiment are the carbon sources in constant minimal media and environment. A metabolic phenotype has been considered as a metabolic network that can synthesize all biomass molecules in a given chemical medium with a given efficiency (Wagner, 2011). In other words, a metabolic phenotype can be defined as a metabolic network that can sustain a specific grow rate in a given environment. Adaptation consists in finding

new genotypic variants with higher grow rate, i.e. with better efficiency in metabolizing a given carbon source, the sole carbon source in a minimal medium. *E. coli*'s metabolic network has proved to be highly resistant to mutations (Rodrigues and Wagner, 2009). Rodrigues's computational works on *E. coli*'s metabolic networks showed how those networks supporting life in one environment can have very different essential reactions. These capabilities to tolerate mutations without significant loss of function, makes this bacterium an ideal candidate for studying the effect of CGV, and ultimately phenotypic robustness, on evolvability.

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0.3.2 Article I

Article Published in *Journal of Experimental Zoology: Part B, Molecular and Developmental Evolution*

RESEARCH ARTICLE

Enhancing Effect of Phenotype Mutational Robustness on Adaptation in *Escherichia coli*

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ABSTRACT Theoretical and computational studies predict a positive role for widespread phenotype resistance to genetic mutation, or "phenotype mutational robustness," in enhancing adaptation to novel environments through the accumulation of cryptic genetic variation. However, this has not been verified through experimental evolution in biological systems at the level of whole organisms. In a short-term evolution experiment of about 250 generations, we studied the adaptive performances of independently evolving populations of the bacterium *Escherichia coli* in two new nutritional environments, represented by minimal media with either lactate or glycerol as the sole carbon source. At the start of the experiments, all populations expressed identical phenotype, while differing for the amount of cryptic genetic variation, artificially produced by mutagenesis. We found that cryptic genetic variation can promote significantly faster adaptation to a new nutritional environment in *E. coli*. The scale of this effect varies between the two environments, and correlates with an estimation of the phenotype robustness of the ability to grow in a given medium, based on survival rate after mutagenesis in the same medium. *J. Exp. Zool. (Mol. Dev. Evol.) 326B:31–37, 2016.* © 2015 Wiley Periodicals, Inc.

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Widespread phenotype resistance to the impact of genetic mutations, or "phenotype mutational robustness" (Kitano, 2004; Stelling et al., 2004; Wagner, 2005), is a necessary condition for the accumulation of neutral genetic variation (Hayden et al., 2011; Wagner, 2011). This so called "cryptic genetic variation" (CGV) has no effect on the phenotype in a particular genetic or environmental context, but can eventually be expressed in the phenotype as a consequence of genetic mutations or environmental change (Gibson and Reed, 2008).

Since evolutionary adaptation by natural selection requires phenotypic variation, phenotype robustness may seem to be a quality of the organism's genotype–phenotype map that would oppose the process of adaptation. However, somewhat counter-intuitively, robustness can effectively enhance adaptation to novel environments by increasing the number of different phenotypes accessible through mutation (Wagner, 2008; Draghi et al., 2010). This effect has been demonstrated in theoretical studies using computational models (Matias Rodrigues and Wagner, 2009; Barve and Wagner, 2013) and with experimental studies on ribozymes (Hayden et al., 2011), but has not been verified through experimental evolution in more complex evolving systems, such as whole organisms. The possibility of extending this principle of the positive effect of robustness on adaptation to whole organisms by theoretical reasoning is limited by the need for specific assumptions on the features of the organism's genotype–phenotype map. These include the level of epistasis, pleiotropy, and neutrality, for which, despite substantial theoretical modeling (Orr, 2000; Wagner et al., 2008; Pavlicev et al., 2009; Wagner and Zhang, 2011), there are few observational data (Grüneberg, '38; Albert et al., 2008; Rohner et al., 2013).

We designed an experiment to investigate the role of robustness in the adaptation of the bacterium *Escherichia coli* by measuring the effects of CGV in adapting to novel

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environments, represented by minimal media with a single carbon source, different from the original strain's native carbon source which is glucose. The two phenotypic traits under scrutiny were the instantaneous population growth rate in either glycerol or lactate, which can be interpreted as measures of population absolute fitness in these environments (Elena and Lenski, 2003). Our results provide evidence of an enhancing effect of CGV on the process of adaptation to a novel environment, with quantitative differences between the two traits that match an independent measure of trait robustness, based on the survival rate after mutagenesis in the two environments.

MATERIALS AND METHODS

Outline of the Experiment

The experiment consisted in generating *E. coli* populations with different levels of CGV starting from the same genotype and then allowing them to grow in new environments, whereas the level of adaptation (in terms of growth rate) was recorded through time (Fig. 1). We treated different sub-clones of genotype BW30270 with Ethyl-methanesulfonate (EMS), regulating the time of exposure to the mutagen in order to obtain lines with, on average, either 12 or 24 randomly distributed mutations per genome. Control lines (0 mutations), obtained from the same BW30270 clone, were not exposed to the mutagen. Subsequently, all lines underwent stabilizing selection in the native carbon source, to re-establish the original phenotype and to remove phenotypic variation, while preserving the neutral genetic variation produced by the treatment. Combining the level of CGV with the carbon source, five type of lines (treatment groups) were established for the following adaptation experiments. Starting from identical population size ($N = 10^7$), all lines were allowed to grow for 266 generations either in glycerol (lines Gly0, Gly12, and Gly24, with 0 (control), 12 and 24 mutations, respectively) or lactate (lines Lat0 and Lat12, with 0 (control) and 12 mutations, respectively) as the sole carbon sources, with population size varying between 10^7 and 10^{11} during the experiment. The population growth rate was measured at the start of the experiment, after 14 generations and then at intervals of 42 generations. The adaptation experiment was replicated three times for each treatment group, always starting from an independently established line. During adaptation, mutation was the sole source of genetic variation, as BW30270 does not conjugate.

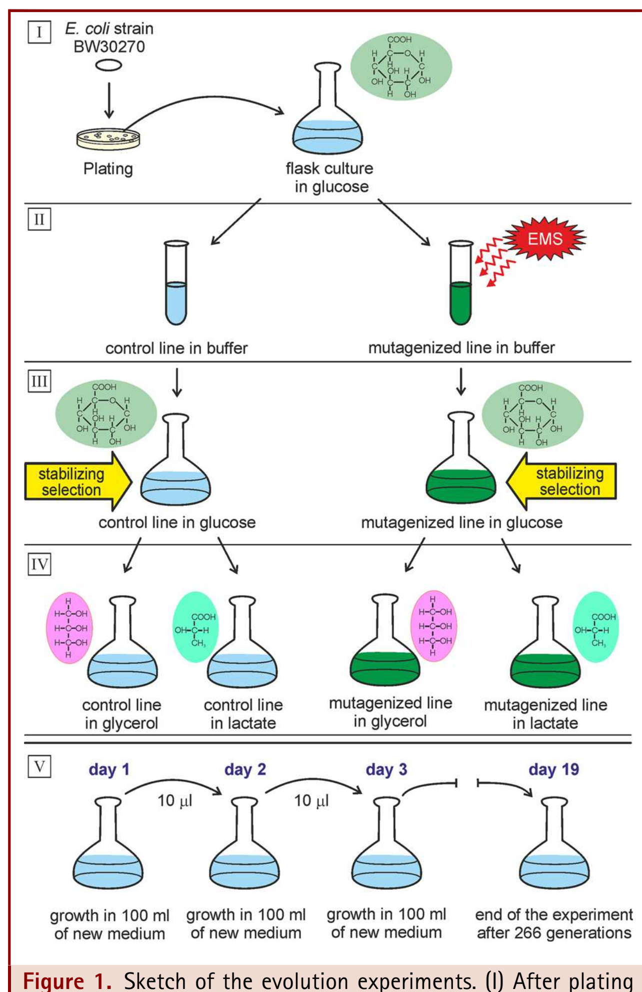

Figure 1. Sketch of the evolution experiments. (I) After plating the original strain, a single colony was randomly selected and sub-cultured in glucose for the following evolution experiments. (II) From this culture, a sub-clone (mutagenized line) was exposed to Ethyl-methanesulfonate (EMS), whereas the control line sub-clone was not exposed to the mutagen. (III) Subsequently, both mutgenized and control lines underwent stabilizing selection for 56 generations in the native carbon source (glucose), to re-establish the original phenotype and to remove phenotypic variation. (IV) Then, sub-samples of each line were transferred to a new nutritional environments (either, glycerol, or lactate) and allowed to growth for 266 generations by daily serial cultures (V), whereas the growth rate was periodically recorded. From step (II), the experiment was replicated three times.

Strain

Evolving cultures were propagated from fresh cultures of the wild-type *E. coli* K12 BW30270 provided by CGSC (Yale University, New Haven, CT, USA).

EMS Mutagenesis

We treated different sub-clones of the genotype BW30270 with the mutagen Ethyl-methanesulfonate (EMS), performing one or two cycles of mutagenesis in order to establish lines with, on average, either 12 or 24 randomly distributed mutations, respectively. The mean number of 12 mutations per genome induced by each cycle of mutagenesis was calibrated on the basis of previously reported mutation rates, based on the counting of revertants (Cupples and Miller, '89).

The mutagenesis protocol followed that by Cupples and Miller ('89). A fresh overnight culture was sub-cultured and grown until it reached a density of 2–3 × 10^8 cells per ml ($A_{600}=0.3$–0.5). The cells were chilled on ice, spun down at 5,000*g*, at 4 °C, for 10 min, washed twice in M9 salts (ForMedium, UK), and then re-suspended in half the original volume of M9 salts. EMS (Sigma–Aldrich, USA) was added in the cold by pipetting 0.07 mL of EMS into 5 mL of re-suspended cells ([EMS] = 1.4%) and incubated on a roller drum at 30 rpm for 15 min at 37 °C. The reaction was stopped by adding 5 mL of sterile sodium thiosulfate at 20% (w/w) (Sigma–Aldrich, USA). After mutagenesis, the cells were spun down, washed twice in M9 buffer, and then re-suspended in the same volume of M9 buffer and plated for viable cells and contaminations. Samples (1 mL) were added to 100 mL of M9 + glucose broth and the cultures were grown overnight. This process was independently carried out for each mutagenized line, for a total of six (three Gly12 and three Lat12). Samples from the three Gly12 mutagenized populations were mutagenized one more in the same conditions (after stabilizing selection, see below) to produce the three Gly24 lines. Gly0 and Lat0 lines undergone the same treatment, but for the addition of the mutagen.

Control of Phenotypic Variation

The interpretation of the differential grow rates in the different lines as a consequence of CGV is conditional on the absence of genetic variation with phenotypic effect on growth rates at the start of the evolution experiments, possibly introduced by mutagenesis.

In order to eliminate possible mutant phenotypes, the mutagenized populations were subjected to a stabilizing selection regime, cultured in M9 minimal medium + glucose (4 gr/l) and sub-cultured daily (the same procedure adopted for the evolving cultures, see below) for 4 days, for a total of 56 generations. Selection preserved most of the neutral genetic variation produced by the treatment, only slightly reduced by a minimal sampling effect ($N=10^7$) at the start of each daily culture.

Fifty-six generations of stabilizing selection are sufficient for reducing variation in growth rates well below the phenotypic effect of an average mutation in *E. coli*, estimated between 3% (Trindade et al., 2010) and 10% (Lenski et al., '91). Absence of significant phenotypic variation of growth rates in glucose after stabilizing selection was nonetheless directly assessed through statistical testing on observed growth rates.

Stabilizing selection in glucose was also aimed at eliminating possible epigenetic effects induced by the mutagenesis. It is known, in fact, that *E. coli*, like other bacteria, can epigenetically change mutation rate and expression profile under stressful condition (Rosenberg et al., 2012). However, this effects are transient (Hastings, 2007), and 56 generations of growth in glucose, which is a standard medium for *E. coli* BW30270 strain, are expected to eliminate any possible epigenetic effects produced by mutagenesis (Foster, 2005). Statistical testing on observed growth rates after stabilizing selection attested the absence of significant phenotypic variation of epigenetic origin.

Absence of significant phenotypic variation was further checked through repeated sampling from the starting population of the evolving cultures. For each type of line (Gly0, Gly12, Gly24, Lat0, and Lat12), we extracted 96 independent samples ($N=50\times10^8$) from the starting populations and measured the initial growth rate in their new medium (glycerol or lactate). For the sampling procedure, 100 µL of a fresh overnight culture (in M9 minimal medium + 0.4% glucose) 10^{-6} diluted, were plated to isolate approximately 200 colonies. After 24 hr of incubation at 37°C, a sample of each colony was randomly picked with a sterile stick and put on a well of a 96-well cell culture microplate, each filled with 150 µL of M9 minimal medium + 0.4% glucose. The plates were incubated overnight. After that, 5 µL of each well were transferred in another well of a 96-well microplate, filled with 150 µL of test-medium (M9 minimal medium + 0.4% lactate or glycerol). The five plates were incubated in an incubator shaker at 37°C, 280 rpm and the optical density (*OD*) of each well was periodically recorded (1 hr) at 600 nm with a microplate reader. Growth rates measures underwent statistical testing.

Evolving Cultures

Cultures were conducted in 100 mL of M9 minimal medium supplemented with 4 g/l of lactate (Sigma–Aldrich, USA) or glycerol (Sigma–Aldrich, USA) in covered 250 mL Erlenmeyer flasks in an incubator shaker at 37°C, 180 rpm. Each day, bacteria were grown overnight from an initial population size of 10^7 cells, until reaching the stationary phase at about of 10^{11} cells- ($A_{600}\leq0.9$–1.0), corresponding to about 14 generations. The day after, they were sub-cultured into fresh medium, using a biosafety cabinet and adopting standard sterile technique practices, restabilizing the initial population size of 10^7 cells. Batch growth and serial passage were conducted for 266 generations for all lactate and glycerol cultures. Lines Gly12, Gly24, Gly0 were tested for adaptation in glycerol as sole carbon source, whereas Lat12 and Lat0 lines were tested in lactate. Throughout the course of evolution, samples of each evolving population were frozen in 15% glycerol and stored at −80°C.

Growth Rate Measurements

Growth rate was measured at generations 0, 14, and then once every 42 generations until generation 266. At each time point examined, a sub-sample of each culture was used to inoculate 50 mL of fresh preheated medium for a batch culture in the same conditions of the evolution experiment. The growth rate was determined by measuring the *OD* of 2 mL growing cultures over time using a spectrophotometer (A_{600}) by periodic sampling ($\Delta t=1$ hr) of each batch culture, and interpolating the Log(*OD*)/Log(2) time series of the exponential phase with a linear model in

order to obtain a growth rate estimation in terms of generations per hour (slope of the regression line).

RESULTS

Mutagenesis and Stabilizing Selection

Starting from the same genotype, mutagenesis generated *E. coli* populations with different levels of genetic variation, with on average 0, 12, or 24 randomly distributed mutations per genome. Then, in order to eliminate possible mutant phenotypes, the mutagenized populations were subjected to a stabilizing selection regime in glucose for 56 generations. Growth rates were measured during stabilizing selection at generations 0, 14, 28, 42, and 56, and no significant differences were detected between mutated and non-mutated lines staring from generation 14 (equivalence tests, 95% confidence intervals of the differences within the measurement error of 0.015). This result also supports the effectiveness of the stabilizing selection at eliminating possible epigenetic effects on the mutation rate in the mutagenized lines induced by the mutagenesis stress.

Absence of significant phenotypic variation after stabilizing selection, either of genetic or epigenetic origin, was further assessed through repeated sampling ($n = 96$) from the starting population of each type of line (Fig. 2). We found no statistically significant differences between mutagenized and non-mutagenized lines, either for glycerol or lactate, neither for the means (equivalence tests, 95%CI of the differences within the measurement error of 0.020), nor for the standard deviations (Levene's tests, all comparisons $P > 0.6$).

Absence of effective phenotypic variation after stabilizing selection is also supported by observed adaptive dynamics (see Discussion).

Evolution Experiments

In the process of adaptation to a novel environment, all the lines with some amount of CGV outperformed the corresponding lines with (almost) no CGV in the same environment (Fig. 3). In glycerol, Gly12 lines showed average growth rates significantly higher than Gly0 lines from generation 98 onwards (one-tailed Student's t-tests, $P < 0.05$, $n = 3$, significant also after Tukey correction for multiple comparisons). A similar result was obtained for Gly24 lines, with average growth rates significantly higher than Gly0 lines from generation 98 onwards (one-tailed Student's t-tests, $n = 3$, $P < 0.05$, also after Tukey correction). In both cases the largest differences were reached at generation 98, when growth rate of Gly12 and Gly24 were 1.36 and 1.49 times that of Gly0, respectively. Likewise, lactate, Lat12 lines showed average growth rates significantly higher than Lat0 lines, although differences were relatively less marked, becoming significant from generation 140 onwards (one-tailed Student's t-tests, $n = 3$, $P < 0.05$, from generation 182 after Tukey correction), reaching the largest difference at generation 224, when growth rate of Lat12 was 1.2 times that of Lat0. In all three comparisons, the difference in growth rates between treated and control lines tended to reduce toward the final part of the experiment, mainly due to a deceleration in the growth rate increase of the treated lines.

Considering the effects of different levels of CGV in the same environment (Fig. 3A), Gly24 showed an average growth rate slightly higher than Gly12 from generation 98 to 182 (one-tailed Student's t-tests, $n = 3$, $P < 0.05$ at generations 98 and 182, but not significant after Tukey correction). After that, this small gap was rapidly filled in subsequent generations, and the two groups of lines converged to almost identical phenotypes.

Comparing adaptation trajectories in glycerol (Fig. 3A) with those in lactate (Fig. 3B), we observed a faster adaptation in the

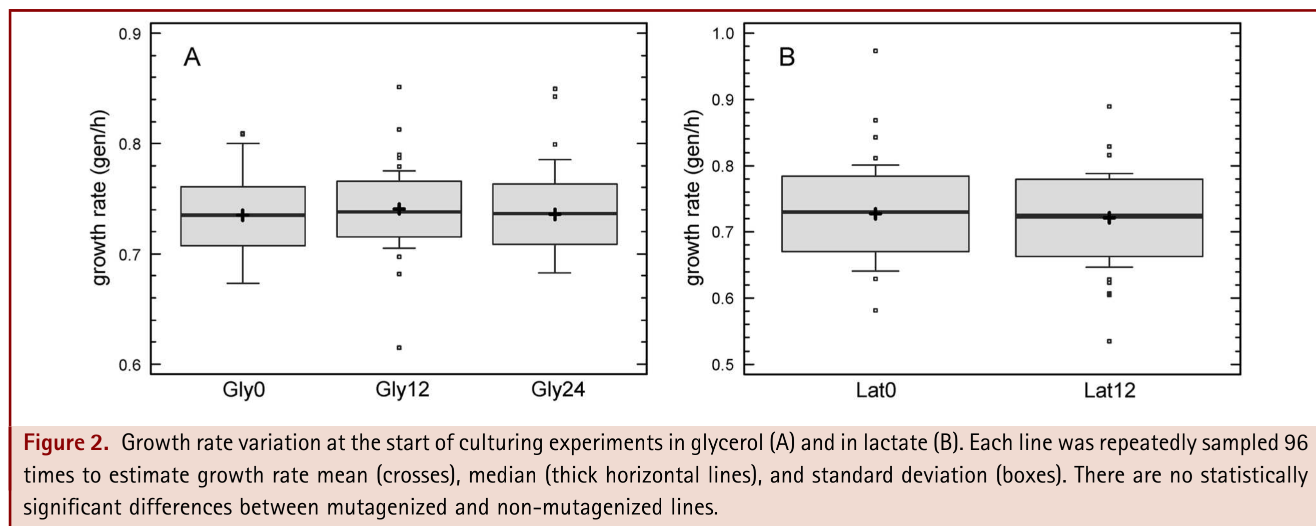

Figure 2. Growth rate variation at the start of culturing experiments in glycerol (A) and in lactate (B). Each line was repeatedly sampled 96 times to estimate growth rate mean (crosses), median (thick horizontal lines), and standard deviation (boxes). There are no statistically significant differences between mutagenized and non-mutagenized lines.

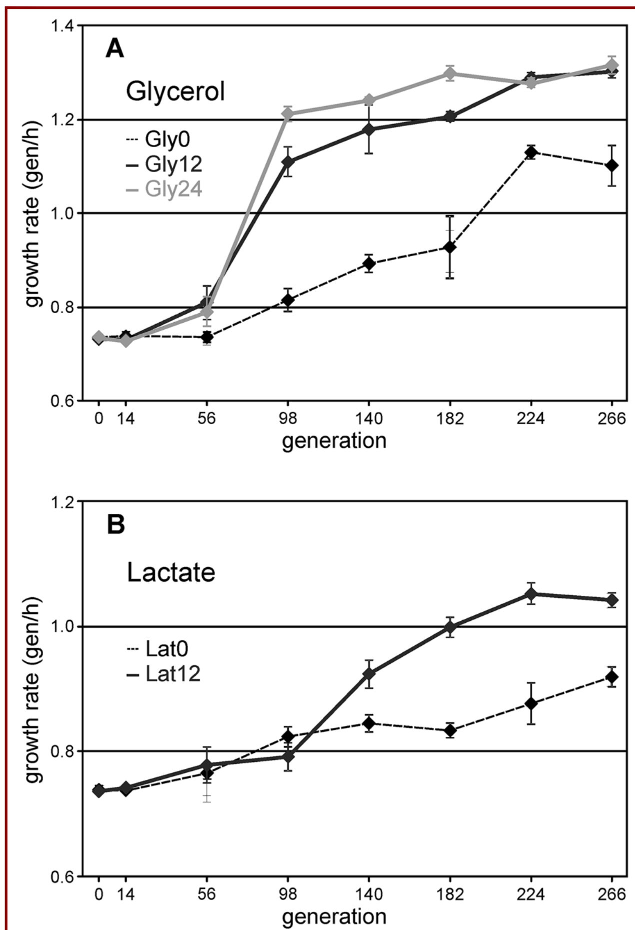

Figure 3. Effect of cryptic genetic variation on adaptation performance in *E. coli*. Average growth rates (diamonds) and standard errors (bars) are based on measurements on three independently evolving lines with the same amount of cryptic genetic variation. (A) Evolution in glycerol. Lines Gly12 and lines Gly24 (12 and 24 neutral mutations per genome on average, respectively) outperformed lines Gly0 (0 neutral mutations per genome). Growth rate differences are significant starting from generation 98. (B) Evolution in lactate. Lines Lat12 (12 neutral mutations per genome on average) outperformed lines Lat0 (0 neutral mutations per genome). Growth rate differences are significant starting from generation 140.

former, for both treated and control lines. Gly12 lines exhibited an average growth rate significantly higher than Lat12 lines from generation 98 onwards (one-tailed Student's t-tests, $n=3$, $P<0.05$, also after Tukey correction), when the largest difference was reached, with growth rate of Gly12 1.40 times that of Lat12. Similarly, growth rates of Gly0 were significantly higher than those of Lat0 from generation 140 onwards (one-tailed Student's t-tests, $n=3$, $P<0.05$, from generation 224 after Tukey correction), reaching the largest difference at generation 224, with growth rate of Gly0 1.29 times that of Lat0.

Survival Rates After Mutagenesis

Differences between the adaptation processes in the two media also emerge from analyzing the survival rate after mutagenesis to about 12 mutations per genome (R_{12}). Assuming the set of genomes of the viable individuals in one medium belong to the (nearly) neutral network of genotypes mapping on the same phenotype (defined by the viability in that medium) (Matias Rodrigues and Wagner, 2009), the proportion of survivors of the mutagenesis can be taken as a rough measure of the robustness of the ability to growth in that medium (Fig. 4). This must be taken as a crude measure of robustness because factors other than mutation can also contribute to observed mortality, as for instance the direct toxicity of the treatment.

Replicating the measure of survival rate on four independently mutagenized samples for each medium, at an average distance of 12 mutations from the original genotype the ability to grow in glycerol resulted a more robust character ($R_{12}=0.73$) than the ability to grow in lactate ($R_{12}=0.56$) (two-tailed Student's t-test, $n=4$, $P<0.005$).

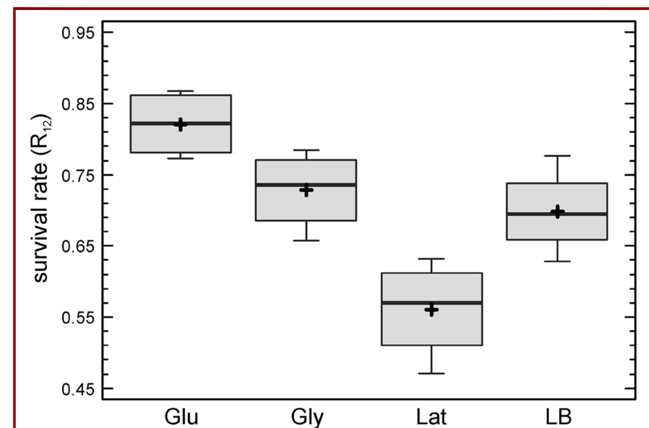

Figure 4. Phenotype robustness estimated as survival rate after mutagenesis in *E. coli*. This is the proportion of viable individuals in a given carbon source after they have undergone a mutagenizing treatment producing on average 12 mutations per genome (R_{12}). The measure assumes that the genomes of the survivors belong to the nearly-neutral genotype network mapping on the same phenotype. Means (crosses), medians (thick horizontal lines), interquartile ranges (boxes), and whole ranges of variation (vertical segments) are based on four independent measures for each substrate. The survival on lactate (Lat) is significantly lower than the survival on glycerol, indicating a lower mutational robustness for the ability to grow on the former substrate. R_{12} in glucose (Glu) and in Luria broth (LB) are shown as reference.

DISCUSSION

In classical models of natural selection, the population rate of change in mean fitness is expected to be proportional to heritable fitness variation (Orr, 2005; Frank, 2012). However, we observed a significant difference in the increase rates of mean fitness between lines that exhibited the same (almost zero) level of fitness variation, but possessed different levels of cryptic genetic variation (CGV). The lines with sizeable CGV exhibited higher *phenotype variability* (Wagner and Altenberg, '96; Willmore et al., 2007) than lines with no CGV, despite starting with the same (almost zero) *phenotypic variation*. This provide evidence in support of more recent views which see an effective role of phenotype robustness in enhancing adaptation, through the accumulation of significant levels of CGV (Wagner, 2012). The two *E. coli*'s metabolic traits under scrutiny appear to show some degree of "innovability" *sensu* Wagner (2011), that is, a propensity to evolve that does not necessary stem from heritable phenotypic variation, or "evolvability" (Pigliucci, 2008).

The roughly sigmoid dynamic of adaptation observed in our study, with the growth rate tending to a plateau, is quite similar to that observed in other experimental evolution studies on *E. coli* (Lenski et al., '91; Fong et al., 2005). Also quantitatively, both growth rate progression and between-lines growth rate variation of Gly0 and Lat0 lines are comparable with those measured in other studies (Fong et al., 2005). However, our specific experimental setting, including direct measurements assessing the absence of effective phenotypic variation between the treatment groups at the start of the evolution experiments, made it possible to expose the counter-intuitive effect of CGV in enhancing adaptation.

There are two, non-mutually exclusive ways in which CGV is thought to be able to promote faster adaptation (Wagner, 2012). Firstly, among the different genotypes with the same phenotype, some variants may be accidentally "pre-adapted" or "exapted" to the new environments (Hayden et al., 2011). Part of the cryptic variation is thus, "unveiled" and immediately converted to effectively advantageous phenotypic variation. Such fortuitous events were not expected to play a significant role in our short-term evolutionary experiment, as genetic variation produced with mutagenesis was relatively modest, less than 1/10 of the genetic variation recorded in wild-type populations of *E. coli* (Zhang et al., 2006). Secondly, the scattering of genotypes through the genotype space allows the population to access a greater number of new phenotypes trough mutation, increasing the probability of finding phenotypes that happen to have higher fitness (Matias Rodrigues and Wagner, 2009). This second mechanism confers a wider-ranging advantage to a population with significant CGV, because it does not depend on the specific mutations accumulated. Genotype scattering, which can be maintained even under a selective regime (Barrick and Lenski, 2013), was expected to affect more consistently the adaptive dynamics of our experiment.

The observed adaptation trajectories in our experiments suggest indeed a dominance of the effects of genotype dispersal. If a phenotype with a growth rate in the order of the value reached toward the end of the experiments (about 1.30 gen/h for glycerol and 1.05 gen/h for lactate) was already present at generation 0, even in one single individual (frequency 10^{-7}), the growth rate would have nearly reached the observed plateau within about 40 generations for glycerol and 70 for lactate (Fig. S1). But, in all lines, after 56 generations, average growth rates are still below 0.85 gen/h and the differences between treated and control lines are all statistically not significant (Fig. 3). The observed pattern is instead completely compatible with the progressive emergence of novel phenotypes with increasingly higher growth rates, in a sort of a stepwise adaptive progression, as described in other studies on bacterial evolution (Lenski et al., '91). This gradual increase in growth rate also makes very unlikely that some residual, undetected standing phenotypic variation might have remained in the treated lines after the stabilizing selection, and thus represents further assessment of the absence of significant phenotypic differences between treated and control lines at the start of the evolution experiments.

From comparing the adaptation dynamics of lines Gly24 with lines Gly12, it appears that the difference in CGV does not affect the final level of adaptation, but only the adaptation dynamics. In fact, both groups of lines tend to level to the same growth rate plateau, although in lines Gly24 the increase in growth rate is faster and the plateau is reached earlier (Fig. 3). This growth rate value seems to correspond to a not uncommon high-fitness phenotype, as not only the average adaptive paths of the two groups reached the same final value, but also those of each of the six individual lines (Fig. S1). In effect, across a relatively small number of generations, as those of our experiment, consistent adaptive patterns can more easily emerge because of some general features of genetic variation, like the dispersal of genotypes through the genotype space, rather than depending on the finding of rare advantageous mutations.

The observed slower adaptation rates in lactate with respect to glycerol can hardly be explained by simply assuming a phenotype optimum in the lactate closer to the starting growth rate value than in the glycerol, such that the slowing down of adaptation rate would depend on the relative proximity of the fitness plateau (*plateau effect*). Actually, longer adaptation experiments on *E. coli*, conducted for up to 600 generations, found a similar growth rate plateau for glycerol and lactate (Fong et al., 2005), although the plateau tends to be reached later in lactate than in glycerol. Conversely, the slower adaptation rate in lactate is in agreement with a lower robustness of the ability to grow in this medium with respect to the ability to grow in glycerol (*robustness effect*), as independently emerged from the different survival rates after mutagenesis in the two environments (Fig. 4).

Overall, this study provides experimental support on the view that phenotype robustness, through the accumulation of cryptic genetic variation, can promote faster adaptation at the level of a whole organismal system, here a bacterium. It also suggests that this can be achieved by allowing genetically more variable

populations to access a greater amount of phenotype variation, and that can be effective even in short-term evolution. Further studies, complementing measures of adaptive performances with a genetic analysis of mutation patterns, will be necessary to clarify the precise dynamics underlying the influence of cryptic genetic variation on adaptation.

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0.4 ChapterII

0.4.1 Introduction to Article II

Contrary to what presented in Article I, works from this point on, started as an attempt to investigate the origin and evolution of phenotypic robustness in living systems. This is a different, under-investigated aspect of phenotypic robustness. In fact, many previous mentioned studies aimed to mark the long-term effects of phenotypic robustness on evolvability and innovability. Indeed, except for some few studies, the mechanisms by which robustness might be established during evolution are far from clear and overall little explored. Phenotypic robustness seems to be an individual quality that should oppose the short-term adaptation process of populations per se even excluding possible fitness costs. Is robustness an adaptation in historical sense, i.e. has it been shaped by natural selection? Or simply a by-product of evolution? A complete treatment of adaptation within evolutionary theory should try to "endogenize" (Okasha, 2006)phenotypic robustness, rather than to treat it as a given. According to this, we adopted a theoretical approach; We tried to fill the gap of a rigorous mathematical theory that should precede any experimental plan or hypothesis. We tried to avoid simple linear reasoning and trade-off based hypothesis. Instead, we focused on what we could directly derive from standard evolutionary models. In particular, we elaborated on two famous models representing two very different approaches to model construction: The Quasi-species model (Eigen et al., 1989), originally conceived as an allele-based modellization, like the majority of evolutionary models in the history of evolutionary biology, and the Price's equation (Price et al., 1970), a phenotype-bases theorem that actually subsumes gene-based theories, since alleles and genotypes can be thought of as phenotypic

characters themselves. The Quasi-species is a multi-allele mutation-selection model that has the form of a dynamical system describing the frequency change of a particular sequence type (i) during time. The term "quasi-species" refers to an ensemble of similar genomic sequences generated by a mutation-selection process. Imagine a sufficiently large population of sequences of length L and of different type i. Denote by x_i the relative abundance of the ith sequence type, thus we have $\sum_i x_i = 1$. The population structure is given by the vector $\vec{x} = (x_0, x_1, ...x_n)$. Denote by f_i the fitness (growth rate) of the ith sequence type. The fitness landscape is given by the vector $\vec{f} = (f_0, f_1, ..., f_n)$. The average population fitness is $\psi = \sum_i x_i f_i$. The probability that genotype j results in genotype i by mutation is given by q_{ji}. $Q = [q_{ji}]$, where Q is the mutation matrix. Each element of Q represents the probability of a sequence j to mutate in a sequence i per replication. The quasi species equation is given by:

$$\dot{x}_i = \sum_{j=0}^{n} x_j f_j q_{ji} - x_i \psi \qquad (1)$$

This means that sequence i is obtained by replicating sequence j at rate f_j times the probability that replication of sequence j generates sequence i. This model is no more than a deterministic mutation-selection multi-allele model describing the frequency change of the ith sequence ($\dot{x}_i$).

Differently, the Price's equation is an "a posteriori" description of the change over time (generations) of the population mean trait value (note that the trait described could be the fitness itself or a variance instead of a mean, or the frequency of an allele) not focused on the dynamic but on the different states of the system at different

arbitrary times. The Price equation is based on a different approach, tracking the change in the mean population value of a given phenotype, not the frequency of a single pheno(geno)type. If a positive covariance between fitness and the phenotypic value of a certain trait exist, we can define adaptation as the increase of the mean of that particular character value over a definite time interval. This approach is very useful if we have to deal with quantitative traits and continuous character values. In addition, the Price equation contains all the evolutionary relevant factors and is a more complete and comprehensive description of the evolutionary process. Here we adopt the Price equation as a model to describe the effects of phenotypic robustness on adaptation considering the following form (Okasha, 2006):

$$\Delta\overline{z} = cov(w_i, z_i') + E[\Delta z_i] \tag{2}$$

Where$\Delta\overline{z}$ is the change in the population mean character value, z_i' is the mean of the offspring's character values, w_i the relative fitness, and $E[\Delta z_i]$ is the transmission bias, namely the change in mean character value due to other factors rather than selection, i.e genetic or environmental mutations, drift etc...Beyond the transmission bias, what really matters in evolution is the covariance between the parent's fitness w_i (offspring number) and the offspring phenotype, z_i'. Price equation is a theorem rather than a theory describing what is actually going on rather than make a simplified model of basic properties of the system. Indeed, almost every evolutionary phenomenon can be find in such equation which can be properly decomposed to describe it. Irrespective of the model, either quasi-species or Price, interpretation of our results is based on three principal key premises, that should correspond to situations found in the majority of the biological cases. First, we consider an infinitesimal model (Barton et al., 2016) perspective, where each phenotypic trait is the result of a very high number of genetic determinants (Turelli, 2017). In other

words, we consider that a huge number of loci can affects each trait of an organism, although to a different degree. The infinitesimal model finds its origin in a seminal paper R.A. Fisher, where he showed that, if many genes affect a trait, then the random sampling of alleles at each gene produces a continuous, normally distributed phenotype in the population (Aymler, 1918). As the number of genes grows very large, the contribution of each gene becomes correspondingly smaller, leading in the limit to Fisher's famous "infinitesimal model" (Barton et al., 2016). Despite the revival of interest (both theoretical and empirical) in "evolutionary quantitative genetics" in recent decades, the infinitesimal model itself has received little attention. However, recent advances in genome wide association studies (GWAS) highlight the possibility that virtually all the genome can affect every trait, that the genetic determinants are widespread through the genome and are highly interconnected such as that even apparently non-related peripheral factors can have a tiny effect on a given trait. This phenomenon has recently been marked as the *omnigenetic* model (Boyle et al., 2017). One important consequence of this model is that the high number of genetic determinants dramatically increase the phenotypic mutation rate of a given trait (even if with tiny effects). In the following work, we will show how this can affect our interpretation of the role of phenotypic robustness on adaptive dynamics. Second, we adopted the universal pleiotropy view, which is a natural consequence of the infinitesimal model, where each locus can affects virtually all traits (Boyle et al., 2017). It is reasonable to think that an organism is the result of the interaction of its interdependent parts rather than simply the resulting sum of independent factors. There are arguably millions of traits one can describe in a complex organism, but the number of genes is generally much lower. Inevitably, exactly for the same principle that a phenotype corresponds to multiple genotypes, there are genes that must affect multiple traits. This phenomenon of one gene (or one mutation) affect-

ing multiple traits is known as pleiotropy (Allen Orr, 2000; Orr, 2005). Pleiotropy is a central topic in genetics and has broad implications for evolution (Aymler, 1918; Allen Orr, 2000; Wagner and Zhang, 2011; Barton et al., 2016). Pleiotropy is the cause of trade-offs among the adaptations in different traits, because a mutation that is advantageous to one trait may be disadvantageous for another trait. The quantitative modeling of this idea led to the so called ”cost of complexity” hypothesis, which posits that complex organisms are inherently less evolvable or adaptable to changing environments with respect to simple organisms, because mutations have more pleiotropic effects (Fisher 1930). Recent advances in GWAS highlighted the fact that universal pleiotropy might seem the most likely scenario. Both the infinitesimal model and the universal pleiotropy are more and more supported by empirical evidence as suggested by recent works (Boyle et al., 2017). Third, and most important point, in this work we elaborated on a particular aspect of the G-P map, namely the many to one relationship between genotypes and phenotypes, which is usually not taken into account in the standard interpretation of these evolutionary models. Accounting for phenotypic robustness in the quasi-species and Price models lead us to the results presented and discussed in the following article.

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0.4.2 Article II

Article in preparation, to be submitted to an evolutionary biology journal (e.g., *J. Teor. Biol.*)

Effects of phenotypic robustness on adaptive evolutionary dynamics

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Abstract

Though the ubiquity of phenotypic robustness underlying molecular, metabolic and developmental processes is not a topic of major debate, the mechanisms by which robustness might be established during evolution are far from clear and overall little explored. With the aim of contributing to the understanding of the origin and evolution of phenotypic robustness in living systems, we adopted a theoretical approach, elaborating on standard deterministic population genetic models of evolutionary dynamics. Preliminary results showed that, under common selective regimes, a high level of phenotypic robustness is a necessary condition (although not sufficient) for adaptation to take place. This appears as a threshold effect, i.e. as a minimum level of phenotypic robustness under which evolution by natural selection cannot occur, even in the case of sizable positive selection coefficients and in absence of any drift effects. This ongoing work represents a first attempt to formally include phenotypic robustness in the more inclusive framework of a theory of adaptation, by providing an explanation for the evolution of this basic feature of living organisms and showing how a key feature of the genotype-phenotype map can directly affect the role of natural selection in evolutionary dynamics.

1 Introduction

Since evolution by natural selection requires phenotypic variation (Orr, 2005), the widespread phenotypic robustness to mutations may seem to be a quality of the organism's genotype-phenotype map that would oppose the process of adaptation (Draghi et al., 2010). Indeed, phenotypic robustness is expected to slow down the adaptation process, making the occurring of new beneficial mutations more rare. However, somewhat counter-intuitively, theoretical and computational studies predict a positive role for phenotypic robustness in enhancing long-term adaptation to novel environments, through the accumulation of cryptic genetic variation (Gibson and Reed, 2008; Hayden et al., 2011). This view is also

supported by a recent experimental study, showing that phenotypic robustness can promote significantly faster adaptation to new nutritional environments in *E. coli* (Rigato and Fusco, 2016). Although the ubiquity of phenotypic robustness underlying molecular, metabolic and developmental processes is not a topic of major debate, the mechanisms by which robustness might be established during evolution are far from clear and overall little explored (Wagner, 2011). For example, Wagner (2011) highlighted the role of slow environmental changes as a mechanism for the emergence of phenotypic robustness in any one environment (Wagner, 2011). Some attempts have also been made to connect the above mentioned long term benefits of phenotypic robustness to its origin (Wagner, 2005). However neither long-term benefits of phenotypic robustness, nor environmental fluctuations can provide a completely satisfactory explanation for the widespreadness of phenotypic robustness and for its origin and conservation in living systems (Kitano, 2004). How such a feature of living systems can be maintained throughout generations without short-term benefits and in a continuously changing environment? Indeed, phenotypic robustness seems to be an individual quality that should oppose the short-term adaptation process of populations *per se*, even excluding possible fitness costs. Are there any constraints or general principles that govern the degree of robustness of evolving systems? Is it an adaptation in historical sense, i.e. has it been shaped by natural selection? Is robustness a by-product of evolution or a necessary condition for life? A complete treatment of adaptation within evolutionary theory should try to "endogenize" (Okasha, 2006) phenotypic robustness, rather than to treat it as a given. With the aim of contributing to an understanding of the origin and evolution of phenotypic robustness in living systems, we adopted a theoretical approach, elaborating on two classical mathematical models of evolutionary dynamics, the quasi-species equation (Eigen et al., 1989) and the Price equation (Price et al., 1970). Both are deterministic models. The quasi-species model it is a multi allele mutation-selection dynamical system, describing the frequency change of a particular genotype i during time. Differently, the Price equation is an "a posteriori" description of the change over time (generations) of the population mean character value (note that the character described could be the fitness itself) and at variance with the quasi-species model is not focused on the

dynamic but on the different states of the system at different arbitrary times. The two models allow to approach the problem of robustness with different perspectives. With both models, our analyses are based on three key premises that should apply to the majority of the relevant biological cases. First, we considered an infinitesimal model (Barton et al., 2016) perspective, where each phenotypic trait is the result of a very high number of genetic determinants (Turelli, 2017). In other words a huge number of loci can affects each trait of an organism (although if with different magnitude effects). Second, we adopted the "universal pleiotropy view", where virtually each locus can affects all traits (Wagner and Zhang, 2011). It is reasonable to think that an organism is the result of the interaction of its interdependent parts rather than simply the resulting sum of independent factors. Both the infinitesimal model and the universal pleiotropy are supported by empirical evidence (Boyle et al., 2017). Third, and most important, we highlighted the many to one relationship between genotypes and phenotypes which is usually not taken into account in standard evolutionary models. In fact, phenotypic robustness is an emergent property of the genotype-phenotype map structure deriving from the fact that many genotypes maps on the same phenotype. Taking into account theses three key feature of the g-p map in the above-mentioned models, here we show that, counterintuitively, a certain level of phenotypic robustness is likely to be not only a favorable but also a necessary condition (although not sufficient) for adaptation to occur. This appears as a threshold effect, i.e. as a minimum level of phenotypic robustness under which evolutionary adaptation cannot occur, even in the case of sizably selection coefficients or differentials and in absence of any drift effect.

2 Phenotypic robustness and phenotypic stability

Phenotypic robustness is a property of the genotype-phenotype map. Here, for the derivations to follow, we will adopt a narrow, quantitative definition of *phenotypic robustness* (ρ), that is the probability that, across one replication/generation, mutation of a given genotype g takes to a genotype g' that exhibits the same phenotype of g:

ρ :=probability that a mutation has no phenotypic effect

From this definition of robustness, a definition of *phenotypic stability* (ϕ_{pp}) follows. This is the probability that the replication of a given genotype g takes to a genotype that exhibits the same phenotype of g. Indicating with η_g the mutation probability per genome per replication, phenotypic stability results to be the sum of the probabilities of two mutually exclusive events, namely i) that there is no mutation $(1-\eta_g)$ and ii) that in case of mutation the mutant genotype maps of the same phenotype $(\rho\eta_g)$, thus:

$$\phi_{pp} = (1-\eta_g) + \rho\eta_g \tag{1}$$

3 Quasi species model analysis

The quasi-species model (Eigen et al., 1977) is a single locus, multi allele, mutation-selection model where each allele differs from the others by at least a single point mutation.

Imagine a sufficiently large population of genomes of size G and of different type i. Sufficiently large means that we can neglect the role of drift. Denote by x_i the relative abundance of the ith sequence type, thus we have $\sum_i x_i = 1$. The population structure is given by the vector $\vec{x} = (x_0, x_1, ...x_n)$. Denote by f_i the fitness (growth rate) of the ith sequence type. The fitness landscape is given by the vector $\vec{f} = (f_0, f_1, ..., f_n)$. The average population fitness is $\psi = \sum_i x_i f_i$. The probability that genotype j results in genotype i by mutation is given by q_{ji}. $Q = [q_{ji}]$, where Q is the mutation matrix. Each element of Q represents the probability of a sequence j to mutate into a sequence i per replication (see Nowak, 2006). The quasi species equation is given by:

$$\dot{x}_i = \sum_{j=1}^{n} x_j f_j q_{ji} - x_i \psi \tag{2}$$

This means that sequence i is obtained by replicating sequence j at rate f_j times the probability that replication of sequence j generates sequence i.

This model is no more than a deterministic mutation-selection multi-allele model describing the frequency change of the ith sequence ($\dot{x}_i$).

3.0.1 Introducing the genotype-phenotype dualism, a phenotypic version of the quasi species model

Since the principle of the quasi-species dynamic holds for every mutating and reproducing entity, we can use the quasi-species model to track phenotypic frequency changes instead of the genotypic ones. Defining x_p as the frequency of a given phenotype we can write a phenotypic version of the quasi-species model as:

$$\dot{x}_p = \sum_{p'=1}^{n} x_{p'} f_{p'} \phi_{p'p} - x_p \psi \tag{3}$$

.

Where $f_{p'}$ is the fitness of the p' phenotype, $\phi_{p'p}$ is the phenotypic mutation probability of p' into p and ψ is the population mean fitness ($\sum_{p'=1}^{n} x_{p'} f_{p'}$). We can decompose equation [3] to highlight the two main contribution to the frequency change of p, yielding to:

$$\dot{x}_p = x_p f_p \phi_{pp} + \sum_{p' \neq p} x_{p'} f_{p'} \phi_{p'p} - x_p \psi \tag{4}$$

.

Equation [4] is the phenotypic version of the quasi species model assuming different sequences mapping on the same phenotype. The first term of the right hand side of [4] is the contribution of non mutant phenotypes, while the second one is the sum of the contribution of mutations from different phenotypes p'. The term ϕ_{pp}, is the phenotypic stability which contains the robustness term ρ.

3.0.2 Phenotypic robustness is a necessary condition for adaptation to occur I

Considering equation [4], adaptation occurs when for an advantageous phenotype p ($f_p > \psi$), $\dot{x}_p > 0$, i.e. when:

$$x_p f_p \phi_{pp} + \sum_{p' \neq p} x_{p'} f_{p'} \phi_{p'p} - x_p \psi > 0 \quad (5)$$

Dividing both therms by ψ, we have:

$$x_p w_p \phi_{pp} + \sum_{p' \neq p} x_{p'} w_{p'} \phi_{p'p} - x_p > 0 \quad (6)$$

where w_p is the relative fitness of a given phenotype p. Under the assumption that the mutational contribution from different phenotypes is reasonably negligible ($\sum_{p' \neq p} x_{p'} w_{p'} \phi_{p'p} \simeq 0$), we can write:

$$x_p (w_p \phi_{pp} - 1) > 0 \quad (7)$$

yielding to:

$$w_p \phi_{pp} > 1 \quad (8)$$

Inequality [8] is the necessary condition to be satisfy for adaptation to occur. Since the phenotypic stability term ϕ_{pp} contains the robustness term, we can ask what's the minimum level of robustness required to satisfy [8] substituting [1] into [8] we have:

$$w_p((1-\eta_g)+\rho\eta_g)>1 \tag{9}$$

Rewriting the relative fitness term as $w_p = (1+s_p)$, were s_p is the selection coefficient of the advantageous phenotype p ($s_p > 0$), we get:

$$(1+s_p)((1-\eta_g)+\rho\eta_g)>1 \tag{10}$$

and

$$\rho > \frac{(1+s_p)\eta_g - s_p}{(1+s_p)\eta_g} = \rho_c \tag{11}$$

The right-hand side of inequality [11] is the minimum level of phenotypic robustness required for adaptation to occur or to be maintained, that we indicate as the *critical robustness* (ρ_c). This depends only on the mutation probability η_g and on the selection coefficient s_p. As a rough proxy, we can see that as the mutation rate increases, higher levels of phenotypic robustness are required for adaptation to occur, while as the selection coefficient increases, lower levels of phenotypic robustness are required (Fig.1). ρ_c can vary from $-\infty$ to 1. When $\rho_c < 0$, no robustness is required for adaptation. This is the case of most evolutionary models where we have great selection coefficients and very low mutation rates, however we can show that this is a very narrow condition (see discussion). In fact, we can study when $\rho_c > 0$, and ask if this is at least a realistic condition. As $0 < \eta_g < 1$, the condition for ρ_c to be positive is:

$$s_p < \frac{\eta_g}{1-\eta_g} \tag{12}$$

This means that some robustness is necessary for adaptation when $s_p < \frac{\eta_g}{1-\eta_g}$.

It is interesting to analyze the two limit cases:

1) For $\eta_g \to 0$, no robustness is necessary for adaptation. Most evolutionary models assume this condition since they are focused on small genotypes (one locus, few loci and independent effects). In other words, they consider few genes affecting one trait and that these gene effects are independent from other genes affecting another trait.

2) For $\eta_g \to 1$, some robustness is always necessary, independently from s_p. In this particular case ρ_c equals to $\frac{1}{(1+s_p)}$

Since $\frac{\eta_g}{1-\eta_g}$ increases nearly exponentially from 0 to infinity with η_g, it is more likely that some robustness is required for adaptation, and when $\eta_g \to 1$, the condition for adaptation to occur is approximately:

$$\rho > \rho_c = \frac{1}{(1+s_p)} \tag{13}$$

This means that the phenotypic robustness needed for a particular advantageous phenotype to spread throughout the population is inversely related to its selective advantage (s_p) in that particular moment. This is true indeed if we consider that almost the whole genome can affect the phenotype of each trait. In this case the genotypic mutation probability equals to the genome mutation probability per generation. We know that at least for eucaryotes and viruses, the mutation probability per genome per generation is near to one in most cases (Drake et al., 1998). Inequality [13] highlights a strict condition for adaptation to occur. Let's remark that this condition holds even in the presence of a positive selection coefficient and

in the absence of any drift effect, since we assumed a large population size. In other words the selection efficiency can be heavily affected by the levels of phenotypic robustness.

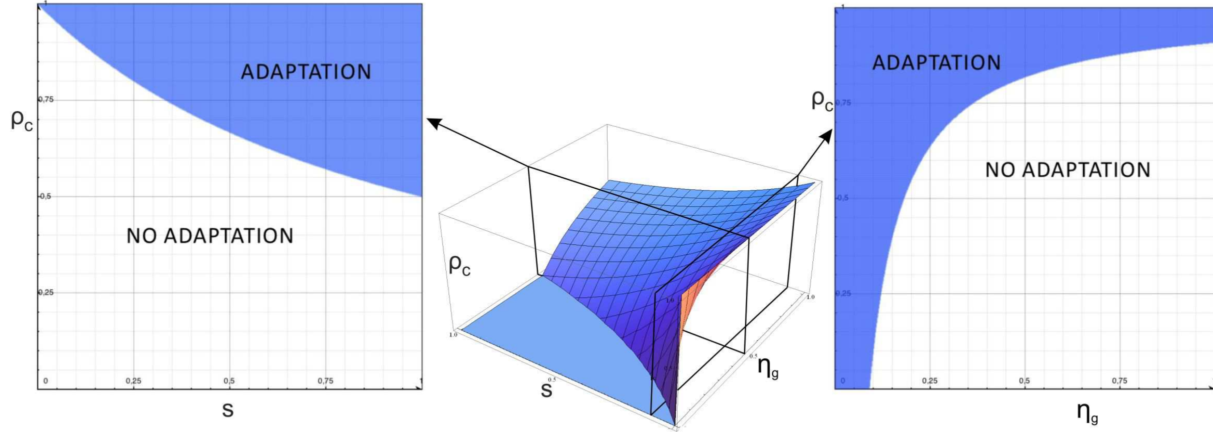

Figure 1: Center: three dimensional representation of the critical robustness ρ_c, blue surface, for different combinations of s and η_g; Left: critical robustness (transition line between white and blue areas) under different selection coefficients, with fixed $\eta_g = 0.5$. The blue area represents the parameter space where adaptation can occur, while the white one where adaptation cannot occur. Right: critical robustness (transition line between white and blue areas) under different genotypic mutation probability (0 to 1) with fixed $s_p = 0.1$.

3.0.3 A more accurate model of ϕ_{pp}

A more accurate modeling of ϕ_{pp} could be the following:

$$\phi_{pp} = \sum_{k=0}^{G} \nu(k)\zeta(k) \tag{14}$$

where $\nu(k)$ is the proportion of neutral genotypic (i.e. with the same phenotype p) variants for each set of mutants with k mutations, and $\zeta(k)$ is the probability to have exactly k mutations ($\zeta(k)$), with $0 < k < G$ where G is the genome size. The sum $\sum_{k \neq 0} \zeta(k) = \eta_g$. η_g is the previously defined probability to have a mutated genome over a generation or replication. $\nu(1)$ can be thought to be a phenotypic robustness proxy, and is the definition of robustness adopted by Wagner (Wagner, 2011). We can then generalize ϕ_{pp} as follow:

Since

$$\phi_{pp} = \sum_{k=0}^{G} \nu(k)\zeta(k) = \vec{\nu} \cdot \vec{\zeta} \tag{15}$$

We can write ϕ_{pp} in vector notation:

$$\phi_{pp} = |\vec{\nu}||\vec{\zeta}| \cos\theta \tag{16}$$

where θ is the angle between the mutation probability vector and the robustness vector. Recalling the condition for adaptation to occur, $\phi_{pp} w_p > 1$, we can apply the new definition of ϕ_{pp} to write it as a function of ν:

$$|\vec{\nu}||\vec{\zeta}| \cos\theta > \frac{1}{w_p} \tag{17}$$

Making explicit the selection coefficient we have:

$$|\vec{\nu}| > \frac{1}{|\vec{\zeta}| \cos\theta (1 + s_p)} \tag{18}$$

This last inequality holds for any model $\nu(k)$ and $\zeta(k)$.

More interesting are those cases in which we can assign a function to both $\nu(k)$ and $\zeta(k)$. Following Wagner (2011), we can assign a function to $\nu(k)$. In addition we can assign two explicit functions to $\zeta(k)$, the Binomial distribution (1) and the Poisson distribution (2). We can use the second, computationally more tractable, distribution because when $G \to \infty$, the Binomial distribution can be approximated to an Poisson distribution. We will treat both in this paragraph.

1) Binomial distribution of mutations.

In this case we have:

$\nu(k) = \nu(1)^k$; $\zeta(k) = \binom{G}{k}\mu_b^k(1-\mu_b)^{(G-k)}$

where $\nu(1)$ is the proportion of neutral variants at a distance of one point mutations $(k = 1)$ and μ_b is the mutation rate per bp per replication or generation. This model assumes that each genotype with phenotype p has $\nu(1)$ neutral neighbors (genotypes with the same phenotype p) at a distance of one point mutation. We can thus rewrite ϕ_{pp} as:

$$\phi_{pp} = \sum_{k=0}^{G} \frac{G!\mu_b^k(1-\mu_b)^{(G-k)}\nu(1)^k}{(G-k)!k!} \tag{19}$$

Since:

$\sum_{k=0}^{G} \frac{G!\mu_b^k(1-\mu_b)^{(G-k)}\nu(1)^k}{(G-k)!k!} = (\mu_b\nu(1) + 1 - \mu_b)^G$ (Newton binomial formula), we have:

$$(\mu_b\nu(1) + 1 - \mu_b)^G > \frac{1}{w_p} \tag{20}$$

$$\nu(1) > \frac{(1+s_p)^{1/G}\mu_b - (1+s_p)^{1/G} + 1}{(1+s_p)^{1/G}\mu_b} \tag{21}$$

We managed to write the entire inequality in therms of $\nu(1)$ which is the proportion of neutral variants at one point mutation distance (local robustness). In this case we can highlight the fact that the minimum amount of robustness required for adaptation depends not only on the selection coefficient and the mutation rate, but also on the genome size.

2) Poisson distribution of mutations:

In this case we have:

$\nu(k) = \nu(1)^k; \eta(k) = \frac{\mu_G^k e^{-\mu_G}}{k!}$

where μ_G is the mean mutation rate per genome per generation, which correspond to $G\mu_b$ in the binomial model.

Thus

$\phi_{pp} = \sum_{k=0}^{G} \frac{\mu_G^k e^{-\mu_G} \nu(1)^k}{k!}$

Since:

$\sum_{k=0}^{G} \frac{\mu_G^k e^{-\mu_G} \nu(1)^k}{k!} = \frac{e^{\mu_G(\nu(1)-1)} \Gamma(1+G,\, \mu_G \nu(1))}{G!}$

and

$\lim_{G \to \infty} \frac{\Gamma(1+G,\, \mu_G \nu(1))}{G!} = 1$ (see Appendix)

We have:

$$e^{\mu_G(\nu(1)-1)} > \frac{1}{w_p} \tag{22}$$

$$\nu(1) > \frac{\mu_G - \ln(1 + s_p)}{\mu_G} \tag{23}$$

In this case the minimum amount of robustness required for adaptation does not depends from the genome size.

4 Price equation analysis

Untill now we dealt with the minimum level of robustness required for adaptation to occur in the sense of increasing the frequency of a particular (favourable) p phenotype. A different approach could be to track the change in the mean population phenotype. If a positive covariance between fitness and the phenotypic value of a certain character exist, we can define adaptation as the increase of the mean of that particular character value over a definite time interval. This approach is very useful if we have to deal with quantitative traits and continuous character values differently from what happens with the quasi-species model where we have discrete values.

4.0.1 Phenotypic robustness is a necessary condition for adaptation to occur II

We explored the role of the phenotypic stability ϕ_{pp} and phenotypic robustness ρ considering the following form of the Price's equation (Okasha, 2006):

$$\Delta\overline{z} = Cov(w_i, z_i') + E[\Delta z_i] \tag{24}$$

where $\Delta\overline{z}$ is the change in the population mean character value, z_i' is the mean of the offspring's character values, w_i the relative fitness, and $E[\Delta z_i]$ is the average of the difference between parents and offspring character values, the so called "transmission bias".

We can study how robustness affects each term of the Price equation considering that phenotypic stability ϕ_{pp} affect the mean character value of offsprings z_i'

z_i' can be written as:

$$z_i' = \phi_{pp} z_i + (1 - \phi_{pp}) \frac{\sum z_j}{W_i(1 - \phi_{pp})} \tag{25}$$

$$z_i' = \frac{W_i \phi_{pp} z_i + \sum z_j}{W_i} \tag{26}$$

where $\sum z_j$ is the sum of the character values of the $W_i(1 - \phi_{pp})$ offspring with mutant phenotypes and W_i is the absolute fitness.

We show that there is a minimum amount of phenotypic robustness under which $\Delta\overline{z}$ cannot be greater than zero for any particular character even if a positive covariance between fitness and that character value exist ($Cov(w_i, z_i) > 0$):

Substituting [26] into [24] leads to:

$$\Delta\overline{z} = Cov(w_i, \frac{W_i \phi_{pp} z_i + \sum z_j}{W_i}) + E[\frac{W_i \phi_{pp} z_i + \sum z_j}{W_i} - z_i] \tag{27}$$

Assuming that phenotypic stability ϕ_{pp} is approximately constant across the studying population:

$$\Delta\overline{z} = \phi_{pp}[Cov(w_i, z_i) + E[z_i]] - E[z_i] + E[\frac{\sum z_j}{W_i}] + Cov(w_i, \frac{\sum z_j}{W_i}) \tag{28}$$

For any particular character z we can ask when $\Delta\overline{z} > 0$. From [22] this happens when:

$$\phi_{pp} > \frac{E[z_i] - E[\frac{\sum z_j}{W_i}] - Cov(w_i, \frac{\sum z_j}{W_i})}{E[z_i] + Cov(w_i, z_i)} \tag{29}$$

Given that $\phi_{pp} = (1 - \eta_g) + \rho\eta_g$ we can rewrite the above inequality as:

$$\rho > \frac{\eta_g(E[z_i] + Cov(w_i, z_i)) - Cov(w_i, z_i) - E[\frac{\sum z_j}{W_i}] - Cov(w_i, \frac{\sum z_j}{W_i})}{\eta_g(E[z_i] + Cov(w_i, z_i))} \tag{30}$$

Inequality [30] represents the minimum level of phenotypic robustness for adaptation to occur. To simplify the discussion we can also assume that $Cov(w_i, \frac{\sum z_j}{w_i}) = 0$ and obtain:

$$\rho > \frac{\eta_g(E[z_i] + Cov(w_i, z_i)) - (E[\frac{\sum z_j}{W_i}] + Cov(w_i, z_i))}{\eta_g(E[z_i] + Cov(w_i, z_i))} \tag{31}$$

Considering also that $E[\frac{\sum z_j}{W_i}] -> 0$ if phenotypic mutations are rare, we have:

$$\rho > \frac{\eta_g(E[z_i] + Cov(w_i, z_i)) - (Cov(w_i, z_i))}{\eta_g(E[z_i] + Cov(w_i, z_i))} \tag{32}$$

We can divide both numerator and denominator by $E[z_i]$, yielding:

$$\rho > \frac{\eta_g(1 + Cov(w_i, z_{i_r})) - (Cov(w_i, z_{i_r}))}{\eta_g(1 + Cov(w_i, z_{i_r}))} \tag{33}$$

Where z_{i_r} is the value of the ith parent phenotype relative to the parent population mean phenotype (This equals to set the population mean phenotypic value to 1). Furthermore given that $Cov(w_i, z_{i_r})$ can be interpreted as a the selection differential S, we can rewrite the above inequality as:

$$\rho > \frac{\eta_g(1+S) - S}{\eta_g(1+S)} = \rho_c \tag{34}$$

This inequality is analogous to [6], at least formally.

5 Comparing the two model results

S is the covariance between the relative fitness w_i and the relative character values z_{i_r}. In both models some levels of phenotypic robustness is required for adaptation to occur even in the case of sizable selection coefficient (s_p) or differential (S) and in absence of any drift effect.

In particular the minimum levels of phenotypic robustness required are:

1) $\rho_c = \frac{(1+s_p)\eta_g - s_p}{(1+s_p)\eta_g}$ (according to the quasi-species model)

2) $\rho_c = \frac{(1+S)\eta_g - S}{(1+S)\eta_g}$ (according to the Price equation)

The only difference between the these two main results is the selection coefficient s_p versus the selection differential S, both expressing the selection strength. This difference arises from the fact that

the quasi-species model focuses on the change in the frequency of an advantageous phenotype p (depending on its selection coefficient s_p), while the Price equation focuses on the change of the population mean phenotypic value (depending on the selection differential S). The relation between the selection coefficient and the selection differential that can be expressed as follow:

$$S = Cov(1 + s_i, z_i) \tag{35}$$

which equals to

$$S = Cov(s_i, z_i) \tag{36}$$

Despite differences, the two analyses on different evolutionary models yield the same qualitative result, namely that in order for adaptation to occur, a minimum level of phenotypic robustness is required.

6 Discussion

Previous studies marked the role of phenotypic robustness in enhancing evolutionary adaptation through the effect of cryptic genetic variation (Hayden et al., 2011; Rigato and Fusco, 2016), in particular on long term effects on evolvability or innovability (Wagner, 2008). However, in a short-term context, phenotypic robustness is thought to oppose the process of adaptation through its buffering effects on positive mutations. Here we showed that, counterintuitively, not only phenotypic robustness can boost the adaptation process but also that it could be a necessary condition for adaptation to occur and to be maintained during evolution. This appears as a threshold effect, i.e. as a minimum level of phenotypic robustness under which evolutionary adaptation cannot occur, even in the case of sizably selection coefficients and in absence of any drift effect. The phenotypic mutational threshold we observed is analogous to the mutational threshold of the quasi-species model (Eigen et al., 1989; Wilke et al., 2001). The only difference is that here we consider genotypes and phenotypes in two distinct levels rather than only the genotype (sequence) level. In addition we showed that this threshold emerged also from the analysis of

the Price equation in an analogous form. The fact that different independent evolutionary models gave the same analytical results confers consistency to our claim, however one may ask if these findings can be applied to the majority of real biological cases. To answer this question, the key point to highlight is that the minimum amount of robustness required is very high (or at least greater than zero) in the case of small selection coefficients (or differentials) and relative high mutation rates. We argue that this is the case of most living systems. First, even if selection coefficients can vary widely between taxa, populations, times, and many other factors, it is widely accepted or assumed in most evolutionary models that actually, biological selection coefficients tend to be very small (Orr, 2005). For example, experimental measurements of s usually span between 10^{-4}-10^{-1} (Tamuri et al., 2012; Nielsen and Yang, 2003; Mathieson and McVean, 2013). A selection coefficient of 0.1 can be considered to be a high value, namely a strong selection acting on that population. This is the widely accepted darwinian idea that adaptation proceed through small evolutionary steps. Second, the universal pleiotropy view have heavy implications on the organisms's genotype mutation rate. According to this model every mutation in the genome can potentially affect the phenotype of every trait and eventually the organism's fitness ($Omnigenetic$ model)(Boyle et al., 2017). This means that the whole genome mutation rate should be considered as potentially affecting each trait. Accordingly we should consider the entire individual genome as a single allele for each trait. This might seem a non-orthodox view, however it is in accordance with many empirical findings also deriving from GWAS studies (Boyle et al., 2017).Thus, differently from the mutation rate of a single gene, the genome mutation rate is inherently high, usually, at least for eucaryotes and viruses, in the order of many mutations per generation per genome (Drake et al., 1998). In this scenario, a minimum amount of robustness ρ_c, is likely to be always required to sustain adaptation. To give an example, using representative real data on the genome mutation rates (μ_G) (Ridley, 2000), and a great selection coefficient of $s = 0.1$, we can calculate ρ_c for different life forms using equation [11]. The result is that ρ_c values are typically high, $\rho_c = 0.85$ for a virus ($G = 10^4; \mu_G = 1$), $\rho_c = 0.90$ for an eucaryote ($G = 3.6 \times 10^8; \mu_G = 4$), but is negative, $\rho_c = -90$, for a typical bacterium ($G = 2 \times 10^6; \mu_G = 10^{-3}$),

meaning that no robustness is required in this case. However if we consider the higher mutation rate (from three to ten-fold the basal) that bacteria experience during a stressful condition (and thus adaptation) (Foster, 2007), higher and positive levels of phenotypic robustness are required as well. Also, for lower and more common selection coefficients ($s < 0.1$), the ρ_c values are increasingly higher, tending to 1.0 in all cases. For example for a bacterium in a stressful condition with a ten-fold mutation rate ($\mu_G = 10^{-2}$), and a common selection coefficient of $s = 10^{-3}$, the minimum level of robustness required is $\rho_c = 0.89$. We can conclude that phenotypic robustness is a necessary and favorable condition for adaptation to occur, potentially contributing to explain its origin and the reason why we observe these very high levels of robustness and redundancy in living systems.

7 Appendix

Here we show that:

$$\lim_{G\to\infty} \frac{\Gamma(1+G,\, \mu_G \nu(1))}{G!} = 1$$

We start from the definition of the Γ function; Renaming $\mu_G \nu(1) = x$, we have:

$$\frac{\Gamma(1+G,x)}{G!} = \frac{\int_x^\infty e^{-t} t^G \,\mathrm{d}t}{G!}$$

For the integral properties we can write:

$$\frac{\int_x^\infty e^{-t} t^G \,\mathrm{d}t}{G!} = \frac{\int_o^\infty e^{-t} t^G \,\mathrm{d}t - \int_o^x e^{-t} t^G \,\mathrm{d}t}{G!}$$

Using the definition of complete Γ function:

$$\frac{\int_o^\infty e^{-t}t^G\,\mathrm{d}t - \int_o^x e^{-t}t^G\,\mathrm{d}t}{G!} = \frac{\Gamma(1+G)}{G!} + \frac{\int_o^x e^{-t}t^G\,\mathrm{d}t}{G!}$$

and since $\Gamma(1+G) = G!$, we have:

$$\frac{\Gamma(1+G)}{G!} + \frac{\int_o^x e^{-t}t^G\,\mathrm{d}t}{G!} = 1 - \frac{\int_o^x e^{-t}t^G\,\mathrm{d}t}{G!}$$

Now we have to show that

$$\lim_{G\to\infty} 1 - \frac{\int_o^x e^{-t}t^G\,\mathrm{d}t}{G!} = 1$$

Considering that $\forall x$:

$$0 \leq \int_o^x e^{-t}t^G\,\mathrm{d}t \leq \int_o^x t^G\,\mathrm{d}t = \frac{x^{G+1}}{G+1}$$

also the following condition holds $\forall x$:

$$1 - \frac{x^{G+1}}{(G+1)!} \leq 1 - \frac{\int_o^x e^{-t}t^G\,\mathrm{d}t}{G!} \leq 1$$

Given that

$$\lim_{G\to\infty} 1 - \frac{x^{G+1}}{(G+1)!} = 1$$

necessarily imply that

$$\lim_{G\to\infty} 1 - \frac{\int_o^x e^{-t}t^G\,\mathrm{d}t}{G!} = 1$$

$\forall x$ and then $\forall\ \mu_G$ and $\nu(1)$, and thus:

$$\lim_{G\to\infty} \frac{\Gamma(1+G,\ \mu_G\nu(1))}{G!} = 1$$

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0.5 Chapter III

0.5.1 Introduction to Article III

In Article II we showed the effects of phenotypic robustness on evolutionary dynamics, in particular that there is a critical level of phenotypic robustness required for adaptation to occur. This was shown through the analysis of deterministic models, thus our results hold under the assumption of a non-stochastic scenario. However, evolutionary processes are a combination of deterministic and stochastic mechanisms and virtually every aspect of evolution is somehow affected by randomness. Accounting for stochasticity in evolutionary models have been shown to be crucial for a better and more comprehensive understanding of evolutionary dynamics (Rice, 2008). This is the case for example of the genetic drift model (Ohta, 1992), in which is shown that the effect of random sampling can produce non-adaptive directional evolutionary changes. As a first attempt, we tried to consider stochastic evolutionary models in our analysis, in particular we focused on the diffusion approximation model (Ohta, 1992), which is the most powerful method devised for combining different deterministic and stochastic mechanisms (Rice, 2004). However, we realized that even this model lack of sufficient generality for two main reasons. First, the diffusion approximation model is a closed system with non-changing parameters. This means that stochasticity on parameters is non taken into account. Second, it has very narrow boundary condition limits: in fact to be mathematically tractable the model assumes that three main evolutionary forces, migration, selection and mutation are of similar magnitude and sufficiently weak that allele frequencies are not likely to change by more than the amount of 1/2N per generation (Ohta, 1992; Rice, 2004). Since our boundary conditions are more extended, virtually to all possible parameter values,

we decided to study the evolutionary consequences of stochasticity through evolutionary computer simulations. Computer simulations have become a useful tool for the mathematical modeling of many natural systems including biological, and more specifically, evolutionary systems. A simulationis the imitationof the operation of a real-world process or system overtime. The act of simulating something first requires that a model be developed; this model represents the key characteristics, behaviors andfunctionsof the selected physical or abstract system or process. The model represents the system itself, whereas the simulation represents the operation of the system over time. We first developed a model representing the stochastic and the deterministic processes occurring each generation, thus it can be seen as a stochastic-deterministic dynamical system. Model rules and functions define the relationships between elements of the modeled system. The main elements of the model are internal variables and parameters. An internal variable is a changing variable during time according to the internal model rules or to external inputs, while a parameter is defined as a non-changing value of the model. When parameters can change during time they actually become variables. In this case the dynamical system is defined as "non-sufficient", meaning that is no more mathematically tractable during time or at best only instantaneously (this is indeed why we need computer simulations). At the beginning of each simulation, initialization parameters and variables must be specified, corresponding to the definition of the initial conditions. At each time point, the dynamical system can be defined by the so called state variables. A state variable is a value describing a particular aspect of the system during time and is usually calculated with specific rules from parameters and internal variables. Finally, the observed behaviors are emergent properties of the dynamical system.

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0.5.2 Article III

Article in preparation, to be submitted to an evolutionary biology journal (e.g., *JEB, BMC Evol. Biol, Proc. R. Soc. B*)

A heuristic model on the role of robustness in adaptive evolution

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Abstract

A previous study (Rigato and Fusco, in prep.) showed that including phenotypic robustness in standard population genetic models can give us a simple explanation for the widespread resistance to genetic and environmental perturbations, namely that, counterintuitively, a high level of phenotypic robustness is not only a favourable but also a necessary condition for adaptation to occur. However, these results hold under the assumptions of fixed parameters and large, nearly infinite, population sizes. Here, we built and analyzed a simple heuristic individual-based computational model to test for the effects of stochasticity on these deterministic results. We examined how phenotypic robustness affects adaptive evolution under different population sizes, mutation rates, and different selection regimes, and then we examined how phenotypic robustness evolve under common scenarios in constant and variable environments. Overall, our simulations confirm the consistency of the deterministic model predictions, however we highlight that a deeper analysis of the population size effects and environmental interactions should be carried on to assess their role on the origin and evolution of phenotypic robustness.

1 Introduction

Complex living systems are inherently robust to internal and external changes. In particular, mutational phenotypic robustness is widespread across organisms at various level of organization, from molecules to individuals (Wagner, 2011). Phenotypic robustness is a key feature deriving from the genotype-phenotype map structure, namely from the fact that many genotypes map on the same phenotype, leading to a reduction of the probability that a genetic mutation produces a novel phenotype. Adaptive evolution requires heritable phenotypic variation for selection to act upon, and the standing paradigm that emerged from Modern Synthesis argued that random genetic mutations of fixed phenotypic effects are one of the most important source of heritable phenotypic variation fuelling adaptive evolution (Huxley, 1942;

Kutschera and Niklas, 2004; Pigliucci et al., 2010). Thus, phenotypic robustness may seem to act as a buffer on mutations and consequently on phenotypic variation and adaptive evolution (Draghi et al., 2010). So far, phenotypic robustness has received little attention in standard population genetic models, and only recently long-term effects of robustness on evolvability and innovability have started to be studied more in depth. However, except for some few works, the mechanisms by which robustness might be established during evolution are far from clear and overall little explored (Wagner, 2011). In a previous study, we showed that including phenotypic robustness in standard population genetics and evolutionary models (the Quasi-species model and the Price equation, Price et al. (1970); Eigen et al. (1989)) can explain the widespread phenotype resistance to genetic and environmental perturbations. Specifically, and counterintuitively, that a certain (sizable) level of phenotypic robustness is not only a favourable but also a necessary condition (although not sufficient) for adaptation to occur. This appears as a threshold effect, i.e. as a critical level of phenotypic robustness under which evolutionary adaptation cannot occur, even in the case of significant positive selection coefficients and in absence of any drift effect. These theoretical results were obtained under the assumptions of fixed evolutionary parameters (e.g., mutation rates) and large, virtually infinite, population sizes. In other words, the models we used to obtain the analytical results are deterministic, and this can potentially affect the generality of previous results. However, evolution is certainly not a completely deterministic process and stochasticity has been shown to play a crucial role in evolutionary dynamics, leading to the formulation of many important stochastic models like the genetic drift model (Kimura et al., 1968) or the probabilistic version of the Price's equation (Rice, 2008). Here we tested for the effects of stochasticity on the predictions of our deterministic derivations, to assess their generality and soundness with respect to assumption violation. We built and put to work a simple heuristic individual-based computational model, comparing adaptive evolution in populations of different sizes and fixed or changing parameters (variables) such as the mutation rate, phenotypic robustness and the selection coefficient. We examined how phenotypic robustness affects adaptive evolution under different population sizes, mutation rates, and different selection regimes, and

then we examine how phenotypic robustness evolve under common scenarios in constant and variable environments.

2 The Model

This model description follows the Overview, Design concepts and Details protocol for describing individual-and agent-based models (Grimm et al., 2005, 2006; Gilbert, 2008). The model is implemented in NETLOGO v. 5.0.3, (Tisue and Wilensky, 2004) and is designed to simulate the effects of different levels of phenotypic robustness ρ, $(0 < \rho < 1)$ on adaptive dynamics. As proposed by Rigato and Fusco (in prep.) we adopted a narrow, quantitative definition of phenotypic robustness, that is the probability that, across one replication, mutation of a given genotype g takes to a genotype g' that exhibits the same phenotype of g, in other words, the probability that a mutation has no phenotypic effect. The model consists of a population of entities (or individuals), each assigned with initial fitness and robustness values. Each entity produces offspring according to its fitness value. Each single offspring can exhibit a mutated genotype with probability μ, and, conditional on the mutated genotype, a mutated phenotype with probability $(1 - \rho)$. Magnitude of the phenotypic mutation can be fixed or modeled with a random variable, depending on the simulation. There are no fitness costs directly associated to phenotypic robustness.

2.1 Purpose

The main purpose of the model is to explore the consequences of phenotypic robustness in adaptive evolution, testing the effects of stochasticity with respect to the deterministic predictions of Rigato and Fusco (in prep.). This is done by simulating finite population persistence and phenotypic evolution under constant or changing environmental conditions (according to the simulation).

2.2 Entities, state variables and timing

Entities of the model are asexual individuals. Each individual i has a genotype (not modeled) and a phenotype that includes its absolute fitness W_i, and phenotypic robustness ρ_i. Different genotypes can

map on the same phenotype. Depending on the simulation, state variables are: population average relative fitness ($\overline{w}$), relative fitness variance (σ_w^2) and average robustness ($\overline{\rho}$). Relative fitness is computed with respect to a hypothetical maximum fitness value attainable in a given environment (W_{opt}), thus, $w_i = W_i/W_{opt}$. The model is time-discrete, one time step corresponding to one generation, and generations do not overlap.

2.3 Process overview

See a schematic diagram in Figure 1. Each cycle (generation) starts with a parent population of N individuals. Parents reproduce according to their fitness and die. Offspring initially inherit their parent's genotype and phenotype, but immediately the genotype can mutate with probability μ, and this can have a phenotypic effect with probability $(1-\rho)$. If the resulting offspring population is larger than N, this is reduced to size N through random elimination of the exceeding entities. These are the parents of the succeeding generation. In simulation with changing environment, fitness values are updated before parents reproduce.

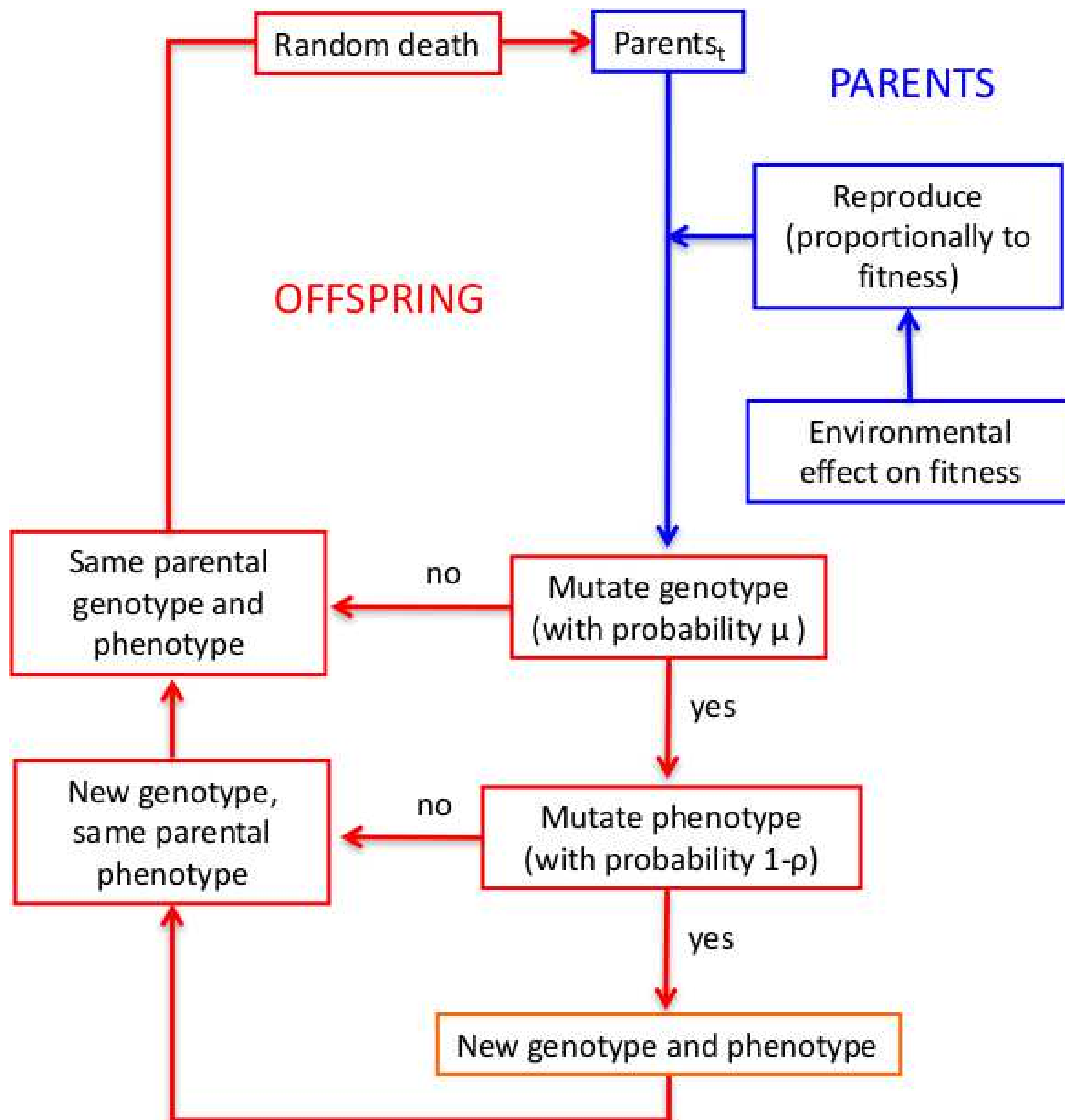

Figure 1: Schematic diagram of the process overview (see text).

2.4 Design concepts

Evolution (changes in population mean and variance values of phenotypes) and other population dynamics (e.g., stability, bottlenecks and extinction) emerge from the combined effects of heredity (mutation

and phenotypic robustness), natural selection (differential fecundity fitness of individuals) and demographic (population size) processes. Stochasticity has effects on i) genotype and phenotype mutation (with their magnitude), ii) survival and reproduction and iii) environmental changes.

2.5 Initialization

Initialized parameters and their initial values depend on the simulation (see below).

2.6 Input

The model does not have any external input; parameters are updated according to internal model rules.

2.7 Sub-model ”environmental-change”.

Environmental change is simulated as a periodic indiscriminate negative effect on entity's fitness. The magnitude of the negative effect is described as a fixed negative factor (X). To simulate the negative environmental effect, the negative factor (X), is added to the individual absolute fitness values (W_i) before reproduction. Changes happens with a period T, in generation time unit ($T_{min} = 1\ generation$).

2.8 Sub-model ”reproduction”.

In each generation, each individual i produces W_i offspring and die.

2.9 Sub-model ”mutation”.

The genotype can mutate with probability μ , and if a genetic mutation occurs, the phenotype can mutate with probability $(1 - \rho)$. Depending on the simulation, this sub-model can be activated for fitness only or phenotypic robustness as well. In the latter case they operate independently. If the phenotype mutates, the new phenotype value is updated according to the following rules:

For fitness values:

1) in simulations with fixed selection coefficients, s, the new mutated fitness is set to $W_{updated} = \overline{W}(1+s)$. s can be positive with probability $p = 0.17$ (Allen Orr, 2000), and negative with probability $(1 - p)$.

2) in simulations with variable selection coefficients, s, the new mutated fitness value is set to a value ranging from 0 to the maximum attainable absolute fitness W_{max}, with equal probability. Accordingly, fitness mutant gains decrease as the population approach to the optimum, as implied by the Fisher's geometric model.

For robustness values:

1) the new mutated robustness value is set to a value ranging from 0 to 1 with equal probability.

2.10 Sub-model "maximum population size"

In each generation, if offspring population is larger than N, the population is reduced to size N through random elimination of the exceeding entities. The surviving entities are going to be the parents of the succeeding generation.

3 Simulations

The deterministic model proposed by Rigato and Fusco (in prep.) predicted a minimum level of phenotypic robustness for adaptation to occur, i.e. for the mean population fitness to increase. This minimum level depends on the magnitude of the selection coefficient and on the genotypic mutation probability according to the following equation:

$\rho_c = ((1+s)\mu - s)/((1+s)\mu)$

where ρ_c is the critical amount of robustness required, μ the genotype mutation probability, and s the selection coefficient of a given mutant phenotype. To test the consistency of this prediction we run four simulations. Simulations went on for 100-500 generations. Each simulation included several runs, each characterized by different initialization parameters (equal for all individuals or not), and a number of replicas for each run.

3.1 Simulation 1

It tested the existence of a ρ_c, by fixing for all runs the initialization parameters of the selection coefficient and the genotype mutation probability. Different runs were initialized with different fixed parameters such as the population size N and phenotypic robustness ρ. Different combinations of N and ρ constitute the separate runs, each replicated three times. Individual fitness is the only changing internal variable, and the average population relative fitness is the only state variable. This simulation allows to verify the existence of a maximum robustness level under which the mean population fitness (state variable) does not increase significantly during time (generations).

3.2 Simulation 2

It tested how stochasticity can affect the structure of the relation between ρ_c, genotypic mutation probability and the selection coefficient s, predicted by Rigato and Fusco (in prep.), in populations of nearly constant finite size N. We initialized all runs with the same fixed population size of 500, but different fixed values of s, and ρ, each run in three replicas. As a result of the simulation, for each combination of s and , an observed ρ_c was selected as the minimum level of ρ under which adaptation did not occur with that particular combination of s and .

3.3 Simulation 3

This simulation allowed to test the existence of a critical robustness level under which the mean population relative fitness does not increase during time in a fixed environment, with constant population size, mutation rate but variable selection coefficients among individuals, in a more realistic scenario that considers a population adapting through phenotypic mutations of declining magnitude effect in approaching the optimum and adapting with different (not fixed) selection coefficients. Simulation 3 tested the existence of a ρ_c, by fixing for all runs the initialization parameter of genotype mutation probability. Among runs, different fixed initialization parameters are population size N and phenotypic robustness ρ. Different combinations of N and ρ constitute the separate runs, each replicated ten times. Individual fitness

is the only changing internal variable while the distribution of the selection coefficients is an emergent property of the system, namely a new state variable like the average population relative fitness. The latter derives from the random assignment of fitness to newly mutated phenotypes (see sub-model ”mutation”).

3.4 Simulation 4

To assess the evolvability of phenotypic robustness under different conditions, we simulated the fixed initialization conditions of Simulation 3, but allowing phenotypic robustness to evolve during adaptation. This means that, like fitness, phenotypic robustness is a changing internal variable. Population average phenotypic robustness is the new state variable as the population average relative fitness. We simulated a constant and a variable environment (see ”environment” sub-model). Evolution of phenotypic robustness were compared between different initialization fixed parameter values of N and under constant and variable environments.

4 Results

4.1 Simulation 1

- Fixed parameters (equal among runs): $s = 0.1$; $\mu = 1.0$; $W_{opt} = 30$
- Fixed parameters (different among runs): $N = (20, 50, 100, 250, 500, 1000)$;
  $\rho = (0.7, 0.75, 0.80, 0.85, 0.90, 0.95, 0.99)$
- Internal variables: W_i (initial value=10 for all individuals)
- State variables: $\overline{w}$
- Generations: 500
- Runs: 42
- Replicas: 3 per run

If the relative population mean fitness significantly increases after an arbitrary time, adaptation is said to have occurred. Note that as the environment is fixed, the population mean fitness cannot exceeds the maximum attainable relative fitness, $w_{max} = 1$. As illustrated in Figure 2, with a large population size of $N = 1000$, adaptation occurred after 500 generations only in populations with $\rho > 0.85$. In fact, the mean fitness gain is larger than zero in populations with $\rho > 0.9$ (Student's t test, $p < 0.001$), while is significantly negative for population with $\rho < 0.85$ ($\rho < 0.001$). Thus the robustness threshold is located between 0.85 and 0.9. This value is very close to the deterministic model expected value of 0.9. In population of intermediate size ($N = 500, 250$) the same pattern is observed, as populations with ρ between to 0.9 and 0.99 have a positive increase in relative fitness after 500 generations ($p < 0.001$), otherwise the population mean fitness declines ($p < 0.001$). In populations with N between 1000 and 250, populations with $\rho = 0.99$ were always outperformed by population with $0.90 < \rho < 0.95$. It seems that the effect of robustness above the critical level is that to enhance the adaptation rate till another critical point under which the boosting effect disappear. This could be due to a trade-off effect between the need to have new phenotypic mutations, and the possibility for these mutations to spread through the population, possibility guaranteed by high levels of phenotypic robustness. With $\rho = 0.99$, probably there are less positive phenotypic mutations respect to the possibility for these mutations to adapt. At $N = 100$ the adaptation rate slowed down dramatically and only populations with $\rho > 0.95$ could adapt. However, the fitness decrease appear to be inversely proportional to the level of robustness. At $N = 100$ populations with $\rho = 0.99$ started to outperformed those with $0.90 < \rho < 0.95$. This is probably due to the fact that the efficacy of selection was weakened by the effect of random drift. This effect become more evident for populations of sizes $N = 50$ and $N = 20$. In these cases no significant adaptation was observed for all populations. However the rate of fitness loss was inversely proportional to the amount of robustness. In particular it seems that populations with very high robustness levels ($\rho = 0.99$) did not decrease substantially in their population mean fitness values. Population with a size of $N = 50$ and $N = 20$ got extinguished at some point time in some replicas (not visible in the graphs),

however a clear buffering effect against the fitness decrease appeared in populations with $\rho = 0.99$. This buffering effect seems to be very strong, possibly allowing the population to traverse long periods in small population size without significant fitness decreases due the effect of genetic drift. Overall we noted that, as expected, phenotypic robustness boosted the adaptation rate in populations with $\rho > \rho_c$, or buffered the population mean fitness decrease in populations with $\rho < \rho_c$.

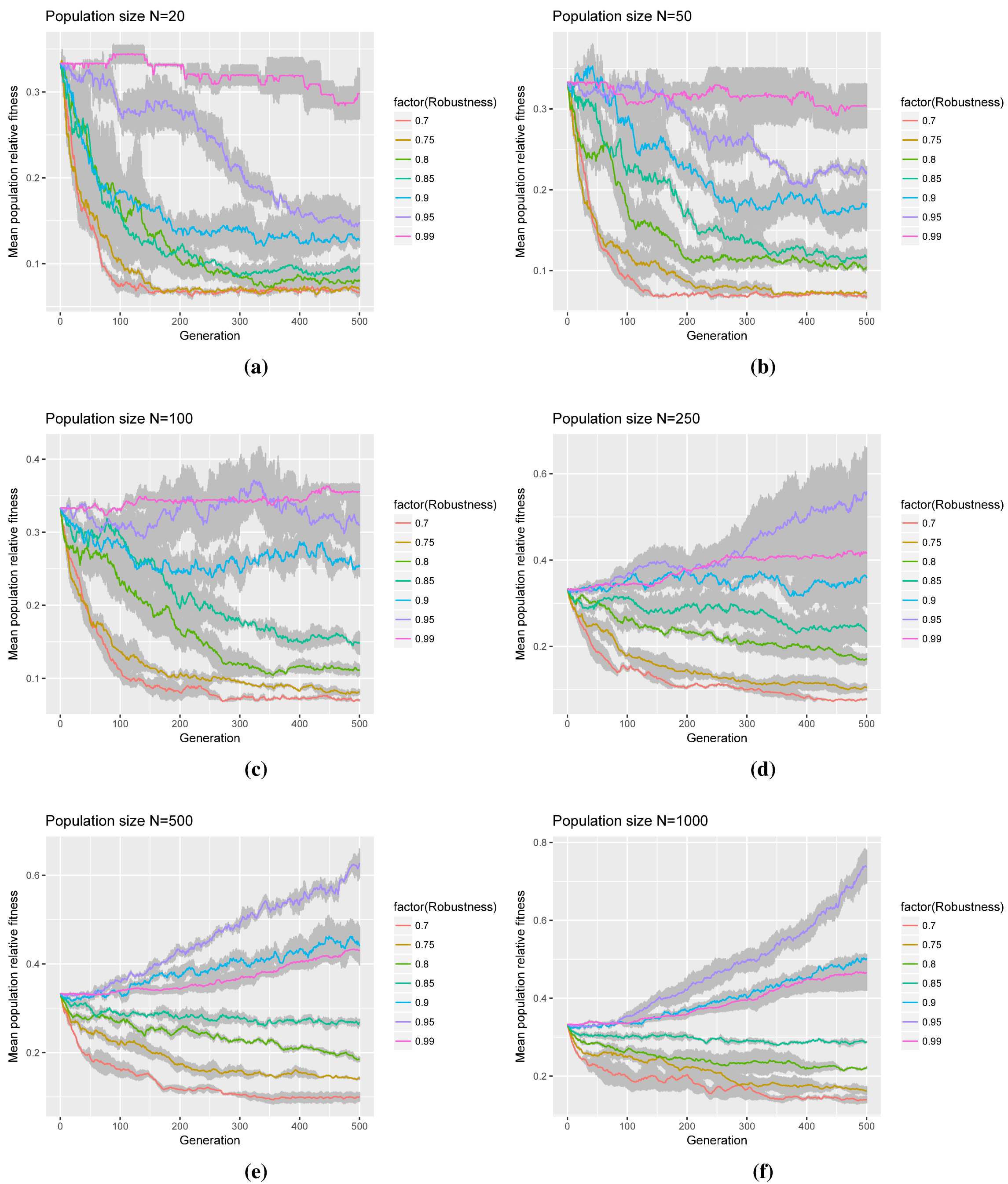

Figure 2: Adaptation dynamics of populations with different levels (different coloured lines) of phenotypic robustness (0.7, 0.75, 0.8, 0.85, 0.9, 0.95, 0.99) and different population sizes (20, 50, 100, 250, 500, 1000) during 500 generations. Single coloured lines are the means of three independent evolutionary histories. The grey zones are the standard error of the three replicas. Adaptation is said to have occurred if at the end of the 500 generations, populations achieved a mean fitness significantly greater than the starting relative fitness of 0.33.

4.2 Simulation 2

- Fixed parameters (equal among runs): $N = 500$; $W_{opt} = 30$
- Fixed parameters (different among runs): $\mu = (0.0010, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 0.99)$;
  $\rho = (0.0010, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 0.99)$
  $s = (0.0010, 0.0050, 0.01, 0.02, 0.04, 0.1)$
- Internal variables: W_i (initial value=10 for all individuals)
- State variables: $\overline{w}$
- Generations: 500
- Runs: 726
- Replicas: 3 per run

As shown in Figure 3, the ρ_c values obtained through simulation are very close to the corresponding points on the surface of the expected ρ_c values in the parameter space defined by and s. This means that even in the case of a finite, and relatively small population size, the minimum values of phenotypic robustness required for adaptation to occur is comparable to those predicted by the deterministic model through the explored parameter space.

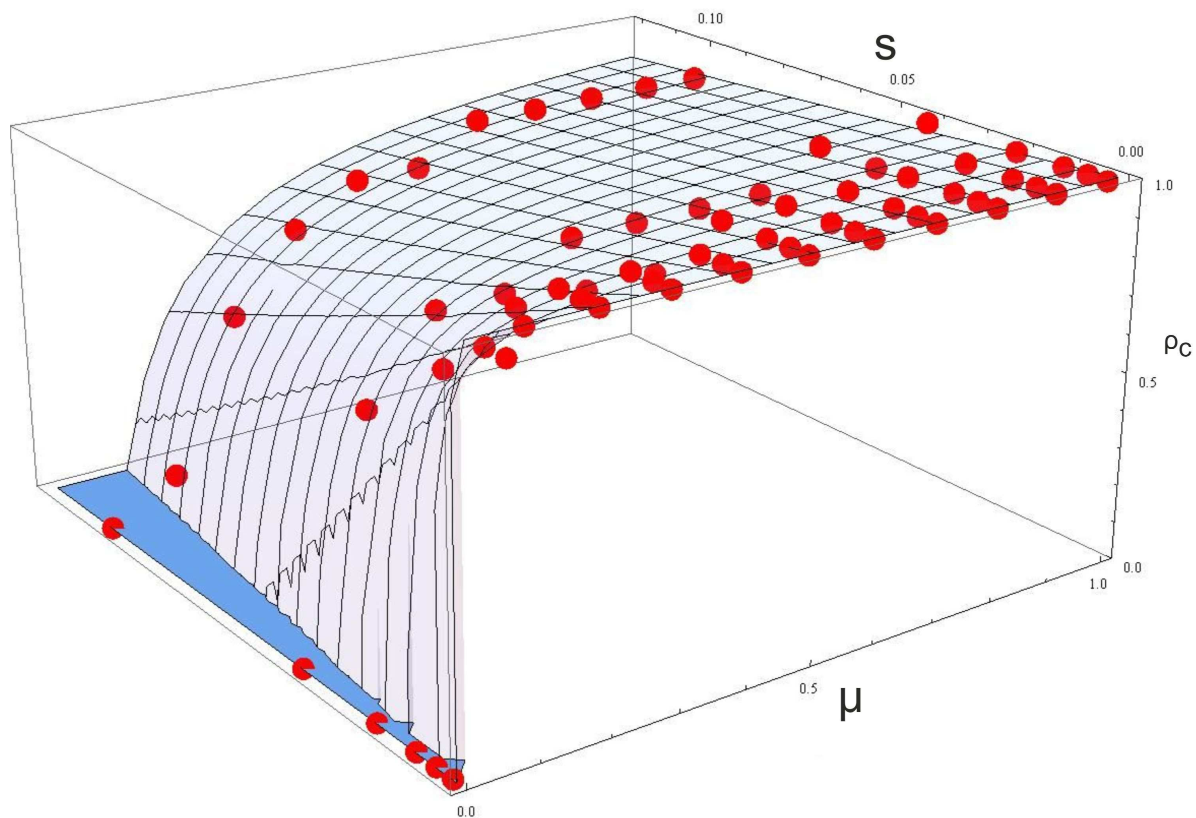

Figure 3: Surface (light-blue) of the expected values of ρ_c (Rigato and Fusco, in prep.), and observed values of ρ_c (red dots) from simulation 2. μ is the mutation probability, s is the selection coefficient.

4.3 Simulation 3

- Fixed parameters (equal among runs): $\mu = 1.0$; $W_{opt} = 30$
- Fixed parameters (different among runs): $N = (20, 50, 100, 250, 500, 1000)$;

  $\rho = (0.7, 0.75, 0.80, 0.85, 0.90, 0.95, 0.99)$
- Internal variables: W_i (initial value=20 for all individuals)
- State variables: $\overline{w}$

- Generations: 100
- Runs: 42
- Replicas: 10 per run

With a large population size of 1000, adaptation occurred after 500 generations only in populations with a phenotypic robustness value greater than 0.85. The mean increase in fitness is different from zero and positive in populations with robustness values of 0.9, 0.95 and 0.99, ($p < 0.001$), while is significantly negative for population with $\rho < 0.90$ ($p < 0.001$). Thus, the robustness threshold is located between 0.85 and 0.90, which is again very close to the deterministic model expected value of 0.9 in the case of fixed s. In populations of intermediate size ($N = 500, 250, 100$) the same pattern was observed, as populations with ρ equals to 0.9 or 0.95 have a positive increase in relative fitness after 500 generations ($p < 0.001$), while the population mean fitness of the others declined ($p < 0.001$). At variance with what was observed in Simulation 1, populations with a size of $N = 50$ still adapt within 500 generations with ρ levels of 0.95 and 0.99, while at $N = 20$ a very small but significant increase in fitness was observed with $\rho = 0.99$ only. In addition a buffering effect of high levels of robustness is more evident in small populations with variable selection coefficients than with fixed coefficients. It seems that if the selection coefficients are not fixed, the positive effect of robustness on adaptation appears to be less sensitive to the stochastic effect of a finite population size. In addition, we noted that, as expected, phenotypic robustness boosted the adaptation rate in populations with $\rho > \rho_c$, or buffered the population mean fitness decrease in populations with $\rho < \rho_c$. Populations with phenotypic robustness of 0.99 always increase significantly more ($p < 0.001$) than populations with the minimal amount of robustness required for adaptation to occurs, $\rho = 0.9$ and 0.95. This happened also for simulations with intermediate/small population sizes. However, as can be noted graphically in Figure 3, in the very short term (< 100 generation) populations with $\rho = 0.9$ or 0.95 sometimes outperformed those with $\rho = 0.99$.

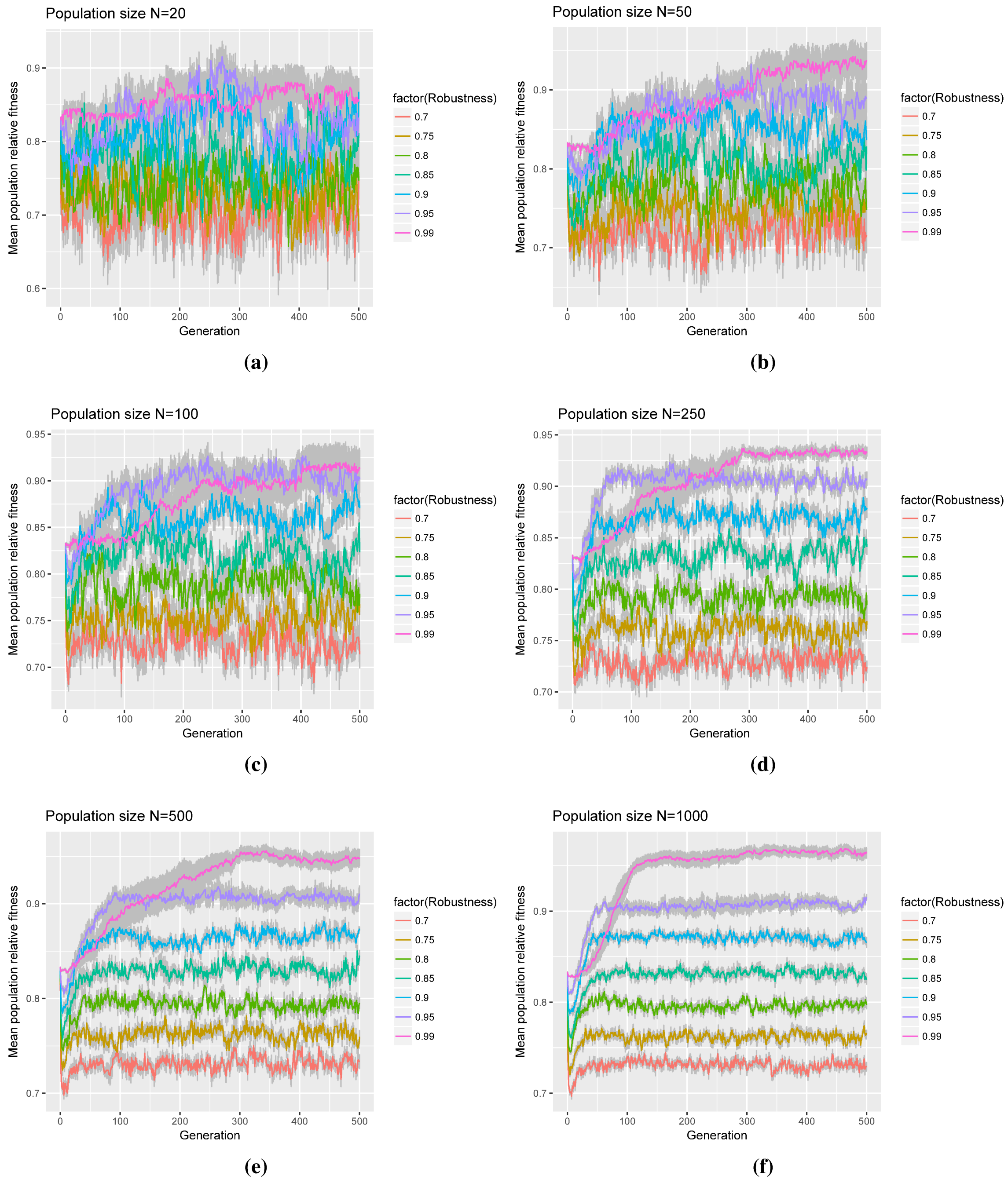

Figure 4: Adaptation dynamics of populations with different levels (coloured lines) of phenotypic robustness (0.7, 0.75, 0.8, 0.85, 0.9, 0.95, 0.99) and different population sizes (20, 50, 100, 250, 500, 1000) during 500 generations (simulation 3). Single coloured lines are the means of ten independent evolutionary histories. The grey zones are the standard error of the three replicas. Adaptation is said to have occurred if, at the end of the 500 generations, populations achieved a mean fitness significantly greater than the starting relative fitness of 0.83.

4.4 Simulation 4

- Fixed parameters (equal among runs): $\mu = 1.0$; $W_{opt} = 30$
- Fixed parameters (different among runs): $N = (1000); X = (0, 1, 2, 3); T = (1)$
- Internal variables: W_i (initial value=10 for all individuals); ρ (initial value=0.3 for all individuals)
- State variables: $\overline{w}$
- Generations: 100
- Runs: 4
- Replicas: 100 per run

We performed the simulation in different variable oscillating environment, with the same period but different effect magnitude on fitness (X); specifically, the simulated environments were fixed (X=0), moderately variable (X=1), intermediate variable (X=2), highly variable (X=3) (see Supplementary Appendix 1 for details). The period for each environmental variation is 1 generation. In other words, in each generation an environmental effect of size X occurred on the absolute fitness W_i. In all populations we observed a significant increase in the average population mean fitness from the starting relative fitness value of 0.3 as in the mean robustness (Fig. 4). According to an intuitive expectation, we observed a higher speed in the increase of phenotypic robustness in less variable environments than in the more variable ones. The effect appears to be proportional as showed in Figure 4. We observed also a higher final mean level of phenotypic robustness in populations adapting in more stable environments. In other words the minimum amount of robustness required for adaptation to occur is lower in case of a very unstable, oscillating, difficult-to-adapt-to environments, while can be very high when populations are reaching the fitness optimum in a more stable or constant environment. In addition, as a general result, we observed that phenotypic robustness increased during adaptation and the increase in phenotypic robustness preceded the increase in the relative population mean fitness value (Fig. 5). The reason why

phenotypic robustness precede adaptation is because it is a necessary condition for adaptation to occur. This is also evident from simulation 3, since populations with lower levels of robustness cannot adapt more than populations with higher robustness levels.

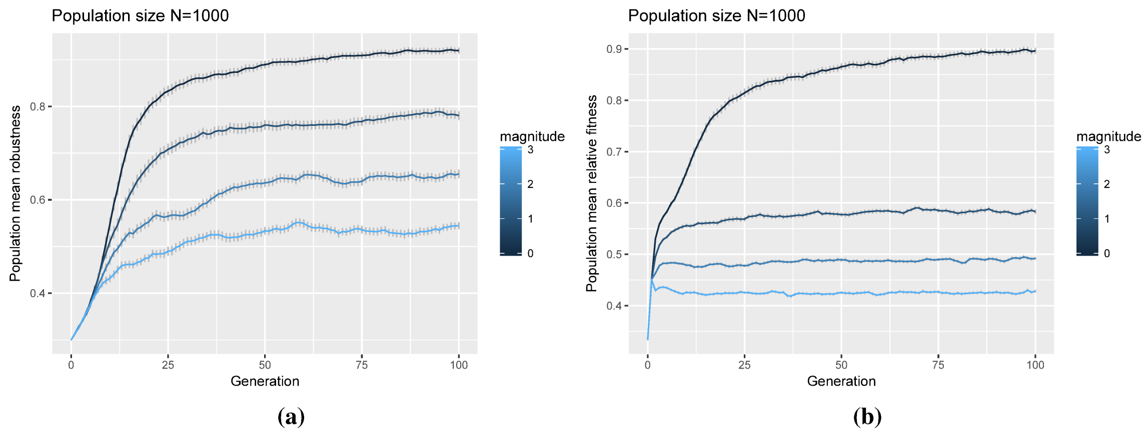

Figure 5: Adaptation dynamics of four populations with different environmental effect magnitudes (different coloured blue lines, X=0, 1, 2, 3) on robustness (left) and fitness (right), during 100 generations. Single coloured lines are the means of 100 independent evolutionary histories.Bars are the standard errors of the 100 replicas.

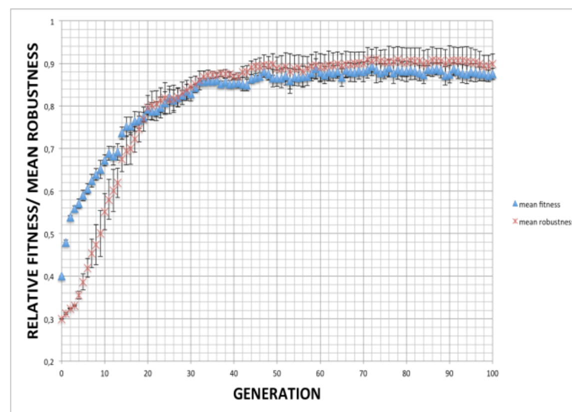

Figure 6: Adaptation dynamic of fitness (blue triangles) and robustness (red crosses), of a population of $N = 1000$ in a fixed environment during 100 generations.

5 Discussion

As a general result, here we showed that a minimum level of phenotypic robustness required for adaptation to occur is exists also in case of stochastic effects due to the finite dimension of populations. In

addition, the critical levels predicted from the deterministic model, nearly perfectly match that ones from the stochastic scenarios in a finite population of larger size. As the population size decreases, the robustness threshold effects tend to disappear, and also extinction events become more frequent. Also, the theoretical relationship between ρ_c, μ and s appears to be robust to the stochastic effects in population of not too small size. We didn't explore in depth the case where the selection efficiency is outperformed by the drift effect. This case seems to appear in simulation 1 and 3 when we experimented a selection coefficient of 0.1 with a constant population size of 50, or even more with $N = 20$. In this case, it is reasonable to assume that even if the level of robustness is sufficient for adaptation to occur, the role of drift outperformed the selection efficiency itself because $s < 1/Ne$ (Ohta, 1992). On the contrary when $s > 1/Ne$, selection efficiency is affected by the level of phenotypic robustness. Even if $s > 1/Ne$, low levels of phenotypic robustness cannot allow the adaptation process to occur. Related to this finding, in simulation 1 and 3, we observed also a boosting effect of phenotypic robustness on adaptation for levels above ρ_c, or a buffering effect on fitness decline for values under ρ_c or above ρ_c but with very small population sizes ($N = 20$ and 50). It is important to note that we designed the simulation intentionally without considering possible difference in the probability of different neutral genotypes to find new phenotypes. In fact, other studies showed that phenotypic robustness could lead to the accumulation of cryptic genetic variation eventually boosting the adaptation process through this mechanism (Hayden et al., 2011; Rigato and Fusco, 2016). Note that the boosting effect of phenotypic robustness we observed does not derive from the above-mentioned mechanism, but actually derives from the fact that a positive mutant phenotype needs a minimum level of phenotypic robustness to spread through the population. Higher levels of phenotypic robustness allow more and more small positive mutations to persist and spread through the population. In other words, higher phenotypic robustness levels allow a wider range of small sized positive mutant phenotypes to increase their frequency in population. Given a fixed phenotypic robustness, positive phenotypic mutants under a critical selection coefficient (s_c), cannot persist. This is clear if we allow entities to have different selection coefficients as performed in Simulation 3. In this case, as the

size of positive selection coefficients decreases when approaching the optimum, populations with lower levels of phenotypic robustness not only adapt slower, but they also reach a sub-optimal mean population fitness. This is because approaching the fitness optimum, the low levels of phenotypic robustness do not allow positive phenotypes with shrinking selection coefficient sizes to spread through the populations. Finally, we showed that phenotypic robustness, if allowed to be evolvable itself, can increase during adaptation, in particular its increase precedes the population mean fitness increase. This is because as the population approach to the fitness optimum, phenotypic robustness is a necessary condition to allow positive phenotypes with shrinking selection coefficient sizes to spread through the populations. Thus, approaching the fitness optimum, increasingly higher levels of phenotypic robustness are required for adaptation to occur. In an unchanging environment we expect phenotypic robustness to reach a value near to 1, however organisms live in an ever-changing world. Periodic changes contribute to lower the mean levels of phenotypic robustness in populations as shown in Simulation 4. These results suggest that the levels of phenotypic robustness we observed in real populations could be a resulting equilibrium between the effects of the ρ_c and the changing environment. Overall, our simulations confirm the consistency of the deterministic model predictions. However, a deeper analysis of the environmental interactions should be carried on to assess their precise role on the origin and evolution of phenotypic robustness.

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0.6 Chapter IV

0.6.1 Introduction to Article IV

Although both empirical and theoretical approaches to scientific research are essential to scientific progress, the interactions between the two practices seems to be problematic in the biological scientific community (Fusco, 2015). In an apology for "non-mathematical biology," E.O. Wilson (2013) went so far as to asses that "The annals of theoretical biology are clogged with mathematical models that either can be safely ignored or, when tested, fail." Not subscribing to Wilson's point of view and in an attempt to put a bridge between our theoretical and empirical studies in evolutionary biology, we tried to formulate some risky theoretical predictions to test on real biological data. The problem in the genomic era is the difficulty to extract full value from the large amounts of data becoming available. It seems easier to keep doing what we are doing on a larger and larger scale than to try and think critically and ask deeper questions. Given the already available huge amount of genomic data for a vast array of taxa representing a broad spectrum of the biological diversity, to test our predictions, we adopted a comparative approach based on phylogeny (Garland Jr et al., 1993; Blomberg and Garland, 2002; Blomberg et al., 2003). The Phylogenetic comparative methods (PCMs) use information on the species historical relationships (phylogenies) to test evolutionary hypotheses on adaptation. The comparative method has deep roots in evolutionary biology; For example, in "The Origin of Species" Charles Darwin used differences and similarities between lineages as a major evidence of the process of descent with modification. In fact, closely related lineages share many traits and trait combinations meaning that they are not independent. Thus, the development of explicitly phylogenetic comparative methods

are needed (Felsenstein, 1988).These statistical methods were primarily developed to control for phylogenetic history when testing for adaptation (Pagel and Lutzoni, 2002). Although most studies that employ PCMs focus on extant organisms, many methods can also be applied to extinct taxa and can incorporate information from thefossil record (Fusco et al., 2012). Phylogenetic comparative approaches can complement other ways of studying adaptation, such as studying natural populations, experimental studies, and mathematical models as used in this particular case. Making interspecific comparisons allow to assess the generality of evolutionary phenomena by considering independent evolutionary events. Such an approach is particularly useful when there is little or no variation within species. Felsenstein (Felsenstein (1988))proposed the first general statistical method in 1985 for incorporating phylogenetic information, i.e., the first that could use any arbitrary topology(branching order) and a specified set of branch lengths. The logic of the method is to use phylogenetic information (and an assumed Brownian motionlike model of trait evolution) to transform the original tip data (mean values for a set of species) into values that are statistically independent and identically distributed. Successively other methods were developed such as the Phlylogenetic Generalized least-squaresmodel (PGLS), used in this work, which is now probably the most commonly used PCM (Grafen, 1989).This approach is used to test whether there is a relationship between two (or more) variables while accounting for the fact that lineage are not independent. The method includes the generalized least squares (GLS or OLS) as a special case, and as such the PGLS estimator is also unbiased, consistent, efficient, and asymptotically normal. The PGLS consider the V matrix of expected variance and covariance of the residuals given an evolutionary model and a phylogenetic tree. Therefore, it is the structure of residuals and not the variables themselves that show phylogenetic signal. A number of models have been proposed for the structure of V such as Brownian

motion (Felsenstein, 1988) Ornstein-Uhlenbeck, (Hansen, 1997) and Pagel's lambda model. (Pagel and Lutzoni, 2002)(see article IV for detailed descriptions).When a Brownian motion model is used, PGLS is identical to the independent contrasts estimator (Grafen, 1989). It is important to mark that also the GLS is a specific PGLS assuming a Brownian motion and a star phylogeny. In this case the phylogenetic signal is absent and the PGLS is a normal GLS (OLS). In PGLS, the parameters of the evolutionary model are typically co-estimated with the regression parameters. PGLS can only be applied to questions where the dependent variable is continuously distributed. In the following article we used evolutionary models to fit our data and detect the phylogenetic signal. Successively we adopted the PGLS method to verify our theoretical predictions on the relationships between couples of continuous variables on a genomic dataset.

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0.6.2 Article IV

Article in preparation, to be submitted to an evolutionary biology journal (e.g., *JEB, BMC Evol. Biol, Proc. R. Soc. B*)

Phylogenetic comparative analysis of the effects of phenotypic robustness on genome evolution

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Abstract

Previous theoretical results predicted a positive and permissive role of phenotypic robustness on adaptive evolution, namely that a minimum level of phenotypic robustness is required for adaptation to occur, correlating with organismal complexity. Phenotypic robustness can be achieved by various mechanisms but is not very clear how phenotypic robustness is expressed among organisms. Since genome evolution is one of the major topic under debate in the evolutionary biology research program, here we proposed two possible genomic candidate mechanisms of robustness, that are also genomic features: the amount of non-coding neutral DNA, and the alternative splicing level. We tested if the genomic proportion of neutral DNA and the alternative splicing level are positively correlated with the amount of organismal complexity defined as the proteome size. We adopted a phylogenetic comparative approach, based on the phylogenetic relationship of 210 eukaryotic taxa and showed that according to the theoretical expectations, the two genomic robustness proxies can explain part of complexity evolution.

1 Introduction

Previous theoretical studies predicted a positive and permissive role of phenotypic robustness in adaptive evolution, specifically that a minimum level of phenotypic robustness is required for adaptation to occur (Rigato and Fusco, in prep). This minimum level increases with the genome mutation probability, and decreases with the magnitude of the selection coefficients. Genome mutation probability is positively correlated to genome size, whereas the magnitude of the selection coefficients is expected to decrease with organismal complexity. In fact, complexity is generally associated to adaptation processes with small selection coefficients, because complex organisms are supposed to pay a so called ”cost of complexity” (Allen Orr, 2000). Thus, theoretically, we should expect that higher levels of phenotypic

robustness are required for adaptation to occur in more complex organisms due to the expected smaller positive selection coefficients. Here we tried to test these predictions using available genomic data. Phenotypic robustness can be achieved by various mechanisms and an exact understanding of the phenomenon in biological organisms is still lacking (de Visser et al., 2003; Kitano, 2004; Masel and Siegal, 2009; Siegal and Leu, 2014). We focused on two possible candidate mechanisms that affect two very debated genomic features: the amount of neutral DNA (as the proportion of non-coding and non-selected DNA), and the splicing index (expressed as the proteins/genes ratio). To test for the goodness of the two candidate mechanisms, according to the theoretical expectations, we tested if organismal complexity, defined as the proteome size (Schad et al., 2011), can be explained by the amount of neutral DNA and the splicing level. Specifically, the three main predictions are:

i) The splicing index should be higher in organisms with larger genomes. In fact, it has been proposed that alternative splicing was a very important step towards higher efficiency in eukaryote evolution, because information can be stored much more parsimoniously. This is also known as the ”economic hypothesis” (Black, 2003). Several proteins can be encoded by a single gene, rather than requiring a separate gene for each, thus allowing a more rich proteome from a genome of limited size. Independently from complexity, if the genome sizes are constrained, we should expect a higher splicing index in organisms with larger genomes. Note that larger genomes do not automatically imply higher complexity levels, because of the neutral DNA and the alternative splicing phenomenon itself. In other words, we should expect a higher selective pressure for higher level of alternative splicing in organisms with larger genomes because of the constrains in the genome size.

ii) Alternative splicing should explain part of organismal complexity. Independent from the mutational model assumed, we should expect this since a reduction of the proportion of the selected genome sites should decrease the probability to have a phenotypic mutation. In other words, maintaining the

same genome and proteome sizes, higher splicing levels should increase the proportion of neutral sites and thus the probability that a mutation gives the same phenotype.

iii) The proportion of neutral DNA should explain part of organismal complexity. The hypothesis that the neutral DNA could act as a mutational buffer at the phenotypic level is a very old and still debated d hypothesis (Yunis and Yasmineh, 1971; Hsu, 1975; Patrushev and Minkevich, 2008; Gregory, 2005). In this scenario, the neutral DNA reduces the probability that a genetic mutation leads to a phenotypic mutation. Thus, neutral DNA should contribute to the total robustness level, representing a good proxy that is expected to explain, at least in part, the organismal complexity (proteome size).

To test these three predictions, we adopted a phylogenetic comparative approach based on the genomic data of 210 eukaryotic taxa, spanning the main unicellular and pluricellular lineages of the group.

2 Materials and Methods

2.1 Source dataset

Genomic data were mined from the genomic database Genome (NCBI, https://www.ncbi.nlm.nih.gov/genome/). In particular, we mined all genomes sequenced at the level of complete genome, and those for which an estimation of the gene number and the protein number was provided, either derived from manual or automatic annotations. In case of more than one genome per taxon, the best annotated genome was chosen manually. Raw data of interest were: Taxon name, Genome size (bp), Gene number estimation, Proteome size (protein number estimation). We selected 210 eukaryotic taxa for which with a complete genome and full genome data were available, spanning from unicellular eukaryotes, to pluricellular plants, animals and fungi.

2.2 Neutral DNA estimation

Since no general annotations of the non-conserved (neutral) portion of the genomes are available, and in consideration of the major debate on the so called garbage DNA in recent years (Graur et al., 2015), we derived a rough figure of the proportion of the neutral sites in each genome on the basis of the estimated gene number. Considering that the average protein size for eukaryotes is 300-400 AA (Brocchieri and Karlin, 2005), this means that the mean size of the totality of the exons of a given gene is roughly 900-1200 bp (each AA corresponding to a single codon). Thus, considering the degeneracy of the third codon position, we arrived at 1000 bp as the mean number of potentially selected sites per eukaryotic gene. Multiplying the estimated gene number of a given taxon times 1000 bp, we obtained the estimated number of the sites potentially under selection in its genome. Subtracting this number from the total genome size and dividing by the genome size, we obtained an estimate of the proportion of potentially neutral sites. This gross calculation has obviously several limitations, however, since regression analyses span several orders of magnitude, we expect that errors in these estimates do not significantly affect the results.

2.3 Splicing index definition and estimation

For each taxon, we defined a splicing index simply as the protein-number/gene-number ratio. This is a measure of how many polypeptides can be encoded on average by a single gene.

2.4 Proteome size

We defined the proteome size as the estimated number of different protein types annotated for each genome, derived both from different genes and from alternative splicing of the same gene. In most cases, the estimation was obtained by automatic bioinformatics methods.

2.5 Phylogenetic tree topology and dating

The phylogenetic tree representing the phylogenetic relationships among the 210 eukaryote taxa, was obtained by using the automatic tree builder of the taxonomic database Taxonomy (NCBI, https://www.ncbi.nlm.nih.gov/taxonomy/). The function *Taxonomy Common Tree* generates a tree topology for a selected group of organisms, but without node dating. For each node of the tree we assigned a dating in million years (Ma), provided by the Time Tree project (http://www.timetree.org/), using the *Evolutionary Timeline function* (Hedges et al., 2006). According to the nodes dating, the length of the tree branches was adjusted manually using Mesquite v. 5.0.3 tree handling functions (Maddison and Maddison, 2001).The resulted phylogeny is illustrated in Figure 1.

2.6 Phylogenetic signal

Fitting data with ordinary latest square model (OLS) tacitly assumes evolutionary independence among taxa. This equals to assume a star phylogeny. Since taxa are evolutionary related, assuming a star phylogeny can be more or less appropriate, according to the amount of phylogenetic signal exhibited by a given character. In the absence of any phylogenetic signal, the OLS regression is a more appropriate assessment of directional change in trait evolution. Phylogenetic signal is the measure of the tendency for related organisms to resemble each other, and this has implications for understanding how traits evolve, and how data are best analyzed in the context of a phylogeny (Blomberg and Garland, 2002; Revell et al., 2008). Phylogenetic signal was ascertained for all analyzed characters using the Lambda model. The model and the parameter were fit via maximum likelihood using the function *fitContinuous* in the R package geiger (Harmon et al., 2009). This function fits various likelihood models for continuous character evolution. The function returns parameter estimates, (approximate) confidence intervals based on the Hessian of the likelihood function, likelihoods and AICc values. We set the *model* argument to ”lambda”. Model ”lambda”, also known as Pagel’s lambda, multiplies all internal branches of the tree by lambda, leaving tip branches as their original length. As the parameter lambda approaches zero, the

model becomes speciational (star phylogeny, BMStar). For this reason, we used the model lambda with fixed parameter of $\lambda = 0$ to fit the BMStar model. AICc values for the two models were compared to assess the significance of the phylogenetic signal.

2.7 Evolutionary model fitting

In a phylogenetic context, regression analyses have to be adjusted on the basis of the evolutionary dynamics of characters. Hence, for each character, we fit five evolutionary models, corresponding to as many distinct evolutionary dynamics: i) Brownian motion (BM), ii) accelerating-decelerating (ACDC), iii) single stationary peak (OU), iv) constant trend (Trend), and v) white (BMStar).

i) Under BM, evolutionary changes are independent, non-directional, and occur at a constant instantaneous rate, β, throughout the phylogeny. Character evolution is modelled as a Brownian motion.

ii) The accelerating-decelerating (ACDC) model (Blomberg et al. (2003); also known as early-burst model (EB), Harmon et al. (2010)) is similar to BM, except that the Brownian rate parameter decreases or increases exponentially over time as $\beta(t) = \beta e^{rt}$, where t is time, β_0 is the rate at the root of the tree, and r modulates the change in the BM rate. When r is negative, traits evolve rapidly at first, but slowdown over time, as designed in some models of adaptive radiation (Harmon et al., 2010). Positive r values indicate accelerating evolutionary rates, as might occur after key evolutionary innovations or mass extinctions (Blomberg et al., 2003).

iii) The single stationary peak model is an Ornstein-Uhlenbeck (OU) process in which a trait value acts as an evolutionary attractor. In macro-evolutionary studies, this attractor has been interpreted as the phenotypic center of an adaptive zone (Felsenstein, 1988; Garland Jr et al., 1993; Hansen, 1997; Butler and King, 2004). In addition to a rate parameter, this model has a parameter α that measures the strength of attraction to the optimum. When attraction to the optimum is absent ($\alpha = 0$), this process reduces to BM. With increasing values of α, the influence of the optimum is more pervasive; at very high values, taxa are pulled so strongly to the optimum that the phylogenetic signal is erased (Felsenstein, 1988; Hansen,

1997).

iv) The constant trend (Trend) model considers BM with a uniform trend in character values over time resulting from a constant bias in the direction of evolutionary change (Pagel and Lutzoni, 2002), analogous to a phylogenetic regression that includes time since the root as a predictor of character values. In addition to the BM rate, this model also has a parameter, μ, indicating the directional bias in evolution, equivalent to the slope of trait values with respect to time.

v) Finally, ”white” (or BMStar) model can be thought of as an extreme version of an OU process in which the strength of attraction to the optimum is infinitely strong. The resulting trait will be independently distributed among species, equivalent to BM on an unstructured (star) phylogeny (BMStar). Because species values are independent, this model predicts that traits in sister taxa will be no more similar than in distant relatives, and thus bear no phylogenetic signal. For this reason, this model was used as a null model for comparison with the lambda model in phylogenetic signal analysis. Models were fit via maximum likelihood using the function *fitContinuous* in the R package geiger (Harmon et al., 2009) with the *model* argument set to ”BM,” ”EB,” ”OU,” ”trend,” and ”white” respectively. BM is the simplest of the candidate models, with only one additional parameter. Each of the remaining models has three parameters. Akaike information criterion scores (AIC_c) were used to balance log-likelihoods and model complexity, and for convenience these were converted to Akaike weights that represent the proportional support received by candidate models (Burnham et al., 2011).

2.8 OLS and PGLS analyses

Focal analyses of this work are the regression analyses among the selected characters to test the three predictions on the effects of phenotypic robustness on genome evolution. We performed phylogenetic generalized least squares (PGLS) and ordinary least squares (OLS) regressions of the three trait comparisons: i) Splicing index vs. Genome size, ii) Proteome size vs. Splicing index, and iii) Proteome size vs. Neutral DNA. In the absence of phylogenetic signal, the OLS regression is a more appropriate assess-

ment of directional change in trait evolution. Both PGLS and OLS regressions were computed using the Generalized least square function ("gls"), fit by REML in the R package ape (Paradis et al. 2004). In this case, we fit the PGLS with six different correlation structures expected under different evolutionary models. The six correlation structures are: CorMartins, CorPagel, CorBlomberg, CorGrafen, CorBrownian, CorPagel with fixed $\lambda = 0$. CorMartins is the correlation structure expected under the OU model (see above, Evolutionary model fitting), CorPagel under the lambda model, CorBlomberg under the ACDC model and CorGrafen is a particular correlation structure under a model that appropriately modify the tree branch lengths. CorBrownian is expected under the Brownian model and CorPagel with fixed =0, is expected under the speciational model (BMStar). For each PGLS model we obtained the Akaike information criterion scores (AIC_c) to find the best model with the best correlation structure. We used the best fitting PGLS models for comparison with the OLS models. Note that the PGLS under the BMstar (CorPagel (fixed $\lambda = 0$)) correlation structure, correspond to the OLS.

3 Results

Genomic traits vary considerably across the selected taxa, and can vary significantly even between strictly related taxa. Mean, range and standard deviation of the traits under study are listed in Table 1.

Table 1: Summary statistics for the mean, range and standard deviation of the analyzed trait values for 210 eukaryotic taxa.

Trait	N	Mean	Range	SD
Genome size (Mb)	210	6.46E+02	2.188-3598.000	9.85E+02
Gene number	210	1.98E+04	1883-97830	1.60E+04
Protein number	210	2.43E+04	1831-114100	2.15E+04
Splicing index	210	1.137	0.6021-2.1190	0.283
ncDNA proportion	210	0.763	0.09288-0.99660	0.214

3.1 Phylogenetic signal of traits

We tested all the trait values for the phylogenetic signal to assess the appropriateness of the OLS analysis. Results are summarized in Table 2. We allowed the lambda model to find the best parameter and compared the fitted lambda model with the null model of a star phylogeny (BMstar). We found high values of phylogenetic signals for all traits and they all resulted statistically significant over the null model with AICc weight values of 1.0 for all traits, and evidence ratio of nearly infinite for all traits (in favor of lambda). The best estimation for the trait Genome size was =1 (LogLik -1584.4), this mean a very high phylogenetic signal, corresponding to a full Brownian motion model. Also, the other traits obtained high values of and thus high phylogenetic signals ranging from 0.63 to 0.95 (see Table 2). However, the correlation is lower than that expected under a full Brownian motion model, entailing that a component of the evolutionary change can be explained by other factors rather than a simple Brownian motion. Overall, the strict dependency of the character values on phylogeny suggests that the OLS analysis might be not fully appropriate to test for correlations among these traits.

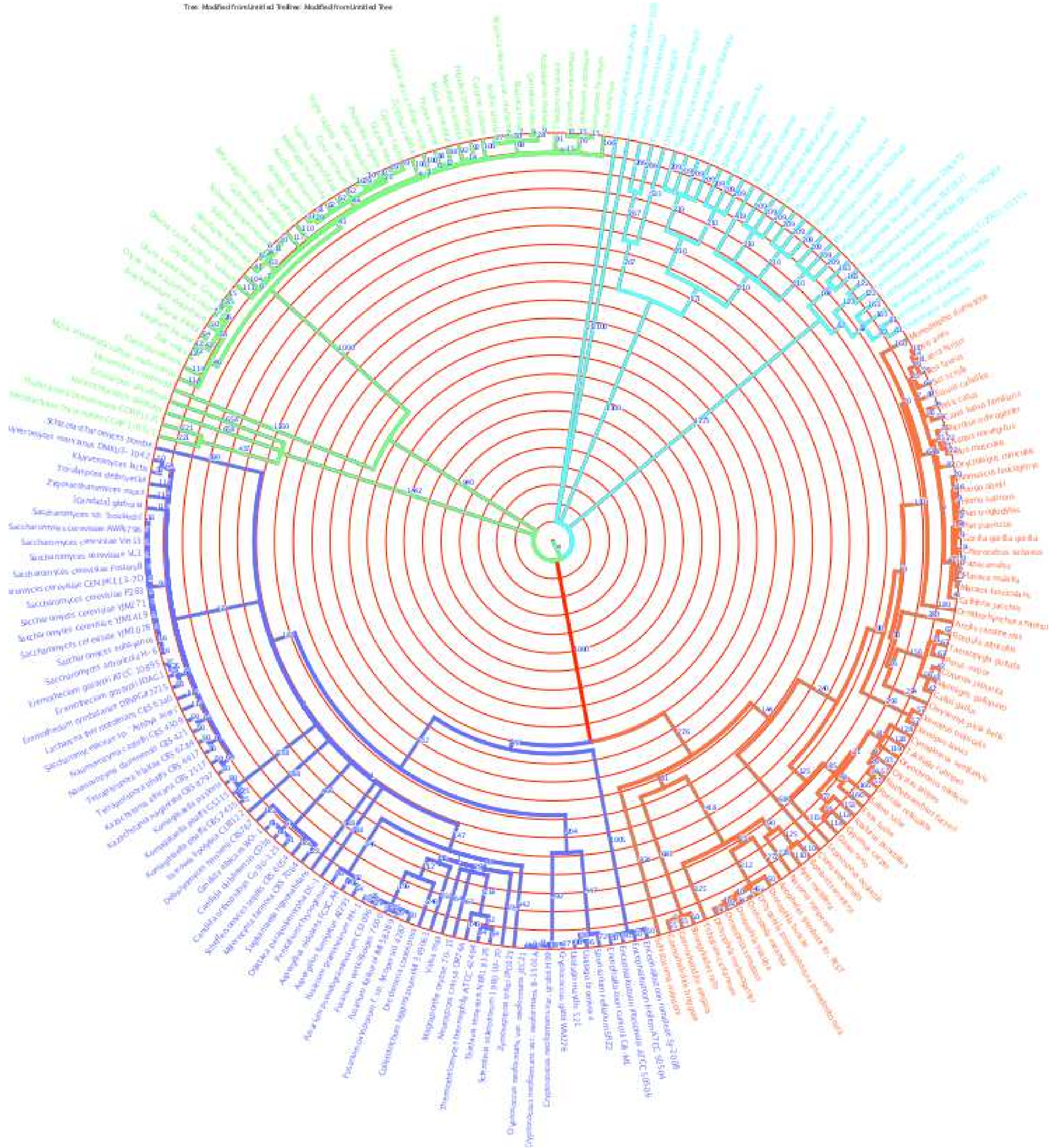


Figure 1: Phylogenetic tree of 210 eukaryotic taxa. In red the animals, in blue the fungi, in green the green algae and plants, other taxa are in turquoise. Branch lengths are in Ma (data from TimeTree, see materials and methods for details.

Table 2: Statistics of significant tests for phylogenetic signal in five traits as calculated with the *fitContinuous* function of the R package Geiger (Harmon et al. 2009). The phylogenetic tree is shown in Figure 2. Significant results for the AICc comparison between the Lambda (Lambda) model and a Brownian motion model under a star like phylogeny (BMStar) indicate the presence of phylogenetic signal for all analyzed traits. λ-statistics indicate the amount of phylogenetic signal relative to a star-like phylogeny expectation of 0.00 (Pagel et al. 2003).

Trait	lambda estimation	LogLik lambda	AICc lambda	AICc Star	Probaility lambda	Probability BMStar	Evidence Ratio
Genome size (Mb)	1	-1584.3682	3176.9315	3495.9995	1	0	∞
Gene number	0.642914	-2227.7304	4463.6559	4691.5717	1	0	∞
Protein number	0.64442	-2289.8959	4587.9870	4791.5717	1	0	∞
Splicing index	0.627412	40.7148	-73.2344	70.1664	1	2.66454E-15	3.753E+14
Neutral DNA proportion	0.947444	252.0901	-495.9850	-46.7461	1	0	∞

3.2 Evolutionary model fitting of traits

As phylogenetic signal analysis showed that trait evolution is not fully comparable to Brownian motion model, we fitted other evolutionary models to find the best fitting model that can explain the departure from a pure Brownian model. AICc values of the fitting models for each trait are provided in Table 3. The lowest AICc values are those of the OU model for all traits with an evidence ration of 1 in all cases (Table 4). The Genome size and the neutral DNA proportion have α estimates of 0.0028 and 0.0016 respectively. These values can be considered the results of a weak attraction to the optimum. Gene number, Protein number and the Splicing index have α estimates differing from the preceding for an order of magnitude. This means that for these traits, the attraction to the optimum, or the influence of selection is roughly ten times greater than that expected for the Genome size and the neutral DNA traits. These results are in agreement with the phylogenetic signal estimation, in particular showing that traits with high values (Genome size =1, Neutral DNA =0.94) have low α values and traits with lower values (Gene number =0.64, Protein number =0.64, Splicing index =0.63) have higher α values. Thus the evolution of these traits seems to be influenced by a weak evolutionary attractor, meaning that their evolution is not totally neutral, but they might be influenced by selection in some way. This supports the significance of a search for adaptive correlations between these traits.

Table 3: Model fitting results for the five analyzed trait values. Best models are those with lower AICc values. See text for parameter explanation. See Table 4 for comparisons of model fit statistics.

Trait	BMStar AICc	BM AICc	ACDC AICc	OU AICc	Trend AICc	Trend parameter(m)	ACDC parameter(r)	OU parameter (a)
Genome size (Mb)	3495.9995	3241.4431	3243.5232	3187.2927	3230.7197	99.99	0.0001	0.00276
Gene number	4691.5717	4705.9924	4708.0768	4566.7136	4693.0107	98.8	0.0003	0.00923
Protein number	4791.5717	4867.6925	4869.7774	4716.3670	4854.4265	99.99	0.0004	0.01131
Splicing index	70.1664	129.6058	131.6893	13.9356	117.0566	97.62	0.0010	0.01118
Neutral DNA proportion	-46.7461	-343.0086	-340.9304	-372.0943	-351.9285	99.31	0.0011	0.00165

Table 4: Comparing the fit among models presented in Table 3. The five columns are Akaike weights computed from AICc scores. Among evolutionary models the OU model is the only supported for al traits. An Akaike weight of 1.0, means an evidence ratio equal to ∞ .

Trait	BMStar Akaike wheights	BM Akaike wheights	ACDC Akaike wheights	OU Akaike wheights	Trend Akaike wheights
Genome size (Mb)	0	0	0	1	0
Gene number	0	0	0	1	0
Protein number	0	0	0	1	0
Splicing index	0	0	0	1	0
Neutral DNA proportion	0	0	0	1	0

3.3 OLS and PGLS analyses

We first tested the three predicted relationships by fitting each comparison with OLS (see OLS in Table 6). We found a statistically significant relationship between the Splicing index and the Genome size ($R^2 = 0.30$, $p < 0.0001$) (Table 6, Fig. 2a). Thus, Genome size account for 30% of the total Splicing index variance assuming a star phylogeny as the phylogenetic model. A statistically significant relation was also found for the other two comparisons, Proteome size vs. Splicing index and Proteome size vs. Neutral DNA, with an $R^2 = 0.41$ ($p < 0.0001$) and $R^2 = 0.49$ ($p < 0.0001$) respectively (Table 6, Fig. 2b,c). This means that both the amount of splicing and the proportion of neutral DNA explain a sizable percentage of the proteome size variance. Moreover, if we consider a linear combination of the two predictor factors together, these explain 50% of the total variance in proteome size ($R^2 = 0.56$, $p < 0.0001$). For each relationship, we fitted six different PGLS models differing for the correlation structure imposed. Model fitting results for the three trait comparisons are presented in Table 5. For the trait pair Splicing index and the Genome size, we found the CorPagel to be the best correlation structure with an evidence ratio of $6.85\ 10^{15}$ over the CorGrafen which is the second-best correlation structure. The same hold for the Proteome size vs. Splicing index trait with CorPagel as the best correlation struc-

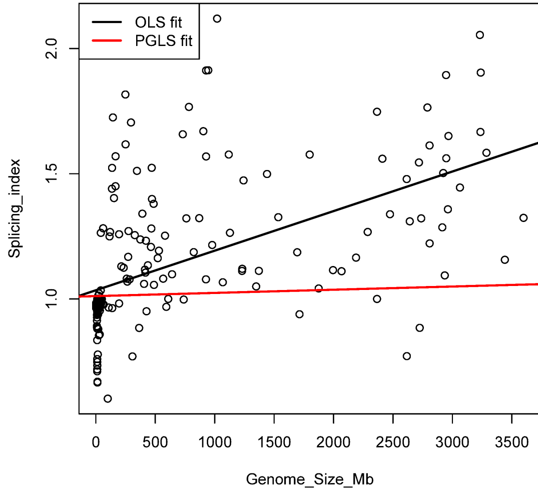
ture with an evidence ratio of 1.0 over the second best CorGrafen. On the opposite, an evidence ratio of 1.0 was obtained for the CorGrafen over the corPagel for the trait pair Proteome size vs. Neutral DNA. Overall, these results suggest that the relationships analyzed evolved somehow in between a pure Brownian model (BM) and a speciational model (BMStar). This means that it is necessary to take into account the phylogeny when studying relations between these trait pairs. For each relationship, the best fitted PGLS was chosen for the comparison with OLS (Table 6). After the PGLS analysis for the Splicing index and the Genome size relationship, the positive relation found with OLS disappeared (Fig. 2a). A simple AICc comparison gave the best support to the PGLS (CorPagel) model. This means that is very likely that the genome size is not a predictor of the splicing index level and that the relation we found in OLS was due to the effect of their common ancestry. On the contrary, for the other two relationships PGLS analysis resulted in a lower but still significant regression slope (Fig. 2b,c). In both cases the PGLS model with the relative best correlation structure (CorPagel, CorGrafen) have a higher significant support over the corresponding OLS (evidence ratios=1). These results suggest that the splicing index and the neutral DNA proportion are significant predictors of the proteome size and consequently of the organismal complexity level.

Table 5: Model fitting results for the three trait comparisons analyzed (PGLS) with six different correlation structures. The best model have the lowest AICc value and was chosen for comparison with the OLS analysis.Note that the PGLS with the BMStar correlation structure equals to an OLS. See Table 6 for result comparisons of OLS and PGLS analysis.

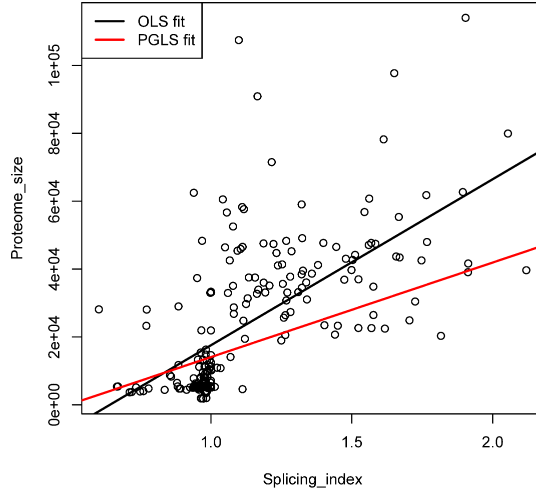
Trait	BMStar AICc	BM AICc	ACDC AICc	OU AICc	corGrafen AICc	corPagel AICc
Splicing index vs. Genome size	-5.99	129.87	139.84	-3.00	-72.93	-73.59
Proteome size vs. Splicing index	4679.91	4807.90	4818.17	4681.91	4548.30	4546.82
Proteome size vs. Neutral DNA	4648.06	4868.93	4879.30	4681.91	4579.83	4584.19

Table 6: Statistics for phylogenetic generalized least squares (PGLS) regression and ordinary least squares (OLS) of the three trait comparisons. The chosen PGLS is that with the best correlation structure (specified). The phylogenetic tree is shown in Figure 1.

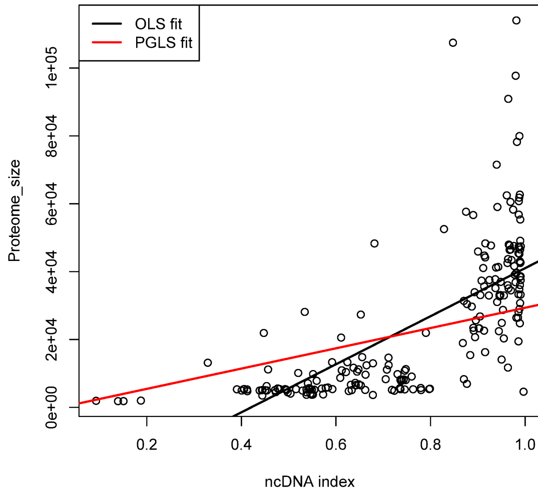
Trait	Linear model	AICc	Slope	R^2	P values
Splicing index vs. Genome size	OLS	-5.99	0.0002	0.30	0
	PGLS (corPagel)	-73.59	0.0000		0.6507
Proteome size vs. Splicing index	OLS	4679.91	48930.7100	0.41	0
	PGLS (corPagel)	4546.82	27866.1200		0
Proteome size vs. Neutral DNA	OLS	4648.05	70829.5600	0.49	0
	PGLS (corGrafen)	4579.83	298847.7600		0.0009



(a)



(b)



(c)

Figure 2: OLS fit versus PGLS fit of linear models on Splicing index vs. Genome size (a), Proteome size vs. Splicing index (b), Proteome size vs. Neutral DNA (c), N=210, all regressions are significant except for the PGLS on Genome size versus Splicing index. See Table 6 for detailed statistics.

4 Discussion

Genome evolution is one of the main debated topics in evolutionary biology, and here we tested for three evolutionary adaptive relationships among some genomic features predicted by previous theoretical work (Rigato and Fusco, in prep). Simple OLS analyses provided strong significant support for all the three predictions. This means that under the OLS assumptions, the neutral DNA and the splicing index appears to be a predictor of the organismal complexity (proteome size). However, in this scenario, the relation between the splicing index and the proteome size could be due only to the relation between the genome size and the splicing index itself, conferring support to the economic hypothesis for the origin of splicing. However, an analysis of the data under a phylogenetic framework, resulted in high levels of phylogenetic signal (lambda) for all traits and the evolutionary model fitting resulted in the fact that evolution of all traits is likely to be characterized by some levels of selective pressure (OU model). In other words, this means that adaptive relations may exist in these genomic features but, due to the phylogenetic signal, they might be distorted if phylogeny is not taken into account. In fact the PGLS analysis under the best found correlation structures, supported only two relations out of three, in particular the relation between the genome size and the splicing index disappeared under the PGLS analysis (Fig. 3a). This means that is very likely that the genome size is not a predictor of the splicing index level and that the relation we found in the preceding OLS was due to the effect of common ancestry. The PGLS resulted as the best fitted model in all comparisons conferring a stronger support to the fact that the splicing index and the neutral DNA proportion are good candidate mechanisms for the phenotypic robustness since they are positively related with complexity. In addition, if neutral DNA and the splicing index are robustness components, they should precede the escalation of complexity in evolution, since their increase allow to access higher levels of phenotypic robustness and thus higher levels of complexity. This is also the reason why we regressed the proteome size as the response variable, while we adopted the neutral DNA and the splicing index as predictor variables. Comparative studies already indicated that alternative splicing preceded

multicellularity in evolution, and suggested that this mechanism might have been co-opted to assist in the development of multicellular organisms (Irimia et al., 2007). Similarly, we suggest that the neutral DNA proportion increases should preceded any increases in organismal complexity because higher levels of robustness allows to explore higher levels of complexity. Surely the neutral DNA proportion and the splicing index are not the only source of robustness and other robustness candidate sources have been shown to be positively related with complexity proxies (like proteome size), as for instance the mean level of protein disorder (Schad et al., 2011). Possibly even more factor contributing to robustness could be tested in the future. The neutral DNA proportion and the splicing index evolution can also partly contribute to explain the puzzle of the genome size evolution. This is because in most eukaryotes a great proportion of the genome is composed by neutral DNA (the mean of our eukaryotic dataset is 76%), and because the splicing index influence how much information can be stored in the encoding genome. Simple linear explanations of the genome size problem without a phylogenetic framework have been proposed since long, the last and most famous being that provided by Lynch (Lynch and Conery, 2003), showing that nearly 60% of variance in genome size can be explained by the variance in the effective population size (with a taxon sampling of N=21). However, these results has been subsequently confuted by a phylogenetic analysis provided on the same dataset by Whitney and Garland (Whitney and Garland Jr, 2010). Genome size is a complex trait that is unlikely to be explained by univariate analyses (Charlesworth and Barton, 2004; Gregory, 2005). Phylogenetic comparative methods should be combined with multivariate models that are capable of distinguishing the contributions of highly correlated predictor variables. We suggest that the relation between the neutral DNA proportion, the splicing index and the complexity should be taken into account in such analysis aiming to explore the problem of the genome size evolution. These first results could be improved in a near future by using better proxies of complexity, such as the complexity dimensions (n) of a Fisher geometric model, which in principle can be derived from genetic population data (i.e. dN/dS estimantion) (Orr, 2006), or the organismal cell type number. Here we found strong support for our two main predictions even accounting

for the effect of phylogeny, however a wider and a deeper analysis should be provided in the future considering a multivariate phylogenetic analysis and different robustness and complexity proxies.

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