Evolvability in the context of antibiotic resistance

Timo J.B. van Eldijk

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The research presented in this thesis was carried out at the University of Groningen in the research group Modelling Adaptive Response Mechanisms (MARM) at the Groningen Institute of Evolutionary Life Sciences (GELIFES) and in the Molecular Genetics (MolGen) group at the Groningen Biomolecular Sciences and Biotechnology Institute (GBB).

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Cover image: A strain of the bacterium *Lactococcus lactis* that has evolved high-level resistance to the antibiotic ciprofloxacin (from the end of the experiment shown in Chapter 6 of this thesis, plasmid-free population B). The image shows a magnification of approximately 1000X. The bacteria were cultured in medium containing 160 µg/ml ciprofloxacin and subsequently photographed using a 100x oil immersion objective, an Amscope 2x camera adapter and a Cannon 77D camera.

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Chapter 1

Introduction

Timo J.B. van Eldijk

The emergence and spread of antibiotic resistance represent a major challenge to public health. Every year an estimated 700,000 people are killed globally by antibiotic-resistant infections, and, according to some estimates, this number will rise to 10 million by the year 2050 (Willyard, 2017). Inadequate prescribing practices, the agricultural use of antibiotics, and the improper disposal of wastewater containing antibiotics have all contributed to an alarming rise in antibiotic resistance (Martens & Demain, 2017; Nadimpali et al., 2017). In addition, the problem of antibiotic resistance is exacerbated by a low discovery rate of new antibiotics (Luepke et al., 2017).

The rise of antibiotic resistance is an example of 'evolution in action': bacterial populations adapt to a toxic environment by evolving resistance. However, luckily, bacterial populations are not always able to adapt to survive antibiotic exposure. What determines if a bacterial population can adapt and develop resistance? To answer this question, we need to understand what mechanisms enable bacterial populations to undergo adaptive evolution, along with the factors that constrain their adaptive potential. In other words, we need to understand 'evolvability' in the context of antibiotic resistance. Therefore, this PhD thesis will focus on understanding evolvability, using the evolution of antibiotic resistance as a model system. I will combine experimental evolution and simulation models to gain fundamental insights into the mechanisms and organismal features that determine evolvability. Meanwhile, these insights might also help to solve a major societal challenge.

Defining evolvability

The exact definition of evolvability has been much debated in the literature (Wagner, 2007; Pigliucci, 2008). Numerous definitions have been provided, yet these definitions can roughly be split into two categories, those based on the work of Houle (1992) and those based on Wagner & Altenberg (1996). Evolvability as defined by Houle concerns the standing genetic variation and covariation within a population (Houle, 1992; Pigliucci, 2008). In evolutionary quantitative genetics this standing genetic variation, quantified using narrow-sense heritability, determines the response of a population to selection (Houle, 1992; Pigliucci, 2008). In the short term, the response to

selection may indeed be predominantly determined by the standing genetic variation. However, when considering evolution on longer timescales the influx of new mutations cannot be neglected, as the evolutionary trajectory of a population is ultimately determined by the mutations that occur (Tenallion et al., 2012; Lässig et al., 2017). In short, evolvability *sensu* Houle is a concept more focused on short-timescales, which overlaps with the already existing concept of heritability. Therefore, under this definition evolvability does not provide an interesting extension of evolutionary theory. Wagner & Altenberg (1996) made an important conceptual advance by stating that the capacity of a biological system to undergo adaptations dependent on both the variation present within that system but also the variability of that system, that is, the capacity of the system to produce new variation (Pigliucci, 2008). When describing evolvability including variability, evolvability is a rich concept that goes beyond the standard treatments of classical evolutionary theory.

Throughout this thesis, I will be defining evolvability as the capability of a biological system to undergo adaptive evolution. For a detailed motivation for using this definition the reader is referred to **Chapter 2** of this thesis. Whilst this definition is derived from that of Wagner & Altenberg (1996), it is different in two crucial ways. Importantly this definition encompasses both (standing) variation and variability, and also many other mechanisms and organismal features that impact adaptive evolution. The definition I use also deliberately focuses on adaptive evolution. This focus is motivated by the potential applications of evolvability research; I am ultimately interested in understanding and predicting adaptive processes, such as the evolution of antibiotic resistance.

Evolutionary rescue theory

A particularly useful theoretical modelling framework to describe evolvability in the context of antibiotic resistance is evolutionary rescue theory. Evolutionary rescue models describe a population facing a drastically altered environment, for example a bacterial population facing antibiotics (Ramsayer et al., 2013; Alexander et al., 2014; Bell, 2017). When antibiotics are introduced, the individuals in the population die faster than they can

reproduce, leading to a steady decrease in population size. If nothing happens, this decrease will continue until the population is extinct. If a resistant mutant arises that can grow in the presence of antibiotics, this mutant can rescue the population, allowing the population to recover. Here two scenarios are usually considered: Resistant rescue mutants can either be already present in the population (standing genetic variation) or they can arise during the rescue process because of de-novo mutation. These two scenarios echo the two commonly considered aspects of evolvability: variation and variability.

In **Chapter 7** of this thesis, I will use simulations to expand evolutionary rescue theory to a multispecies context, simulating how species abundances change when a whole bacterial community undergoes evolutionary rescue as a consequence of antibiotic exposure. Evolutionary rescue theory also formed an important inspiration for **Chapters 3 and 4** of this thesis. In evolutionary rescue theory one of the key parameters that determines the probability of evolutionary rescue is the mutation rate, the higher the mutation rate the higher the probability of rescue. This emphasizes the role of mutation rates as a key mechanism underlying evolvability.

The mechanisms underlying evolvability

In **Chapter 2** of this thesis, I systematically explore the mechanisms and features that underlie evolvability. In short, these determinants can be divided into three categories, based on how they impact evolvability. The first category of determinants are features or processes that generate and/or maintain variation, for example the mutation rate (Tenallion et al., 2016; Sprouffske et al., 2018). The second category consists of those determinants that shape the effect of variation on fitness. An example is the structure of the gene-regulatory network. The structure of this gene-regulatory network can shape the way mutations can impact the phenotype, thereby impacting the potential for adaptation (Crombach & Hogeweg, 2008). The third category of determinants are those that shape the process of selection. An example is the generation time: a shorter generation time speeds up adaptive evolution (Tomas et al., 2010; Gandon & Michalakis, 2002). It is worth noting that determinants of evolvability can fit into more than one

category, since some features affect evolvability in more than one way. An example of this is horizontal gene transfer: it allows variation to be recombined in new ways (generating and maintaining variation), but it also allows beneficial variants to spread more quickly (shaping the process of selection) (Koonin, 2016; Croll & McDonald, 2012). In this thesis, I will focus on three determinants of evolvability: mutation rates, the gene regulatory network structure, and horizontal gene transfer.

The mutation rate

One of the clearest mechanisms underlying evolvability is the mutation rate. Mutation rates are known to vary widely, both between different species and between individuals of the same species (Conrad et al., 2011; Sung et al., 2016). There is also overwhelming evidence that mutation rates are subject to selection (Sniegowski et al., 1997; Tenallion et al., 2001; Tenallion et al., 2016). Normally, when a population is well adapted, there is selection for a low mutation rate, because most mutations will have a detrimental effect on fitness. The situation is different for populations that are initially poorly adapted, such as in microbial evolution experiments in which "mutator" strains arise. These mutator strains have an elevated mutation rate, for example due to DNA mismatch repair defects (Sniegowski et al., 1997; Tenallion et al., 2016). In these experiments, the population is undergoing rapid adaptation to a new environment, associated with the spread of mutator strains. These mutator strains produce more mutations, many of which are maladaptive. However, when adapting to a new environment the fitness cost of these maladaptive mutations is more than compensated by the faster occurrence of beneficial mutations (Sniegowski et al., 1997; Tenallion et al., 2001; Tenallion et al., 2016). Mutator alleles are indirectly selected through the beneficial mutations they produce. In other words, when a population is poorly adapted the increased influx of beneficial mutations offered by an elevated mutation can to some extent outweigh the detrimental effects of an elevated mutation rate (Sniegowski et al., 1997; Tenallion et al., 2001; Tenallion et al., 2016; Sprouffske et al., 2018). The interplay between the beneficial and detrimental effects selects for an optimal mutation rate.

Considering that a higher mutation rate can be selectively advantageous under certain environmental conditions, it does not seem surprising that in some cases the mutation rate can be regulated depending on the environmental state: in some bacteria the mutation rate is elevated in stressful conditions through a process known as stress-induced mutagenesis (Bjedov et al., 2003; Foster, 2007). In **Chapter 3** I will experimentally explore how an environmental parameter, in this case temperature can shape the mutation rate toward antibiotic resistance. I aim to see if the manipulation of temperature, can be used as an intervention to lower the mutation rate and can thus be used to combat the evolution of antibiotic resistance (i.e. lower the probability of evolutionary rescue). In **Chapter 4** I will use individual-based simulation models to see under what circumstances one might expect condition-dependent mutation rates to evolve.

Gene regulatory network structure

Traditional evolutionary models often fail to consider the complex relationship between the genotype and the phenotype. A wide range of underlying mechanisms, including complex network of developmental pathways and gene interactions are involved in translating the genotype to the phenotype. Explicitly considering this gene regulatory network brings to light another interesting mechanism underlying evolvability. The structure of the gene regulatory/developmental network can bias the effect of genotypic mutations on the phenotype. So, whilst genetic mutations occur at random, their effect on the phenotype is biased through the structure of the gene-regulatory network (Wagner & Zhang, 2011; Hogeweg, 2012). In the literature, this effect is sometimes referred to as developmental bias. A well-known example of developmental bias are the two eyespots on the wings of the butterfly *Bicyclus anynana*. Mutations can easily change the colour composition of both eyespots together, but they cannot easily change the colour composition of the two eyespots independently (Allen et al., 2008). Depending on the direction of selection such a developmental bias could inhibit or facilitate adaptive evolution.

A more extreme case of the gene regulatory network structure shaping evolvability is what I have termed a ‘mutational transformer’. This

phenomenon was first described by Crombach & Hogeweg (2008) and further investigated by Cuypers et al. (2017). They considered an environment that alternates between two alternative states and each state of these two states requires a different optimal phenotype. They also explicitly modelled the underlying gene-regulatory network. The population adaptively tracks the environment, this is where the population undergoes evolutionary adaptation each time the environment shifts to the alternative state (Botero et al., 2015; Cuypers et al., 2017). During the first few fluctuations, many different mutations are required to shift to the new optimal phenotype when the environment switches. However, after the population has experienced many such fluctuations, mutations altering the gene regulatory network structure occur that shorten the mutational paths between the two optimal phenotypes, creating a mutational transformer. Thus, when the environment shifts to the alternative state, these shortened mutational paths allows for more rapid adaptation. In a mutational transformer the gene regulatory network is structured in such a way that a single or very few mutations allow the switch to an alternative adaptive phenotype, facilitating rapid adaptation. However, how mutational transformers evolve and function is not well understood. In **Chapter 5** of this thesis, I examine the evolution of mutational transformers in a simple gene regulatory network model, elucidating their evolution and inner workings.

Horizontal gene transfer

In bacteria, horizontal gene transfer can occur through various mechanisms. In this thesis, I focus on conjugation, since many clinically relevant antibiotic resistance genes are horizontally transferred using conjugation (Robicsek et al., 2006; Litrup et al., 2017; Yui et al., 2019; Venketesan et al., 2023). In conjugation, extrachromosomal pieces of DNA called 'plasmids' are exchanged between bacterial cells through cell-to-cell contact (Lopatkin et al., 2017). Here it is important to note that genes on plasmids can also be transferred to the chromosome and vice versa through recombination (Heap et al., 2012). Carrying plasmids often comes at a cost to the host cell, which needs to maintain, replicate, and express an extra piece of DNA. Furthermore, since plasmids are pieces of DNA that replicate separately from

the bacterial chromosome, they can act as independent 'selfish' evolutionary entities (San Millan & MacLean; 2017; Ghally & Gillings; 2021). For example, some plasmids are maintained due to very high rates of horizontal transmission, essentially persisting as an infection, to the detriment of the host cells (Brockhurst & Harrison, 2022).

However, there is also another side to plasmids, several hypotheses can be found scattered throughout the literature on how plasmid-based horizontal gene transfer might enhance the evolvability of the host cell. First of all, horizontal gene transfer creates new combinations of genes, generating novelty by recombining existing variation (Hall & Kerney, 2012; Kingston et al., 2015). Second, horizontal gene transfer can bring together genes from independent clonal lineages, alleviating 'clonal interference' (Cooper, 2007). In 'clonal interference', beneficial genes from different clonal lineages compete, this competition of beneficial can hinder adaptation. Third, if beneficial genes are spread horizontally as well as vertically this can increase the rate of spread of beneficial genes, thereby enhancing the rate of adaptation (Chu et al., 2018). Fourth, horizontal gene transfer creates a reservoir of genetic variants that can be easily accessed. This kind of reservoir dynamic, facilitates the maintenance of genetic variation, thereby enhancing evolvability (Croll & McDonald, 2012; Woods et al., 2020; van Dijk, 2020). However, the link between horizontal gene transfer through plasmids and evolvability is poorly understood at best (Koonin, 2016). In **Chapter 6,** I will use experimental evolution to study the impact of plasmids on the de-novo evolution of antibiotic resistance.

The evolution of evolvability

If we consider the mechanisms and features underlying evolvability, it is evident that these differ widely across the tree of life and are subject to evolution (Conrad et al., 2011; Sung et al., 2016; Martinez-Padilla et al., 2017). However, an important question remains: Have these mechanisms been shaped by selection because of their effects on evolvability? In other words, is evolvability a selected (evolved) property of a biological system or are differences in evolvability simply a side effect of other selective pressures or genetic drift (Tenallion et al., 2001; Woods et al., 2011; Nelson & Masel,

2018)? For mutation rates, there is a good body of empirical and modelling evidence that they are indeed subject to selection for evolvability (Sniegowski et al., 1997; Tenallion et al., 2001; Tenallion et al., 2016), although these mechanisms are subtle because they tend to rely on indirect selection. For most other determinants of evolvability, such evidence is currently lacking. In **Chapters 4 and 5** I will use simulation models to explore the evolution of evolvability in response to fluctuating environments.

This thesis

This thesis aims to understand evolvability in the context of antibiotic resistance. As a starting point, I have developed a mechanistic framework that categorizes the determinants of evolvability and provides several concrete recommendations on how to study evolvability (**Chapter 2**). By focusing on the underlying mechanisms of evolvability, I break down the problem of understanding evolvability into manageable chunks. Each of my chapters will focus on a particular determinant of evolvability: mutation rates, the genotype-to-phenotype map, and horizontal gene transfer. Throughout I use two complementary approaches: microbial experiments (**Chapters 3 and 6**) and simulation models (**Chapters 4, 5 and 7**). Theory and experiment interact in a synergistic way. Microbial experiments provide new observations that aid the development of theoretical models. On the other hand, the hypotheses and ideas derived from theoretical models can improve the design of experiments. This combination of the practical and theoretical is echoed throughout this thesis: I hope to show that fundamental theoretical insights into evolvability can help address the very practical problem of antibiotic resistance evolution.

In **Chapter 2,** I first discuss how to define evolvability. Subsequently, I explore the mechanisms and organismal features underlying evolvability and develop a coherent mechanistic framework for categorizing these determinants of evolvability. I argue that a mechanistic view coupled with a clear definition, brings the concept of evolvability into sharper focus. This leads to several concrete recommendations on how to study evolvability. For example, I recommend that researchers should explicitly account for the timescale on which determinants act. Furthermore, I urge researchers to consider what I

have termed “the scope” of a determinant. Some determinants, such as mutation rates, affect evolvability in a wide range of environments. Other determinants, such as the re-structuring of the genotype-to-phenotype map, may only affect evolvability in a more restricted set of environments.

In **Chapter 3,** I examine an empirical example of a condition-dependent mutation rate in the bacterium *Escherichia coli*. I experimentally assess the impact of elevated temperatures on the mutation rate towards antibiotic resistance for three different antibiotics. Such elevated temperatures are often encountered by bacterial populations since fever (an elevation of body temperature) is a common response to infection. I show that even a relatively small change in temperature can alter the mutation rate by an order of magnitude. However, the nature of the change and the underlying mechanism differ depending on the antibiotic. These results might pave the way for a new way to mitigate the emergence of antibiotic resistance: the choice of an antibiotic and the decision of whether to suppress fever could be coordinated to minimize the mutation rate towards antibiotic resistance.

In **Chapter 4,** I use an individual-based simulation model to study the evolution of condition-dependent mutation rates in a changing environment. I consider a model where the mutation rate can potentially be changed/regulated according to the mismatch between an individual’s phenotype and its environment. I show that both condition-dependent and constant mutation-rate strategies can evolve. Which strategy emerges is strongly dependent on the rate of environmental change. I also show that condition-dependent mutation rates enhance evolvability: they allow populations to better track a changing environment.

In **Interlude 1,** I worked in a team collaborating with researchers studying fatty-acid synthesis in a parasitic wasp. These researchers had found that a particular metabolic pathway was only expressed when it was induced by the environment, a phenomenon known as phenotypic plasticity. They also found that this inducible metabolic pathway was stably maintained over long evolutionary timescales, whilst it was only very rarely expressed. This was surprising since one would expect such an adaptation to be degraded by mutations and subsequently lost, since selection against degrading

mutations only occurs very rarely. To help explain this I explore an individual-based simulation model of how such a phenotypically plastic trait evolves and is maintained over long timescales. I show that the structure of the gene regulatory network can evolve to become robust to mutation. This explains how a phenotypically plastic adaptation can be maintained over long timescales even when it is rarely expressed.

In **Chapter 5,** I consider a model where the phenotype of an individual is determined by a simple gene regulatory network. A population of these individuals must adapt to an environment that switches/changes between two alternative states (for example the presence or absence of an antibiotic). I show that even in this very simple model, the structure of the gene-regulatory network can evolve to bias the phenotypic effects of mutations towards adaptive outcomes, thus facilitating evolvability. I examine in detail the mechanisms underlying such mutational transformers and show that two distinct mechanisms emerge depending on the nature of gene interactions.

In **Chapter 6,** I use a 31-day evolution experiment (approximately 237 generations) to examine the impact of plasmids on the de-novo evolution of antibiotic resistance. I exposed plasmid-bearing and plasmid-free populations of the bacterium *Lactococcus lactis* to an increasing concentration of the antibiotic ciprofloxacin. Over the course of the experiment, the populations managed to evolve a high level of ciprofloxacin resistance. Meanwhile, I also evolved plasmid-bearing and plasmid-free control populations in the absence of the antibiotic. I found that, in the context of this experiment, plasmids did not have a noticeable impact on evolvability. This may be explained by the relatively limited timescale of the experiment. I find indications that there is selection against the plasmid, but only in the presence of the antibiotic ciprofloxacin. I also used whole-genome sequencing to examine the mutations underlying ciprofloxacin resistance.

In **Chapter 7,** I use a simulation model to expand evolutionary rescue theory to a community context. I model a whole ecological community undergoing evolutionary rescue in response to a drastically deteriorated environment, for example, a gut microbial community facing antibiotic treatment. I study how species abundances change during the evolutionary rescue process. I

show that community-wide evolutionary rescue leads to a very rapid loss of rare species from the community.

Bringing everything together in **Chapter 8,** I briefly summarise the insights gained into evolvability and its evolution and how some of these might eventually be harnessed to combat the evolution of antibiotic resistance. I also outline the way forward for evolvability research. I argue that a mechanistic approach is essential, using the synergy between theory and experiment to formulate a coherent theory of evolvability.

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Chapter 2

Capturing the facets of evolvability in a mechanistic framework

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Abstract

“Evolvability” – the capability to undergo adaptive evolution – is a key concept for understanding and predicting the response of biological systems to environmental change. Evolvability has various facets and is applied in many ways, easily leading to misunderstandings among researchers. To clarify matters, we first categorize the mechanisms and organismal features underlying evolvability into determinants providing variation, determinants shaping the effect of variation on fitness, and determinants shaping the selection process. Second, we stress the importance of timescale when studying evolvability. Third, we distinguish between evolvability determinants with a broad and a narrow scope. Finally, we highlight two contrasting perspectives on evolvability: general evolvability and specific evolvability. We hope that this framework facilitates communication and guides future research.

Evolvability is an important yet elusive concept

Understanding adaptation to changing environments is more important than ever. Climate change, antibiotic resistance, and viral vaccine evasion represent major societal challenges: is an endangered species able to adapt to environmental change? Will a bacterial pathogen evolve antibiotic resistance? Can a virus evade vaccine-based immunization? At the core of these issues lies a common element: the capability of organisms to adapt – evolvability [1]. Evolvability research sheds new light on genomic architecture [2], the structure of regulatory networks [3,4], and many other features of **biological systems** (see Glossary). It has yielded surprising new insights, such as: adaptive evolution can proceed at a similar pace as ecological change, resulting in intricate and unexpected eco-evolutionary dynamics [5,6], evolvability and **robustness** do not conflict but mutually reinforce each other [3,7,8]; and organisms with high evolvability can “generalize” over environments [9,10]. Furthermore, evolvability research may add new perspectives to formulating a predictive theory of evolution (see Box 1 and Outstanding Questions).

Evolvability is studied by diverse approaches. For instance, Johansson et al. [11] inspect the genetic variance-covariance matrix; Woods et al. [12] compare the speed of adaptation of bacterial strains; and Martín-Serra et. al [13] focus on morphological integration and modularity. These approaches, though all valid, are widely disparate: all aim to understand evolvability, yet each focuses on a different facet. This plurality is also reflected in the fact that evolvability has been defined in many different ways (see Box 1). We aim to highlight the different facets of evolvability and how they relate to each other, to facilitate a more nuanced and cohesive discourse on the topic. Throughout, we define evolvability as the capability of a biological system to undergo adaptive evolution (see Box 1 for a justification).

Toward a mechanistic approach to evolvability

Evolvability is often viewed in terms of outcomes (e.g. speed of adaptation). As the same outcome can be achieved in many ways, it is useful to study evolvability by a mechanistic approach [14]: viewing evolvability not as a phenomenon *per se* but as a product of the mechanisms and organismal features that underlie it. A mechanistic perspective also clarifies discussions on the evolution of evolvability [1,14]: while questions regarding the evolution of "the capability to undergo adaptive evolution" easily turn abstract, they become more obvious and transparent when translated into questions regarding the evolution of concrete mechanisms (e.g. the mutation rate).

Categorizing the determinants of evolvability

We refer to the mechanisms and organismal features that govern evolvability as determinants of evolvability. These affect different aspects of adaptive evolution, and consequently shape evolvability in different ways. We here identify three ways in which determinants can shape evolvability, based on what aspect of adaptive evolution they affect, and categorize them accordingly (figure 1). Firstly, determinants may affect evolvability by *providing variation*. The mutation rate is the most obvious example of such a determinant [2,15–17]. Secondly, determinants may affect evolvability by

*influencing the effect of variation on fitness***.** For example, developmental biases may predispose mutations towards being beneficial [18–21]. Thirdly, determinants may affect evolvability by *shaping the selection process* – shorter generation times, for example, may speed up adaptation [22,23].

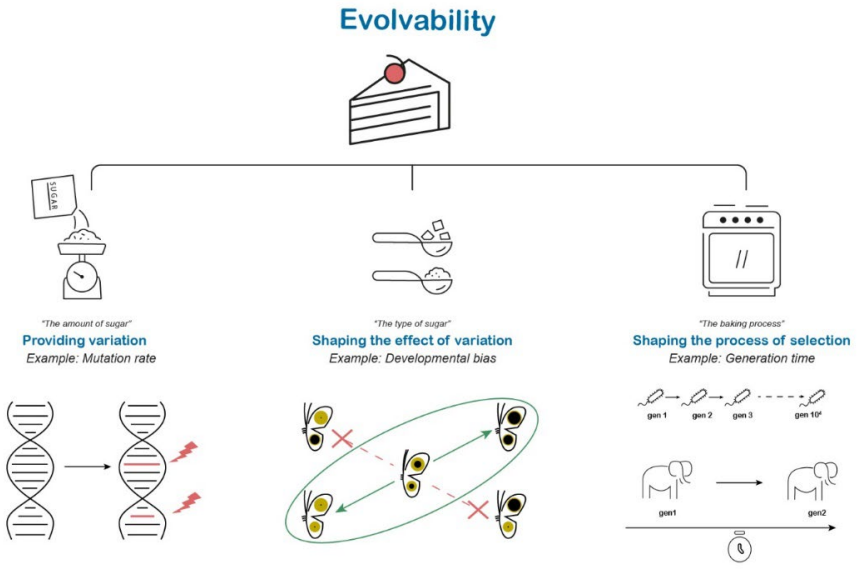

Figure 1: The way that mechanisms and organismal features affect evolvability can be classified into three categories. Each one contributes to evolvability in a different way. This can be compared to the process of baking a cake: the end result depends on several fundamentally different aspects - the amount of ingredients, the quality of ingredients and the baking process. We suggest that evolvability is analogously affected by three different classes of determinants: those providing variation ("the amount of sugar"), those shaping the effect of variation on fitness ("the type of sugar"), and those shaping the selection process ("the baking process"). An example of a determinant providing variation is the mutation rate, where mutation encapsulates a wide variety of phenomena ranging from point mutations to genome rearrangements. Developmental biases are examples for determinants that shape the effect of variation on fitness. Consider for instance the developmental system underlying the eyespot pattern on the wings of the butterfly *Bicyclus anynana*. This system is organized in such a manner that mutations can easily change the colour composition of the two wing eyespots in the same direction, whilst mutations changing the colour composition in opposite directions are extremely rare (depiction based on [40]). Depending on whether the selective pressure favours eyespots with the same colour composition or not, this bias may facilitate or impede evolvability. Finally, an example of a determinant that shapes the selection process is generation time: a shorter generation time allows faster adaptation – in absolute time, bacteria evolve faster than elephants.

Category 1: providing variation

Heritable variation serves as the raw material for evolution. Hence, our first category refers to those mechanisms that generate and maintain variation. For example, mutations generate variation in many ways, ranging from point mutations to genome rearrangements. Interestingly, mutation rates vary widely between organisms as well as within genomes [24–26], and they can be regulated based on the environment (e.g. stress-induced mutagenesis, [27]) – indicating that evolvability can evolve through the evolution of the mutation rate. Examples of determinants maintaining variation include **evolutionary capacitor**s such as heat shock proteins. HSP-90 in *Saccharomyces cerevisiae*, for instance, acts as a chaperone protein aiding correct protein folding. Chaperoning can shield sequence mutations from selection, thus maintaining variation. This can later be released under stressful conditions [28,29]. **Developmental canalization** can affect evolvability in a similar manner, in that it allows the accumulation of **cryptic genetic variation** [30]. Furthermore, capacitors may also be behavioural in nature, as parental care and thermoregulatory behaviour also allow cryptic genetic variation to accumulate [31,32]. Horizontal gene transfer may also be viewed as a category 1 determinant, as it allows variants to be maintained that would otherwise be lost from the population, for instance by establishing an "accessory genome" [33,34] or through the so-called rescuable gene hypothesis [35]. Not all heritable variation is genetic: epigenetic inheritance, inheritance of environmental features, and cultural inheritance can also affect adaptive evolution [36,37]. Hence, category 1 also includes mechanisms providing non-genetic heritable variation.

Category 2: shaping the effect of variation on fitness

The mapping from mutation to fitness is affected by a variety of mechanisms: mutations may be random with respect to the genotype, but their effects on the phenotype and consequently fitness are often not [8]. Through features such as genomic, developmental, and regulatory architecture, the genotype-to-phenotype-to-fitness map can bias the fitness effects of mutations [19,38]. Category 2 thus contains determinants that influence the effect of variation on fitness. Examples can be found in the evo-devo literature

[19,21,39,40], which describes various biases introduced through the developmental process (**developmental bias,** for an example see the butterfly *Bicyclus anynana* in Fig. 1). The effect of variation on fitness can also be biased by genomic and regulatory architecture [4,41]. In yeast, for example, genes for which upregulation is selectively favoured in a higher-temperature environment are grouped on the same chromosome. Therefore, a duplication of this chromosome suffices to achieve upregulation of all relevant genes; without such genome organisation, many independent mutations would be required to obtain an equivalent high-temperature adaptation [42].

Category 3: shaping the selection process

Starting from the same variation, evolution can still proceed at a very different pace and/or can lead to very different outcomes. Thus, category 3 contains determinants that impact evolvability not by shaping variation, but rather by shaping how the selective process acts on this variation. Examples are organismal features influencing population structure (e.g. dispersal tendency or mating patterns), as population structure may strongly affect adaptive evolution [43]. For instance, limited dispersal is hypothesized to have aided the rapid evolution of eusociality in diverse clades of insects [44]. Two other examples of category 3 determinants are generation time and the mode of reproduction. In coevolutionary host-pathogen arms races, the shorter generation time of pathogens provides them with an evolvability advantage, as they can evolve faster per time unit than their host [23]. Considering the Red Queen hypothesis, it becomes evident that hosts need other adaptations (e.g. sexual reproduction or a variation-generating immune system) to cope with pathogens on a longer-term perspective [45]. In the coevolution of hosts and their symbionts, the rapid evolution and/or diversification of the symbiont is often not in the interest of their host. Accordingly, the hosts of various symbiotic systems reduce the symbiont's evolvability by actively interfering with the symbiont's sexual reproduction [46].

How a mechanistic categorisation aids our understanding of evolvability

Some determinants of evolvability can be classified into more than one category. This is a deliberate feature of the proposed categorisation, as it highlights that a determinant can affect evolvability in different ways. The categorisation prompts the researcher to critically consider how mechanisms and processes shape adaptive evolution. An illustrative example can be found in the literature on evolvability and plasticity. Some have argued that plasticity impedes evolvability: plastic responses shield organisms from selection, preventing genetic adaptation (category 3) [47]. Others have argued that plasticity allows the accumulation of cryptic genetic variation (category 1) [10,48], thus potentially enhancing evolvability, because plastic traits are only expressed under particular environmental conditions. Finally, arguments derived from **Gene Regulatory Network models** conclude that the evolution of plasticity can restructure the genotype-phenotype map in such a way that random mutations are more likely to produce adaptive phenotypes (category 2) [49,50]. Our categorisation of determinants thus showcases these often subtle but nevertheless crucial distinctions.

The effect of **modularity** on evolvability provides another example. Inspection of the underlying mechanisms reveals that modularity has not one but two impacts on evolvability. Firstly, it facilitates innovation by allowing pre-existing modules to be combined in different configurations, thus providing variation (category 1) [51]. Secondly, it also allows individual modules to vary independently, without affecting the functionality of the entire system (reducing antagonistic pleiotropy): this makes deleterious mutations less impactful, thus creating an adaptive bias that shapes the fitness effects of variation (category 2) [52]. This reduced impact of deleterious mutations (category 2) may also allow organisms to tolerate higher mutation rates (category 1) - showing that determinants in different categories can interact in a reciprocal manner: the processes that provide variation (category 1), bias the fitness effects of variation (category 2), and shape the selection process (category 3) are not independent of each other.

Our view on the determinants of evolvability is suited to different approaches to evolution and evolvability. The first category (providing variation) contains not only mechanisms that provide new mutations, but also mechanisms that facilitate major innovations (however, the relationship between evolvability and major innovations is not yet well understood). Similarly, the second category considers not only instances of genotypic and developmental biases, but also includes broader ideas such as phenotypic accommodation and the theory of facilitated variation [20,53–55]. Finally, the third category considers not only genetic mechanisms (such as horizontal gene transfer, which also allows beneficial variants to spread more quickly [56]) but also - amongst others - niche construction, where organisms shape their own selective environment [57].

Explicitly considering timescale resolves apparent incongruencies

Determinants differ in the timescale on which they act – thus, when comparing evolvability across biological systems, the outcome is crucially dependent on timescale (see figure 2). Considering timescale can help to resolve several apparent discrepancies.

This is exemplified by comparing determinants that provide variation (category 1): consider the impact of standing genetic variation [58] and the impact of mechanisms generating variation [59] on evolvability ([1], see Box 1). In the short term, adaptation is more strongly influenced by standing genetic variation, whereas mechanisms generating and maintaining variation are of greater significance when considering longer-term evolutionary trajectories [60].

Some approach evolvability in terms of speed of evolution [61], whilst others approach it in terms of attained level of adaptation [38,62]. Both aspects are relevant [63], but they are often two sides of the same coin, which becomes apparent when explicitly considering timescale. Consider, for example, different modes of inheritance. Epigenetically inherited traits can provide fast adaptation - yet this adaptation is often relatively inaccurate, given that the relative instability of epigenetic marks impedes the reliable maintenance

of a certain optimal phenotype. In contrast, genetic adaptation proceeds more slowly, but in view of the high fidelity of genetic inheritance it may, in the long term, result in a higher level of adaptation. Therefore, epigenetic inheritance confers higher evolvability in the short term, and genetic inheritance confers higher evolvability in the long term [36].

Another discrepancy that can be resolved by considering timescale is the debate over the evolvability benefits of sexual reproduction, with both sexual and asexual reproduction being linked to increased evolvability [1,64]. All other things being equal, the response to selection (and hence the rate of adaptive evolution) is higher under asexual reproduction, as in case of sexual reproduction selection can only act upon the additive component of genetic variation [65] (category 3). Therefore, asexual reproduction facilitates evolvability in the short term. In the longer term, sexual reproduction confers a higher evolvability, as the slower speed of evolution is outweighed by the ability to better explore the fitness landscape and reach global rather than local peaks. The claims that sexual reproduction increases evolvability, and the claims that asexual reproduction increases evolvability can thus both be true, just at different timescales (see figure 2). Overall, the above examples show that the effects and relative importance of determinants vary over time. Therefore, explicitly considering timescale is crucial when studying evolvability.

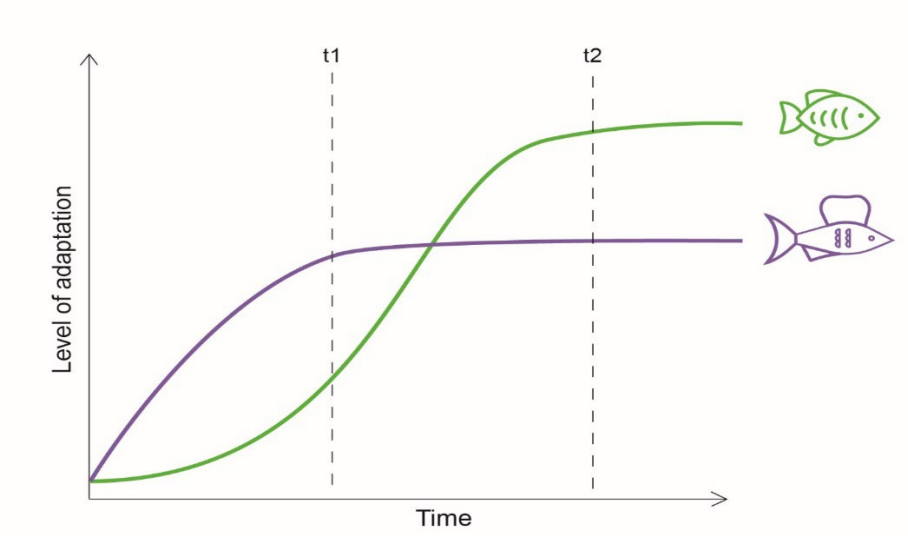

Figure 2: When studying evolvability it is important to explicitly consider timescale; observing at different times can lead to different conclusions. Suppose that we observe the ability of two fish species to adapt to a new food source. Our conclusions on which of the two is more evolvable (is better able to adapt to the new selective challenge) will depend on the time at which we observe their level of adaptation. At time t1, the purple fish species is more adapted (and hence seems more evolvable), but at time t2, the green fish species is more adapted (and hence seems more evolvable). This occurs because rates of adaptation are not constant across time - thus, being explicit about the timescale of observation can resolve apparent discrepancies when comparing the evolvability of different organisms.

Accounting for environmental context shows that determinants differ in scope

Evolvability is the capability to undergo adaptive evolution; it is therefore necessary to consider in relation to which environmental challenge such adaptation arises. This reveals an additional property of determinants: their scope. Some determinants affect evolvability across many different environmental challenges; we consider these to have a *broad scope*. For example, mutation rates impact evolvability in virtually all environments. Other determinants have a *narrow scope* as they only shape evolvability in a restricted set of environments. For example, the grouping of temperature-relevant genes on one chromosome in yeast [42] only enhances evolvability

to a change in temperature; it does not impact adaptation to other environmental challenges.

The scope of determinants pushes the researcher to consider the range of environments in which a determinant is relevant. Determinants relevant for adaptation to one environment may not be as relevant when considering adaptation to another. For example, in the radiation of Darwin's finches, developmental biases in beak development have been implicated in their adaptation to different seed sizes [66,67]. However, evolvability with regards to beak shape will not be relevant with regards to other environmental challenges, such as temperature regulation or predator escape. By contrast, a higher mutation rate will affect evolutionary adaptation with regards to many different environmental challenges.

Two perspectives on evolvability

A very different distinction does not refer to the determinants of evolvability, but to the scholars studying evolvability. Depending on their scientific discipline, research question, or model system, scholars differ in whether they view evolvability as "general" or "specific" with regards to environmental challenges (figure 3). Scientists adopting a *specific evolvability* perspective refer to the capability of a biological system to undergo adaptive evolution *to a specific environment* or a specific challenge – the main focus therefore is on how well a biological system can meet a specific selective target. This perspective is useful when studying adaptation to a particular challenge, e.g. when exploring the capability of bacteria to evolve resistance to a particular antibiotic, the capability of a virus to evolve resistance to a vaccine, or the ability of an endangered species to evolve adaptations to a specific anthropogenic threat [68,69]. In contrast, scientists adopting a *general evolvability* perspective view evolvability as the capability of a biological system to adapt to a wide spectrum of environments or of challenges – thus effectively considering evolvability *irrespective of the environmental context*. This perspective is useful when considering adaptation to unpredictable environments, and is also frequently used in studies exploring the link between evolvability and diversification [62,70,71].

Whilst specific and general evolvability are both useful conceptualizations of evolvability, insights gained from one do not necessarily translate into insights about the other. Depending on the chosen perspective, the same observation can lead to different conclusions (figure 3), it informs what questions are asked, and affects how results are interpreted. Consider a population that is able to adapt rapidly to a specific challenge, such as a bacterial strain quickly evolving resistance to a particular antibiotic. From the perspective of specific evolvability this strain has a high evolvability, whilst viewed from the perspective of general evolvability this single instance of rapid adaptation says nothing about the ability of the strain to adapt to other challenges (heat stress, pH stress, etc.). The distinction between general and specific evolvability should not be confused with the scope of a determinant: the latter is a property of a determinant, whereas the former concerns two different ways of viewing evolvability.

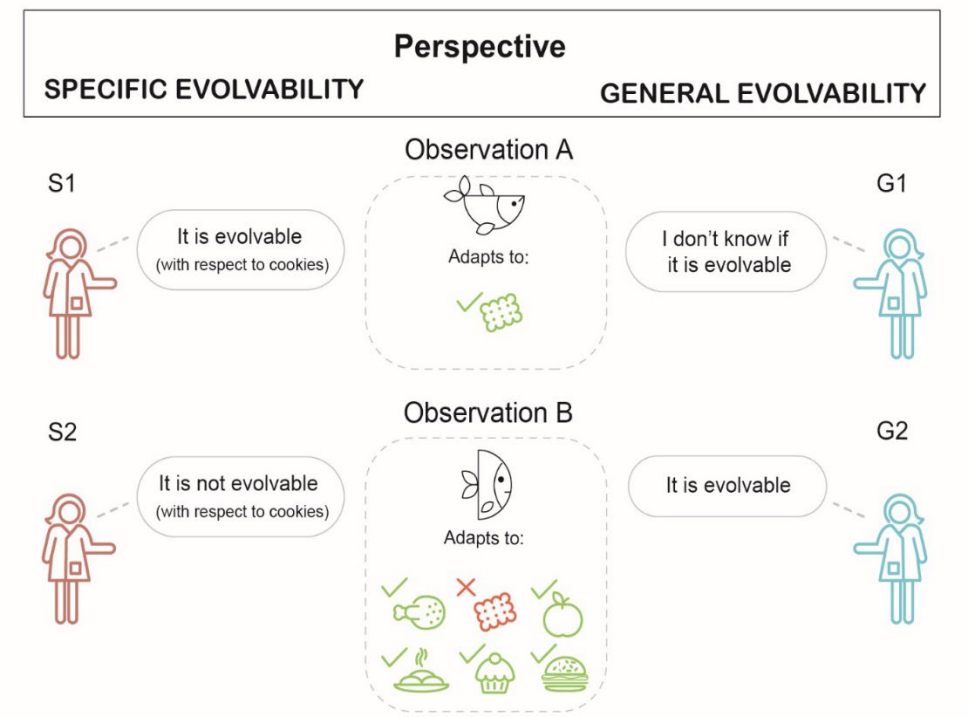

Figure 3: Specific and general evolvability represent two different perspectives on evolvability. This influences questions and interpretation of results in evolvability research. Different scientists can reach different conclusions from the same observations. This figure illustrates how scientists with different perspectives (on the left: specific evolvability, on the right: general evolvability) interpret the same observations regarding adaptation very differently. Observation A: A fish species can easily adapt to using cookies as a food source. From the specific evolvability perspective (S1), this is interpreted as high evolvability with respect to cookies. From the general evolvability perspective (G1), it is not possible to draw conclusions, since no information is available regarding the ability to adapt to other food sources (environments). Observation B: A fish species cannot adapt to using cookies as food source, but (e.g. due to modular mouth parts) can undergo adaptation to a wide range of other food sources (environments). From a specific evolvability perspective (S2), this is interpreted as a lack of evolvability with regards to cookies. From a general evolvability perspective (G2), the ability to adapt to a wide range of different environments (ability to deal with dietary shifts) indicates a high evolvability. Notice that the two perspectives characterize scholars of evolvability, rather than the determinants of evolvability. The two perspectives should therefore not be confused with determinants acting at short vs long timescales, or with determinants having narrow vs broad scope.

Concluding remarks

Evolvability is an intricate concept with many facets. Different facets are at centre stage in different lines of research. Furthermore, evolvability is conceptualized in two different ways: specific and general evolvability. Being aware of these differences is crucial for fostering an integrated and structured view on evolvability research.

Throughout we argue that evolvability should not be studied as a phenomenon per se, but as a product of the mechanisms underlying it. Moreover, it is useful to clearly distinguish between determinants that provide variation, shape the effect of variation on fitness and shape the selection process. A structured mechanistic approach clarifies debates in the literature and provides a sound basis for studying the evolution of evolvability.

Evolvability cannot be quantified by a single number. Both speed of evolution and level of adaptation are relevant, but they are not independent. Scholars should explicitly consider this when conducting evolvability research.

We hope that the proposed mechanistic approach facilitates communication across disciplines, helps to address major questions regarding evolvability (see Outstanding Questions), and provides guidelines for designing future studies on evolvability.

Box 1. Definitions of evolvability

Here we will briefly discuss some definitions of evolvability, as they provide a good overview of the diversity of approaches in the field of evolvability research [63,72,73]. One important early definition revolves around the additive genetic coefficient of variation. It defines evolvability as the ability to respond to selection as governed by the presence or absence of standing genetic variation (often assessed in the G matrix) [38,58,74]. Another important perspective was given by Wagner and Altenberg [59], who made a distinction between variation and variability, i.e. the propensity of characters to vary. Evolvability is then considered not as the currently present variation but instead as the ability to generate new variation. A third, different perspective on evolvability considers the ability to generate major innovations [75,76]. For a comprehensive review of these developments, the reader is referred to [1]. Note that these definitions of evolvability are reflected in our first category of determinants, as they all view evolvability as determined by the presence or provisioning of variation.

One additional aspect of the definitions of evolvability (also called "evolutionary potential" or "adaptive capacity") is the relationship between variation and adaptation. While earlier treatments (as discussed in [1]) define evolvability as the ability of a biological system to evolve, irrespective of whether evolution is adaptive or not, recent definitions tend to restrict the concept to *adaptive* processes. For example, Payne and Wagner [14] combine the aspects of variation and adaptation when they define evolvability as "... the ability of a biological system to produce phenotypic variation that is both heritable *and adaptive*". Other definitions in this vein have been provided by [7,38,60,77–81].

Following the general trend in the field, we here focus on adaptive evolution as well. When defining evolvability as the "capability of a biological system to undergo adaptive evolution" we do not mean the presence or absence of this capability, but rather we consider its degree in a continuous fashion. Adopting an adaptive perspective does by no means imply that non-adaptive processes (e.g. genetic drift) are irrelevant; in fact, many of the determinants

of evolvability reflect such processes (e.g. mutation). However, there are at least two reasons for focusing on adaptive evolution. First, many applications of evolvability (e.g. evolution of antibiotic resistance, adaptation to anthropogenic change) consider the adaptation to environmental challenges. Second, relating the rate and outcome of evolution to underlying selection pressures provides a yardstick, making it possible to differentiate between organismal and environmental features [38] and allowing comparisons across organisms. Both features are important first steps toward a predictive theory of evolution [82].

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Glossary

Biological system: We here define a biological system to be any biological entity that can be subject to evolution by natural selection.

Cryptic genetic variation: Standing genetic variation that has little effect on phenotypic variation under normal conditions, but generates variation under changed conditions. The release of this variation can facilitate (or hamper) adaptation and thus impact evolvability.

Developmental bias: The developmental mechanisms underlying a trait can introduce biases in the variation in the phenotype, even if the underlying mutations are unbiased. These biases can be (but need not be) aligned with the direction of selection, in which case they facilitate adaptive evolution.

Developmental canalization: Robustness to genetic or environmental perturbations frequently exhibited by developmental systems, leading to a stable phenotypic outcome.

Evolutionary capacitor: Mechanism that prevents the expression of genetic variation under some conditions, thus allowing the accumulation of **cryptic genetic variation**, and 'releases' this variation under other conditions, thus exposing it to selection.

Gene regulatory network (GRN) model: Model that explicitly represents the genotype-to-phenotype-map, as a complex network of regulatory interactions between genes. GRN models have been studied extensively in the context of evolutionary developmental biology and evolvability.

Modularity: The ability of subsets of a system ("modules") to function independently of other parts of the system (see [51]). Modularity can impact evolvability in various ways: for example, independent modules can be easily combined in different ways, and furthermore do not interfere with each other's functioning.

Phenotypic plasticity: The expression of different phenotypes by the same genotype in response to environmental conditions. The impact of phenotypic plasticity on evolvability is subject to much debate (see for example [10,47–50]): their relationship is complex and not yet well understood.

Robustness: The capability of the state of a biological system to persist under (environmental or genetic) perturbation. For example, robustness may refer to the ability to maintain a certain phenotype in the face of environmental fluctuations or genetic mutations. Intuitively one might consider evolvability (the ability to change) and robustness (the ability to withstand change) to be opposed, however, it has been shown that they can be two sides of the same coin [3,7].

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Chapter 3

Temperature dependence of the mutation rate towards antibiotic resistance

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Abstract

Environmental conditions can influence mutation rates in bacteria. Fever is a common response to infection that alters the growth conditions of infecting bacteria. Here we examine how a temperature change, such as is associated with fever, affects the mutation rate towards antibiotic resistance. We used a fluctuation test to assess the mutation rate towards antibiotic resistance in *Escherichia coli* at two different temperatures: 37 °C (normal temperature) and 40 °C (fever temperature). We performed this measurement for three different antibiotics with different modes of action: ciprofloxacin, rifampicin, and ampicillin. In all cases, the mutation rate towards antibiotic resistance turned out to be temperature dependent, but in different ways. Fever temperatures led to a reduced mutation rate towards ampicillin resistance and an elevated mutation rate towards ciprofloxacin and rifampicin resistance. This study opens a new avenue to mitigate the emergence of antibiotic resistance by coordinating the choice of an antibiotic with the decision of whether or not to suppress fever when treating a patient. Hence, optimised combinations of antibiotics and fever suppression strategies may be a new weapon in the battle against antibiotic resistance.

Introduction

Antibiotic resistance represents a major societal challenge (1). The emergence of antibiotic resistance is at its core an evolutionary process, with recent research showing that antibiotic resistance can evolve within a patient within just three days (2). More specifically, it may be viewed as an 'evolutionary rescue' process (3, 4); when treatment commences, a bacterial population needs to rapidly evolve resistance before it is driven to extinction by antibiotic exposure. The probability that the bacterial population evolves resistance is dependent on the rate of occurrence of mutations that confer resistance. The higher the mutation rate towards resistance, the greater the probability that antibiotic treatment fails due to the emergence of resistance (3, 4).

Mutation rates in bacteria are not constant but are instead dependent on environmental conditions such as temperature (5, 6). Most biochemical

processes increase with temperature, so it has been speculated that mutation rate would be positively associated with temperature (7, 8). In addition, bacterial mutation rates can be regulated to become elevated in stressful environments, a phenomenon known as stress-induced mutagenesis (9). Pathogenic bacteria may therefore express a different mutation rate when their (human) host responds to an infection with fever – an increase in the body temperature above 38.3 °C (10) – which can create mildly stressful conditions for an infecting bacterial population. This leads to a question that, to our knowledge, has not been addressed before: How does the increase in temperature associated with fever influence the mutation rate towards antibiotic resistance? If in bacteria mutation rates towards antibiotic resistance were indeed elevated at fever temperatures, fever suppression (using widely available antipyretic drugs such as acetaminophen (10)) could be a successful tactic to lower the mutation rate and thus combat the emergence of resistance.

Therefore, we here examine the temperature dependence of the mutation rate towards antibiotic resistance in *Escherichia coli*. We assessed the mutation rate towards resistance at two temperatures: 37 °C (representing a normal body temperature) and 40 °C (representing a typical fever temperature). We performed these measurements using a fluctuation-test approach to determine the in-vitro mutation rate towards antibiotic resistance for three different antibiotics, each with a different mode of action: ciprofloxacin (a topoisomerase and DNA-gyrase inhibitor) (11), rifampicin (a DNA-dependent RNA polymerase inhibitor) (12) and ampicillin (a peptidoglycan transpeptidase inhibitor) (13).

Results

Temperature dependence of the mutation rate

We used a fluctuation-test approach to assess the mutation rate towards antibiotic resistance (14, 15, 16, 17). As shown in Figure 1, the mutation rate depended on temperature, yet the direction of this dependency differed between antibiotics. The mutation rate towards ciprofloxacin and rifampicin resistance was higher at 40 °C than at 37 °C. In contrast, the mutation rate

towards ampicillin resistance was higher at 37 °C than at 40 °C. The patterns of temperature dependence were for each antibiotic robust to using different methods for quantifying mutation rates, including different estimators of final population size (results for CFP fluorescence see supplement 2.4), different estimation methods (maximum likelihood & generating function) and different lifetime models (Luria-Delbrück model with exponential lifetimes, Haldane model with constant lifetimes and inhomogeneous model; see supplement 2.5).

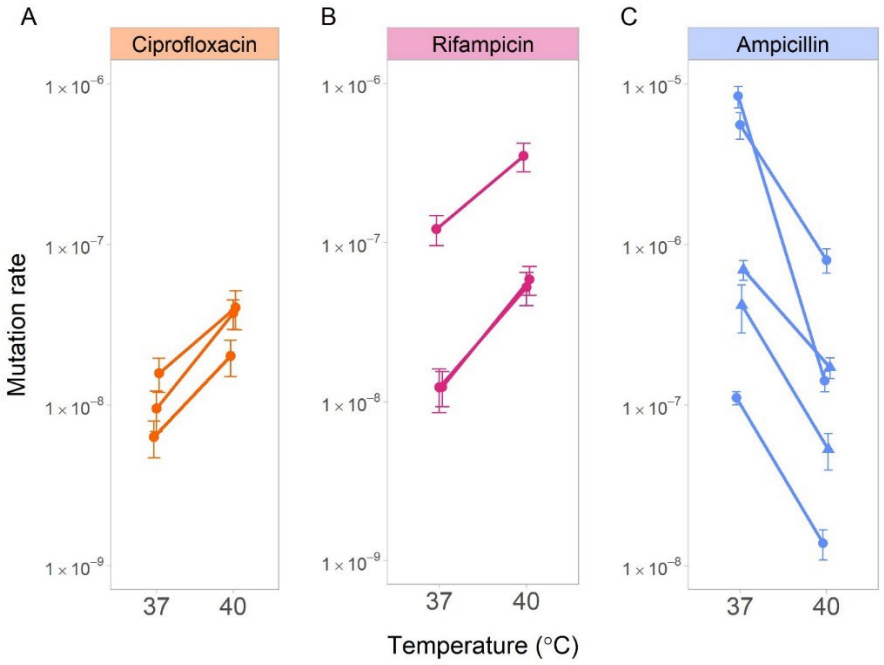

Figure 1. Effect of temperature on the mutation rate towards antibiotic resistance. At least three replicate experiments were conducted for each of the three antibiotics: **(A)** ciprofloxacin, **(B)** rifampicin, and **(C)** ampicillin. Points show the estimated mutation rate for a given experiment and temperature, with error bars corresponding to ± 1 standard error. Lines join the result for 37 °C and 40 °C for a single experiment; the difference between the mutation rate at 37 °C and 40 °C was statistically significant for all experiments (for ciprofloxacin the three p-values ranged between 0.001 and 0.035; for rifampicin, the three p-values ranged between 0.0002 and 0.003; for ampicillin, the five p-values were all below 0.009). Circular points are for experiments performed at the University of Groningen, Netherlands; triangular points are for those performed at the University of Montpellier, France (protocol and sample size for these experiments deviate slightly, see supplement 1.3).

The estimation of population sizes was based on CFU counts. We used the estimation method maximum likelihood, and the Luria-Delbrück exponential lifetime model (default settings in FLAN (18)).

Sequencing

To confirm that the colonies growing on plates containing antibiotics are indeed resistant mutants, a small set of mutants (three per temperature per antibiotic, 18 in total) was fully sequenced. We managed to clearly identify mutations in resistance genes for most mutants (13/18; see supplement 2.3).

Temperature-dependent antibiotic efficacy

To determine whether the observed changes in mutation rate (i.e. the rate of appearance of resistant phenotypes) were due to temperature-dependent antibiotic efficacy, we assessed antibiotic efficacy at the two experimental temperatures, also accounting for the liquid culture temperature (see supplement 2.6). For ampicillin, we found that it was consistently more effective at 40 °C than 37 °C. For rifampicin, the results were inconclusive due to an unexpectedly low sample size. For ciprofloxacin, we did not find a consistent effect of temperature on antibiotic efficacy. Instead, we found that the efficacy increased slightly when the protocol included a temperature change, i.e. when the antibiotic incubation temperature was different from the liquid culture temperature. In our fluctuation-test protocol, such a change in temperature does not occur, therefore this does not impact our mutation-rate estimates.

Discussion

We found that the mutation rate towards antibiotic resistance was temperature-dependent. A three-degree temperature change, such as the one associated with fever, altered the rate at which resistant mutants appeared by almost an order of magnitude for all three antibiotics considered. All else being equal, a higher rate of appearance of mutants with a resistant phenotype (mutation rate towards antibiotic resistance) makes evolutionary rescue more probable (3). This would thus increase the probability that a bacterial population successfully evolves resistance, which in turn could lead to treatment failure (2, 19). Furthermore, the average timeframe in which resistance evolves will be shorter. These effects should

be especially pronounced if the standing genetic variation at the start of infection is relatively low, as would be expected due to the usually small size of the initial inoculum (3, 20). Besides the mutation rate, other parameters that determine the probability of evolutionary rescue, such as the rate of population decay or the establishment probability of mutants (see (3)), may also be affected by temperature, a possible avenue for further study.

For ciprofloxacin (a topoisomerase inhibitor) (11) and rifampicin (a DNA-dependent RNA-polymerase inhibitor) (12) we found that fever temperatures led to an increase in the mutation rate. These findings are consistent with the general finding that mutation rates are higher under suboptimal conditions. Such stress-induced mutagenesis could be an evolved response to facilitate evolutionary rescue under stressful conditions (9). However, in the case of temperature it could also be a side-effect of the fact that all biochemical processes (including those leading to mutation) are accelerated at higher temperatures (5, 8). Regardless of the mechanism, this suggests that all else being equal, resistance towards these antibiotics would be more likely to evolve under fever temperatures.

For ampicillin (a transpeptidase inhibitor) (13), contrary to our expectations, fever temperatures led to a decrease in the mutation rate towards resistance. This result was replicated in two different labs (Groningen and Montpellier), demonstrating its robustness. The observed effect of temperature on the mutation rate (i.e. rate of appearance of resistant phenotypes) could be due to a difference in the rate at which mutations appear in the genome (genomic mutation rate) or to a difference in the proportion of genomic mutations that convey resistance. The proportion of mutations that convey resistance could be influenced by temperature if the efficacy of an antibiotic is temperature-dependent (i.e. mutations that convey resistance at one temperature do not convey resistance at another temperature). Therefore, we conducted an experiment which showed an increase in the efficacy of ampicillin at 40 °C (see supplement 2.6, including results for ciprofloxacin and rifampicin). Thus, the surprising result for ampicillin may largely be explained by this increase in efficacy of ampicillin at the higher temperature, as some of the mutations conveying resistance at 37

°C may not be able to do so at 40 °C, leading to a decrease in the rate of occurrence of resistant mutants. A recent study by Cruz-Loya et al. (21) also found indications for the temperature-dependent efficacy of ampicillin, showing that the presence of ampicillin caused a greater reduction in growth rates at higher temperatures. They hypothesised that this may be due to a synergistic effect between antibiotic-induced cell wall damage and the increased membrane permeability that occurs at higher temperatures. Regardless of the exact mechanism of temperature dependence, our experiments demonstrate that the rate of appearance of mutants resistant to ampicillin is temperature dependent, possibly due to the temperature-dependent efficacy of the antibiotic. It is ultimately the rate of appearance of resistant mutants that determines the probability of resistance evolution. Therefore, all else being equal, ampicillin resistance is less likely to evolve under fever temperatures.

The difference in the mutation rates observed at 37 °C and 40 °C is consistent between different repeats of the same experiment, and for ampicillin even showed replicability across labs. However, for all antibiotics, the estimates of the mutation rate differed considerably (sometimes by an order of magnitude) between replicate experiments. We can only speculate that these differences resulted from small variations (across replicates) in environmental conditions in the lab or small differences in the physiological state of the pre-culture cells from which the dilute cell suspension was made.

It would be worthwhile in the future to investigate how the findings presented here translate to different antibiotics and bacterial species. Furthermore, it would be important to study how our current *in vitro* results translate to the *in vivo* context, as fever has wider effects than merely increasing temperature (such as stimulating immune function (22)). Finally, the antibiotic concentrations considered here were chosen so as to maximise the ability to detect changes in the mutation rate and are therefore lower than those typically used in a clinical setting; future work should reveal whether this pattern holds with higher, clinically relevant concentrations.

The temperature dependence of mutation rates towards antibiotic resistance implies that the evolution of antibiotic resistance (i.e. evolvability

in the context of antibiotic resistance (23)) is influenced by temperature. This study opens up a new way to combat the emergence of antibiotic resistance: decisions regarding fever suppression could be leveraged to minimise the mutation rate in order to slow down or prevent the evolution of antibiotic resistance.

Methods

Fluctuation test

We used *Escherichia coli* strain REL4548-CFP-lux, a strain from Lenski's long-term evolution experiment (24, 25, 25), transformed by chromosomic insertion of a CFP reporter gene (27) and a chromosomic insertion of lux genes (28) (see supplement 1.1). We estimated the mutation rate towards antibiotic resistance using a Luria-Delbrück fluctuation test (14, 15, 16, 17). Three experiments were performed for each antibiotic in Groningen; an additional two were conducted for ampicillin in a different lab in Montpellier (the protocol for these additional two experiments deviates slightly and is described in detail in the supplement 1.3). For each experiment and each temperature (37 °C and 40 °C), 40 replicate populations, all originating from one dilute cell suspension, were grown in liquid culture. When populations were in the mid-exponential phase, population density and the number of antibiotic-resistant mutants were assessed for each population. Population density was estimated using two complementary methods: by counting colony forming units (CFU) and by measuring the fluorescence of cyan fluorescent protein (CFP; CFP fluorescence-based results shown in supplement section 2.4). The number of resistant mutants in each population was assessed by counting the number of colonies that were able to grow when the population was plated on an antibiotic-containing agar plate. These antibiotic-containing agar plates were incubated at the same temperature as the populations were initially grown at; i.e., a population grown at 37 °C was plated at 37 °C. Finally, the R package FLAN was used to infer mutation rates using several different estimation methods (17) (shown in supplement 2.5). We also used the FLAN package to conduct a fluctuation analysis test comparing the mutation rates at the two temperatures. There was a significant day effect for mutation rates, so the experiments for each

antibiotic were analysed in a pairwise manner – i.e. for each comparison, mutation rate estimates were obtained for both temperatures on the same day and from an identical starting culture (with the exception of one of the two ampicillin experiments performed in Montpellier).

Temperature-dependent efficacy

We observed differences in the mutation rate (rate of appearance of resistant phenotypes). This effect could be due to a difference in the rate at which mutations appear in the genome (genomic mutation rate) or due to a difference in the proportion of genomic mutations that convey resistance. The proportion of mutations that convey resistance could be influenced by temperature if the efficacy of an antibiotic is temperature-dependent; in that case, mutations that convey marginal resistance at one temperature may be ineffective in conveying resistance at another temperature. Therefore, we conducted experiments to test for temperature-dependent antibiotic efficacy. The experiment was carried out as above with the following alteration: when assessing the number of resistant mutants in a population (grown in liquid culture at 37 °C or 40 °C), each population was split in half, and half of the population was grown on antibiotic-containing plates at 37 °C and the other half was grown on antibiotic-containing plates at 40 °C. This allowed us to assess whether the temperature at which the antibiotic-containing plates were incubated, affected the number of observed mutants. If an antibiotic is equally efficacious independent of temperature, the temperature at which the antibiotic-containing plates are incubated should not affect the number of mutants observed in a particular population. The number of mutants found at each temperature from each population was compared using a Wilcoxon signed-rank test, taking into account the liquid culture temperature (see supplement 2.6).

Data availability

The data supporting the findings of this study is available at:
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Author contributions

This study was conceptualized by T.J.B.v.E. with input from E.A.S., G.M., F.J.W. and G.S.D. The initial ampicillin experiments conducted in Montpellier (FR) were conducted and designed by T.J.B.v.E. and G.M. The main experiments (based on the previously developed design) were conducted in Groningen (NL) by E.A.S. under the supervision of T.J.B.v.E. and G.S.D. During this time resources were provided by O.P.K. Analysis was performed by E.A.S. and T.J.B.v.E. with input from F.J.W., G.M. and G.S.D. All authors contributed to the writing of the manuscript.

Declaration of interests

The authors declare no competing interests.

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Supplementary material

Detailed Methods

Strain & media

The bacterial strain used in this experiment was *Escherichia coli* REL4548-CFP-lux. The ancestor of this strain was isolated in the long-term evolution experiment where *E. coli* REL606 adapted for 10000 generations to DM25 medium (Lenski et al., 1991; Lenski & Travisano, 1994; Elena et al. 1998). Subsequently, this strain was transformed with a chromosomally integrated constitutive high expression CFP marker (Gallet et al. 2012) and kindly provided by Romain Gallet. Subsequently, this strain was transformed with a chromosomally integrated lux reporter genes according to the protocol described by Howe et al. (2010). The plasmid used for the chromosomal integration of lux reporter genes can be found here https://www.addgene.org/69150/. The resulting strain REL4548-CFP-lux, which we used in our experiment, constitutively expresses CFP and lux reporter genes, allowing for close monitoring of cell density and metabolic activity respectively.

CFP fluorescence was measured using a BMG Labtech CLARIOstar with excitation = 431 ± 15 nm and emission = 472 ± 15nm. All experiments were conducted using Davis minimal media containing 1 mg/mL glucose (DM1000). For plating, this medium was supplemented with 1.6% (w/v) agar and, when required, antibiotics were added to give the following final concentrations: rifampicin 10 µg/mL, ciprofloxacin 0.013 µg/mL, and ampicillin 1.5 µg/mL. These concentrations were chosen by plating 200 µL of culture on a range of antibiotic concentrations and selecting a concentration that resulted in a countable number of mutants, thereby maximizing power to detect changes in the mutation rate. Rifampicin stocks were made by dissolving rifampicin (Sigma) in 0.1N HCl; ciprofloxacin stocks were made by

dissolving ciprofloxacin (Sigma) in 0.1N HCl; and ampicillin stocks were made by dissolving ampicillin sodium (Sigma) in sterilized distilled water.

Fluctuation test

To measure the mutation rate at two different temperatures, a fluctuation test was performed. To set up the experiment, *E. coli* REL4548-CFP-lux was grown to stationary phase overnight at 37 °C (incubator: INCU-line, IL 56 Premium; agitation: Grant-Bio PMS 1000i, shaking = 540 PM). For the ampicillin fluctuation tests, the stationary-phase culture was diluted with a factor 10^6 to create the diluted cell suspension needed to initialize the fluctuation test. For ciprofloxacin and rifampicin, the stationary-phase culture was refreshed after approximately 24 hours. This was done by diluting the culture with a factor 10 and growing it for another 2.5 hours at 37 °C. Refreshing the overnight culture ensured that all cells were in the exponential growth phase prior to the start of the experiment, minimizing the lag time and thereby reducing possible differences in final population densities caused by stochastic variations in lag time. The refreshed culture was then adjusted to an OD_{600} value of 0.5 and diluted with a factor 2.5×10^5, creating the diluted cell suspension needed to initialize the fluctuation text

In all experiments, the diluted cell suspension was then used to initialize the fluctuation test, by establishing 40 populations in each of three 96-well plates (Greiner bio one, clear), for a total of 120 populations. To minimize the effect of evaporation only the inner wells of the plate were used, with the wells bordering the edge of the plate filled with medium. Each population consisted of 200 μL of diluted culture, with each population containing approximately 100 cells for the ampicillin experiments and approximately 200 cells for rifampicin and ciprofloxacin experiments. These initial cell numbers per population are somewhat higher than the single cell assumed by the fluctuation test, yet they are low enough to ensure that the probability that mutations are present at the start of the experiment is small (hence ensuring statistical independence between resistance emergence events). These somewhat higher initial cell numbers allow to limit stochastic population extinction upon starting lineages, and to limit the differences in

the final population density (after a fixed growth period) caused by early stochastic growth dynamics. The first 96-well plate was used to monitor cell growth in a plate reader (shaking = 400 RPM, double orbital). This monitor plate served as a rough indication of when the populations were in mid-exponential phase, this plate was not directly used to gather any data. The other two plates were grown under agitation (Grant-Bio PMS 1000i; ciprofloxacin and rifampicin shaking = 700 RPM; ampicillin shaking = 540 RPM) in identical incubators (INCU-line, IL 56 Premium) at 37 °C and 40 °C respectively.

When the populations were roughly in mid-exponential phase (determined using CFP fluorescence) the number of mutants in each population was assessed by plating the entire population on agar plates containing antibiotics. These agar plates were incubated for five or six days at 37 °C or at 40 °C (at the temperature at which the populations were grown in the 96-well plate). Subsequently, the number of mutants on each agar plate was counted. The final population densities at the time of plating were determined in two ways: through CFU-counting and CFP fluorescence measurements. For CFU counting a 2 μL sample was taken from a subset of 12 of the populations (for one of the AMP experiments a subset of 4 populations was used), this sample was subsequently diluted with a factor of 10^5 and plated on agar plates without antibiotics, this provided an estimate of the mean final population densities. For CFP fluorescence all replicate populations were measured in plate reader (BMG Labtech CLARIOstar, excitation = 431 ± 15 nm, emission = 472 ± 15nm), and using a calibration curve the final number of cells for each replicate population could be estimated. This experiment was repeated three times for each antibiotic in Groningen (NL).

The R-package FLAN (Mazoyer et al. 2016) was used to analyze the data, using the “mutestim” function to compute mutation rate estimates and using the “flan.test” function to perform a fluctuation analysis test to compare the mutation rate between the two temperatures. Several different estimation methods and lifetime models implemented in the FLAN package were used.

Fluctuation tests ampicillin conducted in Montpellier

For ampicillin, two additional fluctuation test experiments were conducted in Montpellier (FR). The experiments shown are a subset of pilot experiments used to create a fine-tuned protocol, hence the protocol for these two experiments deviates slightly from the one described above for the experiments conducted in Groningen. Nonetheless, these experiments are included as they illustrate that the patterns observed for ampicillin replicate across two different labs.

First of all, populations created from the dilute cell suspension had a culture volume of 250 μl. To determine the population densities only a subset of 8 populations (instead of 12) were sampled, diluted with a factor 10^5 and plated on agar plates without antibiotics. No CFP measurements of final population density were performed. For the first ampicillin experiment conducted in Montpellier the experiments for the two temperatures 37 °C and 40 °C were conducted on two different days, the number of replicate populations also differed between these two days. For 37 °C, 14 populations were plated on antibiotic plates. For 40 °C, 30 populations were plated on antibiotic plates. For the second ampicillin experiment conducted in Montpellier the experiments for the two temperatures, 37 °C and 40 °C , were performed on the same day. For each temperature a total of 52 populations was plated on antibiotic plates to asses the number of mutants in each population.

Supplemental results

Several additional experiments were conducted to verify that some of assumptions of the fluctuation test experiments hold. This included comparing the growth rates at 37 °C and 40 °C and comparing the plating efficiency at 37 °C and 40 °C. Additionally, a limited set of mutants was fully sequenced to confirm that the colonies growing on antibiotic containing plates do indeed represent genetic mutants. Furthermore, the mutation rates were estimated using CFP fluorescence as a measure of final population density (instead of CFU counting). In addition, the mutation rates were estimated using several different estimation methods and lifetime models implemented in the FLAN package. Finally, an experiment was conducted to assess temperature-dependent antibiotic efficacy.

Comparing growth rates

A fluctuation test assumes a pure birth process, hence differences in growth rates between the two treatments can cause discrepancies (i.e., a different total number of divisions between the treatments). Therefore, the growth rate of *E. coli* REL4548-CFP-lux was at measured at 37 °C and 40 °C using CFP fluorescence. An overnight culture was diluted 10^6-fold and was used to create populations in a 96-well plate in a similar way to the fluctuation test. This plate was subsequently grown until stationary phase in the plate reader. This was repeated on two separate days: on the first day the 96-well plate was incubated at 37 °C, and on the second day the 96-well plate was incubated at 40 °C . To prevent any effect of evaporation on the wells on the edge of 96-well plate were excluded from the analysis, resulting in 54 replicate populations for each temperature. To compare growth rates between the two temperatures the linear portion of each population's log density dynamics (corresponding to exponential growth) was plotted (figure S1). Visual inspection as well as a statistical test (figure S2) indicated that the growth rate was not different between 37 °C and 40 °C (p = 0.7881).

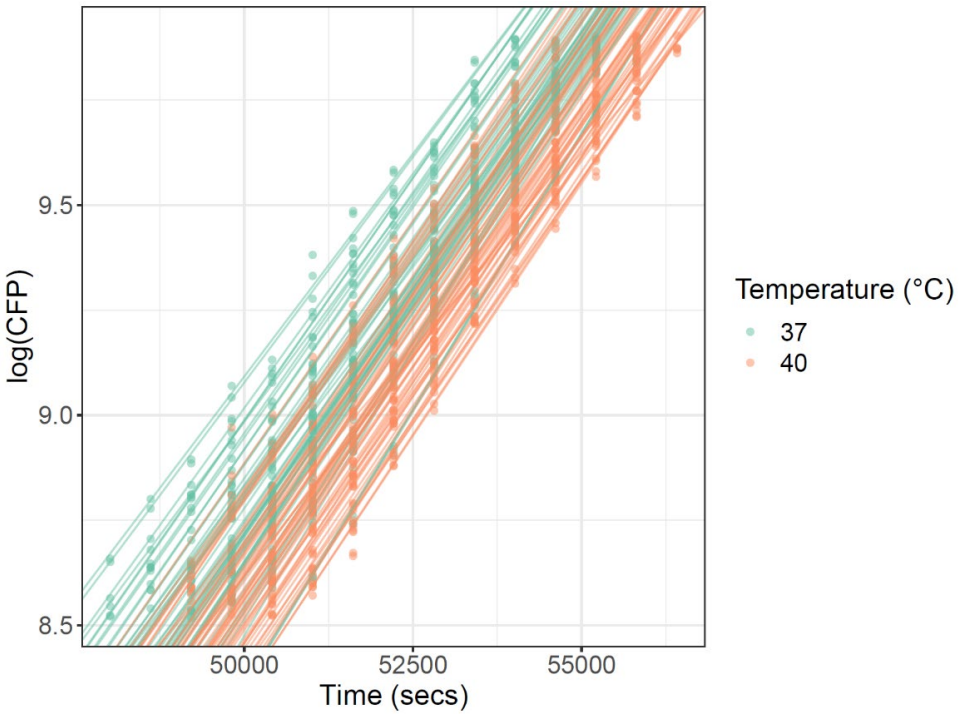

Figure S1. Linear portion of the log density dynamics (log (CFP)), including the linear models fit to these dynamics.

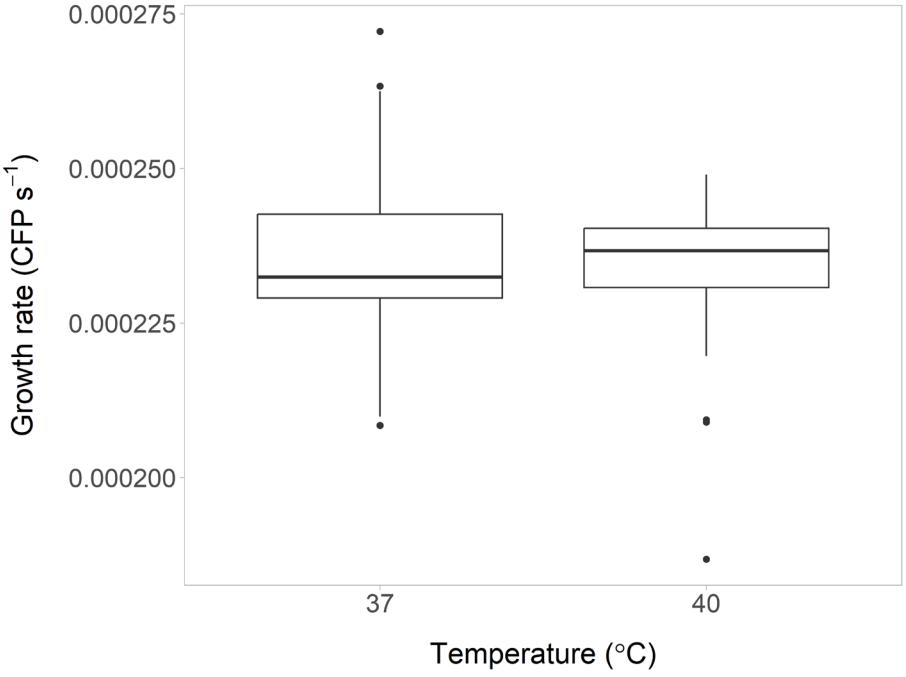

Figure S2. Temperature did not significantly affect growth rate. The linear portion of the log density dynamics was extracted and the slopes compared between temperatures using a *t*-test (p = 0.7881). Vertical line shows the median, box shows the interquartile range.

Comparing plating efficiency

Differences in plating efficiency between the two different temperatures could cause discrepancies in estimating final population densities. Therefore, the plating efficiency of cells plated on standard agar plates (without antibiotics) was compared between the two temperatures. Specifically, an overnight culture was grown to stationary phase prior to being diluted 10^6-fold. 25 µl of this dilution was then plated on 40 non-selective DM1000 agar plates. Subsequently, half of the plates were grown at 37 °C and half at 40 °C , resulting in 20 replicates per temperature. Plates were photographed and counted as for the fluctuation test. The resulting data is shown in figure S3, a Wilcoxon rank sum test showed no significant difference in plating efficiency between 37 °C and 40 °C (p = 0.3935).

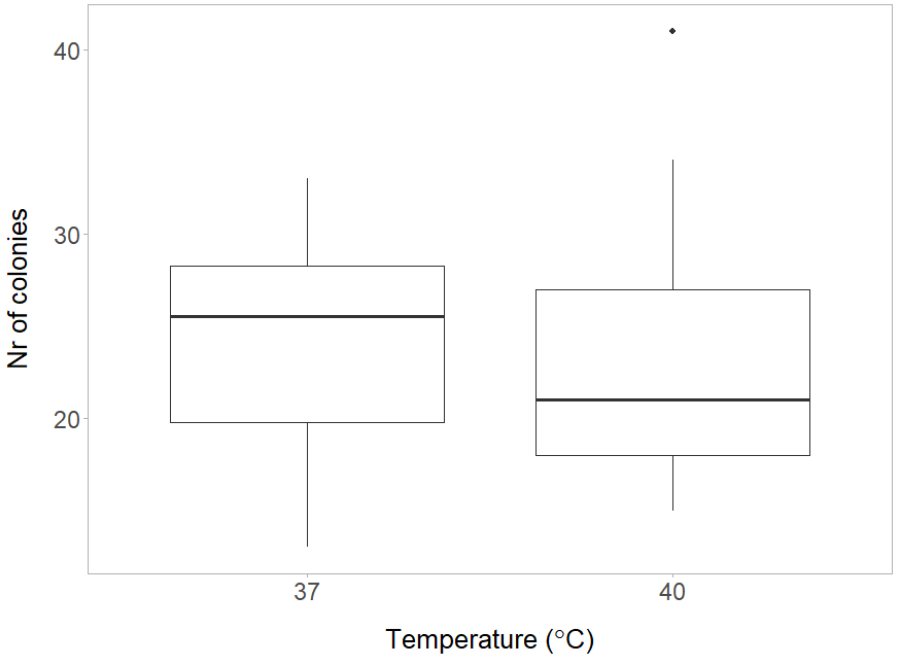

Figure S3. Comparing the plating efficiency of cells plated on standard agar plates (without antibiotics) between the two temperatures 37 °C and 40 °C. Vertical line shows the median, box shows the interquartile range.

Sequencing results

To see if the colonies observed on the agar plates with antibiotics do indeed have mutations conferring resistance, a small subset of mutants was fully sequenced (three per temperature per antibiotic, 18 in total) and their genomes were screened for relevant resistance mutations. Subsequently, the mutants and the wild-type REL4548-CFP-lux strain were grown in DM1000 and DNA was extracted using the GenElute Bacterial Genomic DNA Kit (Merk). The extracted DNA was quantified using a nanodrop and subsequently shipped to BGI Europe for sequencing, where sequencing was performed on a DNBseq machine using a read length of 150 bp, sequencing about 10 million reads per sample. The resulting reads for the starting strain and the mutants were aligned to the REL606 reference sequence (NC_012967.1, resulting in approximately 300X coverage) and the mutations were analysed using Breseq (Detherage & Barrick, 2014). The differences between the reference and the starting strain were subtracted from those in the mutants so that only mutations that occurred during the experiment were observed. The results are summarized in Table S1. Mutations in known resistance associated genes were identified in 13/18 mutants tested.

Table S1. Resistance mutations identified using Breseq in fully sequenced mutants and references to sources identifying and studying similar mutations.

Anti-biotic	Temper-ature	Nr	Mutation	Position	Gene mutated	Citation
Amp	37	1	A→C L350F (TTA→TTC)	95,266	Peptidoglycan glycosyltransferase FtsI (*ftsI*)	Li et al. 2019
Amp	37	2	G→A L714L (CTG→CTA)	433,084	Endopeptidase La (lon)	Nicoloff et al. 2013
Amp	37	3	G→T E96D (GAG→GAT)	3,464,981	2-component system response regulator OmpR (*ompR*)	Jordan et al. 2022
Amp	40	1	+AA coding (498/1089 nt)	1,003,577	porin OmpF (ompF)	Jordan et al. 2022
Amp	40	2	-	-	-	-
Amp	40	3	Δ2 bp coding (264-265/1353 nt)	3,464,288	2-component system sensor histidine kinase EnvZ (*envZ*)	Jordan et al. 2022
Cipro	37	1	-	-	-	-
Cipro	37	2	-	-	-	-
Cipro	37	3	Δ1 bp coding (773/1089 nt)	1,003,302	porin OmpF (*ompF*)	Vinue et al. 2015; Kishi & Takei 2009; Forst et al., 1989
Cipro	40	1	-	-	-	-
Cipro	40	2	Δ9 bp intergenic (-9/+585)	1,004,083	porin OmpF/asparagine--tRNA ligase (*ompF/asnS*)	Vinue et al. 2015; Kishi & Takei 2009; Forst et al., 1989
Cipro	40	3	Δ314 bp	3,464,348	2-component system response regulators (*envZ, ompR*)	Vinue et al. 2015; Kishi & Takei 2009; Forst et al., 1989
Rif	37	1	T→C I530T (ATC→ACC)	4,162,444	DNA-directed RNA polymerase subunit beta (*rpoB*)	Weinstein & Zaman 2018
Rif	37	2	G→T R529L (CGT→CTT)	4,162,441	DNA-directed RNA polymerase subunit beta (*rpoB*)	Weinstein & Zaman 2018
Rif	37	3	-	-	-	-
Rif	40	1	T→A I572N (ATC→AAC)	4,162,570	DNA-directed RNA polymerase subunit beta (*rpoB*)	Weinstein & Zaman 2018
Rif	40	2	T→A I572N (ATC→AAC)	4,162,570	DNA-directed RNA polymerase subunit beta (*rpoB*)	Weinstein & Zaman 2018
Rif	40	3	T→A L511Q (CTG→CAG)	4,162,387	DNA-directed RNA polymerase subunit beta (*rpoB*)	Weinstein & Zaman 2018

Estimation of mutation rates using CFP fluorescence

A calibration curve was constructed to translate CFP fluorescence values to colony forming units (CFU). To construct this calibration curve, a stationary phase culture was repeatedly diluted two-fold and CFP emissions for each dilution read using the plate reader (BMG Labtech CLARIOstar, excitation = 431 ± 15 nm, emission = 472 ± 15nm, shaking = 400 RPM, double orbital). To collect CFU data, a separate dilution was made so that there was a sufficient volume of bacterial culture for plating. From this diluted culture, six replicates of each of three concentrations (0.5×10^{-6}, 1×10^{-6}, and 2×10^{-6}) of 200 µL each were plated on non-selective DM1000 agar and grown at 37 °C for approximately 24 hours before counting as above. Since all of these dilutions gave a countable number of cells, each replicate was converted to the number of cells in an undiluted population and the mean calculated. This was then used to calculate the expected number of cells in each of the dilutions for which CFP emissions had been measured. A linear model was computed between CFU as estimated above and CFP emissions, giving a calibration curve (figure S4). Final population densities were also estimated using CFP fluorescence. The calibration curve is shown in figure S4 was then used to infer final population densities for the fluctuation test experiments, using CFP fluorescence measurements. The obtained results are shown in figure S5, as can be observed they are in agreement with those obtained when estimating final population density using classical CFU counting.

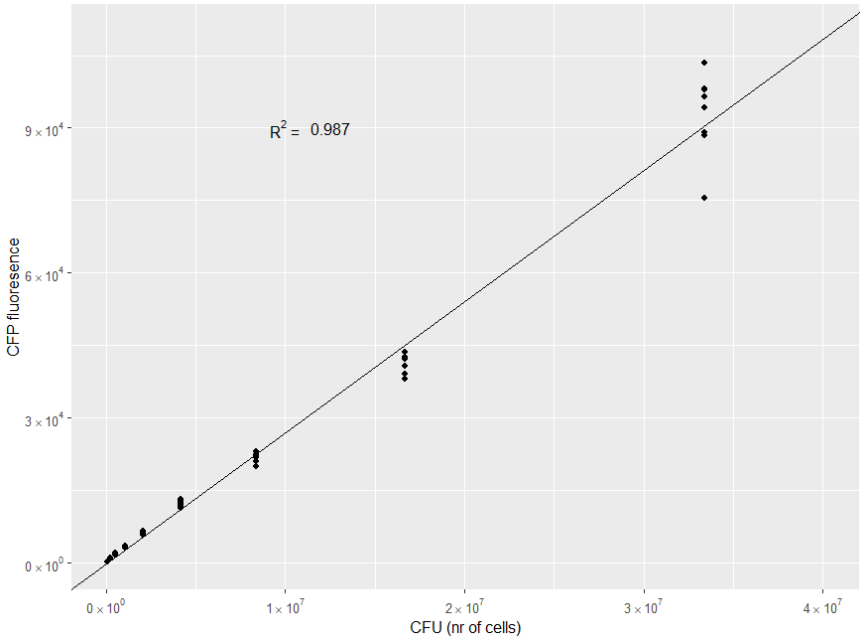

Figure S4. Calibration curve between CFP fluorescence and colony forming units (CFU)

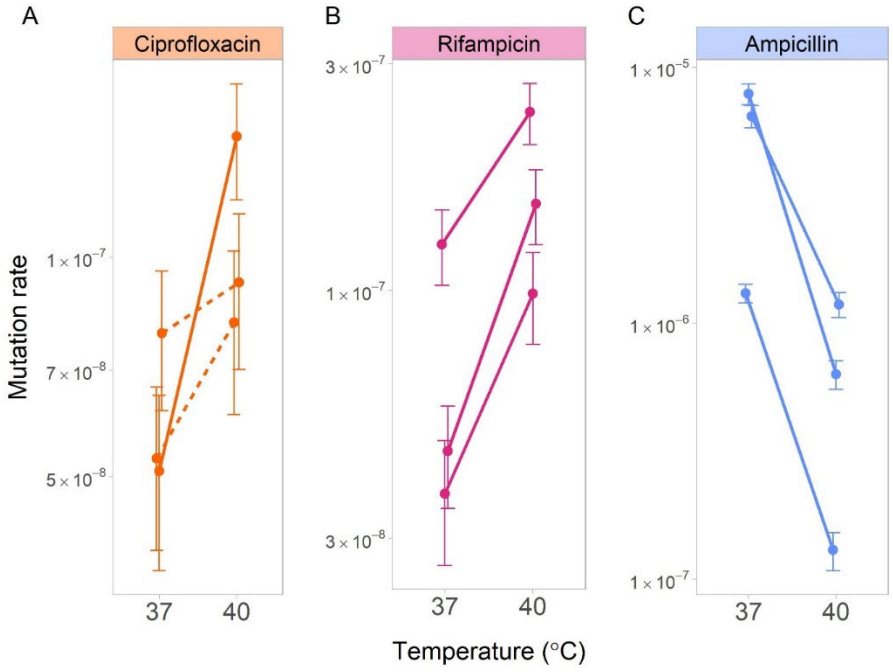

Figure S5. Mutation rates estimated using CFP fluorescence to assess final population density for each population: **(A)** ciprofloxacin, **(B)** rifampicin, and **(C)** ampicillin. Points show the estimated mutation rate for a given experiment and temperature, with error bars corresponding to ± 1 standard error. Lines join the result for 37 °C and 40 °C for a single experiment; significant differences between mutation rate at 37 °C and 40 °C ($p < 0.05$) are shown as solid lines, non-significant differences are shown as dotted lines. All experiments shown were performed at the University of Groningen, Netherlands. The mutation rates were estimated using the FLAN package, using the estimation method maximum likelihood, and using the Luria-Delbrück exponential lifetime model (default settings).

Different procedures for estimating mutation rates

Several procedures for inferring mutation rates are implemented in the FLAN package (17). These include two different estimation methods (maximum likelihood and generating function) and three different lifetime models (Luria-Delbrück exponential lifetime model, Haldane constant lifetime model and Inhomogeneous model). Figure 1 in the main text is based on the default setting in FLAN (maximum likelihood, Luria-Delbrück model). Figures S6 to S10 show the results when applying the other combinations of estimation method and lifetime model to our data. The figures show that our conclusions are robust to the method chosen: in all cases, the differences between the mutation rate at 37 °C and 40 °C were in the same direction and significant for all experiments ($p < 0.05$).

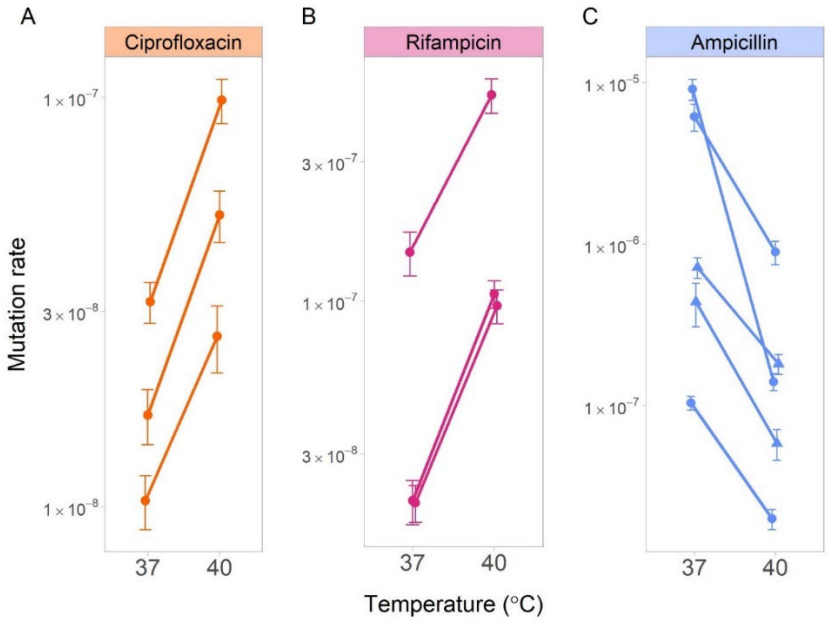

Figure S6. As Figure 1 in main text , but using the estimation method maximum likelihood, and using the Haldane constant lifetime model.

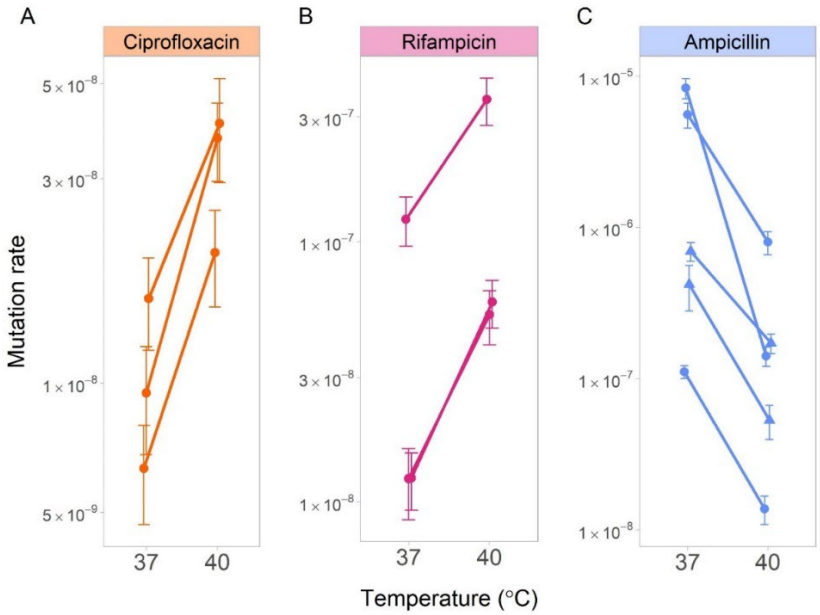

Figure S7. As Figure 1 in main text , but using the estimation method maximum likelihood, and using the inhomogeneous lifetime model.

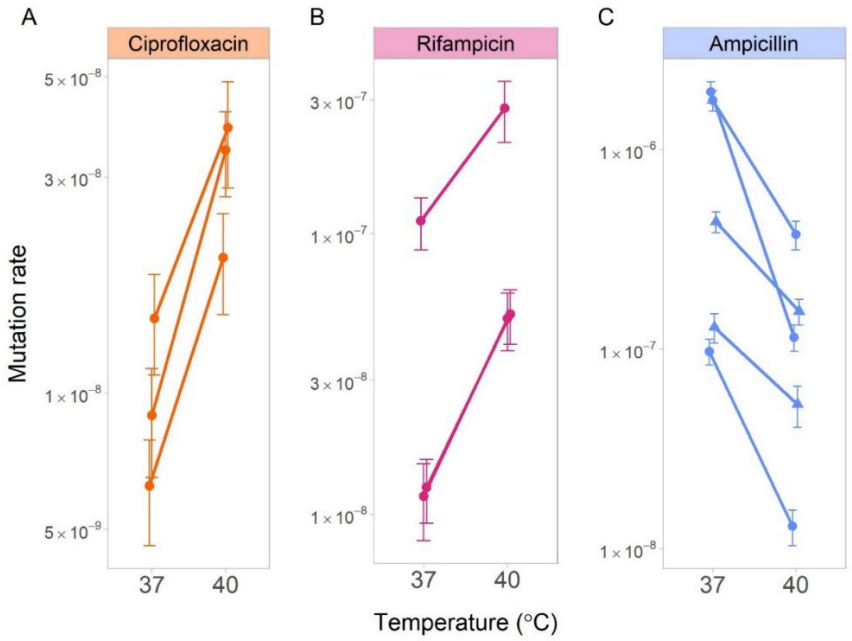

Figure S8. As Figure 1 in main text , but using the estimation method generating function, and using the Luria-Delbrück exponential lifetime model.

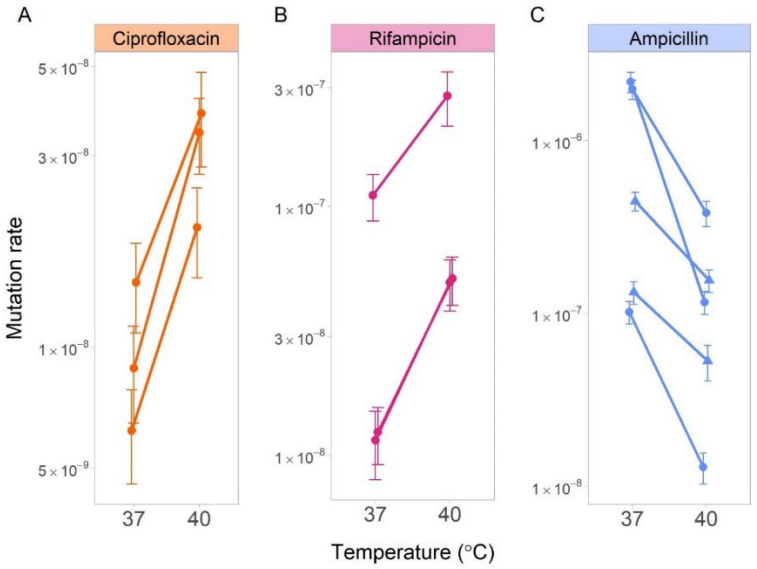

Figure S9. As Figure 1 in main text , but using the estimation method maximum likelihood, and using the Haldane constant lifetime model.

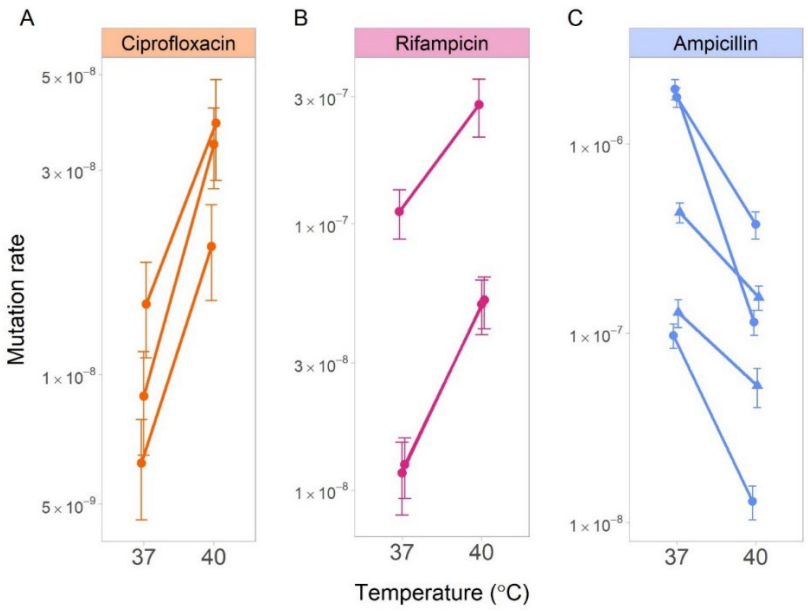

Figure S10. As Figure 1 in main text, but using the estimation method maximum likelihood, and using the inhomogeneous lifetime model.

Temperature-dependent antibiotic efficacy

Temperature-dependent changes in the mutation rate observed in the fluctuation test could be due to changes in the rate at which mutations occur in the genome (genomic mutation rate) or, alternatively, they could be caused by differences in the proportion of mutations that convey resistance at each temperature. The latter effect could be caused by temperature-dependent efficacy of the antibiotic, implying that mutations that convey resistance at one temperature are not able to convey resistance at the other temperature. To explore the temperature-dependent efficacy of each of the three antibiotics, we conducted a fluctuation test as described above. However with an important change, that when assessing the number of resistant mutants in a population (grown in a 96-well plate in liquid culture at 37 °C or 40 °C), each population was split in half, and half of the population was grown on antibiotic-containing plates at 37 °C and the other half was grown on antibiotic-containing plates at 40 °C. Therefore, for each liquid culture temperature, there were two different antibiotic agar plate

culture incubation temperatures. For each population, the number of mutants on each of the two antibiotic agar plates should be equal if there is no temperature dependent efficacy of the antibiotic, and if we ignore the production of de novo mutations during growth on the agar plates, as is classically done in fluctuation test data analysis. If on the other hand the efficacy of the antibiotic is different per temperature, some of the mutants that are resistant at one temperature should not be resistant at another temperature, leading to a difference in the observed number of mutants on the two agar plates originating from the same liquid culture population. Only those liquid culture populations in which at least one mutant was detected were included in the analysis, since being able to detect the impact of antibiotic agar incubation temperature is conditional on the presence of mutants in the liquid culture populations. For each liquid culture temperature, the impact of antibiotic agar incubation temperature was assessed using a Wilcoxon signed-rank test.

For ciprofloxacin (figures S11 and S12) it was found that for both liquid culture temperatures, the antibiotic agar incubation temperature significantly impacted the number of mutants detected (for liquid culture 37 °C $n = 22$ and $p = 0.0394$, for liquid culture 40 °C $n = 13$ and $p = 0.0422$). However, as can be seen in figure S12, the sign of this effect was different for each liquid culture temperature. There is thus no consistent effect of temperature on the efficacy of ciprofloxacin, instead it seems that a change in temperature between the liquid culture phase and the growth on antibiotic agar increases the efficacy of ciprofloxacin. We hypothesize that a change in temperature causes a mild-stress on the cells, subsequently ciprofloxacin works more effectively to kill these mildly stressed cells. We note that such a change in temperature does not occur in our original fluctuation-test protocol as antibiotic agar plates are incubated at the same temperature as the liquid culture from which they originate. Therefore, the effect of a change in temperature on the efficacy of ciprofloxacin should not impact our mutation rate estimates obtained using the fluctuation test.

For rifampicin the sample size was unexpectedly small (for liquid culture 37 °C $n = 2$, for liquid culture 40 °C $n = 4$) and hence no statistical testing could

be conducted, the data is shown in figure S13. For ampicillin (figures S14 and S15) a consistent effect of antibiotic agar incubation temperature was detected regardless of liquid culture temperature (for liquid culture 37 °C $n = 17$ and $p = 0.0003$, for liquid culture 40 °C $n = 14$ and $p = 0.0024$). This clearly indicates that the efficacy of ampicillin is temperature dependent, and/or that de novo mutations, on the plates, contribute to the outcome, and they are produced at different rates in different temperatures.

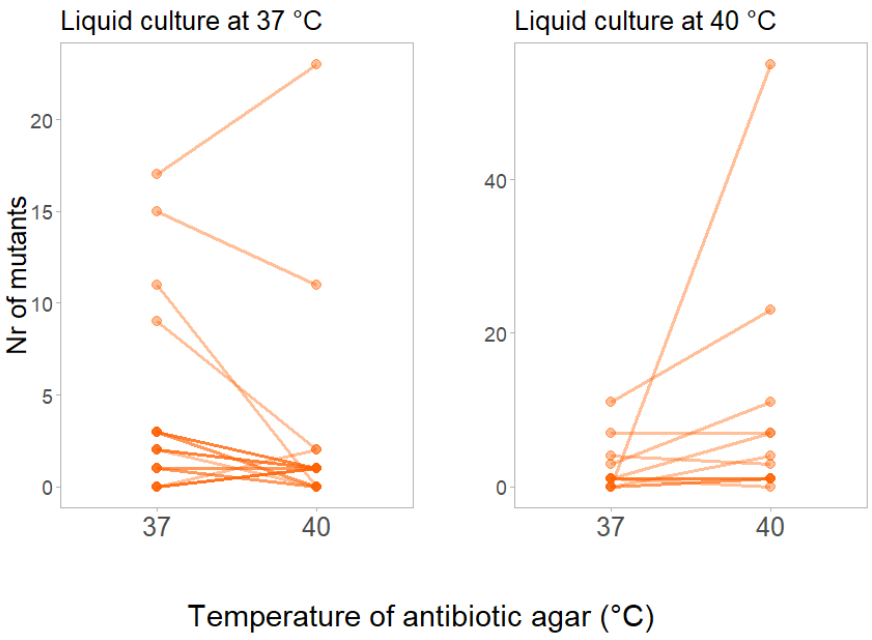

Figure S11. The number of ciprofloxacin resistant mutants detected in each population at each antibiotic agar incubation temperature, lines connect samples originating from the same liquid culture. The left panels shows populations grown in liquid culture at 37 °C. The right panel shows populations grown in liquid culture at 40 °C.

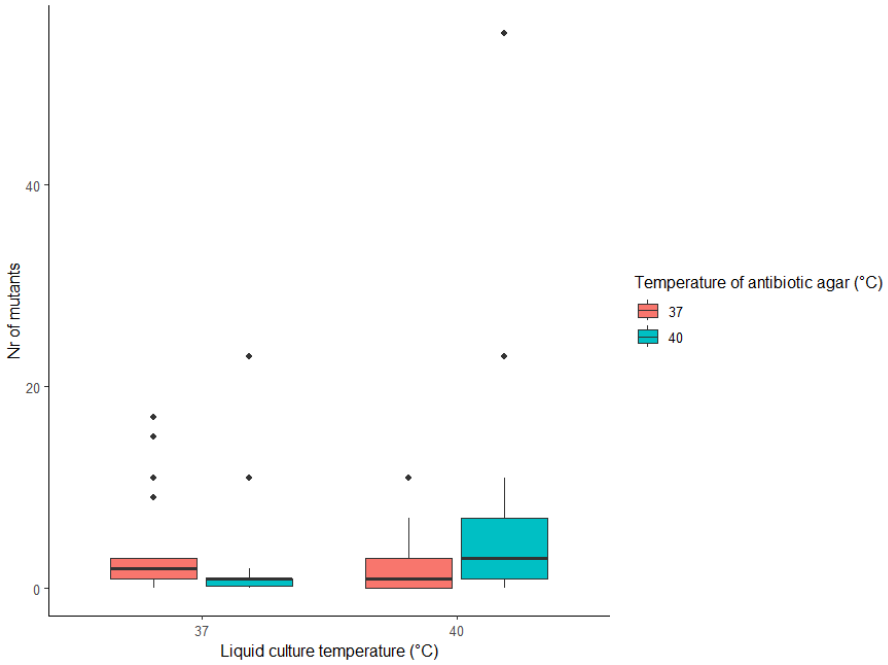

Figure S12. Boxplot showing the number of ciprofloxacin resistant mutants detected depending on the antibiotic agar incubation temperature. This plot shows the data populations grown in liquid culture at 37 °C (left) and at 40 °C (right).

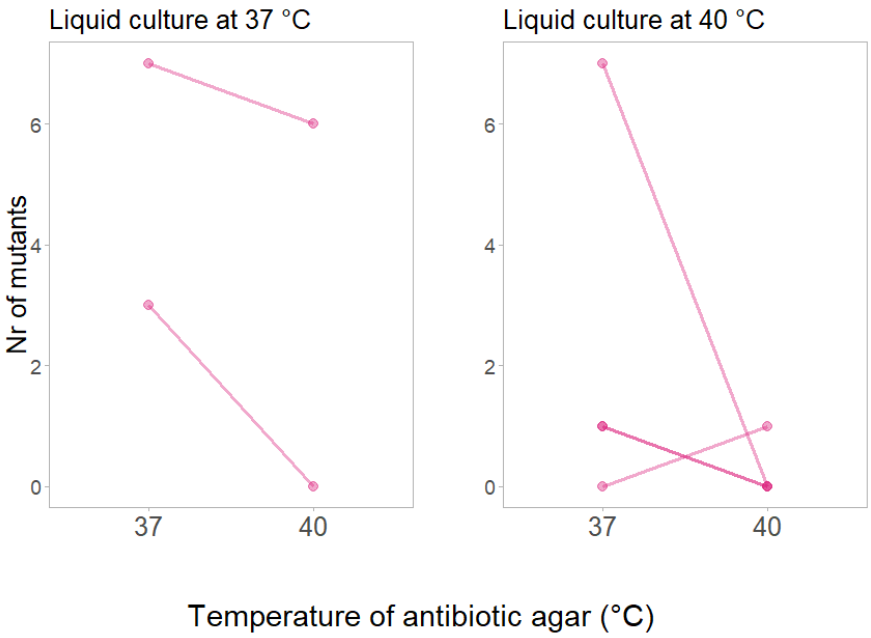

Figure S13. The number of rifampicin resistant mutants detected in each population at each antibiotic agar incubation temperature, lines connect samples originating from the same liquid culture. The left panels shows populations grown in liquid culture at 37 °C. The right panel shows populations grown in liquid culture at 40 °C.

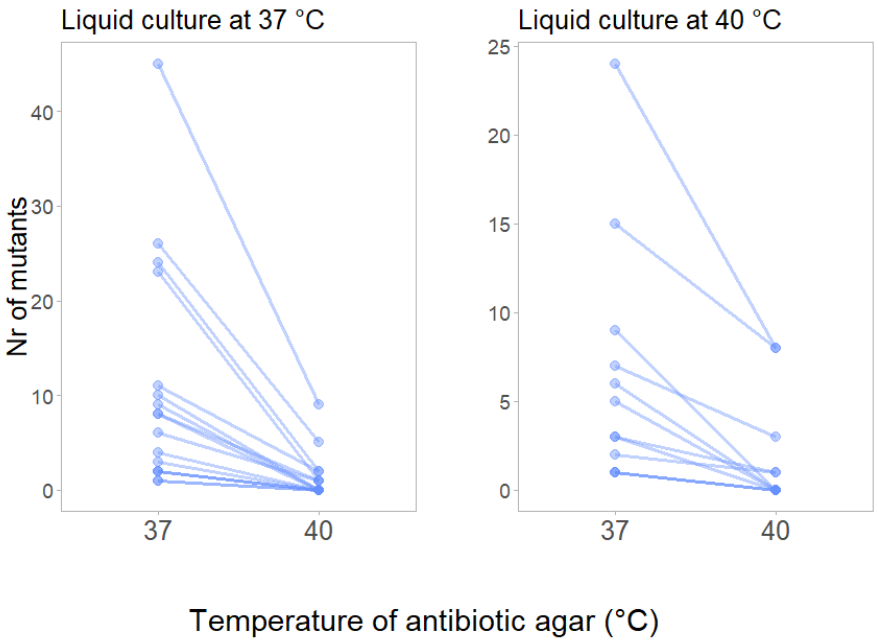

Figure S14. The number of ampicillin resistant mutants detected in each population at each antibiotic agar incubation temperature, lines connect samples originating from the same liquid culture. The left panels shows populations grown in liquid culture at 37 °C. The right panel shows populations grown in liquid culture at 40 °C.

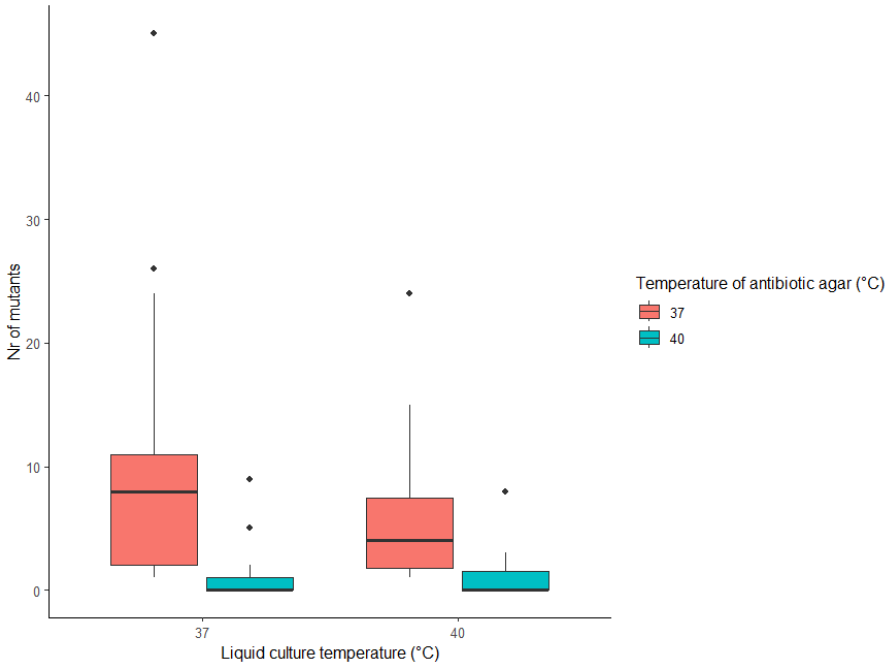

Figure S15. Boxplot showing the number of ampicillin resistant mutants detected depending on the antibiotic agar incubation temperature. Shown for populations grown in liquid culture at 37 °C (left) and at 40 °C (right).

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Chapter 4

The evolution of condition-dependent mutation rates in changing environments

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In preparation

Abstract

Empirical studies have demonstrated that mutation rates can be affected by the condition of an individual or its environment. It has been proposed that condition-dependent mutation rates could be selectively favoured as in times of maladaptation the production of many new mutants might enhance the ability of a population to undergo adaptive evolution (i.e. they enhance evolvability). However, models studying the evolution of condition-dependent mutation rates are lacking. Here we used an individual-based simulation approach to examine the evolution of condition-dependent mutation rates in a changing environment. All simulations converged to one of two alternative outcomes: a condition-dependent mutation rate (which is low when the phenotype matches the environment and high otherwise) or a constant mutation rate at an intermediate level. Populations where a condition-dependent mutation rate evolved were substantially better adapted to their (changing) environment. The likelihood of the two evolutionary outcomes depended on the frequency of environmental change, the initial mutation rates at the start of a simulation, and the model assumptions on the mutation rate of the mutator loci. When this mutation rate was self-referent (i.e., determined by the mutator loci themselves), condition-dependent mutation rates evolved much more readily than when the mutation rate of mutator loci was externally given. We conclude that condition-dependent mutation rates can indeed evolve and that populations with condition-dependent mutation rates have an enhanced evolvability, that is, an enhanced ability to adapt to changing conditions.

Introduction

The study of mutational processes is crucial to understanding evolution, as mutation provides the variation upon which selection acts. Since mutation rates affect what genetic variation is available, they shape the ability of biological systems to undergo adaptive evolution. In other words, mutation rates are key determinants of evolvability (Badeau & Packard, 2003; Jones et al., 2007; Riederer et al., 2022).

The rate of mutation is subject to evolution. This is illustrated by the spread of mutator strains in the Lenski long-term evolution experiment, leading to bacterial strains with an elevated mutation rate (Sniegowski et al., 1999). Many researchers have studied the evolution of mutation rates both in models (Sniegowski et al., 2000; Badeau & Packard, 2003; Andre & Godelle, 2006; Desai et al., 2007; Lynch et al., 2016) and empirically, for example by using evolution experiments (Sniegowski et al., 1999; Colgrave & Collins, 2008; Couce et al., 2017; Sprouffske et al., 2018). In general, the optimal mutation rate strongly depends on the degree of adaptation. When a population is well adapted, a low mutation rate is advantageous, as most mutations are deleterious. However, when a population is maladapted, an increased mutation rate can be beneficial, as it leads to the more rapid production of better-adapted variants. Alleles leading to an increased mutation rate can "hitchhike" along with any beneficial variants they produce. This is especially effective in asexual populations where the linkage between mutator alleles and the adaptive mutations they produce can be more easily maintained.

An organism's condition can influence its mutation rate. An example of this is stress-induced mutagenesis: various stressors are known to result in increased mutation rates. There is widespread empirical evidence for such stress-induced mutagenesis in many bacterial species, such as *Escherichia coli*, *Bacillus subtilis* and *Staphylococcus aureus* (Cirz et al., 2007; Foster, 2007; Debora et al. 2010; Ram & Hadany, 2012; Ha & Edwards, 2021). A stress-related elevation of the mutation rate has also been demonstrated in sexually reproducing eukaryotes, such as *Saccharomyces cerevisiae* and *Drosophila melanogaster* (Heidenreich, 2007; Agrawal & Wang, 2008). Stress-induced mutagenesis has even been demonstrated in human cancer cells (Cipponi et al., 2020).

Stress-induced mutagenesis may be a non-adaptive side effect of stressful conditions (Ram & Hadany 2012). For instance, a stressor or stress response could impact DNA or the enzymes responsible for maintaining and replicating DNA, thereby leading to an increase in mutation rates, a phenomenon more accurately described as stress-associated mutagenesis (Bjedov et al., 2003; Tenallion et al., 2004; Mac Lean et al., 2013). There are, however, at least

two adaptive explanations for stress-induced mutagenesis. First, when an organism is stressed, the costs of maintaining replicative fidelity could outweigh the benefits, inducing selection to switch off fidelity-enhancing mechanisms under stressful conditions (Ram & Hadany 2012). Second, stress may be an indicator that the organism is not well-adapted to its local conditions. As mentioned above, elevating the mutation rate may facilitate adaptation under such circumstances (Sniegowski et al., 1999; 2000).

We are here mainly interested in the second explanation, which is based on the idea that selection should shape mutation rates in such a way that they keep pace with the level of adaptation: in times of low stress (= a high level of adaptation), the mutation rate should be low, whereas in times of high stress (= a low level of adaptation), the mutation rate should be elevated. According to this logic, the mutation rate should be condition-dependent: it should be low under favourable conditions and high under unfavourable conditions. Here, 'condition' could either refer to the state of the environment or to the state of the organism, which by mechanisms like the stress-response system may indicate a lack of adaptation.

Whilst the empirical evidence for condition-dependent mutation rates is abundant, the condition-dependence of mutation rates is much less studied from a theoretical point of view. The earliest models focused exclusively on the effect of deleterious mutations under condition-dependent mutation rates (Agrawal 2002; Baer 2008; Shaw & Baer 2011). In contrast, Ram & Hadany (2012, 2014, 2019) and Ram et al. (2018) also considered beneficial mutations and showed that stress-induced mutagenesis might be favoured by selection under a wide array of circumstances. However, the models by Ram & Hadany (2012, 2014, 2019) considered the loci determining the relationship between an individual's condition and its mutation rate to be fixed and not evolvable. In other words, they did not explicitly model the evolutionary emergence of the relationship between an individual's condition and its mutation rate.

Here we use an individual-based simulation approach to better understand the evolution of condition-dependent mutation rates. We model individuals with a phenotype constantly adapting to a changing environment. We

explicitly consider both beneficial and deleterious mutations. Furthermore, contrary to the previous models by Ram & Hadany (2012, 2014, 2019), we aim to explicitly model the evolutionary dynamics of the relationship between an individual condition and the mutation rate. We thus model this relationship in a flexible manner: In our model, the current mutation rate is determined by two mutation rate loci and a third locus that controls the degree of mismatch between the phenotype and the environment at which individuals switch between the two mutation rates. We allow these loci to dynamically evolve during our simulations.

We address two research questions. First, can condition-dependent mutation rates evolve from scratch and what circumstances are favourable for their evolution? In particular, how is the evolution of condition-dependent mutation rates related to the degree of environmental change? Second, how do condition-dependent mutation rates affect the speed of adaptation in a changing environment? To what extent does a condition-dependent mutation rate enhance the evolvability of a population?

Most previous models of the evolution of mutation rates assume (often implicitly) that the mutator loci (i.e., the loci controlling the mutation rate) mutate according to an externally given fixed mutation rate (e.g., Agrawal 2002, Baer 2008; Shaw & Baer 2011; Ram et al., 2018; Ram & Hadany 2012, 2014, 2019). Here, we also consider the option that the mutation rate at the mutator loci is "self-referent" in the sense that this mutation rate is affected by the mutator loci themselves. This assumption is not unrealistic. Consider, for example, the mechanisms of stress-induced mutagenesis in bacteria, where error-prone polymerases are expressed when a certain level of stress is reached (Maslowska et al., 2019). The expression of error-prone polymerases should increase all mutation rates, including those at the mutator loci. We, therefore, investigate whether and how the evolution of a condition-dependent mutation rate is affected by the model assumptions on the mutation rate at the mutator loci (externally given versus self-referent).

Methods

Model overview

We use an individual-based simulation approach to study the evolution of a population in an environment that may randomly change from one generation to the next. Each individual has a genetically determined phenotype *P*. The expected number of offspring of an individual is negatively related to the mismatch between the individual's phenotype *P* and the current state of the environment *E*. Figure 1A illustrates the change in the environment and the tracking of the environment by the average phenotype in the population due to the joint action of selection, mutation, and genetic drift.

The mutation rate is determined genetically. We consider two scenarios: a constitutive mutation rate and a condition-dependent mutation rate. In the first scenario, the mutation rate is determined by the allele on a mutator locus; this allele is also inherited from parent to offspring, subject to mutation. In the second scenario, the mutation rate is dependent on the condition of the individual harbouring this mutation, where the individual's 'condition' is determined by the mismatch of the individual's phenotype *P* and the current state of the environment *E*. As illustrated in Figure 1B, condition-dependent mutation rates are encoded by three loci: a threshold locus and two mutator loci. The allele at the threshold locus determines the degree of mismatch between *P* and *E* for which the individual 'feels' to be in a 'good' condition (small mismatch) or in a 'bad' condition (large mismatch). The allele on mutator locus *A* determines the mutation rate when in good condition, while mutator locus *B* determines the mutation rate in bad condition. Again, the alleles at all three loci are transmitted from parent to offspring, subject to mutation.

In both scenarios, the alleles at the mutation-relevant loci are also affected by mutation. We consider two variants for the mutation rate at these loci: (a) a constant, externally given (and hence not evolving) mutation rate; or (b) a 'self-referent' mutation rate, which is identical to the mutation rate at the

locus determining the phenotype *P* (and hence determined by the mutation-relevant loci themselves).

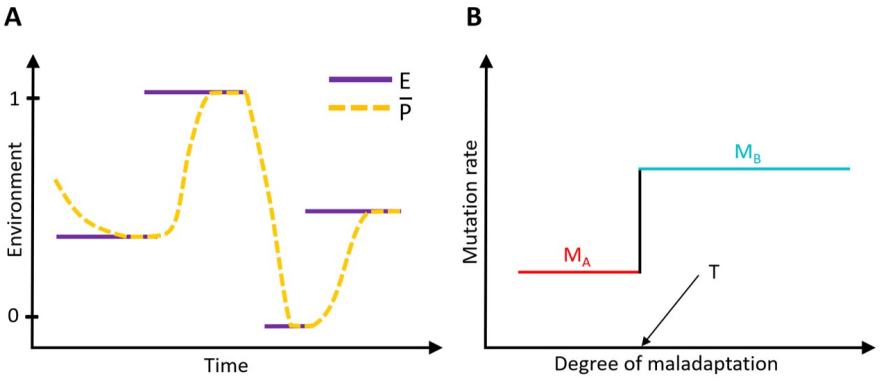

Figure 1. Illustration of the model assumptions. (A) We consider a randomly changing environment *E* (purple line segments) that keeps constant for some generations but switches to a new state with a fixed probability. Natural selection tends to reduce the mismatch between the genetically determined phenotype of individuals and the current environment. As a result, the mean phenotype $\bar{P}$ tends to track the environment. **(B)** Our model allows mutation rates to be condition dependent. In this case, the mutation rate of an individual is governed by three heritable traits (M_A, M_B, T): the mutation rate is M_A if the mismatch between the individual's phenotype *P* and the current environment *E* (the individual's degree of maladaptation) is smaller than the threshold *T*, and it is M_B otherwise.

Model assumptions

Environmental change: The environment takes on values between 0 and 1. The environmental state *E* remains constant throughout a generation and is the same for all members of the population. From one generation to the next, the environmental state changes with a probability χ. Hence the expected duration of environmental stasis is $D = \frac{1}{\chi}$. Whenever an environmental change occurs, a new environmental state is drawn at random from the uniform distribution on the interval [0,1]. Unless stated otherwise, $\chi = 0.01$ or equivalently $D = 100$.

Phenotypes, mismatch, and fitness: Each individual has a genetically determined (see below) phenotype *P*, which is a number between 0 and 1.

The expected reproductive success ('fitness') $F(P,E)$ of individuals with phenotype *P* in an environment in state *E* is negatively related to the mismatch $|P-E|$ and proportional to the Gaussian

$$F(P,E) = exp(-s \cdot (P-E)^2),$$

where the parameter *s* measures the strength of selection. For all simulations reported in the main text, we chose $s = 10$, corresponding to a standard deviation of $\sqrt{0.05} = 0.224$ of the Gaussian.

Selection and reproduction: We consider a population with discrete, non-overlapping generations and a fixed population size of *N* individuals. In all simulations reported, we chose$N = 1{,}000$. Inheritance is asexual; hence, offspring inherit the phenotype of their parents unless a mutation occurs. A new generation is produced as follows: for each of the *N* positions, a parent is drawn from the previous generation by means of a weighted lottery (with replacement), where the probability of an individual *i* with phenotype P_i to be chosen is proportional to the individual's fitness $F(P_i,E)$. This procedure ensures that the expected number of offspring of each individual is proportional to $F(P_i,E)$.

Inheritance: Individuals are haploid and have either two or four gene loci, each of which harbouring infinitely many alleles (corresponding to real numbers). All parental alleles are transmitted from parent to offspring, subject to mutation. One locus encodes the phenotype *P* of an individual, while the other loci determine the mutation rate at the *P* locus. The alleles at the *P* locus are elements of the unit interval [0,1]; each allele directly corresponds to the phenotype it encodes. In the case of an unconditional mutation rate, the second locus encodes the mutation rate *M* at the *P* locus. To be more specific, the alleles at the *M* locus are real numbers *L* that encode the logit of the mutation rate: $L = ln(\frac{M}{(1-M))}$ and therefore $M = \frac{1}{(1+exp(-L))}$. Encoding mutation rates by their logit has the convenient property that a given reduction in the logit has a smaller and smaller effect on the mutation rate the closer the mutation rate is to zero. In the case of a condition-dependent mutation rate, the mutation rate at the *P* locus is determined by

three parameters (M_A, M_B, T), as indicated in Fig. 1B. The mutation rates M_A $M_B$and threshold locus *T* are encoded by their logit.

Mutation at the *P* locus: Whenever a new offspring is produced, it inherits the parental alleles, unless a mutation occurs. At the *P* locus, a mutation occurs with a probability that is determined by the mutation-related loci. If a mutation occurs, the new allelic value is given by $P_{new} = P_{old} + \delta P$, where the mutational step size δP is drawn from a normal distribution with mean zero and standard deviation 0.05. As *P* is limited to the unit interval, a negative value of P_{new} is set to zero, while a value of P_{new} exceeding 1 is set to 1.

Mutation at the mutator loci: The alleles encoding the mutation rate are also inherited with mutation. In the model variant with externally given mutation rates, each of the loci encoding the mutation rate mutates with a constant probability $\mu = 0.001$. In the model variant with self-referent mutation rates, the mutation rate at the mutation-rate determining loci is the same as the mutation rate at the *P* locus (which in turn is determined by the alleles at the mutation-rate determining loci). If a mutation occurs at a locus determining a mutation rate (*M*, M_A or M_B), the new logit value (*L*, L_A or L_B) is given by $L_{new} = L_{old} + \delta L$, where the mutational step size δL is drawn from a normal distribution with mean zero and standard deviation 0.5. For the mutation rate, this implies that

$$M_{new} = \frac{M_{old}}{M_{old} + (1 - M_{old}) \cdot e^{-\delta L}}.$$

Mutations at the threshold locus *T* are modelled similarly to mutations at the *M* loci.

Initialisation: All simulations start with a monomorphic population, with all individuals having allele $P = 0.5$ at the locus determining the phenotype. Unless stated otherwise, all mutation rates are initialised at the value 0.001 and the threshold locus is initialised at $T = 0.5$. We will later study the effect of other initialisations on the evolutionary outcome.

Simulation details: All simulations were run for 100,000 generations. For each initialisation and parameter setting, 100 replicate simulations were run.

The simulation programme was written in C++, and the simulation data were processed and analysed in *R*.

Results

Evolution of a constitutive mutation rate

Figure 2 shows what constitutive mutation rate evolved in 100 replicate simulations for different average times of environmental stasis $D = \frac{1}{\chi}$, or, equivalently, for different rates χ of environmental change. In a rapidly changing environment ($\chi = 0.1;\ D = 10$), the highest possible mutation rate $M = 1.0$tends to evolve, while the evolved mutation rate approaches zero in a slowly changing or constant environment ($\chi \leq 0.001;\ D \geq 1000$). The increase of the mutation rate with the rate of environmental change is not unexpected, as a high mutation rate allows evolution to keep pace with fast environmental change. To verify that a higher mutation rate does indeed lead to better adaptive tracking of a rapidly changing environment, we investigated the average mismatch between phenotype and environment for a range of fixed (i.e., non-evolving) constitutive mutation rates. For the case $D = 100$, the results are shown in Figure 3. The mismatch between phenotype and environment is highest for very low and very high mutation rates and minimal for a constitutive mutation rate of about 0.1. This is in agreement with Figure 2, where the evolved constitutive mutation rates were also close to 0.1 for $D = 100$.

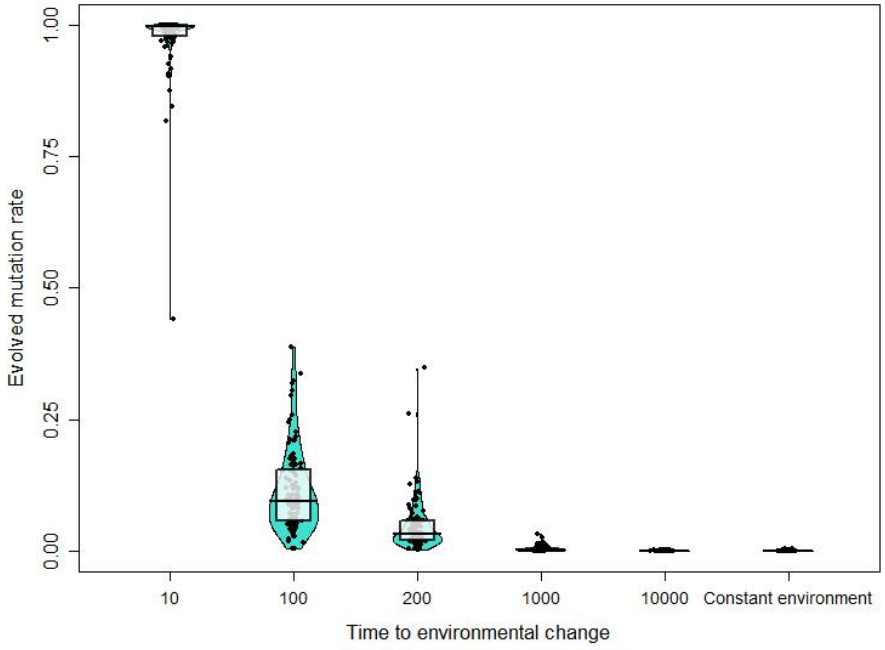

Figure 2. Effect of the average time of environmental stasis on the evolution of a constitutive mutation rate. For six values of $D = \frac{1}{\chi}$, a violin plot summarizes the results of 100 replicate simulations, with the solid lines indicating the median and the boxes indicating the interquartile range. Each point corresponds to the average mutation rate that had evolved at the end of a simulation.

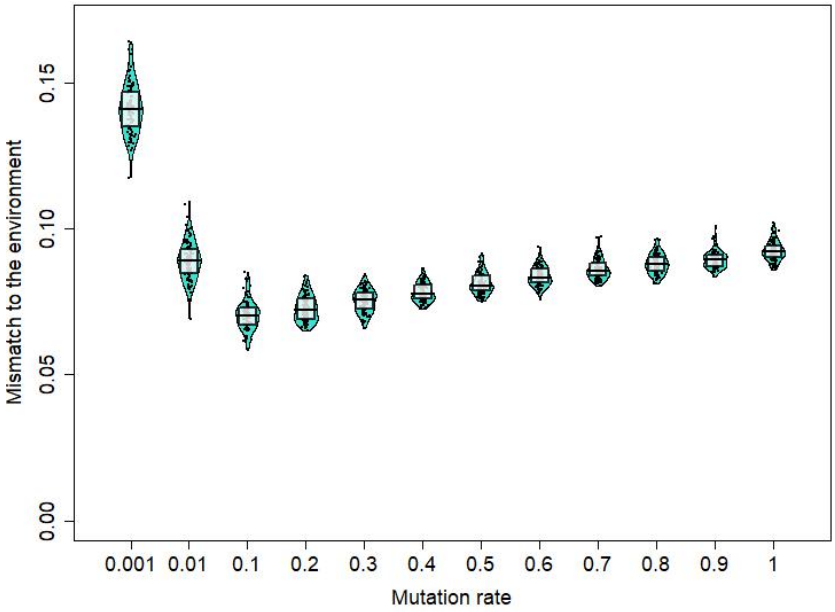

Figure 3. Effect of a non-evolving constitutive mutation rate on the average mismatch between phenotype and environment. For 12 values of a fixed, non-evolving constitutive mutation rate and in an environment that changes on average once every 100 generations ($D = 100$), each violin plot summarizes the results of 100 replicate simulations. Each point corresponds to the time-averaged mismatch between phenotype and environment, calculated over the last 25,000 generations, of a simulation.

Evolution of condition-dependent mutation rates

Figure 4 shows the outcome of 100 replicate simulations that allowed for the evolution of condition-dependent mutation rates. All simulations were initialised with mutation rates $M_A = M_B = 0.001$ and a threshold value $T = 0.5$. In the course of evolution, two distinct mutation rate strategies emerged, that differ in the value of the threshold, which either converged to zero or to one (Fig. 4C). The first strategy, which is illustrated in Fig. 4D, is characterized by a low value of the threshold *T*, a low value of mutation rate M_A, and a high value of mutation rate M_B. Individuals with this strategy express a low mutation rate (M_A) when adapted almost perfectly (when the mismatch $|P - E|$ is smaller than the threshold and, hence, close to zero), but switch to a high mutation rate (M_B) as soon as the mismatch is larger than the threshold. This is thus a condition-dependent mutation rate strategy. The second strategy, which is illustrated in Fig. 4E, is characterized by high values of the threshold and intermediate values for mutation rate M_A. In this case, the intermediate mutation rate M_A is expressed under almost all conditions, since the mismatch $|P - E|$ is only in extreme cases larger than the threshold (which is close to one). Hence, the mutation rate M_B is almost never used. In other words, this second strategy is not really condition dependent and effectively corresponds to the constitutive mutation rate strategy M_A. In line with this, the evolved value of M_A is similar to the mutation rates that evolved in the constitutive mutation rate model for the same value of the parameter *D* (see Figure 2, $D = 100$). The two strategies form distinct clusters: this can be seen in Figure 4AB, when values of M_A and M_B are colour-coded according to the corresponding threshold value.

In the Supplement, we show corresponding simulations, but for weaker selection (i.e., lower values of *s*). Now, the evolution of the mutation rate proceeds more slowly and in a more stochastic fashion. However, both the condition-dependent strategy and the constant mutation strategy still evolve, as shown in Figure S1.

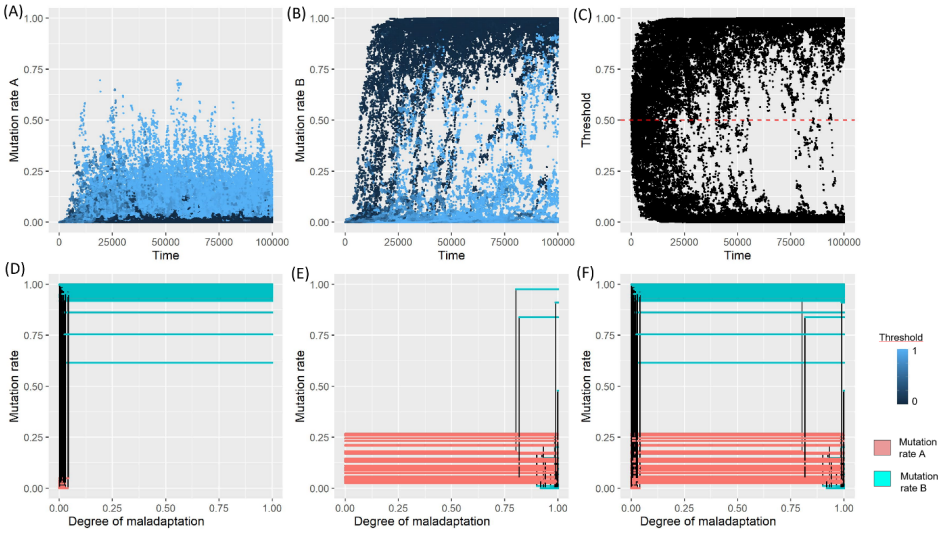

Figure 4. Evolution of condition-dependent mutation rates. Evolution of **(A)** the mutation rate M_A, **(B)** the mutation rate M_B, and **(C)** the threshold T in 100 replicate simulations of the condition-dependent mutation rate model that were all initialised at $M_A = M_B = 0.001$ and $T = 0.5$. Points show the average population value at a particular timepoint for a particular replicate. The threshold either converges to zero or to one. The mutation rates in (A) and (B) are colour-coded in such a way that the rates evolving for low threshold values appear black, while rates evolving for high threshold values appear blue. The distinct clusters in (A) to (C) indicate that there are two distinct evolutionary outcomes (which are plotted as in Fig. 1B): **(D)** a condition-dependent mutation-rate strategy with a low threshold T; here, the low mutation rate M_A (magenta) is expressed when the mismatch between phenotype and environment (the degree of maladaptation) is very low, while the high mutation rate M_B (cyan) is expressed otherwise; **(E)** a mutation-rate strategy with a high threshold T; this is effectively a constitutive mutation-rate strategy, where an intermediate mutation rate M_A (magenta) is expressed under almost all conditions, as mismatches $|P - E|$ close to one occur very rarely. In (D) and (E), each simulation is represented by the average value of M_A, M_B, and T; (D) shows all those simulations resulting in $\bar{T} < 0.5$, while (E) shows the simulations for which $\bar{T} > 0.5$. **(F)** shows all 100 replicate simulations. Average duration of environmental stasis $D = 100$.

Effect of environmental change rate on condition-dependent mutation rates

Figure 4 considered the default setting $D = 100$, where the environment remains constant for, on average, 100 generations. Figure 5 shows the outcome of 100 replicate simulations for six different values of *D*. When the average time of environmental stasis is below 1,000, both the condition-dependent strategy and the constant mutation strategy consistently emerge. This is particularly evident in the strongly bimodal distribution of mutation rate M_B and the threshold T (Figure 5BC). When the environment remains constant or changes very rarely (every 10,000 generations), mutation rate M_A evolves to very low values, while the evolution of M_B and *T* does not show a clear pattern. If environmental change is very rare, there is no selection for a high mutation rate (see Figure 2). In this scenario, the mismatch between phenotype and environment will typically be very small, so that selection on M_B and *T* is very weak. Accordingly, most of the mutation strategy is never expressed and M_B and *T* will largely evolve by genetic drift.

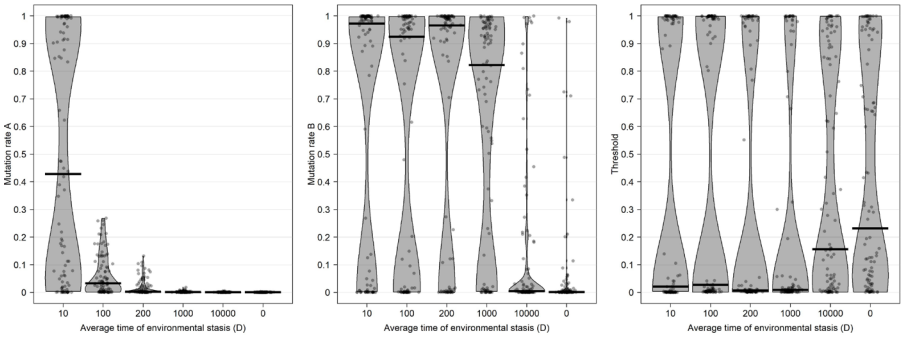

Figure 5. Effect of the average time of environmental stasis on the evolution of condition-dependent mutation rates. For six values of $D = \frac{1}{\chi}$, three violin plots show **(A)** the evolved mutation rate M_A, **(B)** the evolved mutation rate M_B, and **(C)** the evolved threshold *T* in 100 replicate simulations of the condition-dependent mutation rate model that were all initialised at $M_A = M_B = 0.001$ and $T = 0.5$. Each point corresponds to the average value at the end of one replicate simulation. The solid lines indicate the median.

Initialization effects the evolution of condition-dependent mutation rates

Whenever evolution can have different outcomes, the likelihood of each outcome typically depends on the initial conditions. Figure 6 shows that this general principle also applies to the evolution of condition-dependent mutation rates. The colour of the small squares corresponds to the average threshold value that evolved in 900 simulations: almost all of these values were either close to zero (blue) or close to one (yellow). As explained above, the evolved value of T indicates the evolved mutation-rate strategy: low threshold values are associated with a condition-dependent mutation-rate strategy, while high threshold values are associated with a constitutive mutation-rate strategy. Most of the simulations result in a condition-dependent mutation rate if the threshold is initialised at a low value ($T = 0.1$) or if the mutation rates M_A and M_B are initialised in such a way that $M_A < M_B$.

The emergence of the two alternative mutation-rate strategies can intuitively be understood as follows. At the start of a simulation, any change in the environment selects for an increase the mutation rate, as the initialised mutation rates are relatively low. If mutation rate M_A is initially higher than mutation rate M_B, the effective mutation rate can be rapidly increased by increasing the threshold value: with a higher threshold, the relatively high mutation rate M_A is expressed more often. When the threshold approaches one, the resulting mutation-rate strategy is effectively non-condition-dependent. In this case, mutation rate M_B is (almost) never expressed and hence selection cannot effectively operate on it. Mutation rate M_A evolves to higher values, in a fashion similar to the constitutive mutation rate model. .If, in contrast, mutation rate M_A is initially lower than mutation rate M_B, a similar effect operates in the opposite direction. Now, there is initial selection to lower the threshold value, as this increases the effective mutation rate. In this case, a condition-dependent mutation-rate strategy evolves where the threshold evolves to a low value, mutation rate M_A evolves to a low value and mutation rate M_B evolves to a very high value.

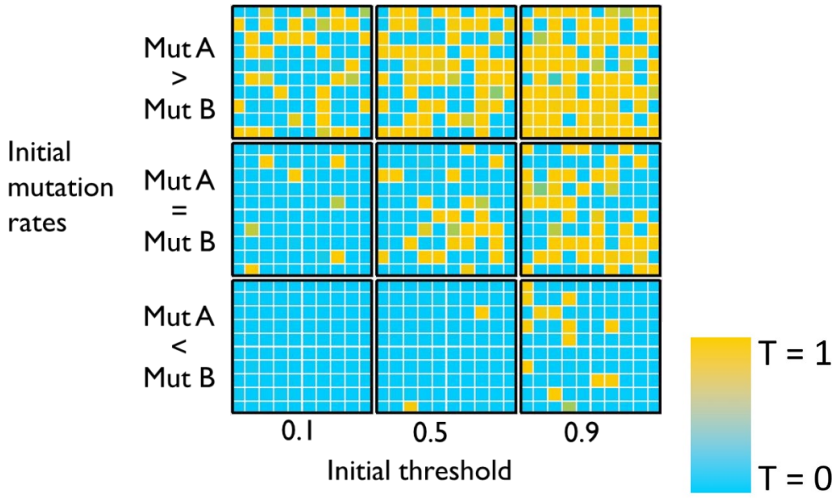

Figure 6. Effect of initial conditions on the evolution of condition-dependent mutation rates. 100 replicate simulations (small coloured squares lined in white) were run for each of nine parameter combinations (larger squares lined in black), which correspond to different sets of initial conditions for the parameters M_A, M_B, and T of the condition-dependent mutation rate model. In the horizontal direction, the threshold T was initialised at the values 0.1, 0.5, and 0.9, respectively. In the vertical direction, the mutation rate M_B was initialised at the values 0.001, 0.01, and 0.1, while M_A was initialised at 0.01 in all cases. Each simulation is represented by a small square that is coloured according to the average value of the threshold T that evolved in that simulation. Squares coloured yellow represent simulations that evolved a threshold value close to 1, indicating a constitutive mutation-rate strategy. Squares coloured blue represent simulations that evolved a threshold value close to zero, indicating a condition-dependent mutation-rate strategy. A condition-dependent mutation rate evolved in most simulations that were initialised with a low threshold ($T = 0.1$) and where at the same time the initial values of M_A and M_B satisfied $M_A \leq M_B$. In contrast, a constitutive mutation rate evolved in most simulations that were initialised at a higher threshold ($T \geq 0.5$), while the mutation rates were initialised with $M_A > M_B$. Average duration of environmental stasis $D = 100$.

Self-referent mutation rates

Until now we assumed that the three loci determining the mutation rate mutated with a fixed mutation probability ($\mu = 0.001$). Now, we consider a self-referent mutation rate: the loci encoding the mutation rate mutate with the same probability as the *P* locus (whose mutation rate is encoded by these loci). A self-referent mutation rate can create an evolutionary runaway effect: the higher the mutation rate, the faster it can change. The accelerated evolutionary dynamics becomes apparent when comparing Figure 7 (self-referent mutation rate) with the corresponding Figure 4ABC (fixed mutation rate at the mutator loci). A self-referent mutation rate also alters the likelihood of the two mutation-rate strategies. As shown in Figure 8, a self-referent mutation rate results much more often in a condition-dependent mutation-rate strategy than a fixed mutation rate at the mutator loci (Figure 6).

In Supplementary Figure S2, we examine the impact of a self-referent mutation rate on the evolution of a constitutive mutation rate. Again, we find an acceleration of the evolutionary dynamics, as observed in the condition-dependent model.

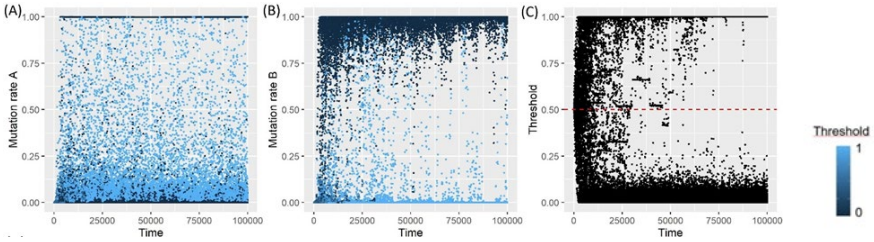

Figure 7. Evolution of condition-dependent mutation rates when the mutation rate at the mutator loci is self-referent. Evolution of **(A)** the mutation rate M_A, **(B)** the mutation rate M_B, and **(C)** the threshold *T* in 100 replicate simulations of the condition-dependent mutation rate model that were all initialised at $M_A = M_B = 0.001$ and $T = 0.5$. Points show the average population value at a particular timepoint for a particular replicate. The threshold either converges to zero or to one. The mutation rates in (A) and (B) are colour-coded in such a way that the rates evolving for low threshold values appear black, while rates evolving for high threshold values appear blue. Average duration of environmental stasis $D = 100$.

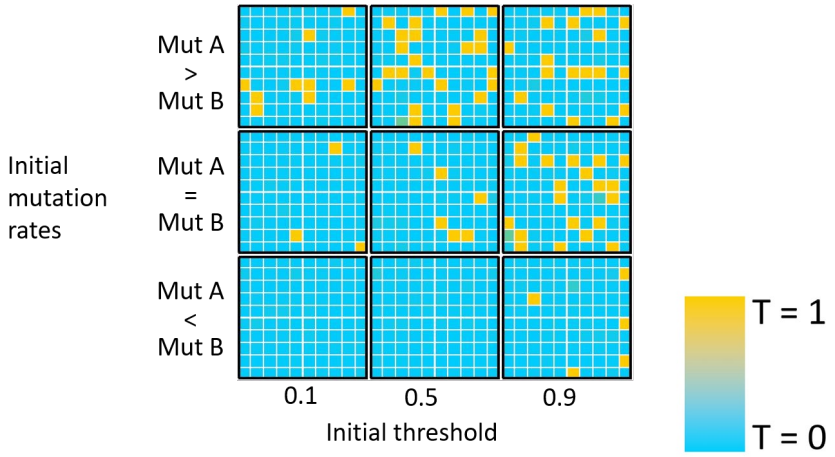

Figure 8. Effect of initial conditions on the evolution of condition-dependent mutation rates when the mutation rate at the mutator loci is self-referent. The figure corresponds in all aspects to Figure 6, but now the mutation rates are self-referent. Now, the likelihood of evolution of the condition-dependent mutation-rate strategy (simulations corresponding to blue squares) is conspicuously higher than in the case of a fixed mutation rate at the mutator loci.

Implications for population fitness

The different mutation strategies influence adaptive tracking of the changing environment. Figure 9 shows how the time-averaged mismatch between phenotype and environment depends on the model version and the evolved mutation-rate strategy. s: the constitutively expressed self-referent mutation rate, the constitutively expressed non-self-referent mutation rate, the condition-dependent self-referent mutation rate (split based on whether a condition-dependent mutation strategy evolved in a given replicate simulation or not, i.e. based on whether the average threshold value was below or above 0.5), the condition-dependent non-self-referent mutation rate (split in the same way). A condition-dependent mutation rate strategy leads to better adaptive tracking of the environment: the simulations in which a condition-dependent mutation strategy evolved show a clearly decreased average mismatch between ecological trait and environment.

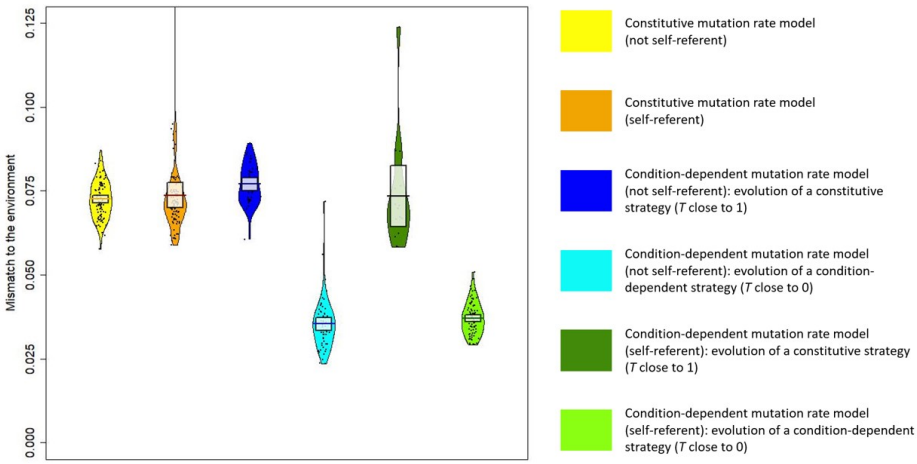

Figure 9. Time-averaged mismatch between phenotype and environment for different evolved mutation-rate strategies. Each point corresponds to the time-averaged mismatch between phenotype *P* and environmental state *E* in the final 25,000 generations of a single simulation. For four model variants, the violin plots summarize the outcome of 100 replicate simulations. The two left-most violin plots show the results for the constitutive mutation rate model, either with a non-self-referent mutation rate (yellow) or a self-referent mutation rate (orange). The middle pair of violin plots and the right-most pair of plots each represent 100 simulations for the condition-dependent mutation rate model, either with non-self-referent (middle pair) or with self-referent (right-most pair) mutation rate. In the latter two model variants, the simulation outcomes are split according to whether a condition-dependent mutation-rate strategy evolved (as indicated by a small evolved threshold $\bar{T} < 0.5$: cyan or light-green plots) or whether a constitutive strategy evolved (as indicated by a large evolved threshold $\bar{T} > 0.5$: dark-blue or dark -green plots). This figure reveals that a condition-dependent mutation-rate strategy leads to better adaptive tracking of the changing environment than a constitutive mutation rate. Average duration of environmental stasis $D = 100$.

Discussion

This study provides four main findings. Firstly, we show that elevated mutation rates evolve under fast environmental change, which facilitate better tracking of the environment. Secondly, when we allow for a condition-dependent mutation rate to evolve, two different outcomes emerge: switching between a high and low mutation rate depending on individual condition, or constitutively expressing only one mutation rate. Thirdly, condition-dependent mutation rates allow for better adaptation in changing environments. Fourthly, it matters a lot whether mutation rates are self-referent or not, i.e. whether mutator alleles affect their own mutation rate. Self-referent mutation rates speed up the dynamics of mutation rate evolution and increase the likelihood that a condition-dependent mutation rate will evolve.

Constitutive mutation rates

As a standard of comparison, we first studied the evolution of a constitutive mutation rate. in relation to the frequency of environmental change. The simulations revealed that, for a given rate of environmental change, the time-averaged mismatch between phenotype and environment is minimised at a particular, intermediate mutation rate. In the simulations, the mutation rate tended to evolve towards this 'optimal' value. Moreover, higher mutation rates evolved in more rapidly changing environments. This finding is in agreement with earlier models of mutation rate evolution (Sniegowski et al., 2000; Badeau & Packard, 2003; Andre & Godelle, 2006; Desai et al., 2007; Lynch et al., 2016). The evolution of elevated mutation rates in this model is also consistent with experimental evidence showing that elevated mutation rates can even evolve in response to a single environmental change (Sniegowski et al., 1997). Our results here are also consistent with the experimental results obtained by Sprouffske et al. (2018) who experimentally showed that only a modestly elevated mutation rate conveys adaptive benefits, whilst highly elevated mutation rates do not. This echoes the observation of an 'optimal' mutation rate in our model.

Condition-dependent mutation rates

When we allowed for the evolution of a condition-dependent mutation rate, we found that replicate simulations could result in two different outcomes: a mutation rate that is indeed strongly dependent on the condition of an individual (i.e., the mismatch between phenotype and environment), or a mutation rate that is essentially independent of an individual's condition and, hence, constitutive. The condition-dependent mutation-rate strategy shows a low mutation rate when the

individual is well adapted to the current environmental conditions and a very high mutation rate when there is the slightest mismatch between phenotype and environment. The emergence of this condition-dependent strategy is in agreement with general results on mutation rate evolution, which show that elevated mutation rates can be advantageous during times of maladaptation, as they allow for the increased production of potentially beneficial variants (Sniegowski et al., 2000; Andre & Godelle, 2006; Sprouffske et al., 2018). Simulations in which condition-dependent mutation rates evolved also show closer tracking of the environment. Why then do some replicates evolve a non-condition-dependent mutation-rate strategy, which is associated with a worse performance?

To address this question, we examined under which initialisation conditions each strategy evolves. The likelihood of the two mutation strategies is strongly dependent on the initial values of the mutation rates. During the early stages of the simulation, the fluctuating environment creates a strong selective pressure to increase the effective mutation rate, in order to keep up with the changing environment. One way the effective mutation rate can quickly be increased is by modifying the threshold locus to predominantly express the highest of the two mutation rate loci. If the highest value mutation rate locus is the mutation rate expressed under low mismatch (mutation rate A) then the threshold locus will evolve towards a high value, leading to the continuous expression of only a single mutation rate. Subsequently drift will act on the non-expressed mutation rate locus, subsequently, it becomes costly to decrease the threshold again, as it risks expressing the now maladapted mutation rate locus. Overall, this has thus effectively trapped the population into a regime in which only a single mutation rate locus is adapted and used: condition dependence is lost. This explains why some replicates evolve a non-condition-dependent mutation strategy despite this strategy being associated with worse performance.

Self-referent mutation rates

Most models on the evolution of condition-dependent mutation rates implicitly assume that alleles at the mutator loci mutate according to a fixed, externally given rate (Agrawal 2002, Baer 2008; Shaw & Baer 2011; Ram et al., 2018; Ram & Hadany 2012, 2014, 2019). Contrary to most previous models, we also considered the possibility that the effects of the mutator loci are ‘self-referent’, that is, that the mutator loci affect their own mutation rate. Such self-reference had a considerable impact on mutation rate

evolution. A mutation rate locus with a higher value can change more rapidly; this can create a runaway effect that speeds up the dynamics of mutation rate evolution. With a self-referent mutation rate, almost all simulations evolved a condition-dependent mutation-rate strategy, with the lower-performing non-condition-dependent mutation-rate strategy only evolving in a few replicates. Overall, we found that the evolutionary dynamics of self-referent and non-self-referent mutation rates are strikingly different: when studying the evolution of mutation rates and in particular when studying the evolution of condition-dependent mutation rates, it is thus important to consider if the mutation rate is self-referent or not.

The exploration of self-referent mutation rates is grounded in biology: mutation rates have been shown to vary strongly across the genome (Hodgkinson & Eyre-Walker, 2011; Monroe et al., 2022) – so genes that affect the mutation rate in one particular part of the genome without affecting the mutation rate of their own genetic basis likely exist (not self-referent). However, some mechanisms enhancing the mutation rate, such as error-prone polymerases (Maslowska et al., 2019), will likely also affect the accuracy of replication of the genes in which they are encoded (self-referent). In nature, self-reference may also not be a categorical trait, as how much of the genome is affected by a mutation rate modifier can likely vary both plastically and along a continuous scale. However, since we here show the evolutionary dynamics of self-referent and non-self-referent mutation rates to be starkly different, it is worth considering to what extent the mutation rate modifier considered in a particular study is self-referent or not - something that has been so far largely ignored in both the theoretical and empirical literature on mutation rates.

Modelling approach

In general, our results align well with those obtained by Ram & Hadany (2012), who, to our knowledge, conducted the only other theoretical study concerning the evolution of condition-dependent mutation rates. They concluded that condition-dependent mutation rates should evolve under a wide range of circumstances as long as at least some mutations are beneficial. In our model, the occurrence of beneficial mutations is most likely

when the environment changes and the population becomes maladapted. Accordingly, condition-dependent mutation rates do not evolve in a static (or nearly static) environment. Note that the modelling approach we used here is in many ways different from that of Ram & Hadany (2012). Ram & Hadany (2012) analysed the fixation probability of individuals with a fixed condition-dependence of the mutation rate in a population of individuals that express a constitutive mutation rate. Our model follows the evolution of a flexible condition-dependent mutation rate, which allows both condition-dependent and constant (not condition-dependent) mutation strategies to emerge. Furthermore, in the model of Ram & Hadany (2012) the mutation rate responds to the presence of deleterious alleles, whereas in our model the mutation rate is linked to the mismatch of the ecological trait and the environment. Whilst the modelling of the condition-dependent mutation rate in our model is more flexible than in previous models, it is worth noting that our implementation still represents a simplification. We use only three evolving loci to model conditional strategies, this significantly restricts the shape of the function relating individual condition to the mutation rate. Future models could use more loci, thereby allowing for more complex functions to evolve.

Mutation rates in our model are also implemented in a rather simplified way – a necessary feature of simulation models. It is nevertheless worthwhile to consider to what extent this implementation may reflect real-life mutation processes. The high mutation rates that evolved in our models are rather unrealistic, highlighting a drawback of this modelling approach: an individual's adaptation to its environment is determined by a single evolving locus, which is of course a strong simplification. In a real-world scenario, this may be complicated by, e.g., traits under constant stabilizing selection (such as traits required to maintain basic physiological functioning) or by pleiotropic relationships between traits. The absence of such complications in our models facilitates a clearer analysis but also leads to an underestimation of the effect of deleterious mutations. This in turn leads to the evolution of unrealistically high mutation rates. Nevertheless, the choice not to model such additional traits or relationships was made deliberately to keep the model simple, allowing us to observe effects governing mutation

rate evolution more easily. In the future, we aim to extend our model by including extra traits that are not subject to fluctuating selection.

Mutation rates and the evolution of evolvability

Mutation rates are linked to a key concept in evolutionary biology: evolvability, the capability of a biological system to undergo adaptive evolution. Since mutation rates affect at what rate variation is generated, they are a crucial determinant of evolvability (Badeau & Packard, 2003; Jones et al., 2007; Riederer et al., 2022). Thus, our modelling results shed some light on evolvability and its evolution. Firstly, we show that in fluctuating environments, increased mutation rates and thus an increased ability to track the environment evolves – in other words, evolvability itself is here evolving. Secondly, we demonstrate that populations employing a condition-dependent mutation strategy show better tracking of a changing environment, i.e. they have greater evolvability. This is interesting considering that determinants of evolvability can affect evolvability in various, fundamentally different ways. These include generating variation (e.g. through increased mutation rates) as well as shaping the fitness effects of this variation (Riederer et al., 2022). Under a condition-dependent mutation strategy, mutations mostly occur in maladapted individuals, where they are more likely to be adaptive. Overall, this highlights how condition-dependence in mutation rates can enhance evolvability by ensuring that the variation created through mutation is more likely to be beneficial. It is also clear that in our simulations the condition-dependent mutation rate strategy emerges because of its effect on evolvability. Thus our results lend support to the so-called “evolvability hypothesis” as discussed by MacLean et al. (2013), which states that condition-dependent mutation rates in bacteria evolved due to their effect on evolvability. We demonstrate that evolvability can itself be shaped by selection, leading to the evolution of condition-dependent mutation rates.

Acknowledgements:

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Supplementary material

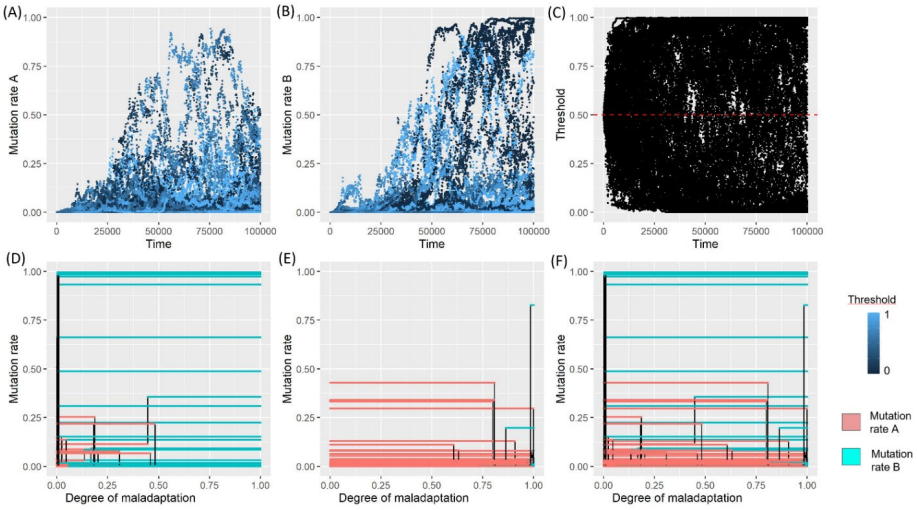

Figure S1. Evolution of condition-dependent mutation rates under weak selection. The figure corresponds in all aspects to Figure 4 in the main text, but now the selection strength is reduced from the default value $s = 10$ to $s = 0.1$, corresponding to a standard deviation of $\sqrt{5} = 2.24$ of the Gaussian fitness function. Average duration of environmental stasis $D = 100$. This figure shows that even under relatively weak selection both condition-dependent and non-condition-dependent mutation strategies still emerge.

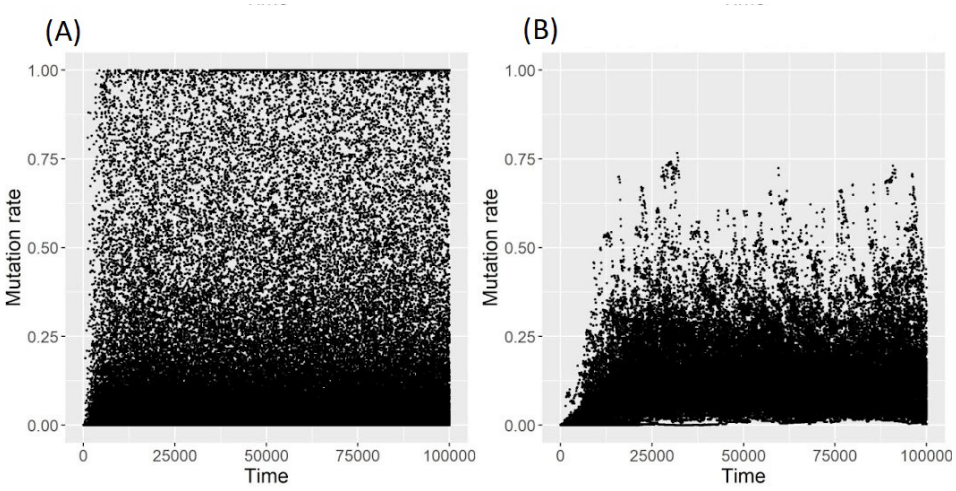

Figure S2. Evolution of a constitutive mutation rate when (A) the mutation rate is self-referent and (B) the mutation rate at the mutator locus is fixed. Each panel shows 100 replicate simulations. Average duration of environmental stasis $D = 100$. Assuming a self-referent mutation rate drastically accelerates the evolutionary dynamics. Panel A shows that the value of a self-referent mutation rate rapidly fluctuates and also attains higher values when compared to non-self-referent mutation rate shown in panel B.

Interlude 1

The evolutionary persistence of a plastic phenotypic switch in the absence of selection

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*These authors contributed equally to the manuscript (shared first authorship)

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Introduction

During my PhD we collaborated with researchers from Louvain University (BE) on a study on the apparent reverse evolution of lipogenesis in parasitic wasps (Visser et al., 2021). We contributed by formulating a simulation model to contextualize and corroborate the empirical section of this project. Together with my colleagues, I developed an individual-based model that showed how adaptive plastic responses can maintain functionality against deleterious mutation for very long periods of time. Our conclusions from this model helped to explain the apparent disappearance and re-evolution of complex traits (such as lipogenesis) and resolve the apparent conflict of these observations with Dollo's law of irreversibility (Chamberlain, 1905; Gould, 1970).

Caroline Nieberding, Bertanne Visser, and their collaborators detected the presence of fatty acid synthesis (lipogenesis) in the parasitic wasp of the species *Leptopilina heterotoma*. These parasitic wasps were never recorded to express lipogenic activity before and were thought to have lost this complex metabolic trait 200 million years previous as Ellers and Visser pointed out in a previous publication (Visser et al, 2010). The overall absence of this trait in parasitoid Hymenoptera is explained by the fact that parasitic wasps' development takes place inside fat-rich insect larvae (Visser et al, 2010). Therefore, these wasps do not need to produce their own fat as they can easily gain this resource from their host; and since lipogenesis is a costly trait to maintain, they have gradually lost it. In the same publication, however, the authors remark that in some cases host-generalist species seemed to have re-evolved lipogenesis from scratch. This discovery seemed to contradict one of the few law-like principles in evolutionary biology: Dollo's law of irreversibility (Chamberlain, 1905). This principle articulated by 19th-century Belgian paleontologist Louis Dollo states that: "an organism never returns exactly to a former state, even if it finds itself placed in conditions of existence identical to those in which it has previously lived." (Gould, 1970), meaning that an organism will never re-evolve the same complex adaptation again, once they have lost it. To reconcile Dollo's law of irreversibility and the reappearance of the apparently lost lipogenic

metabolism, Ellers, Nieberding and Visser speculated that lipogenesis was never actually lost in parasitic wasps, but instead kept dormant in the vast majority of cases and plastically reactivated only when strictly necessary. In Visser et al., (2021), it was indeed shown that lipogenesis reappears when these wasps are raised in larval hosts under starvation. These starved hosts have very low body-fat percentages and thus do not present the parasitic wasps with the fat-rich environment in which they normally develop, forcing them to re-express lipogenesis. This explanation, however, requires that a plastic response can maintain functionality over long periods of time even if selection is not acting on it. One would normally expect that, in the absence of selection deleterious mutations would accumulate and gradually deteriorate the expressed trait. However, we argued that when considering a network of genes and their non-linear interactions, instead of single genes that only interact linearly, the effects of deleterious mutations would be greatly reduced. Interconnected and nonlinear genomes are able to evolve mutational robustness (Payne and Wagner, 2014; Masel and Trotter, 2010; Edlund and Adami, 2004) allowing unselected traits to remain functional for much longer periods of time. To corroborate this argument, we designed an individual-based simulation to test if indeed it was possible for a plastic phenotype-switch to maintain correct functionality (i.e. on in rare fat-poor environments, off in frequent fat-rich environments). The results from this model show that the on–off switch underlying plasticity can evolve mutational robustness and be maintained in the genome for hundreds to thousands of generations. This helped to solidify the conceptual background necessary to support the claims and explanation of the empirical findings. This work resulted in a publication (see Visser et al., 2021). Below, I give an account of the modelling aspects of this article.

Model description

We consider the general situation where phenotypic plasticity is only sporadically adaptive and ask the question of whether and under what circumstances plasticity can remain functional over long evolutionary time periods when the regulatory processes underlying plasticity are gradually

broken down by mutations. We consider a regulatory mechanism that switches on or off a pathway (like fat synthesis) in response to environmental conditions (e.g., host fat content).

Fitness considerations

We assume that the local environment of an individual is characterized by two factors: fat content F and nutrient content N, where nutrients represent sugars and other carbohydrates that can be used to synthesize fat. Nutrients are measured in units corresponding to the amount of fat that can be synthesized from them. We assume that fitness (viability and/or fecundity) is directly proportional to the amount of fat stored by the individual. When fat synthesis is switched off, this amount is equal to F, the amount of fat in the environment. When fat synthesis is switched on, the amount of fat stored is assumed to be $N - c + (1 - k)F$. This expression reflects the following assumptions: (1) fat is synthesized from the available nutrients, but this comes at a fitness cost c; (2) fat can still be absorbed from the environment but at a reduced rate $(1 - k)$. It is adaptive to switch on fat synthesis if $N - c + (1 - k)F$ is larger than F, or equivalently if $F < (N - c)/k$. The right-hand side of this inequality is a straight line, which is illustrated by the blue line in Figure 1. The three boxes in Figure 1 illustrate three types of environmental conditions. Red box: low-fat environments. Here, $F < (N - c)/k$ is always satisfied, implying that fat synthesis should be switched on constitutively. Yellow box: high-fat environments. Here, $F > (N - c)/k$, implying that fat synthesis should be switched off constitutively. Orange box: intermediate-fat environments. Here, fat synthesis should be plastic and switched on if for the given environment (N, F) the fat content is below the blue line and switched off otherwise. The simulations reported here were all run for the parameters $k = \frac{1}{2}$ and $c = \frac{1}{4}$. We also investigated many other combinations of these parameters; in all cases, the results were very similar to those reported here.

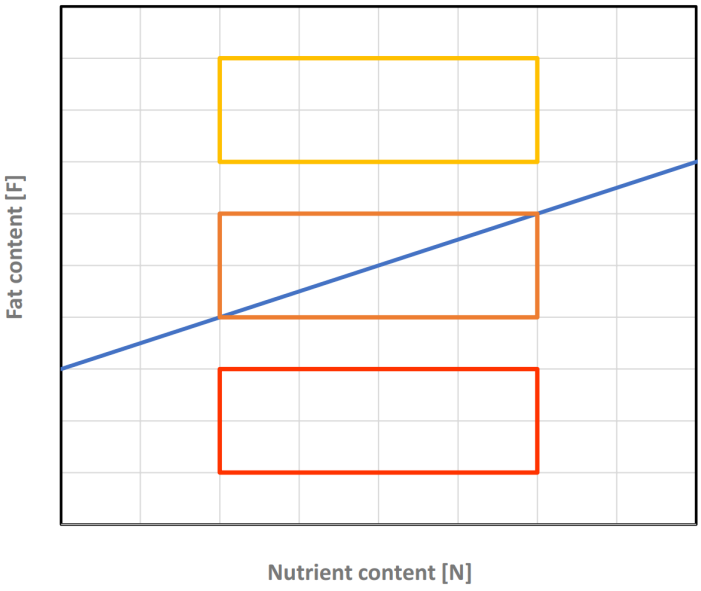

Figure 1. Environmental conditions encountered by the model organisms. For a given combination of environmental nutrient content N and environmental fat content F, it is adaptive to switch on fat synthesis if (N, F) is below the blue line (corresponding to $F < (N - c)/k$) and to switch it off otherwise. The three boxes illustrate three types of environment: a low-fat environment (red) where fat synthesis should be switched on constitutively; a high-fat environment (yellow) where fat synthesis should be switched off constitutively; and an intermediate-fat environment (orange) where a plastic switch is selectively favored.

Gene regulatory networks

In our model, the switching device was implemented by an evolving gene regulatory network (GRN, as in van Gestel and Weissing, 2016). As shown in Figure 2, the networks considered had two receptor (or input) nodes, several (levels of) processor nodes, and an effector node. The two receptor nodes sense the fat and nutrient content in the local environment, respectively. The effector node switches on fat synthesis if the combined weighted inputs from all nodes connected to the effector node exceeds a threshold value T and switches it off otherwise. We ran simulations for GRNs of varying complexity, but for simplicity, we here focus on the simplest possible network, consisting of just two receptor nodes and an effector node. If F and N are the local fat

and nutrient content and w_F and w_N are the corresponding weighing factors, fat synthesis is switched on if $w_F F + w_n N > T$ (and off otherwise). Hence, this simple GRN is characterised by the weighing factors w_F and w_N and the threshold T. Simulations (shown in Figure 3) are based on the most straightforward possible network, consisting of two receptor nodes (sensing the fat and the nutrient content in the local environment, respectively) and an effector node that switches on fat synthesis if the combined weighted input of the two receptor nodes exceeds a threshold value T and switches it off otherwise. Hence, fat synthesis is switched on if $w_F F + w_n N > T$ (and off otherwise). The GRN is characterized by the weighing factors w_F and w_n and the threshold T. These parameters are transmitted from parents to offspring, and they evolve subject to mutation and selection. For the simple GRN described above, the switching device is 100% adaptive when the switch is on (i.e., $w_F F + w_n N > T$) if $F < (N - c)/k$ and off otherwise. A simple calculation yields that this is the case if $w_n > 0$ and $w_F = -k w_n$ and $T = c w_N$

Evolution of the GRN

For simplicity, we consider an asexual haploid population with discrete, nonoverlapping generations and fixed population size of 10,000. Each individual has several gene loci, each locus encoding one parameter of the GRN. In the case of the simple network described above, there are three gene loci, each with infinitely many alleles. Each individual harbors three alleles, which correspond to the GRN parameters w_F, w_n and T , and hence determine the functioning of the genetic switch. In the simulations, each individual encounters a randomly chosen environment (N, F). Based on its (genetically encoded) GRN, the individual decides on whether to switch on or off fat synthesis. If the synthesis is switched on, the individual's fitness is given by $N - c + (1 - k)F$; otherwise, its fitness is given by F. Subsequently, the individuals produce offspring, where the number of offspring produced is proportional to the individual's fitness. Each offspring inherits the genetic parameters of its parent, subject to mutation. With probability μ (per locus) a mutation occurs. In such a case the parental value (in the case of a simple network: the parent's allelic value w_F, w_n or T) is changed to a mutated value($w_F + \delta$, $w_n + \delta$ and $T + \delta$) where the

mutational step size δ is drawn from a normal distribution with mean zero and standard deviation σ. In the reported simulations, we chose $\mu = 0.001$ and $\sigma = 0.1$. The speed of evolution is proportional to $\mu \cdot \sigma^2$, implying that the rate of change in Figure 3 (both the decay of plasticity and the rate of regaining adaptive plasticity) are positively related to μ and σ.

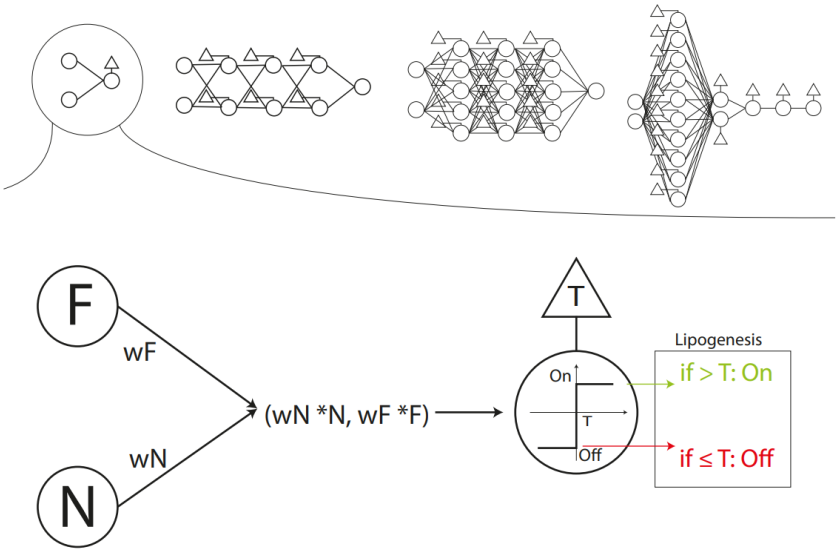

Figure 2. GRNs of varying complexity were simulated. The results reported here refer to the simplest GRN considered (encircled). Bottom: In-detail illustration of the functioning of the simplest GRN. Two local concentrations of fat and nutrients F and N feed into two separate connections and are multiplied by the respective connections weights: w_F and w_n. The values of the two weighted inputs are then summed together. If the sum is larger then the threshold value T the switch for plastic lipogenesis is switched on, otherwise it is kept off.

Preadaptation of the GRNs

Starting with a population with randomly initialized alleles for the GRN parameters, we first let the population evolve for 10,000 generations in the intermediate-fat environment (the orange box in Figure 1). In all replicate simulations, a "perfectly adapted switch" (corresponding to $w_n > 0$ and $w_F = -kw_n$ and $T = cw_N$) evolved, typically within 1,000 generations. Still, the evolved GRNs differed across replicates, as they evolved different values

of $w_n > 0$. These evolved networks were used to seed the populations in the subsequent "decay" simulations.

Evolutionary decay of the GRNs

For the decay experiments reported in Figure 3, we initiated a large number of monomorphic replicate populations with one of the perfectly adapted GRNs from the preadaptation phase. These populations were exposed for an extended period of time (1,000,000 generations) to a high-fat environment (the yellow box in Figure 1), where all preadapted GRNs switched off fat synthesis. However, in some scenarios, the environmental conditions changed back sporadically (with probability q) to the intermediate-fat environment (the orange box in Figure 1), where it is adaptive to switch on lipogenesis in 50% of the environmental conditions (when (N, F) is below the blue line in Figure 1). In Figure 3, we report on the changing rates $q = 0$ (no changing back; red), $q = 0.001$ (changing back once every 1,000 generations; purple), and $q = 0.01$ (changing back once every 100 generations; pink). When such a change occurred, the population was exposed to the intermediate-fat environment for t generations (Figure 3 is based on $t = 3$).

Throughout the simulation, the performance of the network was monitored every 100 generations as follows: 100 GRNs were chosen at random from the population, and each of these GRNs was exposed to 100 randomly chosen environmental conditions from the intermediate-fat environment (orange box in Figure 1). From this, we could determine the average percentage of "correct" decisions (where the network should be switched on if and only if $F < (N - c)/k$. 1.0 means that the GRN is still making 100% adaptive decisions; 0.5 means that the GRN only makes 50% adaptive decisions, as would be expected by a random GRN or a GRN that switches the pathway constitutively on or off. This measure for performance in the "old" intermediate-fat environment was determined for 100 replicate simulations per scenario and plotted in Figure 3 (mean ± standard deviation).

Results

If plasticity of fat synthesis arose in the common ancestor of parasitic wasps, and wasps are generally exposed to lipid-rich hosts, the question arises whether a switching device that is not used for extensive periods of time should be lost during the course of evolution. To investigate this, we ran individual-based simulations that monitored the sustained functionality of a switching device (a gene regulatory network that could decay by mutation) that is only sporadically used in evolutionary time. Figure 3 shows that the switching device rapidly disintegrates (red simulations) if it is never used (see the methods section for model assumptions, modeling details, and simulation settings). However, even very infrequent use (pink: every 100 generations; purple: every 1000 generations) suffices to keep the switching device largely intact. Interestingly, the switching device does not erode gradually, but instead slowly evolves an improved performance over evolutionary time (i.e., the percentage of correct decisions increases with the increasing number of generations). An inspection of the evolving gene regulatory networks (GRNs) reveals that they become more and more robust (i.e., less and less affected by mutational decay), in line with earlier findings on network evolution (Wagner, 2013). The simulations in Figure 3 are representative of all networks and parameters considered. Whenever $q = 0$, the performance of the regulatory switch eroded in evolutionary time, but typically at a much lower rate in the case of the more complex GRNs. Whenever $q = 0.01$, the performance of the switch went back to levels above 90% and even above 95% for the more complex GRNs. Even for $q = 0.001$, a sustained performance level above 75% was obtained in all cases. Intriguingly, in the last two scenarios the performance level first drops rapidly (from 1.0 to a much lower level, although this drop is less pronounced in the more complex GRNs) and subsequently recovers to reach high levels again. Apparently, the GRNs have evolved a higher level of robustness, a property that seems to be typical for evolving networks (Masel and Trotter, 2010; Edlund and Adami, 2004; Wagner, 2008). For the simple GRN studied in Figure 3, this outcome can be explained as follows. The initial network was characterized by the genetic parameters $w_n > 0$, $w_F = -kw_n$ and $T = cw_N$ (see above), where w_n was typically a small positive number. In the course

of evolutionary time, the relation between the three evolving parameters remained approximately the same, but w_n (and with it the other parameters) evolved to much larger values. This automatically resulted in an increasingly robust network, since mutations with a given step size distribution affect the performance of a network much less when the corresponding parameter is large in absolute value. We also considered alternative network structures (see Figure 2), and obtained very similar results.

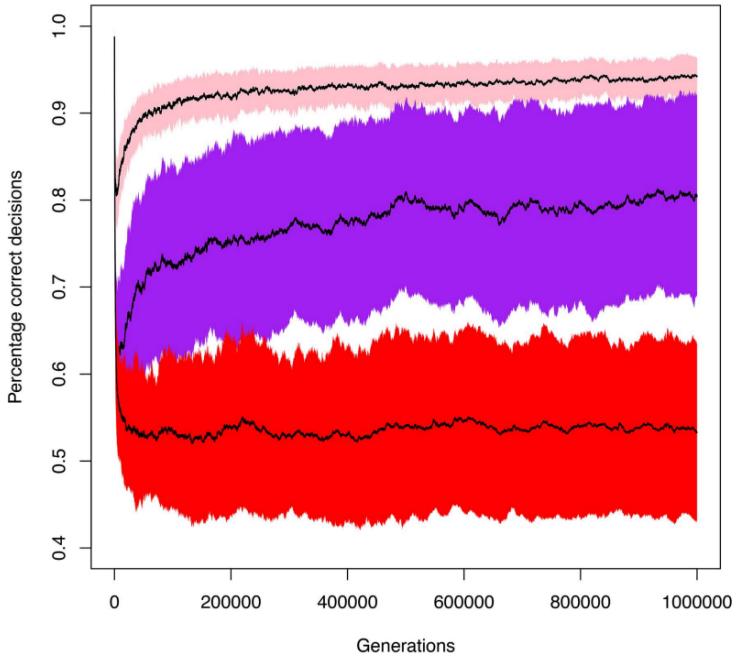

Figure 3. Simulation results comparing the average performance for different scenarios. We first evolved replicate GRNs in a variable environment where it is adaptive to switch on a metabolic pathway (fat synthesis) under low-fat conditions and to switch it off under high-fat conditions. In generation 0, a monomorphic population was established, where all 10,000 individuals were endowed with the same well-performing GRN (different across replicates). Subsequently, the population evolved subject to selection, mutation ($\mu = 0.001$ per gene locus), and genetic drift in a fat-rich environment, where it is adaptive to constitutively switch off the metabolic pathway. Every 100 generations, we monitored the performance of a sample of GRNs (percentage correct decisions) in the original (fat-variable) environment: 1.0 means that the GRN is still making 100% adaptive decisions; 0.5 means that the GRN only makes 50% adaptive decisions, as would be expected by a random GRN or a GRN that switches the pathway constitutively on or off. The colored graphs show the average performance (± standard deviation) of the GRNs for three scenarios (100 replicates per simulation). Red: the population never again encounters the fat-variable environment; performance converges to 0.5, corresponding to constitutively switching off fat synthesis and hence the loss of adaptive plasticity. Pink: the individuals encounter a fat-variable environment on average every 100 generations; after an initial rapid drop in performance, a sustained high performance (> 90% correct decisions) of the GRNs is regained after about 100,000 generations. Purple: the individuals encounter a fat-variable environment on average every 1000 generations; after an initial rapid drop in performance, an intermediate performance (> 75% correct decisions) is regained gradually.

Discussion

We showed that a switch underlying plastic responses evolves mutational robustness and can withstand decay if it remains unused for extended periods of time. Another modeling study found that adaptive plasticity will be maintained in the genome for 108 generations (Masel et al, 2007). Our simulation study shows that non-switching rapidly evolves in a fat-rich environment (leading to the loss of plasticity), but once the device has evolved mutational robustness, only incidental 'switching on' of the trait is sufficient for plasticity to be maintained within the genome. Plasticity itself can thus be highly robust to mutational change, which can apply also to other traits and systems. Our results further revealed large differences in the slopes of reaction norms between families, suggesting that there is genetic variation for plastic expression of fat 8 synthesis. The plasticity of fat synthesis itself may thus evolve according to the local fat availability of host populations in the wild. Phenotypically plastic organisms can incur different types of costs (Auld et al, 2010). In our simple model, we only consider the cost of phenotype-environment mismatching, that is, the costs of expressing the 'wrong' phenotype in a given environment. When placed in a high-fat environment, the preadapted GRNs in our simulations take the 'right' decision to switch off lipogenesis. Accordingly, they do not face any costs of mismatching. Yet, the genetic switch rapidly decays (as indicated in Figure 3 by the rapid drop in performance when tested in an intermediate-fat environment), due to the accumulation of mutations. It is not unlikely that there are additional fitness costs of plasticity, such as the costs for the production and maintenance of the machinery underlying plasticity (Auld et al, 2010). In the presence of such constitutive costs, plasticity will be selected against when organisms are living in an environment where only one phenotype is optimal (as in the high- and low-fat environments in Figure 1). This would obviously affect the evolutionary dynamics in Figure 3, but the size of the effect is difficult to judge, as the constitutive costs of plasticity are notoriously difficult to quantify. In the case of the simple switching device considered in our model, we consider the constitutive costs of plasticity as marginal, but these costs might be substantial in other scenarios.

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Chapter 5

The evolution of mutational transformers speeds up adaptation in a changing environment

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*These authors contributed equally to the manuscript (shared first authorship)

In preparation

Abstract

At the genetic level, mutations are generally assumed to be random with respect to the 'demands' of natural selection. Yet, it has been demonstrated that mutations are often biased with respect to their phenotypic effects. Such biases can be important, as they allow the rapid adaptation of a population to changing conditions. Here, we demonstrate that adaptation-enhancing biases can readily evolve in a fluctuating environment. To this end, we consider a population that is exposed to a stochastically changing environment. The environment can be in two states for which different phenotypes are adaptive. The phenotype of an individual is determined by a gene regulatory network that cannot sense the environmental state and can therefore only adapt to environmental change through mutation and selection. In our model, the mutation process at the genetic level is random and not evolvable. Yet, even small networks rapidly evolve a structure that biases the phenotypic effects of mutations in such a way that adaptive evolution after environmental change is speeded up considerably. We term the mechanism responsible for this increase in evolvability a "mutational transformer". A mutational transformer is a network configuration where the phenotypic effects of genetic mutations are distributed in such a way that substantial adaptation to new conditions can be achieved via a single or few genetic mutations. We show that in our model mutational transformers are based on two distinct mechanisms: mutation amplification and mutation canalisation.

Introduction

Evolvability concerns *"the capability of a biological system to undergo adaptive evolution"*(Payne & Wagner, 2019; Pigliucci, 2008; Riederer, Tiso, van Eldijk, & Weissing, 2022). The mechanisms that govern evolvability are subject to evolution themselves; hence, evolvability is far from a static property but is shaped by selection and other evolutionary processes. For instance, mutation rates can evolve to increase the production of variation under stressful conditions, thereby enhancing evolvability (Metzgar & Wills, 2000; Sprouffske, Aguílar-Rodríguez, Sniegowski, & Wagner, 2018; Woods et al., 2011). Evolvability can also change over the generations through the

evolution of mechanisms that amplify, buffer, or bias the effect of (genetic) variation on fitness (Kokko et al., 2017; Pavlicev & Hansen, 2011; Uller, Moczek, Watson, Brakefield, & Laland, 2018), phenomena that are sometimes referred to as developmental bias and/or canalisation (Crombach & Hogeweg, 2008; Riederer et al., 2022; Waddington, 1942). These mechanisms alter the genotype-to-phenotype map that describes how changes at the genetic level translate into phenotypic differences. Any alteration of this map can have implications for the dynamics and outcome of evolutionary processes (Greenbury, Louis, & Ahnert, 2022; McGuigan & Aw, 2017; Takeuchi & Hogeweg, 2012).

Some genotype-to-phenotype maps enhance evolvability more than others (Riederer et al., 2022). This may simply be a coincidence or a side-effect of other processes. However, it is worth considering the possibility that the structure of the genotype-to-phenotype map has been selected for evolvability, so as to facilitate rapid adaptation to frequently encountered environmental changes. In several empirical systems, there is evidence for this, as after a commonly encountered environmental change, a highly adaptive phenotype can be achieved via a single or very few mutations with large phenotypic effect. A first example concerns the rapid emergence of antibiotically resistant subpopulations that quickly arise in bacterial populations grown from a single susceptible clone ('heteroresistance'). This extremely fast acquisition of resistance is associated with the spontaneous tandem amplification of specific resistance-enhancing genes (Andersson, Nicoloff, & Hjort, 2019; Band & Weiss, 2019; Hjort, Nicoloff, & Andersson, 2016; Nicoloff, Hjort, Levin, & Andersson, 2019; Pereira, Larsson, Hjort, Elf, & Andersson, 2021). A second example concerns the adaptation of the yeast *Saccharomyces cerevisiae* to heat stress. Rapid adaptation to a higher-temperature environment can be provided by the duplication of a single chromosome. This duplication simultaneously upregulates a multitude of genes that provide adaptation to a higher temperature (Yona et al., 2012). In these and other examples, the structure of the genotype-to-phenotype seems to have evolved to facilitate rapid adaptation. The idea that the structure of the genotype-to-phenotype map evolves to facilitate the

generation of adaptive variation has also been proposed and extensively discussed in the theory of facilitated variation (Solopova et al., 2012).

But how can the genotype-to-phenotype map evolve in a way that random genetic mutations have phenotypic effects that speed up adaptive evolution? A key theoretical study on this question was performed by Anton Crombach and Paulien Hogeweg (Crombach & Hogeweg, 2008), who considered the evolution of gene regulatory networks (GRNs) that generated expression patterns, which in turn resulted in a specific phenotypic effect. A population of individuals evolved in an environment that alternated between two states, each selecting for a different target GRN expression pattern. The authors observed that throughout evolution the GRNs re-organized to allow for increasingly rapid switching between the two target expression patterns. Moreover, the increasing rate of adaptation was accompanied by a reduction in the number of mutations required to switch between expression patterns. These findings clearly demonstrate that structural features can evolve to enhance evolvability: whilst the frequency and type of genetic mutations remain random and constant, the GRNs evolve to be structured in such a way that a single or very few mutations are sufficient to switch to a phenotype that is well-adapted to the new environment. In a follow-up study, Hogeweg and colleagues (Cuypers, Rutten, & Hogeweg, 2017) showed that such a mutational configuration can also evolve in GRNs that are able to sense the environment and, hence, can evolve phenotypic plasticity. The results of (Crombach & Hogeweg, 2008) and (Cuypers et al., 2017) showcase clear instances of the evolution of developmental biases toward an adaptive phenotype. We here term the underlying configuration a "mutational transformer": the GRN is structure transforms the phenotypic impact of mutations in such a way that a single or very few mutations allow for a switch to a different adaptive phenotype, facilitating rapid adaptation. The name indicates that a population has evolved to use mutation (and not plasticity) to rapidly switch between alternative adaptive phenotypic states.

The studies of Hogeweg and colleagues provide proof of principle that mutational transformers can evolve. However, the GRNs and their phenotypic effects in (Crombach & Hogeweg, 2008) and (Cuypers et al., 2017) are quite complex, making it difficult to see how the mutational

transformers arise and how they function. Moreover, it remains unclear whether such complexity is essential for mutational transformers to evolve. Here, we therefore take a minimal modelling approach that allows us to study in detail whether, when, and how the distribution of phenotypic effects of random genetic mutations evolves under changing environmental conditions. To this end, we consider simple heritable GRNs consisting of a small number of interacting nodes and a single phenotypic output value. The performance of a network is determined by how well this output value matches the current state of the environment. In line with (Crombach & Hogeweg, 2008), we evolve these GRNs in an environment that randomly alternates between two different states. The GRNs considered cannot sense their environment, making it impossible to evolve phenotypic plasticity. Accordingly, individuals whose phenotype matches one environment will inevitably be mismatched to the other. Thus, the only way in which an individual's phenotype can track environmental change is through mutation and genetic evolution of their GRN. Over a long period of evolution, in which the population has been repeatedly selected to adapt to a transition between environmental states, we expect the evolution of a mutational transformer, that is, the evolution of networks that rely on a smaller number of mutations to change their phenotype from one optimal phenotype to the other. In a first step, we will demonstrate that mutational transformers do indeed evolve even in very simple networks, consisting of merely two nodes and encoded by just three gene loci. This allows us to investigate the evolution and functioning of a mutational transformer in considerable depth and detail. Subsequently, we study how the complexity of the GRN influences the evolution of a mutational transformer. Besides varying the number of nodes, we also investigate whether non-linear interactions in the gene regulatory network allow for the evolution of more efficient mutational transformers.

Model

Model overview

The model structure is illustrated in Figure 1. Each simulation follows a population of fixed size (1,000 individuals) over a period of 1 million

generations. Generations are discrete and non-overlapping and reproduction is asexual. The individuals are living in an environment that can be in one of two states (E1 or E2). The state is constant throughout a generation (and the same for all individuals), but it switches stochastically to the other state once in a while. Each individual has a phenotype *y* that is determined by a heritable gene regulatory network. Phenotype $y = +0.5$ is optimal in environment E1, while $y = -0.5$ is optimal in environment E2. The 'fitness' (expected reproductive success) of an individual is negatively related to the distance of its phenotype y from the optimal value y_{opt} of its environment.

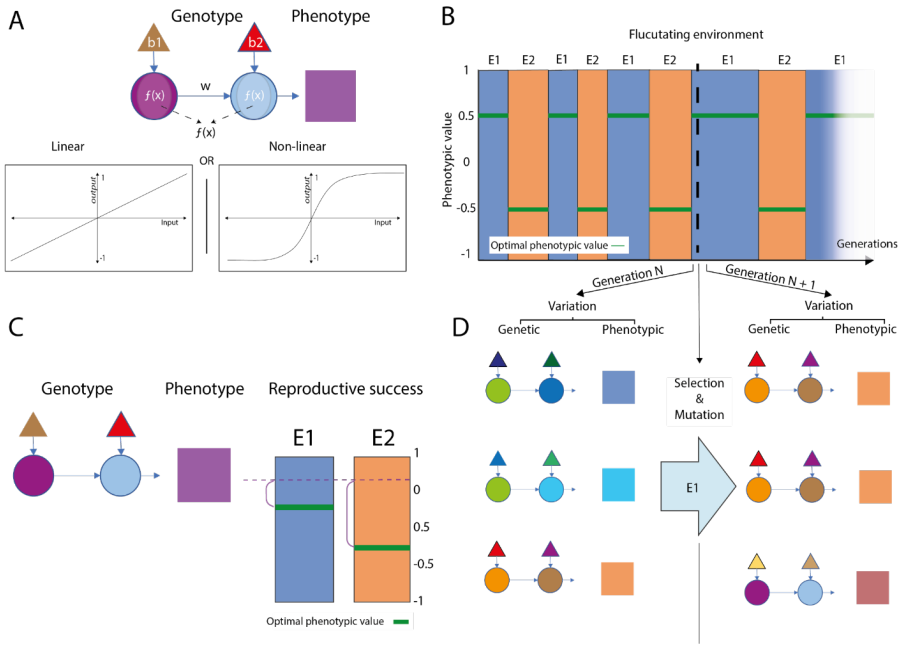


Figure 1. Graphical illustration of the model. A. Determination of the phenotype of an individual. Each individual harbours a heritable gene regulatory network (GRN) that determines the individual's phenotype. The panel shows the simplest network, which consists of two connected nodes (coloured circles). The working of the network is characterised by two baseline activation levels *b*1 and *b*2, the connection weight *w*, and the expression function *f*(*x*), which is either the identity function (left) or a sigmoidal function (right). The phenotype *y* of an individual is fully determined by these ingredients. The parameters *b*1, *b*2, and *w* are encoded by alleles at three gene loci and transmitted from a parent to its offspring, subject to rare mutations. All three loci have the same mutation rate and the mutational step size is normally distributed around zero. **B.** The environment fluctuates stochastically between two states (*E*1 and *E*2). The duration of the periods between environmental switch events is geometrically distributed, with a mean value of 100 generations. In each state, there is a different optimal phenotypic value (indicated in green). **C.** Individuals are selected to minimise the mismatch between their phenotype *y* and the optimal phenotype y_{opt} in the current environment: the expected reproductive success of an individual is given by a Gaussian function of this mismatch and therefore decreases with the squared mismatch between *y* and y_{opt}. **D.** Selection and mutation determine (together with genetic drift) determine how the genotype distribution (and the corresponding phenotype distribution) change from one generation to the next.

Each individual harbours a GRN that is encoded by *N* gene loci (where *N* depends on the network architecture). The alleles at these loci are real numbers whose values represent the strength of gene-gene interactions and basal gene-activity levels (Figure 1A). Together, the GRN alleles of an individual determine the individual's phenotype. These alleles are transmitted from parent to offspring, subject to rare mutations. At the start of each generation, 1,000 offspring are produced. In a first step, a parent is assigned to each offspring by drawing an individual from the previous generation (with replacement) by means of a weighted lottery where the weighing factor of each potential parent is proportional to the fitness of that individual. This procedure ensures that the 'fitness' of each individual in the parental population is proportional to the individual's expected reproductive success. Each newly produced offspring inherits all GRN alleles from its parent. Subsequently, mutation takes place: at each GRN locus, a mutation takes place with a certain mutation probability that is fixed and the same for all loci. If a mutation takes place, the parental allele *a* is changed by adding a small number (the mutational step size) to it. For all loci, the mutational step sizes are drawn from the same normal distribution with mean zero and a small mutational variance. After mutation has taken place, the offspring's phenotype is determined, which, in turn, determines the fitness of the offspring.

This procedure results in an evolutionary trajectory that is governed by the interplay of selection, mutation, and genetic drift. At the genetic level, the mutation process is completely random, that is, not affected by the state (or the state dynamics) of the environment: the mutation rate and the distribution of mutational step sizes remain constant throughout each simulation. However, the phenotypic effect of a mutation depends on the allelic values at the GRN loci (see below). Accordingly, the distribution of phenotypic mutational effects can evolve. We are interested in the evolution of this distribution and the corresponding evolution of the 'evolvability' of the population, which we quantify by the speed with which the new phenotypic optimum is approached after each change of the environment.

Environmental change

In each generation, the environment is in one of two states (E1 or E2). At the start of each generation, the environment switches from the previous state to the alternative state with probability $\chi = 0.01$. Hence the duration of environmental stasis is geometrically distributed with mean value $\chi^{-1} = 100$ generations.

Fitness

The optimal phenotype in environmental state E1 is $y_{\mathrm{opt}} = 0.5$, while it is $y_{\mathrm{opt}} = -0.5$ in state E2. The fitness of an individual with phenotype *y* decreases with the mismatch $y - y_{\mathrm{opt}}$ with the environmental optimum in a Gaussian manner:

$$F(y) = exp(-S \cdot (y - y_{\mathrm{opt}})^2).$$

The parameter *S* quantifies the strength of selection. In all our simulations $S = 2$.

Gene regulatory networks

The phenotype of an individual is encoded by a gene regulatory network. There are various ways to model GRNs. We here follow (van Gestel & Weissing, 2016), where the flow of information through the network (via gene activation and inhibition) is modelled in close analogy to that of artificial neural networks. The GRNs considered are multilayered and feedforward: they consist of one initial node, multiple layers of internal nodes, and one output node. The type of network will be indicated by the notation n_1-n_2-n_3-1, where n_k is the number of nodes in layer *k*, and the 1 to the right denotes the output node. For Instance, 1-2-2-2-1 refers to a network with one initial layer of one node and three internal layers with two nodes per layer. The simplest network considered does not have internal layers and is of type 1-1. This network is shown in Fig. 1A.

Each node *j* of the GRN has an activation state x_j and produces an output $z_j = f(x_j)$, where *f* is the so-called transfer function of the node. The activation

state of a node *j* is given by the node's baseline activation b_j and a weighted sum of the outputs of the nodes in the previous layer:

$$x_j = b_j + \sum_i w_{ij} \cdot z_i = b_j + \sum_i w_{ij} \cdot f(x_i),$$

where *i* ranges over all nodes of the previous layer. The baseline activation values b_j and the connection weights w_{ij} are real numbers that are transmitted from parents to their offspring (subject to rare mutations). Together with the transfer function, they fully determine the functioning of the GRN and the resulting phenotype: The initial nodes have no inputs from other nodes. Accordingly, their activation state is $x_i = b_i$ and its output is $z_i = f(x_i)$. Together with the weighing factors w_{ij}, where *j* ranges over the nodes of the second layer, this determines the activation states x_j and the output $z_j = f(x_j)$ of these nodes. These again determine the activation states and the output values of the nodes in the third layer, and so on. In the end, the output node ω is reached. As before, the activation level x_ω of this node is determined by this node's baseline activation level b_ω and a weighted sum of the outputs of the nodes of the previous layer. In our model, the output of the terminal node $z_\omega = f(x_\omega)$ corresponds to the phenotype of the individual ($y = z_\omega = f(x_\omega)$).

The simplest network of type 1-1 (illustrated in Fig. 1A) has only two nodes: the input node 0 and the output node ω. Hence, there are only three heritable parameters: the baseline activation levels b_0 and b_ω and the weighing factor $w_{0\omega}$, which, for simplicity, we call *w*. In view of the above rules, the phenotype of an individual with a 1-1 network is therefore given by:

$$y = f(x_\omega) = f(b_\omega + w \cdot z_0) = f(b_\omega + w \cdot f(b_0)).$$

We will consider two transfer functions. The simplest is the identity function $f(x) = x$. In this case, the phenotype of an individual is given by the relationship $y = b_\omega + w \cdot b_0$. The second is the sigmoidal function (illustrated in Fig. 1A) $f(x) = \frac{x}{(1+|x|}$).

Inheritance and mutation

Individuals are haploid and have an allele at all GRN loci, that is, at the loci encoding the baseline activation values b_j and the connection weights w_{ij}. The alleles are real numbers that are transmitted from parents to their offspring, subject to mutation. The per-locus mutation probability is $\mu = 0.01$. If a mutation occurs, a mutational step size is drawn from a normal distribution $N(0, \sigma)$ with mean sigma and mutational standard deviation $\sigma = 0.1$. This step size is added to the parental value, yielding the new mutated value.

Quantifying evolvability

To assess whether long-term evolution in a fluctuating environment enhances evolvability, we need to quantify the rate of adaptation to a new environmental state after a change in the environment. To this end, we operationally define the "time to adaptation" as the number of generations it takes until the mean fitness of the population reaches a threshold value of 0.9. In two situations, no "time to adaptation" is recorded: (a) if the threshold value is not reached before another change in environmental state occurs, and (b) if immediately after a change in environment the mean fitness of the population already exceeds the threshold value. However, we record how often situations (a) and (b) do occur.

Results

Network structure and the evolution of mutational transformers

The example simulation in Figure 2 illustrates that, in the course of long-term evolution, the time to adaptation substantially decreases even in the case of a simple 1-1 GRN with identity transfer function. The substantial decrease in the time to adaptation from the start of the simulation (Fig. 2A) to the end of the simulation (Fig. 2B) suggests that a "mutational transformer" has evolved, that is, a change in the way how random mutations at the genetic level translate into changes of the phenotype.

To check for the generality of this result, we run multiple replicate simulations for a wide range of GRN architectures. GRNs varied in the number of layers, the number of nodes per layer, and the type of transfer function. Figure 3 shows the results for four network architectures (1, 1-1, 10-2-1-1-1 and 5-5-5-1) and both transfer functions. In each case, the time to adaptation was reported in relation to the duration of evolution. If a mutational transformer evolves, the time to adaptation should decrease in the course of evolution. This does not happen for the trivial "network" 1 (consisting of just one output node and therefore arguably not really a network). As shown in Fig. 3A, the time to adaptation did not noticeably decrease in the course of evolution, neither for the identity nor for the sigmoidal transfer function. In contrast, there is a pronounced decrease in the time to adaptation in GRNs of type 1-1 (Fig. 3B), both in the case of an identity transfer function (for which this phenomenon is illustrated by Fig. 2) and even more so in case of a sigmoidal transfer function

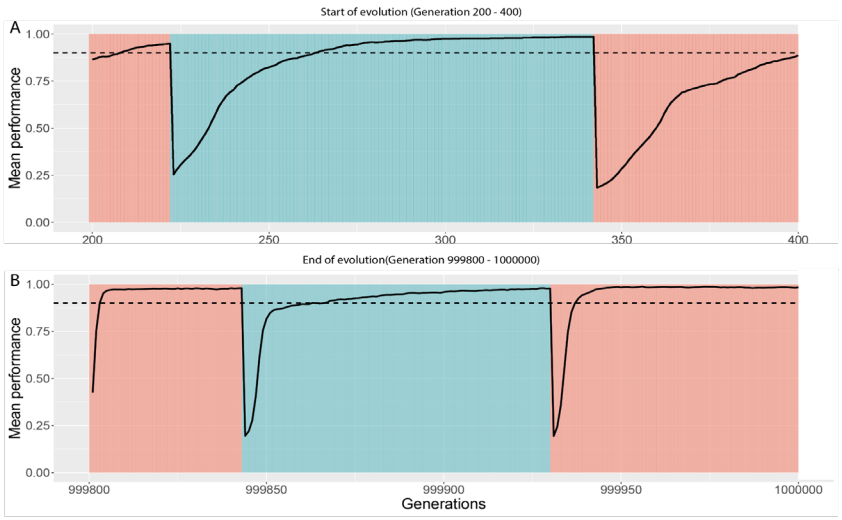
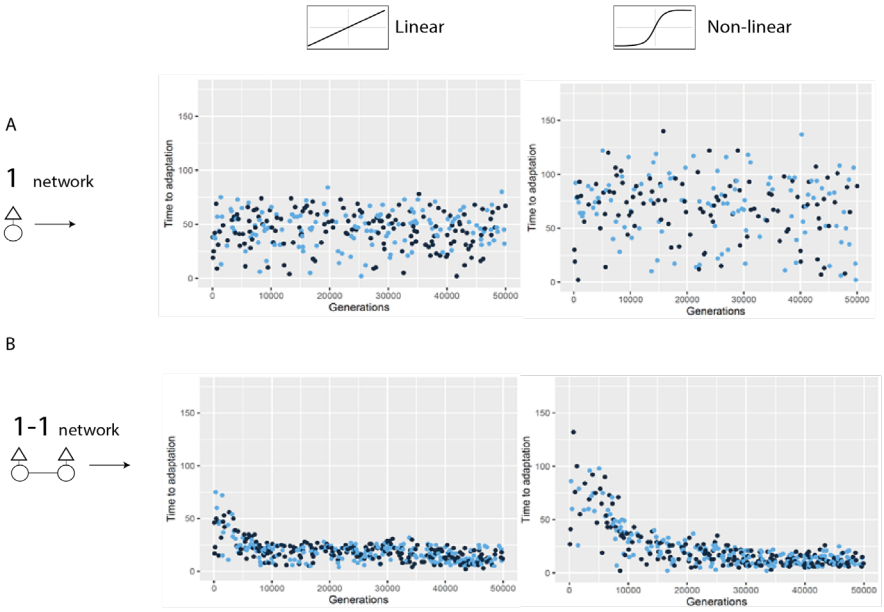
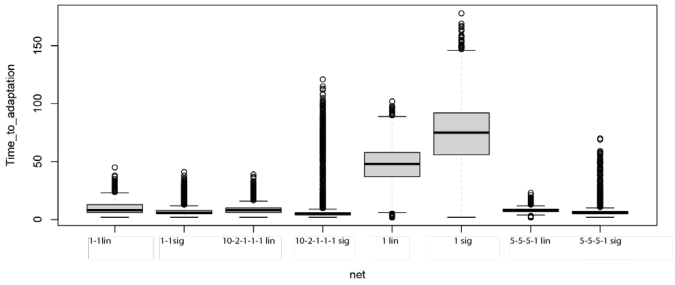


Figure 2. Time to adaptation at (A) the start and (B) the end of long-term evolution. For an example simulation based on a 1-1 network with identity transfer function, the graphs show the drop and increase in mean fitness after two instances of environmental change. The state of the environment is indicated by the background colour (blue: $y_{\mathrm{opt}} = 0.5$, red: $y_{\mathrm{opt}} = -0.5$). The dashed line indicates the threshold value of 0.9 used to quantify the time to adaptation. In the early part of the simulation (Panel A: generations 200 to 400), the mean fitness of the population recovers much more slowly after environmental change than in the end of the simulation (Panel B: the final 200 generations).

The boxplots in Fig. 3CD show, for the GRNs considered, the time to adaptation in the second half of the evolutionary trajectory. In all cases, the initial time to adaptation was typically 50 generations or more. With the exception of the trivial “network” 1, the time to adaptation decreased in all cases to a median value between 5 and 10 in the second half of the simulations (the final 500,000 generations). In other words, all non-trivial GRNs allowed for the evolution of a faster rate of adaptation (i.e., higher evolvability). There is no clear relationship between network complexity and the evolved time to adaptation. However, for any network architecture considered, the evolved time to adaptation was markedly shorter for the sigmoidal transfer function than for the identity function. We will now investigate in more detail how the reduction in adaptation time was achieved. For this, it will be useful to consider the two transfer functions separately.


Linear
Non-linear
A
1 network
Time to adaptation
Generations
B
1-1 network
Time to adaptation
Generations


C
Time_to_adaptation
1-1lin
1-1sig
10-2-1-1-1 lin
10-2-1-1-1 sig
1 lin
1 sig
5-5-5-1 lin
5-5-5-1 sig
net

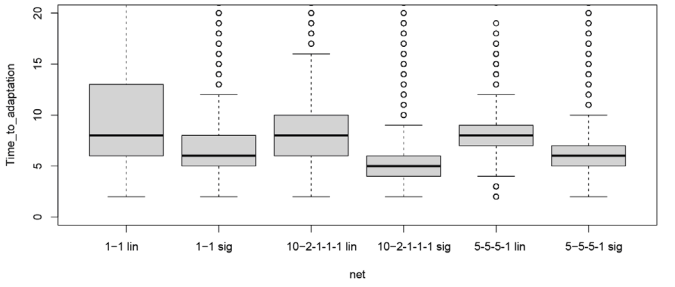
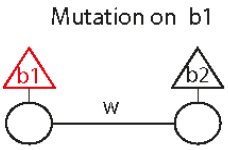
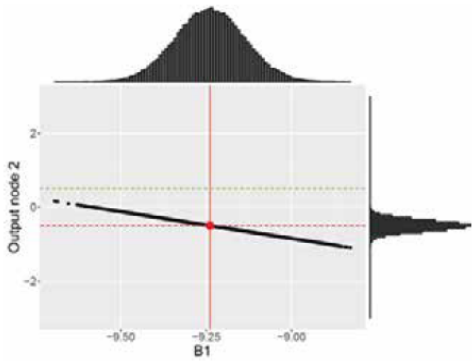

D
Time_to_adaptation
1−1 lin
1−1 sig
10−2-1-1-1 lin
10−2-1-1-1 sig
5-5-5-1 lin
5−5-5-1 sig
net

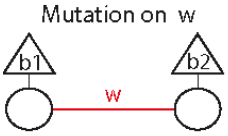
Figure 3. Evolution of the time to adaptation for GRNs differing in complexity and transfer function. A. For the trivial "network" with just one output node, no evolutionary trend in the time to adaptation was observed, neither in the case of the identity transfer function (left panel) nor in the case of a sigmoidal transfer function (right panel). Point colours indicate the two different environments. **B.** For the 1-1 network, the time to adaptation markedly decreased in the initial phase of evolution (first 10,000 generations), for both transfer functions. **C.** Evolved time to adaptation during the last 500,000 generations of the simulation for GRNs differing in architecture (1, 1-1, 10-2-1-1-1 and 5-5-5-1) and transfer function (identity or sigmoidal). In the boxplots, the data of 7-10 replicate simulations are combined. With the exception of the trivial "network" 1, a fast adaptation rate evolved in all GRNs. **D.** A zoomed-in version of panel C, showing only those types of GRN that evolved a fast rate of adaptation. This panel shows that the GRNs with a sigmoidal transfer function evolved a faster rate of adaptation than GRNs with the same architecture but an identity transfer function.

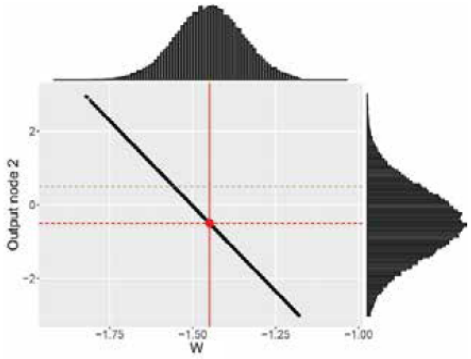
A


Mutation on b1
b1
b2
w

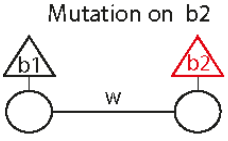

Output node 2
2
0
-2
-9.50
-9.25
-9.00
B1

B


Mutation on w
b1
b2
w


Output node 2
2
0
-2
-1.75
-1.50
-1.25
-1.00
W

C


Mutation on b2
b1
b2
w

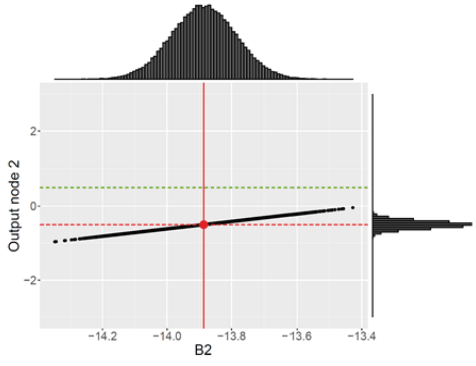

Output node 2
2
0
-2
-14.2
-14.0
-13.8
-13.6
-13.4
B2

Figure 4. Phenotypic effects of mutations at the three loci of a 1-1 GRN that evolved a fast rate of adaptation. Data in (A-C) reflect a mutational analysis of a 1-1 GRN with linear transfer function that had evolved a fast rate of adaptation. The panels show the phenotypic effects of random mutations at **A.** the *b*1 locus, **B.** the *w* locus, and **C.** the *b*2 locus. The allelic values before mutation are indicated by the solid red line. The phenotype produced by the unmutated GRN (indicated by the dotted red line) matches the current environmental optimum (which happens to be equal to -0.5). The dotted green line indicates the alternative optimum (0.5), the "desired" value after a change in environment. While the mutational effects on the phenotype are relatively small in case of the *b*1 and the *b*2 locus, the *w* locus acts as a mutation amplifier: the other phenotypic optimum can be reached with just one or two mutations, which is the defining property of a mutational transformer.

Mutational amplifiers cause mutational transformation in linear GRNs

To understand the inner workings of a network that evolved a fast mutation rate, we first study the simplest type of GRN: a network with two nodes and a linear transfer function (1-1). The behaviour of this GRN is controlled by only three loci: *b1*, *w*, and *b2*. When analysing the results of many replicate simulations, we observe that in this linear GRN, either the *w* locus or the *b1* locus evolves to become responsible for the fast adaptation; this locus is the sensitive locus that acts as a mutational transformer. This is illustrated by Figure 4, which shows for an evolved example network the phenotypic effects of genetic mutations at each of the three loci. While the phenotypic effects of mutations at the *b1* and *b2* locus are very small (Fig. 4AC), the sensitive *w*-locus acts as a "mutation amplifier": mutations at this locus have a large effect on the phenotype, allowing the rapid evolution of the phenotype to a new optimum, should the environment require this. In fact, the distribution of phenotypic effects induced by small-scale and normally distributed mutations at the *w*-locus encompass both phenotypic optima. Hence, if a GRN is adapted to one phenotypic optimum, the other phenotypic optimum can be produced by just one or two mutations. To summarise these findings: The amplification of small mutations on the sensitive locus to large phenotypic changes, allows the alternate optimal phenotype to be reached through a single mutation, thereby facilitating rapid adaptation when the environment changes, creating a mutational transformer.

These results can be explained by the fact that for the network considered the phenotype of an individual is given by $y = w \cdot b1 + b2$ (see Methods). This shows that *b*1 and *w* have a similar effect on the phenotype and explains why both corresponding loci can be sensitive. The phenotypic effect of mutations at the *w* locus are amplified by a factor *b*1, the current value of *b*1. Hence, the allele at the *b*1 locus regulates the phenotypic effects of *w*-mutations, and *vice versa*.

Non-linear GRNs evolve more refined mutational transformers

For all network architecture considered, the evolved time to adaptation was markedly shorter for the sigmoidal transfer function than for the identity

function (Figure 3D). We hypothesise that non-linear gene interactions allow for the evolution of a more refined mutational transformer. To understand this, we now examine the simplest nonlinear GRN that managed to evolve a mutational transformer (i.e. 1-1, see Figure 5). Figure 5A illustrates that in the initial phase of evolution (in generations 200 to 400, left panel) it takes more than 50 generations before, after a change of the environment, the mean fitness of the population has regained the value 0.9, while the time to adaptation is much faster (between 5 and 10 generations) in the final stage of evolution (right panel). This is achieved by a marked differentiation of the alleles *b1*, *w*, and *b2* at the three GRN loci (Fig. 5B). This raises the question: how does the evolution of these loci translate to the evolution of a mutational transformer? To investigate this, we record the phenotypes produced by 1000 different mutations at each locus of the best-performing GRN at a certain time point (Figure 5C). Initially all loci are similarly sensitive to mutations and the distribution of the phenotypes produced is unimodal. Most mutations will only slightly alter the value of the currently expressed phenotype. This explains why, at the start of the simulation, the recovery of the population after an environmental change is slow and gradual: after an environmental change, many mutations are required to shift to the new optimal phenotype.

Towards the end of the simulation, when the population is able to recover rapidly from environmental change (i.e. the mutational transformer has evolved), we observe that the phenotype is highly sensitive to mutations at a particular, sensitive, locus. We observed this same pattern in the linear GRNs, where a single gene evolved to be sensitive to mutations. However, instead of being unimodal as for the 1-1 GRN with an identity transfer function (Figure 4), the distribution of phenotypes produced by mutations at the sensitive locus of non-linear 1-1 GRN is bimodal, that is, with two peaks (Figure 5C, panels 3 and 4). The two peaks coincide with the two optimal phenotypes showing that the GRN has evolved to bias the effect of random mutations toward these phenotypes. The distribution of phenotypic effects of mutations on the sensitive locus is shaped such that the phenotype to which the population is currently adapted is most frequently produced. The second peak in the distribution of the phenotypic effects of mutations

corresponds with the optimal phenotype of the alternative environment. This mechanism seems to be symmetrical: after adaptation to an environmental change, the new optimal phenotype is now produced more often. If mutation leads to an intermediate phenotype that is between the two different optimal phenotypes, mutations are equally likely to produce either of the two optimal phenotypes.

In all non-linear GRNs considered (1-1, 10-2-1-1-1 and 5-5-5-1), a sensitive locus with similar properties evolved and it always occurred in the initial part of the GRN (either at the b_0 locus or at one of the loci encoding the weighing factor w_{0j} of the connection between the initial node and a node j in the first processing layer).

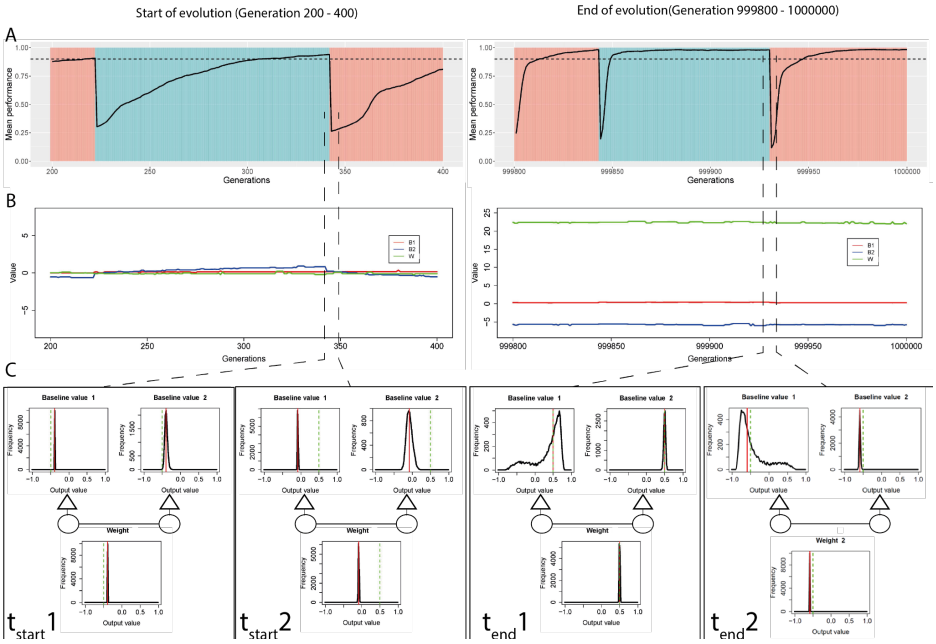


Figure 5. Evolution of a refined mutational transformer. The figure shows a representative simulation of the evolution of a 1-1 GRN with sigmoidal transfer function. **A.** Evolutionary trajectory of mean population fitness at the start (generations 200 to 400, left panel) and the end (final 200 generations, right panel) of the simulation. The state of the environment is indicated by the background colour (blue: $y_{\mathrm{opt}} = 0.5$, red: $y_{\mathrm{opt}} = -0.5$). **B.** Evolved allelic values at the three network loci at the start (left panel) and the end (right panel) of the simulation. **C.** Distributions of the phenotypic effects of mutations at four different time points during the simulation. The first two time points are early in the simulation, shortly before ($t_{\mathrm{before}}^{\mathrm{early}}$) and after ($t_{\mathrm{after}}^{\mathrm{early}}$) an environmental change. The other time points are from a later stage in the simulation, shortly before ($t_{\mathrm{before}}^{\mathrm{late}}$) and after ($t_{\mathrm{after}}^{\mathrm{late}}$) an environmental change. A comparison of the phenotypic-effect distributions at the early time points with those at the later time points reveals that for two of the GRN loci genetic mutations have a small effect on the phenotype, while the GRN locus encoding *b*1 is "sensitive": mutations at this locus produce a broad spectrum of phenotypes. Moreover, the distribution of phenotypic effects at the sensitive locus is strongly asymmetric and bimodal, with a sharp peak close to the current environmental optimum and a second maximum close to phenotypic optimum at the other environmental state.

The inner workings of a refined mutational transformer: a mutation canaliser

The mutational transformers that evolve in non-linear GRNs represent a more refined solution when compared to the mutational amplifiers observed in linear GRNs. This is highlighted by the faster recovery of performance after an environmental change (Figure 3 C-D). The non-linear GRNs evolve their architecture, not only to put the two optimal phenotypes in the range of a single mutation step, but also to canalise the phenotypic outcomes of mutations towards particular phenotypic values. We term this more refined mechanism underlying the evolution of a mutational transformer in nonlinear GRNs a mutation canaliser.

The behaviour of the non-linear 1-1 GRN is governed by only three parameters which allow for numerical analysis (Figure 6). This allowed us to study how mutations on the sensitive locus affect not only the output of the second node (phenotype) but also how they affect the output of the first (intermediate) node. Applying normally distributed mutations to the evolved value of locus *b1* leads to a slightly skewed distribution of node 1 output values. The values of the *b2* and *w* loci have evolved so that when the skewed distribution of outputs of node 1 is fed into node 2 it is transformed to a bimodal distribution of phenotypes (output values). As can be seen in Figure 6 A, the ability of node 2 to transform the asymmetrical distribution of outputs of node 1 into a bimodal distribution of phenotypes relies on the presence of a non-linear (sigmoidal) transform function. This shows why in our minimal model the ability to canalise mutations towards particular phenotypic values is contingent on the presence of a non-linear transfer function. As we previously noted the mechanism of a mutational canaliser seems to be symmetrical, after mutation to the other environmental optimum a bimodal distribution of phenotypes is maintained. Figure 6 B shows that this symmetry is a consequence of the sigmoid transfer function. If we apply the average mutation to the sensitive locus (*b1* =+ 0.1 in Figure 6 B) this causes the bimodal distribution to “flip”. The main phenotypic peak now sits on the alternative optimum and the smaller peak on the current optimum. This, therefore, shows that canalisers not only have evolved to easily move to an alternative adaptive phenotype but that they have also

evolved an architecture that allows them to easily revert back to the previous adaptive phenotype as well.

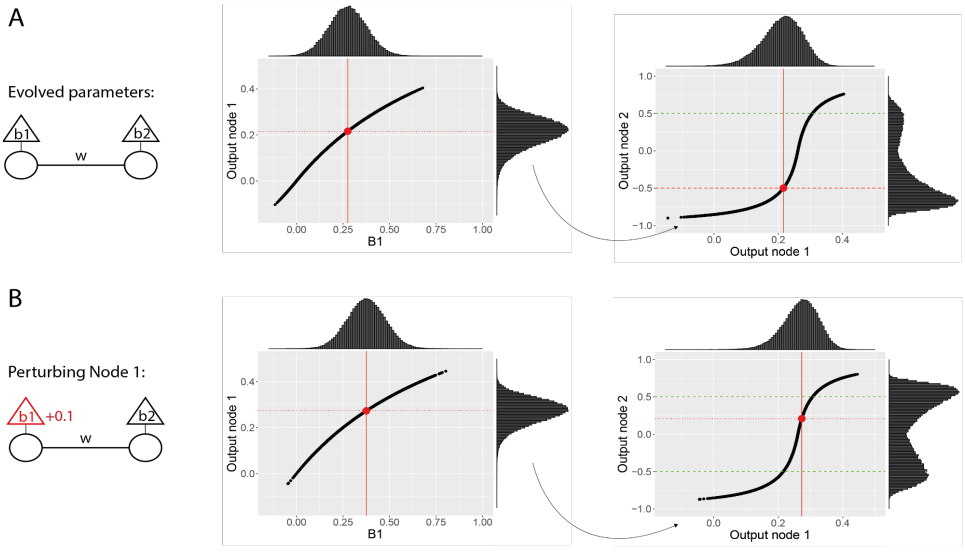

A
Evolved parameters:
b1
b2
w
Output node 1
B1
Output node 2
Output node 1
B
Perturbing Node 1:
b1
+0.1
b2
w
Output node 1
B1
Output node 2
Output node 1

Figure 6. Transformation of normally distributed genetic mutations into a bimodal distribution of phenotypic effects. The plots in each panel (A, B) show how different 1-1 networks transform a normal distribution of mutations on the sensitive locus (*b1* in this case) into a (different) distribution of phenotypes. A represents an evolved and adapted GRN, and B shows the same GRN but now with a mutated/perturbed value of its sensitive locus, The values of the sensitive locus are on the x-axis of the plots on the left column, while the values of the phenotype are projected on the y-axis of the plots on the right column. The plots on the left show the mapping of mutations on *b1* to outputs of node 1, the solid red line corresponds with the non-mutated original value of *b1*, while the dashed red line shows the value of the corresponding original output. The plots on the right show how the distribution of outputs of node 1 (now on the x-axis) is transformed into a distribution of phenotypes (outputs of node 2, y-axis). Also in this case the red and dashed line mark the non-mutated input and output values, while the dashed green lines show the values of the two environmental optima. **A:** The mutations-to-phenotype mapping of an evolved network. The distribution of outputs of node 1 produced by mutations is skewed but not bimodal. The distribution of phenotypic values in node 2 instead is clearly bimodal, with two peaks corresponding to the two optimal phenotypic values of the two environmental states. Therefore, mutations on node 1 of an evolved network will preferentially produce adaptive outcomes. In the evolved network we can see how node 1, although being the one sensitive to mutations that cause the mutational transformer (see Figure 5C), does not create the bimodal distribution of phenotypes. It instead skews the distribution of mutations. The asymmetry in these outputs is then used in node 2 to create a bimodal distribution of phenotypes. **B:** The mutations-to-phenotype mapping of a network for where the sensitive locus (*b1*) is perturbed (adding the average mutation step = 0.1 to its evolved value). Node 1 still skews the distribution of outputs but towards slightly lower values. This causes the creation of a bimodal distribution of outputs in node 2 but now with the two peaks inverted. If before the main peak of the bimodal distribution sat on the environmental optimum value of 0.5, now it sits on the other optimum value -0.5. At the same time, the smaller peak now sits at 0.5 instead of -0.5. This shows that the architectures of these GRNs evolved to be able to match the environmental optimum and, as well, rapidly switch to the other environmental optimum via a single mutation.

Discussion

First of all, we have demonstrated that even in a very simple model of a GRN the genotype-to-phenotype map can evolve to facilitate rapid adaptation to fluctuating environments. Even with such a simple genotype-to-phenotype map consisting of just three loci, evolution can lead to a mapping, such that adaptation to an environmental change occurs very fast and through a single mutation. Building on the work of those who studied this phenomenon before us (Crombach & Hogeweg, 2008; Cuypers et al., 2017), we term this phenomenon a mutational transformer, where the phenotypic state rapidly changes between alternative adaptive states through mutation.

Mutational transformers evolve in both GRNs with both linear and non-linear gene interactions. Whilst both types of mutational transformers facilitate rapid adaptation, the time to adaptation is shorter for non-linear GRNs. This points to the fact that these two different types of GRN lead to the evolution of mutational transformers that function through two distinct mechanisms. Both mechanisms enhance the evolvability of GRNs. However, they do so in two distinct ways.

Linear GRNs rapidly switch phenotype by amplifying the phenotypic effects of small mutations on a specific locus. Thus, these GRNs evolve to reach a relatively wide range of phenotypes through a single mutation; we term this type of mechanism a mutation amplifier. A mutational amplifier increases the amount of phenotypic variation that is created through mutation, providing a wider range of phenotypic variance on which selection can act. We show that the range of phenotypic variation is tuned to the specific distance between the two adaptive phenotypes encountered during evolution. Whilst the range of phenotypic variation is tuned, the phenotypes generated through mutation are not biassed towards the particular adaptive values. This also highlights a key drawback of mutation amplifiers; whilst they accelerate evolution when a population is maladapted (not on a fitness peak), they lead to the production of a lot of deleterious phenotypes when a population is well adapted (on a fitness peak). These properties of mutation amplifiers are reminiscent of results obtained by those studying the evolution of mutation rates, here it has been shown that high mutation rates

are advantageous only when a population is maladapted (Sprouffske et al., 2018).

On the other hand, non-linear GRNs (sigmoid transfer function) evolve a more refined mutational transformer. In these GRNs, the distribution of phenotypes generated through mutation is targeted towards the two adaptive phenotypes encountered during evolution. The non-linear interactions are essential to this strategy: they allow normally distributed mutations to create a bimodal distribution of phenotypes. In other words, the genotype-to-phenotype map evolves to canalise the effects of mutations towards adaptive outcomes; we term this mechanism a mutation canaliser. A mutational canaliser, biases variation towards particular adaptive outcomes, shaping the way mutations affect fitness. This more refined mechanism explains why non-linear GRNs adapt faster in response to a change in environment than their linear counterparts. Interestingly the mechanism behind the mutation canaliser seems to closely resemble the phenomenon described by Crombach and Hogeweg (Crombach & Hogeweg, 2008), where the evolution of the genotype-to-phenotype map increased the frequency of beneficial mutations.

When comparing the mutation amplifier and the mutation canaliser two key differences are apparent. Since the mutation canaliser preferentially produces adaptive outcomes it has a much lower production of deleterious phenotypes when the population is well-adapted (on a fitness peak) compared to the mutation amplifier. However, this lower production of deleterious phenotypes comes at a cost: mutation canalisers are only expected to enhance adaptation when environmental fluctuations are repetitive (i.e. previous adaptive challenges are representative of future adaptive challenges), whereas a mutation amplifier is also expected to enhance adaptation in more irregularly fluctuating environments. In other words, mutational amplifiers have a broader scope than mutational canalisers (see also (Riederer et al., 2022)). Whilst above we compare and contrast the two caricatures of the mutation amplifier and the mutation canaliser, it is worth noting that in our opinion mutation amplification and mutation canalization are not mutually exclusive, it is possible to amplify the phenotypic effects of mutations whilst also somewhat biassing the effects of

mutation towards particular adaptive outcomes. In our model the environmental fluctuations are repetitive, switching between two different adaptive values, therefore previous adaptive challenges are representative of future adaptive challenges. It is clear that mutation amplifiers can enhance adaptation in such repetitively fluctuating environments. However, given that the phenotypic variation generated by a mutation amplifier is not directed towards a particular phenotype, mutation amplifiers are also expected to enhance adaptation in environments that fluctuate in a more irregular manner.

We think that a mechanism similar to the mutational amplifier and the mutational canaliser might be responsible for the pattern we observed in some empirical examples described above, such as heteroresistance, yeast thermal adaptation, and evolution of lactose metabolism. The rapid switching to an adaptive phenotype using a single or very few mutational steps closely resembles the behaviour of mutational transformers in our model. In each of these three empirical cases it is very likely that populations have previously repeatedly faced the relevant environmental change (antibiotics presence/absence, different growth temperatures, loss of lactose metabolising plasmid). Therefore we strongly suspect that in these cases the structure of the underlying GRN is not a fluke, it is instead the product of the evolution of evolvability, which has led to the creation of a mutational transformer. Furthermore, the mutational canaliser is reminiscent of many of the examples of developmental bias described in the literature. Consider for example how the structure of the GRN shapes the available variation in *Bicyclus anynana* (Allen, Beldade, Zwaan, & Brakefield, 2008), where the structure of the underlying GRN seems to favour the production of particular phenotypic variants over others.

Our results are largely aligned with those obtained by Crombach and Hogeweg (Crombach & Hogeweg, 2008), and Cuypers et al. (Cuypers et al., 2017), who studied similar questions using a different set of models. Their models used more complex GRNs and explicitly modelled a wide set of biological mechanisms, aiming to more closely mimic biological complexity. We instead set out to create a minimal model of a mutational transformer, which allowed us to uncover in detail the evolution of the underlying

mechanisms. We have demonstrated the evolution of mutational transformers across a wide range of different network architectures. It can be concluded that a minimal complexity of two nodes (three loci) is required, a network with a single node is not able to evolve a mutational transformer. Surprisingly, complexity beyond two nodes does not seem to improve the functioning (i.e. time to adaptation) of the mutational transformer. We demonstrate that mechanisms that shape the effect of mutations on the phenotype can readily evolve in very simple systems.

The fact that mutational transformers readily evolve in our extremely simple GRN model leads us to expect they may be a rather widespread phenomenon, in organisms facing fluctuating environments. Mutational transformers might be especially prevalent when organisms face repetitively fluctuating environments, where past adaptive challenges are likely to be similar to future adaptive challenges. Future work should explore exactly if and how mutational transformers can evolve under more diverse and complex environmental regimes (e.g.: more than two alternating environmental optima) or environments where the phenotype is represented by more than one dimension). In a more general sense, our results indicate that the genotype-to-phenotype map can easily evolve to shape the phenotypic effects of mutation towards adaptive outcomes, even with minimal complexity. This outcome seems to contradict the assumptions of standard evolutionary theory, which assigns little relevance to the exact nature of the genotype-to-phenotype map. However, recent work has shown that explicitly considering the structure of the genotype-to-phenotype map greatly influences the outcomes of evolutionary models (Milocco & Salazar-Ciudad, 2020; van Gestel & Weissing, 2016). Our results are in line with these findings, we demonstrate that even a simple model of a genotype-to-phenotype map produces dynamics rarely observed in more classical models i.e. the evolution of evolvability. All in all we show in detail how mutational transformers evolve and function. We argue that mutational transformers can greatly impact evolutionary dynamics and that their emergence represents a clear example of the evolution of evolvability.

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Chapter 6

The impact of plasmids on the de-novo evolution of ciprofloxacin resistance in *Lactococcus lactis*

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In preparation

Abstract

Many relevant antibiotic-resistance genes are found on plasmids. Whilst plasmids play an important role in the spread of antibiotic resistance, the role that plasmids play in the de-novo development of resistance is less well understood. However, it has been hypothesised that plasmids can enhance evolvability and hence speed up the evolution of antibiotic resistance. Here we use experimental evolution to study the impact of plasmids on the de-novo evolution of ciprofloxacin resistance. As our model system, we use *Lactococcus lactis* MG1363 and the pLP712 plasmid which both do not contain any resistance genes at the start of the experiment. Over the course of 31 days of experimental evolution, both plasmid-free and plasmid-bearing populations managed to evolve high levels of ciprofloxacin resistance. Plasmid-bearing strains did not evolve resistance faster and their final resistance level was equal to that of their plasmid-free counterparts. Hence, we did not find evidence that plasmids enhance the rate of resistance evolution. Instead, we found evidence for ciprofloxacin-dependent selection against the plasmid, as in populations exposed to ciprofloxacin the plasmid frequency decreased drastically over the course of the experiment. Using next-generation sequencing we also found several new genes that may play a role in ciprofloxacin resistance. These new genes provide potential new avenues to better understand ciprofloxacin resistance. The finding that exposure to ciprofloxacin seems to induce selection against plasmids could in the future be used to combat plasmids encoding resistances to other antibiotics

Introduction

Antibiotic resistance represents a massive public health problem (WHO, 2014). To effectively combat antibiotic resistance, it is necessary to understand the mechanisms underlying resistance. Here it seems that plasmids play a crucial role: many clinically relevant antibiotic-resistance genes are located on plasmids. Plasmids are small circular extrachromosomal pieces of DNA that have their own origin of replication and can thus replicate independently from the chromosome (Caratolli, 2011). Plasmids can be

horizontally transferred between bacterial cells through a process known as conjugation, where a connection is formed between two cells through which the plasmid is transferred. By recombination and transposable elements, genes can move between the plasmid and the chromosome. Plasmids often impose a fitness cost on the host bacterial cell (San Millan et al. 2017). Infamous are so-called multi-resistance plasmids, such as those commonly found in *Staphylococcus aureus*, where genes encoding resistances to multiple antibiotics are located on a single plasmid (Shearer et al. 2011; Liu et al. 2013; Dun et al. 2019; Hao et al. 2019). This is especially worrying since plasmids can facilitate the spread of antibiotic resistance, both geographically and across different bacterial species (Waters, 1999; Rozwandowicz et al, 2018). It is clear that plasmids play an important role in the spread of antibiotic resistance. However, before resistance can spread it must first arise. The exact process of how resistance arises and how this process may be impacted by plasmids is much less well understood. Here we investigate the role of plasmids in the evolutionary process that transforms a naïve bacterial population, harbouring plasmids without any resistance genes into a resistant population harbouring plasmids containing resistance genes.

So how might plasmids impact the de-novo evolution of antibiotic resistance? It has been hypothesised that plasmids can enhance the evolvability of bacteria, that is, the ability of bacteria to undergo adaptive evolution (Ramsay & Firth 2017; Riederer et al. 2022). One key study showed that a strain with resistance precursor genes located on a plasmid (and not on the chromosome) was able to evolve resistance more quickly(San Millan et al 2016). Since each cell had more than one copy of the plasmid, this increased the mutational target size, making mutations conferring resistance more probable. However, for the de-novo evolution of resistance this mutational target size effect seems of somewhat limited relevance since it requires precursor genes to already be located on a plasmid, which may not always be the case.

There is an additional way in which plasmids might enhance bacterial evolvability because some plasmids can facilitate horizontal gene transfer between cells. Several hypotheses have been put forward as to how plasmid-

based horizontal gene transfer might enhance evolvability. First of all, horizontal gene transfer can recombine existing genetic variation, leading to new combinations of genes (Hall & Kerney, 2012; Kingston et al., 2015). This generation of new variation and evolutionary novelty can accelerate adaptation, thus increasing evolvability. A second hypothesis concerns the rate of spreading beneficial mutations in a population. If beneficial mutations can spread through intercellular gene transfer (horizontally) as well as through cell division (vertically) the rate of spread of beneficial genes is accelerated, thereby increasing the rate of adaptation (Chu et al., 2018). Third, horizontal gene transfer can create a reservoir of genetic variation. This kind of reservoir dynamic also allows variation to be maintained that would otherwise be lost. This maintenance of variation due to horizontal gene transfer enhances evolvability (Croll & McDonald, 2012; Woods et al., 2020; van Dijk, 2020). Fourth, plasmid-mediated horizontal gene transfer can alleviate "clonal interference". In clonal interference different beneficial mutations compete with one another because they occur in different asexual lineages, and this competition can hinder adaptation (Gerish & Lenski, 1998). Plasmid-mediated horizontal gene transfer allows for recombination between different lineages, which can bring beneficial mutations together in the same lineage, thereby preventing clonal interference and accelerating adaptation (Cooper, 2007; Winkler & Kao, 2012). Whilst some theoretical models underpin these different hypotheses (Gerish & Lenski, 1998; van Dijk, 2020), most hypotheses lack experimental verification. Furthermore, it is unclear exactly under what conditions different evolvability-enhancing mechanisms might operate: do plasmids always enhance evolvability or do they only do so under a limited set of circumstances? All in all, mechanisms by which plasmids might enhance evolvability are currently poorly understood. Thus, experiments studying the impact of plasmids on evolvability are clearly needed.

We aimed to uncover which role plasmids play in the de-novo evolution of antibiotic resistance using experimental evolution. We experimentally evolved resistance to ciprofloxacin in populations of *Lactococcus lactis* with and without plasmids. We also evolved control populations in a medium without ciprofloxacin. Previous experimental studies aiming to study the

impact of plasmids on resistance evolution have used engineered plasmids of some sort. These plasmids already contain a particular resistance gene or a resistance precursor gene. We take a different, more open-ended approach, using a naturally occurring metabolic plasmid that does not carry any known resistance precursor genes. Our model system aims to simulate a situation in which a bacterium containing naturally occurring plasmids that do not encode antibiotic resistance genes comes into contact with a new antibiotic, as we hope to uncover the role that plasmids play in the de-novo evolution of resistance.

We address two research questions. Do plasmids enhance evolvability in the context of antibiotic resistance evolution? To this end, we compare the rate of resistance evolution between plasmid-bearing and plasmid-free populations. If as hypothesized plasmids enhance evolvability we would expect resistance to evolve faster in plasmid-bearing populations. How do plasmids influence the evolutionary trajectories that lead to resistance? To address this question we use whole genome sequencing to investigate the mutations that underpin ciprofloxacin resistance to see if the presence of a plasmid impacts the evolutionary trajectories leading to resistance. If plasmids enhance evolvability in the context of antibiotic resistance evolution, then we might expect plasmids to impact the combination of mutations underpinning resistance.

Material and methods

Strains & media

We choose to work with *L. lactis* since it is a well-studied model organism that is safe to work with (generally regarded as safe), yet is relatively closely related to *Streptococcus pneumoniae*, an important pathogen that has acquired problematic resistance to ciprofloxacin (Sahm et al., 2000; Mrazek et al. 2002; Wegeman et al. 2007; Patel et al. 2011), meaning that any new insights gained into ciprofloxacin resistance in *L. lactis* are likely to be of some clinical relevance. Another reason for using *L. lactis* concerns the fact that plasmids seem to have played an important role in past adaptation of *L. lactis* to the milk environment, meaning that there are many well-studied naturally

occurring plasmids available (Wegeman et al. 2012; Tarazanova et al. 2016). The plasmid we use, pLP712 is capable of high-frequency horizontal transfer through conjugation and genetic exchange with the chromosome has also been shown (Gasson, 1983; Wegeman et al. 2012; Tarazanova et al. 2016). The pLP712 plasmids plays an important role in wild-type *L. lactis*, since it carries genes that are essential for growing in milk (Wegeman et al. 2012; Tarazanova et al. 2016).

In this experiment, we used two different *L. lactis* strains: As a plasmid-free strain we used the plasmid-cured strain MG1363 (Gasson et al, 1983; Wegeman et al. 2007; Linares et al. 2010;). Our plasmid-bearing strain was created by transforming MG1363 with the pLP712 plasmid (Gasson et al, 1983; Wegeman et al. 2012; Tarazanova et al. 2016). These two strains formed the starting point for our evolution experiment, they are henceforth referred to as our two starting strains. Both strains were obtained from the laboratory collection of the Molecular Genetics group at the University of Groningen (The Netherlands). Using PCR, we tested both strains for the presence/absence of the pLP712 plasmid and the presence of the sex factor (see supplementary figure S1). The sex factor is a chromosomally integrated mobile genetic element that contains the genes required for conjugation, the presence of this element is necessary to allow for the conjugative transfer of the pLP712 plasmid. The sex factor has also been shown to facilitate chromosomal integration of the pLP712 plasmid (Gasson et al.1992; Wegeman et al. 2012; Wels et al. 2019). We confirmed that our plasmid-bearing strain contained both the sex factor and the pLP712 plasmid. Our plasmid-free was shown to contain the sex factor, but indeed lacks the pLP712 plasmid. Both strains were regrown from a single colony before starting the experiment, to assure minimal standing genetic variation within the two starting strains. All experiments were conducted in CDMPC medium (Goel et al 2012; Solopova et al. 2017; Price et al. 2017). The treatment culture medium was supplemented with ciprofloxacin (Sigma) dissolved in 0.1N HCl to achieve the following final concentrations over the course of the evolution experiment: 2.5 ug/ml, 5 ug/ml, 10 ug/ml, 20 ug/ml/ 40 ug/ml, 80 ug/ml and 160 ug/ml. During the experiment cells were cultivated in 96-well plates in a culture volume of 200ul, all cultures were incubated at 30 °C.

Determining initial resistance

Before starting the experiment, the initial resistance of both the plasmid-free and the plasmid-bearing starting strain was determined. The strains were grown in several concentrations of ciprofloxacin, whilst measuring the optical density (OD600) of the strains in a plate reader (BMG Clariostar). The following concentrations of ciprofloxacin were tested: 2.5 µg/ml, 5 µg/ml, 10 µg/ml, 16 µg/ml, 18 µg/ml, 20 µg/ml / 40 µg/ml, 80 µg/ml, and 160 µg/ml. As a control, the strains were also grown in medium without ciprofloxacin. This experiment was repeated twice, both leading to the conclusion that no growth occurred at a concentration of 10 ug/ml or higher. On the basis of this data 2.5 ug/ml was chosen as the initial concentration to start the evolution experiment, as this concentration was well below the inhibitory concentration and hence still allowed for adequate growth. The data from these experiments was also used to assess the growth rate of the two starting strains (plasmid-free and plasmid-bearing) in medium without antibiotic and in medium containing the sub-inhibitory concentration of ciprofloxacin (2.5 ug/ml). We used eight replicate growth curves per strain (obtained across two days, four per treatment per day). Growth rates were estimated by fitting a linear model to the exponential growth phase (OD600 between 0.05 and 0.3) of the log-transformed OD600 growth curve.

Experimental evolution protocol

The evolution experiment followed a sequential batch culture protocol, in which a total of 32 populations were evolved, all originating from our two starting strains. These 32 populations were spread across four different treatments each with eight replicate populations: eight plasmid-free control populations, eight plasmid-bearing control populations, eight plasmid-free populations exposed to ciprofloxacin and eight plasmid-bearing populations exposed to ciprofloxacin. The control populations served to control for any adaptation to the medium that might occur throughout the experiment. To start the experiment an overnight culture of the two starting strains was prepared (in selective medium for the plasmid-bearing strain), subsequently on day 0, 1 ul of this overnight culture was used to initialise the populations. For all populations, the first transfer occurred on day 1 and in total the

populations were transferred 31 times (until day 31). The control populations were grown in medium without antibiotics and approximately every 24 hours 1ul of each population was transferred to 200ul of fresh medium, with the first transfer occurring on day 1. The populations exposed to ciprofloxacin were initialised using the overnight cultures and were initially grown in medium containing 2.5 ug/ml on day 0. Subsequently, every 24 hours 1ul of each population was transferred to two separate wells, one containing the current ciprofloxacin concentration and one well containing twice the current ciprofloxacin concentration. If after 24 hours the population had managed to grow in the double concentration (i.e. Optical Density (OD600) > 0.3) 1 ul of the double concentration well was used for transfer and the double concentration then became the current ciprofloxacin concentration of that population. If in 24 hours the population had not managed to grow in the double concentration (i.e. OD600 < 0.3), the current concentration remained unchanged and the well with the current concentration was used for continued cultivation. In this way the population is always growing at the highest concentration of ciprofloxacin to which it is resistant, therefore the current concentration provides a measurement of the level of ciprofloxacin resistance of a particular population. If the current concentration reached the maximum concentration of 160 ug/ml the population was cultivated at this maximum concentration for the remainder of the experiment. Every third transfer as well as on the final day (day 31), a glycerol stock was prepared from all the populations, with the first stock being made on day 3. At the end of the experiment, all evolved populations were grown in CDMPC medium without ciprofloxacin inside a plate reader (BMG Clariostar) to determine their growth rate. This allowed us to assess the cost of evolving resistance.

Resuscitation of failed populations

During the experiment some populations failed to grow after transfer, for these populations the resuscitation protocol was initiated. The initiation of resuscitation occurred a total of 10 times during the experiment. It occurred both when the ciprofloxacin concentration had been increased (4 times) and when the concentration had remained the same, with the latter likely due to

pipetting error (1 time in control populations, 5 times in populations exposed to antibiotics). If a population (control or exposed to antibiotics) failed to grow then the current concentration was halved (if applicable) and the population was resuscitated using 1 ul of culture from the previous day (inoculated 48 hours ago) using the old culture with the lowest ciprofloxacin concentration available. This old culture was also turned into a glycerol stock. For subsequent resuscitation attempts 1 ul of this glycerol stock was used, each time halving the current concentration, this was continued until the population managed to grow again in the current concentration. If during the resuscitation process the population did not manage to grow at the lowest concentration of 2.5 ug/ml a final attempt was made to resuscitate the population at this concentration using 10 ul of the glycerol stock. On the final day of the experiment (day 31) a single population failed to grow in both the double and current concentration, but as the experiment ended, resuscitation was not possible. For this population the glycerol stock made on day 30 was used for all final analyses. A separate test was made after day 31 that confirmed that this population was able to grow in half the current concentration, and hence this was denoted as the final resistance level for this population.

Sequencing & analysis

For sequencing the evolved strains and the two starting strains were grown overnight in CDMPC medium without ciprofloxacin. Subsequently, using 5 ml of these overnight cultures, DNA was extracted using the gen elute bacterial genomic DNA kit (Sigma), using the gram-positive protocol (including incubation with lysozyme). Subsequently, the DNA was sequenced by Eurofins on the Illumina platform, generating at least 5 million 150 bp paired end-reads per sample. These reads were subsequently analysed in BRESEQ (Detherage & Barrick, 2014) using the pLP712 reference genome (GenBank FJ649478.1) and the MG1363 reference genome (GenBank AM406671.1) For the MG1363 reference genome 71 insertion sequences were manually annotated as mobile elements, with a version of the reference genome containing these additional annotations kindly provided by A. M. Veenstra. The BRESEQ analysis used the following filters: polymorphism bias cutoff =

0.05, polymorphism minimum variant coverage = 10 and polymorphism total variant coverage = 20. Using the GD tools available in BRESEQ the mutations present in the starting strains were subtracted from those in the evolved strains so that only those mutations that occurred during the experiment could be analysed.

Results

Growth rates in the absence of the antibiotic

We compared the growth rates of our two starting strains (plasmid-free and plasmid-bearing) in medium without the antibiotic. The growth rate was estimated by fitting a linear model to the linear portion of the log-transformed OD600 data; model fits are shown in supplementary figure S2. The resulting growth rate estimates are plotted in Figure 1. In the absence of the antibiotic, the plasmid-bearing strain has a significantly higher growth rate than the plasmid-free strain (t-test, $p = 0.007$), with the growth rate of the plasmid-bearing strain being approximately 5% higher than that of the plasmid-free strain. In other words, in the absence of the antibiotic having this plasmid seems to increase the bacterial growth rate.

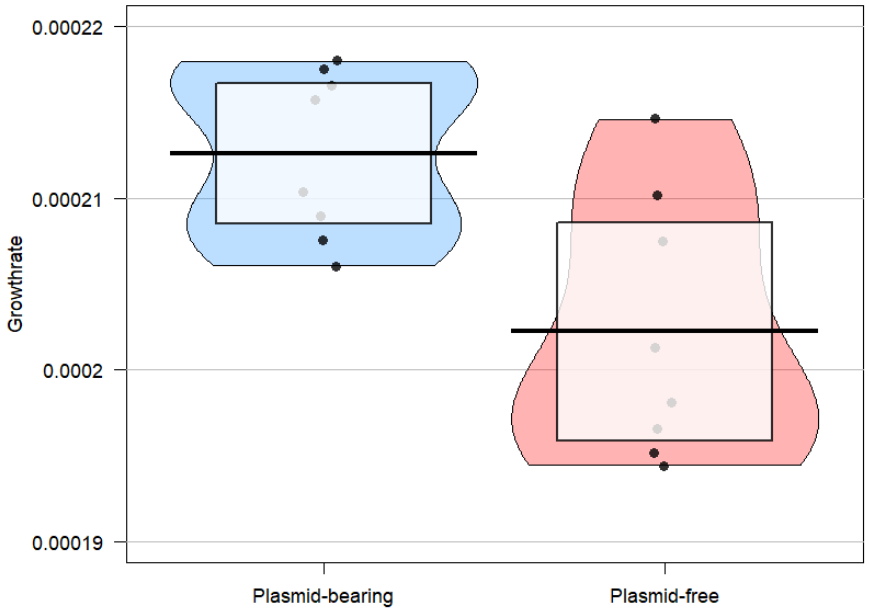

Figure 1. Growth rates of plasmid-free and plasmid-bearing strains in the absence of ciprofloxacin. The growth rates of 8 populations of the plasmid-bearing and the plasmid-free strain were estimated by the slope of the linear portion of the log-transformed OD600 trajectory (as shown in Supplementary Figure S2). Black lines show the mean value and the boxes show the 95% confidence interval of the growth rate of the two strains. In the absence of ciprofloxacin, the growth rate of the plasmid-bearing strain is significantly higher than that of the plasmid-free strain (t-test, p =0.007).

Growth rates in the presence of the antibiotic

Next, we assessed the growth rate of the two starting strains (plasmid-free and plasmid-bearing) in medium containing a sub-inhibitory concentration of ciprofloxacin (2.5 ug/ml). The model fits used to obtain these growth rate estimates are shown in supplementary figure S3. The growth rate estimates are plotted in Figure 2. At this sub-inhibitory concentration of ciprofloxacin the plasmid-free strain has a significantly higher growth rate (t-test, p = 0.004), with the growth rate of the plasmid-free strain approximately 10% higher than that of the plasmid-bearing strain. It thus seems that in this case having a plasmid lowers the growth rate.

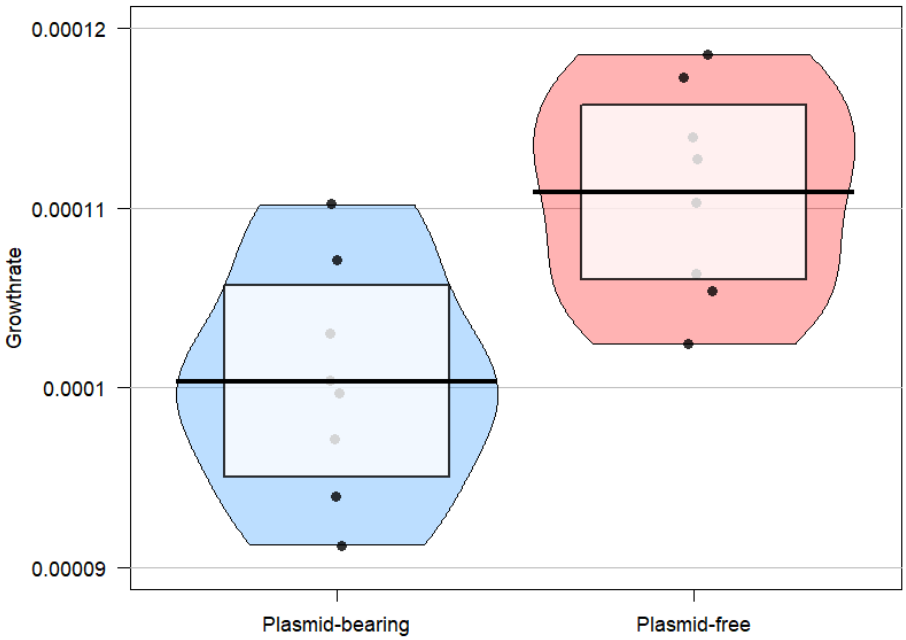

Figure 2. Growth rates of plasmid-free and plasmid-bearing strains in the presence of ciprofloxacin. The growth rates of 8 populations of the plasmid-bearing and the plasmid-free strain at a sub-inhibitory concentration of ciprofloxacin (2.5 ug/ml) were estimated by the slope of the linear portion of the log-transformed OD600 trajectory (as shown in Supplementary Figure S3). Black lines and boxes indicate the mean value and the 95% confidence interval of the growth rate of the two strains. At this sub-inhibitory concentration of ciprofloxacin the growth rate of the plasmid-free strain is significantly higher than that of the plasmid-bearing strain (t-test, p =0.004).

Resistance trajectories during experimental evolution

Figure 3 shows the mean resistance trajectories of the eight plasmid-free and the eight plasmid-bearing populations over the course of the evolution experiment. First of all, both the plasmid-free and plasmid-bearing populations manage to evolve high levels of resistance. Second, the final resistance level of the plasmid-bearing populations lags behind that of the plasmid-free populations. When it comes to the maximum level of resistance two plasmid-free populations managed to reach this level by the end of the experiment (160 ug/ml), whilst none of the plasmid-bearing populations managed to reach this maximum. Figure 4 shows the log-transformed final resistance level achieved in the populations exposed to ciprofloxacin. A t-test comparing the mean final resistance level of the plasmid-bearing and the plasmid-free populations indicated no significant difference ($p=0.475$). It seems that the presence of the plasmid did not impact the final resistance level evolved during the experiment.

Next, we assessed the rate of resistance evolution in each of the populations exposed to ciprofloxacin. To this end, a linear model was fitted to the log-transformed resistance trajectory of each population. The slope of this linear model fit (slope of resistance increase) served as an indicator of the rate of resistance evolution. For populations that showed a very drastic decrease in resistance during resuscitation, only the trajectory before the decline was used to fit the linear model. Model fits and resistance trajectories for plasmid-fee and plasmid-bearing populations are shown in Supplementary Figures S4 and S5, respectively. Figure 5 shows the resulting slopes of resistance increase, obtained using these linear model fits. A t-test comparing the mean slope of resistance between the plasmid-free and plasmid-bearing populations did not produce a significant result ($p=0.757$). In other words, there is no evidence that plasmids did impact the rate of resistance evolution during the experiment.

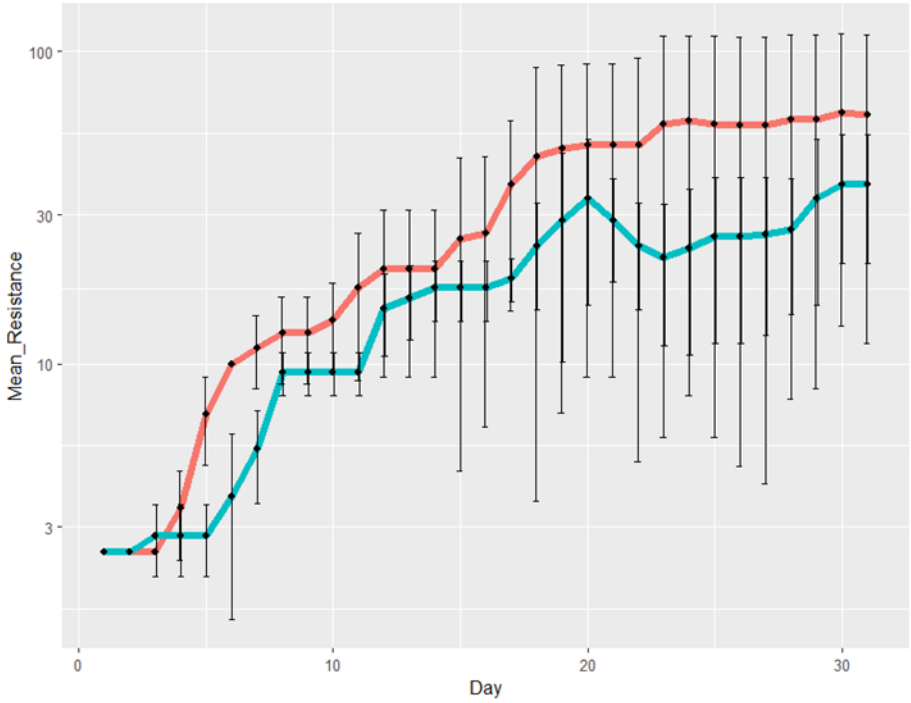

Figure 3. Average resistance trajectory of plasmid-free (red) and plasmid-bearing (blue) populations. Mean ciprofloxacin resistance trajectories in ug/ml of the eight plasmid-free (in red) and the eight plasmid-bearing populations (in blue) for each day of the experiment, shown on a logarithmic scale. The error bars indicate the 95% confidence interval of the resistance level.

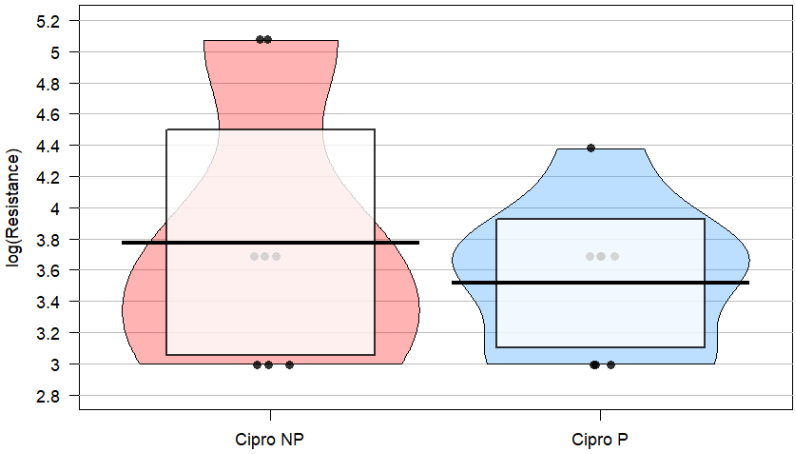

Figure 4 Final resistance level achieved at the end of evolution. The logarithm of the final resistance levels achieved in the different populations exposed to ciprofloxacin at the end of the experiment (day 31). The left violin shows the plasmid-free populations and the right violin shows the plasmid-bearing populations. Black lines indicate the mean, boxes indicate the 95% confidence interval of the growth rate.

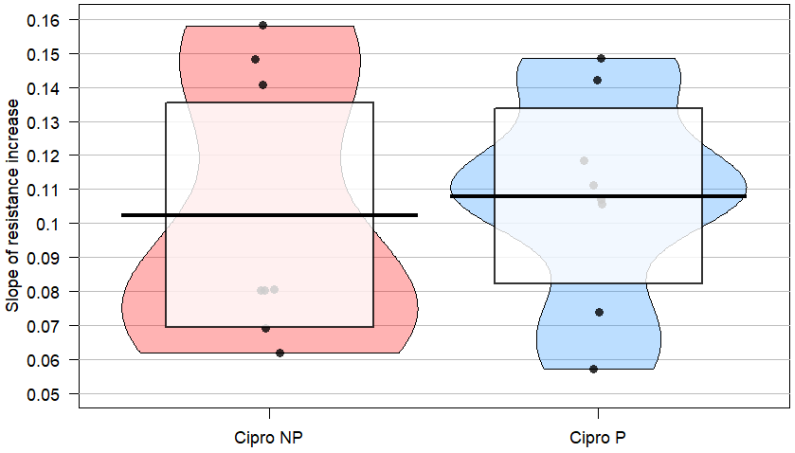

Figure 5. Rate of resistance increase over the course of evolution. The estimates of the rate of increase are based on the linear model fits shown in supplementary figures S4 and S5. The left violin shows the plasmid-free populations, the right violin shows the plasmid-bearing populations. Black lines indicate the mean, boxes indicate the 95% confidence interval of the slope.

Sequencing results of evolved strains

Based on the sequencing results, the mutations that occurred during the experiment in the evolved strains were analysed. Mutations were assessed at the gene level, therefore multiple mutations in the same gene were counted as a single mutation. Only mutations with an estimated within-population frequency of 10% or above were included in the analysis.

First, we compared the number of mutations that occurred in the evolved populations during the experiment, which is shown in Figure 6. It is evident that more mutations occurred in populations evolving resistance to ciprofloxacin, whilst the presence or absence of a plasmid did not influence the number of mutations that occurred. This impression was confirmed by using a Kruskal-Wallis test which revealed significant differences between the treatments ($p = 0.00005$) (non-parametric tests were used as it concerns count data). A post-hoc Dunn test did not find an indication for an effect of the presence or absence of the plasmid (Control NP-Control P: $p=0.828$ and Cipro NP-Cipro P: $p=1.0$). There was however a significant difference in the number of mutations between those populations exposed to ciprofloxacin and the control populations (Control NP-Cipro NP: $p= 0.007$, Control NP-Cipro P: $p= 0.001$, Control P-Cipro NP: $p= 0.010$, Control P-Cipro P: $p= 0.002$).

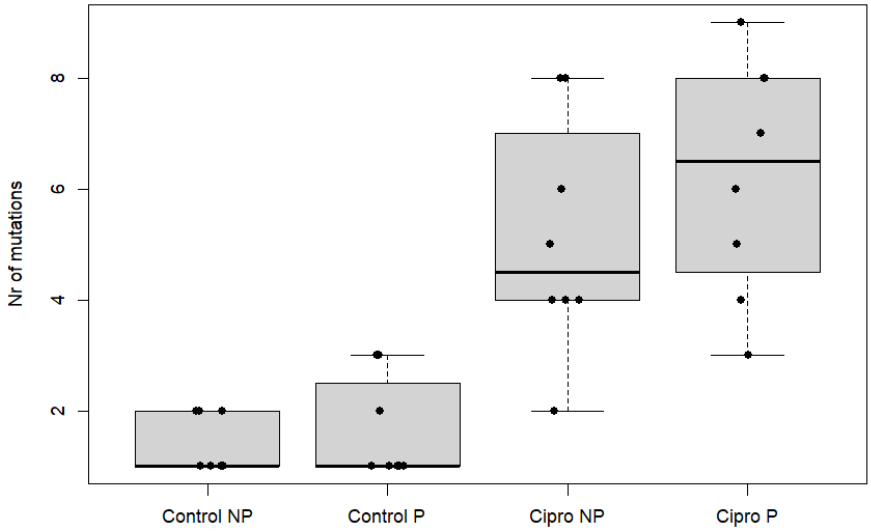

Figure 6. Number of mutations occurring during the evolution experiment. Mutations were counted at the gene level and only mutations with a frequency of 10% or higher were included. From left to right the following treatments are displayed: control plasmid-free, control plasmid-bearing, ciprofloxacin exposed plasmid-free and ciprofloxacin exposed plasmid-bearing. Populations exposed to ciprofloxacin displayed more mutations at the end of the experiment. The presence or absence of the plasmid does not seem to impact the number of mutations that occur for both the control and ciprofloxacin-exposed treatments. Black lines indicate the median number of mutations, boxes indicate the interquartile range.

The mutation patterns associated with resistance in the strains exposed to ciprofloxacin are shown in Figure 7. For this analysis, we only considered mutations that occurred independently in at least two of the populations. Mutations in the *tgt* gene that also frequently occurred in control populations are also removed from the analysis, as they most likely represent adaptation to the medium. These criteria ensure that the analysed mutations are likely involved in modulating ciprofloxacin resistance. The frequencies of different mutations are also plotted in supplementary figure S6

When examining Figure 7, it seems that the presence or absence of the plasmid does not impact the patterns of mutation in the evolved strains. Furthermore, we did not find any mutations in the pLP712 plasmid. However,

several interesting observations can be made by examining the mutation patterns of the chromosome. Mutations in the *rpoB* gene are most commonly observed (in 8 out of 16 evolved populations), second most commonly are mutations in the *parC* gene and the *llmg_2080* gene (6/16) and the *rpoC* gene (5/16). The *rpoB* and *rpoC* genes encode the β and the β' subunits of the RNA polymerase. Mutations in these genes occur in 13 out of 16 populations, interestingly it seems that either *rpoB* mutates or *rpoC,* these mutations do not co-occur. Mutations in the llmg_2080 gene seem to be very common; this mutation is of note since this gene is not traditionally associated with ciprofloxacin resistance. Interestingly mutations in the *llmg_2080* gene do not co-occur with mutations in the *rpoC* gene, but they do frequently co-occur with mutations in the *rpoB* gene. This observation supports the aforementioned hypothesis that there are two separate mutation pathways leading to resistance.

The genes traditionally associated with ciprofloxacin resistance are *parC* and *gyrA* and to a lesser extent *parE* and *gyrB* (Lupien et al. 2013; Shariati et al. 2022). These genes produce the DNA topoisomerase IV and the DNA gyrase enzymes, which are the direct targets to which ciprofloxacin binds. Mutations in these genes do occur in several of the evolved populations (*parC*: 5 out of 16, *gyrA*: 3 out of 16, *parE*: 2 out of 16 and *gyrB*: 3 out of 16). However, these mutations are far from ubiquitous and they are not clearly associated with a high level of resistance. The main observation that can be made from the mutational patterns is that there seems to be a wide variety of mutational patterns that lead to ciprofloxacin resistance. These patterns seem to suggest that ciprofloxacin resistance depends on mutations in multiple genes.

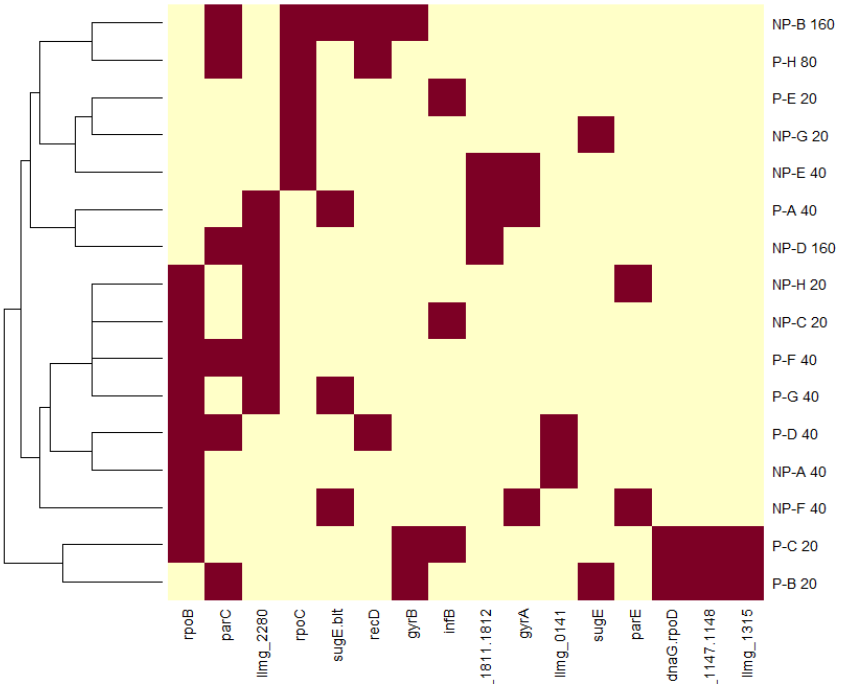

Figure 7. Visualisation of the mutation patterns that are associated with resistance. It shows the occurrence of different mutations identified in the evolved populations exposed to ciprofloxacin at the end of the evolution experiment. Mutations were analysed at the gene level and only those with a within-population frequency of 10% were counted. Furthermore, only mutations that occurred in two or more populations are included in this plot. Mutations in the *tgt* gene that also frequently occurred in the control populations are also not included. Plasmid-free populations are marked as NP, whilst plasmid-bearing populations are marked as P. The numbers show the level of resistance each population managed to achieve. The populations are sorted vertically based on the similarity of their mutation patterns (as indicated by the dendrogram).

Finally, we used the sequencing results to assess the plasmid abundance in the evolved plasmid-bearing populations. To estimate plasmid abundance, we used the percentage of the total sequencing reads that mapped to the plasmid, as reported by BRESEQ. As a baseline, we used the sequencing results from the starting plasmid-bearing strain, which has been regrown (on medium selecting for plasmid presence) from a single colony. When this strain was sequenced, 3.1 % of sequencing reads mapped to the plasmid. In the literature, the copy number of pLP712 is reported to be between 1-2

copies per cell (Wegeman et al. 2012; Tarazanova et al. 2016). If we consider the relative sizes of the MG1363 genome (2529478 bp) and the pLP712 plasmid (55395 bp), we arrive on the following: (55395/2529478) * 100 = 2.2 %. This would be the expected percentage of reads mapping to the plasmid if each cell had a single plasmid. Therefore, if we then use this to estimate the copy number per cell, we obtain 3.1%/2.2% = 1.4 copies per cell in the starting plasmid-bearing strain.

Figure 8 shows the percentage of reads mapped to the plasmid for the evolved plasmid-bearing populations. When examining this figure, it seems that the control plasmid-bearing populations largely maintain the plasmid at the same abundance as the starting strain. However, when we examine the plasmid-bearing populations that were exposed to ciprofloxacin it is immediately evident that most populations (6/8) clearly display a marked decrease in plasmid abundance over the course of the experiment. Interestingly, one of the ciprofloxacin-exposed populations completely lost the plasmid from the population. Furthermore, another ciprofloxacin-exposed population completely lost the sex factor that is necessary for conjugation. To confirm these impressions we performed two one-sample Wilcoxon tests, comparing the plasmid abundance at the start of the experiment with the plasmid abundance in the evolved control populations and the evolved ciprofloxacin-exposed populations. These tests showed evidence of a change in plasmid frequency for the ciprofloxacin-exposed populations ($p= 0.04$). For control populations this test did not provide any evidence for a change in plasmid frequency ($p= 0.844$). A Wilcoxon test comparing the difference in plasmid abundance between the control populations and the ciprofloxacin-exposed populations did not yield a significant result ($p=0.105$), but note the violation of the homoscedasticity assumption.

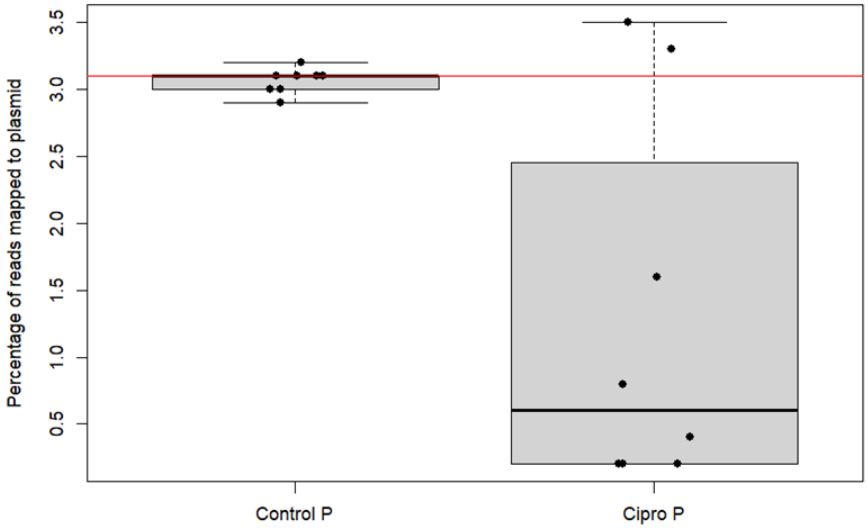

Figure 8. Percentage of reads mapped to the plasmid in the evolved plasmid-bearing populations. The percentage of reads mapped to the plasmid for the plasmid-bearing starting strain is shown by a red line. It can be observed that most of the control populations maintain the plasmid at about the same abundance as the starting strain. The populations exposed to ciprofloxacin on the other hand showed a marked decrease in the plasmid abundance. Black line shows the median, box shows the interquartile range.

Cost of resistance

Evolving resistance often comes at the cost of a lower growth rate in the absence of the antibiotic. To assess this cost of evolving resistance and to see if this cost differed between plasmid-free and plasmid-bearing populations, we examined the growth rates of the evolved populations. The populations were grown in medium without antibiotics and their growth curves (OD600) were obtained. The growth rate was estimated by fitting a linear model to the linear portion of the log-transformed OD600 data, model fits are shown in supplementary figure S7. The resulting growth rates of the different evolved populations are shown in Figure 9. A one-way ANOVA confirmed that there were significant differences in growth rate between the different treatments ($p < 0.0001$). A subsequent post-hoc Tukey test did not show any indication that the presence or absence of the plasmid impacted the growth rate of the evolved populations (Control NP-Control P: $p=0.728$ and Cipro NP-

Cipro P: p=0.957). The fact that no difference in growth rate is detected here, whilst such a difference was present in the starting strains, might be due to the strong decrease in plasmid abundance in the plasmid-bearing populations exposed to ciprofloxacin. Whether a population evolved in the presence of ciprofloxacin did significantly impact the growth rate in the absence of antibiotic (Control NP-Cipro NP, Control NP-Cipro P, Control P-Cipro NP and Control P-Cipro P: for all cases $p < 0.00001$). The populations that evolved ciprofloxacin resistance had a lower growth rate when cultivated in the absence of this antibiotic when compared to the control. In other words, it seems that evolving ciprofloxacin resistance comes at the cost of a lower growth rate in the absence of this antibiotic.

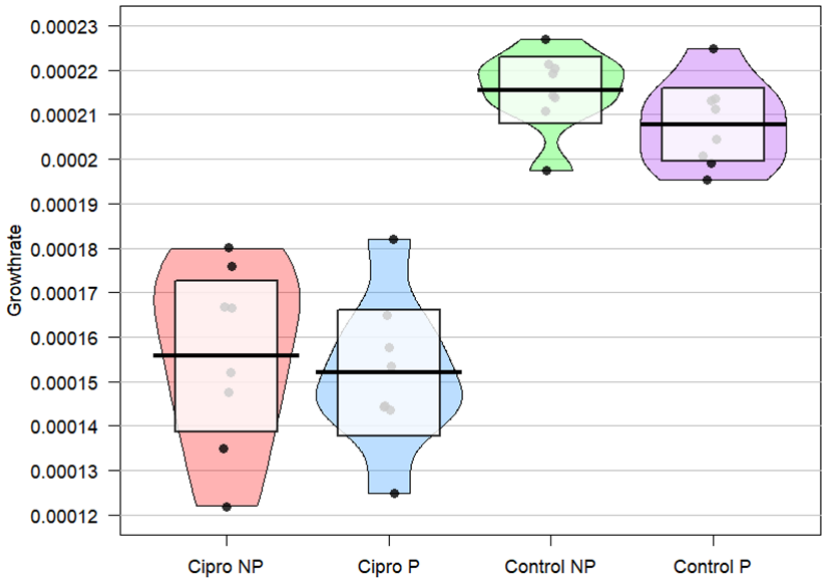

Figure 9. The growth rates of the evolved populations measured in the absence of antibiotics. The following treatments are displayed (left to right): ciprofloxacin exposed plasmid-free (Cipro NP), ciprofloxacin exposed plasmid-bearing (Cipro P), control plasmid-free (Control NP), control plasmid-bearing (Control P). The black lines indicate the mean, the boxes the 95% confidence interval.

Discussion

Over the course of the evolution experiment the populations manage to rapidly evolve ciprofloxacin resistance. Before discussing the results in detail, it is important to emphasise that our experiment is not aiming to understand the role that plasmids play in the spread of antibiotic resistance, a process that has already been studied extensively. We are interested to see the impact that plasmids play in the development of resistance. Hence, we use a plasmid and a bacterial strain that both do not have any resistance genes at the start of the experiment. Our experimental evolution approach allows us to examine, from several different angles, the role that plasmids play in this de-novo evolution of ciprofloxacin resistance.

Considering all the outcomes of our experiment, we did not find any evidence that the presence of the plasmid enhanced evolvability in the context of antibiotic resistance. The presence of a plasmid does not seem to result in a significantly higher final evolved resistance level at the end of the experiment. Furthermore, when we analysed the slope of the increase in resistance during the experiment, this was also not significantly impacted by the presence of the plasmid. If anything, it seems that the presence of the plasmid might be detrimental when evolving resistance: we observed strong selection against the plasmid in populations exposed to ciprofloxacin. This is evidenced by the strong decrease in plasmid frequencies in populations exposed to ciprofloxacin, whilst plasmid frequencies remained stable in the control populations.

Whole genome sequencing revealed that the plasmid also did not influence the mutational patterns that underly ciprofloxacin resistance. This result might provide some explanation as to why the hypothesised effect of plasmids on evolvability was not observed. The hypothesised evolvability impact of a plasmid would involve bringing together resistance genes from different clonal lineages into a single lineage. In our experiment, this would likely involve the transfer of genes between the chromosome and the plasmid, which we did not observe. Perhaps the timescale of our experiment may have been too short for such relatively rare mutational events to occur. Furthermore, we only evolved resistance to a single antibiotic, perhaps a

plasmid would enhance evolvability in a more complex selective environment. Regardless, contrary to our initial hypothesis, it seems that in the current experiment the presence of the pLP712 plasmid did not enhance evolvability in the de-novo evolution of ciprofloxacin resistance in *L. lactis*.

As highlighted before, in the plasmid-bearing populations that evolve ciprofloxacin resistance the frequency of the plasmid in the populations strongly decreases, with one of the populations even losing the plasmid entirely. It appears that there is strong selection against the plasmid when populations are exposed to ciprofloxacin. On the other hand, when populations are grown without ciprofloxacin (control) it seems that there is no selection against the plasmid. It seems that the plasmid is only costly in the presence of ciprofloxacin. This conditional cost of the plasmid also comes to light when we examine the growth of the plasmid-free and plasmid-bearing starting strains, from which the evolved populations were created. In the absence of antibiotic, the plasmid-bearing strain has a faster growth rate than the plasmid-free strain. However, when both strains are exposed to a sub-inhibitory concentration of ciprofloxacin the plasmid-bearing strain grows slower than the plasmid-free strain Somehow the growth rate of the plasmid-bearing strain is disproportionately affected by the presence of ciprofloxacin. This could explain the lower baseline level of resistance and the strong selection against the plasmid observed in the plasmid-bearing populations exposed to ciprofloxacin.

To further explain why the plasmid is only costly in the presence of ciprofloxacin it is necessary to examine the mechanism of action of this antibiotic. Ciprofloxacin works by inhibiting the DNA topoisomerase IV and the DNA gyrase enzymes, which serve to release mechanical stress on the DNA during replication and transcription. When these enzymes are poisoned by ciprofloxacin they bind to the DNA and then are unable to disassociate, this blocks DNA synthesis and causes double-stranded DNA breaks (Lupien et al. 2013; Oijkic et al., 2020; Shariati et al. 2022). We hypothesise that, under normal circumstances, the replication rate of cells is not limited by the presence of enough DNA topoisomerase IV and DNA gyrase or the stability of replication forks and hence the plasmid is not costly. However, in the presence of ciprofloxacin these factors limit the rate of replication. As

functioning DNA topoisomerase IV and DNA gyrase are scarce and replication forks are unstable, maintaining and replicating (which requires the formation of an extra replication fork) extra DNA in the form of a plasmid suddenly becomes very costly to a host cell.

We also made some interesting observations regarding the mutational patterns underlying ciprofloxacin resistance in *L. lactis*. To our knowledge, these mutation patterns have not been described before, while they may be especially relevant since *L. lactis* is closely related to *S. pneumoniae*, a pathogen that has acquired problematic resistance to ciprofloxacin (Sahm et al., 2000; Mrazek et al. 2002; Wegeman et al. 2007; Patel et al. 2011). The classic mutations associated with ciprofloxacin resistance in S. pneumoniae and many other bacteria are in the *parC* and *gyrA* genes and to a lesser extent *gyrB* and *parE* which encode the DNA gyrase and topoisomerase IV enzymes (Lupien et al. 2013; Oijkic et al., 2020; Shariati et al. 2022). These mutations also occur in some of our evolved populations, with 11 out of 16 populations having a mutation in one of these four genes. The three populations that evolved a very high level of resistance (80 ug/ml or higher), all have a *parC* mutation. However, these mutations do not occur in all our populations and some populations manage to reach quite high levels of resistance (40 ug/ml) without mutating any of these genes.

The most commonly observed mutations in the evolved populations are in the *rpoB* or *rpoC* genes (13/16 populations), which encode subunits of the RNA polymerase. In *Escherichia coli* it has been described that *rpoB* mutations can lead to ciprofloxacin resistance, by upregulating *mdtK* dependent antibiotic efflux (Pietsch et al. 2016; Brandis et al, 2021). Based on the high frequency of *rpoB* and *rpoC* mutations we suspect a similar mechanism might be operating in *L. lactis*. *RpoB* and *rpoC* mutations do not seem to co-occur. This could be due to chance, but it tentatively suggests that there might be two separate mutational paths that can lead to resistance, were either the β or the β' subunit of the RNA polymerase mutates. Another frequently occurring mutation are mutations in the *llmg_2280* gene (6/16 populations), this mutation did cooccur with mutations in the *rpoB* gene but never with mutations in the *rpoC* gene. The *llmg_2280* gene encodes a small 69 amino acid long protein of the *YozE*

family (NCBI Reference Sequence: WP_011677077). Similar proteins have been described from *Staphylococcus aureus* and *Bacillus subtillis* and these proteins may have a function that involves binding to RNA (Swapna et al. 2012). Based on our results *llmg_2280* represents an interesting new candidate gene that clearly plays a role in ciprofloxacin resistance in *L. lactis*. Future studies should aim to investigate the possibility that *YozE* family proteins play a role in ciprofloxacin resistance in other bacterial species.

Another interesting group of mutations involves mutations in the *sugE* gene (2/16) and its adjacent intergenic region (4/16). The *sugE* gene encodes a protein from the small drug-resistance protein family, many proteins of this family are known to be involved in antibiotic efflux (Bay et al. 2008; He et al. 2011). *SugE* has been associated with resistance to several antimicrobials, but experiments have not previously linked it to ciprofloxacin resistance or resistance to other fluoroquinolones (Chung & Saier 2002; He et al. 2011). Some populations (3/16) also had mutations in the *recD* gene, which encodes for a protein involved in the repair of double stranded DNA breaks (Dillingham et al. 2008). In summary when considering the mutation patterns that lead to ciprofloxacin resistance *L. lactis* it becomes clear that resistance is a highly multigenic phenotype. Many different mutations interact to create ciprofloxacin resistance and the underlying mechanisms are likely quite diverse; involving target modifications (*parC*, *gyrA*, *gyrB*, *parE*), antibiotic efflux (*rpoB*, *rpoC*, *sugE*) and perhaps even other mechanisms (*recD*, *llmg_2280*). Our results also indicate that in *L. lactis* ciprofloxacin resistance is a costly phenotype: the populations that evolved resistance had a lower growth rate compared to the control populations strains when grown in the absence of ciprofloxacin (see figure 9).

All in all, we did not find any support for our initial hypothesis that plasmids enhance evolvability in the context of antibiotic resistance. We also did not observe any impact of plasmid the mutational patterns that underly ciprofloxacin resistance. Nonetheless, our evolution experiment enabled us to make several new observations. First of all, we showed that the pLP712 plasmid was only costly in the presence of ciprofloxacin. This raises the interesting possibility of using ciprofloxacin to create selection against certain plasmids. Perhaps ciprofloxacin could be used to rid pathogen

populations of plasmids containing resistances to other antibiotics. We are also the first to describe the mutation patterns in *L. lactis* that lead to ciprofloxacin resistance. We show that there are several different mutational paths that can lead to resistance, which go beyond the *parC* and *gyrA* mutations typically considered. Our results tentatively suggest that ciprofloxacin resistance is a highly multigenic phenotype involving several different mutations. Finally, we also identify *llmg_2208* and by association other *YozE* family genes, as new genes of interest that may play a role in ciprofloxacin resistance.

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Supplementary material

PCR for pLP712 and Sex factor

PCR reactions were performed using a standard colony PCR protocol. To create a template 200 ul of glycerol stock of each strain was centrifuged and the pellet was resuspended in 25ul miliq water, 0.6ul of template was added to each reaction. For all PCR reactions an annealing temperature of 50 °C was used together with an extension time of 120 seconds, the reaction was run for 30 cycles.

To test for the presence of pLP712 the following primers based on the PrtM gene were used, which resulted in a 860bp product:

prtM_Forward: CGCCTTAAAGTATTATTGGC

prtM_Reverse: TAACTATCTAGCGCATCCGC

To test for the presence of the sex factor genes (required for conjugation) the following primers based on the TraD gene were used, which resulted in a 794 bp product.

traD_Forward: GAATATTATGCAGCTCAATGCC

traD-Reverse: CAATCTTACCAATATTGGCG

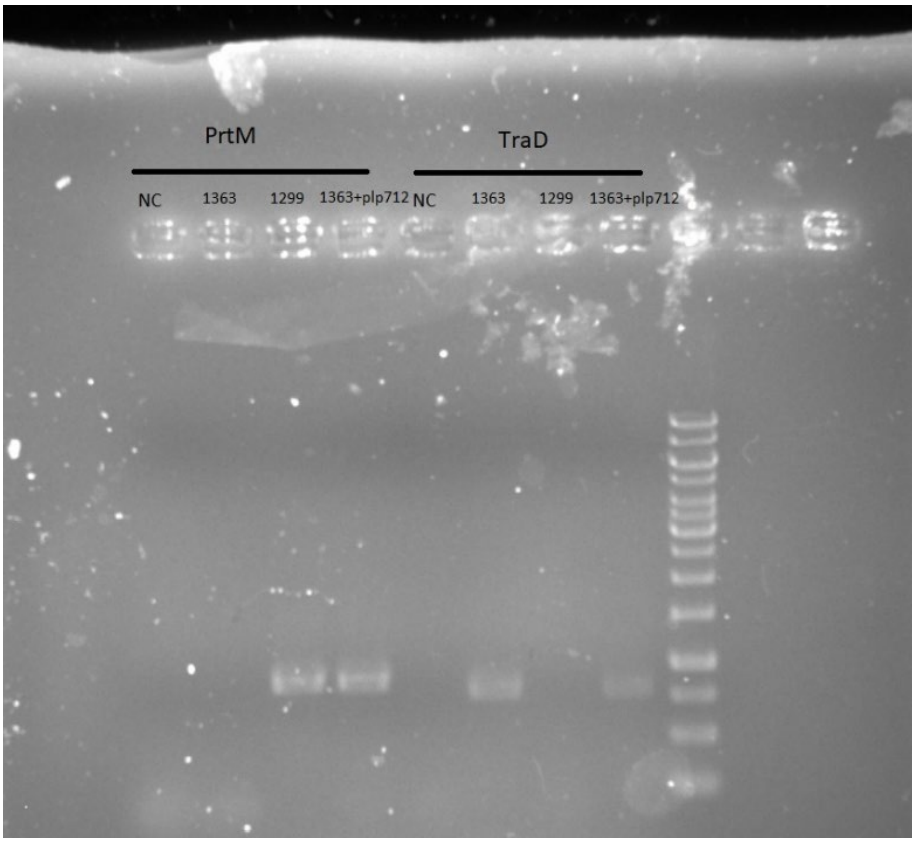

Figure S1 Electrophoresis gel showing PCR results to test for the presence of the pLP712 plasmid (PrtM) and the sex factor (TraD) (required for conjugation). NC stands for negative control, 1363 indicates strain MG1363 (plasmid-free strain in the experiment), 1299 indicates strain MG1299 (which is know to contain pLP712, but lacks the sex factor genes), 1363+pLP712 indicates strain MG1363 transformed with the pLP712 plasmid (plasmid-bearing strain in the experiment). 1 Kb gene ruler was included in the electrophoresis to verify fragment size, showing the PCR products match expected sizes (PrtM: 860bp, TraD: 794bp).

Linear model fits for growth rate estimations of starting strains

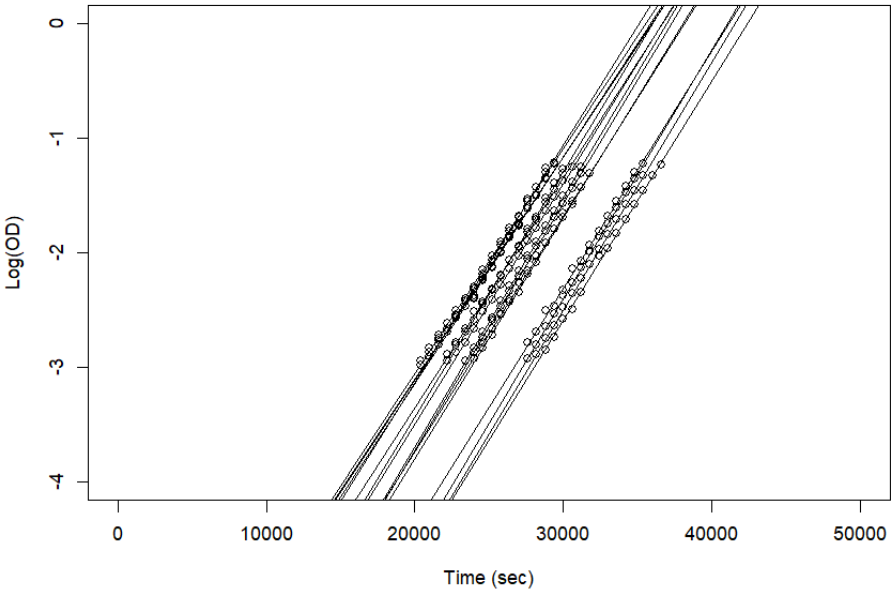

Figure S2. Linear model fits on log-transformed OD600 growth curve data, used to estimate growth rates for the two starting strains in the absence of antibiotic.

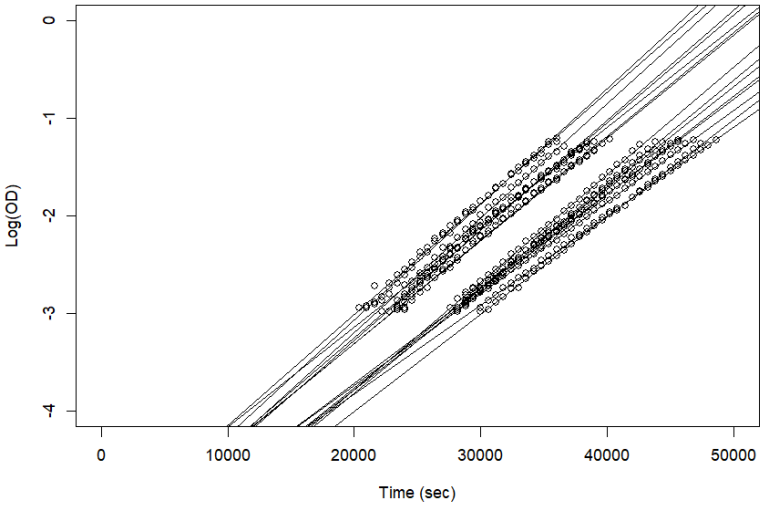

Figure S3. Linear model fits on log-transformed OD600 growth curve data, used to estimate growth rates for the two starting strains at a sub-inhibitory concentration of ciprofloxacin (2.5 ug/ml).

Resistance trajectories for populations exposed to ciprofloxacin

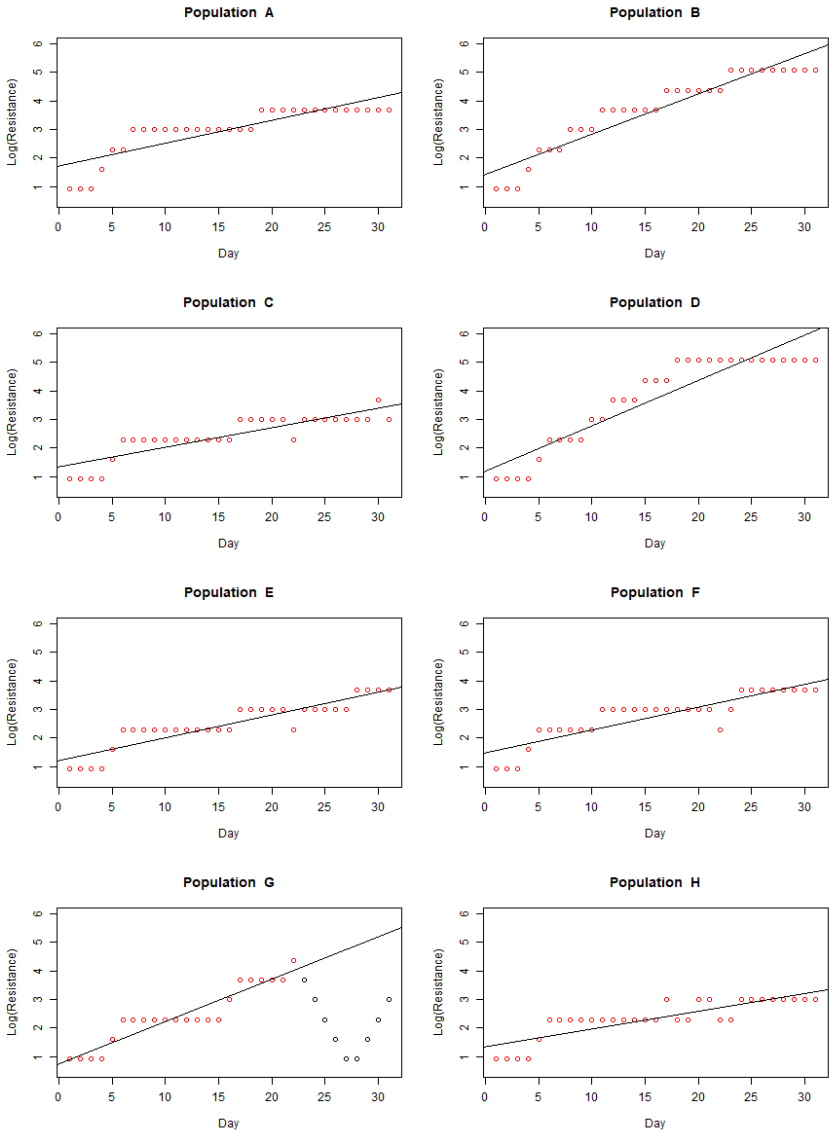

Figure S4 This figure shows the log-transformed resistance levels of each of the individual plasmid-free populations exposed to ciprofloxacin. For each population a linear model-fit is shown, the slope of this model fit served as an indicator of the rate of resistance evolution. For populations that showed a drastic decline in resistance (due to resuscitation), only the trajectory before the decline was used to fit the linear model. Points used to fit the linear model are plotted in red, excluded points are plotted in black.

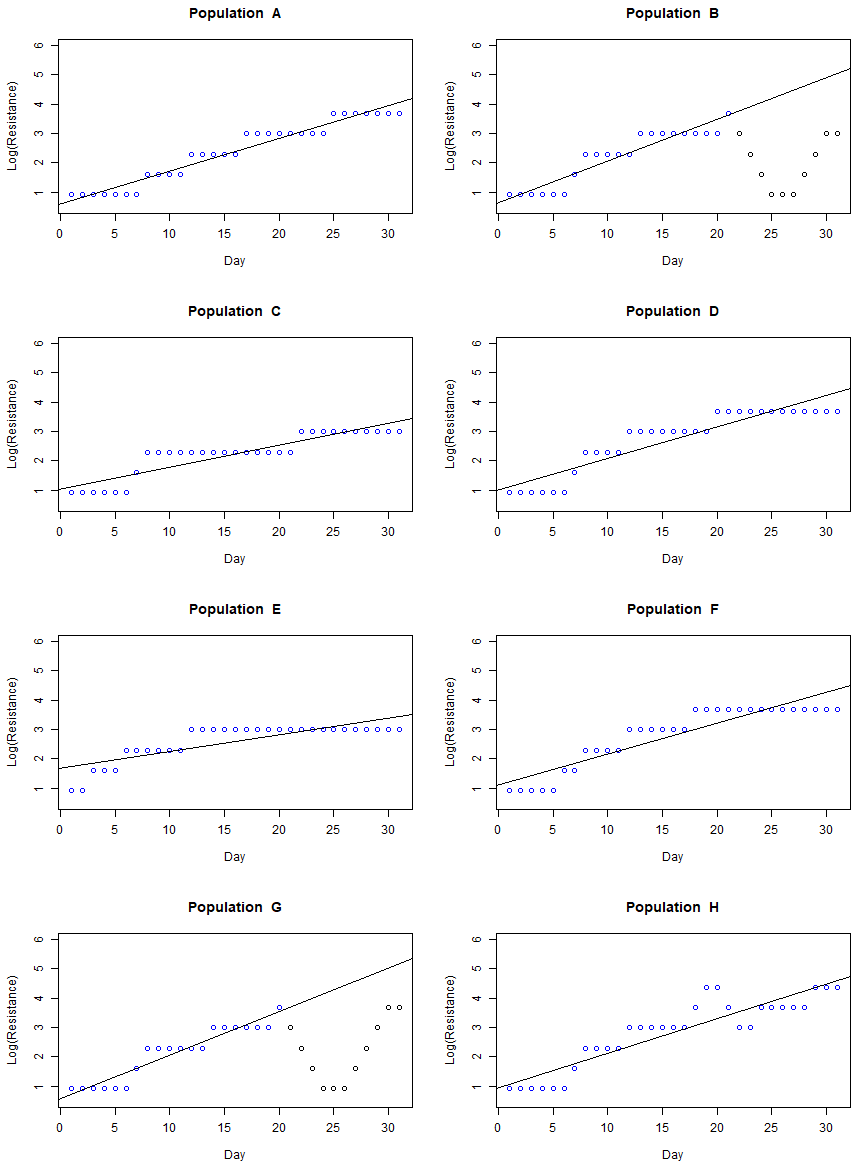

Figure S5 This figure shows the log-transformed resistance levels of each of the individual plasmid-bearing populations exposed to ciprofloxacin. For each population a linear model-fit is shown, the slope of this model fit served as an indicator of the rate of resistance evolution (figure 5 in main text). For populations that showed a drastic decline in resistance (due to resuscitation), only the trajectory before the decline was used to fit the linear model. Points used to fit the linear model are plotted in blue, excluded points are plotted in black.

Frequencies of mutations in populations exposed to ciprofloxacin

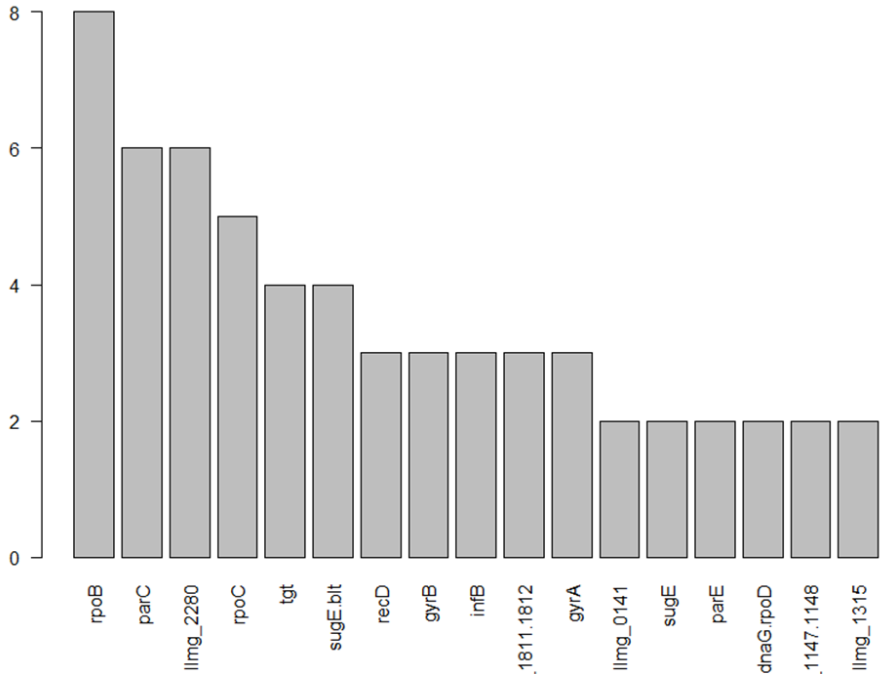

Figure S6 This figure shows the frequency of different mutations identified in the evolved populations exposed to ciprofloxacin at the end of the evolution experiment. Mutations were analysed at the gene level and only those with a within population frequency of 10% were counted. Furthermore, only mutations that occurred in two or more populations are included in this plot.

Linear model fits for growth rate estimations of evolved strains

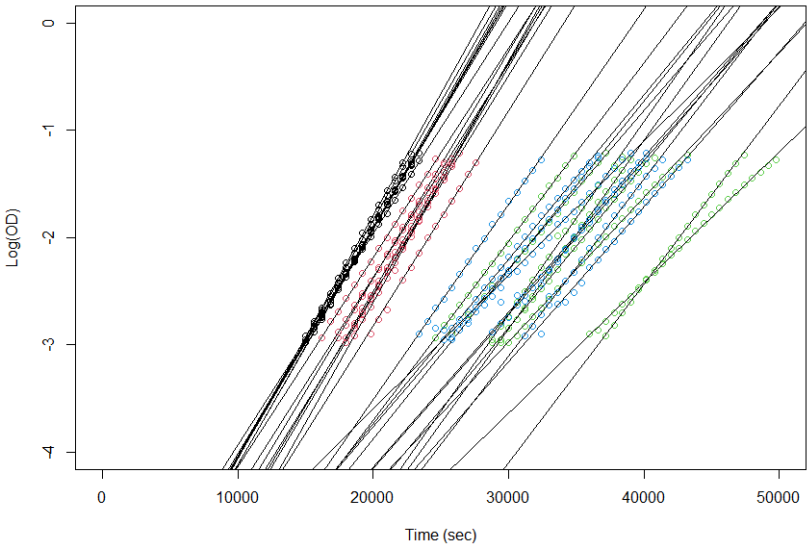

Figure S7 Linear model fits on log-transformed OD600 growth curve data (a single growth curve per evolved population), used to estimate growth rates in the absence of antibiotic for the evolved populations. Plasmid-free control populations are shown in black, plasmid-bearing control populations are shown in red, plasmid-free populations exposed to ciprofloxacin are shown in green and plasmid-bearing populations exposed to ciprofloxacin are shown in blue.

Chapter 7

Uniting community ecology and evolutionary rescue: Community-Wide Rescue

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Abstract

Most ecological communities are facing changing environments, particularly due to global climate change. When migration is impossible, adaptation to these altered environments is necessary to survive. Yet, we have little theoretical understanding how ecological communities respond both ecologically and evolutionarily to such environmental change. Here we introduce a simple eco-evolutionary model, the Community-Wide Rescue (CWR) model, in which a community faces environmental deterioration and each species within the community is forced to undergo adaptation or become extinct. We assume that all species in the community are equivalent except for their initial abundance. This individual-based simulation model thus combines community ecology and evolutionary rescue theory. We show that under Community-Wide Rescue a rapid loss of rare species occurs. This loss occurs because rare species face competition and a limited supply of mutations. The rapid loss of rare species provides a testable prediction regarding the impact of Community-Wide Rescue on species abundance distributions in ecological communities.

Introduction

Many ecosystems face abrupt human-induced environmental change and evolutionary adaptation might be the only way to avoid extinction when migration is difficult (Vitousek et al. 1997, IPCC 2014). Understanding precisely how ecological communities respond to abruptly changing environments is therefore paramount. This calls for models that predict how an ecological community composed of many different species adapts to such a deteriorated environment (Hoffman and Sgró, 2011). Such models of community-wide adaptation are not only relevant from the perspective of global change, but they are also important to understand the response of any community to environmental change, such as the microbiome of a medical patient undergoing a prolonged treatment with antibiotics. In this case, not just a single pathogenic bacterium faces a changed environment, but a complex community consisting of many thousands of species (Arumugam et al. 2011, Cho and Blaser 2012), must adapt to avoid extinction. Whilst many

models exist that study how a population of a single species, or a community composed two species, adapts to environmental change (Hoffman and Sgrò, 2011, Martin et al. 2013, Osmond & De Mazancourt 2013, Northfield & Ives 2013 , Cortez & Yamamichi 2019), fewer models exist that describe the response of an entire community composed of a multitude species to an altered environment, though there are some examples (De Mazancourt et al. 2008, Bell 2017, Lasky 2019). Furthermore, empirical results, describing community wide adaptation, such as those presented by Low-Décarie et al. (2015), Bell et al. (2019) and Roodgar et al. (2019), are clearly calling for such models.

Evolutionary rescue theory models situations in which a population can only escape extinction if it adapts. In a classical evolutionary rescue scenario, where the environment in which a population resides deteriorates, the population starts declining as a result. Extinction can then only be averted if a mutant establishes that has a positive growth rate in the new environment; i.e. the population is rescued. This process results in the well-known U-shaped curve of population size over time (Gomulkiewicz & Holt 1995, Gonzalez et al. 2012, Orr and Unckless 2014). Most models of evolutionary rescue focus on deriving the probability of the occurrence of such a rescue event given a certain initial population size, a rate of population decline, and a mutation rate. Evolutionary rescue theory could even be a useful tool to predict the emergence of antibiotic resistance (Martin et al. 2013; Alexander et al. 2014).

Here, we explore a new scenario in which not a single population, but a whole community composed of many different species faces a deteriorated environment, causing the populations of each species to decline. Only those species in which a rescue mutant with a positive growth rate establishes, remain in the community. In other words, evolutionary rescue occurs on a community-wide basis.

We present a parsimonious model of this Community-Wide Rescue (CWR) process. It describes the change in species abundances, during and after community-wide evolutionary rescue. We assume that all species are

equivalent; they all start with the same negative growth rate and all have the same fixed mutation rate towards a phenotype with a positive growth rate. These assumptions are inspired by those made in the neutral model of biodiversity (Hubbell, 1997). The neutral model has been shown to be able to explain various patterns of species abundances, and has become a baseline model for community diversity patterns when species differences or species asymmetries are ignored (Alonso et al. 2006, Rosindell et al. 2011, Wennekes et al. 2012, Scheffer et al. 2018). However, because we include an explicit mutational process that introduces a different growth rate, our Community-Wide Rescue model is not strictly neutral. We compare our results with those of two neutral models, in which the community dynamics are solely governed by ecological drift, that serve as null models.

The aim of this paper is to construct and explore a simple model for the CWR process, and to examine how under this model CWR affects the patterns of species abundances within a community. We quantify these patterns using Rank Abundance Curves (RAC, also known as rank abundance diagrams or distributions, RAD, McGill et al. 2007). It is well known that many different mechanisms can generate similar RACs, and hence RACs should be interpreted with caution (Chave et al. 2002). We aim to see if this general pattern also holds for our CWR model, or if perhaps RACs are informative about the (past) occurrence of CWR. We show that CWR causes a loss of rare species from the community due to competition and a limited supply of mutations. However, RACs produced by the CWR process could equally well have been produced by a neutral model. In addition, as RACs proved uninformative, we also examined the rate at which CWR changes the relative species abundances (i.e. alters the RAC) and compare this to the rate at which ecological drift alters species abundance patterns. We show that CWR causes an extremely rapid loss of rare species. Such insights are crucial to understand the effects of global change on ecological communities.

Methods

Our model of the CWR process is a continuous-time individual-based stochastic model, where birth, death, and mutation events are simulated using the Doob-Gillespie algorithm (Gillespie 1976). We assume that all species are equivalent except for their initial densities. This assumption is unlikely to hold in a natural community, but its simplicity allows us to focus on the key ingredients of the CWR process. Furthermore, we consider a single closed community, i.e. there is no immigration. It is worth noting that this implies that the observed dynamics are transient in nature, when time goes to infinity all species will eventually go extinct due ecological drift. This assumption allows us to more clearly see the effect of CWR in a single (local) community. In the CWR model, the community consists of several species, each with an initial abundance that is drawn using the sampling formula for standard neutral communities (Etienne 2005). Initially, all individuals of each species have the same negative growth rate. We call an individual with this negative growth rate a "resident". The initial community thus represents a community immediately after a drastic environmental change, in which the populations of all species are declining and unless adaptation occurs extinction is inevitable for all species. However, each resident individual can undergo a mutation to become a mutant individual, this occurs with a rate μ (note that this process implicitly assumes haploid inheritance). Again, the value for μ is the same, regardless of the species to which an individual belongs. All mutants have the same positive growth rate. Hence, we assume the simplest possible model of evolutionary rescue, as posited by Orr & Unkcless (2008) and Martin et al. (2013): only a single mutational step is required to achieve a positive growth rate and this mutation has a constant fitness effect. μ could for example represent the mutation rate toward antibiotic resistance, see also Martin et al. (2013).

The growth rates of the residents and mutants are implemented as follows. The death rates for the residents and the mutants are equal and given by d. We assume that the birth rate for both mutants and residents depends on total community size (i.e. total number of individuals in the community of all species combined),

Equation 1: $b = b_0(1 - \frac{N_{tot}}{K})$

where b_0 is the rate of birth in a pristine community (no other individuals present). This parameter b_0 is different between residents and mutants (hence, we have $b_{0,\,res}$ and $b_{0,\,mut}$). We assume that $b_{0,\,res} < d$ so that the resident always has a negative growth rate and $b_{0,\,mut} > d$, so that the mutants always have a positive growth rate. Parameter K is the number of individuals at which the birth rate is equal to 0, and N_{tot} is the total number of individuals (summed across species, including both residents and mutants) in the community. It is important to note that K is not the sole parameter controlling the carrying capacity of the community, this is determined by the interplay of b_0, d, and K and is given by $K^*(1- d / b_0)$. Our model deviates from standard neutral models in that we do not impose a zero-sum constraint (otherwise the community cannot decline), and that instead we have community-wide density-dependent birth. Haegeman and Etienne (2008) showed that community-level density-dependence in immigration and birth does not affect the predictions on the species abundance distributions, so we do not strongly deviate from a standard neutral model in this sense. The default parameter set for simulating the CWR model was $b_{0,res} = 0.05$, $b_{0,mut} = 0.6$, $d = 0.1$, $K = 16000$, and $\mu = 0.0005$. The initial species abundances for all simulations were generated with the sampling formula for standard neutral communities as derived by Etienne (2005) using a community size of 16000, a fundamental biodiversity number, θ, of 200 and a migration parameter, I, of 40. In this neutral model, the fundamental biodiversity number controls the species abundance distribution in the regional species pool, whilst the migration parameter governs the frequency of migration from the regional species pool to the local species pool. For a more complete description the reader is referred to Etienne & Olf (2004) and Etienne (2005). Here this model is simply used to generate a reasonable initial species abundance distribution. The exact same initial species abundance distribution was used for all simulations, unless stated otherwise. All simulations, plots and analysis were performed using R version 3.5.1 (R Core Team, 2014). All new

simulation code is provided in the CWERNI R-package that is available at: https://github.com/DeadParrot69/CWERNI.

To answer the question whether an endpoint RAC from a CWR community can be distinguished from a RAC generated by a neutral community, we used a simulation, fitting and re-simulation approach. First, we simulated a community using CWR, with the default parameters. Subsequently, we fitted a neutral model to the RAC using the SADISA-package (Haegeman and Etienne 2017). From this fit we obtain a log-likelihood, which in essence is a measure of the goodness of fit of the neutral model on the RAC generated using CWR. To generate a distribution of log-likelihoods with which to compare the log-likelihood of the neutral model fit on the CWR RAC, the parameters obtained from the neutral model fit were used to perform 500 neutral model simulations (Etienne 2005). Then, the SADISA-package (Haegeman and Etienne 2017) was used on each of these neutral simulations to fit a neutral model. This created a distribution of log-likelihoods for these neutral model simulations. Subsequently we determined whether the log-likelihood obtained from the neutral model fit on the CWR RAC falls outside or inside the distribution of the log-likelihoods obtained through neutral model fits on neutral model simulations. Instead of the log-likelihood we also looked at the distribution of two different diversity indexes, the Shannon and Rényi entropy of the simulated communities. This process was repeated ten times each time with a newly drawn neutral starting community. We note that the model underlying the SADISA estimates is subtly different from that used to perform the re-simulations. The SADISA estimator makes an independent species assumption, whilst the code used for the simulations instead assumes a zero-sum assumption, but it has been shown that the RACs that these model produce are indistinguishable (Haegeman and Etienne 2008, 2017).

In order to place the rate of rare species loss due to CWR into context, we compared it to the rate of rare species loss in a local community due to ecological drift in two truly neutral models. The first is a simple neutral model (SN) without a CWR process, where the birth and death rates are equal to those of the mutant in the CWR model. This model is thus a neutral model of

the local community without immigration or speciation; it only describes the loss of species through ecological drift.

We expected the CWR model to show a decrease in total community size before recovery (due to the negative growth rate of the residents). Such a decrease in local community size, can accelerate the rate of rare species loss through ecological drift. Therefore, we also constructed a neutral model similar to SN, but where the basic birth probability for all species is set to a value less than the death probability for a predetermined time interval. This induces a steady decrease in total community size from the start of the simulation until the end of the interval. We chose the length of the interval, such that the community size decrease is similar to that observed during CWR. We call this model the variable-birth neutral model (VBN).

We simulated the three models (CWR, SN, and VBN) for 100 units of time. This was a sufficient number for all residents to go extinct in the CWR model, see also supplementary material figure 6. When there are no more residents in the community the evolutionary rescue process is considered complete, hence we chose to simulate for 100 units of time. Each model was simulated 500 times. The SN model was simulated using the parameters $b_0 = 0.6$, $d = 0.1$, and $K = 16000$ (i.e. the same parameters as the mutants in the CWR model). For the VBN model we set the basic birth rate of all the species in the community (b_0) equal to $b_{0,\,res}$, during the first twenty units of time. After this time interval, which was tuned so as to create a decrease in total community size similar or perhaps even slightly larger in nature than that in the CWR community, we set the basic birth rate equal to $b_{0,\,mut}$. The other parameters were the same as in the SN model. To study the RAC of a community at different stages of CWR, we plotted the resulting RACs at different points in time: $t = 15$, $t = 30$, $t = 50$, $t = 75$ and $t = 100$.

In models examining evolutionary rescue, the mutation rate and the establishment probability of the mutant are known to determine the probability of evolutionary rescue (Martin et al. 2013). Therefore, to gain more insight into our CWR model, we wanted to examine the effect of the mutation rate (μ) and mutant birth rate ($b_{0,\,mut}$), on the CWR process. by

respectively varying the mutation rates (μ = 0.00005, 0.0005, 0.005, 0.05) and the mutant birth rate ($b_{0,\ mut}$ = 0.2, 0.4, 0.6, 0.8), and leaving all other parameters the same as in the default parameter set. Again, we ran 500 independent simulations for each set of parameters.

In our CWR model we assumed that $b_{0,\ res} < d$ so that the resident always has a negative growth rate. If this condition is not satisfied, one is no longer modelling evolutionary rescue. However, one can imagine a scenario in which $b_{0,\ res} > d$, for example when a bacterial community is confronted with sub-inhibitory concentrations of antibiotics. In such a community the species are not doomed to go extinct, but residents are simply replaced by fitter mutants, in essence a community-wide selective sweep. Such situations might be much more common than strict evolutionary rescue scenarios, so examining this situation could extend the applicability of our model. Therefore, we also studied a selective sweep model, derived from our CWR model, in which the only difference is that $b_{0,\ res} > d$, resulting in both a resident and a mutant with a positive net growth rate, whilst the mutant still has a higher net growth rate than the resident. We performed 500 simulations of this model using the parameter set $b_{0,\ res}$ = 0.3 and all other parameters the same as in the default CWR model parameter set.

Results

The loss of rare species in the CWR community (figure 1A, 1B) is much faster than in the neutral (SN) community (figure 1C, 1D). In other words, the CWR process causes a very rapid loss of rare species, when compared to the rate of rare species loss from a local community due to ecological drift. Furthermore, the rate of rare species loss in the CWR model is also much larger than in the VBN model (figure 1E, 1F). Because the VBN model has a variable carrying capacity tuned to create a decrease in total community size similar to the one observed in the CWR model, we can conclude that the rapid loss of rare species in the CWR model is not just due to ecological drift being accelerated by a decrease in total community size. In addition, the observed rapid loss of rare species occurs consistently in a relatively wide range of community sizes (K=16000 - K=1000, see supplementary materials figures 12-14)

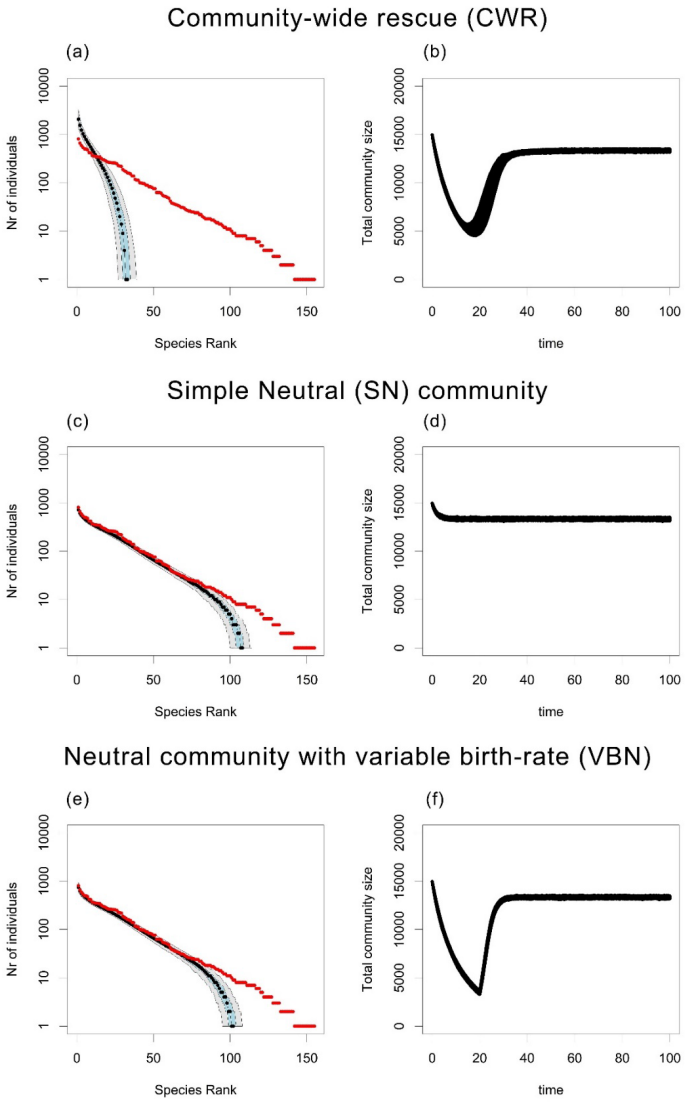

Figure 1: RACs and total community size under the CWR, SN and VBN models. Panels A, C, E show the RACs produced after 100 units of time by the CWR model, the SN model and the VBN model, where the median is shown in black, the 25th and the 75th percentile are shown in blue and the 5th and the 95th percentile are shown in grey, the initial community is plotted in red. Panels B, D and F show the accompanying trajectories of total community size for each simulation over time. All plots are based on 500 simulations. Parameters for A and B: $b_{0,\,res}$ = 0.05, $b_{0,\,mut}$ = 0.6, d = 0.1, K = 16000, and μ = 0.0005, for C and D: b_0 = 0.6, d = 0.1 and K = 16000, for E and F: b_0 = 0.05 for t between 0 and 20, b_0 = 0.6 for t between 20 and 100, d = 0.1, and K = 16000.

The same pattern is evident if one examines the figures showing the RACs at different time points for each of the three models (figure 2, supplementary materials figures 7 and 8). Furthermore, by closely examining figure 2 one can see exactly at which point during the CWR process the loss of rare species occurs. During the first stage of CWR a community-wide decline occurs that does not greatly alter the shape of the RAC (Figure 2A). It is only as the first mutants begin to invade and the total community size starts to rebound (figure 2F) that the shape of the RAC begins to change and that the loss of rare species starts to occur (figure 2B). The loss of rare species continues after the community size has stabilized (figure 2C, 2D). Once the residents have disappeared from the population, the shape of the RAC is fairly stable (figures 2D, 2E, 2F).

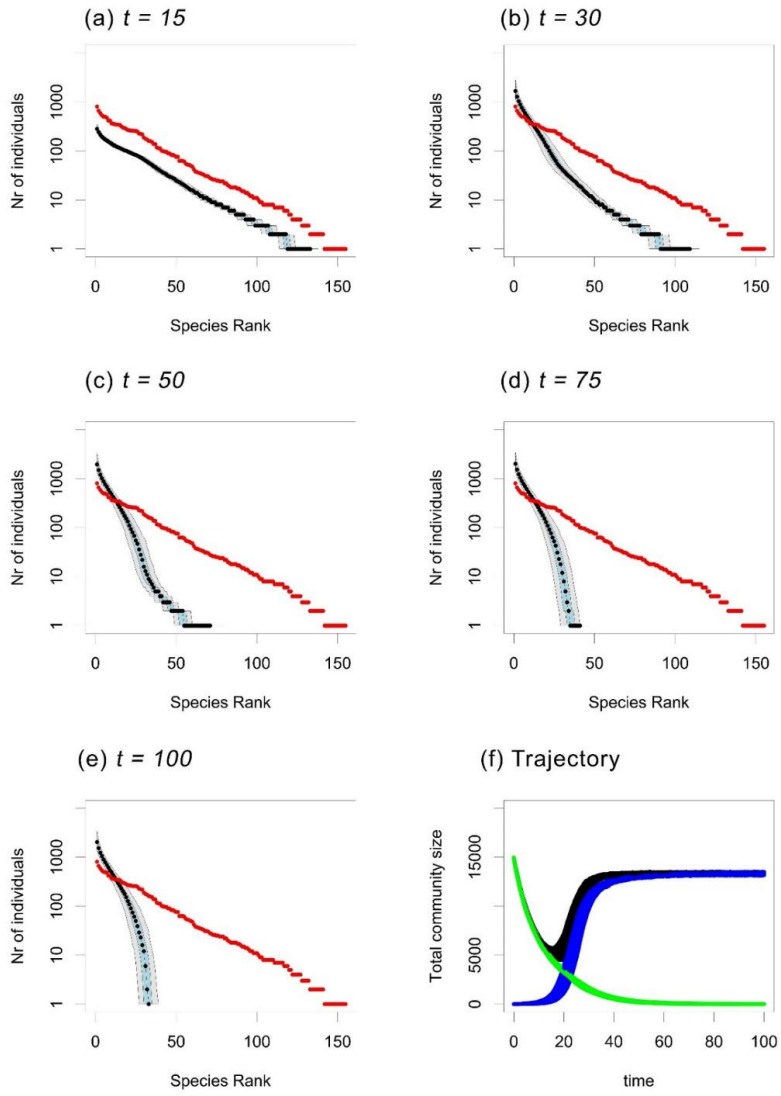

Figure 2: Time trajectory of the RAC under the CWR model. Plots based on 500 CWR simulations using the (default) parameters $b_{0,res} = 0.05$, $b_{0,mut} = 0.6$, $d = 0.1$, $K = 16000$ and $\mu = 0.0005$. Panels A, B, C, D, and E. show the RAC of the community at *t = 15, t= 30, t = 50, t = 75* and *t = 100* respectively, were the median is shown in black, the 25th and the 75th percentile are shown in blue and the 5th and the 95th percentile are shown in grey, and the input community is plotted in red. Panel F shows the trajectories of the total community size (black), the total number of residents in the communities (green) and the total number of mutants in the community (blue).

The mutation rate has a strong influence on the results (figure 3). If the mutation rate is very high, rescue becomes so likely that all species undergo rescue and there is no loss of rare species beyond the effects of normal ecological drift in a neutral community without speciation/immigration (figure 3A, 3B). By contrast, if the mutation rate is very low, almost none of the species in the community undergo rescue (figure 3E) and in some cases not a single rescue mutant manages to establish itself in the community (figure 3F). Therefore, intermediate mutation rates seem to be required for CWR to impact the RAC and create a loss of rare species greater than that produced by ecological drift alone. In other words, the rate of rare species loss during CWR depends on the mutation rate.

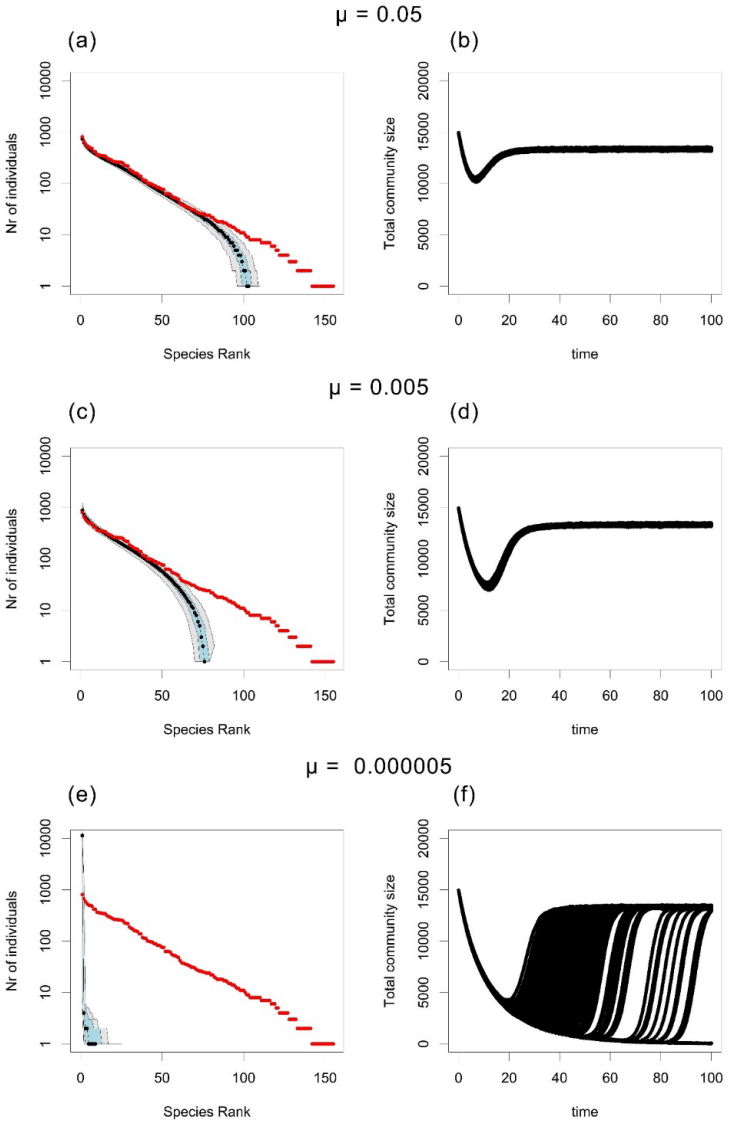

Figure 3: RACs (A, C, E) and total community size trajectories (B, D, F) under the CWR process, for different mutation probabilities (μ), for each different mutation rate 500 simulations were performed. In the RAC plots the median is shown in black, the 25th and the 75th percentile are shown in blue, the 5th and the 95th percentile are shown in grey and the initial community is plotted in red. Simulations were performed using the parameters $b_{0,\,res} = 0.05$, $b_{0,\,mut} = 0.6$, $d = 0.1$, and $K = 16000$. For A and B μ = 0.05, in C and D μ = 0.005 and in E and F μ = 0.000005. The total community size trajectories were plotted for of each of the 500 simulations, hence the separation of these trajectories at low mutation rates (F).

Increasing $b_{0,\,mut}$ i.e. increasing the fitness advantage of the mutant, does not seem to influence the loss of rare species, as the RAC's obtained after the CWR process, for different values of $b_{0,\,mut}$ are indistinguishable (figure 4). However, increasing $b_{0,\,mut}$ does seem to increase the speed of the rescue process. In particular, if $b_{0,\,mut}$ is higher, the recovery phase of the rescue process proceeds much faster, due to the higher maximal growth rate of the mutant. It should be noted that increasing $b_{0,\,mut}$ also increases the net carrying capacity of the rescued population, because despite a constant K, the net carrying capacity is the density where the birth probability is equal to the death probability. The net carrying capacity is given by $K*(1- d / b_0)$. Despite this increased carrying capacity, the recovery phase is still much faster in the simulations with a high $b_{0,\,mut}$.

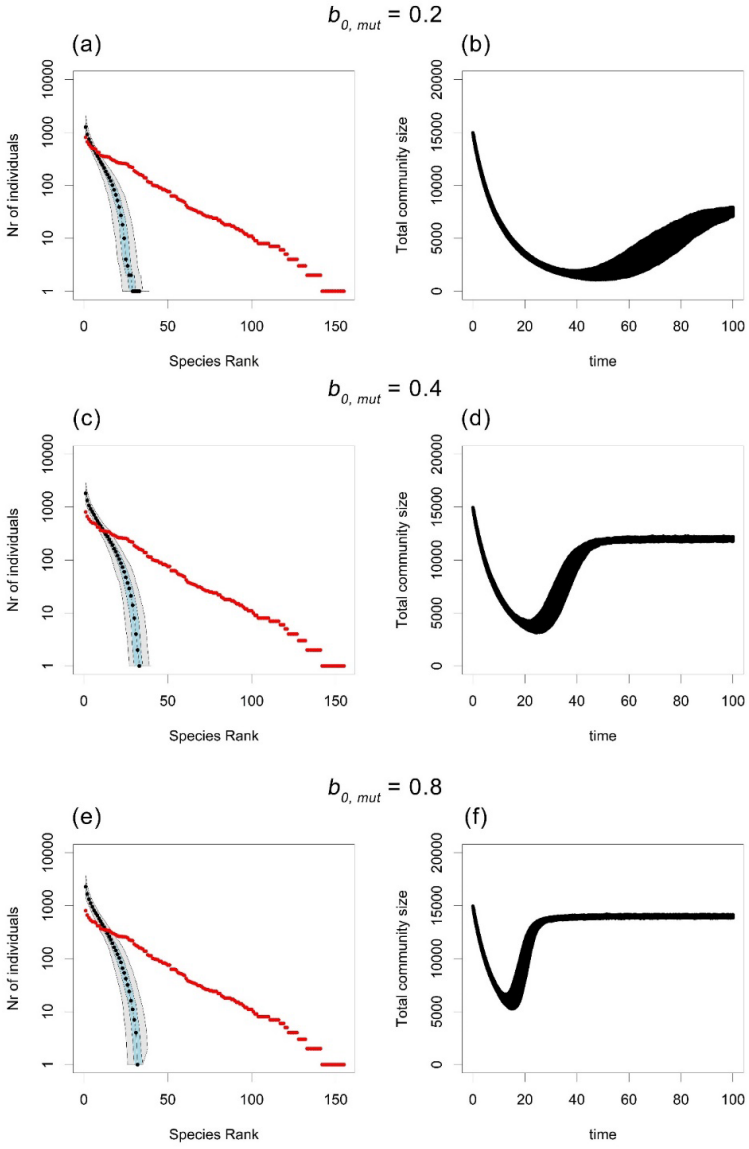

Figure 4: The RAC under CWR processes with different mutant birth probabilities ($b_{0,\,mut}$). All simulations were performed using the parameters $b_{0,\,res}$ = 0.05, d = 0.1, K = 16000, and μ = 0.0005. In panels A and B $b_{0,\,mut}$ = 0.2, for C and D $b_{0,\,mut}$ = 0.4 and in E and F $b_{0,\,mut}$ = 0.8. Panels A, C and E show the RAC's after 100 units of time, where the median is shown in black, the 25th and the 75th percentile are shown in blue and the 5th and the 95th percentile are shown in grey, with the input community plotted in red. Panels B, D, and F display the trajectories of total community size over time. All plots are based on 500 simulations.

In the selective sweep model, the residents have a positive net growth rate, i.e. instead of CWR, the resident with a positive growth rate is replaced by a mutant with an even higher growth rate. As can be seen in figure 5B, there is only a very minor decrease in the total community size during this replacement process. However as can be seen in figure 5A, the rate of rare species loss in the selective sweep model is much higher than in the neutral SN and VBN models. In other words, when compared to ecological drift, community wide adaptation can cause a very rapid loss of rare species, just like CWR.

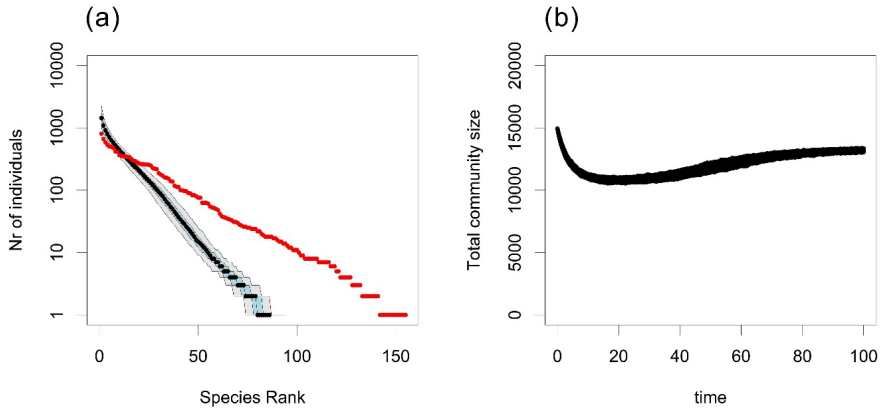

Figure 5: The RAC (A) and the total community size (B) for simulations of the CWR model where the strict conditions of CWR are relaxed and reflect a scenario where the net growth rate of the residents is positive, yet still lower than that of the mutants. This causes a selective sweep during which the residents are replaced by the mutants, because the mutants have a higher fitness. In the RAC plot the median is shown in black, the 25th and the 75th percentile are shown in blue and the 5th and the 95th percentile are shown in grey, and the input community is plotted in red. These plots are based on 500 simulations. The parameters used were $b_{0,\,res} = 0.3$, $b_{0,\,mut} = 0.6$, $d = 0.1$, $K = 16000$ and $\mu = 0.0005$.

The fitting and re-simulation approach using the log-likelihoods of neutral model fits showed that the log-likelihood of a neutral model fit on the CWR model results consistently fell within the distribution of log-likelihoods obtained from neutral model simulations (supplementary material figure 9A). A similar result was obtained when instead of log-likelihoods, the values of the Shannon entropy and the Rényi entropy of the RACs were used; the values of the Shannon entropy and the Rényi entropy estimated from the

CWR RAC consistently fell inside those estimated on neutral model simulations simulated using neutral model parameters estimated from the CWR RACs (supplementary material figure 9B, 9C). Both of these results imply that there is no information in an endpoint RAC alone that would allow one to determine whether that RAC had been created by a neutral process or a CWR process.

Discussion

We have shown that a single endpoint RAC does not allow one to determine whether that RAC had been created by a neutral process or a CWR process. This conclusion is in accordance with the general pattern in the literature; whilst some non-neutral processes, such as trait based environmental filtering (Jabot, 2010), can be detected by examining species abundances, many different non-neutral processes can generate surprisingly similar RACs (Chave et al. 2002).

The most striking outcome of our modelling effort is that CWR (figure 1A, 1B) causes a very rapid loss of rare species, when compared to ecological drift (figure 1C, 1D). This holds even if one accounts for the increase in ecological drift due to a decrease in total community size as in the VBN model (figure 1E, 1F). Furthermore, this result is shown for a wide range of community sizes (K=16000 - K=1000, see supplementary materials figures 12-14). In a neutral model governed by ecological drift, rare species are more likely to go extinct simply due to their lower abundance. However, in the CWR model rare species have a higher probability of going extinct, because their low abundance also means that they will have a lower probability of producing a beneficial mutant before going extinct. In other words, for rare species the supply of beneficial mutations is limited by their low abundance. This dependence of the probability of rescue on the initial population density is well characterized in standard models of evolutionary rescue and has also been demonstrated empirically (Bell & Gonzalez 2009, Martin et al. 2013). Low abundance causes a low probability of a beneficial mutant occurring, because mutation occurs on a per-capita basis, so during the same time interval a mutation is less likely to occur in a small population. Furthermore,

the time to extinction for rare species is lower, so there is less time for a mutant to arise before the rare species goes extinct.

However, there is another effect, hypothesized by Bell (2017), which contributes to the loss of rare species: competition. This represents a crucial difference between our model and standard models of evolutionary rescue (Martin et al. 2013). In our model the birth rate of all species is governed by the total number of individuals in the community (regardless of their species), all species compete with each other (community-level density dependence). So, a species that has undergone rescue will increase the total number of individuals in the community. This causes the birth-rate of the remaining species to decrease. For the species that have not yet undergone rescue, this accelerates their decay, decreasing the time available to find a mutant before going extinct. In other words, the evolutionary rescue of one species, promotes the extinction of its competitors (Bell 2017). Rare species that do manage to produce a mutant will tend to do so relatively late in the simulation, because their low abundance gives them a low probability of producing a mutant per unit of time. On the other hand, species with a high abundance that manage to produce a mutant will tend to do so relatively early on in the simulation, thereby promoting the extinction of the rare species through competition. It is interesting to contrast these results with those of De Mazancourt et al. (2008), who showed that on a community level biodiversity can inhibit adaptation, due to competitive interactions. In our model, the fact that rare species fail to adapt is also partly driven by competitive interactions, in that sense reaffirming the general result that competition can inhibit adaptation. However, the crucial difference is that in the model of De Mazancourt et al. (2008) these competitive interactions are driven by explicit assumptions about the ecology of each species, whilst in our model species are ecologically equivalent except for their initial abundance.

In our model, the limited supply of beneficial mutations at low abundance and competition between the species together disproportionally promote the extinction of rare species during CWR. These two effects are also crucial to understand how changing the mutation rate impacts the CWR process

(figure 3). From standard models of evolutionary rescue it follows that a high mutation rate results in a high probability of rescue (Martin et al. 2013). Furthermore, in our model a high mutation rate implies that mutations occur at very similar times for different species, limiting the competitive advantage of common species that rescue early. Therefore, if the mutation rate is too high, almost all species undergo rescue and very little rare species loss occurs (figure 3A, 3B). For very low mutation rates the opposite holds true and very few species undergo rescue (figure 3E, 3F). It should be noted that at very low mutation rates, in some cases not even a single species undergoes rescue. So, in other words, a lower mutation rate causes a greater loss of rare species, yet if the mutation rate is too low no rescue occurs and the entire community goes extinct.

The influence of the mutant birth rate ($b_{0,\,mut}$) on the CWR process (figure 4) is not as straightforward. Based on standard models of evolutionary rescue, increasing the mutant birth rate should increase the fixation probability of the mutant and thereby increase the probability of rescue. Furthermore, increasing the mutant birth rate should also increase the competitive advantage of those species that rescue early. However, contrary to our expectations, we observed that an increase in the mutant birth rate does not cause an increase in the loss of rare species. Instead, an increase in the mutant birth rate only seems to affect the speed of the CWR process. This is in part due to the fact that an increase in the mutant birth rate also increases the overall carrying capacity of the community. This increase could offset the competitive advantage of the species that rescue early. Because they grow faster, the equilibrium community size is also larger. However, this increase in the community size does not influence the fixation probability of the mutant as derived in classical models of evolutionary rescue. Therefore, the fact that increasing the mutant birth rate does not increase the loss of rare species indicates that competition between early and late rescuing species is the more dominant mechanism responsible for the loss of rare species. This emphasizes the added value of our current modelling approach for understanding evolutionary rescue in a multi-species context.

We also created a different model based on the CWR model where we allowed the residents to have a positive growth rate ($b_{0,\,res} > d$). Relaxing this assumption implies that this model does not reflect a strict evolutionary rescue scenario, as this requires a decaying resident population. This model represents a community wide selective sweep, during which residents with a positive growth rate are replaced by mutants with an even higher growth rate. When comparing this selective sweep model (figure 5A, B) to the neutral SN and VBN model it is evident that the community wide selective sweep causes a rapid loss of rare species when compared to ecological drift. However, the rate of rare species loss is lower than in the CWR model. Hence, one might conclude that community wide adaptation in general leads to a loss of rare species, implying that our findings from the CWR model are more generally applicable. Furthermore, as there is no evolutionary rescue process in our selective sweep model, the only mechanism responsible is the competition between species that have found the high fitness mutant. Hence the fact that competition alone is enough to cause the rapid rare species loss in the selective sweep model also indicates that competition is a more dominant mechanism of rare species loss in the CWR model.

As emphasized before, our CWR model assumes a simple model of evolutionary rescue. Most notably, rescue requires only a single mutation step, with a fixed positive fitness effect. For some situations these assumptions should provide a reasonable approximation. For example, the evolution of resistance to certain antibiotics requires only a single or very few mutations. In addition, the mechanisms underlying resistance can be quite similar across different species (Hooper & Jacoby 2015). However, obviously these simple assumptions do not hold under all biological circumstances. So how would a more complex assumptions regarding mutation affect the outcome of our CWR model? Allowing multiple mutational steps of varying fitness effects would serve to make the competition during the rescue process more asymmetrical. Therefore, this would be expected to cause an even greater loss of rare species compared to our current CWR model.

The CWR model presented here assumes that all species are (initially) equal, differing only in their initial abundances, an assumption inspired by the

neutral theory of biodiversity. Evidently this assumption is unlikely to strictly hold in natural communities, yet it allows us to create a relatively simple model. Furthermore, our model does not consider immigration and speciation. Future CWR models could include mutation probabilities, birth probabilities, and death probabilities that differ across species, and include migration and speciation. It will be interesting to see whether demographic rescue, by immigration, will counteract or aid evolutionary rescue by mutation.

It is striking that the change in the shape of the RAC produced by the CWR process i.e. one devoid of rare species is a pattern commonly observed by ecologists in "stressed" or disturbed communities (Bazzaz 1975, Halloy and Barratt 2007, Webb et al. 2011). Additionally, antibiotic treatment also seems to cause a similar loss of rare species in the microbiome of patients, which persists long after the treatment (Sommer and Dantas 2011). Interestingly, a study of benthic foraminifera during the Palaeocene-Eocene thermal maximum by Webb et al. (2009) showed a decrease in richness, an increase in kurtosis, and a decrease in evenness during the Palaeocene-Eocene thermal maximum, i.e. a change in the shape of the RAC that would also be consistent with a CWR scenario.

However, it is important to realize that there are countless other ecological explanations that may account for the loss of rare species in stressed environments. For example, rare species tend to be more specialized and are hence more sensitive to disturbance (Davies et al. 2004). Or the loss of a single keystone species can in turn lead to the loss of many rare species that may depend on it (Rapport et al. 1985). Thus, if rapid loss of rare species is observed, that is much faster than would be expected due to ecological drift, this does not per se imply an underlying CWR process.

However, this does not mean that CWR is a hypothetical process with little relevance, to real ecological communities. Experimentalists are examining evolutionary rescue in a community context. The examples include microbiomes adapting to antibiotic treatment (Roodgar et al. 2019); soil microbial communities adapting to herbicides (Low-Décarie et al. 2015) and

lacustrine plankton communities adapting to acidification (Bell et al. 2019). There are many more situations in which CWR could be considered as a potential mechanism for rare species loss, as many ecosystems face irreversible human induced environmental change on a community-wide level (Vitousek et al. 1997).

All in all, the current CWR model represents an initial exploration of CWR and could be considered as a baseline model regarding the effect of community wide evolutionary rescue on species abundances. Yet, this simple model provides a clear testable prediction regarding the effect of CWR on species abundances: Community Wide Rescue causes a very rapid loss of rare species.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author contributions

TJBvE was primarily responsible for the conception of the idea, programming, simulation, analysis and writing of this manuscript. KB contributed to the development of the ideas and provided extensive input regarding the writing of the manuscript. RSE contributed to the development of the ideas, proposed modelling strategies, suggested analyses and provided extensive input regarding the writing of the manuscript.

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Supplementary material

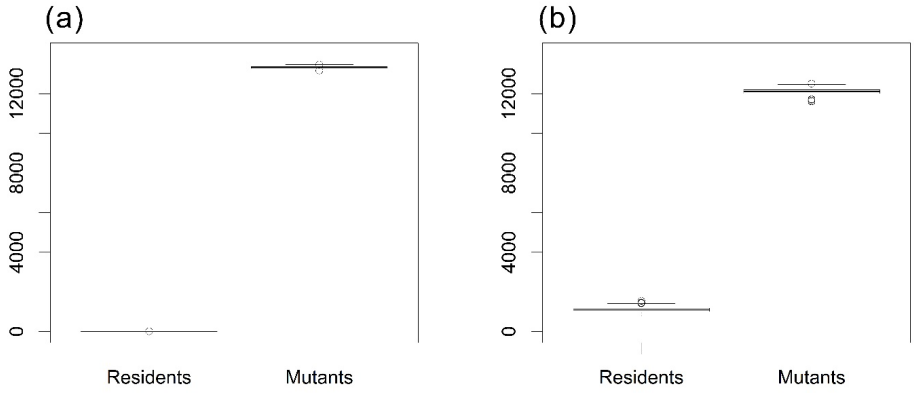

Figure 6: Panel A shows a boxplot of the amount of residents and mutants at the end of each of the 500 CWR simulations using the default parameters, this plot corresponds to figure 1A and 1B. This boxplot looked the same for all other CWR simulations i.e. all residents had gone completely extinct in all simulations (figures 2, 3 and 5), except for those simulations performed under the selective sweep scenario. The distribution of residents and mutants at the end of 500 CWR simulations for the selective sweep scenario (figure 4) is shown in panel B.

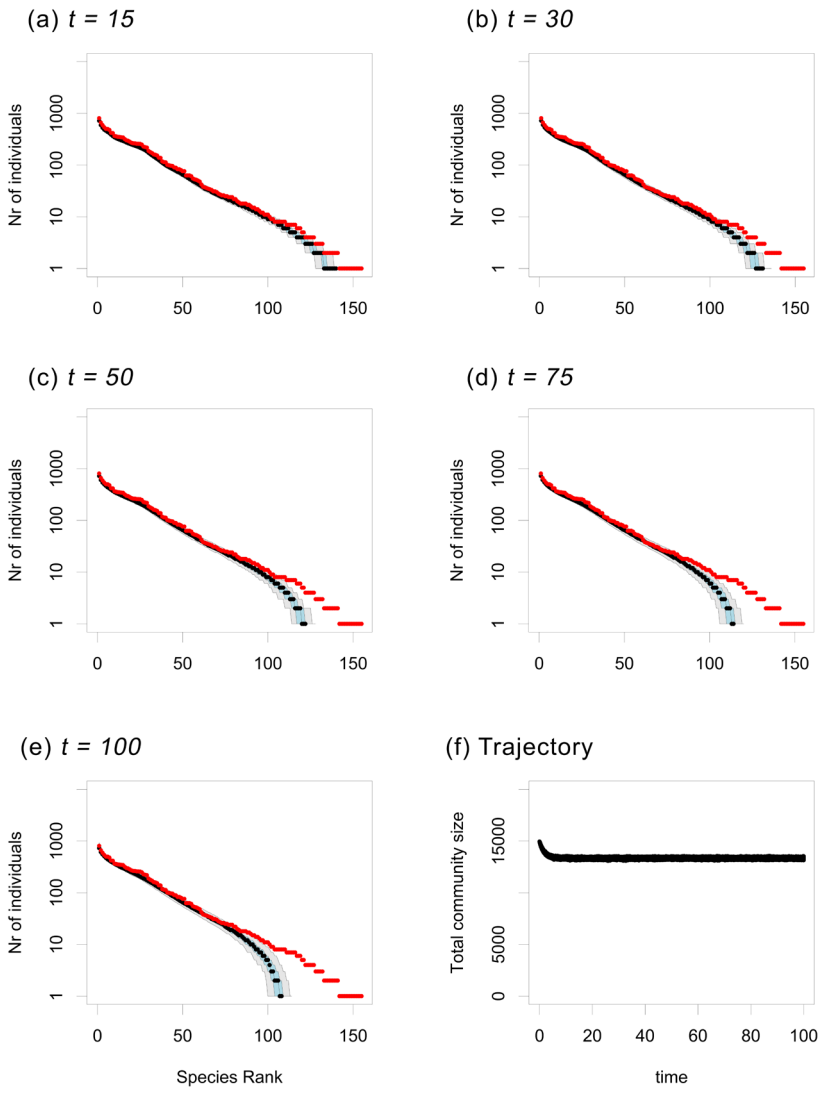

Figure 7: Time trajectory of the RAC under the SN model. Panels A, B, C, D, and E. show the RAC of the community at *t = 15, t= 30, t = 50, t = 75* and *t = 100* respectively, were the median is shown in black, the 25th and the 75th percentile are shown in blue and the 5th and the 95th percentile are shown in grey, and the input community is plotted in red. Panel F shows the trajectories of the total community. Plots are based on 500 SN simulations using the parameters.

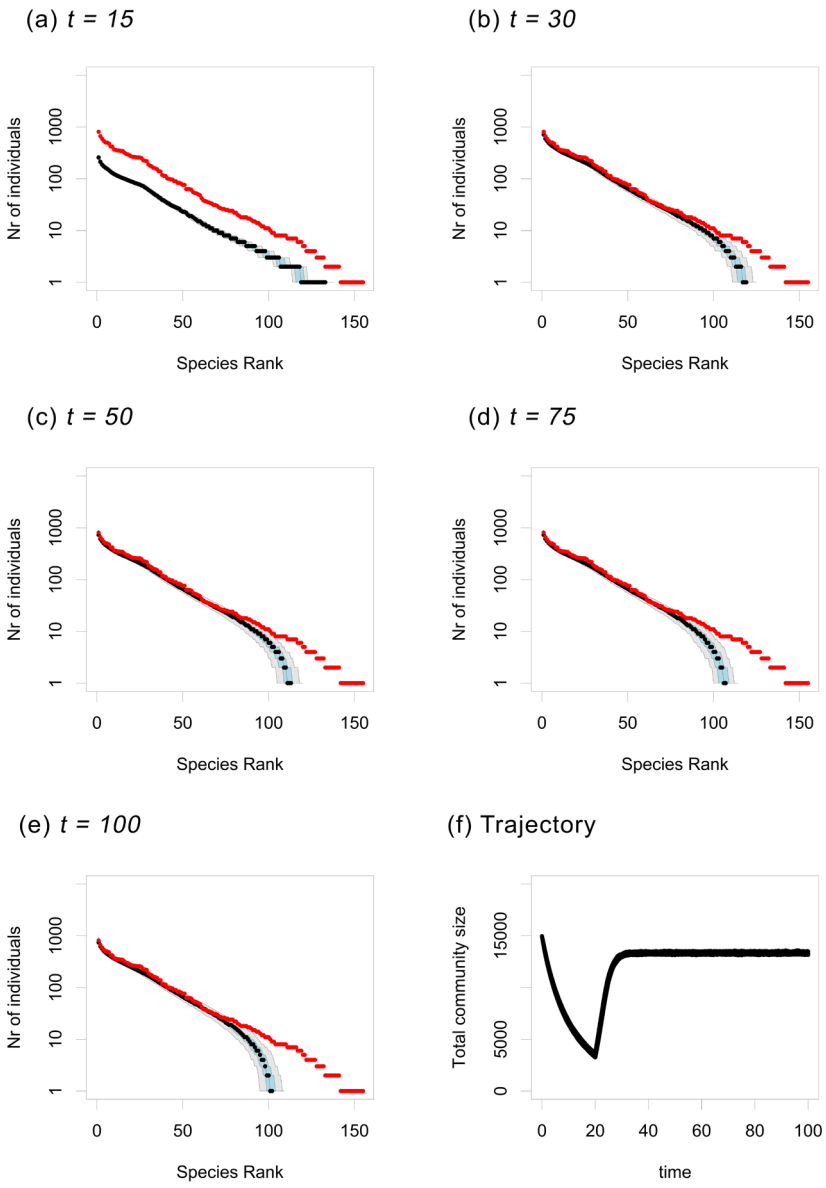

Figure 8: Time trajectory of the RAC under the VBN model A, B, C, D, and E. show the RAC of the community at *t = 15, t= 30, t = 50, t = 75* and *t = 100* respectively, were the median is shown in black, the 25th and the 75th percentile are shown in blue and the 5th and the 95th percentile are shown in grey, and the input community is plotted in red. Panel F shows the trajectories of the total community. Plots are based on 500 VBN simulations using the default parameters.

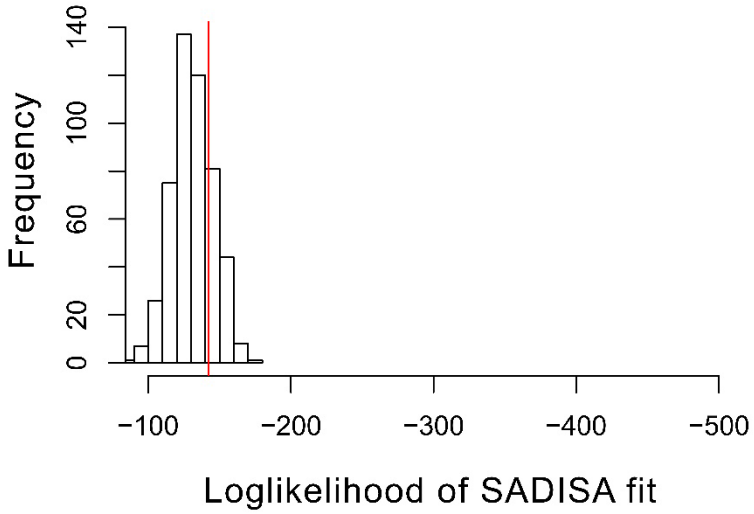

Figure 9: Distribution of log-likelihoods under neutral simulations with parameters derived from the neutral fit to the CWR simulation data. The log-likelihood of the neutral model fit on the CWR simulation is shown in red. In total this analysis was repeated ten times, each time producing the same result, here only a single example is shown.

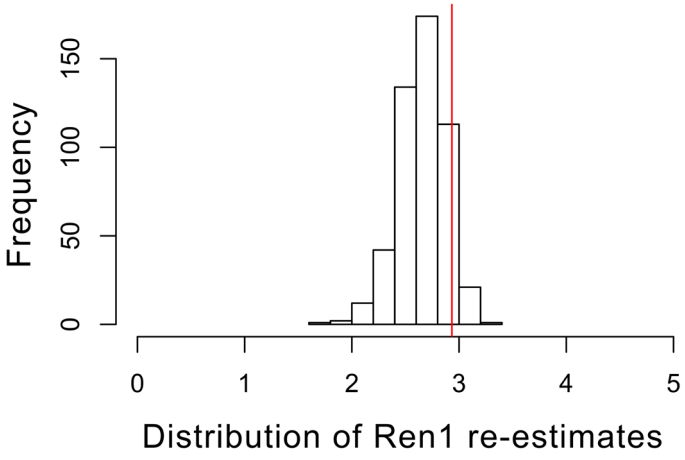

Figure 10: Distribution of the Shannon entropy under neutral simulations with parameters derived from the neutral fit to the CWR simulation data. The value of the Shanon entropy (Reny entropy, $\alpha = 1$) with of the CWR simulation RAC is shown in red. In total this analysis was repeated ten times, each time producing the same result here only a single example is shown.

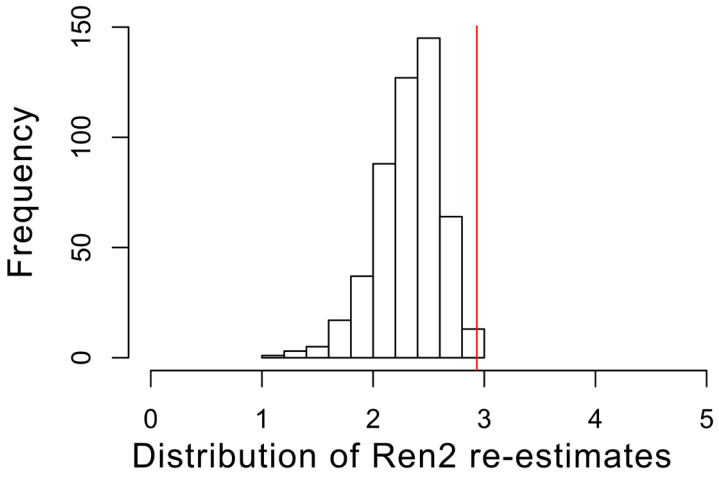

Figure 11: Distribution of log-likelihoods under neutral simulations with parameters derived from the neutral fit to the CWR simulation data. The value of the collision entropy (Reny entropy, $\alpha = 2$) with of the CWR simulation RAC is shown in red. In total this analysis was repeated ten times, each time producing the same result here only a single example is shown.

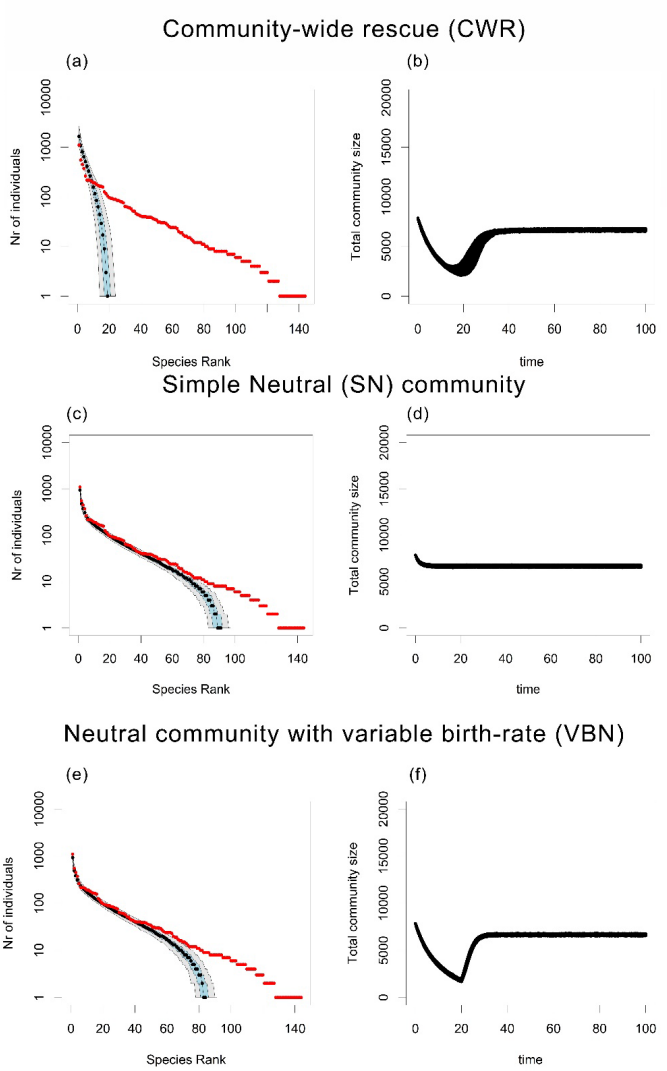

Figure 12: RACs and total community size under the CWR, SN and VBN models for K = 8000. Panels A, C, E show the RACs produced after 100 units of time by the CWR model, the SN model and the VBN model, where the median is shown in black, the 25th and the 75th percentile are shown in blue and the 5th and the 95th percentile are shown in grey, the initial community is plotted in red. Panels B, D and F show the accompanying trajectories of total community size for each simulation over time. All plots are based on 500 simulations. Parameters for A and B: $b_{0,\,res}$ = 0.05, $b_{0,\,mut}$ = 0.6, d = 0.1, K = 8000, and μ = 0.0005, for C and D: b_0 = 0.6, d = 0.1 and K = 8000, for E and F: b_0 = 0.05 for T between 0 and 20, b_0 = 0.6 for T between 20 and 100, d = 0.1, and K = 8000.

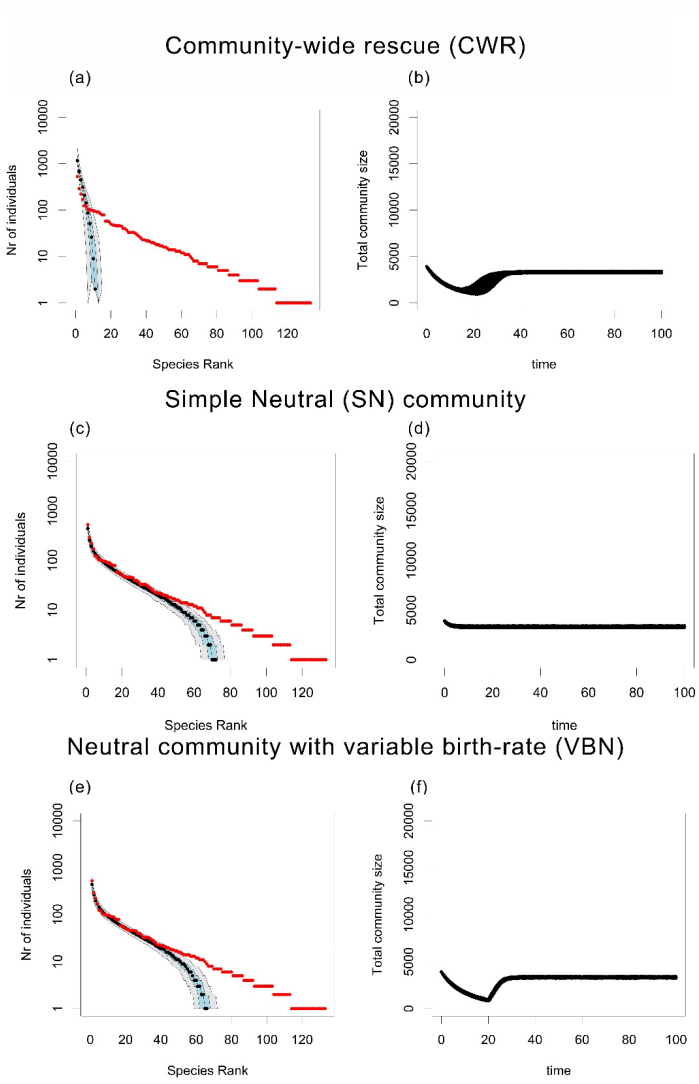

Figure 13: RACs and total community size under the CWR, SN and VBN models for $K = 4000$. Panels A, C, E show the RACs produced after 100 units of time by the CWR model, the SN model and the VBN model, where the median is shown in black, the 25th and the 75th percentile are shown in blue and the 5th and the 95th percentile are shown in grey, the initial community is plotted in red. Panels B, D and F show the accompanying trajectories of total community size for each simulation over time. All plots are based on 500 simulations. Parameters for A and B: $b_{0,\,res} = 0.05$, $b_{0,\,mut} = 0.6$, $d = 0.1$, $K = 4000$, and $\mu = 0.0005$, for C and D: $b_0 = 0.6$, $d = 0.1$ and $K = 4000$, for E and F: $b_0 = 0.05$ for T between 0 and 20, $b_0 = 0.6$ for T between 20 and 100, $d = 0.1$, and $K = 4000$.

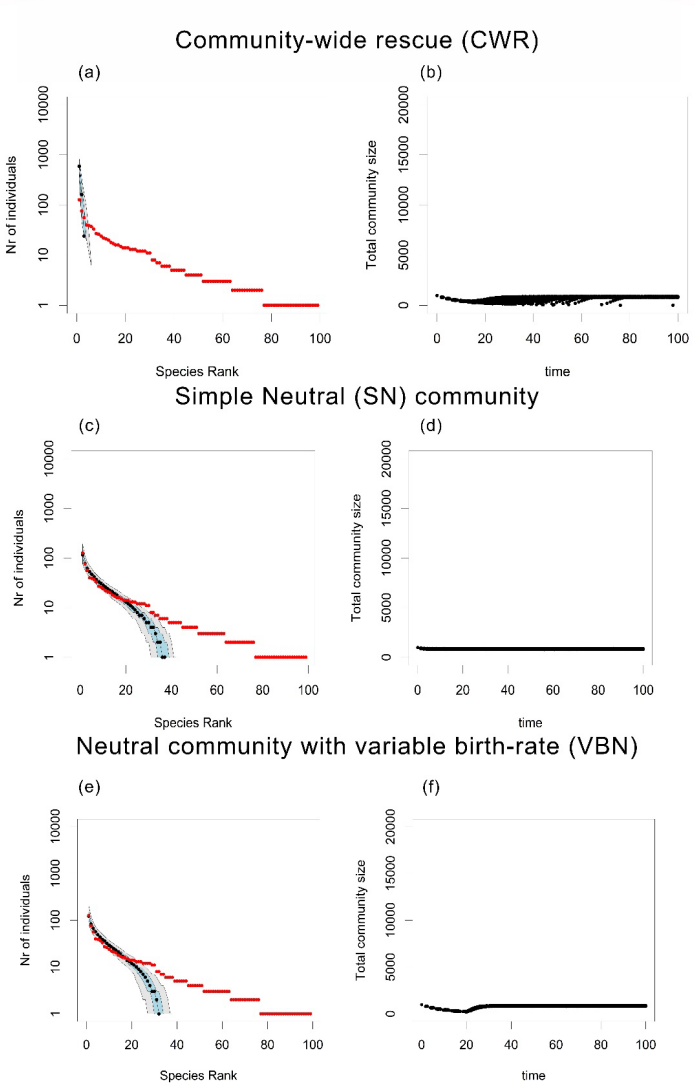

Figure 14: RACs and total community size under the CWR, SN and VBN models for K = 1000. Panels A, C, E show the RACs produced after 100 units of time by the CWR model, the SN model and the VBN model, where the median is shown in black, the 25th and the 75th percentile are shown in blue and the 5th and the 95th percentile are shown in grey, the initial community is plotted in red. Panels B, D and F show the accompanying trajectories of total community size for each simulation over time. All plots are based on 500 simulations. Parameters for A and B: $b_{0,\,res}$ = 0.05, $b_{0,\,mut}$ = 0.6, d = 0.1, K = 1000, and μ = 0.0005, for C and D: b_0 = 0.6, d = 0.1 and K = 1000, for E and F: b_0 = 0.05 for T between 0 and 20, b_0 = 0.6 for T between 20 and 100, d = 0.1, and K = 1000.

Chapter 8

Discussion

Timo J.B. van Eldijk

In this thesis, I have studied evolvability, the ability of biological systems to undergo adaptive evolution, in the context of antibiotic resistance. Throughout I took a mechanistic approach, combining theory and experiment. The purpose of this final chapter is twofold: I will first briefly reflect on how some of my findings might be applied to combat the emergence of antibiotic resistance and improve antibiotic treatment. Second, I will reflect on what I perceive to be the way forward for evolvability research.

Applying evolvability insights to combat antibiotic resistance

Before discussing in detail how the insights into evolvability I obtained in this thesis might be applied, a general word of caution is due. Whilst I have extensively studied the evolution of antibiotic resistance in this thesis, I have exclusively done so using theoretical models and controlled experiments in the laboratory. Hence, the insights gained throughout this thesis are of a more fundamental nature. The process of translating such insights into clinical improvements requires many decades of further experiments, modelling and clinical trials. Nonetheless, I think fundamental insights are essential to facilitate the practical progress of the future. We need to look no further than the history of antibiotics to find an illustrative example: Alexander Fleming's fundamental work on penicillin in 1929 initially received little attention. Yet, it was essential to the work of Ernst Boris Chain, Howard Florey, and Norman who more than a decade later figured out how to successfully isolate penicillin, turning it into a practically utilisable drug (Gaynes et al. 2017). The fundamental insights of today are essential for tomorrow's practical breakthroughs. In this first section of my discussion, I hope to sketch out how the insights about evolvability in the context of antibiotic resistance, that I obtained in this thesis, may in the future be translated into practice. My purpose is to inspire those with more knowledge of practical medical aspects to pick up the gauntlet.

Chapter 3 – Temperature dependent mutation rates

When populations are well adapted to their environment, a low mutation rate is advantageous, since most mutations are deleterious. However, when a population is poorly adapted, a higher mutation rate can be advantageous, as the positive effects of beneficial mutations can outweigh the negative effects of deleterious mutations. Therefore, elevated mutation rates can evolve when a bacterial population is maladapted, a prime example of the evolution of evolvability (Sniegowski et al., 1997; 2000). However, some bacteria have also evolved a more refined solution to optimise their mutation rate. In this case, the mutation rate is regulated depending on the conditions. A good example of such a condition-dependent mutation rate is stress-induced mutagenesis, which has been described in several different bacterial species (MacLean et al., 2013). When the bacterial cell experiences stress (indicative of maladaptation) the mutation rate is upregulated, for example by using different error-prone DNA polymerases for DNA replication.

Stress-induced mutagenesis may be very relevant in the context of antibiotic resistance: the mutation rate towards antibiotic resistance determines the probability that a bacterial population will evolve resistance. Higher mutation rates make it easier to evolve resistance. However, environmental conditions can influence mutation rates in bacteria. Fever, a change in body temperature in response to infection can alter the growth conditions of infecting bacteria. Therefore in Chapter 3, I used laboratory experiments to examine the effect of fever temperatures on the mutation rate towards antibiotic resistance for three different antibiotics in *Escherichia coli*. I show that in all cases the mutation rate toward resistance was influenced by fever temperatures, however, the nature of this relationship differed per antibiotic. For two antibiotics, rifampicin and ciprofloxacin, fever temperatures lead to an increased mutation rate. For another antibiotic, ampicillin, fever temperatures lead to a decreased mutation rate. Stress-induced mutagenesis could provide an explanation for the observation that the mutation rate towards resistance increases at fever temperatures for the antibiotics ciprofloxacin and rifampicin. The different result for ampicillin is

likely due to the temperature-dependent efficacy of this antibiotic, which I demonstrated using a separate experiment.

Together these results suggest that coordinating the choice of antibiotic and the choice to use fever-suppressing drugs when treating a patient could be used to lower the mutation rate towards resistance. The findings from Chapter 3 are perhaps the most promising when it comes to translation into medical practice. They open a clear new avenue of research regarding the effect of fever suppression on the evolution of resistance, potentially paving the way for improved treatments. However, a word of caution is due. It remains to be seen how the results from Chapter 3 translate to different bacteria and antibiotic concentrations. Furthermore, a change in temperature might also alter other parameters that affect the probability of resistance evolution, such as the rate of killing of a non-resistant population exposed to antibiotics (Mackowiack et al., 1981; Martin et al., 2013). In addition, it is unclear how these *in vitro* results might hold up *in vivo*. For example, the experiments leave the body's own antimicrobial defence strategies out of consideration. It is well known that fever can play an important role in stimulating the immune system. In other words, there are still a lot of unanswered questions regarding the exact mechanism by which fever might affect the evolution of antibiotic resistance. While we wait for these mechanisms to be unravelled by follow-up fundamental research, we may take a more empirical approach in order to translate the results from Chapter 3 into improved treatments. Data on the impact of fever suppression on the outcomes of severe infections treated with antibiotics has already been gathered, using both observational studies and randomly controlled trials (Young et al. 2015; Schnell-Chapele 2018). Overall analyses of these data did not show a consistent positive effect of fever suppression on treatment outcomes (Young et al. 2015; Schnell-Chapele 2018). However, in Chapter 3 I showed that the impact of fever suppression on the mutation rate differed per antibiotic. Therefore, I would suggest in the future that such data should be analysed whilst taking into account the exact antibiotic used in the antimicrobial treatment. In addition, future studies could gather real-time data on the impact of within-patient and between-patient evolution of

resistance (for example using the methodology of Tueffers et al., 2019). It could be that antibiotic-specific fever suppression strategies can prevent the evolution of resistant strains, without necessarily improving within patient treatment outcomes.

Chapter 4 – Condition-dependent mutation rates

Condition-dependent mutation rates have been empirically demonstrated in several different species of bacteria (including in Chapter 3 of this thesis), but some controversy surrounds the interpretation of these results (MacLean et al., 2013). Some have argued for a non-adaptive explanation, where the increase in the mutation rate is simply a side-effect of suboptimal functioning under stressful conditions. Others have argued that the increase in the mutation rate under stressful conditions represents an evolved response, which optimises the mutation rate to facilitate adaptation and enhance evolvability (MacLean et al., 2013). Therefore, in Chapter 4, I set out to examine under what circumstances we would expect condition-dependent mutation rates to evolve. I used an individual-based simulation model with an environment that changes at different speeds, ranging from a change every ten generations to a change every ten thousand generations. Such an environment could represent a bacterial pathogen population which, over the course of generations, faces different within-patient conditions (and possibly antibiotics). Each individual has three evolving loci that determine the mutation rate as a function of the fitness of the individual in the current environment (i.e. degree of maladaptation).

The results of the model show that both condition-dependent and condition-independent mutation strategies emerge, under a wide range of different environments, ranging from environments that change every ten generations to environments that change every thousand generations. I also considered two scenarios for the mutation rate of the mutator loci: a 'self-referent' mutation rate that is determined by the mutator loci themselves, and an externally determined (not self-referent) mutation rate. Previous models of the evolution of condition-dependent mutation rates only considered non-self-referent mutation rate loci. However, I would argue that

many empirical examples, such as stress-induced mutagenesis, are to some extent self-referent. After all, error-prone DNA polymerases are also used to replicate the genes in which they are themselves encoded. When the mutation rate loci are assumed to be self-referent, the condition-dependent mutation strategy emerges at a much higher frequency. In addition, I also show that condition-dependent mutation rates lead to better tracking of a changing environment i.e., they improve evolvability. Considering these results I conclude that condition-dependent mutation rates can evolve under a large array of circumstances. This lends some credence to the idea that the empirically observed condition-dependent mutation rates are indeed evolved responses and not merely a side-effect. However, some caution is due, as the model in Chapter 4 makes several important simplifying assumptions. The environment considered is relatively simple. In reality, the environment faced by infecting bacteria is much more complex. The individuals in the model adapt using only a single trait. Additional traits under stabilising selection are not considered in the model and hence some of the deleterious effects of mutations are likely underestimated. The model therefore possibly overestimates the range of conditions in which condition-dependent mutation rates can arise.

I conclude that condition-dependent mutation rates can evolve under a wide range of circumstances and can enhance adaptation to the environment (i.e. enhance evolvability). Therefore, I would argue that evolved condition-dependent mutation rates are likely to be widespread in bacteria. I hypothesize that the empirical observations of condition-dependent mutation rates in several bacterial species can be explained by selection for evolvability. Furthermore, given that condition-dependent mutation rates can enhance evolvability I would also expect them to play an important role in the evolution of antibiotic resistance. Effectively applying this insight will obviously require many more years of research, however, I believe that it highlights a relevant research direction: interfering with condition-dependent mutation rates could be a promising strategy to combat the evolution of antibiotic resistance. One way to utilise the presence of condition-dependent mutation rates is to modify the environment to

downregulate the stress response and minimize the mutation rate, thereby decreasing the probability of resistance evolution. In Chapter 3 I studied a particular implementation of this idea, however, there may be many other ways to modify the environment. For example, the availability of carbon sources, the environmental pH and quorum sensing (a proxy for cell density) can impact the stress response (Joelsson et al., 2007; Arcari et al., 2020; Tapia et al., 2020). Thus, these factors could perhaps be engineered to downregulate the stress response, lowering the mutation rate, and slowing the evolution of resistance. Alternatively, it may be possible to use molecules that directly interfere with the mechanisms underlying the stress response, such as N6-(1-naphthyl)-ADP, thereby preventing the upregulation of mutation rates (Lee et al., 2005). The molecular mechanisms behind condition-dependent mutation rates are only well understood in a few model bacterial species (Tenallion et al., 2004; MacLean et al., 2013). Therefore, future research into these fundamental mechanisms should be pursued with the aim of uncovering new methods to lower the mutation rate towards resistance.

Chapter 5 – Mutational transformers

Many standard evolutionary models commonly assume a relatively straightforward relationship between the genotype and the phenotype. For example, quantitative genetic models commonly assume that traits are governed by many unlinked genes with infinitesimally small effects (Hill, 2010). However, in reality, there is a complex network of genetic interactions and regulation that translate a genotype into a phenotype. It has been demonstrated that taking into account this complex gene regulatory network can drastically improve the predictions of trait evolution when compared to more traditional quantitative genetics approaches (Milocco & Salazar-Ciudad, 2020). A hallmark study by Crombach & Hogeweg (2008) showed that explicitly modelling gene regulatory networks is also essential when trying to understand evolvability. They modelled virtual cells that had to adapt to an environment that alternated between two states. However, after a while, adaptation occurred much more rapidly when the environment

changed. The structure of the gene regulatory network had evolved and transformed the phenotypic impact of some mutations in such a way that relatively few mutations were required to achieve rapid adaptation to new environmental conditions. In other words, Crombach and Hogeweg demonstrated that evolvability can evolve through a restructuring of the gene regulatory network.

I was greatly inspired by their work as it shows how evolvability can evolve through the structure of gene regulatory networks. Furthermore, the alternating environment they studied was reminiscent of the environment faced by bacterial pathogens facing intermittent antibiotic treatment as they move from host to host. Therefore, in Chapter 5 I aimed to further investigate the phenomenon discovered by Crombach & Hogeweg (2008) which I have termed "mutational transformation": A gene regulatory network is structured in such a way that some mutations have a high phenotypic impact, allowing to switch to a different adaptive phenotype in a few mutational steps, thus facilitating rapid adaptation. As the Crombach-Hogeweg model is very complex, making it difficult to understand the exact mechanisms underlying mutational transformation, I investigated very simple gene-regulatory-network models, allowing me to explore these mechanisms in detail. In Chapter 5, I show that mutational transformers can evolve even in very simple gene regulatory network models. I uncovered two distinct mechanisms underlying mutational transformation: mutation amplifiers and mutation canalisers. A mutation amplifier enlarges the phenotypic effects mutation but the distribution of phenotypic effects remains unbiased. A mutation canaliser biases the effects of mutations towards particular (adaptive) phenotypic outcomes. These two mechanisms impact evolvability in different ways, to use the terminology from Chapter 2; they have a different scope. Mutation amplifiers can enhance evolvability even when the environment changes erratically. On the other hand, mutation canalisers can only enhance evolvability towards environments that have already occurred repeatedly in the past. Based on these two mechanisms, I expect that mutational transformers will also evolve in many different types of changing environments, not just environments that alternate between two states.

Mutational transformers can easily evolve in a model that makes relatively few assumptions about the exact nature of the gene regulatory networks. Furthermore, in view of the plausible underlying mechanisms, mutational transformers should be able to evolve and operate in many different types of changing environments. This leads to the expectation that mutational transformers are widespread in organisms that face changing environments. When it comes to the evolution of antibiotic resistance bacterial pathogens face just such an environment. As pathogenic bacteria transfer between hosts, they alternatingly face environments with and without antibiotics, each time undergoing evolutionary adapting to a change in the environment. Therefore, I argue mutational transformers likely play a role in the evolution of resistance. There is even some empirical evidence that supports this assertion. Consider for example the phenomenon known as "heteroresistance", where some bacterial strains can very rapidly evolve high levels of resistance through gene duplications (Andersson et al., 2019). This somewhat resembles a mutation canaliser: the effect of gene duplications seems biased towards an adaptive outcome.

I think that taking into account mutational transformers in the context of antibiotic resistance could help to improve treatment. If we can identify mutational transformers for the evolution of resistance to certain antibiotics, bacteria causing an infection could be screened for their presence. This information could be used to evaluate how easily a certain pathogen can evolve resistance to a certain antibiotic (i.e. evolvability in the context of antibiotic resistance). Subsequently, this information might help us optimise the choice of the antibiotic that will be used to combat a particular infection. However, we can only account for mutational transformers if we can reliably identify them. Currently empirically identifying mutational transformers in bacteria is very difficult. It requires extensive analysis of the phenotypic effects of mutations and evolutionary trajectories, a difficult and labour-intensive process. While empirical examples exist that suggest the presence of mutational transformers, as far as I know, no one has yet managed to unequivocally identify an empirical mutational transformer. Nonetheless, in the future the painstaking research aimed at empirically studying mutational

transformers and their inner workings should be undertaken. Experimental evolution (similar to the methodology used in Chapter 6) could be used to examine evolutionary trajectories, whilst targeted mutagenesis could be used to explore the distribution of phenotypic effects. I hope that coupled with modelling efforts (such as the one presented in Chapter 5) such research can lead to ways to identify mutational transformers quickly and reliably. It could turn out that in the future some mutational transformers can be identified simply on the basis of the structure of a gene regulatory network or the presence of particular genes. In this case, sequencing methods could be employed to rapidly identify relevant mutational transformers in bacterial pathogens, potentially enhancing antibiotic treatment.

Chapter 6 – Horizontal gene transfer

Many clinically relevant antibiotic resistance genes are located on extrachromosomal elements called plasmids. Plasmids can be horizontally transferred between cells through a process known as conjugation. Plasmids can play an important role in the spread of antibiotic resistance. However, before antibiotic resistance can spread, it must first arise. The role of plasmids in the de-novo evolution of resistance is much less well studied. It has been hypothesised that plasmids might facilitate the evolution of resistance (i.e. enhance evolvability), for example by bringing together resistance genes from separate clonal lineages. Therefore, in Chapter 6 I used experimental evolution to study the impact of plasmids on the de-novo evolution of antibiotic resistance. I used a plasmid and a bacterial strain that did not contain any antibiotic resistance genes at the start of the experiment. Subsequently, I evolved populations with and without plasmids in the presence of an increasing concentration of the antibiotic ciprofloxacin. This experiment showed no significant effect of plasmids on the rate of resistance evolution. It seems that in the context of this experiment, plasmids did not enhance evolvability, perhaps due to the relatively short timescale of the experiment.

However, the experiment I conducted in Chapter 6 did yield several other interesting outcomes. I found that in *Lactococcus lactis*, ciprofloxacin

exposure creates selection against the plasmid. I hypothesise that having extra DNA in the form of a plasmid is costly when evolving resistance to a DNA-damaging antibiotic. Further study is required to see if this finding holds up in other bacterial species and with other plasmids, which I would expect based on the hypothesised mechanism. If ciprofloxacin can indeed effectively create selection against plasmids this could be utilised in the fight against antibiotic resistance, since many clinically relevant resistance genes are located on plasmids. If, for example, an infecting bacterial population contains plasmids that provide resistance against beta-lactam antibiotics, a pre-treatment with ciprofloxacin could be used to decrease the abundance of this resistance plasmid. This might then increase the effectiveness of a subsequent treatment with a beta-lactam antibiotic. However, some words of caution are due, depending on the clinical situation such a pre-treatment might not be feasible, for example, if an infection is immediately life-threatening. Furthermore, a pre-treatment might also create selection for plasmid-borne resistance genes to be integrated into the chromosome, especially if these resistance genes also convey some resistance against ciprofloxacin. The chromosomal integration of resistance genes would make resistance genes more persistent and could perhaps even lead to an increase in resistance levels. Thus, many more experiments and trials would be required to see if an anti-plasmid pre-treatment can be a successful strategy to increase the efficacy of antibiotic treatment.

Chapter 7 – Microbial communities

When a patient receives antibiotic treatment, the effects of this treatment will not just be limited to infecting pathogenic bacterial cells. The microbial community inhabiting the patient's gut will also face the antibiotic and will be forced to adapt to the antibiotic or face extinction. Evolutionary rescue theory is a modelling framework that has previously been used to describe the probability that a single bacterial species adapts when it is exposed to an antibiotic. In Chapter 7, I expand this modelling framework to a multispecies context. I model a community-wide evolutionary rescue process, with the aim of understanding how this process changes species abundance

distributions. The model makes several simplifying assumptions, most importantly it assumes that besides differences in their initial abundance, all species in the community are ecologically equivalent. Real bacterial communities obviously have complex ecological interactions, that are ignored in this model. The model in Chapter 7 is meant to serve as a null model. Only if the changes in the species abundance distribution observed in a real-life bacterial community do not match the model predictions, complex ecological interactions need to be invoked to explain the observed patterns. The results in Chapter 7 show that community-wide rescue leads to a rapid loss of rare species. I also show that the mutation rate modulates the degree of rare-species loss: the higher the mutation rate, the fewer rare species are lost. In other words, the lower the evolvability of the community with respect to a certain antibiotic, the larger the number of rare species that will be lost during the rescue process.

The effect of antibiotic treatment on the gut microbiome can cause unwanted side effects, such as antibiotic-associated diarrhoea and an increased risk for gastrointestinal infections. It has been demonstrated that antibiotic treatment can lead to changes in the gut microbiome that can persist for months after treatment has ended (Patangia et al., 2022). Currently, a fully mechanistic and functional understanding of the human gut microbiome is lacking; we are unable to predict the exact effects an antibiotic treatment might have, or when side effects will occur. However, we do know that rare species might be especially important for the functioning of microbial communities since they can ensure that functioning is maintained under a broad set of conditions (Jousset et al. 2017). Therefore, maintaining as much of the rare species in the gut microbiome as possible during antibiotic treatment might allow us to minimise side effects. Here the insights from Chapter 7 come in handy. Sequencing-based methods could be used to quantify the loss of rare species experienced by gut microbial communities during antibiotic treatment. This allows us to assess the evolvability of gut microbial communities with respect to different types of antibiotics. This information about the evolvability of the gut microbiome should be combined with information about the evolvability of specific

pathogens. Compiling this information would allow us to optimise the choice of antibiotic, choosing an antibiotic to which the gut microbiome can adapt, whilst the pathogen cannot. Thus we can effectively eliminate the pathogen, whilst minimising the negative effects of antibiotic treatment on the gut microbiota. There is likely some degree of trade-off here, if the gut microbiome can more easily adapt to an antibiotic it is likely a pathogen is also better able to adapt to said antibiotic. However, even if such a trade-off exists, the choice of antibiotic can still be optimised along this trade-off. It should also be noted that the composition of healthy gut microbiomes differs between individuals, thus when assessing gut microbiome evolvability, one might also need to account for different types of gut microbial communities (also called enterotypes) (Rininella et al., 2019). Some words of caution are due, many labour-intensive clinical experiments would need to be conducted in order to compile the relevant information. Furthermore, the loss of rare species can only be used to assess microbiome evolvability if the model in Chapter 7 is a decent approximation of the microbiome dynamics during antibiotic treatment. This may not be the case given the simplifying assumptions this model makes, such as assuming that all species are ecologically equivalent. However, this assumption of ecological equivalence may be more reasonable when we divide the microbiome into broad functional groups. In this case, within a group of microbes performing the same function, all species could be considered ecologically equivalent. Thus, instead of estimating the evolvability of the entire microbiome, we could expand the model in Chapter 7 to estimate the evolvability of certain functional groups within the microbiome independently. In addition, the changes in the microbiome during antibiotic treatment may not be the outcome of an evolutionary rescue process and could just be due to pre-existing differences in resistance amongst the different species. A good next step would be to see if the model in Chapter 7 can be used to quantitatively reproduce the changes in species abundance patterns observed in the gut microbiome during antibiotic treatment, using already available datasets (Patangia et al., 2022).

Towards a theory of evolvability and its evolution

In this next section, I will briefly outline what I perceive to be the way forward for evolutionary biology. I will first argue why I think a theory of evolvability is essential to advance evolutionary biology. Subsequently, I will explain why I think that such a theory can best be obtained by taking a mechanistic approach.

The goal of evolutionary biology is to explain and predict evolutionary processes. To some extent, current evolutionary theory has been successful at achieving this goal. Consider for example the numerous animal and plant breeding programmes that have successfully used quantitative genetics models to improve yield and disease resistance. However, many evolutionary models make important simplifying assumptions about the processes that generate variation that limit their predictive and explanatory power. For example, most quantitative genetics models make simplifying assumptions regarding the genetic underpinnings of traits, ignoring the complex gene regulatory networks underlying traits. In a similar vein, many standard models simply lack mutational processes altogether. When these processes are considered, mutation rates are often assumed to be constant. These and other simplifying assumptions about the processes that generate variation likely affect the explanatory and predictive power of evolutionary models, especially on longer timescales. In the short term, the response to selection is mostly governed by standing genetic variation. On longer timescales, however, the generation of variation is what ultimately determines evolutionary trajectories! Consider as an example the outcomes of the famous Lenski long-term evolution experiment. This elegant experiment follows the evolution of several bacterial populations originating from a single clone for thousands of generations as they evolve in a very simple constant environment (Sniegowski et al., 1997; 2000; Blount, 2016; Tenallion et al., 2016; Leon et al., 2018). Many of the key findings that have emerged from this experiment, such as the emergence of elevated mutation rates and the evolution of citrate metabolism, can only be adequately explained when the mechanisms underlying the generation of variation are explicitly

considered (Sniegowski et al., 1997; 2000; Blount, 2016; Tenallion et al., 2016; Leon et al., 2018).

By taking into account evolvability, i.e. the capability of biological systems to undergo adaptive evolution, we can expand the explanatory and predictive power of evolutionary models. Studying evolvability shifts focus to the processes that underly the generation of variation. These processes are currently often neglected but may strongly affect the course and outcome of evolution. A good example is provided by Milocco & Salazar-Ciudad (2020), who showed that explicitliy accounting for developmental processes strongly impacted evolutionary predictions. Furthermore, when we consider evolvability it becomes evident, that mutation rates are unlikely to be constant across environments. As I have shown in Chapters 3 and 4 of this thesis, mutation rates vary depending on the conditions. In other words, a solid theory of evolvability has the potential to enhance the explanatory and predictive power of evolutionary models.

Yet, I think the study of evolvability has even more to offer. If one considers that evolvability is the product of underlying mechanisms, such as the structure of gene regulatory networks and mutation rates, it becomes immediately evident that these underlying mechanisms must themselves be shaped by evolution. In some cases the mechanisms enhancing evolvability may evolve simply as a side-effect of selection on other traits, in other cases, evolvability may be the target of selection. For example, there is extensive evidence that in Lenski's long-term evolution experiment, elevated mutation rates evolved due to selection for evolvability (Sniegowski et al., 1997; 2000; Blount, 2016; Tenallion et al., 2016; Leon et al., 2018). I have also observed the evolution of evolvability in this thesis: In the model in Chapter 4 condition-dependent mutation rates evolve due to selection for evolvability. In Chapter 5 I showed that the structure of gene regulatory networks can respond to selection for evolvability, by biasing the phenotypic effects of mutations towards adaptive outcomes. However, currently the evolution of evolvability is still poorly understood, with most insights based on simplified models. Furthermore, the degree to which evolvability mechanisms in

empirical systems are shaped by selection for evolvability remains mostly unknown. Regardless, to truly enhance current evolutionary theory, we need to understand how evolvability evolves.

In summary, I think that evolvability is a key concept to enhance the predictive and explanatory power of evolutionary theory. To obtain long-term predictions about evolution we need to go beyond merely predicting what happens when selection acts on variation. We need to understand the mechanisms that generate and maintain variation. We need to grasp how the gene regulatory networks shape the phenotypic impact of mutations. We need to account for the subtle organismal features that shape the process of selection. In other words, we need a coherent general theory of evolvability and its evolution. It is therefore not surprising that evolvability plays an important role in the discussion regarding a potential Extended Evolutionary Synthesis (EES; Laland et al., 2014; Laland et al., 2015). If such a synthesis were to come about, I think that evolvability would be one of its most central concepts, as it relates to a wide array of EES concepts such as mutational bias, phenotypic plasticity and epigenetic inheritance (Crombach & Hogeweg, 2008; Laland et al., 2015; Cuypers et al., 2017). However, currently the extended evolutionary synthesis best be described as a loose agglomeration of interesting ideas, rather than a coherent synthesis.

So how might the scientific community go about creating a theory of evolvability and its evolution? I by no means have a definitive answer to this question. However, given the current state of the art, I consider it unlikely that a general theory of evolvability and its evolution can easily be created. The problems at hand are too poorly understood and underlying mechanisms are too idiosyncratic.

I have shown several times in this thesis that seemingly minor mechanistic details of biological systems can greatly impact how these systems evolve and function. For example, I showed in Chapter 3 that the exact type of antibiotic can influence the relationship between temperature and the mutation rate towards resistance. Fever temperatures decreased the mutation rate towards ampicillin resistance, but increased the mutation rate

towards ciprofloxacin and rifampicin resistance. In Chapter 4, I considered the assumption of whether mutation rate loci mutate according to a separate fixed rate (not self-referent), or mutate according to their own value (self-referent). This assumption drastically impacted the dynamics and outcome of evolution: when mutation rate loci mutated in a self-referent manner evolution proceeded much faster and a condition-dependent mutation strategy evolved much more frequently. In Chapter 5, I showed that the nature of gene interactions (linear or non-linear) altered the kind of mutational transformer that evolved. In Chapter 6, I expected to show the impact of plasmids on the rate of ciprofloxacin resistance evolution. However, I failed to foresee that due to its particular mechanism of action ciprofloxacin would create selection against plasmids. I believe these results represent a more general truth: when it comes to evolvability, the l devil is hiding in the mechanistic details. The impact of mechanistic details illustrates why with the current state of the art developing a general theory on evolvability is not feasible. Therefore, I think that for now we best resign ourselves to a mechanistic approach. However, I would argue that this resignation is perhaps a blessing in disguise: to some extent, it frees us of the conceptual shackles of previous evolutionary models. I think this freedom is essential since a theory of evolvability and its evolution might represent a significant change from current general evolutionary models. Thus for now we should focus on studying the specific idiosyncratic mechanisms, allowing a great diversity of mechanistic models to flourish, so that eventually we can distil solid general principles from a multitude of mechanistic models. Perhaps, the mechanistic models presented in this thesis could then be viewed as tiny pebbles paving the road towards a theory of evolvability and its evolution.

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Summary

The rise of antibiotic resistance represents a major societal challenge. It has been estimated that currently, at least 700,000 people die annually because of antibiotic resistance. According to some estimates, this number may rise to 10 million by 2050. A better understanding of antibiotic resistance is crucial to combat this looming threat. In essence, the emergence of antibiotic resistance is an example of adaptive evolution: when bacteria are faced with antibiotics this creates a strong selection for resistance. However, luckily for humans, bacterial populations are not always able to adapt and develop resistance. What determines if a bacterial population can evolve resistance? To answer this question, we need to consider "evolvability": the ability of organisms to undergo adaptive evolution. In this thesis, I therefore study the evolvability of bacteria in the context of antibiotic resistance. To this end, I apply a combined experimental and theoretical approach. I strive to gain general insights about evolvability, whilst at the same time applying these insights to better understand the evolution of antibiotic resistance.

In **Chapter 1,** I briefly introduce the concept of evolvability, and I motivate my definition of evolvability. I also explain and motivate the mechanistic approach used throughout this thesis. Subsequently, I briefly introduce three mechanisms underlying the evolvability of bacteria: condition-dependent mutation rates, the architecture of gene-regulatory networks, and horizontal gene transfer. All three mechanisms are studied in this thesis.

In **Chapter 2,** I provide a theoretical framework for understanding evolvability. This framework outlines how evolvability can be understood through its underlying mechanisms. I classify the many mechanisms and organismal features that determine evolvability into three categories: determinants that provide variation, determinants that shape the relationship between genetic variation and phenotypic fitness effects, and determinants that determine how fitness differences lead to evolutionary change. Each category of determinants shapes evolvability in a different way. These differences are often not considered, which has led to miscommunication and confusion in the scientific literature. The proposed

framework also leads to concrete recommendations on how evolvability should be studied. For example, researchers should specify the timescale on which the determinant under scrutiny is supposed to act. Moreover, one should consider the scope of a particular determinant: some determinants impact adaptation in many different environments, whilst others act only in a more restricted set of environments.

Chapter 3 addresses the condition dependence of mutation rates. Using laboratory experiments, I assessed the impact of environmental temperature on the mutation rate toward antibiotic resistance in the bacterium *Escherichia coli*. I show for three different antibiotics that a small temperature change from 37 °C to 40 °C, as would be associated with fever, can alter the mutation rate towards resistance by almost an order of magnitude. For some antibiotics, an increase in temperature led to an elevated mutation rate, whilst for others this led to a decrease in the mutation rate. Regardless, antibiotic resistance is expected to evolve much faster under the temperature at which the mutation rate is the highest. This highlights a potential new way to mitigate the evolution of antibiotic resistance by selectively using fever suppression to minimise the mutation rate towards resistance.

In **Chapter 4,** I use a simulation model to study whether and when one would expect the evolution of condition-dependent mutation rates. It has been hypothesised that an increase in the mutation rate under stressful conditions enhances evolvability since it provides extra variation during times of maladaptation, thus accelerating adaptive evolution. I show that stress-related mutation rates do indeed evolve under a wide array of circumstances. Furthermore, I show that condition-dependent mutation rates enhance the ability of populations to adapt to changing environments and that this evolvability-enhancing effect drives their evolution. These results illustrate that evolvability can itself evolve.

Interlude 1 shows a modelling contribution that was added to an empirical study of fat metabolism in certain parasitoid wasps. This study found empirically that fat metabolism was still maintained within a lineage, even though it had supposedly been lost millions of generations ago. It turns out

that the wasps had maintained the ability to switch on fat metabolism under certain rarely occurring circumstances. The model explains how such a switch can evolve to become robust to mutation, allowing phenotypic plasticity to be maintained even if it is only sporadically exposed to selection.

In **Chapter 5,** I simulate the evolution of a very simple gene-regulatory network in a changing environment. I show that the structure of the gene-regulatory network can easily evolve to bias the phenotypic effect of mutations towards adaptive outcomes, thereby accelerating adaptation in the changing environment. I term the underlying mechanism a mutational transformer: it transforms the phenotypic effects of random genetic mutations in such a way that they promote an adaptive outcome. Depending on model details, two different types of transformers evolved in my simulations. The evolution of mutational transformers shows that evolvability can evolve through the modification of gene-regulatory networks.

Chapter 6 reports on an experiment that investigates the impact of horizontal gene transfer on evolvability in the context of antibiotic resistance. It has been hypothesised that horizontal gene transfer can enhance evolvability, for example by bringing together favourable mutations within a single lineage, which can accelerate adaptation. Using experimental evolution, I examined the impact of horizontally transferable plasmids on the de-novo evolution of ciprofloxacin resistance in *Lactococcus lactis*. During the 31-day (approximately 200 generations) experiment, the bacterial populations evolved high-level resistance. Contrary to expectations, the presence of plasmids did not accelerate the rate of resistance evolution. However, this outcome may be specific for the experimental system considered, as plasmid abundance decreased strongly in populations exposed to ciprofloxacin. Apparently, exposure to ciprofloxacin had created selection against the plasmid. This serendipitous finding may be of clinical relevance. Using next-generation sequencing I also analysed the mutations underlying resistance, revealing several previously unknown genes that may be involved in resistance.

In **Chapter 7,** I modelled how species abundances change when a community of bacteria composed of many different species (for example, a gut microbiome) is exposed to an antibiotic. I used a modelling framework known as evolutionary rescue theory. The question was how the pattern of species abundances in the community changes when each component species is selected to evolve antibiotic resistance. My simulations show that a community-wide rescue process leads to the rapid loss of rare species from the community. This model may help us better understand how bacterial communities change when exposed to antibiotics.

Chapter 8 constitutes an overarching discussion of this thesis. This chapter addresses two main themes. First, I consider on a chapter-by-chapter basis how the insights from this thesis may eventually be applied to improve antibiotic treatment in a clinical context. Second, I outline my vision for the future of evolutionary biology. A coherent theory of evolvability and its evolution is essential to better understand, explain, and predict evolutionary processes. I argue that such a theory can best be obtained by taking a mechanistic approach.

Samenvatting

De toename van antibioticaresistentie vormt een grote maatschappelijke uitdaging. Er wordt geschat dat op dit moment jaarlijks minstens 700.000 mensen sterven als gevolg van antibioticaresistentie. Volgens sommige schattingen kan dit aantal tegen 2050 zijn opgelopen tot 10 miljoen. Een beter begrip van antibioticaresistentie is cruciaal om deze dreiging het hoofd te bieden. In wezen is het ontstaan van antibioticaresistentie een voorbeeld van adaptieve evolutie: wanneer bacteriën worden geconfronteerd met antibiotica leidt dit tot een sterke selectie op resistentie. Gelukkig voor de mens zijn bacteriepopulaties niet altijd in staat om zich aan te passen en resistentie te ontwikkelen. Wat bepaalt of een bacteriepopulatie resistentie kan ontwikkelen? Om deze vraag te beantwoorden moeten we kijken naar "evolueerbaarheid": het vermogen van organismen om adaptieve evolutie te ondergaan. In dit proefschrift bestudeer ik daarom de evolueerbaarheid van bacteriën in de context van antibioticaresistentie. Hiervoor gebruik ik een gecombineerde experimentele en theoretische benadering. Ik streef ernaar algemene inzichten te verwerven over evolueerbaarheid om hierdoor de evolutie van antibioticaresistentie beter te begrijpen.

In **Hoofdstuk 1** introduceer ik het concept van evolueerbaarheid. Ik verklaar en motiveer ook de mechanistische benadering die in dit proefschrift gebruikt wordt. Vervolgens introduceer ik drie mechanismen die ten grondslag liggen aan de evolueerbaarheid van bacteriën: omgevings-afhankelijke mutatiesnelheden, de architectuur van genregulatienetwerken en horizontale genoverdracht. Alle drie de mechanismen worden in dit proefschrift nader bestudeerd.

In **Hoofdstuk 2** geef ik een theoretisch raamwerk voor het begrijpen van evolueerbaarheid. Dit raamwerk schetst hoe evolueerbaarheid begrepen kan worden aan de hand van de onderliggende mechanismen. Ik deel de mechanismen en eigenschappen die evolueerbaarheid bepalen in verschillende categorieën in: determinanten die variatie verschaffen, determinanten die de relatie tussen genetische variatie en fenotypische fitnesseffecten vormgeven, en determinanten die bepalen hoe

fitnessverschillen leiden tot evolutionaire verandering. Elke categorie van determinanten draagt op een andere manier bij aan evolueerbaarheid. De verschillende manieren waarop determinanten evolueerbaarheid bepalen worden vaak niet in beschouwing genomen, wat heeft geleid tot miscommunicatie en verwarring in de wetenschappelijke literatuur. Het door mij geschetste raamwerk leidt tot een aantal concrete aanbevelingen over hoe evolueerbaarheid bestudeerd zou moeten worden. Ten eerste moet men rekening houden met de tijdschaal waarop bepaalde determinanten werken. Daarnaast is ook de reikwijdte van een bepaalde determinant belangrijk: sommige determinanten beïnvloeden adaptatie in veel verschillende omgevingen, terwijl andere slechts in een beperktere reeks omgevingen relevant zijn.

Hoofdstuk 3 behandelt de omgevings-afhankelijkheid van mutatiesnelheden. Met laboratoriumexperimenten heb ik de invloed van omgevingstemperatuur op de mutatiesnelheid naar antibioticaresistentie in de bacterie *Escherichia coli* onderzocht. Ik laat voor drie verschillende antibiotica zien dat een kleine temperatuurverandering van 37 °C naar 40 °C (zoals bij koorts), de mutatiesnelheid naar resistentie met een orde van grootte kan veranderen. Voor sommige antibiotica leidde een verhoging van de temperatuur tot een verhoogde mutatiesnelheid, terwijl dit voor andere antibiotica tot een verlaging van de mutatiesnelheid leidde. Hoe dan ook, antibioticaresistentie zal naar verwachting veel sneller evolueren bij een hogere mutatiesnelheid. Dit wijst op een mogelijke nieuwe manier om de evolutie van antibioticaresistentie te beperken door selectief gebruik te maken van koortsonderdrukking om de mutatiesnelheid naar resistentie te minimaliseren.

In **Hoofdstuk 4** gebruik ik een simulatiemodel om te bestuderen of en wanneer men de evolutie van toestandsafhankelijke mutatiesnelheden zou kunnen verwachten. In de literatuur werd de hypothese geopperd dat een toename in de mutatiesnelheid onder stressvolle omstandigheden de evolueerbaarheid verbetert, omdat dit in tijden van maladaptatie een grotere genetische variatie oplevert, waardoor adaptieve evolutie versneld wordt. Ik laat zien dat stress-gerelateerde mutatiesnelheden inderdaad evolueren onder een breed scala aan omstandigheden. Verder laat ik zien

dat toestandsafhankelijke mutatiesnelheden het vermogen van populaties om zich aan te passen aan veranderende omgevingen vergroten en dat dit effect op evolueerbaarheid verantwoordelijk is voor de evolutie van toestandsafhankelijke mutatiesnelheiden. Deze resultaten illustreren dat evolueerbaarheid zelf kan evolueren.

Intermezzo 1 toont een modelleringbijdrage die werd toegevoegd aan een empirische studie naar vetmetabolisme bij bepaalde soorten parasitaire wespen. Op basis van meerdere studies had men verondersteld dat deze wespen hun vetmetabolisme al miljoenen generaties geleden kwijt waren geraakt. Nu bleek uit nieuwe experimenten dat de wespen het vermogen hadden behouden, maar dat zij hun vetstofwisseling alleen onder bepaalde, zelden voorkomende omstandigheden inschakelen. Het model verklaart hoe zo'n zelden gebruikte schakelaar evolutionair behouden kan blijven omdat deze, via evolutie, robuust wordt voor mutatie. Hierdoor kan deze fenotypische plasticiteit behouden blijven, zelfs als deze slechts sporadisch wordt blootgesteld aan selectie.

In **Hoofdstuk 5** bestudeer ik met behulp van een simulatiemodel de evolutie van een genregulatie netwerk in een omgeving die over de generaties sterk verandert. Ik laat zien dat de structuur van een genregulatie netwerk gemakkelijk kan evolueren om de fenotypische effecten van mutaties te beïnvloeden in de richting van adaptieve uitkomsten, hetgeen adaptatie versnelt. Ik noem het onderliggende mechanisme een mutatietransformator: het transformeert de fenotypische effecten van willekeurige genetische mutaties zodanig dat ze een adaptieve uitkomst bevorderen. Afhankelijk van de details van het model evolueerden er twee verschillende soorten transformatoren in mijn simulaties. De evolutie van mutatietransformatoren laat zien dat evolueerbaarheid kan evolueren door de modificatie van genregulatienetwerken.

In **Hoofdstuk 6** bestudeer ik de invloed van horizontale genoverdracht op evolueerbaarheid in de context van antibioticaresistentie. Er wordt verondersteld dat horizontale genoverdracht de evolueerbaarheid kan vergroten, bijvoorbeeld door het samenbrengen van gunstige mutaties, hetgeen adaptatie kan versnellen. Met behulp van experimentele evolutie

heb ik de invloed onderzocht van een horizontaal overdraagbare plasmide op de de-novo evolutie van ciprofloxacine resistentie in de bacterie *Lactococcus lactis*. Tijdens het experiment van 31 dagen (ongeveer 200 generaties) evolueerden de bacteriële populaties een hoge mate van resistentie. Tegen de verwachting in versnelde de aanwezigheid van plasmiden de snelheid van de resistentie-evolutie niet. Dit resultaat kan echter specifiek zijn voor het experimentele systeem in kwestie, want tijdens het experiment nam het aantal plasmiden sterk af in populaties die blootgesteld werden aan ciprofloxacine. Blijkbaar leidde blootstelling aan ciprofloxacine tot selectie tegen het plasmide. Deze toevallige bevinding kan van klinisch belang zijn. Met behulp van "*next-generation sequencing*" analyseerde ik ook de mutaties die ten grondslag liggen aan resistentie. Hierbij werden verschillende, voorheen onbekende genen gevonden die mogelijk betrokken zijn bij resistentie.

In **Hoofdstuk 7** heb ik gemodelleerd hoe de soortenrijkdom verandert als een gemeenschap van bacteriën, die bestaat uit veel verschillende soorten (bijvoorbeeld een darmmicrobioom), wordt blootgesteld aan een antibioticum. Ik gebruikte een modelleerraamwerk dat bekend staat als evolutionaire reddingstheorie. Ik modelleer hoe elke soort in de gemeenschap onafhankelijk van elkaar antibioticaresistentie ontwikkelt door mutatie en hoe dit proces de patronen van soortenrijkdom verandert. De modelresultaten laten zien dat een dergelijk proces leidt tot een zeer snel verlies van zeldzame soorten uit de gemeenschap. Dit model kan ons helpen beter te begrijpen hoe bacteriegemeenschappen veranderen wanneer ze worden blootgesteld aan antibiotica.

Hoofdstuk 8 vormt een overkoepelende discussie van dit proefschrift. Dit hoofdstuk behandelt twee hoofdthema's. Ten eerste beschouw ik per hoofdstuk hoe de fundamentele inzichten uit dit proefschrift uiteindelijk kunnen worden toegepast om de behandeling met antibiotica in een klinische context te verbeteren. Ten tweede schets ik mijn visie op de toekomst van de evolutiebiologie. Een coherente theorie van evolueerbaarheid en de evolutie daarvan is essentieel om evolutionaire processen beter te begrijpen, te verklaren en te voorspellen. Ik

beargumenteer dat zo'n theorie het beste kan worden verkregen door een mechanistische benadering te kiezen.

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About the author

Timo van Eldijk was born on the 24th of July 1995 in Utrecht, The Netherlands. Timo completed his high school education at the Anna van Rijn College in Nieuwegein. Here he became fascinated by evolution, as evidenced by a rather extensive fossil collection accrued during this time. He made his first steps into the academic world in Utrecht in the year 2011, by joining the Junior College Utrecht. In 2013 Timo started his bachelor's studies in biology at Utrecht University, which included a brief stay at the University of Bonn to study the scales of ancient butterflies. Timo received his bachelor's degree in 2016 with the highest distinction. Subsequently, he enrolled in the Erasmus Mundus Master's Programme in Evolutionary Biology (MEME). It was at this time that he became particularly interested in the evolution of antibiotic resistance. During his master's studies, Timo studied at the University of Uppsala, the University of Montpellier, and the University of Groningen. In 2018, he graduated with the highest distinction. From 2019 to 2023, Timo conducted his PhD research at the University of Groningen, resulting in this PhD thesis. In March 2024, Timo will start as a postdoctoral researcher at the University of Oldenburg, where he will continue his research into the evolution of antibiotic resistance.

Publication list

Klompmaker*, A. A., **Van Eldijk***, **T. J. B.**, Winkelhorst, H., & Reumer, J. W. F. (2023). A non-marine horseshoe crab from the Middle Triassic (Anisian) of The Netherlands. *Netherlands Journal of Geosciences*, *102*, e1. *(* joint first authorship)*

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