Epigenetic and Genetic Contributions to Adaptation in *Chlamydomonas*

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Abstract

Epigenetic modifications, such as DNA methylation or histone modifications, can be transmitted between cellular or organismal generations. However, there are no experiments measuring their role in adaptation, so here we use experimental evolution to investigate how epigenetic variation can contribute to adaptation. We manipulated DNA methylation and histone acetylation in the unicellular green alga *Chlamydomonas reinhardtii* both genetically and chemically to change the amount of epigenetic variation generated or transmitted in adapting populations in three different environments (salt stress, phosphate starvation, and high CO_2) for two hundred asexual generations. We find that reducing the amount of epigenetic variation available to populations can reduce adaptation in environments where it otherwise happens. From genomic and epigenomic sequences from a subset of the populations, we see changes in methylation patterns between the evolved populations over-represented in some functional categories of genes, which is consistent with some of these differences being adaptive. Based on whole genome sequencing of evolved clones, the majority of DNA methylation changes do not appear to be linked to *cis*-acting genetic mutations. Our results show that trangenerational epigenetic effects play a role in adaptive evolution, and suggest that the relationship between changes in methylation patterns and differences in evolutionary outcomes, at least for quantitative traits such as cell division rates, is complex.

Introduction

Evolutionary adaptation occurs when the population growth rate increases as a result of natural selection sorting heritable variation across individuals in fitness related traits, and the origin of this variation across individuals is usually characterised using genetic differences (Mayr, 1982; Hartl and Clark, 1997; Orr, 2005). However, it is now widely appreciated that heredity is not based on DNA sequence alone (Rassoulzadegan et al., 2006; Richards, 2006; Bonduriansky and Day, 2009; Jablonka and Raz, 2009; Crews et al., 2012; Daxinger and Whitelaw, 2012; Donelson et al., 2012; Salinas and Munch, 2012; Kelly, 2014; Taudt et al., 2016). Information not directly encoded in the DNA sequence can also be transmitted between generations. For example, non-genetic information can be transmitted when DNA or its associated proteins are modified, as is the case for DNA methylation and histone modifications (Cubas et al., 1999; Manning et al., 2006; Chinnusamy and Zhu, 2009; Johannes et al., 2009; Bossdorf et al., 2010; Verhoeven et al., 2010; Ou et al., 2012; Song et al., 2012; Verhoeven and van Gurp, 2012; Cortijo et al., 2014; Lauria et al., 2014). Collectively, these modifications are called epigenetic changes. It has now been established that epigenetic changes can be passed not only through mitotic cell division but also from parent to offspring (Johannes et al., 2009; Öst et al., 2014; Cortijo et al., 2014; Gaydos et al., 2014; Ragunathan et al., 2014; Audergon et al., 2015). Mutation accumulation experiments have shown that spontaneous epigenetic changes occur much like genetic mutations. However, one key difference is that epigenetic mutations occur at a faster rate, but may be less stable than genetic mutations. For example, rates of change in DNA methylation patterns have been estimated to be about five orders of magnitude higher than genetic mutations rates (Becker et al., 2011; Schmitz et al., 2011; van der Graaf et al., 2015).

The evidence for transmission of epigenetic variation opens up the question of how epigenetics affects evolutionary adaptation. Theoretical models and simulations predict that epigenetic variation has the potential to affect the rate and outcomes of adaptation (Day and Bonduriansky, 2011; Klironomos et al., 2013; Kronholm and Collins, 2016). Previous empirical research has either focused on adaptive plastic responses, and shown that plastic phenotypic changes have an epigenetic component (Bossdorf et al., 2010; Herrera et al., 2012; Verhoeven and van Gurp, 2012), or investigated adaptation occurring by independent epigenetic changes in wild populations indirectly by population genetic methods (Paun et al., 2010; Silveira et al., 2013).

Here, we investigate the effects of epigenetic variation directly using experimental evolution. This allows us to study how epigenetic variation affects adaptation over timescales that are long enough for novel adaptive genetic mutations to occur and increase in frequency in populations. Previously, adaptation on this timescale has been investigated and explained in purely genetic terms (Barrick et al., 2009; Blount et al., 2012; Dettman et al., 2012; Wong et al., 2012). We carried out laboratory experiments in four different environments using the unicellular green alga *Chlamydomonas reinhardtii.* We manipulated the production and transmission of epigenetic variation either genetically or chemically. Specifically, we genetically

manipulated epigenetic variation by generating a *sir2* mutant (see methods) to change the extent of histone acetylation. *SIR2* is a NAD-dependent histone deacetylase that is conserved from archaea and bacteria to animals and plants (Frye, 2000). In all organisms studied, *SIR2* is involved in transcriptional silencing (Tanny et al., 1999; Blander and Guarante, 2004; North and Verdin, 2004; Smith et al., 2008). The populations made up of *sir2* mutants were less able to produce epigenetic variation than wild-type populations, but could transmit that variation. We chemically manipulated epigenetic variation by periodically subjecting evolving populations to chemical treatments that prevent either DNA-methylation, or both methylation and histone deacetylation. The treatment "demet" contained demethylating agents 5-aza-deoxycytidine, L-Ethionine, and Zebularine and treatment "demet + acet" contained 5-aza-deoxycytidine, L-Ethionine, and histone deacetylation inhibitor Trichostatin A. Published studies have shown that the concentrations used for 5-aza-2-deoxycytidine and L-Ethionine are effective for demethylation in *C. reinhardtii* without decreasing growth (Feng and Chiang, 1984), and we confirmed the lack of effect on growth here. Thus the chemically-treated populations had reduced levels of epigenetic variation compared to the untreated populations, with both reduced production and transmission of epigenetic variation. Since the 5-aza-deoxycytidine and Zebularine can be mutagenic, we included a UV-treated strain to account for an increased supply of genetic mutations.

The selection experiment consisted of four strains (CC-2937, UV irradiated CC-2937, *sir2* mutant, complemented *SIR2* mutant), three chemical treatments (demet, demet + acet, control), and four selection environments (high salt, high CO_2, low phosphate, control). Each selection environment imposes different selection on evolving populations. The experiment was run for approximately 200 asexual generations. Since the UV-irradiated CC-2937 was used to account for changes (in this case an increase) in mutational supply, it is treated as a "strain" throughout the experiment. Throughout this study, we refer to strains as "strains", chemical treatments as "treatments" and selection environments as "environments". See Figure 1A for a schematic of the selection experiment, and methods for details of strains and environments. We expect that high salt and low phosphate environments are stressful environments and adaptation to these environments should increase growth rates, as in batch culture fitness should be proportional to maximum population growth rate *r*. However, the high CO_2 environment is an enriched environment. While counter intuitive, previous work has revealed that evolution in high CO_2 environments either does not improve on the plastic response, or reverses it to decrease growth (Collins and Bell, 2004; Schaum and Collins, 2014). This strategy evolves rapidly and repeatedly, and is associated with an increase in competitive ability and cell condition, so that the best current interpretation is that the reduction in population growth rate is adaptive for chlorophytes in nutrient-rich, high CO_2 environments (Schaum and Collins, 2014; Collins, 2016). To understand the roles of genetic and epigenetic differences in adaptation to a range of selection environments, we isolated clones from the CC-2937 control and demet chemical treatments and resequenced their genomes and methylomes after the selection experiment. If it is the case that epigenetic changes are used in adaptation, we expect that manipulating the amount of epigenetic variation available for the populations we will reduce adaptation in the different environments (Figure 1B). Furthermore, if we cluster the strains based

on epigenetic changes we should see similarities in the between strains that come from the same environment (Figure 1B).

We find that reducing the production or transmission of epigenetic variation available to the populations can reduce growth rate evolution when populations adapt to novel environments. We also observed that when the methylation patterns of evolved populations were compared, populations evolved in the high salt clustered together based on methylation differences, and among the genes containing methylation differences gene functions related to aminoglycan catabolism were enriched in all environments, membrane depolarization in high CO_2, and transmembrane transport in high salt. Differences in methylation patterns were not associated with nearby genetic mutations, and have the potential to be adaptive.

Results

Environmental and chemical treatment effects on initial population growth rates

To investigate the effects of epigenetic transmission on adaptation as generally as possible, we used three environments that exerted different selection pressures on the populations. Each one of these is a full evolution experiment. Here, the high NaCl environment exerted strong selection (indicated by a large initial drop in population growth rates), while low phosphate was a more benign environment (indicated by a small initial drop in population growth rates) and high CO_2 was an enriched environment (indicated by an initial increase in population growth rates). See Table 1 for list of initial (ancestral) growth rate responses to all environments for the different strains. This is consistent with previous experiments in *C. reinhardtii* showing growth declines in high NaCl and low phosphate environments (Collins and de Meaux, 2009; Lachapelle and Bell, 2012; Lachapelle et al., 2015), and positive or no change in growth in high CO_2 environments (Collins and Bell, 2004), and confirms that our environments exert different intensities of selection on evolving populations.

The initial effects of the different chemical treatments are listed in Table 2. The effects of the chemical treatments were environment and strain dependent. While variability was high, averaged over all strains and environments, the demet treatment decreased growth by -6% and the demet + acet treatment had a stronger effect as it decreased growth by -15% relative to the control treatment.

Population extinctions during the selection experiment

Populations were evolved for approximately 200 generations in their selection environments. Of the 432 populations in the selection experiment, 19 went extinct. Extinction events were not randomly distributed

among environments (Chi square goodness-of-fit test, χ^2 = 35.95, df = 3, p = 7.68*10^{-8}). There were 2 extinctions in the control environment, 1 in the high CO_2 environment, 16 in the low phosphate environment, and no extinctions in the high NaCl environment. In the low phosphate environment, strains had different extinction rates, with 13 populations of CC-2937, 2 populations of CC-2937 UV, 1 population of LM3 *sir2*, and no populations of LM3 c*SIR2* going extinct (Chi square goodness-of-fit test, χ^2 = 27.5, df = 3, p = 4.63*10^{-6}). CC-2937 may have had a higher extinction rate because its relatively fast growth rate led to rapid phosphate depletion in the culture. Furthermore, phosphate depletion often caused CC-2937 cells to become sticky and clump together, which decreased their ability to be transferred in the experiment. UV irradiation increases mutation rates in *C. reinhardtii* (see supplementary material), and although deleterious mutations will be more common with UV irradiation than without UV irradiation, so will beneficial ones, such that selection is likely to be more effective in the large populations used here. Thus, a lower extinction rate in the UV-treated CC-2937 strain is consistent with evolutionary rescue made possible by an increased mutational supply, and shows that in this experiment, the evolutionary potential of the UV-treated strain is different from the wild type within environments. Chemical treatment did not have a significant effect on extinctions in the low phosphate environment (Chi square goodness-of-fit test, χ^2 = 3.88, df = 2, p = 0.1441).

Decreasing epigenetic transmission affects growth rate evolution

We have visualized the direct response to selection, which is calculated by dividing the growth rate of populations evolved and measured in one of the three novel environments (high NaCl, low phosphate, high CO_2) by the growth rate of the populations evolved in the control environment but measured in the novel selection environments. Populations were matched by chemical treatments (Figure 2). However, the statistical analysis has been performed on absolute growth rates (Figure S1), because this allows statistical testing of differences between control and evolved populations. In this section we analyse the effects of epigenetic manipulations on adaptation using linear models. Since each selection environment was a complete evolution experiment, we discuss the results for each environment separately. The statistical model includes terms for strain effect, effect of selection (whether population evolved in the one of the three novel environments or the control environment), and the effect of chemicals and the interactions of these terms. We tested the full model first, and then dropped non-significant terms. The 3-way interaction of selection × chemical × strain tests the effect of epigenetic manipulation on adaptation varied across different strains. The interaction of selection × chemical tests the effect of epigenetic manipulation of adaptation and the selection × strain tests the effect of different strains on adaptation. See Table 3 for a summary of evolutionary outcomes in terms of growth over all strains, chemical treatments, and selection environments. In all cases, "growth rate" indicates population growth rate (increase in cell number over time) and not an increase in size off individual cells. We discuss the direct responses to selection here, and the indirect (correlated) responses to selection, which are evolutionary changes that occurred but were not directly acted on by natural selection during the evolution experiment (Travisano et al., 1995), in the supplementary material.

High NaCl environment. In general, populations adapted to the high NaCl environment, and adaptation was affected by the ability to produce and transmit epigenetic information. In the high NaCl environment, growth rates were initially low and an increase in growth rate is adaptive (Lachapelle et al., 2015). The 3-way interaction of selection × chemical × strain was not significant. However, the selection × chemical interaction was significant ($F_{2,196}$ = 5.953, p = 0.0031), as populations subjected to control or only demet epigenetic manipulation were able to adapt to high NaCl regardless of strain, while the more severe demet + acet epigenetic manipulation impeded adaptation to high salt (Figure 2A). The chemical × strain effect was not significant, but the selection × strain effect was ($F_{3,196}$ = 3.628, p = 0.0140). The different strains responded to selection in a different manner with more adaptation in the UV-treated strain and in the complemented mutant (Figure 2A).

In terms of the specific evolutionary responses for the wild-type strain CC-2937, populations evolved in high NaCl had an average of 21% (chemical control) and 15% (demet treatment) higher growth rates in the high NaCl environment than CC-2937 populations evolved in the control environment. The UV-treated CC-2937 populations evolved in high NaCl had 72%, 58%, and 15% higher average growth rates in high NaCl than populations selected in the control environment, for the chemical control, demet and demet + acet treatments respectively. The higher growth rates of the UV-treated strain reflects the increased genetic variation in the UV-treated populations. For the *sir2* mutant, the chemical control, demet, and demet + acet populations had direct responses to selection of 24%, 37%, and 12% respectively. For the complemented *sir2* populations, the control, demet, and demet + acet populations increased growth rate by 35%, 25%, and 19%. While there were slight differences between the *sir2* mutant and the complemented strain, the effect of the *sir2* mutation was not significant (contrast: t = 1.26, df = 196, p = 0.2093). Overall, we see that decreasing epigenetic variation decreased or impeded adaptation to the high salt environment.

Low phosphate environment. While populations generally adapted to the low phosphate environment, but the ability to produce and transmit epigenetic information did not statistically affect adaptation. In the low phosphate environment neither the 3-way interaction nor any of the 2-way interactions were significant. Although previous work with *C. reinhardtii* detected substantial growth rate evolution in a low phosphate environment (Collins and de Meaux, 2009), the direct response to selection in this experiment was only 14% on average over all other strains and treatments (Figure 2B, effect of selection: $F_{1,186}$ = 10.35, p = 0.0015). The effect of strain was significant ($F_{1,186}$ = 9.19, p = 1.06×10^{-5}), but the main effect of chemical was not. The wild type CC-2937 strain populations evolved in low P had 28% (control), 10% (demet), and 15% (demet + acet) higher growth rates as corresponding populations evolved in the control environment. UV-treated CC-2937 populations evolved in low P grew 12% (control), 18% (demet), and 0% (demet + acet) faster than their respective controls. The *sir2* mutant populations grew 7% (control), 9% (demet), and 40% (demet + acet) faster than their control populations. For the complemented mutant populations, growth rates of the evolved populations were 25% (control), 8% (demet), and 8% (demet + acet) faster than control

populations, the effect of the sir2 mutation was not significant (contrast: $t = -0.4$, $df = 186$, $p = 0.69$). These results suggest that epigenetic variation plays a smaller role in adaptation to low phosphate environment than to high NaCl. Alternatively, given the increased variability and smaller responses to selection than in high NaCl environment (14% in low P vs 28% in high NaCl), we may lack the statistical power to detect a trend associated with manipulating epigenetic variation chemically in the low phosphate environment.

High CO_2 environment. In the high CO_2 environment, the effects of decreasing the production and transmission of epigenetic information resulted in populations evolving higher growth rates than the control chemical populations. Based on other studies on evolution in high CO_2 environments for unicellular chlorophytes, we suggest that the higher growth rates are maladaptive. High CO_2 is initially an improved environment relative to the control environment for *C. reinhardtii*, and in previous studies wild type *C. reinhardtii* did not evolve to increase their growth rate beyond the initial plastic response in high CO_2 environments (Collins and Bell, 2004; Collins et al., 2006). Thus, we did not expect relative growth rate to increase in this environment in the wild-type populations. In addition, some unicellular chlorophytes eventually decrease their growth rates over several hundred generations of growth in high CO_2 environments where growth initially increased (Schaum and Collins, 2014), suggesting that eventually slowing growth in chronically enriched environments can be adaptive (Collins 2016).

In the high CO_2 environment 3-way interaction of selection × chemical × strain was significant ($F_{6,188} = 2.41$, $p = 0.0288$), indicating that adaptation depended on chemical treatment and strain. As expected, the high CO_2-evolved wild type strain that is not chemically treated does not increase its growth rate relative to the control-selected population growing in the high CO_2 environment (Figure 2C) and their plastic response to changes in CO_2 was lost or diminished (see absolute growth rates in supplementary material). The wild type CC-2937 populations evolved in high CO_2 had a change in growth of −18% (control), −9% (demet), and 19% (demet + acet) compared to populations evolved in control environment. The UV-treated CC-2937 populations had a change in growth of −13% (control), −13% (demet), and 19% (demet + acet) compared to their respective controls. These results are consistent with studies suggesting that slower growth than predicted from the short-term (ancestral or control) response may be adaptive under chronically elevated CO_2 or other chronic environmental enrichment (Schaum and Collins, 2014; Collins, 2016). In contrast, when the most extreme epigenetic manipulation is used (demet + acet chemical treatment) in the CC-2937 genetic background, populations evolved in high CO_2 evolve higher growth than the plastic response of control populations. This has never been reported for chlorophytes evolved under the moderate levels of CO_2 enrichment used here. It is also possible that the higher growth rate in the demet + acet treatments simply reflects a different, but adaptive, strategy than seen in the control treatments. The *sir2* mutant populations had a change in growth of −13% (control), −21% (demet), and −9%. And finally, the complemented mutant had a change in growth of 10% (control), 13% (demet), and 5% (demet + acet) compared to populations evolved in control environment. The effect of the *sir2* mutation was significant in control ($t = -4.24$, $df =$

188, $p = 3.48 \times 10^{-5}$), demet ($t = -4.96$, $df = 188$, $p = 1.54 \times 10^{-6}$), and suggestive in demet + acet ($t = -1.88$, $df = 188$, $p = 0.0619$) treatment. The strains with the LM3 genetic background react differently, the *sir2* mutant does have lowered plastic response but the complemented mutant does not. In summary, populations with the CC-2937 genetic background have likely adapted to high CO_2, while the demet + acet treatment may alter the evolutionary trajectory populations as they may have adapted using a different mechanism.

Manipulating epigenetic effects also affected the indirect responses to selection. Indirect responses were smaller than direct responses, and are detailed in the supplementary material.

Effects of UV-irradiation and changes in mutational supply

Our UV-irradiation treatment increased mutational supply in UV-treated CC-2937 strain. Analysis of the possible effects of mutational supply on adaptation has to be done using absolute growth rates and this can be found in supplementary material. However, we found little evidence that adaptation was limited by mutational supply in populations that did not go extinct. There was some evidence that conditionally neutral mutations accumulated in the UV-treated strain but deleterious mutations did not prevent adaptation either.

Effects of decreasing epigenetic transmission in the control treatment

To measure how much transmitted epigenetic patterns affect growth rate of populations evolved in the control chemical treatment that had never undergone chemical treatment, we used an assay of phenotypic stability in the face of chemical treatment. This was done by subjecting chemical control populations and ancestors to a single round of chemical treatment (demet + acet), followed by a growth cycle to allow epigenetic marks to be re-established, and then measuring their growth rates. If epigenetic patterns have not contributed to the evolved phenotypes (and adaptation in purely genetic), then a single round of chemical treatment should have the same magnitude of effect in the ancestor and the evolved populations. Conversely, if changes to epigenetic patterns have played a role in evolution, then we expect that the change in phenotype in the evolved populations differ from the ancestor. Our reasoning is that chemical treatment will remove epigenetic marks; if adaptation is primarily genetic, then the phenotype should be stable except for any effects of the drugs themselves, which will also be evident in the ancestor. On the other hand, if adaptation involved inherited epigenetic information, the adapted phenotype should be less (or more) stable in the face of chemical treatment then the ancestral phenotype is. The toxic effects of drugs (if any) should remain constant or attenuate over time, so changes in response to chemical treatment over time indicates that there have been changes in epigenetic marks that effect phenotype in the evolved populations. For this experiment we used CC-2937 populations evolved in the control treatment, and control, high CO_2, and high NaCl environments, and their ancestor. Evolved low phosphate populations were excluded because many of them had gone extinct. Results discussed below are shown in Figure 3.

We find evidence that the changes to epigenetic patterns that are transmitted between generations affect growth rate evolution in our experiment. The effect of the chemical treatment on growth rate is environment-specific in the CC-2937 ancestor (environment × chemical interaction, $F_{4,33} = 2.578$, $p = 0.0555$). Chemical treatment had negative effects on growth rate in all environments (Figure 3). For populations evolved in the control environment, there was a significant interaction between the chemical treatment and population ($F_{8,36} = 2.56$, $p = 0.0255$), where the chemical treatment decreased growth in all but one of the evolved populations relative to the ancestor. If the one outlier population (Figure 3) is removed from the analysis, the interaction is no longer significant. However, as the data come from replicate measurements made on independently grown and treated subcultures, it is likely that this reflects variation in evolutionary outcomes instead of measurement error. This suggests that epigenetic configurations changed during the selection experiment in the control environment, and that the stability of the adapted phenotype requires direct transmission of these epigenetic marks, such that the epigenetic configuration underlying the evolved phenotype cannot be re-established from genetic information alone. In contrast, in the high CO_2 environment, most populations did not respond to chemical treatment differently than the ancestor as the interaction between chemical treatment and population was not significant ($F_{8,36} = 1.34$, $p = 0.27$, Figure 3). This suggests that in these populations adapted primarily through genetic changes (though these genetic changes could in turn direct epigenetic patterns). In the high NaCl environment, the responses to the chemical treatment did not differ between evolved populations and the ancestor ($F_{7,28} = 0.63$, $p = 0.72$, Figure 3). However, there is a non-significant trend for the chemical treatment to have less effect on the high NaCl-evolved populations than on the ancestor, indicating that epigenetic configurations may have changed during adaptation. Together, the data from all three environments shows that while the phenotypic effect of epigenetic marks can evolve over hundreds of generations, the frequency with which this occurs is environment-specific, and is likely less important than genetic variation during directional adaptation (in the selection environments) than under stabilizing or reduced selection (in the control environment). Thus, while manipulating the production and transmission of epigenetic information affects evolutionary outcomes in environments where adaptation occurs in this experiment, we also show that the role of directly-transmitted epigenetic changes, when they can occur, is low by the time populations have undergone a significant change in fitness. This is consistent with modelling work showing that directly transmitted epigenetic marks can aid in the exploration of a fitness landscape, and contribute to the early stages of adaptation, but are often ultimately replaced by genetic mutations later in adaptation (Klironomos et al., 2013; Kronholm and Collins, 2016).

Phenotypes of evolved populations

Manipulating sources of variation on which selection can act also has the potential to affect the evolution of traits other than growth rate. If this is the case, we expect that either the *sir2* mutant, the chemical treatments, or both, affect the trait values of the populations at the end of the experiment relative to the rescued mutant and the control chemical treatment. Overall, we find that cell size, cell shape and chlorophyll content

changed during the experiment in most environments (Figure S3-S5). However, effects of epigenetic manipulation on trait evolution was environment and phenotype dependent. The *sir2* mutation affected responses to selection for cell size in high CO_2 and for chlorophyll content in high NaCl, and the chemical treatments affected responses to selection for cell shape in high CO_2 and high NaCl, and chlorophyll content in low phosphate. Many of the effects on trait evolution were small, at least on the timescale of this experiment. Overall, effects of epigenetic manipulation on traits other than population growth were environment and trait dependent. Detailed description of the results is given in Supplementary Material.

Genome sequencing

We used evolved populations of the strain CC-2937 from the control and demet chemical treatments to examine the genetic and epigenetic basis of adaptation. We isolated single clones from our evolved populations and sequenced the genomes and methylomes of clones from the control, high CO_2, and high NaCl environments, from both the control and demet chemical treatments. The low phosphate environment was excluded because of extinctions. We aimed to sequence the genomes and methylomes of 3 clones from each of the three environments and chemical treatments. However, due to failures in library construction in bisulfite sequencing, methylomes for some clones are missing. For control environment and control treatment 2 methylomes were missing, leaving 1; and demet treatment 1 was missing leaving 2. For high NaCl environment demet treatment 2 methylomes were missing leaving 1 (Table S2). We also sequenced the methylomes of the ancestor in all three environments to originally assess the ancestral methylation states (but see results below) and 3 methylomes of the ancestor in control and 3 methylomes of the ancestor in demet chemical treatments to assess the effectiveness of the demethylation treatment.

Numbers of genetic mutations in control and demet chemical treatments

In the resequenced control chemical treatment clones we detected 77 mutations in total, with a mean number of 9 mutations in the control environment clones, a mean of 5 mutations in the high CO_2 environment clones, and a mean of 11 mutations in the high NaCl environment clones. Numbers of mutations for each clone are presented in Table 4. The nine resequenced clones from the demet chemical treatment had 3594 genetic mutations in total, with a mean of 428 mutations in the control environment clones, 571 mutations in the high CO_2 environment clones, and 201 mutations in the high NaCl environment clones. We observed over 46 times more mutations in the demet treatment than in the control chemical treatment. This can be explained by the mutagenicity of the cytosine analogs, 5'-aza-deoxycytidine and zebularine (Umen and Goodenough, 2001), where the modified cytosine tends to be replaced with guanine during DNA replication. This is consistent with C↔G mutations in the two different treatments; there were 7 C↔G mutations out of 57 SNPs (12 %) in the control chemical treatment, but 3152 C↔G mutations out of 3544 SNPs (89 %) in the demet chemical treatment. For the control chemical treatment the majority of the mutations were in UTRs (untranslated region) and introns (49), with 26 mutations in coding regions, including 6 non-synonymous

mutations and 3 indels causing frameshifts. For the demet chemical treatment mutations in UTRs and introns together contained most mutations (1745). In coding regions there were 1526 mutations, 1138 non-synonymous, 379 synonymous, and 4 indels causing frame-shifts. A breakdown of mutations in different functional regions is shown in Table 5.

The bioinformatics pipeline for calling mutations was the same for the control and demet treatments and identical thresholds were applied. Both treatments were sequenced in the same run. We validated 20 mutations by Sanger sequencing (supplementary material) and in each case we confirmed the mutations. Thus, it is not likely that differences in the quantity and identity of mutations are due to sequencing errors.

Across all environments the number of mutations in the demethylation treatment was correlated with the number of generations those populations went through during the experiment (r = 0.89, n = 9, p = 0.0012), but the control chemical treatment showed the opposite trend (r = -0.86, n = 9, p = 0.002) with more mutations in populations that had gone through fewer generations. Here the effect of environment itself is confounded with the number of generations as we do not have enough data to test the effect of generation number within each environment, and there is little variation in generation number within environments. However, given that the biological mechanism of more cell divisions in the presence of mutagen leads to more mutations is reasonable, and we do not observe this pattern in the control treatment, suggest that it is the number of generations that drive the relationship.

The number of mutations did not account for variation in growth rate (among all of the lines, effect of number of mutations on growth rate: $F_{1,12}$ = 0.64, p = 0.438). This is consistent with genetic mutations in the demet lines being neutral or nearly neutral, and with deleterious mutations being removed by natural selection during the experiment. Alternatively, deleterious mutations may be offset by beneficial (compensatory) mutations.

Genetic changes in clones from the control chemical treatment

Since control treatment populations adapted in the high salt and high CO_2 environments, some of the mutations in these clones are probably beneficial. However, there are multiple mutations in each clone, which makes pinpointing the mutations responsible for phenotypic changes impossible in an asexual population. Many of the genes with mutations are of unknown function or annotated based on homology. Mutations that occurred in sequenced chemical control clones are listed in supplementary table S3.

For mutations that occurred in annotated genes, there are some candidates for adaptive mutations. For example, in the control environment, clone P3G11 has a deletion of one amino acid that preserves the reading frame in Cre17.g723600, which is an intraflagellar transport protein 81, which may be involved in cell motility. In the high NaCl environment clone P10C5, there is a deletion that causes a frameshift in

Cre03.g160050, which is annotated as flagellar associated protein. Another mutation of potential interest is a SNP in 3' UTR of Cre14.g629650 (NIK1) which is a nickel transporter. In clone P12E4 there is an insertion of 1 bp in the intron Cre17.g732150 which is a flagellar associated protein, as in clone P10C5. Another interesting mutation is a SNP in the intron of Cre02.g078400, a gene with a Bestrophin RFP-TM chloride channel domain. In the high CO_2 environment there are no mutations in genes with functions obviously related to high CO_2.

Genetic changes in clones from the demet chemical treatment

The large number of mutations precludes discussing each one individually, so we concentrate on mutations with multiple hits over different clones in genes with functions that are more obviously related to the selective environment. For clones evolved in the control environment we observed several mutations in mitochondrial genes. In particular we observed five non-synonymous mutations in cytochrome b, two non-synonymous mutations in NADH dehydrogenase subunit 5, two non-synonymous mutations in subunit 4, two in subunit 1, two mutations in RrnS4, which is gene producing an RNA of the S4 portion of small subunit rRNA and two non-synonymous mutations in rtl, a reverse transcriptase like protein.

For the clones evolved in the high CO_2 environment we also observed mutations in mitochondrial genes. We observed four non-synonymous mutations in cytochrome b with one non-sense mutation causing an early stop codon. Another five non-synonymous mutations in NADH dehydrogenase subunit 5 with one non-sense mutation. There were also five mutations in total in RrnS4, which is gene producing an RNA of the S4 portion of small subunit rRNA. Notable mutations in the nuclear genome were two non-synonymous mutations in Cre01.g024400 which is a component of the TRAPP complex and is predicted to be involved in meiosis, three mutations in gene Cre03.g200250 which is an enzyme that catalyzes the reaction of isocitrate to glyoxylate and succinate, one mutation was in 5' UTR region and two other were in clone P4C7, one of them intronic and one synonymous mutation.

For clones evolved in high NaCl we never observed genes with more than two mutations, in contrast to clones evolved in high CO_2 and control environments. In mitochondrial genes, we only observed one non-synonymous mutation in cytochrome b, no mutations in NADH dehydrogenase subunit 5, and only one in RrnS4. Other non-synonymous mitochondrial mutations were one mutation in rtl, and one mutation in NADH dehydrogenase subunit 2. For nuclear mutations, we observed one intronic and one non-synonymous mutation in Cre09.g400850, which is a putative polycystin cation channel.

Since the demethylation treatment contained a large number of mutations we also looked at the function of genes that were overrepresented in terms of having multiple mutations across different environments. In the control and high CO_2 environments we see an over representation of mutations in genes related to microtubule based movement (GO terms 0070018, 0070017, and 006928) (Table 6). These mutations could

reflect changes in swimming behaviour if swimming is costly for *C. reinhardtii*. In all environments, we also observed mutations in genes related to cyclic nucleotide metabolism and biosynthesis (GO terms 0009187, and 0009190). These changes may reflect adaptation to laboratory conditions and the growth media as these terms are significant in all environments including the control (Table 6). In control and high NaCl environments, terms for oxidative phosphorylation and electron transport were also significant. In high CO_2 and high NaCl environments GO terms for detecting external and abiotic stimulus, as well as those for detection and response to mechanical stimulus were significant (Table 6). In the high NaCl environment, the GO term for transmembrane transport was the most significant term, which may be related to osmoregulation in the high salt environment.

Bisulfite sequencing and general features of DMRs

To examine the methylation changes that happened during adaptation, we compared the methylation patterns of the evolved clones. Methylation levels were low in *C. reinhardtii* in this experiment, as expected (Feng et al., 2010; Lopez et al., 2015). The mean value of CG methylation was 1.3 %, CHG methylation was 0.3 %, and CHH methylation was 0.4 % for the ancestor in the control environment and treatment. It is unclear how to interpret the biological effects of differences in methylation of single cytosines (Wibowo et al., 2016), so we focused our analysis of differential methylation patterns on large contiguous stretches of methylation. Differentially methylated regions (DMRs) were found by pairwise comparison of all the evolved samples and the ancestor to each other, and regions that had differences in methylation were designated as DMRs. See methods for detailed criteria required for a region to be defined as DMR.

We first considered DMRs that were detected when all clones were included in the analysis. We detected 924 such DMRs, with a median length of 61 bp (ranging from 9 bp to 1150 bp in length). Most DMRs (72 %) were located within genes and 28 % were intergenic, which contrasts with results in *Arabidopsis* where 20 % of DMRs were located within genes (Hagmann et al., 2015; Wibowo et al., 2016) but is consistent with *C. reinhardtii* having mainly genic methylation (Feng et al., 2010; Lopez et al., 2015). Of the within- gene DMRs, 54 % were in exons, 26 % in introns 1.8 % in 5' UTRs, and 19 % in 3' UTRs.

Clustering of clones based on methylation changes

When we clustered clones based on DMRs (Figure 4), the ancestor samples and the evolved clones separate with high bootstrap support. The ancestor samples have higher methylation rate in the observed DMRs (Figure 4). This may be attributable to storage effects on the ancestor, as the ancestor had to be stored on agar slants. Based on hierarchical clustering, clones P11B4, P11B11, and P12E4 from the high NaCl environment cluster together with bootstrap support of 100% (Figure 4), suggesting that the DMRs unique to these clones are specifically involved in adaptation to high salt. The remaining clones cluster by chemical treatment, indicating that the demethylation treatment affected methylation patterns in this experiment,

although bootstrap support for the branch separating the remaining evolved clones by chemical treatments is 89%. Among the ancestor samples, the branch separating the chemical treatments has a bootstrap support of 100%.

Epigenetic changes among the control and demet treatment clones

Because the difference in ancestral and control evolved methylation patterns is so large (Figure 4), and may represent a storage effect rather than evolutionary differences relevant to this experiment, we considered the 542 DMRs that were polymorphic among the evolved clones and used parsimony to determine the ancestral state of the DMR. For each DMR, we assigned the DMR ancestral state to the most common state among the clones from the chemical control treatment of all environments. To resolve ties we included all the evolved clones. Among the evolved clones, most DMRs occurred only in one clone (Figure 5A), but some were present in multiple clones. Certain DMRs were also present across multiple clones that had evolved in different environments (Figure 5B). DMRs in the evolved clones had similar distribution of annotations as all DMRs (Figure 5C).

We observed 143 derived DMR changes in the clone from control chemical treatment and control environment. Among the control treatment clones from the high CO_2 environment there were on average 87 DMR changes per clone, and on average 149 changes per clone in the high NaCl treatment. Changes per clone are listed in Table 4. In the clones from the demet chemical treatment, we observed 70 DMRs on average in the control environment, 73 changes on average in the high CO_2 environment, and 123 changes in the high NaCl environment. While the total number of DMR changes was lower in the demet (483) than in the control treatment (852), this difference was only marginally significant (Wilcoxon-Mann-Whitney rank sum test, W = 34, p-value = 0.073). We also compared the mean methylation levels of DMRs in control and demet clones over all environments in different sequence contexts (Figure 5D). Methylation rates in CG context were 19.8 % for the control clones, and 10.2 % for clones strains (paired sample t-test, t = 24.8, df = 541, $p < 2.2*10^{-16}$). For CHG context methylation rates were 6.1 % and 7.4 % (paired sample t-test, t = -0.96, df = 45, p-value = 0.347), for control and demet. samples respectively. For CHH context methylation rates were 4.9 %. and 5.5 % (paired sample t-test, t = -0.40, df = 48, p-value = 0.688). This shows that the demethylation treatment did reduce overall methylation levels in the CG context. The very low methylation rate in the first place limits our ability to detect differences in methylation in the other sequence contexts.

Next we investigated whether DMRs occurred evenly across the two chemical treatments. We observed that out of the 542 DMRs present in the evolved clones a change in 500 of them occurred in the control chemical treatment, and a change in 223 of them occurred in the demet chemical treatment. Of those changes, 319 were unique to the control treated lines, 181 occurred in both treatments, and 42 changes were unique to the demet chemical treatment. We tested whether we had expected amounts of unique changes by permuting (n = 1000) the clone labels, 95 % quantiles were: overlapping changes 174 – 253, 86 – 292 for changes unique

to control clones, and 64 – 240 for changes unique to demet clones. Thus, there was an over representation of changes unique to control clones and under representation of changes unique to demet clones. Most of the DMRs were gains in methylation. However, DMRs that changed in multiple clones were often losses of methylation, especially in the demet treatment clones (Table 7). These results are consistent with the demethylating effect of the chemical treatment and the demet clones being less likely to use changes in methylation patterns to adapt as a result.

No indication of genetic control of DMRs

In clone P12E4, which evolved in high NaCl, there is a mutation in chromosome 16 at position 3227089 that is close to three DMRs in that region. No other genetic mutations were within 1kb up or downstream of the DMRs. There was only a single case of over overlap between genetic mutations and DMRs among the demet clones. In clone P4C7, which evolved in high CO_2, a DMR occurred on chromosome 14, position 3546199 that was downstream and within 1 kb of a genetic mutation. Thus across all clones there were only two cases where a genetic mutation was near a DMR. While we cannot exclude the possibility that some of the mutations are *trans*-acting or *cis*-acting over very long distances the vast majority of the DMRs appear independent of DNA sequence changes. Furthermore, clones from the demet treatment contained nearly the same number of DMR changes as did clones from the control chemical treatment, despite having many more genetic mutations. This supports the interpretation that many of the DMR differences were not directly caused by genetic mutations.

Enrichment of gene ontology annotations for DMRs

To examine the potential effects of the DMRs on phenotype, we identified GO terms that were enriched in DMRs that were polymorphic among the evolved clones regardless of chemical treatment in each of the three environments. In the control environment, only a few GO terms were enriched ($p = 0.00032$), these are involved in aminoglycan (GO:0006026) and chitin catabolic processes (GO:0006032). Enrichment of these terms was due to DMRs on gene Cre10.g451600 and Cre10.g458350, which are both annotated as chitinases (E.C 3.2.1.14). Based on computational predictions, *C. reinhardtii* has a chitin degradation pathway, but there is no experimental support for this yet. In the high CO_2 environment, the GO term 0006026 was again enriched ($p = 0.00020$), again due to polymorphic DMRs in genes Cre10.g451600 and Cre10.g458350. The DMR in gene Cre10.g451600 was also polymorphic in the lines evolved in the high salt environment. While the physiological role of the putative chitinase genes is unknown, these results indicate that the methylation changes observed in these genes possibly contribute to adaptation to shared laboratory conditions since changes were observed in all of the environments.

Specific changes to high CO_2 included enrichment of GO term for membrane depolarization during action potential (GO:0086010, $p = 0.00036$). This was due to DMRs in genes Cre07.g333535 and Cre11.g467528,

which are both annotated as voltage gated Ca^{2+} channels. In the high salt environment no GO terms reached the cut-off value of 0.01. The GO term 0055085, transmembrane transport, had the lowest lowest p-value ($p = 0.013$). DMRs in genes that were responsible for enrichment of this GO term were Cre05.g234645 (sodium/hydrogen exchanger), Cre06.g260100 (adenosine 3'-phospho 5'-phosphosulfate transporter), Cre07.g327750 (ion transport protein), Cre07.g333535 as well, and Cre17.g725150 (xenobiotic-transporting ATPase).

For DMRs that were polymorphic among the ancestor samples, we did not find any significantly enriched GO terms that were represented by more than one gene.

Discussion

Based on theoretical models of adaptation with epigenetic variation (Klironomos et al., 2013; Kronholm and Collins, 2016), reducing the amount of epigenetic variation either genetically or chemically should decrease adaptation. Our study broadly supports these predictions; we see evidence for epigenetic contributions to adaptation in the high NaCl and high CO_2 environments, which are the two environments where a large direct response to selection occurred. Our major findings are summarized in Table 8.

Effects of epigenetic manipulation on adaptation in the selective environments

The results from the high NaCl environment most closely match the prediction that reducing the amount of epigenetic variation available with the chemical treatments decreased adaptation. This effect was not likely mediated by SIR2 dependent mechanisms as the *sir2* mutation had no consistent effects in high NaCl. We also observed that clones from the high NaCl environment clustered together based on their methylation changes. Taken together these data support the conclusion that epigenetic changes are important in adapting to this environment.

In the low phosphate environment, the evolutionary response in growth rate was small and no consistent effect of the chemical treatments or the *sir2* mutation was observed. In the CC-2937 strain many populations went extinct and this reduces our power to detect the effects of the chemical treatments for the CC-2937 strain. Overall, it may be that our power to detect an effect of epigenetic mechanisms is small due to higher variation in evolutionary responses, or there is little or no epigenetic regulation of phosphate metabolism in *Chlamydomonas*.

In contrast, the evolutionary response to high CO_2 was to decrease population growth rates. While this may seem counter-intuitive, previous studies of single-celled chlorophytes evolving in high CO_2 environments show that high growth rates result in low competitive fitness, and that this can associated with poor mitochondrial function (Schaum and Collins, 2014). Therefore the evolution or maintenance of low growth

rates may be adaptive in high CO_2 environments, where rapid growth both compromises the ability of cells to withstand other challenges, and decreases competitive ability. Indeed, lineages with slower population growth rates evolved in high CO_2 environments are generally better competitors than faster-growing lineages evolved in those same environments (Collins, 2010; Schaum and Collins, 2014). This evolutionary reversal of a plastic increase in cell division rates has been called "Prodigal Son dynamics". Modelling studies suggest that Prodigal Son dynamics may occur when cells cannot evolve mechanisms to cope with the consequences of maintaining an overall increase in metabolism in enriched environments (Collins, 2016). Thus, if low growth rate is adaptive in high CO_2, we observed an adaptive evolutionary response in our experiment. We do not show in this study that higher growth rates are maladaptive, so it is also possible that increased growth in high CO_2 environments is adaptive for *Chlamydomonas*, but this interpretation goes against all of the empirical evidence to date, so we consider it to be unlikely here. The demethylation chemical treatment alone did not reduce adaptation but the demethylation + acetylation had a consistent adaptation reducing effect in the CC-2937 background. In terms of methylation patterns, strains from the high CO_2 environment cluster together with strains from the control treatment. This is consistent with our observation that demethylation alone had little effect, and suggests that changes in DNA methylation do not contribute to adaptation that is specific to high CO_2. However, other epigenetic modifications, such as histone acetylation, may do so. The plastic response to high CO_2 was diminished in the *sir2* mutant strain but not in the complemented strain, suggesting that SIR2 mediated silencing may be important for plastic response to high CO_2, but that the evolutionary response of the LM3 background is different from CC-2937 background.

Potential side effects of the chemical treatments

The chemicals 5-aza-deoxycytidine and Zebularine are mutagenic, as is evident by our sequencing results. This raises the possibility that an increased input of deleterious mutations, or mutational meltdown, could explain cases where little or no adaptation occurred. However, UV-treated populations, which also have extremely high mutation rates, adapted in a similar manner in all environments showing that increased mutation loads did not impede adaptation in this experiment. Thus, the decreased rates of adaptation in the chemical treatments are unlikely to be caused by an increased input of deleterious mutations, and are more likely to be caused by the effects of the drugs on the production and transmission of epigenetic information.

Epigenetic mechanisms (methylation, acetylation) have many cellular functions, some of which are related to normal functioning of the cell, so that chemically manipulating epigenetic marks could conceivably have general toxic effects on cells. However, general toxic effects are unlikely to be driving our results. First, the chemical treatments had no systematic effect on ancestral fitness in the control environment during pilot studies. Second, the effects of the chemical are strongly dependent on the environment. If the chemical treatments were acting through cytotoxic effects, we would expect them to have an effect in the same direction across the environments, which was not the case for the ancestors. This environmental dependence suggests that effects on growth are caused by changes in cellular function due to the modification of

644 epigenetic marks rather than general cytotoxicity. Finally, the chemical treatments change growth rates, but do not systematically lower them, in the populations evolved in the control treatment. This strongly suggests
646 that the growth effects seen in the chemical treatments are due to the modification of epigenetic marks rather than cytotoxicity.

648

If cytotoxic effects exist, they could also have demographic effects, such as depressing population sizes or
650 slowing down cellular division rates, which would result in the chemically treated populations going through fewer generations over the experiment. We examined this possibility but found no evidence that the chemical
652 treatments caused demographic effects large enough within environments to explain variance in evolutionary outcomes (see Supplementary Information). Since we cannot find any reasonable indication that the effects
654 of the chemical treatments are only due to cytotoxicity or demographics effects, we conclude that the differences in growth associated with these treatments are likely attributable to their effects on the
656 transmission of epigenetic information between transfers.

658 *DNA methylation changes*

660 In both this and other studies, the role of changes to DMR patterns relative to genetic change appears both species and environment specific. Among the sequenced clones from chemical control treatment, we
662 observed the most genetic mutations and DMR changes in the high NaCl environment. Many more DMR changes occurred than genetic mutations, which is consistent with changes in DMRs being more common
664 than genetic mutations in *C. reinhardtii*. This is in line with mutation accumulation experiments that have shown rapid changes in methylated positions, with a rate for gain of methylation reported at 2.56×10^{-4}, and
666 loss 6.30×10^{-4} per CG site per generation in *Arabidopsis* (van der Graaf et al., 2015). However, there are also reports that DMRs can change at similar rates to genetic mutations (Becker et al., 2011). In *Arabidopsis*,
668 for example, natural variation in methylomes shows that DMR patterns tend to be stable and often under genetic control (Dubin et al., 2015; Hagmann et al., 2015). In contrast, our results show that for *C.*
670 *reinhardtii* the role of changes in DMR patterns relative to genetic variation is environment specific. Comparing our results with those from *Arabidopsis* indicates that the role of changes to DMR patterns is also
672 species specific.

674 Like genetic changes, we see evidence for limited "parallel evolution" (Bailey et al., 2015) of changes in DMR patterns between replicate populations evolving in the same environments. We observed some DMR
676 changes that occurred in parallel over many, but not all, lines within selection environments. These parallel changes suggest that changes to methylation patterns have the potential to be adaptive, but our study cannot
678 separate the possibilities of the changes either being environmentally induced from that of the changes being random and under positive selection (or some combination of these two). This would be an interesting
680 direction for future work, and would require a detailed comparison of the epigenetic changes associated with plastic and adaptive evolutionary responses in the same environments, as well as a reasonably accurate
682 epigenotype-phenotype map.

Previous studies on natural variation in DNA methylation have suggested that most methylation variants are controlled by DNA sequence (Dubin et al., 2015; Hagmann et al., 2015). One example of this would be a SNP or a transposable element insertion determining whether a downstream sequence gets methylated or not. However, there is no evidence that genetic mutations caused the observed methylation changes in our study. We observed only two cases across the 13 sequenced lines where a genetic mutation was within 1 kb of a region that had a DMR change. In the chemical control lines we observed many more DMRs than genetic mutations, so if genetic mutations were indeed responsible for a high proportion of DMR changes, they would have to control multiple DMRs over long and variable distances in *trans*.

Effect of epigenetic variation on adaptation

Theoretical models predict that adaptation from epigenetic variation happens in two steps: first a population adapts using epigenetic variation, and then epigenetic variation is replaced by genetic changes over a long period of time (Klironomos et al., 2013; Kronholm and Collins, 2016). Our observations are in line with some, but not all, of the model predictions. In our experiment, manipulating the epigenetic system slows adaptation, which is consistent with the model prediction that epigenetic changes, which occur at a faster rate, are available first to natural selection. Consistent with the prediction of epigenetic changes preceding genetic ones, we observed more DMR changes than genetic mutations in the chemical control lines. However, this study did not include a timecourse to monitor the rate at which genetic and epigenetic changes were fixed in populations, nor the rate at which epigenetic changes disappeared. While our study did not test the timescale on which epigenetic changes were replaced with genetic changes as predicted by models, the outcome of test for phenotypic stability in the control chemical treatment populations is certainly consistent with epigenetic changes being replaced with genetic changes during adaptation. Recently, Wang et al. (2015) also demonstrated that a fission yeast mutant with uncontrolled heterochromatin spreading reverted back via rapid epigenetic adaptation.

Conclusion

Epigenetic variation can contribute to adaptation, although the extent to which it does so depends on the selection environment. These results highlight the need to consider epigenetic variation during microevolution, even on timescales where genetic mutations can be used. While this study shows that epigenetic variation can contribute to adaptation, it also points out the need to better characterize epigenetic mutations in a way that will allow them to be included in standard theory. In particular, understanding the link between genetic mutations and patterns of epigenetic change is required in order to advance our mechanistic understanding of how phenotypes evolve.

Materials and methods

Chlamydomonas strains

We used four different *Chlamydomonas* strains in the selection experiment: the strain CC-2937, UV-treated CC-2937, a *sir2* mutant and a complemented mutant (the rationale for treating the UV-treated CC-2937 as a strain is detailed in the "selection experiment" section). The *sir2* mutant was generated in the genetic background hereafter called LM3 by insertional mutagenesis (see below). A complemented *SIR2* line was constructed by transforming the genomic region encompassing the *SIR2* gene into the mutant background. The LM3 strain has no cell wall, which allows for easier transformation. CC-2937 is a standard wild-type strain of *Chlamydomonas reinhardtii*, obtained from the *Chlamydomonas* Resource Center.

In order to manipulate the genetic mutational supply available, we treated the UV CC-2937 strain with UV-radiation every other transfer, using a UV-lamp placed 5 cm above the plate for 1 minute. This produced an irradiance of 33.75 W / m^2, giving a radiant exposure of 2025 J / m^2. This radiation dose was chosen based on preliminary experiments that showed an increased number of mutants appearing in a culture but did not substantially increase mortality (see supplementary material).

sir2 mutant and complementation

The *sir2* mutant was found using a screen for components involved in transgene silencing. Briefly, the screen was performed by using a reporter plasmid containing a 500 bp repetitive region from the 3' end of the L1 transposon, which was cloned upstream of a bleomycin/zeocin resistance cassette driven by the RBCS2 promoter (pMTBRBle-L1-3'-2F). The forward primer used to amplify the L1-region was: TTAGATCTATTGGAGACAACGCGCTGTACC and the reverse primer was: TTAGATCTGCCTTGCTCTTGTCCGGATGG. The plasmid also contained an *ARGININOSUCCINATE LYASE* gene for transformant selection. The plasmid was transformed into the *cw*15- 325 *arg*- strain, and a clone was selected that had silenced expression of the zeocin resistance gene, and was therefore sensitive to zeocin. The presence of silenced zeocin resistance cassette was checked via PCR and using the histone acetylation inhibitor Trichostatin A, which increased zeocin resistance. Insertional mutagenesis was performed by transforming the zeocin sensitive strain with a linearized pKanAPHVIII plasmid which contained a paromomycin resistance cassette driven by PSAD regulatory elements. Mutants were selected for both zeocin and paromomycin resistance. All transformations were performed using the glass-beads method (Kindle et al., 1991). The site of the insertion was determined by inverse PCR and sequencing which showed an insertion in intron 2 of the gene Cre10.g462200 (Figure S6), which belongs to the SIR2 family of class IV sirtuins. The site of insertion was confirmed by PCR amplification across the region of the insertion and Southern blotting. Complementation of the *sir2* mutant was performed with a 6172 bp genomic fragment encompassing the entire *sir2* gene and including 1.2 kb of the upstream promoter region. This was amplified by PCR using Pfusion polymerase (NEB) and oligonucleotides gSIR2F (attaatGAGCGATGTCGGTGGCCCC) and gSIR2R (attaatTTTGCGGTACCGGTCCCACG), and cloned into the *Ase* I site of the pMTH vector encoding a hygromycin resistance gene driven by a PSAD promoter

for transformant selection. Mutant strains were transformed, selected with hygromycin, and tested for complementation of the mutant phenotype by loss of expression of the zeocin resistance gene by qRT-PCR (Figure S7) and by comparison of growth on media containing different zeocin concentrations (Figure S8).

Chemical treatments

Two different chemical treatments were used to decrease the transmission rates of epigenetic information during our evolution experiment. To lower methylation rates, we used mixture of 5-aza-2-deoxycytidine at 0.2 mM and zebularine at 0.2 mM, both of which are cytosine analogs that cannot be methylated (Jones, 1985; Cheng et al., 2003), along with L-Ethionine at 0.2 mM, which blocks methylation by competing with methionine for the transfer of methyl groups to DNA (Moore and Smith, 1969). We refer to this treatment as "demet". In the second chemical treatment we used 5-aza-2-deoxycytidine and L-Ethionine as before, but added a histone deacetylase inhibitor Trichostatin A (TSA) (Marks et al., 2000) at 0.1 μM. We refer to this treatment as as "demet + acet". These concentrations of 5-aza-2-deoxycytidine and L-Ethionine have been shown to demethylate DNA in *C. reinhardtii* , after treating cells with 0.2 mM of the drugs, the same concentration used in this study, no methylation was detected in chloroplast DNA in vegetative cells and approximately 45 % reduction in methylation occurred in gametes and zygotes, which are heavilty methylated in Chlamydomonas (Feng and Chiang, 1984). Since the 5-aza-2-deoxycytidine stocks had to be dissolved in DMSO, a DMSO blank was included in the control chemical treatment.

Selection environments

Four different selection environments were used, each of which imposed a different selection pressure on evolving populations. The environments were: high CO_2, high salt, and low phosphate, and a control environment that reflects standard laboratory conditions.

The experiment was done in customized incubators (Infors, Basel, Switzerland). For control environments, the CO_2-level was set at 420 ppm and temperature was set to +25 °C. In all environments the base growth media was Sueoka high salt media (HSM) (Sueoka, 1960) with 20 mM Tris added (HSMT) buffered at pH 7.0. Populations were cultured in 200 μl of media under constant light. This reflects the usual culturing conditions for these stains. We used AeraSeal breathable sealing films (Alpha Laboratories, Hampshire, UK) to cover the 96-well plates to allow even gas exchange across all wells.

The selection environments were modified as follows: in the high CO_2 environment we increased CO_2 levels to 2000 ppm, in the high NaCl environment we added 43 mM of NaCl to the base media and in the low phosphate environment we decreased the phosphate available in the HSMT by 50-fold, from 13.56 mM to 0.2712 mM. Phosphate is added to HSMT as potassium salt, so we added KCl to the low phosphate media to achieve the level of 22 mM K^+ ions as in the control media.

794

Selection experiment

796 The selection experiment consisted of four strains (CC-2937, UV irradiated CC-2937, *sir2* mutant, complemented *SIR2* mutant), three chemical treatments (demet, demet + acet, control), and four selection 798 environments (high salt, high CO_2, low phosphate, control), for a total of 48 treatments. We used the control environment populations as an evolving control, and compared growth rates of the populations from the 800 other environments to these populations. We wanted to investigate how important epigenetic effects are for adaptation in general in different environments, so having an evolving control that adapts to the shared lab 802 environment allows us to investigate specific adaptation to the different environments. We treated the UV-irradiated CC-2937 as a strain throughout the experiment. UV-irradiation is used here to increase the genetic 804 variation produced. Each combination of strain, chemical treatment, and selection environment had 9 independent replicate populations. The selection experiment was carried out on 96 well plates (Corning, NY, 806 USA) using a split plot design, with the different chemical treatments randomized on the columns (plots) of each plate and genotypes randomized within plots, with blank wells present in a unique pattern within each 808 plate, both to monitor possible migration between wells, and to serve as a unique identifier for each plate. Edges of plates were filled with water to minimize edge effects. All populations were founded from single 810 cells, so that very little genetic variation was present within populations at the beginning of the experiment. All populations were acclimated to the selection environment for four days, after which a population of 5000 812 cells was used to start the selection experiment.

814 Populations were propagated by batch transfer. During each transfer we transferred 40 µl of control and low phosphate populations, 50 µl of the high NaCl, and 20 µl of the high CO_2 populations to a new plate with 200 816 µl of fresh medium. In the first four transfers 40 µl of the high NaCl populations and 20 µl of control populations were transferred but this amount was increased to 50 µl and 40 µl respectively in the subsequent 818 transfers. The populations were transferred twice a week, and population size measured at each transfer. During the experiment there was an interruption in the compressed air supply on transfers 7 and 8, during 820 this interruption the lights were turned off and the plates sat in the incubator for seven days. After this the experiment resumed normally. The selection experiment was run for 62 transfers (roughly 200 generations). 822 The selection experiment was not designed to give us detailed understanding of adaptation to each different environment, but to use different environments (three different selection experiments) to let us examine the 824 general role of epigenetic effects in adaptation.

826 *Standard curves for optical density and cell counts*

At the end of the selection experiment we constructed a standard curve for optical density and cell counts for 828 each of the populations both in the environment it evolved in and in the control environment and all other environments for populations evolved in the control environment. Dilution series were made on 96-well

plates and the cultures we measured spectrophotometrically. All spectrophotometric measurements were done using absorbance at 750 nm with an EL808 plate reader (BioTek, Potton, UK). Cell numbers were measured in these cultures by flow cytometry (see supporting information for details). Standard curves were constructed by fitting a linear regression to the data (for all standard curves median $R^2 = 0.9902$). In all subsequent experiments cell numbers were estimated by transforming absorbances into cell numbers using these curves.

Fitness measurements

Control and selection environments used for fitness assays were the same as during the selection experiment. Populations were acclimated to the environment in which fitness was measured for one transfer and then transferred to a fresh medium. Growth curves in the environment of interest were made by measuring the cell density over 140 hours. Three replicate fitness measurements were performed for each population. The fitness of evolved control populations was measured in all environments. Populations evolved in the high CO_2, high salt, and low phosphate environments were measured in their selection environment and in the control environment. In all cases, chemical treatments used for the fitness assays are the same for each population as they experienced during the selection experiment unless otherwise noted.

Statistical analysis

To calculate fitness, we fitted growth curves to the cell number data. We used the baranyi growth curve model in the R package “nlstools” (Baty and Delignette-Muller, 2012) without lag phase. The curves were fitted using the Levenberg-Marquardt algorithm implemented in the R package "minpack.lm" (Elzhov et al., 2013). Some populations experienced a lag phase, while others did not; in order to fit the same growth model to all populations, we removed the data points within the lag phase when it were present. We extracted the maximum growth rate, *r*, from the growth model for each population and used this as a measure of fitness. Relative fitness measures were calculated by standardizing the absolute fitness for each population by the mean fitness of control populations measured in that environment and chemical treatment. For example, the relative fitness of populations of strain CC-2937 evolved in the high salt environment and demet chemical treatment was calculated by standardizing their absolute growth rate by the mean absolute growth rate of CC-2937 populations evolved in the control environment and demet chemical treatment. By comparing the same chemical treatments to each other, any growth inhibiting effects that the chemicals themselves have are controlled for. Fitness data was analyzed using linear models in R (R Core Team 2013), we fitted fitness as a response variable and assay environment, selection environment, chemical treatment, and strain and their interactions as explanatory variables. First we fitted all terms and their interactions and then dropped non-significant interactions one by one to get to the final model. Contrasts were calculated using the R package “contrast”. Throughout the study, responses are reported as mean ± SEM.

Sample preparation for genome and bisulfite sequencing

After the evolution experiment we chose three populations randomly from different experimental plates from control, high CO_2, and high salt environments, and the control chemical treatment and from the demethylation (demet) treatment. We focused only on populations on the CC-2937 background, and low phosphate environment was excluded because of not enough populations were alive. We plated cultures of these populations and picked single colonies from each at random that were stored for further experiments, hereafter called clones. This came to 18 evolved clones and the CC-2937 ancestor, 19 clones in total for genome re-sequencing. In addition, we grew the ancestor in the three environments and in the control environment with and without demet treatment. For comparing the control and demet chemical treatments we used three independent replicate cultures. In total for bisulfite sequencing we had 22 different clones, since five were lost due to failed library construction (Table S2). We grew the clones to high density in liquid culture in the same environmental conditions that they had experienced during the evolution experiment and extracted DNA using phenol-chloroform extraction.

Genome re-sequencing and bisulfite sequencing was done at the Beijing Genomics Institute (BGI-Hong Kong) with the Illumina HiSeq 2000 platform using paired-end sequencing. For re-sequencing Libraries were prepared by fragmenting DNA by sonication, ligating adapters, size selecting DNA, and PCR amplified. Insert size in libraries was 500 bp. *Chlamydomonas* has a high GC content, so PCR conditions in library preparation were modified to accommodate high GC content following Aird et al. (2011). For bisulfite sequencing, after fragmenting DNA methylated sequencing adaptors were ligated and DNA was bisulfite treated with the ZYMO EZ DNA Methylation-Gold kit, following manufacturer's instructions. Insert size in libraries was 100-300 bp. Libraries were sequenced with the Illumina HiSeq 2000 platform using paired-end sequencing, read length was 90 bp. We obtained approximately 4 Gb of clean sequence for each sample.

Read mapping for DNA resequencing and genotyping

In general for read mapping and genotyping we followed the approach of Ness et al. (2012). We mapped reads to the available *Chlamydomonas* reference genome using BWA (Li and Durbin, 2009) and realignment of reads near indels was done using GATK 3.1-1 (McKenna et al., 2010; DePristo et al., 2011). Genotypes were called jointly for all samples with GATK Unified Genotyper with heterozygosity set to 0.01, minimum base quality to 10, and sample ploidy set to diploid.

Mutations that had happened during the evolution experiment were identified as different genotype calls in the ancestor and a sample. We processed vcf files produced by GATK using Wormtable 0.1.5a2 (Kelleher et al., 2013) and a custom Python script. We filtered low quality genotype calls by discarding all cases where read depth was less than five, genotype quality less than 30 for either ancestor or the sample. All

heterozygous positions were also discarded; we let Unified Genotyper run in diploid mode even though *Chlamydomonas* is haploid, as we observed that heterozygous genotype calls were indicative of read mapping errors. After a list of putative mutations had been produced by the filtering step, we checked mutations manually in IGV (Thorvaldsdóttir et al., 2013); visual inspection of read alignments in IGV revealed any potential sequencing or mapping errors. This allowed us to produce a manually curated list of mutations. For the control chemical treatment we manually checked all mutations and for the demethylation chemical treatment we checked 18 % of mutations prioritizing those with the lowest genotype qualities.

Read mapping for bisulfite sequencing and methylated base calling

For read mapping of bisulfite treated reads we used BWA-meth (Pedersen et al., 2014). BWA-meth uses *in silico* conversion of C's to T's in both reference genome and reads. Methylation status of C's is recovered by comparing mapped converted reads to the original reads. Calling of methylated cytosines was performed using BisSNP 0.82.2 (Liu et al., 2012). Based on a methylation bias plot, we trimmed 4 bp from both 5' and 3' ends of the reads. We let BisSNP call methylated cytosines in CG, CHG, and CHH contexts. Minimum base quality was set to 20 and minimum mapping quality to 60. All samples were called together making use of GNU parallel (Tange, 2011) to parallelize the task. We processed the vcf file using PyVCF 0.6.8 with a custom python script to extract methylated bases for downstream analysis.

Calling methylated regions (MRs) and differentially methylated regions (DMRs)

In order to identify differentially methylated regions in a statistically robust manner we followed the approach of Hagmann et al. (2015). First we identified contiguous methylated regions in each clone using a Hidden Markov Model (Molaro et al., 2011; Hagmann et al., 2015). The model considers each three sequence contexts, CG, CHG, and CHH separately with different methylation rate distributions, the model is trained on genomewide data to identify regions of high and low methylation. We set maximum distance between adjacent cytosines within an MR to 100 bp and trimmed those sites off the ends of the region that had methylation rate < 10 %. After training the model, the methylation rates within a region were summed to give a score for that region. Then whether the observed score was higher than expected by chance was tested by computing an empirical distribution of scores by permuting cytosines across the genome to obtain a p-value for that region. These p-values were corrected for false discovery rate (FDR) and those highly methylated regions that had FDR < 0.05 were kept as MRs.

Next we selected regions to be tested for differential methylation. For two clones every region could in principle be tested for differing methylation. However, we want to group clones based on differentially methylated regions, so identical regions need to be compared to each other. MRs are not necessarily identical in among clones, and for multiple clones this would result in a very large number of tests among all pairwise combinations of clones. Thus, we followed the rules defined by Hagmann et al. (2015) to select regions for

testing. The start and end coordinates of each MR across all clones formed a set of breakpoints and each combination of coordinates defined a segment to be tested for differential methylation. Then using the following rules segments were discarded to reduce the number of tests. Segment was kept if at least one clone was in high methylation state throughout this segment. To detect quantitative methylation differences entirely methylated segments in more than one clone were also compared to each other. Segments were discarded from pairwise comparison if less than two clones contained at least 8 cytosines covered by at least 3 reads each. Segments were discarded if they overlapped more than 70 % with a previously tested segment. Pairwise tests were not performed if both clones were in low methylation state in the region. To prevent testing regions with coverage imbalance clones were excluded if the number of positions covered by at least 3 reads was less than half of the maximum number of such positions of all clones in the same region.

Statistical analysis of DMRs

For data visualisation we used the "ggplot2" and "gplots" R packages. DMR clustering was performed with the "fastcluster" R package (Müllner, 2013). Bootsrap values for DMR clustering were calculated with the "pvclust" R package (Suzuki and Shimodaira, 2006).

Gene ontology term enrichment test

We extracted gene ontology (GO) terms from the *Chlamydomonas* genome annotation and supplemented these with annotations from the Uniprot database. We used reciprocal BLAST to identify matching genes between the *Chlamydomonas* genome annotation and the Uniprot database. All proteins which were annotated as from *Chlamydomonas* in the Uniprot database were blasted against proteins from the *Chlamydomonas* genome annotation and the best hit was identified. Then all proteins from the *Chlamydomonas* genome annotation were blasted against the Uniprot set and best hit was identified. If the both best hits were against the same proteins, the loci were designated as a pair and GO terms were propagated from one database to another. GO terms for proteins encoded by the organelle genomes were extracted from the Uniprot database. We used a GO term enrichment test, implemented in the R package "topGO" (Alexa et al., 2006), using the classic algorithm. P-values for the GO terms were calculated using Fisher's exact test.

For GO enrichment among the genes with genetic mutations, we included genes that had multiple mutations across the whole experiment among the demet treated strains. Parallel mutations in the experiment could potentially mean that those genes were involved in adaptation. We performed separate tests for the three different environments, while pooling all mutations that occurred in clones from the same environment. For GO enrichment among the DMRs, we included DMRs overlapping genes that were polymorphic at least in one environment. Separate tests were performed for all environments.

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Tables

Table 1. Initial effects of the different environments on population growth rate of the ancestors. Comparisons are shown in percentages relative to ancestor in control environment.

Environment	Genotype	Difference to control (%)	±SE of difference (%)
High NaCl	CC-2937	-80	11
High NaCl	sir2	-45	11
High NaCl	cSIR2	-70	17
Low P	CC-2937	-27	12
Low P	sir2	-19	13
Low P	cSIR2	-35	12
High CO2	CC-2937	26	14
High CO2	sir2	60	13

High CO2	cSIR2	68	13

Table 2. Initial effects of the chemical treatments to the population growth rate of the ancestors in the four environments assayed. Comparisons are shown in percentages relative to the control chemical treatment in the appropriate environment and ancestor.

Environment	Genotype	Treatment	Difference to control (%)	±SE of difference (%)
Control	CC-2937	demet	9	10
Control	CC-2937	demet + acet	−40	14
Control	sir2	demet	−17	10
Control	sir2	demet + acet	−37	11
Control	cSIR2	demet	−32	17
Control	cSIR2	demet + acet	−36	13
High NaCl	CC-2937	demet	61	51
High NaCl	CC-2937	demet + acet	2	36
High NaCl	sir2	demet	−49	7
High NaCl	sir2	demet + acet	−8	10
High NaCl	cSIR2	demet	−41	49
High NaCl	cSIR2	demet + acet	15	51
Low P	CC-2937	demet	−7	10
Low P	CC-2937	demet + acet	0	9
Low P	sir2	demet	−7	14
Low P	sir2	demet + acet	−31	16
Low P	cSIR2	demet	27	20
Low P	cSIR2	demet + acet	−13	14
High CO2	CC-2937	demet	−5	9
High CO2	CC-2937	demet + acet	−25	9
High CO2	sir2	demet	−4	9
High CO2	sir2	demet + acet	0	7
High CO2	cSIR2	demet	−9	6
High CO2	cSIR2	demet + acet	−9	6

1200

Table 3. Summary table of epigenetic effects.

1202 The effect of reduced epigenetic transmission by chemical treatment on adaptation for strain CC-2937 was calculated as the ratio of growth rate of a population selected and assayed in the focal environment in a given

1204 chemical treatment relative to growth rate of a population selected and assayed in the same environment but in the control chemical treatment. Effect of the *sir2* mutation, which reduces the epigenetic mutation rate,

1206 was calculated as growth rate of the mutant relative to the growth rate of the complemented line.

Effect of reducing epigenetic contribution	Control environment	High NaCl	Low phosphate	High CO_2
Direct responses				
demet vs. control	0.83	1.04	0.97	0.99
demet + acet vs. control	0.83	0.76	0.92	0.98
sir2 mutation vs cSIR2	0.95	1.09	0.88	0.66

1208 Table 4. Genetic mutations and observed derived DMR changes in the evolved clones. All sequenced clones were of CC-2937 background.

Clone	Genetic mutations	DMRs	Environment	Chemical treatment
P1B3	8	143	Control	Control
P2B8	10	NA	Control	Control
P3G11	10	NA	Control	Control
P1D2	461	72	Control	Demet
P2D9	433	68	Control	Demet
P3B7	391	NA	Control	Demet
P4C5	8	77	High CO_2	Control
P5F6	3	88	High CO_2	Control
P6E8	5	97	High CO_2	Control
P4C7	642	61	High CO_2	Demet
P5B10	595	60	High CO_2	Demet
P6E2	475	99	High CO_2	Demet

P10C5	11	87	High NaCl	Control
P11B4	11	192	High NaCl	Control
P12E4	11	168	High NaCl	Control
P10C7	201	NA	High NaCl	Demet
P11B11	239	123	High NaCl	Demet
P12G10	162	NA	High NaCl	Demet

Table 5. Observed mutations in evolved clones for control and demethylation chemical treatments by functional categories. All sequenced clones were of CC-2937 background.

	Control chemical treatment			Demethylation chemical treatment		
Category	All	SNP	Indel	All	SNP	Indel
UTR	14	13	1	729	719	10
5' UTR	6	6	0	143	143	0
3' UTR	8	7	1	586	576	10
Intron	35	24	11	1016	992	24
Coding region	26	19	7	1526	1517	9
Non-synonymous	6	6	NA	1138	1138	NA
Synonymous	13	13	NA	379	379	NA
Frameshift	3	NA	3	4	NA	4
Inframe	4	NA	4	5	NA	5
Intergenic	2	1	1	303	296	7
rRNA	0	0	0	27	27	0
tRNA	0	0	0	2	2	0
Total	77	57	20	3594	3544	50

Table 6. Results of gene ontology (GO) enrichment test for genetic mutations for the demethylation treatment in the different environments. For each of the top ten most significant GO terms, shown are the number of genes that have been annotated this GO term, number of significantly enriched genes, expected number of genes and p-value for significant enrichment. All sequenced clones were of CC-2937 background.

	Control Environment				

GO ID	Term	Annotated	Significant	Expected	p-value
GO:0007018	Microtubule-based movement	75	11	1.19	1.8E-08
GO:0006928	Movement of cell or subcellular component	81	11	1.29	4.1E-08
GO:0009187	Cyclic nucleotide metabolic process	93	11	1.48	1.8E-07
GO:0009190	Cyclic nucleotide biosynthetic process	93	11	1.48	1.8E-07
GO:0007017	Microtubule-based process	127	11	2.02	4.1E-06
GO:0035556	Intracellular signal transduction	259	12	4.12	0.00071
GO:0042773	ATP synthesis coupled electron transport	12	3	0.19	0.00077
GO:0006119	Oxidative phosphorylation	13	3	0.21	0.00099
GO:0009165	Nucleotide biosynthetic process	232	11	3.69	0.00100
GO:1901293	Nucleoside phosphate biosynthetic process	233	11	3.71	0.00103
	High CO_2 environment				
GO:0009187	Cyclic nucleotide metabolic process	93	9	1.86	8.7E-05
GO:0009190	Cyclic nucleotide biosynthetic process	93	9	1.86	8.7E-05
GO:0006928	Movement of cell or subcellular component	81	8	1.62	0.00019
GO:0050982	Detection of mechanical stimulus	7	3	0.14	0.00026
GO:0009612	Response to mechanical stimulus	8	3	0.16	0.00041
GO:0070588	Calcium ion transmembrane transport	19	4	0.38	0.00047
GO:0035556	Intracellular signal transduction	259	14	5.19	0.00058
GO:0009581	Detection of external stimulus	9	3	0.18	0.0006
GO:0009582	Detection of abiotic stimulus	9	3	0.18	0.0006
GO:0007018	Microtubule-based movement	75	7	1.5	0.00068
	High NaCl environment				
GO:0055085	Transmembrane transport	411	11	3.87	0.0012
GO:0009187	Cyclic nucleotide metabolic process	93	5	0.88	0.0017
GO:0009190	Cyclic nucleotide biosynthetic process	93	5	0.88	0.0017
GO:0050982	Detection of mechanical stimulus	7	2	0.07	0.0018

GO:0009612	Response to mechanical stimulus	8	2	0.08	0.0024
GO:0009581	Detection of external stimulus	9	2	0.08	0.003
GO:0009582	Detection of abiotic stimulus	9	2	0.08	0.003
GO:0042773	ATP synthesis coupled electron transport	12	2	0.11	0.0054
GO:0006119	Oxidative phosphorylation	13	2	0.12	0.0064
GO:0022904	Respiratory electron transport chain	16	2	0.15	0.0096

Table 7. Results on how often DMRs among the evolved lines gained or lost methylation in different DMR frequency classes in the control and demet chemical treatments. All sequenced clones were of CC-2937 background.

DMRs in control treatment			
Frequency class	Gain	Loss	Frequency of loss
1	256	3	0.01
2	117	13	0.10
3	82	29	0.26
DMRs in demet treatment			
Frequency class	Gain	Loss	Frequency of loss
1	129	4	0.03
2	23	3	0.12
3	9	1	0.10
4	6	12	0.67
5	9	11	0.55
6	2	14	0.86

Table 8. Summary of the major findings.

	Prediction	Observation
High NaCl	Evolutionary adaptation to a stressful environment by increasing growth rate. Reducing epigenetic variation lowers adaptation	Evolution of higher growth rate. Demet and demet + acet treatments reduced adaptation. Sequenced strains from high NaCl clustered together based on their methylation changes. SIR2 mutation had no consistent effect.
Low P	Evolutionary adaptation to a stressful	Evolution of higher growth rates but

	environment by increasing growth rate. Reducing epigenetic variation lowers adaptation	no consistent effects of chemical treatments or the SIR2 mutation
High CO_2	Evolutionary adaptation by reducing growth rate as a result of losing the plastic response to high CO2. Reducing epigenetic variation lowers adaptation (increases growth rate)	Plastic response to high CO_2 was diminished or lost in most strains, in the demet + acet treatment this response was retained. The complemented SIR2 mutant increased its growth rate in contrast to other strains. Sequenced strains from high CO_2 clustered together with control strains based on their DNA methylation changes.
Chemical treatments	Reducing epigenetic variation reduces adaptation	Chemical treatments reduced adaptation. Multiple lines of evidence suggest that cytotoxic effects unlikely to have caused the observed effects of the chemicals.
Methylation changes	Most methylation changes are under genetic control.	More methylation changes than genetic mutations in the sequenced control treatment clones. Both shared methylation changes that suggest environmental induction and but many unique changes that suggest random methylation changes. Genetic mutations unlikely to have caused methylation changes.

Figures

Figure 1. A) Schematic representation of the selection experiment. Four different environments were used. In each environment, there three different chemical treatments applied to each of four strains. Each population was grown in nine replicates, resulting in a full factorial experiment with 4*3*4*9 = 432 populations in total. Populations were grown in 200 µl of media on 96-well plates and transferred every three and four days for approximately 200 generations. The chemical treatments were randomized among the columns of the plate and strains were randomized within columns, such that each strain occurred once in each column. After batch transfers for ~ 200 generations, fitness assays were performed for each population in the environment they evolved and in the control environment. Populations evolved in the control environment were measured in all other environments. Fitness assays were performed in a fully randomized design. B) Expected results of the experiment. Top row shows the expected effect of manipulating epigenetic variation (either chemically or genetically) during a adaptation. If epigenetic changes contribute to adaptation we expect treated populations in the high NaCl and low phosphate environments have lower relative growth rates than control treatment after the selection experiment. In the high CO_2 environment, based on previous studies, we expect that initial plastic response to increase growth rate in high CO_2 will diminish in the control treated

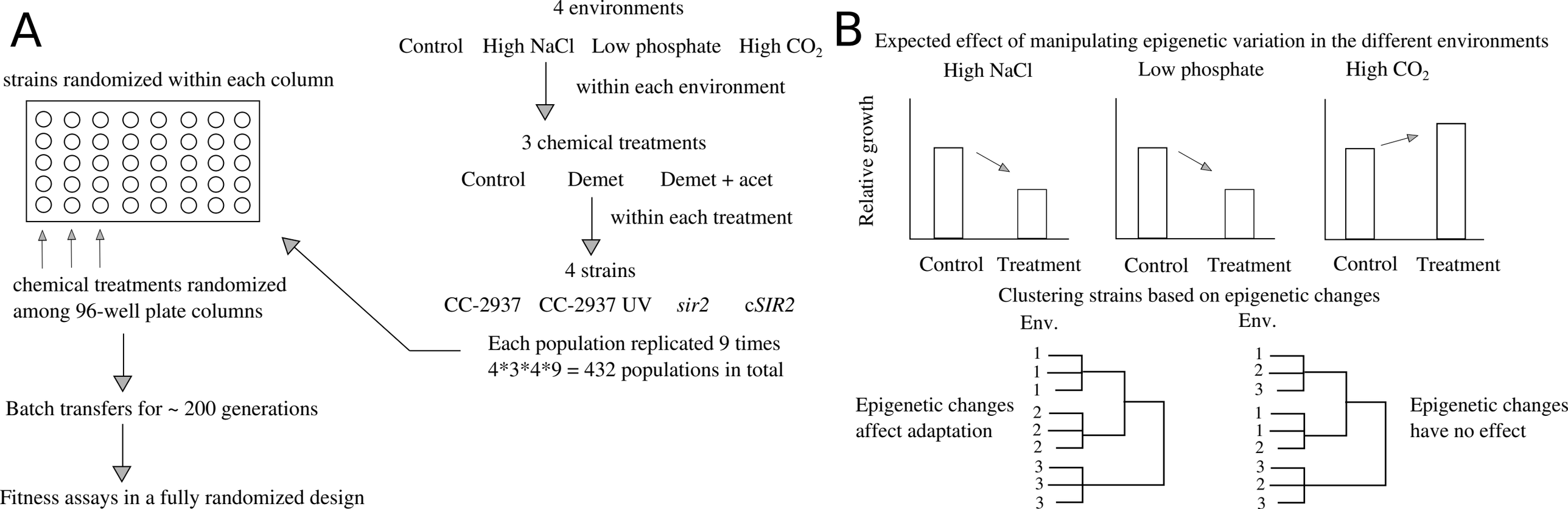
populations. If this response is due to epigenetic changes, then in the treated populations this response may remain, resulting in increased relative growth rate. Bottom row shows expected results after clustering the evolved strains based on epigenetic changes. If epigenetic changes contribute to adaptation strains should cluster by the environment but if not then clustering is expected to be random.

Figure 2. Direct responses to selection in the different environments. Relative growth rates were calculated by taking the growth rate of populations evolved in one of the three environments (high NaCl, low P, and high CO_2) measured in the environment they evolved in, over growth rate of corresponding population evolved in the control environment but measured in the novel environment. Error bars indicate ± SEM. Dashed line indicates relative growth rate of one. (A) Populations evolved in the high NaCl environment. (B) Populations evolved in the low phospate environment. (C) Populations evolved in the high CO_2 environment.

Figure 3. The effects of "knocking out" epigenetic transmission with the demet + acet chemical treatment in the ancestor and populations evolved in the control treatment. At the end of the experiment, we measured growth rates of populations that had the CC-2937 background and that had evolved in the control chemical treatment, both in the control and demet + acet chemical treatments. We calculated the effect of the demet + acet treatment as a contrast between these two growth measurements (growth in demet + acet treatment - growth in control treatment). The effect of the demet + acet chemical treatment is plotted on the horizontal axis. An effect of zero means that the demet + acet has no effect on growth, negative values indicate the the demet + acet treatment reduces growth relative to control and positive values indicate that growth is increased relative to control. If there have been no epigenetic changes during the experiment that contribute to phenotype, then the chemical treatment should have the same effect in the ancestor and the evolved populations. Populations are stacked on the vertical axis, point are estimates of the effects and error bars are one standard error. Facets show the different environments. The low phosphate environment was excluded from this experiment as most control treated populations had gone extinct. Vertical lines show the values for the ancestor.

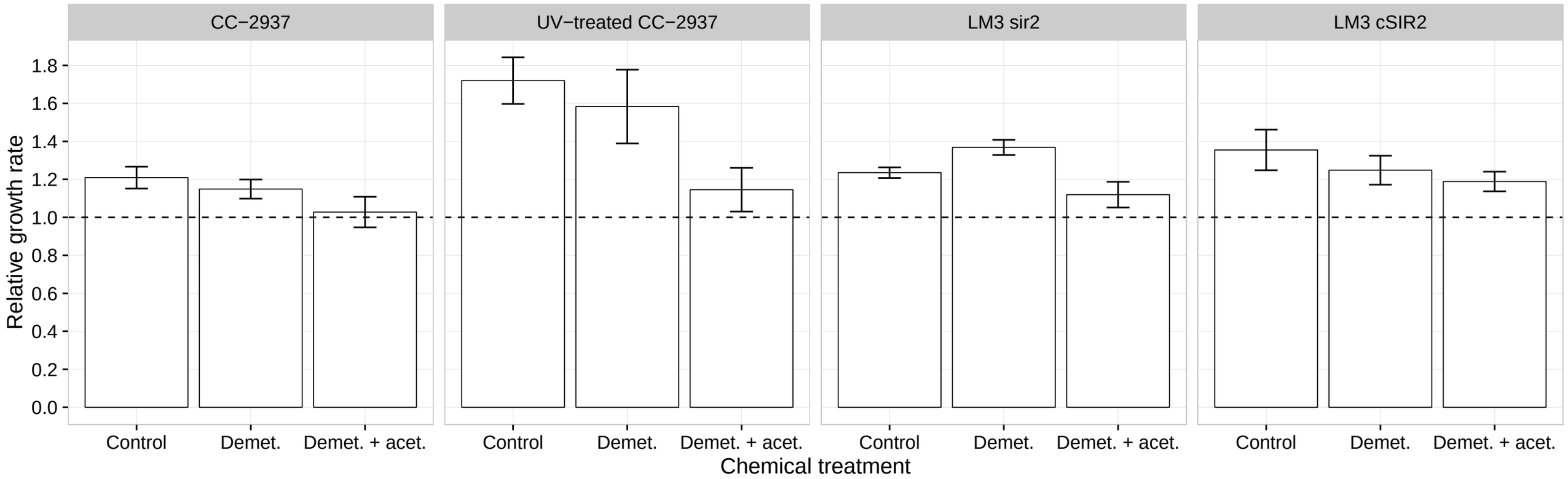
Figure 4. Clustering of clones and ancestors based on DMRs in CG context. In the heatmap DMRs are on rows and samples in columns. Black lines in the heatmap represent missing data. Numbers on sample dendrogram are bootstrap values. Coloured boxes above the heatmap show clone treatments.

Figure 5. Properties of DMRs among the evolved clones. (A) Frequency distribution of the derived DMRs among the evolved clones. Classes show the number of clones the DMRs are present out of 13 sequenced clones. (B) Venn diagram of DMR count overlaps among the evolved clones in different environments. (C) Distribution of annotations among the DMRs in the evolved clones and DMRs among the evolved clones and the ancestor. (D) Mean methylation frequencies of cytosines in within DMRs among the evolved clones that come from different chemical treatments and different sequence contexts.


A
4 environments
Control
High NaCl
Low phosphate
High CO2
within each environment
3 chemical treatments
Control
Demet
Demet + acet
within each treatment
4 strains
CC-2937
CC-2937 UV
sir2
cSIR2
Each population replicated 9 times
4*3*4*9 = 432 populations in total
strains randomized within each column
chemical treatments randomized among 96-well plate columns
Batch transfers for ~ 200 generations
Fitness assays in a fully randomized design
B
Expected effect of manipulating epigenetic variation in the different environments
High NaCl
Low phosphate
High CO2
Relative growth
Control
Treatment
Clustering strains based on epigenetic changes
Env.
Epigenetic changes affect adaptation
Epigenetic changes have no effect

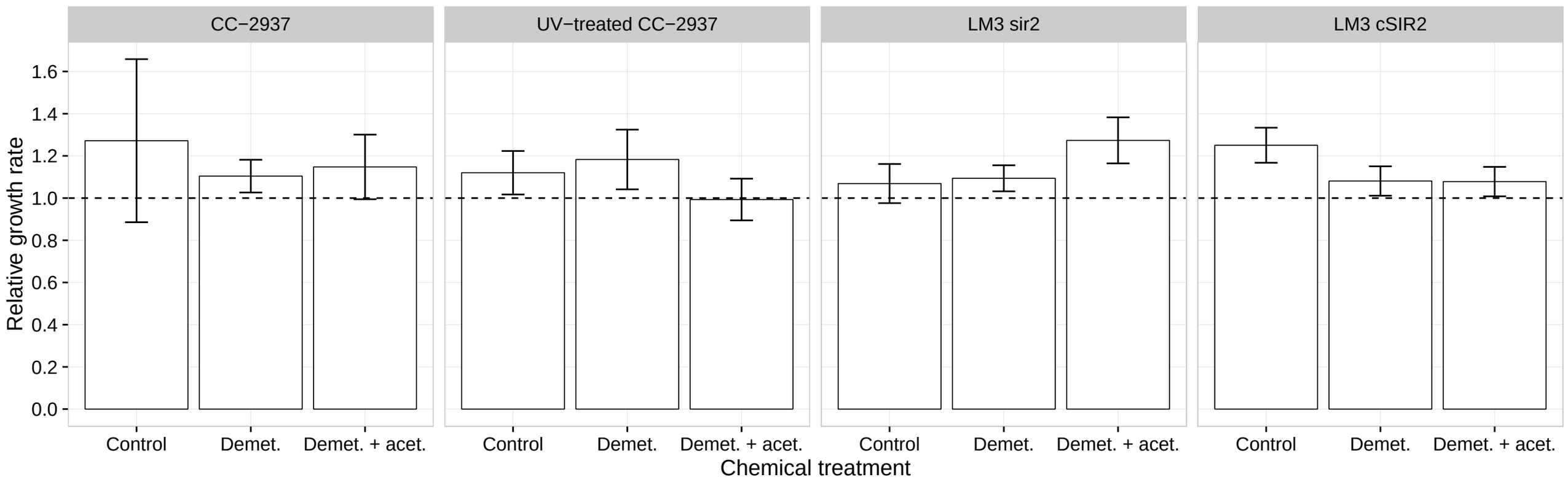
A

Direct response to selection in high NaCl



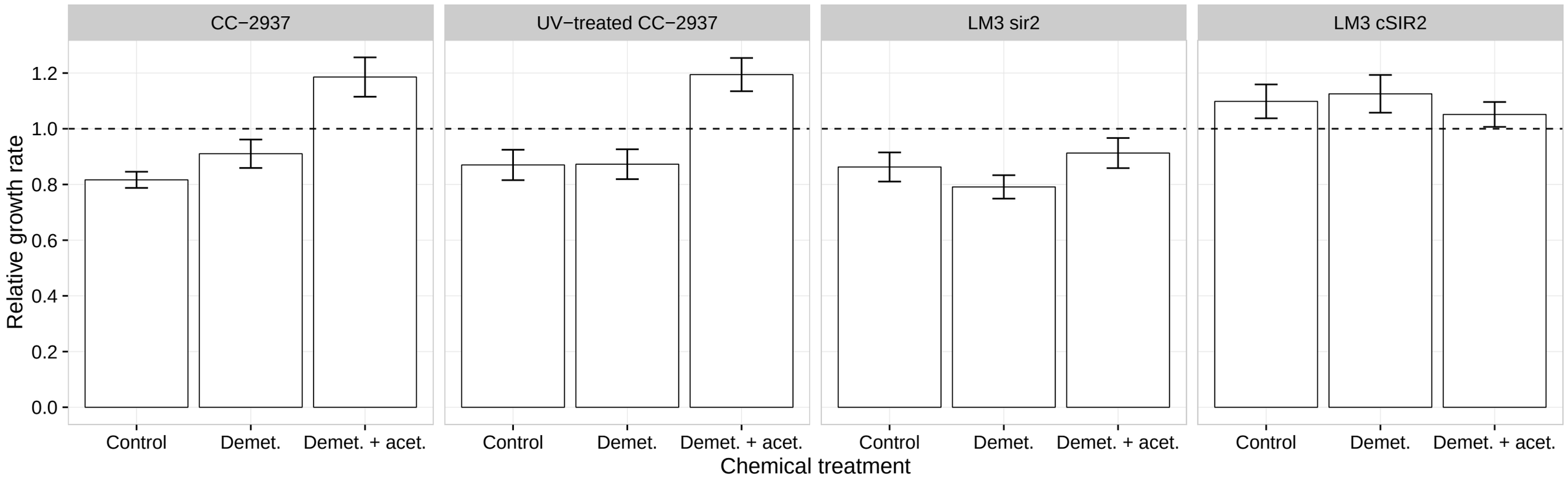
B

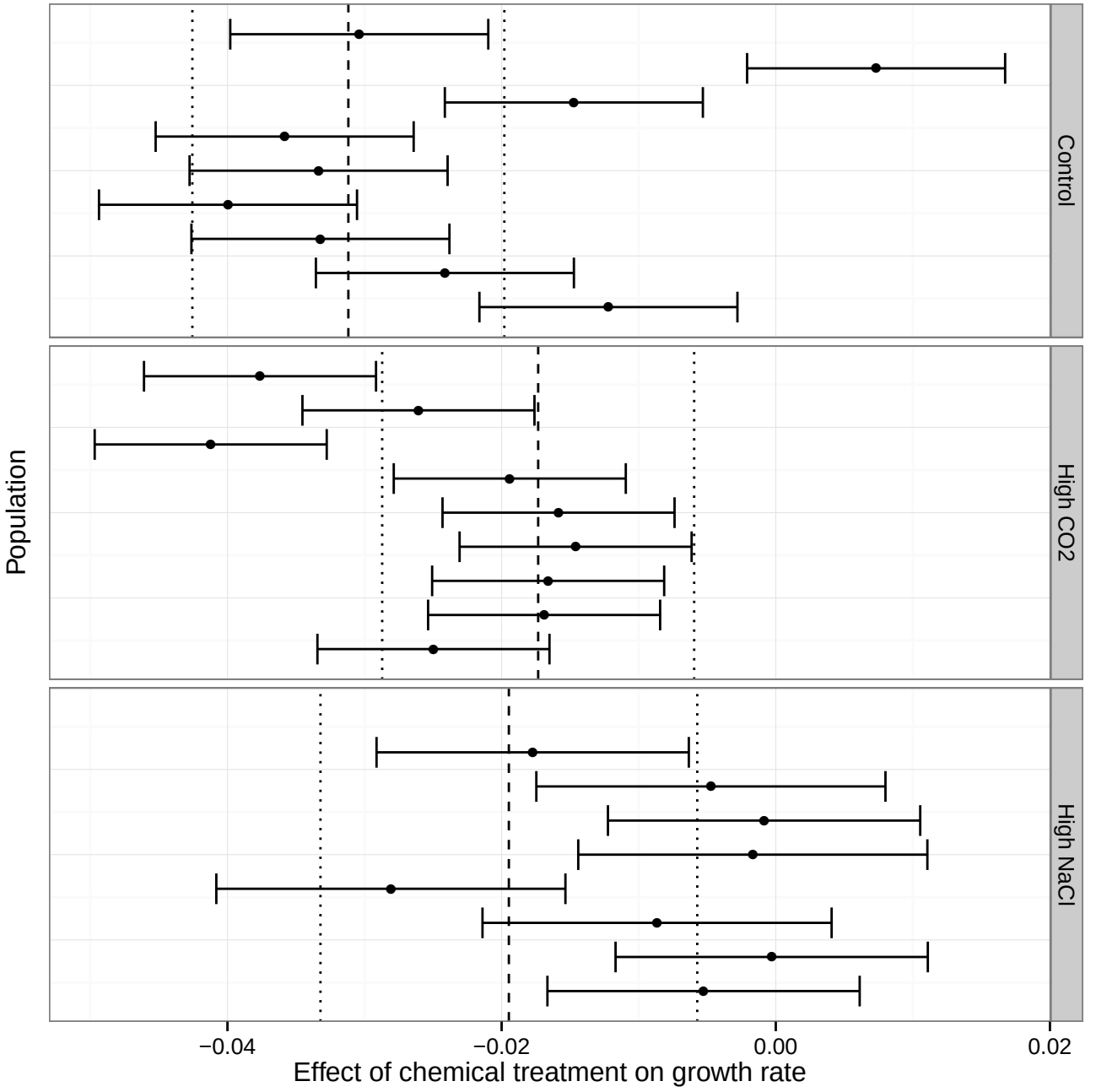
Direct response to selection in low P



C

Direct response to selection in high CO2




Control
High CO2
High NaCl
Population
−0.04
−0.02
0.00
0.02
Effect of chemical treatment on growth rate

Color Key

0 20 40 60 80

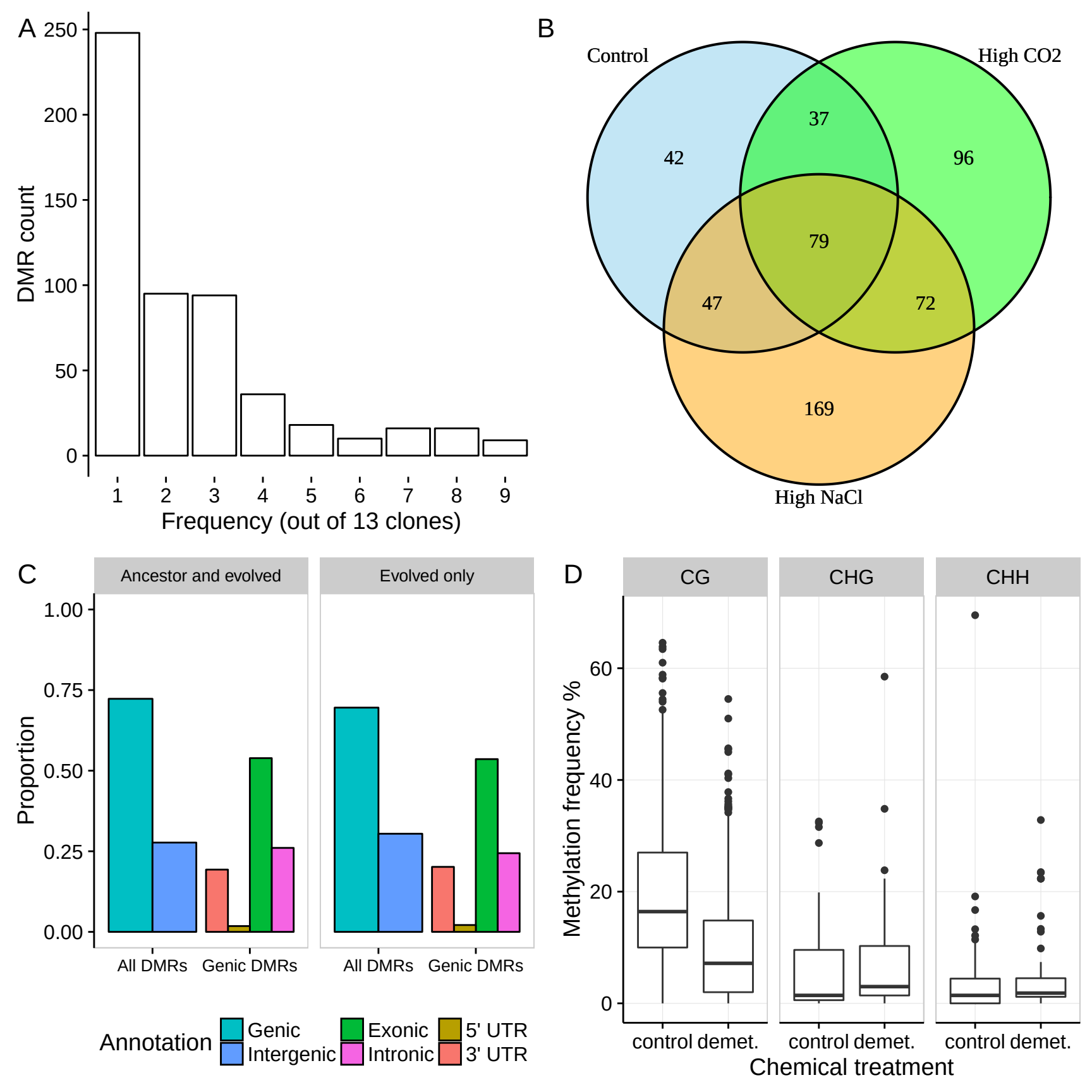
Methylation frequency (%)

100
100 91 87 89 90 100 100
83 88 88 56 100 63 96 97 70

Ancestor
Evolved
Control
High CO2
High NaCl
Control
Demet.

Treatment
Environment
History

P11B4
P11B11
P12E4
P1B3
P5F6
P6E8
P4C5
P10C5
P6E2
P5B10
P1D2
P4C7
P2D9
Demet
CC2937hco2
CC2937cont
CC2937hsalt
Control


A
DMR count
250
200
150
100
50
0
1
2
3
4
5
6
7
8
9
Frequency (out of 13 clones)
B
Control
High CO2
High NaCl
42
37
96
79
47
72
169
C
Ancestor and evolved
Evolved only
Proportion
1.00
0.75
0.50
0.25
0.00
All DMRs
Genic DMRs
All DMRs
Genic DMRs
Annotation
Genic
Intergenic
Exonic
Intronic
5' UTR
3' UTR
D
CG
CHG
CHH
Methylation frequency %
60
40
20
0
control
demet.
control
demet.
control
demet.
Chemical treatment