1   Fitness effects of new mutations in *Chlamydomonas reinhardtii* across two stress

2   gradients

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4   Running title: Fitness effects of new mutations under stress

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1 **Abstract**

2 Most spontaneous mutations affecting fitness are likely to be deleterious, but the strength of

3 selection acting on them might be impacted by environmental stress. Such stress-dependent

4 selection could expose hidden genetic variation, which in turn might increase the adaptive

5 potential of stressed populations. On the other hand, this variation might represent a genetic load

6 and thus lead to population extinction under stress. Previous studies to determine the link

7 between stress and mutational effects on fitness, however, have produced inconsistent results.

8 Here, we determined the net change in fitness in 29 genotypes of the green algae

9 *Chlamydomonas reinhardtii* that accumulated mutations in the near absence of selection for

10 approximately 1,000 generations across two stress gradients, increasing NaCl and decreasing

11 phosphate. We found mutational effects to be magnified under extremely stressful conditions,

12 but such effects were specific both to the type of stress as well as to the genetic background. The

13 detection of stress-dependent fitness effects of mutations depended on accurately scaling relative

14 fitness measures by generation times, thus offering an explanation for the inconsistencies among

15 previous studies.

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18 **Key words:** Chlamydomonas, Stress-dependent fitness effects, Mutation accumulation, Stress,

19 Green algae

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1 **Introduction**

2 Spontaneous mutation is a fundamental biological process that generates the abundant variation

3 we observe in nature. Understanding the fitness effects of new mutations is central, therefore, to

4 many problems in evolutionary biology. Whilst knowledge of both the rate and fitness effects of

5 spontaneous mutation has increased for several species (Drake et al., 1998; Lynch et al., 1999;

6 Eyre-Walker and Keightley, 2007), our understanding of the extent by which mutational effects

7 depend on the environment is limited. Environment-specific fitness effects of genotypes (GxE

8 interactions) are commonly observed for standing genetic variation in natural populations

9 (Kassen and Bell, 2000; Kraemer and Kassen, 2015). Such GxE interactions may be caused by

10 recently arisen mutations that have environment-specific fitness effects. Alternatively, GxE

11 interactions may be actively maintained by selection, for example in the form of local adaptation

12 to different environments (Belotte et al., 2003). Tests for selection acting on standing genetic

13 variation in natural populations are usually difficult to perform and thus the degree to which GxE

14 interactions are adaptive is largely unknown. A related idea is whether stress might release

15 cryptic genetic variation, which selection can subsequently act upon (Latta et al., 2015). Such a

16 release of variation might, for example, be caused by mutations with effects that are too small to

17 be selected under benign conditions, but effectively selected under stressful conditions (Lenski

18 and Remold, 2001; Kishony and Leibler, 2003). This phenomenon may manifest itself as an

19 increase in the expressed genetic variation among genotypes when tested in a stressful

20 environment compared to a more benign environmental control.

21 It is widely believed that the vast majority of new mutations that affect fitness are

22 deleterious (Keightley and Lynch, 2003; Loewe and Hill, 2010, but see Rutter et al., 2010) and it

23 has often been suggested that the deleterious effects of new mutations may be amplified in

24 stressful environments (Agrawal and Whitlock, 2010; Kondrashov and Houle, 1994; Korona,

25 1999; Martin and Lenormand, 2006). If this is the case, inference of the properties of new

26 mutations, typically made in relatively benign laboratory conditions, may provide a misleading

27 view of their fitness effects in natural populations, where conditions are generally assumed to be

28 more stressful. There is some empirical support for the existence of stronger fitness effects of

29 spontaneous mutations in stressful environments, with examples in *E. coli* (Remold and Lenski,

30 2001; Cooper et al., 2005), *S. cerevisae* (Szafraniec et al., 2001; Jasnos et al., 2008), *Drosophila*

31 (Kondrashov and Houle, 1994; Fry and Heinsohn, 2002; Wang et al., 2009; Young et al., 2009),

32 *C. elegans* (Vassilieva et al., 2000) and *C. briggsae* (Baer et al., 2006). However, the generality

1   of such observations has been brought into question by a number of studies, particularly in

2   microbial systems (Korona, 1999; Kishony and Leibler, 2003; Jasnos et al., 2008;), showing that

3   some forms of environmental stress can actually reduce the effects of deleterious mutations

4   (reviewed in Agrawal and Whitlock, 2010).

5   Previous studies of the phenotypic effects of mutations under stressful conditions have

6   typically examined the performance of genotypes in pairs of environments that are categorically

7   different, for example environments with or without a antibiotic (Kishony and Leibler, 2003), or

8   high *versus* low temperature (Szafraniec et al., 2001). The genetic basis of performance in such

9   environments may differ dramatically in ways that are unrelated to the severity of the stress, and

10 this may explain some of the inconsistency seen in previous studies. For example, suppose that

11 there are large differences in the number of genes expressed in different environments, but that

12 the number of genes expressed is uncorrelated with stress. If we compare performance in

13 arbitrary pairs of environments, then any differences in performance may simply reflect

14 differences in the size of the mutational target for fitness in these environments, rather than

15 effects of stress *per se*. Furthermore, extreme laboratory environments are unlikely to represent

16 conditions generally encountered by natural populations. Rather, changing conditions are likely

17 to occur due to environmental deterioration across a gradient, stretching from benign, to slightly

18 stressful, to detrimental and finally lethal. Moreover, different stressors might elicit different

19 responses, depending on which aspect of the organism's physiology they act on.

20   Here, we utilize lines derived in the absence of (most) selection during the course of a

21 mutation accumulation (MA) experiment (Morgan et al., 2014). Specifically, we study 29

22 spontaneous MA lines derived from two genetically distinct strains of the unicellular green algae

23 *Chlamydomonas reinhardtii*, along with the ancestral strains they were derived from, to

24 investigate change in the fitness effects of *de novo* mutations as two environmental stressors are

25 increased along a continuum. *Chlamydomonas reinhardtii* shares characteristics with both

26 microbial as well as multicellular species. For example, its generation time and effective

27 population size are close to those of *E. coli* and *S. cerevisiae* (Ness et al., 2012), whereas its

28 genome size and structure are comparable to multicellular eukaryotes. In this study, we use

29 genotypes generated by ca. 1,000 generations of mutation accumulation to address the following

30 questions: 1) Are mutational effects ameliorated or exaggerated under stressful conditions? 2)

31 Can we observe GxE interactions in genotypes derived from a mutation accumulation regime? 3)

32 Does increasing stress lead to a release of cryptic variation?

1 We found that severe stress can amplify the effects of new mutations, but such condition

2 dependence was impacted both by the type of stress as well as the genetic background. The

3 detection of such subtle effects depended on accurate scaling of fitness by ancestral generation

4 times, thus providing a partial explanation for the inconsistency among results from previous

5 studies.

6

Materials and Methods

8 Mutation accumulation lines and ancestral strains

9 We studied MA lines and their ancestors derived from two genetically distinct laboratory

10 strains of *C. reinhardtii*. Full details of the production of the MA lines are provided elsewhere

11 (Morgan et al., 2014). There were 15 and 14 MA lines from ancestral strains CC-2344 and CC-

12 2931, respectively. Briefly, the MA lines were generated under conditions where the

13 effectiveness of natural selection is reduced by bottlenecking to a single cell at regular intervals

14 (approximately every 12 divisions) for ~1,000 generations. Full genome sequencing showed that

15 the MA lines have accumulated substantial numbers of new mutations (Ness et al. 2015). They

16 typically show reduced growth rate compared to their ancestors when assayed in a benign

17 laboratory environment (Morgan et al., 2014). The ancestors and their descendant MA lines had

18 all been cryopreserved in liquid nitrogen prior to this study.

19

20 Reconditioning of MA lines prior to the fitness assays

21 Prior to the fitness assays, we allowed cryopreserved cultures of all strains to thaw in a

22 water bath at 35°C for 5 minutes. We transferred the cultures to 30ml universal tubes containing

23 10ml of Bold's liquid media (Bold, 1942) and incubated them in standard growth conditions

24 (25°C under constant white light illumination) for 7 days. We then transferred 100µl of each MA

25 line culture into a new universal tube containing 5ml of Bold's media. At the same time, we

26 created replicates of the two ancestors by inoculating 15 samples of each into separate tubes of

27 media. These cultures were incubated under standard growth conditions for four days. Finally,

28 we transferred 2µl of each culture to individual wells containing 200µl of Bold's media on a 96

29 well plate. Each MA line was randomly paired with one of the replicate cultures of the

30 corresponding ancestor, and these two cultures were allocated at random to adjacent wells. In

1   order to avoid edge effects (Morgan et al., 2014), we only used the central 60 wells of each plate,

2   and filled the outer wells with Bold's media to maintain humidity and to reduce evaporation

3   from the central wells. We repeated this procedure three times, to create three independent

4   conditioning plates with a different random allocation of genotypes on each plate. Subsequently,

5   we incubated these plates for three days prior to the fitness assays.

6

7   <u>Manipulating environmental stress</u>

8   We created two environmental stress gradients by modifying the concentrations of

9   components of standard Bold's media that are known to affect the performance of *C. reinhardtii*:

10   NaCl and phosphate (Bell, 1992; Goho and Bell, 2000; Moser and Bell, 2011). For the phosphate

11   gradient, we altered the concentration of the dipotassium phosphate (K_2HPO_4) and the

12   monopotassium phosphate (KH_2PO_4) components so that the media contained 400% (0.6 g/L),

13   200% (0.3 g/L), 100% (0.15 g/L), 50% (0.075 g/L), 25% (0.038 g/L), 10% (0.015 g/L), and 5%

14   (0.0075 g/L) of the concentration of phosphate of standard Bold's media (0.15 g/L). For the NaCl

15   gradient, we supplemented standard liquid Bold's medium with between 0 and $6gl^{-1}$ of NaCl in

16   $1gl^{-1}$ increments. Higher concentrations of NaCl investigated in pilot studies largely failed to

17   support growth. Natural genotypes of *C. reinhardtii* vary in their performance over these ranges,

18   and growth is significantly reduced in the most stressful environments we investigated (the

19   lowest phosphate or the highest NaCl).

20   Before measuring the fitness of the lines in the different assay conditions we first allowed

21   them to acclimatize in order to ensure that we measured fitness, rather than differences in

22   plasticity in response to a new environment. We set up three replicate 96 well plates per

23   environmental condition (i.e., level of phosphate or NaCl) by adding 200µl of liquid media. We

24   initiated the fitness assays by adding 2µl of each culture from one conditioning plate. A different

25   conditioning plate was used to initiate each replicate plate for a given environment. These plates

26   were incubated under standard conditions for four days to allow the lines to acclimatize to the

27   assay conditions.

28   After acclimatizing the lines, we estimated the maximum growth rates of the MA lines

29   and their ancestors in 96 well plates containing the appropriate assay media. 2µl of each culture

30   was then transferred to the corresponding well of a second 96 well plate containing the same

31   growth media. This maintained the randomization of the lines across the 96 well plates and

32   pairing with their ancestors for the assay. These plates were incubated for four further days under

1   standard growth conditions, and the absorbance of each well at 650nm was measured three times

2   each day to provide estimates of culture density. Absorbance values below 0.01 do not provide

3   reliable estimates of cell density, and this places a lower threshold on the culture density that we

4   can estimate using this method. However, this threshold corresponds to a cell density of about 1

5   x 10^5 cells per ml, which is well below the cell density at which exponential growth ceases

6   (approximately 1 x 10^7 cells per ml), and so this method allows us to estimate the maximum

7   growth rate of cultures once they pass this detection threshold.

8

9   The maximum growth rate of each culture was calculated as

10

11    $(\ln(\text{absorbance}_2) - \ln(\text{absorbance}_1))/(t_2 - t_1),$ (1)

12

13   where t_1 and t_2 are the times in hours of the first two assays, at which an absorbance above 0.01

14   detection threshold of the plate reader was measured for a culture, which corresponds to the

15   period of fastest growth under these assay conditions. absorbance_1 and absorbance_2 are the

16   absorbance values at these two time point, respectively. Since cultures in different environmental

17   conditions differed in their time to reach the absorbance threshold, the actual values of t_1 and t_2

18   differed for different cultures. Cultures that failed to grow were assigned a growth rate of 0.

19   For logistical reasons, it was not possible to assay both stress gradients concurrently.

20   Instead, we first assayed all MA lines across the NaCl gradient, and carried out the assays for the

21   phosphate gradient two weeks later.

22

23   <u>Statistical analyses</u>

24   Data sets were analyzed separately for each stressor. To examine effects of mutation

25   accumulation and environmental stress on growth rate, we fitted linear mixed models using the

26   packages lme4 (Magezi, 2015) nlme in R (R Development Core Team, 2009). *p*-values were

27   assigned using the Satterthwaite-approximation, based on SAS Proc mixed theory (Schaalje et

28   al., 2002), as implemented in the lmerTest package in R.

29   Specifically, we investigated the relationship between relative fitness of MA genotypes

30   and the level of environmental stress by fitting linear mixed models accounting for random

31   effects due to plates and ancestry. We based calculations of relative fitness on the average

32   growth of all pseudoreplicates of the ancestor per plate rather than using the individual values of

7

1 each paired ancestor, because some ancestral samples failed to grow. All model coefficients can

2 be found in the supplemental methods file.

3   Genotype-by-environment interactions for all genotypes between each possible pair of

4 test environments were further investigated by partitioning GxE into two components: the

5 genetic correlation of growth and the variation of the genetic standard deviation expressed by

6 genotypes between the two environments (Robertson, 1959; Kassen and Bell, 2000; Kraemer

7 and Kassen, 2015; Barrett et al., 2005). The genetic correlation of growth describes

8 the GxE component that determines the consistency of performance of genotypes across

9 environments. For example, if test environments are highly similar, the rank order positions of

10 fitness of genotypes in the two environments are expected to be similar, leading to a high genetic

11 correlation. Very dissimilar environments, on the other hand, are likely to elicit different fitness

12 responses such that the rank order of fitness of genotypes changes substantially from one

13 environment to another, leading to a low genetic correlation of growth. The second component

14 of GxE interactions describes how the variance in performance among genotypes changes

15 between the two environments and will be generated if more variation is expressed in one

16 environment than the other.

17

18 Total GxE interactions are described by

19

20 GxE = (1- genetic correlation of growth) + variation of the genetic standard deviation,   (2)

21

22 and formally by the Robertson equation (Robertson, 1959):

23

24 $\sigma^2 GE = \sigma_{G1} * \sigma_{G2} * (1 - \rho_{G1G2}) + \frac{1}{2} * (\sigma_{G1} - \sigma_{G2})^2,$ (3)

25

26 where σ_{G1} and σ_{G2} are the genetic standard deviations of the fitness of all genotypes grown in

27 environment 1 and 2 specifically and ρ_{G1G2} is the genetic correlation of growth between the two

28 environments. Thus, overall GxE, as well as the variation of the genetic standard deviation, are

29 expected to increase with increasing environmental dissimilarity, while the genetic correlation of

30 growth is expected to decrease. A schematic showing GxE variation due to either the genetic

8

1   correlation of growth or due to the variation of the genetic standard deviation is shown in Supp.

2   Fig. 1.

3   Since GxE measures in these correlations are the results of comparisons of pairs of

4   environments and thus not necessarily independent data points, we assessed the significance of

5   GxE patterns across environments using a permutation test in which, instead of assuming a

6   normal distribution of residuals, we generated a distribution based on the data. Test results were

7   subsequently compared to this distribution to assess significance. Specifically, we generated GxE

8   component estimates for each pair of environments based on the Robertson equation with the

9   asreml package in R and regressed these on environmental difference (the difference in either

10   NaCl or Phosphate concentration between the two focal environments). We then generated

11   10,000 permuted data sets by randomly assigning the environmental difference scores to the

12   pairwise component estimates without replacement and repeated the regression analysis on each.

13   We subsequently utilized the resulting distribution regression statistics as a null-hypothesis to

14   test for significant effects of total GxE, genetic correlation of growth and variation of the genetic

15   standard deviation with respect to differences between environments

16   The expressed genetic variation was calculated based on the relative fitness values of MA

17   genotypes under each stress level using the lme4 package in R (Magezi, 2015). Confidence

18   intervals around values of expressed genetic variation were calculated assuming a chi-squared

19   distribution.

20

Results

22   <u>1. Overall effect of the environments on growth rate</u>

23   The growth rates of the MA lines and their ancestors were significantly reduced by

24   decreasing phosphate and increasing NaCl concentrations (linear mixed models, effect of stress

25   treatment on growth rate, all $p < 0.001$, Table 1, Figure 1). Across the gradient of decreasing

26   phosphate concentration, growth was reduced by 65%, while increasing NaCl concentration

27   reduced growth by 84%. Although the MA lines grew on average 20% more slowly than their

28   ancestors across all conditions, we found no evidence that the stress treatments impacted MA

29   line growth rates differently compared to paired ancestral pseudoreplicates (linear mixed models,

30   interaction between type (MA or ancestor) and stress level, all $p > 0.05$, Table 1, Figure 1). Thus,

1   the magnitude of the average reduction in maximum growth rate due to accumulated

2   spontaneous mutations appears to be unaffected by stress.

3

4   <u>2. Does environmental stress affect relative fitness?</u>

5   The evolutionary dynamics of a new mutation is not determined by its effect on growth

6   rate *per se*, but instead by its effect on relative fitness compared to its ancestral genotype under

7   the same environmental conditions (Chevin, 2011). Here, we focus on the growth rate of MA

8   lines, relative to the growth rate of their ancestors, as a proxy for relative fitness (1 - selection

9   coefficient [*s*]) of each MA line genotype under a given stress level. For a specific MA line

10   genotype, we first calculated ΔG = its growth rate - average growth rate of all its ancestral

11   pseudoreplicates on the same plate. Since both growth rates are measured on a log scale, this

12   provides a measure of relative fitness in units of time. However, in evolutionary biology we are

13   generally interested in relative fitness per generation. This distinction becomes particularly

14   important in studies such as this where fitness is compared among environments where the

15   generation time varies. To obtain a measure of relative fitness per generation (*s*), we then scaled

16   ΔG by the ancestral generation time, measured under the same conditions, by multiplying it with

17   ln(2) divided by the average ancestral growth rate on the same plate (Chevin, 2011). This

18   measure of relative fitness should be directly comparable across our selection environments as

19   well as providing estimates that can be compared to other organisms with different generation

20   times. In contrast to results obtained using raw growth rates, we found that stress exacerbated the

21   effects of mutations: we detected a weak but significant effect of increasing NaCl stress on

22   relative fitness (linear mixed model, effect of stress level on relative fitness of all MA genotypes

23   nested within their respective ancestor, $p < 0.01$, Table 2, Fig. 2).

24   Although MA line genotypes derived from one of the ancestors, CC-2344, also showed a

25   slight decrease in relative fitness under the highest phosphate stress, we did not detect a

26   significant effect of stress level on relative fitness under this stress regime (linear mixed model,

27   effect of stress level on relative fitness of all MA genotypes derived from both ancestors, all $p =$

28   0.15 and $p = 0.55$ respectively, Table 2, Fig. 2).

29   MA genotypes under NaCl stress showed a significant interaction between stress levels

30   and genotype (Table 2, $p < 0.05$), indicating that MA lines respond differently to NaCl stress. We

1   further investigated this GxE interaction between the individual MA genotypes and stress level

2   by separately analyzing data from the MA line genotypes derived from the two ancestors. Only

3   MA genotypes derived from ancestor CC-2344 assayed under NaCl stress exhibited a significant

4   genotype-by-environment interaction (linear mixed models, interaction of stress level with MA

5   line genotype, $p < 0.05$, all other interactions $p > 0.05$, Table 3). However, we did not detect any

6   such effects for the two MA lines under phosphate stress (linear mixed models, interaction of

7   stress level with each MA line genotype, $p > 0.05$, Table 3). We also performed all analyses

8   based on relative fitness unscaled by ancestral generation time (1-s). Importantly, we did not

9   detect any effect of stress level on fitness, nor any significant GxE interactions between genotype

10   and stress level (Supp. Fig. 2, all $p > 0.05$).

11

12   3. How do GxE interactions change between environments with different stress levels?

13   To investigate how GxE interactions vary between environments, they can be partitioned

14   into their components, genetic correlation of growth and the variation of the genetic standard

15   deviation, according to the Robertson equation (Robertson, 1959). To investigate how GxE

16   might be correlated with increasing stress levels, these components can be plotted against

17   measures of environmental dissimilarity between each pair of environments tested, for example

18   against the difference in NaCl or phosphate concentrations. Overall GxE interactions and the

19   variation of the genetic standard deviations are expected to increase with increasing differences

20   between environments, while the genetic correlation of growth, or how consistent MA genotypes

21   behave across environments, is expected to decrease. Here, we focus on MA genotypes derived

22   from ancestor CC-2344 under NaCl stress, which showed significant GxE interactions (Table 3,

23   Fig. 3). We do not see a clear pattern with increasing differences between environments for

24   neither total GxE interactions nor the genetic correlation of growth. The variation of the genetic

25   standard deviation, however, increases significantly with increasing environmental differences

26   (Permutation tests (N= 10,000) of GxE ($p > 0.05$), genetic correlation of growth ($p > 0.05$) and

27   variation of the genetic standard deviation ($p < 0.01$) against differences in NaCl concentrations,

28   Fig. 3).

29

30   4. Does genetic variance for relative fitness depend on the environment?

31   Whilst an examination of GxE interactions provides some information about changes in

32   genetic variance across environments, we can also calculate the level of genetic variance among

1   MA genotypes within each environment directly. Regardless of stressor and the ancestor of the

2   MA genotypes, we did not observe any systematic change in the level of expressed genetic

3   variation as environmental stress increased (Fig. 4, linear mixed model, effect of stress level onto

4   expressed genetic variation: $p = 0.36$, effect of stress level onto the variation of the expressed

5   genetic variation: $p = 0.35$). Likewise, there was no significant impact of stress level on

6   evolvability (the variance scaled by the mean, $p = 0.85$).

7

8   **Discussion**

9   Assessing the effect of mutations on fitness across gradients of environmental stress

10   provides a more complete picture of the consequences of new mutations relevant to natural

11   populations than an experiment done in a single environment. Here, we assayed fitness responses

12   of MA lines carrying significant mutational load (Ness et al., 2012; Morgan et al., 2014) across

13   two distinct stress gradients, increasing NaCl concentration and decreasing phosphate

14   concentration. In agreement with previous work (Bell, 1992; Goho and Bell, 2000; Moser and

15   Bell, 2011), both gradients significantly and substantially reduce the fitness of both the MA lines

16   and their ancestors.

17   Previous studies of mutational effects under deteriorating environmental conditions have

18   often assessed fitness based either directly on maximum growth rates (Fudala and Korona, 2009;

19   Kishony and Leibler, 2003; Szafraniec et al., 2001) or on Malthusian growth parameters per unit

20   of time (Cooper et al., 2005; Jasnos et al., 2008; Remold and Lenski, 2001). However, such time-

21   based measures of fitness do not take into account the likely impact of stressful conditions on

22   generation time (Chevin, 2011). Thus, to avoid overestimation of per-generation selection

23   strength, relative fitness should be scaled by the ancestral generation time under identical

24   conditions.

25   The results of our study underscore the importance of this distinction. Basing our

26   analyses on growth rates alone, we found no evidence that *de novo* mutations changed growth

27   rates across gradients of the two stressors, as evident by a lack of a significant interaction

28   between the decrease in growth rate and whether or not genotypes had accumulated mutations.

29   Similarly, analyses based on Malthusian fitness parameters showed no effects of stress level

30   treatments on relative fitness. However, relative fitness corrected by ancestral generation times

31   under the same conditions was impacted by environmental deterioration along one of the two

32   stress gradients we investigated. In other words, the increase in generation time caused by

12

1   stress means that the relatively uniform effects of mutations on growth rates seen across the

2   gradient translates into a greater reduction in absolute fitness per generation since generation

3   time is longer in the more stressful environments. This in turn implies that we would see a

4   greater change of gene frequencies between generations in stressful environments. Specifically,

5   we observed a significant decrease in relative fitness in the highest NaCl concentration

6   investigated. This environment causes an on average 84% decrease in growth rate and is close to

7   the lethal limit for most MA lines. While there is some suggestion that high phosphate stress

8   causes a decrease in relative fitness in MA line genotypes derived from one of the two ancestors,

9   this effect was not significant. The highest phosphate stress caused a smaller fitness decrease in

10   the ancestors (65%) than our high NaCl stress (84%), so this difference may simply reflect a

11   difference in the severity of stress that our gradients imposed. A systematic comparison of

12   mutational effects and severity of the stressful environment remains to be undertaken to

13   disentangle these factors. Overall, these results suggest that changing environmental conditions

14   can exacerbate mutational effects, but that this effect is not necessarily linear and only detectable

15   under near-lethal circumstance. Moreover, in a broader sense, this result may help to explain the

16   inconsistency of results obtained in some previous studies, which only investigated mutational

17   effects under one control and one stressful environment (Baer et al., 2006; Kishony and Leibler,

18   2003; Remold and Lenski, 2001).

19   We also tested whether increased environmental stress affected the expressed genetic

20   variation both between and within environments. Only MA genotypes derived from one of the

21   two ancestors showed significant GxE interactions, and only under NaCl stress. When we

22   partitioned these interactions into their components, they seemed to be mainly driven by an

23   increase of the variation of the genetic standard deviation of genotype fitness across increasingly

24   different NaCl concentrations. However, all other MA genotypes and stressors investigated did

25   not show any significant genotype-by-environment interactions. Likewise, we saw no systematic

26   change of the expressed genetic variation within environments across both stress gradients. Both

27   these findings are consistent with previous results (Latta et al., 2015), in which only a minority

28   of test strains and environments yielded significant GxE interactions. Our results are in part

29   consistent with theoretical expectations formulated by Martin and Lenormand (Martin and

30   Lenormand, 2006), who found that stress does not impact the average mutational effect (though

31   it might very well interact with the effects of individual mutations). In contrast to their findings

32   we do not detect a significant effect of stress on the expressed genetic variation. It is noteworthy,

1 however, that lines and stress conditions that had the strongest effect on relative fitness in our

2 experiment also show an increase of expressed genetic variation under deteriorating conditions.

3 Thus, changes in expressed genetic variation under environmental deterioration might be both

4 depending on the genetic background as well as the exact nature of the stress. We find our results

5 of limited effects of non-density dependent stressors on selection to be in accordance with

6 expectations formulated by Agrawal and Whitlock (2010). However, we did not contrast density

7 and non-density dependent stressors explicitly here.

8   Although mutations mostly had a constant effect across stress levels, fitness was affected

9 both by the type of stress and the ancestor from which a MA genotype was derived. Thus,

10 accurately predicting fitness responses based on the number of mutations might require a

11 mechanistic understanding of the gene in which a given mutation resides. For example,

12 mutational effects might be amplified if the gene carrying them is part of the core genome,

13 whereas the effects of mutations in condition-dependent genes will be harder to detect, and

14 depend on the assay conditions. Likewise, effects of mutations in highly connected genes may be

15 impacted by the gene's epistatic interactions (Draghi and Whitlock, 2015). Moreover, the effect

16 of a mutation might depend on the genetic background in which the mutation occurs. For

17 example, if the ancestral genotype were close to a fitness peak in its native environment, most

18 mutations are expected to be deleterious (Fisher, 1958), therefore, measuring fitness in a novel

19 stressful environment where the ancestor is not well adapted will alter our interpretation of a

20 mutation's effect on fitness. Lastly, different stresses will impact the physiological state of the

21 cell differently and thus might have different interactions with the mutations present.

22   In summary, these results suggest that new mutations obtained by mutation accumulation

23 may have environment-specific effects on relative fitness. The implications of this are two fold.

24 First, a stress-mediated release of cryptic genetic variation is possible, especially under highly

25 detrimental conditions. Such a release may, on the one hand, increase adaptiveness of a

26 population under stress by increasing variation between individuals. On the other hand, however,

27 it may represent a genetic load that might decrease the fitness of a stressed population below a

28 recovery threshold. Secondly, interactions between genotypes and test environments were both

29 ancestor- and stress-specific, thus making generalizations about the adaptive nature of GxE

30 interactions difficult. The environments we used have shown GxE interaction amongst natural

31 isolates of *C. reinhardtii* (Bell, 1992; Goho and Bell, 2000; Moser and Bell, 2011) but, overall,

32 we found GxE interactions in our study to be of limited importance. This provides a hint at the

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1   importance of selection in maintaining GxE in natural populations: mutations responsible for

2   much of the GxE interactions seen in genotypes isolated from nature seem to be actively

3   maintained by selection, rather than due to the accumulation of conditionally deleterious

4   mutations that are neutral in the environment that they arise in (except for highly detrimental

5   conditions). More broadly, it implies that our understanding of the distribution of mutational

6   effects gained from laboratory studies (Kassen and Bataillon, 2006; Latta et al., 2015; Sanjuan et

7   al., 2004; Wloch et al., 2001; Zeyl and DeVisser, 2001) may be representative of the situation in

8   more natural conditions.

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Table 1: Linear mixed model analyses of maximum growth rate.

Genotype Stressor	Model	Random effect	DF	N		F-value	*p*-value
CC-2344 Phosphate	Growth = Stress level x Type (MA/Ancestor)	Plate	14	630	Stress level Type Stress level x Type	17.78 33.29 0.4	< 0.0001 < 0.0001 0.876
CC-2931 Phosphate	Growth = Stress level x Type (MA/Ancestor)	Plate	14	588	Stress level Type Stress level x Type	26.31 29.08 1.03	< 0.0001 < 0.0001 0.405
CC-2344 NaCl	Growth = Stress level x Type (MA/Ancestor)	Plate	14	630	Stress level Type Stress level x Type	11.85 31.58 1.61	< 0.0001 < 0.0001 0.142
CC-2931 NaCl	Growth = Stress level x Type (MA/Ancestor)	Plate	14	588	Stress level Type Stress level x Type	43.17 96.86 1.49	< 0.0001 < 0.0001 0.179

Table 2: Linear mixed model analyses of relative fitness of all MA line genotypes under either phosphate or NaCl stress.

Data set	Model	Random effects	DF	N		F-values	*p*-value
All Phosphate	Relative fitness = Stress level x Genotype	Plate Ancestor	104	609	Stress level	0.91	0.487
					Genotype	2.98	< 0.001
					Stress level x Genotype	0.78	0.923
All NaCl	Relative fitness = Stress level x Genotype	Plate Ancestor	104	609	Stress level	5.21	0.005
					Genotype	6.89	< 0.001
					Stress level x Genotype	1.41	0.016

Table 3: Linear mixed model analyses of relative fitness for data sets partitioned by stressor and ancestor.

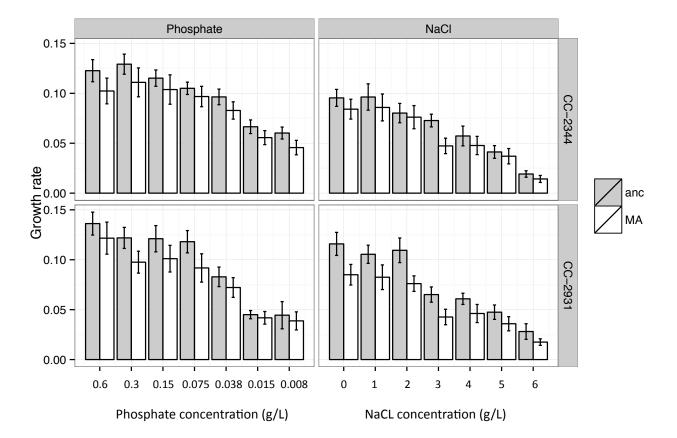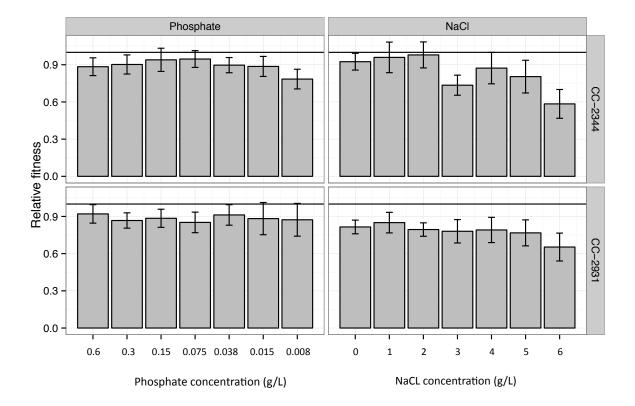| Data set	Model	Random effect	DF	N		F-value	*p*-value
2344 Phosphate	Relative fitness = Stress level x Genotype	Plate	14	315	Stress level	1.11	0.403
					Genotype	4.14	< 0.001
					Stress level x Genotype	1.21	0.147
2931 Phosphate	Relative fitness = Stress level x Genotype	Plate	14	294	Stress level	0.28	0.936
					Genotype	2.18	0.012
					Stress level x Genotype	0.97	0.547
2344 NaCl	Relative fitness = Stress level x Genotype	Plate	14	315	Stress level	6.71	0.002
					Genotype	4.64	< 0.001
					Stress level x Genotype	1.60	0.004
2931 NaCl	Relative fitness = Stress level x Genotype	Plate	14	294	Stress level	0.76	0.614
					Genotype	6.91	< 0.001
					Stress level x Genotype	0.92	0.656

Figure legends

Figure 1: Mean growth rates (h^{-1}) of the MA line genotypes and their respective ancestors under seven levels of two different stress regimes (400% ($0.6gl^{-1}$), 200% ($0.3gl^{-1}$), 100% ($0.15gl^{-1}$), 50% ($0.075gl^{-1}$), 25% ($0.038gl^{-1}$), 10% ($0.015gl^{-1}$), and 5% ($0.0075gl^{-1}$) KH_2PO_4; and 0 to $6gl^{-1}$ of NaCl in $1gl^{-1}$ increments, ordered by increase in stress level. Error bars show 95% confidence intervals.
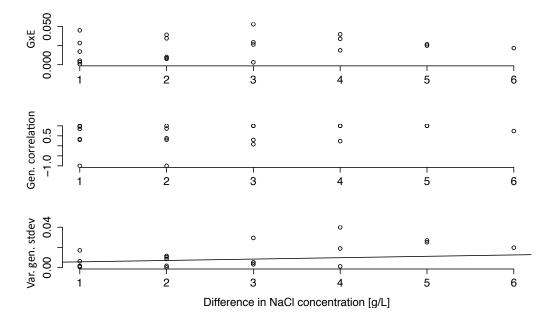
Figure 2: Mean relative fitness of MA line genotypes derived from two ancestral strains under decreasing phosphate 400% ($0.6gl^{-1}$), 200% ($0.3gl^{-1}$), 100% ($0.15gl^{-1}$) , 50% ($0.075gl^{-1}$), 25% ($0.038gl^{-1}$), 10% ($0.015gl^{-1}$), and 5% ($0.0075gl^{-1}$) KH_2PO_4; and increasing NaCl (0 to $6gl^{-1}$ of NaCl in $1gl^{-1}$ increments) conditions, ordered by increase in stress level. Error bars indicate 95% confidence intervals.
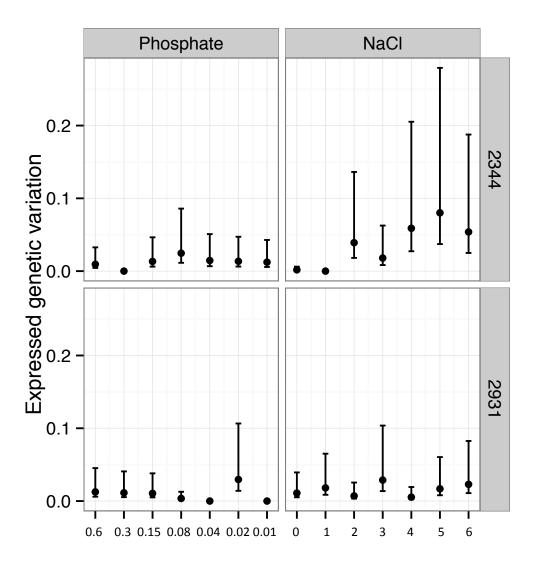
Figure 3: Overall GxE interactions, genetic correlation of growth and variation of the genetic standard deviation based on the relative fitness of MA genotypes derived from ancestor strain CC-2344 plotted against the differences in NaCl concentrations of the pairs of test environments. Lines indicate a significant relationship between the variables after permutation tests.

Figure 4: Mean expressed genetic variation of all MA genotypes under different levels of NaCl or phosphate stress, ordered by increase in stress level. Error bars indicate 95% confidence intervals.

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