Evolution in changing environments

heavy metal adaptation
in experimental yeast populations
as a case study

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heavy metal adaptation in experimental yeast populations as a case study

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Thesis

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Chapter 1

General introduction

Evolution on human time scales

In late 1878, Charles Darwin received a letter from the British entomologist Albert Brydges Farn. Farn shared with Darwin his observation that over the past 25 years, a black variety of the Annulet moth (*Charissa obscurata*) had dramatically increased in frequency on the smoke-blackened chalk slopes over a lime-kiln. He explicitly made the link with Darwin's then recently published theory of natural selection (Darwin 1859), and wrote to him "*Do these variations point to the "survival of the fittest"? I think so*" (Farn 1878). In retrospect, this letter was probably the first documented record of natural selection acting on human time scales (Hart et al. 2010). However, Darwin does not seem to have fully appreciated the significance of the observation, because he never replied to Farn.

Over the next century and a half, much research effort would be directed at gaining a better understanding of the phenomenon reported by Farn — industrial melanism — in the moth species *Biston betularia*. Until the mid of the 19th century, *B. betularia* was described consistently as having white wings speckled with black dots, explaining why it was commonly called the peppered moth (Figure 1.1A). In 1848, the first black — melanic — morph (*f. carbonaria*; Figure 1.1B) was reported near Manchester, and by the end of the 19th century this morph had in some locations almost completely displaced the original variety (Cook

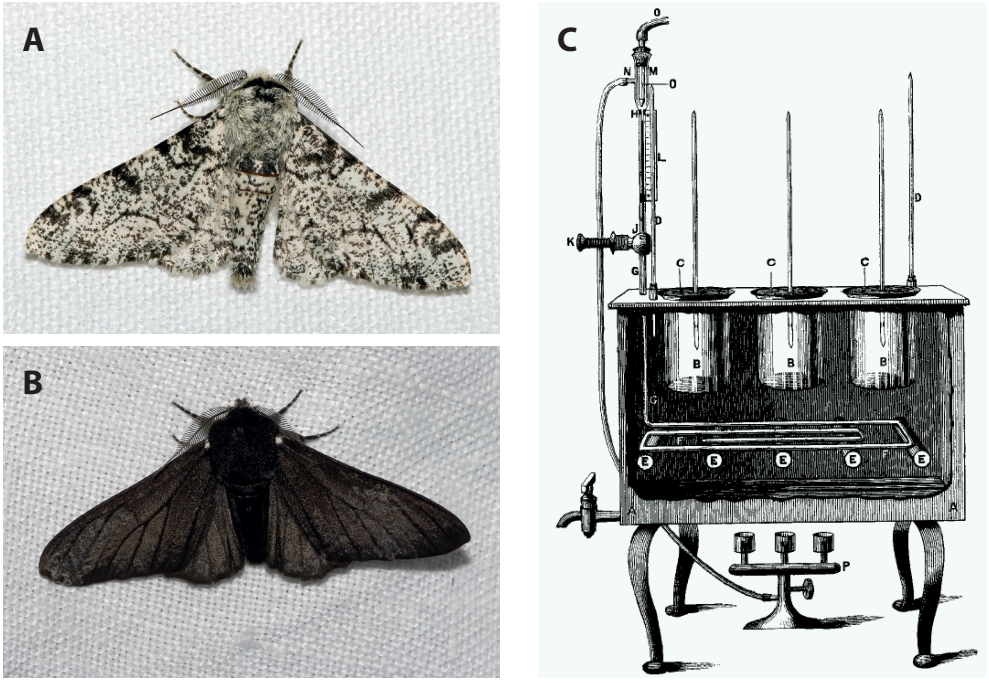

Figure 1.1: Early models of evolution in action. A) *B. betularia f. typica* (standard morph). B) *B. betularia f. carbonaria* (melanic morph). C) Dallinger's incubator.

2003). Several studies have now shown that this displacement was caused by widespread pollution, which killed the light-colored lichens covering the birch tree trunks on which the night flying moth *B. betularia* normally rests during the day, thus increasing the visibility of the original variety (*f. typica*) to its bird predators (Bishop et al. 1975). This resulted in very strong selection for the melanic morph, although other factors such as migration, frequency dependent selection, and an inherent advantage to the melanic morph via increased immunity or heavy metal chelation likely also played a role (Cook 2003; Riley 2013). The strongest proof for the link between pollution and industrial melanism came in the second half of the 20th century, when stronger regulations led to a gradual decrease in air pollution, and, accordingly, a decrease in melanic morph frequency. As a result, melanic frequencies have now been restored almost to their original pre-industrial levels (Cook and Saccheri 2013). The genetic basis of the melanic morph has recently been traced back to a single mutational event that occurred around the year 1819, which fits in remarkably well with the observed phenotypic dynamics (van't Hof et al. 2011; van't Hof et al. 2016).

While the rise and fall of industrial melanism in *B. betularia* is the first, and arguably the best, documented case of evolution occurring on human time scales (i.e. within less than a few hundred years), we now have abundant evidence that contemporary evolution is in fact quite common (Endler 1986; Thompson 1998; Stockwell et al. 2003). For example, natural selection repeatedly drove changes in beak shape and body size in two populations of Darwin's finches over the course of just 30 years (Grant and Grant 2002), adaptation to a novel freshwater environment occurred in three spine stickleback within a few decades (Lescak et al. 2015), and suppression of a parasite-induced sex ratio bias evolved in butterflies in as little as ten generations (Charlat et al. 2007). Many current evolutionary challenges are the result of human activities, and such challenges generally fall into one of two broad categories (Carroll et al. 2014). The first category concerns cases where rapid evolution occurs, but is considered undesirable. This is the case for the evolution of drug and pesticide resistance in public health and agricultural settings (Palumbi 2001), but also for evolution resulting from overharvesting or the introduction of exotic species (Stockwell et al. 2003; Darimont et al. 2009). The second category concerns cases where no or little evolution is thought to occur, but adaptation would be considered desirable. This is the case for biodiversity loss as a result of habitat degradation and fragmentation, as well as for yield loss as a result of climate change (Stockwell et al. 2003; Carroll et al. 2014).

Cases from this second category have traditionally been the focus of conservation biology. This field is strongly based in ecology and quantitative genetics and is concerned primarily with adaptation in small populations of relatively long-lived, complex organisms (Alexander et al. 2014). It asks important questions about whether and how populations can adapt to human-induced threats, such as climate change and pollution: are populations able to adapt, or will they become extinct? What are the important factors determining their fate? For example, does it matter how fast the environment changes? What measures can we take to

ensure long-term population persistence? Because of conservation biology's traditional focus, these questions are normally phrased in terms of phenotypic plasticity, range shifts, and the maintenance of genetic diversity (Przybylo et al. 2000; Charmantier et al. 2008; Chen et al. 2011; Merilä and Hendry 2014). Although the field makes use of several population genetic concepts, it does not usually consider evolution to be a process that can play an appreciable role on the time scales relevant for conservation (Ashley et al. 2003; Stockwell et al. 2003). However, as illustrated by the examples discussed above, as well as a long list of other cases, evolution is not just a long-term process, but can in fact occur readily on what have traditionally been regarded as ecological time scales (Hendry and Kinnison 1999; Hendry et al. 2008; Hoffmann and Sgro 2011). As such, evolution may play an important role in adaptation to changing environments, particularly if changes are large and persistent, because under such circumstances phenotypic plasticity and range shifts cannot compensate sufficiently for fitness losses, and evolution is the only adaptive process with potentially appreciable effects (Reed et al. 2011). Thus, a more thorough understanding of the potential and limitations of evolution for adaptation in populations exposed to deteriorating conditions is urgently needed.

In this thesis, I investigate how the rate of directional environmental change affects the course and outcome of evolution using experimental evolution of cadmium (Cd), nickel (Ni), and zinc (Zn) tolerance in yeast as a model system. Experimental evolution has been defined as research in which populations are studied across multiple generations under defined and reproducible conditions (Garland and Rose 2009). This is a very broad definition, and indeed there are many studies that fall into this category. However, the definition highlights an important property that these studies have in common: traditionally, evolutionary biology was mostly an observational science, relying on looking into the past by describing patterns in extant species and interpreting these to discern the possible role of each evolutionary force. As a result, findings from evolutionary studies were always fully "realistic" in that they considered the evolutionary process under the full complexity of its natural ecological settings. However, such realism necessarily trades off with precision and generality (Levins 1968; Morin 1998). Because in nature many different factors are at play simultaneously, the observational approach makes it hard to reliably ascribe findings to particular explanatory factors. Conversely, by actively manipulating conditions and replicating experiments, it is much easier to draw conclusions about cause and effect and thus increase our understanding of the mechanisms underlying particular observations. The baker's yeast, *Sacchoromyces cerevisiae*, is an important model organism in many disciplines, including experimental evolution (Landry et al. 2006; Zeyl 2006; Replansky et al. 2008; Botstein and Fink 2011). It has several attractive properties that make it particularly well suited for addressing the questions that this thesis attempts to answer, as I will discuss in more detail below. Few experimental evolution studies have used heavy metals as a selective agent (but note Adamo et al. 2012; Kucukgoze et al. 2013; Gerstein et al. 2015). However, metals are ubiquitous

components of the natural world, which are broadly relevant because some of them are required in small amounts by virtually all organisms. Yet, they can also be highly toxic, and as such metal pollution constitutes an important societal problem (Boyd 2010; Bánfalvi 2011).

In the following sections, I will begin by giving a short overview of the history, major themes, and current status of the field of experimental evolution, with an emphasis on microbial experimental evolution. Within this context, I will also discuss the choice for yeast as a model. Next, I will give some background information about heavy metals, the roles that they play in living organisms, and the mechanisms that have evolved for dealing with these elements, with a particular focus on *S. cerevisiae* and Cd, Ni, and Zn. Then, I will discuss the existing theory regarding evolution under different rates of directional environmental change, where possible in direct relation to the study system, to make more explicit the hypotheses that I want to address. Finally, I will briefly summarize each of the subsequent chapters.

Experimental evolution

History

While artificial selection is probably as old as humanity, the use of natural selection experiments — in which selection results from the "struggle for existence" (Malthus 1826) in an imposed environment, rather than a predefined criterion according to which the experimenter picks particular individuals — has become popular only relatively recently. The first such experiment, which shows a remarkable resemblance to current studies, was published in 1887 by William Dallinger (Figure 1.2C; Dallinger 1887). Dallinger cultured three species of protist in a custom-built incubator for a period of seven years, during which he gradually increased the temperature from 16°C to a final 70°C, a temperature at which the original organisms were unable to persist. The evolved organisms appeared to thrive at the final temperature, but could no longer grow under the original conditions. These findings directly demonstrated for the first time the power of evolution on limited time scales.

After Dallinger's experiment, few studies of this sort would be conducted for almost a century. At least part of the explanation for this hiatus is probably the way in which the modern synthesis (i.e. the reconciliation of Darwinian natural selection and Mendelian inheritance) was presented by R.A. Fisher in 1930. Darwin, in his publications on adaptation by natural selection, emphasized repeatedly that he considered evolution to be extremely gradual (Darwin 1859, 1871). This view initially appeared to be fundamentally at odds with the particulate nature of heredity proposed by Gregor Mendel (Mendel 1865). The two theories were only formally united when Fisher showed that it requires remarkably few loci inheriting according to Mendelian principles to arrive at the type of continuous trait distribution consistent with Darwinian evolution (Fisher 1922, 1930). However, in his synthesis, Fisher not only showed that evolution can proceed by very small steps, he also suggested that evolution should generally

proceed by very small steps. His reasoning was based on the assumption that mutations simultaneously affect all traits (universal pleiotropy). From this follows that mutations of large phenotypic effect are more likely to be detrimental, because they have a higher chance of decreasing an organism's fit to its environment in at least one respect. Fisher illustrated this argument with the analogy of a microscope: when an image is already close to focus, you are more likely to improve sharpness with a random turn on a fine knob than with a random turn on a coarse knob. This micro-mutationist view was very influential throughout much of the 20th century, and was supported by key figures in the field such as Theodosius Dobzhansky, Hermann J. Muller, and Julian Huxley (Orr 2005). Therefore, cases of natural selection in the wild were generally considered exceptions, and for a long time hardly any natural selection experiments were attempted (but see Novick and Szilard 1950; Atwood et al. 1951), because people did not expect to actually be able to witness evolution on experimental time scales (Garland and Rose 2009; Bataillon et al. 2013).

It was only in the 1980s that this view began to change (Orr 2005; Tenaillon 2014). At that time, theoretical work by Motoo Kimura brought to the attention that the probability that a mutation will contribute to adaptation depends not only on whether it is beneficial, but also on its effect size (Kimura 1983). Mutations of small effect have a larger probability of being beneficial, but they also have a larger probability of being lost due to genetic drift. Consequently, adaptation should mostly proceed by mutations of intermediate effect. In fact, Kimura's argument would later be extended to predict that over the entire course of the adaptive process, evolution should make use of mutations of exponentially distributed effects, with large effect mutations fixed first (Orr 1998, 1999). Experimental work from this period pointed in similar directions. Quantitative trait loci (QTL) analyses on an increasing number of species revealed that a considerable proportion of all natural trait variation is attributable to relatively few loci of large effect (Tanksley 1993; Kearsey and Farquhar 1998; Orr 2001). Additionally, the development of a universally applicable measure of the strength of selection (Lande and Arnold 1983) allowed for the first time the large-scale assessment of the significance of natural selection in the wild, which revealed that selection is often strong (Endler 1986; Conner 2001; Kingsolver et al. 2001; Hereford et al. 2004). At the same time, the first laboratory evolution experiments using *Drosophila melanogaster*, *Escherichia coli*, and *S. cerevisiae* showed that evolutionary adaptation can indeed be observed at relatively short time scales (Paquin and Adams 1983; Rose 1984; Bennett et al. 1990). For the microorganisms these experiments additionally revealed that, contrary to what was commonly assumed, they are often subject to complex evolutionary dynamics resembling to a considerable extent the dynamics observed in larger organisms (Helling et al. 1987).

Major themes

The field of microbial experimental evolution in its current form finally took off with the start of the long-term evolution experiment (LTEE), which has been running in the lab of

Richard Lenski at Michigan State University ever since February 1988 (Lenski et al. 1991; Maddamsetti et al. 2015). The set-up of this experiment is deceptively simple, but captures several important properties shared by most experiments in the field today. Twelve replicate population of *E. coli* are grown in glucose limited liquid media, and a small part of each population is transferred to a new flask every single day. Nutrients are limiting, and as such each population goes through ~6.64 generations on a daily basis, adding up to over 66,000 generations as of October 2016. Like all microorganisms, *E. coli* can be stored in suspended animation at -80˚C and revived whenever required. This has allowed researchers to build up a "frozen fossil record" over the years, making it possible to directly compare performance of evolved isolates with that of their ancestors (be it distant or more recent), and to replay evolution from various starting points. The large effective population sizes in the experiment ($\sim 3.3 \times 10^7$) imply that sufficient variation for selection to act upon can be generated by *de novo* mutations, and that selection is generally strong relative to drift.

The LTEE, and an increasing number of other studies with conceptually similar set-ups (see Figure 1.2), have greatly increased our understanding of the evolutionary process. Important insights have been obtained on many different levels, ranging from the rate at which mutations arise to the conditions that favor the evolution of multicellularity (for an overview see Elena and Lenski 2003; Buckling et al. 2009; Garland and Rose 2009; Kawecki et al. 2012). Some of the most fundamental topics that have been addressed include the generation of genetic variation (i.e. mutation rates, mutation spectra, recombination, and sex (Cooper et al. 2007; MacLean and Buckling 2009; Wielgoss et al. 2013; Levy et al. 2015; McDonald et al. 2016)), how this variation translates into phenotype and fitness (i.e. pleiotropy, ploidy, dominance, epistasis, evolvability, and robustness (Draghi et al. 2010; Khan et al. 2011; Woods et al. 2011; Kryazhimskiy et al. 2014; Selmecki et al. 2015)), and how the combination of mutation, selection, and drift plays out over time. Several general patterns have emerged. First, adaptation usually occurs — at least initially — via the fixation of a limited number of mutations of relatively large effect. Second, increase in fitness and the number of beneficial mutations that get fixed slow down over time, but adaptation does not come to an end even after tens of thousands of generations (Wiser et al. 2013; Tenaillon et al. 2016). Third, mutations do not sweep to fixation one by one but instead compete with each other for prolonged periods of time (clonal interference (de Visser and Rozen 2006; Kao and Sherlock 2008)). Fourth, while chance plays an appreciable role in adaptation, particularly in small populations (Rozen et al. 2008), evolution in replicate populations is often remarkably parallel at the fitness, phenotypic, gene, and even nucleotide level (Bailey et al. 2015). Finally, the processes that generate heritable variation, as well as genetic architecture, may themselves evolve (e.g. mutations rates and evolvability can evolve (de Visser 2002; Woods et al. 2011)).

In all of the above studies, researchers have attempted to keep the environment as constant as technically feasible in order to make general inferences about the evolutionary process. However, in nature environments are seldom homogeneous or constant, and this can have important

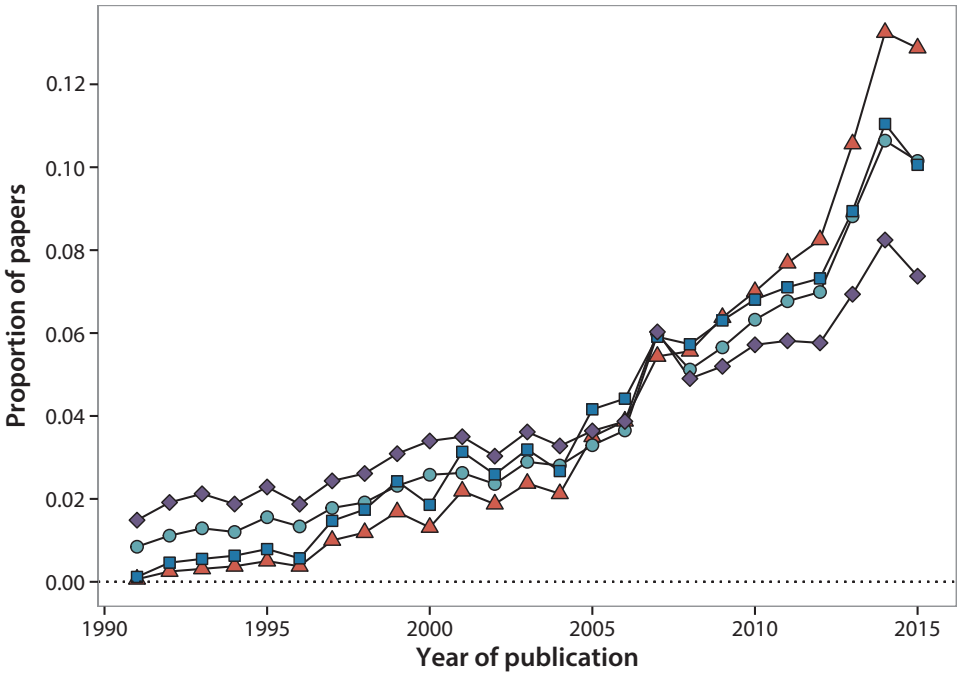

Figure 1.2: Experimental evolution continues to increase in popularity. Proportion of all experimental evolution studies since Lenski et al. 1991 that was published in each year, based on a search in ISI Web of Knowledge. Red: number of studies for keyword "experimental evolution". Blue: idem, corrected for overall increase in number of publications. Green: number of studies for keywords "experimental evolution" or "selection experiment". Purple: idem, corrected for overall increase in number of publications. Approach based on Buckling et al. 2009.

consequences for evolution. Even experimental conditions that appear homogeneous at first sight often contain different spatial or nutritional niches, and such heterogeneity generally leads to the evolution of diverse specialists, particularly when trade-offs are strong (Rainey and Travisano 1998; Kawecki and Ebert 2004; Le Gac et al. 2012; Herron and Doebeli 2013; Traverse et al. 2013; Frenkel et al. 2015). By contrast, conditions that fluctuate over time select mostly for one generalist type that has the highest geometric mean fitness across all environments (Reboud and Bell 1997; Turner and Elena 2000; Cooper and Lenski 2010), although bet-hedging or increased phenotypic plasticity may also evolve under such circumstances (Levins 1968; Beaumont et al. 2009; Leggett et al. 2013). Even more complex patterns of adaptation arise when organisms perform actions that affect the reproductive success of others (West et al. 2007; Damore and Gore 2012). Microorganisms can, for example, create new niches by excreting by-products that others can use (Finkel 2006; Rozen et al. 2009), collectively break down antibiotics or complex sugar molecules (Koschwanez et al. 2011; Artemova et al. 2015), secrete freely exploitable chelating molecules that serve in iron acquisition (Buckling et al. 2007), produce harmful toxins to

which they themselves are resistant (Inglis et al. 2009; Pieczynska et al. 2016), and divide labour amongst identical or closely related genotypes (Diard et al. 2013; Kim et al. 2016). Such actions may lead, depending on the details of the system, to dynamics such as positive or negative frequency-dependent selection, the evolution of cheating and multicellularity, and co-evolutionary arms races. Interspecific interactions have similarly been shown to give rise to complex co-evolutionary dynamics (Hall et al. 2011b; Meyer et al. 2012; Brockhurst and Koskella 2013; Hillesland et al. 2014), and the nature of such interactions can even evolve with time (e.g. commensalism can change to exploitation (Hansen et al. 2007) and amensalism can change to antagonism (Andrade-Dominguez et al. 2014)).

Current developments

Over the past decade, whole genome sequencing has become an increasingly popular tool in experimental evolution studies due to falling costs (Brockhurst et al. 2011; Conrad et al. 2011; Barrick and Lenski 2013; Jerison and Desai 2015). Before this time, researchers generally relied on phenotypic data, the tracking of a few genetic markers over time, and labour intensive genetic analyses, to make inferences about the genomics of adaptation. The first studies that directly investigated changes at the whole genome level were necessarily limited to small genomes such as those of viruses (Wichman et al. 1999; Sanjuan et al. 2007) or to a low number of evolved isolates (Velicer et al. 2006; Barrick et al. 2009; Araya et al. 2010), while other pioneering studies made clever use of techniques such as microarrays, which were originally developed for other purposes (Gresham et al. 2006; Gresham et al. 2008). By contrast, laboratory evolution studies nowadays increasingly often include whole-genome sequencing data for a large number of replicate clones, populations, and/or time points, resulting in much more detailed insight into the evolutionary process (Tenaillon et al. 2012; Wong et al. 2012; Kryazhimskiy et al. 2014; Tenaillon et al. 2016). For example, in one recently published study researchers sequenced whole population samples isolated at 12 different time points from 40 replicate evolution lines, which revealed extensive clonal interference between different mutational cohorts, i.e. groups of mutations that move synchronously through the population (Lang et al. 2013). Although such high level of replication is not yet — financially — feasible in all cases, this so-called evolve-and-resequence (E&R) approach is increasingly becoming the norm in experimental evolution (Long et al. 2015; Schlotterer et al. 2015).

Model organisms

A wide range of different organisms is used for laboratory evolution studies, and each of these has its own advantages and disadvantages. Among the most frequently used model systems are *D. melanogaster*, *S. cerevisiae*, *E. coli*, *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*, and various bacteriophages. When studying complex phenomena such as behavior, development, and other life-history traits, multicellular organisms with complex life cycles such as mice,

nematodes, and fruit flies are the preferred choice (Kawecki et al. 2012). However, an important drawback of these organisms is that they have relatively long generation times, and populations of more than a few thousand individuals cannot be practically maintained in the laboratory. Consequently, adaptation in studies with such organisms occurs mainly from standing genetic variation, and the outcome is subject to considerable stochasticity. By contrast, microbes, while lacking some of the more complex properties of larger organisms, have short generation times and large population sizes (Buckling et al. 2009). As such, they are ideal for investigating more general evolutionary questions about how mutation, selection, and drift play out over time (see **Major themes** section for examples), which are applicable to all organisms, but cannot be studied at the same level of replication or over the same evolutionary time scales in, for example, flies. An added benefit of microorganisms is that they are easy to maintain in the laboratory and can be stored frozen for future analysis. Furthermore, many tools are available for their genetic manipulation. Finally, microorganisms generally have small, well-annotated genomes, which greatly reduces the cost and complexity of whole-genome sequencing analysis.

Within this range of model systems, the baker's yeast *S. cerevisiae* occupies a special place. Yeast has all the attractive properties of other microorganisms, but it is also a eukaryote and can reproduce both sexually and asexually (Zeyl 2006; Voordeckers and Verstrepen 2015; Fisher and Lang 2016). As such, it is the model of choice when addressing questions about the effect of ploidy, the evolution of sex, speciation, and the relative importance of *de novo* mutation versus standing genetic variation for adaptation (Anderson et al. 2010; Burke et al. 2014; Selmecki et al. 2015; McDonald et al. 2016). The fact that it is a eukaryote implies that it is more closely related to plants and animals than bacteria and viruses. Consequently, observed evolutionary changes are more likely to be relevant for such higher organisms as well, which is particularly useful when one is interested in the genetic basis of a particular trait, such as tolerance to a specific stressor. Experimental evolution studies with yeast also have more direct applications, because *S. cerevisiae* is used extensively in biotechnology (Cakar et al. 2012; Amirnia et al. 2015; Voordeckers et al. 2015; Gonzalez-Ramos et al. 2016).

Heavy metals

Effects of heavy metals and mechanisms for dealing with them

Heavy metals, which have been defined as metals and metalloid elements with an atomic weight of 21 or more that may have harmful effects on biological systems, play an important role in both essential biological processes and toxicity due to their highly reactive chemical properties (Bánfalvi 2011). Trace amounts of some of these elements, such as Cu, Fe, Mn, Ni, and Zn, are essential nutrients for many living organisms, and a substantial part of all proteins requires such metal ions as cofactors for proper functioning (Gadd 1993;

Bleackley et al. 2011). However, at elevated concentrations these same metals are toxic. Other elements, such as Cd, Pb and Hg, are not required for biological function, but instead interfere with processes in which essential metals are involved because of their chemical similarity to these elements (Goyer 1997).

The toxic effect of a metal depends on its physicochemical properties, several environmental factors, and the affected organism (Gadd 1993; Giller et al. 1998). Nonetheless, there are some common ways in which metals may interfere with biological function. First, metal toxicity may be the result of the —indirectly triggered — formation of reactive oxygen species, which results in oxidative stress. Another common mode of action is the inhibition of enzyme function, either through the binding to thiol groups in cysteine residues, or through the competitive displacement of crucial cofactors. Such inhibition has particularly far-reaching consequences when the enzyme is involved in mismatch repair — as is the case for Cd and the Msh2p-Msh6p complex — because in that case it will increase mutation rates. Other encountered toxicity mechanisms include changes in membrane fluidity and protein folding (Hall 2002; Wysocki and Tamas 2010).

As high levels of metals exert strong selective pressures and are found in many natural and human influenced habitats, such as serpentine soils and mine spoil heaps, tolerance mechanisms have evolved in various species, including humans (Stokes et al. 1973; Macnair 1987; Chang and Leu 2011; Warringer et al. 2011; Chang et al. 2013; Cappa and Pilon-Smits 2014; O'Brien and Buckling 2015; Schlebusch et al. 2015). Ways for cells to cope with the harmful elements include binding to externally excreted compounds or to cell walls, reduced uptake, increased export, intracellular sequestration by means of chelating agents, and sequestration in specific parts of the cell, primarily the vacuole. In practice, metal tolerance often depends on a combination of several of these mechanisms, although the relative importance of each in a specific case may be hard to determine (Hall 2002; Wysocki and Tamas 2010).

Heavy metal homeostasis in *S. cerevisiae*

In *S. cerevisiae*, a wide range of proteins is involved either directly or indirectly in metal homeostasis. Deletion and overexpression mutant screens have identified hundreds of genes that affect metal tolerance or content (Eide et al. 2005; Jin et al. 2008; Ruotolo et al. 2008; Arita et al. 2009; Yu et al. 2012). Some of these genes are involved in the general stress response, while others can be categorized either as common metal-responsive genes or as metal-specific response genes (Wysocki and Tamas 2010). Transcriptome analysis supports this picture, and suggests that the chemical properties of a metal largely determine the changes in gene expression that it elicits, with a clear distinction between transcriptional responses to essential and non-essential metals (Jin et al. 2008).

For Cd and Zn, some of the key players involved in metal uptake, sequestration, and export in *S. cerevisiae* have been identified (Figure 1.3; Wysocki and Tamas 2010; Bleackley and MacGillivray 2011). In fact, these two metals share many pathways because of their

chemical similarity. Cd is a non-essential metal, and as such there are no transporters dedicated specifically to its uptake. Instead, it enters the cell via transporters involved in the uptake of the essential metals calcium (Mid1p), manganese (Smf1p), iron (Fet4p), and zinc (Zrt1p). The main route of Cd entry is through the high-affinity transporter Zrt1p, while evidence for Cd uptake through Mid1p and Fet4p is limited at present. Once Cd has entered the cytosol, there are several ways for yeast to deal with the metal. First, it may be detoxified through chelation to gluthatione (GSH) or to the metallothioneins Cup1p and Crs5p, although metallothioneins are probably of minor importance for Cd. Second, Cd can be excreted, either in its elemental form by the high-specificity transporter Pca1p, or as a Cd-GS conjugate by the multidrug transporter Yor1p. Interestingly, *S. cerevisiae* lab strains carry a non-functional version of *PCA1*, suggesting that these strains should be more sensitive to Cd than their natural counterparts (Adle et al. 2007). Finally, Cd can be sequestered into the vacuole, again either in its elemental form or as a conjugate, by the transporters Zrc1p or Bpt1p and Ycf1p, respectively.

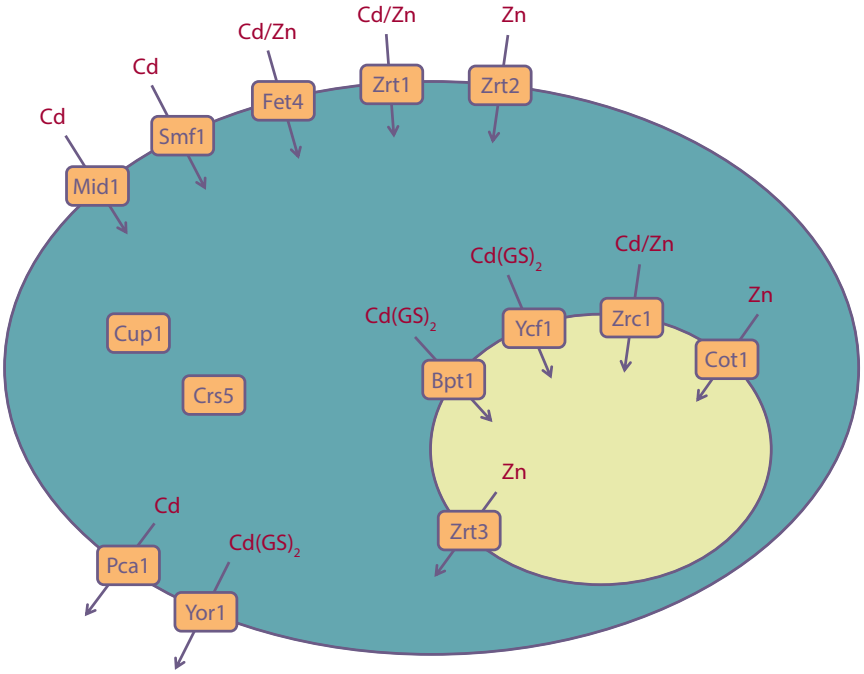

Figure 1.3: Key players involved in Cd tolerance and Zn homeostasis in yeast. Large green ellipse represents a yeast cell, small yellow ellipse represents the vacuole. Metals can enter or exit the cell and the vacuole via a number of transporters, which are represented by orange shapes with purple arrows. Some transporters are important either for Cd or for Zn, while others are important for both metals due to the chemical similarity of these elements. Metals can be transported either in their elemental form, or as gluthatione (GS) conjugates. Cup1p and Crs5p are important metallothioneins and localize to the cytoplasm. See text for details.

The genetic elements involved in Zn homeostasis overlap to a large extent with those involved in Cd tolerance. Like for Cd, the main route of Zn entry is through Zrt1p, although this high affinity transporter is expressed only under Zn deficiency conditions to prevent Zn over-accumulation. Under Zn replete conditions the low-affinity transporter Zrt2p is responsible for Zn uptake, and low amounts of Zn can also enter the cell through the iron transporter Fet4p (Wysocki and Tamas 2010; Bleackley and MacGillivray 2011). To maintain constant levels of Zn in the cytosol, several vacuolar transporters act either to remove Zn from the cytosol (Zrc1p and Cot1p), or to remobilize it from vacuolar storage (Zrt3p). Crs5p has a very high affinity for Zn, suggesting that chelation by this metallothionein may also play a role in Zn homeostasis.

Compared with Cd and Zn, surprisingly little is known about the way in which yeast cells deal with Ni. It has been suggested that Ni is transported into the cell via several broad-specificity divalent metal transporters, because mutant strains with reduced magnesium uptake are resistant to Ni (Joho et al. 1991; Bleackley and MacGillivray 2011). Ni may also enter the cell via iron transporters, because it was found to interfere with iron uptake (Chen et al. 2005). Additionally, several studies point towards a role for the vacuolar H^+-ATPase in sequestering Ni into the vacuole, possibly as a histidine conjugate (Joho et al. 1995; Nishimura et al. 1998; Arita et al. 2009). Because Ni interferes with Zrc1p-dependent Zn transport, it is also possible that Ni is transported into the vacuole by this protein.

Parallels with other organisms

A striking number of the transporters involved in Cd and Zn homeostasis in yeast has a human and/or plant ortholog with a similar function (Hanikenne et al. 2005; Thorsen et al. 2009). Zrt1p, Zrt2p, and Zrt3p belong to the Zrt- and Irt-like (ZIP/ SLC39) family of proteins, which are responsible for Zn transport into the cytoplasm in both humans and plants (Lin and Aarts 2012; Jeong and Eide 2013). Similarly, Smf1p is a member of the neutral resistance-associated macrophage protein (Nramp) family, which also has human and plant orthologs involved in metal transport into the cytoplasm (Portnoy et al. 2000; Nevo and Nelson 2006). Zrc1p and Cot1p are part of the cation diffusion facilitator (CDF/ SLC30/ MTP) family, and their human and plant orthologs are the Mn transporter SLC30A10 and the vacuolar Zn transporter MTP1, respectively (Lin and Aarts 2012; Tuschl et al. 2012). Ycf1p, Bpt1p, and Yor1p are ATP-binding cassette (ABC) transporters, and are similar to the human cystic fibrosis transmembrane conductance regulator (CFTR) and *Arabidopsis thaliana* ABCC1 and ABCC2, which also transport Cd conjugates into the vacuole (Paumi et al. 2009; Lin and Aarts 2012). Finally, Pca1p is a P-type ATPase. In *A. thaliana*, proteins from this family are responsible for Cd and Zn export from the cytoplasm, either into the xylem (HMA4) or into the vacuole (HMA3).

The above molecular parallels indicate a strong resemblance between metal tolerance mechanisms in yeast and higher eukaryotes. However, there are also important differences.

In animals, vacuoles do not play a significant role in metal sequestration, and instead these organisms rely more heavily on the action of metallothioneins for metal detoxification (Blindauer and Leszczyszyn 2010; Bleackley and MacGillivray 2011). Furthermore, animals and plants are multicellular. Consequently, they have evolved mechanisms for maintaining metal homeostasis not only at the single cell level, but also at the organismal level. The most striking example of such homeostasis is a phenomenon in plants called metal hyperaccumulation. Broadly speaking, plants can be divided into four different categories with respect to metal tolerance (Lin and Aarts 2012). First, they can be sensitive, which means that they are unable to deal with high metal concentrations and thus have reduced performance when growing on metallicolous soils. Second, they can be resistant, indicating that they keep metals out of their roots by manipulating external metal bioavailability, reducing metal uptake, and/or increasing metal export. Third, they can be tolerant, in which case they mitigate toxic metal effects by manipulating internal metal bioavailability and sequestering metals into the root vacuoles, thus preventing root-to-shoot translocation. Finally, the most interesting category concerns metal tolerant hyperaccumulators. These plants combine a higher uptake of metals from the soil with increased root-to-shoot transport and enhanced chelation and compartmentalisation in the shoot (Verbruggen et al. 2009; Rascio and Navari-Izzo 2011). Different ecological factors have been proposed to have driven the — repeated — evolution of this curious phenomenon, the most popular one being the presence of grazers and pathogens that are deterred by high metal concentrations in the shoot (Boyd 2007; Cappa and Pilon-Smits 2014).

Evolution of metal hyperaccumulation

An important outstanding question with respect to metal hyperaccumulation is how it evolved, because the trait involves dozens of changes in gene expression, and many of these changes are likely individually detrimental. One possible scenario is that the evolution of this trait started with a higher copy number of the vacuolar Zn transporter MTP1, which increased expression of this gene in the shoot (Shahzad et al. 2010). This would have increased shoot metal tolerance, but simultaneously conveyed a Zn deficiency signal to the roots, thus upregulating the ZIP transporters involved in metal uptake from the soil. Subsequently, an increase in copy number of the HMA4 transporter responsible for root-to-shoot transport could have established hyperaccumulation, while at the same time further increasing ZIP transporter expression (Hanikenne et al. 2008; Guimaraes et al. 2009). Other expression changes might then have resulted either from internal feedback mechanisms, or from evolutionary fine-tuning of the trait once it reached appreciable frequencies in the population. All in all, this scenario is rather speculative. Crucially, however, it includes only steps that are beneficial by themselves under a scenario of combined selection for increased tolerance and enhanced herbivore and pathogen determent, and such evolutionary accessibility of mutational pathways is an important prerequisite for the origin of a novel trait.

Clearly, metal hyperaccumulation in the strict sense cannot evolve in yeast, because it requires the presence of differentiated structures. However, many of the key genes that play a role in this trait in plants (e.g. ZIP4, HMA4, and MTP1) have orthologs in yeast, and a single cell equivalent of metal hyperaccumulation involving increased uptake, chelation, and sequestration in the vacuole could theoretically constitute a strategy to deal with high metal concentrations. Like for plants, the evolution of such a trait could be driven by the presence of grazers. However, for yeast this scenario would additionally require a spatially structured environment: only in that case could metal hyperaccumulation protect clone mates from grazing, because a fungivore might be deterred by the high metal concentrations it encountered when consuming part of the colony. Metal hyperaccumulation in yeast could also be a social trait if it significantly reduces the external metal concentration. More specifically, it might be a mutually beneficial trait (West et al. 2007), because some individuals will likely pay the cost of hyperaccumulating the metal, while all cells in the neighbourhood can benefit from the reduction in external metal concentration conferred by the trait. In that case, hyperaccumulation should evolve primarily in relatively low-density, spatially structured environments, where clone mates have preferential access to the "metal-free" space, and the scope for invasion by cheaters is limited. Indeed, it was previously shown that the expression of social metal tolerance traits can have important consequences for eco-evolutionary dynamics. In the bacterium *P. fluorescens*, collective periplasmic reduction of mercury to its non-toxic form by a plasmid-encoded resistance operon led to negative frequency-dependent selection between resistant and sensitive subpopulations across a wide range of metal concentrations (Ellis et al. 2007). Similarly, in the presence of high concentrations of copper, cheating *P. aeruginosa* cells, which do not excrete the costly copper-chelating siderophore molecules required for resistance, repeatedly evolved de novo and invaded experimental populations (O'Brien et al. 2014).

Models of evolution in directionally changing environments

Quantitative genetic models

In nature, environments frequently change in one specific direction, and such change may have important consequences for the persistence and evolution of the organisms inhabiting these environments. Among the first fields to investigate how different rates of directional environmental change affect evolution was conservation biology. This discipline is motivated primarily by concerns about population persistence in the face of human-induced climate change and pollution (Alexander et al. 2014). Accordingly, its main research question is: are populations able to adapt, or will they become extinct? Because conservation biology focuses on comparatively small populations of large, sexually reproducing organisms in which *de novo* mutations are rare, this question is generally addressed using quantitative

genetic models (Figure 1.4A; orange distributions). Such models in their simplest form assume that individuals in a population vary according to one (or a few) continuous traits that are determined by a large number of independently segregating loci of small effect (Lynch and Lande 1993; Gomulkiewicz and Holt 1995). The population as a whole can then be described by a Gaussian (or multivariate Gaussian) distribution with a given mean and variance (or variance-covariance matrix when multiple traits are considered (Gomulkiewicz and Houle 2009; Chevin 2013)). When the population mean is not equal to the optimal trait value, selection will act on the additive genetic variance to shift the trait distribution in the direction of the optimum. Fitness decreases with increasing distance to the optimum, and if the mismatch between the population and the optimum is sufficiently large, the population will become extinct (Burger and Lynch 1995). When there is a sudden large change in the environment, the optimum will make a big jump, and when the environment changes more gradually, the optimum will move more slowly. There is some consensus that overall the maximum rate at which the population mean can track the optimum is 0.1 *haldanes* (phenotypic standard deviations per generation (Kinnison and Hendry 2001; Kopp and Matuszewski 2014)). Consequently, when environmental change is fast enough for it to temporarily reduce absolute mean population fitness below 1, a "race" between population decline and evolutionary adaptation will occur. When this race is "won" by evolution, we speak of evolutionary rescue (Bell and Collins 2008; Gonzalez et al. 2013). Several empirical studies have investigated the factors determining the probability of rescue, and in line with theoretical predictions these studies have found that rescue is promoted by higher genetic variance, larger population sizes, and a lower rate of change (Carlson et al. 2014).

Discrete phenotypic models

While the above models are useful for understanding whether populations will persist, they provide little information about the genetic basis of adaptation and largely ignore *de novo* mutations (Tenaillon 2014). Specifically, they make no explicit predictions about the order, type, and number of mutations that are selected over the course of the adaptive process under different rates of directional environmental change. However, for a thorough understanding of the long-term consequences of evolution such information is crucial, particularly when individual mutations have large effects (Agrawal et al. 2001; Tenaillon 2014). One way to overcome this limitation is to consider the properties of adaptive walks — that is, series of allelic substitutions caused by natural selection — when the optimal phenotype changes either gradually or more abruptly in response to changes in the environment, as in the quantitative genetic models described in the previous paragraph (Figure 1.4A; black arrows). Several theoretical studies have used this so-called moving optimum framework to ask how the rate of directional environmental change affects: i) the order in which mutations of different phenotypic effect are fixed (Kopp and Hermisson 2007), ii) the expected time to fixation for mutations of different phenotypic effect (Kopp and Hermisson 2009a), and

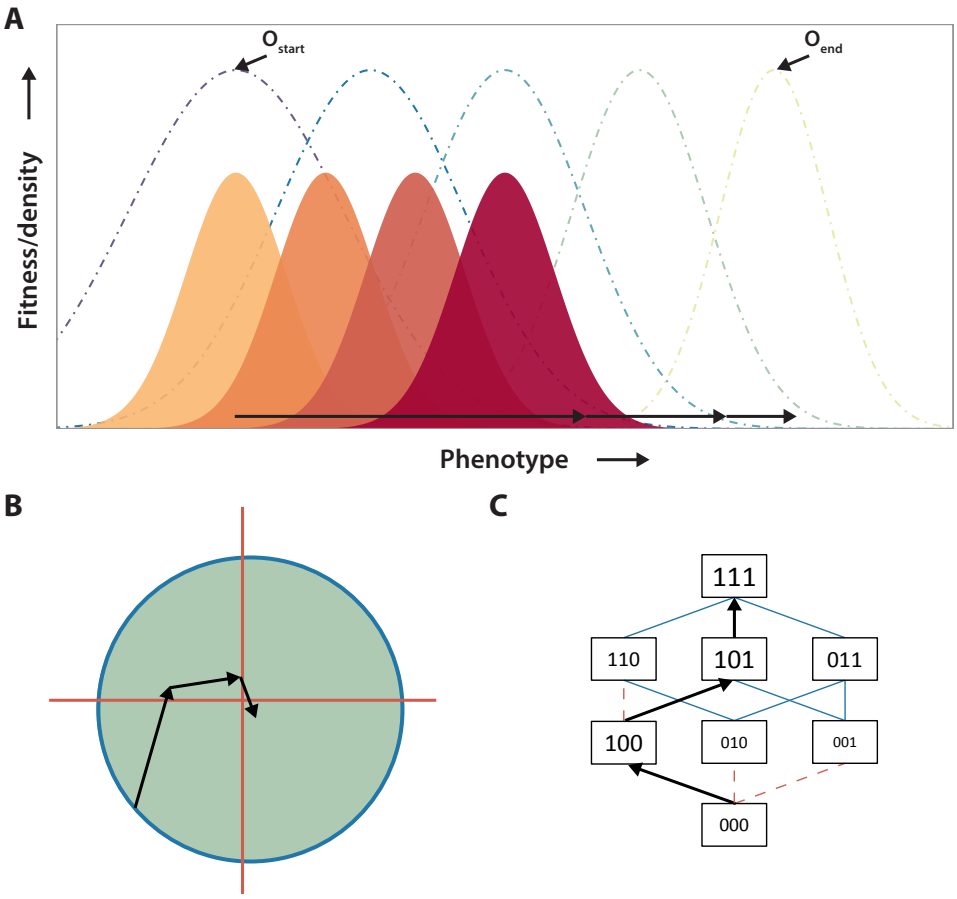

Figure 1.4: Models used for understanding evolutionary adaptation in changing environments. A) One-dimensional moving optimum model. Fitness (dot-striped lines) decreases with distance to the optimum phenotype. Over time, the optimum phenotype (O) shifts from O_{start} to O_{end}, either gradually or more abruptly. The strength of selection decreases with the width of the fitness function. In quantitative genetic models evolving populations (distributions) are characterised by a mean and variance, and track the moving optimum in response to selection. When the mismatch between a population and the optimum phenotype is large, populations may decrease in size and go extinct unless they are rescued by evolution. Moving optimum models are also used for investigating the properties of individual mutations fixed over the course of adaptive walks (black arrows). B) Fisher's geometric model with two phenotypic dimensions (red axes). All mutations that fall within the green area are beneficial. Over the course of an adaptive walk, several mutations (black arrows) are fixed that bring the population progressively closer to the optimum (intersection of red lines). In changing environments, the location of the optimum changes over time. C) Local fitness landscape with three loci. 0s and 1s indicate the presence of alternative alleles at each locus (position), and font size of the strings reflects fitness. The original genotype, 000, may evolve to the genotype residing at the fitness peak, 111, but only the mutational pathway indicated by black arrows is accessible when populations evolve via natural selection of single-mutation steps. Some mutations decrease fitness (red dotted lines), and there is reciprocal sign epistasis at the level of genotypes 000, 010, 001, and 011 because for locus 2 and 3, the sign of the mutational effect depends on the allele that is present at the other locus. Upon a change in the environment, genotype fitnesses change, and this can fundamentally alter the height and/or shape of the fitness landscape.

iii) the distribution of phenotypic effects over the entire course of the adaptive process (Kopp and Hermisson 2009b). The main prediction following from these studies is that gradual environmental change should lead to the repeated fixation of mutations of intermediate phenotypic effect. By contrast, abrupt environmental change should lead to the fixation of mutations of exponentially distributed phenotypic effects, with large effect mutations being fixed first (Orr 1998, 2002). Recently, these models have been extended to multiple phenotypic dimensions, to yield what the authors termed "Fisher's geometric model with a moving optimum" (Matuszewski et al. 2014). That is, as in Fisher's classic geometric model (Figure 1.4B; (Fisher 1930)), they assumed that adaptation proceeds in a multidimensional continuous phenotype space characterized by universal pleiotropy (mutations simultaneously affect all traits), where an adapting population repeatedly takes mutational steps that bring it progressively closer to the optimum. However, in contrast to the original model, they let the location of the optimum change across environments, so that the population has to track the optimum in order to remain adapted. Notably, these model extensions showed that the general predictions from the one-dimensional model also hold in the more realistic context of multidimensional trait space.

One thing that all of the models described so far have in common is that they make predictions about evolution based on the relationship between a particular phenotype and fitness. This may be a logical — and convenient — choice in the case of large organisms, where the main phenotype under selection is sometimes known and/or easy to determine. For example, in animals environmental change often selects for differences in morphology or life-history traits (Stockwell et al. 2003), and such macroscopic changes can frequently be predicted from ecological or physiological principles. Conversely, in the case of microorganisms, the — microscopic —phenotype under selection is often unknown and/or hard to determine. Thus, phenotypic models are especially hard to test empirically when working with such organisms. An additional problem with phenotypic models for understanding evolution in changing environments is that a phenotype is by definition a product of a genotype and its environment. To see this, consider adaptation under Fisher's geometric model. A genotype that occupies one point in phenotype space in environment A may well occupy a very different point in phenotype space in environment B. Furthermore, the scaling between phenotypic distance and fitness may — and in the case of different intensities of the same selection pressure most likely will — change dramatically across environments. Models that only shift the location of the optimal phenotype in response to a change in the external environment are thus likely to make inaccurate predictions about evolution under such circumstances.

Genotypic models

The most obvious way to overcome these limitations is to use models that directly relate genotype to fitness. Such models have been used extensively for understanding adaptation, and date back to another founding father of population genetics: Sewall Wright. Wright

introduced the concept of the fitness landscape, which is a multidimensional genotype-fitness map (a hypercube in mathematical terms) that has one dimension for each locus at which variation exists, plus an additional dimension for fitness (Figure 1.4C; (Wright 1931; Wright 1932)). At a given time, a population will be mostly monomorphic and occupy a particular point in genotype space, and it will then move to connected points of higher fitness via mutation and selection, until it has reached a point where no further beneficial mutations are available, a so-called fitness peak. A major advantage of this model is that it explicitly incorporates the possibility that the effect of a mutation depends on the genetic background in which it occurs, that is, it allows for epistatic interactions between mutations (de Visser and Krug 2014). Such epistatic interactions can take various forms, the most consequential one of which is reciprocal sign epistasis. Reciprocal sign epistasis occurs when for a pair of interacting loci, mutations have a positive or negative fitness effect depending on the allele that is present at the other locus (Weinreich et al. 2005; Poelwijk et al. 2007). This type of epistasis gives rise to one of the most important properties of the fitness landscape, which sets it apart from the phenotypic models in yet another respect: the existence of multiple optima. A landscape with many local optima is considered to be rugged, and on such landscapes, evolution will be severely constrained, because many mutational pathways to genotypes of higher fitness are inaccessible. This has important consequences for evolution. For example, populations can get "stuck" on suboptimal fitness peaks, and evolutionary trajectories can be highly contingent upon the chance fixation of initial mutations (Salverda et al. 2011).

While the properties of the fitness landscape are studied mostly for single, constant environments, such analyses can in principle be readily extended to multiple environments by allowing the topography of the landscape to change across environments (Lindsey et al. 2013; Taute et al. 2014; de Vos et al. 2015). Understanding the rules according to which this happens is crucial for predicting the course and outcome of evolution in changing environments. For example, if the intensity of a selection pressure changes, will this usually result in a change in sign for each of the individual mutational effects, and thus in a change in the shape of the fitness landscape? Or will mutational effects mostly change in magnitude, so that the same evolutionary pathways remain accessible, and the landscape will only change in height? And what if different selection pressures are considered? Are there any general physical or biological principles that may help us predict how the landscape topography changes across environments?

Changing fitness landscapes

These questions are obviously difficult to answer. However, in this thesis I will make one of the first attempts at elucidating some of the principles according to which the fitness landscape changes across environments, as well as the consequences that this has for evolution, within the context of my particular model system. Specifically, I propose that there is a fundamental difference between how a change in concentration of the non-essential heavy metal Cd on

the one hand, and the presumably essential heavy metals Ni and Zn on the other hand, affects the topography of the fitness landscape. Because Cd is toxic at all concentrations, I would expect that the same evolutionary solution, reducing the internal bioavailable Cd concentration to a minimum, will be favoured at all concentrations. Such a solution could take many forms, ranging from a reduction in uptake to increased chelation and storage in the vacuole. However, crucially, each of these solutions is likely to be more beneficial at higher metal concentrations, because under these circumstances the detrimental effects of the metal will be more pronounced. I therefore predict that for Cd, the height of the fitness landscape increases with concentration, but that its shape remains roughly constant. This should cause the same evolutionary pathways to be followed under gradual and abrupt increase in metal concentration, but adaptation to be delayed under gradual change.

By contrast, for the essential metals Ni and Zn, internal metal concentrations need not be reduced to a minimum, but rather controlled within specific physiological limits, because these metals are required in small quantities. This implies that different evolutionary solutions should be favoured at different external metal concentrations. For example, strongly reducing metal uptake is likely to be beneficial at high metal concentrations, but may be detrimental at lower metal concentrations, where it may result in metal deficiency. The predicted consequence of such differences in optimal strategy across concentrations is that for Ni and Zn, the shape of the fitness landscape should change considerably with metal concentration. This should cause different evolutionary pathways to be followed under gradual and abrupt increase in metal concentration. Additionally, because the mutations selected at lower metal concentrations confer a suboptimal fitness at high concentrations, fitness increase at high concentrations should be delayed under gradual change.

Aims and outline of this thesis

The aim of this thesis was to investigate how the rate of directional environmental change affects the course and outcome of evolution using experimental evolution of Cd, Ni, and Zn tolerance in the baker's yeast *S. cerevisiae* as a model system.

In **Chapter 2** I present phenotypic results from a laboratory evolution experiment in which *S. cerevisiae* was cultured for 500 generations in the presence of either gradually increasing or constant high concentrations of the heavy metals Cd, Ni, and Zn. I determined relative fitness of evolved populations from different time points at different concentrations of the selective metal in order to assess how evolutionary dynamics and outcomes depended on the rate of environmental change and the nature of the selection pressure. Specifically, I addressed the hypothesis that for Cd, the same genotypes are selected at all metal concentrations, but fitness differences are larger at high concentrations ("magnitude GxE"), whereas for Ni and Zn, different genotypes are selected at different metal concentrations ("reranking GxE"). My

results supported this hypothesis, and, moreover, showed that increase in fitness, as assayed at the final, high metal concentration, was delayed under gradual change for all metals. However, fitness of evolved populations from the final time point of the experiment was the same following gradual and abrupt change.

To understand how the rate of directional environmental change affects evolution at the genomic level, I performed whole-genome sequencing analysis on single clones isolated from all replicate populations at the final time point of my evolution experiment (**Chapter 3**). This revealed that adaptation to the selective environments occurred via a complex combination of SNPs, small indels, whole-genome duplication and other large-scale structural changes. Furthermore, my results confirmed the phenotypic results and showed that for Cd, mutations in the same genes were selected under gradual and abrupt change, whereas for Ni and Zn, mutations in different genes were selected in response to different rates of environmental change. Additionally, I found that genomic evolution was more repeatable following abrupt change in the case of Ni and Zn, as predicted by my GxE framework.

In **Chapter 4**, I reconstructed local fitness landscapes for each metal by deleting all repeatedly mutated genes — as identified by whole genome sequencing — both by themselves or in combination (deletion was used to mimic the loss-of-function effect of the mutations that I found). Fitness assays on these landscapes at different metal concentrations were then used to evaluate how the height and shape of each landscape changed as a function of concentration. For Cd, I found that the height, but not the shape of the landscape, changed across concentrations. Conversely, for Ni, the shape of the landscape changed considerably across concentrations, and I made predictions about the consequences that this likely had for the selective dynamics of mutations in my evolution experiment. Deep sequencing of evolved population samples generally confirmed these predictions, demonstrating the power of landscape reconstruction approaches for understanding evolutionary dynamics.

In **Chapter 5**, I summarize the main findings from the experimental chapters, and put these results into a broader context. Additionally, I suggest some future research directions.

Chapter 2

Dynamics of adaptation in experimental yeast populations exposed to gradual and abrupt change in heavy metal concentration

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Abstract

Directional environmental change is a ubiquitous phenomenon that may have profound effects on all living organisms. However, it is unclear how different rates of such change affect the dynamics and outcome of evolution. We studied this question using experimental evolution of heavy metal tolerance in the baker´s yeast *Saccharomyces cerevisiae*. To this end, we grew replicate lines of yeast for 500 generations in the presence of (i) a constant high concentration of cadmium, nickel or zinc, or (ii) a gradually increasing concentration of these metals. We found that gradual environmental change leads to a delay in fitness increase compared to abrupt change, but not necessarily to a different fitness of evolutionary endpoints. For the non-essential metal cadmium this delay is due to reduced fitness differences between genotypes at low metal concentrations, consistent with directional selection to minimize intracellular concentrations of this metal. In contrast, for the essential metals nickel and zinc different genotypes are selected at different concentrations, consistent with stabilizing selection to maintain constant intracellular concentrations of these metals. These findings indicate diverse fitness consequences of evolved tolerance mechanisms for essential and non-essential metals, and imply that the rate of environmental change and the nature of the stressor are crucial determinants of evolutionary dynamics.

Introduction

Directional environmental change is happening all around us. Two prominent current examples of such change are human-induced pollution and global climate change. Understanding how these phenomena affect living organisms and thus ultimately our planet's biodiversity is crucial. Are organisms able to adapt or will they become extinct? Does it matter how fast the world changes? Which factors limit possible adaptive responses? These questions are often addressed in terms of physiological or behavioural changes (phenotypic plasticity) and shifts in species' spatial or temporal range (Przybylo et al. 2000; Charmantier et al. 2008; Chen et al. 2011; Merilä and Hendry 2014). However, in the case of more severe and longer-term environmental change, these mechanisms do not suffice and the only way for populations to persist is through mutation and selection, that is, the genetic tracking of the environment via adaptive evolution (Visser 2008; Bell and Gonzalez 2009). So, ultimately, to answer these questions, we need to know how evolution is affected by different rates of environmental change.

An approach that is particularly powerful for studying the effect of environmental change is microbial experimental evolution. It has provided support for many longstanding hypotheses and helped quantify fundamental processes (for a recent review, see Barrick and Lenski 2013). However, most of this work was performed in constant environments that impose an initially strong selection pressure (Collins 2011). Principles derived from such studies may very well have only limited applicability to evolution in more slowly changing environments.

Here, we investigate how gradual and abrupt environmental change affect evolutionary dynamics and outcomes using experimental evolution of heavy metal tolerance in the baker´s yeast *Saccharomyces cerevisiae*. Heavy metals are important pollutants that primarily originate from mining and industry and play a key role in both essential biological processes and toxicity due to their highly reactive chemical properties (Boyd 2010; Bánfalvi 2011). While some of these metals, such as cadmium (Cd), lead (Pb), and mercury (Hg), are non-essential and have harmful effects even at low concentrations, others, such as manganese (Mn), iron (Fe), nickel (Ni), and zinc (Zn), are essential micronutrients and are only harmful at high concentrations (Gadd 1993; Wysocki and Tamas 2010). In most living organisms, including yeast, essential metals are required as cofactors for certain enzymes and for maintaining protein structure (Waldron et al. 2009). Non-essential metals interfere with processes in which essential metals are involved because of their chemical similarity to these elements (Goyer 1997). The toxic effect of a metal depends on several factors (Gadd 1993; Giller et al. 1998), but common modes of action include induction of oxidative stress, inhibition of enzyme function and impaired DNA repair (Hall 2002; Wysocki and Tamas 2010). *S. cerevisiae* exhibits extensive natural variation for metal tolerance (Vadkertiová and Sláviková 2006; Warringer et al. 2011), and combines large population sizes with short generation times (Zeyl 2006), making it ideally suited for our studies. We grew replicate lines

of yeast for approximately 500 generations in the presence of either increasing or constant high concentrations of one non-essential metal, Cd, and two essential metals, Ni and Zn, and monitored changes in fitness relative to the ancestor over time.

We made a number of predictions about the effect of gradual and abrupt environmental change on evolution. Gradual and abrupt are relative qualifications, while the quantitative difference in the rate of environmental change will matter (Matuszewski et al. 2014). However, as a necessary first step we infer qualitative predictions based on the general nature of the relationship between metal concentration and relative fitness for different genotypes, that is, the predominant kind of genotype-by-environment (GxE) interaction. GxE interactions may be of two general types, which we hypothesize to have fundamentally different consequences for evolutionary dynamics and outcomes. *Magnitude* GxE refers to the case where the magnitude of fitness differences between genotypes changes across environments, but ranked performance remains the same. Conversely, *reranking* GxE implies that the ranked performance of genotypes changes across environments (Figure 2.1). In the case of magnitude GxE, the same mutations are selected under gradual and abrupt change. However, weaker selection pressures (and thus smaller fitness differences) at low concentrations imply that selection will be delayed under gradual change. As the same mutations are selected under both rates of change, the same evolutionary trajectories may be followed, so that evolution ultimately leads to the same adapted genotypes and hence the same end fitness. In contrast, in the case of reranking GxE, different mutations are selected under gradual and abrupt change. Mutations selected at low concentrations will have a suboptimal fitness at high concentrations, which will similarly cause a delay in fitness increase (as assayed in the final environment) for populations exposed to gradual change. As different mutations are selected under gradual and abrupt change, different evolutionary pathways will be followed. This may

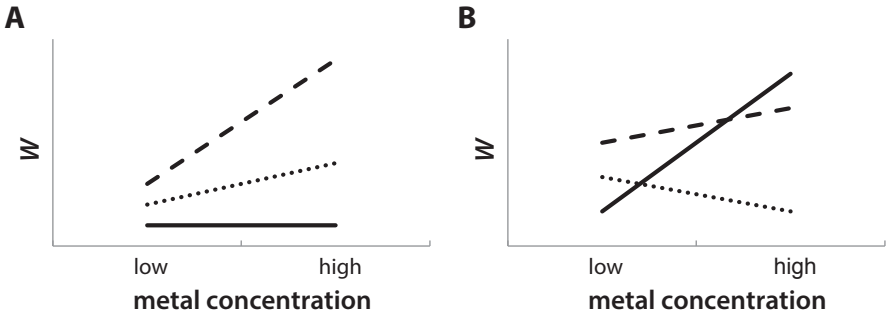

Figure 2.1: Illustration of the hypothetical relationships between fitness (W) at low versus high metal concentrations. Each line represents a different genotype. (A) Magnitude GxE; fitness ranking is the same at all concentrations, but fitness differences are larger at high concentrations. This type of GxE is expected for genotypes selected with non-essential metals, where directional selection will minimize intracellular metal concentrations. (B) Reranking GxE; fitness ranking changes across metal concentrations. This type of GxE is expected for essential metals, where stabilizing selection maintains low intracellular metal concentrations.

result in different end genotypes, with potentially different fitnesses, depending on epistatic interactions between involved mutations.

We anticipate that the relative importance of magnitude and reranking GxE is different for different selection pressures and regimes of environmental change. More specifically, in the context of our experiments, we predict that the magnitude GxE relationship is more important in the case of the non-essential metal Cd, as the same evolutionary solution, that is, reducing the internal Cd concentration, will be favoured at all concentrations (but more strongly so at high metal concentrations). This is consistent with directional selection with increasing intensity. In contrast, for Ni and Zn, being essential heavy metals, different solutions are likely to be favoured at different concentrations, as the internal metal concentration need not be reduced to a minimum but rather controlled within specific physiological limits. Consequently, we expect reranking GxE to play a more prominent role for these metals. This is consistent with stabilizing selection to maintain low, but non-zero intracellular metal concentrations.

To test the aforementioned hypotheses, we determined the fitness of evolved isolates from different time points relative to the ancestor at different concentrations of the selective metal. We used this information to establish the general relationship between metal concentration and relative fitness for our isolates, to test how treatment affected timing of fitness increase, and to test whether treatments led to different evolutionary endpoints in terms of fitness.

Materials and methods

Strains

The strains used in this study are *MAT**a*** haploids derived from *S. cerevisiae* strain BY4743 and were previously transformed at the *HO* locus with cassettes KanMX4 and NatMX4 to create ancestral strains W20 and W24, respectively (Goldstein and McCusker 1999; Wloch-Salamon et al. 2008). These cassettes confer resistance to the antibiotics geneticin and nourseothricin, respectively, and are presumed to be neutral with respect to fitness in the absence of antibiotics. Single colonies of W20 (*MAT**a**/his3/leu2/LYS/ura3/met/ΔHO:KanMX*; genR) and W24 (*MAT**a**/his3/leu2/lys/ura3/MET/ΔHO:NatMX*; natR) were used as the starting point for each of our selection lines.

Selection experiment

Yeast was grown in 10 mL liquid 0.5x YPD medium (5 g yeast extract, 10 g peptone, 10 g D-glucose L^{-1}, pH set to 6.0 with HCl) at 28°C and 300 rpm for a total of 75 transfers. Every 24h, 1% of the culture was transferred to a new tube, which resulted in a total of ~500 yeast generations. Every fifth transfer, a sample was taken from each population and stored at -80°C in 20% v/v glycerol. Before the experiment, selection lines were cultured in medium without additional metals for two transfers to acclimatise the strains to the culture conditions. Six

replicate populations (three for W20 and three for W24, tubes alternating to increase the scope for detection of cross-contamination) were grown under each of seven different selection regimes: control (no metals added, lines R1-6), increasing levels of Cd (0.2-3 μM $CdCl_2$, lines CI1-6), constant high levels of Cd (3 μM $CdCl_2$, lines CH1-6), increasing levels of Ni (16.7-250 μM $NiCl_2$, lines NI1-6), constant high levels of Ni (250 μM $NiCl_2$, lines NH1-6), increasing levels of Zn (23.3-350 μM $ZnCl_2$, lines ZI1-6), and constant high levels of Zn (350 μM $ZnCl_2$, lines ZH1-6). Appropriate metal concentrations were determined in a pilot experiment and chosen so that the highest levels caused a ~90% reduction in yield (OD_{600}) after 3x24h of growth of the ancestral strains (average from four replicates). We found this measure to be a reliable indicator of the highest selection pressure that still allows for long-term population persistence. Reductions in yield were relatively transient; after five transfers into the main experiment, yields of the lines exposed to constant high concentrations had, on average, already recovered to over 70% of the yield of the lines grown in the absence of metal. For the increasing concentration treatment, the initial concentration was one-fifteenth of the constant high concentration, and at every fifth transfer this concentration was increased, so that after 70 transfers the same level was reached as for the constant high concentrations. We screened for cross-contamination between the lines approximately every ten transfers by plating out dilutions of each culture on 0.5x YPD agar plates containing either 200 μg mL^{-1} G418 (geneticin) or 100 μg mL^{-1} nourseothricin. Although a small number of colonies of the opposite marker type was detected occasionally, we were never able to confirm cross-contamination upon re-plating.

After 75 transfers under the conditions described above, we continued selection with 131-μl samples taken from the -80°C stocks (equivalent to 100 μl of culture) for 25 extra transfers in the presence of the final metal concentrations used in the previous experiment. After a few transfers, we discovered that the frequency of petite colonies in 10 out of 12 Cd lines was high (up to 100%). Because fitness effects of petite mutations are thought to be considerable and we were unable to remedy this problem, we restricted ourselves to the control, Ni, and Zn lines for this part of the experiment.

Fitness assays

Competition assays (Lenski et al. 1991) were used to determine the relative fitness of the (evolved) populations. In brief, 24h cultures grown from 10 μl of the -80°C stocks of the evolved populations and the ancestral strain of opposite marker type were mixed in equal proportions (50 μl of each to a total volume of 10 mL 0.5x YPD). Both prior to and after 24h competition, appropriate dilutions were plated on geneticin and nourseothricin plates. The counted number of colonies for each strain was then used to calculate the relative number of doublings.

Competitions were performed in independent blocks for each of the three metals. Evolved isolates from different time points (transfer 0, 25, 50, and 75: T_0-T_{75}) were assayed in the presence of different concentrations of the metal that they had been exposed to during the selection experiment (no metal, one-third and two-thirds of the final concentration, final [or constant

high] concentration). In the same assays, fitness of control populations that were isolated at the same time points was determined in the presence of all three metals at all concentrations.

In a second round of assays we determined the fitness of the populations from T_{75} (except for the Cd lines as explained above) and T_{100} at the final concentration of the metal they had been cultured with. As before, all control lines were included in the assays.

Data analysis

Relative fitness of the evolved populations, W_{ij}, was calculated as the relative number of doublings over 24h compared to the ancestral competitor of opposite marker type, where i corresponds to the evolved population and j to the ancestral population (Lenski et al. 1991).

All statistical analyses were separately performed for each metal. To identify the factors explaining fitness differences, we fitted general linear mixed models with log-transformed relative fitness as the response variable. Metal concentration at which fitness was determined, evolutionary treatment (three levels: control, increasing concentrations, and constant high concentration), and time point of isolation (four levels: T_0, T_{25}, T_{50}, and T_{75}) were used as fixed effects, whereas strain (W20 or W24), replicate evolving population within strain, and time point within replicate population were incorporated as random effects to account for non-independence. We started by fitting models with interactions between all fixed effects and performed sequential backwards elimination to find the minimal adequate model. Parameter estimates from these analyses were used to infer evolutionary dynamics. To compare evolutionary outcomes, models were refitted on the relevant subset of the data. Changes in fitness resulting from the extra transfers (T_{75}-T_{100}) for the Zn and Ni lines were analysed with the same approach. However, in this case we excluded the effects of metal concentration and time point within replicate population, because fitness was determined only at the final metal concentration.

To determine whether fitness ranking changes with metal concentration, we calculated Kendall's W (Sokal and Rohlf 1995) using relative fitness of evolved isolates from all time points, treatments, and populations at the three metal concentrations used in the assays. A W of 1 indicates perfect concordance between fitness ranks at different concentrations, whereas a W of 0 corresponds to complete discordance. To assess whether W was significantly different from 1, we used χ^2 tests.

To assess whether adaptive dynamics differed between increasing and constant high treatments, we compared fitness increase over time for both treatments, as assayed in the final environment. For Ni and Zn we also incorporated information about isolates from T_{100}, where we added the difference in fitness between T_{75} and T_{100} to the fitness difference between T_0 and T_{75} to obtain the fitness increase of T_{100} relative to T_0. Previous studies have shown that over long bouts of evolution in stable environments, increase in fitness occurs in a stepwise manner that is approximated by a hyperbolic or power law function (Wiser et al. 2013). However, when considering the selection of a single mutant allele in haploid organisms,

increase in fitness is better approached by a logistic function (Charlesworth et al. 2010). Because our evolutionary experiment is comparatively short and thus likely to involve few mutations, a logistic model might be more appropriate in our case. Besides, such a model would also better account for the "lag-phase" before fitness increase that we anticipated for our increasing treatment. To test this notion, we fitted a hyperbolic ($\Delta W = a \times t / (b + t)$) and a logistic ($\Delta W = a / [1 + e^{(b - t)/c}]$) model to the data for each treatment separately, where ΔW is fitness increase compared to the initial time point of the same replicate population, t is time in number of transfers and a, b, and c are model parameters. In all models, we allowed for different asymptotes per replicate population by means of a random effect. The Akaike information criterion (AIC) was used to determine which of the two models better explained the data for each of the treatments. In each case, parameter estimates and variances from the best model were used to calculate the time at which 50% of the total increase in fitness over 75 transfers was reached ($T_{50\%}$). More specifically, we randomly drew six values for each parameter (assuming a normal distribution) to calculate six $T_{50\%}$ values per treatment, which were then compared to six similarly obtained $T_{50\%}$ values for the opposite treatment using a Wilcoxon rank sum test. This procedure was repeated 1000 times, and reported values are averaged over these repetitions.

All statistical analyses were performed in R (R Core Team 2013). Data used in the analyses are deposited in the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.84c25 (Gorter et al. 2015).

Results

We let replicate lines of *S. cerevisiae* evolve for 500 generations in the presence of (i) a constant high concentration of Cd, Ni or Zn (termed CH, NH, and ZH, respectively), (ii) a gradually increasing concentration of these metals (termed CI, NI, and ZI), or (iii) in the absence of these metals (control lines; termed R). For all selection lines, competitive fitness in the presence of the final, high concentration of the selective metal strongly increased over the course of the experiment, indicating that they adapted to the selective environment (Figure 2.2 and Figure S2.1–S2.6; relative fitness ± SEM of evolved isolates from the final time point: CI: 2.43 ± 0.14; CH: 2.42 ± 0.16; NI: 1.27 ± 0.02; NH: 1.27 ± 0.04; ZI: 1.10 ± 0.05; ZH: 1.22 ± 0.03). Control lines had an increased fitness when exposed to high levels of Cd, but not Ni and Zn (with Cd: 1.24 ± 0.05; with Ni: 1.03 ± 0.02; with Zn: 1.02 ± 0.03). However, in all cases fitness was substantially lower than fitness of lines that had evolved in the presence of metal.

Effect of metal concentration on fitness

Although relative fitness of evolved isolates from different treatments and time points was increased at all metal concentrations, we found strong evidence for GxE interactions. That is,

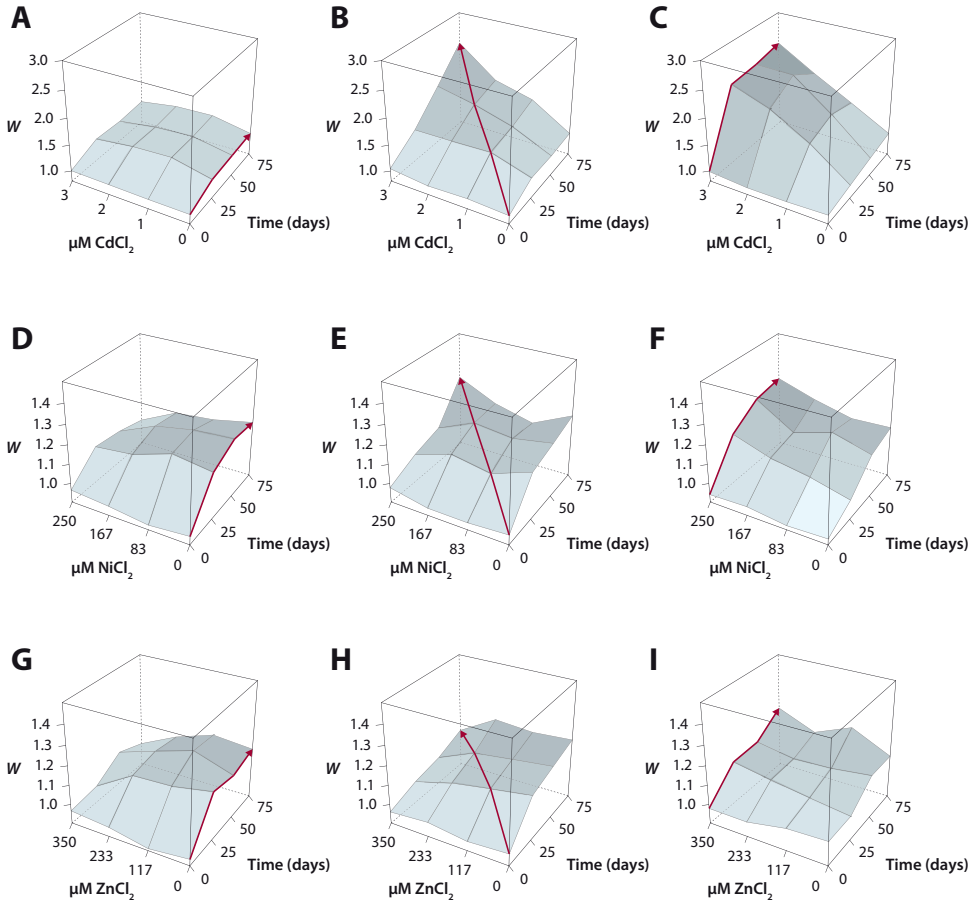

Figure 2.2: Average relative fitness (*W*) of six replicate evolution lines isolated from different time points assayed at different metal concentrations. A) Control lines (R) assayed with Cd; B) lines exposed to increasing concentrations of Cd (CI); C) lines exposed to constant high concentrations of Cd (CH); D) control lines (R) assayed with Ni; E) lines exposed to increasing concentrations of Ni (NI); F) lines exposed to constant high concentrations of Ni (NH); G) control lines (R) assayed with Zn; H) lines exposed to increasing concentrations of Zn (ZI); I) lines exposed to constant high concentrations of Zn (ZH). Red arrows indicate the metal concentration of the selective environment at each time point; lines evolving under increasing metal concentrations have previously been exposed to metal concentrations equal to or lower than those indicated by the red arrows. Note the different scale of the z-axis for Cd.

the effect of metal concentration significantly depended on the combination of evolutionary regime and time point of isolation (treatment x time x Cd concentration: $F_{6,204} = 15.07$, $P < 0.0001$; treatment x time x Ni concentration: $F_{6,204} = 4.93$, $P = 0.0001$; treatment x time x Zn concentration: $F_{6,204} = 6.66$, $P < 0.0001$; Figure 2.2 and Figure S2.1–S2.6).

To discriminate between magnitude and reranking GxE (see Figure 2.1), we calculated Kendall's coefficient of concordance (*W*), using the relative fitness of evolved isolates from

all time points, treatments, and populations at each metal concentration. A W of 1 indicates perfect concordance between the rankings of isolates at all concentrations, such that we interpret cases where W is significantly different from 1 as cases of reranking. For Cd, we found no significant reranking of evolved isolates ($W = 0.84$, $\chi^2 = 26.24$, df = 53, P = 0.99). This indicates that overall the same genotypes do best at all metal concentrations. Consistently, the relative fitness of evolved isolates varied much more at high than at low metal concentrations (Levene's test for equality of variances: $F_{2,159} = 34.45$, P < 0.0001), as expected in the case of magnitude GxE. Conversely, for Ni, W was significantly lower than 1 ($W = 0.51$, $\chi^2 = 77.59$, df = 53, P = 0.016), reflecting significant reranking of fitness (i.e. different genotypes do best at different concentrations). Finally, for Zn, we did not detect significant reranking ($W = 0.60$, $\chi^2 = 64.40$, df = 53, P = 0.14). However, in this case variance in fitness of evolved isolates in each metal environment was not significantly larger than that of unevolved isolates (Levene's test for equality of variances: P > 0.05 for all metal concentrations). Because unevolved isolates are essentially clonal, variance in fitness between them may be interpreted as noise, implying that we lack statistical power to test for reranking in the case of Zn.

Adaptive dynamics

To compare observed adaptive dynamics across treatments, we estimated changes in metal reaction norms, which are plots of relative fitness against metal concentration, over evolutionary time. We considered three distinct patterns: (1) adaptation to the general culture conditions, reflected by an increase in the intercept of the metal reaction norm but no change in slope; (2) metal-specific adaptation, reflected by an increase in slope of the metal reaction norm; and (3) adaptation to metal- free conditions, reflected by a decrease in slope of the reaction norm. Interestingly, although environment-specific adaptation occurred in multiple cases, there was no evidence for strong trade-offs, where fitness is lower than that of the ancestor in non-selective environments.

We observed very clear differences in the pattern of adaptation for each of the treatments (Figure 2.2 and Figure S2.1–S2.6). First, evolution in the presence of constant high concentrations of Cd, Ni or Zn lead to metal-specific adaptation within the first 25 transfers (increase in slope: CH: P < 0.0001; NH: P = 0.0058; ZH: P = 0.0018), which was accompanied by significant general adaptation to the culture conditions for Cd and Ni, but not for Zn (increase in intercept: CH: P = 0.0015; NH: P = 0.030; ZH: P = 0.89). Further adaptation to the general culture conditions took place for Ni and Zn between T_{25} and T_{50} (increase in intercept: NH: P = 0.0005; ZH: P = 0.048), which compensated for the earlier metal-specific adaptation in the case of Zn (reduction in slope: ZH: P = 0.048). Notably, further metal-specific adaptation was absent after T_{25}.

Under conditions of increasing metal concentrations, the order of adaptation was reversed, that is, general adaptation took place before metal-specific adaptation. During the initial transfers (T_0–T_{25}), intercepts increased for all three metals (P < 0.0001 in all cases). This was

accompanied by a decrease in slope for Ni and Zn (P = 0.032 and P = 0.0059, respectively), indicating that adaptation was specific to metal-free conditions in these cases. Metal-specific adaptation occurred between T_{25} and T_{75} for Cd (increase in slope: T_{25}–T_{50}: P = 0.0001; T_{50}–T_{75}: P = 0.028), and between T_{50} and T_{75} for Ni (P = 0.0083), while no significant increase in slope was observed for Zn at any time during the experiment.

During the first 25 transfers, control lines showed the exact same pattern of adaptation as the lines evolved under increasing concentrations of metal (increase in intercept: P ≤ 0.0001 for all metals; decrease in slope: P = 0.043 for Ni, P < 0.0001 for Zn). However, no significant change in either intercept or slope occurred after this time.

We expected that increase in fitness, as assayed in the final high-metal environment, would be delayed under increasing concentrations compared to constant high concentrations. The metal-specific adaptation in the early stages of evolution experiments with high concentrations, but not experiments with increasing concentrations, already provides indirect evidence for such a trend. However, a more direct test involves the actual pattern of fitness increase in the final environment. As such pattern may assume various shapes, we fitted both a hyperbolic and a logistic model for each metal treatment and tested which one better fitted the data. For all increasing treatments, as well as for the constant high treatment for Zn, hyperbolic models were unable to converge on realistic parameter estimates, indicating that such models poorly describe the dynamics. Conversely, for constant high concentrations of Cd and Ni, the hyperbolic model explained the data better than the logistic model (Cd: hyperbolic model AIC = 18.63, logistic model AIC = 20.75. Ni: hyperbolic model AIC = -61.60, logistic model AIC = -59.29). For all treatments, we used parameter values from the best model to calculate the time at which 50% of the fitness increase at T_{75} was realised. For Cd and Ni we found this time to be significantly larger for increasing than constant high concentrations, while for Zn there was no difference between treatments (Table 2.1).

Fitness of evolutionary outcomes

Fitness of T_{75} isolates in the final high-metal environment depended on the evolutionary regime for all metals (Cd: $F_{2,14}$ = 46.79, P < 0.0001; Ni: $F_{2,14}$ = 26.00, P < 0.0001; Zn: $F_{2,14}$ = 7.78, P = 0.0053). However, there was no significant difference in endpoint fitness between constant high and increasing concentrations for any of the metals (Cd: P = 0.97; Ni: P = 0.84; Zn: P = 0.063), and for Zn only the control and constant high treatment were truly different (P = 0.0048).

To determine whether the absence of differences in fitness between T_{75} isolates from the different metal treatments was transient, we continued evolution in the presence of the final high-metal concentrations of Ni and Zn for another 25 transfers (we did not continue evolution for the Cd lines because of the high frequency of petite mutants). These additional ~170 generations resulted in an average increase in fitness at 250 μM Ni of 0.13 for all Ni lines (effect of time: $F_{1,9}$ = 17.41, P = 0.0024; Figure S2.7), and this effect was the same for both treatments. In contrast, fitness at 350 μM Zn did not increase during continued evolution in

Table 2.1: Estimated time at which 50% of the fitness increase at T_{75} was realised for each of the treatments ($T_{50\%}$).

Treatment	$T_{50\%}$ (± SEM)	*W*	*P*
increasing Cd	42.56 ± 1.35	36	0.0022
constant high Cd	2.45 ± 1.67		
increasing Ni	53.43 ± 2.05	36	0.0022
constant high Ni	22.31 ± 1.77		
increasing Zn	37.74 ± 2.55	8.62	0.26
constant high Zn	43.23 ± 1.72		

Note: For Cd and Ni, but not Zn, $T_{50\%}$ was significantly larger for the increasing than for the constant high treatment, as indicated by Wilcoxon test statistics (*W*) and associated *P* values.

the presence of Zn (effect of time: $F_{1,10} = 0.99$, P = 0.34; Figure S2.7). Importantly, continued evolution did not result in fitness divergence between treatments for either metal.

Discussion

Gradual environmental change delays adaptation without an effect on final fitness

We investigated how gradual and abrupt environmental change affect evolutionary dynamics and outcomes using experimental evolution of heavy metal tolerance in *S. cerevisiae*. In line with our expectations, we found that gradual change in metal concentration leads to a delay in fitness increase compared to abrupt change. However, relative fitness of isolates from the final time point of our experiments (~500 generations) was no different between treatments and this did not change upon ~170 generations of continued evolution in the presence of high concentrations of metal.

We explain these results in different ways for the different selective metals. For Cd, the difference in relative fitness between isolates increased with metal concentration, but their rank order did not change significantly (magnitude GxE). In this case the delay in fitness increase under gradual change was likely caused by the fact that the probability to survive drift decreases and the time to fixation increases with decreasing selection coefficients (Kopp and Hermisson 2007). While the duration of our experiment was somewhat arbitrary, the absence of fitness differences between endpoints is also consistent with this explanation, because magnitude GxE predicts that populations will follow the same mutational pathways and will thus end up on the same fitness peaks, irrespective of the rate of change (note, however, that the relative chance of realisation of different mutational pathways may depend on the relative benefit of different mutations at different concentrations (Gillespie 1984)).

For Ni, different isolates did best at different metal concentrations (reranking GxE). In this

case, we explain the delay in fitness increase under gradual change by the fact that this regime initially selected for genotypes with a suboptimal fitness at higher metal concentrations. As a consequence, different evolutionary pathways have likely been followed under gradual versus abrupt change. In this case, the fact that final fitness of both regimes does not differ may be explained in several ways. Firstly, it could be that they still moved towards the same fitness peak because the underlying fitness landscape in the final environment is relatively smooth. Alternatively, they may have approached different peaks of comparable height. A third potential explanation, namely that the absence of fitness differences is transient, seems less likely given that ~170 additional generations in the presence of high concentrations of Ni did not lead to any divergence in fitness between the two treatments.

For Zn, we found evidence for GxE, but were unable to distinguish between magnitude and reranking GxE. Moreover, we did not detect a significant delay in fitness increase in the slowly changing environment, even if the qualitative pattern of adaptation resembled that for Ni (i.e. adaptation to the general culture conditions, metal environment, and metal-free environment occurred in the same order and during the same periods for Ni and Zn treatments). We suggest that these observations are the result of the smaller evolutionary response to Zn, causing observed differences to be non-significant. If so, increased replication of the assays should uncover dynamics that resemble those observed for Ni. As to the reasons for the smaller evolutionary response to Zn than to Ni, we can only speculate. One possibility is that Zn plays a more pivotal role in yeast metabolism than Ni (Bleackley et al. 2011), implying that selection for Zn tolerance mechanisms is subject to more stringent constraints.

Our GxE framework is consistent with previous studies

Previous work on evolution in directionally changing environments has introduced a moving optimum model (Kopp and Hermisson 2007, 2009a). This model posits that the optimal phenotype linearly changes as a function of the selection pressure's intensity, and predicts that gradual change results in the fixation of mutations of intermediate rather than large phenotypic effect. However, its assumptions are rather restrictive. Magnitude GxE implies a fixed rather than a moving optimum, and although reranking GxE is in principle consistent with the model, there are numerous other ways in which the optimum can change instead of linearly in a single phenotypic direction. We therefore argue that the moving optimum model is a specific case of reranking GxE. Furthermore, a practical problem with the model is that it is hard to test empirically, because the key phenotype under selection is often unknown.

A remarkable outcome of our study is that the observed delay in adaptation under slowly changing conditions does not affect final fitness. In contrast, a previous study that applied different rates of environmental change found that gradual change resulted in higher growth rates in the final environment (Collins and De Meaux 2009). The authors explained this using the moving optimum model, and argued that because mutations of intermediate

effect are more abundant than mutations of large effect, more diverse evolutionary pathways are initiated under gradual change. This should allow populations to more thoroughly explore the fitness landscape, facilitating them to locate higher fitness peaks. We consider it unlikely that this is a general result, because pathways associated with smaller-effect first-step mutations may just as well lead to lower fitness peaks (Rozen et al. 2008; Jain et al. 2011). Indeed, others who applied different rates of change have reported that populations surviving abrupt change grow faster (Lindsey et al. 2013) or reach higher levels of antibiotic resistance (Perron et al. 2008). It should be noted though that in both these cases the rapid environmental change was much more abrupt than in our experiments, causing most populations to become extinct. An extreme form of the magnitude GxE scenario, where at high stress levels the absolute fitness of most (adaptive) genotypes falls below the threshold required for population persistence, may explain these observations.

Effect of mutation supply rate

Our results likely depend on demographic parameters such as population size (N) and mutation rate (μ). Mutation supply rates (i.e. $N\mu$) have profound consequences for evolutionary dynamics and outcomes (Szendro et al. 2013), and may importantly differ between our treatments. Constant high metal concentrations initially caused a strong reduction in population size. Such transient reductions in population size are the focus of evolutionary rescue (ER) studies, which address whether evolution may save — often abruptly — declining populations from extinction (Bell and Gonzalez 2011; Gonzalez et al. 2013). Conditions under which adaptation occurs through ER are predicted to both increase the fraction of mutations that are beneficial (Bell and Gonzalez 2011) as well as reduce the fraction of such mutations that are sufficient for long-term population survival (Gonzalez et al. 2013). Regardless, the high metal concentrations that strongly reduced population size are likely to have simultaneously increased mutation rates. The metals that we used, particularly Cd, are known mutagens (Leonard et al. 1986; Fletcher et al. 1994; Jin et al. 2003). Additionally, heavy metals may induce the general stress response (Wysocki and Tamas 2010), which can increase eukaryotic mutation rates (Shor et al. 2013). Finally, the type of mutations used for adaptation under gradual and abrupt change may be altogether different (Yona et al. 2012). Whatever the joint effect of these factors, populations experiencing a sudden change in the environment do not seem to have been mutationally limited, because they considerably increased in fitness over the first 25 transfers.

GxGxE

It may be argued that the distinction between magnitude and reranking GxE is arbitrary, as most forms of GxE will change into reranking GxE at some concentration of the stressor. However, what is important is not whether reranking happens, but whether it happens under relevant environmental conditions. Others have made the distinction between magnitude and

reranking GxE before (Haldane 1946; Bowman 1972; Fry et al. 1996), and in fact it is an important contemporary concept in plant and animal breeding (Calus 2006; Des Marais et al. 2013). An analogous categorisation in population genetics is that of magnitude and sign epistasis (Poelwijk et al. 2007), where the effect of a mutation depends on the genetic background rather than the environment. The consequences of these types of epistasis are well characterised (de Visser et al. 2011), and several recent studies have combined epistasis (GxG) and GxE into a single GxGxE framework (Remold 2012; de Vos et al. 2013; Flynn et al. 2013; Goulart et al. 2013; Lindsey et al. 2013). Because we lack genetic information for our isolates, we cannot say how GxG interactions change between environments in our case. However, reranking GxE can in itself explain why different evolutionary pathways are followed under different dynamics of environmental change. Furthermore, it is a key prerequisite for the types of GxGxE that may cause gradual and abrupt change to result in different evolutionary endpoints (Lindsey et al. 2013).

Significance of the nature of the selection pressure

Our results provide support for both magnitude GxE (for Cd) and reranking GxE (for Ni). As we argue in the introduction, a likely explanation for this difference is the nature of the stressors. Cd is a non-essential heavy metal and thus toxic at all concentrations. In this case, a single evolutionary 'solution' is called for at all concentrations, i.e. a mechanism that minimizes intracellular metal concentrations. By contrast, Ni and Zn are essential heavy metals, thus calling for different 'solutions' at different external concentrations. It is unclear what the relative importance of magnitude and reranking GxE is for different stressors and different types of environmental change. A recent meta-analysis showed that the majority of reported QTLxE interactions in plants is of the magnitude type (Des Marais et al. 2013). Conversely, considerable reranking GxE was observed from the change in topography of an artificially constructed fitness landscape in *E. coli* between three different environments (Flynn et al. 2013). Although we currently cannot say which type of GxE prevails in nature, we hypothesize that more similar environments are more often associated with magnitude GxE. Also, selection pressures with a negative impact on fitness at all intensities, such as antibiotics, are probably more often associated with magnitude GxE than selection pressures, such as temperature, that may have positive or negative effects and thus call for different solutions at different levels of the "stressor".

In summary, we have shown that gradual and abrupt environmental change lead to different evolutionary dynamics, with a faster response under abrupt change, but not necessarily to a different fitness of endpoints. These findings imply that the rate of environmental change and the nature of the stressor are crucial determinants of evolutionary dynamics, with the potential to affect the persistence of populations in the short- and/or long-term. Whole-genome sequencing will be a next step to take to identify the mutations underlying the adaptive dynamics that we observed.

Acknowledgements

We would like to thank Bertha Koopmanschap for assistance with the competition assays, Joost van den Heuvel for advice on statistics, and Mark Zwart, Sander van Doorn, and two anonymous reviewers for useful comments on earlier versions of the manuscript.

Appendix: supplemental figures

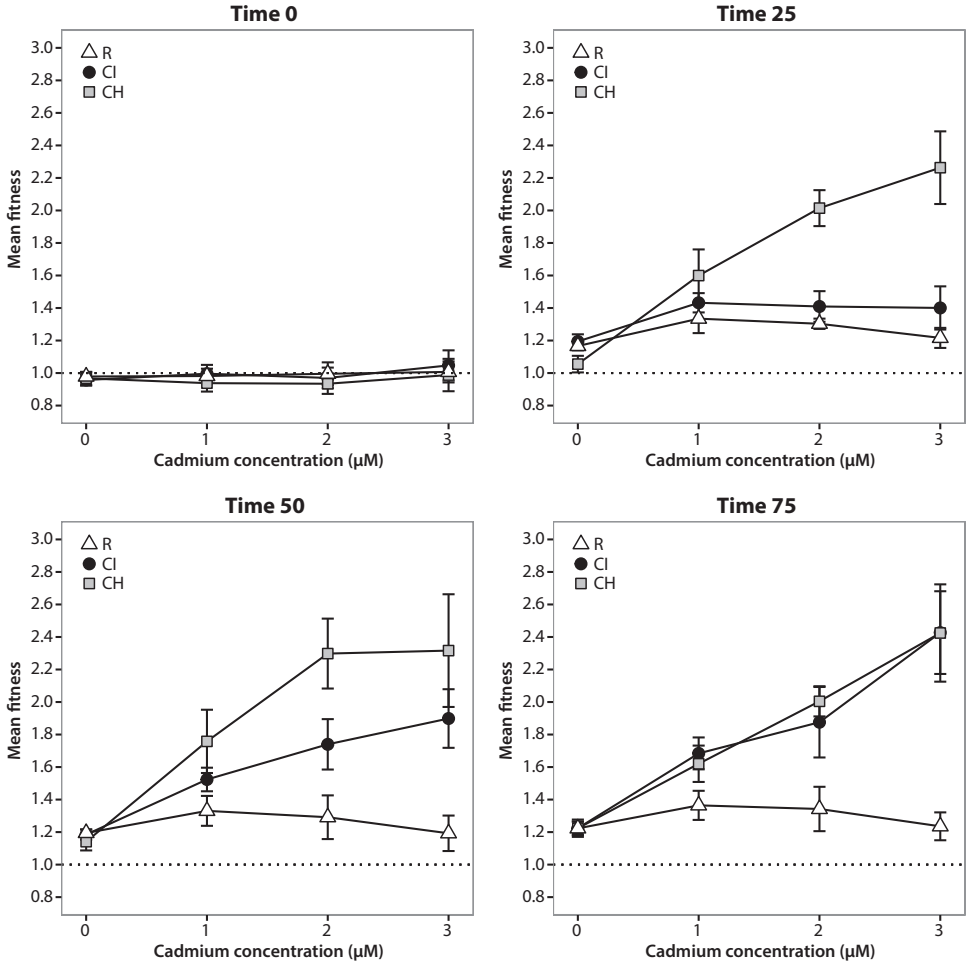

Figure S2.1: Relative fitness of control lines (R), increasing Cd lines (CI), and constant high Cd lines (CH) from different time points at different Cd concentrations. Error bars represent standard error of the mean.

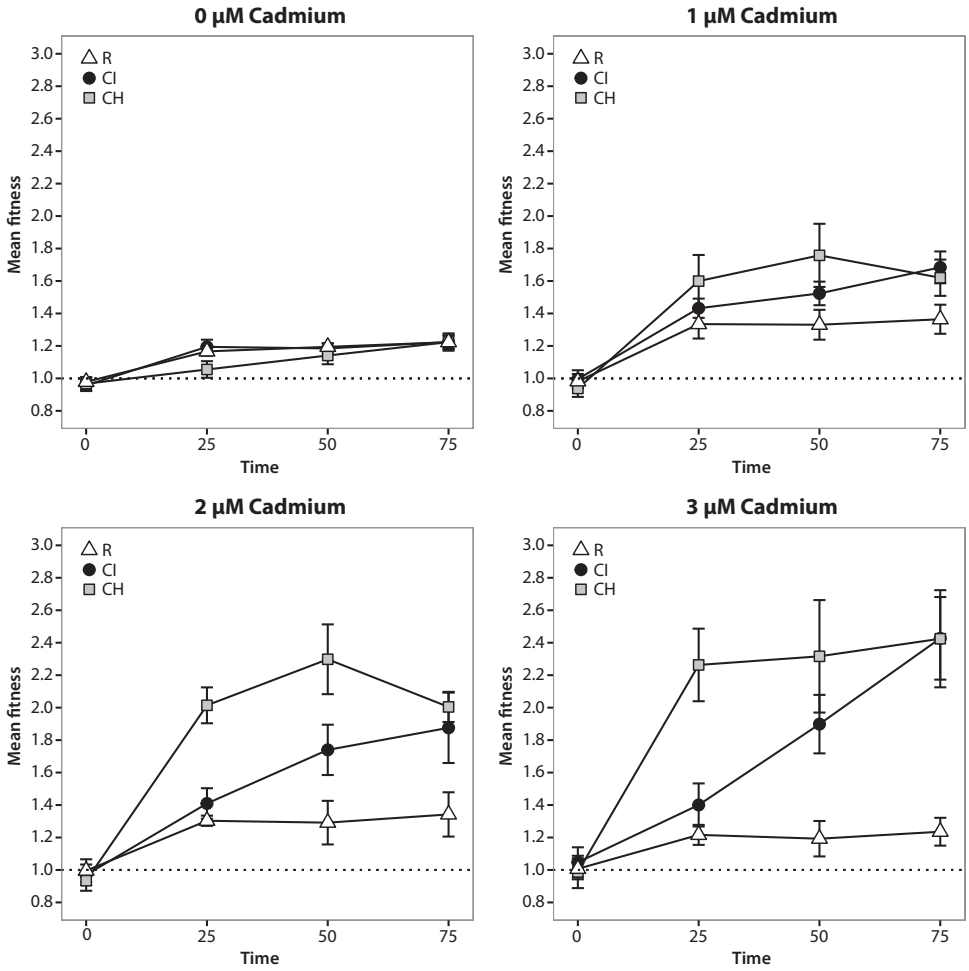

Figure S2.2: Relative fitness of control lines (R), increasing Cd lines (CI), and constant high Cd lines (CH) at different Cd concentrations over time. Error bars represent standard error of the mean.

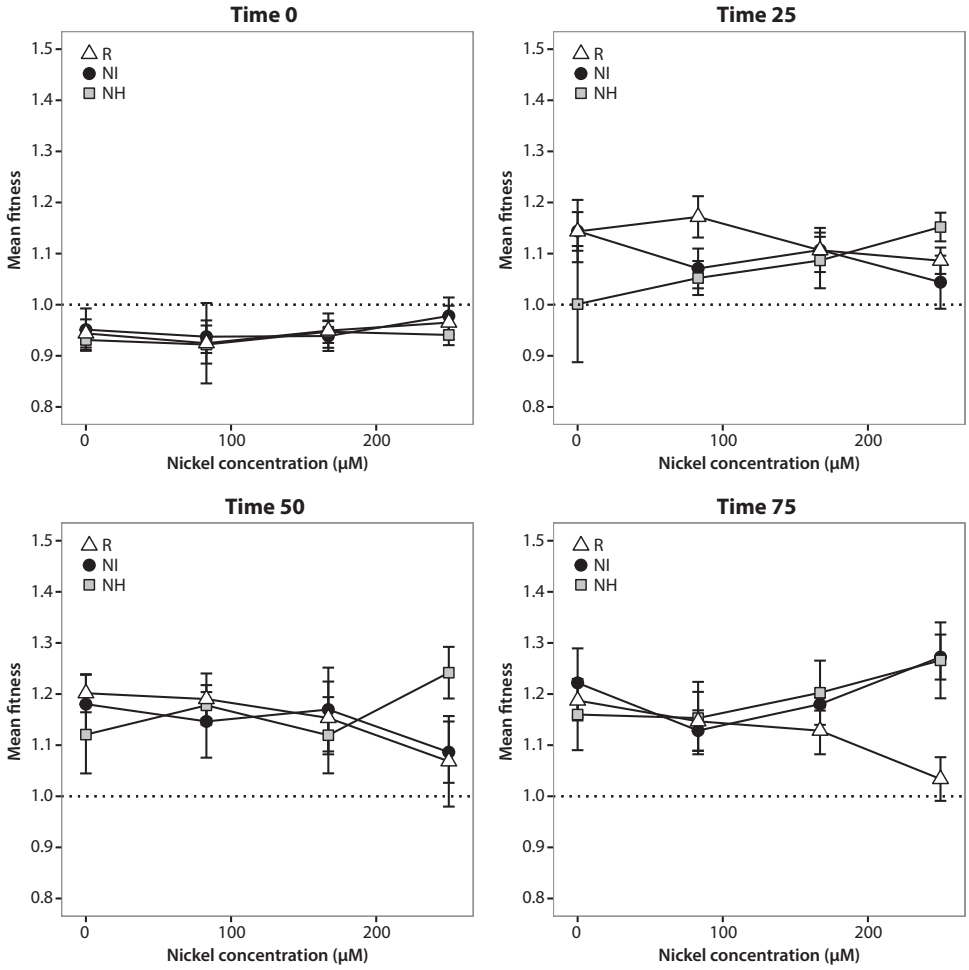

Figure S2.3: Relative fitness of control lines (R), increasing Ni lines (NI), and constant high Ni lines (NH) from different time points at different Ni concentrations. Error bars represent standard error of the mean.

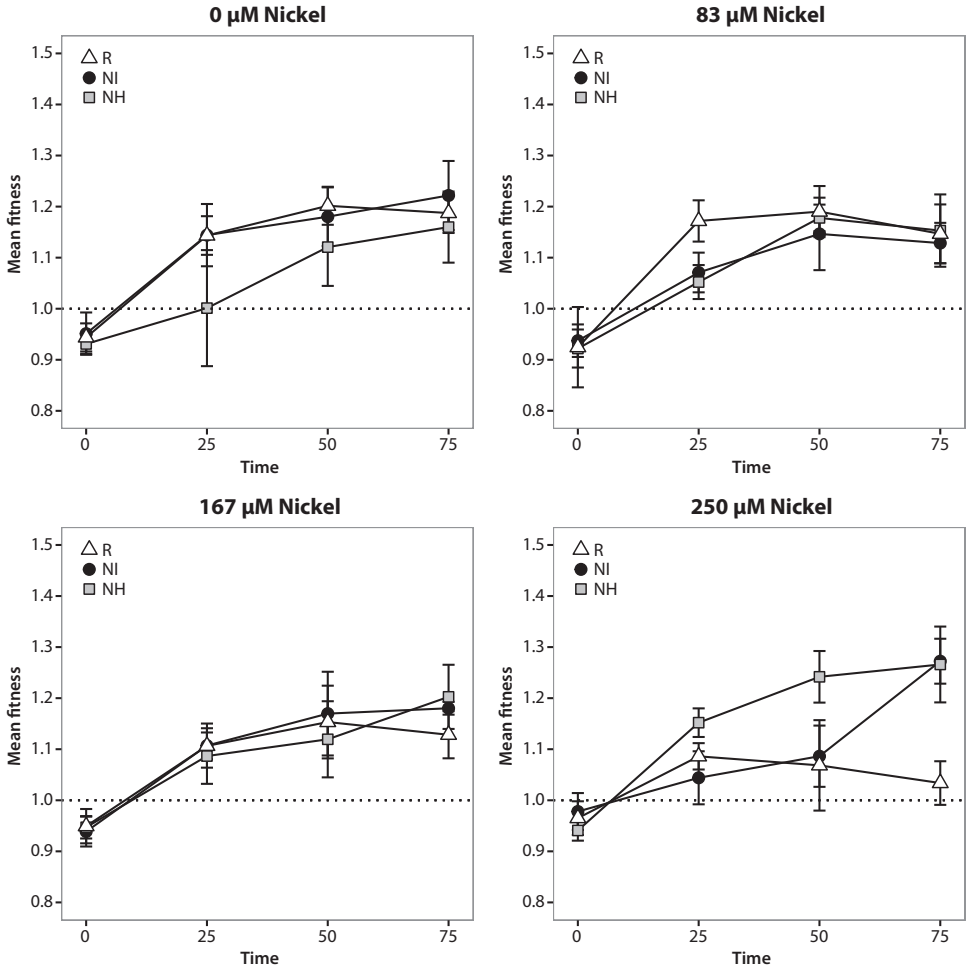

Figure S2.4: Relative fitness of control lines (R), increasing Ni lines (NI), and constant high Ni lines (NH) at different Ni concentrations over time. Error bars represent standard error of the mean.

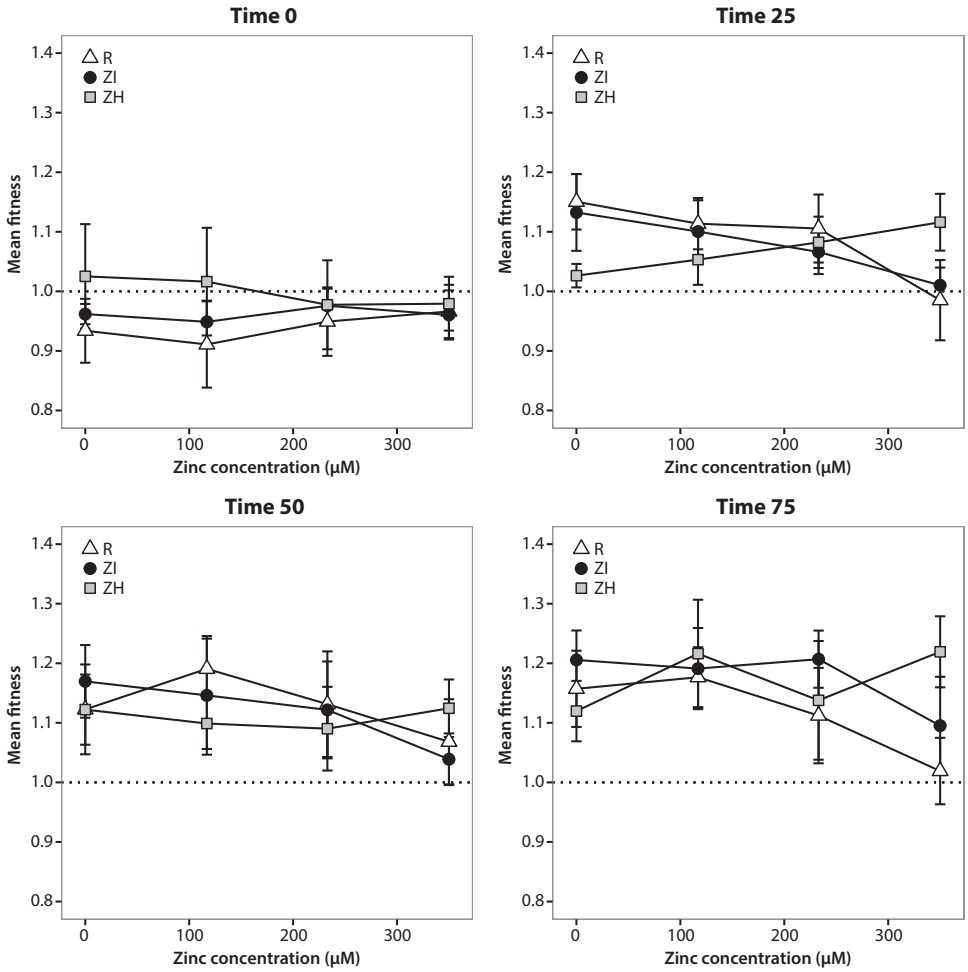

Figure S2.5: Relative fitness of control lines (R), increasing Zn lines (ZI), and constant high Zn lines (ZH) from different time points at different Zn concentrations. Error bars represent standard error of the mean.

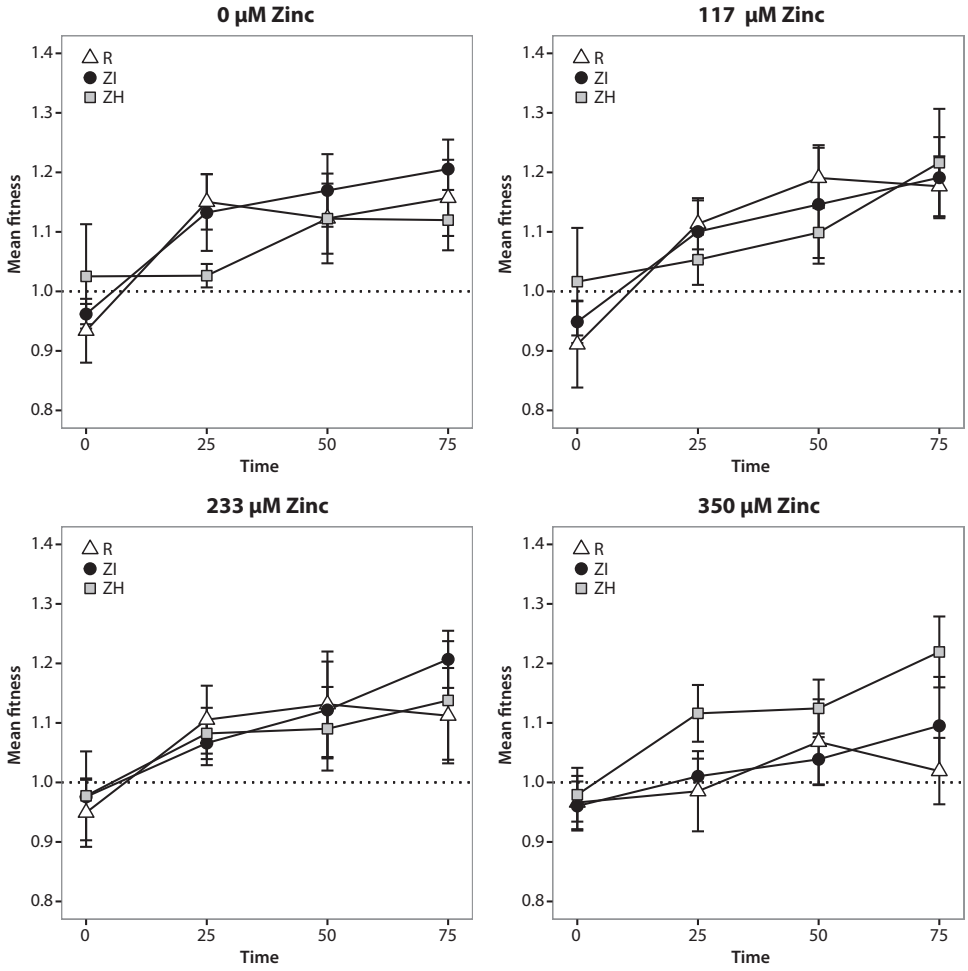

Figure S2.6: Relative fitness of control lines (R), increasing Zn lines (ZI), and constant high Zn lines (ZH) at different Zn concentrations over time. Error bars represent standard error of the mean.

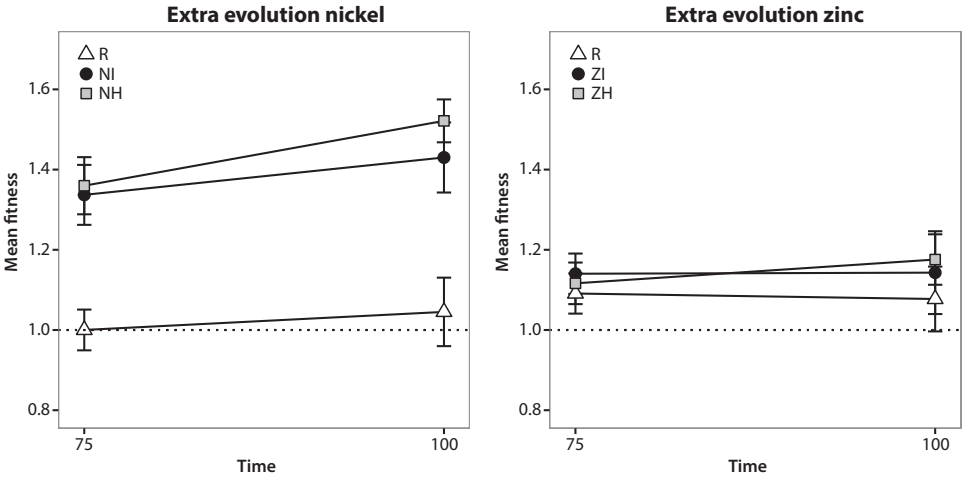

Figure S2.7: Relative fitness of isolates from the final time point of the main experiment (T75) and the continued evolution experiment (T100), as assayed in the constant high metal environment. R = control lines; NI = increasing Ni lines; NH = constant high Ni lines; ZI = increasing Zn lines; ZH = constant high Zn lines. Error bars represent standard error of the mean.

Chapter 3

Genomics of adaptation depends on the rate of environmental change in experimental *Saccharomyces cerevisiae* populations

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In preparation for submission.

Abstract

The rate of directional environmental change may have profound consequences for evolutionary dynamics and outcomes. Yet, most experimental evolution studies focus exclusively on how populations adapt to a sudden large change in the environment, after which the environment is kept constant. We previously cultured replicate *Saccharomyces cerevisiae* populations for 500 generations in the presence of either gradually increasing or constant high concentrations of the heavy metals cadmium, nickel, and zinc. Here, we investigate how each of these treatments affected the genomics of adaptation. Whole genome sequencing of evolved clones revealed that adaptation occurred via a complex combination of SNPs, small indels, whole genome duplications and other large-scale structural changes. For the non-essential metal Cd mutations in the same genes were used for adaptation to both gradual and abrupt increase in concentration. Conversely, for the essential metals Ni and Zn mutations in different genes were used for adaptation to different rates of change. Moreover, evolution was more repeatable following a sudden change in the environment, particularly for Ni and Zn. Our results show that the rate of environmental change and the nature of the selection pressure are important drivers of evolutionary dynamics and outcomes. This implies that if we are to fully understand and anticipate the consequences of important societal problems such as climate change and pollution, we need to take into account not only the total magnitude of the projected environmental change, but also its rate.

Introduction

In nature, organisms typically live in changing environments. Long-term population persistence in the face of such change requires either migration to more favourable habitats, or *in situ* phenotypic or genetic adaptation (Burger and Lynch 1995). One specific scenario of environmental change that is of particular current relevance is directional environmental change, with global climate change and human-induced pollution as prominent examples. The rate of directional environmental change may have profound consequences for population persistence (Gonzalez and Bell 2013; Lindsey et al. 2013), as well as for evolutionary dynamics and outcomes (Collins and De Meaux 2009; Toprak et al. 2012; Morley et al. 2015). Yet, most experimental evolution studies focus exclusively on how populations adapt to a sudden large change in the environment, after which the environment is kept constant (Collins 2011; Barrick and Lenski 2013).

Several predictions exist about how different rates of environmental change affect the course and outcome of evolution. Most of these predictions fall within the framework of the so-called moving optimum model. This model regards adaptation to directionally changing environments as the evolution of a quantitative trait under stabilizing selection with a moving optimum (Kopp and Hermisson 2007, 2009a; Kopp and Hermisson 2009b). The main prediction from this model is that adaptation to gradual change occurs through the repeated fixation of mutations of intermediate phenotypic effect, while adaptation to a sudden large change in the environment occurs through the fixation of fewer mutations of relatively large phenotypic effect. Because mutations of smaller phenotypic effect are more abundant, this should cause evolution to follow more diverse pathways under gradual change. Additionally, it has been suggested that because genetic interactions between mutations of smaller effect are weaker (Schenk et al. 2013; Schoustra et al. 2016), evolution will be less constrained under gradual change, so that under this regime populations will reach higher fitness (Collins and De Meaux 2009).

While the moving optimum model is useful from a conceptual perspective, it has the drawback of focussing on the relationship between an — often unknown — phenotype under selection and fitness. This makes it problematic to perform dedicated empirical tests. Furthermore, it ignores cases where the optimal phenotype does not change linearly with the intensity of the selection pressure (but note Matuszewski et al. 2014). To overcome these limitations, we recently proposed to consider evolution in directionally changing environments within a genotype-by-environment (GxE) framework (Gorter et al. 2016). Specifically, we distinguished between two alternative scenarios, magnitude and reranking GxE. Under magnitude GxE, the same genotypes will be selected at different intensities of the selection pressure, but selection is stronger at higher intensities. This should cause evolution to use the same mutations for adaptation under gradual and abrupt change. Conversely, under reranking GxE, different genotypes will be selected at different intensities of the selection

pressure. This should cause different mutations to be used for adaptation under different rates of environmental change. Additionally, because under gradual change different mutations are selected at different periods during the adaptive process, the window of selection for each individual mutation will be smaller, thus leading to more diverse evolutionary outcomes. Which of these two scenarios applies, and to what extent, will depend on the nature of the selection pressure (Gorter et al. 2016).

In the present study, we investigate how different rates of environmental change affect the genomics of adaptation in experimentally evolved populations of the baker's yeast *Saccharomyces cerevisiae*. We previously cultured replicate yeast populations for 500 generations in the presence of either gradually increasing or constant high concentrations of the heavy metals cadmium (Cd), nickel (Ni), and zinc (Zn) (Gorter et al. 2016). Heavy metals are important environmental contaminants that are required for the maintenance of protein structure and function, but they can also be highly toxic (Boyd 2010; Wysocki and Tamas 2010). Cd is a non-essential heavy metal, which means that it is toxic at all concentrations. Conversely, Ni and Zn are essential heavy metals: they are needed in small amounts, and are toxic at high concentrations only. We suggested that this difference in essentiality would correspond with different types of GxE (Gorter et al. 2016). That is, for Cd we expected the magnitude GxE scenario to apply, because the same evolutionary solution — reduction of the internal Cd concentration — should be favoured at all concentrations, but more strongly so at high concentrations. On the other hand, for Ni and Zn, we expected the reranking GxE scenario to apply, because different evolutionary solutions are required at different external metal concentrations to maintain a low internal metal concentration.

Fitness assays on the evolved populations at different metal concentrations supported this distinction between magnitude and reranking GxE for non-essential (Cd) and essential (Ni, Zn) metals, respectively (Gorter et al. 2016). Moreover, these assays showed that gradual and abrupt change led to similar fitness improvements at the final high metal concentration. Here, we analyse the genomes of single clones isolated at the final time point of our long-term evolution experiment to test three specific predictions. First, we test the prediction following from the moving optimum model that gradual environmental change leads to the selection of more mutations (of presumably smaller phenotypic effect) than rapid change. Second, we test the prediction following from the GxE framework that mutations in the same genes are involved in adaptation to Cd under both regimes, while mutations in different genes are used for adaptation to Ni and Zn. Third, we test the prediction following from the GxE framework that gradual environmental change leads to less parallel evolution than rapid change in the case of Ni and Zn. To our knowledge, our study provides the first direct test of the effect of different rates of environmental change on genomic evolution in a living organism.

Materials and methods

Strains

The ancestral strains used for our evolution experiments, W20 (*MAT**a**/his3/leu2/LYS/ura3/met/ΔHO:KanMX*) and W24 (*MAT**a**/his3/leu2/lys/ura3/MET/ΔHO:NatMX*), are *MAT**a*** haploids derived from S. cerevisiae strain BY4743. These strains were previously transformed at the *HO* locus with cassettes KanMX4 and NatMX4 to confer resistance to the antibiotics geneticin (G418) and nourseothricin, respectively (Goldstein and McCusker 1999; Wloch-Salamon et al. 2008). Populations initiated from single clones of the ancestral strains were evolved in a previously performed long-term selection experiment (Gorter et al. 2016). In brief, six replicate populations were grown in 10 mL 0.5xYPD medium for ~500 generations (75 daily transfers of 1% of the population) under each of seven different treatments: control (no metals added, lines R1-6), increasing levels of Cd (0.2-3 μM $CdCl_2$, lines CI1-6), constant high levels of Cd (3 μM $CdCl_2$, lines CH1-6), increasing levels of Ni (16.7-250 μM $NiCl_2$, lines NI1-6), constant high levels of Ni (250 μM $NiCl_2$, lines NH1-6), increasing levels of Zn (23.3-350 μM $ZnCl_2$, lines ZI1-6), and constant high levels of Zn (350 μM $ZnCl_2$, lines ZH1-6). After 75 transfers, a single clone was isolated from each replicate population by plating out dilutions and growing up a single colony in liquid culture for storage at -80°C.

Determination of cell ploidy

DNA content of evolved clones was determined by flow cytometry. Yeast was grown to saturation and stained with propidium iodide (PI) using a slightly modified version of a previously published protocol (Zhang and Siede 2004) and counting 50,000 events on a FACSAria III BD. The ancestral haploid strains, as well as a related diploid strain, were used as reference points for determining the ploidy level of the evolved clones.

Whole genome sequencing

Ancestral strains and evolved clones were grown up overnight from frozen. Genomic DNA was isolated using a protocol based on a previously published method (Amberg et al. 2005) and phenol-chloroform extraction. Paired-end libraries were generated according to the TruSeq DNA™ Sample preparation LT protocol (Illumina Inc, San Diego CA, USA) and sequenced on a MiSeq V3 (ancestors) or HiSeq2000 (evolved clones) sequencer. Mean read length, distance between reads, and coverage were 291 bp, 440 bp, and 76x, respectively, for the ancestral strains, and 101 bp, 282 bp, and 54x for the evolved clones.

SNP and indel analysis

We used the CLC Genomics Workbench 8.5.1 for SNP and small indel calling. Reads were trimmed to remove adapter sequences and low quality regions (Q<30), and discarded if very short or long (<50 bp, >1000 bp). Mapping to the reference *S. cerevisiae* S288C genome

was performed using standard settings, except that we used a similarity fraction of 0.9 and randomly mapped non-specific matches. Unmapped reads were aligned to the KanMX4 and NatMX4 sequences as a control to check for cross-contamination between samples. In a few cases, one or two reads mapped to the opposite marker sequence, but we found no substantial sign of cross-contamination.

Variant detection for the evolved clones was performed using the fixed ploidy variant detector, specifying the ploidy of each sample as determined by flow cytometry and using a minimum variant probability of 95%. We required a minimum coverage of 10 and ignored variants in regions covered by non-specific matches. Variants were filtered against control reads, i.e. the mapping of the corresponding ancestral strain, and kept if fewer than 2 control reads supported the variant. Annotation of variants was performed using the *S. cerevisiae* CDS track and variants in promoter regions were identified using SnpEff (Cingolani et al. 2012). Promoter regions were defined as regions 500 bp upstream of the transcription start site of a gene. Variants were further filtered manually to exclude events where coverage for the ancestral strain was <10, or <5 forward or reverse reads supported the variant (lower thresholds for clones with low coverage and/or high ploidy to ensure that the maximum probability of observing this number of reads in this orientation by chance is < 0.05).

The PROVEAN tool (Choi et al. 2012) was used to predict functional consequences of amino acid changes in multihit genes. The GO Slim Mapper (2016) was used to group the putative functional consequences of all nonsynonymous mutations per treatment into broad categories. Cluster frequency output from the mapper was adjusted manually to account for the fact that some genes were hit multiple times, that is, GO terms were counted more than once if the gene was hit more than once, and the top 3 terms per treatment were compared to assess functional overlap between treatments.

Copy number variation analysis

For the copy number variation analysis, mapping was performed separately. Briefly, reads were trimmed with trimmomatic (version: 0.32), cleaned from phiX contamination with bowtie2 (version: 2.2.3) and mapped to the reference sequence using bwa-mem (version: 0.7.6a-dev-r434) with default settings. Control-FREEC (Boeva et al. 2012) was used to identify CNVs and large-scale chromosomal copy number alterations with default settings, except for some parameters recommended for yeast and fungi: breakPointThreshold: 0.6, window: 400bp, telomeric: 10kb. For each sample, mean ploidy level as determined by FACS analysis (see above) was used as the reference copy number. Control-FREEC calculates normalized ratios between sample and control read coverage for each individual window and then segments the genome into regions of equal copy number. To identify the most reliable and important copy number variation events, we filtered putative CNVs based on associated Kolmogorov-Smirnov test values ($P < 1 \times 10^{-16}$), and only report events where the total gain or loss comprises >25% of the length of the chromosome. Events where total gain or loss is >75%

are interpreted as whole chromosome duplication/loss. CNV-seq was used to plot the whole genome CNV events (Xie and Tammi 2009).

Engineering of reconstruction mutants

The starting material for our reconstruction mutants are the BY4741 *Δsmf1::KanMX* and *Δfet4::KanMX* strains from the yeast deletion collection (Giaever et al. 2002; Giaever and Nislow 2014). We amplified the HphMX cassette, which confers hygromycin resistance, from strain W26 (*MAT**a**/his3/leu2/LYS/ura3/MET/ΔHO:HphMX*) using primers Repl-F (CAGACGCGTTGAATTGTCCC) and Repl-R (CTGGGCAGATGATGTCGAGG). The resulting PCR product was used for gene replacement in the *Δfet4::KanMX* deletion mutant to yield *Δfet4::HphMX*. Primers located upstream and downstream of *SMF1* and *FET4* (primers A + D from the deletion collection) were used to amplify both gene disruptions, which were then transformed into W24 and its nearly isogenic counterpart of opposite mating type W22 (*MATα/his3/leu2/LYS/ura3/met/ΔHO:NatMX*). Mating followed by selection on SC-met-lys plates was used to generate diploids.

To overexpress *GEX1* we used the low copy number centromeric plasmid pAG26 (Goldstein and McCusker 1999), which contains the HphMX4 cassette. We amplified *GEX1* under the control of its native promoter (-959 bp) from W20 genomic DNA using primers AscI_GEX1_F (TAATGGCGCGCCTGATCAGTTCTAGGTATAAGCATAA) and PacI_GEX1_R (CCGGGTTAATTAATGTTGCACTCACACTACGGA). PCR product was digested with *AscI* and *PacI*, and the plasmid was digested with the same enzymes followed by CIP (calf intestinal phosphatase) treatment. Ligation products were transformed into *E. coli* and colonies were screened for the desired insertion by restriction digestion. Sanger sequencing confirmed correct sequence identity, and the resulting plasmid was transformed into yeast. An overview of all reconstruction mutants can be found in Table 3.1.

Fitness assays

We used competition assays (Lenski et al. 1991; Gorter et al. 2016) to determine the relative fitness of reconstruction mutants. Strains were pregrown in 0.5x YPD for 24h from frozen and competed with a control strain for 24h in the absence and presence of 3 μM $CdCl_2$. For most assays, the competitor used was W3 (*MAT**a**/his3/leu2/lys/ura3/MET*), which is isogenic with W24 but does not contain the NatMX4 cassette. For assays with strains harbouring the pGEX1 plasmid, the competitor was W3 transformed with the empty pAG26 plasmid (Goldstein and McCusker 1999), and hygromycin was added during both pregrowth and competitions. Both before and after 24h of competition, appropriate dilutions of each competition were plated on 0.5x YPD to determine the total number of colonies. Replica plating was used to determine which subset of colonies was nourseothricin resistant. This information was used to calculate the relative number of doublings, or relative fitness (W_{ij}), of each mutant. All competitions were performed in triplicate.

Table 3.1: Overview of all reconstruction mutants

Strain	Details
haploid	W24
haploid pGEX1	W24 + pGEX1
haploid *Δsmf1*	W24 *Δsmf1::KanMX*
haploid *Δfet4*	W24 *Δfet4::HphMX*
haploid *Δsmf1/Δfet4*	W24 *Δsmf1::KanMX Δfet4::HphMX*
diploid	W22 x W24
diploid pGEX1	W22 x W24 + pGEX1
diploid *SMF1/Δsmf1*	W22 *Δsmf1::KanMX* x W24
diploid *SMF1/Δsmf1* + pGEX1	W22 *Δsmf1::KanMX* x W24 + pGEX1
diploid *Δsmf1/Δsmf1*	W22 *Δsmf1::KanMX* x W24 *Δsmf1::KanMX*
diploid *Δsmf1/Δsmf1* + pGEX1	W22 *Δsmf1::KanMX* x W24 *Δsmf1::KanMX* + pGEX1
diploid *Δsmf1/Δsmf1/FET4/Δfet4*	W22 *Δsmf1::KanMX Δfet4::HphMX* x W24 *Δsmf1::KanMX*
diploid *Δsmf1/Δsmf1/Δfet4/Δfet4*	W22 *Δsmf1::KanMX Δfet4::HphMX* x W24 *Δsmf1::KanMX Δfet4::HphMX*

Statistical analyses

Genomic data

For all statistical analyses on SNP and indel count data, we limited ourselves to the data from the metal treatments, and used generalized linear models with metal, rate of change, and the interaction between these effects as explanatory variables. When the response variable was a count, we assumed a Poisson error structure and used a log link function; when the response variable was a proportion, we assumed a binomial error structure and used a logit link function. We performed sequential backward elimination to find the minimal adequate model, and in each case report statistics from model comparison. To test for under- and overdispersion, we used the dispersiontest function in the R package AER (Kleiber and Zeileis 2008). In case of significant overdispersion, we switched to quasipoisson and quasibinomial models, respectively.

For statistical analyses on the total number of mutations including CNV events, we took a conservative approach and considered a change in overall copy number as a single event. Each case of partial or whole chromosome loss/gain was counted as an additional event, irrespective of exact copy number. To determine the effect of chromosome length on the likelihood of a CNV event, we used a generalized linear binomial model with chromosome length as the explanatory variable, and proportion of lines containing a CNV event at that chromosome as the response.

Reconstruction mutants

To determine the fitness effects of (combinations of) *Δsmf1* (continuous effect, with heterozygous state intermediate between wildtype and homozygous state), *Δfet4* (idem), diploidisation (yes/no), and the addition of pGEX1 (yes/no), we used general linear models with relative fitness as the response variable. We started by fitting models with the main effects plus all two-way interactions, and used sequential backward elimination to find the minimal adequate model. We also fitted models in which we treated *Δsmf1* and *Δfet4* as factors with different levels (i.e. wild type vs. mutant; wild type vs. heterozygous vs. homozygous; wild type vs. homozygous haploid vs. heterozygous diploid vs. homozygous diploid), but none of these models was better at explaining the data than our additive (continuous) model. Fitness in the absence and presence of Cd was analysed separately.

All statistical analyses were performed in R (R Core Team 2013).

Results

We previously evolved 42 haploid populations of the baker's yeast *S. cerevisiae* for ~500 generations in the presence of either gradually increasing or constant high concentrations of the heavy metals Cd, Ni, or Zn (Gorter et al. 2016; see Material and Methods for details). Fitness assays of the evolved populations in the presence of different metal concentrations showed: (i) no effect of the rate of environmental change on total fitness improvement in the presence of the final high metal concentration, (ii) quantitative effects of metal concentration on fitness for Cd, but qualitative effects leading to fitness reranking for Ni and Zn (reranking GxE). At the final time point of this experiment, we isolated a single clone from each replicate population for genomic analysis.

Diploidisation

A major change that is commonly observed in evolution experiments with haploid yeast strains is diploidisation (Oud et al. 2013; Hong and Gresham 2014; Voordeckers et al. 2015). To assess whether this occurred in our populations, we used flow cytometry (Figure S3.1). This indicated that indeed the majority of evolved clones had become diploid. Deviating ploidy levels were observed in the majority of the lines that had been exposed to high Zn, which remained haploid, and in a subset of the lines that had been exposed to increasing Cd concentrations, which attained an even higher ploidy level (Figure 3.1).

Number of mutations

Next, we isolated genomic DNA from all evolved clones, as well as from the ancestral strains with which the evolution experiment was initiated, and used paired-end whole genome

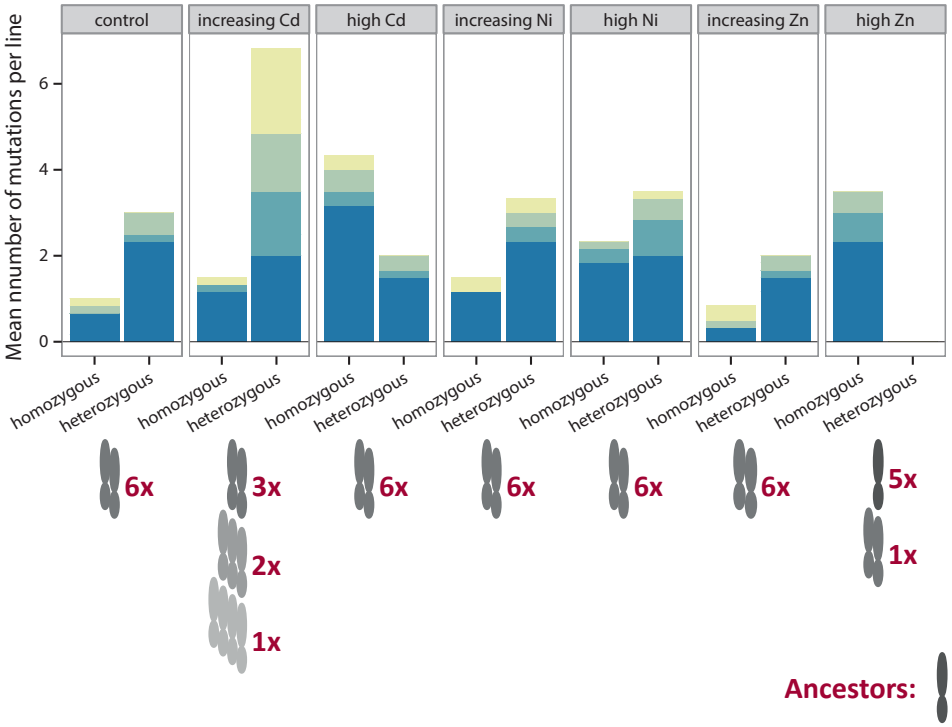


Figure 3.1: Mean number of SNPs and small indels per line per treatment. Shading reflects the type of mutation. Blue = nonsynonymous, teal = synonymous, green = promoter, yellow = intergenic. Chromosome symbols indicate the number of lines of a given ploidy per treatment. Ancestors were haploid, and most evolved lines were diploid.

sequencing to identify additional genetic changes underlying adaptation to each of the selective environments. SNP and small indel calling revealed that evolved clones had acquired on average 5.1 such mutations: 2.1 homozygous and 3.0 heterozygous ones (Figure 3.1). The average number of SNPs and indels per clone was affected by the metal used (χ^2 = 20.41, df = 2, P < 0.0001), with Cd clones containing the highest and Zn clones containing the lowest number of such mutations (7.3 and 3.2 mutations, respectively). This finding may be explained by the relative mutagenic effects of each of these metals, which are highest for Cd and lowest for Zn (LEONARD ET AL. 1986; FLETCHER ET AL. 1994; JIN ET AL. 2003). The rate of environmental change had no effect on the total number of SNPs and indels (χ^2 = 0.02, df = 1, P = 0.88), i.e. we did not find support for the prediction from the moving optimum model that gradual environmental change leads to the selection of more mutations than abrupt change.

Because the metals that we used have a different mutagenic effect, it is possible that our treatments differentially affected the relative fraction of functional and non-functional mutations. If this were the case, our treatments may have had a different effect on the number of adaptive substitutions than on the total number of mutations. To assess this possibility,

we tested whether the number of putatively functional (i.e. nonsynonymous and promoter) mutations depended on treatment as described above. This revealed that the qualitative effect for the total number of mutations also held for the number of putatively functional mutations: metal had a significant effect on this (χ^2= 9.69, df = 2, P = 0.0079; Cd clones contained the highest and Zn clones contained the lowest number of such mutations), but rate of change did not (χ^2= 1.20, df = 1, P = 0.27). The same was true for the total number of mutations when diploidisation and other large scale chromosomal changes were included in the definition (see below for further discussion; effect of metal: χ^2 = 21.65, df = 2, P < 0.0001; effect of rate of change: χ^2 = 2.78, df = 1, P = 0.10). In conclusion, we find a robust effect of only metal on the number of mutations.

Timing of diploidisation

To estimate when diploidisation occurred, we used the relative fractions of homozygous and heterozygous mutations as a proxy for the time spent in the haploid and diploid phase, respectively. The fraction of mutations per line that was homozygous depended on the combined effects of metal and rate of change ($F_{2,30}$ = 5.01, P = 0.013). For the high Cd as well as the high Zn treatment, this fraction was significantly larger than for all other treatments (Figure 3.1), suggesting that diploidisation took place at a later time point under these treatments, or not at all (i.e. for most of the high Zn clones).

Identity of SNPs and small indels

While all synonymous and promoter mutations occurred in unique genomic regions (i.e. never in the same gene, or in the same 500 bp region upstream of a gene), a substantial part of all nonsynonymous mutations occurred in genes that were mutated in two or more replicate lines (Figure 3.2; Table S3.1). Such parallel evolution at the gene level is commonly observed in microbial experimental evolution studies and provides strong evidence for the adaptive benefit of these mutations (Barrick et al. 2009; Kvitek and Sherlock 2011; de Kok et al. 2012; Tenaillon et al. 2012; Toprak et al. 2012; Lang et al. 2013; Kryazhimskiy et al. 2014; Tenaillon et al. 2016). The majority of the mutations that we found in these multi-hit genes are loss-of-function mutations (i.e. stop codon or frameshift mutations), while the effect of all other mutations is predicted to be deleterious based on changes in sequence similarity to a set of homologous protein sequences (PROVEAN score < -2.5 in all cases (Choi et al. 2012); Table S3.2). Null mutants of several of these genes have previously been reported to exhibit increased resistance to the metals that we used (*Δsmf1*/*Δfet4*: + Cd resistance (Ruotolo et al. 2008), *Δvip1*/*Δbul1*/*Δvtc4*: + Ni resistance (Ruotolo et al. 2008; Arita et al. 2009)) or have increased overall performance in rich nutrient media similar to the medium that we used (*Δwhi2* (Qian et al. 2012)).

Based on our GxE framework, our second prediction was that the same mutations would be selected at increasing *versus* constant high concentrations of Cd, while different mutations

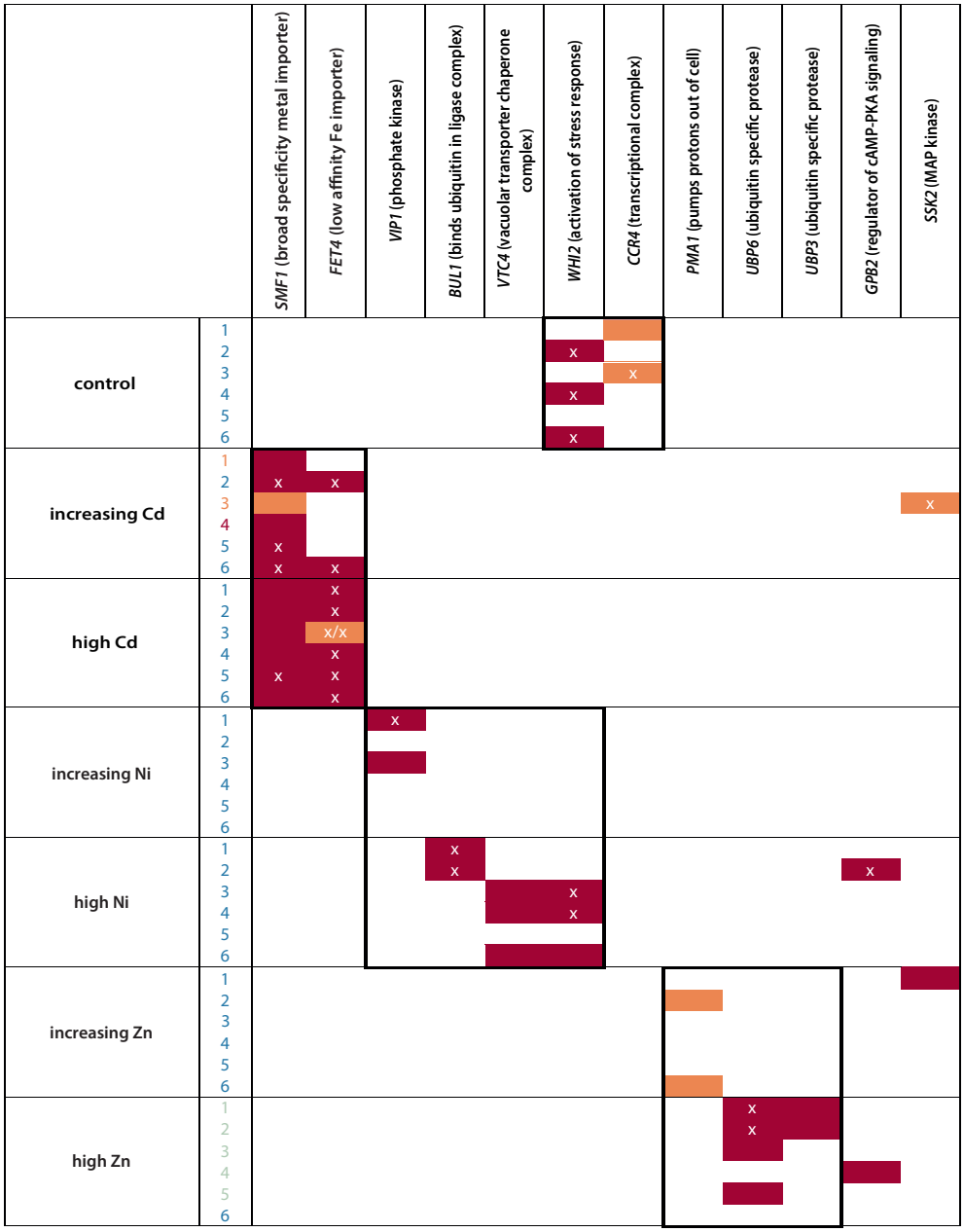


Figure 3.2: Mutations in multi-hit genes per evolved clone. Multi-hit genes are genes in which two or more replicate lines have a mutation. Red shading: homozygous. Orange shading: heterozygous. x symbols signify stop codon or frameshift mutations. Thick black boxes group mutations per metal. The colour of the line number reflects ploidy: green = haploid, blue = diploid, orange = triploid, red = tetraploid.

would be selected at different rates of change for Ni and Zn. To assess this prediction, we determined the fraction of putatively functional (i.e. nonsynonymous or promoter) mutations that was in a gene that was also hit in the populations exposed to the other regime of change for the same metal. For Cd, this fraction was significantly larger than for both other metals (Figure 3.2; $F_{2,33}$= 78.33, P < 0.0001). In fact, the majority of Cd lines had acquired mutations in the same two genes, *SMF1* and *FET4*, irrespective of the rate of change, while lines exposed to increasing *versus* constant high concentrations of Ni and Zn did not share any mutations at the gene level. This same pattern also held at the functional level: while two out of the three top GO Slim terms (ion transport and cellular ion homeostasis) were shared between increasing and constant high levels of Cd, none out of three were shared between increasing and constant high levels of Ni or Zn (Table S3.3).

Repeatability of evolution

The third prediction we wanted to test was that evolution is more repeatable at high concentrations of Ni and Zn than at increasing concentrations of these metals. For the Cd treatments, we had no clear *a priori* expectations about relative repeatability; that is, the same mutations are predicted to be selected under both rates of change for this metal, but the relative likelihood of their fixation depends on their relative benefit at different concentrations (Gillespie 1984). To quantify repeatability, we used the fraction of putatively functional mutations that occurred in multi-hit genes. Neither the interaction between metal and rate of change (χ^2 = 1.31, df = 2, P = 0.52) nor metal per se (χ^2 = 3.20, df = 2, P = 0.20) had a significant effect on repeatability. Conversely, rate of environmental change had a significant effect on repeatability across the three metals (χ^2 = 4.76, df = 1, P = 0.029), with more rapid change increasing the fraction of putatively functional mutations found in multi-hit genes. In line with our expectations, this increase was particularly pronounced in the case of Ni and Zn (fraction of putatively functional mutations in multi-hit genes at increasing versus constant high concentrations: Cd: 0.30 vs. 0.39; Ni: 0.08 vs. 0.30; Zn: 0.14 vs. 0.35). In fact, the difference in repeatability for the Cd lines appears to be driven almost entirely by several putative promoter mutations that were present in a single copy in poly(A) stretches of the triploid and tetraploid lines from the increasing treatment, which are unlikely to be functional mutations. Excluding these mutations from our analyses changes the fractions for increasing *versus* constant high Cd concentrations to 0.40 vs. 0.39.

Large scale copy number variations

Relative read depth analysis on mapped reads from ancestral and evolved clones revealed that, in addition to whole-genome copy number changes, several clones had acquired other large-scale structural changes (Figure 3.3, Figure S3.2, and Table S3.4). These changes were most frequent in clones that had evolved in the presence of increasing levels of Cd: four out of six clones from this treatment had lost or gained (large parts of) entire chromosomes (Figure

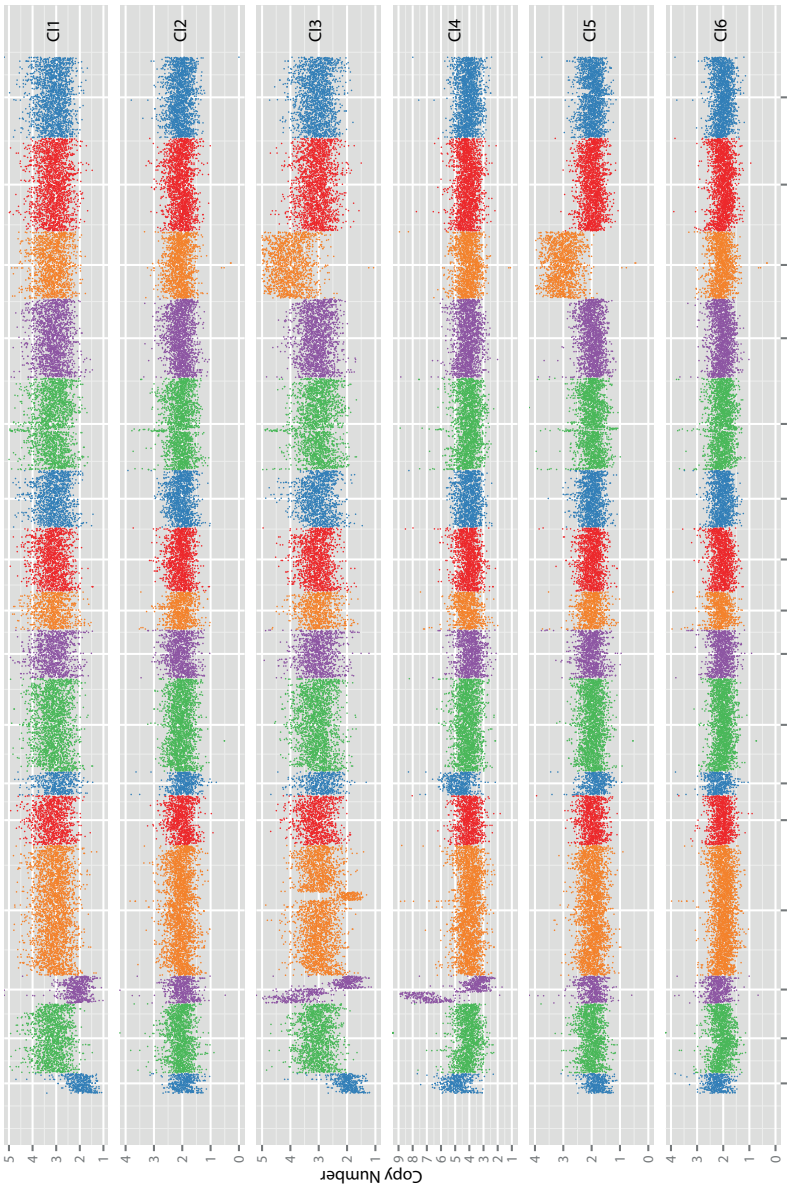


Figure 3.3: Copy number variation across the genome in evolved clones from the increasing Cd treatment. Values are calculated based on FACS analysis (to determine the overall mean copy number for each clone) and relative read depth analysis (to determine the copy number of each specific genomic region). Each dot represents a 400 bp window.

3.3). The same chromosomes (I, III, and XIV) repeatedly featured in these events, although the direction of change (loss or gain) was not always consistent. An interesting case in this respect is chromosome III: in two replicate lines, the leftmost part of this chromosome was present at a higher copy number than the rightmost part, while a copy of the entire chromosome was lost in another case. This raises the possibility that there is selection for a higher copy number of gene(s) residing on the left part of this chromosome, which is counteracted by selection for a lower copy number of gene(s) residing on the right part of this chromosome. We used the Yeastmine tool (Balakrishnan et al. 2012) to identify candidate genes matching these criteria. This revealed that the left part of chromosome III is home to *GEX1*, a gluthatione exchanger that increases Cd resistance upon overexpression (Dhaoui et al. 2011). Conversely, there is no gene in the right part of chromosome III whose deletion is known to increase Cd resistance, although this region does contain several genes whose deletion increases resistance to other metals such as manganese, nickel, and zinc (Ruotolo et al. 2008; Dziedzic and Caplan 2011; Chesi et al. 2012). Perhaps a more likely explanation for the repeated loss of the right part of chromosome III is that it contains the mating type locus. Loss of this locus allows cells to mate with other cells of the same mating type (Lemoine et al. 2005), which may be advantageous when polyploidisation is a selectable trait.

While CNVs were less common in the other treatments, one of the clones evolved in the presence of increasing Ni concentrations acquired an extra copy of chromosome XII (Table S3.4, Figure S3.2). This chromosome contains the *EMP70* gene, which increases Ni resistance upon overexpression (Hegelund, et al. 2010). Visual inspection of relative read depth plots pointed towards two additional putative cases of CNVs that were neglected by our stringent settings, but likely represent real events (clone CI3, chromosome IV 884000-981200 (loss); clone ZI3, chromosome XII 594400-654000 (gain)). Consistent with previous studies (Kumaran et al. 2013), we found that shorter chromosomes were more likely to feature in CNV events (χ^2 = 9.33, df = 1, P = 0.0022). This may be explained by the fact that the copy number change of a shorter region involves fewer genes and is thus less likely to confer antagonistic pleiotropic effects.

Mutant reconstruction

Our results suggest that adaptation to the selective environments was mediated by a combination of SNPs, small indels, whole-genome duplication, and other large-scale structural changes. To investigate the role that each of these types of genetic change, as well as their interactions, play in adaptation, we focused on four genetic changes that occurred repeatedly in the Cd lines: loss-of-function mutations in the metal importers Smf1p and Fet4p, diploidisation, and additional copies of the left arm of chromosome III. To approximate the effects of each of these four changes, we knocked out *SMF1* and *FET4* in the ancestral genetic background, mated *MAT**a*** strains with their nearly isogenic *MATα* counterpart to create diploids, and used a centromeric — low copy number — plasmid to introduce extra copies of *GEX1* under

the control of its native promoter. Additionally, we generated mutant strains in which these mutations were combined in the same way as in the evolved clones, plus several potential evolutionary intermediates, to assess whether the effect of each of the mutations depended on the presence of the other – relevant – mutations.

Competition assays with a differently marked ancestor revealed that, in the absence of Cd, neither of the knockout mutations had an effect on fitness (Figure 3.4, *Δsmf1*: $F_{1,29}$ = 0.56, P = 0.46; *Δfet4*: $F_{1,28}$ = 0.12, P = 0.73), while diploidisation provided a fitness advantage ($F_{1,30}$ = 34.20, P < 0.0001), and introduction of pGEX conferred a fitness disadvantage ($F_{1,30}$ = 32.91, P < 0.0001). Conversely, in the presence of the final high concentration of Cd, *Δsmf1* increased relative fitness (Figure 3.4; $F_{1,28}$= 47.88, P < 0.0001), irrespective of the genetic background. This effect was codominant, i.e. fitness increase in the heterozygous state was half of that in the homozygous state (an additive [continuous] model performed no worse than a model with separate factor levels). Conversely, we did not find a significant increase in fitness for the *Δfet4* mutation ($F_{1,27}$ = 3.12, P = 0.089), which may be explained by its relatively subtle effect in combination with the large error in our measurements, or by the fact that the hygromycin marker that these mutants carry (non-significantly) reduces fitness in the presence high concentrations of Cd (t = -3.544, df = 2, P = 0.071; data not shown). The effects of diploidisation and pGEX1 introduction were mutually dependent ($F_{1,28}$ = 11.44, P = 0.0021): by itself, diploidisation is neutral, and pGEX1 introduction detrimental, but the combination of these mutations provides a strong selective advantage in the presence of Cd.

Discussion

We studied how the rate of directional environmental change affects genomic evolution using experimental evolution of Cd, Ni, and Zn tolerance in *S. cerevisiae*. Whole genome sequencing revealed that evolved clones carried combinations of SNPs, small indels, whole genome duplication, and other large-scale structural changes. Based on previously performed fitness assays of evolved isolates (Gorter et al. 2016) and predictions from theory, we used this genomic information to test the following predictions: (i) gradual environmental change leads to the selection of more mutations than sudden change, (ii) different mutations are used for adaptation under different rates of environmental change for Ni and Zn, but not Cd, and (iii) evolution is more repeatable following sudden change for Ni and Zn. Our results do not support the first prediction, and instead show that gradual and abrupt change led to the selection of similar numbers of mutations. By contrast, we did find support for our second prediction: mutations in the same genes were used for adaptation to both gradual and abrupt increase in concentration of the non-essential metal Cd. Conversely, mutations in different genes were used for adaptation to different rates of change for the essential metals Ni and Zn. Finally, in support of our third prediction, we found that evolution was more repeatable

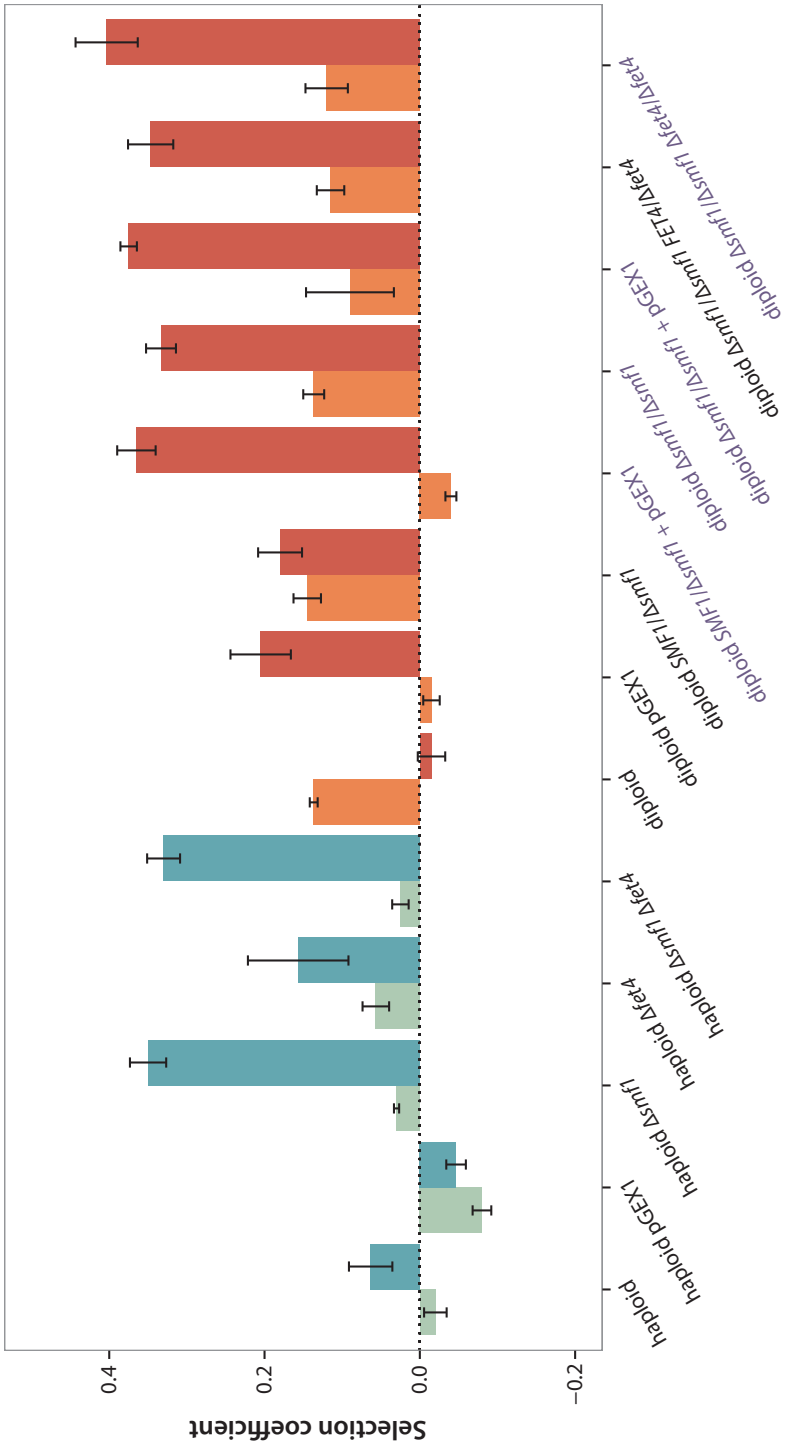


Figure 3.4: Mean selection coefficient (±SEM) of reconstruction mutants in the absence (light colours) and presence (dark colours) of 3 µM Cd. Green = haploid strains, orange = diploid strains. Purple labels: mutation combinations same as in evolved clones.

following a sudden change in the environment, particularly for Ni and Zn. These results are in line with our previous phenotypic results (Gorter et al. 2016), and show that the rate of environmental change and the nature of the selection pressure are important determinants of evolutionary dynamics and outcomes.

Our results can be explained by the properties of the metals that we used. Cd is a non-essential metal, which means that it is toxic at all concentrations. Consequently, we predicted that for this metal the same evolutionary solution, reducing the internal metal concentration to a minimum, would be favoured under both regimes of environmental change. Indeed, evolution of Cd tolerance repeatedly occurred through loss-of-function mutations in two of the main metal importers through which Cd can enter the cell, Smf1p and Fet4p (Wysocki and Tamas 2010). While in theory upregulation of Cd export should confer the same phenotype, laboratory strains of *S. cerevisiae* carry a non-functional version of the Cd exporter Pca1p (Adle et al. 2007), which presumably constrains adaptation via this route. In our previous study, we found that fitness increase was delayed under gradual increase in Cd concentration (Gorter et al. 2016). This implies that even if the same mutations were selected under both rates of change, this happened more slowly under gradual change because selection is weaker at lower external metal concentrations.

In contrast to Cd, yeast requires small amounts of the essential metals Ni and Zn (Gadd 1993; Bleackley and MacGillivray 2011). We thus hypothesized that different evolutionary solutions would be favoured at different external concentrations of these metals to maintain optimal metal homeostasis. This might lead to different evolutionary outcomes following gradual and abrupt change if the mutations that are selected at earlier stages of adaptation to gradually increasing metal concentrations prevent the secondary selection of mutations with benefits at high metal concentrations only. This can happen either because of epistatic interactions between mutations, i.e. mutations are not beneficial in each other's presence (de Visser et al. 2011), or because of genetic correlation between metal environments , i.e. mutations that are selected at intermediate concentrations also confer a — more moderate — benefit at high concentrations (Samani and Bell 2010). In line with this, for Ni and Zn we found no overlap between the mutations used for adaptation to gradual and abrupt change, when defined at either the gene or the functional (GO Slim) level. Furthermore, the function of the genes involved in adaptation to Ni and Zn was less directly related to metal transport and processing, but rather to processes such as ubiquitination and vacuolar transport. This is consistent with the requirement to keep the levels of these metals within specific limits, rather than to reduce them to a minimum.

A notable finding in our study is that evolution in gradually changing environments is less repeatable. That is, the fraction of putatively functional mutations that was in multi-hit genes was significantly smaller under gradual change. This effect was particularly pronounced for the essential metals Ni and Zn, and can be understood by thinking of evolution under gradual change as evolution on continuously changing fitness landscapes. The time window for selection

of individual beneficial mutations will be reduced under such circumstances, and consequently the final outcome of the selective process will be more divergent. A previous study in which *E. coli* was exposed to increasing concentrations of the antibiotic rifampicin similarly found genotypic evolution to be more repeatable following faster environmental change (Lindsey et al. 2013). Conversely, a virus undergoing a gradual or abrupt shift to a novel host used more diverse mutations for adaptation when it was transferred to this host at once (Morley et al. 2015). However, this study did not involve any intermediate environments in the true sense, but instead changed the ratio between permissive and novel host cells over time. In such patchy environments, evolution is likely constrained by trade-offs between performance on each of the individual hosts, thus selecting for the subset of generalist mutations that simultaneously increase performance on both resources (Bailey et al. 2015).

Some of our results are consistent with predictions from the moving optimum model. This model predicts that different mutations are selected at different rates of environmental change, and that adaptation is more divergent under gradual than abrupt change. This is indeed what we found for Ni and Zn. However, this is not the case for Cd, underscoring that the model is applicable to only a subset of all scenarios of directional environmental change. Moreover, the rate of change did not affect the number of mutations, while this is one of the main predictions following from the model. We cannot exclude the possibility that the mutations used for adaptation to increasing Ni and Zn confer a smaller change in some important trait under selection (Kopp and Matuszewski 2014) than those used for adaptation to constant high concentrations of these metals. However, we found no indication for this, because the mutations used under different rates of change fall in different functional categories. Finally, a more gradual change in the environment does not result in better adaptation (Gorter et al. 2016). All in all, our results provide limited support for the moving optimum model as a unifying framework explaining adaptation to directionally changing environments.

A result we did not anticipate, and that substantially complicated our analyses, was that most of our evolved lines had become diploid; some lines evolved in the presence of Cd even became triploid or tetraploid. This likely occurred via failed cytokinesis or spurious mating between yeast cells of the same mating type (Ono et al. 1990; Lemoine et al. 2005; Jin et al. 2011; Storchova 2014). Several previous experimental evolution studies using a variety of selective environments have also reported diploidisation (Oud et al. 2013; Hong and Gresham 2014; Voordeckers et al. 2015). A systematic screen revealed that benefits from a reduced surface-to-volume ratio of diploid cells, caused by their larger size, are likely of limited general importance in the presence of toxins (Zorgo et al. 2013). Instead, it appears that diploidy is the ancestral state upon which yeast converges repeatedly (Mable and Otto 2001; Gerstein et al. 2006; Selmecki et al. 2015). Even if diploidisation was common in our evolution lines and provides a benefit in the absence of metals (Figure 3.4), it probably occurred as a secondary adaptation, because most of the — likely driving — mutations in multi-hit genes were present in the homozygous state (Figure 3.2; but note that loss of

heterozygosity can have the same result (Andersen et al. 2008; Llorente et al. 2008; Ratcliff et al. 2015)). Diploidisation also has consequences for subsequent evolution: it essentially doubles the mutation rate, but at the same time reduces the efficiency of selection (Otto and Gerstein 2008). Predicting the joint effect of these processes is complicated, although diploidisation may provide an immediate advantage in the presence of mutagens by masking partially recessive deleterious mutations (Mable and Otto 2001; Thompson et al. 2006), and can significantly change the spectrum of mutations used for adaptation (Anderson et al. 2003; Anderson et al. 2004).

In addition to a shift in ploidy level, we observed several other large-scale copy number changes. These changes were most frequent in triploid and tetraploid lines, consistent with polyploidy being an unstable transient state in which organisms lose chromosomes over time to revert back to a diploid state (Fujiwara et al. 2005; Gerstein et al. 2006; Gerstein et al. 2008; Sheltzer et al. 2011; Storchova 2014). CNV events comprising parts of chromosomes were generally bordered by retrotransposons (six out of seven times), suggesting that these events arose through homologous recombination between such elements (Dunham et al. 2002; Mieczkowski et al. 2006). In fact, some of the specific breakpoints that we observed were previously identified as mitotic recombination hotspots (Lemoine et al. 2005). The fitness effects of aneuploidy and large chromosomal changes strongly depend on environmental conditions, and often result from a change in expression of one or a few key genes (Tan et al. 2013; Sunshine et al. 2015). The copy number changes involving the left arm of chromosome III that we observed under increasing Cd show a strong resemblance to naturally occurring large-scale rearrangements that involve the *CUP1* and *CUP2* loci involved in copper tolerance (Chang et al. 2013). That is, these rearrangements are also variable in length, but always contain the *CUP* loci, and are bordered by retrotransposons. Several previous experimental evolution studies have found that gene amplification can contribute considerably to adaptation (Brown et al. 1998; Sun et al. 2009; Araya et al. 2010; Hong and Gresham 2014). Although we did not detect amplifications at the single gene level, these findings, together with our analyses of constructed strains overexpressing *GEX1* (Figure 3.4), do support our hypothesis that the chromosome III CNVs were selected because they increase *GEX1* expression.

To determine how the different types of observed mutation (i.e. SNPs/small indels, polyploidisation, and CNVs) combine to increase fitness, we constructed mutants carrying combinations of four frequent mutations found in the Cd evolution lines. Fitness assays confirmed that all four mutations contributed to performance in the selective environment. Crucially, mutational effects depended significantly on the genetic background, the assay environment, and the interaction between these factors (i.e. GxGxE (Remold 2012; de Vos et al. 2013; Flynn et al. 2013; Lindsey et al. 2013)). This implies that evolutionary trajectories may be highly contingent on the specific scenario of environmental change, even when the same mutations are used for adaptation. In our case, in the absence of Cd *Δsmf1*

and *Δfet4* did not affect fitness, while diploidisation had a beneficial, and increased *GEX1* expression a detrimental effect. By contrast, in the presence of high concentrations of Cd *Δsmf1* conferred a large fitness increase. *Δfet4* and diploidisation did not have a significant effect in this environment, while introduction of extra copies of *GEX1*, hypothesized to have driven selection of the chromosome III CNVs, had a detrimental effect in isolation. Conversely, the combination of diploidisation and increased *GEX1* expression did provide an advantage. In all of these cases it should be noted though, that the results may depend to some extent on the reduction in yield from Cd, which was considerably smaller in our current study relative to Gorter et al. 2016 (see Supplement). It seems therefore plausible that the fitness increase conferred by both *Δsmf1* and *Δfet4* was larger under the original assay and evolution conditions.

The above information allows us to speculate about the evolutionary paths that were likely taken by the populations challenged with Cd. The most plausible scenario for the constant high Cd populations is that mutations in *SMF1* were followed by mutations of more subtle effect in *FET4* and finally diploidisation (chromosome III CNVs were not found in response to this treatment): most *FET4* mutations were homozygous, and the overall homozygous/heterozygous ratio under this treatment suggests that diploidisation occurred relatively late. By contrast, we infer that evolution under gradually increasing concentrations also started with mutations in *SMF1*, but that diploidisation followed relatively quickly: under this treatment, a much larger fraction of all mutations is heterozygous. In line with this, diploidisation is beneficial in the absence of Cd (Figure 3.4), and hence likely also at low Cd concentrations. This early diploidisation may then have set the stage for further increases in ploidy level and associated CNVs, which are beneficial at high Cd concentrations only. The fact that lines from the increasing Cd treatment had either mutations in *FET4* or polyploidy/copy number changes suggests reciprocal sign epistasis between these mutations (Poelwijk et al. 2007). This could also explain why we did not observe any CNV events in response to the constant high Cd treatment, because all lines evolved under this treatment contained mutations in *FET4*. Alternatively, the high Cd lines may have already gone through a phase of "quick and dirty" CNV-mediated adaptation (Yona et al. 2012), and reverted back to the more stable diploid state, or not reached this stage yet because it is contingent on diploidisation, which was delayed under this treatment.

In summary, we have shown that the rate of directional environmental change has crucial consequences for the molecular evolution of yeast adapting to non-essential and essential heavy metals. The identity of the mutations used for adaptation, as well as the repeatability of evolution, depended on a combination of the rate of change and the nature of the selection pressure. Given the fundamental relevance of these factors and the application of three rather different metals, our results are likely relevant for a broad range of other organisms and stressors as well. For instance, we predict that our results for the non-essential metal Cd (i.e. same mutations selected irrespective of the rate of change) also apply to other situations

where the selective agent has detrimental effects at all intensities or the total magnitude of environmental change is small, because in these cases we expect only limited fitness reranking between environments. Analogously, the findings for the essential metals Ni and Zn (i.e. different mutations selected following gradual and abrupt change, evolution more repeatable under abrupt change) are likely applicable to other cases where the selective agent has positive or negative effects depending on the intensity or the environmental change is large, because in these cases fitness reranking should be more prominent. In general, our results imply that if we are to fully understand and anticipate the consequences of important societal problems such as climate change and pollution, we need to take into account not only the total magnitude of the projected environmental change, but also its rate.

Acknowledgements

We thank R. Korona and K. Tomala for providing deletion strains and the pAG26 plasmid, R. Akkers and M. Zwart for practical advice, and G. Sanchez, E. Schijlen, and J. van Haarst for assistance with whole genome sequencing.

Appendix A1: Supplemental figures and tables

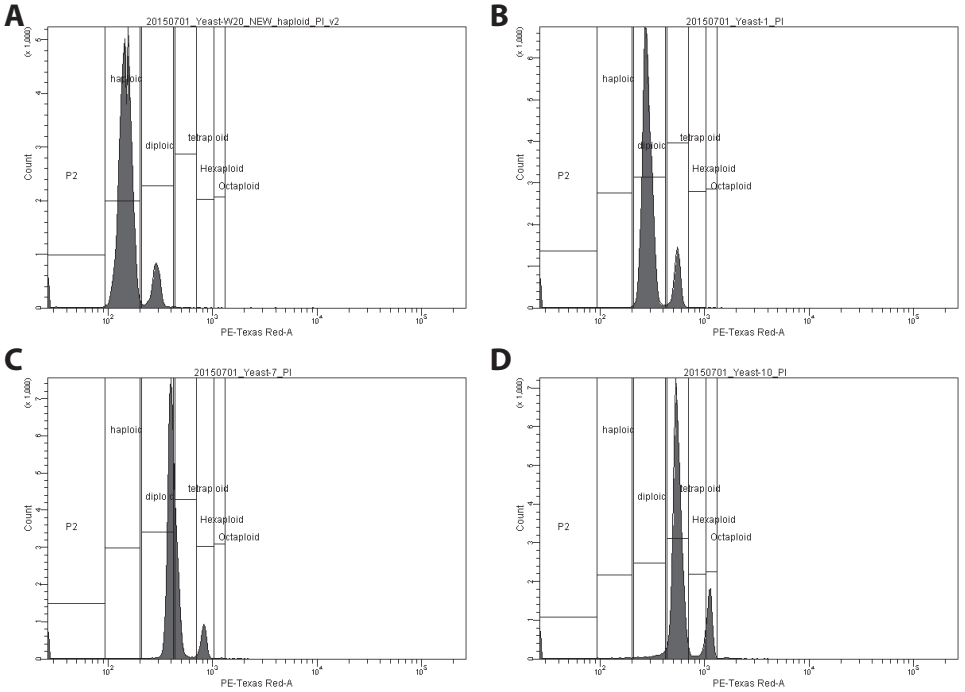
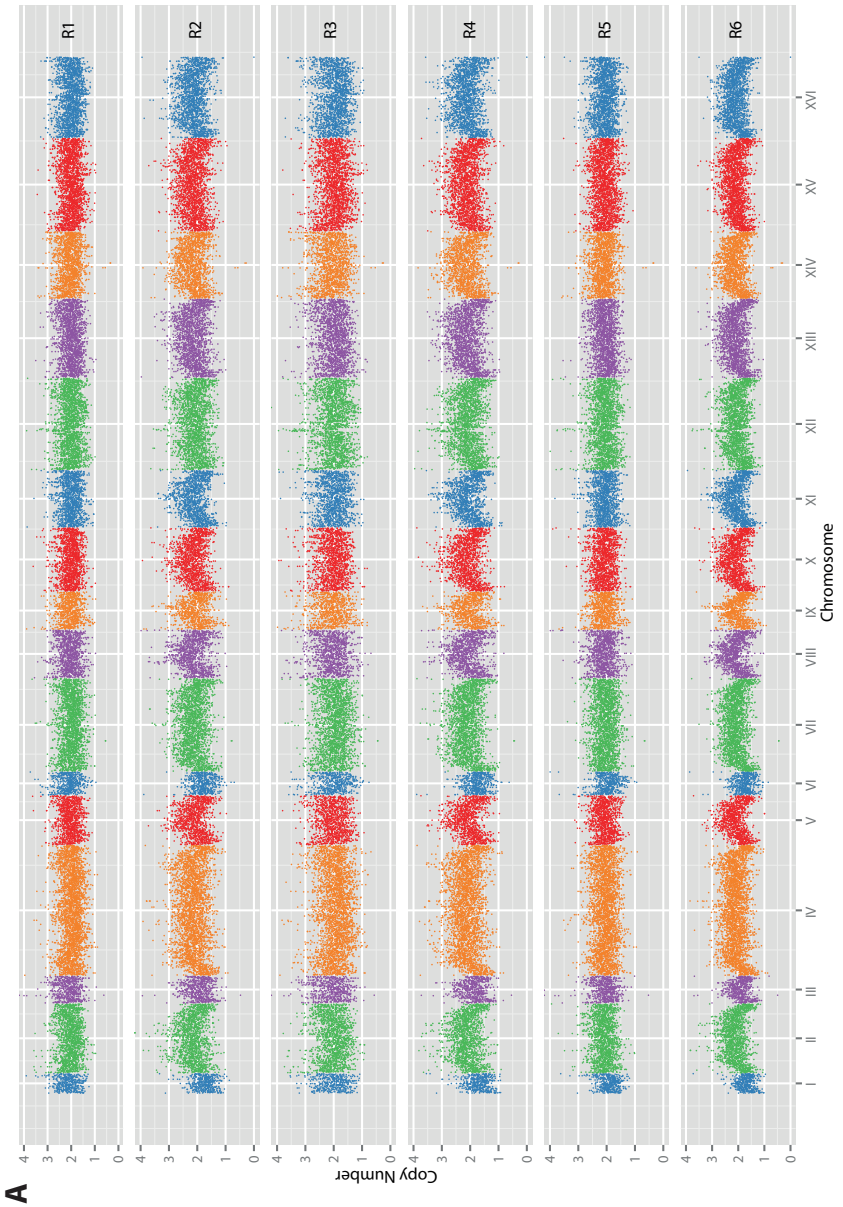


Figure S3.1: Example peak patterns from FACS analysis. A) Haploid control strain (W20). B) Diploid strain (R1). C) Triploid strain (CI1). D) Tetraploid strain (CI4).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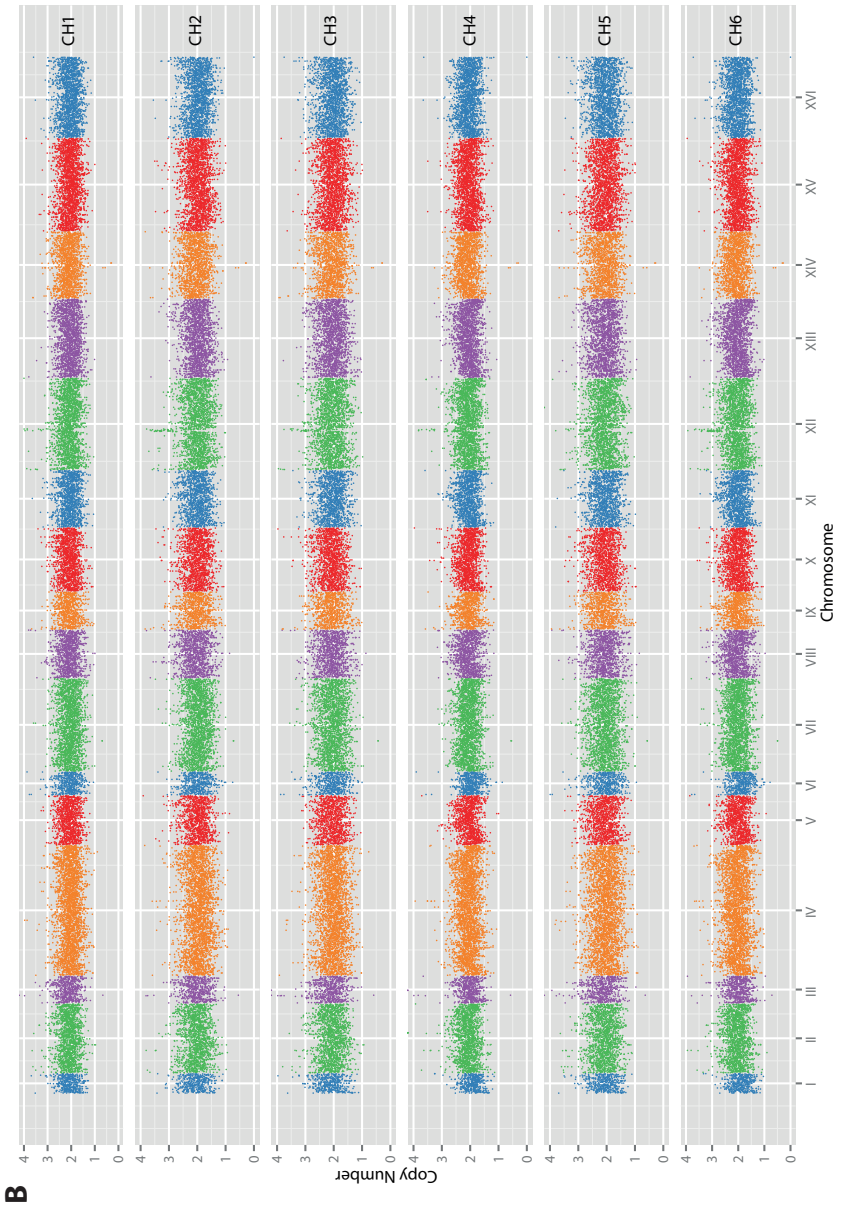
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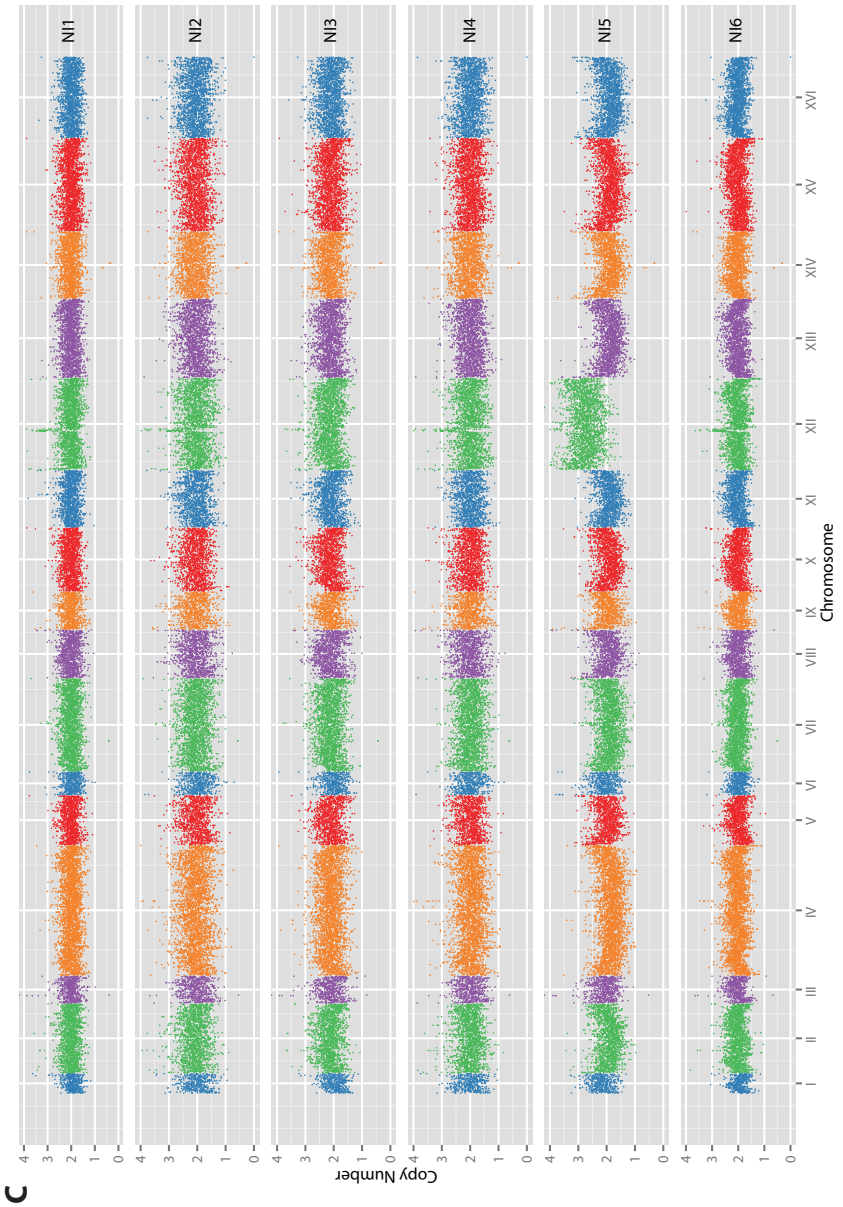
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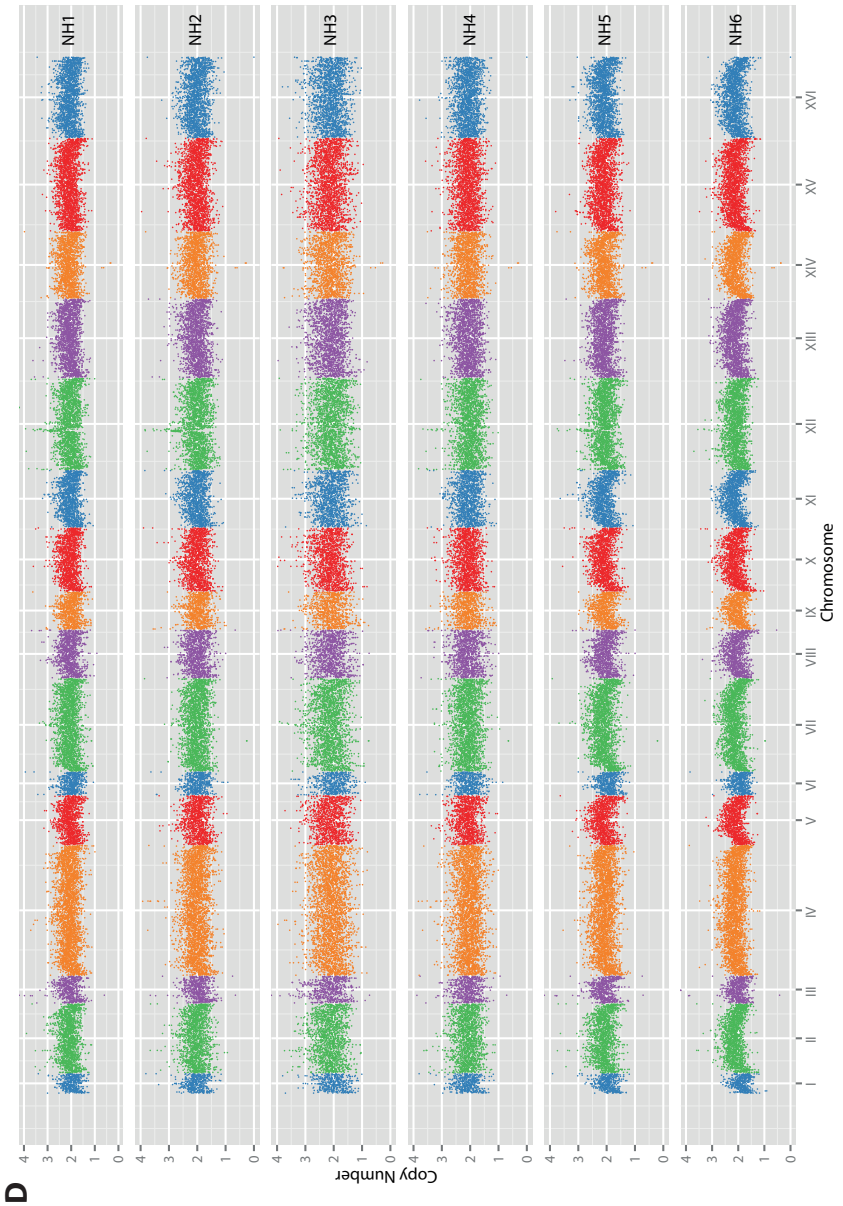
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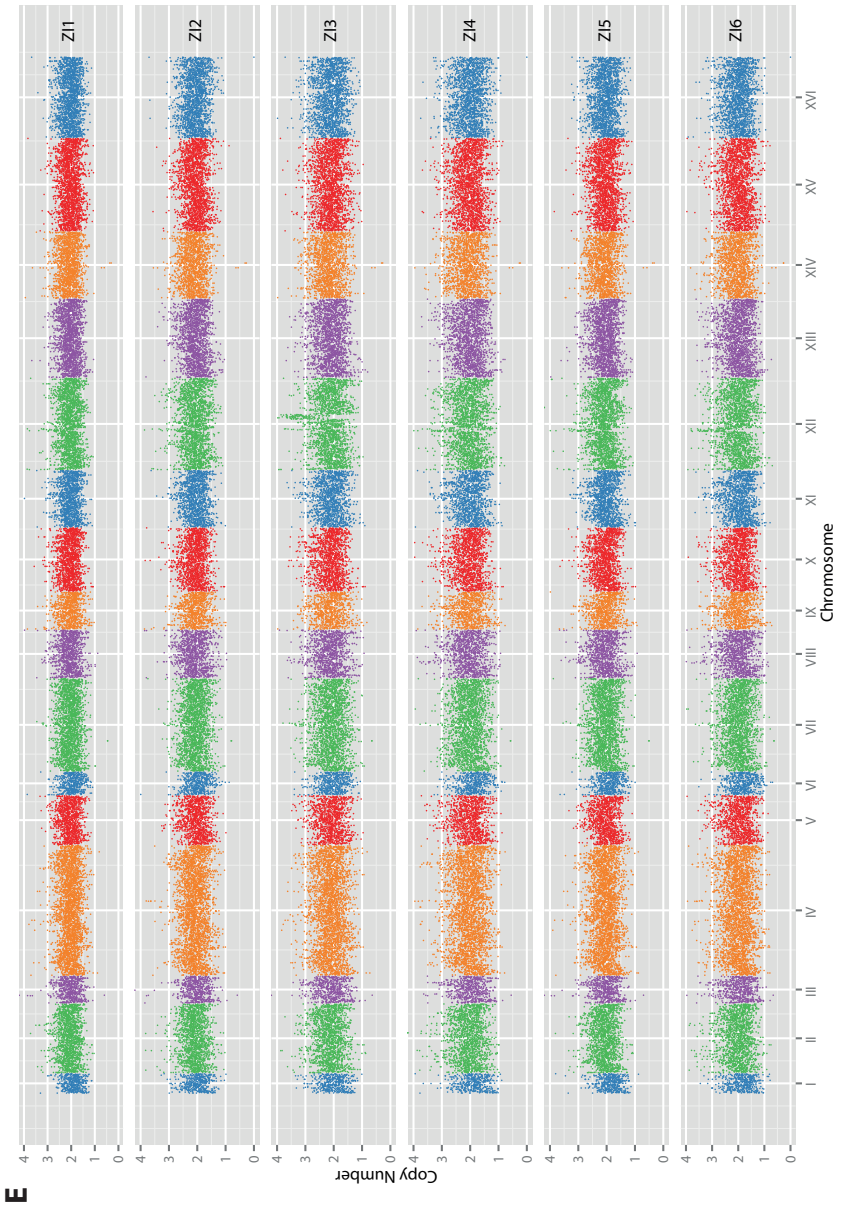
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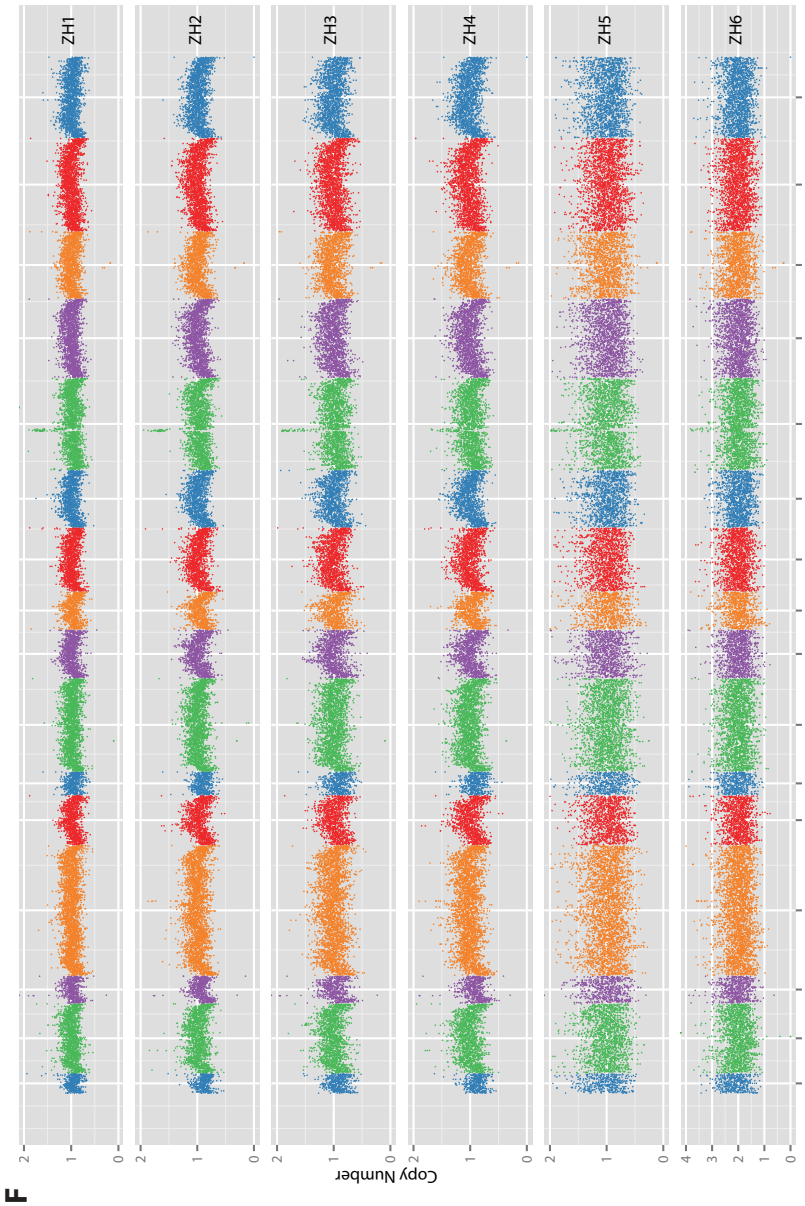

Figure S3.2: Copy number variation across the genome in evolved clones from different treatments: A) control (R1 - R6), B) high Cd (CH1 - CH6), C) increasing Ni (NI1 - NI6), D) high Ni (NH1 - NH6), E) increasing Zn (ZI1 - ZI6), F) high Zn (ZH1 - ZH6). Values are calculated based on FACS analysis (to determine the overall mean copy number for each clone) and relative read depth analysis (to determine the copy number of each specific genomic region). Each dot represents a 400 bp window.

Table S3.1: SNPs and small indels in evolved clones

Line	Chromosome	Region	Type	Reference	Allele	Zygosity	Genotype	Promoter region change	Coding region change	Amino acid change
CI1	III	127715	SNV	T	G	Heterozygous	G/G/T			
CI1	Mito	20366	SNV	A	C	Homozygous	C/C/C		Q0065:c.[244-142A>C]; Q0045:c.[244-142A>C]; Q0070:c.[244-142A>C]	
CI1	X	64146	SNV	G	T	Heterozygous	G/G/T		YJL197W:c.342G>T	YJL197W:p.Lys114Asn
CI1	X	465318	SNV	G	A	Heterozygous	A/G/G		YJR016C:c.891C>T	
CI1	XV	90283	SNV	C	A	Homozygous	A/A/A		YOL122C:c.1137G>T	YOL122C:p.Leu379Phe
CI1	XV	202498	SNV	G	T	Heterozygous	G/G/T		YOL067C:c.21C>A	
CI1	XV	780245	SNV	A	T	Heterozygous	A/A/T			
CI2	II	99114	SNV	A	T	Heterozygous	A/T		YBL066C:c.1002T>A	YBL066C:p.Asp334Glu
CI2	II	485846	SNV	C	A	Heterozygous	A/C		YBR123C:c.846G>T	
CI2	IV	616386	SNV	G	A	Heterozygous	A/G	YDR085C-236		
CI2	Mito	29341^ 29342	Insertion	-	C	Homozygous	C/C			
CI2	XIII	914235^ 914236	Insertion	-	A	Homozygous	A/A		YMR319C:c.303_304insT	YMR319C:p.Ile102fs
CI2	XV	90945^ 90946	Insertion	-	A	Homozygous	A/A		YOL122C:c.474_475insT	YOL122C:p.Ala159fs

Line	Chromo-some	Region	Type	Refe-rence	Allele	Zygosity	Geno-type	Promoter region change	Coding region change	Amino acid change
CI2	XVI	270237	SNV	G	T	Hetero-zygous	G/T		YPL150W:c.2050G>T	YPL150W:p.Gly684Cys
CI3	II	280617	Deletion	T	-	Hetero-zygous	-/T/T			
CI3	IV	1276301^ 1276302	Insertion	-	C	Hetero-zygous	-/-/C			
CI3	VI	74486	Deletion	A	-	Hetero-zygous	-/A/A	YFL032W-386, YFL031W-693, YFL033C-59		
CI3	VI	220462	Deletion	T	-	Hetero-zygous	-/T/T	YFR031C-356		
CI3	VIII	190365	Deletion	T	-	Hetero-zygous	-/T/T	YHR042W-178, YHR041C-501		
CI3	X	436490	Deletion	T	-	Hetero-zygous	-/T/T	YJR001W-312		
CI3	XII	5823	SNV	A	G	Hetero-zygous	A/A/G			
CI3	XIII	645835	Deletion	T	-	Hetero-zygous	-/T/T		YMR191W:c.180delT	YMR191W:p.Phe61fs
CI3	XIV	560682	Deletion	A	-	Hetero-zygous	-/A/A			
CI3	XIV	681191	Deletion	T	-	Hetero-zygous	-/T/T		YNR031C:c.4243delA	YNR031C:p.Ile1415fs
CI3	XV	90283	SNV	C	G	Hetero-zygous	C/G/G		YOL122C:c.1137G>C	YOL122C:p.Leu379Phe
CI4	II	426983^ 426984	Insertion	-	A	Hetero-zygous	-/-/-/A		YBR090C:c.75_76insT	YBR090C:p.Arg26fs

Line	Chromosome	Region	Type	Reference	Allele	Zygosity	Genotype	Promoter region change	Coding region change	Amino acid change
CI4	IV	283286	Deletion	A	-	Heterozygous	-/A/A/A	YDL100C-110, YDL099W-133		
CI4	IV	604994	SNV	A	C	Heterozygous	A/A/A/C		YDR080W:c.987A>C	
CI4	IV	1204283	Deletion	T	-	Heterozygous	-/T/T/T	YDR364C-73		
CI4	V	117970	Deletion	C	-	Heterozygous	-/C/C/C			
CI4	VI	97864^ 97865	Insertion	-	T	Heterozygous	complex			
CI4	VII	73941	SNV	C	G	Heterozygous	C/C/C/G			
CI4	XII	196592	Deletion	T	-	Heterozygous	-/T/T/T	YLR026C-120		
CI4	XV	90572	SNV	C	T	Homozygous	T/T/T/T		YOL122C:c.848G>A	YOL122C:p.Gly283Asp
CI4	XV	270739	SNV	G	T	Heterozygous	G/G/G/T		YOL028C:c.632C>A	YOL028C:p.Pro211Gln
CI5	II	714105	SNV	A	C	Heterozygous	A/C		YBR247C:c.351T>G	YBR247C:p.Phe117Leu
CI5	VI	253327	SNV	T	A	Heterozygous	A/T		YFR052W:c.823T>A	YFR052W:p.*275Lys
CI5	VII	110617	Deletion	A	-	Heterozygous	-/A			
CI5	XII	5823	SNV	A	G	Heterozygous	A/G			
CI5	XII	492784	SNV	C	A	Heterozygous	A/C		YLR163C:c.471G>T	YLR163C:p.Met157Ile

Line	Chromosome	Region	Type	Reference	Allele	Zygosity	Genotype	Promoter region change	Coding region change	Amino acid change
CI5	XIII	381833	SNV	G	A	Heterozygous	A/G		YMR053C:c.1066C>T	
CI5	XIV	88044	SNV	A	G	Heterozygous	A/G		YNL289W:c.148A>G	YNL289W:p.Thr50Ala
CI5	XV	90147^90148	Insertion	-	C	Homozygous	C/C		YOL122C:c.1272_1273insG	YOL122C:p.Ile425fs
CI6	IV	827023	SNV	A	G	Heterozygous	A/G		YDR181C:c.333T>C	
CI6	IX	387243	SNV	A	G	Heterozygous	A/G			
CI6	V	257691	SNV	T	G	Heterozygous	G/T		YER052C:c.268A>C	
CI6	VIII	158821	SNV	G	A	Heterozygous	A/G		YHR024C:c.372C>T	
CI6	XIII	913515	Deletion	T	-	Homozygous	-/-		YMR319C:c.1024delA	YMR319C:p.Arg342fs
CI6	XV	89865	SNV	C	A	Homozygous	A/A		YOL122C:c.1555G>T	YOL122C:p.Glu519*
CI6	XVI	131712	SNV	C	T	Heterozygous	C/T		YPL222W:c.1551C>T	
CH1	VIII	484601	SNV	C	T	Heterozygous	C/T	YHR188C-764, YHR190W-244		
CH1	X	504130	SNV	G	A	Homozygous	A/A		YJR039W:c.195G>A	
CH1	X	519954	SNV	T	C	Homozygous	C/C		YJR045C:c.1649A>G	YJR045C:p.Glu550Gly
CH1	XI	162598	SNV	T	G	Homozygous	G/G			

Line	Chromosome	Region	Type	Reference	Allele	Zygosity	Genotype	Promoter region change	Coding region change	Amino acid change
CH1	XI	483645	Deletion	A	-	Homozygous	-/-		YKR023W:c.227delA	YKR023W:p.Thr79fs
CH1	XIII	913515	Deletion	T	-	Homozygous	-/-		YMR319C:c.1024delA	YMR319C:p.Arg342fs
CH1	XIV	54649	SNV	C	T	Homozygous	T/T		YNL308C:c.1249G>A	YNL308C:p.Glu417Lys
CH1	XV	91137	SNV	T	C	Homozygous	C/C		YOL122C:c.283A>G	YOL122C:p.Asn95Asp
CH2	IV	374260	SNV	A	C	Heterozygous	A/C		YDL044C:c.1030T>G	YDL044C:p.Phe344Val
CH2	IV	826709^826710	Insertion	-	T	Heterozygous	-/T		YDR181C:c.646_647insA	YDR181C:p.Ser216fs
CH2	IX	222088	SNV	G	T	Heterozygous	G/T		YIL074C:c.403C>A	YIL074C:p.Gln135Lys
CH2	VII	968892	Deletion	T	-	Heterozygous	-/T	YGR238C-205		
CH2	XIII	914236	Deletion	A	-	Homozygous	-/-		YMR319C:c.303delT	YMR319C:p.Phe101fs
CH2	XV	20803	SNV	C	T	Heterozygous	C/T		YOL158C:c.509G>A	YOL158C:p.Arg170His
CH2	XV	91139	SNV	C	T	Homozygous	T/T		YOL122C:c.281G>A	YOL122C:p.Gly94Asp
CH3	II	631646	SNV	G	T	Heterozygous	G/T		YBR203W:c.2479G>T	YBR203W:p.Asp827Tyr
CH3	V	325518	SNV	C	A	Heterozygous	A/C		YER082C:c.419G>T	YER082C:p.Arg140Ile
CH3	XIII	913736	Deletion	T	-	Heterozygous	-/T		YMR319C:c.803delA	YMR319C:p.Lys268fs

Line	Chromosome	Region	Type	Reference	Allele	Zygosity	Genotype	Promoter region change	Coding region change	Amino acid change
CH3	XIII	913514^913515	Insertion	-	T	Heterozygous	-/T		YMR319C:c.1024_1025insA	YMR319C:p.Arg342fs
CH3	XV	91112	SNV	C	T	Homozygous	T/T		YOL122C:c.308G>A	YOL122C:p.Gly103Asp
CH3	XV	964043	SNV	G	A	Heterozygous	A/G		YOR341W:c.3057G>A	
CH4	VII	394958^394959	Insertion	-	T	Homozygous	T/T			
CH4	VIII	151580	Deletion	T	-	Homozygous	-/-	YHR023W-86, YHR022C-A-274		
CH4	XIII	914235^914236	Insertion	-	A	Homozygous	A/A		YMR319C:c.303_304insT	YMR319C:p.Ile102fs
CH4	XV	90603	SNV	C	T	Homozygous	T/T		YOL122C:c.817G>A	YOL122C:p.Ala273Thr
CH4	XV	768697	SNV	G	A	Heterozygous	A/G		YOR229W:c.289G>A	YOR229W:p.Asp97Asn
CH4	XVI	221736	SNV	T	C	Homozygous	C/C		YPL174C:c.1038A>G	
CH4	XVI	302995	Deletion	A	-	Homozygous	-/-	YPL131W-114		
CH5	VII	937934	Deletion	A	-	Homozygous	-/-		YGR221C:c.1059delT	YGR221C:p.Thr354fs
CH5	XIII	914235^914236	Insertion	-	A	Homozygous	A/A		YMR319C:c.303_304insT	YMR319C:p.Ile102fs
CH5	XIV	483237	Deletion	A	-	Homozygous	-/-	YNL076W-319		
CH5	XV	91212	SNV	T	A	Homozygous	A/A		YOL122C:c.208A>T	YOL122C:p.Arg70*

Line	Chromosome	Region	Type	Reference	Allele	Zygosity	Genotype	Promoter region change	Coding region change	Amino acid change
CH6	IV	767153	SNV	C	G	Homozygous	G/G		YDR153C:c.819G>C	YDR153C:p.Gln273His
CH6	XIII	92422	SNV	C	T	Homozygous	T/T		YML088W:c.188C>T	YML088W:p.Thr63Ile
CH6	XIII	403810	SNV	C	T	Homozygous	T/T		YMR066W:c.2270C>T	YMR066W:p.Ser757Phe
CH6	XIII	581917	SNV	A	T	Homozygous	T/T		YMR162C:c.2005T>A	YMR162C:p.Ser669Thr
CH6	XIII	914236	Deletion	A	-	Homozygous	-/-		YMR319C:c.303delT	YMR319C:p.Phe101fs
CH6	XV	91157	SNV	A	C	Homozygous	C/C		YOL122C:c.263T>G	YOL122C:p.Val88Gly
NI1	I	37644	SNV	T	C	Heterozygous	C/T		YAL058W:c.181T>C	YAL058W:p.Ser61Pro
NI1	II	682947	SNV	G	A	Heterozygous	A/G		YBR231C:c.144C>T	
NI1	IV	1361934	SNV	C	G	Heterozygous	C/G		YDR451C:c.248G>C	YDR451C:p.Arg83Pro
NI1	IX	295261	SNV	C	A	Heterozygous	A/C		YIL031W:c.2629C>A	YIL031W:p.Leu877Ile
NI1	VII	870554	SNV	G	C	Heterozygous	C/G		YGR187C:c.863C>G	YGR187C:p.Pro288Arg
NI1	X	697407	SNV	T	C	Heterozygous	C/T		YJR142W:c.267T>C	
NI1	XI	524106	SNV	A	C	Heterozygous	A/C	YKR045C-137		
NI1	XII	133163	SNV	C	T	Heterozygous	C/T		YLL008W:c.1435C>T	YLL008W:p.Gln479*

Line	Chromosome	Region	Type	Reference	Allele	Zygosity	Genotype	Promoter region change	Coding region change	Amino acid change
NI1	XII	938472	Deletion	G	-	Homozygous	-/-		YLR410W:c.932delG	YLR410W:p.Ala312fs
NI1	XII	1051058	SNV	T	C	Homozygous	C/C		YLR454W:c.7061T>C	YLR454W:p.Leu2354Ser
NI1	XIII	779394	SNV	G	A	Heterozygous	A/G	YMR256C-267		
NI2	II	517804	SNV	C	T	Homozygous	T/T		YBR140C:c.8825G>A	YBR140C:p.Trp2942*
NI2	II	783365	SNV	C	T	Heterozygous	C/T		YBR290W:c.775C>T	YBR290W:p.Gln259*
NI2	IV	842546	SNV	G	C	Heterozygous	C/G		YDR191W:c.210G>C	YDR191W:p.Leu70Phe
NI2	XII	711148	SNV	G	T	Heterozygous	G/T		YLR287C:c.911C>A	YLR287C:p.Pro304Gln
NI2	XIII	25343	Deletion	G	-	Heterozygous	-/G		YML123C:c.458delC	YML123C:p.Ala153fs
NI2	XIV	688148..688150	MNV	GAA	TTC	Heterozygous	complex		YNR033W:c.514_516delGAAinsTTC	YNR033W:p.Glu172Phe
NI3	IX	51644	SNV	G	T	Heterozygous	G/T			
NI3	Mito	5209	SNV	T	G	Homozygous	G/G			
NI3	XII	881254	SNV	C	A	Homozygous	A/A		YLR381W:c.1532C>A	YLR381W:p.Ala511Asp
NI3	XII	939005	SNV	A	C	Homozygous	C/C		YLR410W:c.1465A>C	YLR410W:p.Asn489His
NI4	V	411981	SNV	T	G	Heterozygous	G/T		YER125W:c.1793T>G	YER125W:p.Met598Arg

Line	Chromosome	Region	Type	Reference	Allele	Zygosity	Genotype	Promoter region change	Coding region change	Amino acid change
NI4	XI	2719	SNV	T	G	Heterozygous	G/T		YKL223W:c.331T>G	YKL223W:p.*111Glu
NI4	XII	135041	SNV	A	G	Heterozygous	A/G		YLL007C:c.1259T>C	YLL007C:p.Leu420Ser
NI5	IV	63424	SNV	C	T	Homozygous	T/T		YDL220C:c.1595G>A	YDL220C:p.Cys532Tyr
NI5	VII	1040233	SNV	C	G	Heterozygous	C/G		YGR274C:c.2863G>C	YGR274C:p.Val955Leu
NI6	Mito	29505	SNV	C	T	Homozygous	T/T			
NI6	VIII	130695	SNV	A	G	Heterozygous	A/G			
NI6	XII	846727	SNV	G	T	Homozygous	T/T		YLR360W:c.625G>T	YLR360W:p.Glu209*
NH1	IV	391618	SNV	A	G	Heterozygous	A/G		YDL035C:c.440T>C	YDL035C:p.Phe147Ser
NH1	VIII	85392	SNV	G	T	Heterozygous	G/T	YHL009W-A-517, YHL009W-B-517, YHL009C-332		
NH1	XII	817000	SNV	G	A	Heterozygous	A/G		YLR343W:c.907G>A	YLR343W:p.Gly303Ser
NH1	XIII	817936	SNV	G	A	Homozygous	A/A		YMR275C:c.646C>T	YMR275C:p.Gln216*
NH2	I	40840	Deletion	C	-	Heterozygous	-/C		YAL056W:c.1582delC	YAL056W:p.Val529fs
NH2	II	592691	SNV	T	G	Heterozygous	G/T		YBR181C:c.6+78A>C	

Line	Chromosome	Region	Type	Reference	Allele	Zygosity	Genotype	Promoter region change	Coding region change	Amino acid change
NH2	XIII	818356	SNV	C	A	Homozygous	A/A		YMR275C:c.226G>T	YMR275C:p.Gly76*
NH3	III	294222	SNV	C	T	Heterozygous	C/T		YCR097W:c.334C>T	YCR097W:p.Leu112Phe
NH3	VII	1075611	SNV	T	C	Heterozygous	C/T		YGR290W:c.[130T>C]; YGR289C:c.[203A>G]	YGR290W:p.[Ser44Pro]; YGR289C:p.[Asp68Gly]
NH3	X	412738	SNV	A	C	Homozygous	C/C		YJL012C:c.662T>G	YJL012C:p.Val221Gly
NH3	XIV	776880	SNV	G	T	Heterozygous	G/T			
NH3	XV	411805	SNV	G	A	Homozygous	A/A		YOR043W:c.936G>A	YOR043W:p.Trp312*
NH3	XV	897758	SNV	A	G	Homozygous	G/G		YOR310C:c.603T>C	
NH3	XVI	908607	SNV	G	T	Heterozygous	G/T		YPR185W:c.1390G>T	YPR185W:p.Asp464Tyr
NH4	II	422796	SNV	A	T	Heterozygous	A/T		YBR086C:c.246T>A	
NH4	III	241057	SNV	T	C	Homozygous	C/C		YCR072C:c.1296A>G	
NH4	VII	51100	SNV	G	A	Heterozygous	A/G		YGL238W:c.1549G>A	YGL238W:p.Ala517Thr
NH4	VII	521554	SNV	T	G	Homozygous	G/G		YGR015C:c.523A>C	YGR015C:p.Ser175Arg
NH4	X	412808	SNV	T	C	Homozygous	C/C		YJL012C:c.592A>G	YJL012C:p.Thr198Ala
NH4	XIV	348767	SNV	C	A	Heterozygous	A/C	YNL150W-484, YNL151C-246		

Line	Chromosome	Region	Type	Reference	Allele	Zygosity	Genotype	Promoter region change	Coding region change	Amino acid change
NH4	XIV	753455	SNV	G	A	Heterozygous	A/G		YNR065C:c.246C>T	
NH4	XV	412199	SNV	A	T	Homozygous	T/T		YOR043W:c.1330A>T	YOR043W:p.Lys444*
NH4	XV	1038773	SNV	T	C	Homozygous	C/C		YOR373W:c.1940T>C	YOR373W:p.Leu647Ser
NH5	I	135803	SNV	G	A	Heterozygous	A/G	YAL009W-51, YAL010C-138		
NH5	II	217048	SNV	C	T	Heterozygous	C/T		YBL006C:c.82G>A	YBL006C:p.Ala28Thr
NH5	V	302808	SNV	G	T	Homozygous	T/T		YER072W:c.3G>T	YER072W:p.Met1?
NH5	VII	179567	SNV	T	C	Heterozygous	C/T		YGL173C:c.547A>G	YGL173C:p.Asn183Asp
NH5	VIII	328832	SNV	C	A	Heterozygous	A/C		YHR108W:c.530C>A	YHR108W:p.Ala177Glu
NH5	XIV	404979	SNV	T	G	Heterozygous	G/T		YNL118C:c.586A>C	
NH5	XIV	668467	SNV	C	G	Heterozygous	C/G		YNR021W:c.91C>G	YNR021W:p.Gln31Glu
NH6	IV	869816	SNV	G	T	Heterozygous	G/T		YDR208W:c.1593G>T	YDR208W:p.Leu531Phe
NH6	X	412283	SNV	G	A	Homozygous	A/A		YJL012C:c.1117C>T	YJL012C:p.Arg373Cys
NH6	XI	530729	SNV	G	T	Homozygous	T/T	YKR051W-217		
NH6	XV	411008	SNV	C	T	Homozygous	T/T		YOR043W:c.139C>T	YOR043W:p.Gln47*

Line	Chromosome	Region	Type	Reference	Allele	Zygosity	Genotype	Promoter region change	Coding region change	Amino acid change
NH6	XV	778735	SNV	A	T	Heterozygous	A/T		YOR234C:c.144T>A	
R1	I	111362	SNV	C	A	Heterozygous	A/C		YAL021C:c.1998G>T	YAL021C:p.Leu666Phe
R1	XIV	22916	SNV	C	T	Heterozygous	C/T		YNL328C:c.359G>A	YNL328C:p.Arg120Lys
R2	IV	630673..630674	Replacement	AA	TGC	Homozygous	TGC/TGC			
R2	IX	116152	SNV	G	A	Heterozygous	A/G		YIL128W:c.2347G>A	YIL128W:p.Asp783Asn
R2	XII	952986	SNV	T	G	Heterozygous	G/T		YLR413W:c.1831T>G	YLR413W:p.Phe611Val
R2	XV	411836	Deletion	T	-	Homozygous	-/-		YOR043W:c.967delT	YOR043W:p.Phe323fs
R3	I	112127	Deletion	A	-	Heterozygous	-/A		YAL021C:c.1233delT	YAL021C:p.Phe411fs
R3	VII	222723	SNV	C	A	Heterozygous	A/C		YGL150C:c.2851G>T	YGL150C:p.Asp951Tyr
R3	X	673099	SNV	G	T	Heterozygous	G/T		YJR133W:c.109G>T	YJR133W:p.Ala37Ser
R4	II	639131	SNV	T	C	Heterozygous	C/T		YBR208C:c.3080A>G	YBR208C:p.Asp1027Gly
R4	IV	975919	SNV	T	C	Heterozygous	C/T	YDR259C-137		
R4	V	402956	SNV	T	A	Heterozygous	A/T		YER122C:c.1397A>T	YER122C:p.Asp466Val
R4	XIV	173932^173933	Insertion	-	A	Heterozygous	-/A		YNL251C:c.383_384insT	YNL251C:p.Leu128fs

Line	Chromosome	Region	Type	Reference	Allele	Zygosity	Genotype	Promoter region change	Coding region change	Amino acid change
R4	XV	412031	Deletion	A	-	Homozygous	-/-		YOR043W:c.1162delA	YOR043W:p.Thr388fs
R5	XIV	256069	SNV	C	T	Heterozygous	C/T		YNL207W:c.717C>T	
R5	XIV	320377	SNV	G	T	Heterozygous	G/T		YNL167C:c.983C>A	YNL167C:p.Thr328Asn
R5	XVI	506236	SNV	A	G	Homozygous	G/G		YPL023C:c.77T>C	YPL023C:p.Leu26Ser
R6	V	35221	SNV	G	T	Heterozygous	G/T		YEL062W:c.815G>T	YEL062W:p.Gly272Val
R6	X	609768	Deletion	G	-	Heterozygous	-/G	YJR095W-5		
R6	XIV	106467	SNV	C	G	Heterozygous	C/G		YNL283C:c.229G>C	YNL283C:p.Gly77Arg
R6	XIV	273898	SNV	G	A	Heterozygous	A/G	YNL193W-470, YNL194C-283		
R6	XV	411238^ 411239	Insertion	-	T	Homozygous	T/T		YOR043W:c.369_370insT	YOR043W:p.Asp126fs
R6	XVI	55301	SNV	G	A	Homozygous	A/A	YPL258C-148, Gene_tW_ CCA_P-868		
R6	XVI	72391	SNV	T	C	Heterozygous	C/T		YPL253C:c.616A>G	YPL253C:p.Thr206Ala
ZI1	I	16988	SNV	G	T	Homozygous	T/T			
ZI1	XIV	684676	SNV	A	G	Homozygous	G/G		YNR031C:c.758T>C	YNR031C:p.Val253Ala

Line	Chromosome	Region	Type	Reference	Allele	Zygosity	Genotype	Promoter region change	Coding region change	Amino acid change
ZI2	IV	977680	SNV	A	G	Heterozygous	A/G		YDR261C:c.1530T>C	
ZI2	VII	480473	SNV	C	T	Heterozygous	C/T		YGL008C:c.2194G>A	YGL008C:p.Ala732Thr
ZI2	XII	290199	SNV	T	C	Heterozygous	C/T	YLR081W-13		
ZI2	XIII	308124	SNV	T	G	Heterozygous	G/T		YMR017W:c.636T>G	YMR017W:p.Cys212Trp
ZI2	XV	177540	SNV	T	A	Heterozygous	A/T		YOL081W:c.6471T>A	YOL081W:p.Asn2157Lys
ZI3	IV	1297195	SNV	G	A	Heterozygous	A/G		YDR415C:c.967C>T	YDR415C:p.Pro323Ser
ZI3	X	167335	SNV	C	T	Heterozygous	C/T		YJL130C:c.5033G>A	YJL130C:p.Cys1678Tyr
ZI3	XVI	24689	SNV	A	C	Heterozygous	A/C		YPL274W:c.1752A>C	YPL274W:p.Arg584Ser
ZI4	IV	569995	SNV	A	C	Homozygous	C/C	YDR060W-654, YDR059C-225		
ZI4	XIII	507284	SNV	G	T	Homozygous	T/T		YMR119W-A:c.289G>T	YMR119W-A:p.Ala97Ser
ZI4	XVI	452330	Deletion	A	-	Heterozygous	-/A		YPL057C:c.728delT	YPL057C:p.Phe243fs
ZI5	VI	158026	SNV	C	A	Homozygous	A/A			
ZI5	XIV	447262	SNV	A	T	Heterozygous	A/T	YNL094W-349, YNL095C-422		
ZI6	VII	481568	SNV	C	G	Heterozygous	C/G		YGL008C:c.1099G>C	YGL008C:p.Glu367Gln

Line	Chromosome	Region	Type	Reference	Allele	Zygosity	Genotype	Promoter region change	Coding region change	Amino acid change
ZI6	XII	295847	SNV	C	A	Heterozygous	A/C		YLR083C:c.248G>T	YLR083C:p.Cys83Phe
ZH1	V	341331	SNV	T	G	Homozygous	G		YER091C:c.837A>C	
ZH1	V	470138	SNV	C	A	Homozygous	A		YER151C:c.2287G>T	YER151C:p.Val763Phe
ZH1	VI	165108	Deletion	T	-	Homozygous	-		YFR010W:c.42delT	YFR010W:p.Val16fs
ZH1	XIII	346850	SNV	G	A	Homozygous	A	YMR037C-333		
ZH1	XIV	41245	SNV	C	T	Homozygous	T		YNL317W:c.627C>T	
ZH2	II	479843	SNV	G	T	Homozygous	T		YBR119W:c.417G>T	YBR119W:p.Lys139Asn
ZH2	V	470668	SNV	G	A	Homozygous	A		YER151C:c.1757C>T	YER151C:p.Ala586Val
ZH2	VI	166438	SNV	G	T	Homozygous	T		YFR010W:c.1372G>T	YFR010W:p.Glu458*
ZH3	IX	108761	SNV	C	G	Homozygous	G		YIL129C:c.4477G>C	YIL129C:p.Glu1493Gln
ZH3	VI	166403	SNV	G	T	Homozygous	T		YFR010W:c.1337G>T	YFR010W:p.Gly446Val
ZH3	XV	273395	SNV	C	T	Homozygous	T		YOL027C:c.330G>A	
ZH3	XV	273398	SNV	A	C	Homozygous	C		YOL027C:c.327T>G	
ZH4	I	40585	SNV	G	C	Homozygous	C		YAL056W:c.1327G>C	YAL056W:p.Gly443Arg

Line	Chromo-some	Region	Type	Refe-rence	Allele	Zygosity	Geno-type	Promoter region change	Coding region change	Amino acid change
ZH4	II	571335	SNV	A	G	Homo-zygous	G	YBR166C-135		
ZH4	IV	247140	SNV	G	T	Homo-zygous	T		YDL119C:c.473C>A	YDL119C:p.Ala158Glu
ZH4	IV	585615	SNV	G	A	Homo-zygous	A		YDR069C:c.2105C>T	YDR069C:p.Ala702Val
ZH4	XVI	788124^ 788125	Insertion	-	G	Homo-zygous	G		YPR125W:c.164_165insG	YPR125W:p.Glu56fs
ZH5	IV	406032	SNV	T	C	Homo-zygous	C		YDL025C:c.1175A>G	YDL025C:p.Asp392Gly
ZH5	VI	166013	SNV	G	T	Homo-zygous	T		YFR010W:c.947G>T	YFR010W:p.Arg316Ile
ZH6	Mito	36298	Deletion	A	-	Homo-zygous	-/-	Q0105-242, Q0110-242, Q0115-242, Q0120-242		
ZH6	V	142064	SNV	C	T	Homo-zygous	T/T		YEL007W:c.173C>T	YEL007W:p.Ser58Leu

Table S3.2: PROVEAN scores

Gene	Line	Amino acid change	PROVEAN score
CCR4	R1	L666F	-3.86
GPB2	ZH4	G443R	-6.43
PMA1	ZI2	A732T	-3.79
PMA1	ZI6	E367Q	-2.95
SMF1	CI1; CI3	L379F	-4.00
SMF1	CI4	G283D	-7.00
SMF1	CH1	N95D	-5.00
SMF1	CH2	G94D	-7.00
SMF1	CH3	G103D	-7.00
SMF1	CH4	A273T	-3.07
SMF1	CH6	V88G	-6.92
SSK2	ZI1	V253A	-2.50
UBP3	ZH1	V763F	-4.20
UBP3	ZH2	A586V	-4.00
UBP6	ZH3	G446V	-8.86
UBP6	ZH5	R316I	-8.00
VIP1	NI3	N489H	-4.92
VTC4	NH3	V221G	-6.94
VTC4	NH4	T198A	-4.92
VTC4	NH6	R373C	-8.00

Table S3.3: Main GO Slim terms per treatment

Treatment	GOID	GO term	Rank	Count	Total	%	Gene(s)
CI	6811	ion transport	1	8	19	42	FET4,SMF1
CI	6873	cellular ion homeostasis	2	6	19	32	SMF1
CI	6468	protein phosphorylation	3	3	19	16	PCL1,SSK2,YPL150W
CH	6811	ion transport	1	15	28	54	DNF3,FET4,SMF1,ENB1
CH	55085	transmembrane transport	2	8	28	29	SSC1,FET4
CH	6873	cellular ion homeostasis	3	6	28	21	SMF1
NI	51603	proteolysis involved in cellular protein catabolic process	1	5	21	24	CNE1,BSD2,RSP5, ULP2,CTF3
NI	33043	regulation of organelle organization	2	4	21	19	CDC13,RSP5,ULP2,CTF3
NI	8150	biological process unknown	3	4	21	19	HGH1,YKL223W,YLR287C, FMP27
NH	7005	mitochondrion organization	1	6	23	26	BUL1,WHI2,ATG13
NH	10324	membrane invagination	2	5	23	22	VTC1,VTC4,ATG13
NH	61025	membrane fusion	3	4	23	17	VTC1,VTC4
ZI	6811	ion transport	1	3	11	27	PMA1,SAM3
ZI	55086	nucleobase-containing small molecule metabolic process	2	2	11	18	URA2,IRA2
ZI	55085	transmembrane transport	3	2	11	18	PMA1
ZH	70647	protein modification by small protein conjugation or removal	1	6	14	43	UBP3,UBP6
ZH	51603	proteolysis involved in cellular protein catabolic process	2	5	14	36	DOA4,UBP6
ZH	51049	regulation of transport	3	2	14	14	UBP3

Table S3.4.: Major copy number variation events per evolved clone

Line	Chromosome	Start	End	Length	Total fraction	Copy number	Status
CI1	I	1200	160400	159200	0.89	2	loss
CI1	I	164800	211200	46400	0.89	2	loss
CI1	III	7200	84000	76800	0.95	2	loss
CI1	III	90800	316000	225200	0.95	2	loss
CI3	I	2400	160400	158000	0.89	2	loss
CI3	I	165200	211200	46000	0.89	2	loss
CI3	III	0	15600	15600	0.28	5	gain
CI3	III	15600	87600	72000	0.28	4	gain
CI3	III	168800	316620	147820	0.47	2	loss
CI3	XIV	0	14800	14800	0.98	4	gain
CI3	XIV	14800	25200	10400	0.98	5	gain
CI3	XIV	26800	55600	28800	0.98	4	gain
CI3	XIV	56800	356000	299200	0.98	4	gain
CI3	XIV	359600	414800	55200	0.98	4	gain
CI3	XIV	419200	602400	183200	0.98	4	gain
CI3	XIV	606400	727600	121200	0.98	4	gain
CI3	XIV	727600	741600	14000	0.98	5	gain
CI3	XIV	741600	783200	41600	0.98	4	gain
CI4	I	31200	48400	17200	0.85	6	gain
CI4	I	49600	132000	82400	0.85	6	gain
CI4	I	132000	229200	97200	0.85	5	gain
CI4	III	0	8400	8400	0.39	8	gain
CI4	III	8400	51200	42800	0.39	7	gain
CI4	III	51200	84800	33600	0.39	8	gain
CI4	III	90800	128000	37200	0.39	8	gain
CI4	VI	0	147600	147600	0.82	5	gain
CI4	VI	149200	183600	34400	0.82	5	gain
CI4	VI	229200	269600	40400	0.82	5	gain
CI5	XIV	0	54000	54000	0.99	3	gain
CI5	XIV	56800	358000	301200	0.99	3	gain
CI5	XIV	359600	414800	55200	0.99	3	gain
CI5	XIV	415200	770400	355200	0.99	3	gain
CI5	XIV	770400	783200	12800	0.99	4	gain
NI5	XII	0	11600	11600	0.94	4	gain
NI5	XII	11600	52800	41200	0.94	3	gain
NI5	XII	56000	113200	57200	0.94	3	gain

Line	Chromosome	Start	End	Length	Total fraction	Copy number	Status
NI5	XII	116800	216400	99600	0.94	3	gain
NI5	XII	216800	451200	234400	0.94	3	gain
NI5	XII	482000	562400	80400	0.94	3	gain
NI5	XII	563600	651200	87600	0.94	3	gain
NI5	XII	657200	688000	30800	0.94	3	gain
NI5	XII	688400	1011600	323200	0.94	3	gain
NI5	XII	1013200	1064000	50800	0.94	3	gain

Appendix A2: Relative fitness of evolved clones

To relate the results in our current paper to those in Gorter et al. 2016, we assessed whether the single clones that we used for sequencing were representative of the previously assayed population samples. To this end, we determined clone fitness relative to a differently marked ancestor in the presence of the final (or constant high) concentration of the metal used in the evolution experiment in triplicate as previously described (Gorter et al. 2016). Control strains were competed in the absence of additional metals. Mean clone fitness as assayed for the current study was different from population fitness as assayed for the original study, and this depended on the evolutionary treatment (Figure S3.3; general linear mixed model with log transformed fitness as the response variable, and evolution line as a random effect: assay

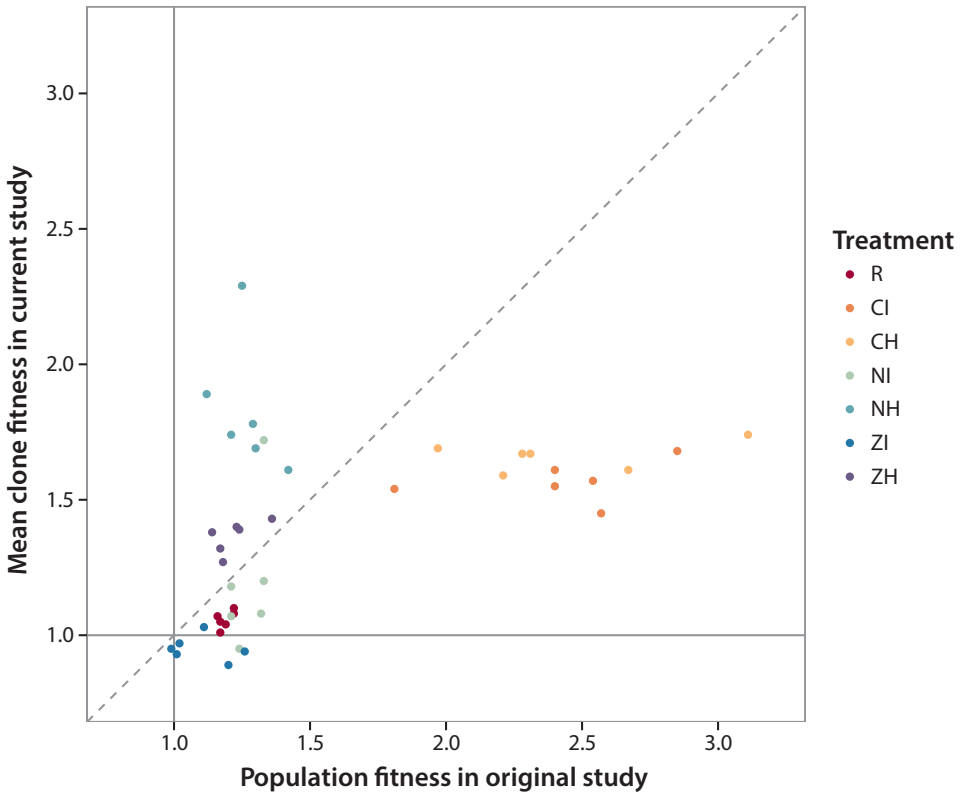

Figure S3.3: Relative fitness of population samples as assayed for our original study versus single clone mean relative fitness as assayed for the current study. Cadmium had a smaller reductive effect in the current study, decreasing relative fitness values. Nickel and zinc had a stronger reductive effect in the present study, increasing relative fitness values for constant high, but not gradually increasing treatments. Colours correspond with different treatments.

x treatment: $F_{6,35}$ = 24.69, P < 0.0001). That is, fitness was lower in the current assays for the increasing and high Cd treatments, as well as for the increasing Ni and Zn treatments. Conversely, fitness was higher in the current assays for the high Ni and Zn treatments.

Fitness of evolved clones may depend on the presence of other isolates from the same population (Cowen et al. 2001; Lee et al. 2010). However, in our case we consider it more likely that the systematic discrepancy between fitness values is caused by a change in medium composition, which in turn has affected the relative reductive effect of each of the metals. YPD is a rich, undefined medium, and subtle changes in such media can have crucial consequences (Joho et al. 1988; O'Keefe et al. 2006; Corbacho et al. 2011). In a pilot assay performed prior to this study, clone fitness correlated strongly with population fitness (data not shown). However, in the assays performed for the study itself, cell densities were highly reduced compared with the assays performed for Gorter et al. 2016. Moreover, Cd had a much weaker reductive effect than before, whereas the opposite was true for Ni and Zn. This observation is consistent with the fitness values that we measured: a lower effective metal concentration is expected to reduce the benefit of resistant mutants relative to the sensitive ancestor, and *vice versa*. The fact that populations exposed to increasing concentrations of Ni and Zn had in fact a lower fitness can be explained by the pleiotropic effects of mutations selected at different metal concentrations under a reranking GxE scenario: mutations selected at intermediate concentrations (encountered during gradual change) will to some extent also increase fitness at constant high concentrations as a correlated effect (Kassen and Bell 2000; Samani and Bell 2010). Likewise, mutations selected at constant high concentrations will confer a niche expansion to even higher concentrations. When the effective metal concentration is increased beyond the original constant high concentration, assays will then indicate a larger fitness increase for mutants selected at high concentrations (Perron et al. 2008). The drop in relative fitness below 1 that we observed for the isolates evolved at gradually increasing Zn concentrations may represent an exceptionally strong, but not unheard of, case of such pleiotropy (Anderson et al. 2003).

Chapter 4

Analyses of local fitness landscapes explain dynamics of adaptation in directionally changing environments

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Abstract

The fitness landscape is a concept that is widely used for understanding and predicting evolutionary adaptation. The topography of the fitness landscape depends critically on the environment, with potentially far-reaching consequences for evolution under changing conditions. However, few studies have assessed directly how empirical fitness landscapes change across conditions, or validated the predicted consequences of such change. We previously evolved replicate yeast populations in the presence of either gradually increasing or constant high concentrations of the heavy metals Cd, Ni, and Zn, and analysed their phenotypic and genomic changes. Here, we reconstructed the local fitness landscapes underlying adaptation to each metal by deleting all repeatedly mutated genes both by themselves and in combination. Fitness assays revealed that the height and/or shape of each local fitness landscape changed considerably across metal concentrations, with distinct qualitative differences between non-essential and essential metals. This change in topography had particularly crucial consequences in the case of Ni, where a substantial part of the individual mutational fitness effects changed in sign across concentrations. Based on the Ni landscape analyses, we made several predictions about which mutations had been selected when during the evolution experiment. Deep sequencing of population samples from different time points generally confirmed these predictions, demonstrating the power of landscape reconstruction approaches for understanding evolutionary dynamics, even under complex scenarios of environmental change.

Introduction

One of the central aims in evolutionary biology is understanding adaptation. An important concept that is used to aid in this is the fitness landscape (Wright 1932; Orr 2005), which is a multi-dimensional genotype-fitness map consisting of a single orthogonal axis for each locus at which variation exists, plus an additional axis for fitness. The added value of the fitness landscape for understanding adaptation lies primarily in its explicit incorporation of epistasis, where mutations combine to affect fitness in a way that is not expected from their individual effects. Mutational effects may change in magnitude or sign depending on the genetic background in which they occur, and this can have crucial consequences for evolution. A change in sign will render some evolutionary pathways to fitter genotypes inaccessible, and several such interactions can together act to separate peaks of higher fitness, thus potentially constraining evolution to a limited subset of genotype space (Weinreich et al. 2005; Poelwijk et al. 2007).

The prevalence and overall nature of epistasis play a key role not only in understanding and predicting adaptation, but also in theories on the evolution of sex, speciation, robustness, and evolvability (Kondrashov 1988; Whitlock et al. 1995; Kondrashov and Kondrashov 2001; de Visser and Krug 2014). As such, a significant body of work has been dedicated to characterizing the topography of the fitness landscape for several empirical examples (Kogenaru et al. 2009; de Visser and Krug 2014). These studies generally combine a limited number of mutations in all possible ways and then determine (a proxy of) fitness for each individual genotype. These studies have found ample evidence for the presence of (sign) epistatic interactions and the landscape ruggedness ensuing from them, particularly within enzymes and when individual mutational effects are large (Schenk et al. 2013; de Visser and Krug 2014). Some authors have gone even further and used the acquired topographical information to make predictions about the course and outcome of evolution (Weinreich et al. 2006; Lozovsky et al. 2009), and in the case of the antibiotic resistance enzyme TEM β-lactamase these predictions were partly confirmed using an *in vitro* evolutionary approach (Salverda et al. 2011).

While empirical fitness landscape reconstructions appear promising for predicting evolution under constant conditions, they have one important shortcoming. The pathways that are predicted to be followed as well as their eventual outcomes generally involve the selection of multiple mutations, which can take hundreds of generations. However, in nature environments are seldom constant for such extended periods of time. Traditionally, ecology and evolution were thought to occur on different time scales and selection was thought to maximize fitness in the mean environment (Carroll et al. 2007). However, it is increasingly recognized that there is in fact considerable overlap between these time scales, so that ecology matters for evolution, particularly in rapidly evolving microbes (Bohannan and Lenski 2000; Bell 2013). This implies that if we are to fully understand evolution in more realistic settings, we

need to take into account how selection pressures change, and thus how the fitness landscape changes, in response to changes in the environment (Mustonen and Lassig 2009).

Several previous studies have addressed the question how the topography of the fitness landscape changes across environments. Most of these are based on fitness landscapes of single enzymes. Measuring landscapes in the presence of different selection pressures and at different intensities of the same selection pressure has shown that the shape of the fitness landscape can change dramatically even when environmental differences are limited (Hayden and Wagner 2012; de Vos et al. 2013; Flynn et al. 2013; Mira et al. 2015). These analyses indicate potentially far-reaching consequences of different regimes of environmental change for evolution. First, different concentrations of the same chemical may select for different genotypes (Ogbunugafor et al. 2016). Second, fluctuating conditions can open up new pathways to higher fitness that are inaccessible in constant environments (de Vos et al. 2015), and such knowledge may be applied in antibiotic cycling strategies to prevent the evolution of highly resistant bacteria (Tan et al. 2011; Goulart et al. 2013). By contrast, no predictions from landscape reconstructions exist about the effect of different rates of directional environmental change on evolutionary dynamics, despite the fact that such environmental change is common in nature, with pollution and climate change as prominent examples. Furthermore, no study to date has evaluated yet how useful fitness landscape reconstructions are for predicting evolution in changing environments by directly exposing organisms to the involved scenarios of change and determining the phenotypic and genetic consequences.

We previously evolved replicate populations of *Saccharomyces cerevisiae* for 500 generations in the presence of either gradually increasing or constant high concentrations of the non-essential heavy metal Cd, and the essential heavy metals Ni and Zn (Gorter et al. 2016). We found that the rate of directional environmental change as well as the nature of the selection pressure had crucial consequences for evolution. Fitness increase under gradual change was delayed, but eventually populations exposed to both rates of change reached the same fitness. Whole genome sequencing of single clones showed that this was likely due to mutations in the same genes being selected more slowly under gradual change for the non-essential metal Cd. Conversely, mutations in different genes were found in response to both rates of change for the essential metals Ni and Zn (Gorter et al. unpublished). This suggests that genotype-by-environment (GxE) interactions across different concentrations of the same metal were important within the context of our experiments, and, moreover, that the nature of this GxE was different for the non-essential metal Cd versus the essential metals Ni and Zn.

In addition to these general observations, we noted that several mutations always occurred in particular combinations in our evolved clones (Table 4.1). In response to the Cd treatments, mutations in *SMF1* and *FET4* were selected repeatedly, but mutations in *FET4* never occurred without those in *SMF1*. Similarly, in response to the constant high Zn treatment,

Table 4.1: Coding mutations in multihit genes per evolved clone.

Line		*SMF1*	*FET4*	*VIP1*	*BUL1*	*VTC4*	*WHI2*	*UBP6*	*UBP3*
increasing Cd	1	X							
	2	X[a]	X[a]						
	3	X[b]							
	4	X							
	5	X[a]							
	6	X[a]	X[a]						
high Cd	1	X	X[a]						
	2	X	X[a]						
	3	X	X[a,c]						
	4	X	Xa						
	5	X[a]	Xa						
	6	X	X[a]						
increasing Ni	1			X[a]					
	2								
	3			X					
	4								
	5								
	6								
high Ni	1				X[a]				
	2				X[a]				
	3					X	X[a]		
	4					X	X[a]		
	5								
	6					X	X[a]		
increasing Zn	1								
	2								
	3								
	4								
	5								
	6								
high Zn	1							X[a]	X
	2							X[a]	X
	3							X	
	4								
	5							X	
	6								

[a] = stop codon or frameshift mutation, [b] = heterozygous, [c] = double heterozygous. Adjusted from Gorter et al. unpublished. Note that most evolution lines contained several additional mutations apart from the ones listed here, including diploidisation. However, each of these mutations occurred in unique genomic regions, so that we have no direct evidence that they were adaptive. Additionally, the large majority of the mutations listed here was homozygous, implying that diploidisation was a secondary mutation, and that the mutations listed here were among the first to fix and drive adaptation.

we found mutations in *UBP6* and *UBP3*, but those in *UBP3* were found only in combination with mutations in *UBP6*. This suggests that in both cases the selection of one mutation was contingent upon the presence of the other, indicating epistasis (GxG). In response to the constant high Ni treatment, mutations in *VTC4* and *WHI2* were found in combination multiple times. Mutations in *WHI2* have previously been reported to decrease Ni tolerance in isolation (Arita et al. 2009), raising the possibility that they were selected specifically in the background of mutations in *VTC4*, which would similarly suggest contingency/epistasis. Finally, in response to high Ni we either found mutations in *VTC4* and *WHI2* in combination, or in *BUL1* in isolation. This also suggests epistasis, although in this case mutations appear to have prevented rather than enabled each other's selection.

The above findings can potentially be explained using a GxE framework that we envisioned previously, which relies on the distinction between magnitude GxE and reranking GxE. Magnitude GxE refers to the case where the same genotypes are selected at different metal concentrations, but selection is stronger at higher concentrations (Gorter et al. 2016). For the fitness landscape, this implies that it can have any shape, i.e. epistatic interactions (GxG) of various nature are possible. Additionally, the overall height of the landscape will increase with metal concentration (GxE) as fitness differences between genotypes become larger. However, individual mutational steps do not change in sign across environments, and as such the overall shape of the landscape will be comparable across concentrations (that is, magnitude epistasis will remain magnitude epistasis, sign epistasis will remain sign epistasis). While this does not preclude a significant GxGxE interaction per se (identical ranking can be associated with varying individual fitness differences), it does imply that the variance associated with such interaction is likely limited. By contrast, reranking GxE refers to the case where different genotypes are selected at different concentrations. In this case, individual mutational steps will commonly change in sign across environments, and as such the overall shape of the landscape will change considerably across concentrations (i.e. magnitude epistasis can change into sign epistasis and vice versa). This implies a significant GxGxE interaction by definition. Because under reranking GxE mutations are beneficial at some concentrations but not others, evolution will be directed along different pathways depending on the dynamic of environmental change.

Our previous findings (Gorter et al. 2016) are consistent with the predictions from our GxE framework. Specifically, our results for Cd may be explained using a magnitude GxE scenario, because we found mutations in the same genes in response to both gradual and abrupt change. Conversely, our results for Ni and Zn may be explained using a reranking GxE scenario, because in these cases we found mutations in different genes in response to gradual and abrupt change. In addition, we repeatedly found mutations in specific combinations, suggesting that epistasis was important within the context of adaptation to all three metals. Taken together, these findings led us to hypothesize that, depending on the selective metal, the height and/or shape of the fitness landscape change considerably across concentrations,

with important consequences for the mutational pathways used under each of our treatments. Specifically, for Cd we expect that the *height*, but not the shape, of the landscape changes across concentrations. Conversely, for Ni and Zn we expect that the *shape*, and possibly the height, of the landscape changes across concentrations, and that this causes different evolutionary pathways to be followed under different regimes of environmental change.

Here, we set out to test these predictions using two complementary approaches. First, we generated fitness landscapes for each metal by constructing mutants with all possible combinations of the parallel mutations that we found in our evolved clones. Because most of these mutations likely confer a reduction- or loss-of-function (Gorter et al. unpublished), we used deletion mutants to approximate their effects. We then measured the fitness of each mutant at different metal concentrations, which allowed us to test our hypotheses about how the height and/or shape of each landscape changes with concentration, and make predictions about the likely consequences that this had for evolutionary dynamics and outcomes. Second, for the more extensive Ni landscape, we then tested these predictions using deep sequencing of population samples isolated from our evolution experiment at different times points. Our results confirm that the height and/or shape of the fitness landscape change considerably across metal concentrations, depending on the metal used. Moreover, the landscape reconstructions correctly predicted which mutations were selected at what time in our evolving populations, demonstrating the power of our approach and theoretical GxE framework for understanding evolution.

Materials and methods

Strains

We used a set of haploid strains from the *S. cerevisiae* deletion collection (Giaever et al. 2002; Giaever and Nislow 2014) as the basis for our reconstruction mutants. In these strains single ORFs are replaced with a KanMX cassette, which confers geneticin (G418) resistance (Goldstein and McCusker 1999). The strains used as the basis for the deletion collection are essentially isogenic with the ancestral strains from our evolution experiment (Gorter et al. 2016). That is, they are based on the closely related haploid BY4730, BY4739, BY4741, and BY4742 strains, which differ only in mating type and the presence of the *HIS* auxotrophic marker, while our ancestral strains were derived from the diploid BY4743 strain (=BY4741/BY4742 (Brachmann et al. 1998; Wloch-Salamon et al. 2008)).

All single mutants in our study as well as the "ancestral" strain are *MAT**a*** strains from the deletion collection. Conversely, double, triple, and quadruple mutants were generated via gene replacement followed by multiple rounds of mating, sporulation and selection. Specifically, we amplified the HphMX cassette, which confers hygromycin resistance and shares its promoter and terminator sequences with the KanMX cassette, from strain W26 (*MAT**a**/his3/leu2/LYS/*

ura3/MET/ΔHO::HphMX) using primers Repl-F (CAGACGCGTTGAATTGTCCC) and Repl-R (CTGGGCAGATGATGTCGAGG). The resulting PCR product was transformed into a set of *MATα* strains to replace the KanR gene with the HphR gene, and these strains were then crossed to *MAT**a*** strains containing a different deletion to yield double heterozygous diploids. Sporulation followed by tetrad dissection and replica plating onto geneticin and hygromycin was used to isolate haploid double deletion mutants. Finally, we performed mating assays with tester strains W23 (*MATα/his3/leu2/lys/ura3/MET/ΔHO::NatMX*) and W24 (*MAT**a**/his3/leu2/lys/ura3/MET/ΔHO::NatMX*) to determine the mating type of each mutant.

This entire procedure (i.e. mating, sporulation, and selection) was repeated to generate triple and quadruple mutants. However, in this case a resistance phenotype is insufficient to ascertain strain genotype. We thus performed colony PCR on candidate strains using the deletion collection confirmation primers in combination with a primer internal to the HphR gene (Hph-3': TCGTCCATCACAGTTTGCCA) to identify the desired mutants. To generate the competitor strain for the fitness assays, we replaced the KanR gene in the *ΔHO::KanMX* mutant with the NatR gene (amplified from W24 using the same approach as for HphR/W26), which confers nourseothricin resistance.

An overview of all reconstruction strains can be found in Table 4.2.

Assays

To determine the relative fitness of the reconstruction mutants, we performed competition assays in triplicate as described previously (Lenski et al. 1991; Gorter et al. 2016). Briefly, strains were pre-grown from frozen in 0.5x YPD medium, and then cultured together with the competitor strain in the presence of different concentrations of the appropriate metal (0,1,2, and 3 μM $CdCl_2$; 0, 83, 167, and 250 μM $NiCl_2$; 0, 117, 233, and 350 μM $ZnCl_2$) for 24h. Plating on geneticin and nourseothricin before and after competition was used to determine the relative number of doublings, or relative fitness (W_{ij}), of each strain.

Similar assays were also used to confirm the absence of any genetic background effects on fitness within the context of our experiments. In this case, we determined fitness of the *ΔHO::KanMX* and *ΔHO::NatMX* reconstruction mutants relative to the ancestral strains from our evolution experiment, W24 (*MAT**a**/his3/leu2/lys/ura3/MET/ΔHO::NatMX*) and W20 (*MAT**a**/his3/leu2/LYS/ura3/met/ΔHO::KanMX*), respectively, in the presence of all concentrations of all metals in triplicate. These assays revealed no effect of genetic background on fitness (i.e. relative fitness no different from 1: $F_{1,35} = 0.28$, $P = 0.60$), in any of the assay environments (effect of metal, concentration, and metal x concentration: $P > 0.30$ in all cases).

To determine the relative reductive effect of metal on yield, we cultured pre-grown control strains (W20 and W24 mixed in equal volumes) in the presence of different metal concentrations for 24h. Based on the reduction in yield observed during the fitness assays, concentrations were chosen such that they were likely to include most of the relevant dose-response curve for each metal (Cd: 0, 1, 2, 3, 4, 5, and 6 μM; Ni: 0, 28, 56, 83, 167, 250 μM;

Table 4.2: Overview of all reconstruction mutants.

Strain	Genotype	Phenotype	Details (source or parental strain pair)
ancestor	*MAT**a** ΔHO::KanMX*	genR	from deletion collection
Δsmf1	*MAT**a** Δsmf1::KanMX*	genR	from deletion collection
Δfet4	*MAT**a** Δfet4::KanMX*	genR	from deletion collection
Δsmf1Δfet4	*MAT**a** Δsmf1::KanMX Δfet4::HphMX*	genR hphR	*MAT**a** Δsmf1::KanMX x MATa Δfet4::HphMX*
Δvip1	*MAT**a** Δvip1::KanMX*	genR	from deletion collection
Δbul1	*MAT**a** Δbul1::KanMX*	genR	from deletion collection
Δvtc4	*MAT**a** Δvtc4::KanMX*	genR	from deletion collection
Δwhi2	*MAT**a** Δwhi2::KanMX*	genR	from deletion collection
Δvip1 Δbul1	*MAT**a** Δvip1::KanMX Δbul1::HphMX*	genR hphR	*MAT**a** Δvip1::KanMX x MATa Δbul1::HphMX*
Δvip1 Δvtc4	*MAT**a** Δvip1::KanMX Δvtc4::HphMX*	genR hphR	*MAT**a** Δvip1::KanMX x MATa Δvtc4::HphMX*
Δvip1 Δwhi2	*MAT**a** Δvip1::KanMX Δwhi2::HphMX*	genR hphR	*MAT**a** Δvip1::KanMX x MATa Δwhi2::HphMX*
Δbul1 Δvtc4	*MAT**a** Δbul1::KanMX Δvtc4::HphMX*	genR hphR	*MAT**a** Δbul1::KanMX x MATa Δvtc4::HphMX*
Δbul1 Δwhi2	*MAT**a** Δbul1::KanMX Δwhi2::HphMX*	genR hphR	*MAT**a** Δbul1::KanMX x MATa Δwhi2::HphMX*
Δvtc4 Δwhi2	*MAT**a** Δvtc4::KanMX Δwhi2::HphMX*	genR hphR	*MAT**a** Δvtc4::KanMX x MATa Δwhi2::HphMX*
Δvip1 Δbul1 Δvtc4	*MAT**a** Δvip1::KanMX Δbul1::HphMX Δvtc4::KanMX*	genR hphR	*MAT**a** Δvip1::KanMX Δbul1::HphMX x MATa Δvtc4::KanMX*
Δvip1 Δbul1 Δwhi2	*MAT**a** Δvip1::KanMX Δbul1::HphMX Δwhi2::KanMX*	genR hphR	*MAT**a** Δvip1::KanMX Δbul1::HphMX x MATa Δwhi2::KanMX*
Δvip1 Δvtc4 Δwhi2	*MAT**a** Δvip1::KanMX Δvtc4::HphMX Δwhi2::KanMX*	genR hphR	*MAT**a** Δvip1::KanMX Δvtc4::HphMX x MATa Δwhi2::KanMX*
Δbul1 Δvtc4 Δwhi2	*MAT**a** Δbul1::KanMX Δvtc4::HphMX Δwhi2::KanMX*	genR hphR	*MATa Δbul1::KanMX Δvtc4::HphMX x MATa Δwhi2::KanMX*
Δvip1 Δbul1 Δvtc4 Δwhi2	*MAT**a** Δvip1::KanMX Δbul1::HphMX Δvtc4::KanMX Δwhi2::HphMX*	genR hphR	*MAT**a** Δvip1::KanMX Δbul1::HphMX x MATa Δvtc4::KanMX Δwhi2::HphMX*
Δubp3	*MAT**a** Δubp3::KanMX*	genR	from deletion collection
Δubp6	*MAT**a** Δubp6::KanMX*	genR	from deletion collection
Δubp3 Δubp6	*MAT**a** Δubp3::KanMX Δubp6::HphMX*	genR hphR	*MAT**a** Δubp3::KanMX x MATa Δubp6::HphMX*
competitor	*MAT**a** ΔHO::NatMX*	natR	*MAT**a** ΔHO::NatMX*

Zn: 0, 15, 29, 58, 117, 233, and 350 μM). Both prior to and after growth we determined the number of colonies for each replicate to establish the relative reduction in yield. This reduction was then scaled to the change in yield of a control sample grown in the absence of metals to express yield in the presence of metals as a percentage of yield in their absence. All assays were performed in duplicate. An equivalent number of observations was taken from the data generated for our original paper (Gorter et al. 2016), and analysed in the same way.

Data analysis

Fitness scaling between experiments/studies

To determine the effect of metal concentration on yield under the original and the present conditions, we fitted regressions to the yield data for all metals separately under both conditions, forcing the intercept through 100 (= percentage yield at 0 μM metal). The slopes of these regression lines were then used to calculate the conversion relationship between metal reductive effect in the original and the present assays (Table S4.2). This analysis confirmed that cadmium had a lower (~50%) reductive effect in the current assays, while Ni and Zn had a stronger reductive effect (~200% and ~120%, respectively). Specifically, 3 μM Cd, 250 μM Ni, and 350 μM Zn in the original assays (= final high concentrations) are equivalent to 6.7 μM Cd, 130 μM Ni, and 293 μM Zn in the current assays, respectively.

To translate our measured fitness values to the original conditions, we fitted a quadratic regression of log-transformed fitness on metal concentration for each mutant separately, and used the previously determined metal conversion relationships to predict fitness of each mutant under the original assay conditions. We then added the residuals from the model to the predicted fitness values to arrive at the converted "raw" data points that we used for further analysis. Log transformation was employed to correct for increasing variance, while the quadratic terms allowed for the modelling of the non-linear effects of metal concentration on fitness that we observed for a substantial part of all mutants. The regressions for the single mutants were also used to predict their fitness across the complete range of metal concentrations used in the experiment.

Statistical analyses

We used general linear models to evaluate the GxGxE interactions within each reconstructed fitness landscape. First, we fitted models with log-transformed fitness as the response variable, and mutant genotype (4 or 16 levels for Cd/Zn and Ni, respectively), metal concentration (4 or 3 levels for Cd/Zn and Ni, respectively), and the interaction between these effects as explanatory variables. We used these models to assess the significance of each individual mutational step at each metal concentration. Next, we reformulated the models to test our hypotheses about GxGxE. That is, we replaced mutant genotype with separate factors for each

mutation, and fitted models with all individual mutations (2 levels each), metal concentration (as above), and the interactions between these effects (that is, up to three-way interactions for Cd/Zn, and up to five-way interactions for Ni) to the data. In each case, we used model simplification to find the minimal adequate model and report statistics from deletion tests. In these analyses, log transformation of fitness was used not only to correct for increasing variance, but also because it enables direct testing of a multiplicative null model of epistasis.

To investigate whether loss-of-function mutants of *VTC4* had a different fate than amino acid change mutants of this gene, we fitted linear regressions of frequency on time for each mutant separately. We then tested whether these slopes were positive/negative more often for either type of mutant using Fisher's exact test.

All statistical analyses were performed in R 3.0.2 (R Core Team 2013)

Sequencing

To investigate evolutionary dynamics in our previously performed long-term laboratory evolution experiment (75 transfers/500 generations; for details see Gorter et al. 2016), we revived frozen whole population samples isolated at different transfer time points (T=25, T=50, and T=75) from all lines evolved in the presence of either gradually increasing or constant high concentrations of $NiCl_2$ (lines NI1–6: 16.7–250 μM, and lines NH1–6: 250 μM). Genomic DNA was isolated according to standard procedures, and the *VIP1*, *BUL1*, *VTC4*, and *WHI2* genes were amplified using an optimized PCR protocol with the Phusion High-Fidelity DNA polymerase (NEB) and the deletion collection confirmation primers located upstream and downstream of each gene. As a control, three separate PCRs were performed for each gene on genomic DNA isolated from the ancestral strain W20. For each replicate population, as well as the three control reactions, cleaned up PCR products from all genes were mixed in equimolar ratios. Libraries were prepared from these mixtures using the Nextera XT DNA library preparation kit (Illumina Inc.), and these were then sequenced by Illumina MiSeq with 2 x 300 bp paired end reads, by the Cologne Centre for Genomics.

Sequencing reads were analysed using the CLC Genomics Workbench 8.5.1. Reads were trimmed to remove adapter sequences and low quality regions (Q<30) and mapped to the reference sequence of each of the four genes (*VIP1*, *BUL1*, *VTC4*, and *WHI2*) using standard parameter settings. Average coverage per gene across populations was high, ranging from 6622x for *BUL1* to 8294x for *VTC4*. We used the low frequency variant detector to call SNPs and small indels that were present in more than 1% of the reads and annotated the variants using the *S. cerevisiae* CDS track. We tried to further reduce the threshold for variant calling, but this yielded variants in the control reactions. Such variants are likely due to PCR and/or sequencing errors, or alternatively may represent very low frequency variants maintained by drift or mutation-selection balance under standard culture conditions, and are therefore not relevant to our selection regimes.

Results

We previously evolved replicate populations of *S. cerevisiae* for 500 generations in the presence of either gradually increasing or constant high concentrations of the heavy metals Cd, Ni, or Zn (Gorter et al. 2016) and found that adaptation to these selection regimes occurred repeatedly through specific combinations of reduction- or loss-of-function mutations in a limited number of genes (Table 4.1; Gorter et al. unpublished). To explain these observations, and gain more insight into evolutionary dynamics in directionally changing environments in general, we here reconstructed the underlying local fitness landscape for each metal by deleting all repeatedly mutated genes per metal both by themselves and in combination. This yielded landscapes of 4 (Cd and Zn: two genes → 2^2 combinations) or 16 (Ni: four genes → 2^4 combinations) different genotypes. To assess how the effect of each mutation depended on the genetic background as well as the environment, we then determined the fitness of each mutant relative to a differently marked control strain in the presence of different concentrations of the selective metal. Specifically, we assayed each mutant in the presence of the final, or constant high, metal concentration used in the selection experiment, as well as two intermediate concentrations (33% and 67% of the final concentration), and in the absence of additional metals.

Assay problems

When performing these assays, we observed that all three metals had a very different effect compared with the assays performed for our original study (Gorter et al. 2016). That is, the same concentrations of Ni and Zn reduced overall yield (number of colonies after competition) more strongly in our current assays, while Cd had a smaller reductive effect. This difference in reductive effect may be due to subtle differences in the composition of our — undefined — media, which can have large consequences (Joho et al. 1988; O'Keefe et al. 2006; Corbacho et al. 2011). Since we were unable to remedy this problem, we chose to correct our measurements for this effect. To do so, we determined the dose-response relationship to each metal — that is, the relative reduction in yield as a function of metal concentration — for a pair of control strains. These same strains had also been assayed at different metal concentrations as part of our previous study, which allowed us to determine the same dose-response relationship under the original conditions. We used these relationships to calculate metal-specific conversion factors between the old and new conditions (Table S4.2), which enabled us, in combination with regression of fitness against metal concentration for each mutant, to translate our fitness measurements to the original conditions (see Materials and Methods for details). In the case of Ni, the increased reductive effect at the highest assay concentration (in 80% of all competitions the less fit competitor formed fewer than five colonies) introduced a very large measurement error. We therefore excluded all data from this condition from our analyses, and instead used the data collected

at lower Ni concentrations to calculate fitness under the original conditions in the presence of the final, high Ni concentration, and at 50% of this concentration.

Cadmium

We used the corrected fitness landscapes to test several of our hypotheses about the effect of the rate of directional environmental change on evolution. For Cd, we anticipated that the height of the fitness landscape would increase with metal concentration, but that its shape would remain roughly constant across concentrations. This should result in the same pathways being followed under gradual and abrupt change. In support of this prediction, we found that the total variance in fitness (~ height of the fitness landscape) increased significantly with Cd concentration (Figure 4.1; Levene's test for equality of variances: $F_{3,12} = 12.31$, P = 0.0006). This was largely due to the increased fitness of *Δsmf1* strains at higher metal concentrations (i.e. GxE: *Δsmf1* x Cd concentration: $F_{3,38} = 89.12$, P < 0.0001). As anticipated, the *Δfet4* mutation was beneficial only in the background of *Δsmf1* (i.e. GxG: *Δfet4 x Δsmf1*: $F_{1,38}$ =

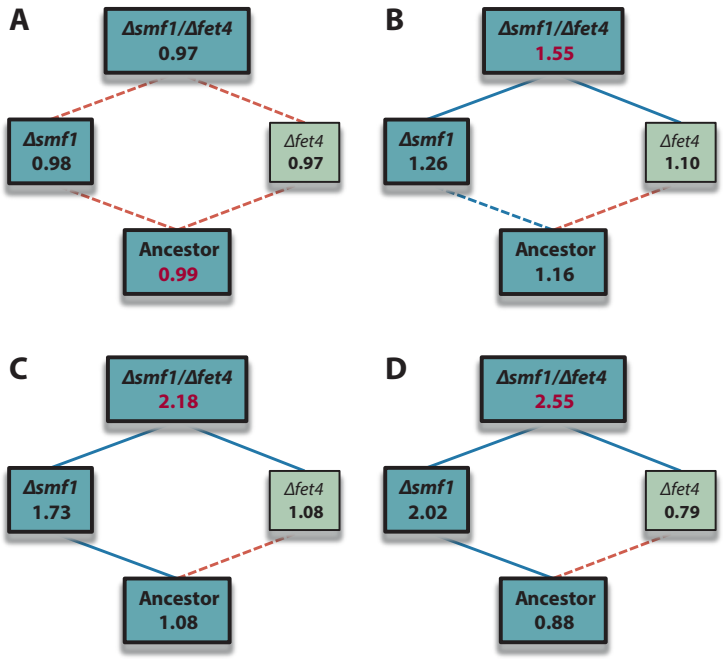

Figure 4.1: Predicted fitness of reconstruction mutants in the presence of 0 (A), 1 (B), 2 (C), or 3 (D) μM $CdCl_2$. Edge colour reflects how the addition of each mutation (i.e. from bottom to top) affects fitness (blue = increase, orange = decrease). Solid lines represent significant changes, dotted lines non-significant ones. After sequential Bonferroni correction everything remains significant. Bold, darker boxes correspond with (combinations of) mutations found in clones isolated from the long-term evolution experiment. Red fitness values represent global optima.

20.60, P < 0.0001). However, we found no evidence that the shape of the fitness landscape changed across concentrations: the GxGxE interaction was non-significant (*Δfet4* x *Δsmf1* x Cd concentration: $F_{1,32}$ = 2.86, P = 0.052), and, moreover, the fitness ranking of all four genotypes was the same at all metal concentrations.

As a result, none of the individual mutational steps changed in sign across Cd concentrations, and the *Δsmf1Δfet4* double mutant consistently represented the global fitness optimum in the presence of Cd (Figure 4.1). This optimum is accessible via a single pathway only, leading to the straightforward prediction that evolution under both gradually increasing and constant high concentrations of Cd will lead to the initial selection of loss-of-function mutations in *SMF1*, followed by selection of loss-of-function mutations in *FET4*. Because fitness differences are larger at high Cd concentrations, this process should be faster following abrupt change. Indeed, we previously found that all six populations exposed to constant high Cd concentrations contained mutations in both *SMF1* and *FET4* (Table 4.1). Conversely, only two of the populations exposed to increasing Cd concentrations contained this combination of mutations, while the other four populations from this treatment contained a mutation in *SMF1* in isolation. This may indicate that these populations had not yet acquired a mutation in *FET4* due to delayed adaptation.

Zinc

For Zn, we predicted that the shape of the fitness landscape would change across concentrations, and that this would have consequences for the evolutionary pathways followed under different regimes of environmental change. In contrast to Cd, the total variance in fitness was similar at different Zn concentrations (Figure 4.2; Levene's test for equality of variances: $F_{3,12}$ = 0.83, P = 0.50). This implies that the overall height of the fitness landscape was comparable across concentrations. In contrast to our expectations, the shape of the landscape did not change significantly with concentration (i.e. GxGxE: *Δubp3* x *Δubp6* x Zn concentration: $F_{3,32}$ = 0.77, P = 0.52). Moreover, neither the GxE interactions nor the GxG interaction were significant (P > 0.10 in all cases), and instead the minimal model predicted that both the *Δubp3* and the *Δubp6* mutation confer a fitness decrease, while the highest Zn concentration consistently increases mutant fitness (P < 0.02 in all cases).

Nonetheless, between 233 and 350 μM Zn all four genotypes changed in rank, and 50% of the mutational steps changed in sign. Accordingly, the global optimum changed from the ancestral genotype to the *Δubp6* single mutant. Taken together, these observations suggest that at least part of the explanation for the lack of significant GxGxE may be that for the Zn landscape fitness differences are small relative to measurement error. *Δubp6* increased fitness marginally at the high concentration at which mutations in this gene were selected during the evolution experiment. By contrast, for *Δubp3* there was no indication that it increased fitness in any environment, either by itself or in combination with *Δubp6*. In fact, a previous study reported that this mutation decreases zinc tolerance (Dziedzic and Caplan 2011),

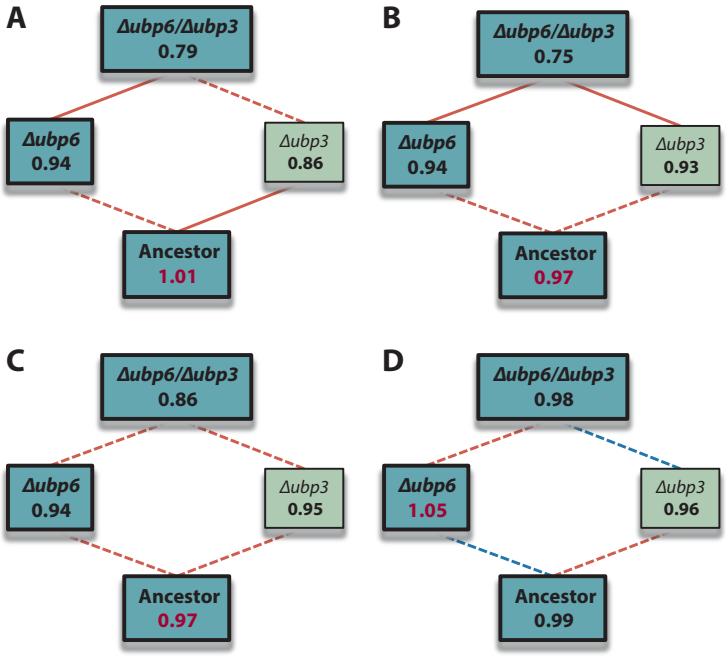

Figure 4.2: Predicted fitness of reconstruction mutants in the presence of or 0 (E), 117 (F), 233 (G), or 350 (H) μM $ZnCl_2$. Edge colour reflects how the addition of each mutation (i.e. from bottom to top) affects fitness (blue = increase, orange= decrease). Solid lines represent significant changes, dotted lines non-significant ones. After sequential Bonferroni correction only the comparisons at 117 μM remain significant. Bold, darker boxes correspond with (combinations of) mutations found in clones isolated from the long-term evolution experiment. Red fitness values represent global optima.

making it hard to explain why we found mutations in *UBP3* in response to our high Zn selection regime. One possibility is that the amino acid changes in our evolution lines altered rather than abolished *UBP3* function (neither of the two observed mutations in this gene is a stop codon or frameshift mutation), in which case the deletion mutant phenotype does not accurately reflect their effect.

Nickel

For Ni, we had the same hypotheses as for Zn, that is, we predicted a change in the shape of the fitness landscape as a function of concentration, with distinct consequences for the evolutionary pathways followed under different rates of change. Because it is composed of four rather than two genes, the results for the Ni landscape were considerably more complex (Figure 4.3). However, the same general statistics that we used to characterize differences between landscapes for Cd and Zn can also be obtained for Ni. First, the height of the Ni landscape increased with concentration, but this was not significant (Levene's test for equality of variances:

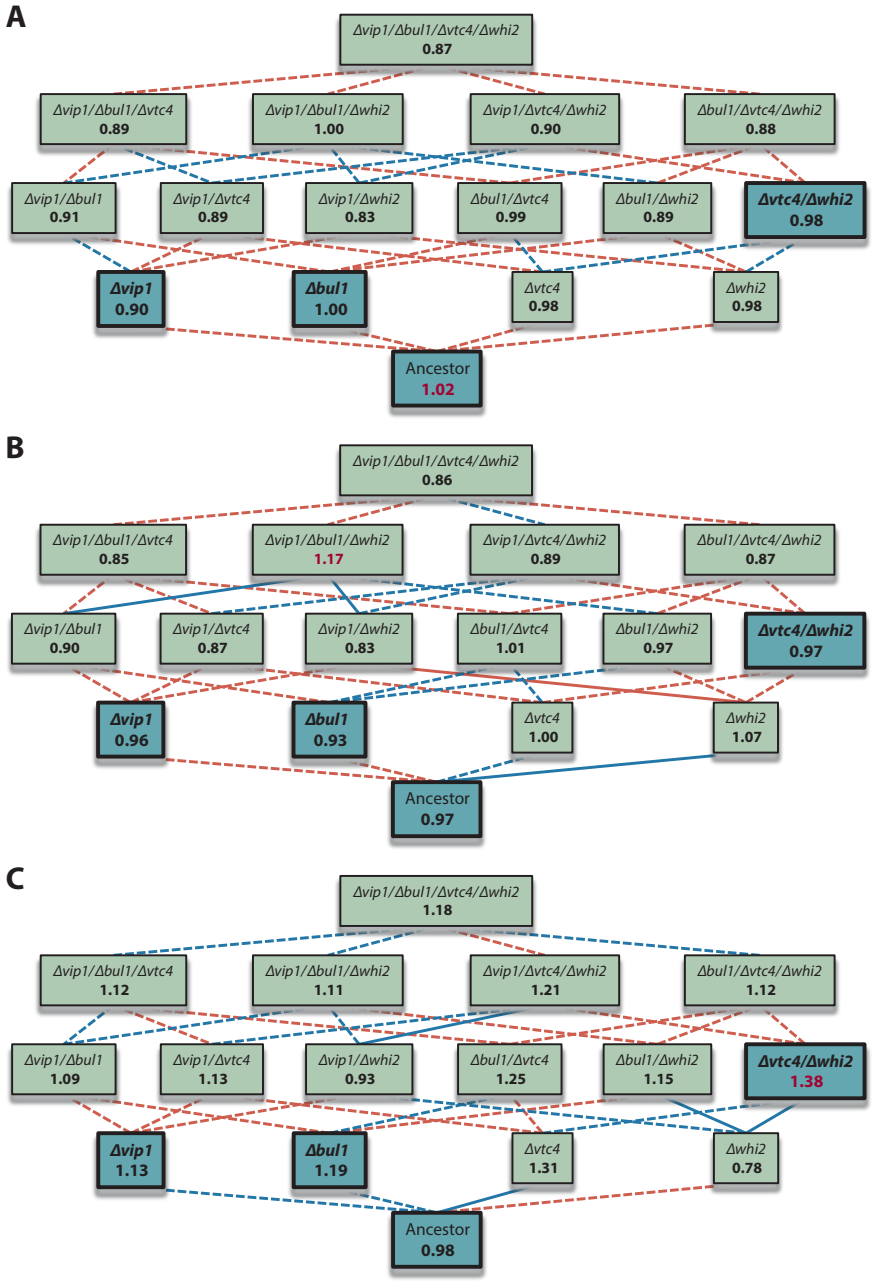

Figure 4.3: Predicted fitness of reconstruction mutants in the presence of 0 (A), 125 (B) or 250 (C) μM $NiCl_2$. Edge colour reflects how the addition of each mutation (i.e. from bottom to top) affects fitness (blue = increase, orange = decrease). Solid lines represent significant changes, dotted lines non-significant ones. After sequential Bonferroni correction, only the changes from *Δwhi2* to *Δvtc4Δwhi2* and *Δbul1Δwhi2* at 250 μM remain significant. Bold, darker boxes correspond with (combinations of) mutations found in clones isolated from the long-term evolution experiment. Red fitness values represent global optima.

$F_{2,45}$ = 1.84, P = 0.17). Second, the shape of the landscape changed across concentrations, as evidenced by a significant GxGxGxE interaction (*Δvip1* x *Δvtc4* x *Δbul1* x Ni concentration: $F_{2,108}$ = 3.74, P = 0.027). In addition to this interaction, the minimal model for this landscape contained two GxGxG interactions involving *Δwhi2*: *Δbul1* x *Δvtc4* x *Δwhi2* ($F_{1,108}$ = 7.75, P = 0.006), and *Δvip1* x *Δbul1* x *Δwhi2* ($F_{1,108}$ = 4.74, P = 0.032). These patterns appear complex, but can be explained for a large part by the mutations in *VIP1*, *VTC4*, and *BUL1*, each of which significantly increased fitness at 250 μM Ni only. However, the effects of these mutations were almost entirely redundant: the presence of one of these mutations conferred a similar fitness increase as the presence of two or three of them (effect of number of mutations: $F_{2,36}$ = 0.51, P = 0.61), providing a clear example of diminishing returns epistasis. Additional effects that were present at all concentrations included a decreased fitness of the *Δbul1Δvtc4Δwhi2* mutant and an increased fitness of the *Δvip1Δbul1Δwhi2* and *Δvtc4Δwhi2* mutants, compared with expectations based on the fitness of single and double mutants. However, for the latter two mutants, this appears to be due almost entirely to their fitness effects at one specific concentration, 125 μM in the case of *Δvip1Δbul1Δwhi2*, and 250 μM in the case of *Δvtc4Δwhi2*.

Between the two Ni environments (i.e. 125 μM versus 250 μM Ni), the majority of all sixteen genotypes changed in rank, and, crucially, 41% of the individual mutational steps changed in sign. This cannot be explained by the increased dimensionality of the landscape: when considering the sub landscapes composed of two mutations each (ancestor, both single mutants, double mutant), the majority of all genotypes still changed in rank. Moreover, 63% of the mutational steps changed in sign on average, and for the *Δvtc4Δwhi2* combination that we found in our evolution experiment this percentage was even higher (75%). Consistently, both complete landscapes had a different global optimum, which was accessible from the ancestor in the case of 250 μM only. Taken together, these results suggest that evolutionary dynamics will be very different under different regimes of environmental change for Ni. Specifically, at intermediate Ni concentrations only mutations in *WHI2* should be selected (and possibly mutations in *VTC4* followed by mutations in *BUL1*, although these mutations confer only very marginal fitness increases). Conversely, at high Ni concentrations mutations in *VIP1*, *BUL1*, and *VTC4* should be selected, while mutations in *WHI2* should be selected against. In this environment, the *Δvtc4Δwhi2* mutant represents the global fitness optimum, suggesting that at high Ni concentrations selection of mutations in *WHI2* is contingent upon the presence of mutations in *VTC4*. These predictions are in good agreement with our sequencing results for clones from the constant high Ni treatment: we repeatedly found mutations in either *BUL1* in isolation, or in *VTC4* and *WHI2* in combination (Table 4.1). The fact that we did not find any mutations in *VIP1* may be explained by the smaller fitness benefit of *Δvip1* relative to *Δbul1* and *Δvtc4*.

Our reconstruction approach appears to adequately explain the evolutionary outcomes in response to constant high Ni concentrations. However, why mutations in *VIP1* rather than *BUL1*, *VTC4*, and *WHI2* were found in response to increasing Ni concentrations (Table 4.1)

is not immediately clear. Mutations in *WHI2* may have been selected and subsequently lost again, because they are beneficial at intermediate Ni concentrations only. Conversely, *Δvip1* confers a fitness increase at 250 μM only, and there its benefit is smaller than that of *Δbul1* and *Δvtc4*. A possible explanation for finding mutations in *VIP1* in our evolved clones is that mutations in this gene start to increase fitness at lower concentrations than mutations in *VTC4* or *BUL1*. To assess this possibility, we plotted the predicted fitness as a function of (original) metal concentration for all four single mutants (Figure 4.4), which indeed revealed that *Δvip1* becomes beneficial at a lower concentration (166 μM) than *Δbul1* (182 μM). This suggests that

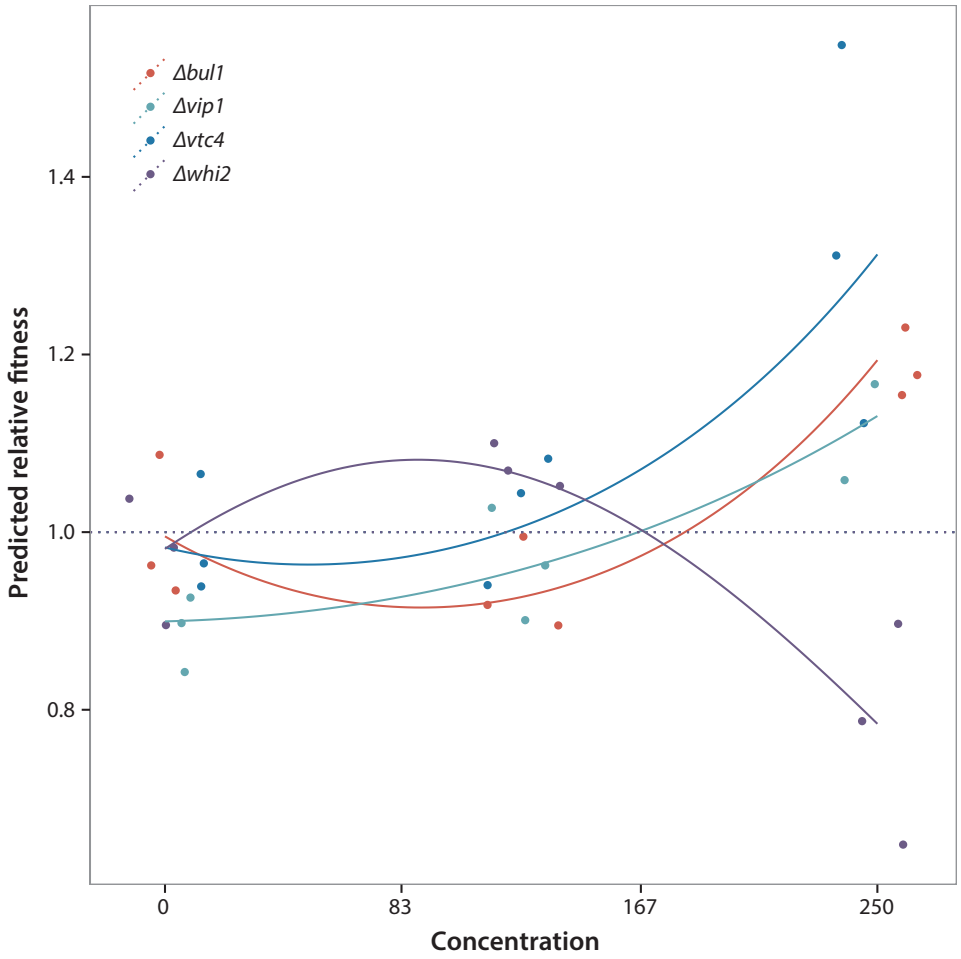

Figure 4.4: Predicted relative fitness of single-gene deletion mutants as a function of Ni concentration. Predictions are based on quadratic regressions of log-transformed fitness on metal concentration for each mutant separately. Coloured dots represent transformed replicate fitness measurements for each mutant, and are jittered for increased visibility.

mutations in *VIP1* were selected starting from transfer T=45, while mutations in *BUL1* were selected from transfer T=50, and conferred a higher fitness than *VIP1* from T=60 onwards. By contrast, mutations in *VTC4* are predicted to both become beneficial at an earlier time point, and confer a larger benefit than both other mutants over the entire concentration range up to 250 μM. The mutations in *VTC4* in our evolved clones were never stop-codon nor frame-shift mutations, raising the possibility that these mutations confer a smaller benefit than loss-of-function mutations in the same gene, which would explain why we found mutations in *VIP1* rather than *VTC4* in response to increasing Ni concentrations. However, if that were the case it is unclear why we did not find loss-of-function mutations instead in our evolution experiments, because such mutations should have increased fitness more strongly. One possibility is that *Δvtc4* has antagonistic pleiotropic effects that do not play out over the course of our fitness assays. For example, it was recently reported that *Δvtc4* causes genomic instability (Bru et al. 2016), which may have detrimental effects in the long term.

Sequencing

The aim of our landscape reconstructions was to increase our understanding of how the height and shape of the fitness landscape change in response to different rates of directional environmental change, and how this in turn affected the evolutionary trajectories followed under our metal-selection regimes. Our reconstructions were able to explain a substantial part of our whole-genome sequencing results (Table 4.1), but also generated new predictions about the underlying dynamics in our selection experiments. These predictions were relatively straightforward for Cd, but more subtle for Ni, with different mutations predicted to be selected at different times under increasing *versus* constant high concentrations of this metal. To test these predictions and thus assess the validity of our reconstruction approach, we amplified *VIP1*, *BUL1, VTC4*, and *WHI2* from whole population samples isolated at different time points (T=25, T=50, and T=75) from all evolution lines exposed to increasing and constant high concentrations of Ni. Deep sequencing of the resulting PCR product was then used to determine the relative frequency of different alleles at these four loci over time. This analysis revealed extensive clonal interference within and between loci and showed that evolutionary dynamics under gradually increasing and constant high Ni concentrations were fundamentally different (Figure 4.5; Table S4.1).

Under gradually increasing Ni concentrations, mutations in *VIP1* were selected repeatedly, but, as predicted by our landscape analyses, this happened only during the final stage of the experiment. At T=50, no mutation in *VIP1* had reached the detection threshold of 1% yet (although one of the mutations that we found at T=75 was present at 0.13%), but at T=75 half of the populations contained such mutations at frequencies ranging from 43-77%. In line with our finding that *Δwhi2* confers a fitness advantage at low Ni concentrations, we found a loss-of-function mutation in this gene in isolation at T=25 in one instance (at a frequency of 12%). At T=50 this mutation was no longer detectable, consistent with such mutations being

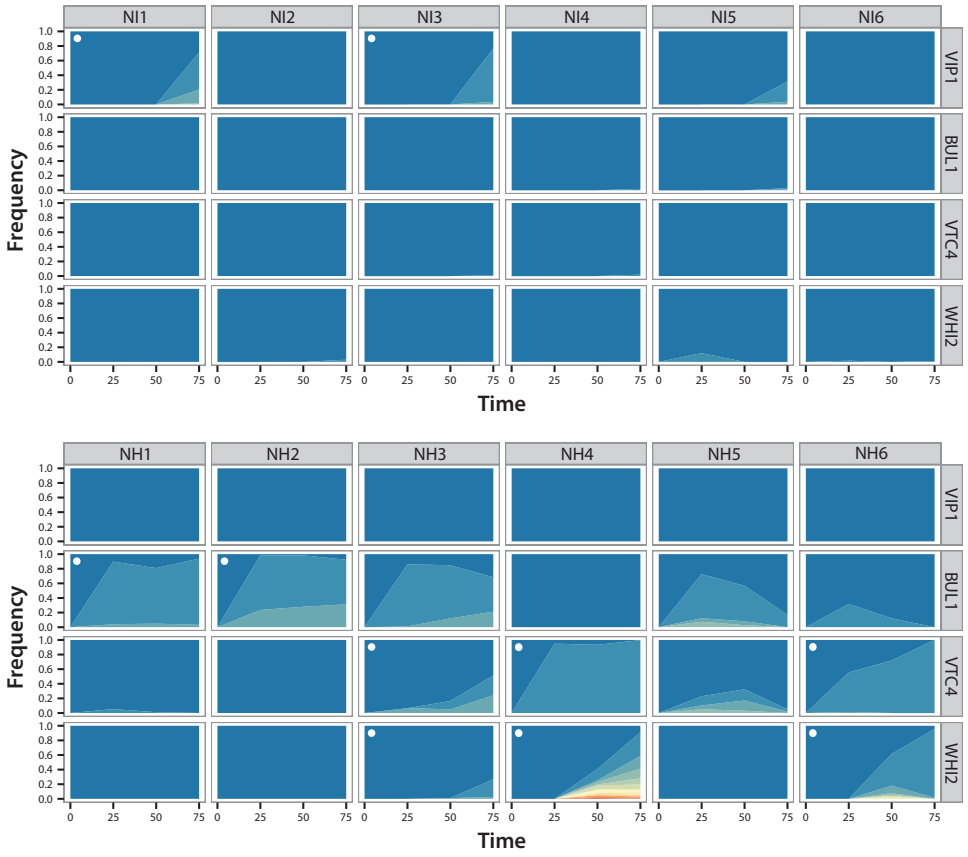

Figure 4.5: Evolutionary dynamics of replicate yeast populations exposed to increasing (NI) or constant high (NH) concentrations of Ni at the repeatedly mutated loci *VIP1*, *BUL1*, *VTC4*, and *WHI2*. Colours depict different alleles and their frequency over time (in transfers). Blue shading represents the wild type allele. White dots indicate the presence of a mutation in the corresponding gene in the sequenced clone from that population. In all cases, the mutations that we previously identified in the sequenced clones were also present in the corresponding population samples from T=75 at appreciable frequencies

detrimental at higher Ni concentrations. Furthermore, mutations in *BUL1*, *VTC4*, and *WHI2* were present at T=75 at very low frequencies (<3%) in two replicate lines each, in the same and different populations than the *VIP1* mutations (Table S4.1). This confirms that *BUL1* and *VTC4* mutations are also under selection at this final time point, and suggests that this has been so for a shorter time than for *VIP1*. Mutations in *WHI2* were present in the two populations that had no mutations in any of the other genes. Because these lines contain other mutations that are known to increase Ni tolerance (loss-of function of *BSD2* (NI2) and *VPS38* (NI6) (Arita et al. 2009)), it is possible that *WHI2* mutations were selected specifically in their background, analogous to their selection in the background of *VTC4* mutations.

In contrast to the gradually increasing treatment and consistent with their absence in the sequenced clones from T=75, we never found mutations in *VIP1* in the populations exposed to constant high Ni concentrations. Conversely, mutations in *BUL1* and/or *VTC4* were present in all populations from the first sequenced time point (T=25, although at low frequency in some populations), and in four populations (NH1, NH3, NH5, and NH6; Figure 4.5 and Table S4.1) alleles of these two genes showed clonal interference. This competition generally persisted for many transfers, with comparatively small increases in the relative frequency of *VTC4* alleles, suggesting that overall such mutations have a small selective advantage relative to *BUL1* alleles. Interestingly, all loss-of-function mutants of *VTC4* decreased in frequency over time, while amino acid change mutants increased in frequency with a single exception (Fisher's exact test: P = 0.048). This supports our previous hypotheses that these two classes of mutations are fundamentally different, and that *Δvtc4* has detrimental effects in the long term. In population NH5, both *BUL1* and *VTC4* mutants eventually decreased in frequency, implying that they were outcompeted by another, more beneficial mutant (this is also the population that contained the single *VTC4* amino acid change mutant that decreased in frequency). A likely candidate gene for this is *VTC1*, which we found to be mutated in the evolved clone from this line. Our landscape reconstructions predicted (nearly reciprocal) sign epistasis between *Δvtc4* and *Δbul1* at 250 μM Ni, which can explain why we never found mutations in both genes in the same evolved clone. However, in population NH3, the joint frequency of such mutations clearly exceeds 1 at T=75 (1.2). Because *Δvtc4* marginally increases fitness in the *Δbul1* background, this suggests that mutations in *VTC4* were selected in a *BUL1* mutant background in at least some cases.

One of the main predictions from our landscape analyses was that *WHI2* mutations would only be selected in the background of *VTC4* mutations at high Ni concentrations. The deep sequencing results support this: mutations in *WHI2* never occurred before *VTC4* mutations rose to appreciable frequencies, and after their addition, *VTC4* mutants increased in frequency relative to *BUL1* mutants when they were in competition. Remarkably, in population NH4 we simultaneously found up to 14 different alleles of *WHI2*. Although no *WHI2* allele had reached the threshold of 1% yet at T=25 for this population, 5 WHI2 alleles found at T=50 were present at this time at lower frequencies, suggesting that they were selected from a very early stage.

Discussion

The fitness landscape is a concept that is widely used for understanding and predicting evolutionary adaptation (Wright 1932; Orr 2005; de Visser and Krug 2014). The topography of the fitness landscape depends critically on the environment, which has potentially far-reaching consequences for evolution under changing conditions (Taute et al. 2014). However, few studies have assessed directly how empirical fitness landscapes change across

conditions, or validated the predicted consequences of such change. We previously evolved replicate yeast populations in the presence of either gradually increasing or constant high concentrations of the heavy metals Cd, Ni, and Zn, and analysed their phenotypic (Gorter et al. 2016) and genomic changes (Gorter et al. unpublished). Here, we reconstructed the local fitness landscapes underlying adaptation to each metal by deleting all repeatedly mutated genes both by themselves and in combination. Fitness assays revealed that the height and/or shape of each local fitness landscape changed considerably across metal concentrations, with distinct qualitative differences between non-essential and essential metals. This change in topography had particularly crucial consequences in the case of Ni, where a substantial part of the individual mutational fitness effects changed in sign across concentrations. Based on the Ni landscape analyses, we made several predictions about which mutations had been selected when during our evolution experiment. Deep sequencing of population samples from different time points generally confirmed these predictions, demonstrating the power of landscape reconstruction approaches for understanding evolutionary dynamics, even under complex scenarios of environmental change.

Change in landscape topography across concentrations depends on the selective metal

Our results are consistent with our hypotheses as outlined in the introduction. Based on a previously developed GxE framework (Gorter et al. 2016), we predicted that for the non-essential — that is, unconditionally toxic — heavy metal Cd the height of the fitness landscape would increase with metal concentration, but that its overall shape would not change. The rationale behind this is that the same evolutionary solution, reducing the internal Cd concentration to a minimum, should be favoured at all external metal concentrations, but more strongly so at high concentrations. Indeed, our results show that, while the mutations in the Cd landscape interact epistatically (that is, the benefit of *Δfet4* was contingent upon the presence of *Δsmf1*), the nature of this interaction does not change across concentrations, so that the same adaptive trajectory is accessible under both gradual and abrupt change. However, fitness differences between genotypes were larger at high concentrations, implying that selection should be faster following abrupt change. These findings are in good agreement with our previous phenotypic and genomic analyses, and suggest that for Cd, the exact scenario of environmental change (e.g. gradual, sudden, fluctuating) may affect the timing of selection, but will have limited effect on the outcome of evolution.

By contrast, for the essential — that is, required at low concentrations and toxic at high concentrations only — heavy metals Zn and Ni, we predicted that the shape of the fitness landscape would change substantially across metal concentrations. The reason for this is that in this case different evolutionary solutions should be favoured at different external metal concentrations to maintain a constant internal metal concentration. For Zn we found limited evidence for this hypothesis. That is, fitness ranking of genotypes changed across

Zn concentrations, but the GxGxE interaction was not significant, possibly because fitness differences were small relative to measurement error. Alternatively, it is possible that the fitness correction that we used was imprecise. However, qualitative results were the same (i.e. no significant GxGxE interaction, *Δubp3* detrimental at all concentrations) for analyses based on the uncorrected fitness measurements (data not shown), suggesting that our conclusions do not depend strongly on the way in which we corrected fitness.

For Ni we found strong evidence that the shape of the fitness landscape changed across concentrations. That is, in the Ni landscape mutations interacted epistatically (GxG), and these interactions changed significantly across concentrations (GxGxE). At the final high Ni concentration (250 μM), *Δwhi2* was contingent upon *Δvtc4*, while *Δvip1*, *Δbul1*, and *Δvtc4* mutually excluded each other. However, between 125 μM and 250 μM Ni, almost half of the individual mutational effects changed in sign, which has important consequences for the predicted evolutionary dynamics. Extension of this analysis to other concentrations suggests pervasive changes in the fitness ranking of genotypes, implying that in the case of Ni evolutionary dynamics and outcomes depend strongly on the exact scenario of environmental change. For example, if we had chosen to perform our experiments at 80% of the current concentration (200 μM $NiCl_2$), we would likely have found mutations in *VIP1*, rather than *BUL1* or *VTC4*. Similarly, our deep sequencing results showed that mutations in both *BUL1* and *VTC4* were present at low frequencies at the final time point of several of the increasing Ni populations. This suggests that under continued exposure to constant high Ni concentrations it will be only a matter of time before mutations in *VIP1* are outcompeted by these, now fitter, mutants. In our current study, we considered only a limited number of loci, based on the presence of mutations in multiple populations. However, it seems plausible that similar patterns exist for other loci as well, so that under gradually increasing Ni concentrations several mutations were selected but subsequently lost again as the concentration increased.

Landscape analyses correctly predict evolutionary dynamics despite uncertainties

We used deep sequencing of population samples from different time points to test the predictions from our landscape reconstructions about evolutionary dynamics in response to gradually increasing and constant high Ni concentrations. For practical reasons, these predictions were based on several simplifying assumptions. First, we used knock-out constructs to approximate the effect of each mutation. Second, we used a — crude — measure based on relative yield to translate our measurements to the original evolutionary conditions, and assumed a quadratic relationship between concentration and fitness across all concentrations. Finally, we did not differentiate between significant and non-significant fitness differences between genotypes when making predictions. For the Cd landscape, fitness ranking of all genotypes, and thus accessibility of mutational pathways, was the same at all Cd concentrations, and our predictions would have been similar if we had made different

assumptions. Conversely, for the Ni landscape, fitness ranking, and thus accessibility of mutational pathways, changed considerably across concentrations, and our predictions would have been fundamentally different if we had made different assumptions. Nonetheless, the deep sequencing results for the populations evolved in the presence of Ni fit our predictions remarkably well. Most notably, the reconstructions correctly predicted the timing of selection of mutations in *VIP1* and *WHI2* in response to gradually increasing Ni concentrations, as well as the interference between mutations in *BUL1* and *VTC4* in response to constant high Ni concentrations. This shows the validity of our approach: based on analyses of constructed fitness landscapes we could predict which mutations were selected at what time, even under complex scenarios of change. Despite the fact that our analyses suffer from an undeniable degree of incompleteness and inaccuracy, we believe that our approach may well serve as a basis for models exploring a much wider variety of scenarios of change.

Extensive clonal interference within and between loci

Our deep sequencing results revealed extensive clonal interference within and between loci. Clonal interference between mutations at different loci is commonly observed in microbial evolution experiments (Barrick and Lenski 2013; Lang et al. 2013), and several studies have reported clonal interference between alleles of the same locus (Lee and Marx 2013; Hong and Gresham 2014; Maharjan et al. 2015). The repeated occurrence of mutations in the same genes is a strong indication for the selective advantage of such mutations (Elena and Lenski 2003). However, the simultaneous presence of up to 14 alleles of the same gene — *WHI2* — within a single population is remarkable and quite puzzling at first sight. If these mutations are so beneficial, why did we not observe one of them going to fixation before the others occurred? The answer to this question potentially lies in the fact that at high Ni concentrations mutations in *WHI2* are only beneficial in the background of mutations in *VTC4*, which themselves provide a large benefit. When the frequency of *VTC4* mutants in the population is low, few *WHI2* mutations will occur in genotypes also carrying *VTC4* mutations, but when they do, they will rise to appreciable frequencies relatively quickly. By contrast, when the frequency of *VTC4* mutants in the population is high, many double mutants will occur, but their selective advantage relative to the rest of the population will be small, so that they will need many transfers to rise in frequency. The combination of these effects may have led to the apparently simultaneous invasion of multiple double mutants as *VTC4* was spreading through the population, followed by a period with only limited further increase in frequency once *VTC4* (and other mutants with comparable fitness) came to dominate the population.

Epistatic interactions may indicate functional relationships

Our fitness landscape analyses revealed substantial epistasis among the mutations that contributed to adaptation under our selective regimes. These interactions are not only interesting for their evolutionary consequences, they also provide valuable information about

the functional relationship between the genes involved (Segre et al. 2005; Collins et al. 2007; Costanzo et al. 2010). Specifically, synergistic epistasis refers to the case where the combined effect of mutations is more extreme than expected from their individual effects. This implies that when mutations have a positive effect, their joint benefit is larger than expected, whereas when mutations have a negative effect, their joint detrimental effect is larger than expected. For loss-of-function mutants, synergistic epistasis may indicate that mutations affect parallel pathways (Segre et al. 2005; de Visser et al. 2011): if loss of function of a given pathway increases (decreases) fitness, then additional loss of function of a parallel "back-up" pathway may completely abolish the involved function, thus further increasing (decreasing) fitness beyond what is expected from loss of function of each of the individual pathways.

We observed two cases of positive synergistic epistasis: the *Δsmf1Δfet4* and *Δvtc4Δwhi2* double mutants both have a higher fitness than expected from the single mutant fitnesses. Indeed, Smf1p and Fet4p function in parallel, because they are two of the main importers through which Cd can enter the cell, with a more central role for Smf1p (Wysocki and Tamas 2010). For the *VTC4-WHI2* pair it is harder to explain the epistatic effects that we found. Vtc4p is part of the vacuolar transporter chaperone complex that is involved in the sorting of V-type ATPases, which drive Ni transport to the vacuole via the generation of a proton gradient (Nishimura et al. 1998; Muller et al. 2003; Luo et al. 2016). Conversely, Whi2p is required for full activation of the general stress response (Kaida et al. 2002), a seemingly unrelated function. One way in which these two genes may interact is that mutations in *VTC4* first act to reduce the toxic effects of Ni exposure. Then, mutations in *WHI2* can be selected because they restore the unstressed physiological state of the cell — such adaptations are frequently among the first to be selected in laboratory evolution experiments (Philippe et al. 2007; Harrison et al. 2015; Rodriguez-Verdugo et al. 2016). For the *Δubp6Δubp3* double mutant we also expected to find synergistic epistasis, but we found no evidence for this. In fact, a previous study reported antagonistic epistasis — where the combined effect of mutations is less extreme than expected from their individual effects, potentially because the mutations are in the same pathway — between these mutations, although this was based on their individually deleterious effect in the absence of metal (Collins et al. 2007). Consistent with such antagonistic epistasis, Ubp6p and Ubp3p both act to deubiquitinate proteins targeted for degradation, with a more central, downstream role for the proteasome-associated Ubp6p (Finley et al. 2012; Isasa et al. 2015). Taken together, this information reinforces our conclusion that the *UBP3* deletion mutant probably does not accurately reflect the effect of the — missense — mutations that we found in this gene in our evolution experiment.

A final prominent case of epistasis in our study was the antagonistic (diminishing returns) epistasis between *Δvtc4*, *Δbul1*, and *Δvip1*. Interestingly, *Δvtc4* was previously found to interact synergistically with both *Δbul1* and *Δvip1* to decrease fitness in the absence of metal beyond what was expected from their individual deleterious effects (Costanzo et al. 2010), suggesting that these genes function in related but not identical pathways. One way to

explain this discrepancy with our results is that these three mutants interact synergistically with respect to both Ni tolerance and growth reduction. However, at the Ni concentrations in our evolution experiments, one mutation is sufficient for increasing Ni tolerance, so that the combined effect on growth causes the observed antagonism with respect to fitness. In line with this, at the highest Ni concentration that we used for our assays (but that we left out of our further analyses for reasons outlined in the Results) we found that the triple mutant represented the global optimum in the Ni landscape. All in all, our results suggest that the nature of epistatic interactions can have very different causes, making it hard to explain such interactions from a single mechanistic principle applied at one level of organisation.

Conclusions

Our reconstruction mutants are a biased sample of the local fitness landscape because they contain only mutations with a positive effect in at least one genetic background at one metal concentration (Blanquart and Bataillon 2016; du Plessis et al. 2016). However, these mutations are among the ones that presumably provide the largest benefit in the relevant environments. As such, they likely play a dominant role in adaptation to these environments, which is what we aimed to predict here. While we generated landscapes for the evolution of tolerance to just three metals, our results are potentially relevant for a much broader range of selection pressures and regimes of environmental change. Specifically, some selection pressures may resemble Cd in that they select for the same evolutionary "solutions" at all intensities, while others may resemble Ni and Zn in that they select for different "solutions" at different intensities. For both types of selection pressure, we would predict the same qualitative consequences of directional environmental change that we observed here. More detailed knowledge of the nature of a given selection pressure is thus crucial for predicting the consequences of different scenarios of environmental change for evolutionary dynamics and outcomes.

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Appendix: supplemental tables

Table S4.1: Frequency of mutated alleles at different time points (in transfers) per evolved line and gene. Underlined italic alleles are the same as those in the previously sequenced clones from T=75.

Line	Gene	Time	Region	Type	Reference	Allele	Frequency	Coding region change	Amino acid change
NI1	VIP1	75	606	SNV	G	T	1.39	606G>T	Lys202Asn
NI1	VIP1	75	932	Deletion	G	-	19.04	932delG	Ala312fs
NI1	VIP1	75	1363	SNV	G	T	50.78	1363G>T	Glu455*
NI2	WHI2	75	279	SNV	C	G	2.99	279C>G	Tyr93*
NI3	VIP1	75	1078	SNV	A	T	3.43	1078A>T	Ile360Phe
NI3	VIP1	75	1465	SNV	A	C	72.99	1465A>C	Asn489His
NI3	VTC4	75	1276	SNV	G	A	1.03	1276G>A	Glu426Lys
NI4	BUL1	75	1385	SNV	T	G	1.86	1385T>G	Val462Gly
NI4	VTC4	75	758	SNV	G	T	1.9	758G>T	Arg253Ile
NI5	BUL1	75	403	SNV	G	T	1.04	403G>T	Glu135*
NI5	BUL1	75	1160	SNV	C	T	1.84	1160C>T	Pro387Leu
NI5	VIP1	75	1304	SNV	G	A	27.7	1304G>A	Gly435Asp
NI5	VIP1	75	326^327	Insertion	-	A	3.61	326_327insA	Ala112fs
NI5	WHI2	25	541	SNV	C	T	11.97	541C>T	Gln181*
NI6	WHI2	25	178..179	Deletion	TC	-	1.61	178_179delTC	Leu61fs
NI6	WHI2	75	178..179	Deletion	TC	-	1.08	178_179delTC	Leu61fs
NH1	BUL1	25	646	SNV	C	T	85.67	646C>T	Gln216*
NH1	BUL1	50	646	SNV	C	T	76.08	646C>T	Gln216*
NH1	BUL1	75	646	SNV	C	T	90.67	646C>T	Gln216*
NH1	BUL1	25	1017	SNV	G	T	4.1	1017G>T	Trp339Cys
NH1	BUL1	50	1017	SNV	G	T	4.85	1017G>T	Trp339Cys
NH1	BUL1	75	1017	SNV	G	T	3.21	1017G>T	Trp339Cys
NH1	VTC4	25	1631	Deletion	C	-	5.35	1631delC	Thr546fs
NH1	VTC4	50	1631	Deletion	C	-	1.16	1631delC	Thr546fs
NH2	BUL1	25	226	SNV	G	T	74.91	226G>T	Gly76*
NH2	BUL1	50	226	SNV	G	T	70.35	226G>T	Gly76*
NH2	BUL1	75	226	SNV	G	T	61.08	226G>T	Gly76*
NH2	BUL1	25	1765	SNV	G	T	23.61	1765G>T	Gly589*
NH2	BUL1	50	1765	SNV	G	T	27.95	1765G>T	Gly589*
NH2	BUL1	75	1765	SNV	G	T	31.23	1765G>T	Gly589*
NH3	BUL1	25	454	SNV	G	C	1.09	454G>C	Asp152His
NH3	BUL1	50	454	SNV	G	C	12.05	454G>C	Asp152His

Line	Gene	Time	Region	Type	Reference	Allele	Frequency	Coding region change	Amino acid change
NH3	BUL1	75	454	SNV	G	C	21.21	454G>C	Asp152His
NH3	BUL1	25	1204	SNV	C	T	84.98	1204C>T	His402Tyr
NH3	BUL1	50	1204	SNV	C	T	72.77	1204C>T	His402Tyr
NH3	BUL1	75	1204	SNV	C	T	47.11	1204C>T	His402Tyr
NH3	VTC4	50	5	SNV	A	C	11.43	5A>C	Lys2Thr
NH3	VTC4	75	5	SNV	A	C	27.17	5A>C	Lys2Thr
NH3	VTC4	25	662	SNV	T	G	6.64	662T>G	Val221Gly
NH3	VTC4	50	662	SNV	T	G	4.99	662T>G	Val221Gly
NH3	VTC4	75	662	SNV	T	G	24.21	662T>G	Val221Gly
NH3	WHI2	50	670	SNV	A	T	1.04	670A>T	Arg224*
NH3	WHI2	75	670	SNV	A	T	2.24	670A>T	Arg224*
NH3	WHI2	75	936	SNV	G	A	24.44	936G>A	Trp312*
NH4	VTC4	25	592	SNV	A	G	94.95	592A>G	Thr198Ala
NH4	VTC4	50	592	SNV	A	G	93.36	592A>G	Thr198Ala
NH4	VTC4	75	592	SNV	A	G	99.77	592A>G	Thr198Ala
NH4	WHI2	50	19	SNV	C	T	1.68	19C>T	Gln7*
NH4	WHI2	75	19	SNV	C	T	8.21	19C>T	Gln7*
NH4	WHI2	50	64	SNV	C	T	1.09	64C>T	Gln22*
NH4	WHI2	75	64	SNV	C	T	1.76	64C>T	Gln22*
NH4	WHI2	50	221	Deletion	T	-	1.17	221delT	Leu76fs
NH4	WHI2	50	283	SNV	G	T	1.43	283G>T	Val95Phe
NH4	WHI2	75	283	SNV	G	T	1.58	283G>T	Val95Phe
NH4	WHI2	50	414	Deletion	A	-	1.71	414delA	Phe140fs
NH4	WHI2	75	414	Deletion	A	-	1.76	414delA	Phe140fs
NH4	WHI2	50	440	SNV	C	G	1.38	440C>G	Ser147*
NH4	WHI2	75	440	SNV	C	G	1.06	440C>G	Ser147*
NH4	WHI2	50	519	SNV	T	A	2.46	519T>A	Tyr173*
NH4	WHI2	75	519	SNV	T	A	1.07	519T>A	Tyr173*
NH4	WHI2	50	703	SNV	G	T	5.25	703G>T	Glu235*
NH4	WHI2	75	703	SNV	G	T	6.24	703G>T	Glu235*
NH4	WHI2	75	745	Deletion	A	-	2.29	745delA	Lys250fs
NH4	WHI2	50	974	Deletion	C	-	3.31	974delC	Pro326fs
NH4	WHI2	75	974	Deletion	C	-	13.24	974delC	Pro326fs
NH4	WHI2	50	1171	SNV	A	T	15.54	1171A>T	Lys391*
NH4	WHI2	75	1171	SNV	A	T	32.43	1171A>T	Lys391*
NH4	WHI2	50	1248	Deletion	A	-	2.35	1248delA	Lys417fs

Line	Gene	Time	Region	Type	Reference	Allele	Frequency	Coding region change	Amino acid change
NH4	WHI2	75	1248	Deletion	A	-	17.28	1248delA	Lys417fs
NH4	WHI2	50	1330	SNV	A	T	3.44	1330A>T	Lys444*
NH4	WHI2	75	1330	SNV	A	T	2.08	1330A>T	Lys444*
NH4	WHI2	75	276^277	Insertion	-	T	1.02	276_277insT	Tyr93fs
NH4	WHI2	75	278..280	MNV	ACA	TTT	1.09	278_280 delACA insTTT	Tyr93_Ile94 delins PhePhe
NH5	BUL1	25	2349	SNV	T	G	7.47	2349T>G	Tyr783*
NH5	BUL1	50	2349	SNV	T	G	2.8	2349T>G	Tyr783*
NH5	BUL1	25	2381	SNV	T	G	4.82	2381T>G	Leu794*
NH5	BUL1	50	2381	SNV	T	G	5.3	2381T>G	Leu794*
NH5	BUL1	25	2755	SNV	C	T	60.43	2755C>T	Arg919*
NH5	BUL1	50	2755	SNV	C	T	48.54	2755C>T	Arg919*
NH5	BUL1	75	2755	SNV	C	T	16.72	2755C>T	Arg919*
NH5	VTC4	25	189	SNV	C	A	12.57	189C>A	Cys63*
NH5	VTC4	50	189	SNV	C	A	15.25	189C>A	Cys63*
NH5	VTC4	75	189	SNV	C	A	2.93	189C>A	Cys63*
NH5	VTC4	25	875	SNV	C	T	5.16	875C>T	Ser292Phe
NH5	VTC4	50	875	SNV	C	T	14	875C>T	Ser292Phe
NH5	VTC4	75	875	SNV	C	T	2.12	875C>T	Ser292Phe
NH5	VTC4	25	1027	SNV	C	T	5.2	1027C>T	Gln343*
NH5	VTC4	50	1027	SNV	C	T	3.13	1027C>T	Gln343*
NH6	BUL1	25	2118	Deletion	A	-	31.88	2118delA	Lys708fs
NH6	BUL1	50	2118	Deletion	A	-	12.02	2118delA	Lys708fs
NH6	VTC4	25	1117	SNV	C	T	54.16	1117C>T	Arg373Cys
NH6	VTC4	50	1117	SNV	C	T	71.94	1117C>T	Arg373Cys
NH6	VTC4	75	1117	SNV	C	T	99.95	1117C>T	Arg373Cys
NH6	VTC4	25	1303	SNV	C	T	1.42	1303C>T	Gln435*
NH6	WHI2	50	139	SNV	C	T	43.42	139C>T	Gln47*
NH6	WHI2	75	139	SNV	C	T	94.09	139C>T	Gln47*
NH6	WHI2	50	457	SNV	G	T	1.32	457G>T	Glu153*
NH6	WHI2	50	502	SNV	G	A	1.45	502G>A	Glu168Lys
NH6	WHI2	50	936	SNV	G	A	2.42	936G>A	Trp312*
NH6	WHI2	50	1271	SNV	G	A	2.61	1271G>A	Trp424*
NH6	WHI2	50	770^771	Insertion	-	T	10.22	770_771insT	Leu258fs
NH6	WHI2	75	770^771	Insertion	-	T	1.49	770_771insT	Leu258fs

Table S4.2: Relationship between metal reductive effect in the original (old) assays vs. in the current (new) assays.

$CdCl_2$	$[Cd_{old}] = 0.4485167\ [Cd_{new}]$	$[Cd_{new}] = 2.229571\ [Cd_{old}]$
$NiCl_2$	$[Ni_{old}] = 1.925134\ [Ni_{new}]$	$[Ni_{new}] = 0.5194444\ [Ni_{old}]$
$ZnCl_2$	$[Zn_{old}] = 1.192971\ [Zn_{new}]$	$[Zn_{new}] = 0.8382432\ [Zn_{old}]$

Chapter 5

General discussion

Directional environmental change in the form of global climate change and human-induced pollution is one of the most pressing problems facing society today (IPCC 2014; United Nations 2016; WHO 2016). While species can sometimes adapt to such change by means of phenotypic plasticity and range shifts (Przybylo et al. 2000; Charmantier et al. 2008; Chen et al. 2011; Merilä and Hendry 2014), there is considerable concern that these mechanisms are insufficient for long-term population persistence for at least some species. Evolutionary adaptation could potentially offer a solution, but it is unclear what the potential and limitations of this process are for populations exposed to deteriorating conditions. More specifically, we have limited knowledge about the factors promoting evolutionary population rescue (Gonzalez et al. 2013; Alexander et al. 2014; Carlson et al. 2014), and we know even less about the way in which directional change affects evolutionary dynamics and outcomes (Collins 2011).

In this thesis, I investigated how the rate of directional environmental change affects the course and outcome of evolution using experimental evolution of Cd, Ni, and Zn tolerance in the baker's yeast *S. cerevisiae* as a model system. To this end, I cultured replicate populations of yeast in the presence of either gradually increasing or constant high concentrations of these metals for 500 generations, and determined the effect of each treatment on evolution at the fitness (**Chapter 2**) and genomic (**Chapter 3**) level. Additionally, I generated several mutants based on genomic information from Chapter 3, and determined their fitness at different metal concentrations (**Chapter 4**). I used these data to address a number of hypotheses, which were based on a genotype-by-environment (GxE) framework that I developed to aid my thinking about evolution in changing environments. This framework is based on the concept of the fitness landscape, and distinguishes between two general patterns of change in landscape topography across conditions. Magnitude GxE refers to the case where the *height* of the fitness landscape increases along an environmental gradient, while the shape of the landscape remains roughly constant. This scenario predicts that evolution will use the same mutations for adaptation under gradual and abrupt change, but that adaptation will be delayed under gradual change. By contrast, reranking GxE refers to the case where the *shape* of the fitness landscape changes along an environmental gradient. This scenario predicts that evolution will use different mutations for adaptation under gradual and abrupt change, and similarly predicts that adaptation will be delayed under gradual change. However, it additionally predicts different evolutionary outcomes following gradual and abrupt change, and lower repeatability of evolution under gradual change. I anticipated that the magnitude GxE scenario would apply to evolution in the presence of the non-essential metal Cd, because in that case the same evolutionary solutions should be favoured at all external metal concentrations. By contrast, I anticipated that the reranking GxE scenario would apply to evolution in the presence of the essential metals Ni and Zn, because in that case different evolutionary solutions should be favoured at different external metal concentrations.

My results provide support for these hypotheses at several different levels and show that:

i) the *height*, but not the shape, of the fitness landscape changes across Cd concentrations (**Chapter 4**), ii) the *shape* of the fitness landscape changes across Ni concentrations (**Chapter 4**), iii) mutations in the same genes are used for adaptation to different rates of environmental change for Cd, but not Ni and Zn (**Chapter 3**), iv) evolution is less repeatable under gradual change for Ni and Zn (**Chapter 3**), and v) increase in fitness is delayed under gradual change, but final fitness is the same following both regimes of change (**Chapter 2**). This multi-level evidence makes a strong case for the usefulness of my GxE framework for understanding evolution in changing environments, and shows that the rate of directional environmental change and the nature of the selection pressure have crucial consequences for evolutionary dynamics and outcomes.

Dynamics in my evolution experiments

While the approach that I took to understanding evolutionary dynamics in my long-term selection experiment was quite extensive, I still had to make a number of assumptions and inferences based on a limited data set. I sequenced single clones from the final time point of the experiment (**Chapter 3**), and performed deep sequencing on a handful of genes at a selection of extra time points (**Chapter 4**). Consequently, I will no doubt have missed many variants that — transiently — contributed to adaptation, while the allele frequency dynamics that I inferred lack temporal resolution. Several recent studies have sequenced whole population samples isolated from long-term laboratory evolution experiments at multiple time points, and such studies frequently uncover complex dynamics of adaptation that could not have been predicted from phenotypic data or genomic information on single evolved clones (Kvitek and Sherlock 2013; Lang et al. 2013; Maddamsetti et al. 2015; Voordeckers et al. 2015; McDonald et al. 2016). It therefore seems likely that I have also missed some of the more subtle dynamics in my experiments. It would thus be interesting to obtain more sequencing data from my experiment for whole populations at multiple time points. This would be particularly interesting for the populations evolved under gradually increasing concentrations of Ni and Zn, because it seems likely that different mutations were selected for at lower concentrations of these metals. Such information would also be useful for creating fitness landscapes that are less biased towards mutations that increase fitness at the highest metal concentrations: the fitness landscapes analysed in **Chapter 4** are based on mutations in clones from the final time point, and at that time all lines were exposed to high metal concentrations. More extensive and/or unbiased landscapes involving mutations from earlier stages of adaptation would allow for a more thorough testing of my hypotheses about the topography of the fitness landscape, and I expect that it will provide additional evidence for reranking GxE in the case of Ni and Zn.

An interesting question that I did not directly address in this thesis is how different rates of directional environmental change affect within-population diversity, and how this diversity

changes over time (Rainey et al. 2000; Kassen 2014). Based on my GxE framework, I would predict that gradual change leads to higher within-population diversity for Ni and Zn, because in these cases different mutants were selected at different times over the course of the evolutionary process. Because the concentration changed relatively rapidly in the gradual treatments, environments likely changed before selective sweeps were completed. The next environment would then have selected for other mutants that now had a higher fitness, while mutants from the previous environment would have decreased in frequency only gradually due to genetic correlation between concentrations (or increased in frequency less rapidly than the now fittest mutants; Samani and Bell, 2010). Once such increased diversity would have been established, it should have been maintained for as long as the environment kept changing at similar rates. In that respect, it would be particularly interesting to see how the 25 additional transfers at constant high metal concentrations following the main evolution experiment (**Chapter 2**) affected within-population diversity: if my hypotheses were correct, diversity should have decreased during this time for the populations evolved under gradual change. Testing these predictions would be most straightforward with more extensive whole-genome sequencing data. However, determining relative fitness of several clones from each population might be a reasonably accurate, less costly, alternative, although in that case it will not be possible to distinguish between different mutants with a similar fitness (Habets et al. 2006).

Other scenarios of change

In this thesis, I tried to gain more insight into the principles according to which the fitness landscape changes across conditions, as well as the consequences that this has for evolution. For the scenarios of change that I investigated, my results match my hypotheses remarkably well. However, it is unclear how good my framework is at predicting evolution under other scenarios of change. Ideally, I would like to obtain improved fitness estimates for more mutants at more concentrations to paint a clearer picture of the way in which the topography of the local fitness landscape changes across metal concentrations. One first step in this respect would be to construct individual point/frameshift mutants, because I found that the deletion mutants that I used did not always accurately reflect the effect of the mutations found in my evolution experiment (**Chapter 4**). Better characterized fitness landscapes would allow me to simulate evolution under different scenarios of change, such as faster or slower increases in concentration, or fluctuation conditions. Predictions from such simulations could be tested empirically with evolution experiments. Another related interesting research avenue would be to generate fitness landscapes *in silico* with different contributions of reranking GxE (for example by multiplying fitness values from a simulated fitness landscape with factors taken from a distribution with higher (= more reranking GxE) or lower (= less reranking GxE) variance), and to investigate theoretically how this property impacts evolution. Such

approaches could reveal interesting patterns that are not immediately obvious from qualitative reasoning, and these patterns could then similarly be evaluated using empirical data.

A related question is to what extent my results can be extrapolated to other selection pressures. Are there any underlying physical or biological principles that may help us predict how the landscape topography changes across environments? I found that the effect of different rates of change was fundamentally different for the non-essential metal Cd on the one hand, and the essential metals Ni and Zn on the other hand. The crucial distinction between these elements is that Cd is toxic at all concentrations, whereas Ni and Zn are needed in small amounts (Gadd 1993; Wysocki and Tamas 2010; Bleackley and MacGillivray 2011). In the first case I expect directional selection with increasing intensity, and in the second case I expect stabilizing selection with a moving optimum (Kopp and Matuszewski 2014). For other abiotic factors, this general distinction likely also exists. Specifically, I would predict that toxins and radiation lead to patterns that resemble those for Cd, while nutrients (very high concentrations are often toxic), temperature, light, pressure, and disturbance frequency (for example via wind or currents) lead to patterns that resemble those for Ni and Zn. Testing these predictions requires extensive research effort. However, a first approach might be to, for a certain selection pressure, pick several beneficial mutants and determine the distribution of their fitness effects (DFE) at different intensities of that selection pressure (Eyre-Walker and Keightley 2007; MacLean and Buckling 2009; Kraemer et al. 2016). This should reveal the relative importance of magnitude GxE (= stretching of the DFE tail) and reranking GxE (= reranking of the mutants in the DFE tail across environments). Picking the mutants at different intensities of the selection pressure should reduce the bias towards mutations that increase fitness at a given intensity only, and may additionally provide information about the beneficial mutation rate and spectra at different intensities of the selection pressure (Shor et al. 2013; Zhu et al. 2014). In practical terms, beneficial mutations might be identified, for example, from increased yield in a millifluidics set-up (Cottinet et al. 2016).

It is relatively straightforward to make predictions about the applicability of my framework to other abiotic selection pressures. However, making such predictions for biotic selection pressures is more complicated. What happens to predators if the composition of a prey population shifts over time? How do the dynamics of parasite host range shifts depend on the rate at which the composition of the host population changes? What if a new competing species gradually invades the niche of a resident species? Under such scenarios, evolutionary dynamics are potentially more complicated than under abiotic scenarios of change. Nonetheless, there may be some general underlying principles. For example, I would predict that evolutionary outcomes depend significantly on the strength of the interaction, as well as the number of individuals of the other species that an individual encounters during its life: if this number is high, such as for a predator that consumes many prey over the course of its lifetime, a shift in population composition may have effects comparable to abiotic directional change under a reranking GxE scenario, because prey is also a nutrient. However,

if the number of individuals encountered by an individual over its lifetime is low, such as for a parasite that can infect just a single host, the perceived environment will be patchy — and thus fluctuating — with changes in relative patch size over time, rather than directional in the sense that I explored. Things are likely to be even more complicated when species coevolve. When this happens, it may depend on the nature of the coevolutionary process whether a magnitude or reranking GxE scenario applies. Specifically, an escalating arms race scenario, in which both coevolutionary partners acquire more mutations that increase their performance (e.g. resistance and infectivity) over time (Flor 1956; Sasaki 2000), bears a clear resemblance to the magnitude GxE scenario, because in that case, the same genotypes (i.e. the ones with the highest number of resistance/infectivity mutations) will always do best irrespective of the genetic makeup of the partner species. By contrast, a fluctuating selection scenario, in which rare genotypes have an advantage (Agrawal and Lively 2002), implies that different mutations are selected over time, and is thus more similar to the reranking GxE scenario. Arms race dynamics may be more important upon newly formed interactions (or interactions that have been taken out of their original context), whereas fluctuating selection dynamics may be more important in the case of more firmly established interactions (Gomez and Buckling 2011; Hall et al. 2011a; Gomez et al. 2015).

Genetics of adaptation

Whether the same or different mutations are selected in replicate experiments and at different intensities of the same selection pressure depends not only on environmental conditions, but also on the genetic architecture of the trait(s) under selection. For example, many different genes are involved in metal homeostasis (Jin et al. 2008; Ruotolo et al. 2008; Arita et al. 2009), and as such there are numerous ways in which a cell can potentially adapt to changes in external metal concentration. Mutations with subtly different effects on metal homeostasis may be favored most strongly at concentrations that are only slightly different, which increases the scope for reranking GxE. By contrast, for some other selection pressures, the mutational target size may be considerably smaller, or the DFE across environments may be such that one or a few mutations consistently drive adaptation. For example, a recently published study reported that various different mutations contributed to adaptation in replicate yeast populations growing under glucose or phosphate limitation, while a single mutation (amplification of a sulphate transporter) repeatedly drove adaptation to sulphate limitation (Payen et al. 2016). Interestingly, a considerable part of the specific mutations used for adaptation in these experiments could be predicted from the DFEs as determined in the presence of each selection pressure. This type of analysis alludes to an intriguing direction for future evolutionary studies: evolutionary systems biology. This emerging discipline (Papp et al. 2011; Soyer and O'Malley 2013) asks a fascinating question: if we had a complete

picture of a biological system such as a cell or a microbial community, and the way in which different mutations alter this system, could we then predict evolution? Approaches to addressing this question might still be unfeasible in most cases. However, ever larger datasets are being generated using high-throughput phenotyping and whole genome sequencing, and computational and modeling approaches are increasingly successful at combining such data across multiple organizational levels. For example, a recent model that integrated flux balance analysis with eco-evolutionary dynamics correctly predicted the evolution of cross-feeding in the *E. coli* long-term evolution experiment (Grosskopf et al. 2016).

In **Chapter 1**, I listed the key players involved in metal tolerance for Cd and Zn, and mentioned that we have only limited information about the genes involved in Ni tolerance. Interestingly, for Cd I repeatedly found loss-of-function mutations in two of the four importers for this metal (Smf1p and Fet4p; **Chapter 3**). These transporters are used mainly for manganese and iron, while the other two transporters are used mainly for Zn and calcium (Ca). Cd displaces Zn and Ca from metalloproteins and zinc finger proteins (Wysocki and Tamas 2010), which may explain why I found mutations in *SMF1* and *FET4* rather than in the other two genes: such mutations would likely have further offset the internal Cd to Zn/Ca ratio, and thus increased displacement of Zn and Ca by Cd. Why I did not find mutations in or close to any of the other key players involved in tolerance (Figure 1.3) is not immediately clear. However, one reason may be that such mutations would have had to confer a gain-of-function in order to increase metal tolerance, for example via gene amplification or changes in the promoter sequence. Loss-of-function mutations in negative regulators of these genes could in theory confer a similar benefit, but such mutations may have more severe pleiotropic effects, because they also interact with a large number of other proteins. In general, loss-of-function mutations occur at higher frequencies than gain-of-function mutations (Lind et al. 2015), and are thus more likely to contribute to adaptation.

For Zn, I did not find any mutations in the key players listed in **Chapter 1**. This can potentially be explained by the essential nature of this metal: loss-of-function mutations in for example the metal importers Zrt1p and Zrt2p would probably have resulted in Zn deficiency. The mutations that I found under the high Zn treatment — loss-of-function mutations in deubiquitinating enzymes — instead seem to deal with the toxic effects of Zn excess by enhancing protein degradation rather than with the element itself (**Chapter 3**; Finley et al. 2012; Isasa et al. 2015). Interestingly, under gradually increasing Zn concentrations two replicate lines acquired heterozygous mutations in the plasma membrane proton pump Pma1p. Deletion of this gene is lethal, but a more subtly impaired function provides tolerance to a wide range of chemicals because it decreases the proton-motive force across the plasma membrane that is required for their uptake (Cyert and Philpott 2013).

For Ni, I had no clear *a priori* expectations about the mutations used for tolerance evolution, but from the functions of the genes that were mutated repeatedly in response to this treatment it appears that what applies to Zn also applies to Ni: I found no mutations in (promoters of)

transporter genes or chelating molecules, but instead found mutations that may have helped to mitigate the toxic effect of excess Ni (**Chapter 3**). This fits in well with the fact that Ni is also an essential metal.

For most of the strains from my evolution experiments I have at least some idea about how their mutations might contribute to metal tolerance. However, to confirm these hypotheses, I would have to measure metal content of evolved strains, and/or determine (free) metal content of the medium in which these strains were cultured. This could be done using atomic absorption spectroscopy and bioassays (Codina et al. 1993; Bánfalvi 2011), respectively. For the *SMF1* and *FET4* mutants, I would expect to find that internal Cd concentrations are significantly reduced. Conversely, for strains in which the *GEX1* gene is present at a higher copy number (Chapter 3), I would expect that the free Cd concentration in the medium is reduced, because such a mutation is predicted to increase gluthatione export and thus metal chelation in the external environment (Dhaoui et al. 2011). One of the potentially most interesting mutants that I found is the *VTC4-WHI2* double mutant (**Chapter 3**). *Δvtc4* increases Ni tolerance (Bishop et al. 2007; Ruotolo et al. 2008), whereas *Δwhi2* increases Ni accumulation (Yu et al. 2012). This raises the possibility that the double mutant is comparable to the metal hyperaccumulators described in **Chapter 1**, which also combine increased tolerance with increased accumulation. For the other mutants, I lack a clear functional understanding. Nonetheless, it would be interesting to measure metal content for each of them to gain additional insight into the general mechanisms via which they increase tolerance. In all cases, it would be important to quantify the effect on — internal or external — metal concentration under the conditions used in my evolution experiments: if a mutation reduces metal concentration only slightly under such conditions, it is unlikely that this mutation was selected for its effect on metal concentration.

Metal hyperaccumulation

One of the original aims of this thesis was to assess under which conditions metal hyperaccumulation can evolve. As explained in **Chapter 1**, this trait cannot evolve in yeast in the same sense as in plants, because yeast does not possess differentiated structures where metals can accumulate. However, a single-cell equivalent could potentially evolve, and the *VTC4-WHI2* double mutants that I found are candidate hyperaccumulators. I probably did not find (more) hyperaccumulators because I performed my evolution experiments in the absence of grazers or spatial structure, while these are the main factors predicted to drive the evolution of hyperaccumulation (**Chapter 1**). In a pilot I tested a set-up for a selection experiment on agar plates with *D. melanogaster* larvae as grazers (Bahadorani and Hilliker 2009). However, in doing so I encountered several difficulties, the most important of which was my inability to reliably reduce yeast numbers via grazing. An additional problem was that the larvae appeared

unable to distinguish between hyperaccumulating and non-hyperaccumulating yeast. Specifically, I used a strain in which the *CKA1* gene was deleted — this gene encodes a negative regulator of the vacuolar Cd transporter Ycf1p (Paumi et al. 2008) — as a hyperaccumulator, and made patches of this strain and a non-hyperaccumulating control strain (pre-grown in the presence versus absence of Cd) on agar plates. I then let *Drosophila* larvae graze the yeast from the plates and determined the relative reduction that this caused for each strain (Figure 5.1). The *Δcka1* strain was more tolerant to Cd, but there was no difference in reduction from grazing between the strains. Moreover, experiments in which I mixed yeast with a broad range of Cd concentrations showed that larvae were only deterred by extremely high Cd concentrations (in the millimolar range, which is well above the highest concentrations tolerated by yeast). In these experiments grazing was in fact beneficial for the non-accumulating strain, probably because it dispersed this strain to uncolonized patches of agar (Coluccio et al. 2008). I obtained similar but inconsistent results for Ni and Zn. All in all, these results suggest that *Drosophila* larvae may not be sensitive enough to the types and concentrations of metals that I used to select for hyperaccumulating strains. In addition, the system that I developed was very hard to control experimentally, implying that it is unsuitable for testing the types of hypotheses that I was interested in. Possibly, other yeast-consuming organisms such as *Drosophila* adults, *Caenorhabditis elegans*, rotifers or ciliates such as *Paramecium* and *Tetrahymena* are more appropriate grazers for testing my hypotheses (Tamaru et al. 1993; Joubert et al. 2006; Felix and Braendle 2010; Lewis and Collins 2010).

My second hypothesis about the evolution of metal hyperaccumulation in yeast was that it is a social trait because it reduces external metal concentrations (**Chapter 1**). If this would be the case, hyperaccumulation might lead to phenomena such as density- and frequency

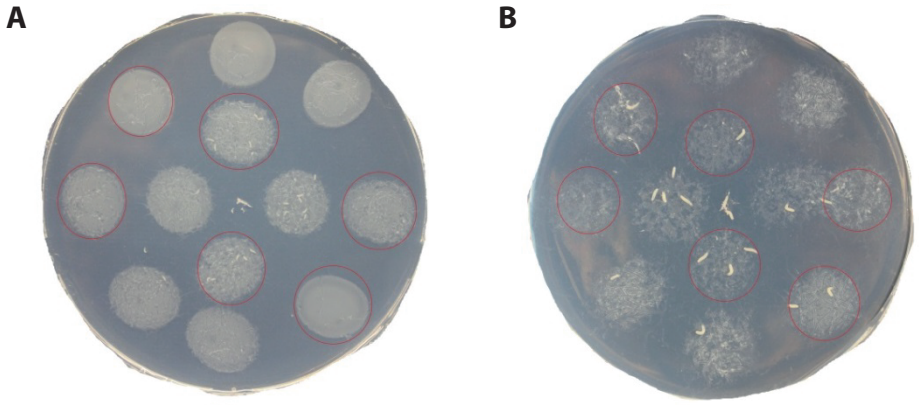

Figure 5.1: Agar plates with patches of non-hyperaccumulating yeast (without Cd) and hyperaccumulating yeast (with Cd, red circles) and grazing *D. melanogaster* larvae after 24h (left) and 48h (right). Photos taken by Sebastian Müller.

dependence, depending on the exact experimental conditions. Density dependence can occur when each hyperaccumulating cell reduces metal concentration by a given amount, and many cells together do so more efficiently, thus increasing population growth rate (Duxbury and Mcintyre 1989; van der Heide et al. 2010). Frequency dependence can occur when only part of the population hyperaccumulates metal and rare types outcompete common types or *vice versa* (Ellis et al. 2007; O'Brien et al. 2014; O'Brien and Buckling 2015). I used the *Δcka1* strain to test this, but did not find any evidence for either phenomenon. There are several possible explanations for this finding. First, it could be that the strain that I used is not a hyperaccumulator. Second, it could be that hyperaccumulation is not a social trait. Third, it could be that the conditions that I used did not allow for observing density- or frequency dependence. To address these hypotheses, and investigate more thoroughly whether there are any circumstances under which metal hyperaccumulation in yeast can be regarded as a social trait, these pilot studies should be repeated with different strains under conditions that maximize the possibility of observing a benefit of hyperaccumulation for other cells. A particularly useful set-up for this might be one where potential hyperaccumulators are grown at very high densities, and then removed before a non-hyperaccumulating strain is grown in the same environment (Romano and Kolter 2005). The rationale behind this set-up is, that each hyperaccumulating cell will reduce metal concentration by a given amount, and growing many cells in a small area thus maximizes the reduction of local metal concentration. By removing the hyperaccumulators, and replenishing nutrients, the beneficial effect of metal hyperaccumulation can then be separated from the effect of competition between strains. If I would observe that hyperaccumulation confers a non-excludable benefit (i.e. a benefit that others can also make use of) because it enhances growth of the non-hyperaccumulator strain, this would be a useful starting point for further investigations into the conditions under which hyperaccumulation can evolve. Importantly, a non-excludable benefit by itself is insufficient for the trait to evolve , and several other criteria have to be met (Gore et al. 2009; Vega and Gore 2014). For example, as a first requirement, the cost of hyperaccumulation needs to be outweighed at least by the benefit of the trait when yeast is growing at high densities.

Strengths and weaknesses of the model system

I used the baker's yeast *S. cerevisiae* for my experiments because it has all of the advantages of other microorganisms (e.g. large population sizes, short generation times, easy handling and cryogenic storage), but is also a eukaryote and thus in many respects more similar to higher organisms such as plants and animals. In my experiments I encountered several problems (or interesting phenomena, depending on the perspective) that were more or less specific to yeast. First, the main problem that I came across was that fitness assays were sometimes irreproducible, particularly between batches of media. This problem is not specific to yeast,

and might have been found with any organism cultured in undefined nutrient media (O'Keefe et al. 2006; Corbacho et al. 2011). It is possible that the consequences of variation in medium composition were particularly pronounced in my experimental set-up because metals have steep reaction norms (Joho et al. 1988; Gadd 1993), such that small changes in effective concentration have far-reaching consequences for fitness. The main lesson from these issues is that microbial experiments that involve quantitative measurements should be performed under defined conditions wherever possible. An additional explanation for the irreproducibility of my results may be the error introduced by the set-up of my competition assays, in which relative fitness was determined by plating competing strains on selective media. A solution to this might be to use fluorescent markers in combination with a FACS set-up instead, because this method introduces less sampling error.

The second problem that I encountered was the high frequency of petite colonies in the majority of the lines evolved in the presence of Cd after they had been frozen and thawed several times. This problem is more specific to yeast, because petite formation is a sign of mitochondrial malfunction, and obligate aerobes with such mutations are not viable (interestingly, however, there are clear similarities between petite metabolism and the Warburg effect, which is also characterised by an increase in aerobic glycolysis, and is a hallmark of human cancer cells (Diaz-Ruiz et al. 2011)). In my case, the increased petite frequency was likely a pleiotropic effect of the loss-of-function mutations in the manganese transporter Smf1p: such mutations reduce internal Mn concentrations (Supek et al. 1996; Jensen et al. 2003), and Mn is an important anti-oxidant (Reddi et al. 2009). Freeze-thaw stress leads to the formation of reactive oxygen species (Park et al. 1998), which can damage mitochondria and/or select for respiratory deficient mutants (Davermann et al. 2002; Gibson et al. 2008). At lower anti-oxidant concentrations such as those caused by *SMF1* mutations, freezing and thawing can then dramatically increase petite frequencies.

A third problem that I encountered was the extensive polyploidisation and copy number variation in my evolved lines. Such changes are in fact quite common in yeast evolution experiments (Gerstein et al. 2006; Selmecki et al. 2015; Venkataram et al. 2016), but introduce unwanted "noise" because they make it harder to obtain a clear picture of evolutionary dynamics at the SNP level. Particularly complicating in this respect is my observation of extensive and sustained within-population variation in ploidy over the course of the experiment: FACS analyses on whole population samples from different time points of the lines evolved in the presence of Ni indicated that these populations consisted of a mixture of cells of different ploidy, ranging from haploid to tetraploid (Figure 5.2). This result is quite puzzling, and may indicate a problem with the methods that I used, for example with sample storage or -preparation. However, variation in ploidy generally increased with time spent evolving, suggesting that the result may not be an artifact. Possibly, genome size is even more dynamic in yeast than is often assumed. More and more evidence is accumulating that rapid changes in genome size are important in wild yeast strains (Chang et al. 2013;

		n	2n	4n
haploid control	1	**79**	15	1
	2	**77**	19	2
diploid control	1	8	**75**	14
	2	6	**78**	11

		T=25			T=50			T=75		
		n	2n	4n	n	2n	4n	n	2n	4n
increasing Ni	1	**50**	**40**	7	5	**68**	**22**	2	**50**	**42**
	2	**27**	**53**	14	4	**55**	**34**	4	**52**	**38**
	3	**48**	**43**	6	6	**70**	**20**	4	**55**	**35**
	4	**30**	**56**	10	**25**	**59**	9	10	**72**	14
	5	**45**	**43**	9	4	**70**	**22**	3	**61**	**31**
	6	**33**	**54**	10	**21**	**62**	13	3	**67**	**25**
high Ni	1	**68**	**26**	1	3	**55**	**36**	2	**45**	**43**
	2	**60**	**33**	3	7	**55**	**32**	7	**50**	**29**
	3	**65**	**30**	1	4	**49**	**40**	3	**54**	**37**
	4	**48**	**42**	5	**21**	**55**	**19**	7	**37**	**45**
	5	**59**	**35**	2	7	**55**	**32**	4	**52**	**33**
	6	**49**	**40**	7	**13**	**47**	**31**	4	**32**	**53**

Figure 5.2: FACS data for whole population samples of lines evolved in the presence of Ni. Numbers indicate the percentage of the total area from the FACS peak pattern within the haploid (n), diploid (2n), and tetraploid (4n) window, respectively. Haploid and diploid control strains have clear peaks in the expected ranges, but evolving populations are most likely mixtures of strains of different ploidy. Specifically, after 25 transfers, the populations appear to be mixtures of haploid and diploid strains, whereas after 50 and 75 transfers, the populations generally appear to be mixtures of diploid and tetraploid strains. These populations also frequently contain extra peaks beyond the tetraploid window. Bold indicates that a percentage is more than 3 standard deviations higher than the mean of that peak for the diploid, haploid, and diploid control strains, respectively, and serves to show that a population likely contains a significant subpopulation of a particular ploidy.

Storchova 2014; Berman 2016; Zhu et al. 2016). There is also strong evidence that *S. cerevisiae* underwent a whole genome duplication event followed by massive gene loss at some point in its evolutionary past (Kellis et al. 2004). Yet, the type of dynamics that I observed and that others also repeatedly find (Gerstein et al. 2008; Hong and Gresham 2014; Voordeckers et al. 2015) suggests that this event may not be so unique after all. Nonetheless, these dynamics make my results harder to interpret, because they imply that I may not be dealing with just a single duplication event in my evolved lines. However, it is interesting to note the parallels between these dynamics and those observed in cancer evolution, which are frequently driven by mutations in the *MSH2* gene of which the paralog is also involved in mismatch repair in yeast (Gorringe et al. 2005; Sprouffske et al. 2012; Burrell et al. 2013; Dewhurst et al. 2014; Gerlinger et al. 2014). This parallel also brings to mind that even if dynamic genomes have a higher fitness under stressful

conditions, they will often be unable to undergo successful meiosis, and as such can be considered evolutionary dead ends (Avelar et al. 2013).

Taken together, the problems described in the previous paragraphs point to some specifics of yeast that complicate the direct translation of my results to higher eukaryotes. In particular, large scale copy number variation and dynamic genome size are less likely to play an appreciable role in the —transgenerational — evolution of metal tolerance in higher organisms, because such organisms have to go through meiosis every single generation, and meiotic products of organisms with aberrant genome size are often inviable (Charles et al. 2010). Multicellular organisms are also generally less tolerant to aneuploidy, possibly because they require a tighter between-cell coordination for proper development (Boveri 1902, 1904; Torres et al. 2008), and have evolved mechanisms to keep fast-proliferating aneuploid "cheaters" in check (Aktipis et al. 2015). However, some of the genes in which I found mutations have homologs in higher eukaryotes. The most notable example of this is the metal transporter Smf1p, in which I repeatedly found loss-of-function mutations in lines evolved in the presence of Cd. I would expect that similar mutations in its plant and animal counterparts also increase metal tolerance. Other experimental evolution studies with yeast have repeatedly found mutations in highly conserved pathways such as the Ras/PKA and TOR/Sch9 pathway (Kvitek and Sherlock 2013; Hong and Gresham 2014; Venkataram et al. 2016; in fact I also found several mutations in the Ras/PKA pathway, but most of these genes were mutated only once), which are also present in animals (Abraham 2002; Stork and Schmitt 2002; Diaz-Ruiz et al. 2011). This implies that yeast evolution studies can indeed uncover mutations that are potentially broadly relevant.

Relevance of microbial experimental evolution

A common criticism of experimental evolution studies with microbes — and thus also of my study — is that their set-up is ecologically unrealistic, and that they therefore provide little information about how evolution proceeds in nature (Buckling et al. 2009). In most cases, microbial experimental evolution studies look at extremely large asexual populations (for example, in my case the effective population size was ~6.6 x 10^7) initiated from a single clone that are suddenly placed into a novel environment (Barrick and Lenski 2013). Under such circumstances, adaptation occurs from *de novo* mutation (but note that millions of new mutants arise on a daily basis), and — at least the initial — mutations that become fixed confer very large fitness benefits. Because such mutations are rare, evolution is often highly repeatable, particularly at the gene level. Large-effect mutations interact more strongly (Schenk et al. 2013), and, as such, epistatic interactions between initial mutations are very important for determining the course and outcome of evolution in microbial evolution experiments (Salverda et al. 2011). Accordingly, the essence of evolutionary dynamics in

these experiments is generally captured best by genotypic models based on the concept of the fitness landscape, which concern only a limited number of loci but precisely characterize the interactions between them (de Visser and Krug 2014). By contrast, evolution in natural populations is generally thought of as taking place in relatively small, sexually reproducing populations, where the process of adaptation is driven by selection on standing genetic variation (Alexander et al. 2014; Kopp and Matuszewski 2014). Such populations can adapt to changing conditions, but are limited by the genetic variation that is present and will go extinct when the environment changes too suddenly. Genetic variation is thought to be conferred mainly by many loci of small effect, between which epistatic interactions are of minor importance (Hill et al. 2008). Consequently, the process of adaptation under such circumstances is generally captured best by phenotypic models that view adaptation as a statistical process in which the mean population phenotype changes over time (Lynch and Lande 1993; Burger and Lynch 1995; Gomulkiewicz and Holt 1995).

The fundamental difference between these two scenarios raises an important question: to what extent can results from microbial evolution experiments be used for understanding adaptation in nature? I would argue that such experiments are in fact quite useful, for two main reasons. First, not all adaptation outside the laboratory takes place in small, sexually reproducing populations with standing genetic variation. Microbial populations can be very large and are often founded from a single clone, for example when microbes disperse to a different habitat or infect a novel host (Moxon and Murphy 1978; Diard et al. 2013; O'Rourke et al. 2015). Microorganisms cause ~30% of deaths world-wide (Levy et al. 2015) and play crucial roles in nutrient cycling and the biotechnological industry. Therefore, microbial evolution is highly relevant in itself.

Second, the distinction between the evolutionary scenarios that I sketched may be more related to differences in the ecological settings that are normally assumed under each scenario than to inherent differences between microorganisms and larger organisms. In one recent study, the authors evolved highly outcrossed yeast strains under standard laboratory conditions for ~500 generations, and found that selection merely changed allele frequencies of variants that were already present in the population, even if effective population sizes were large enough by far for *de novo* mutations to arise (Burke et al. 2014). This suggests that when there is sufficient standing genetic variation and a population is reasonably well adapted to its environment, evolutionary adaptation will occur from standing genetic variation, irrespective of the type of organism or the effective population size. However, when the environment is more stressful, some *de novo* mutations may confer larger fitness benefits than the existing standing genetic variation, and such mutations will thus be more important for adaptation under such circumstances. For example, I would predict that repeating the experiment of Burke et al. (2014) in the presence of Cd would lead to the selection of *de novo* mutations in *SMF1* and *FET4*, just like in my study. Such adaptation from *de novo* mutations is likely also important for natural populations of larger organisms, but because they have smaller effective

population sizes, mutations may not arise in time to save them from extinction (larger organisms generally have higher mutation rates (Drake et al. 1998; Lang and Murray 2008; Keightley et al. 2014), but this is probably insufficient to completely offset the effect of reduced population size). Nonetheless, there is abundant evidence that mutations of large effect contribute to between population variation in nature (Tanksley 1993; Kearsey and Farquhar 1998; Orr 2001, 2005), and the same adaptive phenotypic changes in different populations or species are often caused by one or a few mutations of large effect in the same gene, rather than by many different mutations of small effect in different genes (Conte et al. 2012). All in all, the above suggests that for all organisms initial adaptation to highly novel environments — such as those imposed in microbial evolution experiments — occurs via the rapid fixation of relatively few mutations of large effect, which often arise *de novo*. However, after populations become reasonably well adapted to their new environments, further adaptation becomes more of a subtle process in which allele frequencies change gradually over time, and many more or less equivalent mutations contribute to further fitness increase.

If the nature of the adaptive process indeed depends on external conditions as hypothesized in the previous paragraph, one could ask which of these two general types of evolutionary dynamics is more important in nature. Do organisms usually find themselves in environments to which they are adapted reasonably well? Although the answer to this question clearly depends on environmental specifics, I would say that they usually do. However, even if populations find themselves in this "fine-tuning" regime most of the time, the relatively short periods in which they find themselves in the "rapid adaptation" regime are likely to be much more consequential in terms of the amount of evolutionary change realized. The initial stages of microbial evolution experiments, in which adaptation occurs from *de novo* mutations of large effect, are thus highly relevant because they focus on the type of evolution that matters the most. Furthermore, microbial evolution experiments also directly support the notion that after a large change in the environment, evolutionary adaptation is rapid at first, and then slows down. Interestingly, similar changes in evolutionary rates over time are also observed in nature over geological time scales. In fact, such changes are at the heart of the punctuated equilibrium theory (Eldredge and Gould 1972), which states that short periods of rapid change —which coincide with speciation — alternate with longer periods of evolutionary stasis. Rates of phenotypic change within species and higher taxonomic groups are also generally found to decrease with the time interval over which they are measured (Gingerich 1983; Stockwell et al. 2003). However, this superficially similar pattern is likely primarily due to changes in the direction of selection over time. For example, in two populations of Darwin's finches on the Galapagos islands, selection led to substantial changes in beak shape and body size over the course of just a few years (Grant and Grant 2002). However, over the course of 30 years, the direction of selection changed several times, such that the apparent rate of change over this period was much lower than over shorter periods of time.

This raises the question of whether evolution will ever result in lasting change: what if I

would place my evolved populations — or the LTEE lines for that matter — back in their "original" environments again? Would they then evolve back to their original state? At the molecular level, this seems highly unlikely, particularly because many of the mutations that I found confer a loss-of-function phenotype and would thus require one specific nucleotide change for reversion. However, at the phenotypic level, evolution can be remarkably reversible (Teotonio and Rose 2000; Velicer et al. 2006; Ratcliff et al. 2012; Laan et al. 2015), although particular mutations may inhibit such reverse evolution (Ogbunugafor et al. 2016). Overall, whether a phenotypic change is reversible appears to depend strongly on the biological details as well as the evolutionary history of the system under study (Teotonio and Rose 2001; Tan et al. 2011). Nonetheless, irreversibility may generally be more likely when a mutation causes a loss of function — particularly when entire genes or genomic regions are lost (Collin and Miglietta 2008; Morris et al. 2012) — or when interactions with secondary genetic changes render reversion of the initial mutation detrimental (Bull and Charnov 1985). This latter scenario is potentially more likely in the case of evolutionary innovations such as the acquisition of new defence compounds or the use of a novel resource (Ehrlich and Raven 1964; Blount et al. 2012), because such qualitatively new traits can open up new niches and thus rapidly select for secondary genetic changes that interact with the primary innovative mutation.

Conclusions

Directional environmental change is a ubiquitous phenomenon with important consequences for all living organisms. However, few studies have directly addressed how such change affects evolution. To fill this lacuna, I have studied how the rate of directional environmental change affects the course and outcome of evolution using experimental evolution of heavy metal tolerance in yeast as a model system. My results show that the rate of environmental change and the nature of the selection pressure have crucial consequences for the types of mutations that are used for adaptation, as well as the rate at which these mutations are selected. These findings imply that if we are to fully understand and anticipate the consequences of important societal problems such as climate change and pollution, we need to take into account not only the total magnitude of the projected change, but also its rate. My results are explained remarkably well by a genotype-by-environment framework that I developed, which captures some of the fundamental principles according to which the fitness landscape may change across environments. This framework can be readily extended to other selection pressures and scenarios of change. Therefore, my work provides a firm basis for future studies aimed at understanding evolution in changing environments. While some of the specifics of my model system and experimental set-up preclude a straightforward extrapolation of my results to smaller populations of larger organisms, there are in fact more parallels between evolution

under both these settings than might be immediately obvious. Microbial laboratory evolution experiments may be simplifications of reality, but it is exactly for this reason that they capture the essence of adaptation to directionally changing environments in a thus far unmatched manner.

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Summary

Directional environmental change in the form of global climate change and human-induced pollution is one of the most pressing problems facing society today. While species can sometimes adapt to such change by means of phenotypic plasticity and range shifts, there is considerable concern that these mechanisms are insufficient for long-term population persistence for at least some species. Evolutionary adaptation could potentially offer a solution, but it is unclear what the potential and limitations of this process are for populations exposed to deteriorating conditions. Specifically, we have limited knowledge about the factors promoting evolutionary population rescue, and we know even less about how directional change affects evolutionary dynamics and outcomes.

In this thesis, I investigated how the rate of directional environmental change affects evolutionary dynamics and outcomes using experimental evolution of heavy metal tolerance in the baker's yeast *Saccharomyces cerevisiae* as a model system. Heavy metals are important pollutants that primarily originate from mining and industry and play a key role in both essential biological processes and toxicity due to their highly reactive chemical properties. While some of these metals, such as cadmium (Cd), lead (Pb), and mercury (Hg), are non-essential and have harmful effects even at low concentrations, others, such as manganese (Mn), iron (Fe), nickel (Ni), and zinc (Zn), are essential micronutrients for a wide range of organisms and are harmful only at high concentrations. Baker's yeast is the most widely studied eukaryote model organism and shares numerous features with both plants and animals. Due to its short generation time, large population sizes, and readily manipulated genetics it is also used extensively in experimental evolution studies. In such studies, organisms are cultured under controlled conditions for many generations, mostly to study fundamental evolutionary questions, but increasingly also to characterize the evolutionary response of an organism to a particular selection pressure and thus increase insight into the genetic architecture of the trait under selection.

I cultured replicate populations of yeast for 500 generations in the presence of either gradually increasing or constant high concentrations of Cd, Ni, and Zn, and analysed the evolutionary response at the fitness (**Chapter 2**) and genomic (**Chapter 3**) level. Additionally, I generated several mutants carrying various combinations of mutations based on the genomic information from **Chapter 3**, and determined their fitness at different metal concentrations (**Chapter 4**). I used these data to address a number of hypotheses, which were based on a genotype-by-environment (GxE) framework that I developed to aid my thinking about evolution in changing environments. This framework is based on the concept of the fitness landscape, and distinguishes between two general patterns of change in landscape topography across conditions. Magnitude GxE refers to the case where the *height* of the fitness landscape increases along an environmental gradient, while its shape remains roughly constant. This implies that the fitness ranking of genotypes is the same at all concentrations,

but that fitness differences between genotypes are larger at high metal concentrations. This scenario predicts that the same mutations will be selected under gradual and abrupt change, but that adaptation will be delayed under gradual change. By contrast, reranking GxE refers to the case where the *shape* of the fitness landscape changes along an environmental gradient. This implies that the fitness ranking of genotypes changes across metal concentrations. This scenario predicts that different mutations will be selected under gradual and abrupt change, and that adaptation will be delayed under gradual change. Moreover, it predicts different evolutionary outcomes following gradual and abrupt change, and lower repeatability of evolution under gradual change.

I anticipated that the relative importance of magnitude and reranking GxE would depend on the nature of the stressor. That is, I expected that magnitude GxE would be more important for the non-essential metal Cd, because in that case the same evolutionary solution — minimizing internal Cd concentrations — should be favoured at all concentrations, but more strongly so at high concentrations. This is consistent with directional selection with increasing intensity. On the other hand, for the presumably essential metals Ni and Zn, I expected the reranking GxE scenario to be more important, because in these cases different evolutionary solutions should be favoured at different external metal concentrations to maintain a constant internal metal concentration. This is consistent with stabilizing selection to maintain low, but non-zero intracellular metal concentrations.

My results provide support for these hypotheses at several levels. In **Chapter 2**, I determined relative fitness of populations isolated at different time points from the evolution experiment in the presence of different concentrations of the selective metal. These phenotypic assays showed that for Cd, the fitness ranking of isolates was the same at all metal concentrations, but that fitness differences were larger at high concentrations. Conversely, for Ni and Zn, the fitness ranking of isolates changed across metal concentrations. For all metals, this resulted in a delay in fitness increase under gradual relative to abrupt change. However, fitness of evolved populations from the final time point of the experiment was the same following gradual and abrupt change.

In **Chapter 3**, I performed whole-genome sequence analysis on a single clone isolated from each replicate population at the final time point of the evolution experiment. This revealed that adaptation to the selective environments occurred via a complex combination of SNPs, small indels, whole-genome duplications, and other large-scale structural changes. Furthermore, these analyses confirmed the phenotypic results of **Chapter 2** and showed that for Cd, mutations in the same genes were selected under gradual and abrupt change, whereas for Ni and Zn, mutations in different genes were selected in response to different rates of environmental change. Additionally, I found that evolution was less repeatable at the genomic level following gradual change in the case of Ni and Zn, as predicted by my GxE framework.

Finally, in **Chapter 4**, I reconstructed local fitness landscapes for each metal by deleting all repeatedly mutated genes — as identified by whole genome sequencing — both by themselves

and in combination. I used deletions because most mutations that I found in the evolved lineages were predicted to result in loss of function. Fitness assays on these landscapes at different metal concentrations were then used to evaluate how the height and shape of each landscape changed as a function of concentration. For Cd, I found that the *height*, but not the shape, of the landscape changed across concentrations. Conversely, for Ni, the *shape* of the landscape changed considerably across concentrations, and I made predictions about the consequences that this likely had for the selective dynamics of mutations in my evolution experiment. Deep sequencing of evolved population samples from different time points supported these predictions, demonstrating the power of landscape reconstruction approaches for understanding evolutionary dynamics.

Taken together, this multi-level evidence makes a strong case for the usefulness of my GxE framework for understanding evolution in changing environments. Moreover, my results confirm that the rate of environmental change and the nature of the selection pressure can have crucial consequences for the types and selective dynamics of the mutations that are used for adaptation. These findings imply that if we are to fully understand and anticipate the consequences of important societal problems such as climate change and pollution, we need to take into account not only the total magnitude of the projected change, but also its rate.

My results are explained remarkably well by the GxE framework that I developed, which captures some of the generic principles that determine how local fitness landscapes may change across environments. This framework can be readily extended to other selection pressures and scenarios of change. Therefore, my work provides a potentially useful basis for future studies aimed at understanding evolution in changing environments. Although some of the specifics of my model system and experimental approach preclude a straightforward extrapolation of my results to smaller populations of larger organisms, there are in fact more parallels between evolution under both these settings than may seem obvious. While microbial laboratory evolution experiments make simplifying assumptions about reality, it is exactly for this reason that they capture the essence of adaptation to directionally changing environments in a thus far unmatched manner.

Nederlandse samenvatting

Gerichte omgevingsverandering in de vorm van wereldwijde klimaatverandering en door de mens veroorzaakte vervuiling is een van de meest urgente maatschappelijke problemen van tegenwoordig. Hoewel soorten zich soms kunnen aanpassen aan deze verandering door middel van fenotypische plasticiteit en verschuiving van hun verspreidingsgebied, bestaat er grote zorg dat deze mechanismen ontoereikend zijn voor het voortbestaan op de lange termijn van althans sommige soorten. Evolutionaire aanpassing zou hiervoor een oplossing kunnen bieden, maar het is onduidelijk wat de mogelijkheden en beperkingen van dit proces zijn voor populaties die zijn blootgesteld aan verslechterende omstandigheden. We hebben slechts beperkte kennis van de factoren die evolutionaire redding bevorderen, en weten nog minder van hoe gerichte omgevingsverandering evolutionaire dynamiek en uitkomsten beïnvloedt.

In dit proefschrift heb ik onderzocht hoe de snelheid van gerichte omgevingsverandering evolutionaire dynamiek en uitkomsten beïnvloedt met behulp van experimentele evolutie van zware metalen tolerantie in de bakkersgist *Saccharomyces cerevisiae* als modelsysteem. Zware metalen zijn belangrijke vervuilende stoffen die voornamelijk afkomstig zijn uit de mijnbouw en industrie en spelen een belangrijke rol in zowel essentiële biologische processen als toxiciteit vanwege hun zeer reactieve chemische eigenschappen. Sommige van deze metalen, zoals cadmium (Cd), lood (Pb) en kwik (Hg), zijn niet essentieel en hebben zelfs bij lage concentraties schadelijke effecten. Andere metalen, zoals mangaan (Mn), ijzer (Fe), nikkel (Ni) en zink (Zn), zijn echter essentiële micronutriënten voor een breed scala aan organismen en zijn alleen schadelijk bij hoge concentraties. Bakkersgist is het meest bestudeerde eukaryote modelorganisme en deelt tal van eigenschappen met zowel planten als dieren. Vanwege zijn korte generatietijd, grote populatieomvang, en gemakkelijk te manipuleren genetica wordt gist ook veel gebruikt in experimentele evolutie studies. In dergelijke studies worden organismen gedurende vele generaties gekweekt onder gecontroleerde omstandigheden, voornamelijk om fundamentele evolutionaire vragen te bestuderen, maar in toenemende mate ook om de evolutionaire reactie van een organisme op een bepaalde selectiedruk te karakteriseren en dus inzicht te verkrijgen in de genetische architectuur van de eigenschap onder selectie.

Voor mijn onderzoek heb ik gerepliceerde gistpopulaties gedurende 500 generaties opgegroeid in de aanwezigheid van ofwel geleidelijk toenemende, ofwel constant hoge, concentraties Cd, Ni en Zn, en de evolutionaire respons van deze populaties op het niveau van zowel fenotype (**Hoofdstuk 2**) als genoom (**Hoofdstuk 3**) geanalyseerd. Daarnaast heb ik, op basis van de genomische informatie uit **Hoofdstuk 3**, verscheidene mutanten geconstrueerd met verschillende combinaties van adaptieve mutaties. Van deze mutanten heb ik vervolgens de *fitness* bepaald in de aanwezigheid van verschillende metaalconcentraties (**Hoofdstuk 4**). Deze gegevens heb ik gebruikt om een aantal hypothesen te toetsen, die gebaseerd waren op een genotype-bij-omgeving (*GxE*) denkkader dat ik heb ontwikkeld om mijn denken over evolutie in veranderende omgevingen te ondersteunen. Dit denkkader

is gebaseerd op het concept van het fitnesslandschap, en maakt onderscheid tussen twee algemene patronen van verandering in landschapstopografie als gevolg van verandering in de externe omgeving. *Magnitude GxE* verwijst naar de situatie waarin de hoogte van het fitnesslandschap toeneemt langs een milieugradiënt, terwijl de vorm van het landschap vrijwel onveranderd blijft. Dit impliceert dat de fitnessrangschikking van genotypen gelijk is bij alle concentraties, maar dat fitnessverschillen tussen genotypen groter zijn bij hoge metaalconcentraties. Dit scenario voorspelt dat dezelfde mutaties worden geselecteerd bij geleidelijke *versus* abrupte omgevingsverandering, maar dat aanpassing langzamer zal verlopen bij geleidelijke verandering. *Reranking GxE* daarentegen verwijst naar de situatie waarin de vorm van de fitnesslandschap verandert langs een milieugradiënt. Dit houdt in dat de fitnessrangschikking van genotypen verandert tussen metaalconcentraties. Dit scenario voorspelt dat verschillende mutaties zullen worden geselecteerd bij geleidelijke *versus* abrupte omgevingsverandering, en dat aanpassing langzamer zal verlopen bij geleidelijke verandering. Bovendien voorspelt dit scenario verschillende evolutionaire uitkomsten als gevolg van geleidelijke versus abrupte verandering, en een lagere herhaalbaarheid van evolutie bij geleidelijke omgevingsverandering.

Ik verwachtte dat het relatieve belang van *magnitude* en *reranking GxE* af zou hangen van de aard van de stressor. Dat wil zeggen dat ik verwachtte dat *magnitude GxE* belangrijker zou zijn voor het niet-essentiële metaal Cd, omdat in dat geval dezelfde evolutionaire oplossing – het verlagen van de interne Cd concentratie – optimaal zou zijn bij alle metaalconcentraties, maar dat dit met name het geval zou zijn bij hoge concentraties. Dit komt overeen met gerichte selectie met toenemende intensiteit. Aan de andere kant verwachtte ik dat voor de vermoedelijk essentiële metalen Ni en Zn *reranking GxE* belangrijker zou zijn, omdat in deze gevallen bij verschillende externe metaalconcentraties verschillende evolutionaire oplossingen nodig zijn om een constante interne concentratie te handhaven. Dit komt overeen met stabiliserende selectie om een lage intracellulaire metaalconcentratie te behouden.

Mijn resultaten ondersteunen deze hypothesen op verschillende niveaus. In **Hoofdstuk 2** heb ik de fitness in de aanwezigheid van verschillende concentraties van het selectieve metaal bepaald voor populaties die ik op verschillende tijdstippen heb geïsoleerd uit het evolutie experiment. Deze fenotypische tests toonden aan dat voor Cd de fitnessrangschikking van de isolaten hetzelfde was bij alle metaalconcentraties, maar dat fitnessverschillen groter waren bij hoge concentraties. Aan de andere kant veranderde voor Ni en Zn de fitnessrangschikking van isolaten tussen metaalconcentraties. Voor alle drie de metalen resulteerde dit in een vertraagde toename in fitness bij geleidelijke verandering ten opzichte van abrupte verandering. De fitness van geëvolueerde populaties van het uiteindelijke tijdstip van het experiment was echter hetzelfde na geleidelijke en abrupte verandering.

In **Hoofdstuk 3** heb ik hele genoomsequenties geanalyseerd van één kloon per populatie van het uiteindelijke tijdstip van het evolutie-experiment. Hieruit bleek dat aanpassing aan de selectieve omgevingen had plaatsgevonden door middel van een combinatie van SNP's, kleine

indels, duplicaties van het hele genoom en andere grootschalige structurele veranderingen. Bovendien bevestigden deze analyses de fenotypische resultaten van **Hoofdstuk 2** en toonden ze aan dat voor Cd mutaties in dezelfde genen geselecteerd waren bij geleidelijke en abrupte verandering, terwijl voor Ni en Zn mutaties in verschillende genen geselecteerd waren bij verschillende snelheden van omgevingsverandering. Bovendien vond ik dat evolutie minder herhaalbaar was op genomisch niveau bij geleidelijke verandering in het geval van Ni en Zn, zoals voorspeld werd door mijn GxE denkkader.

Tenslotte heb ik in **Hoofdstuk 4** lokale fitnesslandschappen gereconstrueerd door voor elk metaal de herhaaldelijk gemuteerde genen (zoals geïdentificeerd door de analyse van genoomsequenties) zowel apart als in combinatie te verwijderen. De reden dat ik hiervoor deleties gebruikt heb is, dat de meeste mutaties die ik vond vermoedelijk tot een verlies van functie leidden. Vervolgens heb ik fitnessmetingen bij verschillende metaalconcentraties gebruikt om voor al deze landschappen te bepalen hoe ze van hoogte en vorm veranderden als functie van metaalconcentratie. Voor Cd vond ik dat de *hoogte* maar niet de vorm van het landschap veranderde als gevolg van concentratieverandering. Daarentegen vond ik voor Ni dat de *vorm* van het landschap sterk veranderde als gevolg van concentratieverandering. Op grond hiervan deed ik een aantal voorspellingen over de gevolgen die dit waarschijnlijk had gehad voor de selectieve dynamiek van mutaties in mijn evolutie-experiment. *Deep sequencing* van geëvolueerde populatiesamples van verschillende tijdstippen ondersteunde deze voorspellingen, wat de potentie toont van landschapsreconstructies voor het begrijpen van evolutionaire dynamiek.

Alles bij elkaar leveren mijn resultaten sterk bewijs voor de bruikbaarheid van mijn GxE denkkader voor het begrijpen van evolutie in veranderende omgevingen. Daarnaast bevestigen mijn resultaten dat de snelheid van omgevingsverandering en de aard van de selectiedruk belangrijke gevolgen kunnen hebben voor de types en selectieve dynamiek van de mutaties die worden gebruikt voor adaptatie. Dit impliceert, dat als we de gevolgen van maatschappelijke problemen zoals klimaatverandering en vervuiling volledig willen begrijpen, en hierop willen anticiperen, we niet alleen rekening moeten houden met de totale omvang van de verwachte verandering, maar ook met de snelheid waarmee deze verandering plaatsvindt.

Mijn resultaten worden opmerkelijk goed verklaard door het GxE denkkader dat ik heb ontwikkeld. Dit kader vangt enkele van de algemene principes die bepalen hoe lokale fitnesslandschappen veranderen in reactie op veranderingen in de externe omgeving, en kan gemakkelijk worden uitgebreid naar andere selectiedrukken en scenario's van omgevingsverandering. Daarom vormt mijn werk een potentieel bruikbare basis voor toekomstige studies die gericht zijn op het begrijpen van evolutie in veranderende omgevingen. Hoewel sommige van de specifieke kenmerken van mijn modelsysteem en experimentele aanpak een eenvoudige extrapolatie van mijn resultaten naar kleinere populaties van grotere organismen verhinderen, zijn er meer parallellen tussen evolutie onder deze omstandigheden dan misschien vanzelfsprekend lijkt. Laboratorium evolutie-experimenten met micro-

organismen doen sterk vereenvoudigde aannames over de werkelijkheid. Het is echter precies om deze reden, dat ze de essentie van adaptatie in gericht veranderende omgevingen zo goed weten te vangen.

Acknowledgements

So, finally, there is no more excuse for putting it off. I have finished the rest of my thesis, the content, the cover, the layout, so the only thing left to do now really is to write the acknowledgements. The most read part of any thesis, and arguably the hardest part to write. It is easy to get a bit nostalgic over this, and think of the PhD journey as the personal equivalent of an evolutionary trajectory on an ever-changing fitness landscape. What were the crucial choices, people, and opportunities that have led me to this point where I am now? Were there any major contingencies, or would different choices have led to a rather similar "outcome"?

Among the first things that come to mind when thinking about this is my BSc thesis with Peter de Jong, which gave me a first real taste of (co)evolutionary biology and introduced me to Fisher *vs.* Wright Another potentially decisive moment was a lecture by Arjan about how certain factors affect the repeatability of evolution: now that was a much more fascinating topic than anything else I had ever encountered before during my studies! Fons noticed my enthusiasm, and thus linked me to Arjan and Merijn for my first MSc thesis. This thesis, and the two other research projects that followed, then further fueled my interest in evolution and genetics, and led me to want to do a PhD in these fields. Lucky for me, when I told Arjan about this, it turned out that there was some money from the Genetics group already, and that they were willing to give me the chance to do a PhD off this.

During my PhD, a large number of people supported me in one way or another and thus directly or indirectly contributed to "this thing called thesis". Without these people, I surely would not be at "this point where I am now". The most important among them are of course my advisors. Arjan, thank you for your endless enthusiasm, for your always open door, and for teaching me how to explain myself clearly with your never-ending questions. Bas, thank you for making the Genetics group such a great place to work in, for your sharp insights into almost any topic, and for always thinking in opportunities. Mark, thank you for your fresh outlook on the project, and for sharing your insights into the process and politics of science.

Several people from outside the Genetics group have contributed to my project in invaluable ways. I would like to thank Prof. Ryszard Korona and his group at Jagiellonian University (in particular Dominika, Magda, Marcin, Ela, Kasia, and Joanna) for teaching me the basics of yeast genetics during my visit to Kraków, for providing me with strains and plasmids, for tetrad dissection, and for answering my practical questions. Dick de Ridder, Martijn Derks, Joost van den Heuvel, Gabino Sanchez Perez, Elio Schijlen, and Jan van Haarst all helped in one way or another to bring the sequencing analyses to a good end, for which I am grateful. Finally, I would also like to thank the members of my reading committee, Prof. Bart Thomma, Dr. Claudia Bank, Dr. Bertus Beaumont, and Dr. Lidewij Laan, for taking the time and effort to evaluate my thesis.

Day-to-day life in the Genetics group would have been much harder without the practical support from Bertha, Marijke and Wytske. Thank you Bertha and Marijke for always making

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It is hardly possible to imagine a more supportive and inspiring community to do your PhD in than the Genetics group. Thanks to everyone for the great atmosphere and for the countless science and non-science conversations over coffee, lunch, Friday afternoon drinks and other social events. Fons, Duur, Klaas, Bart P. and Sijmen, thank you for the interesting discussions and giving me more insight into what it means to "be in science". Merijn, Bart N., Eric, you preceded me by a few years. You showed me what life as a PhD student is like, and I much appreciated your advice and company both in- and outside the lab. Martijn and Mark Z., TEM-boys *avant la lettre*, it was great having you around when I was once again struggling with my unruly yeast. Although officially your expertise did not extend to my bug, it was nice to have someone understand what I was going through, and help me troubleshoot with the experienced eye of a postdoc. Mark, big thanks as well for your help with arranging the sequencing in Cologne, data analysis discussions, and going through my AmNat paper. Andy and Diego, it is good to see that the TEM-boys are expanding their territory, and I'm curious what your projects will bring you. The same goes for Marjon en Krithi, who now complete "team Arjan". Thank you all, it has been great fun to work with you!

I started my project around the same time as a number of other PhD candidates, and we shared many hours of sitting and working (or chatting) behind our desks and in the lab, discussing life during and after the PhD, drinking beer, having dinner, and attending courses and conferences. Jelle, it was great to have your curly head full of out-of-the-box ideas just across from me, and to be able to openly discuss almost anything with you. Also, thank you for being my paranymph! Tina, it was a lot of fun to organize the Friday drinks together, although I suspect we were equally bad at keeping track of the financial side of them ;). Alex, I really appreciated discussing science and non-science with you, and coming to the lab in the morning to discover the latest addition to your T-shirt collection. Also, thanks for helping me name Heidi and — often unknowingly — lending me your ggplot book (I might have to consider buying my own now…). Claudio, thank you for joining me on the stats course in cold and rainy England (not easy for such a warm-blooded South American, I know!), poker nights, and tasty Venezuelan dinner. Jianhua, I like your refreshing sense of humor, and am happy to have at least one colleague that I can play Mahjong with. Magda, it was always nice to chat with you about the PhD experience, the irrationality of the Dutch system(s), and yeast (finally someone who understood!), and of course to practice being on stage as your paranymph.

Apart from my own PhD cohort, a large number of other people from the Laboratory of Genetics also contributed to my "PhD experience" in one way or another. I would like to thank my students Sebastian, Yorick, Erik L., Nils, and Michiel for their effort and enthusiasm. Joost, thank you for all the sequencing and stats help, I may have been a bit critical at times, but that

does not mean I did not appreciate it (or your dry sense of humor). Valeria, I really like your positive attitude and friendly smile. Robert, thank you for all your advice on cloning and "the future". Ramon, you often manage to put a smile on my face, be it with your real-life banana tree, fighting plants or nighttime photo shoots. I am glad you also want to be my paranymph (oh, and by the way, don't forget to take good care of Arwen!). Other people without whom the PhD would not have been the same are Aina, Ana Carolina, Anneloes, Charles, Corrie, Cris, Erik W., Eveline S., Eveline V., Frank, Gabriella, Gil, José, Justin, Kim, Kristiina, Lennart, Lidia, Margo, Mathijs, Mina, Mohamed, Nihal, Padraic, Paola, Pingping, René, Rolf, Ross, Roxanne, Sabine, Suzette, Tania L., Tânia N., Ya-Fen, Yanli (I hope I am not forgetting anyone, if so, sorry!), and all the students who joined the group over the past few years. Thank you all for the fun, the support, etc.!

Of course, life during a PhD is not only about the PhD, even if it may seem so at times. Over the past few years, I have had the support of many amazing friends. Jos, thanks for sharing good and bad times ever since high school, beers in de Vlaam, Moroccan bazaar and surfing adventures, and escaping from prison together. Joene, although our lives are now a bit different, we still manage every now and then to have an old fashioned evening of chilling out with good records and beer/whisky/wine, which I really appreciate. Your encyclopedic knowledge of all things music continues to amaze me. Inge, I just realized that a significant part of my holidays over the past few years has been with you. Thanks for all the hitchhiking and couchsurfing adventures, for the trips to Ukraine, England, Copenhagen, Munich and Paris, and of course for being such a great friend in general. After we both get back safely from India, I will come visit you and Tony (and Mr. Monkey of course, how could I forget ;)) again in Ghent! Marleen, I feel we've grown much closer over the past few years, partly because of the shared PhD experience, but also because squashing, cooking and chatting together was just so much fun. It's great to know someone who understands what is to love cooking but (sometimes) be so clumsy about it ;)

Karin, Lotte, Inge, Marjolein, Petra (a.k.a. KLIMP(F)), I really liked to be able to leave the PhD behind every now and then and spend an afternoon or weekend hiking or exploring new cities together. Also meeting one-on-one with each of you was always very "gezellig". Roos, we may not have seen each other a lot over these past few years, but when we did it was always nice to catch up. Thanks to the Roadburn clan, Joene, Martine, Rembrandt, Anouk, Laurens, Judith, Willem, Teun, Maarten V., Alexander, and Wout for all the festivals, concerts and other music related activities that we enjoyed together over the past few years. As soon as I have a better idea about the future, I will buy a Roadburn ticket! 0607, it's ten (!) years this year. Still, we manage to see and visit each other now and then, wherever we are now, which I think is very special.

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Curriculum vitae

Florien Anne Gorter was born on the 20th of March 1985 in Renkum, the Netherlands. After obtaining her high school degree from "*Het Pantarijn*" in Wageningen, the Netherlands, in 2002, she decided it was time for a break and took a gap year to work and learn Spanish in Salamanca, Spain. Upon her return to the Netherlands, she started the Bèta-Gamma Bachelor at the University of Amsterdam, hoping to fit her broad interests into a single study. However, she soon realised that this study was not what it seemed, and thus switched to Biology at Wageningen University, because Biology was the subject she liked best after all. She never regretted this decision, and in 2008 obtained her BSc in Plant Biology, with an additional minor in Public Administration and Organisational Science from Utrecht University. During this time, she was also actively involved in several student and volunteer organisations, and spent one year as a full-time board member for youth association and pop venue Unitas.

After obtaining her bachelors from Wageningen, Florien continued to pursue an MSc in Biology at the same university. As part of this degree, she performed three research projects that gave her a taste for science in general, and evolution and genetics in particular. For her first thesis at the Laboratory of Genetics, she worked on the effect of genetic constraints on the *in vitro* evolution of an antibiotic resistance gene in the bacterium *Escherichia coli*. This project yielded important insights into the repeatability of evolution, and sparked her initial interest in experimental evolution. Next, she spent half a year at the University of California, Berkeley, USA, to work on the genetics of a *Barley Stripe Mosaic Virus* strain infecting the grass species *Brachypodium distachyon*. This project provided her with extensive experience in molecular — cloning — techniques and first introduced her to host-pathogen interactions. Finally, during her last MSc thesis at the University of Oxford, UK, she combined the topics of both previous research projects and worked on the experimental coevolution of a bacterium and its viral parasite.

After her graduation in 2010 (*cum laude*), Florien shortly worked as a secretarial officer to finance her subsequent travels to South America, before embarking on a PhD at the Laboratory of Genetics at Wageningen University in 2011. For her PhD, she worked on the experimental evolution of heavy metal tolerance in the baker's yeast *Saccharomyces cerevisiae* under the supervision of Arjan de Visser, Bas Zwaan, and Mark Aarts. The results of this work are presented in this thesis. During her PhD, Florien coordinated the Wageningen Evolution and Ecology Seminars (WEES) committee, which invites researchers from around the world that have leading roles in the fields of evolution and ecology. Currently, she is looking for an exciting postdoc position to continue her work in evolutionary genetics.

Publications

Gorter FA, Scanlan PD, Buckling A (2016) Adaptation to abiotic conditions drives local adaptation in bacteria and viruses coevolving in heterogeneous environments. *Biology Letters*. **12(2)**: 20150879.

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Gorter FA, Bobula J, Aarts MGM, Zwaan BJ, Korona R, de Visser JAGM. Analyses of local fitness landscapes explain dynamics of adaptation in directionally changing environments. *In preparation*.

PE&RC Training and Education Statement

With the training and education activities listed below the PhD candidate has complied with the requirements set by the C. T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises a minimum total of 32 ECTS (= 22 weeks of activities).

Review of literature (4.5 ECTS)
- Experimental evolution of heavy metal tolerance in yeast

Writing of project proposal (4.5 ECTS)
- Molecular and evolutionary genetics of Zn/Ni/Cd tolerance in *Saccharomyces cerevisiae*

Post-graduate courses (10.2 ECTS)
- "*Molecular advances in ecology: finding the middle road between big questions and the newest methods*", PE&RC, Wageningen, the Netherlands (2011)
- Evolutionary biology workshop, Basel University/ETH Zürich, Guarda, Switzerland (2012)
- Introduction to R and statistics, Imperial College, London, UK (2013)
- Practical course on next generation sequencing for population genetics and experimental evolution, Vienna Graduate School of Population Genetics, Vienna, Austria (2014)

Laboratory training and working visits (0.9 ECTS)
- Experimental techniques for working with yeast, Jagiellonian University, Kraków, Poland (2011)

Competence strengthening/skills courses (7.1 ECTS)
- Competence assessment, PE&RC, Wageningen, the Netherlands (2011)
- Scientific writing, PE&RC, Wageningen, the Netherlands (2014)
- Project and time management, PE&RC, Wageningen, the Netherlands (2014)
- Writing grant proposals, PE&RC, Wageningen, the Netherlands (2015)
- Career orientation, PE&RC, Wageningen, the Netherlands (2016)

PE&RC annual meetings, seminars and the PE&RC weekend (2.1 ECTS)
- PE&RC introduction weekend (2011)
- PE&RC day (2012)
- PE&RC last year weekend (2015)

Discussion groups/local seminars or scientific meetings (7.5 ECTS)
- Wageningen Evolution and Ecology Seninars (WEES), participation and chairmanship (2011-2015)
- Experimental Evolution Discussion Group (EEDG), participation (2011-2015)

International symposia, workshops and conferences (26.3 ECTS)
- Cologne Spring Meeting, Cologne, Germany (2012)
- 18th European Meeting of PhD Students in Evolutionary Biology (EMPSEB), Virrat, Finland (2012)
- "*Experimental approaches to evolution and ecology using yeast*", EMBO Conference. Heidelberg, Germany (2012)
- "*Evolution in the laboratory*", symposium on the occasion of awarding an honorary degree to Prof. Richard E. Lenski by Wageningen University (2013)
- 14th Congress of the European Society for Evolutionary Biology (ESEB), Lisbon, Portugal (2013)
- 4th Mind the Gap conference, Vienna, Austria (2013)
- Netherlands Annual Ecology Meeting (NERN). Lunteren, the Netherlands. (2014)

- WE Heraeus Seminar 'Mechanisms, Strategies, and Evolution of Microbial Systems'. Bad Honnef, Germany (2014)
- "*Experimental approaches to evolution and ecology using yeast and other model systems*", EMBO conference, Heidelberg, Germany (2014)
- Gordon Research Seminar + Conference on microbial population biology, Andover, USA (2015)
- 15th Congress of the European Society for Evolutionary Biology (ESEB), Lausanne, Switzerland (2015)
- Royal Dutch Microbiology Association (KNVM) Spring Meeting, Arnhem, the Netherlands (2016)

Lecturing/supervision of practicals/tutorials (18 ECTS)
- Molecular evolution and ecology (2012)
- Evolution and systematics (2013 + 2014)
- Fundamentals of genetics and molecular biology (2015 + 2016)

Supervision of MSc student (3 ECTS)
- Testing the defence hypothesis using *S. cerevisiae* and *D. melanogaster*

The research described in this thesis was carried out at the Laboratory of Genetics at Wageningen University, Wageningen, the Netherlands.

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