Environmental complexity constrains evolutionary adaptation across taxa

Giacomo Zilio[1,2,*], Emilie Aubin[3], Stéphanie Bedhomme[1], Laurence Bolick[4], Ignacio G. Bravo[5], Claude Bruand[6], Delphine Capela[6], Marie Challe[2], Guillaume M. Charrière[7], Gaelle Courtay[7], Marie-Ange Devillez[2], Fiona Elmaleh[7], Stanislas Fereol[1], Rémy Froissart[5], Justina Givens[2], Claire Gougat-Barbera[2], Lynn Govaert[4], Alice Guidot[6], Jeanne Hamet[1], Michèle Huet[3], Philipp Hummer[1], Staffan Jacob[3], Oliver Kaltz[2], Marc Krasovec[8], Martin Guillaume[2], Delphine Legrand[3], Thibault Nidelet[9], Denis Orcel[2], Hervé Philippe[3], Gwenael Piganeau[8], M. Stefania Przybylska[1], Philippe Remigi[6], Laurent Sauviac[6], Fabian Schneider-Nettsträter[4], Diego Segond[9], Celine Serre[9], Delphine Sicard[9], João Gabriel Colares Silveira[2], Jeanne Tonnabel[2], François Vasseur[1], Apolline Vedrenne[9], Edith Vidal[8], Cyrille Violle[1], Marielle Kathrin Wenzel[4], Emanuel A. Fronhofer[2,*], Luis-Miguel Chevin[1,*]

[1] CEFE, Univ Montpellier, CNRS, EPHE, IRD, Montpellier, France
[2] ISEM, Univ of Montpellier, CNRS, EPHE, IRD, Montpellier, France
[3] Station d'Ecologie Théorique et Expérimentale, CNRS, UAR 2029, Moulis 09200, France
[4] Leibniz Institute of Freshwater Ecology and Inland Fisheries, Berlin, Germany
[5] MIVEGEC, Univ Montpellier, CNRS, IRD, Montpellier, France
[6] Univ Toulouse, CNRS, INRAE, LIPME, Castanet-Tolosan, France
[7] IHPE, Univ Montpellier, CNRS, IFREMER, Univ Perpignan Via Domitia, Montpellier, France
[8] Sorbonne Université, Microbial Biodiversity and Biotechnology Laboratory (UMR 8176), University of Perpignan, CNRS, Banyuls sur Mer, France
[9] SPO, Univ Montpellier, INRAE, Institut Agro, Montpellier, France

*Corresponding authors: giacomo.zilio@umontpellier.fr / gcm.zilio@gmail.com, luis-miguel.chevin@cefe.cnrs.fr, emanuel.fronhofer@umontpellier.fr

Abstract

Natural populations are constantly exposed to complex environments, characterized by the simultaneous variation of multiple stressors, such as osmotic and thermal stress, and diverse pollutants. As a consequence, population persistence often depends on the potential to rapidly evolve in the face of these challenges. However, while evolution in response to single stressors has been widely studied, experimental evidence for adaptation to multiple stressors is sparse and remains inconclusive, due to a lack of consistent experimental designs and metrics across systems.

To overcome this limitation, we here used a meta-experimental evolution approach to test how environmental complexity shapes adaptive evolution across a diversity of taxa. We exposed 14 species of bacteria, unicellular eukaryotes, and plants, to single versus multiple stressors for multiple generations, and measured their fitness in the presence or absence of stressors.

We show that across taxa, populations evolving under multiple stressors adapted more slowly, and exhibited fitness costs when exposed back to control environments. The magnitude of these effects varied with the identity of the stressors (*e.g.*, temperature, salinity, copper). Further, all species followed a consistent pattern of greater fitness gains with greater initial fitness reduction at the start of the experiment.

Our results are consistent with the predicted cost of complexity when selection acts on pleiotropic genes. Accounting for the complexity of environmental stress should thus prove crucial to prevent biodiversity loss and its cascading effects on ecosystem functioning.

Introduction

Natural populations live in multifaceted and complex environments, often characterised by the concurrent presence of multiple stressors, *i.e.,* factors that reduce fitness by causing higher mortality or reduced reproduction (1). Anthropogenic activity is one important source of stress, notably imposing changes in temperatures (2) and salinity (3), but also drought or pollution. Hence, multi-stress environments are the new norm in ecosystems worldwide, and understanding their impact on organismal performance (physiology, life history) and ecological outcomes (population growth, biodiversity, ecosystem functioning) is a central problem for basic and applied research. Most evidence of responses to multiple stressors mainly comes from short-term ecological studies (*e.g.*, ecotoxicology), and involves within- or trans-generational plasticity (4, 5). However, genetic evolution can also unfold over ecological timescales (6, 7), and it represents a key mechanism by which organisms may respond to the strong selective pressures imposed by multiple stressors (8–10). It is therefore crucial to investigate how rapidly species can evolve in response to multiple stressors, as adaptation rate determines how effectively organisms will be able to persist under changing environments (11).

Evolutionary theory has long recognized that rates of adaptation can be reduced by a cost of complexity, wherein fewer mutations are available to simultaneously provide an answer to multiple biological challenges (12–14). While this cost was originally formulated with respect to the complexity of the organism in an abstract geometric model of adaptation (12, 13), the number of organismal traits upon which selection acts in any given environment is likely to also reflect the degree of complexity of this environment (defined as increased number of dimensions, as previously argued by (15)). On the one hand, there is abundant evidence for trade-offs that can constrain an organism performance across environments acting via antagonistic pleiotropy, genetic correlations, epistasis, and limitation of resources (16–18), although counter-examples exist (16, 19). On the other hand, a larger number of stressors could also increase the number of genes that may respond to selection. This larger genomic target, which was not considered in traditional theory on the cost of complexity (12, 13), could accelerate adaptation by increasing the overall supply of advantageous mutations. However, the picture may be less clear than the theory anticipates, and adapting to multiple stressors might require specific biological solutions that are unlikely to evolve in response to single stressors (20).

Adaptation to single or multiple stressors may also incur an additional cost: a fitness reduction in environmental conditions where selection did not occur (21, 22), including benign ones (sometimes described as a cost of resistance for adaptation to biocides (23)). Although such fitness costs in non-selective environments are not always quantified and considered (24), they may constrain future evolutionary responses, if the selective environment later changes (14, 25). While some empirical evidence supports costs of adaptation in benign environments (17), other experiments show no relationships, or even positively correlated responses (26–

29). Furthermore, whether or not such costs differ when populations evolve in response to multiple stressors remains little understood.

Providing robust answers to these fundamental questions about limits to adaptation requires moving beyond the specificities of individual species and taxa, and relying on manipulative rather than correlative evidence. Nevertheless, apart from a few notable evolutionary experiments (18, 30–33), we lack consistent and quantitative evidence about how adaptation proceeds in response to single *vs*. multiple-stressor environments. For instance, Brennan *et al.* (30) found faster adaptation under a higher number of stressors in the chlorophyte *Chlamydomonas reinhardtii*. A recent evolutionary experiment with the yeast *Saccharomyces cerevisiae* employed a full factorial design with all possible combinations of 4 stressors (including the 4 single stressors) to explore the role of environmental complexity on adaptation (18). Populations that evolved under multiple stressors attained lower fitness than those evolved in the corresponding single-stressor conditions, and paid fitness costs in environments where they had not undergone selection. In both cases stress levels were not matched, hence introducing potential confounding of stressor intensity and stressor complexity, such that more stressors meant a higher probability to include one that severely reduces fitness. White et al. (34) did control for the total strength of selection while investigating local adaptation of the rotifer *Brachionus plicatilis* under single- *vs.* four-stressor regime, and found more rapid adaptation in the multiple stressor treatment. As each of these studies used somewhat different methodologies and measurements, drawing general conclusions can be difficult. A powerful alternative is offered by meta-experiments: by coordinating similar experimental protocols across systems, they help overcome the idiosyncrasies of any specific study organism, experimental setup or laboratory conditions, thereby enabling broader inference. Such collaborative experiments have been successfully applied in ecology to study, for example, dispersal and plant productivity (35–37). Here, we conducted, to our knowledge, the first evolutionary meta-experiment on the influence of environmental complexity on adaptive evolution across diverse taxa.

Results

Our meta-experiment involved 12 research groups using analogous protocols across 14 species (**Table 1**), ranging from aquatic and terrestrial bacteria to unicellular eukaryotes (ciliates, euglenozoa, chlorophytes, fungi) and plants, with one or more strains for each (23 strains in total, see Supporting Information for details). For each species, we used two sources of stress, combined in four treatments: a control (absence of stressors), two single stressors applied separately, and a multiple stressor combining the two single stressors. Stressor levels differed between experimental systems and reflected species-specific stress responses (**Table 1** and SI). Importantly, however, we adjusted stressor levels for each system so as to obtain similar initial fitness reductions for single and multiple stressor treatments. This experimental design allowed us to separate the cost of complexity from the total magnitude of stress, one main prerequisite of our study.

130 Furthermore, the identity of stressors differed between species (**Table 1**), and were chosen
131 among major threats to ecosystems worldwide that exert prominent selective pressures *in*
132 *natura*: temperature, salt, copper, antibiotics, and resource deprivation (2, 38–40). We
133 assessed evolutionary responses by comparing demographic performance (exponential
134 growth rate r_0 estimated from growth curves as in **Fig. 1;** but see S1-2 for plants protocol) at
135 the beginning and end of the long-term experiments.

136 **Table 1** List of the species used in the study, with biological and experimental details. The Indirect response
137 column indicates whether the species was tested in control conditions after evolution. In the stressor identity
138 column, T indicates Temperature. Unless specified, the different strains within species experienced the same
139 experimental conditions and replication.

Species	Group	Strains	N° of transfers	Time (months)	Indirect response	Stressor identity	Final replicates per evolutionary treatment
Arabidopsis thaliana	Plants	1	5	60	Yes	Resources, Herbivory	Control x7, Single x14, Multi x7
Brassica rapa	Plants	1	3	12	No	Resources, Shade	Control x5, Single x10, Multi x5
Colpidium striatum	Ciliates	1	~ 17	12	Yes	T, Salt, Copper	Control x3, Single x8, Multi x5
Dunaliella salina (all strains)	Chlorophytes	4	36	8	Yes	T, Salt	Control x3, Single x6, Multi x3
Euglena gracilis	Euglenozoan	1	~ 20	5	Yes	T, Salt, Copper	Control x10, Single x14, Multi x14
Ostreococcus tauri	Chlorophytes	1	17	4	No	T, Salt	Control x6, Single x12, Multi x6
Paramecium caudatum	Ciliates	1	~ 48	12	Yes	T, Salt	Control x5, Single x7, Multi x3
Picochlorum costavermella	Chlorophytes	1	12	3	No	T, Salt	Control x6, Single x12, Multi x5
Pseudomonas aeruginosa	Bacteria	1	26	~1	Yes	Salt, Copper	Control x12, Single x20, Multi x9
Ralstonia solanacearum	Bacteria	1	15	~0.5	Yes	T, Salt	Control x5, Single x10, Multi x5
Saccharomyces cerevisiae (all strains)	Fungi	2	26	3	Yes	T, Copper	Control x3, Single x6, Multi x3
Sinorhizobium meliloti	Bacteria	1	20	~0.66	Yes	T, Salt	Control x5, Single x10, Multi x5
Tetrahymena thermophila Strain T1 Strain D16 Strain D19 Strain D21 Strain D3	Ciliates	5	~ 20	~5	Yes	T, Salt, Copper, Salt, Antibiotic Salt, Antibiotic Salt, Antibiotic Salt, Antibiotic	Control x10, Single x15, Multi x15 Control x6, Single x9, Multi x4 Control x6, Single x12, Multi x5 Control x6, Single x12, Multi x6 Control x6, Single x12, Multi x5
Vibrio aestuarianus Strain 12/016 Strain U29	Bacteria	2	22 16	~1.5 ~0.25	Yes	Salt, Copper	Control x3, Single x6, Multi x3

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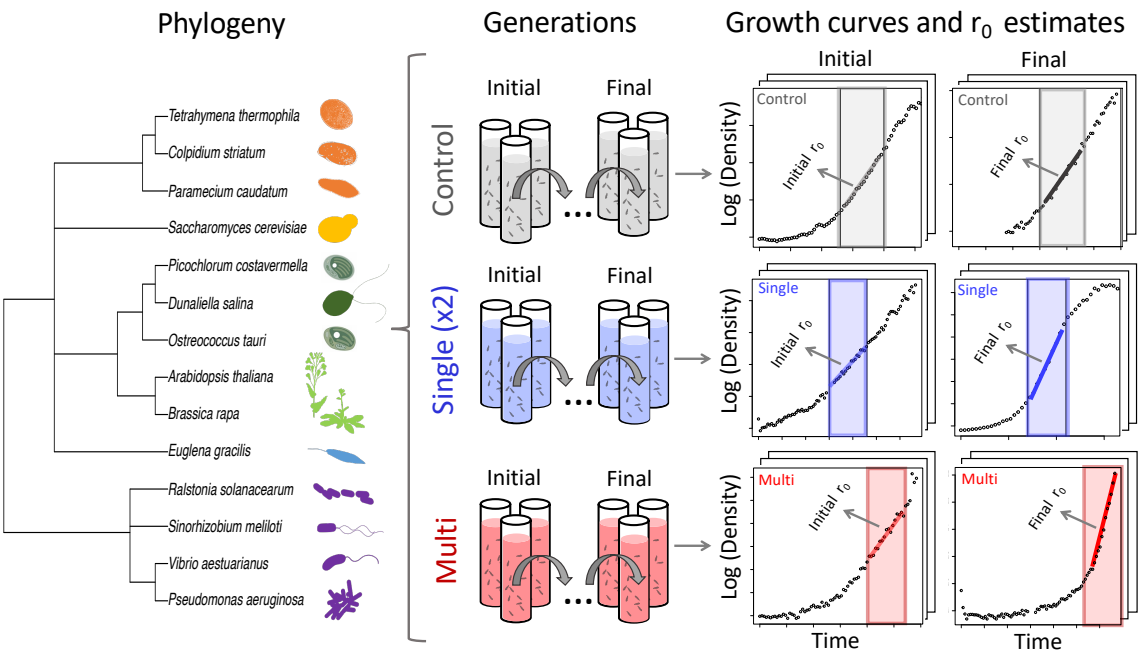


Fig. 1 General experimental design and methodology. Schematic representation of the main procedures of the study, showing three evolutionary replicates per treatment for illustrative purpose. The model organisms (silhouettes in orange: protists; yellow: fungus; dark green: chlorophytes; light green: plants; light blue: Euglenozoa; purple: bacteria) were propagated in conditions of control (grey), single stressors (blue) or multiple stressor (red) by serial transferring. Growth curves with population density over time were measured at the first (Initial) and last (Final) transfer in their own evolutionary treatment, for all unicellular species (12/14). For the final transfer, fitness was also estimated in the absence of stressors (control conditions) for 11 species. The slope estimates correspond to the intrinsic growth rates r_0 for all microbial species, and were used as proxy for fitness in the main analyses. For plants (2/14 species), fitness estimates were obtained using a different experimental protocol (see section S1-S2). The experimental protocols and additional details for each species are provided in the SI.

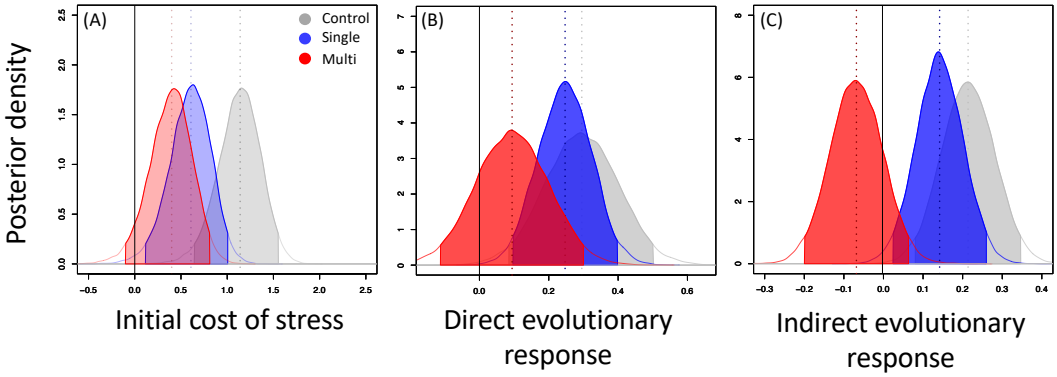
<u>The initial fitness drop was similar between single and multiple stressors</u>

Initial exposure to our environmental stressors reduced fitness by ~55 % on average compared to the control treatment (**Fig. 2A**, Linear Mixed-effect Model (LMM), fitness median and 95% Compatibility Interval (CI): 1.140 [0.643, 1.553]; (41)). There was no clear difference between single and multiple stressors, as suggested by their highly overlapping posterior distributions (LMM, Single: 0.607 [0.115, 1.005]; Multiple: 0.399 [-0.101, 0.811]). This result confirms the adequacy of our experimental conditions, designed to impose similar fitness reductions in single and multiple stressor treatments at the start of the evolutionary phase.

<u>Direct evolutionary response was weaker under multiple stressors</u>

After evolution, fitness had increased in all treatments (**Fig. 2B,** see also **Fig. S1** with raw data on initial and final fitness for each strain), indicating that adaptation occurred during experimental evolution. We quantified the direct evolutionary response (**Fig. 2B**) as the difference between final and initial fitness for each treatment (see also caption). Populations evolving under single stressor showed similar increases in fitness as those in the control

168 treatment (LMM, Single: 0.247 [0.096, 0.398], Control: 0.295 [0.084, 0.501]; **Fig. 2B**). In
169 contrast, the multiple stressor treatment led to only 38% of the fitness increase of single
170 stressor fitness (LMM, 0.094 [-0.112, 0.300]), indicating a general trend of stressor complexity
171 reducing evolutionary rates. Variation in these direct responses was however large (I^2 total
172 mean and 95% compatibility interval; 88.26% [79.95, 94.97]), and mostly due to between-
173 species or between-strain heterogeneity (rather than sampling error), with the between-
174 strain component being smaller (I^2 strains = 31.12% [7.97 %, 75.94 %]; ~ 1/3 of the total I^2)
175 than the between-species (I^2 species = 57.14 % [10.38 %, 85.49 %]; ~ 2/3 of the total I^2).
176 Separate analyses (linear models) conducted on each strain recaptured the same general
177 pattern of smaller fitness increase in multiple stressors than single (lower median values, **Fig.**
178 **S2**) in 13 cases out of 23. Since the fitness measurement in plants differed from that in
179 microbes (S1-2), we also carried out complementary analysis removing the 2 plant species,
180 which led to the same qualitative outcome (**Fig. S6**).



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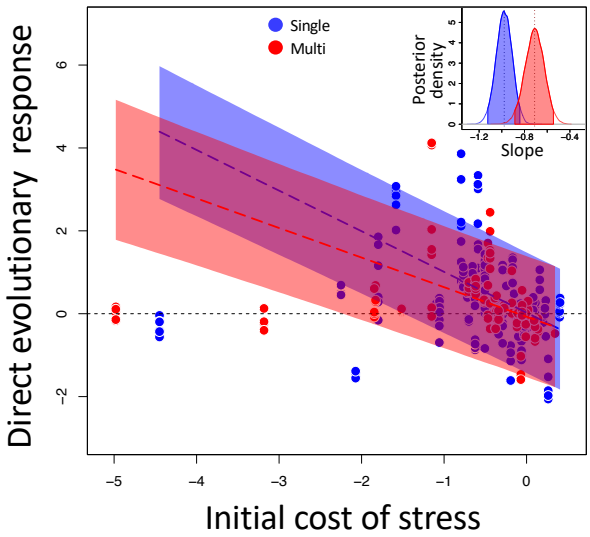
182 **Fig. 2 Posterior distributions of initial fitness, direct and indirect responses.** The dashed lines visualize the
183 median posterior values, and the shaded area the 95% compatibility intervals of the posterior distributions. Note
184 that in a Bayesian framework as used here, increasingly overlapping distributions can be interpreted as little to
185 no effect (or differences), while more separated distributions correspond to stronger effects or differences
186 between treatments. Grey represents the control in the absence of stressors, while blue and red show the single
187 and multiple stressors respectively. (A) Fitness following the initial transfer to the experimental conditions,
188 standardized by centering and reducing within strain. (B) Direct response to selection, assayed in the conditions
189 of experimental evolution, by calculating the difference between final and initial fitness for each treatment. (C)
190 Indirect response to selection, obtained by calculating the difference between the final fitness of populations
191 from the 3 evolutionary treatments when assayed in control conditions, minus the initial fitness in control
192 conditions. The complete dataset (14 species and 23 strains) was used in (A) and (B), and a reduced dataset (11
193 species and 20 strains) in (C).

194 <u>The rule of decline adaptability depended on environmental complexity</u>
195 Consistent with a common observation in experimental evolution, sometimes described as
196 the rule of declining adaptability (42, 43), we found that greater direct evolutionary responses
197 (fitness gain in selective environment) occurred for populations that initially experienced a
198 higher cost of stress (started from lower fitness relative to the control) (**Fig. 3**). This negative
199 relationship between initial cost of stress and fitness gain was strong, and differed between
200 stressor treatments (slope posteriors, insert panel **Fig. 3**), being steeper for single (median
201 and 95% CI slope posterior for Single: -0.979 [-1.123, -0.840]) than for multiple stressor

202 treatments (Multiple: -0.711 [-0.885, -0.544]). This suggests a constraint to adaptation in
203 complex environments that limits the rate of adaptation conditional on the initial
204 maladaptation.



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206 **Fig. 3. The rule of declining adaptability in simple and complex environments.** Relationship between direct
207 response to selection and initial cost of stress (fitness in control – fitness in treatment) and initial fitness, for
208 single (blue) and multiple (red) stressors. Each point represents an independent evolutionary replicate relative
209 to its initial average control (14 species and 23 strains, further details in the Material and Methods). The dashed
210 lines are the median, and the shaded areas the 95% CI of the model predictions.

211 Multiple stressors led to more indirect costs of adaptation

212 We then assayed the fitness of evolved populations under control conditions at the end of
213 the experiment (**Fig. S3**), to investigate costs of adaptation to stressors for 11 out of 14 species
214 (see "Indirect response" column in **Table 1**). We found that evolved populations from single
215 stressor treatments showed a positive change in fitness (difference between final and initial
216 fitness) in control conditions (LMM, 0.141 [0.023,0.259]; **Fig. 2C**), which was comparable to
217 that of control populations (LMM, 0.213 [0.080,0.346]). This indicates that evolution in single-
218 stress conditions did not come at a fitness cost in the absence of stress. In contrast, we
219 detected a cost for evolved multi-stress populations, which showed a tendency for negative
220 change in fitness (LMM, -0.068 [-0.199, 0.063], **Fig. 2C**), and was clearly different from the
221 control (LMM, minimal overlap in 95% CI). The total heterogeneity in these indirect responses
222 closely resembled that for direct response (I^2 total = 87.32% [75.99, 95.57]), with most
223 variation lying between species (I^2 species = 76.92 % [31.83 %, 95.35 %]), and much lower
224 between-strain component (I^2 strain = 10.40% [1.27 %, 49.19 %]). Separate linear models run
225 on each strain revealed the same general pattern of greater fitness decreases in multiple
226 stressors than single (lower median values, **Fig. S4**) in 16 cases out of 20.

227 The relationship between direct and indirect responses varied among stressor types

228 Costs of adaptation can be the result of a trade-off, such that the fitness gain in one
229 environment is proportional to the fitness loss in another (44). Accordingly, we tested for a

quantitative relationship between the direct and correlated responses to selection in our experiment. Working on a subset of 11 species for which both responses were available we found a strong effect of the stressor regimes on their bivariate evolutionary responses (consistent with the small overlap between the red and blue contours in **Fig. 4A**; ΔWAIC= 78395.99 with the model without a treatment effect). We further found an overall positive correlation (median Pearson correlation coefficient and 95% CI: 0.438 [0.431, 0.444]) between direct and indirect responses across single and multiple stressors (**Fig. 4A**), confirming that multiple stressors led to overall weaker fitness across the selective and non-selective environments.

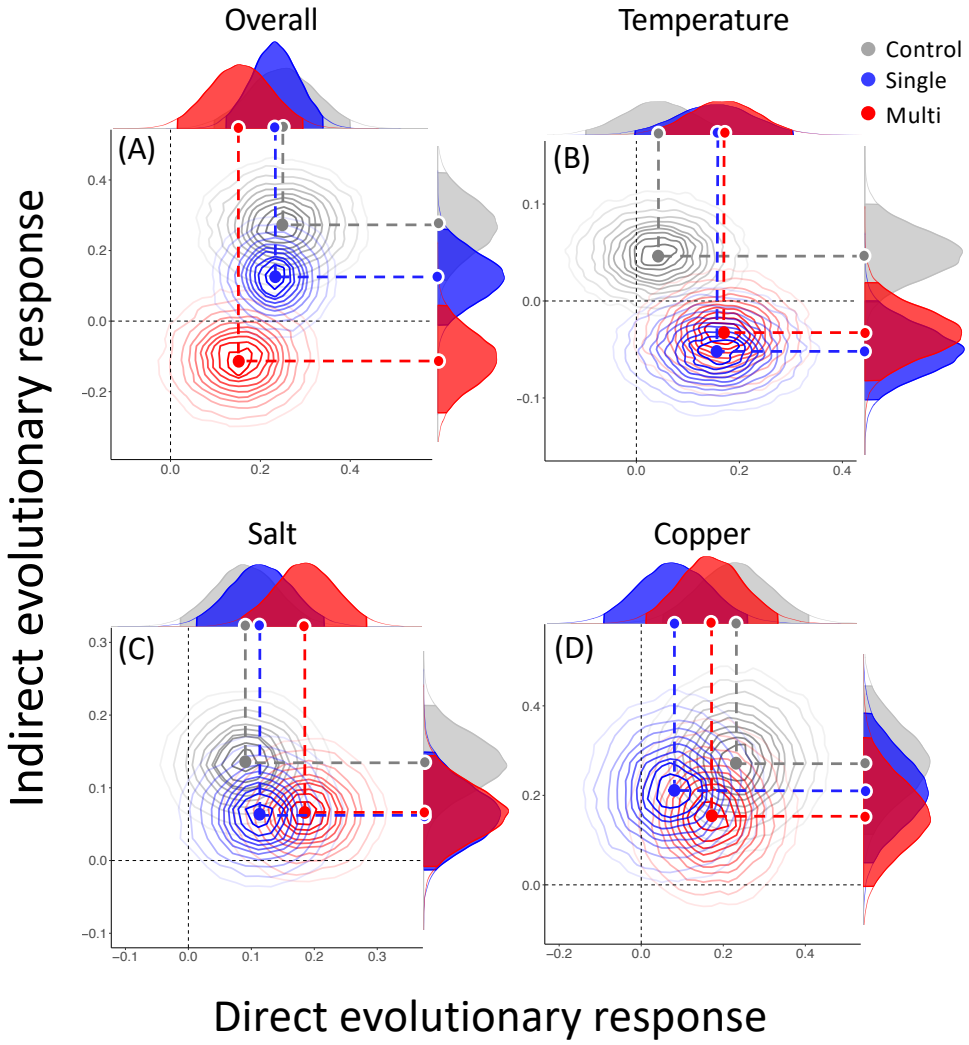

Fig. 4. Joint posterior distributions of the direct (assay in own evolutionary conditions) and indirect (assay in control conditions) responses to selection, for the control, single, and multiple stressor evolutionary treatments. Closed lines are the probability isoclines, spanning from 10% to 95% from darker to lighter. The coloured dots and dashed lines are the median values of the posterior distributions (top- and right-hand side). The overall distribution across stressors (11 species and 20 strains, reduced dataset) is shown in (A), while the next panels show marginal distributions for temperature (B: 8 species and 12 strains), salt (C: 9 species and 13 strains), and copper (D: 6 species and 8 strains). Note that the values of direct and indirect responses in (A) may differ from those in **Fig. 2** because of the reduced dataset used for normalization.

248 To explore whether the nature of the stressors may also play a role in these responses, we
249 conducted additional analysis on reduced datasets associated to the 3 most employed
250 stressors in our study (**Table 1**): temperature, salt and copper. These stressors markedly
251 differed from the overall pattern (**Fig. 4A**), and all had strong impact, regardless of whether
252 they were applied individually or in combination with other stressors (**Fig. 4B-D**; ΔWAIC
253 temperature= 7655.78; ΔWAIC salt= 22150.71; ΔWAIC copper= 19089.98). For temperature,
254 single and multiple stressors led to a similar direct evolutionary response (**Fig. 4B**, LMM Single:
255 0.152 [-0.003, 0.305]; Multiple: 0.168 [0.031, 0.302]), as well as indirect negative response
256 (**Fig. 4B**; -0.051 [-0.102, -0.0001]; -0.033 [-0.082, 0.018]). For salt, we observed a similar direct
257 evolutionary response in the two treatments (0.115 [0.013, 0.215]; 0.184 [0.084, 0.282]), and
258 no difference for indirect responses (0.064 [-0.012, 0.149]; 0.066 [-0.007, 0.144]) as shown in
259 **Fig. 4C**. For copper, single and multiple stressors led to a similar direct evolutionary response
260 (0.080 [-0.090, 0.259]; 0.171 [0.012, 0.333]), and no difference for indirect responses (**Fig.4D**;
261 0.210 [0.048, 0.383]; 0.154 [-0.004, 0.328]).

262 <u>Organismal complexity had little effect on the rate of adaptation</u>
263 Species with higher numbers of protein-coding genes or protein families (as a proxy for the
264 number of functions, and therefore for organismal complexity (45)) might be less constrained
265 in their evolutionary response to multiple stressors. However, we found no signal for an
266 association between direct response to selection and the number of protein-coding genes
267 (after GenBank annotation) in a species, neither for single nor for multiple stressors (LMM,
268 Single: -0.039 [-0.194, 0.118], Multi: 0.007 [-0.146, 0.165]; **Fig. S7A**). We found the same
269 absence of relationship (**Fig. S7B**) when using protein families as explanatory variable (across
270 the 8 species for which this information was available; information from the close relatives
271 *Paramecium tetraurelia* and *Vibrio cholerae* was used for the 2 species *P. caudatum* and *V.*
272 *aestuarianus*): posterior slopes for single and multi were both not different from 0 (LMM,
273 Single median and 95% Compatibility Intervals: 0.042 [-0.224, 0.310]; Multi: 0.022 [-0.224,
274 0.289]).

275 Discussion

276 Understanding how species will adapt to the concurrent presence of multiple stressors is a
277 fundamental open question in evolutionary biology. Using a meta-experimental evolution
278 approach combining different organisms and stressor types and mixtures, we were able to
279 uncover broad and robust patterns regarding adaptation to multiple *vs.* single stressors,
280 decoupling the effect of environmental complexity from that of initial stress.

281 Evolution under multiple stressors led to a smaller fitness gain compared to single stressors,
282 confirming the general prediction that it is more difficult for organisms to adapt to more
283 complex environments. A likely explanation is that pleiotropic interactions limit adaptation
284 more in complex environments (where more traits are likely to be under selection), as
285 empirically found for combination therapy of phages and antibiotics (46). Our results also
286 reinforce findings showing that mixtures of herbicides or antibiotics hindered the evolution

287 of resistance to these compounds. For herbicides, slower adaptation was possible when these
288 were applied in high combined doses (47). For antibiotics, slower bacterial adaptation was
289 found only for certain pathogens (48). Interestingly, resistance evolution in *E. coli* was
290 hindered under combinations of multiple drugs, requiring novel genetic response relative to
291 the application of these same drugs in isolation (49). The resistance evolution was also
292 delayed in *Pseudomonas fluorescens* when it evolved under multiple stressors (combination
293 of antibiotic and predator), compared to one of the two stressors alone (50). On the other
294 hand, our observations are in contrast with previous experimental evolution studies reporting
295 higher fitness (growth rates) in the green alga *Chlamydomonas reinhardtii* (30) and in the
296 copepod *Acartia tonsa* (51) when evolved under multiple stressors. These results were likely
297 explained by the increasing selection intensity with increasing number of stressors in those
298 studies, leading to faster adaptation for the surviving populations, despite higher probability
299 of extinction. Here we strived to limit this effect by having similar stress levels for the single
300 and multiple stressor treatments.

301 The negative relationship that we found between fitness gain and initial fitness is consistent
302 with the "rule of declining adaptability", also found in other experimental evolution studies
303 (29, 42, 43). When observed across time over evolutionary trajectories, this pattern is thought
304 to arise from diminishing returns epistasis, where low-fitness genotypes have access to
305 beneficial mutations with larger fitness gains, and thus adapt faster (52, 53). Such
306 macroscopic or global epistasis, where the fitness effects of mutations only depend on the
307 fitness of the genetic background in which they occur (42, 54), can arise (among other
308 possibilities) from deviations of multiple traits from their optimum phenotype (Fisher's
309 geometrical model of adaptation (12, 55)), caused by either genetic or environmental
310 perturbations (56, 57). Interestingly, Fisher's geometrical model was also at the origin of the
311 prediction of a cost of complexity on the rate of adaptive evolution (12, 13). Initial populations
312 grown in the presence of multiple stressors displayed lower fitness initially (although not
313 formally different from single stressor **Fig. 2A**), and we could have expected to have more
314 evolutionary adaptation overall. However, we detected a different negative slope between
315 multiple and single stressors for the relationship between initial fitness and fitness gain (**Fig.
316 3**). In other words, conditional on the overall stress level (initial fitness decrease) the fitness
317 gain was lower under multiple-stressors evolution, consistent with the predicted constraint
318 to adaptation in complex environments. The fitness gain was however not correlated with the
319 number of coding genes nor protein families (**Fig. S7A-B**), both possible metrics of organism
320 complexity (45, 58).

321 Evolutionary biologists have long been interested in understanding how adaptation to a novel
322 environment affects fitness in other environments where selection did not occur (17, 18).
323 Here, we found that populations that had evolved under multiple stressors tended to exhibit
324 a loss in fitness in control conditions, while those evolved under single stressors showed a
325 fitness increase. This suggests that adaptation to multiple stressors comes at the ecological
326 cost of maladaptation in the ancestral environment. A similar result was documented in the

327 rotifer *Brachionus calyciflorus*, where the loss in fitness in the original environment was
328 greater for the population that adapted to a more complex environment (59). Our results can
329 have implications for pest and pathogen management: not only is it more difficult to adapt to
330 combination treatments (*i.e.*, multiple stressors), but there also is a possible selective
331 disadvantage when these treatments are stopped (costs of adaptation). If general, this
332 pattern could also affect and complicate ecosystem management. If removing (or
333 attenuating) multiple stressors *in situ* potentially leads to loss of fitness because the
334 populations have evolved to adapt to these stressors, such a strategy should be carefully
335 evaluated. A complete removal of all stressors, while leading to a direct increase in
336 demographic performance, might render the population more susceptible to future abiotic
337 and biotic stressors. To inform management decisions, it would therefore be crucial to have
338 some knowledge on how well-adapted the focal population is to its current stressors (or at
339 least for how long it has been exposed to these stressors). Alternatively, stressors could be
340 removed one by one, or sequentially. Using this approach on populations of *B. calyciflorus*
341 that had evolved in the presence of three stressors (temperature, salt and copper), Orr *et al.*
342 (32) showed that removing one of the three stressors led to different consequences:
343 removing the temperature stressor produced fitness increase, whereas the removal of either
344 salt or copper had no substantial impact on fitness.

345 Separate analyses for the most recurrent stressors in our study (*i.e.*, temperature, salt,
346 copper) showed deviations from these general trends (see also **Fig. S5** illustrating the
347 relationship between direct and indirect responses for each strain). Of particular interest in
348 the ongoing global change context is the impact of temperature, as heat-waves and rising
349 temperatures are expected to be a worldwide recurrent stressor affecting all ecosystems (60,
350 61), and they will jointly accompany other stressors (*e.g.*, pollutants, pH, salinity). Mounting
351 evidence is showing that several ectotherm species can respond to thermal selection and
352 evolve their thermal performance curve in the absence of other stressors (62, 63).
353 Experimental evolution studies using temperature in a multiple stressor context are less
354 common, but they seem to indicate strong adaptive potential for increased thermal tolerance
355 in two marine species (30, 51). In contrast, *Drosophila melanogaster* evolved under combined
356 temperature and diet regime showed no heat tolerance adaptation (64). In our study, direct
357 responses were identical in thermally-stressed populations either with or without another
358 stressor, and for direct and indirect responses. Future studies should explore further the
359 impact of stressor identity on the adaptive potential.

360 Another important source of heterogeneity in our results came from the species and strain
361 levels. This highlights another fundamental limit of multiple stressors studies: a single genetic
362 background might not be sufficient to obtain reliable and general results within species (as a
363 third of the variation was explained by the strain variation), and similarly between species
364 within broader taxonomic groups. Thus, in order to understand both short- or long-term
365 responses and adaptation to complex environments it will be crucial to include and consider

genetic variability and inter-specific variation, as also suggested by recent findings on the evolution of thermal (single stressor) in different species and strains of yeast (63).

Perspectives and conclusion

While our study allows general inferences about the evolutionary consequences of environmental complexity, several limitations must be acknowledged. Firstly, natural ecosystems are far more complex than our experimental settings, as organisms often face more than two concurrent stressors (65). The robustness of our results relies on our species sampling, but in the future, such initiative might increase the number of stressors, considering a greater multidimensionality (34). In addition, we only considered constant stress, and it will be important to consider fluctuating environments, pulse occurrence of stressors, sequential stressor exposure (66, 67), and biotic interactions. This would make it possible to study adaptation to even more realistic temporal regimes and magnitude of stressors. Secondly, adaptive genetic evolution can modify the interactive effects multiple stressors have on phenotypes. For instance, initially additive interactions can become antagonistic, or synergistic (32), or be cancelled out with time, and future analysis with tuned experimental design could investigate such cases. Thirdly, adaptive genetic evolution is not the only path to persistence under global change (10). Populations could persist in the face of novel environments also *via* plasticity (68), epigenetic responses (69) - some of which may unfold over many generations, especially in microbes (70) - , as well as dispersal (71). All these aspects and their interactions should eventually be integrated into all global change research frameworks. Finally, although we included data from two plant species representative of larger and longer-lived organisms, our sampling is biased towards microbial species. Nevertheless, bacteria, protists and microalgae are the basis of food webs and will rapidly evolve due to their short generations and fast turnover (59, 72). Their adaptation to multiple stressors has therefore important implications as they might lead to knock-on consequences for other species (73), but also to the whole ecosystem (11). Such facets of multiple stressors are also of concern for public health (74–76), as many pathogens - with even larger population sizes and faster evolution - can alter disease dynamics and the risk of emerging infections (77–79).

Understanding and predicting the consequences of multiple stressors for biodiversity and ecosystem functioning, but also agriculture, public health, and management strategies, remains a pressing challenge (80–83). Our large-scale meta-experimental evolution study highlights the critical need for including evolutionary dynamics into the multiple-stressor research agenda. Beyond demonstrating this proof of principle, we show that meta-experiments can serve as a powerful framework to test predictions, infer generality, and explore system-specific responses across taxa and stressor types.

Material & Methods

<u>Experimental treatments and data collection</u>

406 The evolutionary experiments across all species followed the same rationale and protocol.
407 We used two sources of stress in each experiment, for a total of four treatments: a control,
408 two single stressors applied separately, and a multiple stressor corresponding to the two
409 single stressors combined. For three strains (*Tetrahymena thermophila*, *Euglena gracilis*,
410 *Colpidium striatum*), three single stressors and three multiple stressors (three coupled
411 combinations of the single stressors) were used. Each research group used different stressor
412 types, based on the environmental factors predicted to be the most relevant for their model
413 organism. The precise description of the material and methods and experimental protocols
414 for each species (strain details, stressor levels and/or concentration, number of replicates,
415 generations) are provided in the Supporting Information and in **Table 1**.

416 The levels of the single stressors (within each species) were chosen so that they imposed
417 similar reductions in fitness, quantified by the exponential growth rate of the population as
418 detailed below. For the multiple-stressor treatments, the levels of each individual stressor
419 were adjusted so that the combined impact of multiple stressors was comparable to that of
420 single stressors. During the evolutionary phase, each study organism was propagated for
421 several generations in all treatments, with 3 to 12 independent evolutionary replicates per
422 strain per treatment. Lastly, at the end of the evolutionary experiment, we measured fitness
423 of all 14 species in their own evolutionary treatment, and for 11/14 species also in control
424 conditions.

425 <u>Data inspection and statistical analyses</u>
426 All statistical analyses were conducted in R version 4.2.0 using Bayesian Linear Mixed Models
427 with the "rstan" (version 2.32.7; Stan Development Team, 2025 (84)), "brms" (version 2.22,
428 (85)), and "rethinking" (version 2.21; (41)) R packages.

429 For unicellular organisms, we focused on the exponential growth rate as a fitness estimate.
430 This is an appropriate fitness proxy for populations that have been maintained in near-
431 exponential phase most of their time by frequent dilutions (86), as in the case of our meta-
432 experiment. Firstly, population density was log-transformed and the time series for each
433 individual growth curve was visually inspected to identify the exponential growth phase with
434 linear increase on log scale (shading, **Fig. 1**), as well as to remove potential outliers, and cases
435 in which populations did not grow. Methods that automatically screen growth curves to
436 search for the steepest slope on log scale (exponential growth phase) were also tested (e.g.,
437 growthcurver (87), rstan model fitting (88), LoLinR (89)), but discarded as more prone to
438 spurious results. After this screening phase and prior to model fitting, both log-density and
439 time were standardised by centering and reducing across treatments, within each strain of
440 each species, allowing comparison of the influence of treatments among species with
441 markedly different growth rates. For plants, different approaches and protocols were used,
442 all of which are described in Supporting Information Text (S1-2).

443 We fitted Bayesian linear models to the identified exponential growth phase, and estimated
444 the exponential growth rate (r_0) as the slope of log abundance against time. Models were
445 fitted to the most relevant biological level, namely the replicate experimental evolution line.
446 When the data also included technical replicates (repeated measurements of the same
447 experimental line), these were treated as a random-effect term in the analysis. We used
448 vaguely informative priors for the intercepts and slope parameters (normal and half-normal
449 distribution, respectively), with mean 0 and standard deviation 1. The posterior distributions
450 for the slope parameter provided standardized estimates of the intrinsic growth rates r_0 (**Figs.**
451 **S1 and S3**). In the follow-up analyses, we also included the associated error (standard
452 deviation) of the slope posteriors to effectively weight fitness effect sizes by study precision
453 (42). Such weighting procedure is very conservative, leading to a particularly cautious
454 interpretation of the results.

455 Using the obtained fitness estimates, we conducted six main analyses. In all analyses we
456 included "Species ID" and "Strain ID" as random effects, to take into account that different
457 strains belonged to the same species, and different replicates to the same strain. Following
458 the results of Cinar *et al.* (90), we did not explicitly include the phylogeny in the model. In fact,
459 under weak phylogenetic signal as in our study – with a mean phylogenetic matrix correlation
460 of 0.27 – the model would struggle to separate non-phylogenetic and phylogenetic species
461 effects. The two variability components would collapse into one, reducing the model
462 reliability (91). Thus, in such a context, including only the species level random effect is
463 desirable, and still provides conservative results. The stressor identity was also not included
464 as a random effect because some stressors, such as antibiotics, herbivory and shade, were
465 used in one species only (**Table 1**), leading to divergent transitions and models not converging.
466 In additional tests we evaluated the heterogeneity (I^2) of direct and indirect responses using
467 posteriors of the variances (σ^2) (91, 92). The posteriors were directly extracted from the
468 models for all levels (strain and species), and we calculated I^2 with 95% compatibility intervals
469 for each random effect following Viechtbauer (93). These analyses capture the relative
470 amount of variance due to true effects rather than sampling error, once the fixed effects are
471 taken into account.

472 For the main analyses, we firstly investigated direct responses to selection on the complete
473 dataset (14 species, 23 strains) using a linear multilevel model (mixed effect). We asked
474 whether the change in fitness between the initial and final time point, assayed under the
475 same conditions under which experimental evolution took place, was different in the control,
476 single, or multiple stressors (6 explanatory factors: 3 treatments x 2 time points). The initial
477 fitness for single and multiple stressors was standardized by taking the difference with the
478 corresponding initial control.

479 Secondly, we explored whether the final change in fitness for direct response was related to
480 the initial cost of stress, and whether this relation was affected by the stressor treatment,
481 using a linear multilevel model. For the initial cost of stress, we averaged the initial fitness by

482 strain (separately within single and multiple stressor treatments), from which we subtracted
483 the average initial fitness of the control. For the evolutionary change in fitness, we took the
484 difference between the final and initial fitness estimate of each evolutionary replicate. The
485 associated error was always propagated at each step, and the weighting procedure described
486 above was propagated here on both responses and explanatory variables.

487 Thirdly, we analysed indirect responses to selection, defined as the change in fitness under
488 experimental evolution with stressors assayed under control conditions. We used the same
489 model structure as for direct responses, and for each evolved population (under control,
490 single or multiple stressor conditions) we compared its fitness in control conditions at the end
491 of the experiment against the initial fitness in control conditions (4 explanatory factors: Initial
492 Control, Final Control, Final Multiple in control, and Final Single in control). This analysis was
493 conducted on a reduced dataset (11 species, 20 strains) for which such measurements in the
494 absence of stressors were taken at the end of experimental evolution.

495 Fourthly, we merged the data for direct and indirect responses, focusing on the reduced
496 dataset (11 species, 20 strains). After centering and reducing within strain, we refitted
497 Bayesian linear models to estimate the exponential growth rate (r_0), and have all data on the
498 same scale. Thus, the obtained estimates for the control treatment were the same for direct
499 and indirect response, apart from *A. thaliana*, *D. salina*, and *V. aestuarianus*. The
500 measurements of the control treatments for these 3 species varied between direct and
501 indirect responses due to specific protocol handling and experimental design. Following the
502 same procedure as for the first and second analysis, we investigated direct and indirect
503 response using linear multilevel model (2 analyses). We then fitted bivariate multilevel
504 models to the posterior distributions (change in fitness) obtained from the direct and indirect
505 analyses. We only considered the single- and multiple-stressor treatments. We compared the
506 intercept model with a model having treatment as explanatory factor (*i.e.*, models with *vs.*
507 without effects) using WAIC weights (94). We obtained the relative importance (RI) of the
508 evolutionary treatment explanatory variable by averaging the posterior predictions of the two
509 models. We additionally performed a Pearson correlation analysis across single and multiple
510 evolutionary treatments.

511 Fifthly, we repeated each of the analysis above with respect to specific stressors that were
512 recurrent across our experiments (**Table 1**), namely temperature (x8 species and x12 strains),
513 salt (x9 species and x13 strains), and copper (x6 species and x8 strains).

514 Lastly, we investigated with linear multilevel models whether changes in fitness for direct
515 responses, calculated as described above, could be explained by species complexity, as
516 approximated by the number of protein-coding genes (after GenBank annotation), or the
517 number of protein families (after Pfam annotation, (45)). For *Colpidium striatum*, no data
518 were available for protein-coding genes, and it was therefore excluded for this analysis (x13
519 species and x22 strains). Data for C*olpidium striatum*, *Dunaliella salina*, *Euglena gracilis*, and

520 *Picochlorum costavermella* were not available for the number of protein families, and they
521 were removed from this analysis (10 species). For *Paramecium caudatum* and *Vibrio*
522 *aestuarianus* data were also not available, and we used the 2 closely related species,
523 *Paramecium tetraurelia* and *Vibrio cholerae* respectively.

524 In complementary analyses we additionally tested direct, indirect response and their
525 relationship for each strain running the statistical analyses described above with no random
526 effects (**Figs. S2, S4, and S5**).

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536 Author contributions

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548 Competing interests

549 The authors declare no competing interests.

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Supporting Information for

Environmental complexity constrains evolutionary adaptation across taxa

Giacomo Zilio[1,2,*], Emilie Aubin[3], Stéphanie Bedhomme[1], Laurence Bolick[4], Ignacio G. Bravo[5], Claude Bruand[6], Delphine Capela[6], Marie Challe[2], Guillaume M. Charrière[7], Gaelle Courtay[7], Marie-Ange Devillez[2], Fiona Elmaleh[7], Stanislas Fereol[1], Rémy Froissart[5], Justina Givens[2], Claire Gougat-Barbera[2], Lynn Govaert[4], Alice Guidot[6], Jeanne Hamet[1], Michèle Huet[3], Philipp Hummer[1], Staffan Jacob[3], Oliver Kaltz[2], Marc Krasovec[8], Martin Guillaume[2], Delphine Legrand[3], Thibault Nidelet[9], Denis Orcel[2], Hervé Philippe[3], Gwenael Piganeau[8], M. Stefania Przybylska[1], Philippe Remigi[6], Laurent Sauviac[6], Fabian Schneider-Nettsträter[4], Diego Segond[9], Celine Serre[9], Delphine Sicard[9], João Gabriel Colares Silveira[2], Jeanne Tonnabel[2], François Vasseur[1], Apolline Vedrenne[9], Edith Vidal[8], Cyrille Violle[1], Marielle Kathrin Wenzel[4], Emanuel A. Fronhofer[2,*], Luis-Miguel Chevin[1,*]

[1] CEFE, Univ Montpellier, CNRS, EPHE, IRD, Montpellier, France
[2] ISEM, Univ of Montpellier, CNRS, EPHE, IRD, Montpellier, France
[3] Station d'Ecologie Théorique et Expérimentale, CNRS, UAR 2029, Moulis 09200, France
[4] Leibniz Institute of Freshwater Ecology and Inland Fisheries, Berlin, Germany
[5] MIVEGEC, Univ Montpellier, CNRS, IRD, Montpellier, France
[6] Univ Toulouse, CNRS, INRAE, LIPME, Castanet-Tolosan, France
[7] IHPE, Univ Montpellier, CNRS, IFREMER, Univ Perpignan Via Domitia, Montpellier, France
[8] Sorbonne Université, Microbial Biodiversity and Biotechnology Laboratory (UMR 8176), University of Perpignan, CNRS, Banyuls sur Mer, France
[9] SPO, Univ Montpellier, INRAE, Institut Agro, Montpellier, France

*Corresponding authors: giacomo.zilio@umontpellier.fr / gcm.zilio@gmail.com, luis-miguel.chevin@cefe.cnrs.fr, emanuel.fronhofer@umontpellier.fr

Supporting Information Text and Tables

S1 *Arabidopsis thaliana*

Authors

François Vasseur, M. Stefania Przybylska, Cyrille Violle

Study organism

Arabidopsis thaliana is a small plant from the mustard family (Brassicaceae) widely distributed throughout Europe, Asia, and North America (1). At maturity, *A. thaliana* can reach 10 to 50 cm in height, and can produce a variable amount of seeds, from a few dozen up to more than 5000 seeds. Due to its short life cycle (~ 6 weeks), ease of manipulation, and well-characterised genome, *A. thaliana* is an established model species in several research fields from genetics and molecular investigations, biochemistry, physiology, to ecology and evolution (2, 3). *A. thaliana* can rapidly respond to selection (4), and its evolutionary history, as well as its response to environmental variables, are well studied (5).

Experimental setup and treatments

Before the beginning of the evolution experiment, we created 350 recombinant lines (F2 lines) from random crosses between 358 wild accessions spanning a large range of genetic, geographic, and phenotypic variation (6). Around 2500 seeds per cross were pooled to be used

In November 2017, we set up an experimental evolution project (AraBreed project) in the experimental field of Centre d'Ecologie Fonctionnelle et Evolutive (CEFE), Montpellier, France (43° 38' 18" N, 3° 51' 44" E; Fig. S8A). 28 closed mesocosms with 1.2 m length x 1.2 m width x 1 m height were built in seven rows (Fig. S8A, B). Mesocosms were 1.5 m apart and contained entirely, both belowground and aboveground, in an insect-proof veil. During their installation, we removed the top 30 cm of soil (calcareous clay soil), steam sterilized it to prevent seed bank sprouting, mixed it with blond peat, and placed it back within the mesocosms. Mesocosms were assigned to four evolutionary treatments (Fig. S8A, C): resources single stressor (low Resources/ low Herbivory; R-/H-), herbivory single stressor (R+/H+), multiple resources and herbivory stressors (R-/H+), and a control without stressors (R+/H-). Treatment assignment was random but ensuring that each row of mesocosms contained one replicate of all four treatments (Fig. S8A, C; 4 treatments × 7 replicates). At the end of November 2017, we placed approximately 17500 randomly selected F2 seeds in each mesocosm in a density of around 1.2 seeds/cm2.

Rearing protocol

From 2018 to 2020, we let plant generations spontaneously grow in the mesocosms, with spontaneous seed establishment and germination. In this period, from January to June (*i.e.*, the season at which *A. thaliana* generally grows and sets seeds), we applied the treatments

to the mesocosms and we harvested fruits for seed extraction. We watered and fertilized mesocosms assigned to high resources (R+) with commercial organic fertilizer twice per week. Low resource (R-) mesocosms received no applications of water or fertilizer. In the same period, we introduced intermittently snails (*Helix aspersa*, a generalist herbivore) into the mesocosms assigned to high herbivory (H+). Meanwhile, we sprayed mesocosms assigned to low herbivory (H-) with large-spectrum insecticide every three weeks. Regarding fruit harvest, every three weeks, we harvested one fruit per individual located in different parts of the mesocosm, for a maximum of 100 fruits per mesocosm. We pooled fruits of a given mesocosm in a given year and extracted seeds, which were then stored in air dry condition at 4°C.

Final phenotypic tests and data collection

Population size as direct responses to selective environment

Using pictures of each mesocosm taken in March 2018-2022, the month that roughly corresponded to the peak of vegetative growth, we estimated population size in all 28 mesocosms. For that, we counted the number of plants in the images using the cell counter plug-in in ImageJ.

To obtain the fitness estimates for each mesocosm, we fitted Bayesian linear models to the log-transformed population size data. We used vaguely informative prior (normal distribution), with mean 0 and standard deviation 1. Since populations evolved with natural recruitment, comparing the size of the first generation (G1) to the number of seeds sown initially (G0) was not relevant to estimate initial fitness. We thus calculated the difference in fitness between G2 and G1, and G5 to G4, and considered these values as initial and final fitness, respectively. These fitness estimates were used in the main analyses (direct responses) together with their corresponding standard deviation, which was included as measurement error in the follow-up statistics to effectively weight fitness effect sizes by precision. The description of the main analyses with references of the statistical software and packages are provided in the main text.

Common garden and indirect responses in non-selective environment

We performed a completely randomized common garden experiment between February and July 2021 under shade cloth in the experimental field of Centre d'Ecologie Fonctionnelle et Evolutive (CEFE), Montpellier, France (Fig. S8D; (7). Pots of 0.08 L were filled with a sterilized soil mixture composed of 50 % river sand, 37.5 % calcareous clay soil from the experimental field at CEFE, and 12.5 % blond peat moss. Such conditions represented a non-selective environment for the evolutionary lines, further allowing control for the confounding effect of phenotypic plasticity (8). As detailed below, the data obtained during this common garden were used as indirect fitness responses in the main analyses. 630 individuals were planted in separate pots organized in three blocks: 198 individuals from the same pool of F2 seeds that were used to found populations in the mesocosms (G0), and 432 individuals from the third generation of evolution (G3) in the four treatments (108 individuals × 4 treatments). At the end of the reproduction phase (fruit ripening), inflorescences were cut and photographed. On

inflorescence pictures, all fruits were counted and the length of three fruits per plant were measured using ImageJ. To obtain fitness estimates, we then considered the number of fruits produced per plant weighted by their length (number of fruits * mean fruit length). We fitted Bayesian linear models to the log-transformed fruits' metric using vaguely informative prior (normal distribution), with mean 0 and standard deviation 1. Estimates for G0 and G3, corresponding to the initial and final fitness, were used in the main analyses (indirect responses) together with their standard deviation (measurement error, as described in the population size paragraph above). The main analyses, and references on statistical software and package, are detailed in the main text.

S2 *Brassica rapa*

Authors

Jeanne Tonnabel, Denis Orcel, Marie Challe, François Vasseur, Cyrille Violle

Study organism

Our experimental evolution lines were established using the *Brassica rapa* Wisconsin Fast Plant (WFP) line (Carolina Biological Supply Company, Burlington, North Carolina, USA). This line is particularly well suited for experimental evolution studies, as it was derived from a broad sampling of natural *B. rapa* populations that subsequently underwent artificial selection for a short generation time (~8 weeks), while retaining considerable genetic and phenotypic variation (9, 10). *B. rapa* is a hermaphroditic, insect-pollinated annual herb in the Brassicaceae family. Like other members of the genus, it possesses a genetic self-incompatibility system (11, 12), although this mechanism is leaky, allowing variable levels of self-fertilization. Such incomplete self-incompatibility has been observed in the WFP line as well, where selfing has evolved under experimental evolution conditions (13). Pollination in our experiment was performed by the fly *Phormia terraenovae*, which did not evolve during the course of the study. This species had been validated as an effective pollinator of *B. rapa* in a preliminary experiment (Tonnabel, pers. comm.) and was reared from larvae obtained from a commercial supplier of fishing bait (https://appats-michel.fr/).

Experimental evolution phase

Experimental setup, treatments, and growing protocol

To initiate the experimental evolution populations, plants first underwent three to four generations of selection under strong polygamous mating in five replicate populations. In each generation, seeds were collected from 85 parental plants, each of which had three flowers pollinated with mixed pollen derived from 12 anthers per plant pooled across all 85 individuals. Seeds from the three pollinated flowers of each plant were combined to produce the next generation. The present study used seeds from the third and fourth generations of these lines, hereafter referred to as the 'source population'.

Our protocol followed standard experimental evolution procedures, in which natural selection acts on standing genetic variation present in the source population (14). We subjected this initial standing genetic variation to four treatments: light deprivation (single stressor), nutrient deprivation (single stressor), the combination of both stressors (multiple stressor) and a control (no stressor). Experimental populations were grown in a greenhouse arranged in a 4 × 5 grid. Because of a potential light gradient in the greenhouse, population positions were randomized following specific rules: (i) each row (corresponding to a given distance from the external glass wall) contained one population from each treatment; (ii) treatment assignments were rotated across positions at each generation; and (ii) each population was relocated to a different position between generations.

Using well-mixed seeds from the source population, we established 20 experimental populations, randomly assigning five populations to each of the four treatments in the first generation. All experimental populations were cultivated in a single greenhouse at the CNRS Experimental Plateform in Montpellier. Each population was grown in an individual 80 x 80cm tray containing 100 plants arranged in a 10x10 grid, with individuals spaced 7 cm apart. Two seeds were sown per position, to allow estimation of germination rate based on the absence of plants at a given position, which was later incorporated into individual fitness estimates. After two weeks, when both seeds germinated at a given position and none at other positions, one seedling was transplanted elsewhere within the same population to maximize the total number of plants while retaining a single seedling per position. Germination rate was calculated as the total number of seedlings divided by the total number of seeds sown.

All populations were grown in sterilized, nutrient-poor soil composed of 2/3 sand and 1/3 compost. During the first three weeks of growth, liquid nutrients were supplied at watering to temporarily restore nutrient-rich conditions and allow plants to establish before flowering. We used a liquid fertilizer containing nitrogen, phosphorus and potassium (Universal Liquid Fertilizer, StarJardin). Each population received weekly 10L watering with a concentration of 5mL/L of liquid fertilizer. After this initial period, nutrient availability was manipulated by varying the concentration of liquid fertilizer: 5mL/L in the control and light-deprivation treatments to maintain nutrient-rich conditions, and 2.5mL/L in the double-stress treatment.

At flowering onset, each population tray was enclosed within a mesh cage. A paper cup containing approximately 100 *P. terraenovae* larvae and a glass of sugary water covered with fine mesh (allowing access to the solution while preventing drowning) were placed inside each cage. The high abundance of pollinators ensured that none of the populations experienced pollen limitation. Because pollinators were confined to their respective populations, reproduction occurred independently in each population, preventing any gene flow among them. Pollinator densities were visually monitored and adjusted as needed by adding larvae to maintain similar numbers across populations until all plants had senesced.

Data collection

<u>Seed mass as direct responses to selective environment</u>

At the end of each generation, when plants had died, we harvested every individual, counted its total number of fruits, and stored the fruits separately by plant. Seeds were then manually extracted, and total seed mass was measured using a precision balance (Sartorius, Entris II 220g/0.1mg).

We measured the total seed mass for all plants that germinated in each experimental population. To obtain fitness estimates at the population level (20 independent evolutionary replicates), we fitted Bayesian linear models to the log-transformed seed mass using vaguely

informative prior (normal distribution), with mean 0 and standard deviation 1. The individual plant identity, for which the seed mass was calculated, was included as random term. We obtained estimates of averaged seed mass for first and third generation, corresponding to the initial and final fitness, were used in the main analyses (direct responses) together with their standard deviation (error used in the follow-up statistics to fitness effect sizes by precision). The main analyses, and references on statistical software and package, are detailed in the main text. To verify that total seed mass provided a reliable proxy for seed number, we randomly chose 30 plants per population and counted all produced seeds - or a maximum of 30 fruits for highly fecund individuals - before weighing them at the end of the first generation. A linear regression of total seed number on total seed mass confirmed that seed mass is a reliable predictor of seed production (linear model estimate = 601.4 ± 15.7 SD; $t = 38.4$, $p < 0.0001$; $R^2 = 0.73$).

Seeds from all plants within a population were bulked to establish the next generation, ensuring that the contribution of each individual to the next generation reflected its reproductive success. Each experimental population thus evolved independently under its assigned treatment. In total, three generations of evolution were completed for the 20 experimental populations, corresponding to approximately 6000 plants in total. The fitness assessments took place between October 2023 and January 2024, February and May 2024, and October 2024 and January 2025, respectively.

226 **S3 *Colpidium striatum***
227
228 **Authors**
229 Lynn Govaert, Laurence Bolick, Fabian Schneider-Nettsträter and Marielle Kathrin Wenzel
230
231 **Study organism**
232 Freshwater ciliates are well suited organisms for addressing ecological and evolutionary
233 questions, they have high population densities, short generation times, are easy to maintain,
234 and the microcosms they live in are easy to manipulat (15). *Colpidium striatum* is commonly
235 found in freshwater and frequently used in microcosm experiments(15). An original culture
236 of *Colpidium striatum* was purchased at Carolina Biocompany and maintained for several
237 years in the laboratory kept in 250-mL autoclaved erlenmeyers containing two wheat seeds
238 and filled with 100 mL of autoclaved protozoan medium maintained under standard
239 laboratory conditions (20°C and 24h light condition). Since November 2021, protozoan
240 medium consisted of Volvic water Naturelle with 0.40 g/L alfalfa powder (A-4363-BIO
241 Piowald.com) and fed with the bacteria *Serratia marcescens*. From this culture, a single cell
242 was taken and washed to start a monoclonal culture. Throughout the experiment, *Colpidium*
243 *striatum* reproduces asexually.
244
245 **Experimental evolution phase**
246 <u>Experimental setup and treatments</u>
247 At the end of June 2023, an experimental evolution experiment was started using a clonal
248 culture of *C. striatum*. Initially, a single cell was isolated from a genetically diverse stock
249 culture maintained under standard laboratory conditions (*i.e.,* 20°C and 24h light condition)
250 and grown to a dense population. Then, triplicates of this population were exposed to an
251 increased salinity, copper concentration and temperature as well as all pairwise combinations
252 of these stressors. Specifically, triplicates were kept as a control (0 g/L NaCl, 0 µg/L $CuSO_4$,
253 20°C), exposed to increased temperature (0 g/L NaCl, 0 µg/L $CuSO_4$, 24°C), increased salinity
254 (3 g/L NaCl, 0 µg/L CuSO4, 20°C), increased copper concentration (0 g/L NaCl, 200 µg/L
255 CuSO4), or exposed to an elevated level of each two-stressor combination (Table S3.1). In
256 total, these were 21 cultures that were maintained for one year. The experimental evolution
257 populations were transferred into new medium at a 3-week interval using autoclaved 250-mL
258 Schott bottles with two wheat seeds, which were maintained in a 24h-light environment and
259 were fed with the bacteria *Serratia marcescens*.
260 The concentrations of salt, copper and temperature were chosen based on previous pilot
261 experiments where we observed extinction of *C. striatum* when exposed to 4 g/L NaCl (when
262 not prior acclimated) and showed a large fitness reduction at 26°C (16). In addition, the
263 combination of 3 g/L NaCl and 26°C previously indicated a high risk for population extinction
264 (16).
265 To track the population growth of the ciliates, the cell density was measured by taking videos.
266 For this, the bottles were sampled (250 µL) daily for 8 days, then every 2nd day for another

267 five timepoints. The videos were taken with an Olympus SZX16 stereo-stereomicroscope (1.6-
268 fold magnification) and Hamamatsu Orca Flash 4 video camera. 10 second videos were
269 recorded of an effective sample volume 36.125 µL (height 0.5 mm). The videos were
270 processed with the software ImageJ (version 1.46a) and an adapted version of the BEMOVI R
271 package (17) to extract information on density.
272
273 **Table S3.1** Evolutionary treatments and number of replicates at the start and end of the experiment.

Strain ID	Salinity (g/L)	Copper (µg/L)	Temperature (°C)	Stress	Replicates start/end
C. striatum	0	0	20	Control	3/3
C. striatum	0	0	24	Single Temperature	3/3
C. striatum	3	0	20	Single Salinity	3/2
C. striatum	0	200	20	Single Copper	3/3
C. striatum	3	0	24	Multi S x T	3/0
C. striatum	3	200	20	Multi S x C	3/2
C. striatum	0	200	24	Multi C x T	3/3

274
275 Rearing and transferring protocol
276 Every three weeks, 20% (*i.e.*, 20 mL) of the experimental evolution populations were
277 transferred into new medium using autoclaved 250-mL Schott bottles with two wheat seeds
278 and filled with 80 mL autoclaved protozoan medium (i.e., autoclaved Volvic water and 0.4 g/L
279 alfalfa powder) and were placed in incubators at the respective treatment temperatures with
280 full 24h-light environment. Cultures were fed with the bacteria *Serratia marcescens*. Given
281 that *C. striatum* has an average generation time of 16h, this resulted on average in 32
282 generations between transfers.
283
284 **Final phenotypic tests and data collection**
285 Mid-July 2024, a common garden experiment was started to test for evolutionary changes in
286 the evolved *Colpidium* populations from the different stressor environments. Specifically,
287 each of the populations evolved in a stressor environment was measured in the control
288 environment (i.e., 0 g/L NaCl, 0 µg/L $CuSO_4$ and 20°C) and their original stressor environment.
289 Additionally, the populations evolved in the control environment were measured in all the
290 stressor environments and in the control environment (see Table S3.2).
291
292 **Table S3.2** Overview of selection cultures and common garden environment condition used in the common
293 garden experiment.

		Environment the existing culture needs to go in (common garden environment)					
		Control	Single salinity	Single copper	Single temperature	Multi S x C	Multi T x C
Existing culture (selection environment)	Control	x	x	x	x	x	x
	Single salinity	x	x				
	Single copper	x		x			
	Single temperature	x			x		
	Multi S x C	x				x	
	Multi T x C	x					x

294

295 For the common garden experiment, we used evolved populations of the following stressor
296 combinations (Table S3.2): three replicates of the control, two replicates of the high salt
297 environment (single salinity), three replicates of the high copper environment (single copper),
298 three replicates of the high temperature environment (single temperature), three replicates
299 of the combined high salt and high copper environment (multi salt x copper), and two
300 replicates of the high temperature and high copper environment (multi temperature x
301 copper). Unfortunately, populations from the high temperature and high salinity environment
302 went all extinct by the end of the experimental evolution phase. In total, we had 16 uniquely
303 evolved populations.
304
305 From each of these populations 10 individuals were washed by passing each individual with
306 minimum of liquid through 5 drops of autoclaved protozoan medium to reduce bacterial load
307 coming from the selection cultures. The washing of the individual cells was done using the
308 protozoan medium of their respective stressor environment. Each of the 16 evolved
309 populations was triplicated and exposed to their respective stressor environment and to the
310 control (as described earlier), resulting in a total of 132 experimental units. Experimental units
311 consisted of autoclaved 250-mL Schott bottles with two wheat seeds and were filled with 3
312 mL of the corresponding selection culture (cultured for 1.5 weeks in their respective stressor
313 treatment) and with 97 mL of the corresponding protozoan medium with salinity and copper
314 concentrations modified such that adding the selection culture resulted in salinity and copper
315 concentrations of 3 g/L NaCl and 200 µg/L $CuSO_4$, respectively (Table S3.3). Because
316 transferring the selection cultures into their common garden environments brings additional
317 medium with it, the control condition was set to a concentration of 0.093 g/L NaCl and 6.186
318 µg/L $CuSO_4$, which is known to not have any effects on the ciliate densities from prior
319 experience. For temperature treatments, autoclaved protozoan medium of the respective
320 salinity and copper concentrations was placed in a 24°C incubator to obtain the correct
321 temperature. All autoclaved protozoan medium was enriched with a 5% 36-h bacterial culture
322 of *Serratia marcescens* reflecting an addition of 24,338,256 CFU/mL. Experimental units were
323 given a random ID and placed in either 20 or 24°C incubators depending on the temperature
324 treatment and maintained in a full 24h light environment.
325 To track the population growth of the ciliate populations during the common garden
326 experiment, the cell density was measured by taking videos. For this, we took 150 µL daily for
327 9 days, then 2 days no sample followed and then by every 2 days for three timepoints. The
328 last samples were taken another three days later (in total three weeks). The videos were taken
329 and processed as previously described.
330
331
332
333
334
335

Table S3.3 Overview of salt and copper concentrations for the common garden experiment.

selection environment	common garden environment	Total volume [L]	Culture volume [L]	Medium volume [L]	Salt conc. culture [g/L]	Salt conc. medium [g/L]	Copper conc. culture [µg/L]	Copper conc. medium [µg/L]	Salt conc. end [g/L]	Copper conc. end [µg/L]
control	Control / Single temperature	0.1	0.003	0.097	0	0.093	0	6.186	0.093	6.186
	Single salinity	0.1	0.003	0.097	0	3.093	0	6.186	3	6.186
	Single copper / Multi T x C	0.1	0.003	0.097	0	0.093	0	206.186	0.093	200
	Multi S x C	0.1	0.003	0.097	0	3.093	0	206.186	3	200
Single salinity	Control	0.1	0.003	0.097	3	0	0	6.186	0.093	6.186
	Single Salinity	0.1	0.003	0.097	3	3	0	6.186	3	6.186
Single copper	Control	0.1	0.003	0.097	0	0.093	200	0	0.093	6.186
	Single copper	0.1	0.003	0.097	0	0.093	200	200	0.093	200
Single temperature	Control	0.1	0.003	0.097	0	0.093	0	6.186	0.093	6.186
	Single temperature	0.1	0.003	0.097	0	0.093	0	6.186	0.093	6.186
Multi S x C	Control	0.1	0.003	0.097	3	0	200	0	0.093	6.186
	Multi S x C	0.1	0.003	0.097	3	3	200	200	3	200
Multi T x C	Control	0.1	0.003	0.097	0	0.093	200	0	0.093	6.186
	Multi T x C	0.1	0.003	0.097	0	0.093	200	200	0.093	200

339 **S4 *Dunaliella salina***

340

341 **Authors**

342 Philipp Hummer, Stanislas Fereol and Luis-Miguel Chevin

343

344 **Study organism**

345 *Dunaliella salina* is a well-studied halophile unicellular green alga due to its extreme
346 physiology. It is one of the most salt-tolerant eukaryotes, and is the main primary producer
347 in hypersaline environments (18, 19). It is commonly found in salt lakes, evaporation ponds
348 of salterns, hypersaline coastal waters and salt marshes worldwide (20). *D. salina* can also
349 withstand wide ranges of environmental stresses such as temperature (from -35°C to +40°C)
350 and nitrogen content (20, 21). In standardised laboratory conditions at 24°C and 12/12
351 dark/light cycle (200 µmol m^{-2} s^{-1} light intensity), it reproduces asexually with a growth rate
352 of ~ 1 generation per day (19).

353

354 **Experimental evolution phase**

355 Experimental setup and treatments

356 To start our experiment, we used 4 strains isolated from salterns in southern France
357 (clonal/isogenic strains; for details see Table S4.1) and their combination (mixed strain;
358 standing genetic variation). The mixed strain was established by mixing equal densities of the
359 four different strains. We call these 5 genetic backgrounds "strains" (including the mixed
360 strain) for simplicity.

361

362 **Table S4.1** Coordinates and additional information on the sampling sites.

Site	Strain ID	Saltern	Site name	Latitude	Longitude	Salinity (in g/l)	Salinity (in M)	Mean salinity (in g/l)	Mean salinity (in M)
S1	S1	Gruissan	Tables Gruissan	43.096087	3.086734	280	4.8	280.00	4.8
S3	S3	Gruissan	Lac	43.086220	3.091718	120	2.1	80.00	1.4
S11	S11	La Palme	Nord-Ouest	42.973766	3.028587	130	2.2	133.33	2.3
S13	S13	La Palme	Reprise	42.976025	3.032964	200	3.4	176.67	3.0

363

364 *D. salina* strains were cultivated in flasks, using artificial seawater complemented with NaCl
365 and nutritive F/2 medium, the required salinity was obtained by mixing hypo- (0M) and hyper-
366 saline (4.8M) solutions (Table S4.2). Before being introduced in the evolutionary treatments,
367 the strains were maintained under favourable laboratory conditions of 2M salinity and 24°C.
368 The carrying capacity of each strain was measured (approximately 470k cells/mL for S1, 940k
369 for S3, 830k for S11 and 770k for S13) and adjusted to start populations at similar densities.
370 The measurements of cell concentration during all experimental phases were obtained with
371 the Guava easyCyte HT flow cytometer and the corresponding software guavasoft 3.4 by
372 Luminex. An established pipeline (22) allowed us to discriminate between live and dead *D.*

373 *salina* cells, as well as other particles on the basis of forward scatter (FSC), side scatter (SSC)
374 and Red and yellow Fluorescence.
375
376 **Table S4.2** Artificial seawater composition for a total of 1l of water. The two solutions SS1 and SS2 were prepared
377 separately to avoid unwanted chemical reactions during autoclaving. 20 ml of Guillard's (F/2) Marine Water
378 Enrichment Solution were added before use.

Solution	Compound	Added mass [g] of the compound
SS1 (0.9l)	NaCl (only for the hypersaline solution)	280.512
	Na_2SO_4	3.55
	KCl	0.599
	$NaHCO_3$	0.174
	KBr	0.0863
	H_3BO_3	0.023
	NaF	0.0028
SS2 (0.1l)	MgCl, 6 H2O	9.592
	$CaCl_2$, 2 H2O	1.344
	$SrCl_2$, 6 H2O	0.0218

379
380 For each strain, we established 4 evolutionary treatments in 3 replicates (Table S4.3) by
381 manipulating temperature and salinity. In the control non-stressful treatment, we used 24°C
382 and 2M salinity, in line with previous work in the group (*e.g.*, Leung et al. 2020). For the 3
383 stressful treatments (salinity stress, temperature stress, and the combination of the two),
384 pilot experiments indicated that a temperature of 36°C and salinities of 3.5M or 4M NaCl
385 would cause fitness reduction with little risk of population extinctions.
386 For each strain, the experimental flasks were set up at different salt concentrations by mixing
387 2mL of the strain with 13mL of medium. The flasks were then placed into two incubators at
388 their respective treatment temperatures under 12/12 dark/light cycle (200 µmol m^{-2} s^{-1} light
389 intensity). Note that the 3.5M salt concentration only occurred at 36°C (Table S4.3) where our
390 preliminary tests suggested that 4M would be too stressful. This gave us a total of 75
391 evolutionary replicates: 5 strains x 2 salinities (2M, 4M) x 3 replicates at 24° C, and 5 strains x
392 3 salinities (2M, 3.5M, 4M) x 3 replicates at 36°C. In addition, five media flasks with no cells
393 (for the different conditions) were added to calibrate spectrometer measurements. Air
394 composition was not controlled in the incubator, but carbon dioxide necessary to
395 photosynthesis was provided in dissolved form. At 36°C, we additionally placed a tray filled
396 with water to prevent excessive evaporation. At the first transfer, at the start of the
397 experimental evolution phase, we tracked population growth with daily measurements of
398 population density over eleven days for all replicates of the methods described above.
399
400 Rearing and transferring protocol
401 Each of the 75 evolutionary lines was transferred into new flasks weekly. The standard
402 transfer (for strains S1, S13 and Mix) consisted of diluting 2mL of culture into 13mL of fresh
403 medium at the same salinity (i.e., a dilution rate of 2/15). However, dilution rates had to be

404 adjusted for some strains and transfers to prevent out-dilution, as detailed in Table S4.4.
405 Before each transfer, cell concentration was measured using a flow cytometer (see below) to
406 keep track of densities and reduce the risk of out-diluting the cultures. Nonetheless, all
407 replicates from strain S11 failed to grow and were lost at transfer 5 (Table S4.4).
408
409 **Table S4.3** Evolutionary treatments and number of replicates at the start and end of the experiment.

Strain ID	Salinity	Temperature	Stress	Replicates
S1	2M	24°C	Control	3
S1	4M	24°C	Single Salinity	3
S1	2M	36°C	Single Temperature	3
S1	3.5M	36°C	Multi	3
S1	4M	36°C	Multi	3
S3	2M	24°C	Control	3
S3	4M	24°C	Single Salinity	3
S3	2M	36°C	Single Temperature	3
S3	3.5M	36°C	Multi	3
S3	4M	36°C	Multi	3
S11	2M	24°C	Control	3
S11	4M	24°C	Single Salinity	3
S11	2M	36°C	Single Temperature	3
S11	3.5M	36°C	Multi	3
S11	4M	36°C	Multi	3
S13	2M	24°C	Control	3
S13	4M	24°C	Single Salinity	3
S13	2M	36°C	Single Temperature	3
S13	3.5M	36°C	Multi	3
S13	4M	36°C	Multi	3
Mix	2M	24°C	Control	3
Mix	4M	24°C	Single Salinity	3
Mix	2M	36°C	Single Temperature	3
Mix	3.5M	36°C	Multi	3
Mix	4M	36°C	Multi	3

410
411 **Table S4.4** Dilution rates for the different strains at the two temperatures during the weekly transfers. Variations
412 from the 2/15 dilution are highlighted in bold, and X indicates the strain that failed to grow and was lost.

Transfer week	**S1**		**S3**		**S11**		**S13**		**Mix**	
	24°C	36°C	24°C	36°C	24°C	36°C	24°C	36°C	24°C	36°C
1	2/15	2/15	2/15	2/15	2/15	2/15	2/15	2/15	2/15	2/15
2	2/15	2/15	2/15	**5/15**	2/15	**5/15**	2/15	2/15	2/15	2/15
3	2/15	2/15	2/15	**4/15**	2/15	**4/15**	2/15	2/15	2/15	2/15
4	2/15	2/15	2/15	**4/15**	2/15	**4/15**	2/15	2/15	2/15	2/15
5	2/15	2/15	**4/15**	**4/15**	**X**	**X**	2/15	2/15	2/15	2/15
6	2/15	2/15	**4/15**	**4/15**	**X**	**X**	2/15	2/15	2/15	2/15
7	2/15	2/15	2/15	**4/15**	**X**	**X**	2/15	2/15	2/15	2/15

413
414 **Final phenotypic tests and data collection**
415 At the last transfer, we tracked population growth with daily measurements of population
416 density over eleven days. Each of the populations evolved in a stressor environment was
417 measured in their own stressor environment and in the control environment. All
418 measurements of cell concentration for every evolutionary line (each strain and replicate)
419 were obtained with the Guava easyCyte HT flow cytometer and the corresponding software
420 guavasoft 3.4 by Luminex.
421

422 **S5 *Euglena gracilis and Tetrahymena thermophila* (T1)**
423
424 **Authors**
425 João Gabriel Colares Silveira, Justina Givens, Claire Gougat-Barbera, Marie-Ange Devillez,
426 Giacomo Zilio, Emanuel A. Fronhofer
427
428 **Study organisms**
429 *Tetrahymena thermophila* and *Euglena gracilis* were chosen due to being two low-
430 maintenance unicellular eukaryote species with very distinct life-history traits and feeding
431 habits. *T. thermophila* and *E. gracilis* have respectively generation times of 2-4 hours (23–25)
432 and 10-30 hours (26, 27), and present heterotrophs (28) and mixotrophy (29) life style. *T.*
433 *thermophila* and *E. gracilis* have been extensively used as model unicellular eukaryotes in a
434 variety of scientific works (15).
435
436 **Experimental evolution phase**
437 Experimental setup and treatments
438 This study was designed around a total of 72 clonal stock cultures or "ancestor populations",
439 of *T. thermophila* and 24 of *E. gracilis* lines. Populations of the two species were subjected to
440 eight treatment conditions, each replicated six times. These treatments involved the presence
441 of one or more environmental variables: moderately elevated temperature and the presence
442 of copper ($CuSO_4$) and/or a salinity (NaCl) concentration. All treatments were also conducted
443 under ambient temperatures with the presence of a control.
444 The specific concentrations of $CuSO_4$ and NaCl, as well as the temperature regimes for the
445 evolutionary phase, were chosen through preliminary experiments with the goal of making
446 initial fitness effects homogenous (*e.g.* same fitness loss).
447
448 Rearing and transferring protocol
449 Each line was kept in batch cultures fed with filtered bacterized lettuce medium (BLM), which
450 was comprised of mineral water (Volvic®), shredded dry lettuce (1 g for 1.5 L of water, or 0.6
451 g/L) and a previously prepared liquid culture of the bacterium *Serratia marcescens* (10% of
452 the total volume of the bacterial culture at equilibrium density). The lettuce serves as food
453 resource for the bacterium, which is itself preyed upon by both protist species. Each lineage
454 was transferred to a 100 mL flask as batch cultures for a period of 5 months with 10%
455 bottlenecks. Transfers were done once a week.
456
457 **Final phenotypic tests and data collection**
458 After the experimental evolution phase, we conducted a common garden experiment to
459 eliminate possible transgenerational plastic effects. An aliquot of 5 mL from each evolved
460 stock culture was washed with 10 mL of sterilized shredded lettuce solution medium without
461 *S. marcescens*. This was then centrifuged for 3 minutes under 1100 rpm in a Heraeus
462 Multifuge 3S Centrifuge (Thermo Fisher Scientific Inc., Waltham, Massachusetts, United

States) and the supernatant was discarded. The process was then repeated and the same 5 mL aliquots were transferred to 80 Sarstedt tubes of 25 mL with 15 mL of BLM and no stressors. The tubes were kept in a 25 °C incubator for five days, which was expected to be enough to eliminate the plastic responses.

We then prepared an assay with 16- 12-well Cellvis glass bottom black plates (Cellvis, Mountain View, California, United States), with each well receiving 3 mL of BLM. One aliquot of 50 µL for *T. thermophila* or 150 µL for *E. gracilis* (due to its longer generation time) of each of the Sarstedt tubes was subsequently added to each well of eight plates that would be submitted to experimental conditions (with stressors), as well as to each well of eight more plates, that would be kept under control conditions (no stressors). The well plates were kept under monoxenic conditions (with *S. marcescens*), with BreatheEasy® sealing membranes (Diversified Biotech, Dedham, Massachusetts, United States) preventing contamination. Additionally, the plates were kept in incubators with constant temperature (36 °C for some lineages of *T. thermophila*, 33 °C for some of *E. gracilis* and 25 °C for the rest; according to the scheme shown in Table S5) and a 12:12 hour cycle of light and darkness. The experimental condition plates were submitted to the same treatments used in the first part of the experiment on the ancestor populations, in a way that all possible combinations of stressors were present, including one treatment without any stressors.
The Agilent BioTek Cytation 5 (Agilent Technologies, Santa Clara, California, United States) cell imaging multimode reader was used to record a time-series of cell density in each well for four weeks through 15 second-videos. Moreover, the samples were kept under semicontinuous batch regime, with periodical medium replacements 3 times a week in alternated days for the supplementing of food (10% volume replacement). Sterile conditions were maintained with UV exposure to the plates for 20mins and sterile plastic film to seal wells. Through the videos, the density of cells in each population were estimated using BEMOVI, a package that uses R (version 4.2.3) and ImageJ software (17).
Each population was submitted to the same treatment under which it evolved previously, and under control conditions, so that we could also test if a possible adaptation to the stressors brought fitness cost under no stress. We then ended up with 5 replicates for each of the 8 treatments and species (5 x 8 x 2 = 80). This was replicated for the control condition, adding other 80 lines.

503 **Table S5**: Experiment design of the assays. Stressful conditions are indicated with "+", while the control
504 conditions are represented by "-". Tet = *Tetrahymena thermophila*, Eug = *Euglena gracilis*, ns = no stressor, cu =
505 copper, na = salt, tp = temperature, cs = copper + salt, ct = copper + temperature, st = salt + temperature, cst =
506 copper+salt+temperature, T = temperature, C = copper level, S = salinity.

Species	Treatments	Variables			Nº of	Stressors		
		Temperature (°C)	$CuSO_4$ (mM)	NaCl (g/L)	replicates	T	C	S
Tet	ns	25	-	-	5	-	-	-
Tet	cu	25	0.005	-	5	-	+	-
Tet	na	25	-	7.5	5	-	-	+
Tet	cs	25	0.005	3	5	-	+	+
Tet	tp	36	-	-	5	+	-	-
Tet	ct	36	0.005	-	5	+	+	-
Tet	st	36	-	7.5	5	+	-	+
Tet	cst	36	0.005	3	5	+	+	+
Eug	ns	25	-	-	5	-	-	-
Eug	cu	25	0.005	-	5	-	+	-
Eug	na	25	-	7.5	5	-	-	+
Eug	cs	25	0.015	4.5	5	-	+	+
Eug	tp	33	-	-	5	+	-	-
Eug	ct	33	0.005	-	5	+	+	-
Eug	st	33	-	7.5	5	+	-	+
Eug	cst	33	0.015	4.5	5	+	+	+

507
508
509

510 **S6 *Ostreococcus tauri* and *Picochlorum costavermella***

511

512 **Authors**

513 Edith Vidal, Marc Krasovec and Gwenael Piganeau

514

515 **Study organism**

516 *Ostreococcus tauri* (Mamiellophyceae, Chlorophyta) is a photosynthetic marine
517 picoeukaryote (cell diameter smaller than 2 µm). It was first discovered in the Mediterranean
518 Thau Lagoon (30). *Ostreococcus* species can be dominant during phytoplanktonic blooms in
519 coastal seas, and are the smallest photosynthetic eukaryotic with haploid and very compact
520 genomes of 13 Mb (31, 32). While meiosis has never been observed in the lab, population
521 genomic analyses provided evidence for meiosis and recombination in natural population in
522 *O. tauri* (33).

523 *Picochlorum costavermella* (Trebouxiophyceae, Chlorophyta) is a marine unicellular green
524 alga (cell diameter 1.4µm). This unicellular coccoid microalga has a broader halotolerance and
525 thermotolerance range as compared to *Ostreococcus*, as *P. costavermella* cultures can stand
526 freshwater to hypersaline media changes (34). *P. costavermella* RCC4223 has been isolated in
527 2011 from the estuary of the coastal river "La Massane" (NW Mediterranean Sea, France). *P.*
528 *costavermella* has a compact haploid 13.3Mb genome (34) .

529

530 **Experimental evolution phase**

531 Experimental setup and treatments

532 In preliminary tests, we performed a literature survey about growth rates as a function of
533 temperature and salinity compared to our standard laboratory control conditions (Table
534 S6.1). This survey suggested a temperature range up to 34 and 35°C and a maximum salinity
535 range up to 50 and 80 $g.L^{-1}$, for *O. tauri* and *P. costavermella*, respectively. We performed an
536 initial test on 32°C and 35°C as well as an increased salinity (Table S6.2). These conditions
537 decreased the growth rate of the strains without imposing high mortality (Table S6.3). One
538 incubator per temperature was used, with a lateral light source.

539

540 **Table S6.1** Photoperiod, media and light intensity of the control conditions

Species	All species
Temperature	26°C
Media	L1 (NCMA, Bigelow Lab for Ocean Science, USA)
Hours Day:Night	8:16
Light Intensity	50 $\mu mol.m^{-2}.s^{-1}$
Incubator	Memmert IPP110 ECO PLUS

541

542 **Table S6.2** Conditions of the experimental evolution experiment for each species.

Species	Control		Temperature		Salt		Temperature + Salt		Experiment
O. tauri	26°C	0.60M	32°C	0.60M	26°C	0.86M	32°C	0.86M	Exp1
P. costavermella	26°C	0.60M	35°C	0.60M	26°C	1.37M	35°C	1.37M	Exp1
P. costavermella	26°C	0.60M	35°C	0.60M	26°C	1.03M	35°C	1.03M	Exp2

543

544 **Table S6.3:** Average growth rates and average growth rate decrease (in percent) in control versus stressful
545 experiments over 6 replicates.

Species	Control	Temperature (g | %)		Salt (g | %)		Temperature + Salt (g | %)		Experiment
O. tauri	1.90	1.87	-1.5%	1.83	-55%	1.60	-63%	Exp1
P. costavermella	1.83	1.54	-16%	1.74	-57%	1.05	-95%	Exp1
P. costavermella	1.52	1.00	-34%	1.46	-3.9%	1.26	-17%	Exp2

546
547 The initial fitness measurements were performed for each species from a clonal culture
548 maintained at the Culture Collection of the GENOPHY team. Fitness was estimated by the
549 growth rate, g, the number of divisions per day, t, during the exponential growth phase of the
550 culture. $N(t)$ is the number of cells at day t.
551
552 $$g = \frac{N(t)}{N(0)} e^{1/t}$$
553
554 The cell concentration was followed over 20 consecutive days to assess the range of cell
555 concentrations corresponding to the exponential phase in each condition (for details on cell
556 counting protocol see below). The growth rates measured under the control and stress
557 conditions allowed the exponential growth rate to be estimated from $N(0)= 10^5$ cells to $N(7)$
558 that is 7 days after inoculation. The initial stress response was then estimated by the average
559 growth rate over the seven first days of the experiment (Table S6.3), each well was inoculated
560 with 10^5 cells at the start of each cycle.
561
562 Rearing and transferring protocol
563 We started from a culture that originated from one single cell sorted by dilution. The ancestor
564 culture is thus isogenic, with the exception of variants due to spontaneous mutations. The
565 spontaneous point mutation rate as well as the spontaneous aneuploidy rate is known for
566 these two species (33, 35, 36).
567 The long-term protocol was performed in 24 well plaques (2mL per well). Each well was
568 inoculated with 10^5 cells every seven days in a total volume of 1mL. Cells count was performed
569 using a Beckman-Coulter CytoFLEX flow cytometer (Beckman Coulter, USA) equipped with
570 three lasers emitting light in spatially separated pathways at 405 nm (80 mW), 488 nm (50
571 mW), and 561 nm (30 mW). Subsequent estimations of cell concentrations were calculated
572 using Becton-Dickinson TrucountTM beads. We used the software CytExpert (v. 2.3.1.22),
573 generating listmode (FCS 3.0) files. Phytoplankton cells were analysed unstained and
574 enumerated based on chlorophyll a fluorescence (692 nm maximum emission) and side
575 scatter (SSC). The concentration of cells was used to estimate the volume required to transfer
576 10^5 cells from each cell line into fresh media at each transfer. At the start of the experiment,
577 the cultures were left 7 days under each treatment prior to the first measurement. The
578 number of cells was then estimated every week (corresponding to one cycle) in their own
579 evolutionary treatment for the complete duration of the experiment.
580 In total we established 6 replicates per species and condition, corresponding to a single line
581 in a 24 wells plate. For *P. costavermella* the experiment was restarted (Table S6.2-3, Exp1-

582 Exp2) because of too harsh multiple stress conditions, so that we have: 2 species (*P.*
583 *costavermella* and *O. tauri*) x 4 treatments x 6 biological replicates for 17 cycles (119 days),
584 and 1 species (*P. costavermella*) x 4 treatments x 6 biological replicates for 12 cycles (84 days).
585
586 **Table S6.4:** Number of cycles per experiment.

Species	Control	Temperature	Salt	Temperature + Salt	Experiment
O. tauri	17	17	17	17	Exp1
P. costavermella	17	17	17	0	Exp1
P. costavermella	12	12	12	12	Exp2

587
588 **Final phenotypic tests and data collection**
589 In the last cycle, cells were transferred once more in their own evolutionary treatment and
590 counted by flow cytometry as described, but they were not assayed in control conditions in
591 the absence of stressors.
592

593 ## S7 *Paramecium caudatum*
594
595 **Authors**
596 Oliver Kaltz, Marie-Ange Devillez
597
598 **Study organism**
599 The freshwater ciliate *Paramecium caudatum* represents a classic model system for genetic,
600 developmental and physiological study (37). It has also been used for ecological studies (38,
601 39) and experimental evolution (40, 41). Previous work has shown that *P. caudatum* is highly
602 sensitive to change in temperature and osmotic stress (37, 41–44). For the present study, we
603 used the strain KS3, collected in Germany (45) and kept in the OK lab since 2019. Under
604 standard conditions, we keep *Paramecium* mass cultures at 23°C, in a lettuce medium, made
605 from organic lettuce, Volvic™ mineral water, and supplemented with the bacterium *Serratia*
606 *marcescens* as food resource (46).
607
608 **Experimental evolution phase**
609 Experimental setup and treatments
610 From a series of pilot experiment, testing the KS3 strain over a range of temperatures and salt
611 concentrations, we determined the conditions for the long-term experiment, consisting of a
612 control treatment (23°C), a single-temperature treatment (30°C), a single-salt treatment (2g/L
613 of NaCl), and a combined treatment (28°C and 2g/L of NaCl).
614
615 From a KS3 stationary-phase mass culture at 23°C, we established replicate cultures of 20mL
616 (in 50mL tubes), with a starting density of 100 individuals and exposed to the four above
617 treatments (control, single-stress, double-stress). For the temperature treatments, tubes
618 were placed in incubators set to the appropriate temperature.
619 Over the course of 9 days, population density was tracked in 1- to 2-day intervals, by removing
620 small samples of up to 300µL and counting the number of individuals under a dissecting
621 microscope. After 4 days, we replenished the cultures, by adding 1mL of culture medium. For
622 each treatment, we established four replicates (16 replicates in total).
623
624 Rearing and transferring protocol
625 From a KS3 stationary-phase mass culture at 23°C, we established replicate cultures of 150mL
626 (in 250mL glass bottles) with initially c. 1000 individuals. These were assigned to the four
627 treatments, with 5 long-term replicates per treatment (= 20 lines in total). For the
628 temperature treatments, tubes were placed in incubators set to the appropriate
629 temperature. The long-term culture protocol consisted of weekly transfers of 30mL of culture
630 to a new bottle, to which we added 120mL of freshly prepared medium, adjusted for the
631 appropriate salt concentration. Over the course of 1 year, 48 transfers were accomplished,
632 corresponding to ≥96 population doublings (i.e., asexual generations).
633

634 **Final phenotypic tests and data collection**

635 At the end of the long-term experiment, each evolved line was tested in its own long-term
636 environment and in the control environment. These assays were run in 50mL tubes, and
637 initiated by placing 200 individuals of a given long-term line in 20mL of lettuce medium,
638 adjusted to the appropriate salt concentration. The tubes were then transferred to the
639 appropriate temperature condition. Densities were tracked over 13 days, as described above.
640

641 ## S8 *Pseudomonas aeruginosa*

642

643 **Authors**

644 Jeanne Hamet, Stéphanie Bedhomme

645

646 **Study organism**

647 *Pseudomonas aeruginosa* (PA) is a gram-negative bacterium and opportunistic pathogen,
648 responsible for a growing number of nosocomial infections due to its frequent resistance to
649 antibiotics (47). It is a ubiquitous organism, with a preference for environments close to
650 human activity (48). PA can be observed in planktonic form, but most of the time, it is
651 observed in biofilms (49). This habitat enables it to protect itself from biotic and abiotic
652 environmental stresses (50). In standardized laboratory conditions, at 37°C and classical
653 culture broth (LB Broth), they asexually divide with a generation time of 30 minutes (51).
654 Strain PAO1 (Taxonomy ID: 208964) was chosen here because its genome has been
655 sequenced and it is a model laboratory strain(52). This strain was isolated from a wound (53).

656

657 **Experimental evolution phase**

658 Experimental setup and treatments

659 We designed four evolutionary treatments, a control, copper stressor, salt stressor and salt
660 plus copper stressor. To decide on the concentrations of salt and copper in the three stress
661 treatments, growth curves were realised over a range of salt and copper stress concentrations
662 in a crossed design allowing us to define a 2-dimension niche. A pre-culture was set up from
663 the glycerol stock of the ancestral strain (PAO1) and cultivated in 4mL of LB Broth at 37°C with
664 220 rpm orbital shaking overnight. 2µL of the pre-culture was then transferred to each well
665 of a microtiter plate containing 200µL of LB broth and various concentrations of salt (0, 0.1,
666 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1M Sodium chloride) and copper (0, 0.001, 0.002, 0.003,
667 0.004, 0.005, 0.006 and 0.007M copper(II) sulfate pentahydrate) in triplicate. Sodium chloride
668 and copper(II) sulfate pentahydrate were ordered from Sigma Aldrich. Growth curves (density
669 over time) were obtained monitoring the optical density at 550nm (OD550) during 24h with
670 a Tecan plate reader following the program indicated in Table S8.1.

671

672 **Table S8.1** Description of the 30-minute cycle used to follow the optical density of growing bacterial populations

Length	Action	Description
5s	Absorbance observation	number of points measured : 5 OD 550nm
22min	Waiting time	
5min	Shaking time	Shaking (Double Orbital) amplitude: 2,mm Shaking frequency: 108rpm
2min	Waiting time	

673

674 For each simple and double stress treatment, we chose the experimental evolution stressor
675 concentrations causing a reduction in absolute fitness close to 50% (details in table S8.2).
676
677 **Table S8.2** Description of the 4 evolutionary treatments

Name	Treatment	Concentration
S0	Control	
S1	Salt	0.7M NaCl
S2	Copper	0,00325M $CuSO_4^-$
S3	Salt + Copper	0.5M NaCl - 0,002M $CuSO_{4-}$

678
679 Prior to the experiment, a pre-culture was set up from the glycerol stock of the ancestral
680 strain (PAO1) and cultivated in 4mL of LB Broth at 37°C with 220 rpm orbital shaking for 24
681 hours. All experimental evolution lines are thus derived from a clonal ancestor. To begin the
682 experimental evolution, we added 12µL of bacterial culture to 1188µL of medium in a
683 Deepwell plate. 12 replicate populations were set up for each of the four evolutionary
684 treatments. From these initial mixes, 200µL were transferred to a microtiter plate.
685 Treatments were distributed in a checkerboard pattern with the same positions on each plate,
686 and evolution medium in alternating wells to identify medium contamination or high levels
687 of cross-contamination during transfer. The Deepwell plate was incubated for 24 hours at
688 37°C, with 220 rpm orbital shaking. The microtiter plate was incubated at 37°C, with shaking
689 and OD550 was recorded following the cycle described in Table S8.1.
690
691 Rearing and transferring protocol
692 On following days during experimental evolution, 10µL of each population was transferred to
693 a new plate containing 990µL of fresh medium (10^{-2} dilution, giving approximately 6.6
694 generations/day). When a population had low turbidity, we transferred 20µL of that
695 population (Table S8.3). Each transfer started a new cycle.
696
697 **Table S8.3** List of the 20µL transfers during experimental evolution.

Cycle	Stress	Population
5	Copper	C11
10	Copper	CN2
22	Copper	C2
22	Copper	C3
22	Copper	C6
22	Salt + Copper	CN10
26	Salt + Copper	CN10

698
699 Populations were maintained for 26 cycles, resulting in approximately 173 generations of
700 experimental evolution. At cycle 10 and 26, one line in the salt and copper treatment (S3)
701 went extinct despite transferring 20µL of the evolved population, leaving 46 lines for the final
702 phenotypic assays. Frozen archives of the evolving populations were stored at cycle 1, 4, 10,

703 16, 22 and 26 in the form of glycerol stocks. Each week, we mixed 280µL of homogenate from
704 each evolved population with 350µL of 70% glycerol saline solution, and stored at -70°C.
705
706 **Final phenotypic tests and data collection**
707 At cycle 26, we closely followed the growth of the evolutionary lines in their own
708 environment. We added 2µL of the populations to 198µL of their evolutionary medium in a
709 microtiter plate, and growth curves were obtained by monitoring the OD550 as previously
710 described. We also tested the lines in control condition. We defrosted the glycerol stock from
711 cycle 26, and 10µL of each evolutionary line was transferred to 990µL of their evolutionary
712 medium and incubated at 37°C, with 220 rpm orbital shaking for 24 hours. Afterwards, we
713 added 2µL of each population to 198µL of control medium in a microtiter plate. The
714 populations that did not grow during the final tests are listed in Table S8.4.
715
716 **Table S8.4** Populations that did not grow in the final phenotypic tests.

Evolutionary treatment	Final assay environment	Population
Salt	Salt	Pae_06
Copper	Copper	Pae_14
Salt + Copper	Salt + Copper	Pae_43
Salt + Copper	Salt + Copper	Pae_47

717
718

719 **S9 *Ralstonia pseudosolanacearum***

720

721 **Authors**

722 Delphine Capela, Alice Guidot, Philippe Remigi

723

724 **Study organism**

725 *Ralstonia solanacearum* is a soil bacterium belonging to the beta subclass of Proteobacteria.
726 It is a species complex responsible for the bacterial wilt disease on more than 250 plant
727 species in tropical and subtropical countries (54). The strain GMI1000 isolated from Tomato
728 in French Guyana (55) was used as a model strain in many different studies and its genome
729 was the first *R. solanacearum* genome completely sequenced (56)

730

731 **Experimental evolution phase**

732 Experimental setup and treatments

733 Liquid cultures of GMI1000 strain were carried out in MP synthetic medium which
734 composition is as follows (g liter^{-1}): $FeSO_4$ $7H_2O$, 1.25 10^{-4}; $(NH_4)_2SO_4$, 0.5; $MgSO_4$ $7H_2O$, 0.05;
735 KH_2PO_4, 3.4. The pH was adjusted to 6.5 with KOH (Plener *et al.*, 2010). The MP synthetic
736 liquid medium was supplemented with L-glutamine(10 mM) and oligo-elements (ZnSO4 7
737 H2O 55 µg.L^{-1}, H_3BO_3 28.5 µg.L^{-1}, MnCl2 4·H2O 12.6 µg.L^{-1}, CoCl2 6·H2O 4 µg.L^{-1}, CuSO4 5·H2O
738 3.9 µg.L^{-1}, (NH4)6Mo7O24 4·H2O 2.8 µg.L^{-1}) (57). NaCl was added at final concentrations of
739 75 or 150 mM as needed (see below).

740 The standard rearing temperature of *R. solanacearum* growth in laboratory conditions is 28°C.
741 In preliminary tests conducted before the start of the long-term experiments, we assayed
742 growth at higher temperatures. We observed that growth rate was strongly reduced at 37°C
743 (about 5 folds reduction) but was completely inhibited at 38°C. We therefore decided to keep
744 37°C as the high temperature stress condition. The impact of NaCl addition on GMI1000
745 growth rate was more linear. The addition of NaCl at a concentration of 150 mM in the
746 bacterial culture reduced the growth rate (about 5 folds reduction). We therefore decided to
747 use 75 and 150 mM NaCl as salt stress conditions at 37 and 28°C, respectively.
748 The ancestral strain GMI1000 was revived from glycerol stock on plates containing rich agar
749 medium (bacto peptone 10 g.L^{-1}, casamino acids 1 g.L^{-1}, yeast extract 1 g.L^{-1}) for 2 days at
750 28°C. One individual colony was used to inoculate 100 mL Erlenmeyer flasks containing 20 mL
751 MP-glutamine liquid medium and was incubated overnight at 28°C. After 24 h, optical density
752 at 600 nm (OD_{600}) was measured, and the pre-culture was used to inoculate 100 mL
753 Erlenmeyer flasks containing 20 mL MP-glutamine (at starting OD_{600} = 0.001) in four different
754 evolutionary treatments: control (28°C, 0 mM NaCl), saline stress (28°C, 150 mM NaCl),
755 temperature stress (37°C, 0 mM NaCl), and the combination of two stresses (37°C, 75 mM
756 NaCl).

757

758 Rearing and transferring protocol

759 Five independent cultures were inoculated for each treatment. After 24 hours of growth in

760 these conditions, OD_{600} was measured, the cultures were used to re-inoculate new cultures
761 in the same conditions (starting at OD_{600} = 0.001), and aliquots of the cultures were stored at
762 -80°C in the presence of 20% glycerol. The same protocol was applied for 15 cycles.
763
764 **Final phenotypic tests and data collection**
765 We measured the growth rate of all evolved lines in control and in their own evolutionary
766 condition. The initial growth of the ancestor was tested as well in all four conditions (three
767 stress and control conditions). To standardize these assays, the ancestor strain and the
768 evolved populations were first restarted from frozen glycerol stocks in their evolution
769 medium and incubated for 24 hours at 28°C. These precultures were used to generate, for
770 each population, multiple 100 µl aliquots of bacterial cultures containing 20% glycerol that
771 were stored at -80°C. To initiate a phenotypic test, one entire frozen aliquot per population
772 was thawed in 1ml of its evolution medium and 50 µl or 100 µl of these suspensions were
773 used to inoculate an overnight pre-culture in the evolution conditions (ancestral strain
774 GMI1000 was grown in control). On the next day, the pre-cultures were used to inoculate
775 fresh 20 mL cultures either in control or in evolution medium (ancestral strain GMI1000 was
776 grown in control as well as in all stress conditions). The starting OD_{600} of these cultures were
777 adjusted between 0.000003 and 0.01, so that all populations reached an OD_{600}~0.05-0.1 after
778 ~16 hours (the next morning), from which point OD_{600} was monitored every ~2 hours for ~8-
779 10 hours. After 24 hours, evolved populations grown in control condition were used to
780 inoculate new 20 mL cultures in their evolution treatment and their growth was followed as
781 during the first round of culture, in order to estimate the effect of pre-culture condition on
782 the growth in the stressful environment. The whole experiment was performed at least three
783 times independently for each of the lines.
784

785 **S10 *Saccharomyces cerevisiae***
786
787 **Authors**
788 Apolline Vedrenne, Diego Segond, Delphine Sicard and Thibault Nidelet
789
790 **Study organism**
791 *Saccharomyces cerevisiae* is the most known of yeasts also called baker yeast (58). We worked
792 with three strains: EC1118 (a commercial strain that is used as reference in oenological
793 research) and M1OA, a strain from our collection and collected from vineyards around
794 Montpellier. Under our experimental conditions, yeast divides asexually by budding with a
795 generation time of around 2h (59).
796
797 **Experimental evolution phase**
798 Experimental setup and treatments
799 Two different strains of Saccharomyces cerevisiae (EC1118, and M1OA) were inoculated into
800 deepwell microplates with a starting population of 10^6 cells/mL. Each strain was inoculated in
801 4 different media: an unstressed medium with a temperature of 28°C and no copper, a high-
802 temperature stress medium (33°C) with no copper, a medium with a temperature of 28°C but
803 stressed by a high copper concentration (1g/L) a doubly stressed environment with a high
804 temperature of 33°C and a high copper concentration (1g/L). The based medium was a
805 synthetic must that we use for all our experiments (Bely *et al.*, 1990). It contains mainly 200g
806 of sugars and 200mg of assimilable nitrogen sources, as well as phytosterols and other
807 essential nutrients. These conditions have been chosen after preliminary experiments.
808
809 Rearing and transferring protocol
810 For each strain and each medium, three independent lineages were started. Twice a week,
811 80% of the medium was scattered and replaced by fresh medium. The experiment was carried
812 out for 26 cycles (93 days). Once a week, yeast population growth was determined by
813 cytometry, using an Attune NxT (Life Technologies) with 200 µL/min flux. At the end of the
814 evolutionary phase, we froze all our evolved lines (populations).
815
816 **Final phenotypic tests and data collection**
817 We thawed all the evolved lines, including the ancestral populations, and grew them for 24
818 days in yeast extract peptone dextrose (YPD), a neutral environment. We then inoculated in
819 microplates (at 10^6 cells/ml) each of the lines, including the ancestors, in triplicate in their
820 own evolutionary conditions (no stress, high temperature, high copper concentration and
821 double stress), and in control conditions in the absence of stressors. Ancestors were tested in
822 the four evolutionary conditions. As previously illustrated, we used cytometry and measured
823 population density (OD) during 24h.
824
825

826 **S11 *Sinorhizobium meliloti***

827

828 **Authors**

829 Laurent Sauviac and Claude Bruand

830

831 **Study organism**

832 *Sinorhizobium meliloti* is a soil bacterium belonging to the alpha subclass of Proteobacteria.
833 It is the natural nitrogen fixing microsymbiont of legume plants of the *Medicago* and *Melilotus*
834 genera, including the forage crop alfalfa (*Medicago sativa*) and the model legume *Medicago*
835 *truncatula*. The strain *Sinorhizobium meliloti* CBT2801 is a derivative of the strain Rm2011
836 (60).

837

838 **Experimental evolution phase**

839 Experimental setup and treatments

840 Liquid cultures of *S. meliloti* were carried out in Vincent minimal medium: VMM; 7.35 mM
841 KH_2PO_4, 5.74 mM K_2HPO_4, 1 mM $MgSO_4$, 456 µM $CaCl_2$, 35 µM $FeCl_3$, 4 µM biotin, 48.5 µM
842 H_3BO_3, 10 µM $MnSO_4$, 1 µM $ZnSO_4$, 0.5 µM $CuSO_4$, 0.27 µM $CoCl_2$, 0.5 µM $NaMoO_4$, 10 mM
843 sodium succinate, 18.7mM NH_4Cl pH 7, always supplemented with 100 $\mu g.ml^{-1}$ streptomycin
844 (Sm). NaCl was added at final concentrations of 250 or 400 mM as needed (see below). The
845 standard rearing temperature of *S. meliloti* growth in laboratory conditions is 28°C. In
846 preliminary tests conducted before the start of the long-term experiments, we assayed
847 growth at higher temperatures. Bacteria grew better at 37°C than at 28°C, their growth rate
848 was strongly reduced at 40°C and above, as was the maximal culture density. Moreover,
849 addition of even low salt concentrations at 40°C led to a complete cessation of growth. We
850 observed that at 39.5°C, the strain grew more slowly than at 28°C (~2-fold reduction), while
851 reaching a normal maximal culture density, and supporting the addition of salt (see below).
852 We therefore decided to keep 39.5°C as the high temperature stress condition. NaCl addition
853 at concentrations of 200-250 mM had little effect on growth at 28°C, which was significantly
854 reduced (~2-fold) at concentrations of 350 mM and above. At 39.5°C, addition of 250 mM
855 NaCl further slowed down bacterial growth (~60% reduction) without affecting maximum cell
856 density. We therefore decided to use 250 and 400 mM NaCl as salt stress conditions at 39.5
857 and 28°C, respectively.

858

859 Five independent clones isolated on LB rich medium supplemented with 2.5 mM $MgSO_4$, 2.5
860 mM $CaCl_2$ and 100 $\mu g.ml^{-1}$ Sm were used to inoculate tubes containing 5 mL VMM and
861 incubated overnight at 28°C under agitation. After 24h, optical density at 600 nm (OD_{600}) was
862 measured, and cultures were used to inoculate 100 mL Erlenmeyer flasks containing 20 mL
863 VMM (at starting OD_{600} = 0.001) in four different evolutionary treatments: control (28°C, 0
864 mM NaCl), saline stress (28°C, 400 mM NaCl), temperature stress (39.5°C, 0 mM NaCl), and
865 the combination of two stresses (39.5°C, 250 mM NaCl).

866

867 Rearing and transferring protocol
868 After 24 hours of growth in the four evolutionary conditions, OD_{600} was measured, the
869 cultures were used to re-inoculate new cultures in the same conditions (starting at OD_{600} =
870 0.001), and aliquots of the cultures were stored at -80°C in the presence of 20% glycerol. The
871 same protocol was applied for 20 cycles.
872
873 **Final phenotypic tests and data collection**
874 We measured the growth rate of all evolved lines in control and in their own evolutionary
875 condition. The initial growth of the ancestor was tested as well in all four conditions (three
876 stress and control conditions). The ancestor strain as well as populations evolved for 20 cycles
877 were first restarted from frozen glycerol stocks in 5 ml tubes of VMM (start OD_{600}= 0.001)
878 incubated for 24 hours at 28°C. These precultures were used to inoculate 100 ml flasks
879 containing 20 ml VMM at 28°C. After ~16 hours (overnight) OD_{600} was monitored every ~2
880 hours for ~8-10 hours, to generate the first dataset (fitness of each line in control conditions).
881 24 hours post-inoculation, 20 mL cultures were again inoculated from each of these cultures
882 (start OD_{600}= 0.001), but then incubated in the evolutionary condition of the tested line,
883 except for the ancestor which was grown in the four evolutionary conditions. After this first
884 cycle of 24 hours, this step was repeated, and after 16 hours of growth, OD_{600} was monitored
885 every ~2 hours for ~8-24 hours, to generate the second dataset (fitness of each line in their
886 own evolutionary treatment). The whole experiment was performed at least three times
887 independently for each of the lines.

888

889 ## S12 *Tetrahymena thermophila* (D16, D19, D21, D3)
890
891 **Authors**
892 Delphine Legrand, Staffan Jacob, Michèle Huet, Hervé Philippe
893
894 **Study organism**
895 *Tetrahymena thermophila* is a free-living ciliate living from northern America found in
896 freshwater ecosystems. It is a main actor of aquatic trophic chains, as a grazer of bacteria and
897 prey of mesoplanktonic species (61). It is a historical model species in cellular and molecular
898 biology, which has led to major discoveries like identifications of dynein or telomere structure
899 (28, 62), and has more recently turned into an experimental model in ecological and
900 evolutionary studies, for instance in the fields of dispersal, adaptation and phenotypic
901 plasticity (*e.g.*, (63–65)). The species alternates phases of sexual and asexual reproduction in
902 nature, and can be long-maintained as clonal populations in laboratory. Under standard
903 conditions (24-well plates, 23°C, aqueous 0.3X PPYE medium, 0.6% of Proteose Peptone and
904 0.06% Yeast Extract), the asexual generation time is about 4-6 hours.
905 Here, we used six clonally reproducing *T. thermophila* strains, namely D3, D8, D10, D16, D19
906 and D21, initially collected by Pr Paul Doerder and maintained in our laboratory for ~20 years.
907 These strains were recently sequenced (66), which revealed close genomic proximity between
908 D8, D19 and D21, a probable recent cross between a D8/D19/D21-like strain and a divergent
909 strain leading to D16, and diverging genomes for D3 and D10, the only two strains whose
910 origin seems to match the initial sampling of Pr P. Doerder.
911
912 **Experimental evolution phase**
913 <u>Experimental setup and treatments</u>
914 To obtain the six ancestors' cultures, we filled six 1L Erlenmeyers with 100mL of PPYE 0.3X in
915 which we inoculated 100µL of 7-old stock cultures. Erlens were placed in an incubator at 23°C
916 and gently shaken during seven days to obtain sufficient initial densities to start the
917 experiment.
918 We then established four evolutionary treatments (Control, Salt, the antibiotic
919 Chloramphenicol, Salt + Chloramphenicol) by manipulating salinity and chloramphenicol
920 concentrations (Table S12.1), two pollutants that incur serious threats to aquatic ecosystems
921 (67, 68). Pollutants' concentrations were chosen based on pilot experiments in which we
922 exposed the strains to a gradient of salt concentrations (from 0 to 20g/L) and a gradient of
923 chloramphenicol concentrations (from 0 to 500mg/L). Treatment concentrations (5g/L for
924 NaCl and 40mg/L for chloramphenicol) were fixed so that they reduced but not stopped the
925 growth over all strains. As strains have their own growth capacities, the initial growth
926 reduction was strain-specific (see details on growth measurements below).
927
928

929 **Table S12.1** Description of the four evolutionary treatments. These four treatments were conducted in 0.3X
930 PPYE medium.

Treatment	Concentration
Control	Pollutant-free
Salt	5g/L NaCl
Chloramphenicol	40mg/L
Salt + Chloramphenicol	5g/L NaCl - 40mg/L

931
932 To initiate experimental evolution, we filled three 24-well plates with 2mL of each of the four
933 evolutionary treatments, for a total of 12 managed plates. Each strain was exposed to the
934 four evolutionary treatments in six replicates, leading to 144 evolved populations (6 strains x
935 4 treatments x 6 replicates = 144). Each plate contained two replicates of the six strains,
936 meaning that 12 wells were left empty in a checkerboard configuration to limit cross-
937 contaminations. We randomized the position of each strain between the three plates
938 containing the same evolutionary treatments to control for the effect of the well position. To
939 initiate the experiment, we inoculated 100µL of ancestral populations.
940 To estimate initial growth (fitness), we used growth rate as the fitness proxy. At the time of
941 evolved populations inoculation in 24-well plates, we parallelly inoculated 10µL (~100 to
942 1,000 cells depending on the strain) of ancestral cultures in five technical replicates into 96-
943 well plates pre-filled with 250 µL of each of the four treatments (with randomized position of
944 both strains and treatments). Population growth was quantified by measuring Optical Density
945 (OD) at 450nm every four hours during 20 days (sufficient delay to reach the stationary phase
946 for each strain x treatment condition) using a microplate reader (Tecan Infinite
947 spectrophotometer). OD at 450nm is positively correlated with *T. thermophila* cell density in
948 our laboratory conditions (*e.g.*,(64)).
949
950 Rearing and transfer protocol
951 Evolved populations were maintained in the 24-well plates during five months and a half, with
952 plate transfer using 5% of populations (100µL over the 2mL of each well) every seven days
953 during two months (10 cycles), and then every 14 days until the end of the experiment (7
954 cycles). We indeed observed that some evolved populations had critical densities requiring
955 longer growth periods to ensure their survival. In total, we thus performed 17 cycles. Under
956 standard conditions, the duration of the experiment corresponds to ~800 asexual
957 generations.
958
959 **Final phenotypic tests and data collection**
960 During experimental evolution, two types of data were collected:
961 i) growth rates by spectrophotometry following the protocol described above for the
962 ancestral populations at cycles 17 (final time, 168 days). We placed 10µL of the 144 evolved
963 populations in the four treatments immediately after pipetting (144 evolved lines x 4
964 treatments x 5 replicates = 2,880 growth assays). We further assayed the obtained 144
965 evolved lines in control conditions.

966 ii) cell densities seven days after inoculation in fresh plates by video analysis at cycles 1
967 (ancestors) and 17 (final time, 168 days). To do so, we pipetted 10µl of each evolved
968 population into multichambered counting slides (Kima precision cell) and immediately took
969 15s videos from each chamber under darkfield macroscopy to count the number of cells using
970 the BEMOVI R-package (17). These videos were used to estimate cell densities in the
971 treatment of evolution, so as to check for population extinctions.
972
973 From videos and complementary observation of whole wells under a macroscope, we
974 observed that all populations survived. However, some had very low densities, zero to three
975 cells in the 10µL sampled for video analyses, which corresponds to no more than ~600 cells
976 at the population scale. At such low densities after seven days of growth, we are unable to
977 properly assess growth rates using spectrometry (cell number below the detection threshold).
978 This was the case of all D10 replicates in the multiple stressor treatment. We thus excluded
979 this strain from the final analyses. Besides, ancestors of strain D8 did not grow in the control
980 treatment, meaning that we could not assess its initial fitness. We suspect a pipetting
981 omission when performing the growth assays of D8 ancestor. We thus excluded D8 from final
982 analyses, despite having evolved all populations correctly. In the end, we only kept strains D3,
983 D16, D19 and D21 for the final data analyses.
984

985 **S13 *Vibrio aestuarianus***
986
987 **Authors**
988 Fiona Elmaleh, Gaelle Courtay, Guillaume M. Charrière
989
990 **Study organism**
991 *Vibrio aestuarianus* is a gram-negative halophile γ-proteobacteria. It is an actively studied
992 oyster pathogen that has colonized Europe since the second half of the 20th century (69). The
993 pathogenic subspecies *Vibrio aestuarianus francensis,* like the 12/016 strain, is a primary
994 pathogen for adult oysters and was shown recently to carry a wide array of copper-resistance
995 genes when compared to the non-pathogenic environmental stain, like *Vibrio aestuarianus*
996 U29 (69).
997
998 **Experimental evolution phase**
999 Experimental setup and treatments
1000 To start our experiment, we used 2 strains (12/016 and U29) isolated from diseased oysters
1001 or within the environment respectively. *V. aestuarianus* strains were cultivated in LB-0.3M
1002 NaCl at 25°C under agitation for all the experiments. Four evolutionary treatments, a control,
1003 copper stress, salt stress and salt plus copper stress were chosen. To decide on the
1004 concentrations of salt and copper in the three stress treatments, growth curves were
1005 performed over a range of salt and copper stress concentrations in a crossed design allowing
1006 to define a 2-dimension niche. A pre-culture was set up from the glycerol stock of the
1007 ancestral strains (12/016 or U29) and cultivated in 4mL of LB-NaCl Broth at 25°C with 150 rpm
1008 orbital shaking overnight. After adjusting OD600 at 1, 2µL of the pre-culture was then
1009 transferred to each well of a microtiter plate containing 200µL of LB broth and various
1010 concentrations of sodium chloride and/or copper (copper(II) sulfate pentahydrate) in
1011 triplicate. Sodium chloride and copper(II) sulfate pentahydrate were ordered from Sigma
1012 Aldrich. The optical density at 600nm (OD600) was monitored during 24h with a plate reader
1013 with 5 min double orbital agitation just before reading, readings were acquired every 20 min.
1014
1015 These experiments allowed us to establish 4 evolutionary treatments in 3 replicates (Table
1016 S9.1) by testing different concentrations of $CuSO_4$ and NaCl. In the control non-stressful
1017 treatment, we used LB-0.3M NaCl. For the 3 stressful treatments (copper, salt, and the
1018 combination of the two), pilot experiments indicated that for 12/016 strain, $CuSO_4$ at 2.5M,
1019 NaCl at 0.84M, and 1.25M $CuSO_4$ + 1.125M NaCl allowed to obtain about 50% initial reduction
1020 of growth. For U29 strain, $CuSO_4$ at 1.25M, NaCl at 0.84M, and 0.625M $CuSO_4$ + 0.84M NaCl
1021 allowed to obtain about 30% initial reduction of growth rate.
1022 For each strain, the experimental microplates were set up with triplicate for each strain and
1023 for each stress condition. Microplate wells were filled with 180µl of media and inoculated
1024 with 20µl of culture at OD600 equal to 1 to obtain a final OD600 at 0.01 and incubated at 25°C

1025 under double orbital agitation in a microplate reader. This gave us a total of 24 evolutionary
1026 replicates: 2 strains x 4 stressor conditions x 3 replicates (Table S13.1).
1027
1028 **Table S13.1** Evolutionary treatments and number of replicates at the start and end of the experiment.

Strain ID	Salinity	Copper	Stress	Replicates
12/016	0.3M	0	Control	3
12/016	0	1.25M	Copper	3
12/016	0.84M	0	Salt	3
12/016	0.84M	0.625M	Copper + Salt	3
U29	0.3M	0	Control	3
U29	0	2.5M	Copper	3
U29	0.84M	0	Salt	3
U29	1.125M	1.25M	Copper + Salt	3

1029
1030 Rearing and transferring protocol
1031 To start each experiment, a pre-culture was set up from a colony and cultivated in 4mL of LB-
1032 0.3M NaCl at 25°C with 150 rpm orbital shaking for 24 hours. All experimental evolution lines
1033 were derived from the same clonal ancestor. For 12/016 strain, evolution lines were
1034 propagated every 48h (to achieve stationary phase) by 1/100 dilution in a new microplate.
1035 For U29, evolution lines were propagated every 12h (to achieve stationary phase) by 1/100
1036 dilution in a new microplate. Readings of OD600 were acquired every 20min for each well.
1037 After propagation, each microplate was cryopreserved at -80°C after adding 15% of glycerol
1038 per well. For 12/016 strain, the experiment lasted for 46 days (22 cycles), corresponding to ~
1039 180 generations. For U29 strain, the experiment lasted 8 days (16 cycles), corresponding to ~
1040 128 generations.
1041
1042 **Final phenotypic tests and data collection**
1043 As described above, initial and final growth rate was obtained in its own evolutionary
1044 treatment via spectrophotometry at cycles 1 and 22 for strain 12/016, and at cycle 1 and 16
1045 for strain U29. In addition, after defrosting the glycerol stock from the last cycle, 20µL of each
1046 evolutionary line was transferred to 180µL and assayed in the control media (LB-0.3M NaCl,
1047 incubated at 25°C), with double orbital shaking for 24 hours and OD600 recorded every
1048 20min.
1049

Supporting Information Figures

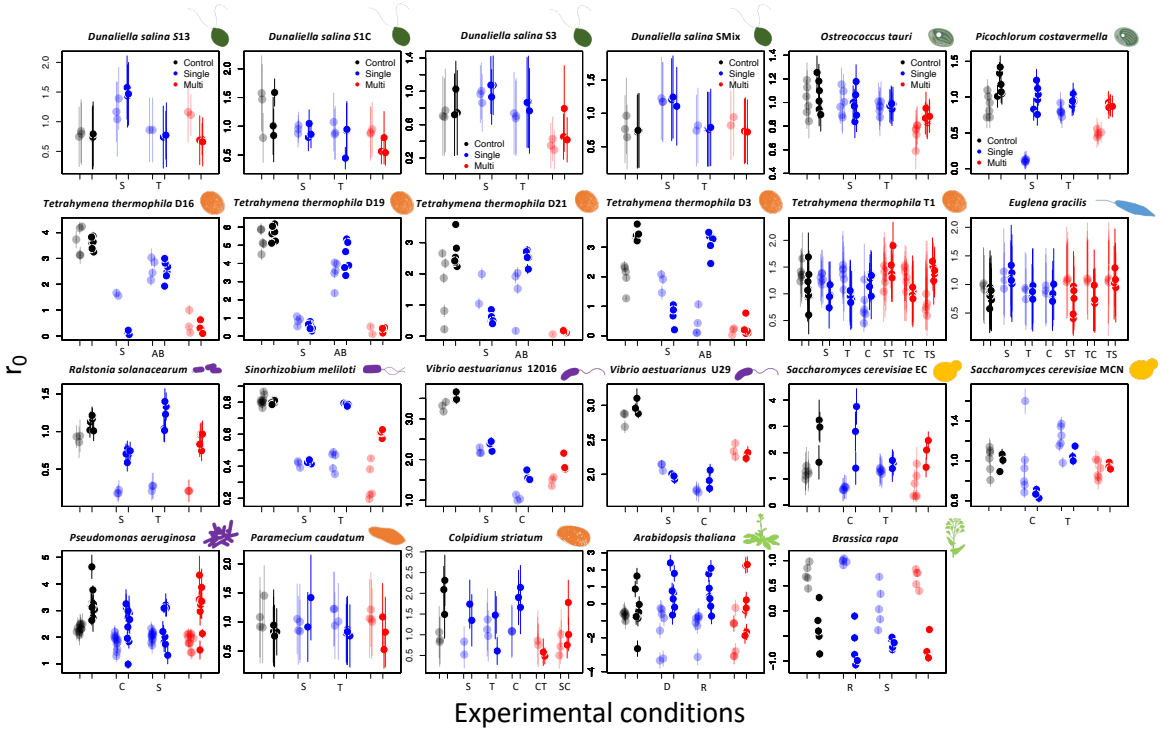

Fig. S1 Fitness estimates (r_0) for each of the 14 species (silhouette) assayed in their own evolutionary conditions; control (grey), single stressors (blue) or multiple stressor (red). Shaded points are fitness estimates after the initial transfer to the experimental conditions, while thick points are the final fitness estimates at the end of the evolutionary experiments. The corresponding initial and final fitness estimates are therefore coupled by experimental treatments. Points and bars represent the fitness estimate and standard deviation for a biological replicate. Different strains of the same species are indicated next to their scientific name, with their silhouette repeated. Capital letters indicate the identity of the single stressors, S (Salt), T (Temperature), C (Copper), AB (Antibiotics), D (Disturbance), R (Resources), or of the multiple stressors when more than one combination was tested (*i.e.*, ST, TC, TS).

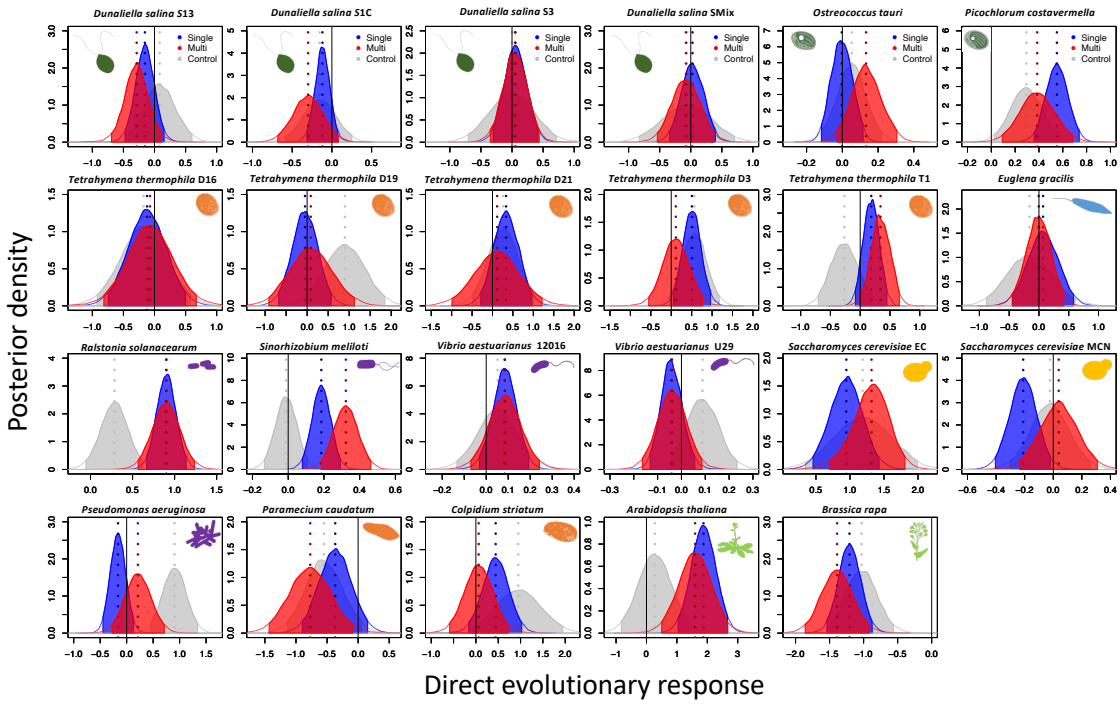

Fig. S2 Posterior distributions for direct evolutionary responses of each strain. Positive values represent a gain in fitness over the evolutionary experiment. In grey is the control in the absence of stressor, in blue and red the single and multiple stressors respectively. The dashed lines visualize the median posterior values, and the shaded area the 95% compatibility intervals. The posteriors were obtained by calculating the difference between final and initial fitness for the control, single and multiple stressor evolutionary treatments respectively (14 species and 23 strains, complete dataset).

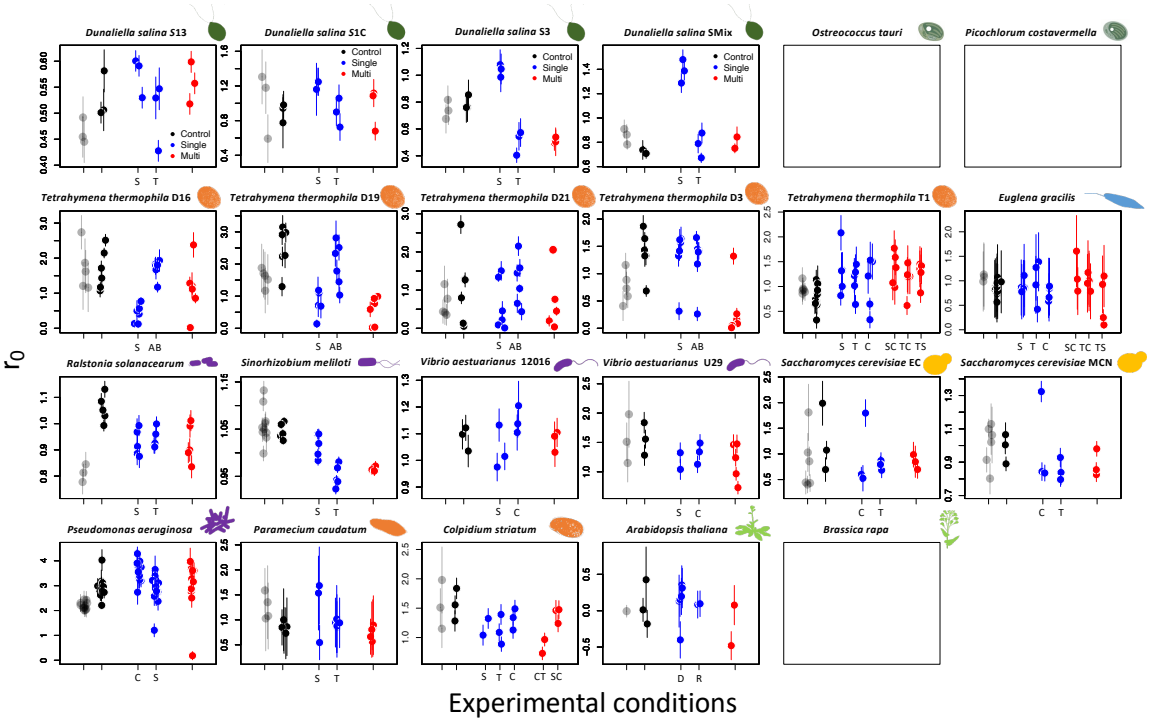

Fig. S3 Fitness estimates (r_0) for 11/14 species (silhouette) assayed in control conditions, the 3 empty plots correspond to species for which such back-to-control conditions were not tested. The evolutionary treatment of origin is indicated by different colours; grey is control, blue is single stressors, and red is multiple stressors. Shaded grey points are the initial fitness estimates in control conditions, while thick points are the final fitness estimates at the end of the evolutionary experiments. Different strains of the same species are indicated next to their scientific name, with their silhouette repeated. Points and bars represent the fitness estimate and standard deviation for a biological replicate. Capital letters indicate the identity of the single stressors experienced during evolution, S (Salt), T (Temperature), C (Copper), AB (Antibiotics), D (Disturbance), R (Resources), or of the multiple stressors when more than one combination was used (*i.e.*, ST, TC, TS).

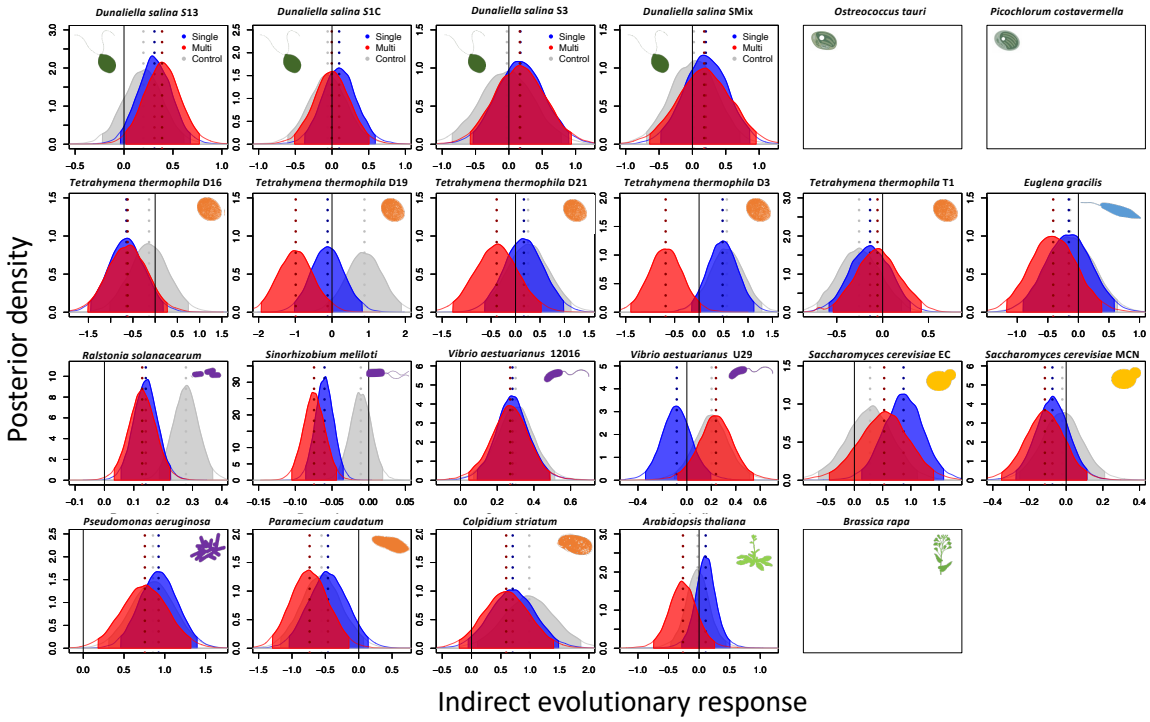

Fig. S4 Posterior distributions for indirect evolutionary responses of each strain. Positive values represent a gain in fitness over the evolutionary experiment. In grey is the control in the absence of stressor, in blue and red the single and multiple stressors respectively. The dashed lines visualize the median posterior values, and the shaded area the 95% compatibility intervals. The posteriors were obtained by calculating the difference between the final fitness of the 3 evolutionary treatments assayed in control conditions and the initial fitness in control conditions (11 species and 20 strains, reduced dataset).

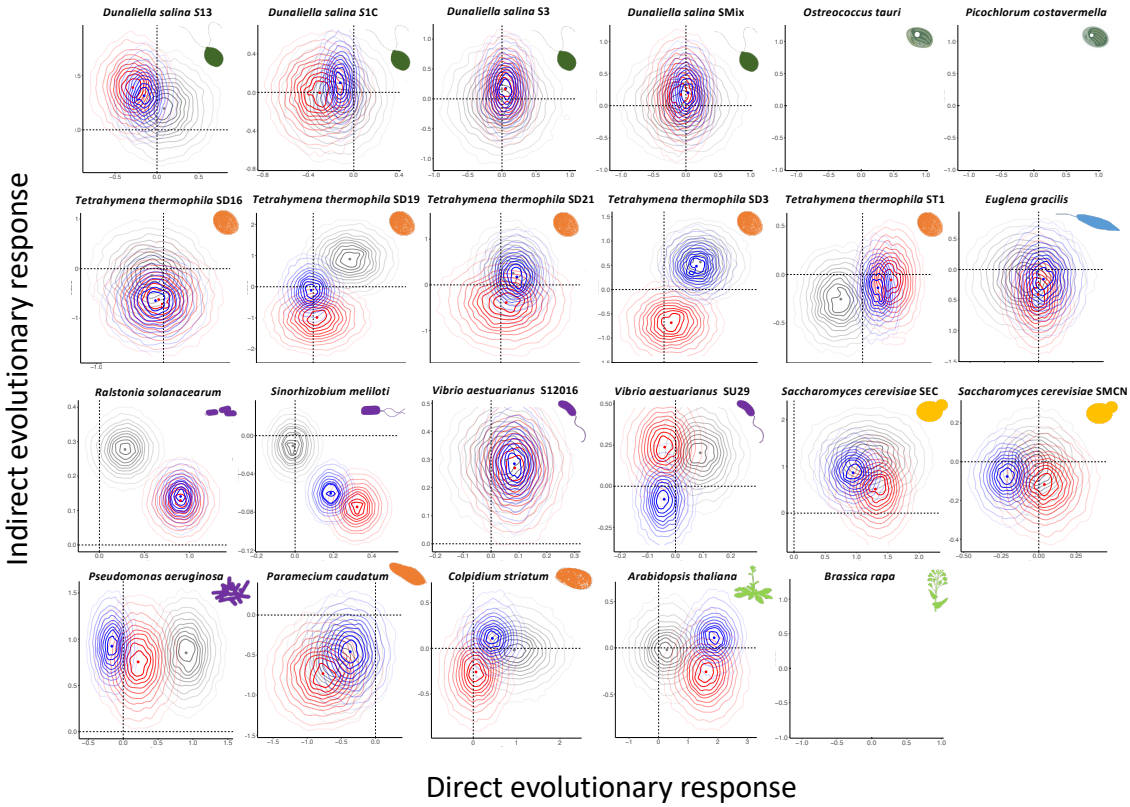

Fig. S5 Density distributions of the direct response to selection (assay in own evolutionary conditions) against the indirect evolutionary response (assay in control conditions) for the control, single and multiple stressor evolutionary treatments (11 species and 20 strains). Closed lines are the probability regions spanning from 10% to 95% from darker to lighter. The points and dashed lines are the median values of the posterior distributions. The direct and indirect responses correspond to the difference between final and initial fitness, with positive values representing a gain in fitness over the evolutionary experiment.

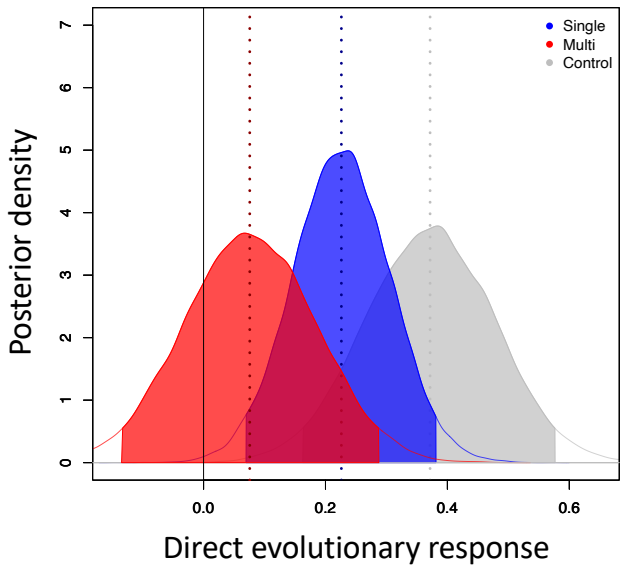

Fig. S6 Posterior distributions for direct evolutionary responses without the two plant species. Posterior distributions of the direct response to selection (fitness estimates from assay in own evolutionary conditions). The posteriors were obtained by calculating the difference between final and initial fitness for the control, single and multiple stressor evolutionary treatments respectively (12 species and 21 strains).

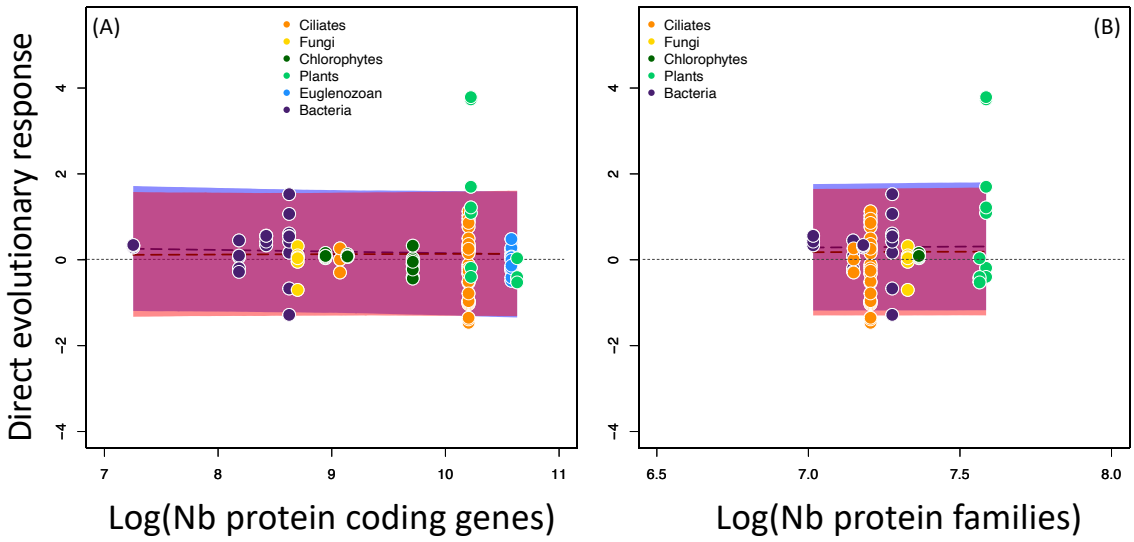

Fig. S7 Direct evolutionary response in fitness as a function of proxies of organismal complexity. (A) Change in fitness of the taxon in function of their complexity defined as the logarithm of the number of protein coding genes. Information for *Colpidium striatum* was not available, and it was removed from this analysis (13 species and 22 strains). (B) Change in fitness of the taxon in function of their complexity (logarithm of the number of protein families). The points are the mean fitness value of an independent evolutionary replicate relative to its average control, and each colour identifies a different taxon. Dashed lines are the median, and the shaded areas the 95% CI of the model predictions for single (blue) and multiple (red) stressors.

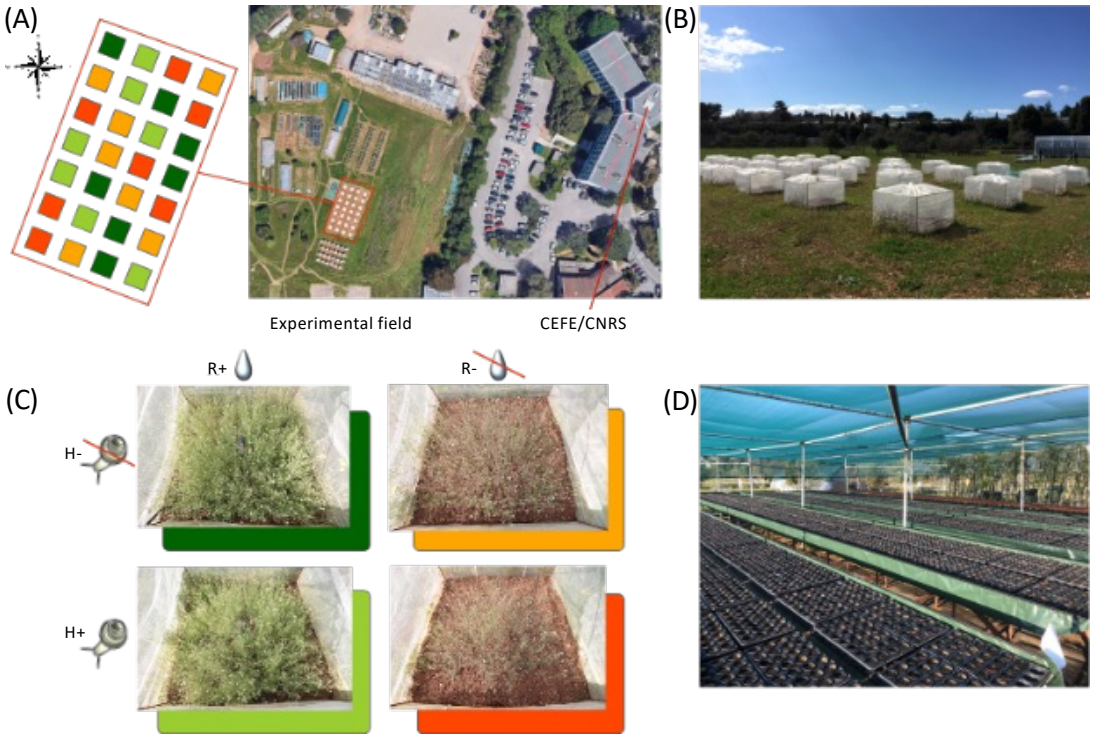

Fig. S8 (A) The localization of the mesocosms of the experiment for *A. thaliana*, with their treatment assignment indicated with different colours: high resource, low herbivory (R+/H-, dark green); high resource, high herbivory (R+/H+, light green); low resource, low herbivory (R-/H-, yellow). (B) Photograph of the mesocosms of the AraBreed experimental evolution project. (C) Photographs of populations representative of the first generation of experimental evolution in four treatments: high resource, low herbivory (R+/H-, dark green); high resource, high herbivory (R+/H+, light green); low resource, low herbivory (R-/H-, yellow); and low resource, high herbivory (R-/H+, red). (D) Photograph of the common garden experiment.

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