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The role of chance and history during evolution in *Chlamydomonas reinhardtii*

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Lay Summary

If we could go back in time and let life unfold a second time, would evolution proceed the same way? For example, would humans evolve again? And if they would, would they end up the same way as they are today? To answer these questions we need to understand whether evolution is mainly a deterministic process or mainly a stochastic process. If evolution is mainly deterministic, then the outcome of evolution should always be the same. But if evolution is mainly stochastic, then the outcome of evolution can be different every time. It is important that we understand under which circumstances evolution is more or less repeatable, as this will affect our understanding of the nature of biodiversity and our ability to make predictions about the outcome of environmental change.

To determine how repeatable evolution is, and what factors decrease or increase the repeatability of evolution, I did experiments in the laboratory using the single-celled green alga *Chlamydomonas reinhardtii*. I maintained many populations of the algae in a range of different environments. After a few months, which corresponds to a few hundred generations of algal evolution, I measured the fitness of the populations that had survived, and the number of populations that had gone extinct. If the populations all reach the same outcome, this is an indication that evolution is highly repeatable, whereas if the populations reach different outcomes, then this is an indication that stochastic factors such as history and chance play an important role in evolution.

I found that the repeatability of evolution can be very high in some cases, but can also be much lower in other cases. For example in large populations, deterministic factors contribute about 80% to evolution, whereas stochastic factors contribute only 20%. However, in small populations, evolution is driven as much by deterministic factors as by stochastic factors. Overall, I found that the repeatability of evolution depends on the size of the population, the mode of reproduction of the population, the environment in which evolution occurs, and the evolutionary history of the population.

I also studied the process of adaptation to a continuously deteriorating environment, by exposing replicate populations of the freshwater algae to increasing concentrations of salt over time until they had reached concentrations typical of marine conditions. I found that rates of extinction are extremely high during continuous environmental deterioration, and that a history of sexual reproduction and phenotypic plasticity play an important role in survival and adaptation. The surviving populations were very different in how well they can grow in seawater.

When evolution is highly repeatable, diversity is lost as all populations end up being the same. By measuring the importance of deterministic factors and stochastic factors during evolution, we can gain a better understanding of when we expect diversity to be lost and when diversity might increase. Hence the results of this thesis can inform us about the nature of the biodiversity we see today and that we predict for the future.

Abstract

The extent to which evolution is repeatable has important implications. If evolution is highly repeatable, the trajectories and outcomes of evolution in different lineages will always be the same. On the other hand, if evolution is not repeatable, then trajectories and outcomes will be diverse. Thus, the repeatability of evolution affects our understanding of the nature of biodiversity and can inform the extent to which evolutionary theory can be used to make predictions. The repeatability of evolution depends on the relative contribution of selection, chance, and history.

To determine what factors affect the importance of chance and history during evolution, I propagated replicated populations of the unicellular green alga *Chlamydomonas reinhardtii* in controlled environments. I measured the change in fitness after a few hundred generations and determined how much variation had arisen among replicate populations and among populations with different histories. I applied a similar approach to study the importance of history in extinctions, and measured rates of extinction in populations with different histories.

I found that evolution is much less repeatable in small than in large populations because history is more constraining and selection less efficient in small than in large populations. There is also a significant effect of sex and recombination on the repeatability of evolution at the fitness level, but this effect is highly dependent on the environment of selection. Sex can increase the importance of chance or history in some environments, but lower their importance in others, thereby leading to convergence or divergence depending on the environment. Thirdly, I found that the importance of history during evolution does not appear to come from the accumulation of past evolutionary selection pressures, but rather comes from only the most recent selection pressure as it determines genetic correlations for growth between different environments and the amount of genetic variance. Finally, I found that extinction risks are extremely high during continuous environmental deterioration, although a history of sexual reproduction and phenotypic plasticity play an important role in adaptation.

By focusing not solely on the effect of treatments on mean trait values, but also on the variance that arises in our evolution experiments, we can gain a better understanding of the contribution that chance and history make to evolution. The repeatability of evolution can therefore inform us about the adaptive vs. stochastic nature of the diversity we see today, and about the specificity or generality of evolutionary outcomes.

Signed Declaration

I declare that the thesis has been composed by myself, that the work is my own, that the work has not been submitted for any other degree or professional qualification, and that any included publications are my own with contributions from co-authors indicated at the beginning of each chapter and below.

Chapter 2
This chapter is a modified version of a manuscript published as
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I conceived the study, designed the experiment, contributed in the laboratory work, carried out the statistical analyses and wrote the manuscript. J Reid contributed in designing the experiment and in the laboratory work. N Colegrave coordinated the study and contributed to writing the manuscript.

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I designed the experiment, carried the laboratory work, did the statistical analyses, and wrote the manuscript. N Colegrave contributed to designing the experiment and writing the manuscript.

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I designed the experiments, carried out the laboratory work, did the statistical analyses, and wrote the manuscript. G Bell conceived the study and contributed to writing the manuscript, N Colegrave contributed to writing the manuscript.

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Signature Date

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1. General introduction

The question of the repeatability of evolution has captivated the attention of many over the last few decades. From Gould's 'replaying the tape of life', to Monod's 'chance and necessity', to Lewontin's 'trial-and-error', many analogies and terms have been used to portray the interplay between natural selection as the deterministic driver, chance as the stochastic driver, and history as the source for unpredictable constraints on further evolutionary change. Many reviews have been written to try and consolidate the different fields and properly define the terminology to use (e.g. Maynard Smith *et al.*, 1985; Antonovics & van Tienderen, 1991; McKitrick, 1993; Garland & Carter, 1994; Burt, 2001; Blomberg & Garland, 2002; Beatty, 2006; Vermeij, 2006). Still, a proper understanding of what effects history and chance can have on lineages over evolutionary timescales relative to the more studied effect of natural selection, evades us. My main aim in this introduction, aside from reviewing the evidence experimental evolution provides, is to propose a more systematic way of thinking about historical and chance effects during evolution.

The extent to which evolution proceeds in a deterministic or a stochastic manner has important implications. If evolution is entirely deterministic, then the evolutionary trajectories taken by different lineages and the final evolutionary outcomes in given conditions will always be the same. In other words, highly deterministic dynamics will reduce the extent of diversification and/or lead to the loss of diversity across independent populations. On the other hand, if evolution is largely stochastic, then evolutionary trajectories and outcomes will be diverse and predictions about the effects of environmental change cannot be made with any certainty at all. Thus, the repeatability of evolution, i.e. the degree to which evolution proceeds in a more or less deterministic manner, affects our understanding of the nature of biodiversity and can inform the extent to which evolutionary theory can be used to make predictions.

1.1 Selection, chance, and history

The fitness landscape (i.e. the regression of individual fitness on genotypic space) is a useful heuristic for thinking about the repeatability of evolution. On a fitness landscape, peaks represent trait combinations of high fitness. The top of each peak is therefore one possible outcome of evolution where no single mutation can increase fitness any further. If there is only one fitness peak, the outcome of evolution should always be the same, as all populations will eventually converge on this genotype. If there are many fitness peaks, the outcome of evolution can be different in different populations, potentially leading to long-term divergence. There is increasing evidence that landscapes do have multiple peaks (Weinreich *et al.*, 2005; Lunzer *et al.*, 2010; Flynn *et al.*, 2013; Lindsey *et al.*, 2013; Szendro *et al.*, 2013; Tufts *et al.*, 2014; Kondrashov & Kondrashov, 2015; Nahum *et al.*, 2015), and whether or not evolution will be repeatable in such cases will depend on the relative contribution of selection, chance, and history.

Selection acts by sorting genetic variation, leading to the increase in frequency of the variants with highest fitness. If every possible single mutant is available every generation, the population will be able to sample all the trajectories available, and selection will lead to the fixation of the mutation with largest beneficial effect every step of the way up a fitness peak (Fisher, 1930; Muller, 1932; Gerrish & Lenski, 1998; Desai & Fisher, 2007), assuming there is always just one mutation with largest effect. Thus, when selection is perfectly efficient, the outcome of evolution should always be the same. Convergent evolution is usually taken as evidence for the importance of selection during evolution (Arendt & Reznick, 2008), although convergence can also occur if there are physical or biological constraints restricting the number of possible outcomes (Wake, 1991).

In most cases however, only a subset of all the potential mutations is available in each generation and chance effects will limit the number of trajectories that can be explored by the population. Chance can also arise from drift, i.e. when allele

frequencies change irrespectively of their fitness effects through demographic stochasticity. Strong effects of chance will therefore increase the probability that different populations explore different trajectories, and if there are multiple different high-fitness genotypes available can lead to long-term divergence (Lenski & Travisano, 1994; Wiser *et al.*, 2013).

Finally, if there is significant epistasis (i.e. non-additive interactions) among loci affecting fitness, the fitness landscape will be rugged, with multiple fitness peaks separated by fitness valleys. In such cases, history can have substantial effects on future evolutionary change, since the fitness effects of novel mutations will depend on the current genetic background of the population. Thus, genetic differences in starting points can reduce the repeatability of evolution in rugged landscapes by altering the accessibility of certain evolutionary paths to different populations (Weinreich *et al.*, 2005).

There are many studies that have found evidence for parallel or convergent evolution at the genetic, phenotypic, and fitness levels. The classic examples include the evolution of the same ecomorphs of the *Anolis* lizards on different islands (Losos, 1998); the evolution of armor plate patterning in threespine sticklebacks through changes in the same gene (Colosimo, 2005); and the evolution of phages to their bacterial hosts in a novel environment through the same nucleotide changes (Bull *et al.*, 1997; Wichman *et al.*, 2000). Still, in many cases, the level of parallelism or convergence is not perfect in the sense that there is some amount of variation among populations that remains unexplained. The unexplained variation could be due to a lack of perfect repeatability of evolution, but also to unaccounted differences among populations in their abiotic or biotic selective environments, unaccounted genetic or demographic differences among populations, or to not measuring the trait or the whole of the traits that are actually under selection (Kaeuffer *et al.*, 2012).

1.2 Evidence from experimental evolution

1.2.1 The approach

Experiments in the laboratory can provide a solution to this problem of having the variance among natural populations being confounded with common and uncontrollable differences in their ecology, demography, or genetics. In the laboratory, the environments are engineered such that the experimenter controls the ecological and genetic factors, keeping certain ones constant and manipulating the ones that are of interest. The functional factors are more difficult to control, but can be addressed by measuring multiple traits and estimating fitness using different methods. It has therefore become possible with the advent of experimental evolution to quantify precisely the degree to which independent lineages adapt the same way to a given environment (Colegrave & Buckling, 2005; de Visser & Krug, 2014).

Experiments aimed at testing the repeatability of evolution are typically set up using model organisms such as viruses, bacteria, algae, yeast, or flies. Model organisms can be easily propagated in the laboratory and therefore permit the maintenance of many replicate populations and the use of high-throughput methods; have short generation times enabling the study of the processes over the relevant evolutionary timescale; are well characterised genetically and/or physiologically enabling the investigation of repeatability at genotypic, phenotypic and fitness levels of organisation; and can often be stored in a suspended state of growth, usually in freezing temperatures or away from direct light, such that evolved populations can be directly compared to their ancestor. A defined environment is then chosen in which to propagate the organism. Many populations are propagated independently in the environment, using many different strains of the organism, and many replicate populations of each of these different starting points. After tens, hundreds, or thousands of generations, the evolved populations as whole or evolved individuals from each population are characterised at the fitness, phenotypic, and/or genotypic level.

In such experiments, the contribution of selection to evolution can be measured by comparing the fitness of the evolved lineages (i.e. whole populations or individuals) to that of their ancestors. In cases where the fitness of the ancestors is low to start with, such as when the environment is stressful, then the efficiency of selection is estimated to be higher the greater the increase in fitness. The contribution of chance can be estimated by comparing the fitness (or phenotype or genotype) of replicate independent lineages. The greater the variance among initially identical independent populations after evolution, the greater the contribution of chance to evolution. Finally, the contribution of history is estimated by comparing the fitness (or phenotype or genotype) of initially different independent populations. The greater the increase in variance among starting points after evolution, the greater the contribution of history to evolution. If variance among starting points actually decreases during evolution, then this is an indication of convergence and that history does not constrain further evolution.

1.2.2 The findings on the repeatability of adaptation

In general, experiments in asexual and initially isogeneic populations of microbes show that changes in fitness occur mainly as a result of selection during adaptation to a novel environment (Travisano *et al.*, 1995; Collins *et al.*, 2006; Flores-Moya *et al.*, 2008; Bell, 2012b; Spor *et al.*, 2013). However, selection is not always the sole contributor to changes in fitness as chance can also contribute and history can constrain the direction of the changes, depending on the trait measured, the environment, and the timescale studied.

History tends to be of lesser importance for the evolution of traits closely related to fitness. Work in the bacterium *Escherichia coli* (Travisano *et al.*, 1995), in the green alga *Chlamydomonas reinhardtii* (Collins *et al.*, 2006; Bell, 2012b), and in the dinoflagellate *Prorocentrum triestinum* (Flores-Moya *et al.*, 2008) shows that while selection is the main contributor to changes in fitness of independent populations, chance and history can sometimes explain as large an amount of change as selection when it is changes in phenotypic traits such as cell size, CO_2 uptake affinity, or responsiveness to changes in the concentration of acetate, that are measured. There is

one study in yeast where historical constraints were apparent after evolution even in traits closely related to fitness (Spor *et al.*, 2013). Six strains of *Saccharomyces cerevisiae* were propagated in each of four different selection regimes created by manipulating both the glucose concentration and the length of the transfer cycle. The starting genotype explained better changes in phenotype than selection regime for most of the life-history and metabolic traits measured. However, the fact that the lines were converging, suggests that perhaps given more time historical effects on fitness would have become undetectable in this case as well.

The importance of history and chance can also depend on the level of organisation that is being considered, either genotypic or phenotypic. In viruses, historical constraints tend to be minimal at the fitness level where populations tend to reach similar fitness, and at the genotypic level where the frequency of parallel genetic changes is often modest to high (Rokyta *et al.*, 2009), especially high when the number of genetic changes is low (Nguyen *et al.*, 2011). In one study where three different species of Leviviridae bacteriophages were propagated in an environment with increasing temperatures, similarly to other studies, history significantly constrained evolution at both fitness and genotypic levels, with one species reaching a significantly different doubling rate than the other two species, and zero or only one parallel genetic change being observed among species (Bollback & Huelsenbeck, 2009). Chance had significant effects at the fitness level, but little effects at the genotypic level where an intermediate to high level of parallel genetic changes was detected (between 22% and 66% at the nucleotide level, and between 33% and 83% at the amino acid level). In contrast, in another study history was found to have minimal effects on the evolution of resistance to the antibiotic rifampicin in *Pseudomonas* spp. at the genotypic level, but had significant effects on phenotypic evolution, where the same mutation was found to have different effects on growth rates in different strains (Vogwill *et al.*, 2014). This result might reflect the severe selection pressure imposed by the high dose of antibiotic and the limited number of mutations with beneficial effects.

The fact that, in general, evolution tends to be repeatable at the fitness level and less at the genotypic or phenotypic level suggests that fitness landscapes tend to have many peaks and that these peaks can be of similar heights, at least locally. In other words, it appears that adaptation to laboratory environments might be based on fitness landscapes that are rugged and correlated, a result of low but detectable levels of epistasis (Kauffman & Levin, 1987). Hence, similar evolutionary outcomes in fitness do not necessarily mean a depletion of genetic and phenotypic diversity, and phenotypic diversity should be cautiously interpreted as resulting from differences in selective pressures, as chance and historical contingency can generate similar patterns of variation among populations.

Another factor that can affect the importance of chance and history relative to that of selection is the environment to which adaptation occurs. Different environments will involve different numbers of genes, and different types of interactions among those genes. In fitness landscapes with one peak, we expect populations to diverge initially as different mutations with different effect sizes arise and fix in different populations. However, as populations climb further up the peak, we expect variance to decrease and eventually for populations to converge on the same outcome (e.g. Melnyk & Kassen, 2011). Alternatively, in rugged fitness landscapes, we expect populations to diverge initially as different mutations fix in different populations, and for this divergence to be maintained if the valleys of low fitness prevent the populations from escaping their peak and converging onto the optimal fitness peak. Thus, chance events such as differences in which mutation fixes first, and history, can have greater influences on evolution in rugged landscapes than in smooth landscapes because different trajectories lead to different outcomes. Of course, if population sizes are large enough that every single and double mutant is generated every generation, or if drift is important, then populations might be able to shift from peak to peak until they all converge on the same optimal peak. However, there is no direct empirical evidence to support the theory of peak shifting and it is therefore unclear what the likelihood is of peak shifts occurring in evolving populations of finite size (although see Nahum *et al.*, 2015).

The importance of the environment in determining the repeatability of evolution was clearly demonstrated in an experiment in the bacterium *Pseudomonas fluorescens*, where independent lines converged both in terms of fitness and in terms of metabolic profiles on the same outcome during evolution in a glucose environment, but diverged during evolution in a xylose environment (Melnyk & Kassen, 2011). Not only did the contribution of chance and history differ after 500 generations, but so did the dynamics of their contributions over time, highlighting the importance of taking into account timescale when interpreting the repeatability of evolution. Thus, history and chance are more or less likely to contribute to adaptation in different environments, where the genetic basis of adaptation differs.

Studies of the contribution of selection, chance, and history have also been carried out in sexual and initially diverse experimental populations where contrasting results have been obtained (Teotonio & Rose, 2000; Teotonio *et al.*, 2002; Joshi *et al.*, 2003; Kawecki & Mery, 2003; Griffiths *et al.*, 2005; Simões *et al.*, 2008; Teotónio *et al.*, 2009; Fragata *et al.*, 2014). More empirical work is needed to understand better how recombination and diversity within populations affect the repeatability of evolution.

1.2.3 The findings on the repeatability of extinctions

One aspect of the repeatability of evolution that is vastly understudied is that of the repeatability of extinctions. In other words, what is the probability that the same population would go extinct again if life were to be rewind at let to happen a second time? Extinctions probably occur as often as new species arise and can have severe impacts on biodiversity and ecosystem functioning. Yet, in spite of their importance, very limited work has been done to determine the contribution of chance and history to extinctions, reflecting in part the difficulty of studying lineages that do not exist anymore and the need to rely on the patchy and biased fossil record.

The same approach as the one used to study the repeatability of adaptation can be used to study the repeatability of extinctions. The difference of course is that while there are hundreds if not thousands of possible outcomes to adaptation, only two, alive or dead, are possible for extinctions. But this should make it easier to

characterise the repeatability of extinction given that qualitative descriptions match quantitative descriptions. Hence, the repeatability of extinction can be described as the degree to which the extinction of a given lineage is the result of chance, its history, or selection. Or to use the words of D. M. Raup (1992), the result of 'bad genes or bad luck'. If extinctions occur mainly as a result of chance, we would expect extinct lineages to be a random subset of surviving lineages. If extinctions occur mainly as a result of history, we would expect lineages from a given clade for example to have a higher proportion of extinctions than expected by chance. And finally, if extinctions occur mainly as a result of selection, we would expect the least fit lineages to be the ones going extinct.

The studies that have looked at the repeatability of extinction have mainly been done using phylogenetic analyses. These analyses quantify the heritability of extinction by detecting non-random clustering of species at risk of extinction, extinct, or of shorter longevity. The repeatability of extinction is estimated to be high if the taxa within a clade experience a greater (or lower) rate of extinction than expected by chance. For example, in planktonic foraminifera, keeled species have consistently gone extinct during episodes of mass extinction while unkeeled species have consistently survived them (Norris, 1991). The evidence for differences in the heritability of extinction extends to angiosperms (Vamosi & Wilson, 2008), birds (Gaston & Blackburn, 1997), marsupials (Johnson *et al.*, 2002), and animals in general (Purvis *et al.*, 2000). Thus it appears that extinctions occur repeatedly in certain clades and much less in others. While this suggests that history plays an important role in the repeatability of extinction, such phylogenetic nonindependence in extinction can arise for reasons other than historical contingency. For example, closely related species often live in similar environments and share similar traits (Purvis, 2008). Hence a correlation with extinction risk can be due to similar selection pressures rather than an inherent proneness to extinction.

The repeatability of extinction could be studied experimentally in the laboratory where the causal link between history and extinction could be determined. There are some experiments that have studied extinction dynamics, but the majority never with

the idea to test for the repeatability of extinction, and therefore never with the proper design to determine the contribution of selection, chance, and history to extinction. Such an experiment would require propagating replicate populations of many significantly different lineages in a severe environment. To determine the role of selection in extinctions, the initial fitness of the lineages would be compared with survivability. To determine the role of chance in extinction, survivability of replicate lines would be compared. And to determine the role of history in extinction, survivability of different lineages would be compared.

The only study to my knowledge that has done this is one by Gonzalez and Bell (2013). In phase one of the experiment they propagated replicate populations of two species of yeast, *Saccharomyces cerevisiae* and *S. paradoxus* in different concentrations of salt for eight transfers. In phase two of the experiment, each surviving populations were transferred to 150 gL^{-1} NaCl, a lethal concentration to both species prior to the start of phase one. The probability of extinction during phase two depended significantly on the salt concentration experienced during phase one. The effect was most pronounced for high salt concentration in *S. cerevisiae* and for low salt concentration in *S. paradoxus* where it increased the probability of survival from about 10% to about 40%. While the contribution of chance, history, and selection were not quantified in a manner that would allow them to be compared directly, this study nonetheless demonstrates that there is a significant contribution of chance, as extinction frequencies were only exceptionally at 100%, and that there is a significant contribution of history with both species and selection environments leading to differences in extinction risks. More studies like this one are needed to test predictions about the repeatability of extinction.

1.3 A new framework for studying historical contingency

Experiments designed to test for contingency upon starting conditions have typically lumped all kinds of differences in starting conditions under the term ‘history’. Here I argue that more precise investigations of different kinds of historical differences

between lineages will inform us better as to the reasons why evolution is or is not repeatable under certain circumstances. History is shaped by demography, ecology, and ancestry. Each of these factors generate different types of differences, and in turn different predictions about their constraining effects on adaptation or extinction in a new environment. Historical constraints are one of the most significant hurdles to generalisations of evolutionary theory: as experiments cannot realistically be carried out with all possible genotypes, most of the time we rely on the outcome in a single genotype being generalizable to any other starting genotype. By structuring our investigations of the role of history in evolution by the more precise types of historical differences, we should be able to arrive at a more informative understanding of historical contingency.

1.3.1 Historical demography

One of the ways historical effects can arise is if the demography of different populations has been different in the past. For example, some populations will have recently been bottlenecked whereas others will have been at a constant size, and some populations will have been small whereas others will have been large in size. Such differences in population growth dynamics and in average population size can have significant effects on the amount of standing genetic variation in the population. For example, large populations will have a higher supply of mutations than small populations, and recently bottlenecked populations will have had their standing genetic variation depleted compared to populations at constant size which will have standing genetic variation maintained at mutation-selection-drift balance. Greater amounts of standing genetic variation as well as population size have been shown to lower extinction risks and increase rates of adaptation (Bell & Gonzalez, 2009; Samani & Bell, 2010; Agashe *et al.*, 2011; Lachapelle & Bell, 2012). Hence, historical demography can significantly affect the evolutionary potential of a population by altering its ability to use and generate genetic variation. To what extent historical demography constrains evolution remains to be determined.

1.3.2 Ecological history

Another way historical effects can arise is if the ecology of different populations has been different in the past. For example, some populations will have been completely isolated whereas others will have received frequent migrants, some populations will have been exposed to a temporally or spatially fluctuating environment whereas others to a constant environment, some populations will have been physiologically acclimated to the component in the new environment whereas others will not, and some populations will have been evolving in an environment similar to the new environment whereas others will have been evolving in an environment very different from the new environment.

A population that is constantly receiving migrants will be flooded with new genetic variation. This new variation, while likely to be neutral or maladaptive to the current conditions, has the potential to be adaptive once the environment changes. Migration has been shown to lower extinction risks and increase rates of adaptation (Morgan *et al.*, 2005; Lagator *et al.*, 2014b; Nahum *et al.*, 2015). Migration can also affect the repeatability of evolution by enabling the spread of chance events that would otherwise remain constricted to independent populations (Nahum *et al.*, 2015).

There is plenty of experimental evidence that differences in the heterogeneity of the environment can favour the evolution of different response strategies (Kassen, 2002). In an elegant theoretical study, Botero *et al.* (2015) demonstrated that differences in ecological conditions can affect not only the type of response strategy that evolves but can also constrain evolution upon further changes in ecological conditions. More specifically, by modifying the timescale of environmental variation and the predictability of environmental conditions the authors demonstrated the evolution of four main types of response strategies. When environmental variation is predictable and fast, reversible phenotypic plasticity (i.e. plasticity that occurs throughout the life of the individual) tends to evolve. With slower rates of environmental variation, plasticity switches from reversible to irreversible (i.e. plasticity that occurs only during development) to lower the costs of phenotypic adjustment. Whereas, if environmental variation is unpredictable and slow, beneficial mutations have time to

arise and fix, and adaptive tracking tends to evolve. With faster rates of environmental variation, mutations become insufficient to track changing conditions, and plasticity inadequate in unpredictable conditions, such that bet-hedging tends to evolve. Extinctions are particularly high when environmental conditions change between those that favour bet-hedging and adaptive tracking, and between those that favour phenotypic plasticity and adaptive tracking, and vice-versa. Thus, differences in ecology can lead to differences in genome architecture and likelihood of adapting or going extinct after further environmental change.

The one experiment that I know that tested for historical constraints from differences in previous environmental heterogeneity was done with lineages of viruses with a history of specialism (one plant species host) or generalism (two plant species hosts) (Bedhomme *et al.*, 2013). The lineages were propagated on the ancestral host to determine if ecological history constrained reverse evolution. Historical ecology had limited effects on adaptation at the phenotypic level, with specialists and generalists adapting to the same degree in general. However, ecological history had significant effects on genotype evolution as genetic distances increased after reverse evolution on the ancestral host. This increase in genetic diversity occurred because adaption occurred through different compensatory mutations, instead of reversions.

A final example of how historical ecology can constrain adaptation and extinction is in the similarity of previous environmental conditions to new conditions. When the previous conditions are similar to the new conditions, it is more likely that the population will be able to tolerate the new conditions either because of physiological acclimation or because of adaptation by natural selection (De Visser & Rozen, 2005). Physiological acclimation can increase the match between the phenotype of the population and the optimal phenotype of the environment, contributing in reducing the rate of population decline following environmental change, and thereby providing an opportunity for genetic adaptation (Chevin & Lande, 2009; Gomez-Mestre & Jovani, 2013). On the other hand, adaptation by natural selection can increase tolerance of new conditions through genetic correlations, where alleles conferring resistance to lower stress provide some amount of tolerance to higher

levels of stress or to other types of stress, contributing to survival in deteriorating conditions (Samani & Bell, 2010; Lachapelle & Bell, 2012; Gonzalez & Bell, 2013). Hence, selection in environments with different levels of similarity to the new environment can constrain further evolution and lead to different outcomes.

1.3.3 Historical effects from differences in ancestry

Finally, a third way historical effects can arise is if the genetic architecture of lineages differs. For example, different lineages can have different mutations, with different degrees of pleiotropic effects for growth in alternative environments, and different lineages can regulate gene expression in different ways, leading to differences in phenotypic plasticity. The genetic background has been shown to affect the potential for adaptation to a novel environment (Blount *et al.*, 2008; Poulicard *et al.*, 2012; Angst & Hall, 2013). The genetic background has also been found to affect the number of mutations fixed during adaptation of the bacterium *Escherichia coli* to the antibiotic ciprofloxacin (Wong & Seguin, 2015), although not during adaptation of the fungus *Aspergillus nidulans* to a standard laboratory medium (Gifford *et al.*, 2011).

Joshi *et al.* (2003) studied the contribution of past selection, ancestry, current selection, and chance to evolution during 20 generations in *Drosophila melanogaster*. They found that ancestry contributed on average 0.09 in larval feeding rate (a trait closely related to fitness) throughout the experiment. The remaining variation was mostly attributable to within population variation. Past selection contributed 0.54 of the variation initially but effects disappeared completely by the end, and chance played an insignificant role. Hence while historical differences in ecology tended to disappear, those attributable to ancestry remained apparent, although to a small degree.

It can be difficult to make predictions about the potential importance of genetic differences for the repeatability of evolution as many mutations fix by chance and have no measurable effect on fitness until the organism is actually propagated in a specific environment. Unless there is prior knowledge about the underlying genetics

and mechanisms of adaptation to a given environment, it might prove difficult to predict exactly when differences in ancestry will affect he repeatability of evolution.

A more precise characterisation of history in experiments measuring the repeatability of evolution will much contribute in structuring the field. At the moment, too few experiments test any specific attribute of history (but see Teotonio & Rose, 2000; Teotonio *et al.*, 2002; Kawecki & Mery, 2003; Collins *et al.*, 2006) and this undermines our ability to conclude on when history will and will not contribute significantly to evolutionary change.

1.4 Thesis overview

I use experimental evolution in the unicellular green alga *Chlamydomonas reinhardtii* to investigate the contribution of chance and history to evolution. In the first two chapters, I measure the repeatability of adaptation. I show that adaptation is less repeatable in small populations than in large populations because selection is less efficient and history more constraining in small populations (Chapter 2); and I show that the repeatability of adaptation is significantly different in asexual populations than in sexual populations, although the effects of recombination are dependent on the specific attributes of the environment and therefore unpredictable (Chapter 3). In Chapter 4 I test for the effect of history on extinction risk, and measure the repeatability of extinction. I show that the extinction risk is not constrained by a history of evolutionary rescue, but rather it is constrained by the most recent selection history, that is the latest environment of selection. This chapter starts to implement the framework presented above, by using lineages that differ in one particular aspect of ecological history, i.e. selection history, and testing how this difference affects the contribution of chance and history to evolution. Finally, in Chapter 5 I demonstrate how experimental evolution can be used to study major ecological transitions. I show that a history of standing genetic variation, sexual reproduction, and plasticity favours survival in a deteriorating environment and play an important role in adaptation. This evolutionary transition occurred through changes in the constitutive and inducible responses to salt.

2. Repeatability of adaptation in experimental populations of different sizes

This chapter is a modified version of a manuscript published as

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I conceived the study, designed the experiment, contributed in the laboratory work, carried out the statistical analyses and wrote the manuscript. J Reid contributed in designing the experiment and in the laboratory work. N Colegrave coordinated the study and contributed to writing the manuscript.

2.1 Abstract

The degree to which evolutionary trajectories and outcomes are repeatable across independent populations depends on the relative contribution of selection, chance, and history. Population size has been shown theoretically and empirically to affect the amount of variation that arises among independent populations adapting to the same environment. Here I measure the contribution of selection, chance, and history in different-sized experimental populations of the unicellular alga *Chlamydomonas reinhardtii* adapting to a high salt environment to determine which component of evolution is affected by population size. I find that adaptation to salt is repeatable at the fitness level in medium ($N_e = 5 \times 10^4$) and large ($N_e = 4 \times 10^5$) populations because of the large contribution of selection. Adaptation is not repeatable in small ($N_e = 5 \times 10^3$) populations because of large constraints from history. The threshold between stochastic and deterministic evolution in this case is therefore between effective population sizes of 10^3 and 10^4. My results indicate that diversity across populations is more likely to be maintained if they are small. Experimental outcomes

in large populations are likely to be robust, and can inform our predictions about outcomes in similar situations.

2.2 Introduction

The importance of chance and history as opposed to selection during adaptation is likely to be affected by population size. In the absence of standing genetic variation, small populations are expected to explore more trajectories than larger populations because of the low supply of beneficial mutations, and variation in what particular mutation arises across populations (Handel & Rozen, 2009; Jain *et al.*, 2011; Szendro *et al.*, 2013). Trajectories and outcomes in small populations are therefore predicted to be less repeatable than in large populations because of the higher contribution of chance. In large populations, the higher supply of mutations will increase the probability of there being multiple different individuals each carrying a different beneficial mutation. Clonal interference (Fisher, 1930; Muller, 1932; Gerrish & Lenski, 1998) will tend to lead to the fixation of the mutations with largest beneficial effect (Rozen *et al.*, 2002; Perfeito *et al.*, 2007) and to a reduction in the number of different trajectories taken by independent lineages (Szendro *et al.*, 2013). As such, adaptation in large populations is predicted to be more repeatable because of the greater efficiency of selection and lower contribution of chance (Handel & Rozen, 2009; Jain *et al.*, 2011; Szendro *et al.*, 2013).

While the effect of population size on the contribution of selection, chance, and history has not, to my knowledge, been empirically determined, smaller population sizes do generally lead to greater among population variation than do large population sizes (Miller *et al.*, 2011), although this effect depends on the environment (Rozen *et al.*, 2008) and timescale (Schoustra *et al.*, 2009).

In this chapter I quantify the contribution of selection, chance, and history to adaptation to a novel environment of initially isogenic, asexual experimental

populations of different sizes. I predict that chance and history will play a greater role in small populations whilst selection will be more efficient in larger populations.

2.3 Material and Methods

2.3.1 Base populations

The experiment was started using six different genotypes of the unicellular green alga *Chlamydomonas reinhardtii*: CC-1690 (wild-type, mating type +); CC-1952 (wt, mt -); backcrossed CC-2342 (strain created in our laboratory by backcrossing to the wild-type CC-2342 a total of 12 times, mt-); backcrossed CC-2344 (same as above using wild-type CC-2344, mt-); backcrossed CC-2931 (same as above using wild-type CC-2931, mt+); dark line DD C8 (obtained from G. Bell, mt+). These genotypes are genetically (Jang & Ehrenreich, 2012) and/or ecologically distinct. I propagated each genotype individually, such that all growth during the experiment was vegetative, and adaptation occurred via *de novo* mutations.

2.3.2 Selection experiment

For each combination of genotype and population size, I had six replicate lines, for a total of 6 x 3 x 6 = 108 independent lines. A single colony from each genotype was expanded in standard growth medium. Six samples from each well-mixed culture were used to initiate each replicate line. The amount of genetic variation is minimal and expected to be the same across replicates. The replicates were then propagated independently. Each line was exposed to a constant novel environment consisting of Bold's minimal medium (Harris, 2009) supplemented with 5 gL^{-1} NaCl. High salt imposes strong osmotic and oxidative stresses in *C. reinhardtii* by disrupting the homeostasis of ions (Na^+, Cl^-, K^+, and Ca^{2+}), degrading proteins, and thus reducing rates of photosynthesis and cell division (Husic & Tolbert, 1986; Neelam & Subramanyam, 2013). I chose 5 gL^{-1} NaCl because salinities between 5 gL^{-1} and 7gL^{-1} NaCl (0.085 M and 0.120 M) reduce growth by about 50% (Reynoso & De

Gamboa, 1982; Moser & Bell, 2011; Lachapelle & Bell, 2012), and induce adaptive responses within short evolutionary timescales (Lachapelle & Bell, 2012).

Population size was manipulated by varying the volume of growth medium in which the lines were growing. Small lines were cultured in 0.1 mL of medium (96-well plate); medium lines in 1 mL (48-well plate); and large lines in 8 mL (6-well plate). Lines were serially transferred using the same relative inoculum size (5%) at the end of each cycle (i.e. every 4 days). This means that the number of cells at the end of a growth cycle and the number of cells transferred are greater in larger volumes than in small volumes. Using the same relative inoculum size ensures that the number of cell divisions within a growth cycle, population density, and the relative amount of spent media transferred are the same across treatments initially, although small differences (i.e. about 1.3 fold difference in cell density at the end of the experiment compared to 10 fold differences in population size) will arise as populations adapt during the experiment. Using $N_e = gN_o$ where N_e is the effective population size, g is number of generations between transfers (here g = 4.3), and N_o is the initial population size (Lenski *et al.*, 1991), the effective population sizes for the small, medium, and large lines at the start of the experiment are approximately 5×10^3, 5×10^4, and 4×10^5 cells respectively. Lines were maintained at 24.5 degrees Celsius, 60% air humidity, 8000 Lux constant light intensity, shaking at 130 rpm with a 3 mm rotation diameter. The experiment lasted 40 cycles (about 200 generations). Note, that since our focus is on general adaptation to the selection environment, rather than any specific adaptation to the salt stress, it was not necessary for us to maintain control lines evolving in the absence of salt.

2.3.3 Fitness assay

To estimate fitness, I calculated the maximum growth rate of ancestral and evolved lines when grown in 5 gL^{-1} NaCl. The ancestors had been maintained in dim light on Bold's agar throughout the experiment, conditions which limit growth and selection (Harris, 2009). Six cultures were setup per ancestor to match the number of evolved lines generated per ancestor per population size treatment. All lines were cultured in

Bold's media for two cycles to minimise physiological differences, and then transferred to 5 gL^{-1} NaCl. Each line was assayed three times.

Growth was monitored during the second growth cycle in 5 gL^{-1} NaCl by measuring optical density at 750 nm every 9 ± 1 hours. I transformed the measurements (log10 of [optical density x 10,000]) to allow the models to be fitted. Growth parameters were extracted from a nonlinear model using nonlinear least-squares, nls in the nlstools R package (Baty *et al.*, 2015). I first fitted a baranyi model (Baranyi & Roberts, 1994; Baranyi *et al.*, 1995). This model returned a fit for 83% of the lines. The remaining lines were fitted with either a baranyi model without Nmax, a baranyi model without lag, or a linear model, as appropriate. Model fits were visually inspected to ensure the proper model had been applied.

2.3.4 Determining the contribution of selection, chance, and history

Generally speaking, the effect of selection is to increase fitness. As such, the difference between the ancestors and evolved lines is the contribution of selection on beneficial alleles and any associated alleles that may be hitchhiking. Note here that I am investigating sources of variation in fitness. Differences between the phenotype or genotype of ancestors and evolved lines could be attributable to factors other than selection. Any variation in fitness among evolved lines descending from the same starting genotype will be the result of chance. Finally if history affects adaptation, I expect lines from different starting genotypes to reach different outcomes. As such, variation in final fitness among starting genotypes is the contribution of history.

More specifically, I quantified components of variation in fitness by calculating sums of squares, which provides a phenomenological description of the structure of variation that is entirely additive (Bell, 2013). The effect of selection was estimated as $mnr(F - I)^2$ where F and I are the final and initial grand mean growth rates respectively, m is the number of lines descending from each ancestor, n is the number of ancestors, and r is the number of assay replicates. The effect of history was estimated as $mr\Sigma(A-F)^2$, where A is the mean growth rate of all lines from a given ancestor. The effect of chance was estimated as $r\Sigma\Sigma(L-A)^2$, where L is the

mean growth rate of each replicates from a given line. Finally, the variation due to error measurement was estimated as $\Sigma\Sigma\Sigma(R\text{-}L)^2$, where R is the growth rate of each replicate. Each sum of squares estimate was divided by the sum of all estimates to obtain the relative contribution of each factor. I prefer this method to alternative variance component based approaches (e.g. Travisano *et al.*, 1995; Collins *et al.*, 2006; Fragata *et al.*, 2014) since my design does not pemit a full additive partition of variation using these methods. Never-the-less a variance componant analysis of our data produced similar results.

2.3.5 Statistical analyses

Variance in growth rates among the starting genotypes was estimated by equating observed and expected mean squares from a nested analysis of variance, with genotype and line within genotype as random effects. To determine if adaptation had occurred, and whether it had occurred to different extents in populations of different sizes, multiple comparisons were done using Tukey HSD following a general linear model on population size (with four levels representing the ancestors, and the small, medium, and large evolved lines), as a fixed effect. To further investigate the effect of population size on growth and its interaction with starting genotype and line, I performed an analysis of variance on the growth of the evolved lines. The model included population size as a fixed factor, starting genotype as a random factor, line within genotype as a random factor, and their interactions.

The significance of the difference in relative contribution of selection, chance, and history between two sizes of populations was determined by a randomisation test. I randomly allocated each evolved line to a population size and initial genotype without replacement, and then calculated the relative sums of squares. I compared the ratio of relative sums of squares for each pairs of population sizes to the observed ratios. The number of times where the random ratios were as large or larger than those observed over the total number of randomisations (10 000) is my significance statistic.

2.4 Results

2.4.1 The ancestors differ in their response to the novel environment

There is a significant amount of variation in growth rates among the six starting genotypes (Figure 2.1; variance among genotypes = 0.26, mean = 1.22).

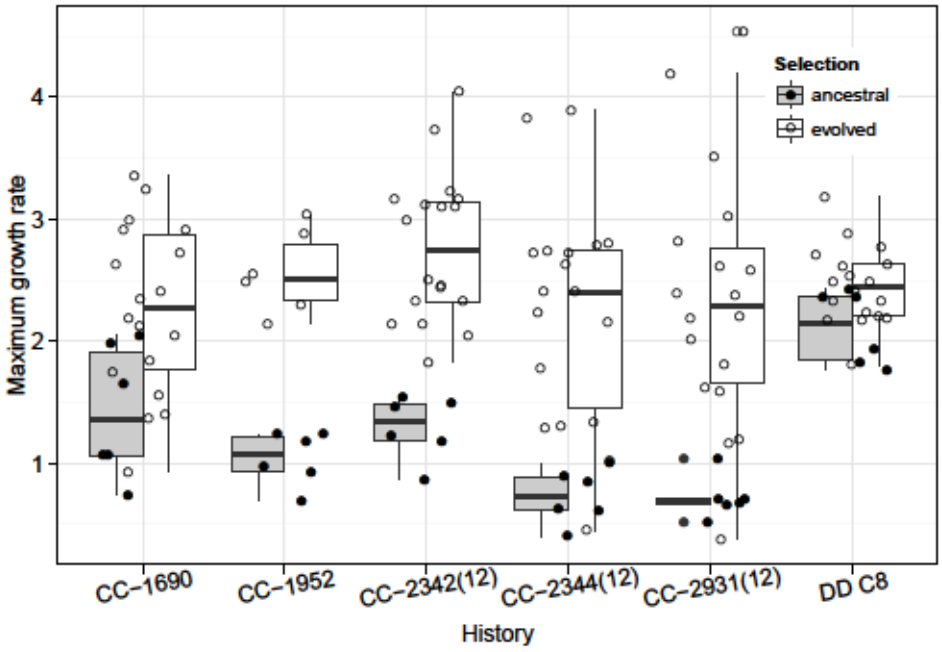

Figure 2.1 Maximum growth rate of ancestors and evolved lines in 5 gL^{-1} NaCl. History corresponds to the different starting genotypes.

2.4.2 Small populations adapt to a lesser degree than larger populations

All six replicate lines of ancestor CC-1952 went extinct in small and medium populations. These lines were not included in the following analyses. Among the surviving lines, all population sizes have greater growth rates on average than their ancestors, meaning that adaptation to 5gL^{-1} NaCl has occurred over the course of 200 generations of evolution (Figure 2.2, effect of population size $F_{3,392} = 88.72$, $P<0.001$; TukeyHSD comparisons between ancestors and small or medium or large evolved lines all have $P<0.001$). The growth rate of small lines is significantly lower than that of the medium and large lines ($P<0.001$ for both comparisons) whilst the growth rates of medium and large lines do not differ ($P = 0.62$).

The growth of each genotype, as well as the growth of each line within genotype varies depending on which size of population they evolved in (effect of population size $F_{2,192} = 70.86$, $P<0.001$; effect of interaction population size : starting genotype $F_{8,192} = 13.02$, $P<0.001$; effect of interaction population size : line within history $F_{50,192} = 3.36$, $P<0.001$).

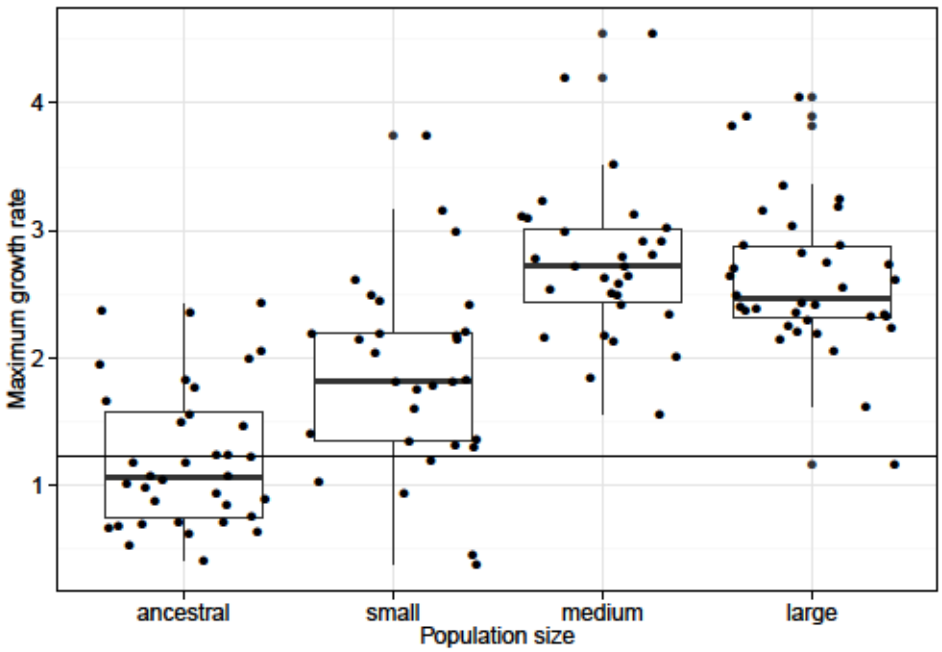

Figure 2.2 Maximum growth rate in 5 gL^{-1} NaCl of the ancestors, and of the small, medium, and large evolved lines.

2.4.3 Population size affects the contributions of selection, chance, and history to evolution

Selection plays a significantly greater role in medium and large lines than in small lines during evolution in 5 gL^{-1} NaCl (Figure 2.3, Table 2.1 and Table 2.2). Selection explains about 80% of the changes in growth rates in medium and large lines, whereas it explains less than 40% in small lines.

History explains less than 4% of the variation in medium and large lines, but explains close to 20% of the variation in small lines. This difference is significant when comparing small to large lines, but not when comparing small and medium lines (Table 2.2). The variance among initial genotypes ($\sigma^2 = 0.26$) is maintained

after evolution in small populations ($\sigma^2 = 0.30$), but much reduced after evolution in medium ($\sigma^2 = 0.13$) and large ($\sigma^2 = 0.016$) populations.

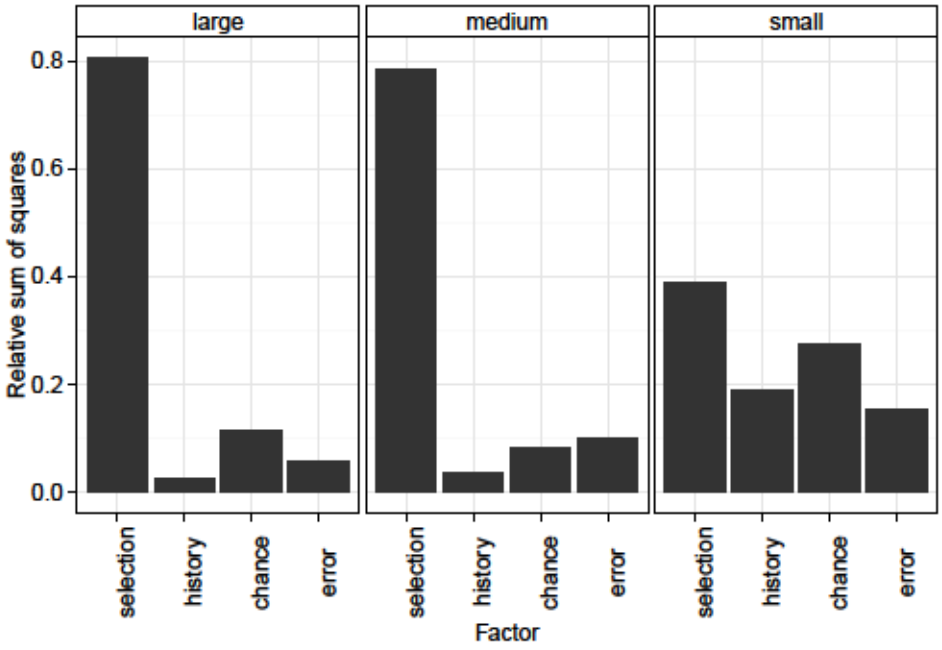

Figure 2.3 Relative contribution of selection, chance, and history after 200 generations of selection in 5 gL^{-1} NaCl.
Error here corresponds to variation among assay replicates.

Finally, chance explains about 10% of the variation in medium and large lines, which is significantly less than the close to 30% that it explains in small lines (Table 2.2).

It is also interesting to look at the absolute amount of variation because it tells us about the diversity that is present for a given component irrespective of mean growth or the amount of variation for another component. Small amounts of variation in growth, whether for low mean growth or high mean growth, means that growth is very similar across lines. The absolute variation among replicate lines with the same starting genotype is very similar for all population sizes (Table 2.1). However, there is two to three times more variation among genotypes evolved in small than in medium and large populations. Finally, the variation between ancestors and evolved lines is more than five times smaller in small lines than in medium and large lines.

Table 2.1 The effect of population size on the contribution of selection, history, and chance to variation in growth rates after 200 generations of evolution in 5 gL^{-1} NaCl.
Error here corresponds to variation among assay replicates.

Population size	Effect	Sum of squares	Total sum of squares	Relative sum of squares
Small	Selection	38.1	98.4	0.387
	History	18.4		0.187
	Chance	27.0		0.275
	Error	14.8		0.151
Medium	Selection	210	267	0.786
	History	9.60		0.0359
	Chance	21.7		0.0810
	Error	26.0		0.0974
Large	Selection	210	261	0.806
	History	6.40		0.0245
	Chance	29.8		0.114
	Error	14.6		0.0560

Table 2.2 Significance of the difference between population sizes in the relative contribution of selection, history, and chance.
P values were determined from a randomisation test.

Factor	Comparison	P values
Selection	Small – Medium	0
	Small – Large	0
	Medium - Large	0.38
History	Small – Medium	0.064
	Small – Large	0.015
	Medium – Large	0.26
Chance	Small – Medium	0.0017
	Small – Large	0.012
	Medium – Large	0.19
Error	Small – Medium	0.042
	Small – Large	0
	Medium - Large	0.0098

We can define the repeatability of adaptation as the ratio of the difference between deterministic and stochastic contributions to evolutionary change over total variation, i.e. $[SS_{selection} - (SS_{chance} + SS_{history})]/ [SS_{selection} + SS_{chance} + SS_{history}]$. A value of one indicates completely deterministic dynamics, and a value of minus one indicates completely stochastic dynamics. Repeatability is -0.087 in small lines, 0.74 in medium lines, and 0.71 in large lines.

2.5 Discussion

I propagated experimental populations of small, medium, or large size ($N_e = 5 \times 10^3$, 5×10^4, and 4×10^5 cells respectively) in a novel environment for 200 generations. By partitioning the variation in growth among lines into selection, chance, and history, I determined which components depend on population size and how this affects the repeatability of evolution at the fitness level. Initial diversity among larger populations was lost as they converged on the same growth rate, whereas diversity among small populations was maintained as they diverged during adaptation. Thus, adaptation is less repeatable in small populations than in larger populations because history is more constraining and selection less efficient in the former.

2.5.1 The transition from stochastic to deterministic dynamics

The main differences in the relative contributions of selection, chance, and history arise between small and medium populations, although we cannot rule out the possibility that a more powerful study would have shown a more continuous effect of population size. This suggests that the transition between stochastic and deterministic dynamics occurs between effective population sizes of 10^3 and 10^4. This is lower than an estimate from microvirid bacteriophages, where the transition occurred between bottleneck sizes of 10^4 and 10^5 (Miller *et al.*, 2011). Stochastic dynamics occur when mutations fix more rapidly than they arise, i.e. when $N_e\mu_b << \ln(N_e s)$ (Desai & Fisher, 2007), and so depend on the effective population size as well as the rate (μ_b) and fitness effects (s) of beneficial mutations. While in *Chlamydomonas reinhardtii*, the estimated mutation rate is 3.23×10^{-10} (Ness *et al.*, 2012) or $6.76 \times$

10^{-11} /site/generation (Sung *et al.*, 2012), the rate per genome could be much greater than in viruses and explain why the transition point was observed at lower N_e. In addition μ_b will depend on the number of genes involved in fitness for a particular environment as well as the specific type of gene interactions, and so the difference may reflect differences in the evolutionary challenge set by different selective environment. Without details of the genetic basis of adaptation in these experiments it is difficult to speculate further.

The greater contribution of selection in medium and large lines than in small lines could be because of higher supply rate or probability of fixing beneficial mutations. It cannot be explained by effects of dilution ratio on the probability of fixing beneficial mutations (Wahl *et al.*, 2002; Raynes *et al.*, 2014) since the dilution ratio was maintained constant across population size treatments in this experiment. Rather, it is likely to result from a reduced supply of beneficial mutations in small lines. Selection was not more effective in large than in medium lines, perhaps because of clonal interference slowing down the rate of fixation of beneficial mutations (Gerrish & Lenski, 1998; de Visser *et al.*, 1999; Colegrave, 2002).

The similar absolute contribution of chance across population sizes contrasts with the prediction that chance should be greater in smaller populations because of their lower supply of mutations and higher degree of drift (Fisher, 1930; Wright, 1931). It is possible that such effects will only occur in much smaller populations than used here.

2.5.2 The importance of historical contingency

Differences in the amount of convergence or divergence in fitness among populations of different sizes could be due to differences in rates of adaptation (Schoustra *et al.*, 2009; Jain *et al.*, 2011) or the ability to cross fitness valleys in rugged fitness landscapes (Szendro *et al.*, 2013). The initial variance among starting genotypes was reduced after evolution in medium and large populations, which is expected if the different histories were converging on the same trait combination. There may be a single fitness peak in this environment and medium and large lines could have climbed it faster than small lines. However, I cannot exclude the

possibility that the lines have reached different peaks of similar heights. However, the maintenance of variance among genotypes evolving in small lines, and the fact that some small lines achieved similar fitness to larger lines suggests that the differences in fitness between small and larger lines are not due entirely to slower rates of adaptation, but result from epistatic interactions. Large and medium lines appear to have ended up on the same peak, whereas small lines have remained trapped on different peaks.

In small populations, the lower supply of mutations can limit the exploration of the fitness landscape and increase the probability of getting trapped on local fitness optima. Larger populations are more likely to find the global fitness optimum because their higher supply of double or double-step mutants makes available a larger proportion of the landscape (Iwasa *et al.*, 2004; Weissman *et al.*, 2009). Convergence in medium and large lines could also have occurred if higher genetic or phenotypic variance within the populations led to the flattening the adaptive landscape, enabling them to move across the landscape more easily than small lines (Whitlock, 1995).

The population sizes investigated here cover a limited range. They are much smaller than most microbial populations (Charlesworth, 2009). However, many isolated microbial populations, such as pathogens initiating an infection, will have their effective population sizes in the range investigated here following environmental change or colonisation of new habitats. While they are of the same order as species such as *Caenorhabditis elegans* with an estimate of 8×10^4 (Charlesworth, 2009) and many plant populations with estimates of 10^3 to 10^4 (Schoen & Brown, 1991), our results are probably only directly relevant to asexual populations without standing genetic variation.

My populations were maintained entirely asexually. In sexual organisms, recombination generally increases the efficiency of selection (Weismann, 1889; Fisher, 1930; Muller, 1932; 1964; Hill & Robertson, 1966; Felsenstein, 1974; Peck, 1994), and should therefore increase repeatability. Thus the threshold between

deterministic and stochastic dynamics seen in my study might be pushed further down in sexual populations. However, whether recombination will reduce the effects of chance and history will depend, in part, on the amount of linkage disequilibrium and the type of gene interactions (Otto *et al.*, 1994; de Visser *et al.*, 2009). Experiments directly examining the effect of sex on the repeatability of adaptation would be valuable.

Another aspect of this system is the lack of initial standing genetic variation. In the short term, adaptation will generally be faster when there is standing genetic variation for fitness (Fisher, 1930). This may affect both the repeatability of adaptation and also the interaction with population size. That is, genetic variation could have a disproportionate effect in small populations which are limited by variation compared to large populations where alleles present at the start will also arise through mutation at some point because of the high supply of mutations. Moreover, the effect might depend on the timescale. Over short timescales, selection will act on the standing alleles rather than the novel mutations because of their greater frequencies (Barrett & Schluter, 2008), while over longer timescales, the contribution of standing genetic variation to adaptation will not be easily distinguishable from that of novel mutations.

2.6 Conclusion

On short evolutionary timescales, my results indicate that adaptation will be repeatable in large populations. If the mechanism of adaptation is well understood, then predictions about outcomes in large populations will be accurate. On the other hand, adaptation will be less repeatable and diversity will be maintained among independent populations if they are of small size. It will therefore be difficult to use evolutionary theory to make predictions about the outcome of environmental change in small populations. The strong effect of history underlines the importance of using different starting genotypes in experiments to investigate the range of potential responses of small populations to environmental change.

3. The effect of sex on the repeatability of evolution in different environments

This chapter is a modified version of a manuscript currently under review as

Lachapelle, J. & Colegrave, N. The effect of sex on the repeatability of evolution in different environments. *Journal of Evolutionary Biology*.

I designed the experiment, carried the laboratory work, did the statistical analyses, and wrote the manuscript. N Colegrave contributed to designing the experiment and writing the manuscript.

3.1 Abstract

The adaptive function of sex has been extensively studied, while less consideration has been given to the potential downstream consequences of sex on evolution. Here I investigate one such potential consequence, the effect of sex on the repeatability of evolution. The repeatability of evolution has important implications for biodiversity, and for making predictions. By comparing the change in fitness, as well as the amount of variance within and among experimental populations of *Chlamydomonas reinhardtii* I find that the importance of selection, chance, and ancestry during evolution is significantly different in sexual populations than in asexual populations. In Bold's minimal medium, sex reduces repeatability overall; in Herbicides sex reduces repeatability among ancestries and increases repeatability within ancestries; in Na_2SO_4 sex increases repeatability among ancestries and reduces repeatability within ancestries; and finally in NaCl sex increases repeatability overall. Thus, sex has important effects on diversity during evolution that are highly dependent on the genetic composition of the population and on the environment. The genetic basis of adaptation is different enough between even relatively simple and similar laboratory environments for recombination to have significantly different effects on evolving

populations. Until we determine the precise mechanism by which the specific environmental attributes mediate the effect of recombination on evolution, we cannot assume that results from experiments in a single environment will generalise to other environments. There is a need for a greater commitment to studying diverse environments for a general and correct interpretation of evolution.

3.2 Introduction

The ubiquity of sexual lineages among eukaryotes is a long-standing problem in biology (Smith, 1978; Bell, 1982). Extensive research has been dedicated to determining the adaptive function of sex, that is the mechanisms for its origin and maintenance over evolutionary time (Lively & Morran, 2014; Becks & Alavi, 2015). However, less consideration has been given to the potential downstream consequences of sex on evolution. While these consequences may or may not have any adaptive significance, they can potentially have important implications for evolution. In this chapter I investigate one potential downstream consequence of sex: the effect of sex on the repeatability of evolution. By altering the repeatability of evolution, sex could have long-term effects on rates of diversification, and consequently on the patterns of diversity that we see today.

The most obvious way that sex and recombination can affect the repeatability of evolution is by increasing the efficiency of selection, either by bringing together beneficial alleles found in different individuals (Weismann, 1889; Fisher, 1930; Muller, 1932), purging the deleterious mutations from the population (Muller, 1964), or releasing beneficial alleles from inferior backgrounds (Hill & Robertson, 1966; Felsenstein, 1974; Peck, 1994). Ample empirical evidence support the idea that sex and recombination increase rates of adaptation to a novel environment (Colegrave, 2002; Kaltz & Bell, 2002; Goddard *et al.*, 2005; Morran *et al.*, 2009; Becks & Agrawal, 2010; Lachapelle & Bell, 2012; Bell, 2012a) and contribute in purging deleterious mutations in constant environments (Zeyl & Bell, 1997; Morran *et al.*, 2009). On the other hand, little is known about the effects sex can have on the

importance of chance and ancestry. There is evidence that recombination increases genetic variation within a population after a single episode of sex (Colegrave, 2002), but none with regards to the effect of sex on diversity among populations over longer evolutionary timescales. Therefore sex has the potential to increase the repeatability of evolution by increasing the contribution of selection, but how it affects the contribution of chance and ancestry remains to be tested empirically.

To determine how sex affects the repeatability of evolution, I propagated diverse asexual and sexual experimental populations of the unicellular green alga *Chlamydomonas reinhardtii* in four different environments. I expect sex will increase the efficiency of selection and therefore increase the repeatability of evolution. I find that sex has important consequences on the repeatability of evolution, and that these effects are highly dependent on the environment, with sex enhancing convergence in some environments and divergence in others. Thus, even in relatively simple and similar laboratory environments, the genetic basis of adaptation is different enough for sex to have different consequences on the repeatability of evolution.

3.3 Material and Methods

3.3.1 Base populations

I generated three genetically different starting points by crossing three different pairs of wild-type strains of *Chlamydmonas reinhardtii*. Ancestry A was generated by using the F1 progeny from a cross between CC-1690 and CC-1691; ancestry B using the F1 progeny from a cross between CC-2342 and CC-2344; and ancestry C using the F1 progeny from a cross between CC-2931 and CC-2937. These strains have been shown to be genetically (Jang & Ehrenreich, 2012) and phenotypically (Malcom *et al.*, 2014) different. The progeny from each cross should retain a fraction of the genetic signature of their two parents and therefore maintain on average the genetic dissimilarity that was present among parents from each ancestry. Thus the different ancestries represent genetically different starting points. Twelve spores

from each ancestry were isolated, for a total of 36. From now on these spores are referred to as the ancestors. Each experimental line was assembled using eight spores from a given ancestry: the asexual lines contained eight spores of a single mating type (I used spores of mating type - for Ancestry A and C, and spores of mating type + for Ancestry B), whereas the sexual lines contained four spores of mating type + and four spores of mating type -. The asexual and sexual lines from a given ancestry thus shared four ancestral spores. The ancestral spores used to assemble the asexual lines do not differ statistically from the ones used to assemble the sexual lines in their growth rates across the four selection environments described below ($F_{1,10}$ = 0.78, P = 0.40). This means that the mode of reproduction treatment is not confounded with differences in starting points.

3.3.2 Selection experiment

For each combination of ancestry and mode of reproduction, I had 6 replicate lines, for a total of 3 x 2 x 6 = 36 independent lines. Each line was propagated in each of four different environments: Bold's minimal medium (referred to as Bold's; Harris, 2009); Bold's minimal medium supplemented with 0.435 μM Atrazine and 0.250 μM S-metalochlor (referred to as Herbicides); Bold's minimal medium supplemented with 7 gL^{-1} Na_2SO_4 (referred to as Na_2SO_4); and Bold's minimal medium supplemented with 5 gL^{-1} NaCl (referred to as NaCl). These environments and concentrations were chosen because they target different aspects of growth (e.g. photosynthesis in the case of Atrazine, synthesis of long chains of fatty acids in the case of S-metalochlor, osmotic and oxidative stresses in the case of NaCl and Na_2SO_4), and because preliminary assays showed that they reduce growth rates to different extents compared to that in the benign environment of Bold's. Each ancestral spore was grown individually from a single colony. Once fully grown, the ancestral spores were pooled together to construct each experimental line, and 24 samples (six replicates in each of four environments) of each mixture were used to initiate each replicate line, which were then propagated independently.

The experiment consisted of vegetative growth cycles interspersed with sexual cycles. The sexual cycles were imposed after about 10, 50, 100, 150, 200, and 260

generations of vegetative growth. The protocol for the sexual cycle was imposed on all lines, even on the asexual lines, which were not expected to mate given that they were composed of spores of only one mating type. Briefly, at the end of a vegetative growth cycle, the spent media was replaced with nitrogen-free media by centrifuging the cultures. The cultures were left static in nitrogen-free liquid media for approximately 24 hours to allow gametogenesis and mating to occur. After this period, the zygotes and 50 μL of culture were transferred to an agar plate, or in the case of the asexual lines 50 μL of culture was transferred to an agar plate. The agar plates were wrapped in aluminium foil and left in the dark for zygote maturation to occur. After four days, mature zygotes were exposed to chloroform vapour for 45 seconds to kill unmated cells, and then placed under the lights for germination. The asexual lines were not exposed to chloroform but put directly under the lights. After two days in the light, the cells were re-suspended in liquid media and transferred back into the vegetative growth cycles. The cultures were then serially transferred every 3-4 days using a 5% inoculum (100 μL into 1900 μL of fresh media). A total of 6 sexual cycles and 60 vegetative cycles were imposed for a total of about 300 generations.

Seven sexual lines (three from the Na_2SO_4 environment and four from the Herbicides environment) went extinct during the experiment because they failed to mate during the sexual cycle. Attempts were made to mate them again whenever this happened but failed repeatedly in these particular cases.

The lines were cultured in 24-well plates, with breathable sealing films to ensure even evaporation and air exchange across the plate (except during mating where the plastic lids were used to ensure optimal light intensity), shaken at 180 r.p.m. with a 3 mm rotation diameter. The cultures were maintained in a growth chamber at 24 degrees Celsius, 60% humidity, and 8000 Lux constant lighting.

3.3.3 Ancestral fitness assay

To estimate the fitness of the ancestral spores used to assemble each selection line, I calculated the maximum growth rate in each of the four selection environments. The

ancestors had been maintained in dim light on Bold's agar throughout the experiment, conditions which limit growth and selection (Harris, 2009). A single colony from each ancestor was grown in Bold's media for two cycles to minimise physiological differences, and then transferred in triplicate to each of the four environments. All cultures were grown for two cycles in the assay environments. Growth was monitored during the second growth cycle in the assay environments by measuring optical density at 750 nm every 8 ± 1 hours. I chose to measure during the second cycle to allow the three replicates one cycle of independent growth and avoid the measurement of initial physiological response to the new environment.

I transformed the optical density measurements (log10 of (optical density x 10 000)) to allow growth models to be fitted. Growth parameters were extracted from a nonlinear model using nonlinear least squares in the 'nlstools' R package (Baty *et al.*, 2015). I first fitted a baranyi model (Baranyi & Roberts, 1994; Baranyi *et al.*, 1995). The lines that could not be fitted using this model were fitted using either a baranyi model without N_{max}, a baranyi model without lag, or a linear model, as appropriate. Model fits were visually inspected to ensure the proper model had been applied. For each combination of environment, ancestry, and mode of reproduction, I identified the fittest ancestral spore as the one with highest maximum growth rate based on the average of the three replicates.

3.3.4 Evolved fitness assays

The evolved lines from each selection environment were assayed in their respective selection environment in separate experiments because of space constraints. For similar reasons, it was impossible for us to assay all 36 ancestral spores and all 36 evolved lines all at once and so I only assayed the fittest ancestral spore, as identified above, along with the evolved lines. This means that my measure of selection is conservative, detecting only the fixation of novel mutations and not sorting of the initial variation.

I assayed four random spores per evolved line. 24 spores (6 lines x 4 spores) were picked from the fittest ancestor to match the number of evolved spores assayed per

ancestry x reproduction mode. All colonies were grown in Bold's liquid media for one growth cycle to minimise physiological differences, and then transferred to the environment in which the evolved lines were selected. Growth was monitored during the second cycle in the assay environment and growth parameters estimated as described above.

3.3.5 Statistical analyses

All analyses were performed in R version 3.2.1. To determine if the ancestral spores used to assemble the sexual lines differ from the ancestral spores used to assemble the asexual lines I fitted a mixed effect model using the lmer function in the R package 'lme4' (Bates *et al.*, 2015). The mode of reproduction (asexual or sexual) was set as a fixed factor, while environment, ancestry, and spore within ancestry were set as random factors. P values were obtained using the R package 'lmerTest' (Kuznetsova *et al.*, 2014) with type III errors in an analysis of variance and Sattertwhaite approximation for degrees of freedom by using the normal approximation.

The effect of recombination on selection was determined individually for each selection environment by fitting mixed effect models using the lmer function, with mode of reproduction (asexual or sexual) and selection (ancestral or evolved) as fixed factors, and ancestry, line within ancestry, and spore within line within ancestry as random factors. I allowed for random intercepts and slopes.

To determine the effect of recombination on ancestry, chance, and diversity within lines, I calculated the difference between evolved variances and ancestral variances. Thus a positive change in variance indicates that there is more variation after evolution than at the start (i.e. divergence over time), whereas a negative change in variance indicates that there is less variance after evolution than at the start (i.e. convergence over time). The evolved variances were extracted from a model with ancestry, line within ancestry, and spore within line within ancestry as random factors. Separate models were fitted for each combination of environment and mode of reproduction. The ancestral variances were extracted from a model with ancestry

and spore within ancestry as random factors. The among-line ancestral variance was set at zero. Note here that the evolved data and the ancestral data come from different fitness assays. Temporal heterogeneity in environmental conditions between assays can lead to differences in growth rates. It is unlikely that temporal heterogeneity would interact with the mode of reproduction treatment, and so the variance estimates for the asexuals and the sexuals should be affected to the same extent. The actual value of the change in variance is likely to be inexact, and values near zero need to be interpreted with reserve.

This approach of using the change in variance differs from the one I used in the previous chapter where I calculated the relative contribution of selection, chance, and ancestry by dividing the evolved variance by the total evolved variance. It is only appropriate to use proportions to compare treatment levels for their effects on selection, chance, and ancestry, when the initial variance is the same across all treatment levels. For example, if lines are isogenic at the start and the same genotype is used across all treatments, then there is no need to correct for initial variance. However, in cases such as in the experiment reported here where lines are diverse at the start, and sexual and asexual lines cannot be assembled using the same genotypes (because of mating type constraints), it is not appropriate to compare evolved variances without correcting for initial variance. Differing amounts of variance can affect the potential for convergence and divergence among histories, among line, within lines. This is why I report the change in variance instead of the proportion of the total variance explained by either chance or ancestry.

To determine the statistical significance of the differences in the change in variance between asexual and sexual populations I did a randomisation test. I randomly allocated each evolved spore to a line, ancestry, and mode of reproduction (keeping spores within their environment of selection), each ancestral spore to an ancestry and mode of reproduction, and then performed the analysis described above to calculate the change in variance. The number of times the random absolute change in variance was as large or larger than the absolute observed change in variance over the total number of randomisations (10,000) is my significance statistic.

3.4 Results

I picked four different environments in which to study the consequences of sex on the repeatability of evolution. The Na2SO4 environment is the most severe with slowest growth rates, followed by NaCl, Herbicides, and Bold's (Figure 3.1; Table 3.1). Not only do the four environments affect the growth of the ancestors to different extents, but they also reveal differing amounts of variance in fitness (Figure 3.1; Table 3.1). The coefficient of variation among spores within ancestries is largest in Herbicides, followed by NaCl, Bold's, and Na2SO4. The coefficient of variation among ancestries is largest in NaCl, followed by Herbicides, Na2SO4, and Bold's. Thus, the four environments affect growth differently and represent a true test of the generality of the consequences of sex on the repeatability of evolution.

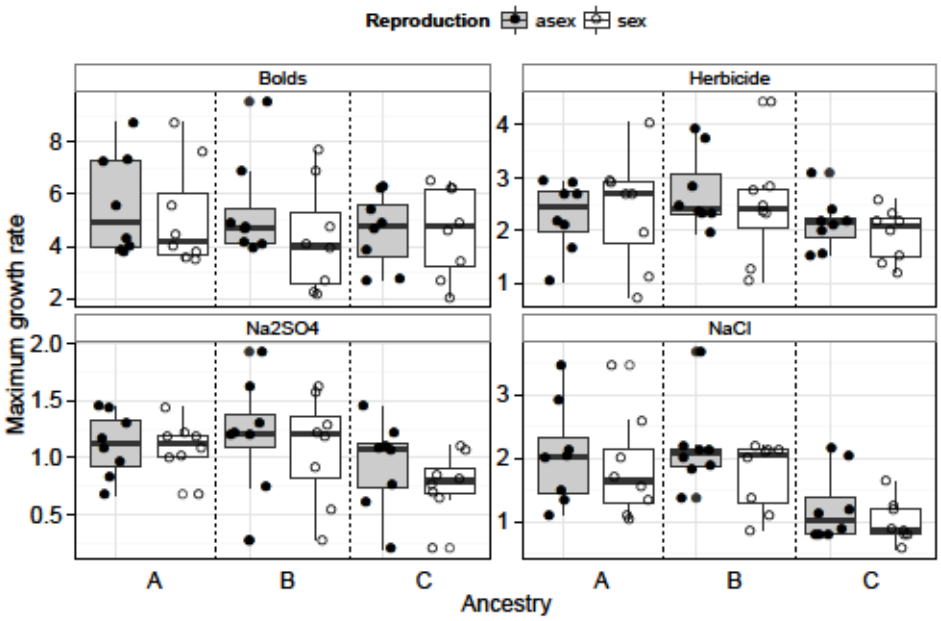

Figure 3.1 Growth rate of the eight ancestral spores used to initiate each asexual and sexual selection lines, in each of the four selection environments.
Each point represents the average of the three assay replicates.

The variance in fitness among ancestral spores within ancestries tends to be greater than among ancestries (Table 3.1), indicating that there is plenty of standing genetic variation available at the start of the experiment for selection to sort. While the fitness of each ancestry might be similar in each environment, the fact that the three

ancestries were generated from different genotypes and that the four different environments reveal differing amounts of variance in fitness, implies that the different ancestries are sufficiently different genetically to validate my test of the consequences of sex on the importance of ancestry.

Table 3.1 Variance among ancestral spores and ancestries in each of the four selection environments.
CV is the coefficient of variation.

Environment	Reproduction	Source	Variance	Mean maximum growth rate	CV
Bolds	asexual	Spore	0.984	5.20	0.191
		History	3.53×10^{-16}		3.62×10^{-9}
	sexual	Spore	1.98	4.67	0.301
		History	0.00		0.00
Herbicide	asexual	Spore	0.285	2.38	0.224
		History	0.0502		0.0942
	sexual	Spore	0.706	2.24	0.375
		History	1.11×10^{-14}		4.70×10^{-8}
Na_2SO_4	asexual	Spore	0.0333	1.08	0.169
		History	0.00		0.00
	sexual	Spore	1.92×10^{-15}	0.982	4.46×10^{-8}
		History	0.0182		0.137
NaCl	asexual	Spore	0.312	1.81	0.309
		History	0.202		0.248
	sexual	Spore	0.302	1.53	0.360
		History	0.171		0.271

3.4.1 The effect of sex on selection

I propagated asexual and sexual replicate experimental populations in each of the four selection environments for about 300 generations. The effect of selection is

estimated by comparing the fitness of evolved spores to that of the fittest ancestral spore, such that the greater the fitness of the evolved spore is relative to its ancestor, the greater the contribution of selection to evolutionary change. The evolved sexual lines have higher growth rates than the evolved asexual lines after evolution in Na_2SO_4 (Figure 3.2, Table 3.2; effect of reproduction:selection interaction $F_{1,63}$ = 18.1, P = 7.15 x 10^{-5}) and in NaCl (effect of reproduction:selection interaction $F_{1,66}$ = 6.87, P = 0.0109). There is no effect of selection or interaction between reproduction and selection after evolution in Herbicides (effect of selection $F_{1,62}$ = 0.535, P = 0.467; effect of reproduction:selection interaction $F_{1,62}$ = 0.149, P = 0.701). There is a significant effect of selection in Bold's, but opposite to expectation with evolved spores having lower growth rates than the fittest ancestral spore (effect of selection $F_{1,66}$ = 35.4, P = 1.11 x 10^{-7}) and no effect of interaction between recombination and selection ($F_{1,66}$ = 2.62, P = 0.110).

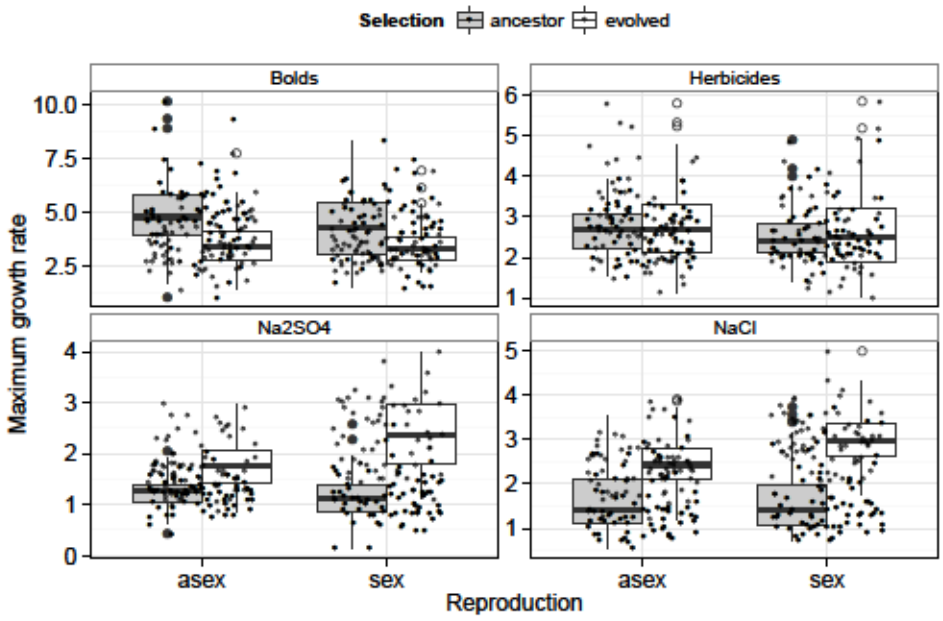

Figure 3.2 Growth rate of ancestral and evolved spores in the corresponding selection environment.
Each point represents the average of the three assay replicates. There are four spores for each of 36 lines (except in Herbicides where there are 32 lines and in Na_2SO_4 where there are 33 lines). The larger data points are part of the boxplot layer and represent outliers.

3.4.2 The effect of sex on divergence of ancestries

If the different ancestries diverged during evolution, then I should see an increase in variance among ancestries, and if the different ancestries converged during evolution, then I should see a decrease in variance among ancestries. Ancestries diverged during evolution in Herbicides and Na_2SO_4, converged in NaCl, whilst no change was observed after evolution in Bold's (Figure 3.3). The sexual populations diverged more than their asexual counterparts in Herbicides ($P < 0.0001$), diverged less than their asexual counterparts in Na_2SO_4 ($P = 0.0054$), whilst sex had no measurable effect in Bold's ($P = 0.26$) and NaCl ($P = 0.26$).

Table 3.2 The effect of recombination on the efficiency of selection at increasing growth rates in each of the four selection environments.
The parameter estimates for the fixed effect are show, where 'Selection has two levels (ancestral and evolved) and 'Reproduction' has two levels (asexual and sexual).

Environment	Effect	Estimate	SE
Bold's	Intercept	4.9	0.22
	Selection (evolved)	-0.63	0.26
	Reproduction (sexual)	-1.4	0.26
	Selection (evolved) : Reproduction (sexual)	0.60	0.37
Herbicides	Intercept	2.7	0.23
	Selection (evolved)	-0.11	0.20
	Reproduction (sexual)	0.16	0.20
	Selection (evolved) : Reproduction (sexual)	-0.11	0.29
Na_2SO_4	Intercept	1.2	0.11
	Selection (evolved)	-0.095	0.12
	Reproduction (sexual)	0.56	0.12
	Selection (evolved) : Reproduction (sexual)	0.71	0.17
NaCl	Intercept	1.6	0.25
	Selection (evolved)	0.86	0.14
	Reproduction (sexual)	0.068	0.14
	Selection (evolved) : Reproduction (sexual)	0.51	0.19

3.4.3 The effect of sex on divergence of replicate lines

If the replicate lines diverged during evolution, then I should see an increase in variance among lines, and if the replicate lines have evolved in parallel, the variance should be equal to zero. Divergence has occurred in all selection environments in this experiment (Figure 3.3). The sexual lines diverged less than their asexual counterparts during evolution in Herbicides ($P < 0.0001$), diverged more than their asexual counterparts during evolution in Na_2SO_4 ($P < 0.0001$) and Bold's ($P = 0.0084$), whilst sex had no measurable effect in NaCl ($P = 0.26$).

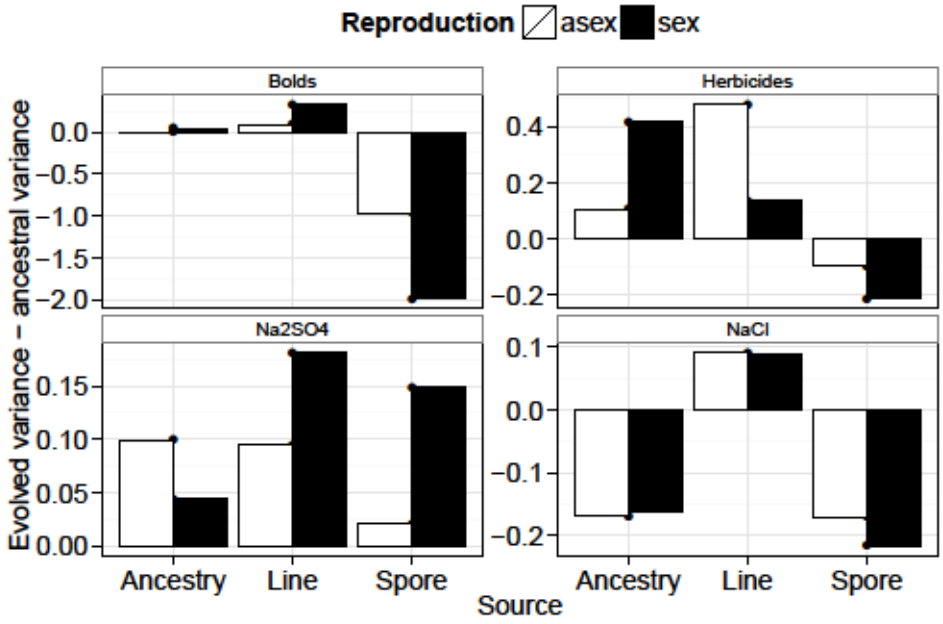

Figure 3.3 Change in variance after evolution in each selection environment in asexual and sexual populations.
Ancestry represents variance among ancestries, Line represents variance among replicate lines within ancestries, and Spore represents variance among spores within lines within ancestries.

3.4.4 The effect of sex on diversity within lines

If diversity within lines increased during evolution, then I should see an increase in variance among spores, and if diversity was lost during evolution, then I should see a decrease in variance among spores. Note that my design for the fitness assays is such that I can separate out variance within lines from variance from measurement error. There is more diversity within lines after evolution in Na_2SO_4, whilst there is less

diversity within lines after evolution in Bold's, Herbicides, and NaCl (Figure 3.3). The sexual lines had a greater increase in diversity within lines than their asexual counterparts after evolution in Na_2SO_4 ($P = 0.028$), a greater decrease in diversity after evolution in Bold's ($P = 0.036$), whilst sex had no measurable effect on diversity within lines in Herbicides ($P = 0.075$) and NaCl ($P = 0.21$).

3.5 Discussion

Most of the research on sex has focussed on the mechanisms for its origin and maintenance over evolutionary time, while much less consideration has been given to the potential downstream consequences sex can have on the repeatability of evolution. I propagated sexual and asexual lines in four different novel environments for 300 generations. By measuring the change in fitness, the change in variance among ancestries and among replicate lines, and the change in diversity within lines, I was able to determine the consequences of sex on the contribution of selection, ancestry, and chance to evolution.

The general prediction is that sex and recombination increase the repeatability of evolution by increasing the efficiency of selection (Burt, 2000; de Visser & Elena, 2007). My results refute this hypothesis. I find that sex has significant consequences for the repeatability of evolution that are far from general, differing in each environment investigated. In Bold's, recombination has no effect on selection and ancestry but increases chance, and hence reduces repeatability overall; in Herbicides recombination has no effect on selection, but increases effects of ancestry and reduces chance; in Na_2SO_4 recombination increases effects of selection and chance, but reduces effects of ancestry; and finally in NaCl recombination increases effects of selection, but has no effect on ancestry or chance, and hence increases repeatability overall. These variable outcomes indicate that the effects of sex are highly dependent on the specific genetic basis of adaptation, the precise mechanism of which remains to be fully determined.

3.5.1 The lack of generality of the effect of sex on the repeatability of evolution

We know from theory that the effects of sex depend on the genetic basis (e.g. the number of genes and their pattern of interaction) of adaptation (Otto *et al.*, 1994; Kondrashov & Kondrashov, 2001; Hadany & Beker, 2003; Watson & Wakeley, 2005; de Visser *et al.*, 2009). The fact that I observed dramatically different effects of sex implies that the genetic basis of adaptation differs significantly between the environments we used, despite the fact that all were simple and relatively similar laboratory environments.

The lack of a general effect of sex is consistent with other findings of the effect of sex on the evolution of herbicide resistance (Lagator *et al.*, 2014b) and with the contrasting results in terms of repeatability of evolution reported for sexual species (Teotonio & Rose, 2000; Teotonio *et al.*, 2002; Joshi *et al.*, 2003; Kawecki & Mery, 2003; Griffiths *et al.*, 2005; Fragata *et al.*, 2014). It most likely reflects differences in linkage disequilibrium as this is an important factor in determining the contribution of chance and ancestry during evolution (Weinreich & Chao, 2005).

The fitness landscape (i.e. the regression of individual fitness on genotypic space) is a useful heuristic for thinking about the contribution of chance and ancestry to evolution. In fitness landscapes, peaks represent trait combinations of high fitness. When there are multiple fitness peaks, the importance of chance and ancestry depends critically on the probability of shifting from sub-optimal to optimal fitness peak. Peak shifts can occur through double-step or double mutants (Gillespie, 1984; Weinreich & Chao, 2005) if the combination of two mutations takes the population to a peak other than the one currently occupied. Recombination will tend to generate such ‘escape’ genotypes if linkage disequilibrium is negative, and will break apart escape genotypes when linkage disequilibrium is positive (Weinreich & Chao, 2005). Differences in linkage disequilibrium can arise because of differences in population size, in the distance to a fitness peak, and/or in the genetic basis of adaptation (Otto *et al.*, 1994; Weinreich & Chao, 2005; de Visser *et al.*, 2009). The four environments in our selection experiment differed with respect to all of these factors and so

provided a strong test of the robustness of recombination to differences in linkage disequilibrium.

Population size will affect the repeatability of evolution in both asexual and sexual population by altering the supply of beneficial mutations and the amount of clonal interference (Gerrish & Lenski, 1998; Lachapelle *et al.*, 2015). In small populations, peak shifting will rely on a stochastic process of sequential fixation of single mutations, whereas in large populations peak shifting can occur by a deterministic process of simultaneous fixation of jointly beneficial mutations (Carter & Wagner, 2002; Iwasa *et al.*, 2004). In sexual populations, recombination can break apart the escape genotypes before they become fixed. Peak shifting then becomes a stochastic process, where deleterious single mutants need to rise to sufficiently high frequency for recombination to combine them and generate the escape genotypes more often than it breaks them apart (Weinreich & Chao, 2005). Differences in population size can therefore affect the effect of recombination by altering the frequency of escape genotypes and thus the stochastic or deterministic nature of peak shifting.

The distance from a fitness peak can also affect the role of recombination during evolution by determining the number of beneficial mutations available, the number of possible trajectories, and the amount of linkage disequilibrium (Otto *et al.*, 1994). For example, as the distance to the peak increases, recombination gains a greater advantage by speeding up the rate at which the population reaches the peak (de Visser *et al.*, 2009). Differences in the type of interactions among genes will also affect the effect of recombination on the repeatability of evolution. Negative epistasis, where the fitness effect of many alleles is lower than predicted by the product of their individual effects, can cause negative linkage disequilibrium and therefore increase the response to selection and the probability of peak shifting (Barton, 1995). Sign epistasis, where the sign of the fitness effect of one mutation depends on what alleles are present at other loci, can also affect the role of recombination by altering the ruggedness of the fitness landscape and the accessibility of certain mutational paths (Weinreich & Chao, 2005).

Hence, while my data does not identify which attribute, population size, distance to a fitness peak, or genetic basis of adaptation, is driving the inconsistency in effects of sex, it suggests that the parameter space used by theoretical studies probably reflects an appropriate if not underestimation of the degree of variation among natural environments. The effects of sex on evolution are highly dependent on the genetic background and the environment and we therefore cannot assume that results from experiments in a single genotype or environment will generalise to other environments. Further experiments need to be carried out to disentangle the role of genetics and different environmental attributes.

3.5.2 The efficiency of selection in initially diverse populations

In initially diverse populations, selection can act on standing genetic variation and on new mutations. One approach to measuring the efficiency of selection when experimental lines are initially diverse is to compare individual evolved spores to individual ancestral spores. I used the fittest ancestral spore as my comparison. If all the evolved spores perform as well as the fittest ancestral spore, sorting has occurred, leading to the fixation of the fittest ancestral spore. If all the evolved spores perform better than the fittest ancestral spore, new mutations (and/or recombination in sexual populations) have contributed to evolutionary change. These inferences assume that sorting will occur before beneficial mutations arise in less fit ancestral spores and become fixed.

An alternative approach to measuring the efficiency of selection in initially diverse populations would be to use population-level fitness estimates. I have opted against population estimates as they depend heavily on the composition of the population, i.e. the number of different genotypes and their respective frequency. Therefore any alteration of the composition through storage and revival of populations for example, would lead to erroneous estimates. Furthermore, contrary to spore-level comparisons, population-level fitness change estimates will detect the action of selection, but will not reveal any information about the contribution of standing genetic variation compared to that of new mutations to evolutionary change.

My results suggests that in Na_2SO_4 and NaCl, new mutations played a role in adaptation as the evolved spores have higher growth rates than the fittest of the ancestral spores. In the Herbicide environment, the growth of the evolved spores is not, on average, any different from that of the fittest ancestral spore. This suggests that adaptation occurred solely through sorting, with no contribution from new mutations. Evolution is more likely to occur from standing genetic variation when the variation is relevant to growth in the new environment, in high enough frequency, and reduced population sizes limit the contribution of novel mutations (Hermisson & Pennings, 2005). Indeed, the coefficient of variation within lines was largest in the Herbicides environment, and population sizes rebounded the quickest amongst all environments, suggesting that the large amount of variance was relevant and sufficient in this environment to lead to rapid evolutionary responses. A rapid response is consistent with adaptation from standing variation that is immediately available and in high frequency (Barrett & Schluter, 2008).

Evolution in the Bold's environment led to lower growth rates than that of the fittest ancestral spore. Bold's is a benign environment where growth rates are high, and beneficial mutations are likely to be rare. In such cases the effect of selection is therefore more to remove deleterious mutations in order to maintain growth rates than to fix beneficial mutations, an effect that I cannot measure with my data. The lower growth rates could be attributable to failure to remove deleterious mutations, but also to inefficient sorting of the standing genetic variation or to a trait other than maximum growth rate being under selection.

When there is initial variance in fitness, it will be sorted quicker the larger it is and lead to an increase in population mean fitness. Variance in fitness is initially high in both the asexual and sexual lines in Bold's (Table 2). As a rough estimate, for a selective advantage of 0.1 (based on the variance present initially in the lines), and an initial frequency of 1/8, I expect the fittest spore to rise to 99% frequency within 45 generations. Diversity was almost completely lost within both the asexual and sexual lines, which is further evidence that sorting did occur. It is therefore unlikely that inefficient sorting in the asexual and sexual lines is responsible for their lower mean

fitness. It is also unlikely that deleterious mutations fixed (either singly or through hitchhiking) given the short evolutionary timescale (300 generations) and the relatively large deleterious effect size that would be needed to produce such drop in growth rate. Ultimately, I cannot exclude the possibility that slower growth rates arose both in asexual and sexual lines because selection in Bold's favours greater competitive ability, higher carrying capacity, or slower growth rates (Schaum & Collins, 2014) instead of faster growth rates.

3.6 Conclusion

Sex has important downstream consequences on diversity within and among populations. I find that sex affects the efficiency of selection, and hence the degree to which fitness increases, which is consistent with what other studies on the adaptive function of sex have found. But I also find that sex affects the contribution of chance and ancestry, and hence the degree to which populations converge or diverge in fitness during evolution. By altering the repeatability of evolution, sex could have long-term effects on rates of diversification, and affect our ability to use evolutionary theory to make predictions about the outcome of environmental change. However, I find that the consequences of sex on the repeatability of evolution are not general, with different consequences in different environments. Even the relatively simple and similar environments used here appear different enough to evolving populations to lead to different effects of sex on patterns of change in diversity. I can only assume that natural environments will differ even more radically. Hence, overall, my results indicate that the effect of sex on evolution of populations is highly dependent on genetic background and environment. More rigorous tests are needed to determine the exact mechanisms by which environmental attributes mediate the effect of recombination. But until then, a greater commitment to using many environments should be given in order to reduce biased and specific results in evolution experiments.

4. The effect of evolutionary rescue history on extinction risk during subsequent severe environmental change

4.1 Abstract

Extinctions can have major impacts on biodiversity and ecosystem functioning. To understand and predict what effect environmental change will have, we need to determine what factors affect the overall probability of populations of going extinct during environmental change and the amount of variance in extinction probability within and among populations. I tested the effect of selection history on extinction risk during environmental change in the green alga *Chlamydomonas reinhardtii*. I exposed multiple spores from multiple populations that have survived zero, one, or two events of evolutionary rescue in the past, to a range of different severe environmental change. I found that the overall extinction risk does not depend on the evolutionary rescue history. Instead, it depends on the most recent selection environment, with adaptation to growing in the dark, as opposed to growing in the light, most severely constraining the number of novel environments in which populations can grow. The repeatability of extinction also differs significantly among selection histories, being especially low in different environments and in different lines from a given selection history. Hence, survival during severe environmental change depends on costs of adaptation to the most recent selection environment, and the amount of variance in resistance to novel environments within and among populations.

4.2 Introduction

We live in a changing world, where changes are usually for the worse and often lead to population extinction (Bürger & Lynch, 1995; Bell & Collins, 2008). Determining what factors favour survival is therefore critical for predicting the outcome of severe

environmental changes. We know from experiments that the probability of survival is higher in larger populations (Bell & Gonzalez, 2009; Willi & Hoffmann, 2009), with higher amounts of genetic variation (Agashe *et al.*, 2011; Lachapelle & Bell, 2012), immigration (Bell & Gonzalez, 2011; Lagator *et al.*, 2014b), and lower rates of environmental change (Perron *et al.*, 2008; Bell & Gonzalez, 2011; Lindsey *et al.*, 2013). However, lineages also differ in the number and type of environmental changes they have survived in the past. Much less is known about the effects such differences in selection history can have on extinction risk during further environmental change (Gonzalez & Bell, 2013; Lagator *et al.*, 2014a). In this chapter I test whether surviving one or more severe environmental changes affects extinction risks during further environmental change.

The survival of a population in an environment that reduces growth rates to below zero, a process called 'evolutionary rescue' (Gomulkiewicz & Holt, 1995), occurs when a variant with a positive growth rate, whether it was present in the population at the time of environmental change or arose after through mutation and/or recombination, rises in frequency through natural selection and restores the growth of the population. It is unclear whether the changes that occur within populations during evolutionary rescue lead to lineages that are more evolutionary constrained or more evolutionary labile. We might expect a history of evolutionary rescue to have an effect on future extinction risks if it consistently affects evolvability or costs of adaptation.

Evolvability is the ability to respond to natural selection through an enhanced ability to generate and/or use genetic variation. High evolvability can arise if there are genes that constitutively increase the genomic mutation rate (Shaver *et al.*, 2002) or modulate the mutation rate (Metzgar & Wills, 2000; Erill *et al.*, 2006), and hence increase the supply of variation; if there are mechanisms that promote gene exchange or recombination such as conjugation, viral infection (Poon & Chao, 2004), and sex (Colegrave, 2002; Lachapelle & Bell, 2012); or if there are mechanisms that change the type of interactions between genes to promote a more modular genome (Weinreich *et al.*, 2006; Colegrave & Collins, 2008). History can alter the

evolvability of a population if the selection environments differ in whether they promote or hinder the survival of evolvable types. For example, a high mutation rate might be deleterious in a benign environment where it can lead to a depression of population fitness, but advantageous in a novel environment where it can increase the probability of generating a beneficial variant. Hence if evolutionary rescue consistently leads to greater evolvability, through such traits being selected directly or indirectly through selection on other traits, the probability of survival should increase with number of events of rescue sustained in the past. Alternatively, if evolutionary rescue consistently leads to lower evolvability, the probability of survival should decrease with number of events of rescue sustained in the past.

Rescue history may also affect extinction risks if it mediates costs of adaptation than can arise because of epistasis, antagonistic pleiotropy, or mutation accumulation. Epistasis is a non-additive type of gene interaction that leads to rugged fitness landscapes. In rugged landscapes, the probability of jumping from one fitness peak to another decreases as the population climbs a peak, because the probability of a mutation with effect size large enough to make the jump decreases (Buckling *et al.*, 2003). Hence specialisation in one environment can be costly as it can reduce the ability to diversify into other environments if the previous environment ceases to exist. Epistasis can also lead to the opposite effect, that is, it can increase fitness in alternative environments when resistance to one stressor leads to a reduction of the costs of resistance to the new stressor (Trindade *et al.*, 2009; Ward *et al.*, 2009; Lagator *et al.*, 2014a).

Pleiotropy occurs when a gene affects more than one trait, such that selection on one of these traits can indirectly change the others. When the effects are antagonistic, then a cost of adaptation ensues and leads to lower fitness in an alternative environment (MacLean *et al.*, 2004), whereas when the effects are positive, an increase in fitness in an alternative environment ensues (Walley *et al.*, 1974; Vogwill *et al.*, 2012; Lagator *et al.*, 2013). Costs of adaptation can also arise through mutation accumulation, when mutations with neutral effects in the current environment but deleterious effects in alternative environments accumulate over time

(Kawecki, 1994; Fry, 1996). Hence a history of evolutionary rescue can affect subsequent extinction risks by mediating the evolution of specialisation in rugged fitness landscapes, or the accumulation of positive or negative genetic correlations.

Few experiments have investigated what effect selection history can have on extinction risk. Gonzalez and Bell (2013) selected replicate populations of two species of yeast, *Saccharomyces cervisiae* and *S. paradoxus* in different concentrations of salt before exposing all surviving populations to an initially lethal concentration of 150 gL^{-1} NaCl. Survivability was increased with prior selection in high concentrations of salt for one species, and by prior selection in low concentrations of salt for the other species. In another study, Lagator *et al.* (2014a) selected replicate populations of the green alga *Chlamydomonas reinhardtii* in one of three herbicides before exposing all surviving populations to the two other herbicides sequentially. Survivability during exposure to the second and third herbicides was either increased, decreased, or not affected, depending on what herbicide in particular was used for the initial selection phase. These experiments indicate that selection history can have a diversity of effects on extinction risks, although more work is clearly needed to understand the circumstances under which the differing effects occur.

In this chapter, I make use of a unique set of lineages of *C. reinhardtii* that have undergone two back-to-back events of evolutionary rescue in the laboratory, and tested whether repeated events of rescue affect the ability to survive further environmental change. I sampled from different time points in the history of these lineages (before any rescue event, after the first event of rescue, and after the second event of rescue), exposed all to a range of different novel environments, and tracked population density and extinction over time. I used multiple lineages from each time point to determine the repeatability of extinction, and multiple novel environments in order to estimate the effect of rescue history on the general proneness to extinction.

4.3 Material and Methods

4.3.1 Selection history

The selection history of the lineages used in this experiment is depicted in **Figure 4.1**. In 1997, experimental lines of the unicellular green alga *Chlamydomonas reinhardtii* were set-up using spores from a cross among standard laboratory strains (CC-124 x [CC-1952 x (CC-1952 x CC-2343)]). Four types of lines were set-up as described in Bell (2005): sexual mass-transfer (obligately sexual propagated by many zygotes); sexual single-zygote (obligately sexual propagated by single zygote); unselected (sexual lines where unmated cells are not killed at transfer); and asexual (obligately asexual lines propagated en masse). These lines were propagated on Bold's minimal medium solidified with agar, phototrophically in the light. I refer to them as the 'light lines'. They have not undergone evolutionary rescue since isolation from nature and act as a control in my experiment.

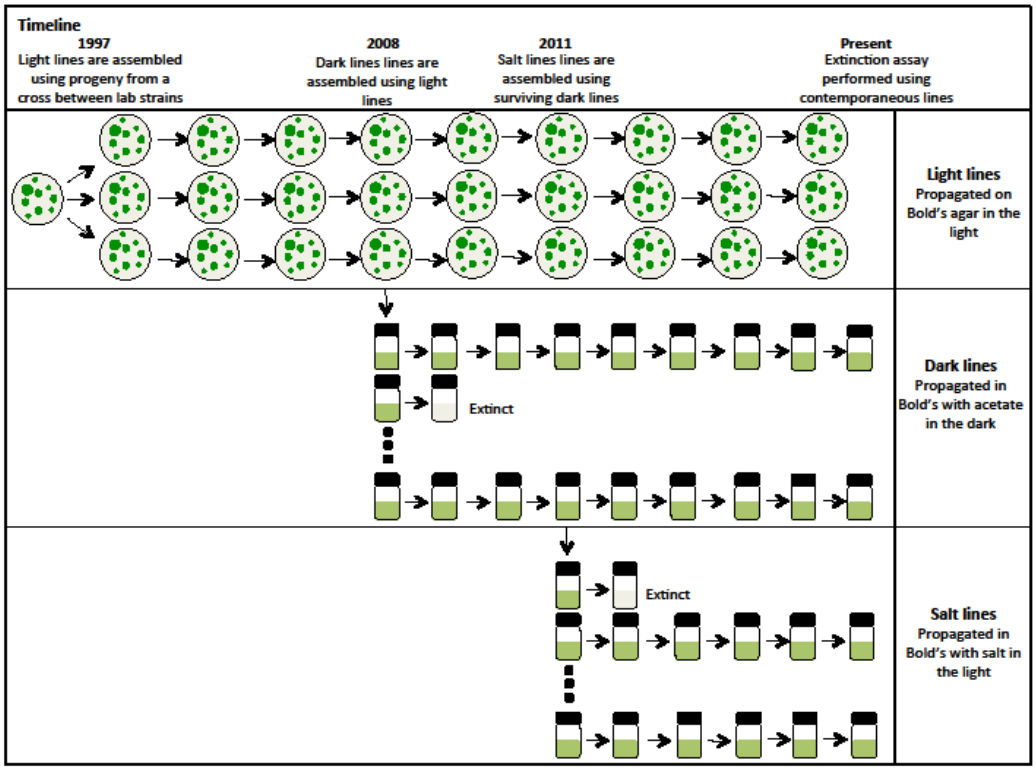

Figure 4.1 Schematic of the selection history of the light, dark, and salt lines.

A decade later, three of the sexual mass-transfer light lines were used to initiate 2880 lines which were propagated in the dark in Bold's minimal medium supplemented with 1.2 gL^{-1} sodium acetate as described in Bell (2012b). Only 241 lines (8.4%) survived. I refer to these lines as the 'dark lines' and they have undergone one event of evolutionary rescue.

In 2011, forty of the dark lines were used to initiate 96 lines which were propagated in steadily increasing concentrations of NaCl as described in Lachapelle and Bell (2012). Ten lines are now surviving in 36 gL^{-1} NaCl. I refer to these lines are the 'salt lines' and they have undergone two events or evolutionary rescue, first the dark, then high salt.

4.3.2 Extinction assay

I isolated four spores from each of five lines from each of the three histories. Since there are only three ancestral lines for the dark lines, I used the three sexual mass-transfer lines as well as two of the asexual light lines, which have been propagated in parallel. Each spore was assayed three times, in each of six environments for a total of 1080 cultures. The six environments are: Bold's minimal liquid media (referred to as 'Bold's'; Harris, 2009); Bold's supplemented with 1.2 gL^{-1} sodium acetate and maintained in the dark (referred to as 'Dark'); Bold's supplemented with 20 gL^{-1} NaCl (referred to as 'NaCl'); Bold's media supplemented with 0.4M Atrazine, a herbicide (referred to as 'Atrazine'); Bold's supplemented with 0.1 μM $CuSO_4$ (referred to as $CuSO_4$); and Bold's buffered to pH4 with a phosphate solution (0.43 gL^{-1} Na_2HPO_4 + 3.36 gL^{-1} KH_2PO_4; referred to as pH4). All cultures were grown phototrophically in the light, except in the Dark environment where all growth had to be heterotrophic.

The concentrations used for the three novel environments Atrazine, $CuSO_4$, and pH4 were determined by running preliminary growth assays with six wild-type strains (CC-1690, CC-1952, CC-2342, CC-2344, CC-2931, CC-2937). My use of wild-type strains in these preliminary assays ensured that my choice of concentration was independent of the biological material used in the extinction assay. The wild-type

strains were grown in a range of different concentrations of Atrazine, $CuSO_4$ and pH, and the concentration that reduced cell densities to just above the detection limit of the spectrophotometer after two growth cycles was chosen. This ensured that the concentration was severe enough to reduce growth, but would not lead to immediate extinctions (which would limit my ability to detect variance in extinction risk).

To start the extinction assay each spore was expanded from a single colony in its home environment (i.e. light lines in Bold's, dark lines in Dark, salt lines in NaCl). I chose to expand the spores into different environments because I could find no single common environment that would not severely disfavour the growth of one history over that of the others. After one cycle of expansion, the spores were transferred to all six assay environments. Cultures were then serially transferred once every 7 days by diluting 10 µL of culture into 190 µL fresh media in 96-well plates, cultured at 26 degrees Celsius, 60% air humidity, and 7150 Lux constant light intensity.

At the end of each growth cycle, every culture was inspected using an inverted microscope to record the presence or absence of living cells. A culture was deemed extinct if the absence of living cells was recorded for two cycles in a row. The cell density was also estimated at the end of each growth cycle by measuring the optical density at 750 nm with a spectrophotometer. The assay was terminated after 11 cycles (about 55 generations) or later in the case of some environments, whenever the number of extinctions had stabilised for two cycles and none of the cultures were on the brink of extinction.

4.3.3 Statistical analyses

All analyses were done in R version 3.2.1. The extinction dynamics were analysed by performing survival analyses using Cox proportional hazards with mixed effects, which assumes Gaussian random effects, with the 'coxme' R package (Therneau, 2015). In all models, I included a 'Censor' variable for spores that had not gone extinct by the end of the assay.

More precisely, to determine if there is a general effect of evolutionary rescue history on extinction risk, I fitted a model with rescue history as a fixed factor, and assay environment, line, and spore within line as random factors.

To compare extinction dynamics between evolutionary recue histories in each environment, I fitted a coxme model with rescue history as a fixed factor, and line and spore within line as random factors. The model was applied to each environment individually, and only in cases where extinctions had occurred in all histories. This is because proper model fitting requires at least one event to have occurred in each level of the fixed factor. Since only two environments out of six had extinctions in all rescue histories, I also computed two-tailed Fisher's exact tests for independence of number of extinction events and rescue history in a contingency table.

To compare the extinction risk between different environments, I fitted a survival model with assay environment as a fixed factor, and line and spore within line as random factors. A separate model was fitted for each rescue history.

Finally, to estimate variance in extinction risk, I fitted a model for each rescue history with line, spore within line, environment, the combination of line and environment, the combination of spore and environment, as random factors. Note that the coxme function does not accept interaction terms for the random factors, and I therefore created two new variables by pasting line and environment or spore and environment together. To determine the significance of the differences in variance between rescue histories, I calculated F ratios. The degrees of freedom were calculated based on an analysis of variance model.

To compare the mean and variance in yield of surviving lines among recue histories I fitted a mixed effect model using the lmer function in the R package 'lme4' (Bates *et al.*, 2015) with assay environment, line, spore within line, the interaction between line and assay environment, and the interaction between spore and environment as random factors. A separate model was fitted for each rescue history. P values were obtained using the R package 'lmerTest' (Kuznetsova *et al.*, 2014) with type III

errors in an analysis of variance and Sattertwhaite approximation for degrees of freedom by using the normal approximation. To determine the significance of the differences in variance between rescue histories, I calculated F ratios. The degrees of freedom were calculated based on an analysis of variance model.

4.4 Results

4.4.1 Extinction risk is independent of evolutionary rescue history

If evolutionary rescue affects the ability to survive further severe environmental change, I would expect extinction risks to depend on the number of prior events of evolutionary rescue. This is not what I observe. The dark lines, which have undergone one event of evolutionary rescue, have the greatest overall extinction risk with 47% of spores going extinct, followed by the control light lines with 40% of spores going extinct, and the salt lines, which have undergone two events of rescue with only 30% of spores gone extinct. The overall extinction dynamics of the dark lines are significantly different from that of the control light lines (56% greater extinction risk, $z = -2.12$, $P = 0.034$), and that of the salt lines (152% greater extinction risk, $z = -5.42$, $P = 5.1 \times 10^{-8}$).

Examination of the extinction risk in each environment in turn supports the trend observed overall, that is that rescue history does not affect survivability (**Figure 4.2**). In the NaCl environment, the dark and light lines have an equal extinction risk ($P = 1.00$), although the dark lines go extinct more rapidly than the light lines ($z = -2.71$, $P = 0.0067$). The extinction risk of both is significantly greater than that of the salt lines (both comparisons $P = 3.2 \times 10^{-22}$). One event of evolutionary rescue therefore does not increase or lower the probability of surviving high salt over no event of evolutionary rescue. In both the Atrazine and $CuSO_4$ environments, the dark lines have a significantly greater extinction risk than the light and salt lines (Atrazine $P = 5.8 \times 10^{-14}$; $CuSO_4$ $P = 3.0 \times 10^{-5}$). The dark lines have again the highest extinction risk in pH4 (dark to salt comparison: $P = 0.013$; dark to light comparison: $P = 4.1 \times 10^{-6}$), although their extinction dynamics are no different from that of the salt lines (z

= -0.45, P = 0.66). The light lines have the lowest extinction risk in pH4 (salt to light comparison: P = 0.038).

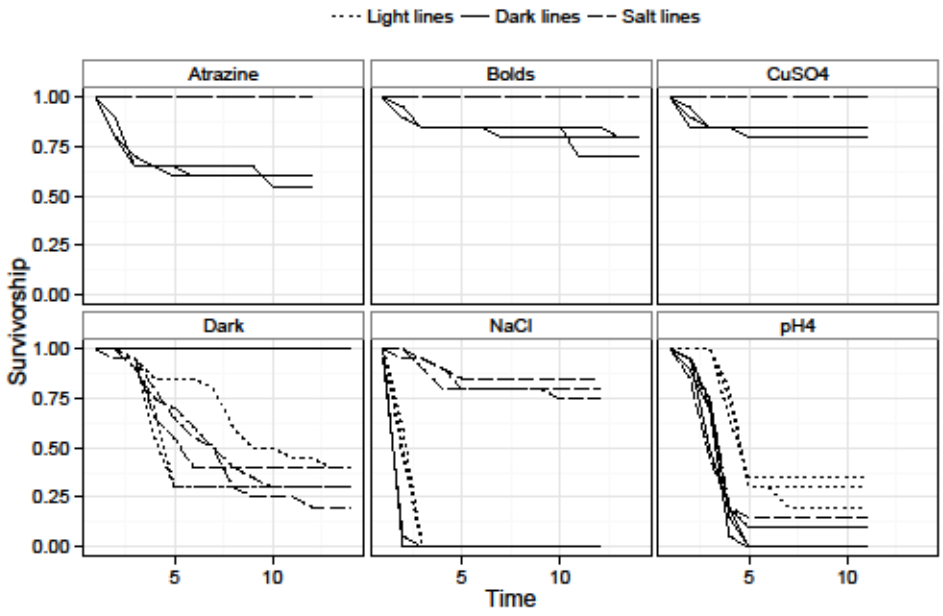

Figure 4.2 **Extinction dynamics of the different selection histories in each novel environment.**
Survivorship corresponds to the proportion of lines alive. Time corresponds to the growth cycle number.

4.4.2 Extinction risk depends on the most recent selection history

If the ability to survive further severe environmental change depends on the most recent environment of selection I would expect evidence of rescue in the dark to be erased by rescue in high salt. One trademark of rescue in the dark is that it reduces survivability in the light as seen by the significant difference in extinction risk between the dark and light lines (**Figure 4.2**; Fisher's exact test, $P = 5.6 \times 10^{-8}$). Light lines and salt lines have the same extinction risk of zero in the light, indicating that rescue in high salt has erased effects of rescue in the dark and restored survivability in the light. A further indication that evolutionary rescue in high salt has erased previous effects of rescue in the dark is the fact that the salt lines have an equal extinction risk in the dark environment to that of the light lines (P = 0.84), which is significantly greater than that of the dark lines (dark line to light line comparison: $P = 7.3 \times 10^{-17}$; dark line to salt line comparison: $P = 4.4 \times 10^{-18}$).

4.4.3 The dark lines are affected both by phototrophic growth and toxic compounds

To determine if the higher extinction risk of the dark lines is due solely to costs of growing in the light, I tested if the extinction risk of the dark lines differs between the Bold's environment and all other novel environments. If extinction risks are the same, this is an indication that the novel compounds have no additional effect and our measure of extinction risk is inflated, whereas if extinction risks are different, this is an indication that the novel compounds have an effect and the dark lines are inherently more prone to extinction. The extinction risk for the dark lines in Atrazine, NaCl, and pH4 is significantly greater than that in Bold's (Figure 4.2; Atrazine: 96% greater extinction risk, $z = -2.92$, $P = 0.0035$; NaCl: 416% greater extinction risk, $z = 11.69$, $P = 0.00$; pH4: 224% extinction risk, $z = 7.22$, $P = 5.0 \times 10^{-13}$) indicating that the herbicide, salt, and acidity themselves are having an effect of survivability. Copper does not affect survivability based on the fact that the extinction risk in $CuSO_4$ is the same as in Bolds (extinction risk is 31% lower in $CuSO_4$, $z = -0.76$, $P = 0.45$).

4.4.4 Repeatability of extinction

The amount of variance in extinction risk provides an estimate of the repeatability of extinction. For example, if all populations from a given history respond the same way to environmental change, i.e. all go extinct or all survive, variance in extinction risk will be low. Whereas if populations from a given history respond in different ways to environmental change, variance in extinction risk will be high, indicating that repeatability of extinction is low. I estimated the variance in extinction risk among lines within rescue histories, among spores within lines, and from the environment and found significant differences (Table 4.1, Figure 4.3).

If rescue history affects the repeatability of evolution, I would expect to find a relationship between number of events of rescue and total amount of variance. The total amount of variance is significantly higher in both the dark and salt lines than in the light lines, but there is no significant difference between the dark and salt lines, indicating that rescue history does not affect the repeatability of extinction.

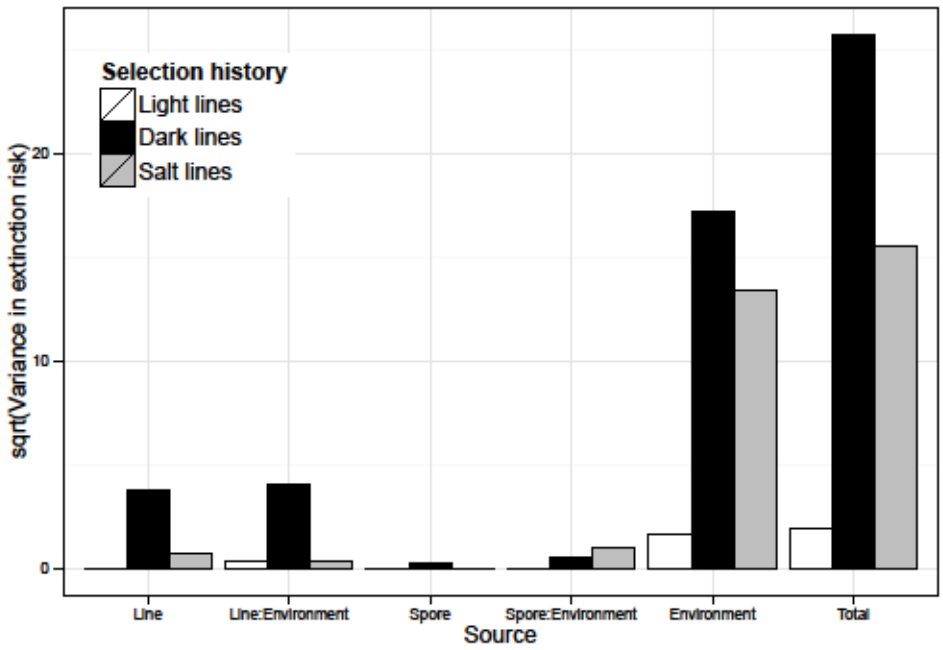

Figure 4.3 Variance in extinction risk depending on selection history.

More specifically, the dark and salt lines have an equal and significantly higher amount of variance than the light lines in terms of variance among lines, from the spore and environment interaction, and among environments. There are no differences among rescue histories in the amount of variance from the interaction of line with environment. And the dark lines have a significantly higher amount of variance among spores than both the light and salt lines. Overall, these results suggest that the repeatability of extinction is lower in populations that have undergone one or two events of rescue compared to control populations. Thus, the outcome of environmental change is much less predictable in the dark and salt lines than in the control light lines.

4.4.5 Variance in yield of surviving populations

To determine if recue history has any effects on the yield of surviving populations, I looked for differences in optical density at the end of the extinction assay (cycle 11) when populations had stabilised. I found that surviving spores from each rescue history all reached equivalent optical densities by the end of cycle 11 except in Atrazine where the light lines reached higher densities than the dark lines (Figure

4.4; $t_{11} = 2.9$, P = 0.014), and in the Dark where the dark lines reach higher densities than the light lines ($t_{12} = -2.9$, P = 0.012) and the salt lines ($t_{12} = -3.2$, P = 0.0079). Hence, overall, selection history has minimal effect on the average yield of surviving lines at the end of the assay. The greater yield of the dark lines in the Dark environment reveals that long-term selection in the Dark increased the capacity for heterotrophic growth that arises spontaneously in unselected populations.

Table 4.1 Significance of differences in variance in extinction risk between rescue histories.

Source	Rescue histories	Df (numerator, denominator)	F ratio	P value
Line	Dark – Light	1, 1	5.20	0.0066
	Dark – Salt	1, 1	9.42×10^{3}	0.26
	Salt – Light	1, 1	1.81×10^{3}	0.015
Line : Environment	Dark – Light	1, 1	10.7	0.17
	Dark – Salt	1, 1	12.7	0.19
	Salt – Light	1, 1	1.18	0.47
Spore	Dark – Light	1, 1	544	0.027
	Dark – Salt	1, 1	552	0.027
	Salt – Light	1, 1	1.02	0.50
Spore : Environment	Dark – Light	5, 5	2.01	9.9×10^{-8}
	Salt – Light	5, 5	2.50×10^{3}	1.7×10^{-8}
	Salt – Dark	5, 5	1.24×10^{3}	0.23
Environment	Dark – Light	5, 5	1.28	0.011
	Dark – Salt	5, 5	10.5	0.40
	Salt – Light	5, 5	8.18	0.019
Total	Dark – Light	13, 13	1.66	2.1×10^{-5}
	Dark – Salt	13, 13	13.1	0.19
	Salt – Light	13, 13	7.92	0.00034

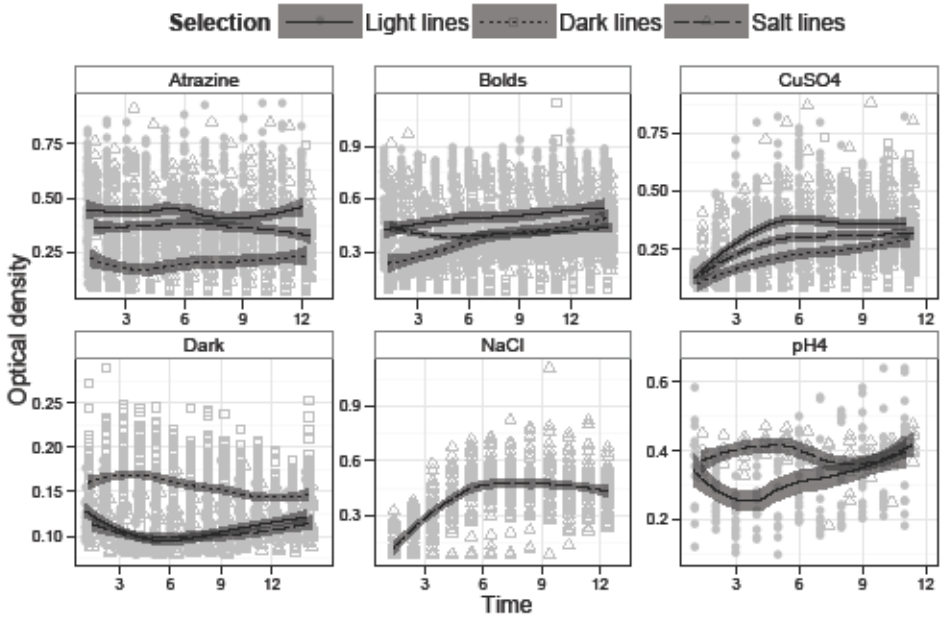

Figure 4.4 Yield over time of the light, dark, and salt lines that survived to the end of the assay.
The trend lines were fitted using local polynomial regression (loess), with 95% confidence intervals in shade.

The amount of variance in yield can provide an estimate of the ability of populations to respond to natural selection, with larger amounts of variance predicted to increase rates of adaptation, and lower amounts of variance predicted to slow or even prevent adaptation. Hence the amount of variance is an indication of the evolvability of populations. There are significant differences among rescue histories in the amount of variance in yield at the end of the assay (Figure 4.5, Table 4.2). Overall, the amount of variance decreases with increasing number of events of evolutionary rescue, although the amount of variance in the light lines is not significantly greater than that in the dark lines.

More precisely, variance among lines is an indication of the ability of metapopulations to respond to natural selection and provides an estimate of the importance of chance and ancestry during evolutionary rescue. Variance among lines is highest for the salt lines. Second, variance among spores is an indication of the ability of independent populations to respond to natural selection and the amount of variation that is generated and maintained within populations. Variance among

spores is highest for the dark lines. Finally, variance among environments is an indication of responsiveness to environmental change. Variance among environments and variance from the line and environment interaction is highest for the light lines, while variance from the spore and environment interaction is highest for the dark lines. Hence the salt lines show greater diversity among lines, the dark lines show greater diversity within lines, and the light lines show greater responsiveness to changes in environments.

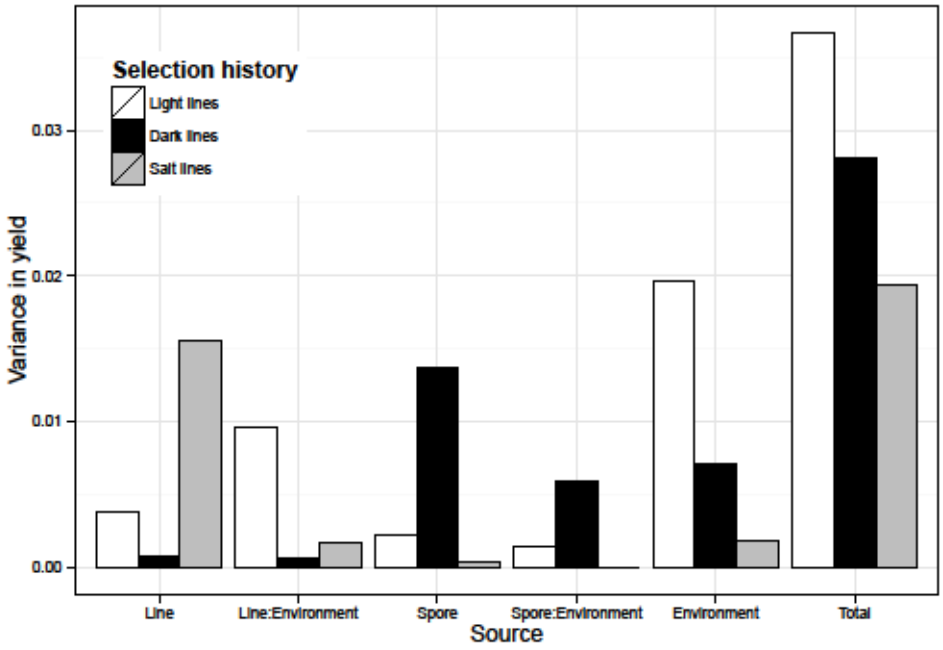

Figure 4.5 Variance in yield at the end of the assay depending on selection history.

Table 4.2 Significance of differences in variance in optical density between rescue histories.

Source	Rescue histories	Df (numerator, denominator)	F ratio	P value
Line	Light – Dark	4, 4	4.07	0.070
	Salt – Light	4, 4	21.1	0.10
	Salt – Dark	4, 4	5.19	0.0059
Line : Environment	Light – Dark	14, 15	5.66	2.4×10^{-5}
	Light – Salt	14, 11	16.1	0.00096
	Salt – Dark	15, 11	2.85	0.043
Spore	Light – Salt	15, 15	6.15	0.00047
	Dark – Light	15, 15	39.0	0.00056
	Dark – Salt	15, 15	6.34	2.8×10^{-9}
Spore : Environment	Dark – Light	32, 43	4.12	1.1×10^{-5}
	Dark – Salt	32, 50	Inf	
	Salt – Light	43, 50	Inf	
Environment	Light – Dark	4, 3	2.74	0.22
	Light – Salt	4, 5	10.8	0.011
	Dark – Salt	3, 5	3.92	0.088
Total	Light – Dark	80, 65	1.31	0.10
	Light – Salt	80, 89	1.89	0.0015
	Dark - Salt	65, 89	1.45	0.041

4.5 Discussion

I made use of lineages with a unique evolutionary history to test for a role of selection history on extinction risk in novel environments. These lineages have undergone two back-to-back events of evolutionary rescue, with their dynamics monitored in the laboratory, and samples from before each event of rescue still available for comparison. I exposed four spores from each of five lines from before any rescue event (light lines), after the first event of rescue (dark lines), and after the second event of rescue (salt lines) to a range of novel and severe environmental changes. The extinction risk in the novel environments was not related to the number

of events of evolutionary rescue. Instead my results suggest that it is the most recent selection history, that is the latest environment of selection, which determines the extinction risk by affecting genetic correlations of growth in alternative environments.

4.5.1 The importance of recent vs. distant selection history

The extinction risk differed significantly between selection histories, being highest for the dark lines and lowest for the salt lines. The difference arises because the dark lines have low survivability in a greater number of environments than both light and salt lines. That is, the dark lines have low survivability in four of the assay environments (light, high salt, herbicide, high acidity), whereas the light lines have low survivability in only three environments (dark, high salt, and high acidity), and the salt lines in only two environments (dark, and high acidity). Even after correcting for the number of novel environments, which differs between selection histories (dark lines have low survivability in 3 out of 4 novel environments; light lines have low survivability in 3 out of 5 environments; and salt lines have low survivability in 1 out of 3 novel environments), the dark lines are still overall more evolutionary constrained, being able to survive in a smaller proportion of environments than either their ancestors or their contemporaries. The extinction risk therefore does not appear to be related to the number of events of evolutionary rescue in these lineages.

The fact that different rescue histories were ordered by their extinction rates differently in different novel environments, and that rescue in one environment erases signs of adaptation to the previous environment, indicates that repeated events of evolutionary rescue do not lead to more or less evolutionary constrained lineages. Rather, it is the last environment of selection that determines the probability of survival during environmental change by defining the sign and magnitude of genetic correlations for growth in novel environments.

I cannot exclude the possibility that the greater extinction risk of the dark lines is due to factors other than its adaptation to the dark. For example, evolutionary rescue in the dark occurred after a sudden change, which has been shown to involve greater

costs than adaptation to gradually changing environments such as in the case of the salt lines (Collins & De Meaux, 2009; Lindsey *et al.*, 2013).

4.5.2 The contribution of genetic correlations and genetic variance to survival

Most of the spores that go extinct do so within the first five cycles (about 25 generations) in the new environment. This is with the exception of the dark environment, where spores tend to go extinct at an almost constant rate. On the other hand, the spores that survive follow a diverse range of dynamics in yield over time, from constant, to steady increase, steady increase followed by a plateau, and U-shaped dynamics (Figure 4.4). Changes in yield over time could be due to physiological acclimation, positive growth rates in initially bottlenecked populations, or possibly although unlikely genetic change. Therefore overall, survival during severe environmental change will depend almost entirely on the presence of spores in the population that can constitutively withstand the novel stressor enough to give time for physiological changes to occur or novel mutations to arise and restore population growth.

The salt lines have the lowest overall extinction risk suggesting that a greater proportion of spores from the salt lines are resistant to a wide range of novel environments. Indeed, among the salt spores that survive, environmental variance is insignificant, indicating that the spores that survive have similar yields in all environments. The low amount of variance in both extinction risk and yield within lines suggests that limited diversity is generated and/or maintained within the salt lines. Hence selection in high salt did not lead to greater evolvability compared to selection in the light or in the dark, but rather has led to more positive genetic correlations for growth in a wide range of environments. Spores from a history of selection in high salt are therefore more likely to survive severe environmental changes than spores with a history of selection in the dark or the light, and rates of adaptation should also be higher in salt meta-populations given the large amount of variation in yield among lines generated by the higher contribution of chance and ancestry during rescue in salt.

Dark spores on the other hand have the highest overall extinction risk suggesting that a lower proportion of dark spores are resistant to a wide range of novel environments. The lower survivability of the dark lines in the light in particular is most likely to have arisen due to loss-of-function mutations accumulating in genes not under selection such as those involved in photosynthesis (Bell, 2012b) rather than due to antagonistic pleiotropy. In the case of low survivability in Atrazine, a herbicide that targets photosystem II, it is possible that the already impaired photosynthetic machinery amplifies the toxic effects of the herbicide. Low survivability in high salt and high acidity is perhaps more likely to have arisen from antagonistic pleiotropy than mutation accumulation as there is no reason to believe that selection in the dark would relax selection for osmotic and oxidative stresses more than selection in the light. In any case, the genetic basis of adaption to the dark appears to be more different from the genetic basis of most other environments than the genetic basis of adaptation to the light or salt. Whether this is because larger regions or regions essential for growth in many environments are under relaxed selection during growth in the dark, or because genes for efficient heterotrophic growth have more negative effects on growth in other environments than genes for photoautotrophic growth or resistance to high salt, remains to be determined. Regardless, the outcome is that lines that successfully adapt to the dark become more evolutionary constrained.

Spores with a history of selection in the dark are therefore less likely to survive severe environmental changes than spores with a history of selection in the light or the dark because of greater negative genetic correlations between growth in the dark and growth in novel environments. The survival of dark meta-populations might be much higher however, as the large amount of variance within and among lines for both extinction risk and yield suggests high evolvability.

There is one limitation to the study presented here in that the history of evolutionary rescue is confounded with the identity of the most recent selection history. In other words, I cannot say whether my results are specific to the sequence of rescue experienced by my lineages (i.e. rescue in the dark followed by rescue in high salt),

or if my results are general to any series of two events of evolutionary rescue. To determine the generality of my results, I would need to use different sets of lineages with different evolutionary rescue histories. These different rescue histories could be created either by changing the order of the rescue environments (e.g. light-to-dark-to-salt, light-to-salt-to-dark) or by changing the identity of the rescue environments (e.g. light-to-dark-to-salt, light-to-herbicide-to-heavy-metals). While such a design would be ideal, it is unrealistic in practice. The lack of replication of rescue histories in this experiment does limit my ability to comment on the generality of the results with regards to the effect of the number of events of evolutionary recue on extinction risk. Nonetheless, this experiment provides one of the most thorough examination of extinction risk to date. By characterizing the rates of extinction of different individuals within populations, of different independent lines within selection environments, and of different selection histories, all of which in multiple different novel environments, I am able to quantify precisely the repeatability of extinction across a whole range of environments. I am therefore conclusively able to demonstrate that selection in some environments significantly increases the general probably of going extinct during subsequent environmental change, and lowers the predictability of extinction risk within and among populations.

4.6 Conclusion

History does affect the subsequent course of evolution by altering genetic correlations for growth in novel environments and the variance within and among populations. Contrary to expectations, repeated events of evolutionary rescue do not make lineages more or less prone to extinction following further environmental change. Rather it is the particular environment to which a lineage has adapted last, i.e. the recent selection history, that determines the probability of survival. During environmental change, the difference between extinction and survival will depend on the presence of resistant spores already in the population, which will be determined by the selection history, on routes for migration among populations, and not likely on novel mutations.

5. Experimental adaptation to marine conditions by a freshwater alga

This chapter is a modified version of a manuscript currently in press as

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I designed the experiments, carried out the laboratory work, did the statistical analyses, and wrote the manuscript. G Bell conceived the study and contributed to writing the manuscript, N Colegrave contributed to writing the manuscript.

5.1 Abstract

The marine-freshwater boundary has been suggested as one of the most difficult to cross for organisms. Salt is a major ecological factor and provides an unequalled range of ecological opportunity because marine habitats are much more extensive than freshwater habitats, and because salt strongly affects the structure of microbial communities. I exposed experimental populations of the freshwater alga *Chlamydomonas reinhardtii* to steadily increasing concentrations of salt. About 98% of the lines went extinct. The ones that survived now thrive in growth medium with 36 gL^{-1} NaCl, and in seawater. My results indicate that adaptation to marine conditions proceeded first through genetic assimilation of an inducible response to relatively low salt concentrations that was present in the ancestors, and subsequently by the evolution of an enhanced inducible response to high salt concentrations. These changes appear to have evolved through reversible and irreversible modifications respectively. The evolution of marine from freshwater lineages is an example that clearly indicates the possibility of studying certain aspects of major ecological transitions in the laboratory.

5.2 Introduction

From time to time, a lineage may become adapted to conditions that lie far outside those that would be tolerated by its ancestors. In most cases this need imply no more than the ability to grow in a specific extreme environment, as in the evolution of antibiotic resistance in bacteria (Davies & Davies, 2010) or heavy metal resistance in plants (Gregory & Bradshaw, 1965). The evolved lineage then flourishes but does not become further modified. In exceptional cases, the novel conditions to which a lineage has become adapted are widespread in nature, and its new ecological attributes then have the potential to lead to an adaptive radiation.

In this chapter, I report the evolution of a marine way of life in the freshwater alga *Chlamydomonas reinhardtii*. It has been suggested that the marine-freshwater boundary is exceptionally difficult to transgress (Lee & Bell, 1999; Vermeij & Dudley, 2000). In plants and yeasts, for example, moving between regions of different salt concentrations requires changes in influx, efflux, and containment of ions, as well as changes in the ability to detoxify reactive oxygen species (Brewster *et al.*, 1993; Mendoza *et al.*, 1994; Zhu, 2000). The pressures that freshwater and high-salt conditions impose on microbes are so different that salt is more important in governing community composition than temperature, pH, substrate, or other physicochemical variables (Lozupone & Knight, 2007). Transitions between the two conditions are consequently infrequent and ancient, as revealed by the large phylogenetic distances between freshwater and marine microorganisms (Logares *et al.*, 2009). High-salt habitats are much more extensive than freshwater habitats, and beside the ocean covering 70% of the surface of the Earth include enclosed seas, inland saline lakes and coastal saltmarshes. Hence, the transition from freshwater to marine conditions both enforces major physiological changes and provides an unparalleled range of ecological opportunities.

Individuals that encounter novel conditions, such as high salt concentration, may be constitutively able to tolerate them and to continue to grow and reproduce. The constitutive response may evolve if there are alleles segregating in the population

that confer different degrees of tolerance. Alternatively, an individual that in its current state is unable to tolerate these novel conditions may be able to modify its state so as to be able to grow and reproduce, a process called phenotypic plasticity. The inducible response may be under genetic control through regulatory elements (e.g. lactase expression in *E. coli* (Dykhuizen & Hartl, 1978; Dykhuizen & Davies, 1980) and the capacity to mount an inducible response may itself evolve (Lande, 2009). Hence, adaptation to a novel environment may be attributable to the evolution of the constitutive response or the induced response or both. Both processes have been shown to play a role in natural populations adapting to changes in the environment (Reale *et al.*, 2003; Charmantier *et al.*, 2008; Gienapp *et al.*, 2008; van de Pol *et al.*, 2012) as well as in facilitating macroevolutionary events such as the origin of new taxonomic groups and of novel traits(Wund *et al.*, 2008; Rajakumar *et al.*, 2012; Standen *et al.*, 2014).

The extent to which the constitutive and inducible responses will evolve will depend on the availability of beneficial variation. A lack of variants with positive growth rates will limit the ability of natural selection to bring the population's mean phenotype toward the new optimal phenotype (Lynch *et al.*, 1991). Not surprisingly, the most common outcome of changes in ecological conditions is therefore extinction (Bürger & Lynch, 1995; Bell & Collins, 2008). In some cases, however, 'evolutionary rescue' may occur (Gomulkiewicz & Holt, 1995), with a population evolving to tolerate conditions that would have been lethal to its ancestor. Rescue is more likely in large populations (Bell & Gonzalez, 2009; Willi & Hoffmann, 2009), in diverse and sexual populations (Agashe *et al.*, 2011; Lachapelle & Bell, 2012; Bell, 2012a), and when environmental deterioration is slow (Perron *et al.*, 2008; Bell & Gonzalez, 2011). Rescue is thought to involve positive genetic correlations of fitness between different levels of stress, such that tolerance of lethal stress is an indirect response to selection at lower levels of stress (Samani & Bell, 2010; Gonzalez & Bell, 2013).

Pre-existing or evolved phenotypic plasticity can also lead to survival. In plastic individuals, the inducible response to changes in environmental conditions can

trigger behavioural, physiological, or morphological changes which may decrease the distance between the phenotype of the individual and the phenotype that maximizes fitness. Phenotypic plasticity can lead to greater genetic variation if it reduces the effectiveness of selection (Draghi & Whitlock, 2012) and reduce the rate of population decline following environmental change, and thereby provides an opportunity for genetic adaptation to occur (Chevin & Lande, 2009; Gomez-Mestre & Jovani, 2013; Schaum & Collins, 2014).

Plasticity may eventually become constitutively expressed, a process called genetic assimilation (Waddington, 1942; Schmalhausen, 1949; Waddington, 1952; 1953; West-Eberhard, 2003; Pigliucci *et al.*, 2006; Crispo, 2007; Lande, 2009; Pfennig *et al.*, 2010). This may occur as the result of selection against plasticity if it is costly to maintain (Snell-Rood *et al.*, 2010), through mutational degradation or drift following long periods of stasis (Masel *et al.*, 2007), or through strong stabilising selection, which reduces genetic variation and thereby attenuates the genetic correlation between plasticity and the mean breeding value (Lande, 2009). The outcome of genetic assimilation is therefore a reduction in plasticity and the constitutive expression of a trait equivalent to that originally produced as a plastic response to the new environment. Genetic assimilation is often difficult to identify because the ancestral reaction norms are not known or because it can occur rapidly (Pigliucci & Murren, 2003). Nevertheless, there is some evidence from natural populations that genetic assimilation may contribute to survival and adaptive radiation following environmental change (Gomez-Mestre & Buchholz, 2006; Bull-Hereñu & Arroyo, 2009; Scoville & Pfrender, 2010).

I propagated experimental lines of the green alga *Chlamydomonas reinhardtii* in gradually increasing concentrations of salt until I obtained lines capable of growing in seawater within about 500 generations. *C. reinhardtii* typically lives in soil and freshwater. The salinity of soil water is expected to vary depending on soil composition and anthropogenic fertilisation, but the salinity of rainwater itself, or the overflow from rivers and lakes, is usually lower than 500 parts per million. The strains used to initiate this experiment have been propagated in the laboratory for

over ten years on medium containing 0.025 gL^{-1} NaCl (0.0004 M). The salinity of seawater on the other hand is about 35 parts per thousand or 35 gL^{-1} (0.6 M), of which about 90% is sodium (Na^+) and chloride (Cl^-). High salinity imposes strong osmotic and oxidative stresses in *C. reinhardtii* by disrupting the homeostasis of ions (Na^+, Cl^-, K^+, and Ca^{2+}) and degrading proteins, and thereby reducing rates of photosynthesis and cell division (Husic & Tolbert, 1986; Neelam & Subramanyam, 2013). In general, salinities between 5 gL^{-1} and 7gL^{-1} NaCl (0.085 M and 0.120 M) are sufficient to reduce the growth of *C. reinhardtii* by about 50%, and salinities higher than between 8 gL^{-1} and 15gL^{-1} NaCl (0.137 M and 0.26 M) are sufficient to suppress growth completely (Reynoso & De Gamboa, 1982; Moser & Bell, 2011; Lachapelle & Bell, 2012). The marine way of life is therefore inaccessible to *C. reinhardtii*. A green alga, identified morphologically as a *Chlamydomonas* sp. was previously isolated off the coast of Japan and characterised for its high salt tolerance (Miyasaka *et al.*, 1998; Tanaka *et al.*, 2007). I use this strain as a comparison for the growth of my salt-selected lines in seawater.

To determine the mechanism of adaptation to high salt, I measured the constitutive and the inducible responses to different salt concentrations by manipulating the acclimation environment. I compared the reaction norms of the salt-selected lines to that of their ancestors and found that both types of response had been modified by natural selection. Plasticity for growth in low salt in the ancestors has been genetically assimilated in the salt-selected lines, and plasticity for growth in high salt has been enhanced. My experiment does not by any means reproduce all of the stages in the colonisation of the oceans by terrestrial or freshwater organisms. It does permit some components of this process to be implemented in the laboratory, however, where the mechanism of adaptation can be elucidated by replicated experiments.

5.3 Material and Methods

5.3.1 Base populations

I isolated one spore from each of 40 different lines that had been propagated independently for two years in the laboratory, growing in the dark on medium supplemented with acetate. These dark lines, from now on referred to as the ancestors, were derived from a previous experiment (Bell, 2005), whose ancestors were derived from a cross among standard laboratory strains (CC-1690 x [CC-1952 x (CC-1952 x CC-2343)]). The lines have not experienced salt concentrations higher than 0.025 gL^{-1} NaCl (4.28×10^{-4} M) during more than ten years of culture in our laboratory.

5.3.2 Selection experiment in ever increasing salt concentration

Details of the initial stages of the selection experiment can be found in Lachapelle and Bell (2012). Briefly, experimental lines varying by their sexuality (asexual, facultatively sexual, or obligately sexual) and initial diversity (low or high) were propagated in an environment where the concentration of salt increased by 1 gL^{-1} NaCl every two growth cycles (i.e. every about 10 generations). The lines that survived longest came from high-diversity, sexually derived ancestors. The two lines able to grow in the highest concentration of salt (up to 30 gL^{-1} NaCl) were used for crosses to continue the selection experiment. It is this continuation of the experiment that I report here. A wild-type strain of opposite mating type to each line (CC-2935 mating type minus) was used to perform the initial cross. The progeny were then mated within and across the F1 families to generate the F2. Gamete fusion and zygote germination followed standard practice (e.g. Lachapelle & Bell, 2012). I grew the progeny in 34 gL^{-1} NaCl for two growth cycles. Only 23 resistant recombinants survived out of about 10^6 cells. The progeny was therefore clearly incapable of growth in 34 gL^{-1} NaCl, and these 23 surviving cells were presumably the ones with the least negative growth rates. I isolated them and propagated each individually, once again in gradually increasing concentrations of salt, starting at 24 gL^{-1} NaCl. The lines were cultured in 48-well plates with 1.4 mL of Bold's medium

supplemented with salt, and transferred every week (two weeks when growth was poor) using a 0.2 mL inoculum. The salt concentration was increased every 2 or 3 growth cycles up until 36 gL^{-1}, at which point it was maintained constant. From the 23 starting lines, 13 survived up to 36 gL^{-1}, and ten have subsequently survived repeated transfers in that concentration. At the time of assay, the surviving lines had been propagated for a total of about 500 generations since the beginning of the selection experiment (Figure 5.1).

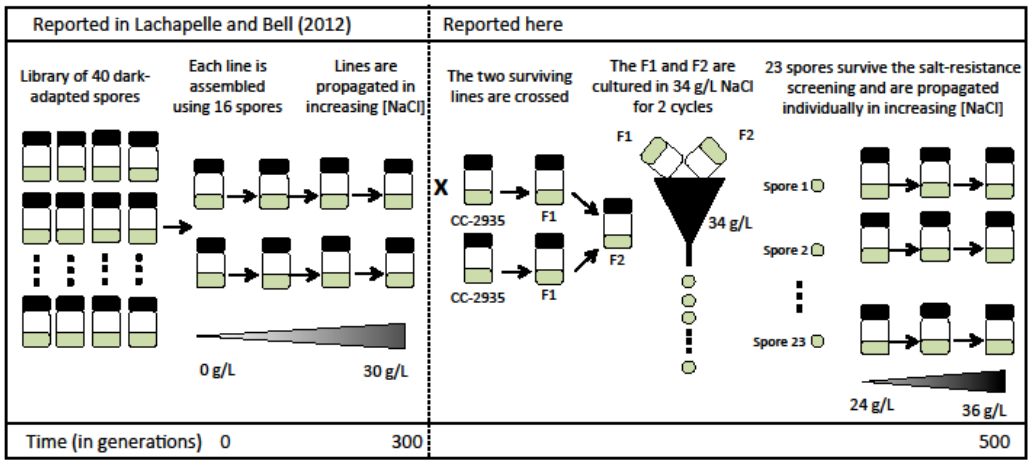

Figure 5.1 Schematic of the history of the salt-selected lines.
Only 10 lines are now surviving in 36 gL^{-1} NaCl.

5.3.3 Seawater growth assay

To determine whether adaptation to high salt had resulted in a transition from freshwater to marine conditions, I assayed the surviving salt-selected lines, the ancestral lines, the wild-type strain that was used to set up the crossing trial, and a related marine chlorophyte (*Chlamydomonas* sp. CW-80, isolated off the coast of Japan; Miyasaka *et al.*, 1998) in seawater. The seawater was collected in August 2013 off the coast of Dunbar, UK, and filter-sterilised two hours after collection. The assay was performed with the same inoculum size and cycle period that the salt-selected lines experienced during the selection experiment. The ancestral lines had been propagated in the dark, using acetate as a carbon source, for the duration of the selection experiment. For the assay, all lines were acclimated in Bold's medium without salt, in the light without acetate, for two cycles before being transferred to the seawater.

Cell density at the end of the first and second cycles in seawater was estimated for two independent replicate cultures using flow cytometry (BD FACSCanto II, BD Biosciences, Oxford, UK). The instrument was calibrated with CS&T beads, and sample acquisition was made using a high-throughput system. Data was acquired and analysed with the BD FACSDiva v6 software. Electronic analysis gates were applied to the forward scatter (pulse area FSC-A and width FSC-W) and side scatter (pulse area SSC-A) plots (proxies for cell size and complexity respectively) to exclude events that are outside expectations for intact *C. reinhardtii* cells, as well as to sort the single cells from clumps of cells. I excluded clumps because I cannot estimate how many cells they contain. Clumps arise as a physiological response to salt in both ancestral and evolved cultures, and should therefore not bias our estimates of growth. All events that were inside the intact and the single-cell gates in a volume of 30 μL acquired at a rate of 1 $\mu L sec^{-1}$ were used to estimate cell density in each culture. Culture samples with cell counts of ten or fewer were not included in further analyses because of the potential for false positives at very low or zero cell density. Cell density at the end of the first cycle was used to estimate cell density at the start of the second cycle. I calculated the rate of increase per week as the natural logarithm of final cell density divided by initial cell density.

5.3.4 Measuring the inducible and constitutive responses to salt

To determine the extent to which the constitutive and the inducible responses to salt were altered in the selection lines, I performed assays comparing the salt-selected lines to the ancestors, and comparing the responses to salt after acclimation in medium lacking salt and in medium containing a stressful but sublethal concentration of NaCl. All assays, unless noted otherwise, were carried out in the light without acetate, as in the extended selection experiment. Note that by 'constitutive' I mean that the phenotype is independent of environmental cues. While constitutive phenotypes are generally associated with genetic changes, it is well recognized that epigenetic changes are mitotically stable (Jablonka & Raz, 2009). A constitutive phenotype can therefore arise from genetic and/or epigenetic changes in asexual populations, and this is investigated as described in the following subsection.

The constitutive response was determined in two ways. First, I compared the growth of the salt-selected lines to the ancestral lines after a period of growth in medium lacking salt. The difference between the two selection histories reflects the direct response to selection and the degree of adaptation that is expressed without need for prior acclimation to salt. The assay was initiated by growing all lines in the light, in medium without salt supplementation, for two growth cycles of one week each. After this period of acclimation, two replicates of each line were transferred to a range of salt concentrations (0, 5, 10, 15, 20, 32, 36, and 40 gL^{-1} NaCl) and grown for two cycles. Fitness was estimated as in the seawater growth assay described above. The difference in responsiveness (i.e. the change in the rate of increase as a function of salt concentration), as well as the amount of variance in growth that could be explained by the history of the lines (i.e. ancestral or salt-selected) was used to determine the degree of change in the constitutive response.

Secondly, I compared the contribution of constitutive and inducible responses to salt. Two replicates of each salt-selected line were acclimated in each of 0, 10, and 36 gL^{-1} NaCl for two growth cycles of one week each before being transferred to a range of salt concentrations (0, 10, 15, 20, 30, 36, and 40 gL^{-1} NaCl). Fitness was estimated as in the seawater growth assay. The variance of growth among lines estimates differences in the constitutive response, and the variance of growth among acclimation environments estimates differences in the inducible response.

I carried out a further assay to determine whether the inducible response to salt in the salt-selected lines is evolved or ancestral, and whether the response of the ancestral lines to salt is due to the salt itself or to photosynthetic growth. I assayed the ancestral lines in the dark and in the light after acclimation in medium lacking salt and in medium containing 5 gL^{-1} NaCl (because most ancestral lines cannot sustain growth in higher concentrations). After acclimation, growth was assayed over a range of salt concentrations (0, 5, 10, 15, 20, and 30 gL^{-1} NaCl).

5.3.5 Characterizing the phenotype of sexual progeny

To examine further the mechanisms responsible for the evolution of the constitutive and the inducible responses to salt, I crossed each of two of the selection lines to an ancestral line to create F1 families, and then crossed within and between these families to create the F2. I chose 8 random spores from each generation of each cross and acclimated them either in medium lacking salt or in medium containing 10 gL^{-1} NaCl. They were then assayed over a range of salt concentrations (0, 28, 36, 44 and 48 gL^{-1}). If genetic changes are responsible for the evolution of the constitutive and/or inducible responses, I expect the sexual progeny to retain tolerance of salt to different extents depending on the number of genes involved and interactions among them. If reversible changes, such as epigenetic changes, are responsible for the evolution of the constitutive and/or inducible responses, I expect tolerance of the salt-selected lines to be annulled by meiosis.

5.3.6 Statistical analyses

Cultures for which estimates of the initial or final cell densities were zero were removed from the analysis to permit model fitting. The removal of some data points led to unbalanced designs in most cases, so I calculated type III sum of squares in all analyses of variance using the R package 'car' (Fox & Weisberg, 2011).

To compare the constitutive response in the high-salt lines to the constitutive response in the ancestors, I fitted a linear mixed-effects model using the lmer function in the R package 'lme4' (Bates *et al.*, 2015), with selection history as a fixed factor, line nested within selection history as a random factor, salt assay concentration (between 0 and 20 gL^{-1} NaCl where the relationship is linear) as a continuous variable, and the interactions as factors. I allowed for random intercepts and random slopes. Type III Wald tests were performed to determine significance of the fixed effects.

To compare the constitutive and inducible response in the ancestors when grown in the dark or in the light, I fitted a linear mixed-effects models using the lmer function,

with acclimation regime (with or without salt) and condition (dark or light) as fixed factors, assay salt concentration as a continuous variable, line as a random factor, and all interactions. I allowed for random slopes and intercepts.

To test the hypothesis that plasticity in the ancestors has been genetically assimilated in the salt lines I fitted a linear mixed-effects model using the lmer function with selection history as a fixed factor, lines nested within selection history as a random factor, assay salt concentration (between 0 and 10 gL^{-1}) as covariate, and all interactions. The data used in this analysis come from the ancestors acclimated with salt (inducible response) and the salt lines acclimated without salt (constitutive response).

To determine the effect of acclimation in different concentrations of salt on the high-salt lines, I fitted an analysis of covariance, with acclimation regime as fixed factor, line as a random factor, assay salt concentration as a covariate, and all interactions. Variance components were then calculated by equating observed and expected mean squares.

To compare the inducible responses in the ancestors to that in the high-salt lines, I fitted a linear mixed-effect model using the function lmer with selection history as a fixed factor, lines nested within selection history as a random factor, assay salt concentration (between 10 and 20 gL^{-1}, or between 20 and 30 gL^{-1}) as a continuous variable, and all interactions. Note here that to look at the evolution of the inducible response I used data from the ancestors acclimated with 5 gL^{-1} NaCl and data from the high-salt lines acclimated with 10 gL^{-1} NaCl.

Finally, to compare the growth of the salt-selected lines and the ancestor to that of the sexual progeny, I calculated confidence intervals for the difference between means, using the *t* distribution for unequal sample sizes.

5.4 Results

5.4.1 Salt-selected lines can grow in seawater

The marine isolate grew well in seawater and could be propagated successfully. The freshwater isolate and all the ancestral lines were incapable of growth in seawater and could not be propagated. The high-salt selection lines had positive growth on average although they varied widely (Figure 5.2: mean r = 0.26, variance among lines = 1.57). About half of the high-salt lines (7/13) have positive growth in seawater, although only 2/6 significantly so (one-tailed t tests for the difference between an estimate and a parametric value; one line could not be tested for significance because of insufficient replication). Some of these lines grew as well as, or even better than, the marine isolate, at least in laboratory conditions.

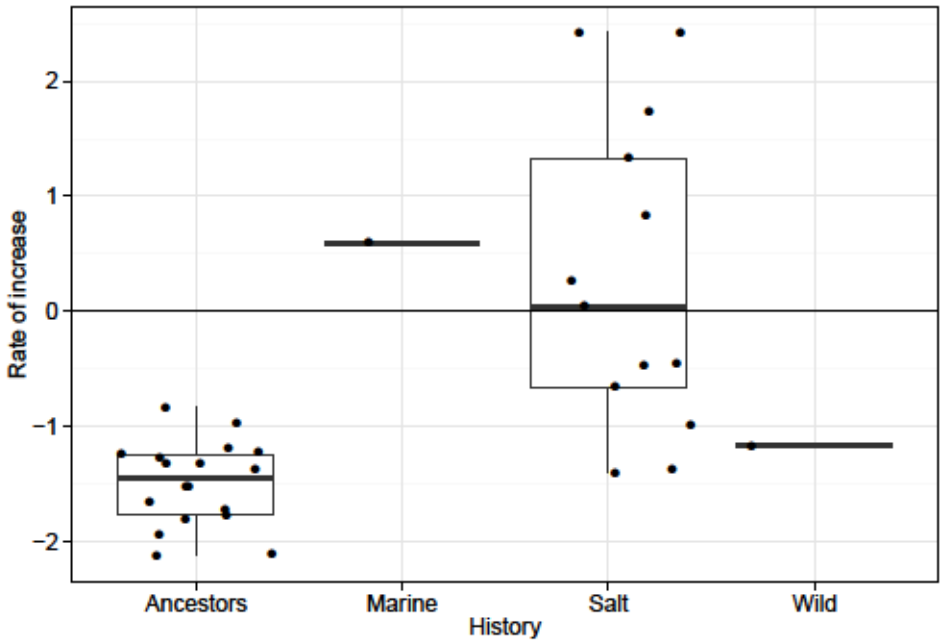

Figure 5.2 Growth of the ancestral lines, the marine green alga strain C.W80, the salt-selected lines, and the wild-type freshwater strain CC2935 in seawater.
Each point is the mean of two assay replicates for a given line. There are 20 ancestral lines, 13 salt-selected lines, and one of each of the marine green alga and the wild-type.

5.4.2 Selection altered the constitutive response to salt

The high-salt lines maintain a high positive rate of increase from 0 gL^{-1} up to 20 gL^{-1} (Figure 5.3: r = 1.75 + 0.02 [NaCl]), whereas growth of the ancestral lines decreases sharply as the salt concentration increases (r = 1.61 - 0.19 [NaCl]). Some ancestral lines have a negative rate of increase at concentrations as low as 5 gL^{-1} NaCl, and the mean rate of increase is well below zero by 10 gL^{-1} NaCl. The difference between the response of the high-salt lines and the ancestral lines to salt is highly significant (effect of interaction history:assay salt concentration: $X^2 = 94.65$, df =1, $P < 0.001$).

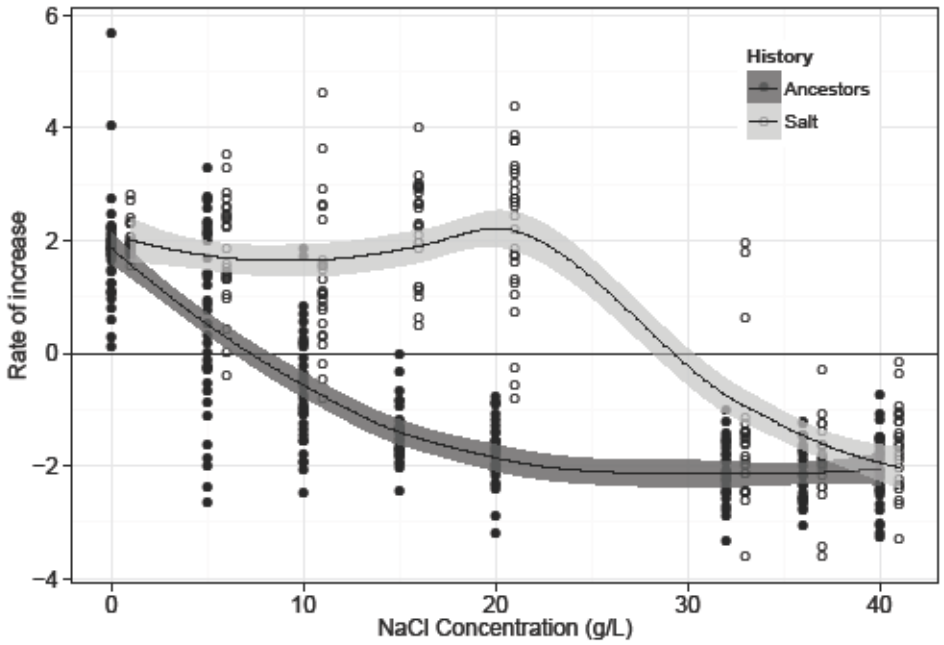

Figure 5.3 Growth of the salt-selected lines and the ancestral lines in different concentrations of salt.
There are 13 salt-selected lines and 20 ancestral lines, each assayed twice. The data points for the salt-selected lines are plotted 1 gL^{-1} NaCl higher than assayed to make it easier to see differences between histories. The trend line was fitted using local polynomial regression (loess), with 95% confidence intervals in shade.

5.4.3 The ancestral lines show an inducible response to salt

Most of the ancestral lines cannot grow in salt concentrations above 5 gL^{-1} when acclimated in medium without salt. When acclimated in 5 gL^{-1} NaCl before assay, however, most ancestral lines are able to grow in salt concentrations as high as 30 gL^{-1} (Figure 5.4). Between 0 and 10 gL^{-1}, where the relationship is linear, the growth

of the ancestral lines decreases significantly more rapidly with increases in salt concentrations when they have been acclimated without salt than when they have been acclimated with 5gL^{-1} NaCl (Table 5.1; effect of interaction between acclimation and concentration: $X^2 = 32.96$, df =1, P < 0.001).

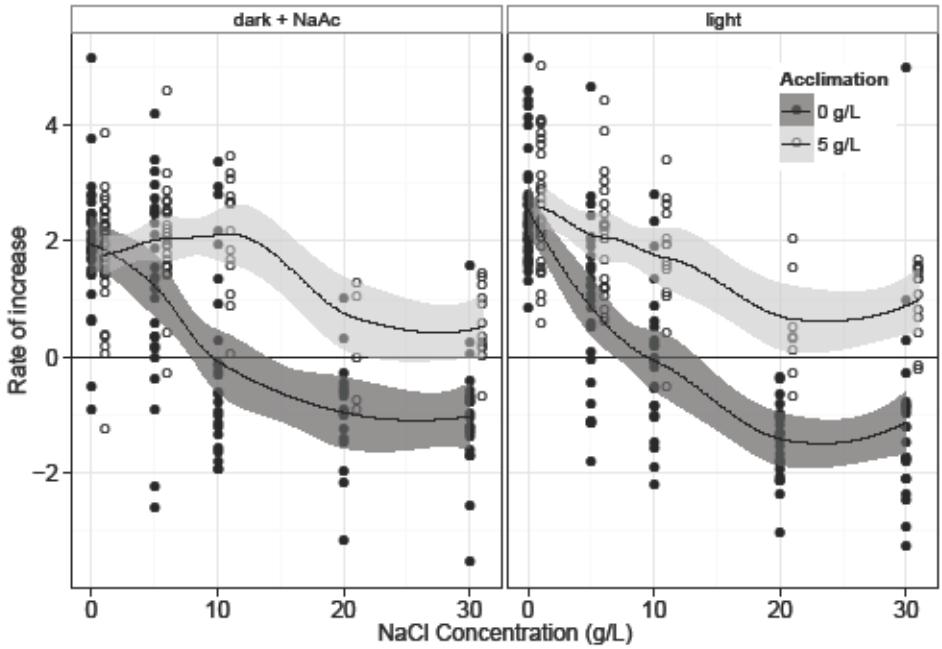

Figure 5.4 The effect of acclimation regime and growing condition (light or dark) on the growth of the ancestral lines in different concentrations of salt.
There are 20 ancestral lines, each assayed twice. The data points for the lines acclimated in 5 gL^{-1} NaCl are plotted 1 gL^{-1} NaCl higher than assayed to make it easier to see differences between acclimation regimes. The trend line was fitted using local polynomial regression (loess), with 95% confidence intervals in shade.

5.4.4 The inducible response of the ancestral lines is expressed in both light and dark conditions

Growth decreases more rapidly with salt concentration when the ancestral lines are grown in the light than when grown in the dark (effect of interaction between growth condition and salt concentration: $X^2 = 10.36$, df =1, P = 0.0013). This is attributable to the higher growth of lines growing in the light than in the dark in medium without salt supplementation, however, and is not due to differences of growth in salt-supplemented media (Figure 5.4). The effect of acclimation without salt or in 5 gL^{-1} NaCl on the response to salt is independent of growing condition (effect of

interaction between acclimation, growth condition and salt concentration: $X^2 = 0.46$, df =1, P = 0.50).

Table 5.1 Effect of acclimation and growing condition (i.e. light or dark) on the response of the ancestral lines to salt concentrations between 0 and 10 gL^{-1}.

Growing condition	Acclimation environment (gL^{-1} NaCl)	Intercept (±se)	Slope (±se)
Light	0	2.2 (0.096)	-0.22 (0.017)
	5	2.2 (0.11)	-0.12 (0.022)
Dark	0	2.3 (0.11)	-0.13 (0.018)
	5	1.9 (0.091)	-0.066 (0.017)

5.4.5 Plasticity for growth in low salt in the ancestors has been assimilated in the high-salt lines

The constitutive response of the high-salt lines to salt concentrations is indistinguishable from the inducible response of the ancestors between 0 and 10 gL^{-1} (selection history : assay salt concentration interaction $X^2 = 0.00$, df =1, P = 0.99).

5.4.6 The high-salt lines have evolved an enhanced inducible response to high salt

The high-salt lines have a strong constitutive response to salt at concentrations up to 20 gL^{-1} (Figure 5.3), but these lines do not appear to be capable of growing at concentrations of 30 gL^{-1} NaCl and higher. Nevertheless, these lines have been propagated in 36 gL^{-1} NaCl for many months without going extinct. Their ability to grow at salt concentrations in excess of 30 gL^{-1} is conferred by an inducible response.

In the lower range of salt concentrations between 0 and 20 gL^{-1}, acclimation in medium containing salt significantly increases the overall rate of increase relative to lines that have been acclimated in medium without salt (Figure 5.5; Table 5.2; effect of acclimation $F_{2,20} = 5.3$, P=0.006). However, acclimation does not significantly affect the slope, meaning that growth decreases at the same rate with increases in salt concentrations whether the lines have been acclimated with or without salt (effect of

acclimation : assay concentration interaction $F_{1,220} = 1.7$, P = 0.19). Note that while growth appears to be higher in no salt than in 10 gL^{-1} NaCl after acclimation in salt, this effect is not significant ($X^2 = 2.70$ df =1, P = 0.10). Comparison of the inducible response of the salt-selected lines to low salt concentrations to the inducible response of the ancestors reveals that it has evolved. Between salt concentrations of 10 and 20 gL^{-1}, growth decreases significantly more rapidly with increases in salt in the ancestors than in the salt-selected lines (selection history : assay salt concentration interaction effect $X^2 = 8.37$ df =1, P = 0.0038), although the intercepts are not statistically different (effect of selection history $X^2 = 3.14$ df =1, P = 0.076).

Table 5.2 Effect of acclimation on the response of the salt-selected lines to a range of different salt concentrations between 0 and 20 gL^{-1}.

Parameter	Acclimation environment (gL^{-1} NaCl)	Estimate (±se)
Slope	0	-0.0014 (0.018)
	10	-0.014(0.018)
	36	-0.027 (0.013)
Intercept	0	1.9 (0.24)
	10	3.0 (0.24)
	36	2.8 (0.17)

Table 5.3 Effect of acclimation on the response of the salt-selected lines to a range of different salt concentrations between 20 and 40 gL^{-1}.

Parameter	Acclimation environment (gL^{-1} NaCl)	Estimate (±se)
Slope	0	-0.18 (0.017)
	10	-0.089 (0.018)
	36	-0.10 (0.012)

In the higher range of salt concentrations between 20 and 40 gL^{-1}, acclimation has a significant effect on the slope of the salt-selected lines, meaning that lines acclimated with salt maintain the same growth with increases in salt concentration, whereas lines acclimated without salt show a steep decline in growth with increases in salt concentration (Figure 5.5; Table 5.3; ANCOVA effect of acclimation : assay concentration $F_{1,215} = 48.4$, P < 0.001). Comparison of the inducible response of the

salt-selected lines to high salt concentrations to the inducible response of the ancestors reveals that it also has evolved. Between salt concentrations of 20 and 30 gL^{-1}, growth is significantly greater overall in the salt-selected lines than in the ancestors (selection history effect $X^2 = 6.58$ df =1, P = 0.010), although the slope is not different (selection history : assay salt concentration interaction effect $X^2 = 2.99$ df =1, P = 0.084).

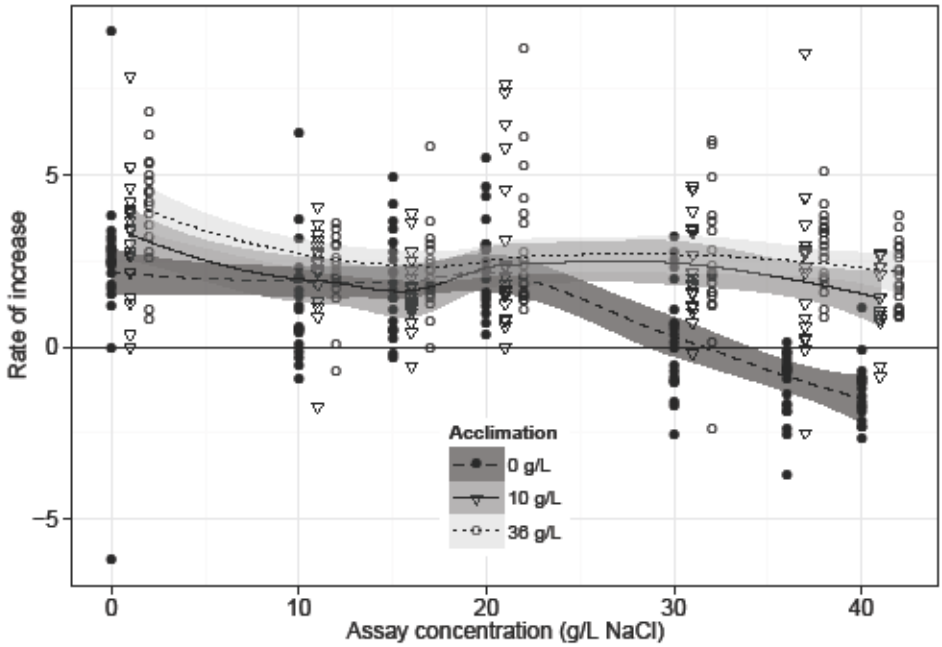

Figure 5.5 The effect of acclimation regime on the growth of the salt-selected lines in different concentrations of salt.
There are 10 salt-selected lines, each assayed twice. The data points for the lines acclimated in 10 gL^{-1} NaCl are plotted 1 gL^{-1} NaCl higher, and the lines acclimation in 36 gL^{-1} NaCl are plotted 2 gL^{-1} NaCl higher than assyed to make it easier to see differences between acclimation regimes. The trend line was fitted using local polynomial regression (loess), with 95% confidence intervals in shade.

5.4.7 Constitutive and inducible responses are affected by meiosis

Without prior acclimation in salt medium, the F1 and F2 progeny grow at the same rate as the ancestors at all salt concentrations, and are unable to grow at concentrations of 28 gL^{-1} or higher (Figure 5.6). This is in contrast to the salt-selected parents, which remain constitutively able to grow in 28 gL^{-1}. Thus, the constitutive ability to grow at high salt concentrations is entirely lost after meiosis and recombination. After acclimation in medium containing 10 gL^{-1} NaCl, the F1

progeny grows as well as the salt-selected parents in concentrations up to 36 gL^{-1} NaCl, and grows better than the salt-selected parent in 48 gL^{-1} NaCl; the F2 progeny does worse than the salt-selected parents in concentrations up to 36 gL^{-1} NaCl, and does better than the salt-selected parents in 48 gL^{-1} NaCl (Table 5.4). Thus the sexual progeny are able to grow at very high concentrations of up to 48 gL^{-1} NaCl, which their salt-selected parents are unable to tolerate.

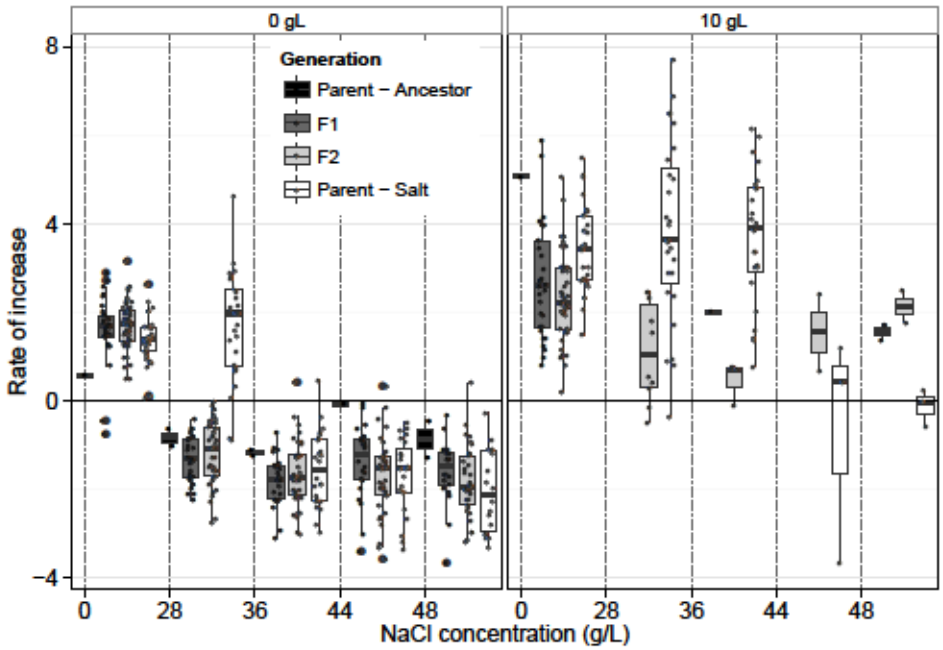

Figure 5.6 Growth of sexual progeny of the salt-selected lines after acclimation without salt or with 10 gL^{-1} salt.
Assay concentrations for which there are fewer than four boxes indicate that none of the spore from the generation missing survived the first cycle in that concentration. The rate of increase during the second cycle could therefore not be calculated. Note that the order of the boxplots on the x axis is the same as in the legend.

5.4.8 Constitutive and inducible responses both contribute to adaptation

In the lower range of assay salt concentrations, the amount of variance in the rate of increase explained by the different lines (i.e. variance in the constitutive responses) is approximately ten times greater than the amount of variance explained by the different acclimation regimes (i.e. inducible responses), with estimates of 0.40 and 0.047 respectively. The amount of variance explained by the interaction of line and acclimation regime is approximately three times greater than the amount explained by line alone (estimate of 1.3). In the higher range of assay salt concentrations, the

amount of variance in the rate of increase explained by the different lines is approximately zero (estimate of -0.94). Acclimation explains a significant amount of the variance (estimate of 0.019), while the interaction of lines and acclimation regime explains about 300 times more of the variance than acclimation alone (estimate of 6.8).

Table 5.4 Difference in the mean rate of increase between the ancestor, salt-selected lines, F1 sexual progeny, and F2 sexual progeny when assayed in different salt concentrations.
Lower and upper confidence intervals were calculated for the difference between means using the t distribution for unequal sample sizes. The assay concentrations that are missing reflect the fact that none of the spores from one of the generations in the comparison survived the first cycle in that concentration.

Acclimation	Comparison	Assay NaCl concentration (gL^{-1})	Lower CI	Difference in mean rate of increase	Upper CI
0 gL^{-1}	Ancestor – F1	0	-2.66	-1.05	0.571
		28	-0.266	0.501	1.27
		36	-0.205	0.642	1.49
		44	-0.494	1.31	3.11
		48	-0.406	0.726	1.86
	Ancestor – F2	0	-2.37	-1.12	0.121
		28	-0.678	0.351	1.38
		36	-0.617	0.467	1.55
		44	-0.172	1.63	3.43
		48	-0.240	0.951	2.14
	Salt parent – F1	0	-0.602	-0.253	0.0963
		28	2.51	3.04	3.57
		36	-0.147	0.271	0.688
		44	-0.849	-0.293	0.263
		48	-1.01	-0.454	0.0988
	Salt parent – F2	0	-0.591	-0.330	-0.0697
		28	2.39	2.89	3.39
		36	-0.315	0.0946	0.504
		44	-0.438	0.0264	0.491

		48	-0.733	-0.228	0.276
	F1 – F2	0	-0.388	-0.0773	0.233
		28	-0.471	-0.151	0.170
		36	-0.533	-0.176	0.181
		44	-0.154	0.319	0.793
		48	-0.221	0.225	0.671
10 gL^{-1}	Salt parent – F1	0	0.131	0.738	1.35
		36	-1.53	1.69	4.92
		48	-2.77	-1.68	-0.589
	Salt parent – F2	0	0.610	1.10	1.59
		28	1.33	2.73	4.12
		36	1.38	3.24	5.10
		44	-8.76	-2.24	4.29
		48	-3.57	-2.26	-0.953
	F1 – F2	0	-0.181	0.364	0.909
		36	-0.845	1.55	3.94
		48	-2.27	-0.581	1.11

5.5 Discussion

5.5.1 Adaptation to marine conditions of growth

New ways of life evolve when organisms adapt to ecological conditions of growth that were not accessible to their ancestors. I have shown that an important ecological transition can occur within 500 generations. Some of the lines that we selected in gradually increasing concentrations of salt are now capable of growth in 36 gL^{-1} NaCl, far beyond what their ancestors could tolerate. In principle, these lines are now capable of growing in the sea.

About 98% of the experimental lines went extinct well before marine conditions were reached. Chronic exposure to a continuously deteriorating environment therefore requires far more than ancestral plasticity for growth in concentrations up to about 20 gL^{-1} NaCl for two growth cycles. The lines that have survived vary

substantially in their ability to grow in seawater. Thus, most populations that experience a profound deterioration in the conditions of growth will simply become extinct. The experimental adaptation to marine conditions that occurred in this freshwater alga give an example of how survival to marine conditions can be achieved to different extents and in different ways.

In the yeasts *Saccharomyces cerevisiae* and *S. paradoxus*, for which the lethal concentration of salt is about 150 gL^{-1} NaCl, population size, the rate of increase in salt concentration, and connectivity with neighbouring populations all affect the probability of surviving the imposed salt regime as well as the probability of surviving a transfer to the lethal concentration (Bell & Gonzalez, 2009; Samani & Bell, 2010; Bell & Gonzalez, 2011; Gonzalez & Bell, 2013). In the bacterium *Serratia marcescens*, tolerance to 90 gL^{-1} NaCl was improved after constant selection in either 80 or 100 gL^{-1} NaCl for 300 generations, but not after selection in a fluctuating environment, most likely because of weaker selection pressure (Ketola & Hiltunen, 2014). Together these results suggest that the rarity of transitions between freshwater and marine conditions may be a consequence of small population sizes, fast rates of increase in salt, fluctuating conditions, or low connectivity between natural populations.

5.5.2 Genetic assimilation of salt tolerance

In my experiment, growth of the evolved lines without acclimation to salt is equal to or greater than the growth of ancestral lines acclimated with salt, at salt concentrations of up to about 20 gL^{-1}. Above this concentration, the evolved lines cannot grow without acclimation. Once acclimated, however, they grow much better than the acclimated ancestral lines in all concentrations above 10 gL^{-1}. These results suggest that the ability to grow at very high salt concentrations evolved in two stages: genetic assimilation at lower concentrations, yielding a constitutive response to conditions lethal to the ancestor, and an enhanced inducible response at higher concentrations that permits growth up to about 40 gL^{-1} NaCl.

Changes in gene expression following long-term exposure to salt have been reported before in *C. reinhardtii* (Perrineau *et al.*, 2014). Short-term acclimation to about 12 gL^{-1} NaCl causes a reduction in photosynthesis, up-regulation of glycerophospholipid signalling, and up-regulation of the transcription and translation machinery. Long-term culture in high-salt medium causes down-regulation of genes involved in the stress response and in transcription and translation. Fatty acid metabolism is also more strongly down-regulated following long-term than short-term acclimation, which suggests that long-term salt stress leads neither to lipid accumulation nor to the synthesis of starch. Selection can therefore alter gene expression for growth in salt.

Genetic assimilation can occur through genetic or epigenetic modifications. Unlike genetic modifications, which are changes in nucleotide sequence that are transmitted from parent to offspring in both asexual and sexual lineages, epigenetic modifications may be preserved in asexual lineages, either of free-living cells or of tissues in a developing body, but are generally removed during meiosis and are therefore not transmitted in sexual lineages (Jablonka & Raz, 2009).

The constitutive tolerance to low salt concentrations was maintained in asexual cultures, but completely lost in the sexual progeny of the salt-selected lines. Indeed, the F1 and F2 progeny have the same phenotype as the ancestor in low salt concentrations after acclimation without salt. If genetic change was responsible for the assimilation of ancestral plasticity in low salt concentrations, I would have expected some of the progeny to have maintained some constitutive tolerance to salt, albeit possibly to lower extents. However, none of the 24 random sexual progeny that I assayed displayed a level of tolerance greater than ancestral. Therefore, I conclude that the assimilation of ancestral plasticity for growth in low salt concentrations is unlikely to be based on genetic changes. Rather the assimilation of ancestral plasticity occurred through reversible changes in my asexually propagated selection lines. The loss of tolerance following meiosis is consistent with an epigenetic basis, although genomic studies will be required to explicitly test this hypothesis.

The inducible response to salt concentrations of up to 40 gL^{-1}, on the other hand, was retained in sexual progeny, albeit more weakly expressed. This is consistent with genetic modification. This could be caused by loss-of-function mutations in a regulatory gene that hindered the binding of a repressor protein. This explanation, however, would require the existence of a cryptic inducible system in the ancestor whose function is obscure. It is more plausible to invoke gain-of-function mutations in an inducible structural gene. This gene is imagined to contribute to the inducible response at low salt concentrations expressed by the ancestor. During serial transfer at gradually increasing salt concentrations, alleles that spread through natural selection because they confer the ability to grow in ambient conditions may indirectly confer the ability to grow in more severe conditions. Adaptation to lethal conditions, resulting in evolutionary rescue, has been attributed to this kind of indirect response to selection in other experiments with algae and yeast (Bell & Gonzalez, 2009; Samani & Bell, 2010; Bell & Gonzalez, 2011; Lachapelle & Bell, 2012; Gonzalez & Bell, 2013). The partial loss of fitness in F1 and F2 hybrid progeny is the expected result of recombination with ancestral alleles, and suggests that such gain-of-function mutations have occurred in more than one gene in our salt-selected lines.

5.5.3 The contribution of plasticity and genetic recombination to evolutionary rescue

In a deteriorating environment, stress provides a continual stimulus capable of eliciting an inducible response. Where such a response exists, as it did in my selection lines, it enables the population to persist for longer and thereby prolongs the period during which genetic adaptation can occur through natural selection. The phenotypic plasticity of the ancestor for low stress is eventually lost after chronic exposure to increasing stress in my asexually propagated lines. The reversibility of this constitutive response to low salt in sexual progeny suggests the assimilation of ancestral plasticity could have arisen through the accumulation of neutral loss-of-function epigenetic modifications. The loss of plasticity would be accelerated if the inducible response were metabolically costly to maintain and/or activate. While I have no way of measuring the cost of maintenance, my data show no evidence of a

cost of activation: the growth of the ancestral lines in medium without salt is the same whether or not they have been previously acclimated with salt (Figure 5.4). Drift could also have played a role in eliminating plasticity, given that the lines were bottlenecked following the first round of crosses. However, it is unlikely that plasticity would have been assimilated in all lines through chance alone.

In this instance of a deteriorating environment, then, the loss of plasticity at low levels of stress is accompanied by the evolution of enhanced plasticity at high levels of stress through genetic modifications. This is consistent with the evolution of enhanced plasticity in fluctuating environments reported by Schaum and Collins (2014). The breadth of conditions that the salt-selected lines can tolerate is much greater than the ancestors, consistent with the 'sidestep niche model' whereby enhanced plasticity contributes in widening the niche after environmental change (Lande, 2009; Gallet *et al.*, 2014). However, I have no evidence that the niche has shifted or is now narrowing. To the contrary, the assimilation of ancestral plasticity in low salt concentrations seems to have contributed in maintaining the larger niche breadth.

The fact that sexual lines were better able to keep pace with the changing environment (Lachapelle & Bell, 2012) indicates that surviving lines were better able to keep track of the moving fitness optimum because of the increased genetic variation generated by recombination. It is possible that the increase in resistance to salt reported here is mostly attributable to recombined variation from the end of this first selection experiment. However, my data does not allow me to make any inferences about the relative contribution of recombination, epigenetic, and genetic modification to the increase in resistance reported here.

Nonetheless, back-crosses of the high-salt lines to the ancestor, or crosses among these families, show that the F1 and F2 continue to grow at salt concentrations of 48 gL^{-1} at the same rate as at lower concentrations, whereas the high-salt lines themselves are unable to grow. This demonstrates the importance of recombination. The enhanced resistance of recombinants cannot be attributed to a more resistant

protein, because the high-salt lines themselves cannot grow at these very high salt concentrations. It is not due to the recombination of improved alleles at different loci, because it is expressed in the F1 of crosses between the ancestor and the selection lines. It might be attributable to the release, through recombination, of an improved structural gene from linkage with a strongly deleterious mutation at some other locus. In this case, it would be necessary to assume further that this mutation is strongly deleterious only at very high salt concentrations, since the F1 and F2 are inferior to the selection lines at salt concentrations of 40 gL^{-1} or less. Population sizes were very low during some stages of the experiment when the salt concentration was increasing. A neutral or mildly deleterious mutation could have therefore fixed by chance, if not by hitchhiking with a beneficial mutation. The uniform phenotype of random spores is also unexpected. Hence, we report that the range of conditions that can be tolerated is substantially extended in the sexual progeny of adapted parents, but we have not identified a simple genetic mechanism that would explain their superiority.

5.6 Conclusion

Experimental evolution has been extensively used to elucidate the mechanism of selection for particular attributes such as the ability to utilize a novel substrate or resist an antibiotic. The evolution of marine from freshwater lineages, of heterotrophs from autotrophs (Bell, 2012a; b; 2013), and of multicellular from unicellular forms (Ratcliff *et al.*, 2012; 2013), are examples that clearly indicate the possibility of studying certain aspects, at least, of major ecological transitions in the laboratory.

Here I reported the adaptation of a freshwater alga to marine conditions within a few hundreds of generations in the laboratory. Continued selection pressure, sexually generated genetic variation, and phenotypic plasticity largely contributed to extending the limits of tolerance and facilitating the ecological transition. In short, the evolution of tolerance to salt involved two different mechanisms: reversible and

irreversible changes. Tolerance to low salt concentrations of unacclimated selection lines was annulled by meiosis, suggesting reversible changes were responsible for the assimilation of ancestral plasticity and adaptation to the limit of tolerance. Tolerance to high salt concentrations of acclimated selection lines was maintained through meiosis, suggesting irreversible genetic changes were responsible for enhancing phenotypic plasticity in the selection lines and extended the range of tolerance to conditions lethal to the ancestor. Both mechanisms contributed to the transition from freshwater to fully marine conditions.

6. General discussion

Chance and history have the potential to increase the stochastic nature of evolution and hence reduce the repeatability of evolution. The goal of this thesis was to identify the factors that affect the importance of chance and history, and to quantify precisely this contribution to evolutionary change. In evolution experiments, variance among populations is often taken as noise blurring the effects of treatments. With a proper experimental design, where the variance can be partitioned into that attributable to measurement error, chance, and history, this noise can become informative. A shift from focussing solely on the effects of treatments on mean trait values to characterising the variance in trait values among populations will increase our understanding of the stochastic nature of biodiversity, and the accuracy of our predictions for the outcome of environmental change.

I have shown that the repeatability of adaptation depends on population size (Chapter 2) and on the mode of reproduction (Chapter 3), that recent selection history imposes stronger constraints on extinction risk than the accumulation of events of evolutionary rescue (Chapter 4), and that a history of phenotypic plasticity and sexual reproduction favours survival during severe environmental deterioration (Chapter 5). In this final section of my thesis, my aim is to discuss the implication these results have for our understanding of the diversity we see today and that we predict for the future.

6.1 The stochastic nature of diversity

The conventional thinking is that diversity results from differences in selection pressures (notion popularised by A. R. Wallace and A. Weismann). Organisms have been modified to optimize functionality, and hence all organismal traits must necessarily be adaptive. The most famous critique of this 'adaptationist programme' is from Gould and Lewontin (1979) who argued that traits are not necessarily

adaptive and instead can arise because of historical constraints, or by chance because of linkage with other traits under selection or drift, for example. Variation in phenotypic traits such as colony morphology (Bell, 2013) and CO_2 uptake strategy (Collins *et al.*, 2006) has been shown to arise from chance and history instead of differences in selection pressures. I have also found that contrary to what most experiments with large and asexual populations of microbes tell us, chance and history can contribute significantly to phenotypic diversity when either of these population attributes is altered.

It has been known for a while now that selection is much more efficient in large populations and chance effects much more prevalent in small populations. Indeed, researchers have exploited this fact and manipulated the population size of experimental populations in order to study either the deterministic or stochastic aspect of evolution. For example, mutation accumulation studies aimed at understanding the distribution of effect sizes of mutations usually propagate single clones in order to eliminate the biasing effects of selection on the fixation of mutations (Haag-Liautard *et al.*, 2007; Denver *et al.*, 2009; Keightley *et al.*, 2009; Ossowski *et al.*, 2010; Sung *et al.*, 2012; Ness *et al.*, 2012). In Chapter 2 I studied populations of different sizes and identified a transition region in effective population sizes (between about 10^3 and 10^4) where evolution switches from being driven mainly by selection, to being driven as much by selection as by history. Whether this result generalises to other environments, and to species other than *C. reinhardtii* remains to be determined. Nevertheless, this result provides a benchmark for which to gage the likely contribution of history to phenotypic diversity.

I also found in Chapter 3 that sex can alter the contribution of chance and history during evolution. Sex is by far the most prevalent mode of reproduction in eukaryotes, although many species such as plants are capable and do reproduce clonally, and bacteria, which are even more abundant than eukaryotes, reproduce asexually. In spite of this diversity in modes of reproduction, the vast majority of long-term evolution experiments have been carried out asexually, leaving a deficit in our understanding of the repeatability of evolution in obligate and facultative sexual

organisms. It is therefore important to carry evolution experiments in both asexual and sexual populations in order to determine whether chance and history contribute differentially to evolution in populations with different modes of reproduction. I found that the effect of sex varies greatly, increasing or decreasing the importance of chance and history during evolution depending on the environment, and hence decreasing or increasing the amount of phenotypic diversity that is adaptive. Overall my results suggest that small and sexual populations, which are often the ones of concern during environmental change due to their lower evolutionary potential, prevalence, and public interest, are going to respond to environmental change in highly unpredictable manners due to the importance of chance and history. While selection always contributes significantly to evolutionary change in sexual and asexual populations of finite size, in some cases as much as half of the phenotypic diversity after a couple hundred generations of evolution has no adaptive value and instead carries the footprint of history and chance.

6.2 A focus on extinction avoidance for an understanding of diversity

Phenotypic diversity depends not only on variability in outcomes of adaptation, but also on differences in extinction rates among populations. For example, if some clades are inherently more prone to extinction than average, whole groups of organisms are likely to disappear during periods of environmental change, leaving ecosystems functionally altered and potentially handicapped. By studying rates of extinction during environmental change in Chapter 4, I found that populations that had most recently adapted to growing in the dark had much lower survivability overall in novel environments than populations that had most recently adapted to the light and to salt. Hence, rates of extinction differ when populations have different selection histories, as the previous selection environment determines the sign and magnitude of genetic correlations in growth in different environments. In addition to a history of different selection environments, in Chapter 5 I found that a history of phenotypic plasticity, sex and recombination can also affect survivability. The

presence of phenotypic plasticity for growth in salt in the ancestors allowed the salt-selected lines to maintain positive growth rates in salt concentrations up to the limit of tolerance, and thereby provided an opportunity for genetic adaptation to occur. Indeed, plasticity for growth in even higher concentrations of salt evolved and led to survival in salt concentrations typical of marine conditions. Survival was also favoured by a history of sex and recombination. Only lines with a history of sex survived to the highest concentrations of salt, and only after crossing these surviving sexual lines furthermore was I able to extend the limit of tolerance. Hence, history plays a significant role in shaping the diversity we see today by differentially favouring the survival of some lineage over others.

Another aspect of populations facing extinction is how surviving and subsequently adapting to severe environmental change can in some cases be the source of major diversification. For example, the adaptation of an artiodactyl lineage to an aquatic way of life led to the adaptive radiation of whales (Thewissen *et al.*, 2001); adaptation to hot, dry conditions through C4 photosynthesis led to radiations of grasses (Edwards *et al.*, 2010); and adaptation to transient water through anhydrobiosis led to the radiation of bdelloid rotifers (Tunnacliffe & Lapinski, 2003). While major ecological transitions have mainly been the domain of study of palaeontologists and phylogeneticists, I have shown in Chapter 5 that they can be studied in the laboratory. Hence, more experimental studies of the repeatability of extinctions and major ecological transitions might shed light on some of the mechanisms responsible for a significant proportion of the biodiversity we see today.

6.3 Generalisations in evolutionary biology

The amount of certainty we can attribute to predictions about the outcome of environmental change in small and sexual populations can only be very low unless we dedicate more research to understanding what attributes of environments mediate the effects of recombination, and what aspects of history mediate the likelihood that

it will constrain further evolution. In order words, we need to determine when experimental results are general and when they are specific.

We often rely on a handful of experiments each done with a single genotype in a single environment to conclude on the plausibility of a theory. The problem with the one genotype – one environment approach is that we are biasing the region of the parameter space that we explore. Theoretical studies often lay out predictions for a range of parameter space, the biologically relevant regions of which need be determined by experiments. If no experiments are done in the environments that we decide to exclude based on bizarre preliminary results for example, then we effectively end up ignoring regions of parameter space and restricting the number of different evolutionary outcomes we deem possible.

There are two possible approaches to solving the problem of generality vs. specificity. One is to design evolution experiments using many different starting points and many different environments. Such an approach will allow us to measure the range of outcomes possible. In Chapter 3 I showed that the effects of sex on the repeatability of adaptation vary greatly depending on the environment of evolution, suggesting that different amounts of linkage disequilibrium in different environments were affecting the outcomes of recombination. Hence, reassuringly, the simple environments used in the laboratory appear to be different enough for the genetic basis of adaptation to differ. While we can only assume that natural environments will differ even more, there is at least significant variation among laboratory environments for us to investigate the generality of effects. Such an approach will not however increase the accuracy of our predictions for any specific genotype or environment.

Another approach would be to test specific attributes of environments (e.g. selection pressure, genetic basis, population size) or of history, such as suggested in the framework presented in the General Introduction. Such an approach would allow a mechanistic understanding that could be applied to any condition that meets the attributes investigated and would therefore increase the accuracy of our predictions.

For example, one environmental attribute that could be investigated is the strength of the selection pressure, or in other words the distance between the position of a population at the time of environmental change and the top of the fitness peak. We might expect that the stronger the selection pressure, the fewer the number of mutations that will lead to survival, and consequently the fewer the number of routes available to high fitness (Lindsey *et al.*, 2013; Vogwill *et al.*, 2014). Hence we would expect selection to be the main contributor to evolutionary change (in surviving populations) in environments with very strong selection pressure, and chance and history to contribute minimally.

6.4 Repeatability of evolution in diverse populations for ecological predictions

Part of the work on the repeatability of evolution is motivated purely by the fundamental desire to know whether evolution is mainly a deterministic process or a stochastic process. This kind of work relates to questions such as 'Given a second chance, would Humans evolve again?'. On the flip side, the study of the repeatability of evolution can make significant contributions to more applied questions. Scientists are being asked to predict the effects the current rates of climate and environmental change will have on species persistence. However, one aspect of natural populations that is most evidently different from most laboratory experimental populations is standing genetic variation: most experimental populations are initiated with a single genotype while natural populations tend to be diverse. There is some evidence that dynamics of evolution can be vastly different in populations with and without standing genetic variation (Lachapelle & Bell, 2012), although much more work on the repeatability of evolution in diverse compared to isogenic starting populations is needed to reflect better the ecological conditions for which predictions need to be made.

When evolution relies mainly on standing genetic variation, the extent of change will depend almost entirely on how much variation there actually is at the start and its

relevance for growth in the new environment. If there are beneficial variants in the population, then selection is predicted to be more efficient than in isogeneic populations because the variation is immediately available and the population does not need to wait for mutations to arise (Barrett & Schluter, 2008). Also, beneficial alleles will be present in more than one copy, which will reduce the possibility that beneficial alleles are lost through chance, and hence reduce the amount of stochasticity during evolution. The amount of standing genetic variation and its relevance for growth in the new environment will depend mainly on the history of the population. It is therefore possible that history will have a greater effect on the repeatability of evolution when populations are diverse compared to when they are isogenic.

In situations where mutations continue to play a significant role during evolution in diverse populations, it is possible that the same mutation will arise repeatedly in different backgrounds. This can increase the number of evolutionary paths that are explored and the probability that the beneficial mutation will arise in a beneficial background, reducing the effect of history and increasing the efficiency of selection. The greater efficiency of selection coupled with the weaker effects of chance should increase the repeatability of evolution in diverse populations with the same starting genetic composition, but might be lower in diverse populations with different starting genetic composition.

There is some indication that evolution can be more repeatable in diverse populations than in initially isogenic populations. Teotónio *et al.* (2009) followed changes in allele frequencies in differentiated populations of *Drosophila melanogaster* undergoing reverse evolution to the common ancestral environment. The initial differentiation phase occurred mostly from changes in the frequency of, and recombination between genotypes already present in the ancestral population. Evolution was highly repeatable during the reverse evolution phase as selection acted to return the allele frequencies to those found in the control populations. In the experiment I presented in Chapter 3, I also made use of diverse starting populations, and presented a statistical approach for measuring the effects of selection when there

are multiple ancestors. However, only a direct comparison of variation in evolutionary outcomes and trajectories between diverse and isogenic populations will tell us whether evolution is more or less repeatable in diverse populations compared to isogenic ones.

I have shown in this thesis that history and chance can make significant contributions to evolutionary change, and lead to noticeably different outcomes in adaptation and extinction rates. To gain a better understanding of the nature of biodiversity and the generality of experimental outcomes, we need to dedicate much more work to unveil the mechanisms by which different environments alter the effect of sex on the repeatability of evolution, different histories constrain evolution, and the applicability of our findings to natural (diverse) populations.

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8. Appendix

8.1 Submitted, accepted, and published papers

8.1.1 Lachapelle J., Reid, J., & Colegrave, N. 2015. *Proc R Soc B*

8.1.2 Lachapelle, J., & Colegrave, N. (*under review*) *J Evol Biol*

8.1.3 Lachapelle, J., Bell, G., & Colegrave, N. 2015. *Evolution*

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Repeatability of adaptation in experimental populations of different sizes

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The degree to which evolutionary trajectories and outcomes are repeatable across independent populations depends on the relative contribution of selection, chance and history. Population size has been shown theoretically and empirically to affect the amount of variation that arises among independent populations adapting to the same environment. Here, we measure the contribution of selection, chance and history in different-sized experimental populations of the unicellular alga *Chlamydomonas reinhardtii* adapting to a high salt environment to determine which component of evolution is affected by population size. We find that adaptation to salt is repeatable at the fitness level in medium ($N_e = 5 \times 10^4$) and large ($N_e = 4 \times 10^5$) populations because of the large contribution of selection. Adaptation is not repeatable in small ($N_e = 5 \times 10^3$) populations because of large constraints from history. The threshold between stochastic and deterministic evolution in this case is therefore between effective population sizes of 10^3 and 10^4. Our results indicate that diversity across populations is more likely to be maintained if they are small. Experimental outcomes in large populations are likely to be robust and can inform our predictions about outcomes in similar situations.

1. Introduction

The repeatability of evolution has important implications. If evolution is repeatable, evolutionary trajectories taken by different lineages and the final evolutionary outcomes in given conditions will be the same. In other words, high repeatability will reduce the extent of diversification and/or lead to the loss of diversity across independent populations. Thus, the repeatability of evolution affects our understanding of the nature of biodiversity [1] and can inform the extent to which evolutionary theory can be used to make predictions [2,3].

Ultimately, the relative contributions of selection, chance and history to adaptation will determine whether trajectories and outcomes are repeatable across independent populations. Using the metaphor of the fitness landscape [4] (i.e. the regression of individual fitness over genotypic space), we describe adaptation as a climb up a fitness peak. In an isogenic population, this will occur through the fixation of novel beneficial mutations. If every possible mutation is generated each generation, selection will lead to the increase in frequency of the one with largest beneficial effect at every step [5–8], assuming there is always a single mutation with largest effect. In such cases, genetic changes will be attributable entirely to selection, and adaptation will be highly deterministic, always following the quickest path up the fitness peak.

In reality, all possible mutations will not be generated and/or established in each generation. Stochasticity in the supply of mutations will increase the probability that different populations fix different mutations, and therefore follow different paths up a fitness peak. If there is only one fitness peak, such as on a 'smooth' fitness landscape, the divergence in evolutionary trajectories will be temporary, as all populations will eventually converge on the same outcome.

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However, if there are multiple peaks, such as on a 'rugged' fitness landscape, this stochasticity can lead to long-term divergence (e.g. [9,10]).

Finally, populations with different evolutionary histories are unlikely to be starting at the same place on the fitness landscape. History can reduce the repeatability of evolutionary trajectories and outcomes among genetically different populations by altering the accessibility of certain paths [11]. We expect history to have minimal impact on the final outcomes of adaptation on smooth landscapes, as all populations will converge on the same peak. By contrast, on rugged landscapes, different populations will remain constrained to the peak nearest their starting location, unless they are able to cross fitness valleys through variance-induced peak shifts [12], drift [4,13], double mutants [14,15] or recombination [15–17]. History could thus potentially cause long-term divergence in adaptive outcomes.

The importance of chance and history as opposed to selection during adaptation is likely to be affected by population size. In the absence of standing genetic variation, small populations are expected to explore more trajectories than larger populations because of the low supply of beneficial mutations, and variation in what particular mutation arises across populations [18–20]. Trajectories and outcomes in small populations are therefore predicted to be less repeatable than in large populations because of the higher contribution of chance. In large populations, the higher supply of mutations will increase the probability of there being multiple different individuals each carrying a different beneficial mutation. Clonal interference [5–7] will tend to lead to the fixation of the mutations with largest beneficial effect [21,22] and to a reduction in the number of different trajectories taken by independent lineages [18]. As such, adaptation in large populations is predicted to be more repeatable because of the greater efficiency of selection and lower contribution of chance [18–20].

Microbial experiments have shown that selection is usually the most important driver of evolutionary change relative to history and chance after at least 200 generations of evolution in a novel environment [23–28]. Similar results have been obtained in sexual and initially diverse experimental populations of *Drosophila* after 20–30 generations [29,30]. While the effect of population size on the contribution of selection, chance and history has not, to our knowledge, been empirically determined, smaller population sizes do generally lead to greater among-population variation than do large population sizes [31], although this effect depends on the environment [32] and time scale [33].

Here we quantify the contribution of selection, chance and history to adaptation to a novel environment of initially isogenic, asexual experimental populations of different sizes. We predict that chance and history will play a greater role in small populations while selection will be more efficient in larger populations.

2. Material and methods

(a) Base populations

The experiment was started using six different genotypes of the unicellular green alga *Chlamydomonas reinhardtii*: CC-1690 (wild-type, mating type+); CC-1952 (wt, mt−); backcrossed CC-2342 (strain created in our laboratory by backcrossing to the wild-type CC-2342 a total of 12 times, mt−); backcrossed CC-2344 (same as above using wild-type CC-2344, mt−); backcrossed CC-2931 (same as above using wild-type CC-2931, mt+); dark line DD C8 (obtained from G. Bell, mt+). These genotypes are genetically [34] and/or ecologically distinct. We propagated each genotype individually, such that all growth during the experiment was vegetative, and adaptation occurred via *de novo* mutations.

(b) Selection experiment

For each combination of genotype and population size, we had six replicate lines, for a total of $6 \times 3 \times 6 = 108$ independent lines. A single colony from each genotype was expanded in standard growth medium. Six samples from each well-mixed culture were used to initiate each replicate line. The amount of genetic variation is minimal and expected to be the same across replicates. The replicates were then propagated independently. Each line was exposed to a constant novel environment consisting of Bold's minimal medium [35] supplemented with 5 gl^{-1} NaCl. High salt imposes strong osmotic and oxidative stresses in *C. reinhardtii* by disrupting the homeostasis of ions (Na^+, Cl^-, K^+ and Ca^{2+}), degrading proteins, and thus reducing rates of photosynthesis and cell division [36,37]. We chose 5 gl^{-1} NaCl because salinities between 5 and 7 gl^{-1} NaCl (0.085 and 0.120 M) reduce growth by about 50% [38–40] and induce adaptive responses within short evolutionary time scales [40].

Population size was manipulated by varying the volume of growth medium in which the lines were growing. Small lines were cultured in 0.1 ml of medium (96-well plate), medium lines in 1 ml (48-well plate) and large lines in 8 ml (6-well plate). Lines were serially transferred using the same relative inoculum size (5%) at the end of each cycle (i.e. every 4 days). This means that the number of cells at the end of a growth cycle and the number of cells transferred are greater in larger volumes than in small volumes. Using the same relative inoculum size ensures that the number of cell divisions within a growth cycle, population density and the relative amount of spent media transferred are the same across treatments initially, although small differences (i.e. about 1.3-fold difference in cell density at the end of the experiment compared with 10-fold differences in population size) will arise as populations adapt during the experiment. Using $N_e = gN_o$, where N_e is the effective population size, g is number of generations between transfers (here $g = 4.3$) and N_o is the initial population size [41], the effective population sizes for the small, medium and large lines at the start of the experiment are approximately 5×10^3, 5×10^4 and 4×10^5 cells, respectively. Lines were maintained at 24.5°C, 60% air humidity, 8000 Lux constant light intensity, shaking at 130 r.p.m. with a 3 mm rotation diameter. The experiment lasted 40 cycles (about 200 generations). Note that since our focus is on general adaptation to the selection environment, rather than any specific adaptation to the salt stress, it was not necessary for us to maintain control lines evolving in the absence of salt.

(c) Fitness assay

To estimate fitness, we calculated the maximum growth rate of ancestral and evolved lines when grown in 5 gl^{-1} NaCl. The ancestors had been maintained in dim light on Bold's agar throughout the experiment, conditions which limit growth and selection [35]. Six cultures were set up per ancestor to match the number of evolved lines generated per ancestor per population size treatment. All lines were cultured in Bold's medium for two cycles to minimize physiological differences, and then transferred to 5 gl^{-1} NaCl. Each line was assayed three times.

Growth was monitored during the second growth cycle in 5 gl^{-1} NaCl by measuring optical density at 750 nm every 9 ± 1 h. We transformed the measurements (log10 of (optical

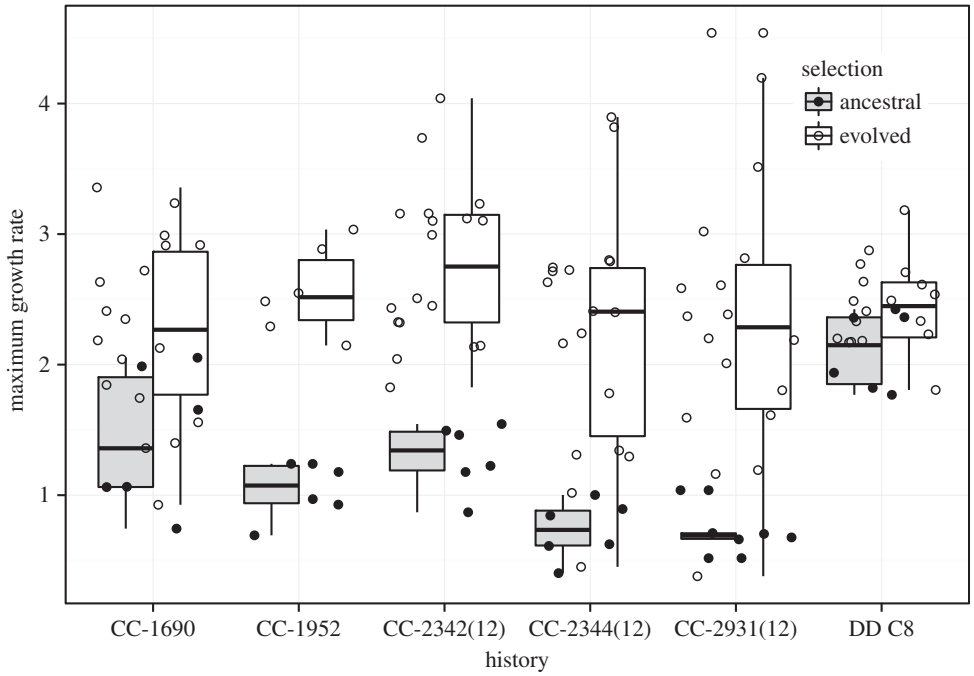

Figure 1. Maximum growth rate of ancestors and evolved lines in 5 gl^{-1} NaCl. History corresponds to the different starting genotypes.

density × 10 000)) to allow the models to be fitted. Growth parameters were extracted from a nonlinear model using nonlinear least squares in the nlstools R package [42]. We first fitted a baranyi model [43,44]. This model returned a fit for 83% of the lines. The remaining lines were fitted with either a baranyi model without N_{max}, a baranyi model without lag or a linear model, as appropriate. Model fits were visually inspected to ensure the proper model had been applied.

(d) Determining the contribution of selection, chance, and history

Generally speaking, the effect of selection is to increase fitness. As such, the difference between the ancestors and evolved lines is the contribution of selection on beneficial alleles and any associated alleles that may be hitchhiking. Note here that we are investigating sources of variation in fitness. Differences between the phenotype or genotype of ancestors and evolved lines could be attributable to factors other than selection. Any variation in fitness among evolved lines descending from the same starting genotype will be the result of chance. Finally, if history affects adaptation, we expect lines from different starting genotypes to reach different outcomes. As such, variation in final fitness among starting genotypes is the contribution of history.

More specifically, we quantified components of variation in fitness by calculating sums of squares, which provides a phenomenological description of the structure of variation that is entirely additive [25]. The effect of selection was estimated as $mnr(F - I)^2$, where F and I are the final and initial grand mean growth rates, respectively, m is the number of lines descending from each ancestor, n is the number of ancestors and r is the number of assay replicates. The effect of history was estimated as $mr\Sigma(A - F)^2$, where A is the mean growth rate of all lines from a given ancestor. The effect of chance was estimated as $r\Sigma\Sigma(L - A)^2$, where L is the mean growth rate of each replicates from a given line. Finally, the variation due to error measurement was estimated as $\Sigma\Sigma\Sigma(R - L)^2$, where R is the growth rate of each replicate. Each sum of squares estimate was divided by the sum of all estimates to obtain the relative contribution of each factor. We prefer this method to alternative variance component-based approaches [23,26,29] since our design does not permit a full additive partition of variation using these methods. Nevertheless, a variance component analysis of our data produced similar results.

(e) Statistical analyses

Variance in growth rates among the starting genotypes was estimated by equating observed and expected mean squares from a nested analysis of variance, with genotype and line within genotype as random effects. To determine whether adaptation had occurred, and whether it had occurred to different extents in populations of different sizes, multiple comparisons were done using Tukey's HSD following a general linear model on population size (with four levels representing the ancestors, and the small, medium and large evolved lines), as a fixed effect. To further investigate the effect of population size on growth and its interaction with starting genotype and line, we performed an analysis of variance on the growth of the evolved lines. The model included population size as a fixed factor, starting genotype as a random factor, line within genotype as a random factor and their interactions.

The significance of the difference in relative contribution of selection, chance and history between two sizes of populations was determined by a randomization test. We randomly allocated each evolved line to a population size and initial genotype without replacement, and then calculated the relative sums of squares. We compared the ratio of relative sums of squares for each pair of population sizes to the observed ratios. The number of times where the random ratios were as large or larger than those observed over the total number of randomizations (10 000) is our significance statistic.

3. Results

(a) The ancestors differ in their response to the novel environment

There is a significant amount of variation in growth rates among the six starting genotypes (figure 1; variance among genotypes = 0.26, mean = 1.22).

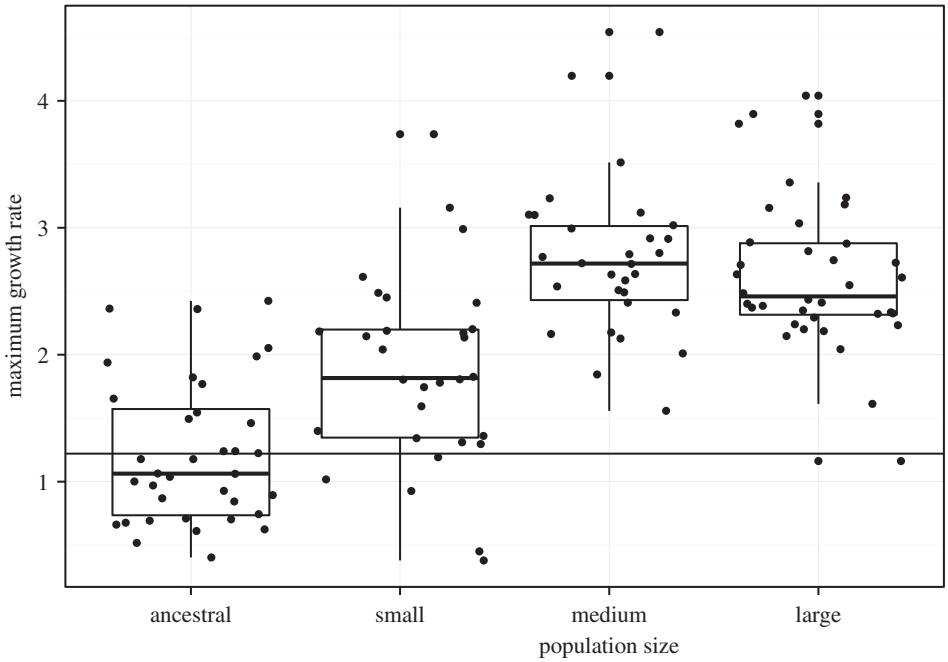

Figure 2. Maximum growth rate in 5 gl^{-1} NaCl of the ancestral line, and of the small, medium and large evolved lines.

(b) Small populations adapt to a lesser degree than larger populations

All six replicate lines of ancestor CC-1952 went extinct in small and medium populations. These lines were not included in the following analyses. Among the surviving lines, all population sizes have greater growth rates on average than their ancestors, meaning that adaptation to 5 gl^{-1} NaCl has occurred over the course of 200 generations of evolution (figure 2; effect of population size $F_{3,392} = 88.72$, $p < 0.001$; TukeyHSD comparisons between ancestors and small or medium or large evolved lines all have $p < 0.001$). The growth rate of small lines is significantly lower than that of the medium and large lines ($p < 0.001$ for both comparisons) while the growth rates of medium and large lines do not differ ($p = 0.62$).

The growth of each genotype, as well as the growth of each line within genotype, varies depending on which size of population they evolved in (effect of population size $F_{2,192} = 70.86$, $p < 0.001$; effect of population size × starting genotype interaction $F_{8,192} = 13.02$, $p < 0.001$; effect of population size × line within history interaction $F_{50,192} = 3.36$, $p < 0.001$).

(c) Population size affects the contributions of selection, chance and history to evolution

Selection plays a significantly greater role in medium and large lines than in small lines during evolution in 5 gl^{-1} NaCl (figure 3, tables 1 and 2). Selection explains about 80% of the changes in growth rates in medium and large lines, whereas it explains less than 40% in small lines.

History explains less than 4% of the variation in medium and large lines, but explains close to 20% of the variation in small lines. This difference is significant when comparing small with large lines, but not when comparing small with medium lines (table 2). The variance among initial genotypes ($\sigma^2 = 0.26$) is maintained after evolution in small populations ($\sigma^2 = 0.30$), but much reduced after evolution in medium ($\sigma^2 = 0.13$) and large ($\sigma^2 = 0.016$) populations.

Finally, chance explains about 10% of the variation in medium and large lines, which are significantly less than the approximately 30% that it explains in small lines (table 2).

It is also interesting to look at the absolute amount of variation, because it tells us about the diversity that is present for a given component irrespective of mean growth or the amount of variation for another component. Small amounts of variation in growth, whether for low mean growth or high mean growth, means that growth is very similar across lines. The absolute variation among replicate lines with the same starting genotype is very similar for all population sizes (table 1). However, there is two to three times more variation among genotypes evolved in small than in medium and large populations. Finally, the variation between ancestors and evolved lines is more than five times smaller in small lines than in medium and large lines.

We can define the repeatability of adaptation as the ratio of the difference between deterministic and stochastic contributions to evolutionary change over total variation. That is, $(SS_{selection} - (SS_{chance} + SS_{history}))/(SS_{selection} + SS_{chance} + SS_{history})$. A value of 1 indicates completely deterministic dynamics, and a value of −1 indicates completely stochastic dynamics. Repeatability is −0.087 in small lines, 0.74 in medium lines and 0.71 in large lines.

4. Discussion

We propagated experimental populations of small, medium and large size ($N_e = 5 \times 10^3$, 5×10^4 and 4×10^5 cells, respectively) in a novel environment for 200 generations. By partitioning the variation in growth among lines into selection, chance and history, we determined which components depend on population size and how this affects the repeatability of evolution at the fitness level. Initial diversity among larger populations was lost as they converged on

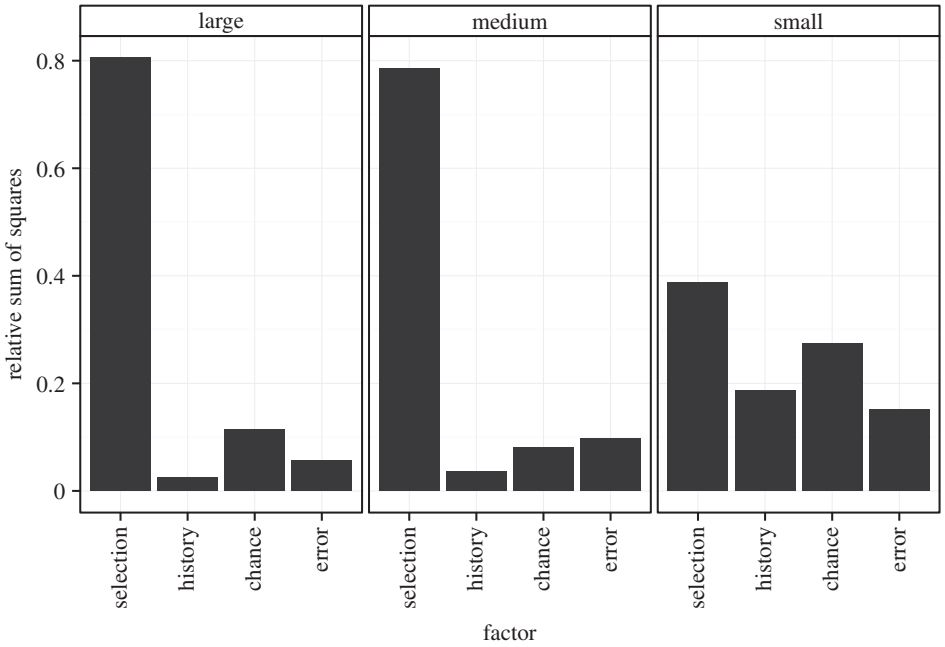

Figure 3. Relative contribution of selection, chance and history after 200 generations of selection in 5 gl^{-1} NaCl. Error here corresponds to variation among assay replicates.

Table 1. The effect of population size on the contribution of selection, history and chance to variation in growth rates after 200 generations of evolution in 5 gl^{-1} NaCl. Error here corresponds to variation among assay replicates.

population size	effect	sum of squares	total sum of squares	relative sum of squares
small	selection	38.1	98.4	0.387
	history	18.4		0.187
	chance	27.0		0.275
	error	14.8		0.151
medium	selection	210	267	0.786
	history	9.60		0.0359
	chance	21.7		0.0810
	error	26.0		0.0974
large	selection	210	261	0.806
	history	6.40		0.0245
	chance	29.8		0.114
	error	14.6		0.0560

the same growth rate, whereas diversity among small populations was maintained as they diverged during adaptation. Thus, adaptation is less repeatable in small populations than in larger populations because history is more constraining and selection less efficient in the former.

(a) The transition from stochastic to deterministic dynamics

The main differences in the relative contributions of selection, chance and history arise between small and medium populations, although we cannot rule out the possibility that a more powerful study would have shown a more continuous effect of population size. This suggests that the transition between stochastic and deterministic dynamics occurs between effective population sizes of 10^3 and 10^4. This is lower than an estimate from microvirid bacteriophages, where the transition occurred between bottleneck sizes of 10^4 and 10^5 [31]. Stochastic dynamics occur when mutations fix more rapidly than they arise—that is, when $N_e\mu_b \ll \ln(N_e s)$ [8]—and so depend on the effective population size as well as the rate (μ_b) and fitness effects (s) of beneficial mutations. While in *C. reinhardtii* the estimated mutation rate is 3.23×10^{-10} [45] or 6.76×10^{-11} per site per generation [46], the rate per genome could be much greater than in viruses, and could explain why the transition point was observed at lower N_e. In addition, μ_b will depend on the number of genes involved in fitness for a particular environment as well as the specific type of gene interactions, and so the difference may reflect differences in the evolutionary challenge set by different selective environments. Without details of the genetic basis of adaptation in these experiments, it is difficult to speculate further.

The greater contribution of selection in medium and large lines than in small lines could be because of higher supply

Table 2. Significance of the difference between population sizes in the relative contribution of selection, history and chance. *p*-values were determined from a randomization test.

factor	comparison	*p*-values
selection	small – medium	0
	small – large	0
	medium – large	0.38
history	small – medium	0.064
	small – large	0.015
	medium – large	0.26
chance	small – medium	0.0017
	small – large	0.012
	medium – large	0.19
error	small – medium	0.042
	small – large	0
	medium – large	0.0098

rate or probability of fixing beneficial mutations. It cannot be explained by effects of dilution ratio on the probability of fixing beneficial mutations [47,48] since the dilution ratio was maintained constant across population size treatments in this experiment. Rather, it is likely to result from a reduced supply of beneficial mutations in small lines. Selection was not more effective in large than in medium lines, perhaps because of clonal interference slowing down the rate of fixation of beneficial mutations [7,49,50].

The similar absolute contribution of chance across population sizes contrasts with the prediction that chance should be greater in smaller populations because of their lower supply of mutations and higher degree of drift [5,13]. It is possible that such effects will only occur in much smaller populations than used here.

(b) The importance of historical contingency

Differences in the amount of convergence or divergence in fitness among populations of different sizes could be due to differences in rates of adaptation [19,33] or the ability to cross fitness valleys in rugged fitness landscapes [18]. The initial variance among starting genotypes was reduced after evolution in medium and large populations, which is expected if the different histories were converging on the same trait combination. There may be a single fitness peak in this environment, and medium and large lines could have climbed it faster than small lines. However, we cannot exclude the possibility that the lines have reached different peaks of similar heights. Yet the maintenance of variance among genotypes evolving in small lines and the fact that some small lines achieved similar fitness to larger lines suggest that the differences in fitness between small and larger lines are not due entirely to slower rates of adaptation, but result from epistatic interactions. Large and medium lines appear to have ended up on the same peak, whereas small lines have remained trapped on different peaks.

In small populations, the lower supply of mutations can limit the exploration of the fitness landscape and increase the probability of getting trapped on local fitness optima. Larger populations are more likely to find the global fitness optimum because their higher supply of double or double-step mutants makes available a larger proportion of the landscape [51,52]. Convergence in medium and large lines could also have occurred if higher genetic or phenotypic variance within the populations led to flattening the adaptive landscape, enabling them to move across the landscape more easily than small lines [53].

The population sizes investigated here cover a limited range. They are much smaller than most microbial populations [54]. However, many isolated microbial populations, such as pathogens initiating an infection, will have their effective population sizes in the range investigated here following environmental change or colonization of new habitats. While they are of the same order as species such as *Caenorhabditis elegans* (with an estimate of 8×10^4) [54] and many plant populations (with estimates of 10^3–10^4) [55], our results are probably only directly relevant to asexual populations without standing genetic variation.

Our populations were maintained entirely asexually. In sexual organisms, recombination generally increases the efficiency of selection [5,6,56–60] and should therefore increase repeatability. Thus, the threshold between deterministic and stochastic dynamics seen in our study might be pushed further down in sexual populations. However, whether recombination will reduce the effects of chance and history will depend, in part, on the amount of linkage disequilibrium and the type of gene interactions [16,17]. Experiments directly examining the effect of sex on the repeatability of adaptation would be valuable.

Another aspect of our system is the lack of initial standing genetic variation. In the short term, adaptation will generally be faster when there is standing genetic variation for fitness [5]. This may affect both the repeatability of adaptation and also the interaction with population size. That is, genetic variation could have a disproportionate effect in small populations which are limited by variation compared with large populations, where alleles present at the start will also arise through mutation at some point because of the high supply of mutations. Moreover, the effect might depend on the time scale. Over short time scales, selection will act on the standing alleles rather than the novel mutations because of their greater frequencies [61], while over longer time scales the contribution of standing genetic variation to adaptation will not be easily distinguishable from that of novel mutations.

On short evolutionary time scales, our results indicate that adaptation will be repeatable in large populations. If the mechanism of adaptation is well understood, then predictions about outcomes in large populations will be accurate. On the other hand, adaptation will be less repeatable and diversity will be maintained among independent populations if they are of small size. It will therefore be difficult to use evolutionary theory to make predictions about the outcome of environmental change in small populations. The strong effect of history underlines the importance of using different starting genotypes in experiments to investigate the range of potential responses of small populations to environmental change.

Data accessibility. The data associated with this manuscript are archived in Dryad (doi:10.5061/dryad.6m150).

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The effect of sex on the repeatability of evolution in different environments

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Abstract

The adaptive function of sex has been extensively studied, while less consideration has been given to the potential downstream consequences of sex on evolution. Here we investigate one such potential consequence, the effect of sex on the repeatability of evolution. The repeatability of evolution has important implications for biodiversity, and for making predictions. By comparing the change in fitness, as well as the amount of variance within and among experimental populations of *Chlamydomonas reinhardtii* we find that the importance of selection, chance, and ancestry during evolution is significantly different in sexual populations than in asexual populations. In Bold's minimal medium, sex reduces repeatability overall; in Herbicides sex reduces repeatability among ancestries and increases repeatability within ancestries; in Na_2SO_4 sex increases repeatability among ancestries and reduces repeatability within ancestries; and finally in NaCl sex increases repeatability overall. Thus, sex has important effects on diversity during evolution that are highly dependent on the genetic composition of the population and on the environment. The genetic basis of adaptation is different enough between even relatively simple and similar laboratory environments for recombination to have significantly different effects on evolving populations. Until we determine the precise mechanism by which the specific environmental attributes mediate the effect of recombination on evolution, we cannot assume that results from experiments in a single environment will generalise to other environments. There is a need for a greater commitment to studying diverse environments for a general and correct interpretation of evolution.

Keywords

Recombination, selection, historical contingency, chance, convergence, divergence, experimental evolution, *Chlamydomonas reinhardtii*

Introduction

The ubiquity of sexual lineages among eukaryotes is a long-standing problem in biology (Maynard Smith, 1978; Bell, 1982) Extensive research has been dedicated to determining the adaptive function of sex, that is the mechanisms for its origin and maintenance over evolutionary time(Lively & Morran, 2014; Becks & Alavi, 2015). However, less consideration has been given to the potential downstream consequences of sex on evolution. While these consequences may or may not have any adaptive significance, they can potentially have important implications for evolution. Here we investigate one potential downstream consequence of sex: the effect of sex on the repeatability of evolution. By altering the repeatability of evolution, sex could have long-term effects on rates of diversification, and consequently on the patterns of diversity that we see today.

The repeatability of evolution depends on whether evolution is mainly a deterministic or stochastic process. If evolution is entirely deterministic, the evolutionary trajectories taken by different lineages and the final evolutionary outcomes in a given environment should always be the same. In other words, highly deterministic dynamics will reduce the extent of diversification and/or lead to the loss of diversity across independent populations. On the other hand, if evolution is largely stochastic, evolutionary trajectories and outcomes will often differ dramatically, even for populations in identical environments. Thus, the repeatability of evolution can affect not only our understanding of the nature of biodiversity, but also the extent to which evolutionary theory can be used to make predictions.

The ultimate outcome of evolution will always be the same if, in a given environment, there is only one combination of traits that maximises fitness, as all populations will eventually converge on this phenotype. However, the outcome of evolution can be different in different populations if there are many different combinations of traits that maximise fitness, potentially leading to long-term divergence. Whether or not evolution will be repeatable in such cases will depend on the relative contribution of selection, chance, and ancestry (Gould & Lewontin, 1979; Gould, 1989; Travisano *et al.*, 1995).

Selection acts by sorting genetic variation, leading to the increase in frequency of the variants with highest fitness. If every possible single mutant is available every generation, the population will be able to sample all the trajectories available. Selection will then lead to the fixation of the mutation with largest beneficial effect every step of the way to the fittest possible genotype (Fisher, 1930; Muller, 1932; Gerrish & Lenski, 1998; Desai & Fisher, 2007), assuming there is always just one mutation with largest effect. Thus, when selection is perfectly efficient, the outcome of evolution should always be the same.

In most cases however, only a subset of all the potential mutations is available in each generation and chance effects will limit the number of trajectories that can be explored by the population. Chance can also arise from drift, i.e. when allele frequencies change irrespectively of their fitness effects through demographic stochasticity. Strong effects of chance will therefore increase the probability that different populations explore different trajectories, and if there are multiple different high-fitness genotypes available can lead to long-term divergence (Lenski & Travisano, 1994; Wiser *et al.*, 2013).

Finally, if there is significant epistasis (i.e. non-additive interactions) between the loci affecting fitness, then evolutionary ancestry can have substantial effects on future evolutionary change, since the fitness effects of novel mutations will depend on the current genetic background of the population. Such effects can reduce the repeatability of evolution by altering the accessibility of certain evolutionary paths to populations at different genetic starting points (Weinreich *et al.*, 2005).

Sex and recombination can potentially affect the repeatability of evolution. The most obvious way is by increasing the efficiency of selection either by bringing together beneficial alleles found in different individuals (Weismann, 1889; Fisher, 1930; Muller, 1932), purging the deleterious mutations from the population (Muller, 1964), or releasing beneficial alleles from inferior backgrounds (Hill & Robertson, 1966; Felsenstein, 1974; Peck, 1994). Ample empirical evidence support the idea that sex and recombination increase rates of adaptation to a novel environment (Colegrave, 2002; Kaltz & Bell, 2002; Goddard *et al.*, 2005; Morran *et al.*, 2009; Becks & Agrawal, 2010; Lachapelle & Bell, 2012; Bell, 2013) and contribute in purging deleterious mutations in constant environments (Zeyl & Bell, 1997; Morran *et al.*, 2009). On the other hand, little is known about the effects sex can have on the importance of chance and ancestry. There is evidence that recombination increases genetic variation within a population after a single episode of sex (Colegrave *et al.*, 2002), but none with regards to the effect of sex on diversity among populations over longer evolutionary timescales. Therefore sex has the potential to increase the repeatability of evolution by increasing the contribution of

142 selection, but how it affects the contribution of chance and ancestry remains to be tested
143 empirically.
144
145 To determine how sex affects the repeatability of evolution, we propagated diverse
146 asexual and sexual experimental populations of the unicellular green alga
147 *Chlamydomonas reinhardtii* in four different environments. We expect sex will increase
148 the efficiency of selection and therefore increase the repeatability of evolution. We find
149 that sex has important consequences on the repeatability of evolution, and that these
150 effects are highly dependent on the environment, with sex enhancing convergence in
151 some environments and divergence in others. Thus, even in relatively simple and similar
152 laboratory environments, the genetic basis of adaptation is different enough for sex to
153 have different consequences on the repeatability of evolution.
154
155
156 **Material and Methods**
157 *Base populations*
158 We generated three genetically different starting points by crossing three different pairs
159 of wild-type strains of *Chlamydmonas reinhardtii*. Ancestry A was generated by using
160 the F1 progeny from a cross between CC-1690 and CC-1691; ancestry B using the F1
161 progeny from a cross between CC-2342 and CC-2344; and ancestry C using the F1
162 progeny from a cross between CC-2931 and CC-2937. These strains have been shown to
163 be genetically (Jang & Ehrenreich, 2012) and phenotypically (Malcom *et al.*, 2014)
164 different. The progeny from each cross should retain a fraction of the genetic signature of

their two parents and therefore maintain on average the genetic dissimilarity that was present among parents from each ancestry. Thus the different ancestries represent genetically different starting points. Twelve spores from each ancestry were isolated, for a total of 36. From now on these spores are referred to as the ancestors. Each experimental line was assembled using eight spores from a given ancestry: the asexual lines contained eight spores of a single mating type (we used spores of mating type - for Ancestry A and C, and spores of mating type + for Ancestry B), whereas the sexual lines contained four spores of mating type + and four spores of mating type -. The asexual and sexual lines from a given ancestry thus shared four ancestral spores. The ancestral spores used to assemble the asexual lines do not differ statistically from the ones used to assemble the sexual lines in their growth rates across the four selection environments described below ($F_{1,10} = 0.78$, $P = 0.40$). This means that the mode of reproduction treatment is not confounded with differences in starting points.

Selection experiment

For each combination of ancestry and mode of reproduction, we had 6 replicate lines, for a total of 3 x 2 x 6 = 36 independent lines. Each line was propagated in each of four different environments: Bold's minimal medium (Harris, 2009); referred to as Bolds); Bold's minimal medium supplemented with 0.435 μM Atrazine and 0.250 μM S-metalochlor (referred to as Herbicides); Bold's minimal medium supplemented with 7 gL^{-1} Na_2SO_4 (referred to as Na_2SO_4); and Bold's minimal medium supplemented with 5 gL^{-1} NaCl (referred to as NaCl). These environments and concentrations were chosen because they target different aspects of growth (e.g. photosynthesis in the case of Atrazine,

synthesis of long chains of fatty acids in the case of S-metalochlor, osmotic and oxidative stresses in the case of NaCl and Na_2SO_4), and because preliminary assays showed that they reduce growth rates to different extents compared to that in the benign environment of Bold's. Each ancestral spore was grown individually from a single colony. Once fully grown, the ancestral spores were pooled together to construct each experimental line, and 24 samples (six replicates in each of four environments) of each mixture were used to initiate each replicate line, which were then propagated independently.

The experiment consisted of vegetative growth cycles interspersed with sexual cycles. The sexual cycles were imposed after about 10, 50, 100, 150, 200, and 260 generations of vegetative growth. The protocol for the sexual cycle was imposed on all lines, even on the asexual lines, which were not expected to mate given that they were composed of spores of only one mating type. Briefly, at the end of a vegetative growth cycle, the spent media was replaced with nitrogen-free media by centrifuging the cultures. The cultures were left static in nitrogen-free liquid media for approximately 24 hours to allow gametogenesis and mating to occur. After this period, the zygotes and 50 μL of culture were transferred to an agar plate, or in the case of the asexual lines 50 μL of culture was transferred to an agar plate. The agar plates were wrapped in aluminium foil and left in the dark for zygote maturation to occur. After four days, mature zygotes were exposed to chloroform vapour for 45 seconds to kill unmated cells, and then placed under the lights for germination. The asexual lines were not exposed to chloroform but put directly under the lights. After two days in the light, the cells were re-suspended in liquid media and transferred back into the vegetative growth cycles. The cultures were then serially

transferred every 3-4 days using a 5% inoculum (100 µL into 1900 µL of fresh media). A total of 6 sexual cycles and 60 vegetative cycles were imposed for a total of about 300 generations.

Seven sexual lines (three from the Na_2SO_4 environment and four from the Herbicides environment) went extinct during the experiment because they failed to mate during the sexual cycle. Attempts were made to mate them again whenever this happened but failed repeatedly in these particular cases.

The lines were cultured in 24-well plates, with breathable sealing films to ensure even evaporation and air exchange across the plate (except during mating where the plastic lids were used to ensure optimal light intensity), shaken at 180 r.p.m. with a 3 mm rotation diameter. The cultures were maintained in a growth chamber at 24 decrees Celsius, 60% humidity, and 8000 Lux constant lighting.

Ancestral fitness assays

To estimate the fitness of the ancestral spores used to assemble each selection line, we calculated the maximum growth rate in each of the four selection environments. The ancestors had been maintained in dim light on Bold's agar throughout the experiment, conditions which limit growth and selection (Harris, 2009). A single colony from each ancestor was grown in Bold's media for two cycles to minimise physiological differences, and then transferred in triplicate to each of the four environments. All cultures were grown for two cycles in the assay environments. Growth was monitored

during the second growth cycle in the assay environments by measuring optical density at 750 nm every 8 ± 1 hours. We chose to measure during the second cycle to allow the three replicates one cycle of independent growth and avoid the measurement of initial physiological response to the new environment.

We transformed the optical density measurements (log10 of (optical density x 10 000)) to allow growth models to be fitted. Growth parameters were extracted from a nonlinear model using nonlinear least squares in the 'nlstools' R package (Baty *et al.*, 2015). We first fitted a baranyi model (Baranyi & Roberts, 1994; Baranyi *et al.*, 1995). The lines that could not be fitted using this model were fitted using either a baranyi model without N_{max}, a baranyi model without lag, or a linear model, as appropriate. Model fits were visually inspected to ensure the proper model had been applied. For each combination of environment, ancestry, and mode of reproduction, we identified the fittest ancestral spore as the one with highest maximum growth rate based on the average of the three replicates.

Evolved fitness assays

The evolved lines from each selection environment were assayed in their respective selection environment in separate experiments because of space constraints. For similar reasons, it was impossible for us to assay all 36 ancestral spores and all 36 evolved lines all at once and so we only assayed the fittest ancestral spore, as identified above, along with the evolved lines. This means that our measure of selection is conservative, detecting only the fixation of novel mutations and not sorting of the initial variation.

257

258 We assayed four random spores per evolved line. 24 spores (6 lines x 4 spores) were
259 picked from the fittest ancestor to match the number of evolved spores assayed per
260 ancestry x reproduction mode. All colonies were grown in Bold's liquid media for one
261 growth cycle to minimise physiological differences, and then transferred to the
262 environment in which the evolved lines were selected. Growth was monitored during the
263 second cycle in the assay environment and growth parameters estimated as described
264 above.

265

266 *Statistical analyses*

267 All analyses were performed in R version 3.2.1. To determine if the ancestral spores used
268 to assemble the sexual lines differ from the ancestral spores used to assemble the asexual
269 lines we fitted a mixed effect model using the lmer function in the R package 'lme4'
270 (Bates *et al.*, 2015). The mode of reproduction (asexual or sexual) was set as a fixed
271 factor, while environment, ancestry, and spore within ancestry were set as random
272 factors. P values were obtained using the R package 'lmerTest' (Kuznetsova *et al.*, 2015)
273 with type III errors in an analysis of variance and Sattertwhaite approximation for degrees
274 of freedom by using the normal approximation.

275

276 The effect of recombination on selection was determined individually for each selection
277 environment by fitting mixed effect models using the lmer function, with mode of
278 reproduction (asexual or sexual) and selection (ancestral or evolved) as fixed factors, and

ancestry, line within ancestry, and spore within line within ancestry as random factors. We allowed for random intercepts and slopes.

To determine the effect of recombination on ancestry, chance, and diversity within lines, we calculated the difference between evolved variances and ancestral variances. Thus a positive change in variance indicates that there is more variation after evolution than at the start (i.e. divergence over time), whereas a negative change in variance indicates that there is less variance after evolution than at the start (i.e. convergence over time). The evolved variances were extracted from a model with ancestry, line within ancestry, and spore within line within ancestry as random factors. Separate models were fitted for each combination of environment and mode of reproduction. The ancestral variances were extracted from a model with ancestry and spore within ancestry as random factors. The among-line ancestral variance was set at zero. Note here that the evolved data and the ancestral data come from different fitness assays. Temporal heterogeneity in environmental conditions between assays can lead to differences in growth rates. It is unlikely that temporal heterogeneity would interact with the mode of reproduction treatment, and so the variance estimates for the asexuals and the sexuals should be affected to the same extent. The actual value of the change in variance is likely to be inexact, and values near zero need to be interpreted with reserve.

This approach of using the change in variance differs from our previous approach (Lachapelle *et al.*, 2015) where we calculated the relative contribution of selection, chance, and ancestry by dividing the evolved variance by the total evolved variance. It is

only appropriate to use proportions to compare treatment levels for their effects on selection, chance, and ancestry, when the initial variance is the same across all treatment levels. For example, if lines are isogenic at the start and the same genotype is used across all treatments, then there is no need to correct for initial variance. However, in cases such as in the experiment reported here where lines are diverse at the start, and sexual and asexual lines cannot be assembled using the same genotypes (because of mating type constraints), it is not appropriate to compare evolved variances without correcting for initial variance. Differing amounts of variance can affect the potential for convergence and divergence among histories, among line, within lines. This is why we report the change in variance instead of the proportion of the total variance explained by either chance or ancestry.

To determine the statistical significance of the differences in the change in variance between asexual and sexual populations we did a randomisation test. We randomly allocated each evolved spore to a line, ancestry, and mode of reproduction (keeping spores within their environment of selection), each ancestral spore to an ancestry and mode of reproduction, and then performed the analysis described above to calculate the change in variance. The number of times the random absolute change in variance was as large or larger than the absolute observed change in variance over the total number of randomisations (10,000) is our significance statistic.

Results

We picked four different environments in which to study the consequences of sex on the repeatability of evolution. The Na_2SO_4 environment is the most severe with slowest growth rates, followed by NaCl, Herbicides, and Bold's (Figure 1; Table 1). Not only do the four environments affect the growth of the ancestors to different extents, but they also reveal differing amounts of variance in fitness (Figure 1; Table 1). The coefficient of variation among spores within ancestries is largest in Herbicides, followed by NaCl, Bold's, and Na_2SO_4. The coefficient of variation among ancestries is largest in NaCl, followed by Herbicides, Na_2SO_4, and Bold's. Thus, the four environments affect growth differently and represent a true test of the generality of the consequences of sex on the repeatability of evolution.

The variance in fitness among ancestral spores within ancestries tends to be greater than among ancestries (Table 1), indicating that there is plenty of standing genetic variation available at the start of the experiment for selection to sort. While the fitness of each ancestry might be similar in each environment, the fact that the three ancestries were generated from different genotypes (see Methods) and that the four different environments reveal differing amounts of variance in fitness, implies that the different ancestries are sufficiently different genetically to validate our test of the consequences of sex on the importance of ancestry.

The effect of sex on selection

We propagated asexual and sexual replicate experimental populations in each of the four selection environments for about 300 generations. The effect of selection is estimated by

348 comparing the fitness of evolved spores to that of the fittest ancestral spore (see
349 Methods), such that the greater the fitness of the evolved spore is relative to its ancestor,
350 the greater the contribution of selection to evolutionary change. The evolved sexual lines
351 have higher growth rates than the evolved asexual lines after evolution in Na_2SO_4 (Figure
352 2; Table 2; effect of reproduction:selection interaction $F_{1,63} = 18.1$, $P = 7.15 \times 10^{-5}$) and in
353 NaCl (effect of reproduction:selection interaction $F_{1,66} = 6.87$, $P = 0.0109$). There is no
354 effect of selection or interaction between reproduction and selection after evolution in
355 Herbicides (effect of selection $F_{1,62} = 0.535$, $P = 0.467$; effect of reproduction:selection
356 interaction $F_{1,62} = 0.149$, $P = 0.701$). There is a significant effect of selection in Bold's,
357 but opposite to expectation with evolved spores having lower growth rates than the fittest
358 ancestral spore (effect of selection $F_{1,66} = 35.4$, $P = 1.11 \times 10^{-7}$) and no effect of
359 interaction between recombination and selection ($F_{1,66} = 2.62$, $P = 0.110$).
360
361 *The effect of sex on divergence of ancestries*
362 If the different ancestries diverged during evolution, then we should see an increase in
363 variance among ancestries, and if the different ancestries converged during evolution,
364 then we should see a decrease in variance among ancestries. Ancestries diverged during
365 evolution in Herbicides and Na_2SO_4, converged in NaCl, whilst no change was observed
366 after evolution in Bold's (Figure 3). The sexual populations diverged more than their
367 asexual counterparts in Herbicides ($P < 0.0001$), diverged less than their asexual
368 counterparts in Na_2SO_4 ($P = 0.0054$), whilst sex had no measurable effect in Bold's ($P =$
369 0.26) and NaCl ($P = 0.26$).
370

371 *The effect of sex on divergence of replicate lines*

372 If the replicate lines diverged during evolution, then we should see an increase in
373 variance among lines, and if the replicate lines have evolved in parallel, the variance
374 should be equal to zero. Divergence has occurred in all selection environments in this
375 experiment (Figure 3). The sexual lines diverged less than their asexual counterparts
376 during evolution in Herbicides ($P < 0.0001$), diverged more than their asexual
377 counterparts during evolution in Na_2SO_4 ($P < 0.0001$) and Bold's ($P = 0.0084$), whilst sex
378 had no measurable effect in NaCl ($P = 0.26$).

379

380 *The effect of sex on diversity within lines*

381 If diversity within lines increased during evolution, then we should see an increase in
382 variance among spores, and if diversity was lost during evolution, then we should see a
383 decrease in variance among spores. Note that our design for the fitness assays is such that
384 we can separate out variance within lines from variance from measurement error (see
385 Methods). There is more diversity within lines after evolution in Na_2SO_4, whilst there is
386 less diversity within lines after evolution in Bold's, Herbicides, and NaCl (Figure 3). The
387 sexual lines had a greater increase in diversity within lines than their asexual counterparts
388 after evolution in Na_2SO_4 ($P = 0.028$), a greater decrease in diversity after evolution in
389 Bold's ($P = 0.036$), whilst sex had no measurable effect on diversity within lines in
390 Herbicides ($P = 0.075$) and NaCl ($P = 0.21$).

391

392

393 **Discussion**

Most of the research on sex has focussed on the mechanisms for its origin and maintenance over evolutionary time, while much less consideration has been given to the potential downstream consequences sex can have on the repeatability of evolution. We propagated sexual and asexual lines in four different novel environments for 300 generations. By measuring the change in fitness, the change in variance among ancestries and among replicate lines, and the change in diversity within lines, we were able to determine the consequences of sex on the contribution of selection, ancestry, and chance to evolution.

The general prediction is that sex and recombination increase the repeatability of evolution by increasing the efficiency of selection (Burt, 2000; de Visser & Elena, 2007). Our results refute this hypothesis. We find that sex has significant consequences for the repeatability of evolution that are far from general, differing in each environment investigated. In Bold's, recombination has no effect on selection and ancestry but increases chance, and hence reduces repeatability overall; in Herbicides recombination has no effect on selection, but increases effects of ancestry and reduces chance; in Na_2SO_4 recombination increases effects of selection and chance, but reduces effects of ancestry; and finally in NaCl recombination increases effects of selection, but has no effect on ancestry or chance, and hence increases repeatability overall. These variable outcomes indicate that the effects of sex are highly dependent on the specific genetic basis of adaptation, the precise mechanism of which remains to be fully determined.

The lack of generality of the effect of sex on the repeatability of evolution

417 We know from theory that the effects of sex depend on the genetic basis (e.g. the number
418 of genes and their pattern of interaction) of adaptation (Otto *et al.*, 1994; Kondrashov &
419 Kondrashov, 2001; Hadany & Beker, 2003; 2005; de Visser *et al.*, 2009). The fact that
420 we observed dramatically different effects of sex implies that the genetic basis of
421 adaptation differs significantly between the environments we used, despite the fact that
422 all were simple and relatively similar laboratory environments.
423
424 The lack of a general effect of sex is consistent with other findings of the effect of sex on
425 the evolution of herbicide resistance (Lagator *et al.*, 2014) and with the contrasting results
426 in terms of repeatability of evolution reported for sexual species (Teotónio & Rose, 2000;
427 Teotonio *et al.*, 2002; Joshi *et al.*, 2003; Kawecki & Mery, 2003; Griffiths & Schiffer,
428 2005; Simões *et al.*, 2008; Fragata *et al.*, 2014). It most likely reflects differences in
429 linkage disequilibrium as this is an important factor in determining the contribution of
430 chance and ancestry during evolution (Weinreich & Chao, 2005).
431
432 The fitness landscape (i.e. the regression of individual fitness on genotypic space) is a
433 useful heuristic for thinking about the contribution of chance and ancestry to evolution. In
434 fitness landscapes, peaks represent trait combinations of high fitness. When there are
435 multiple fitness peaks, the importance of chance and ancestry depends critically on the
436 probability of shifting from sub-optimal to optimal fitness peak. Peak shifts can occur
437 through double-step or double mutants (Gillespie, 1984; Weinreich & Chao, 2005) if the
438 combination of two mutations takes the population to a peak other than the one currently
439 occupied. Recombination will tend to generate such ‘escape’ genotypes if linkage

disequilibrium is negative, and will break apart escape genotypes when linkage disequilibrium is positive (Weinreich & Chao, 2005). Differences in linkage disequilibrium can arise because of differences in population size, in the distance to a fitness peak, and/or in the genetic basis of adaptation (Otto *et al.*, 1994; Weinreich & Chao, 2005; de Visser *et al.*, 2009). The four environments in our selection experiment differed with respect to all of these factors and so provided a strong test of the robustness of recombination to differences in linkage disequilibrium.

Population size will affect the repeatability of evolution in both asexual and sexual population by altering the supply of beneficial mutations and the amount of clonal interference (Gerrish & Lenski, 1998; Lachapelle *et al.*, 2015). In small populations, peak shifting will rely on a stochastic process of sequential fixation of single mutations, whereas in large populations peak shifting can occur by a deterministic process of simultaneous fixation of jointly beneficial mutations (Carter & Wagner, 2002; Iwasa *et al.*, 2004). In sexual populations, recombination can break apart the escape genotypes before they become fixed. Peak shifting then becomes a stochastic process, where deleterious single mutants need to rise to sufficiently high frequency for recombination to combine them and generate the escape genotypes more often than it breaks them apart (Weinreich & Chao, 2005). Differences in population size can therefore affect the effect of recombination by altering the frequency of escape genotypes and thus the stochastic or deterministic nature of peak shifting.

462 The distance from a fitness peak can also affect the role of recombination during
463 evolution by determining the number of beneficial mutations available, the number of
464 possible trajectories, and the amount of linkage disequilibrium (Otto *et al.*, 1994). For
465 example, as the distance to the peak increases, recombination gains a greater advantage
466 by speeding up the rate at which the population reaches the peak (de Visser *et al.*, 2009).
467 Differences in the type of interactions among genes will also affect the effect of
468 recombination on the repeatability of evolution. Negative epistasis, where the fitness
469 effect of many alleles is lower than predicted by the product of their individual effects,
470 can cause negative linkage disequilibrium and therefore increase the response to selection
471 and the probability of peak shifting (Barton, 1995). Sign epistasis, where the sign of the
472 fitness effect of one mutation depends on what alleles are present at other loci, can also
473 affect the role of recombination by altering the ruggedness of the fitness landscape and
474 the accessibility of certain mutational paths (Weinreich *et al.*, 2005).
475
476 Hence, while our data does not identify which attribute, population size, distance to a
477 fitness peak, or genetic basis of adaptation, is driving the inconsistency in effects of sex,
478 it suggests that the parameter space used by theoretical studies probably reflects an
479 appropriate if not underestimation of the degree of variation among natural environments.
480 The effects of sex on evolution are highly dependent on the genetic background and the
481 environment and we therefore cannot assume that results from experiments in a single
482 genotype or environment will generalise to other environments. Further experiments need
483 to be carried out to disentangle the role of genetics and different environmental attributes.
484

The efficiency of selection in initially diverse populations

In initially diverse populations, selection can act on standing genetic variation and on new mutations. One approach to measuring the efficiency of selection when experimental lines are initially diverse is to compare individual evolved spores to individual ancestral spores. We used the fittest ancestral spore as our comparison. If all the evolved spores perform as well as the fittest ancestral spore, sorting has occurred, leading to the fixation of the fittest ancestral spore. If all the evolved spores perform better than the fittest ancestral spore, new mutations (and/or recombination in sexual populations) have contributed to evolutionary change. These inferences assume that sorting will occur before beneficial mutations arise in less fit ancestral spores and become fixed.

An alternative approach to measuring the efficiency of selection in initially diverse populations would be to use population-level fitness estimates. We have opted against population estimates as they depend heavily on the composition of the population, i.e. the number of different genotypes and their respective frequency. Therefore any alteration of the composition through storage and revival of populations for example, would lead to erroneous estimates. Furthermore, contrary to spore-level comparisons, population-level fitness change estimates will detect the action of selection, but will not reveal any information about the contribution of standing genetic variation compared to that of new mutations to evolutionary change.

Our results suggests that in Na_2SO_4 and NaCl, new mutations played a role in adaptation as the evolved spores have higher growth rates than the fittest of the ancestral spores. In

the Herbicide environment, the growth of the evolved spores is not, on average, any different from that of the fittest ancestral spore. This suggests that adaptation occurred solely through sorting, with no contribution from new mutations. Evolution is more likely to occur from standing genetic variation when the variation is relevant to growth in the new environment, in high enough frequency, and reduced population sizes limit the contribution of novel mutations (Hermisson & Pennings, 2005). Indeed, the coefficient of variation within lines was largest in the Herbicides environment, and population sizes rebounded the quickest amongst all environments, suggesting that the large amount of variance was relevant and sufficient in this environment to lead to rapid evolutionary responses. A rapid response is consistent with adaptation from standing variation that is immediately available and in high frequency (Barrett & Schluter, 2008).

Evolution in the Bold's environment led to lower growth rates than that of the fittest ancestral spore. Bold's is a benign environment where growth rates are high, and beneficial mutations are likely to be rare. In such cases the effect of selection is therefore more to remove deleterious mutations in order to maintain growth rates than to fix beneficial mutations, an effect that we cannot measure with our data. The lower growth rates could be attributable to failure to remove deleterious mutations, but also to inefficient sorting of the standing genetic variation or to a trait other than maximum growth rate being under selection.

When there is initial variance in fitness, it will be sorted quicker the larger it is and lead to an increase in population mean fitness. Variance in fitness is initially high in both the

asexual and sexual lines in Bold's (Table 2). As a rough estimate, for a selective advantage of 0.1 (based on the variance present initially in the lines), and an initial frequency of 1/8, we expect the fittest spore to rise to 99% frequency within 45 generations. Diversity was almost completely lost within both the asexual and sexual lines, which is further evidence that sorting did occur. It is therefore unlikely that inefficient sorting in the asexual and sexual lines is responsible for their lower mean fitness. It is also unlikely that deleterious mutations fixed (either singly or through hitchhiking) given the short evolutionary timescale (300 generations) and the relatively large deleterious effect size that would be needed to produce such drop in growth rate. Ultimately, we cannot exclude the possibility that slower growth rates arose both in asexual and sexual lines because selection in Bold's favours greater competitive ability, higher carrying capacity, or slower growth rates (Schaum & Collins, 2014) instead of faster growth rates.

Conclusion

Sex has important downstream consequences on diversity within and among populations. We find that sex affects the efficiency of selection, and hence the degree to which fitness increases, which is consistent with what other studies on the adaptive function of sex have found. But we also find that sex affects the contribution of chance and ancestry, and hence the degree to which populations converge or diverge in fitness during evolution. By altering the repeatability of evolution, sex could have long-term effects on rates of diversification, and affect our ability to use evolutionary theory to make predictions about

554 the outcome of environmental change. However, we find that the consequences of sex on
555 the repeatability of evolution are not general, with different consequences in different
556 environments. Even the relatively simple and similar environments used here appear
557 different enough to evolving populations to lead to different effects of sex on patterns of
558 change in diversity. We can only assume that natural environments will differ even more
559 radically. Hence, overall, our results indicate that the effect of sex on evolution of
560 populations is highly dependent on genetic background and environment. More rigorous
561 tests are needed to determine the exact mechanisms by which environmental attributes
562 mediate the effect of recombination. But until then, a greater commitment to using many
563 environments should be given in order to reduce biased and specific results in evolution
564 experiments.
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566
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723 **Tables**

724 Table 1. Variance among ancestral spores and ancestries in each of the four selection

725 environments. CV is the coefficient of variation.

Environment	Reproduction	Source	Variance	Mean maximum growth rate	CV
Bolds	asexual	Spore	0.984	5.20	0.191
		History	3.53×10^{-16}		3.62×10^{-9}
	sexual	Spore	1.98	4.67	0.301
		History	0.00		0.00
Herbicide	asexual	Spore	0.285	2.38	0.224
		History	0.0502		0.0942
	sexual	Spore	0.706	2.24	0.375
		History	1.11×10^{-14}		4.70×10^{-8}
Na_2SO_4	asexual	Spore	0.0333	1.08	0.169
		History	0.00		0.00
	sexual	Spore	1.92×10^{-15}	0.982	4.46×10^{-8}
		History	0.0182		0.137
NaCl	asexual	Spore	0.312	1.81	0.309
		History	0.202		0.248
	sexual	Spore	0.302	1.53	0.360
		History	0.171		0.271

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729 Table 2. The effect of recombination on the efficiency of selection at increasing growth
730 rates in each of the four selection environments. The parameter estimates for the fixed
731 effect are shown, where 'Selection' has two levels (ancestral and evolved) and
732 'Reproduction' has two levels (asexual and sexual).

Environment	Effect	Estimate	SE
Bold's	Intercept	4.9	0.22
	Selection (evolved)	-0.63	0.26
	Reproduction (sexual)	-1.4	0.26
	Selection (evolved) : Reproduction (sexual)	0.60	0.37
Herbicides	Intercept	2.7	0.23
	Selection (evolved)	-0.11	0.20
	Reproduction (sexual)	0.16	0.20
	Selection (evolved) : Reproduction (sexual)	-0.11	0.29
Na_2SO_4	Intercept	1.2	0.11
	Selection (evolved)	-0.095	0.12
	Reproduction (sexual)	0.56	0.12
	Selection (evolved) : Reproduction (sexual)	0.71	0.17
NaCl	Intercept	1.6	0.25
	Selection (evolved)	0.86	0.14
	Reproduction (sexual)	0.068	0.14

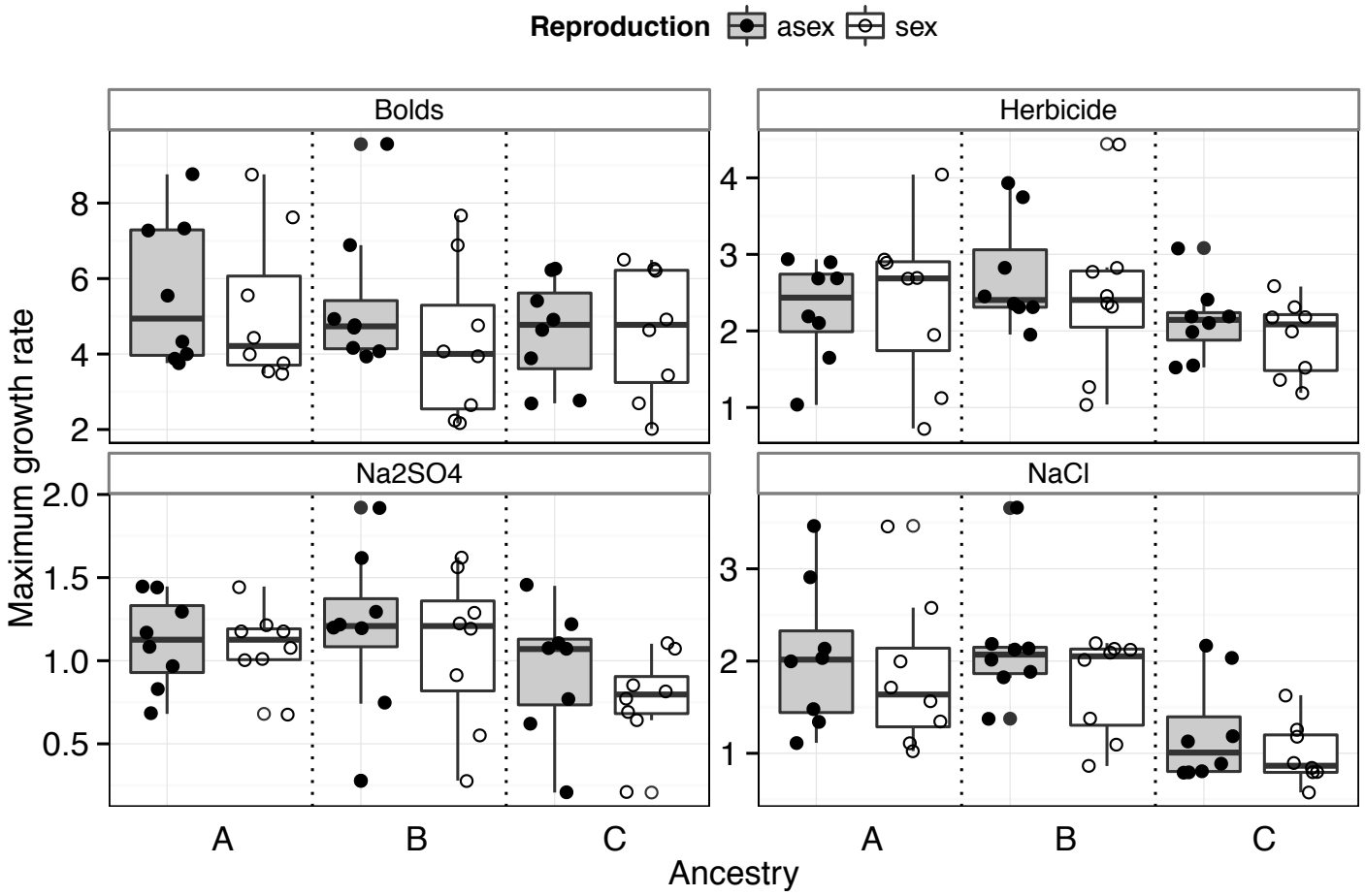
Selection (evolved) : Reproduction (sexual)	0.51	0.19

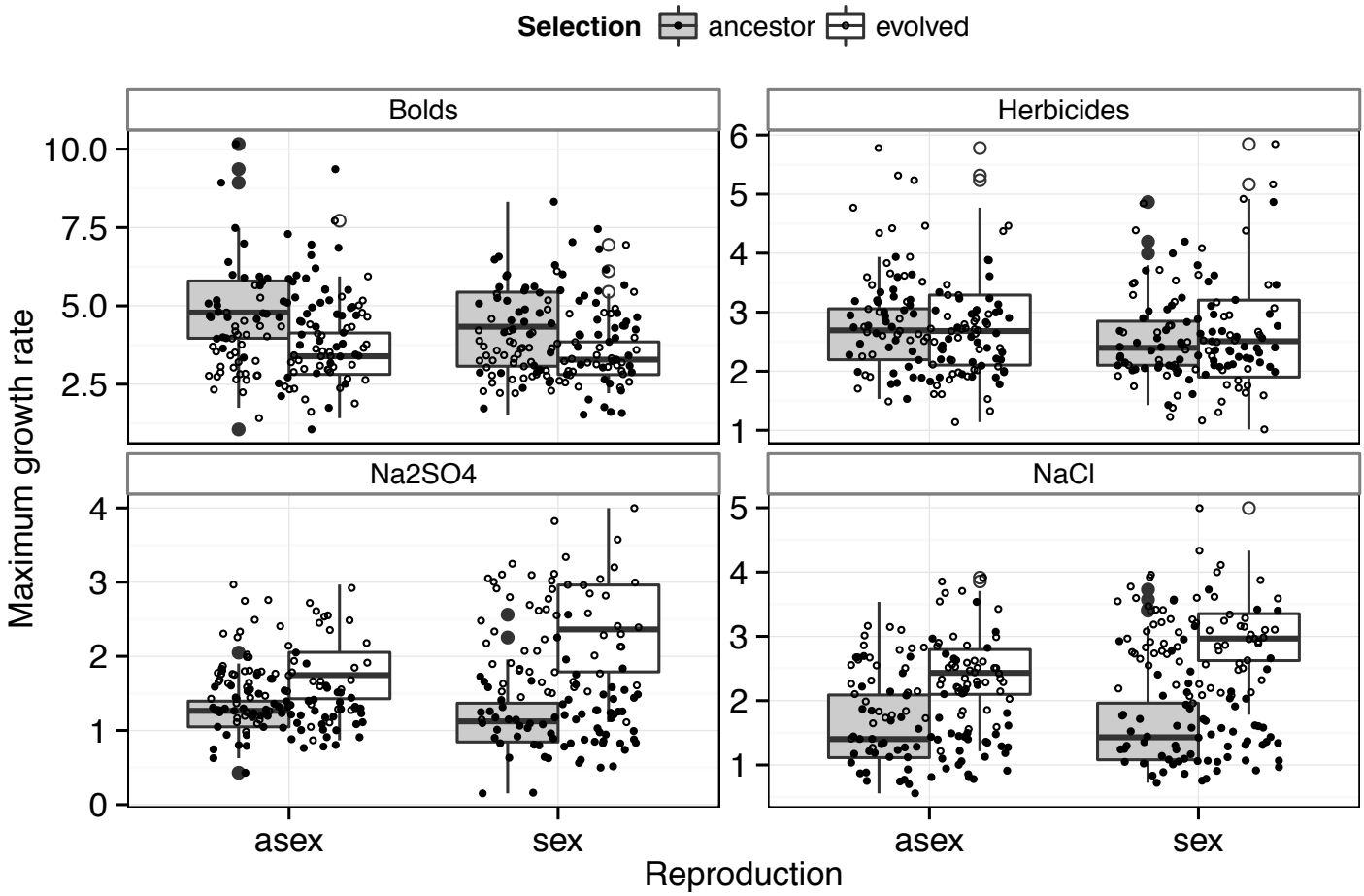
Figure legends

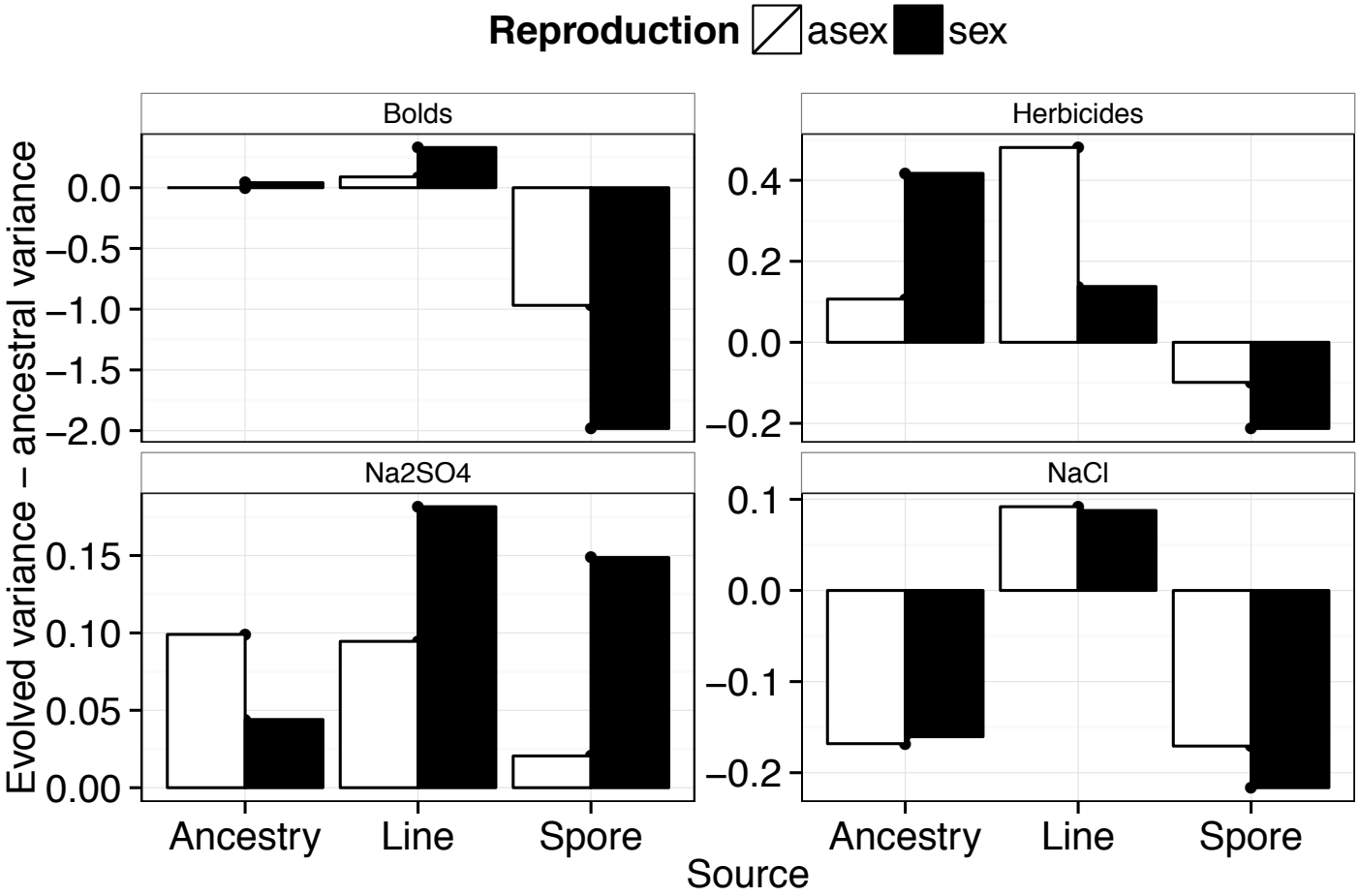
Figure 1. Growth rate of the eight ancestral spores used to initiate each asexual and sexual selection lines, in each of the four selection environments. Each point represents the average of the three assay replicates.

Figure 2. Growth rate of ancestral and evolved spores in the corresponding selection environment. Each point represents the average of the three assay replicates. There are 4 spores for each of 36 lines (except in Herbicides where there are 32 lines and in Na_2SO_4 where there are 33 lines). The larger data points are part of the boxplot layer and represent outliers.

Figure 3. Change in variance after evolution in each selection environment in asexual and sexual populations. Ancestry represents variance among ancestries, Line represents variance among replicate lines within ancestries, and Spore represents variance among spores within lines within ancestries.


Reproduction asex sex
Bolds
Herbicide
Na2SO4
NaCl
Maximum growth rate
Ancestry
A
B
C


Selection ancestor evolved
Bolds
Herbicides
Na2SO4
NaCl
Maximum growth rate
Reproduction
asex
sex


Reproduction
asex
sex
Bolds
Herbicides
Na2SO4
NaCl
Evolved variance − ancestral variance
0.0
−0.5
−1.0
−1.5
−2.0
0.4
0.2
0.0
−0.2
0.15
0.10
0.05
0.00
0.1
0.0
−0.1
−0.2
Ancestry
Line
Spore
Source

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Experimental adaptation to marine conditions by a freshwater alga

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The marine-freshwater boundary has been suggested as one of the most difficult to cross for organisms. Salt is a major ecological factor and provides an unequalled range of ecological opportunity because marine habitats are much more extensive than freshwater habitats, and because salt strongly affects the structure of microbial communities. We exposed experimental populations of the freshwater alga *Chlamydomonas reinhardtii* to steadily increasing concentrations of salt. About 98% of the lines went extinct. The ones that survived now thrive in growth medium with 36 g·L^{-1} NaCl, and in seawater. Our results indicate that adaptation to marine conditions proceeded first through genetic assimilation of an inducible response to relatively low salt concentrations that was present in the ancestors, and subsequently by the evolution of an enhanced inducible response to high salt concentrations. These changes appear to have evolved through reversible and irreversible modifications, respectively. The evolution of marine from freshwater lineages is an example that clearly indicates the possibility of studying certain aspects of major ecological transitions in the laboratory.

KEY WORDS: ***Chlamydomonas*, constitutive and inducible response, evolutionary rescue, phenotypic plasticity, recombination, salt tolerance.**

From time to time, a lineage may become adapted to conditions that lie far outside those that would be tolerated by its ancestors. In most cases, this need imply no more than the ability to grow in a specific extreme environment, as in the evolution of antibiotic resistance in bacteria (Davies and Davies 2010) or heavy metal resistance in plants (Gregory and Bradshaw 1965). The evolved lineage then flourishes but does not become further modified. In exceptional cases, the novel conditions to which a lineage has become adapted are widespread in nature, and its new ecological attributes then have the potential to lead to an adaptive radiation.

Here, we report the evolution of a marine way of life in the freshwater alga *Chlamydomonas reinhardtii*. It has been suggested that the marine-freshwater boundary is exceptionally difficult to transgress (Lee and Bell 1999; Vermeij and Dudley 2000). In plants and yeasts, for example, moving between regions of different salt concentrations requires changes in influx, efflux, and containment of ions, as well as changes in the ability to detoxify reactive oxygen species (Brewster et al. 1993; Mendoza et al. 1994; Zhu 2000). The pressures that freshwater and high-salt conditions impose on microbes are so different that salt is more important in governing community composition than temperature, pH, substrate, or other physicochemical variables (Lozupone and Knight 2007). Transitions between the two conditions are consequently infrequent and ancient, as revealed by the large phylogenetic distances between freshwater and marine microorganisms (Logares et al. 2009). High-salt habitats are much more extensive than freshwater habitats, and beside the ocean covering 70% of the surface of the Earth include enclosed seas, inland saline lakes, and coastal saltmarshes. Hence, the transition from freshwater to marine conditions both enforces major physiological changes and provides an unparalleled range of ecological opportunities.

*These authors have contributed equally.

Individuals that encounter novel conditions, such as high salt concentration, may be constitutively able to tolerate them and to continue to grow and reproduce. The constitutive response may evolve if there are alleles segregating in the population that confer different degrees of tolerance. Alternatively, an individual that in its current state is unable to tolerate these novel conditions may be able to modify its state so as to be able to grow and reproduce, a process called phenotypic plasticity. The inducible response may be under genetic control through regulatory elements (e.g., lactase expression in *E. coli*; Dykhuizen and Hartl 1978; Dykhuizen and Davies 1980) and the capacity to mount an inducible response may itself evolve (Lande 2009). Hence, adaptation to a novel environment may be attributable to the evolution of the constitutive response or the induced response or both. Both processes have been shown to play a role in natural populations adapting to changes in the environment (Reale et al. 2003; Charmantier et al. 2008; Gienapp et al. 2008; van de Pol et al. 2012) as well as in facilitating macroevolutionary events such as the origin of new taxonomic groups and of novel traits (Wund et al. 2008; Rajakumar et al. 2012; Standen et al. 2014).

The extent to which the constitutive and inducible responses will evolve will depend on the availability of beneficial variation. A lack of variants with positive growth rates will limit the ability of natural selection to bring the population's mean phenotype toward the new optimal phenotype (Lynch et al. 1991). Not surprisingly, the most common outcome of changes in ecological conditions is therefore extinction (Burger and Lynch 1995; Bell and Collins 2008). In some cases, however, "evolutionary rescue" may occur (Gomulkiewicz and Holt 1995), with a population evolving to tolerate conditions that would have been lethal to its ancestor. Rescue is more likely in large populations (Bell and Gonzalez 2009; Willi and Hoffmann 2009), in diverse and sexual populations (Agashe et al. 2011; Lachapelle and Bell 2012; Bell 2013a), and when environmental deterioration is slow (Perron et al. 2008; Bell and Gonzalez 2011). Rescue is thought to involve positive genetic correlations of fitness between different levels of stress, such that tolerance of lethal stress is an indirect response to selection at lower levels of stress (Samani and Bell 2010; Gonzalez and Bell 2013).

Preexisting or evolved phenotypic plasticity can also lead to survival. In plastic individuals, the inducible response to changes in environmental conditions can trigger behavioral, physiological, or morphological changes which may decrease the distance between the phenotype of the individual and the phenotype that maximizes fitness. Phenotypic plasticity can lead to greater genetic variation if it reduces the effectiveness of selection (Draghi and Whitlock 2012) and reduce the rate of population decline following environmental change, and thereby provides an opportunity for genetic adaptation to occur (Chevin and Lande 2010; Gomez-Mestre and Jovani 2013; Schaum and Collins 2014).

Plasticity may eventually become constitutively expressed, a process called genetic assimilation (Waddington 1942, 1952, 1953; Schmalhausen 1949; West-Eberhard 2003; Pigliucci et al. 2006; Crispo 2007; Lande 2009; Pfennig et al. 2010). This may occur as the result of selection against plasticity if it is costly to maintain (Snell-Rood et al. 2010), through mutational degradation or drift following long periods of stasis (Masel et al. 2007), or through strong stabilizing selection, which reduces genetic variation and thereby attenuates the genetic correlation between plasticity and the mean breeding value (Lande 2009). The outcome of genetic assimilation is therefore a reduction in plasticity and the constitutive expression of a trait equivalent to that originally produced as a plastic response to the new environment. Genetic assimilation is often difficult to identify because the ancestral reaction norms are not known or because it can occur rapidly (Pigliucci and Murren 2003). Nevertheless, there is some evidence from natural populations that genetic assimilation may contribute to survival and adaptive radiation following environmental change (Gomez-Mestre and Buchholz 2006; Bull-Herenu and Arroyo 2009; Scoville and Pfrender 2010).

We propagated experimental lines of the green alga *C. reinhardtii* in gradually increasing concentrations of salt until we obtained lines capable of growing in seawater within about 500 generations. *Chlamydomonas reinhardtii* typically lives in soil and freshwater. The salinity of soil water is expected to vary depending on soil composition and anthropogenic fertilization, but the salinity of rainwater itself, or the overflow from rivers and lakes, is usually lower than 500 parts per million. The strains used to initiate this experiment have been propagated in the laboratory for over 10 years on medium containing 0.025 $g \cdot L^{-1}$ NaCl (0.0004 M). The salinity of seawater on the other hand is about 35 parts per thousand or 35 $g \cdot L^{-1}$ (0.6 M), of which about 90% is sodium (Na^+) and chloride (Cl^-). High salinity imposes strong osmotic and oxidative stresses in *C. reinhardtii* by disrupting the homeostasis of ions (Na^+, Cl^-, K^+, and Ca^{2+}) and degrading proteins, and thereby reducing rates of photosynthesis and cell division (Husic and Tolbert 1986; Neelam and Subramanyam 2013). In general, salinities between 5 and 7 $g \cdot L^{-1}$ NaCl (0.085 and 0.120 M) are sufficient to reduce the growth of *C. reinhardtii* by about 50%, and salinities higher than between 8 and 15 $g \cdot L^{-1}$ NaCl (0.137 and 0.26 M) are sufficient to suppress growth completely (Reynoso and de Gamboa 1982; Moser and Bell 2011; Lachapelle and Bell 2012). The marine way of life is therefore inaccessible to *C. reinhardtii*. A green alga, identified morphologically as a *Chlamydomonas* sp. was previously isolated off the coast of Japan and characterized for its high salt tolerance (Miyasaka

et al. 1998, 2000; Tanaka et al. 2007). We use this strain as a comparison for the growth of our salt-selected lines in seawater.

To determine the mechanism of adaptation to high salt, we measured the constitutive and the inducible responses to different salt concentrations by manipulating the acclimation environment. We compared the reaction norms of the salt-selected lines to that of their ancestors and found that both types of response had been modified by natural selection. Plasticity for growth in low salt in the ancestors has been genetically assimilated in the salt-selected lines, and plasticity for growth in high salt has been enhanced. Our experiment does not by any means reproduce all of the stages in the colonization of the oceans by terrestrial or freshwater organisms. It does permit some components of this process to be implemented in the laboratory, however, where the mechanism of adaptation can be elucidated by replicated experiments.

Methods

BASE POPULATIONS

We isolated one spore from each of 40 different lines that had been propagated independently for two years in the laboratory, growing in the dark on medium supplemented with acetate. These dark lines, from now on referred to as the ancestors, were derived from a previous experiment (Bell 2005), whose ancestors were derived from a cross among standard laboratory strains (CC-124 × [CC-1952 × (CC-1952 × CC-2343)]). The lines have not experienced salt concentrations higher than 0.025 $g \cdot L^{-1}$ NaCl (4.28×10^{-4} M) during more than 10 years of culture in our laboratory.

SELECTION EXPERIMENT IN EVER INCREASING SALT CONCENTRATION

Details of the initial stages of the selection experiment can be found in Lachapelle and Bell (2012). Briefly, experimental lines varying by their sexuality (asexual, facultatively sexual, or obligately sexual) and initial diversity (low or high) were propagated in an environment where the concentration of salt increased by 1 $g \cdot L^{-1}$ NaCl every two growth cycles (i.e., every about 10 generations). The lines that survived longest came from high-diversity, sexually derived ancestors. The two lines able to grow in the highest concentration of salt (up to 30 $g \cdot L^{-1}$ NaCl) were used for crosses to continue the selection experiment. It is this continuation of the experiment that we report here. A wild-type strain of opposite mating type to each line (CC-2935 mating type minus) was used to perform the initial cross. The progeny were then mated within and across the F1 families to generate the F2. Gamete fusion and zygote germination followed standard practice (e.g., Lachapelle and Bell 2012). We grew the progeny in 34 $g \cdot L^{-1}$ NaCl for two growth cycles. Only 23 resistant recombinants survived out of about 10^6 cells. The progeny was therefore clearly incapable of growth in 34 $g \cdot L^{-1}$ NaCl, and these 23 surviving cells were presumably the ones with the least negative growth rates. We isolated them and propagated each individually, once again in gradually increasing concentrations of salt, starting at 24 $g \cdot L^{-1}$ NaCl. The lines were cultured in 48-well plates with 1.4 mL of Bold's medium supplemented with salt, and transferred every week (two weeks when growth was poor) using a 0.2 mL inoculum. The salt concentration was increased every two or three growth cycles up until 36 $g \cdot L^{-1}$, at which point it was maintained constant. From the 23 starting lines, 13 survived up to 36 $g \cdot L^{-1}$, and 10 have subsequently survived repeated transfers in that concentration. At the time of assay, the surviving lines had been propagated for a total of about 500 generations since the beginning of the selection experiment (Fig. 1).

SEAWATER GROWTH ASSAY

To determine whether adaptation to high salt had resulted in a transition from freshwater to marine conditions, we assayed the surviving salt-selected lines, the ancestral lines, the wild-type strain that was used to set up the crossing trial, and a related marine chlorophyte (*Chlamydomonas* sp. CW-80, isolated off the coast of Japan; Miyasaka et al. 1998) in seawater. The seawater was collected in August 2013 off the coast of Dunbar, UK, and filter-sterilized 2 h after collection. The assay was performed with the same inoculum size and cycle period that the salt-selected lines experienced during the selection experiment. The ancestral lines had been propagated in the dark, using acetate as a carbon source, for the duration of the selection experiment. For the assay, all lines were acclimated in Bold's medium without salt, in the light without acetate, for two cycles before being transferred to the seawater.

Cell density at the end of the first and second cycles in seawater was estimated for two independent replicate cultures using flow cytometry (BD FACSCanto II, BD Biosciences, Oxford, UK). The instrument was calibrated with CS&T beads, and sample acquisition was made using a high-throughput system. Data were acquired and analyzed with the BD FACSDiva version 6 software. Electronic analysis gates were applied to the forward scatter (pulse area FSC-A and width FSC-W) and side scatter (pulse area SSC-A) plots (proxies for cell size and complexity, respectively) to exclude events that are outside expectations for intact *C. reinhardtii* cells, as well as to sort the single cells from clumps of cells. We excluded clumps because we cannot estimate how many cells they contain. Clumps arise as a physiological response to salt in both ancestral and evolved cultures, and should therefore not bias our estimates of growth. All events that were inside the intact and the single-cell gates in a volume of 30 μl acquired at a rate of 1 $\mu l \cdot sec^{-1}$ were used to estimate cell density in each culture. Culture samples with cell counts of 10 or fewer were not included in further analyses because of the potential for false positives at very low or zero cell density. Cell density at the

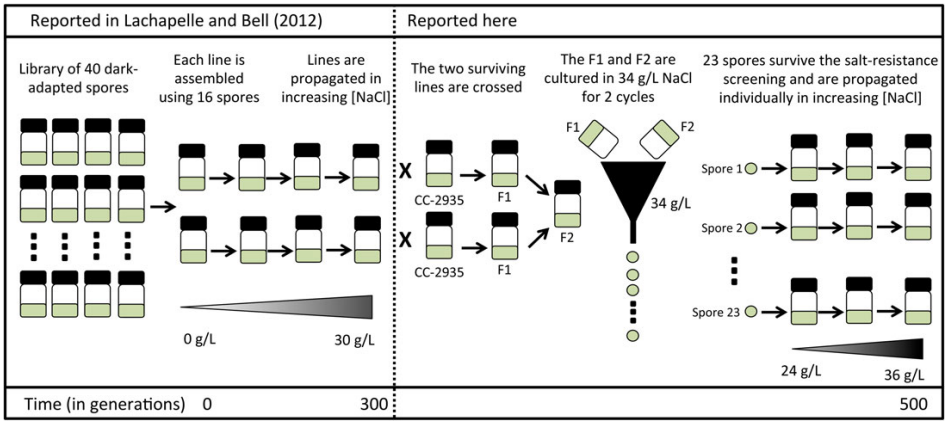

Figure 1. Schematic of the history of the salt-selected lines. Only 10 lines are now surviving in 36 g·L^{-1} NaCl.

end of the first cycle was used to estimate cell density at the start of the second cycle. We calculated the rate of increase per week as the natural logarithm of final cell density divided by initial cell density.

MEASURING THE INDUCIBLE AND CONSTITUTIVE RESPONSES TO SALT

To determine the extent to which the constitutive and the inducible responses to salt were altered in the selection lines, we performed assays comparing the salt-selected lines to the ancestors, and comparing the responses to salt after acclimation in medium lacking salt and in medium containing a stressful but sublethal concentration of NaCl. All assays, unless noted otherwise, were carried out in the light without acetate, as in the extended selection experiment. Note that by "constitutive" we mean that the phenotype is independent of environmental cues. Although constitutive phenotypes are generally associated with genetic changes, it is well recognized that epigenetic changes are mitotically stable (Jablonka and Raz 2009). A constitutive phenotype can therefore arise from genetic and/or epigenetic changes in asexual populations, and this is investigated as described in the following subsection.

The constitutive response was determined in two ways. First, we compared the growth of the salt-selected lines to the ancestral lines after a period of growth in medium lacking salt. The difference between the two selection histories reflects the direct response to selection and the degree of adaptation that is expressed without need for prior acclimation to salt. The assay was initiated by growing all lines in the light, in medium without salt Supplementation, for two growth cycles of one week each. After this period of acclimation, two replicates of each line were transferred to a range of salt concentrations (0, 5, 10, 15, 20, 32, 36, and 40 g·L^{-1} NaCl) and grown for two cycles. Fitness was estimated as in the seawater growth assay described above. The difference in responsiveness (i.e., the change in the rate of increase as a function of salt concentration), as well as the amount of variance in growth that could be explained by the history of the lines (i.e., ancestral or salt-selected) was used to determine the degree of change in the constitutive response.

Second, we compared the contribution of constitutive and inducible responses to salt. Two replicates of each salt-selected line were acclimated in each of 0, 10, and 36 g·L^{-1} NaCl for two growth cycles of one week each before being transferred to a range of salt concentrations (0, 10, 15, 20, 30, 36, and 40 g·L^{-1} NaCl). Fitness was estimated as in the seawater growth assay. The variance of growth among lines estimates differences in the constitutive response, and the variance of growth among acclimation environments estimates differences in the inducible response.

We carried out a further assay to determine whether the inducible response to salt in the salt-selected lines is evolved or ancestral, and whether the response of the ancestral lines to salt is due to the salt itself or to photosynthetic growth. We assayed the ancestral lines in the dark and in the light after acclimation in medium lacking salt and in medium containing 5 g·L^{-1} NaCl (because most ancestral lines cannot sustain growth in higher concentrations). After acclimation, growth was assayed over a range of salt concentrations (0, 5, 10, 15, 20, and 30 g·L^{-1} NaCl).

CHARACTERIZING THE PHENOTYPE OF SEXUAL PROGENY

To examine further the mechanisms responsible for the evolution of the constitutive and the inducible responses to salt, we crossed each of two of the selection lines to an ancestral line to create F1 families, and then crossed within and between these families to create the F2. We chose eight random spores from each generation of each cross and acclimated them either in medium lacking salt or in medium containing 10 g·L^{-1} NaCl. They were then

assayed over a range of salt concentrations (0, 28, 36, 44, and 48 $g \cdot L^{-1}$). If genetic changes are responsible for the evolution of the constitutive and/or inducible responses, we expect the sexual progeny to retain tolerance of salt to different extents depending on the number of genes involved and interactions among them. If reversible changes, such as epigenetic changes, are responsible for the evolution of the constitutive and/or inducible responses, we expect tolerance of the salt-selected lines to be annulled by meiosis.

STATISTICAL ANALYSES

Cultures for which estimates of the initial or final cell densities were zero were removed from the analysis to permit model fitting. The removal of some datapoints led to unbalanced designs in most cases, so we calculated type III sum of squares in all analyses of variance using the R package "car" (Fox and Weisberg 2011).

To compare the constitutive response in the high-salt lines to the constitutive response in the ancestors, we fitted a linear mixed-effects model using the lmer function in the R package "lme4" (Bates et al. 2012), with selection history as a fixed factor, line nested within selection history as a random factor, salt assay concentration (between 0 and 20 $g \cdot L^{-1}$ NaCl where the relationship is linear) as a continuous variable, and the interactions as factors. We allowed for random intercepts and random slopes. Type III Wald tests were performed to determine significance of the fixed effects.

To compare the constitutive and inducible response in the ancestors when grown in the dark or in the light, we fitted a linear mixed-effects models using the lmer function, with acclimation regime (with or without salt) and condition (dark or light) as fixed factors, assay salt concentration as a continuous variable, line as a random factor, and all interactions. We allowed for random slopes and intercepts.

To test the hypothesis that plasticity in the ancestors has been genetically assimilated in the salt lines, we fitted a linear mixed-effects model using the lmer function with selection history as a fixed factor, lines nested within selection history as a random factor, assay salt concentration (between 0 and 10 $g \cdot L^{-1}$) as covariate, and all interactions. The data used in this analysis come from the ancestors acclimated with salt (inducible response) and the salt lines acclimated without salt (constitutive response).

To determine the effect of acclimation in different concentrations of salt on the high-salt lines, we fitted an analysis of covariance (ANCOVA), with acclimation regime as fixed factor, line as a random factor, assay salt concentration as a covariate, and all interactions. Variance components were then calculated by equating observed and expected mean squares.

To compare the inducible responses in the ancestors to that in the high-salt lines, we fitted a linear mixed-effect model using the function lmer with selection history as a fixed factor, lines nested within selection history as a random factor, assay salt concentration (between 10 and 20 $g \cdot L^{-1}$, or between 20 and 30 $g \cdot L^{-1}$) as a continuous variable, and all interactions. Note here that to look at the evolution of the inducible response, we used data from the ancestors acclimated with 5 $g \cdot L^{-1}$ NaCl and data from the high-salt lines acclimated with 10 $g \cdot L^{-1}$ NaCl.

Finally, to compare the growth of the salt-selected lines and the ancestor to that of the sexual progeny, we calculated confidence intervals for the difference between means, using the *t*-distribution for unequal sample sizes.

Results

SALT-SELECTED LINES CAN GROW IN SEAWATER

The marine isolate grew well in seawater and could be propagated successfully. The freshwater isolate and all the ancestral lines were incapable of growth in seawater and could not be propagated. The high-salt selection lines had positive growth on average although they varied widely (Fig. 2 mean $r = 0.26$, variance among lines = 1.57). About half of the high-salt lines (7/13) have positive growth in seawater, although only 2/6 significantly so (one-tailed *t*-tests for the difference between an estimate and a parametric value; one line could not be tested for significance because of insufficient replication). Some of these lines grew as well as, or even better than, the marine isolate, at least in laboratory conditions.

SELECTION ALTERED THE CONSTITUTIVE RESPONSE TO SALT

The high-salt lines maintain a high positive rate of increase from 0 $g \cdot L^{-1}$ up to 20 $g \cdot L^{-1}$ (Fig. 3: $r = 1.75 + 0.02$ [NaCl]), whereas growth of the ancestral lines decreases sharply as the salt concentration increases ($r = 1.61 - 0.19$ [NaCl]). Some ancestral lines have a negative rate of increase at concentrations as low as 5 $g \cdot L^{-1}$ NaCl, and the mean rate of increase is well below zero by 10 $g \cdot L^{-1}$ NaCl. The difference between the response of the high-salt lines and the ancestral lines to salt is highly significant (effect of interaction history:assay salt concentration: $X^2 = 94.65$, df = 1, $P < 0.001$).

THE ANCESTRAL LINES SHOW AN INDUCIBLE RESPONSE TO SALT

Most of the ancestral lines cannot grow in salt concentrations above 5 $g \cdot L^{-1}$ when acclimated in medium without salt. When acclimated in 5 $g \cdot L^{-1}$ NaCl before assay, however, most ancestral lines are able to grow in salt concentrations as high as 30 $g \cdot L^{-1}$ (Fig. 4). Between 0 and 10 $g \cdot L^{-1}$, where the relationship is linear, the growth of the ancestral lines decreases significantly more rapidly with increases in salt concentrations when they have been acclimated without salt than when they have been acclimated with 5 $g \cdot L^{-1}$ NaCl (Table 1; effect of interaction between acclimation and concentration: $X^2 = 32.96$, df = 1, $P < 0.001$).

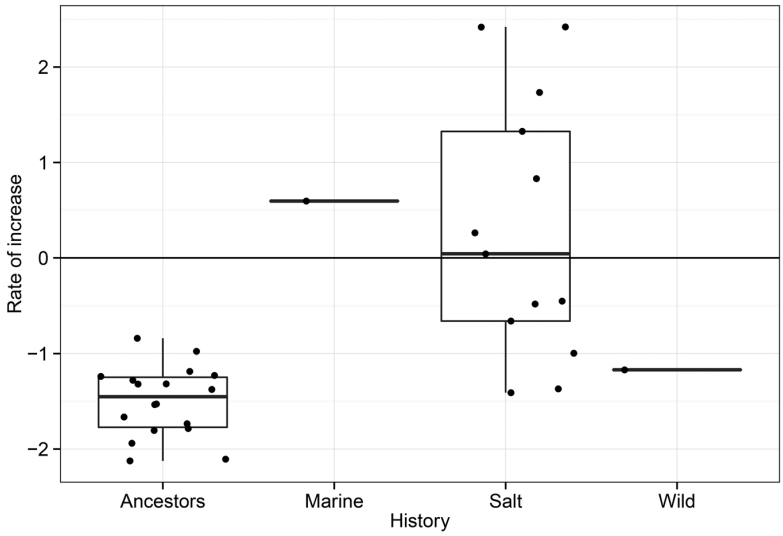

Figure 2. Growth of the ancestral lines, marine green alga strain C.W80, salt-selected lines, and wild-type freshwater strain CC2935 in seawater. Each point is the mean of two assay replicates for a given line. There are 20 ancestral lines, 13 salt-selected lines, and one of each of the marine green alga and the wild type.

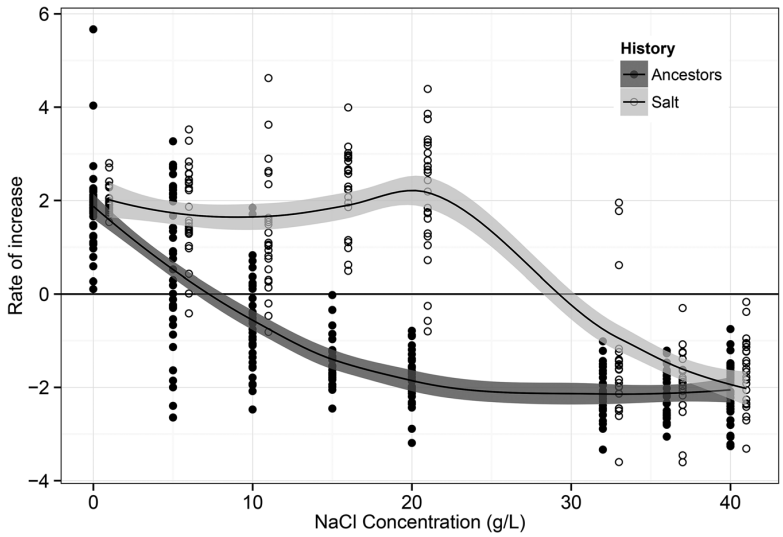

Figure 3. Growth of the salt-selected lines and the ancestral lines in different concentrations of salt. There are 13 salt-selected lines and 20 ancestral lines, each assayed twice. The datapoints for the salt-selected lines are plotted 1 $g \cdot L^{-1}$ NaCl higher than assayed to make it easier to see differences between histories. The trend line was fitted using local polynomial regression (loess), with 95% confidence intervals in shade.

THE INDUCIBLE RESPONSE OF THE ANCESTRAL LINES IS EXPRESSED IN BOTH LIGHT AND DARK CONDITIONS

Growth decreases more rapidly with salt concentration when the ancestral lines are grown in the light than when grown in the dark (effect of interaction between growth condition and salt concentration: $X^2 = 10.36$, df $= 1$, $P = 0.0013$). This is attributable to the higher growth of lines growing in the light than in the dark in medium without salt supplementation, however, and is not due to differences of growth in salt-supplemented media (Fig. 4). The effect of acclimation without salt or in 5 $g \cdot L^{-1}$ NaCl on the response to salt is independent of growing condition (effect of interaction between acclimation, growth condition and salt concentration: $X^2 = 0.46$, df $= 1$, $P = 0.50$).

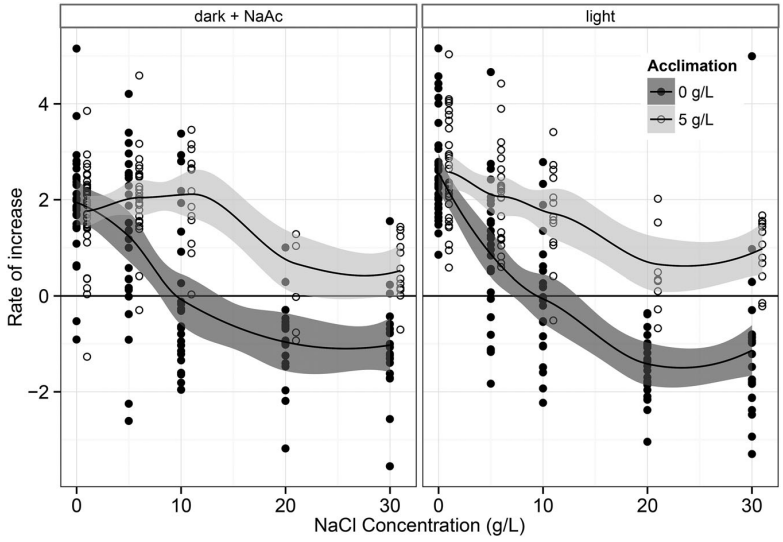

Figure 4. The effect of acclimation regime and growing condition (light or dark) on the growth of the ancestral lines in different concentrations of salt. There are 20 ancestral lines, each assayed twice. The datapoints for the lines acclimated in 5 g·L^{-1} NaCl are plotted 1 g·L^{-1} NaCl higher than assayed to make it easier to see differences between acclimation regimes. The trend line was fitted using local polynomial regression (loess), with 95% confidence intervals in shade.

Table 1. Effect of acclimation and growing condition (i.e., light or dark) on the response of the ancestral lines to salt concentrations between 0 and 10 g·L^{-1}.

Growing condition	Acclimation environment (g·L^{-1} NaCl)	Intercept (±SE)	Slope (±SE)
Light	0	2.2 (0.096)	−0.22 (0.017)
	5	2.2 (0.11)	−0.12 (0.022)
Dark	0	2.3 (0.11)	−0.13 (0.018)
	5	1.9 (0.091)	−0.066 (0.017)

Table 2. Effect of acclimation on the response of the salt-selected lines to a range of different salt concentrations between 0 and 20 g·L^{-1}.

Parameter	Acclimation environment (g·L^{-1} NaCl)	Estimate (±SE)
Slope	0	−0.0014 (0.018)
	10	−0.014 (0.018)
	36	−0.027 (0.013)
Intercept	0	1.9 (0.24)
	10	3.0 (0.24)
	36	2.8 (0.17)

PLASTICITY FOR GROWTH IN LOW SALT IN THE ANCESTORS HAS BEEN ASSIMILATED IN THE HIGH-SALT LINES

The constitutive response of the high-salt lines to salt concentrations is indistinguishable from the inducible response of the ancestors between 0 and 10 g·L^{-1} (selection history:assay salt concentration interaction: $X^2 = 0.00$, df = 1, $P = 0.99$).

THE HIGH-SALT LINES HAVE EVOLVED AN ENHANCED INDUCIBLE RESPONSE TO HIGH SALT

The high-salt lines have a strong constitutive response to salt at concentrations up to 20 g·L^{-1} (Fig. 3), but these lines do not appear to be capable of growing at concentrations of 30 g·L^{-1} NaCl and higher. Nevertheless, these lines have been propagated in 36 g·L^{-1} NaCl for many months without going extinct. Their ability to grow at salt concentrations in excess of 30 g·L^{-1} is conferred by an inducible response.

In the lower range of salt concentrations between 0 and 20 g·L^{-1}, acclimation in medium containing salt significantly increases the overall rate of increase relative to lines that have been acclimated in medium without salt (Fig. 5; Table 2; effect of acclimation: $F_{2,20} = 5.3$, $P = 0.006$). However, acclimation does not significantly affect the slope, meaning that growth decreases at the same rate with increases in salt concentrations whether the lines have been acclimated with or without salt (effect of acclimation:assay concentration interaction: $F_{1,220} = 1.7$, $P = 0.19$). Note that although growth appears to be higher in no salt than in 10 g·L^{-1} NaCl after acclimation in salt, this effect is not significant ($X^2 = 2.70$, df = 1, $P = 0.10$).

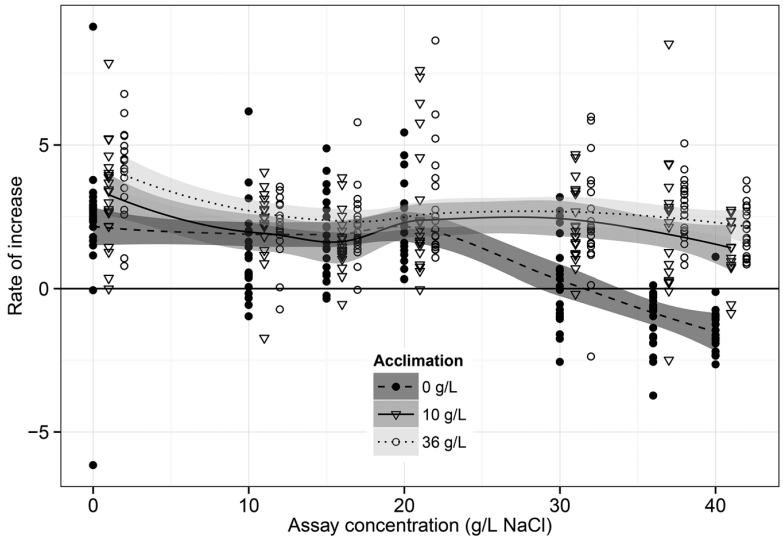

Figure 5. The effect of acclimation regime on the growth of the salt-selected lines in different concentrations of salt. There are 10 salt-selected lines, each assayed twice. The datapoints for the lines acclimated in 10 g·L^{-1} NaCl are plotted 1 g·L^{-1} NaCl higher, and the lines acclimated in 36 g·L^{-1} NaCl are plotted 2 g·L^{-1} NaCl higher than assayed to make it easier to see differences between acclimation regimes. The trend line (dashed for 0 g·L^{-1}, solid for 10 g·L^{-1}, and dotted for 36 g·L^{-1}) was fitted using local polynomial regression (loess), with 95% confidence intervals in shade.

Table 3. Effect of acclimation on the response of the salt-selected lines to a range of different salt concentrations between 20 and 40 g·L^{-1}.

Parameter	Acclimation environment (g·L^{-1} NaCl)	Estimate (±SE)
Slope	0	−0.18 (0.017)
	10	−0.089 (0.018)
	36	−0.10 (0.012)

Comparison of the inducible response of the salt-selected lines to low salt concentrations to the inducible response of the ancestors reveals that it has evolved. Between salt concentrations of 10 and 20 g·L^{-1}, growth decreases significantly more rapidly with increases in salt in the ancestors than in the salt-selected lines (selection history:assay salt concentration interaction effect: $X^2 = 8.37$, df $= 1$, $P = 0.0038$), although the intercepts are not statistically different (effect of selection history: $X^2 = 3.14$, df $= 1$, $P = 0.076$).

In the higher range of salt concentrations between 20 and 40 g·L^{-1}, acclimation has a significant effect on the slope of the salt-selected lines, meaning that lines acclimated with salt maintain the same growth with increases in salt concentration, whereas lines acclimated without salt show a steep decline in growth with increases in salt concentration (Fig. 5; Table 3; ANCOVA effect of acclimation:assay concentration: $F_{1,215} = 48.4$, $P < 0.001$). Comparison of the inducible response of the salt-selected lines to high salt concentrations to the inducible response of the ancestors reveals that it also has evolved. Between salt concentrations of 20 and 30 g·L^{-1}, growth is significantly greater overall in the salt-selected lines than in the ancestors (selection history effect: $X^2 = 6.58$, df $= 1$, $P = 0.010$), although the slope is not different (selection history:assay salt concentration interaction effect: $X^2 = 2.99$, df $= 1$, $P = 0.084$).

CONSTITUTIVE AND INDUCIBLE RESPONSES ARE AFFECTED BY MEIOSIS

Without prior acclimation in salt medium, the F1 and F2 progeny grow at the same rate as the ancestors at all salt concentrations, and are unable to grow at concentrations of 28 g·L^{-1} or higher (Fig. 6). This is in contrast to the salt-selected parents, which remain constitutively able to grow in 28 g·L^{-1}. Thus, the constitutive ability to grow at high salt concentrations is entirely lost after meiosis and recombination. After acclimation in medium containing 10 g·L^{-1} NaCl, the F1 progeny grows as well as the salt-selected parents in concentrations up to 36 g·L^{-1} NaCl, and grows better than the salt-selected parent in 48 g·L^{-1} NaCl; the F2 progeny does worse than the salt-selected parents in concentrations up to 36 g·L^{-1} NaCl, and does better than the salt-selected parents in 48 g·L^{-1} NaCl (Table 4). Thus, the sexual progeny are able to grow at very high concentrations of up to 48 g·L^{-1} NaCl, which their salt-selected parents are unable to tolerate.

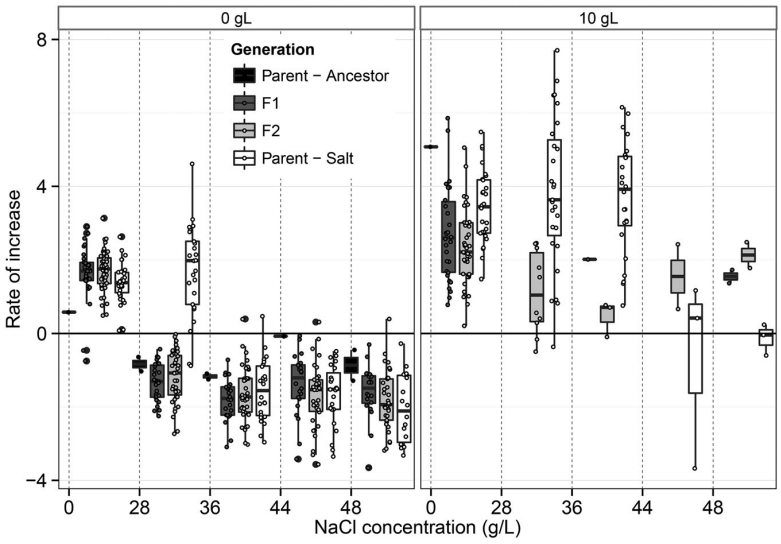

Figure 6. **Growth of sexual progeny of the salt-selected lines after acclimation without salt or with 10 g·L^{-1} salt. Assay concentrations for which there are fewer than four boxes indicate that none of the spores from the generation missing survived the first cycle in that concentration. The rate of increase during the second cycle could therefore not be calculated. Note that the order of the boxplots on the *x*-axis is the same as in the legend.**

CONSTITUTIVE AND INDUCIBLE RESPONSES BOTH CONTRIBUTE TO ADAPTATION

In the lower range of assay salt concentrations, the amount of variance in the rate of increase explained by the different lines (i.e., variance in the constitutive responses) is approximately 10 times greater than the amount of variance explained by the different acclimation regimes (i.e., inducible responses), with estimates of 0.40 and 0.047, respectively. The amount of variance explained by the interaction of line and acclimation regime is approximately three times greater than the amount explained by line alone (estimate of 1.3). In the higher range of assay salt concentrations, the amount of variance in the rate of increase explained by the different lines is approximately zero (estimate of −0.94). Acclimation explains a significant amount of the variance (estimate of 0.019), whereas the interaction of lines and acclimation regime explains about 300 times more of the variance than acclimation alone (estimate of 6.8).

Discussion

ADAPTATION TO MARINE CONDITIONS OF GROWTH

New ways of life evolve when organisms adapt to ecological conditions of growth that were not accessible to their ancestors. We have shown that an important ecological transition can occur within 500 generations. Some of the lines that we selected in gradually increasing concentrations of salt are now capable of growth in 36 g·L^{-1} NaCl, far beyond what their ancestors could tolerate. In principle, these lines are now capable of growing in the sea.

About 98% of the experimental lines went extinct well before marine conditions were reached. Chronic exposure to a continuously deteriorating environment therefore requires far more than ancestral plasticity for growth in concentrations up to about 20 g·L^{-1} NaCl for two growth cycles. The lines that have survived vary substantially in their ability to grow in seawater. Thus, most populations that experience a profound deterioration in the conditions of growth will simply become extinct. The experimental adaptation to marine conditions that occurred in this freshwater alga give an example of how survival to marine conditions can be achieved to different extents and in different ways.

In the yeasts *Saccharomyces cerevisiae* and *S. paradoxus*, for which the lethal concentration of salt is about 150 g·L^{-1} NaCl, population size, the rate of increase in salt concentration, and connectivity with neighboring populations all affect the probability of surviving the imposed salt regime as well as the probability of surviving a transfer to the lethal concentration (Bell and Gonzalez 2009, 2011; Samani and Bell 2010; Gonzalez and Bell 2013). In the bacterium *Serratia marcescens*, tolerance to 90 g·L^{-1} NaCl was improved after constant selection in either 80 or 100 g·L^{-1} NaCl for 300 generations, but not after selection in a fluctuating environment, most likely because of weaker selection pressure (Ketola and Hiltunen 2014). Together, these results suggest that the rarity of transitions between freshwater and marine conditions may be a consequence of small population sizes, fast rates of

Table 4. Difference in the mean rate of increase between the ancestor, salt-selected lines, F1 sexual progeny, and F2 sexual progeny when assayed in different salt concentrations.

Acclimation	Comparison	Assay NaCl concentration ($g \cdot L^{-1}$)	Lower CI	Difference in mean rate of increase	Upper CI
0 $g \cdot L^{-1}$	Ancestor – F1	0	−2.66	−1.05	0.571
		28	−0.266	0.501	1.27
		36	−0.205	0.642	1.49
		44	−0.494	1.31	3.11
		48	−0.406	0.726	1.86
	Ancestor – F2	0	−2.37	−1.12	0.121
		28	−0.678	0.351	1.38
		36	−0.617	0.467	1.55
		44	−0.172	1.63	3.43
		48	−0.240	0.951	2.14
	Salt parent – F1	0	−0.602	−0.253	0.0963
		28	2.51	3.04	3.57
		36	−0.147	0.271	0.688
		44	−0.849	−0.293	0.263
		48	−1.01	−0.454	0.0988
	Salt parent – F2	0	−0.591	−0.330	−0.0697
		28	2.39	2.89	3.39
		36	−0.315	0.0946	0.504
		44	−0.438	0.0264	0.491
		48	−0.733	−0.228	0.276
	F1 – F2	0	−0.388	−0.0773	0.233
		28	−0.471	−0.151	0.170
		36	−0.533	−0.176	0.181
		44	−0.154	0.319	0.793
		48	−0.221	0.225	0.671
10 $g \cdot L^{-1}$	Salt parent – F1	0	0.131	0.738	1.35
		36	−1.53	1.69	4.92
		48	−2.77	−1.68	−0.589
	Salt parent – F2	0	0.610	1.10	1.59
		28	1.33	2.73	4.12
		36	1.38	3.24	5.10
		44	−8.76	−2.24	4.29
		48	−3.57	−2.26	−0.953
	F1 – F2	0	−0.181	0.364	0.909
		36	−0.845	1.55	3.94
		48	−2.27	−0.581	1.11

Lower and upper confidence intervals were calculated for the difference between means using the *t*-distribution for unequal sample sizes. The assay concentrations that are missing reflect the fact that none of the spores from one of the generations in the comparison survived the first cycle in that concentration.

increase in salt, fluctuating conditions, or low connectivity between natural populations.

GENETIC ASSIMILATION OF SALT TOLERANCE

In our experiment, growth of the evolved lines without acclimation to salt is equal to or greater than the growth of ancestral lines acclimated with salt, at salt concentrations of up to about 20 $g \cdot L^{-1}$. Above this concentration, the evolved lines cannot grow without acclimation. Once acclimated, however, they grow much better than the acclimated ancestral lines in all concentrations above 10 $g \cdot L^{-1}$. These results suggest that the ability to grow at very high salt concentrations evolved in two stages: genetic assimilation at lower concentrations, yielding a constitutive response to conditions lethal to the ancestor, and an enhanced inducible response at higher concentrations that permits growth up to about 40 $g \cdot L^{-1}$ NaCl.

Changes in gene expression following long-term exposure to salt have been reported before in *C. reinhardtii* (Perrineau et al. 2014). Short-term acclimation to about 12 $g \cdot L^{-1}$ NaCl causes a reduction in photosynthesis, upregulation of glycerophospholipid signaling, and upregulation of the transcription and translation machinery. Long-term culture in high-salt medium causes downregulation of genes involved in the stress response and in transcription and translation. Fatty acid metabolism is also more strongly downregulated following long-term than short-term acclimation, which suggests that long-term salt stress leads neither to lipid accumulation nor to the synthesis of starch. Selection can therefore alter gene expression for growth in salt.

Genetic assimilation can occur through genetic or epigenetic modifications. Unlike genetic modifications, which are changes in nucleotide sequence that are transmitted from parent to offspring in both asexual and sexual lineages, epigenetic modifications may be preserved in asexual lineages, either of free-living cells or of tissues in a developing body, but are generally removed during meiosis and are therefore not transmitted in sexual lineages (Jablonka and Raz 2009).

The constitutive tolerance to low salt concentrations was maintained in asexual cultures, but completely lost in the sexual progeny of the salt-selected lines. Indeed, the F1 and F2 progeny have the same phenotype as the ancestor in low salt concentrations after acclimation without salt. If genetic change was responsible for the assimilation of ancestral plasticity in low salt concentrations, we would have expected some of the progeny to have maintained some constitutive tolerance to salt, albeit possibly to lower extents. However, none of the 24 random sexual progeny that we assayed displayed a level of tolerance greater than ancestral. Therefore, we conclude that the assimilation of ancestral plasticity for growth in low salt concentrations is unlikely to be based on genetic changes. Rather, the assimilation of ancestral plasticity occurred through reversible changes in our asexually propagated selection lines. The loss of tolerance following meiosis is consistent with an epigenetic basis, although genomic studies will be required to explicitly test this hypothesis.

The inducible response to salt concentrations of up to 40 $g \cdot L^{-1}$, on the other hand, was retained in sexual progeny, albeit more weakly expressed. This is consistent with genetic modification. This could be caused by loss-of-function mutations in a regulatory gene that hindered the binding of a repressor protein. This explanation, however, would require the existence of a cryptic inducible system in the ancestor whose function is obscure. It is more plausible to invoke gain-of-function mutations in an inducible structural gene. This gene is imagined to contribute to the inducible response at low salt concentrations expressed by the ancestor. During serial transfer at gradually increasing salt concentrations, alleles that spread through natural selection because they confer the ability to grow in ambient conditions may indirectly confer the ability to grow in more severe conditions. Adaptation to lethal conditions, resulting in evolutionary rescue, has been attributed to this kind of indirect response to selection in other experiments with algae and yeast (Bell and Gonzalez 2009, 2011; Samani and Bell 2010; Lachapelle and Bell 2012; Gonzalez and Bell 2013). The partial loss of fitness in F1 and F2 hybrid progeny is the expected result of recombination with ancestral alleles, and suggests that such gain-of-function mutations have occurred in more than one gene in our salt-selected lines.

THE CONTRIBUTION OF PLASTICITY AND GENETIC RECOMBINATION TO EVOLUTIONARY RESCUE

In a deteriorating environment, stress provides a continual stimulus capable of eliciting an inducible response. Where such a response exists, as it did in our selection lines, it enables the population to persist for longer and thereby prolongs the period during which genetic adaptation can occur through natural selection. The phenotypic plasticity of the ancestor for low stress is eventually lost after chronic exposure to increasing stress in our asexually propagated lines. The reversibility of this constitutive response to low salt in sexual progeny suggests the assimilation of ancestral plasticity could have arisen through the accumulation of neutral loss-of-function epigenetic modifications. The loss of plasticity would be accelerated if the inducible response were metabolically costly to maintain and/or activate. Although we have no way of measuring the cost of maintenance, our data show no evidence of a cost of activation: the growth of the ancestral lines in medium without salt is the same whether or not they have been previously acclimated with salt (Fig. 4). Drift could also have played a role in eliminating plasticity, given that the lines were bottlenecked following the first round of crosses. However, it is unlikely that plasticity would have been assimilated in all lines through chance alone.

In this instance of a deteriorating environment, then, the loss of plasticity at low levels of stress is accompanied by the evolution of enhanced plasticity at high levels of stress through genetic modifications. This is consistent with the evolution of enhanced plasticity in fluctuating environments reported by Schaum and Collins (2014). The breadth of conditions that the salt-selected lines can tolerate is much greater than the ancestors, consistent with the "sidestep niche model" whereby enhanced plasticity contributes in widening the niche after environmental change (Lande 2009; Gallet et al. 2014). However, we have no evidence that the niche has shifted or is now narrowing. To the contrary, the assimilation of ancestral plasticity in low salt concentrations seems to have contributed in maintaining the larger niche breadth.

The fact that sexual lines were better able to keep pace with the changing environment (Lachapelle and Bell 2012) indicates that surviving lines were better able to keep track of the moving fitness optimum because of the increased genetic variation

generated by recombination. It is possible that the increase in resistance to salt reported here is mostly attributable to recombined variation from the end of this first selection experiment. However, our data do not allow us to make any inferences about the relative contribution of recombination, epigenetic, and genetic modification to the increase in resistance reported here.

Nonetheless, back-crosses of the high-salt lines to the ancestor, or crosses among these families, show that the F1 and F2 continue to grow at salt concentrations of 48 $g \cdot L^{-1}$ at the same rate as at lower concentrations, whereas the high-salt lines themselves are unable to grow. This demonstrates the importance of recombination. The enhanced resistance of recombinants cannot be attributed to a more resistant protein because the high-salt lines themselves cannot grow at these very high salt concentrations. It is not due to the recombination of improved alleles at different loci because it is expressed in the F1 of crosses between the ancestor and the selection lines. It might be attributable to the release, through recombination, of an improved structural gene from linkage with a strongly deleterious mutation at some other locus. In this case, it would be necessary to assume further that this mutation is strongly deleterious only at very high salt concentrations because the F1 and F2 are inferior to the selection lines at salt concentrations of 40 $g \cdot L^{-1}$ or less. Population sizes were very low during some stages of the experiment when the salt concentration was increasing. A neutral or mildly deleterious mutation could have therefore fixed by chance, if not by hitchhiking with a beneficial mutation. The uniform phenotype of random spores is also unexpected. Hence, we report that the range of conditions that can be tolerated is substantially extended in the sexual progeny of adapted parents, but we have not identified a simple genetic mechanism that would explain their superiority.

Conclusion

Experimental evolution has been extensively used to elucidate the mechanism of selection for particular attributes such as the ability to utilize a novel substrate or resist an antibiotic. The evolution of marine from freshwater lineages, of heterotrophs from autotrophs (Bell 2013a,b,c), and of multicellular from unicellular forms (Ratcliff et al. 2012, 2013) are examples that clearly indicate the possibility of studying certain aspects, at least, of major ecological transitions in the laboratory.

Here, we reported the adaptation of a freshwater alga to marine conditions within a few hundreds of generations in the laboratory. Continued selection pressure, sexually generated genetic variation, and phenotypic plasticity largely contributed to extending the limits of tolerance and facilitating the ecological transition. In short, the evolution of tolerance to salt involved two different mechanisms: reversible and irreversible changes. Tolerance to low salt concentrations of unacclimated selection lines was annulled by meiosis, suggesting reversible changes were responsible for the assimilation of ancestral plasticity and adaptation to the limit of tolerance. Tolerance to high salt concentrations of acclimated selection lines was maintained through meiosis, suggesting irreversible genetic changes were responsible for enhancing phenotypic plasticity in the selection lines and extended the range of tolerance to conditions lethal to the ancestor. Both mechanisms contributed to the transition from freshwater to fully marine conditions.

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DATA ARCHIVING

The doi for our data is 10.5061/dryad.rs610.

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