

1 Electronic Supplementary Materials: Appendix A

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3 Supplementary Methods  
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6 The evolution of competitive ability for essential resources  
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8 Joey R. Bernhardt<sup>1\*</sup>, Pavel Kratina<sup>2</sup>, Aaron Pereira<sup>1</sup>, Manu Tamminen<sup>3</sup>, Mridul K.  
9 Thomas<sup>4</sup>, Anita Narwani<sup>1</sup>

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11 <sup>1</sup>Aquatic Ecology Department, Eawag, Überlandstrasse 133, CH-8600 Dübendorf,  
12 Switzerland  
13

14 <sup>2</sup>School of Biological and Chemical Sciences, Queen Mary University of London, Mile  
15 End Road, London E1 4NS, United Kingdom.  
16

17 <sup>3</sup>Department of Biology, University of Turku, Natura, University Hill, 20014 Turku,  
18 Finland  
19

20 <sup>4</sup>Centre for Ocean Life, DTU Aqua, Technical University of Denmark, Kongens Lyngby,  
21 Denmark  
22  
23

24 \*Correspondence to: joanna.bernhardt@eawag.ch.  
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## 29 Supplementary methods

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### 31 *Evolution experiment*

32

33 We obtained a strain of *C. reinhardtii* (CC1690 wild type mt+) from the  
34 Chlamydomonas Center (chlamycollection.org). We then grew this strain in semi-  
35 continuous liquid batch culture on COMBO freshwater medium [1], without vitamins,  
36 silica and animal trace elements, which are unnecessary for growing green algae. We  
37 maintained batch cultures for several months before the start of the evolution  
38 experiment. We then plated the cultures onto agar. From the agar plates we selected  
39 four random colonies, derived from single cells and inoculated them into liquid  
40 COMBO freshwater medium (hereafter referred to as Anc 2, Anc 3, Anc 4 and Anc 5).  
41 We then inoculated the chemostats (28 mL total volume) with one of the four  
42 monoclonal populations or a genetically diverse population of the original CC1690  
43 population. All populations were composed of a single mating type (+), precluding the  
44 possibility of sex during the experiment. Here we use the term 'population' to refer to  
45 each of Anc 2, Anc 3, Anc 4, Anc 5, cc1690 ('ancestors') and their descendant  
46 populations ('descendants'), which are the populations evolved in one of seven  
47 experimental environments. In total there were five ancestral populations, and 32  
48 descendant populations (three were lost to contamination).

49

50 We randomly assigned one chemostat of each of the 5 ancestral populations (Anc 2-5  
51 and CC1690) to one of seven treatments: COMBO, (which we call C, containing 1000  
52  $\mu\text{M}$  N and 50  $\mu\text{M}$  P), nitrogen limitation (N, 10  $\mu\text{M}$  N and 50  $\mu\text{M}$  P), phosphorus  
53 limitation (P, 1000  $\mu\text{M}$  N, 0.5  $\mu\text{M}$  P), light limitation (L, 5  $\mu\text{mol photons m}^{-2}\text{s}^{-1}$  of  
54 light, 1000  $\mu\text{M}$  N and 50  $\mu\text{M}$  P), salt stress (S, 8g/L NaCl, 1000  $\mu\text{M}$  N and 50  $\mu\text{M}$  P),  
55 biotically-depleted medium (i.e. medium previously used to grow seven other species  
56 of phytoplankton, (B)), and a combination of salt stress and biotically-depleted medium  
57 [2] (BS, 8g/L NaCl). We prepared the biotically-depleted medium (B) by growing seven  
58 other species of freshwater algae (*Cosmarium botrytis*, *Kirchneriella subcapitata*,  
59 *Pediastrum boryanum*, *Pediastrum duplex*, *Scenedesmus acuminatus*, *Staurastrum*  
60 *punctulatum*, *Tetraedron minimum*) individually in 4L batch culture in replete COMBO  
61 (Kilham et al. 1998). We then removed the phytoplankton biomass from each batch  
62 culture via filtration, sterilized the filtrate by autoclaving, and mixed the filtrate from

63 each of the seven cultures. The mixture of filtrates is what we called the 'biotically  
64 depleted medium' (B). We stored the biotically-depleted medium in the cold (4°C) and  
65 dark until use during the experiment.

66

67 Each chemostat received daily sterile media replacement at a dilution rate of 56% per  
68 day via an automated peristaltic pump and was continuously mixed and aerated to  
69 prevent heterogeneity in resource availability. Chemostats each contained a magnetic  
70 stir bar at their base and were stirred continuously using a stir plate. The combination  
71 of continuous sterile air inflow and stirring kept the chemostats well mixed and  
72 minimized wall growth, and in this way minimized spatial heterogeneity. We  
73 maintained chemostats at 20°C and illuminated under 90  $\mu\text{mol photons m}^{-2}\text{s}^{-1}$  of light  
74 (except the light-limitation treatment) on an 18h light: 6h dark cycle.

75

76 The biotically-depleted medium was used to investigate the influence that a biodiverse  
77 community may have on the evolution of a species by simultaneously depleting the  
78 availability of multiple dissolved resources. The salt stress treatment consisted of  
79 increasing concentrations of NaCl. We used salt-stress as a point of reference by which  
80 to compare the influence of a resource limitation. Salt stress is known to induce  
81 evolutionary adaptation (i.e. greater salt tolerance) in *C. reinhardtii* [3], and in this way  
82 we could compare adaptation to limiting resources to another type of stress. We  
83 maintained the 'C' cultures in full COMBO medium for the duration of the experiment.  
84 Resource-limitation and salt-stress increased incrementally each month until a final,  
85 highly-stressful concentration was achieved (ESM Figure S3). The experiment ran for  
86 285 days (~285 generations), after which the evolved populations were harvested and  
87 plated onto agar.

88

### 89 **Culture conditions and acclimation**

90 Prior to  $R^*$  assays, we transferred populations from agar plates where they had been  
91 maintained for long-term storage after the selection experiment under low light  
92 (amount) and temperature (12°C) to limit growth to COMBO medium [1] and grew  
93 them at 20°C and 140  $\mu\text{mol light}$  (hereafter "standard conditions") on a 24 hour light  
94 cycle for three days until they reached mid exponential phase. Before the start of each  
95  $R^*$  experiment, we allowed each population to acclimate to a relatively low and high  
96 resource level for three days. The low resource acclimation conditions were set to

97 match the lowest resource level in the  $R^*$  experiments so as to minimize transfer of  
98 nutrients from the nutrient replete culture media to the experimental populations. The  
99 high resource acclimation level was set halfway between the lowest and highest  
100 resource level in the  $R^*$  experiments. The low resource acclimation levels were for light,  
101 nitrogen and phosphorus:  $8\mu\text{mols}/\text{m}^2/\text{s}$ ,  $5\text{ uM N}$  and  $0.5\text{ uM P}$ . The high resource  
102 acclimation levels were for light, nitrogen and phosphorus:  $42\mu\text{mols}/\text{m}^2/\text{s}$ ,  $80\text{ uM N}$ ,  $8$   
103  $\text{uM P}$ .

104

### 105 ***Competitive trait assays***

106 We diluted the 'low' and 'high' resource acclimation cultures to low cell concentrations  
107 in 50 mL falcon tubes with COMBO media containing N and P at one of ten resource  
108 levels. After diluting each population to very low density (measured as 10 chlorophyll-a  
109 relative fluorescence units ("RFU")) at each resource level, we transferred the cultures  
110 to the inner 60 wells of 96 well plates ( $n = 4$  replicates per population per resource  
111 level, 125 uL per well), covered the plates with a Breathe-Easy sealing membrane  
112 (Sigma-Aldrich), and moved them to  $20^\circ\text{C}$  temperature-controlled incubators  
113 (Multitron, Infors HT, Switzerland), which we set to rotate at 100 rpm. We filled outer  
114 wells with COMBO to prevent evaporative losses across the plate. We then tracked  
115 their growth by measuring chlorophyll-a fluorescence in RFU (excitation= $435\text{ nm}$  and  
116 emission= $685\text{ nm}$ ) over time using a Biotek Cytation 5 plate-reader. We used  
117 chlorophyll-a fluorescence because it can be used as a proxy for algal biomass [4],  
118 particularly during exponential growth from low density. We measured RFUs two or  
119 three times a day for three days, long enough to capture the exponential growth phase  
120 at all resource levels. For the  $N^*$  and  $P^*$  experiments, cultures were illuminated at  $140.6$   
121  $\mu\text{mol photons m}^{-2}\cdot\text{s}^{-1}$  of photosynthetically active radiation ('PAR'); for the  $I^*$   
122 experiments light levels were as described below.

123

124 For the nitrogen  $R^*$  experiment, the N levels were: 5, 10, 20, 40, 60, 80, 100, 400, 600  
125 and 1000  $\mu\text{m N}$ . For the phosphorus  $R^*$  experiment, the P levels were 0.5, 1, 2, 4, 6, 8,  
126 10, 20, 35 and 50  $\text{uM P}$ . For the light  $R^*$  experiment, N and P were 1000  $\text{uM N}$  and 50  
127  $\text{uM P}$  respectively, and light was supplied at one of ten levels: 0.25, 1.5, 5, 12.5, 27.5,  
128 50, 82.5, 125, 175 and  $250\mu\text{mol photons m}^{-2}\cdot\text{s}^{-1}$  of PAR. We manipulated light levels in  
129 the light experiment using neutral density filters (Solar Graphics™, Clearwater, Florida),  
130 which alter the total amount of light supplied without changing light spectrum. We

131 mounted the light filters onto opaque frames, which fit over the plates and prevented  
132 unmeasured light from entering the wells from the sides of the plates. We measured  
133 experimental light intensities under the filters using a Skye PAR Quantum sensor.

134

### 135 ***Salt tolerance assays***

136 Similar to the methods for the R\* assays, all ancestral and descendant populations were  
137 first transferred from storage on agar plates to liquid batch cultures and grown in  
138 standard conditions. They were then transferred to liquid culture to start an acclimation  
139 phase in which each of the populations was subjected to one of five levels of NaCl: 0,  
140 2, 4, 6, and 8 g/L for four days. Each of the populations was then diluted to achieve a  
141 final inoculation density of 50 RFU. Populations from each of the acclimation levels  
142 were used to inoculate assay cultures with the same level of salt, or 1 g/L more (i.e. 0  
143 was used to inoculate 0 and 1 g/L, 2 to inoculate 2 and 3 g/L, etc.). For the final growth  
144 rate assays, each population was grown in 10 mL of medium in 6-well plates, with a  
145 single replicate per population x salt level. We estimated salt tolerance by growing  
146 populations over a salt gradient of 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 g/L for nine days. To  
147 estimate growth rates, RFUs were measured once per day for nine days.

148

### 149 *Estimating consumption vectors via stoichiometry*

150 We quantified the consumption vectors for nitrogen and phosphorus for each  
151 population. The consumption vector [5] quantifies the amount of each of these two  
152 resources used by each individual consumes per unit time. We estimated these vectors  
153 by measuring the ratio of phosphorus to nitrogen in the biomass of each population as  
154 it was growing exponentially [6]. Stoichiometry during the exponential phase primarily  
155 reflects the structural pool of nutrients (vs. the storage pool) [7]. We started by  
156 inoculating each population into a 400 mL tissue culture flask with COMBO [1]. We  
157 then allowed these populations to grow for approximately 1.5 days under standard  
158 conditions until they reached their mid-exponential phase. We then harvested the algal  
159 biomass by filtering each culture onto one ashed (400 °C) and pre-massed Whatman ®  
160 glass microfiber filter (grade GF/F 47 mm) and one 25mm Whatman glass microfiber  
161 filter. We then dried the filters dried in an oven overnight at 60 °C, and post-massed  
162 the 47mm filters to obtain an estimate of total dry biomass per mL of culture filtered.  
163 The 47mm filter was used to estimate the elemental carbon and nitrogen content of  
164 the biomass on an Elementar vario PYRO cube EA-IRMS, and the 25 mm filter was used

165 to estimate phosphorus content using Skalar San++Continuous Flow P/N analyser. The  
166 phosphorus samples were first digested and completely oxidized using a  
167 peroxydisulfate solution. We diluted the digested samples 1:20 before being run on  
168 the P/N analyser.

169

### 170 **Estimating cell size**

171 After the final RFU measurements, we fixed the populations in each well by adding a  
172 10% glutaraldehyde fixative (get details) solution, and stored the plates at 4°C until  
173 later analysis. To estimate cell size, we took Brightfield photos of the base of each well  
174 at 10x on a BioTek Cytation 5 imaging plate reader, from which we extracted cell  
175 length (using Gen5 software (BioTek version 2.0), which we converted to biovolume,  
176 assuming the cells were spheres (i.e.  $\frac{4}{3} \times \pi \times \text{radius}^3$ ).

177

### 178 **Quantifying genetic changes associated with selection environments**

179 To gain insight into the genetic responses of *C. reinhardtii* to the selection  
180 environments, we prepared Illumina HiSeq libraries of the ancestors and descendants.  
181 The ancestral populations of all 4 clonal populations and the original cc1690  
182 population were plated onto Sueoka's high salt agar [8] and grown on agar plates for 1  
183 month. We harvested the lawn of cells by scraping the agar and placing the biomass  
184 into microfuge tubes before performing the DNA extraction. The descendant  
185 populations were grown in 50 mL liquid batch cultures in COMBO medium in standard  
186 conditions for one week. Due to low level bacterial contamination, and to ensure that  
187 sequences were highly enriched by *C. reinhardtii*, these cultures were subjected to an  
188 antibiotic treatment of 50 mg/L ampicillin and 50 mg/L tetracycline overnight (<24  
189 hours), before harvesting the cells for DNA extraction [9]. The cells were then harvested  
190 by centrifugation at 4,000 rpm. The DNA extraction protocol was adapted from the  
191 Plant Lab protocol (Institute of Life Sciences, Scuola Superiore Sant'Anna, Pisa Italy and  
192 [10]).

193

194 DNA sequencing libraries were prepared using the Bioo Scientific NEXTflex Rapid  
195 Illumina DNA-Seq Library Prep Kit according to the standard protocol. DNA  
196 sequencing was performed on Illumina HiSeq 2500 version 4 using 125 bp paired ends  
197 (250 sequencing cycles). After sequencing the read quality was verified using FastQC  
198 version 0.11.2. Adaptor and PhiX cleaning were performed using BBDuk version 35.43,

199 using k-mer size 20 for the former. Quality filtering was performed using PRINSEQ  
200 version 0.20.4 with a minimum read length of 50 bp, GC range of 15-85% and  
201 minimum mean quality score 5. The quality-filtered reads were aligned against the *C.*  
202 *reinhardtii* reference version 5.0 [11] using Bowtie2 version 2.2.5. Variants were called  
203 from the resulting BAM files using Freebayes version 1.1.0. The resulting VCF files were  
204 quality-filtered using bcftools version 1.4 to select above SNP quality 20 and excluding  
205 any SNPs closer to 10 bp from any INDEL due to known read mapping errors around  
206 such mutations. The filtered VCF files further processed in R version 3.5.1 using library  
207 Tidyverse version 1.2.1 and Bioconductor package VariantAnnotation version 1.28.11.  
208 The mutations between the ancestors and descendants were determined by  
209 comparing their SNP profiles, determined by comparison to the *C. reinhardtii* cc503  
210 mt+, reference version 5.0, using custom R scripts. The R code for sequence  
211 processing is available at [https://github.com/joeybernhardt/chlamee-r-](https://github.com/joeybernhardt/chlamee-r-star/blob/master/genomics/workflow.R)  
212 [star/blob/master/genomics/workflow.R](https://github.com/joeybernhardt/chlamee-r-star/blob/master/genomics/workflow.R). The DNA sequences have been deposited in  
213 the Sequence Read Archive (SRA) under the BioProject ID PRJNA558172.

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