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Changes in the growth rate of *Chlamydomonas reinhardtii* under long-term selection by temperature and salinity: Acclimation *vs*. evolution

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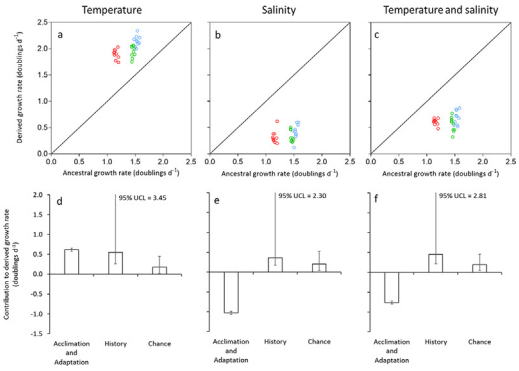
HIGHLIGHTS

- Increased temperature and salinity in freshwater affect phytoplankton growth rate.
- Acclimation allowed growth under selection for salinity.
- Growth rate changed due to acclimation and adaptation under temperature selection.
- Salinity would be a greater challenge than warming for freshwater phytoplankton.

GRAPHICAL ABSTRACT



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ABSTRACT

We investigated the roles of acclimation and different components involved in evolution (adaptation, chance and history) on the changes in the growth rate of the model freshwater microalga *Chlamydomonas reinhardtii* P. A. Dang. exposed to selective temperature and salinity. Three *C. reinhardtii* strains previously grown during one year in freshwater medium and 20 °C were exposed to 5 °C temperature increase and a salinity of 5 g L^{-1} NaCl. Cultures under each selective scenario and in combination (increase of salinity and temperature), were propagated until growth rate achieved an invariant mean value for 6 months (100–350 generations, varying as a function of scenario and strain). The changes of the growth rate under increased temperature were due to both adaptation and acclimation, as well as history. However, acclimation was the only mechanism detected under salinity increase as well as in the selective scenario of both temperature and salinity, suggesting that genetic variability would not allow survival at salinity higher than that to which experimental populations were exposed. Therefore, it could be hypothesized that under a global change scenario an increase in salinity would be a greater challenge than warming for some freshwater phytoplankton.

1. Introduction

Higher temperatures and reduced precipitation are expected to be widespread by the end of the 21st century (Field et al., 2014), causing a reduction in runoff, and this will lead to increasing salinization of epicontinental freshwater bodies (Cañedo-Argüelles et al., 2016; Dudgeon, 2019; Dudgeon et al., 2006; Jeppesen et al., 2020; Reid et al., 2019). Although it is unlikely that warming will exceed the thermal tolerance of epicontinental phytoplankton in mid-latitudes (Staehr and Birkeland, 2006; Sánchez et al., 2008; Huertas et al., 2011), it could induce selection among some ecological groups, altering the structure of phytoplankton communities (Huertas et al., 2011; Kosten et al., 2012; Meerhoff et al., 2012; Willis et al., 2019). On the other hand, an increase in salinity

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can cause the extinction of stenohaline taxa along with changes in phytoplankton community structure (Flöder et al., 2010; Mutshinda et al., 2013).

The short-term, rapid responses of freshwater phytoplankton to higher temperature and salinity have been extensively addressed (Lionard et al., 2005; Weisse et al., 2016; Yvon-Durocher et al., 2017; Shetty et al., 2019). The results of these studies reflect acclimation processes based on the differential expression of the gene pool already present in the cell populations, occurring in hours to days. For this reason, the acclimation responses have a limited predictive potential in the scenario of long-term warming and salinization. Thus, to make better predictions, it is crucial to perform long-term experiments where the selection of new genetic variants could occur (Brennan and Collins, 2015; Lachapelle et al., 2015a,b; Brennan et al., 2017; Yvon-Durocher et al., 2017). In fact, some long-term studies have explored the evolutionary response to salinity and temperature of the model freshwater green microalga *Chlamydomonas reinhardtii* (Lachapelle and Bell, 2012; Lachapelle et al., 2015a,b; Yvon-Durocher et al., 2017). However, it must be highlighted that evolutionary change is not solely the consequence of natural selection, because neutral mutations and genetic drift events arising by chance can occur (Crow and Kimura, 1970; Kimura, 1979; Spiess and Florian, 1989). Simultaneously, a historical footprint can make that variation among strains results in different evolutionary outcomes (Gould and Lewontin, 1979; Blount et al., 2008), requiring many experimental studies to examine multiple samples of a population. The possibility to discriminate the actions of natural selection from those modulated by chance, as well as the limits to evolutionary change imposed by history, has been considered one of the most fascinating questions in evolutionary biology, but which could be addressed only by a *Gedankenexperiment* (Gould, 1989). However, Travisano et al. (1995) successfully tested the contributions of chance and history in the evolution of bacterial populations, by comparing the values of selected phenotypic traits in ancestral and derived populations (Fig. 1). The experimental design of these authors has been applied, with appropriate modifications, to study evolutionary change in other lineages of microorganisms (Flores-Moya et al., 2008; Pérez-Zaballos et al., 2005; Rouco et al., 2011; Flores-Moya et al., 2012) as well as digital organisms (Wagenaar and Adami, 2004; Bundy et al., 2021). Therefore, the strength of a predictive analysis of the future performance of freshwater phytoplankton under a scenario of increased temperature and salinity must not only be based on acclimation responses but, above all, it must consider possible evolutionary changes (disentangling adaptation from chance, and the possible occurrence of constraints imposed by history).

The aim of our study was to evaluate the contribution of adaptation (separating acclimation from selection of favored mutants), chance, and history to the phenotypic change in growth rate of the model freshwater green microalga *Chlamydomonas reinhardtii* P. A. Dang. We chose this microalga because the presence of *C. reinhardtii* in nature is very relevant (Sasso et al., 2018). In addition, *C. reinhardtii* is a model organism of microalgae that has been used previously in evolutionary studies (Collins and Bell, 2006; Hema et al., 2007).

In this case, cultures previously grown in freshwater medium and 20 °C were exposed to a temperature increased by 5 °C and a salinity level of 5 g L^{-1} for a long-term period until the growth rate was invariant after 6 months. This experimental and statistical approach allowed us to assess how much of the observed change in growth rate was due to physiological acclimation and how much to evolutionary change. Moreover, the selection experiments were performed by exposing the cultures to a single selective factor (i.e., increased temperature or salinity) and both selective factors together (increased temperature plus salinity), which allowed us to evaluate the relative contribution of each selective factor to the evolutionary potential of *C. reinhardtii*. Although the study is an oversimplification of the reality, it is a robust way to explore the response of organisms to global change, improving on the limited prediction ability based on short-term responses.

2. Materials and methods

2.1. Experimental organisms and culture conditions

Strains ChlaA and ChlaB of the green microalgae *C. reinhardtii* were provided by the Algal Culture Collection of the Genetics Laboratory, Veterinary School, Complutense University (Madrid, Spain). Strain CCAP 11/45 was obtained from the Culture Collection of Algae and Protozoa, Institute of Freshwater Ecology, Ambleside, UK and it has been maintained in the Department of Botany and Plant Physiology (University of Málaga, Spain) for over 20 years. Details of culture conditions are described in Melero-Jiménez et al. (2019, 2020). In short, cultures were grown in BG11 medium (containing 0.7 g L^{-1} Na^{+} and 0.01 g L^{-1} Cl^{-}; Sigma-Aldrich Chemie, Taufkirchen, Germany), diluted 50% (BG11–50%, so it can be considered as a freshwater medium; Smol and Stoermer, 1999) and were incubated at 20 °C under a continuous photon flux density of 60 μmol m^{-2} s^{-1} over the waveband 400–700 nm (Sylvania Grolux 36W, Erlangen, Germany). Cultures were maintained in mid-log exponential growth by serial transfers of an inoculum to fresh medium every week.

2.2. Experimental design

The rationale for the experimental design is detailed in Travisano et al. (1995), and the adaptation for performance with liquid cultures of cyanobacteria and microalgae was devised by Flores-Moya et al. (2008, 2012). A scheme of the full experiment is shown in Fig. 2. The three experimental strains of *C. reinhardtii* were cultured at 20 °C, in freshwater culture medium almost one year. One cell from each strain was isolated by successive dilutions and re-cloned to ensure genetic homogeneity at the starting

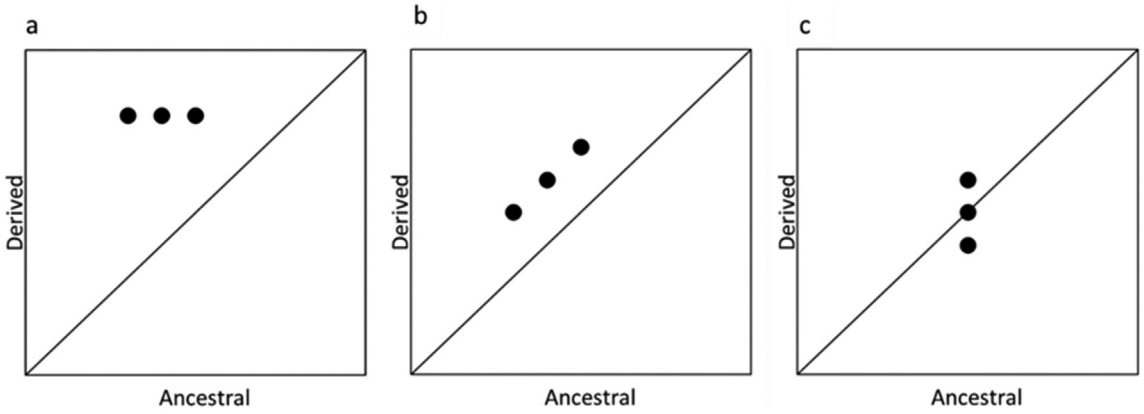

Fig. 1. Schematic representation of phenotypic change determined by acclimation plus adaptation (a), history (b), and chance (c). The effects of chance are demonstrated for a set of clones of a single ancestral phenotype, whereas acclimation plus adaptation and history are illustrated for three independent ancestors. Isoclines (solid line) represent the value if no changes take place. Adapted from Travisano et al. (1995) and Wagenaar and Adami (2004).

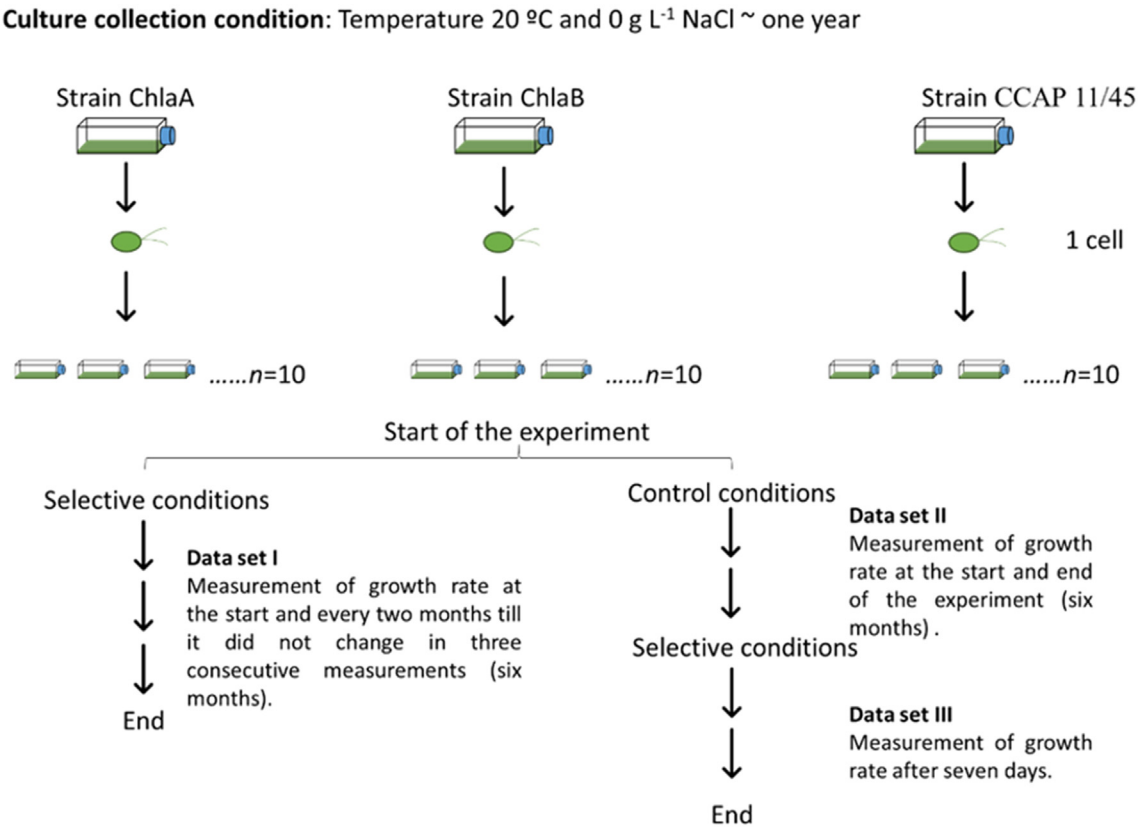

Fig. 2. Schematic representation of the experimental design.

point. Then, 3 sets of 10 independent populations of each strain were founded, their growth rates were measured every two months and, when no significant changes in growth rate was detected in three consecutive measurements (checked by model I, one-way ANOVA), the experiment was finished. For this purpose, every set of 10 populations was used for an independent experiment (selective conditions characterized by a temperature increment of 5 °C, increased salinity (5 g L^{-1} NaCl) and by both increased temperature and salinity). We previously checked that 5 g L^{-1} NaCl was not a lethal condition for the experimental strains of *C. reinhardtii* (Fig. S1). An additional set of 10 populations of each strain was kept at the ancestral conditions (i.e., 20 °C and NaCl-free culture medium) during the experiment (control cultures) (Fig. 2). The experimental cultures were transferred to fresh medium every 7 d (this interval was reduced or increased, depending on the changes in growth rate while running each experiment), to achieve ca. 4 generations before the addition of fresh medium. The number of generations during the experiment was computed in accordance with Novick and Szilard (1950). In each transfer, the inoculum contained 2.5×10^3 cells. The growth rate was checked every 2 months and, when no significant changes in growth rate were detected in three consecutive measurements (checked by model I, one-way ANOVA), the experiment was considered finished.

The effect of pooled acclimation plus adaptation was defined as changes in the mean value of the growth rate; 95% confidence limits were calculated using the Student *t*-distribution. To disentangle acclimation from adaptation, the cells grown under the control conditions were transferred to the three experimental conditions (increase in temperature, increase in salinity, and both factors simultaneously) for 7 d (ca. 4 generations). The adaptation corresponds to the difference between the growth rate of the cells derived from the experiment under selective conditions, and the growth rate of the cells under control conditions and transferred for 7 d to selective conditions (data sets I minus III; Fig. 2). Acclimation corresponds to any increase of the growth rate of the latter with respect to control cells (data sets III minus II; Fig. 2).

The effects of history and chance (at start and at the end of the experiment) were estimated using a two-level nested ANOVA (3 strains and 10 independent populations of each strain, with 3 replicates of the growth rate measurements per population). All statistical tests were performed by using the free software R Core Team (2020). Nested ANOVAS were performed with the R package "nlme". Specifically, the contribution of the history corresponds to the variance among strains, while the contribution of chance was estimated by the variance measured among populations (of the same strain). The homogeneity of the variances was checked with the Bartlett test. The square root of the variance, for history and chance were used to derive units that were comparable to the mean phenotypic change due to acclimation plus adaptation. Approximate 95% confidence limits were calculated for the variance components; these limits are asymmetric with respect to the mean.

2.3. Measurement of growth rate

Growth rate was measured in exponentially growing cultures according to Crow and Kimura (1970) as $\log_e (N_t/N_0)/t$, where N_t and N_0 are the cell number at time $t = 5$ and 0 d, respectively. The values of N_0 and N_t were determined at 6 and 11 d after the transfer of cells to fresh medium because the cultures were at exponential phase during these days. For this purpose, a linear regression ($n = 28$) was computed between cell concentration (CC; units in 10^6 cells mL^{-1}) and absorbance at $\lambda = 750$ nm by using a plate spectrophotometer (EON, BioTek, Winooski, VT, USA), which yielded a slope of 6.3 ($R^2 = 0.992$) for strain CCAP 11/45, 4.4 ($R^2 = 0.981$) for strain ChlaA, and 0.9 ($R^2 = 0.987$) for strain ChlaB.

3. Results

The growth rate of the three strains, in the three experiments, did not change (F ranging from 1.07 to 3.27; $df = 2$ and 27; P ranging from 0.356 to 0.053, respectively) in consecutive measurements carried out at

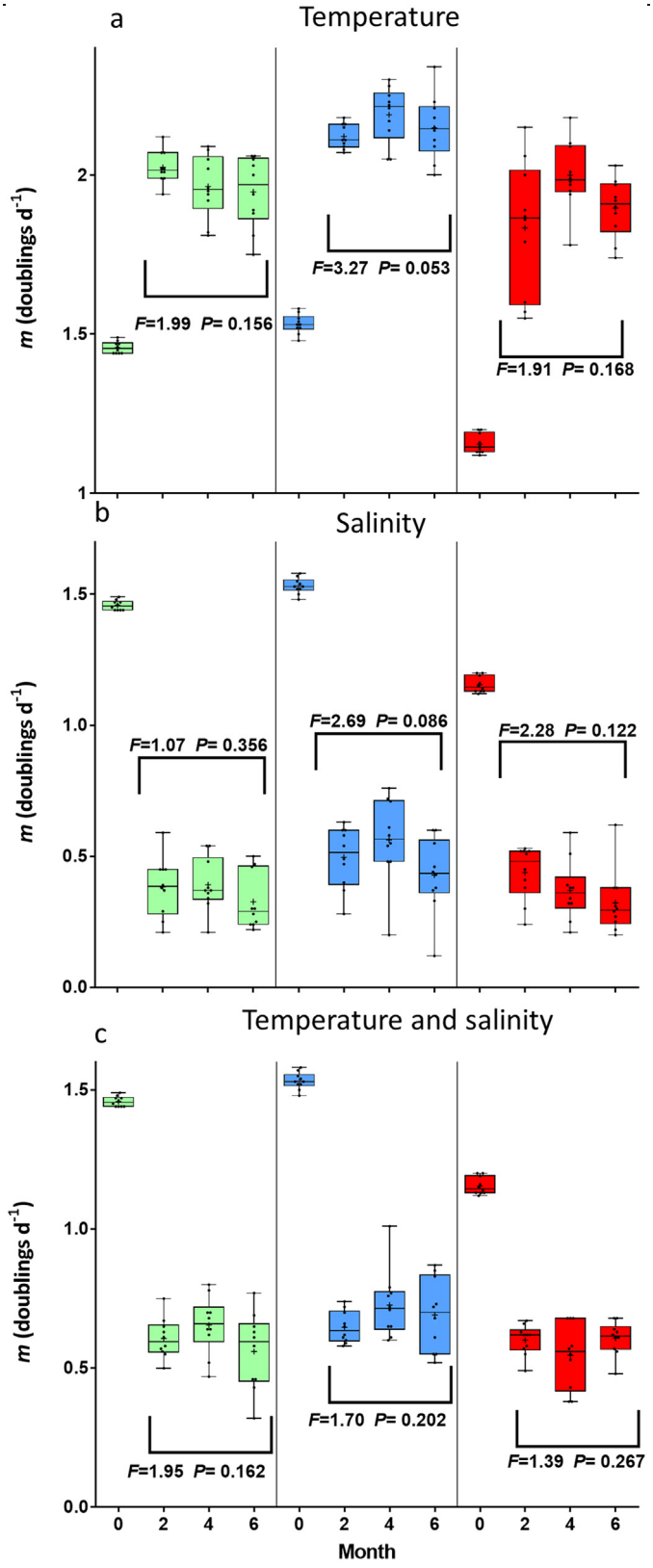

Fig. 3. Growth rate (m) of the strains CCAP 11/45 (green), ChlaB (blue), ChlaA (red) of *Chlamydomonas reinhardtii* at the start of the experiment and every 2 months under selection by temperature (a), salinity (b), and temperature plus salinity (c). Box plots show median, mean (crux), first and third quartiles, while whiskers show the minimum and maximum values ($n = 10$). The F- and P-values correspond to the ANOVAs for comparison of the growth rate scores at months 2, 4 and 6 ($df = 2$ and 27). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2, 4 and 6 months (Fig. 3). Therefore, we considered that the phenotypic changes that could occur were already achieved and, so, the scores of the growth rates after 6 months were used to disentangle the final contribution of acclimation, adaptation, chance, and history. We observed an increment of growth rate at the end of the experiment when the populations where exposed to an increase in temperature. However, population exposition to salinity and to high temperature plus salinity produced a reduction of the final growth rate values.

Ancestral vs. derived growth rates in the three strains of *C. reinhardtii* were plotted for the three experiments (Fig. 4a–c). Acclimation and adaptation were absent (by design) at the start of the experimental selective period. However, the initial scores of growth rate were significantly affected by history ($F = 1952.79$; $df = 2$ and 27; $P < 0.001$) and, to a lesser degree, by chance ($F = 3.92$; $df = 27$ and 60; $P < 0.001$). The final contribution of chance, history, acclimation and adaptation is described separately for each experiment.

3.1. Temperature

After ca. ~350 generations in the three strains, 46% of the phenotypic change was explained as acclimation plus adaptation ($t = -15.078$; $df = 58$; $P < 0.001$; Fig. 4d). From the differences between the growth rate data sets, it was found that acclimation (estimated as data sets III minus II, SI Table S1) accounted for 21% of the phenotypic change while 25% was a consequence of the selection of new genetic variants (estimated from data sets I minus III, SI Table S1).

The contribution of history was 41% ($F = 17.88$; $df = 2$ and 27; $P < 0.001$) while the contribution of chance was 13% ($F = 2.27$; $df = 27$ and 60; $P < 0.001$).

3.2. Salinity

The selective conditions imposed by increased salinity negatively affected the growth rate of the three strains, with mean values lower than those of the ancestral counterparts (Fig. 4b). After ca. ~68 generations in strains CCAP 11/45 and ChlaA, and ca. ~90 generations in strain ChlaB under selective salinity, 65% of the phenotypic change was explained by both acclimation plus adaptation ($t = 26.362$; $df = 58$; $P < 0.001$; Fig. 4e), with acclimation (data sets III–II) accounting for 60%, while adaptation (data sets I–III, SI Table S1) was responsible for the remaining 4%.

The contribution of history to phenotypic change was 23% ($F = 10.35$; $df = 2$ and 60; $P < 0.001$) and the contribution of chance was 13% ($F = 3.27$; $df = 27$ and 60; $P < 0.001$).

3.3. Temperature and salinity

The phenotypic change of the growth rate of *C. reinhardtii* under selective conditions of increased temperature and salinity was more like the results obtained under selective salinity than under selective temperature (Fig. 4c). Thus, after ca. ~100 generations in strains CCAP 11/45 and ChlaA, and ca. ~120 generations in strain ChlaB, 54% of the phenotypic change was explained as acclimation plus adaptation ($t = 19.990$; $df = 58$; $P < 0.001$; Fig. 4f), distributed between 44% as consequence of gene expression and 10% as the selection of new genetic variants (SI Table S1).

The contribution of history was 32% ($F = 14.55$; $df = 2$ and 60; $P < 0.001$) whereas the phenotypic change attributed to chance was 14% ($F = 2.67$; $df = 2$ and 60; $P < 0.001$).

4. Discussion

We applied an experimental approach in order to understand how much of the phenotypic change is temporary (acclimation), and how much could be hereditary because of selection of new genetic variants arising during the long-term exposure of *C. reinhardtii* to increased temperature, salinity or both. Moreover, the design also explores the possible occurrence of evolution that cannot be attributed to natural selection because it is due to neutral

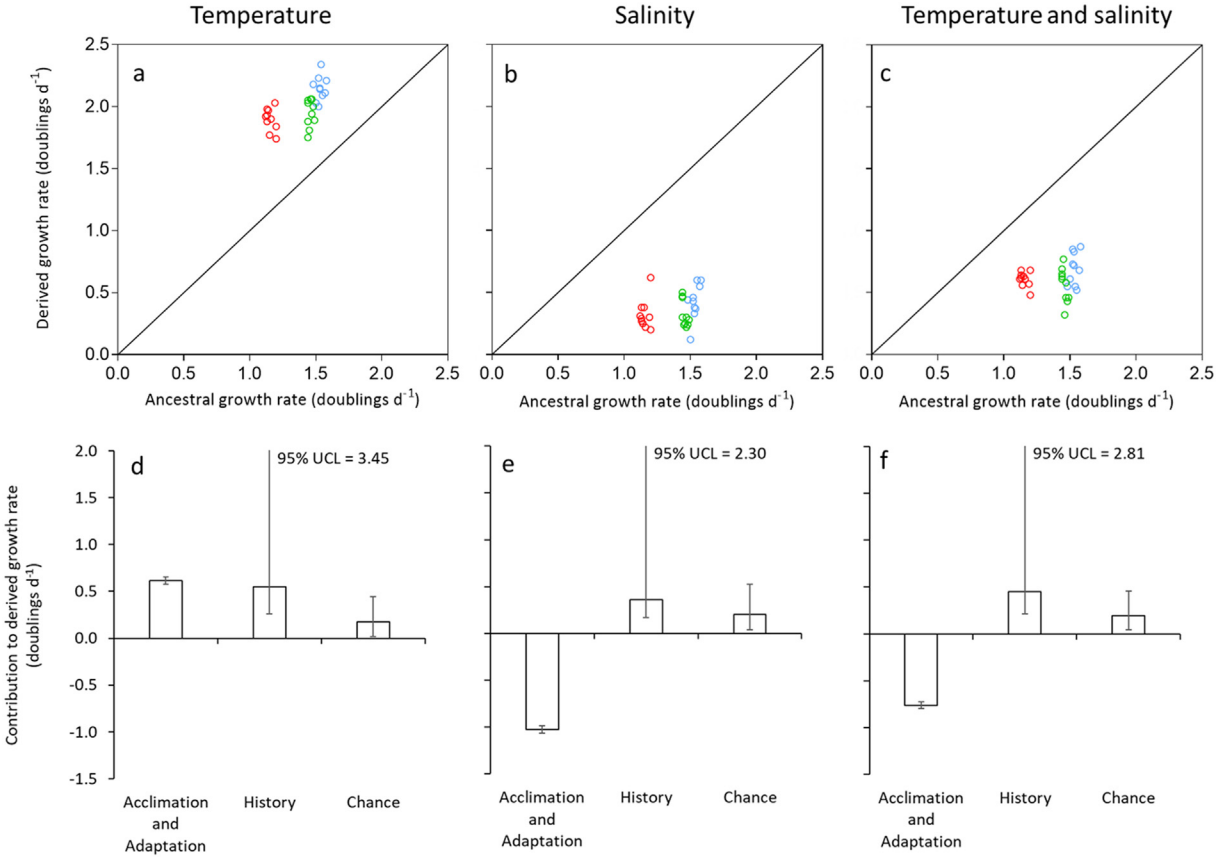

Fig. 4. Ancestral vs. derived scores of growth rate of the strains CCAP 11/45 (green), ChlaB (blue), ChlaA (red), and of *Chlamydomonas reinhardtii*, under selection by temperature (a), salinity (b) and temperature plus salinity (c). Each single symbol corresponds to an independent population in every strain; SD of three growth rate measurements of every population at the end of the experiment were smaller than symbol size. Diagonal line corresponds to the location of hypothetical growth rate values which do not show phenotypic change. Contribution of acclimation plus adaptation, chance and history at the end of the experiment (d–f). Error lines over the bars correspond to 95% confidence limits; they are asymmetric for the contributions of chance and history. UCL: upper confidence limit, overpassing the area of the figure. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

mutations or genetic drift (here called "chance") as well as the limitation of the evolutionary potential among strains due to historical contingency.

The increment of the growth rate value in three strains of *C. reinhardtii* under a temperature increment of 5 °C, after ca. ~350 generations, were due to adaptation and acclimation, as well as history. Since asexually growing clonal cultures were used, the phenotypic changes caused by adaptation are due to new mutations, which occurred during propagation of derived cultures under the new environmental conditions. This could be the consequence of the selection of some mutations that increased growth rate, displacing the wild-type genotypes. Mutations decreasing growth rate would have been eliminated by natural selection. Specifically, we observed that derived cells exposed to temperature increment of the three strains increased their growth rates compared to their ancestors. A similar result has been observed in previous reports where *C. reinhardtii* was exposed to high temperature in short term experiments, and this is to be expected because the optimum temperature of this species is near to 30 °C (Kremer et al., 2018). In this line, we show that the selection of new genetic variants can be as important as acclimation for the response of this species to increased temperature. In addition, historic contingency influences the final growth rate, in agreement with earlier studies with different organisms and environmental variables, where it has been observed that this component conditioned the final phenotype of different strains when they were exposed to new environments (Burch and Chao, 2000; Moore and Woods, 2006; Blount et al., 2008, 2018; Flores-Moya et al., 2012; Jerison et al., 2017). The contribution of chance on growth rate change was very low. According to evolutionary theory, chance includes random genetic drift (Crow and Kimura, 1970) and neutral mutations (Kimura, 1979). Genetic drift events are a consequence of "sampling errors" when the number of individuals in populations becomes relatively low at a given moment (Crow and Kimura, 1970). Because the minimum population inoculum of *C. reinhardtii* in the cultures was 2.5×10^3 cells (relatively large), it can be hypothesized that the role of chance may be due to neutral mutations rather than to genetic drift events. Additionally, it is well established in evolutionary theory that traits that are strongly correlated with fitness (such as growth rate) evolve by adaptation (selection of favorable mutations) whereas traits that are not (or are very weakly) correlated with fitness evolve by chance (Kimura, 1979; Spiess and Florian, 1989), as toxin production (Flores-Moya et al., 2012) or cell size (Flores-Moya et al., 2008). Consequently, it could be supposed that natural selection was strong enough to constrain the phenotypic change of growth rate in the experimental isolates. If this is so, the effect of chance only represents the possible experimental error.

We observed a decrease of growth rate values of *C. reinhardtii* after ca. ~90 generations under a salinity increase. The exposition to this stress agent would be a more relevant challenge because phenotypic changes were based mainly on acclimation (no new mutations appeared that allowed proliferation in more saline conditions). Specifically, adaptation (i.e., selection of favored mutants) accounted for a much lower percentage of total phenotypic change than acclimation. This is not surprising, because microalgae are generally differentiated as freshwater or marine taxa, and few freshwater species can adjust their osmotic potentials (Flöder and Burns, 2004; Kirst and Wiencke, 1995; Thessen et al., 2005). In fact, the growth rates after 6 months of culture at 5 g L^{-1} NaCl were similar to those observed in the initial short experiment at this NaCl concentration (SI Fig. S1). However, Perrineau et al. (2014) exposed *C. reinhardtii* during

1255 generations to 11 g L^{-1} NaCl and they observed that the derived populations reached a similar growth rate as their ancestors in the absence of NaCl. Nevertheless, apart from the difference in the number of generations with this study, they used a medium containing acetic acid that was essential for maintaining the high growth rates of the derived populations at high salinity. On the other hand, it was observed that acclimation (based on down-regulation of genes involved in the stress response and in transcription/translation) was a key response mechanism of *C. reinhardtii* to salinity increment (Perrineau et al., 2014), although the role of adaptation was not explored at the end of the experiment. In this line, we can hypothesize that this species, in the absence of an organic carbon source, as occurs in natural environments, could only face low levels of salinity due to acclimation mechanisms. Therefore, it would be interesting to explore the limits of adaptation to salinity of this species using another eco-evolutionary approach, the ratchet protocol (Melero-Jiménez et al., 2020), and culture media without acetic. Finally, the role of historical contingency on phenotypic change, of derived cells exposed to salinity, was more important than adaptation. These results agree with the suggestions of Blount et al. (2008), who suggested that historical contingency is especially important when it facilitates the evolution of key innovations that are not easily evolved by gradual, cumulative selection.

The effect of the combination of both selective factors corroborated the strong effect that salinity had. We observed that the response of *C. reinhardtii* to an increase in temperature and salinity was correlated with acclimation rather than with adaptation. This is interesting because most global change experiments only take one factor into account. However, global change implies many variables acting at the same time, and much emphasis is placed on temperature, although other variables may have a more significant role. In fact, some changes might actually be beneficial, while others will be 'true' stressors (Collins and Schaum, 2021; Snell-Rood et al., 2015). Our results reveal that for these conditions, the survival challenge is salinity, not temperature, because no adaptation was observed in the salinity experiments, while it was observed with temperature. In fact, there are several examples of the rapid response adaptation of phytoplankton to high temperature (Padfield et al., 2016; Schaum et al., 2018; Schlüter et al., 2014). Otherwise, it has been observed that salinity stress in *Chlamydomonas reinhardtii* can cause reduced cell division, minor size and it can promote palmelloid formation (Hema et al., 2007; Khona et al., 2016; Nakamura et al., 1975; Neelam and Subramanyam, 2013). This negative effect of salinity has also been detected on the growth rate of other freshwater algae species such as *Chlorella vulgaris*, *Chlorella salina*, *Chlorella emersonii* (Talebi et al., 2013) and *Scenedesmus opoliensis* (Demetriou et al., 2007). As a consequence, when both factors acted at same time, salinity modulated the change of growth rate. Consequently, under natural conditions it could be possible that other variables, which vary less than temperature, can affect phytoplankton organisms more than temperature. However, it has to be taken into account that high increases in temperature during short time periods could cause negative effects on phytoplankton populations (Acheampong et al., 2021; Samuels et al., 2021).

Although this study is an oversimplification of environmental conditions, taken together the results from the changes in growth rate under the three simulated scenarios of global change, it could be hypothesized that increase in salinity would be a greater challenge than warming for freshwater phytoplankton.

CRediT authorship contribution statement

IJM-J performed the experiments, analyzed the data, drafted the paper, prepared figures and tables, and reviewed drafts of the paper; EB-E conceived and designed the experiments, analyzed the data, drafted the paper and reviewed drafts of the paper; AF-M and MJG-S conceived and designed the experiments, analyzed the data, contributed reagents/materials/analysis tools, drafted the paper and reviewed drafts of the paper.

Declaration of competing interest

The authors declare no competing interests.

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Data availability

The data sets generated and analyzed in the present study may be available from the corresponding author upon request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.153467.

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