UNIVERSIDADE DE LISBOA
FACULDADE DE CIÊNCIAS
DEPARTAMENTO DE BIOLOGIA ANIMAL

How does Chromosomal Instability affect the tempo and mode of adaptation?

Mestrado em Biologia Evolutiva e do Desenvolvimento

Ana Catarina Silvestre Morais

Dissertação orientada por:
Prof.ª Doutora Margarida Matos e Doutora Lília Perfeito

2015

ACKNOWLEDGEMENTS

I am deeply grateful to everyone that supported me throughout the course of this year.

To Lília, for teaching me how to think evolutionary speaking and how to clarify the questions I want to study.

To everyone in the EGS lab for all the creative and fruitful discussions about the experiments and results.

To Élio for introducing me to this masters that completely shaped my biological reasoning.

To everyone in the Vasco da Gama wing for always helping with inspiration. Especially to Ana and Andreia for also being great friends.

To my dear friends and family. I could never thank you enough for all the love and support you always gave me. To my mom and dad, my aunt and my grandmother. To Cristina, Susana and Cláudia. To Sérgio.

And to everyone who has gone too soon.

RESUMO

A instabilidade genómica ao nível da estrutura cromossómica (instabilidade cromossómica) tem um papel importante na progressão do cancro. Vários estudos sugerem também que a instabilidade cromossómica pode proporcionar saltos gigantescos na paisagem adaptativa durante a adaptação a um novo ambiente. Isto porque o número de passos mutacionais necessários para que daí possam resultar mudanças significativas no fenótipo é menor do que em mutadores ao nível de substituições nucleotídicas simples.

A instabilidade cromossómica gera alterações estruturais nos cromossomas (rearranjos cromossómicos) que podem afetar não só as sequências nucleotídicas de genes específicos como também interferir com a ordem de elementos regulatórios no genoma e, consequentemente, alterar padrões de expressão génica.

Contudo, há uma considerável escassez na literatura de estudos sobre as dinâmicas evolutivas de mutadores da estrutura cromossómica (organismos com elevada instabilidade cromossómica), que em última análise são necessários para uma melhor compreensão do quanto e em que circunstâncias a elevada evolvabilidade, que é intrínseca destes mutadores, poderá ser benéfica ou deletéria a curto e a longo prazo.

Há hipóteses que sugerem que em ambientes constantes, não stressantes, a instabilidade cromossómica paga um custo devido à acumulação de mutações deletérias. No entanto, durante a adaptação a novos ambientes ou em ambientes alternados, estes mutadores da estrutura cromossómica podem contribuir com a variabilidade genética necessária para a adaptação da população. Os efeitos positivos que alguns rearranjos cromossómicos representam para o fitness podem exceder os efeitos pejorativos dos restantes rearranjos resultantes deste tipo de instabilidade.

O objetivo deste trabalho é o estudo dos "trade-offs" evolutivos que condicionam a evolução da instabilidade cromossómica. Através de evolução experimental estudámos a adaptação a curto prazo de estirpes instáveis de *Schizosaccharomyces pombe* em duas condições ambientais: um ambiente constante, não stressante, e um ambiente alternado. Nomeadamente, queríamos determinar se as estirpes instáveis têm uma vantagem adaptativa num contexto de ambiente alternado, em que há mais espaço para adaptação, e se, por outro lado, o balanço entre as mutações deletérias e as mutações benéficas que possam surgir pode constituir um custo para estas estirpes num ambiente constante, em que há menos espaço para adaptação.

O fenótipo de instabilidade característico das estirpes que usámos é consequência de um conjunto de mutações construídas no laboratório que afetam o encapsulamento dos telómeros bem como mecanismos de reparação de DNA e que, em conjunto, originam padrões de rearranjos cromossómicos brutos como translocações, inversões, deleções, amplificações e aneuploidia.

Para responder às questões a que nos propusémos, realizámos ensaios de competição entre as estirpes instáveis e um clone de referência das estirpes controlo, bem como entre as estirpes controlo e um clone referência das estirpes instáveis. As estirpes controlo foram evoluídas em conjunto com as instáveis durante a evolução experimental e estão marcadas com mCherry, uma proteína flourescente, o que permite que as suas frequências sejam seguidas por um equipamento especializado ao longo de alguns ciclos de crescimento e diferenciadas das frequências das estirpes instáveis, que não têm fluorescência. Estes ensaios permitiram-nos determinar o fitness relativo das duas estirpes como função da alteração das suas frequências relativas ao longo das competições. Desta forma, estimámos o fitness das estirpes no início da evolução experimental e depois da adaptação a curto prazo nas duas condições ambientais (ambiente constante e alternado).

Também realizámos experiências para testar se o tamanho populacional das duas estirpes sofreu alterações ao longo da evolução experimental, já que alterações neste parâmetro podem afetar grandemente a eficiência da seleção natural em oposição à ação da deriva genética.

O efeito fundador também pode afetar a ação da seleção natural. No início da evolução experimental usámos diferentes clones de cada estirpe para fundar os replicados populacionais e testámos se haviam diferenças no fitness inicial. Procurámos também perceber se mutações que possam ter surgido nos diferentes clones das estirpes instáveis imediatamente após a criação do seu fenótipo de instabilidade condicionaram a sua evolução. Para isso testámos o efeito do clone no fitness final de cada estirpe para as duas condições ambientais. Fizémos também uma estimativa do papel relativo da seleção, história e deriva na distribuição dos valores finais de fitness de cada estirpe.

Os dados de fitness permitiram-nos responder a várias perguntas específicas: 1) as estirpes adaptaram-se às condições ambientais a que foram expostas (há diferenças entre o fitness inicial e o fitness após a evolução experimental?); 2) qual foi a dinâmica evolutiva das estirpes (houve convergência para os mesmos valores de fitness ou divergência entre os replicados populacionais de cada estirpe?); 3) há diferenças entre as

dinâmicas evolutivas das duas estirpes?; 4) há diferenças entre as dinâmicas evolutivas de cada estirpe na adaptação aos dois tipos de ambiente?.

Concluímos que, após a adaptação a curto prazo em ambiente constante, o fitness das estirpes instáveis aumentou e a adaptação foi caracterizada por uma divergência fenotípica reveladora de uma paisagem adaptativa complexa, composta por vários picos locais. Estas observações suportam a hipótese de que a instabilidade cromossómica pode permitir uma exploração mais abrangente da paisagem adaptativa, aumentando as probabilidades de se atingirem picos mais elevados.

As estirpes controlo, no entanto, sofreram um decréscimo do fitness durante a adaptação em ambiente constante. Não detectámos nenhum padrão de divergência ou convergência entre os replicados populacionais destas estirpes o que, aliado à observação de que o clone teve um efeito significativo nas suas trajectórias evolutivas, constitui evidência para a incapacidade de grandes populações com taxas moderadas de mutação explorarem paisagens adaptativas muito complexas. Assim, os genótipos fundadores dos replicados populacionais destas estirpes terão determinado a paisagem adaptativa passível de ser explorada no início da experiência de evolução tendo em conta as suas limitações em termos de taxa de mutação.

O efeito do clone nos valores de fitness das estirpes controlo foi evidente mesmo no fim da experiência de adaptação. Embora a história não tenha tido um efeito considerável nas distribuições de fitness das estirpes evoluídas em comparação com o efeito da seleção natural, há evidências de que a variabilidade genotípica entre as populações no início da experiência de evolução terá limitado as diferentes populações a vales distintos na paisagem adaptativa.

A adaptação de ambas as estirpes em ambiente alternado foi caracterizada por um "trade-off" na capacidade de exploração de fontes de carbono alternativas.

Em suma, os nossos resultados indicam que as dinâmicas evolutivas que condicionam a adaptação a curto prazo de mutadores da estrutura cromossómica são altamente complexas e dependentes das condições ambientais.

Palavras-chave: instabilidade cromossómica; adaptação; taxa de mutação; evolução da instabilidade cromossómica; evolução experimental; dinâmicas adaptativas.

ABSTRACT

Genomic instability at the level of chromosomal structure (chromosomal instability) plays an important role in cancer progression. It has been suggested by several studies that chromosomal instability could facilitate large leaps in the fitness landscape during adaptation to a novel environment with less mutational steps than single-nucleotide substitution mutators. This is because chromosomal instability generates structural changes in chromosomes (chromosome rearrangements) with the ability to not only affect genetic sequences of particular genes but even interfere with the order of regulatory elements, thus changing gene expression patterns.

Studies addressing the evolutionary dynamics of mutators for chromosomal structure are currently lacking in the literature and are utterly necessary to understand the extent to which the higher intrinsic evolvability of these mutators could be beneficial or deleterious.

It has been hypothesized that in a constant, non-stressful environment, chromosomal instability is expected to pay a cost due to the accumulation of deleterious mutations. However, during adaptation to a novel environment or under fluctuating environments, mutators of chromosomal structure can provide the genetic variation necessary for the population to adapt and the positive fitness effects contributed by individual rearrangements may exceed the detrimental effects.

The aim of this work was to ascertain the evolutionary trade-offs that drive evolution of chromosomal instability. Through an experimental evolution approach, we studied the short-term adaptation of *Schizosaccharomyces pombe* unstable strains to two environmental conditions: a constant non-stressful environment and a fluctuating environment.

The instability phenotype of the strains used in this work is given by a set of mutations in the capping of telomeres and in DNA repair mechanisms that ultimately lead to patterns of gross chromosome rearrangements, including translocations, inversions, deletions, amplifications and aneuploidy.

After short-term adaptation to a constant environment, the fitness of mutator strains increased and adaptation was accompanied by phenotypic divergence, revealing a complex fitness landscape with many local peaks. Furthermore, adaptation under a fluctuating environment revealed a trade-off in the ability to exploit alternative carbon sources.

Altogether, our results indicate that the short-term evolutionary dynamics of mutators for chromosomal structure is highly complex and dependent on the environmental conditions.

Keywords: chromosomal instability; adaptation; mutation rate; evolution of chromosomal instability; experimental evolution; dynamics of adaptation.

Table of Contents

INTRODUCTION

Change is the keystone of evolution. Genomes are dynamic entities that can change as a response to alterations in their immediate environment, and have been repeatedly altered and rearranged since the very beginning of life on the planet [1; 2].

In response to an environmental stress, organisms evolve corresponding adaptive functions that improve their chances of survival and reproduction [3]. These adaptations are achieved through the successive accumulation of beneficial mutations promoted by the action of natural selection [4].

For asexual organisms for which recombination is not an option, mutations are the "raw material" for evolutionary adaptation. It would seem that producing as many mutations as possible would prove to be a good strategy for microbial evolution, especially if we consider the ever-changing, never-constant environments that populations are exposed to in the wild. However, advantageous mutations are rare [5] while many more have deleterious effects [6] and higher mutation rates can bear the cost of more deleterious mutations.

Genetic and genomic bases of adaptation

Adaptation can be achieved, at the nucleotide level, by small-scale nucleotide changes like base insertions, deletions or substitutions [3; 7]. Conversely, adaptive variation can arise from organizational changes to the genome itself through large-scale processes that act at the multi-locus level, known as chromosome rearrangements (CRs) [8; 9]. CRs encompass a variety of events like chromosome duplications and deletions, translocations and inversions, which can be caused by breakage of DNA molecules at two different locations, followed by a rejoining of the broken ends to produce a new chromosomal arrangement of genes [10].

Evolution of high mutation rates

Although the spontaneous mutation rate is generally low [11], higher mutation rates can appear in the so-called mutators through the disruption of mechanisms of DNA replication and/or repair [12], or through mechanisms that disrupt chromosome

segregation. Mutators can be categorized by their strength (the effect on mutation rate) and mutational spectrum (from single-nucleotide to large chromosomal structural variations) [13].

Eukaryotic cells have evolved mechanisms to assure an accurate transmission of chromosomes during mitotic proliferation [14; 15]. Nevertheless, there are many reported examples of instability at the level of chromosome structure in natural isolates at higher frequencies than expected [16; 17; 18; 19]. Laboratory evolution experiments also reported that mutator genotypes were selected in populations undergoing adaptation. In these conditions, they benefit from a higher rate of acquisition of beneficial mutations. [20; 21; 11]. This occurs in asexual populations because the mutator allele remains physically linked, and hence hitchhikes to fixation, with the beneficial mutations it produces [20; 22; 23].

There is clearly a trade-off between the costs of elevated mutation rates in terms of higher genetic load (accumulation of lethal and deleterious mutations resulting in decrease in the rate of population growth) and the potential benefits of increased evolvability once mutators become sufficiently common in a population to be an important contributor to the supply of beneficial mutations [24].

To better understand the interplay between costs and benefits of mutators in microbial adaptation, we need to take into account two main parameters: the population size and the fitness landscape [25]. Large changes in these parameters are expected to greatly affect the adaptive process.

The effect of population size

In small populations of mutators and their nonmutator counterparts, genetic drift can lead to the irreversible accumulation of deleterious mutations (Muller's ratchet – 26; 27), a risk that can be considerably higher for mutators [22]. This cost is negligible in large, maladapted populations or in populations exposed to strong stress. In adaptive conditions such as these, mutators are likely to become fixed in the population [21]. However, adaptation may be limited by the population supply of beneficial mutations: in large or maladapted populations, the supply rate of beneficial mutations is very high and clonal interference may reduce the mutator's benefit of faster adaptation when only one

favorable mutation can become fixed at a time [28; 22]. If, instead, the population is at equilibrium, mutator genotypes are expected to be rare [25].

The effect of the fitness landscape

As mentioned above, the second major factor to consider in the fate of mutators is the fitness landscape. This landscape is simply the relationship between the genotype and fitness. Negative epistatic gene interactions in particular should be considered in the evolutionary dynamics of mutators: a gene favorable in one given genomic context is likely to be unfavorable in other genomic contexts [29]. Specifically, reciprocal sign epistasis (RSE), where there is an intermediate low-fitness genotype between two genotypes, can constrain mutational trajectories and create rugged fitness landscapes with local peaks and valleys [30; 31; 32]. Under RSE, the valleys adjacent to local fitness peaks create barriers to adaptive evolutionary change [33]. The likelihood of a population traversing these valleys depends on the ease with which the population can explore that ridge, subject to its size and mutation rate [33].

The fitness landscape can be altered in different environments. A constant environment, for example, might require different adaptations than a rapidly changing environment, defining new adaptive peaks.

Previous studies have shown that in a constant environment, a minimal mutation rate should be selected since selection will pull large haploid populations towards local fitness optima and mutation should bring populations away from that optima [34; 35; 36; 37].

Conversely, in fluctuating environments, mutation should be advantageous if the genetic variation necessary for a population to adapt to the changing environment is provided [36]. Under temporal and spatial heterogeneity, natural selection favors mechanisms that create and maintain genotypic diversity, thus increasing the long-term adaptability of the population [38; 39].

If environmental conditions change frequently, mutators should rapidly specialize in exploiting the present resources at the expense of other functions that might be needed not in the present but in future environments, a process referred to as “antagonistic pleiotropy” [40; 41; 42]. Thus, mutator populations evolving in a changing environment may suffer from a reduced niche breadth [22].

Short-term fate: lower replication rate and flatter fitness peaks

Studies with digital mutator organisms in direct competition with their low-mutation rate equivalents have demonstrated that, if both populations have a high mutation rate during competition, the mutator organisms win because they adapt to lower but flatter fitness peaks in the adaptive landscape. They become more robust to the effects of deleterious mutations at the expense of a lower replication rate [43]. This suggests that mutator organisms would not only rapidly adapt to the environment but also to the very effects of their increased mutation rate. However, the lower replication rates would make them inferior competitors in direct competition with organisms that evolved with low mutation rates. It remains to be tested if these results can also be observed in real microbes and what would happen in direct competitions where mutation rates are the same as during adaptation for both populations (mutators and nonmutators).

Mutators of Chromosome Structure

While most studies regarding the evolutionary dynamics of mutators have focused on mutators at the level of point mutations, caused by the loss of mismatch repair activity, there is a considerable lack of literature regarding the dynamics of structural mutators that manifest Chromosomal Instability (CIN). CIN is caused by breakdowns in chromosomal segregation mechanisms during cell division or by the fusion of parts of chromosomes to one another [44; 9].

CIN is the most prevalent form of inherent genomic instability and often results in gross chromosomal rearrangements (GCRs) including translocations, inversions, deletions, amplifications and aneuploidy. These have the potential to alter not only genetic sequences of particular genes but also interfere with the order of regulatory elements, therefore changing gene expression patterns in ways not possible through point mutations [45; 46].

GCRs are frequently associated with cancer progression and have been observed in various types of tumours. The majority of solid tumor cells exhibit moderate to drastically elevated CIN [47]. Loeb (2001) [48] first introduced the idea that cancer progression is accelerated by an enhanced mutation rate and later in 2002 Nowak et al.

[49] concluded that it is very likely that CIN mutations contribute to the first phenotypic change in cancer pathways [50]. Despite the association with cancer, extreme mutation rates and genomic instability are thought to be deleterious under constant environments. That is because most of the mutations generated will be highly deleterious [51].

Previous work has shown that GCRs occur at a high frequency in natural yeast populations and in cells adapting to various selective conditions during laboratory short-term evolution experiments [52; 53; 54; 55]. Although most of the rearrangements are probably deleterious, it is possible to detect the appearance of single GCRs after only a few generations of adaptation to a new environment [56; 57]. Altogether these studies indicate a role for GCRs in generating phenotypic variation that could facilitate yeast adaptation to different environments.

It has also been suggested that evolution via karyotypic changes is a more effective way to make large leaps in the fitness landscape within a smaller number of mutational steps, thus conferring the system with a higher intrinsic evolvability in comparison to evolution by point mutations [58]. A matter of discussion is the extent to which this high evolvability could be beneficial or detrimental to cells. One hypothesis is that it depends on the strength of the selective condition: in a non-stressful environment the fitness contributions from all the GCR's variants arising in genomic unstable strains may lead to a slight to highly detrimental effect. However, as the environment becomes more stressful, the positive fitness effects contributed by individual GCRs in a population may start to exceed the detrimental effects, leading to a net selective advantage for instability.

In addition, several studies of short-term yeast adaptation to a strong selective pressure reported the appearance of repeated, independent chromosomal rearrangements throughout the experiments [52]. This suggests that there might be hotspots for genome rearrangements, where rearrangements would be more likely to be beneficial. Theoretical and experimental studies of the evolutionary dynamics of chromosomal unstable strains to a constant *vs* changing selective pressure would be very important to understand the trade-offs between the costs and benefits of these specific mutators.

The aim of this thesis is to study the evolutionary trade-offs that drive the evolution of chromosomal instability. We performed experimental evolution of *Schizosaccharomyces pombe* strains with high levels of chromosomal instability to study the short-term adaptation to two environmental conditions: a constant non-stressful

environment and a fluctuating environment. With this system we addressed two questions: 1. Is there a cost of chromosomal instability in a constant environment where there is little room for adaptation? 2. Is there an advantage of chromosomal instability in a changing environment, where a lot of adaptation is expected?

The instability phenotype of the strains used in this work (HIN strains) is given by a set of mutations in the capping of telomeres and in DNA repair mechanisms that ultimately lead to increased mutation rates throughout the genome with patterns of gross chromosome rearrangements.

We controlled for high population sizes and therefore expect that the mutator strains have a short-term advantage in both environmental conditions since the higher supply of beneficial mutations should be favorable in the initial stages of adaptation. However, clonal interference can be very common in large populations and in populations with high mutation rates [59], so we predict that this will hamper the adaptation rate of our mutator strains comparatively to the nonmutator populations.

Chance events and previous history may also hamper the effect of natural selection and condition evolution in a novel environment. In our study we took into account the relative role of chance, history and selection by comparing the short-term evolution of populations founded by the same clone and populations founded by different clones of the same strain.

MATERIALS AND METHODS

Strains and media

The strains used in this study are listed in the Supplementary Information, Table1. We used strains with different levels of genomic instability.

High Instability (HIN) strains were constructed by Hentges et al. (2014) [60] and have a characteristic genomic instability phenotype given by a set of three mutations: *ctp1d taz1d xlf1.AA*. Because of these three mutations they accumulate damage at the telomeres, repress homologous recombination (HR) and hyper-activate non-homologous end joining (NHEJ). Altogether, this will allow for the generation of multiple chromosome end-fusions that will lead to breakage-fusion-bridge cycles and

subsequently to patterns of gross chromosomal rearrangements like copy number increases of chromosomal segments and fold-back inversions [61; 62].

We constructed a stable counterpart for the HIN strains (ContHIN strains) using one stable parental of HIN and a wild-type strain, as described in the Supplementary Information (1. Construction of Control strains for the High INstability strains).

During the all the experiments carried out for this work, we used standard media and growth conditions [63]. Cultures were always grown until early stationary phase: for 1-2 days at 32ºC at 180 rpm when in liquid medium and for 2-3 days at 32ºC without shaking when in solid plates.

We used three types of medium: rich yeast extract-based medium (YES), glutamate-based medium (PGM) and ammonium-based medium (EMM). YES is a rich, non-selective medium composed of yeast extract, glucose and supplements required for the most common auxotrophies in fission yeast (adenine, histidine, leucine, lysine and uracil). PMG and EMM are minimal, defined media that differ in the source of nitrogen (glutamic acid and ammonia, respectively) but are both composed of potassium hydrogen phthalate, sodium phosphate, glucose and a mix of salts, vitamins and minerals. Solid plates were made by adding 20g/L of agar to any of the above media.

When growing cultures in minimal media we always added the amino acids for which the assay strain is auxotrophic (if any) to the medium. Whenever we needed to test or select for specific auxotrophies, we prepared minimal media with and without the appropriate supplements to which we replica plated single colonies of a given strain and checked for growth under both conditions.

Minimal medium without any supplements nor sources of carbon and nitrogen was also used to dilute cultures between passages of the evolution experiment and the competition assays.

We also used malt extract (ME) medium, a specific medium for nutrient starvation required for conjugation and sporulation [63]. ME is only composed of Bacto-malt extract and the same supplements as YES (adenine, histidine, leucine, lysine and uracil).

For the evolution experiment and all related experiments with the same population replicates we grew cells in liquid culture in VWR 96-well deep well plates (96 deep-well plates) filled with 500uL of medium per well. We also used Corning Incorporated COSTAR 96 Well Cell Culture plates (small 96-well plates) to dilute cultures between

passages and for Fluorescence-Activated Cell Sorting (FACS) analysis during the competition assays.

For long term storage, cells were grown overnight in 5 mL of YES, re-suspended in 1,5 mL of Freezing Medium, a 50:50 mixture of YES and Glicerol (50%), and kept at -80ºC in 2mL cryotubes. The population replicates used in the evolution experiment were frozen in the same way, but were re-suspended in 150uL of Freezing Medium and were transferred to small 96-well plates in the exact same order as they were originally distributed.

Estimation of population sizes

A two-level design was used to evaluate differences in cellular density at early stationary phase between: HIN and ContHIN strains; different media used in the evolution experiment.

Strains were revived from the -80ºC stocks and streaked in YES plates to isolate several colonies. We took 4 isolated colonies of each HIN and ContHIN clones to inoculate 500uL of each of the four minimal media (EMMG, EMMM, PMGG, PMGM) according to the schemes in SI, Table2. After 48h of growth in standard liquid conditions, we transferred 20uL of each culture to 500uL of fresh medium (either EMMG, EMMM, PMGG or PMGM, according to the same plate scheme) (25x dilution). ContHIN strains were previously diluted in 200uL of PMG before passaging to fresh medium (250x dilution). We plated 100uL of each culture in YES plates before dilution and after the following growth cycle, as demonstrated in Figure1.We did three replicates of this assay.

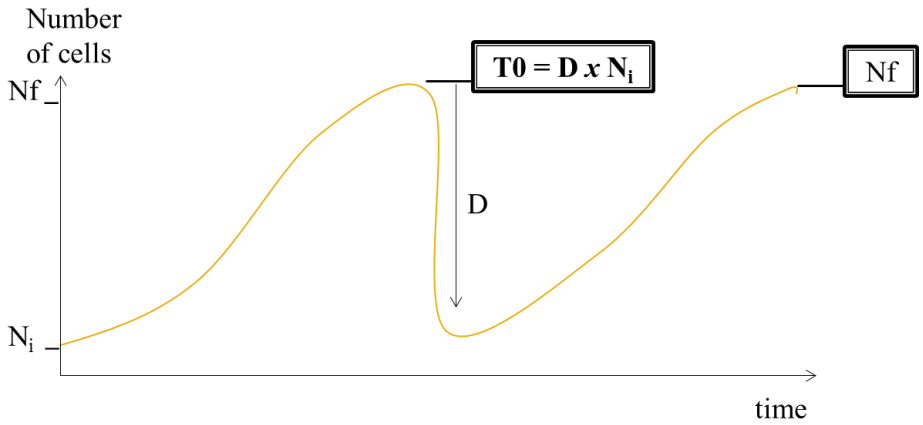


Figure 1: Experimental setup for the estimation of population size after one growth cycle in EMMG, EMMM, PMGG and PMGM (T0) and after dilution (D) and subsequent growth in fresh medium (*Nf*). Cells were always diluted to the same medium.

By counting the number of viable cells (CFUs) in the plates we could estimate the cellular density of cultures in 500uL of each medium before passaging (T0) and at the end of the following 48h-cycle (*Nf*). Assuming exponential growth, we can estimate the number of generations during a single growth cycle:

Equation 1 $$g = log_2(\frac{Nf}{Ni})$$

where and N_i=T0/dilution factor.

In a sequence of serial dilutions, N_i=*Nf* /dilution factor, where *Nf* is the population size achieved in the previous growth cycle. Assuming a constant *Nf* throughout an evolution experiment under serial dilutions, if we apply different dilutions to HIN and ContHIN and thus keep *Ni* constant for each strain, this will affect the number of generations between passages.

We estimated the total number of generations that HIN and ContHIN strains should have at the end of the evolutionary experiments in a constant and in a fluctuating environment under two premises: in a constant environment, cultures are always passaged to the same medium and N_i=N_i /dilution factor and $g = log_2(dilution\ factor)$**;** in a fluctuating environment, the medium between passages is different and we need to estimate *g* for every passage. Therefore, for the constant environment setup, we fixed the number of generations by fixing the dilution factor, and end up with T *x g* generations, where T is the total number of passages. For the fluctuating environment setup, we estimated *g* for every growth cycle based on the sequence of media used during the

evolution assay and taking into account the population sizes each strain achieved after one growth cycle in each medium (see Results and Discussion: Estimation of population sizes): $g = log_2\left(\frac{Nf(in\ the\ current\ medium)}{\frac{Nf\ (in\ the\ previous\ medium)}{dilution\ factor}}\right)$, and ended up with $\sum_0^T g$ generations at the end of the evolution experiment.

At the end of the experimental evolution assay, we plated all HIN and ContHIN population replicates from the two environmental conditions in YES plates to determine the CFUs in each culture and measure the population size of evolved strains.

Evolution Experiment

We propagated each strain (HIN and ContHIN) in two environmental conditions: a constant environment (EMMG) and a fluctuating environment characterized by different combinations of carbon and nitrogen sources. We used EMM and PMG media, which differ in the nitrogen source, and added Glucose or Maltose as alternative carbon sources for each media. In total, we worked with four media: EMM with Maltose (EMMM), EMM with Glucose (EMMG), PMG with Maltose (PMGM), and PMG with Glucose (PMMG). Leucine and Uracil were always added to the medium since HIN and ContHIN strains are auxotrophic for these amino-acids.

Strains were retrieved from the freezer (-80ºC) and streaked in YES plates in order to allow for the isolation of several single colonies. We then distributed the isolated colonies over two 96 deep-well plates with EMMG, one plate for each environmental condition. Each well is considered a population replicate and was founded by a single colony, ensuring that there was no genetic polymorphism within founding populations.

In order to study the effect of history, we used 5 different HIN clones derived from two independent crosses: HIN1 and HIN2, and 5 ContHIN clones derived from the same cross. We propagated 20 replicates of HIN1, 5 to 6 replicates of each of the four HIN2 clones, 20 replicates of one ContHIN clone and 5 to 6 replicates of each of the remaining four ContHIN clones (See SI-Table3).

Strains were spread throughout 24 passages by transferring 20uL of culture (in stationary phase) into 500uL of fresh medium (25x dilution) every other two days. To control for same effective population size, ContHIN cultures were diluted 10x in 200uL

of PMG prior to the transfer to 500uL of fresh medium (250x dilution) (See Results and Discussion: Experimental Evolution).

For the fluctuating environment condition, we created a random cycle of the 4 alternative media (SI-Table4) followed every passage. This way we avoided adaptation to a specific cycle of alternate media.

Samples were stored after 12 and 24 passages (or time-points T12 and T24) at –80ºC and later revived for the competition assays. We also froze the population replicates as they were at the onset of the evolution experiment (T0) for this purpose.

Competitive fitness assays

In order to ascertain the fitness of evolved and ancestral strains (T0 and T24) we performed competitive fitness assays and followed the frequencies of the fluorescently-labelled strain (either the competitor or the strain of interest, depending on the competition) through FACS analysis in a LSR Fortessa equipment.

As the ContHIN strains are marked with mCherry and the HIN strains do not possess any type of fluorescence, we competed the HIN strains from the two evolutionary time-points against a common ContHIN (not evolved) clone (ContHIN ancestor) that was kept at -80ºC for this purpose. We then followed ContHIN (and indirectly, HIN strains') frequencies throughout four passages to ascertain the relative fitness of HIN strains (See SI 1. Fitness Estimates).

ContHIN strains were competed against a HIN ancestral clone (HIN reference), and their fitness was estimated relative to the relative fitness of HIN reference when competed against the ContHIN ancestor.

Strains from each evolutionary time-point were thawed in YES plates, transferred to 96 deep-well plates filled with EMMG, retransferred to fresh EMMG-filled deep-well plates after two days of incubation and left incubating in the same conditions for another two days.

Afterwards, the assay strain was mixed with the competitor and diluted 10-fold onto a small 96-well plate filled with PMG. From this, 20 μL were added to fresh medium (EMMG) in a 96 deep-well plate, which was then incubated at 32ºC with agitation for 48 hours. The remainder of the PMG plate was incubated for at least 2 hours at room temperature and then analyzed by FACS.

When competing population replicates from the constant environment evolutionary setup, we repeated this procedure four times over eight days per competition replicate. When competing population replicates from the fluctuating environment evolutionary setup, we followed a cycle of alternate media that was maintained in all competitions of this kind: EMMG-EMMM-PMGG-PMGM-EMMG. Each competition was replicated three times.

Statistical Analysis

In the competitions under a constant environment, we noticed that the slope of HIN frequencies started to change in signal after the first three timepoints. This is a strong indicative that beneficial mutations appeared during the competitions and could bias our fitness estimates. For this reason, we took only the first three points in the competitions in this environmental condition to estimate fitness.

For each evolutionary timepoint and environmental setup, we calculated the mean fitness between the three competition assays for each population replicate and used this as our unit value for the statistical analysis.

We also estimated fitness trajectories of HIN and ContHIN strains as the difference between mean fitness after adaptation and mean fitness at T0.

We used the Shapiro-Wilk normality test to check if the fitness values of HIN and ContHIN strains at T0 and after adaptation to the two environmental conditions were normally distributed. This test showed a significant deviation from normality for all our datasets and non-parametric tests were used in the following statistical analysis.

We employed Wilcoxon rank sum tests (Wilcoxon) to test the equality of medians between mean fitness distributions between: 1) ancestral and evolved strains; 2) HIN and ContHIN strains at the two assayed evolutionary timepoints; 3) HIN and ContHIN fitness trajectories. We also used the Kolmogorov-Smirnov (KS) test for comparisons between the distributions of mean fitness in the same conditions and tested the homogeneity of variances using the Fligner-Killeen test.

We used the same tests to compare the medians and distributions of population sizes between evolved strains and their ancestors when subject to one growth cycle in each medium.

We tested the effect of clone and cross in the population sizes of HIN and ContHIN strains, as well as in the fitness distributions of these strains, using a One-Way Anova. We used the same test to detect an effect of the medium in the population sizes of the each strain.

RESULTS AND DISCUSSION

Estimation of population sizes

In experiments where populations are serially cultured, the effective population size (*Ne*) depends strongly on the transfer size (N_i) [64]. Specifically, $Ne = N_i \times g$, where g is the time in generations elapsed between passages. The population sampling error associated with each passage follows a Poisson distribution. The bigger this error, the stronger genetic drift will be and natural selection will be less effective. Hence, a higher sampling error at each passage means less beneficial mutations will be represented in the next growth cycle. For the evolution experiment, we wanted to maintain large population sizes to ensure a sufficient supply of mutations and a realistic sampling of the population between passages. From previous experiences in the lab, 10 000 cells is considered as a good threshold for sampling. This represents a standard deviation of 100 individuals (1%).

Since we were working with mutant strains and using different media, we had to test whether we could use the same demographic conditions. To test the growth of the genetically unstable (HIN) and their wild type control (ContHIN) strains we used a two-level design described in the Materials and Methods section to estimate the effect of strain and media in population size. Briefly, strains were grown in each of the media to be used in the experimental evolution. These included minimal media with one of two sources of nitrogen and one of two sources of carbon (in total four different conditions). We then measured the total number of viable cells in the culture (CFUs – colony forming units) after one round of growth.

An analysis of variance showed that the effect of media in the maximum population size of HIN and ContHIN was not statistically significant ($F(3,72)= 0.475$, p-

value=0.7). However, the strain had a significant effect (F(1,72)=18.242, p-value=5.86e-05), with ContHIN having a significantly higher population size in all media than HIN strains (Wilcoxon: W = 1269, p-value = 2.847e-06) (Figure2). We also analyzed the effect of the different carbon and nitrogen sources separately, but did not find any significant effect of either nor of their interaction in the population size (Carbon: F(1,72)= 0.384, p-value=0.538; Nitrogen: F(1,72)=0.852, p-value=0.359; Carbon*Nitrogen: F(1,72)=0.191, p-value=0.663).

Also, the effect of the different HIN and ContHIN clones in population sizes of each strain was not significant (HIN: F(1,32)=0.734, p-value=0.579; ContHIN: F(1,32)=1.870, p-value=0.181), neither was the effect of the cross in the population sizes of HIN strains (F(1,32)=0.258, p-value=0.615).

This indicates that the final population size is equal within each genotype, but different between HIN and ContHIN strains.

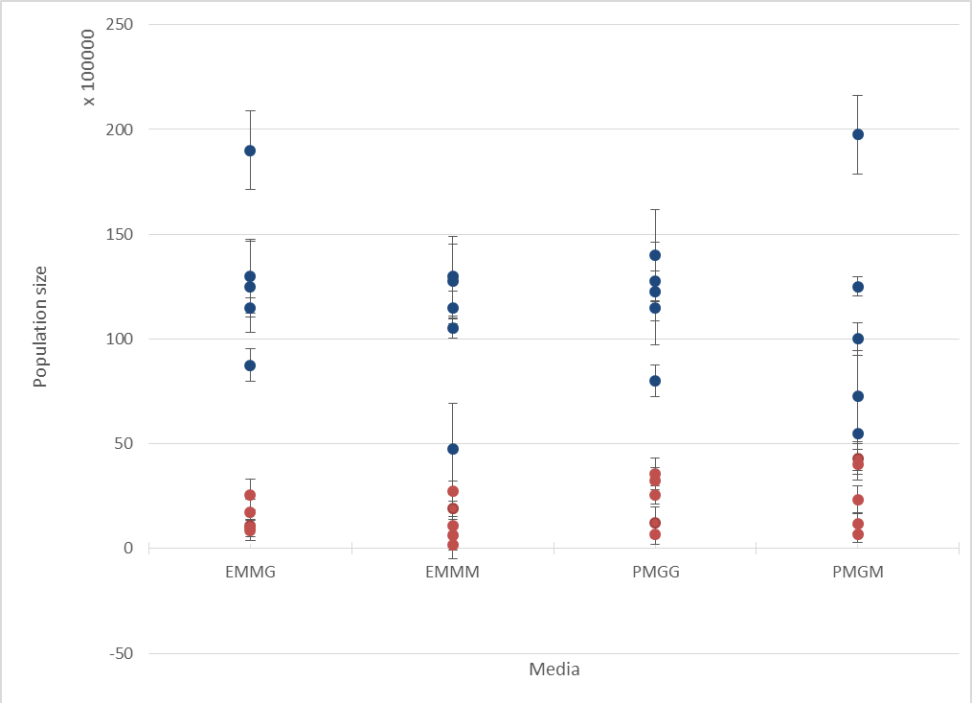


Figure 2: Population size of HIN (red) and ContHIN (blue) clones after one growth cycle in each medium (EMMG, EMMM, PMGG, PMMM): mean of the three assay replicates. The error bars show the standard error.

Experimental Evolution

To study the dynamics of short-term evolutionary adaptation of HIN and ContHIN strains we developed two experimental setups: a setup of evolution under a constant environment and another setup of evolution under a fluctuating environment. For the constant environment condition, strains were evolved under serial dilution to a single medium (EMMG). For the fluctuating environment condition, the medium was changed each passage following a random cycle of four different combinations of carbon and nitrogen sources, one of which was identical to the medium used under the constant environment condition (EMMG, EMMM, PMGG and PMGM). Since the two strains grow to different levels between passages, we could not grow them in the same way. We had to either change N_i or the dilution rate (which affects g). Since the effective population size is more sensitive to N_i, we decided to maintain that value constant (> 10 000 cells) and so at each passage the HIN strains were diluted 25 times and ContHIN 250 times. In both designs we performed a total of 24 passages.

The total number of generations depends on three factors: the final population size that a culture achieves after one growth cycle in the medium prior to a passage, the final population size it achieves after one growth cycle in the medium to which it is passaged next, and the dilution we apply. For the constant environment setup where the medium prior to and posterior to each passage is the same, the total number of generations depends only in the dilution factor applied and the number of passages. This gives a total of 111 generations for HIN strains and 191 generations for ContHIN. For the fluctuating environment condition, we estimated the number of generations after serial dilution to the same sequence of media used in the evolution assay, based on the total population size HIN and ContHIN achieved in average after one growth cycle in each of the four media. This gives an average of 112 generations for HIN strains and 190 generations for ContHIN. For simplification purposes, we consider the same number of generations for each strain under constant and fluctuating environmental conditions.

We founded 5 different HIN clones from two independent crosses HIN1 and HIN2. There were 20 replicates of HIN1, and 5 to 6 replicates of each of the four HIN2 clones. We also used 5 different ContHIN clones with 20 different replicate populations of ContHIN1, and 5 to 6 replicates of each of the other four clones to start the evolution

experiments. This represents a total of 40 HIN lines and 41 ContHIN lines. The HIN clones were derived from two independent crosses, whilst ContHIN clones were derived from the same cross. This design allows for the quantification of the effect of history in fitness trajectories. At the onset of the evolution experiment, we expect HIN1 and HIN2 to have different genomic backgrounds due to differential segregation of mutations in each cross; we also expect the clones from the second independent cross to have different genomic backgrounds between them due to the inherently high mutation rate. We don't expect the clones from ContHIN to not have significantly different genetic backgrounds.

Relative fitness of HIN strains was estimated in competitions with a ContHIN ancestral clone (ContHIN reference) in a constant environment (EMMG) and in an alternate environment, depending on the conditions present during the evolution experiment. Relative fitness of ContHIN strains was estimated relative to the relative fitness of the ContHIN reference (all ContHIN replicates were competed against a HIN reference clone). The competitions in an alternate environment were conducted in a cycle of alternate media that was maintained in all competitions of this kind. The cycle always began with one growth cycle in EMMG (initial EMMG) and ended with one growth cycle in the same medium (final EMMG): EMMG-EMMM-PMGG-PMGM-EMMG.

Adaptation and Divergence in a Constant Environment

At the beginning of the evolution experiment, the fitness median of HIN strains measured in competitions in a constant environment was 0.6831 between population replicates of all the clones (Table1). This was significantly lower than the fitness median of ContHIN strains – 0.9995 (Figure3-A; Table1 and 2). The distribution of fitness values also differed between the two strains (KS: p-value= 5.551e-16).

Table 1: Median and interquartile range of mean fitness of HIN and ContHIN strains relative to the ContHIN ancestral as determined in competitions in a constant environment.

	Median (interquartile range)	
	T0	**Evolved**
HIN	0.6831 (0.5994-0.7543)	1.0320 (1.0050-1.0550)
ContHIN	0.9995 (0.9919-1.0870)	0.7950 (0.7730-0.8191)

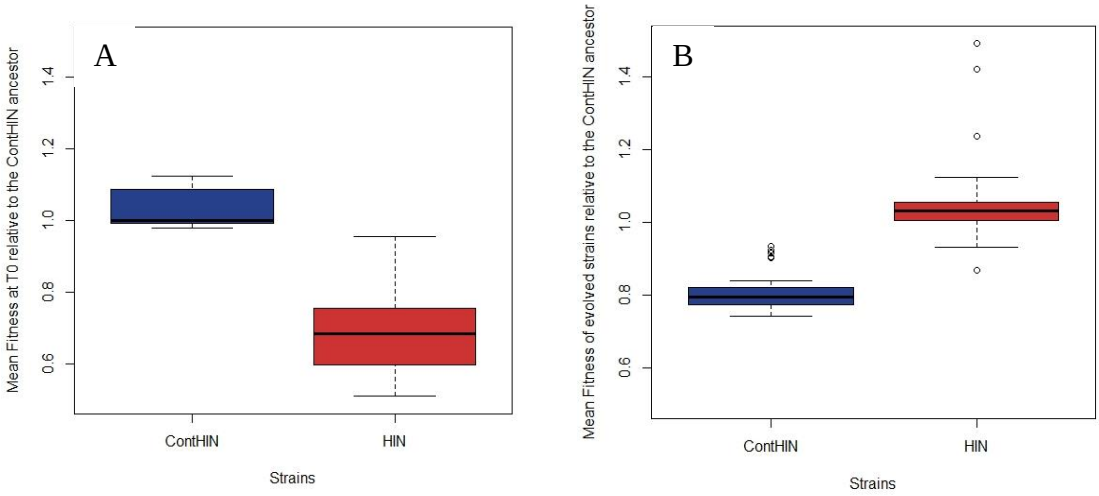


Figure 3: Mean fitness of HIN (red) and ContHIN (blue) strains assayed from three competition replicates in a constant environment (EMMG): A) at the onset of the evolution experiment (T0); B) after evolution in a constant environment (EMMG). Mean fitness of all population replicates is represented as relative to the ContHIN ancestor against which all HIN clones were competed.

Table 2: Wilcoxon rank sum test results for the equality of medians between mean fitness distributions of HIN and ContHIN strains at: the onset of the evolution experiment, after adaptation to a constant environment and in overall fitness trajectories.

T0	**Evolved**	**Fitness trajectories**
W=1640, p-value < 2.2e-16***	W=6, p-value < 2.2e-16***	W=0, p-value < 2.2e-16***

*** p < .001

At the end of the evolution experiment in a constant environment, the fitness median of HIN strains was 1.0320 (Table1), significantly higher than the ContHIN median of 0.7950 (Figure3-B; Table2). Both HIN and ContHIN strains had a significant change in fitness from T0 (Table3). The fitness median of HIN strains increased from 0.6831 to 1.0322 (Figure4; Table1), whilst the fitness median of ContHIN strains decreased from 0.9995 to 0.7950 (Figure4; Table1). The distribution of fitness values around the median of both strains was significantly different between evolved strains and ancestral (Table3).

Table 3: Wilcoxon rank sum test for the equality of medians and Kolmogorov-Smirnov results for comparisons between mean fitness distributions of ancestral and evolved strains as determined in competitions in a constant environment.

	HIN	ContHIN
Wilcoxon	V=0, p-value=1.819e-12***	V=861, p-value=9.095e-13***
Kolmogorov-Smirnov	D=0.95, p-value=8.882e-16***	D=1, p-value=4.441e-16***

*** p < .001

Effect of clone and cross

We wanted to test if founder effects were involved in the evolutionary dynamics of both strains, which may bias our results. Specifically, we wanted to know whether such effects were caused by mutations that appeared shortly after the unstable phenotype was produced. For that, we tested the effect of clone and cross in the mean initial fitness of the strains and after adaptation to the two environmental conditions.

We found a significant effect of both cross and clone for the HIN populations (Table4). However, a post-hoc Tukey's HSD test showed that at 0.05 level of confidence the only significant comparison was between HIN1 and HIN5. As for the ContHIN, the clone had a significant effect in the mean initial fitness of these strains (Table4). Post-hoc Tukey's HSD tests showed that at 0.05 level of confidence all ContHIN clones significantly differed in mean fitness between each other except for ContHIN1 and ContHIN3.

Table 4: One-way ANOVA results for the effect of "clone" and "cross" in mean fitness of HIN and ContHIN strains, at the beginning of experimental evolution, after 110 or 190 generations of adaptation to a constant environment (respectively), and in fitness trajectories.

	HIN			ContHIN		
	T0	**Evolved**	**Fitness trajectories**	**T0**	**Evolved**	**Fitness trajectories**
Clone	0.00452**	0.5053	0.00047***	<2e-16***	0.01367**	0.00116**
Cross	0.00173**	0.1497	0.00116**			

* p < .05
** p < .01
** p < .001

After adaptation to a constant environment, the effects of clone and cross in the distribution of HIN mean fitness were not statistically significant, though both factors affected the fitness trajectories (Table4). The effect of the clone in the ContHIN strains remained after evolution, both in the fitness distributions and in the trajectories (Table4).

Fitness trajectories

The fitness trajectories of HIN and ContHIN were significantly different (Table2), as all HIN replicates increased in mean fitness between time 0 and generation 110 (Figure4 and 5-B) and all ContHIN replicates decreased in mean fitness between time 0 and generation 190 (Figure4 and 5-A).

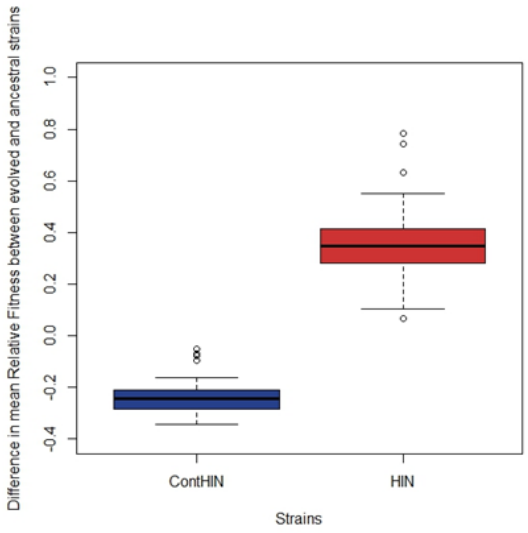
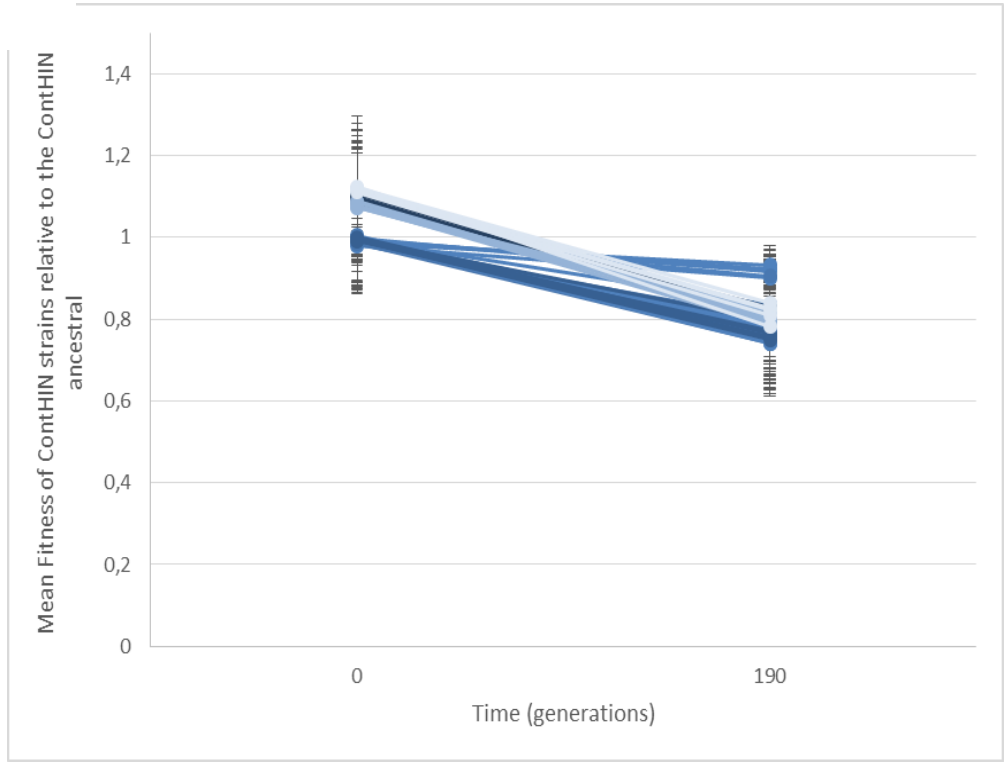


Figure 4: Difference in mean relative fitness between evolved and ancestral ContHIN (blue) and HIN (red) strains after short-term adaptation to a constant environment.

A



B

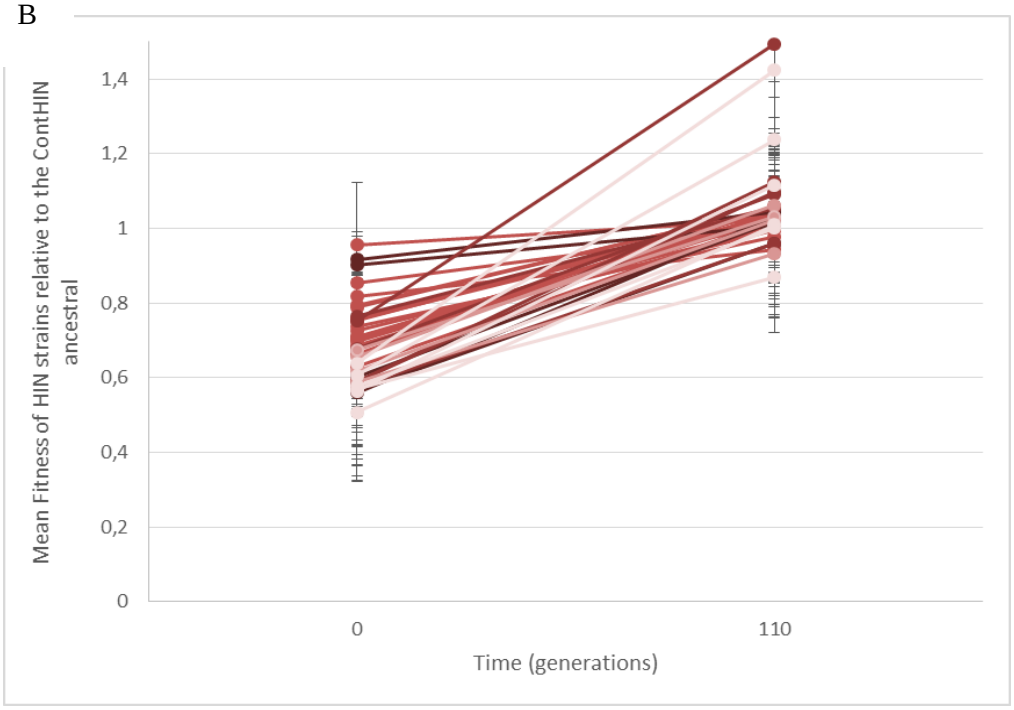


Figure 5: Trajectories of mean fitness of: A) ContHIN population replicates; B) HIN population replicates, during 190 and 110 generations of adaptation to a constant environment (EMMG), respectively. Different shades of red and blue represent different HIN and ContHIN clones. Fitness of all population replicates is represented as relative to the ContHIN ancestor against which all HIN clones were competed. Competitions were carried in a constant environment (EMMG) and the means between the three competition replicates are represented; the error bars show the standard error.

Variance in fitness within and between populations

We tested for overall convergence in fitness values after short-term adaptation of HIN and ContHIN to a constant environment by comparing the between-group variances of each strain between evolved and ancestral population replicates. We also tested if the different clones of each strain are converging or diverging at the same rate by testing the homogeneity of within-group variances for each strain at T0 and after adaptation.

The estimates of within-group and between-group variances for HIN and ContHIN strains are given in Table5 and represented in Figure6. Within-group and between-group variances were estimated for each time-point separately. Within-group variances correspond to the variances of each HIN and ContHIN clone. Between-group variances correspond to the variance of all population replicates of a given strain.

Table 5: Within-group and between-group variances of HIN and ContHIN strains at the beginning of experimental evolution and after adaptation to a constant environment. Between-group variances correspond to the variance of each strain between all population replicates. Within-group variances correspond to the variances of each clone of a given strain (HIN and ContHIN).

			T0	**Evolved**
Between-group	**HIN**		0.011225	0.012417
	ContHIN		0.002835	0.002404
Within-group	**HIN**	HIN1	0.005680	0.000924
		HIN2	0.033565	0.001024
		HIN3	0.007382	0.033880
		HIN4	0.001500	0.003133
		HIN5	0.001963	0.038894
	ContHIN	ContHIN1	0.000045	0.004599
		ContHIN2	0.000164	0.000185
		ContHIN3	0.000012	0.000313
		ContHIN4	0.000044	0.000145
		ContHIN5	0.000034	0.000480

At the beginning of the evolution experiment, the within-group variances of HIN strains (the variances of HIN clones) were all the same (Table6). After adaptation in a constant environment, the between-group variance significantly differed from the variance at T0, increasing from 0.011225 to 0.012417 (Table5 and 6). The within-group variances were also significantly different in evolved HIN strains (Figure6, Table6).

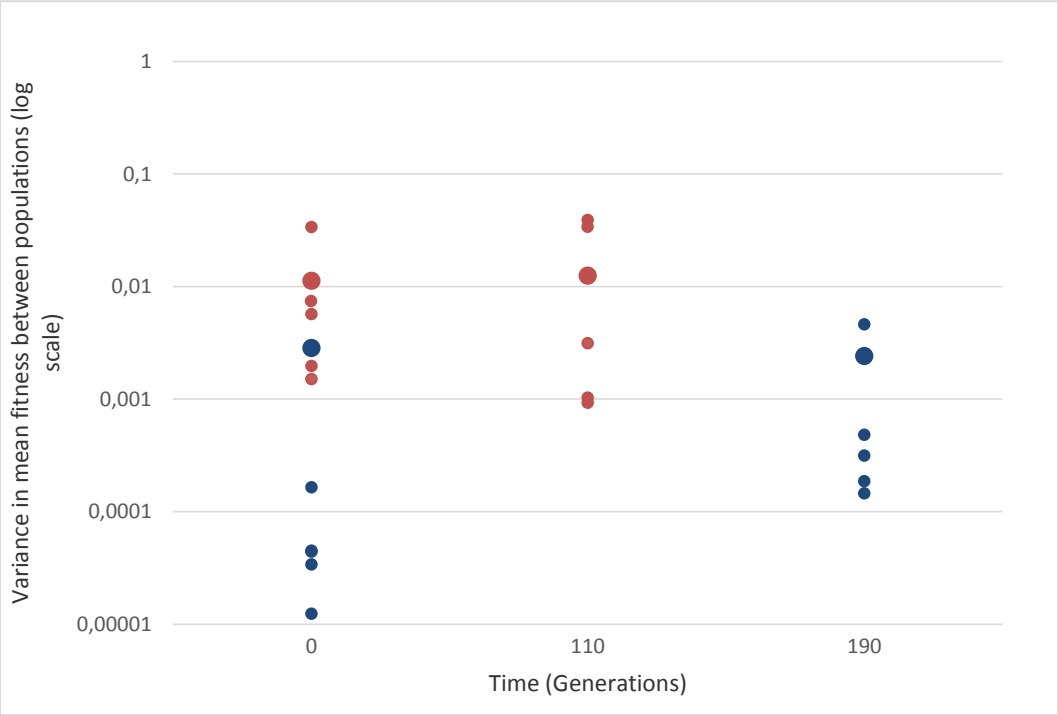


Figure 6: Within-group (smaller circles) and between-group (larger circles) variances of HIN (red) and ContHIN (blue) strains at the beginning of experimental evolution and after adaptation to a constant environment. Within-group variances were estimated as the variances between population replicates of each of the 5 HIN and ContHIN clones. Between-group variances were estimated as the variance between all population replicates of ContHIN and HIN strains.

As for ContHIN, the within-group variances were equal at T0 but significantly differed in all clones after adaptation in a constant environment (Figure6, Table6). However, the between-group variances between ContHIN at T0 and Evolved remained the same (Figure6, Table6).

In general, between- and within-group variances of HIN strains were considerably higher than variances of ContHIN strains, both at T0 and after adaptation in a constant environment (Table5).

Table 6: Fligner-Killeen test results for comparisons of: between-group variances of each strain between population replicates of both time-points; within-group variances for each strain and time-point between the different clones, for the evolution experiment under a constant environment.

	HIN		**ContHIN**	
Between-group	df=1, p-value=0.009747***		df=1, p-value=0.9222	
Within-group	**T0**	**Evolved**	**T0**	**Evolved**
	df=4, p-value=0.2661	df=4, p-value=0.01016*	df=4, p-value=0.06143.	df=4, p-value=0.04428*

. p < .1

* p < .05
*** p < .001

Evolution of population size

During adaptation in the two experimental setups we noticed visible changes in population size in some population replicates. A significant change in population size can affect the effectiveness of selection relative to drift. To test if the population size changed in the course of adaptation to EMMG we compared the population size at the end of the evolution experiment with the population size of the ancestors after one growth cycle in EMMG.

After adaptation to a constant environment, population size was significantly different from the population size of the ancestors for both HIN and ContHIN strains after one growth cycle in EMMG (Wilcoxon: HIN - W = 89.5, p-value = 0.003218; ContHIN - W = 402, p-value = 8.714e-06), with a significant increase in the population size of HIN strains and a significant decrease in the population size of ContHIN strains (SI3.Table7, 8, 9 and 10). However, the median of population sizes at the end of this evolution experiment did not differ between HIN and ContHIN strains (Wilcoxon: W = 1055.5, p-value = 0.3501), neither did the distribution of population sizes (KS: D = 0.22063, p-value = 0.2408). It seems that HIN and ContHIN strains evolved to similar population sizes.

HIN strains adapted to a constant environment

As expected, HIN strains strongly benefited from their increased supply in beneficial mutations during adaptation to a novel environment. In direct competitions with a ContHIN ancestor in the same conditions met during the evolution experiment, evolved HIN strains won. They also evolved to higher population sizes, which indicates that, contrary to what has been suggested in previous studies with digital organisms [43] they did not evolve lower replication rates to become more robust to the effects of deleterious mutations. This suggests that the intrinsic increase in the supply of beneficial mutations is sufficient to outcompete the genetic load of these strains in the early stages of adaptation to a constant environment. It remains to be tested if this increase in fitness would be maintained should the populations become closer to fitness peaks in the adaptive landscape.

The control strains decreased in fitness

The ContHIN strains, contrary to what we were expecting, suffered a significant decrease in fitness during this experiment. This decrease in fitness was sustained by observations that ContHIN strains evolved to lower population sizes, indicating that they had lower replication rates at the end of the evolution experiment.

One possible explanation resides in game theory and is known as the tragedy of the commons [65] which states that individuals (cheaters) overexploiting a common resource for their own gain can drive populations extinct. As Haldane (1932) [66] has previously suggested, individual adaptations do not necessarily lead to traits that are beneficial for the whole population. The cheaters have a higher relative fitness and therefore can invade the population. But as they do so, they decrease the overall density and drive themselves and the population to extinction. This has been reported in microbes in a study with the social bacterium *Myxococcus xanthus* [67] in which cheaters were artificially selected and invaded wild-type strains leading to various competitive fates: persistence at high frequencies with little effect on total population dynamics, persistence after major disruption of total population dynamics, self-extinction with wild-type survival, and total population extinction.

In the current study, all replicate populations of ContHIN strains showed descendent fitness trajectories. If in fact this reflects a cheating strategy, it was replicated in all evolutionary lines. Studies of the within-population diversity may shed some light about the existence of cheaters in all populations, as the cheaters should have higher fitness values than the mean fitness of a given population.

Phenotypic divergence of HIN strains

The between-group variances of HIN strains significantly increased during adaptation to a constant environment, and the within-group variances significantly differed between HIN clones at generation 110. This suggests that populations are diverging to different fitness values, possibly reaching various local optima, while clones are fixing different adaptive mutations at different rates. This adds some weight to the hypothesis that the inherent higher evolvability of chromosomal instability can facilitate large leaps in the fitness landscape, allowing a broader exploration of the fitness landscape, with increased probabilities of reaching global optima [22]. [58]

No clear pattern of convergence or divergence in ContHIN strains

The analysis of between-group variances of ContHIN strains does not suggest any pattern of convergence or divergence in fitness values after adaptation. The within-group variances at generation 190 suggest that the different ContHIN clones are adapting at different rates. This, along with the observations these strains had an overall decline in fitness and that clone had a significant effect in their evolutionary dynamics, constitutes evidence for the hypothesis that large nonmutator populations may be stuck in lower fitness valleys due to the reciprocal sign epistatic interactions between adaptive mutations. Their intrinsic lower mutation rate might be impeding a further exploration of the fitness landscape to overcome these negative epistatic effects and acquire sets of adaptive mutations that increase the general fitness of the population. The valleys they occupy are most likely determined by the founder genotypes and, therefore, there is no phenotypic convergence nor divergence during adaptation.

Adaptation and Divergence in a Fluctuating Environment

As described in the methods, the fluctuating environment experiment was performed in a random sequence of four media with two nitrogen sources (ammonia and glutamate – EMM and PGM respectively) and two carbon sources (Glucose and Maltose – G and M). The four possible combinations were EMMG, EMMM, PMGG and PMGM. At the beginning of the evolution experiment, the fitness median of HIN strains measured in competitions in a fluctuating environment was lowest in EMMG (0.6138) and highest in PMGM (1.2730) (Figure7-A; B and D; Table7). The median of the average fitness values across media was 0.8547 (Table7), significantly lower than the median of the average fitness values across media for the ContHIN strains, 1.0760 (Figure8-A; Table7, 8 and 9). The highest fitness median of ContHIN strains at T0 was also in PMGM (1.2490) and was not significantly different from the fitness median of HIN strains in this medium (Figure7-D; Table8 and 9). After one competition cycle, the fitness median of ContHIN strains in final EMMG at T0 dropped to its lowest level, 0.7624, significantly lower than the fitness median of HIN strains in the same conditions (0.8953) (Figure7-A; Table7, 8 and 9). The difference between the first and the last competitions in EMMG is that in the former, cells were grown in EMMG prior to competition and in the latter cells were grown in PMGM (different nitrogen and carbon sources).

The distribution of fitness values also differed between the two strains in all media and in the average of fitness values across media (KS tests: EMMG, EMMM, PMGG, average of all media - p-values<0.001; PMGM – p-value<0.05; final EMMG – p-value<0.01).

Table 7: Median and interquartile range of mean fitness of HIN strains relative to the HIN ancestral as determined in competitions in a fluctuating environment.

	Median (interquartile range)	
	T0	**Evolved**
EMMG (initial)	0.6138 (0.4953-0.6746)	1.0080 (0.9394-1.0480)
EMMM	0.9602 (0.7833-1.0650)	0.9479 (0.9187-0.9926)
PMGG	0.8151 (0.7610-0.9200)	1.1500 (1.0690-1.1740)
PMGM	1.2730 (1.1670-1.4610)	1.1620 (1.1160-1.2590)
EMMG (final)	0.8953 (0.8005-0.9924)	1.0860 (1.0350-1.1300)
Average of all media	0.8547 (0.7798-0.9152)	1.0740 (1.0250-1.0950)

Table 8: Median and interquartile range of mean fitness of ContHIN strains relative to the ContHIN ancestral as determined in competitions in a fluctuating environment.

	Median (interquartile range)	
	T0	**Evolved**
EMMG (initial)	1.0360 (1.0110-1.1020)	0.8825 (0.8414-0.8971)
EMMM	1.0630 (1.0410-1.1040)	1.7180 (1.5160-1.7950)
PMGG	1.0160 (0.9980-1.1360)	1.0300 (1.0100-1.1160)
PMGM	1.2490 (0.9981-1.7000)	2.8010 (2.3580-2.9800)
EMMG (final)	0.7624 (0.8005-0.9924)	0.4636 (0.3959-0.5105)
Average of all media	1.0760 (0.7798-0.9152)	1.2630 (1.2270-1.3310)

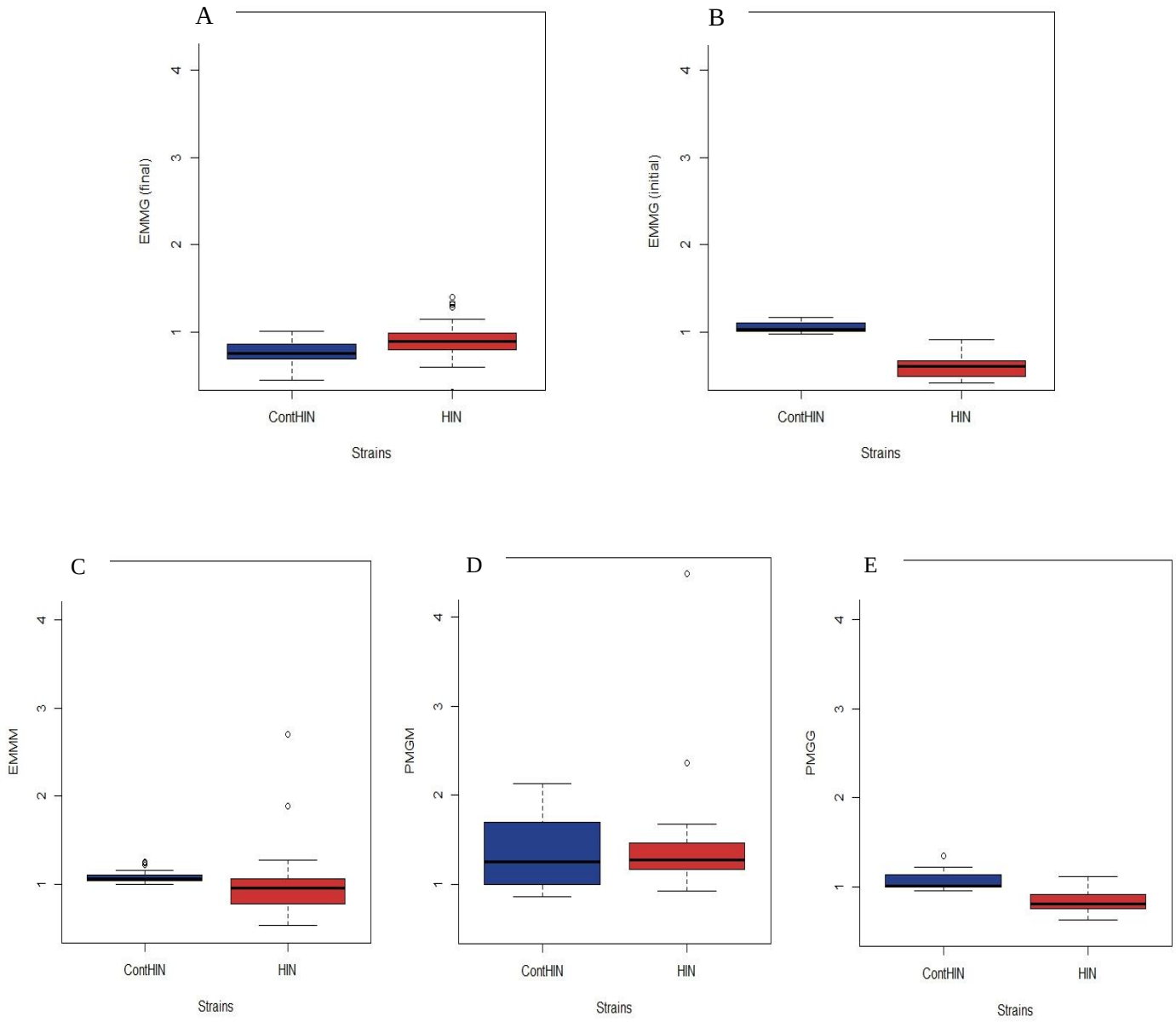


Figure 7: Mean fitness of HIN (red) and ContHIN (blue) strains assayed from three competition replicates in a fluctuating environment at the onset of the evolution experiment in: A) final EMMG; B) initial EMMG; C) EMMM; D) PMGM; E) PMGG. Mean fitness of all population replicates is represented as relative to the ContHIN ancestor against which all HIN clones were competed.

Table 9: Wilcoxon rank sum test results for the equality of medians between mean fitness of distributions of HIN and ContHIN strains at: the onset of the evolution experiment, after adaptation to a fluctuating environment.

	T0	Evolved
EMMG (initial)	W=1638, p-value < 2.2e-16***	W=182, p-value = 6.275e-11***
EMMM	W=1217, p-value = 0.0001211***	W=1638, p-value < 2.2e-16***
PMGG	W=1496, p-value = 1.556e-12***	W=486, p-value = 0.001442**
PMGM	W=785, p-value = 0.7528	W=1638, p-value < 2.2e-16***
EMMG (final)	W=456, p-value = 0.0004878***	W=0, p-value < 2.2e-16***
Average of all media	W=1572, p-value < 2.2e-16***	W=1638, p-value < 2.2e-16***

** p < .01
*** p < .001

Table 10: Wilcoxon rank sum test results for the equality of medians between mean fitness distributions of ancestral and evolved strains (HIN and ContHIN) as determined in competitions in a fluctuating environment.

	HIN	ContHIN
EMMG (initial)	V=0, p-value=3.638e-12***	V=903, p-value=4.547e-13***
EMMM	V=367, p-value=0.7562	V=0, p-value=4.547e-13***
PMGG	V=3, p-value=1.819e-11***	V=455, p-value=0.9704
PMGM	V=603, p-value=0.002361**	V=0, p-value=4.547e-13***
EMMG (final)	V=117, p-value=5.914e-05***	V=900, p-value=2.274e-12***
Average of all media	V=4, p-value=2.547e-11***	V=0, p-value=4.547e-13***

** p < .01
*** p < .001

At the end of the evolution experiment in a fluctuating environment, the fitness median of HIN strains as measured in competitions in a fluctuating environment was lowest in EMMM (0.9479) (Figure9-C; Table7). However, it was not significantly different from the median at T0 (0.9602) (Figure10-C; Table10). The highest median for HIN strains was in PMGM (1.1620), as it was at T0 (Figure9-D; Figure7-D, Table7). However, the median in PMGM was significantly lower for evolved HIN replicates than for the ancestral (Figure10-D; Table10). The biggest difference in HIN strains was between the medians of mean fitness values in initial EMMG which significantly increased from 0.6138 at T0 to 2.8010 at the end of the evolution experiment (Figure10-A; Table7 and 10).

The median of the average HIN fitness values across media was 1.0740, significantly greater than the average at T0 (Figure8-B; Table7 and 10), but significantly lower than the average of fitness values across media for evolved ContHIN strains (1.2630) (Figure8-B; Table8 and 9). The medians of ContHIN fitness values significantly increased from T0 to the end of the evolution experiment in all media except in initial and final EMMG, where they significantly dropped from 1.0360 to 0.8825, and from 0.7624 to 0.4636, respectively (Figure10; Figure8-C; Table8 and 10). The biggest increase in the medians between ancestral and evolved ContHIN replicates was in PMGM (from 1.2490 to 1.0080) (Figure10-D; Table8 and 10).

Evolved HIN strains have significantly higher medians in Glucose media than evolved ContHIN strains, but significantly lower medians in Maltose media (Figure9; Table7, 8 and 9).

The distribution of fitness values around the median of HIN strains was significantly different between evolved and ancestral strains in all media except in EMMM (Table11). As for ContHIN, the distribution of fitness values around the median also differed significantly from ancestral to evolved replicates in all media except for PMGG (Table11).

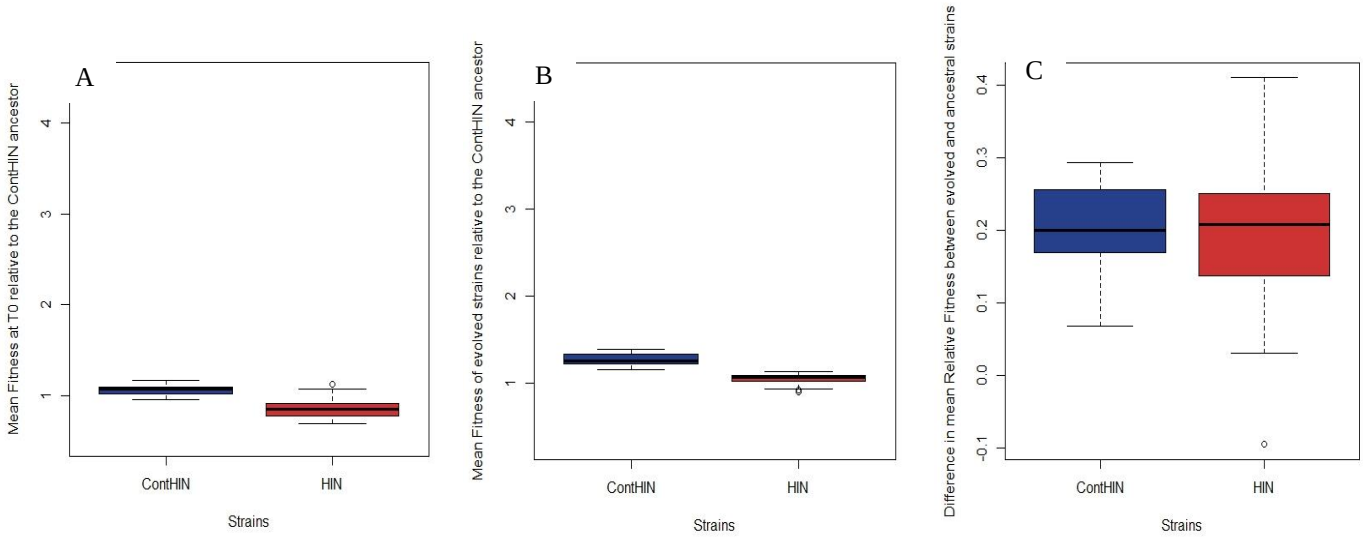


Figure 8: Mean fitness of HIN (red) and ContHIN (blue) strains averaged across all media assayed from three competition replicates in a fluctuating environment at: A) the onset of the evolution experiment; B) the end of adaptation to a fluctuating environment; and C) difference in mean relative fitness between evolved and ancestral ContHIN and HIN strains. Mean fitness of all population replicates is represented as relative to the ContHIN ancestor against which all HIN clones were competed.

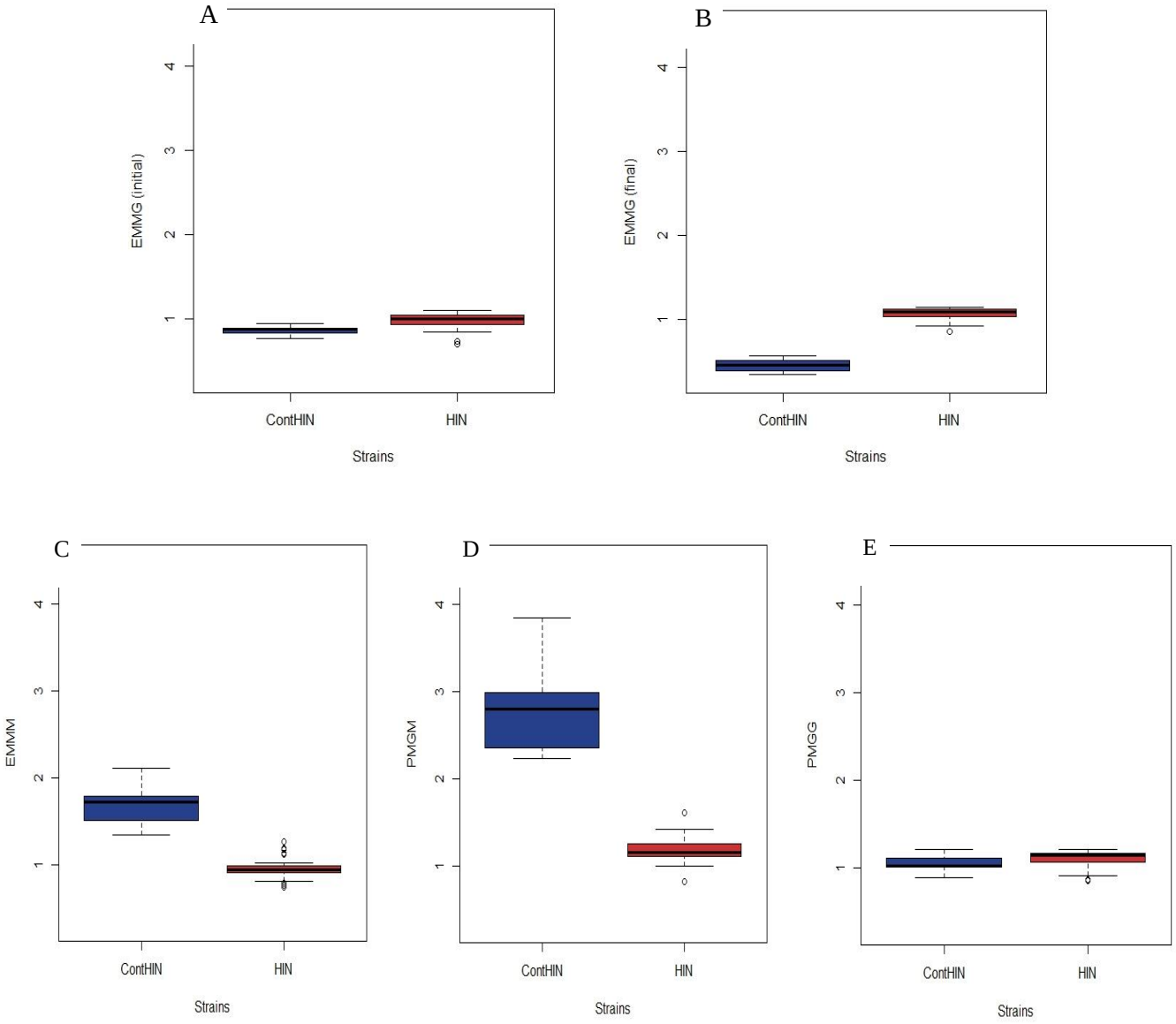


Figure 9: Mean fitness of HIN (red) and ContHIN (blue) strains assayed from three competition replicates in a fluctuating environment in: A) initial EMMG; B) final EMMG; C) EMMM; D) PMGM; E) PMGG. Mean fitness of all population replicates after short-term adaptation to a fluctuating environment is represented as relative to the ContHIN ancestor against which all HIN clones were competed.

Table 11: Kolmogorov-Smirnov test results for comparisons between mean fitness distributions of ancestral and evolved strains as determined in competitions in a fluctuating environment.

	HIN	ContHIN
EMMG (initial)	D=0.89744, p-value=2.22e-16***	D=1, p-value=2.22e-16***
EMMM	D=0.25641, p-value=0.1547	D=1, p-value=2.22e-16***
PMGG	D=0.76923, p-value=1.34e-11***	D=0.19048, p-value=0.4355
PMGM	D=0.33333, p-value=0.02558*	D=1, p-value=2.22e-16***
EMMG (final)	D=0.66667, p-value=1.62e-08***	D=0.92857, p-value=2.22e-16***
Average of all media	D=0.82051, p-value=1.943e-13***	D=0.95238, p-value=2.22e-16***

* $p < .05$
*** $p < .001$

Effect of clone and cross

Similarly to the analysis of the constant environment, we tested whether the founding clone had an effect on the observed evolutionary trajectories. Overall there seems to be an effect of clone in both HIN and ContHIN strains after short-term adaptation in a fluctuating environment (Table12).

At T0 there is an effect of clone in the distribution of mean fitness of HIN strains in EMMG (both initial and final) and in the average of fitness values across all media, but not in the other media conditions (Table12). This effect remained significant after adaptation in a fluctuating environment and in the fitness trajectories in the same media (Table12). As for the ContHIN strains, the clone had a significant effect in T0 and in fitness trajectories in all media conditions, and also in the distribution of fitness values of evolved replicates except in final EMMG (Table12). We also detected an effect of cross in fitness distributions of evolved and ancestral HIN strains in all media except for EMMM and PMGM (Table13). This effect of the cross was not significant in fitness trajectories, with the exception being final EMMG and the averaged fitness values across all media (Table13).

Table 12: One-way ANOVA results for the effect of "clone" in mean fitness of HIN and ContHIN strains, at the beginning of experimental evolution, after 110 or 190 generations of adaptation to a fluctuating environment (respectively), and in fitness trajectories.

	HIN			ContHIN		
	T0	**Evolved**	**Fitness Trajectories**	**T0**	**Evolved**	**Fitness Trajectories**
EMMG (initial)	0.000266***	5.35e-07***	0.0158*	<2e-16***	0.0384*	0.000207***
EMMM	0.268	0.5331	0.28	4.419e-06***	0.000253***	0.00245**
PMGG	0.139	8.35e-05***	0.0393*	2.28e-13***	0.06468.	8.69e-09***
PMGM	0.811	0.564	0.76	0.0004156***	3.96e-06***	0.00074***
EMMG (final)	0.07205 .	0.00224**	0.00167**	0.00752**	0.864	0.0957.
Average of all media	0.000852***	2.77e-05***	0.00167**	2.07e-09***	1.12e-09***	0.000413***

. p < .1
* p < .05
** p < .01
*** p < .001

Table 13: One-way ANOVA results for the effect of "cross" in mean fitness of HIN strains, at the beginning of experimental evolution, after 110 or 190 generations of adaptation to a fluctuating environment (respectively), and in fitness trajectories.

	T0	**Evolved**	**Fitness Trajectories**
EMMG (initial)	0.000107 ***	8.22e-05 ***	0.455
EMMM	0.111	0.9398	0.12
PMGG	0.0138 *	0.00354 **	0.914
PMGM	0.321	0.766	0.7641
EMMG (final)	0.00121 **	0.00134 **	5.03e-05 ***
Average of all media	8.2e-05 ***	0.00198 **	0.0569 .

. p < .1
* p < .05
** p < .01
*** p < .001

Fitness trajectories

Fitness trajectories of HIN and ContHIN were significantly different in all media conditions (Wilcoxon: p-values<0.001). All HIN replicates increased in mean fitness between time 0 and generation 110 in Glucose media, but decreased in PMGM and maintained the same fitness values in EMMM; all ContHIN replicates increased in mean

fitness between time 0 and generation 190 in Maltose composed media, but decreased in EMMG (both initial and final) and maintained the same fitness values in PMGG (Figure10) (Wilcoxon tests to fitness trajectories between HIN and ContHIN strains, p-values <0.001 for all media). However, there is no difference between average of fitness trajectories across all media of HIN and ContHIN strains (Wilcoxon: W = 865, p-value = 0.6688) (Figure 8-C and 11).

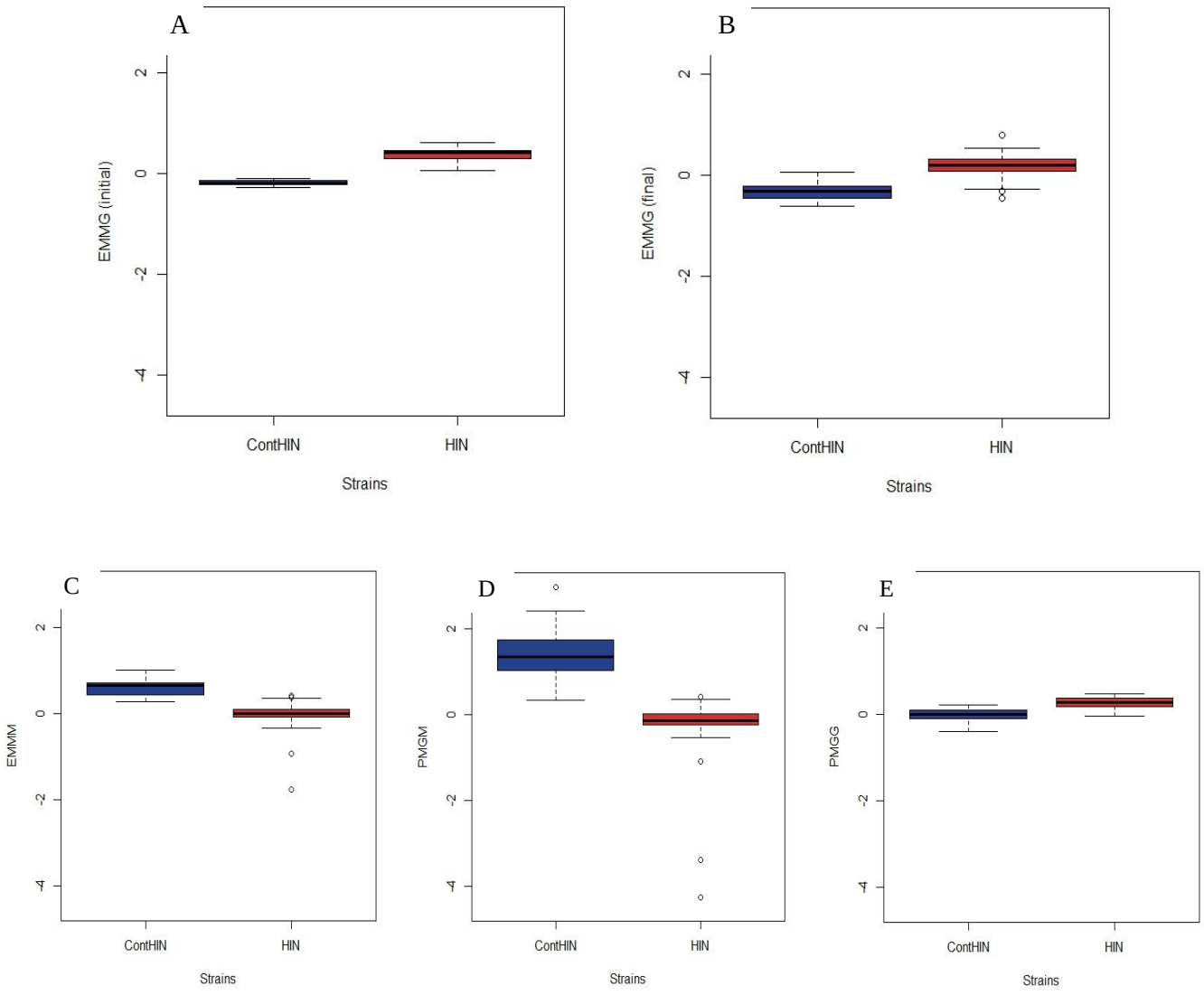


Figure 10: Difference in mean relative fitness between evolved and ancestral ContHIN (blue) and HIN (red) strains assayed from three competition replicates in a fluctuating environment in: A) initial EMMG; B) final EMMG; C) EMMM; D) PMGM; E) PMGG. Mean fitness of all population replicates after short-term adaptation to a fluctuating environment is represented as relative to the ContHIN ancestor against which all HIN clones were competed.

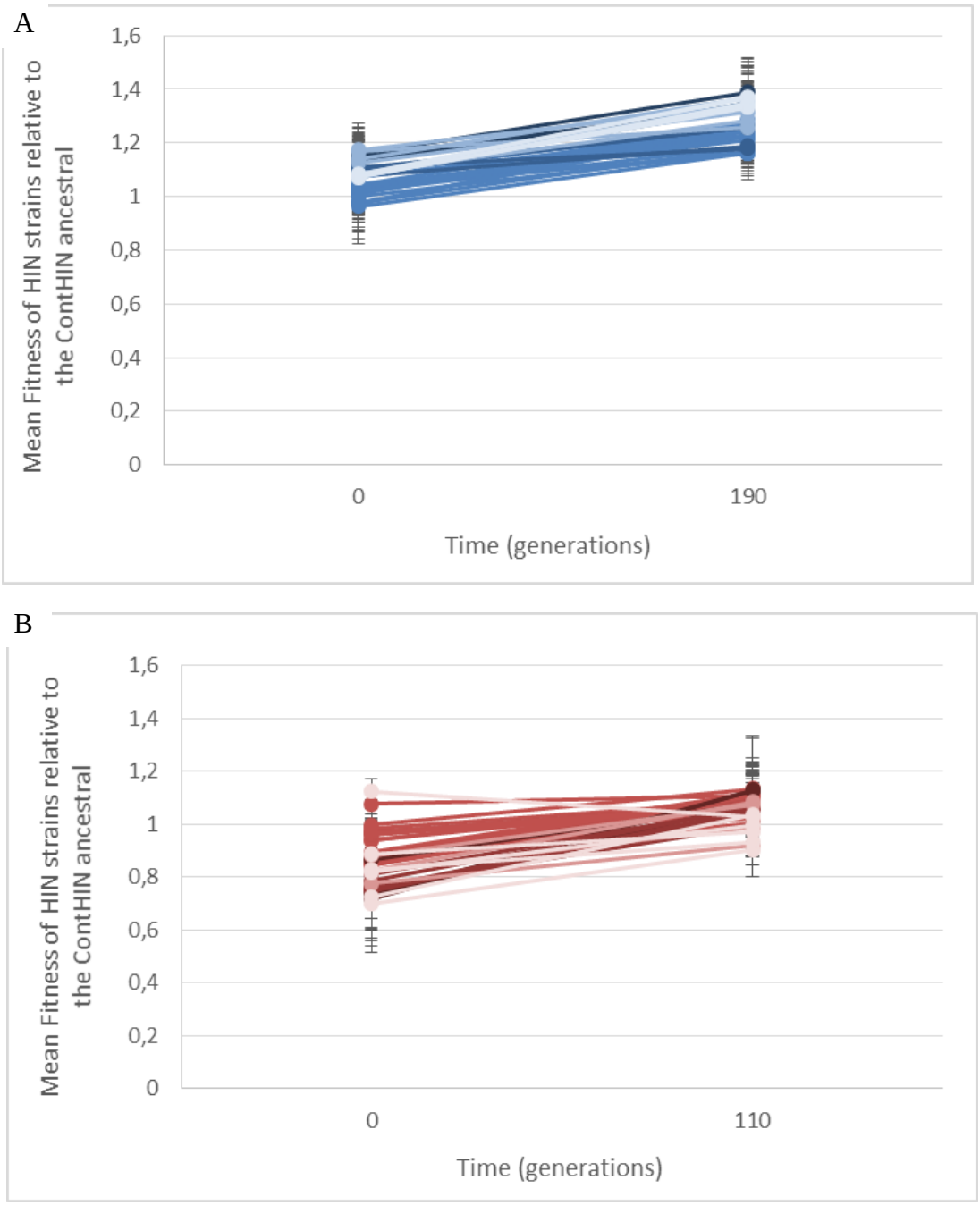


Figure 11: Trajectories of mean fitness averaged from all competition media of: A) ContHIN population replicates; B) HIN population replicates, during 190 and 110 generations of adaptation to a fluctuating environment, respectively. Different shades of red and blue represent different HIN and ContHIN clones. Fitness of all population replicates is represented as relative to the ContHIN ancestor against which all HIN clones were competed. Competitions were carried in a fluctuating environment and the means between the three competition replicates are represented; the error bars show the standard error.

Variance in fitness within and between populations

To test for overall convergence in fitness values after short-term adaptation of each strain to a fluctuating environment, we compared the between-group variances of each strain between evolved and ancestral population replicates for all media conditions.

We also tested the homogeneity of within-group variances for each clone of each strain at T0 and after adaptation to see if the different clones of each strain are converging or diverging at the same rate.

The estimates of within-group and between-group variances for HIN and ContHIN strains are given in Table14 and represented in Figure12. Within-group and between-group variances were estimated for each time-point separately and for all media conditions.

At the beginning of the evolution experiment, the within-group variances of HIN strains (the variances of HIN clones) were all the same in every media condition except in EMMM and in final EMMG (Table14 and 15). After adaptation in a fluctuating environment, the between-group variance significantly decreased from the variance at T0 (from 0.009752 to 0.003357 in the Average of all media) in all media conditions except in PMGG (Table14 and 15; Figure12). The within-group variances were not significantly different for the evolved strains, except in EMMM (Table15).

As for ContHIN, the within-group variances are equal at T0 for initial and final EMMG and PMGG, but significantly differed in EMMM, PMGM and in the Average of all media (Table15). After adaptation to a fluctuating environment, the within-group variances of ContHIN strains were equal in all media except in PMGG (Table15). The between-group variances significantly increased from the variances at T0 in EMMM and significantly decreased in final EMMG (Table14 and 15). Other than that, the variances remained the same as in T0 (Table14 and 15; Figure12).

Table 14: Within-group and between-group variances of HIN and ContHIN strains at the beginning of experimental evolution and after adaptation to a fluctuating environment. Between-group variances correspond to the variance of each strain between all population replicates in each media. Within-group variances correspond to the variances of fitness values of each clone of a given strain (HIN and ContHIN) averaged across all media.

			T0	Evolved
Between-group	**HIN**	EMMG (initial)	0,014661	0,008422
		EMMM	0,136842	0,011857
		PMGG	0,013373	0,008858
		PMGM	0,744379	0,018148
		EMMG (final)	0,044556	0,004328
		Average of all media	0,009752	0,003357
	ContHIN	EMMG (initial)	0,002715	0,001971
		EMMM	0,003312	0,037693
		PMGG	0,008158	0,005866
		PMGM	0,164609	0,160472
		EMMG (final)	0,018517	0,004332
		Average of all media	0,003031	0,004305
Within-group	**HIN**	HIN1	0,004354	0,001130
		HIN2	0,004867	0,000898
		HIN3	0,000530	0,001053
		HIN4	0,001841	0,005945
		HIN5	0,023022	0,023022
	ContHIN	ContHIN1	0,001172	0,001603
		ContHIN2	0,001125	0,001125
		ContHIN3	0,000228	0,001709
		ContHIN4	0,001577	0,001577
		ContHIN5	0,000024	0,000335

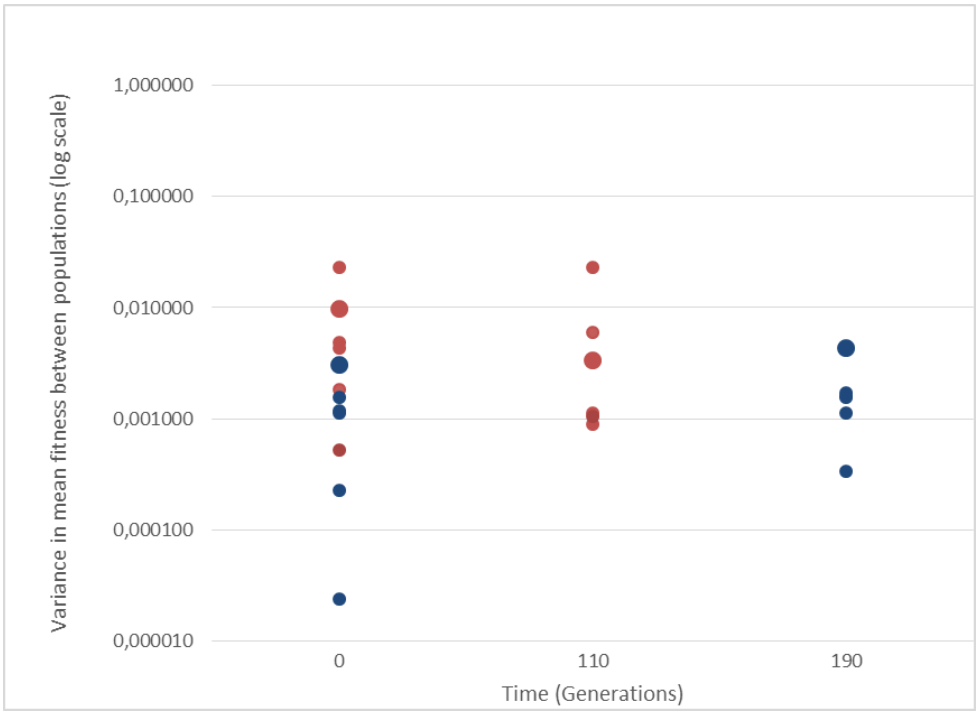


Figure 12: Within-group (smaller circles) and between-group (larger circles) variances of HIN (red) and ContHIN (blue) strains averaged from all competition media at the beginning of experimental evolution and after adaptation to a fluctuating environment. Within-group variances were estimated as the variances between population replicates of each of the 5 HIN and ContHIN clones. Between-group variances were estimated as the variance between all population replicates of ContHIN and HIN strains

Table 15: Fligner-Killeen test results for comparisons of: between-group variances of each strain between population replicates of both time-points; within-group variances for each strain and time-point between the different clones, for the evolution experiment under a fluctuating environment.

		HIN		ContHIN	
Between-group	**EMMG (initial)**	df 1, p-value=0.05316.		df=1, p-value=0.1689	
	EMMM	df=1, p-value=0.0004303***		df=1, p-value=2.115e-05***	
	PMGG	df=1, p-value=0.2165		df=1, p-value=0.677	
	PMGM	df=1, p-value=0.007612**		df=1, p-value=0.4906	
	EMMG (final)	df=1, p-value=4.65e-06***		df=1, p-value=0.0004611***	
	Average of all media	df=1, p-value=0.01238*		df=1, p-value=0.1218	
		T0	**Evolved**	**T0**	**Evolved**
Within-group	**EMMG (initial)**	df=4, p-value=0.2882	df=4, p-value=0.6553	df=4, p-value=0.2302	df=4, p-value=0.1996
	EMMM	df=4, p-value=0.09598.	df=4, p-value=0.0157*	df=4, p-value=0.03479.	df=4, p-value=0.5756
	PMGG	df=4, p-value=0.2382	df=4, p-value=0.1823	df=4, p-value=0.2798	df=4, p-value=0.01546.
	PMGM	df=4, p-value=0.2712	df=4, p-value=0.1506	df=4, p-value=0.01121.	df=4, p-value=0.2443
	EMMG (final)	df=4, p-value=0.03417.	df=4, p-value=0.2866	df=4, p-value=0.2518	df=4, p-value=0.5415
	Average of all media	df=4, p-value=0.1073	df=4, p-value=0.1751	df=4, p-value=0.09178.	df=4, p-value=0.3013

. p < .1
* p < .05
** p < .01
*** p < .001

Evolution of population size

Population size of HIN strains after the adaptation experiment in a fluctuating environment was significantly higher than the population size of the ancestors after one growth cycle in PMGM (the last medium in the random cycle of media for 24 passages, where cultures had grown before plating to count CFU's and freezing to be kept at -80ºC) (Wilcoxon: W = 109.5, p-value = 0.02419) (SI3.Table11 and 12). However, there was a significant decrease in the population size of ContHIN strains (Wilcoxon: W = 374.5, p-value = 0.0001399) (SI3.Table13 and 14).

The median of population sizes at the end of this evolution experiment did not significantly differ between HIN and ContHIN strains (Wilcoxon: W = 791, p-value = 0.5267), neither did the distribution of population sizes (KS: D = 0.16551, p-value = 0.6207).

Carbon source specificity and fitness convergence of HIN strains

The short-term adaptation of HIN and ContHIN strains to a fluctuating environment was specific for the two different carbon sources to which they were adapted: glucose and maltose.

HIN strains had increased fitness levels in all glucose-composed media (EMMG and PMGG), whilst maintaining the same fitness levels or suffering a fitness decrease in all maltose-composed media (EMMM and PMGM, respectively). It seems that our mutator strains rapidly evolved to exploiting glucose at the expense of a better usage of maltose as a carbon source. The distribution of fitness values of HIN strains was significantly narrower after adaptation in all media except for EMMM, to which the strains did not adapt (equal fitness between evolved and ancestral strains). The between-group variances of HIN strains significantly decreased between T0 and generation 110 except in PMGG, but the within-group variances were not significantly different between HIN clones at generation 110 except in EMMM. This suggests that populations are converging to similar fitness values, possibly reaching the same local optima, while the different clones are fixing adaptive mutations at the same rate.

The averaged fitness across all media was higher for the evolved strains, with also narrower distributions of fitness values, which could suggest that HIN strains might be adapting to intermediate fitness values between the four media they were exposed to during the evolution experiment. However, this is not supported by the observation that

the between-group variance of HIN strains in PMGM, the only medium in which they decreased in fitness, also decreased between T0 and evolved strains. Instead, this observation suggests that there is not much room for adaptation in this medium and an antagonistic pleiotropy with glucose-composed media prevailed.

As previously hypothesized, mutators rapidly specialized in exploiting the present resources at the expense of functions needed to exploit the subsequent alternative resources, when environmental conditions changed rapidly [22].

Intriguingly, the population size measured at the end of the evolution experiment, correspondent to one growth cycle in PMGM, was higher on average than the population size of HIN ancestors in this medium. However, this population size may reflect previous growth cycles in the other media during adaptation. To really test if a decrease in fitness in PMGM was accompanied by a decrease in growth rate in this medium, we must measure CFU's after at least two more growth cycles in PMGM.

It remains to be tested if in the long-run, these HIN populations will become extinct under similar evolutionary conditions due to successive reductions of niche breadth in maltose-composed media or if their inherent instability will create the means to reach other fitness peaks in the adaptive landscape to escape the effects of antagonistic pleiotropy between the two carbon sources. It would also be interesting to repeat this evolution experiment starting from another medium, one composed by maltose instead, and see if the first growth cycle in the cycle of random environments is determinant for the nature of this trade-off between carbon sources.

Carbon source specificity and evolutionary dynamics of ContHIN strains

ContHIN strains also adapted differently in carbon and maltose-composed media. Contrary to HIN strains, however, they had an increase in mean fitness in all maltose composed media (EMMM and PMGM) and maintained the same fitness or suffered a fitness decrease in all glucose-composed media (PMGG and EMMG, respectively). Similarly to the HIN strains, ContHIN also evolved to better exploit a carbon resource (in this case, maltose) at the expense of a better usage of an alternative resource. The distribution of fitness values was significantly wider after adaptation in EMMM and PMGM and significantly narrower in EMMG. This suggests a possible convergence of

fitness values in EMMG, similarly to what was observed in adaptation under a constant environment (EMMG), but an overall divergence of fitness values in EMMM and PMGM that might have been responsible for the general increase in fitness in these media.

Within-group variances of ContHIN were equal at T0 for initial and final EMMG and PMGG, but significantly differed in the maltose media EMMM and PMGM. This means that more phenotypic diversity was present in the ancestral populations regarding the exploitation of maltose as a carbon source. This is very important, especially for ContHIN strains that have a wild-type mutation rate and strongly depend on the initial diversity for the supply of beneficial mutations necessary for adaptation to a rapidly changing environment. The between-group variances of ContHIN strains significantly increased between T0 and generation 190 in EMMM but significantly decreased in final EMMG and remained the same for the other media. The within-group variances were not significantly different between ContHIN clones at generation 190 except in PMGG. This indicates that there is no clear pattern of convergence or divergence of fitness values but rather hints of a complex adaptive landscape at the early stages of adaptation.

The averaged fitness accross all media was also higher for the evolved ContHIN strains, with wider distributions of fitness values, which could suggest that these strains might be adapting to intermediate fitness values between the four media transitions. The observation that the between-group variance of ContHIN strains decreased between T0 and evolved strains in EMMG, the only medium in which they decreased in fitness, does not support this hypothesis. Instead, this suggests that adaptation to a rapidly changing environment is hindered by antagonistic pleiotropy independently of the rate supply of mutations in the populations.

The effect of clone in adaptation of ContHIN strains was not eroded in the evolved populations, which reinforces the idea that the genotypic diversity between populations at the onset of this experiment is constraining the mutational trajectories during adaptation.

Strangely, the population size of ContHIN strains after the last growth cycle in PMGM during the adaptation experiment was significantly lower than population size of the ancestors after one growth cycle in the same medium, although fitness of ContHIN strains had dramatically increased in this medium. This reveals a previously predicted trade-off between growth-rate and yield in heterotrophic organisms that can give rise to two ecological strategies: a fast growing, low yield competitive strategy and a slow

growing, high yield cooperative strategy [68; 69; 50]. Although strong selection regularly favors higher growth rates [69], the populations usually need to have evolved for a sufficiently long time in a selective environment for the trade-off to become manifest. As can be seen in standard laboratory strains, organisms that are not well adapted might be able to improve in growth rate without any associated cost in yield [70]. Therefore, further studies are needed to associate the decreased population sizes but increased fitness of ContHIN in PMGM to a trade-off between growth rate and yield in this medium. Namely the measurement of growth kinetics of the evolved strains in competition for a shared resource. It would also be interesting to measure the population size of evolved strains after one growth cycle in the other three media (EMMG, EMMM and PMGG) and compare them to the ancestor population sizes in the corresponding media to see to what extent did this trade-off appear during the short-term adaptation to a rapidly changing environment.

Effect of the previous environment on current fitness

Competitions to estimate fitness in the fluctuating environment condition were conducted in a cycle of alternate media that began and ended with one growth cycle in EMMG. We estimated fitness associated with each medium and also averaged the fitness values obtained in all media throughout the competition, but it is possible that individual fitness values from a given medium are affected by the previous growth cycle in a different medium. In the case of final EMMG, the previous medium in the competitions differs both in the carbon and nitrogen source (PMGM). To test for this, we compared the fitness values obtained in the initial EMMG with fitness values from the final EMMG. The median of fitness values of HIN and ContHIN strains at T0 and at the end of the evolution experiment significantly differed between initial and final EMMG (Wilcoxon tests: at T0: HIN - V = 51, p-value = 1.29e-07; ContHIN - V = 860, p-value = 2.711e-08; at the end of the evolution experiment: HIN - V = 15, p-value = 4.984e-10; ContHIN - V = 903, p-value = 4.547e-13). This suggests that we must take into account the previous transitions during a competition and cannot assume a given fitness value to reflect evolution in that medium alone. In the light of this conclusion, the most accurate fitness measurement in this work corresponds to the averaged fitness values between all media.

Cost of Adaptation to a fluctuating environment

We wanted to test if strains that were adapted in a fluctuating environment condition suffered a fitness cost relative to strains that were adapted in a constant environment condition. For that, we compared the mean fitness of strains that were evolved in a constant environment (EMMG) with the initial EMMG fitness of strains that evolved in a fluctuating environment. As the fitness in initial EMMG was assessed based on the change in frequency in the first growth cycle in the competitions, we took the first slope of the first growth cycle from the constant environment competitions to assess the equivalent of the fitness in initial EMMG from the competitions in a fluctuating environment.

At the beginning of the adaptation experiment, the fitness in initial EMMG as obtained in both competitions (in a constant environment and in a fluctuating environment) was equivalent for HIN strains (Wilcoxon: V = 410, p-value = 1), but not for ContHIN strains (Wilcoxon: V = 10, p-value = 7.882e-08).

The median of initial EMMG fitness values of HIN strains that evolved in a constant environment was 1.0898, significantly lower than 1.00125, the median of the initial EMMG fitness values of HIN strains that evolved in a fluctuating environment (Wilcoxon: V = 717, p-value = 1.04e-05). The medians of evolved ContHIN strains from both environmental conditions were also significantly different (Wilcoxon: V = 1, p-value = 1.819e-12). The medians of ContHIN strains were always higher in competitions under a fluctuating environment.

It would appear to be a cost of adaptation to a fluctuating environment for the HIN strains. However, we must remember that under adaptation in a fluctuating environment, HIN strains spent considerably less time in EMMG than they did in the constant environment setup. Therefore, we might be comparing fitness values from different stages of the adaptive process. To be able to quantify the putative cost of adaptation to a fluctuating environment, we should prolong this experiment until HIN strains spent the same amount of generations in EMMG as they did during adaptation to a constant environment. However, and as the dynamics are so similar between both conditions, we can say that there is a strong suggestion for the existence of such a cost in our experiments with HIN strains.

As for the ContHIN strains, we cannot draw conclusions about such cost because at the onset of the experimental evolution there was a significant difference in fitness as measured in the two competition setups.

The role of selection, history and chance

Both in the short-term adaptation experiments in a constant and fluctuating environment, we observed a significant effect of clone in fitness trajectories of HIN and ContHIN strains. To better understand the role of history in the divergence of the two strains, we quantified the effect of natural selection, history and chance for the two environmental conditions by partitioning the overall sum of squares between evolved and ancestral strains (Figure13). The effect of selection in the adaptation of each strain was estimated as the sum of squared deviations among all population replicates of HIN or ContHIN strains. If the initial grand mean fitness of a given strain is I and the final grand mean is F, this is equivalent to $nc(F-I)^2$, where n is the number of clones and c is the average number of population replicates per clone. The effect of history can be quantified by the sum of squared deviations between the mean score of each clone (C) derived from a given strain and the grand mean score of that strain: $\sum(C-F)^2$, and the effect of chance can be estimated by $\sum\sum(R-C)^2$, where R is the score (mean fitness) of each population replicate.

In both experimental designs (adaptation in constant and fluctuating environments), the largest component of fitness is natural selection (Figure13). The effects of drift are barely observable in adaptation to a constant environment, and the effect of history is even less noticeable (Figure13-A). Although previous tests have shown an effect of clone and cross in fitness trajectories, this effect is small when compared with natural selection. In some environments of the fluctuating environment setup, chance had a much more prominent role than selection. Namely, in the adaptation of ContHIN strains to PMGG and in the adaptation of HIN strains to EMMM and PMGM (Figure13-C and D). History also had a lower but evident role in the adaptation of ContHIN strains in PMGG and of HIN strains in EMMM. For the average of fitness values across all media in the competitions, the predominant force is still natural selection, with ContHIN strains being slightly more affected by history than HIN strains (Figure13-B).

Selection can explain almost all the variance reported in this study for the short-term adaptation of HIN and ContHIN strains under a constant and a fluctuating environment. The only exceptions are the rare cases in which we did not see any significate differences in fitness between evolved and ancestrals. This was the case with the evolution of ContHIN strains in PMGG after 24 growth cycles in a fluctuating environment, and the evolution of HIN strains in EMMM in the same conditions. In both cases, chance played the most relevant role. The other case is with HIN strains in PMGM, the exact medium in which these strains decreased in fitness. It is possible that the reduced efficiency of selection and overrepresentation of chance events as the main force behind adaptation in these particular examples is a consequence of observed reduction in niche breadths in both HIN and ContHIN populations during growth in these media. These niche reductions most likely result from antagonistic pleiotropy between advantageous mutations in Glucose but disadvantageous in Maltose, in the case of HIN strains, and advantageous mutations in Maltose but disadvantageous in Glucose, in the case of ContHIN. Further experiments to measure CFUs of evolved strains after two growth cycles in these media are necessary to validate the observed reductions in population sizes.

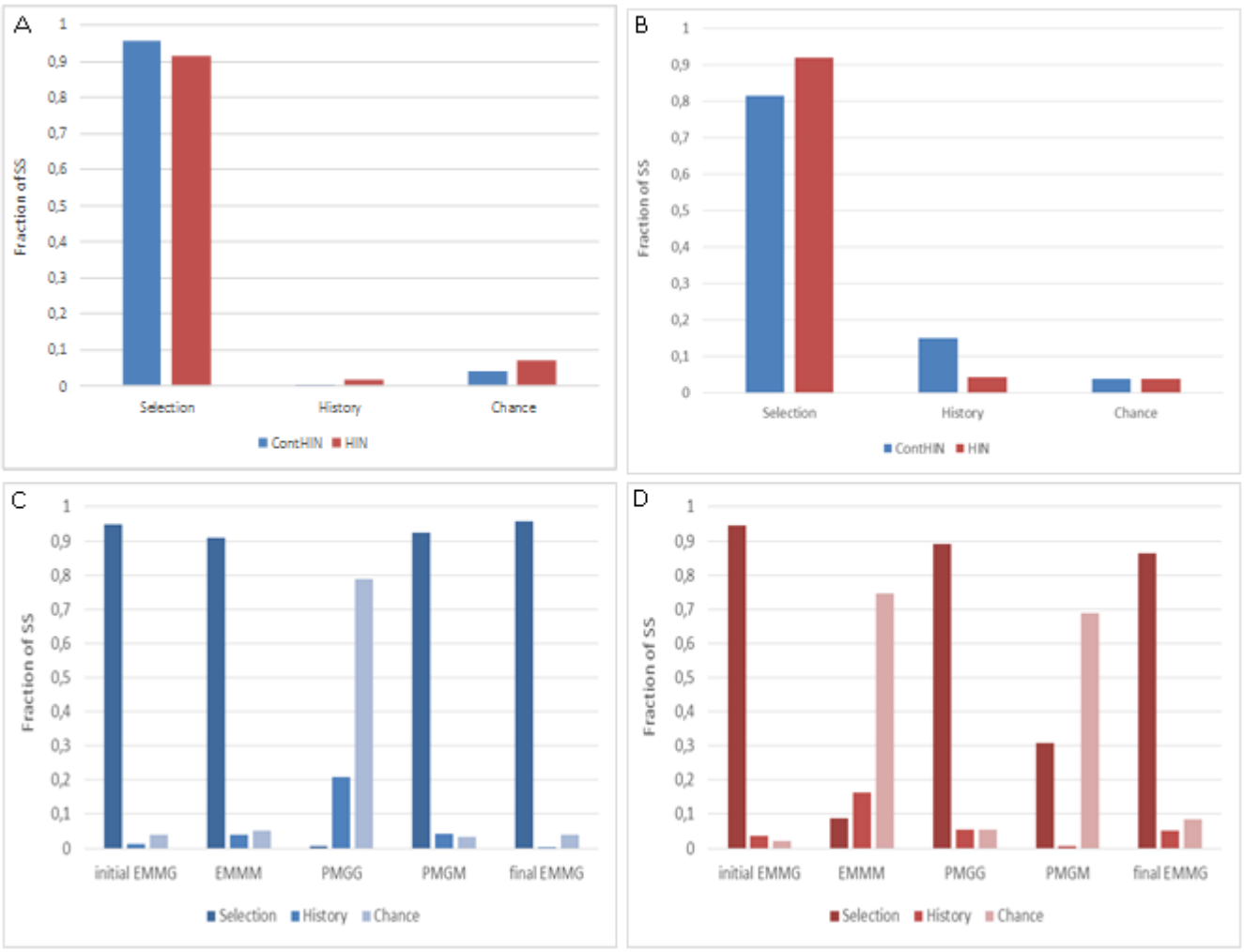


Figure 13: The contributions of selection, history and chance, measured as the fraction of sum of squared differences (Fraction of SS), to the divergence of the evolved HIN and ContHIN strains under adaptation to: A) a constant environment; B) a fluctuating environment (average of all media); C) of ContHIN strains in a fluctuating environment for all the 5 media of each competition (initial EMMG, EMMM, PMGG, PMGM and final EMMG); D) of HIN strains in a fluctuating environment for all the 5 media of each competition (initial EMMG, EMMM, PMGG, PMGM and final EMMG).

CONCLUSION AND FUTURE PERSPECTIVES

The short-term evolutionary dynamics of mutators for chromosomal structure is highly complex and dependent on the environmental conditions. In a constant environment, the increased supply of beneficial mutations exceeded the genetic load of these mutator strains that had an overall increase in fitness. Adaptation in these conditions was accompanied by phenotypic divergence, revealing a complex fitness landscape with many local peaks. However, in a fluctuating environment, we observed an antagonistic pleiotropy between exploitation of alternative carbon sources. The long-term dynamics of these strains remain to be tested. It would be interesting to see what happens as populations get closer to an optimum fitness level in the constant environment. It would also be interesting to see the extent to which a trade-off between carbon sources in the fluctuating environment could lead mutators to extinction.

In the light of our results in a constant environment, it is fair to ask if there are regions in the genome where rearrangements are more likely to be beneficial. Previous studies of short-term yeast adaptation to a strong selective pressure reported the appearance of repeated, independent chromosomal rearrangements throughout the experiments [52]. Next Generation Sequencing methods have been developed that allow for a rapidly identification of translocation breakpoints (as well as tandem duplications, inversions and other complex events) [71] could be used coupled with experimental studies such as the present work to answer this question.

In the future, we would like to repeat the competitive fitness assays for the populations that evolved in a fluctuating environment. As in the competition setup used in this study we could not attribute individual fitness values to a single medium, but rather to the media transitions during the competition, we would do competitions for each the four media used during the evolution experiment. This could help us clarify the patterns of divergence and convergence of our mutator and control strains under a fluctuating environment condition.

Our results suggest that in large asexual populations with lower mutation rates, adaptation to a constant environment is hindered by clonal interference and reciprocal sign epistasis. Whole-genome sequencing would allow us to identify the mutations that arose during the evolution and further competitive fitness assays would permit a clear distinction between the fitness of individual mutations and the fitness of double mutants.

REFERENCES

[1] Lashin et al. (2012) Theories of biological evolution from the viewpoint of the modern systemic biology. Russian Journal of Genetics 48(5):481–496.

[2] Özlem et al. (2013) Genomic Rearrangements and Evolution. Current Progress in Biological Research: Chapter 2.

[3] Nadeau and Jiggins (2010) A golden-age for evolutionary genetics? Genomic studies of adaptation in natural populations. Cell 147(1):484–492.

[4] Fisher (1930) The Genetic Theory of Natural Selection. Oxford: Oxford Univ. Press.

[5] Eyre-Walker and Keightley (2007) The distribution of fitness effects of new mutations. Nature 8:610–618.

[6] Sturtevant (1937) Essays on evolution. I. On the effects of selection on mutation rate. Q. Rev. Biol. 12:467–477.

[7] Barrick et al. (2009) Genome evolution and adaptation in a long-term experiment with Escherichia coli. Nature 461:1243–1249.

[8] Chang et al. (2013) Dynamic Large-Scale Chromosomal Rearrangements Fuel Rapid Adaptation in Yeast Populations. PLoS Genet 9(1):e1003232.

[9] Itan and Tannenbaum (2012) Effect of Chromosomal Instability on the MutationSelection Balance in Unicellular Populations. PLoS ONE 7(5): e26513.

[10] Griffiths et al. (1999) Modern Genetic Analysis. New York: W. H. Freeman.

[11] Sniegowski et al. (1997) Evolution of high mutation rates in experimental populations of E. coli. Nature 387:703–705.

[12] Friedberg et al. (1995) DNA repair and mutagenesis. ASM Press, Washington, D.C.

[13] Couce et al. (2013) Mutational Spectrum Drives the Rise of Mutator Bacteria. PLoS Genetics 9(1):e1003167.

[14] Hartwell et al. (1982) The fidelity of mitotic chromosome reproduction in S. cerevisiae. Rec. Adv. Yeast Mol. Biol. 1:28–38.

[15] Musacchio and Salmon (2007) The spindle-assembly checkpoint in space and time. Nat Rev Mol Cell Biol. 8(5):379–93.

[16] Jyssum (1960) Observation on two types of genetic instability in Escherichia coli. Acta Pathol. Microbiol. Scand. 48:113–120.

[17] Gross and Siegel (1981) Incidence of mutator strains in *Escherichia coli* and coliforms in nature. Mutat. Res. 91:107–110.

[18] Leclerc et al. (1996) High mutation frequencies among Escherichia coli and Salmonella pathogens. Science 274(5290):1208–11.

[19] Matic et al. (1997) Highly Variable Mutation Rates in Commensal and Pathogenic Escherichia coli. Science 277(5333):1833–1834.

[20] Chao and Cox (1983) Competition between high and low mutating strains of Escherichia coli. Evolution 37:125–134.

[21] Mao et al. (1997) Proliferation of Mutators in A Cell Population. Journal of Bacteriology. 179(2):417–422.

[22] de Visser (2002) The fate of microbial mutators. Microbiology 148:1247–1252.

[23] Maynard-Smith and Haigh (1974) The hitch-hiking effect of a favourable gene. Genet. Res. 23**:**23–35.

[24] Elena and Lenski (2003) Evolution experiments with microorganisms: the dynamics and genetic basis of adaptation. Nature Reviews|Genetics 4:457–469.

[25] Tenallion et al. (1999) Mutators, Population Size, Adaptive Landscape and the Adaptation of Asexual Populations of Bacteria. Genetics 152:485–493.

[26] Muller (1964) The relation of recombination to mutational advance. Mutat. Res. 1:2–9.

[27] Haigh (1978) The accumulation of deleterious genes in a population--Muller's Ratchet. Theor Popul Biol. 14(2):251–67.

[28] Gerrish and Lenski (1998) The fate of competing beneficial mutations in an asexual population. Genetica 102/103:127–144.

[29] Wright (1931) Evolution in Mendelian Populations. Genetics 16(2):97–159.

[30] Poelwijk et al. (2007) Empirical fitness landscapes reveal accessible evolutionary paths. Nature 445:383–386.

[31] Weinreich et al. (2007) Perspective: Sign epistasis and genetic constraint on evolutionary trajectories. Evolution. 59(6):1165–74.

[32] Poelwijk et al. (2011) Reciprocal sign epistasis is a necessary condition for multi-peaked fitness landscapes. J Theor Biol. 272(1):141–4.

[33] Chiotti et al. (2014) The Valley-of-Death: Reciprocal sign epistasis constrains adaptive trajectories in a constant, nutrient limiting environment. Genomics 104(6A): 431–437.

[34] Lieberman and Feldam (1986) Modifiers of mutation rate: a general reduction principle. Theor. Popul. Biol. 30:125–142.

[35] Kondrashov (1995) Contamination of the genome by very slightly deleterious mutations — why have we not died 100 times over. J Theor Biol. 175:583–594.

[36] Ishii et al. (1989) Evolutionarily Stable Mutation Rate in a Periodically Changing Environment. Genetics 121:163–174.

[37] Carja et al. (2014) Evolution in changing environments: Modifiers of mutation, recombination, and migration. PNAS 111(50):17935–17940.

[38] Frank and Slatkin (1990) Evolution in a variable environment. Am Nat 136: 244–260.

[39] Kussel and Liebler (2005) Phenotypic Diversity, Population Growth, and Information in Fluctuating Environments. Science 309:2075–2078.

[40] Funchain et al. (2000) The consequences of growth of a mutator strain of Escherichia coli as measured by loss of function among multiple gene targets and loss of fitness. Genetics 154:959–970.

[41] Cooper & Lenski (2000) The population genetics of ecological specialization in evolving *Escherichia coli* populations. Nature 407:736–739.

[42] Giraud et al. (2001) Costs and benefits of high mutation rates: adaptive evolution of bacteria in the mouse gut. Science 291:2606–2608.

[43] Wilke et al. (2001) Evolution of digital organisms at high mutation rates leads to survival of the flattest. Nature 412:331–333.

[44] Putnam et al. (2005) Saccharomyces cerevisiae as a Model System To Define the Chromosomal Instability Phenotype. Molecular And Cellular Biology 25(16):7226–7238.

[45] Geigl et al. (2008) Defining chromosomal instability". Trends in Genetics 24(2):64–69.

[46] Raeside et al. (2014) Large Chromosomal Rearrangements during a Long-Term Evolution Experiment with Escherichia coli. mBio 5(5):e01377-14.

[47] McGranahan et al. (2012) Cancer chromosomal instability: therapeutic and diagnostic challenges. EMBO Rep. 13:528–538.

[48] Loeb (2001) A mutator phenotype in cancer. Cancer Res. 61:3230–3239.

[49] Nowak et al. (2002) The role of chromosomal instability in tumor initiation. P Natl Acad Sci USA 99:16226–16231.

[50] Nowak et al. (2006) Genetic instability and clonal expansion. J Theor Biol. 241(1):26–32.

[51] Sniegowski et al. (2000) The evolution of mutation rates: separating causes from consequences. BioEssays 22(12):1057–1066.

[52] Dunham et al. (2002) Characteristic genome rearrangements in experimental evolution of Saccharomyces cerevisiae. Proceedings of the National Academy of Sciences of the United States of America 99:16144–9.

[53] Chang et al. (2013) Dynamic Large-Scale Chromosomal Rearrangements Fuel Rapid Adaptation in Yeast Populations. PLoS Genet 9(1):e1003232.

[54] Adams et al. (1992) Adaptation and major chromosomal changes in populations of Saccharomyces cerevisiae. Current Genetics 22:13–19.

[55] Dhar et al. (2011) Adaptation of Saccharomyces cerevisiae to saline stress through laboratory evolution. Journal of Evolutionary Biology 24:1135–53.

[56] Eyre-Walker et al. (2006) The distribution of fitness effects of new deleterious amino acid mutations in humans. Genetics 173:891–900.

[57] Selmecki et al. (2009) Acquisition of aneuploidy provides increased fitness during the evolution of antifungal drug resistance. PLoS Genet 5:e1000705.

[58] Rancatia et al. (2013) Karyotypic changes as drivers and catalyzers of cellular evolvability: A perspective from non-pathogenic yeasts. Seminars in Cell & Developmental Biology 24:332–338.

[59] Fogle et al. (2008) Clonal Interference, Multiple Mutations and Adaptation in Large Asexual Populations. Genetics 180:2163–2173.

[60] Hentges et al. (2014) Cdk1 Restrains NHEJ through Phosphorylation of XRCC4-like Factor Xlf1. Cell Reports.

[61] Zakov et al. (2013) An algorithm approach for breakage-fusionbridge detection in tumor genomes. PNAS 110(14):5546–5551.

[62] Murnane (2012) Telomere dysfunction and chromosome instability. Mutat Res. 730(1-2):28–36.

[63] Forsburg and Rhind (2006). Basic methods for fission yeast. Yeast 23(3):173–83.

[64] Lenski et al. (1991) Long-term experimental evolution in Escherichia coli.I. Adaptation and divergence during 2,000 generations. The American Naturalist. 1991;138:1315–1341.

[65] Hardin (1968) The Tragedy of the Commons. Science 162(3859):1243–1248.

[66] Haldane (1932) The causes of evolution. Cornell University Press, New York.

[67] Fiegna and Velicer (2003) Competitive fates of bacterial social parasites: Persistence and self-induced extinction of myxococcus xanthus cheaters. Proceedings of the Royal Society of London.Series B: Biological Sciences, 270(1523),1534.

[68] Kreft and Bonhoeffer (2005) The evolution of groups of cooperating bacteria and the growth rate versus yield trade-off. Microbiology 151: 637-641

[69] Pfeiffer et al. (2001) Cooperation and Competition in the Evolution of ATP-Producing Pathways. Science 292(5516):504–507.

[70] Fischer and Sauer (2005) Large-scale in vivo flux analysis shows rigidity and suboptimal performance of Bacillus subtilis metabolism. Nature Genetics 37:636–640.

[71] Abel et al. (2010) SLOPE: a quick and accurate method for locating non-SNP structural variation from targeted next-generation sequence data. Bioinformatics26:2684–2688.

SUPPLEMENTARY INFORMATION

1. Supplementary Methods

Construction of Control strains for the High INstability strains (ContHIN)

To use as a control for the genomic instability displayed by the HIN strains, which results from the three mutations mentioned above, we used a parental of the HIN strains that does not have these mutations – MGF 1255. We wanted a Control strain with the same auxotrophic markers and resistance as HIN strains, not only because this allows us to control for the effect of all known genetic markers present in the HIN strains, but also because sharing the same auxotrophies implies that the same supplements are added to the minimal media when growing both HIN and ContHIN strains. However, MGF 1255 has different auxotrophic markers and antibiotic resistance than HIN strains (see SI-Table 1). While HIN strains are auxotrophic for leucine and uracil and resistant to kanamycin and hygromycin (*leu- ura-* hph^r kan^r), MGF 1255 is auxotrophic for adenine, leucine, uracil, histidine and lysine, and resistant to hygromycin (*ade- leu- ura- his- lys-* hph^r). Therefore, we crossed MGF 1255 with the wild type strain SPP2 (which is prototrophic but is not resistant to hygromycin) to get rid of *ade-*, *his-* and *lys-* and selected the progeny with the desired genotype.

Following a protocol to kill vegetative cells, the remaining spores were germinated in YES + Hygromycin plates to select for colonies with resistance to this antibiotic (therefore being hph^r). We replica plated the spores to PMG – Leucine, PMG – Uracil, PMG + (Leucine and Uracil) and YES plates. We selected the colonies that were white in the minimal media (PMG) plates (an indicative that they are not *ade-*), that did not grow in PMG – Leucine nor in PMG – Uracil, and grew only in PMG + (Leucine and Uracil), therefore being *leu-* and *ura-*. We arbitrarily picked 20 of the selected colonies and retrieved them from the YES plates, where they could be found in the exact position that they occupied in all the previous plates for which they were replica plated.

The colonies were re-streaked in YES plates in order to increase the probability that we were selecting isolated colonies that were then re-streaked to obtain more cellular material per clone. We used these last cultures to extract DNA and perform a PCR to

assess the mating type of the 20 clones using the following primers: mat1-P forward primer (MP) and mat1-M forward primer (MM) and the mating type primer (MT1) (SI-Table5). We also used some of the remaining cellular material to confirm the desired auxotrophies by re-streaking each clone again in PMG-Uracil, PMG-Leucine and PMG + (Leucine and Uracil). Three of the 20 isolated clones (clones 1, 11 and 18 from SI-Figure1) were not a match and we randomly chose three h+ and three h- clones from the remaining 17 isolated clones. We grew the clones 2, 3, 4, 11 and 13 (SI-Figure1) in 5mL of YES medium, after which the clones were re-suspended in 1,5 mL of Freezing medium and stored at -80ºC.

We were not able to obtain the resistance to kanamycin since none of the parentals of the HIN strains carried this resistance. However, we can consider the resulting control strain as a stable strain with a common history with the HIN strains, close to the WT and with as many as possible markers shared by the HIN strains (the only exception being the kanamycin-resistance cassette).

Crosses

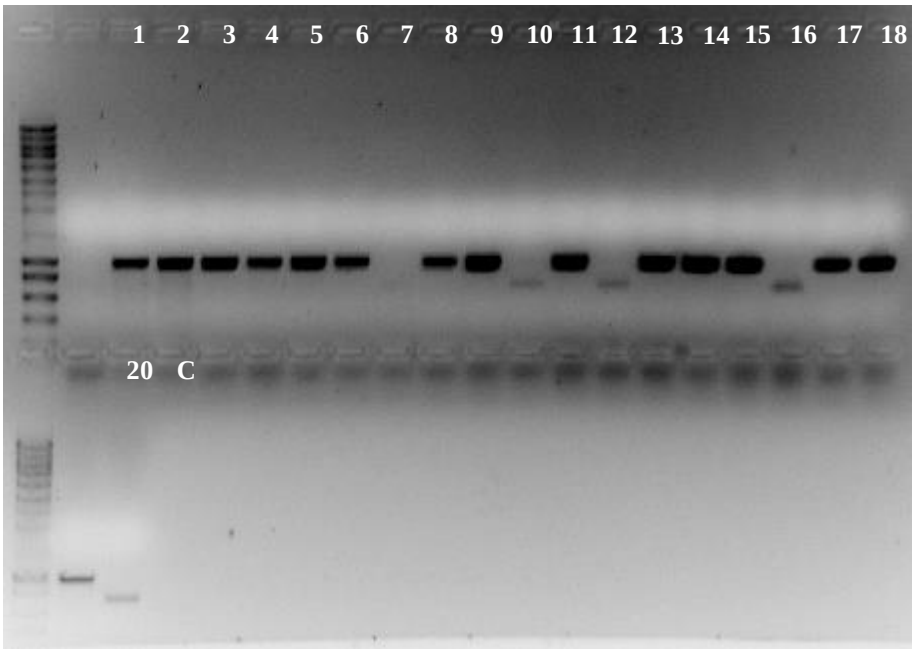
All crosses were performed using a 50:50 ratio of h+ and h- strains, previously washed in PMG to increase the frequency of mating. Each mixture was inoculated in ME plates and incubated for 2 days at 25ºC in order to induce mating [63].

A protocol for killing vegetative cells (cells that did not mate) was then applied using ethanol (20%) and snail juice (*Helix pomatia* extract) overnight at 25ºC with agitation. After that, the remaining meiotic spores were inoculated in YES plates (unless otherwise mentioned).

PCR analysis

For PCR applications, DNA was extracted using a Lithium acetate-based protocol that involves the lysis of yeast colonies in lithium acetate-SDS solution and the subsequent precipitation of DNA with ethanol. Colonies can be retrieved from solid or liquid cultures and suspended in 100uL of 200nM LiOAc, 1%SDS solution. They were left incubating at 70ºC for 10 minutes in this solution after which we added 300uL of

EtOH (100%), spinned down DNA and cell debris at 15000 g for 3 minutes and washed the pellet with 500uL of EtOH (70%). We pelleted DNA and cell debris from this last wash with ethanol and dissolved it in 100uL of H2O, to spin down cell debris and collect supernatant containing genomic DNA that can now be stored at 20ºC and used for PCR. We used a PCR cycle for mating-type assay (SI-Table6) and visualized PCR products using gel electrophoresis.



Supplementary Figure 1: Gel electrophoresis bands of mating type PCR products from the 20 clones selected from replica plating the spores from the cross between MGF 1255 and SPP2. The upper product band corresponds to the h+ mating type (987bp), and the lower corresponds to the h- mating type (729bp). Some clones didn't give a product band. The last band (C) corresponds to genomic DNA used as a positive control for the PCR.

Fitness estimates

The change in frequency of mCherry-labeled ContHIN cells across competition timepoints was used to estimate fitness. If we assume exponential growth, then:

$$\textbf{Equation 1} \qquad \boldsymbol{N(t) = N(0) * W^t}$$

where N(t) is the number of cells at time t and W is the fitness of those cells. We define relative fitness as

$$\textbf{Equation 2} \qquad \boldsymbol{W_R = \frac{W_n}{W_M}}$$

where WR is the relative fitness of the unmarked (n) strain when compared to the reference marked strain (M). By combining equations 1 and 2 we have

$$\textbf{Equation 3} \quad W_R^t = \frac{p(t)[1-p(0)]}{p(0)[1-p(t)]}$$

where p(t) is the frequency of unmarked cells at time t and 1-p(t) is the frequency of ContHIN cells.

Defining the selection coefficient, *s*, as

$$\textbf{Equation 4} \quad s = \ln W ,$$

from equation 3 we get

$$\textbf{Equation 5} \quad st - \ln \frac{1-p(0)}{p(0)} = \ln \frac{p(t)}{1-p(t)} .$$

Equation 5 defines a linear relationship between the natural logarithm of the ratio of frequencies and time (measured as number of generations for each growth cycle of the strain of interest: 8 for ContHIN and 5 for HIN strains). By performing least squares linear regression we can estimate the slope of this line which gives us *s*. We can then estimate fitness using equation 4.

All HIN replicates were competed against a ContHIN reference clone and all ContHIN replicates were competed against a HIN reference clone. Fitness of ContHIN strains was estimated relative to the relative fitness of ContHIN reference when competed against the HIN reference clone. Therefore, by definition, ContHIN reference has a fitness of 1 and the fitness of all the other replicates, either HIN or ContHIN, can be read in comparison to ContHIN reference.

For the competitions in the fluctuating environment setup, we changed the medium at every passage and estimated fitness based on the slope associated with each growth cycle separately, corresponding to the fitness values in each medium in the cycle of alternate media in these competitions. We then estimated the average of all the fitness values measured across all media in each competition.

2. Supplementary Tables

Supplementary Table 1: *Saccharomyces pombe* strains that were used in this study.

Strain Name	**Mating type**	**Genotype**	**Parents**	**Creator**
High INstability 1 (HIN1)	h-	*leu1-32 ura4-D18 ctp1::hph xlf1.T180A.S192A::lox P/M taz1::kan (1clone)*		C.Reis
High INstability 2 (HIN2) (4 clones)	2 clones h+ 2 clones h-	*leu1-32 ura4-D18 ctp1::hph xlf1.T180A.S192A::lox P/M taz1::kan (4clones)*		C.Reis
ContHIN (5 clones)	3 clones h+ 2 clones h-	*leu- ura- hph* ^{r}	MGF 1255 *x* SPP 2	A. C. Morais
MGF 1255	h+	*ade6-M216 his3-D1 leu1-32 ura4-D18 lys4-mCherry-hphMX6R*		A.T. Avelar
SPP 2	h-	*WT*		LP - YGRC

Supplementary Table 2: Scheme showing the distribution of strains in the deep-well plates used to estimate cell density of HIN and ContHIN strains growing in four different media (EMMG, EMMM, PMGG and PMGM). The media were distributed according to the scheme a) and the strains were distributed according to scheme b). Note that there are two replicates of this assay per plate: the first replicate is displayed in the first four lines (A-D) and the second one in the last four lines (E-H). The sequence of the media is the same in both replicates. ContHIN is marked with mCherry and is therefore represented in red.

a)	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	EMMG						EMMG					
B	EMMM						EMMM					
C	PMGG						PMGG					
D	PMGM						PMGM					
E	**R2**						**R2**					
F												
G												
H												

b)	1	2	3	4	5	6	7	8	9	10	11	12
A	HIN1 (clone1)	HIN2 (clone2)	HIN2 (clone3)	HIN2 (clone4)	HIN2 (clone5)		ContHIN (clone1)	ContHIN (clone2)	ContHIN (clone3)	ContHIN (clone4)	ContHIN (clone5)	
B												
C												
D												
E												
F												
G												
H												

Supplementary Table 3: Scheme showing the distribution of strains in the deep-well plates used during the Evolution Experiment. There are 12 wells distributed evenly throughout the plates that were left in blank (no cellular material was inoculated at the starting of the experimental evolution, but the wells were always filled with fresh medium nonetheless) to control for contaminations during the passages. ContHIN is marked with mCherry and is therefore represented in red.

	1	2	3	4	5	6	7	8	9	10	11	12
A	HIN1 (clone1)						ContHIN (clone1)					
B												
C												
D												
E	HIN2 (clone2)						ContHIN (clone2)					
F	HIN2 (clone3)						ContHIN (clone3)					
G	HIN2 (clone4)						ContHIN (clone4)					
H	HIN2 (clone5)						ContHIN (clone5)					

Supplementary Table 4: Random cycle of alternating media used during the evolution experiment in a fluctuating environment.

Media:	**Passage:**
EMMG	T0
PMGM	T1
PMGM	T2
EMMM	T3
PMGG	T4
PMGM	T5
EMMG	T6
PMGM	T7

Media:	**Passage:**
PMGM	T8
EMMG	T9
PMGG	T10
PMGM	T11
EMMG	T12
PMGG	T13
PMGG	T14
EMMM	T15

Media:	**Passage:**
EMMG	T16
EMMM	T17
EMMG	T18
PMGG	T19
PMGG	T20
PMGM	T21
EMMM	T22
EMMG	T23
PMGM	T24

Supplementary Table 5: Primers that will be used in this study for mating-type PCR assay.

Primer name	**Sequence 5' → 3'**
mat1-P forward (MP)	tacttcagtagacgtagtg
mat1-M forward (MM)	acggtagtcatcggtcttcc
MT1 reverse	agaagagagagtagttgaag

Supplementary Table 6: PCR cyles used for PCR-based mating-type assay.

MT-PCR			
		Temperature	Time
1x Hold		92ºC	5min
Nr. of cycles:	30		
	Denaturation	92ºC	30sec
	Annealing	50ºC	30sec
	Extension	72ºC	2min
2x Hold		72ºC	10min
		4ºC	∞

3. Supplementary Data

Supplementary Table 7: Population sizes of HIN replicates after adaptation to a constant environment.

12500000	1800000	2850000	2950000	4100000	5550000
9300000	700000	4050000	950000	1550000	4550000
5100000	6400000	3400000	3200000	1200000	2750000
2850000	6950000	2200000	1000000	1000000	6900000
6850000	4650000	2900000	9050000	450000	5000000
3950000	1350000	2000000	3800000	1150000	
4000000	3750000	5600000	600000	1300000	
4900000	3550000	2600000	750000	9250000	

Supplementary Table 8: Population sizes of HIN ancestors after one growth cycle in EMMG.

390000	1315000	1720000
3050000	1015000	860000
1090000	940000	1105000
630000	3400000	977500
895000	1750000	2575000

Supplementary Table 9: Population sizes of ContHIN replicates after adaptation to a constant environment.

6150000	6850000	1700000	3250000	1900000	5400000
7700000	4300000	1550000	2250000	3950000	5050000
7750000	4750000	1750000	4900000	3000000	4600000
6300000	2400000	4050000	3800000	3150000	850000
2750000	4650000	2350000	2250000	6500000	2250000
9200000	2500000	1750000	3350000	7150000	3250000
8500000	2200000	1500000	3050000	5350000	4700000

Supplementary Table 10: Population sizes of ContHIN ancestors after one growth cycle in EMMG.

17000000	7500000	12500000
8000000	11500000	8750000
12500000	14500000	11500000
5000000	19500000	13000000
15500000	18500000	19000000

Supplementary Table 11: Population sizes of HIN replicates after adaptation to a fluctuating environment.

6100000	2900000	5550000	4750000	1160000	6800000
5600000	8350000	3000000	3200000	2400000	3950000
16500000	10250000	4800000	4950000	896000	5600000
6500000	6350000	4150000	2000000	5750000	2350000
6250000	2200000	3450000	1648000	2250000	2650000
10350000	7000000	6350000	5400000	3500000	4600000
5150000	6100000	3600000	0	1960	

Supplementary Table 12: Population sizes of HIN ancestors after one growth cycle in PMGM.

82500	8550000	2341250
4600000	438000	1200500
251000	950000	4289000
2150000	4100000	694000
28000	3900000	4000000

Supplementary Table 13: Population sizes of ContHIN replicates after adaptation to a fluctuating environment.

146500	110000	3750000	8550000	1176000	2650000
275500	5100000	139000	5800000	3350000	11250000
4850000	8000000	2200000	252000	2550000	5000000
7700000	8100000	7950000	880000	5600000	6600000
6450000	3900000	3350000	3300000	2144000	4450000
5150000	109000	6600000	5200000	6600000	4200000
241000	206500	4600000	5650000	4350000	4500000

Supplementary Table 14: Population sizes of ContHIN ancestors after one growth cycle in PMGM.

7000000	15500000	7250000
7500000	7000000	10000000
8000000	4000000	12500000
12000000	25500000	5500000
9500000	14000000	19750000