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Abstract

The adaptive function of sex has been extensively studied, while less consideration has been given to the potential downstream consequences of sex on evolution. Here we investigate one such potential consequence, the effect of sex on the repeatability of evolution. By affecting the repeatability of evolution, sex could have important implications for biodiversity, and for our ability to make predictions about the outcome of environmental change. We allowed asexual and sexual populations of *Chlamydomonas reinhardtii* to evolve in novel environments and monitored both their change in fitness and variance in fitness after evolution. Sex affected the repeatability of evolution by changing the importance of the effect of selection, chance and ancestral constraints on the outcome of the evolutionary process. In particular, the effects of sex were highly dependent on the initial genetic composition of the population and on the environment. Given the lack of a consistent effect of sex on repeatability across the environments used here, further studies to dissect in more detail the underlying reasons for these differences as well as studies in additional environments are required if we are to have a general understanding of the effects of sex on the repeatability of evolution.

Introduction

The ubiquity of sexual lineages among eukaryotes is a long-standing problem in biology (Smith 1978; Bell 1982). Extensive research has examined the adaptive function of sex, that is the mechanisms for its origin and maintenance over evolutionary time (Lively and Morran 2014; Becks and Alavi 2015). However, less consideration has been given to the potential downstream consequences of sex on evolution, such as changes in genome modularity and architecture, population differentiation, or evolvability. While these consequences may or may not have any adaptive significance, they can potentially have important implications for evolution. Here we investigate one potential downstream consequence of sex: the effect of sex on the repeatability of evolution. By altering the repeatability of evolution, sex could affect the predictability of evolution, and have long-term consequences for rates of diversification.

The repeatability of evolution depends on the relative importance of natural selection as the deterministic driver (Fisher 1930; Muller 1932; Gerrish and Lenski 1998; Desai and Fisher 2007), chance as the stochastic driver (Lenski and Travisano 1994; Wiser et al. 2013), and ancestry as the source for unpredictable constraints (Weinreich et al. 2005) on further evolutionary change. The relative roles of these components in adaptation can be examined experimentally by allowing initially genetically identical replicate populations to evolve in identical environments. If replicates reach similar evolutionary end points then evolution has been highly repeatable, whilst differences in end points indicate the role of chance. If the experiment is repeated with different ancestors, the role of ancestral constraints can also be evaluated.

In general, experiments in asexual and initially isogenic populations of microbes show that fitness changes during adaptation to a novel environment are often repeatable (Travisano et al. 1995; Collins et al. 2006; Flores-Moya et al. 2008; Bell 2012b; Spor et al. 2013). However, differences in ancestry, combined with chance events during adaptation, can have a significant effect on the fitness trajectories of evolving populations in some environments (Melnyk and Kassen 2011) and in small populations (Lachapelle et al. 2015). Chance effects also seem to be more important for the evolution of phenotypic traits than for the evolution of fitness itself (Travisano et al. 1995; Collins et al. 2006; Flores-Moya et al. 2008; Bell 2012b).

In sexual populations, the relative contribution of selection, chance, and ancestry is highly variable from study to study (Teotonio and Rose 2000; Teotonio et al. 2002; Kawecki and Mery 2003; Joshi et al. 2003a; Griffiths et al. 2005; Simões et al. 2008; Teotónio et al. 2009; Fragata et al. 2014). However a direct experimental comparison of the repeatability of evolution of sexual and asexual populations has not, to the best of our knowledge, been done.

There are reasons to expect the repeatability of evolution to be higher in sexual populations since recombination allows selection to act independently at different loci (McDonald et al. 2016). That is, when a beneficial mutation appears in an asexual population, its ultimate fate will be strongly influenced by the genetic background that it arises in. A beneficial mutation that arises in a relatively low fitness background will ultimately be lost, whilst the same mutation arising in a better background may fix

101 (Weismann 1889; Fisher 1930; Muller 1932). As a result, initial chance associations will
102 be relatively important. In contrast, in a sexual population, the beneficial mutation can be
103 selected independently of the background it appears in (Hill and Robertson 1966;
104 Felsenstein 1974; Peck 1994), making such chance associations possibly less important
105 and evolution more deterministic.
106
107 We also expect divergence among individuals in sexual populations to be lower than
108 within asexual populations after evolution. In sexual populations, the variance that is
109 generated by one episode of sex (Colegrave et al. 2002) will increase the efficiency of
110 selection, and recombination will separate beneficial mutations from their background,
111 reducing the spread of groups of linked mutations (McDonald et al. 2016). Whereas in
112 asexual populations, competition between beneficial mutations in different individuals,
113 i.e. clonal interference, will slow the fixation of mutations, and hitchhiking of mutations
114 on the background of the beneficial mutation will lead to a greater number of mutations
115 fixed overall (Kao and Sherlock 2008; Lang et al. 2013; McDonald et al. 2016). Hence
116 we expect there will be fewer mutations segregating at any one point and fewer mutations
117 fixed in sexual populations than in asexual populations.
118
119 To test these hypotheses, we compare the repeatability of evolution between asexual and
120 sexual experimental populations of the green alga *Chlamydomonas reinhardtii* in four
121 different environments. In particular, we focus on how (1) the efficiency of selection; and
122 (2) chance and ancestral constraints differ between asexual and sexual populations. We
123 estimate the efficiency of selection by measuring rates of fitness change; and we estimate

124 the role of chance and ancestral constraints by measuring variance in fitness among
125 independent populations. We find that the effects of sex are highly dependent on the
126 environment, with sex enhancing convergence in some environments and divergence in
127 others.
128
129
130 **Material and Methods**
131 *Base populations*
132 We generated three genetically different starting points by crossing three different pairs
133 of wild-type strains of the haploid green alga *Chlamydmonas reinhardtii*. Ancestry A was
134 generated by using the F1 progeny from a cross between CC-1690 and CC-1691; ancestry
135 B using the F1 progeny from a cross between CC-2342 and CC-2344; and ancestry C
136 using the F1 progeny from a cross between CC-2931 and CC-2937 (Figure 1). These
137 strains have been shown to be genetically (Smith and Lee 2008; Flowers et al. 2015) and
138 phenotypically (Malcom et al. 2015) different, with the average divergence between any
139 pair of strains being about 3% (Smith and Lee 2008; Flowers et al. 2015). Based on
140 population structure analyses, the strains from ancestry A belong to the Laboratory group
141 of strains; strains from ancestry B belong to the West group of strains; and strains from
142 Ancestry C belong to either the Southeast and West groups (CC2391), or the Northeast
143 group (CC2937) (Flowers et al. 2015). The progeny from each cross should retain the
144 genetic signature of their two parents and therefore maintain on average the genetic
145 dissimilarity that was present among parents from each ancestry.
146

147 Twelve spores from each ancestry were isolated at random from each pool of progeny,
148 for a total of 36. From now on these spores are referred to as the ancestors. Each
149 experimental line was assembled using eight spores from a given ancestry: the asexual
150 lines contained eight spores of a single mating type (we used spores of mating type - for
151 Ancestry A and C, and spores of mating type + for Ancestry B), whereas the sexual lines
152 contained four spores of mating type + and four spores of mating type -. The asexual and
153 sexual lines from a given ancestry thus shared four ancestral spores. We started our
154 experiment with genetically diverse populations instead of genetically uniform
155 populations because the sexual populations required at least two genotypes (i.e. one
156 genotype of each mating type), and because the larger the number of genotypes sampled
157 from the F1 progeny, the higher the probability that asexual and sexual populations will
158 share the same amount of genetic variance. Indeed, our growth assays show that the
159 ancestral spores used to assemble the asexual lines do not differ statistically from the
160 ones used to assemble the sexual lines in their growth rates across the four selection
161 environments described below (linear mixed model with Satterthwaite approximations to
162 degrees of freedom: $t_{10} = -0.88$, $P = 0.40$). Hence, the mode of reproduction treatment is
163 not confounded with differences in starting points.
164
165 *Selection experiment*
166 Each ancestral spore was grown individually from a single colony. Once fully grown, the
167 ancestral spores were pooled together to construct each experimental line, and 24 samples
168 (six replicates in each of four environments) of each mixture were used to initiate each
169 replicate line, which were then propagated independently.

For each combination of ancestry and mode of reproduction, we had 6 replicate lines, for a total of 3 x 2 x 6 = 36 independent lines. Each line was propagated in each of four different environments: Bold's minimal medium (referred to as Bold's; Harris 2009); Bold's minimal medium supplemented with 0.435 μM Atrazine and 0.250 μM S-metolachlor (referred to as Herbicides); Bold's minimal medium supplemented with 7 gL^{-1} Na_2SO_4 (referred to as Na_2SO_4); and Bold's minimal medium supplemented with 5 gL^{-1} NaCl (referred to as NaCl). The environments were chosen to represent a random sample of all possible environments, and because they are easily tractable in the laboratory. Bold's is a standard laboratory medium representing fairly benign conditions. NaCl (Lachapelle and Bell 2012; Lachapelle et al. 2015) and Herbicides (Lagator et al. 2014) have been used in the past to study adaptation in *C. reinhardtii*, and target different aspects of growth (i.e. NaCl generates osmotic and oxidative stresses, while Atrazine targets photosynthesis and S-metolachlor targets the synthesis of long chains of fatty acids). We chose to use 5 gL^{-1} of salt because it has been shown to elicit evolutionary responses within 200 generations (Lachapelle and Bell 2012).We used two herbicides instead of only one to generate a more complex target to selection. We chose to use 0.435 μM of Atrazine and 0.250 μM of S-metolachlor after preliminary assays with a range of concentrations showed that this combination reduced fitness significantly, but not to an extent where cell densities become lower than the detection limit of the spectrophotometer. Finally, Na_2SO_4 was chosen randomly as its effects on *C. reinhardtii* are unknown. It occurs naturally in lakebeds, in the form of mirabilite in wet or damp environments and in the form of thenardite in arid environments. Similarly to the herbicides, we chose to use 7 gL^{-1} after preliminary assays with a range of concentrations.

193 Preliminary assays showed that the stressors and the concentrations chosen reduce growth
194 rates to different extents compared to the benign environment of Bold's.
195
196 The experiment consisted of vegetative growth cycles interspersed with sexual cycles.
197 The lines were cultured in 24-well plates, with breathable sealing films to ensure even
198 evaporation and air exchange across the plate (except during mating where the plastic lids
199 were used to ensure optimal light intensity), shaken at 180 r.p.m. with a 3 mm rotation
200 diameter. The cultures were maintained in a growth chamber at 24 degrees Celsius, 60%
201 humidity, and 8000 Lux constant lighting.
202
203 The sexual cycles were imposed after about 10, 50, 100, 150, 200, and 260 generations of
204 vegetative growth. The protocol for the sexual cycle was imposed on all lines, even on
205 the asexual lines, which were not expected to mate given that they were composed of
206 spores of only one mating type. Cultures were visually inspected under a microscope
207 during each sexual cycle to confirm the absence of mating reactions in the asexual lines.
208 Briefly, at the end of a vegetative growth cycle, the spent media was replaced with
209 nitrogen-free media by centrifuging the cultures. The cultures were left static in nitrogen-
210 free liquid media for approximately 24 hours to allow gametogenesis and mating to
211 occur. After this period, the zygotes and 50 μL of culture were transferred to an agar
212 plate, or in the case of the asexual lines 50 μL of culture was transferred to an agar plate.
213 The agar plates were wrapped in aluminium foil and left in the dark for zygote maturation
214 to occur. After four days, mature zygotes were exposed to chloroform vapour for 45
215 seconds to kill unmated cells, and then placed under the lights for germination. The

asexual lines were not exposed to chloroform but put directly under the lights. After two days in the light, the cells were re-suspended in liquid media and transferred back into the vegetative growth cycles. The cultures were then serially transferred every 3-4 days using a 5% inoculum (100 µL into 1900 µL of fresh media). A total of 6 sexual cycles and 60 vegetative cycles were imposed for a total of about 300 generations.

Seven sexual lines (three from the Na_2SO_4 environment and four from the Herbicides environment) went extinct during the experiment because they failed to mate during the sexual cycle. In particular, in the Na_2SO_4 environment, 3 replicate lines from ancestry C went extinct, one during the 2^{nd} sexual cycle, another during the 4^{th}, and the other during the 5^{th}, and in the Herbicides environment, 3 replicate lines from ancestry C and one replicate from ancestry B went extinct, during the 1^{st}, 3^{rd}, 6^{th}, and 4^{th} sexual cycles respectively. Attempts were made to mate them again whenever this happened but failed repeatedly in these particular cases.

Population sizes generally increased throughout the experiment, over the asexual and sexual cycles, except during the asexual cycles in Bold's where they remained stable (data not shown). The minimum population size, i.e. the lowest cell density reached by the end of a growth cycle out of all asexual cycles, was on average 4.2×10^6, 9.8×10^5, 2.8×10^5, and 8.7×10^5 cells, with minimum inoculum sizes hence being 2.1×10^5, 4.9×10^4, 1.4×10^4, and 4.4×10^4 cells for each line in Bold's, Herbicides, Na_2SO_4, and NaCl, respectively. The minimum population size experienced during sexual cycles was on average 2.3×10^6, 1.1×10^6, 5.2×10^5, and 1.6×10^6 cells, with inoculum sizes being 1.1

239 x 10^5, 5.5 x 10^4, 2.6 x 10^4, and 8.1 x 10^4 cells for lines in Bold's, Herbicides, Na_2SO_4,
240 and NaCl, respectively. Our protocol for selecting zygotes from sexual lines (i.e.
241 chloroforming sexual populations to kill unmated cells) did not put the sexual lines at a
242 disadvantage compared to the asexual lines in terms of population sizes, as there was no
243 statistical differences between asexuals and sexuals in the minimum population size after
244 the sexual cycle (analysis of variance: $F_{1,129} = 0.233$, $P = 0.63$).
245
246 *Ancestral fitness assays*
247 We chose maximum growth rate as our measure of fitness. Whilst in principle there are
248 other aspects of growth dynamics that may be components of fitness, previous studies
249 show that in experiments such as these maximum growth rate consistently shows greater
250 selection gradients and evolutionary responses than other life-history traits such as time
251 in lag phase and death rates at stationary phase (Dykhuizen 1990; Vasi et al. 1994; Lenski
252 et al. 1998). Moreover, whilst maximum growth rate should always be positively selected
253 in populations that spend reasonable amounts of time in growth phase, other aspects of
254 fitness may vary in their direction of selection depending on the exact growth dynamics,
255 which vary in different environments. We have nonetheless also estimated fitness using
256 maximum optical density as a measure of yield and obtained the same qualitative results
257 (Supplementary Figure 1 and 2).
258
259 We estimated the fitness of the ancestral spores used to assemble each selection line by
260 measuring maximum growth rates in each of the four selection environments. The
261 ancestors had been maintained in dim light on Bold's agar throughout the experiment,

262 conditions which limit growth and selection (Harris 2009). A single colony from each
263 ancestor was grown in Bold's media for two cycles to minimise physiological
264 differences, and then transferred in triplicate to each of the four environments. All
265 cultures were grown for two cycles in the assay environments. Growth was monitored
266 during the second growth cycle in the assay environments by measuring optical density at
267 750 nm every 8 ± 1 hours. We chose to measure during the second cycle to allow the
268 three replicates one cycle of independent growth and avoid the measurement of initial
269 physiological responses to the new environment.
270
271 To estimate the maximum growth rate from measures of optical density over time, we
272 fitted a nonlinear model using nonlinear least squares in the 'nlstools' R package (Baty et
273 al. 2015). We first fitted a baranyi model (Baranyi and Roberts 1994; Baranyi et al.
274 1995). This type of model fitted 90%, 94%, 67%, and 84% of spores assayed in Bold's,
275 Herbicides, Na_2SO_4, and NaCl respectively. The fit was too poor on the other spores for
276 the model to converge. These spores were therefore fitted using either a baranyi model
277 without N_{max}, a baranyi model without lag, or a linear model, as appropriate. Model fits
278 were visually inspected to ensure the proper model had been applied (for examples of a
279 fits from each type of model see Supplementary Figure 3).
280
281 *Evolved fitness assays*
282 The evolved lines from each selection environment were assayed in their respective
283 selection environment in separate experiments because of space constraints. For similar
284 reasons, it was impossible for us to assay all 36 ancestral spores and all 36 evolved lines

285 all at once and so we assayed the fittest ancestral spore in terms of maximum growth rate,
286 along with the evolved lines.
287
288 We assayed four random spores per evolved line. 24 spores (6 lines x 4 spores) were
289 picked from the fittest ancestor to match the number of evolved spores assayed per
290 ancestry x reproduction mode. All colonies were grown in Bold's liquid media for one
291 growth cycle to minimise physiological differences, and then transferred to the
292 environment in which the evolved lines were selected. Growth was monitored during the
293 second cycle in the assay environment and growth parameters estimated as described
294 above.
295
296 *Statistical analyses*
297 All analyses were performed in R version 3.2.1. To determine if the ancestral spores used
298 to assemble the sexual lines differ from the ancestral spores used to assemble the asexual
299 lines we fitted a mixed effect model using the lmer function in the R package 'lme4'
300 (Bates et al. 2015). The mode of reproduction (asexual or sexual) was set as a fixed
301 factor, while environment, ancestry, and spore within ancestry were set as random
302 factors. P values were obtained using the R package 'lmerTest' (Kuznetsova et al. 2014)
303 with type III sum of squares in an analysis of variance and Sattertwhaite approximation
304 for degrees of freedom by using the normal approximation.
305
306 The effect of recombination on selection was determined individually for each selection
307 environment by fitting mixed effect models using the lmer function, with mode of

reproduction (asexual or sexual) and selection (ancestral or evolved) as fixed factors, and ancestry, line within ancestry, and spore within line within ancestry as random factors. We allowed for random intercepts.

To estimate the constraints from ancestry, the importance of chance, and the change in diversity within lines, we calculated the difference between evolved variances and ancestral variances. Thus a positive change in variance indicates that there is more variation after evolution than at the start (i.e. divergence over time), whereas a negative change in variance indicates that there is less variance after evolution than at the start (i.e. convergence over time). The evolved variances were extracted from a model with ancestry, line within ancestry, and spore within line within ancestry as random factors. Separate models were fitted for each combination of environment and mode of reproduction. The ancestral variances were extracted from a model with ancestry and spore within ancestry as random factors. The among-line ancestral variance was set at zero. Note here that the evolved data and the ancestral data come from different fitness assays. Temporal heterogeneity in environmental conditions between assays can lead to differences in growth. It is unlikely that temporal heterogeneity would interact with the mode of reproduction treatment, and so the variance estimates for the asexuals and the sexuals should be affected to the same extent. The actual value of the change in variance is likely to be inexact, and values near zero need to be interpreted with reserve.

This approach of using the change in variance differs from our previous approach (Lachapelle et al. 2015) where we calculated the relative contribution of selection,

chance, and ancestry by dividing the evolved variance by the total evolved variance. It is only appropriate to use proportions to compare treatment levels for their effects on selection, chance, and ancestry, when the initial variance is the same across all treatment levels. For example, if lines are isogenic at the start and the same genotype is used across all treatments, then there is no need to correct for initial variance. However, in cases such as in the experiment reported here where lines are diverse at the start, and sexual and asexual lines cannot be assembled using the same genotypes (because of mating type constraints), it is not appropriate to compare evolved variances without correcting for initial variance. Differing amounts of variance can affect the potential for convergence and divergence among histories, among line, within lines. This is why we report the change in variance instead of the proportion of the total variance explained by either chance or ancestry.

To determine the statistical significance of the differences in the change in variance between asexual and sexual populations we did a randomisation test. We randomly allocated each evolved spore to a line, ancestry, and mode of reproduction (keeping spores within their environment of selection), each ancestral spore to an ancestry and mode of reproduction, and then performed the analysis described above to calculate the change in variance. The number of times the random absolute change in variance was as large or larger than the absolute observed change in variance over the total number of randomisations (10,000) is our significance statistic.

Results

We picked four different environments in which to study the consequences of sex on the repeatability of evolution. The Na_2SO_4 environment is the most severe with slowest ancestral maximum growth rates, followed by NaCl, Herbicides, and Bold's (Figure 2). To estimate the repeatability of evolution, we measured the strength of both deterministic factors such as selection, and stochastic factors such as chance and ancestry. Note that we use the terms divergence and convergence when referring to an increase and decrease in variance over time, and the term diversity when referring to the amount of variance at a given time point. Below we present the results for each environment in turn.

Repeatability of evolution in Na_2SO_4

We first determine what effect sex has on the efficiency of selection. The effect of selection is estimated by comparing the fitness of evolved spores to that of the ancestral spores, such that the greater the difference, the greater the contribution of selection to evolutionary change. The evolved lines have higher maximum growth rates than their fittest ancestral spore in Na_2SO_4 ($t_{63} = 4.79$, $P = 1.05 \times 10^{-5}$) indicating that selection has been effective in increasing fitness; and the sexual lines have increased their maximum growth rates to a greater extent than their corresponding asexual lines after selection (Figure 3; Table 1; $t_{63} = 4.22$, $P = 7.91 \times 10^{-5}$), indicating that sex increased the effect of selection.

Second, we determine what effect sex has on the constraints of ancestry. The effect of ancestry is estimated by comparing the variance among ancestries before and after

377 evolution, such that the greater the increase in variance among ancestries, the greater the
378 constraints from ancestry. Ancestries diverged during evolution in Na_2SO_4 , indicating
379 that ancestry constrained evolution; but the sexual lines diverged less than their asexual
380 counterparts (Figure 4; $P = 0.0054$), indicating that sex reduced the constraints of
381 ancestry.
382
383 Third, we determine what effect sex has on the importance of chance. The importance of
384 chance is estimated by comparing the variance among replicate lines before and after
385 evolution, such that the greater the increase in variance, the greater the importance of
386 chance. Replicate lines diverged during evolution in Na_2SO_4, indicating that chance had
387 an important contribution to evolutionary change, and the sexual lines diverged more than
388 their asexual counterparts (Figure 4; $P < 0.0001$), indicating that sex increases the
389 importance of chance during evolution.
390
391 Finally, as our experimental lines were initially genetically diverse, we sought to
392 determine if sex had any effect on the maintenance of diversity within lines over long
393 evolutionary timescales. If sex helps to generate diversity within lines during evolution
394 compared to asexual reproduction, then we should see an increase in variance among
395 spores within a line, and if sex leads to a reduction in diversity during evolution, then we
396 should see a decrease in variance among spores. Note that our design for the fitness
397 assays is such that we can separate out variance among spores from variance from
398 measurement error (see Methods). Variance among spores increased during evolution in

399 Na_2SO_4, and increased to a greater extent in sexual lines than in their asexual counterparts
400 (Figure 4; P = 0.0028).
401
402 Hence, after 300 generations of evolution in 7 gL^{-1} Na_2SO_4 sex appears to increase the
403 importance of selection and chance, reduce the constraints of ancestry, and lead to greater
404 increases in diversity within lines (Table 2). We now look at whether the effects of sex on
405 (1) selection, (2) ancestry, (3) chance, and (4) diversity within lines observed after
406 evolution in Na_2SO_4 are the same after evolution in NaCl, Herbicides, and Bold's in turn.
407
408 *Repeatability of evolution in NaCl*
409 The evolved lines have higher maximum growth rates than their fittest ancestral spore (t_{66}
410 = 6.28, P = 3.04 x 10^{-8}), and the sexual lines have higher maximum growth rates than
411 their corresponding asexual lines after selection (Figure 3; Table 1; t_{66} = 2.62, P =
412 0.0108). Ancestries converged during evolution in NaCl, and sexual lines converged to
413 the same degree as the asexual lines (P = 0.26). In terms of chance, replicate lines
414 diverged during evolution in NaCl, but sex again had no measurable effect (Figure 4; P =
415 0.26). Finally, variance among spores was lower after evolution in NaCl, but decreased to
416 the same extent in sexual and asexual lines (NaCl: P = 0.21). Hence, after 300
417 generations of evolution in 5 gL^{-1} NaCl sex appears to increase the importance of
418 selection, but does not alter the constraints of ancestry, the importance of chance, or the
419 amount of diversity within lines (Table 2).
420
421 *Repeatability of evolution in the herbicide mixture*

422 The maximum growth rate of lines evolved in the herbicide mixture is no different from
423 that of their fittest ancestral spore (t_{62} = 0.820, P = 0.416), and there is no effect of sex on
424 selection (Figure 3; Table 1; t_{62} = -0.386, P = 0.701). Upon visual inspection, it appears
425 that the lack of response to selection might be due to contrasting responses in each
426 ancestry. Indeed, by analysing each ancestry on their own, we find that while for ancestry
427 A and C sexual spores have lower fitness than asexual spores after evolution (ancestry A:
428 t_{20} = -2.86; P = 0.0098; ancestry C: t_{17} = -3.14; P = 0.0059); for ancestry B sexual spores
429 have higher fitness than asexual spores after evolution (t_{19} = 3.52; P = 0.0023). This
430 effect of ancestry on the response to selection was also detected in the analyses below.
431
432 Ancestries diverged during evolution in herbicides, and sexual lines diverged more than
433 their asexual counterparts (Figure 4; P < 0.0001). In terms of chance, replicate lines
434 diverged during evolution, and sexual lines diverged less than their asexual counterparts
435 (P < 0.0001). Finally, variance among spores was lower after evolution in the herbicide
436 mixture, but decreased to the same extent in sexual and asexual lines (Herbicides: P =
437 0.075). Hence, after 300 generations of evolution in the herbicide mixture, sex appears to
438 have no effect on the importance of selection and the amount of diversity within lines,
439 increases the constraints of ancestry, and decreases the importance of chance (Table 2).
440
441 *Repeatability of evolution in Bold's*
442 The lines evolved in Bold's have significantly lower maximum growth rates than their
443 fittest ancestral spore (t_{66} = -5.38, P = 1.05 x 10^{-6}), but there is no effect of sex on
444 selection (Figure 3; Table 1; t_{66} = 1.61, P = 0.112). The amount of variance among

445 ancestries remained the same during evolution in Bold's, and sex had no effect (Figure 4;
446 P = 0.26). In terms of chance, replicate lines diverged during evolution, and sexual lines
447 diverged more than their asexual counterparts (P = 0.0084). Finally, variance among
448 spores was lower after evolution in Bold's, and decreased to a greater extent in sexual
449 lines than in their asexual counterparts (P = 0.036). Hence, after 300 generations of
450 evolution in Bold's sex appears to have no effect on the importance of selection or the
451 constraints of ancestry, but increases the importance of chance, and lead to greater
452 reductions in diversity within lines (Table 2).
453
454
455 **Discussion**
456 We propagated sexual and asexual lines in four different novel environments for 300
457 generations. By measuring the change in fitness, and the change in variance among
458 ancestries, among replicate lines, and among spores, we were able to determine the
459 consequences of sex on the roles of selection, ancestral constraints, and chance during
460 evolution. We predicted that sex would increase the importance of selection and reduce
461 the importance of chance, and so increase the repeatability of evolution, and that sex
462 would reduce divergence among spores and so decrease diversity within populations over
463 time.
464
465 In accord with many other experimental studies (Zeyl and Bell 1997; Colegrave 2002;
466 Kaltz and Bell 2002; Goddard et al. 2005; Morran et al. 2009; Becks and Agrawal 2010;
467 Lachapelle and Bell 2012; Bell 2012a), in all of our environments where maximum

468 growth rates increased after evolution (i.e. Na_2SO_4 and NaCl), and hence where we were
469 able to detect an effect of selection, sex led to greater rates of adaptation. In the Bold's
470 and Herbicides environments where maximum growth rates did not increase over
471 evolution, there was no effect of sex.
472
473 Second, we predicted that sex would make chance associations between new mutations
474 and genetic backgrounds less important and thus reduce the importance of chance. This
475 second prediction is clearly not supported in all environments: sex decreased the
476 importance of chance in the herbicide mixture, but increased the importance of chance in
477 Na_2SO_4 and Bold's, and had no effect in NaCl. The lack of generality in the effect of sex
478 on evolution is consistent with findings from another study of the effect of sex on the
479 evolution of herbicide resistance in *C. reinhardtii* (Lagator et al. 2014), and in general
480 with the variable outcomes from different studies of the repeatability of evolution in
481 sexual species (Teotonio and Rose 2000; Teotonio et al. 2002; Kawecki and Mery 2003;
482 Joshi et al. 2003b; Griffiths et al. 2005; Simões et al. 2008; Fragata et al. 2014).
483
484 Third, we predicted that the greater efficiency of selection in sexual populations would
485 lead to less divergence among spores in sexual populations than in asexual populations.
486 We indeed observed a greater reduction of diversity within sexual lines than within
487 asexual lines in three of the four environments (Bold's, Herbicides, and NaCl). This
488 could be because sex reduces the effects of clonal interference (Gerrish and Lenski 1998)
489 and hence lowers the number of variants competing at any one time point. A reduction in
490 diversity within populations could also be favoured by the type of selection regime used

491 in this experiment, i.e. sexual cycles interspersed by tens of generations of vegetative
492 growth. One episode of recombination would contribute in generating variation and
493 separating beneficial mutations from inferior backgrounds, and subsequent asexual
494 generations would give time for selection to lead to the increase in frequency (and
495 perhaps fixation) of the best clone (McDonald et al. 2016).
496
497 Convergence and divergence among spores over evolutionary times is rarely investigated,
498 perhaps because most evolution experiments start with a single clone. By starting our
499 experiment with diverse populations we were able to determine whether diversity within
500 laboratory populations is gained or lost during evolution. We found that the magnitude of
501 the change in variance among spores was in some environments as great or greater (e.g.
502 in Bold's and NaCl) than the change in variance among lines or ancestries, and
503 significantly different between asexual and sexual lines. Hence sex can have important
504 implications for diversity not only among independent populations but also within them.
505
506 *The effect of sex on repeatability depends on the environment*
507 There are situations in which theory predicts that sex might increase divergence between
508 adapting populations (Weinreich and Chao 2005). On a rugged fitness landscape, chance
509 events and/or ancestry might lead a population onto a fitness peak that is less than
510 optimal. Once that peak has been reached, all single mutations will be deleterious, and
511 only the combination of some of these single mutations will be beneficial and take the
512 population to another potentially higher peak. When fitness valleys are shallow, single
513 mutants will be selected out slowly and remain in the population longer. The high

514 frequency of single mutants will generate negative linkage disequilibrium, meaning that
515 recombination will tend to generate multiple mutants. Hence in such cases, sex will
516 reduce the importance of chance by favouring peak shifts and convergence on the optimal
517 fitness peak. On the other hand, when fitness valleys are very deep, single mutants will be
518 selected out rapidly and very few will exist at any one time in the population. The low
519 frequency of single mutants will generate positive linkage disequilibrium, meaning that
520 recombination will instead tend to break apart beneficial combinations. Hence, by
521 hindering peak shifts, sex will increase the importance of chance and history and decrease
522 the repeatability of evolution.
523
524 The theory available therefore predicts either an increase or a decrease in variance among
525 lines during evolution, depending on the value of a number of parameters. For example,
526 differences in linkage disequilibrium can arise not only because of differences in the
527 genetic basis of adaptation, but also because of differences in population size and in
528 initial distance to fitness peaks (Otto et al. 1994; Kondrashov and Kondrashov 2001;
529 Hadany and Beker 2003; de Visser et al. 2009). While our study did find that sex could
530 either increase or decrease variance in fitness among populations, without precise
531 information on the relevant parameter values for our environments (e.g. number of genes
532 involved in fitness, ruggedness of the fitness landscape, distance to optimal fitness, etc.),
533 we are unable to determine if the outcome in any one environment supports that made by
534 theory. This study, which to the best of our knowledge is the first empirical attempt to
535 test these predictions in multiple environments, indicates that different environments,

536 with their different parameter values can lead to vastly different outcomes, although more
537 work is clearly needed to determine precisely which parameters are important.
538
539 The different effects of sex on evolution in different environments could be in part due to
540 the genetic basis of adaptation as it very likely to differ among environments. Growth
541 rates were ancestrally lowest in the Na_2SO_4 environment, followed by NaCl, Herbicides,
542 and Bold's. Therefore, assuming that the optimal fitness in each environment is the same
543 as fitness in the basal medium (i.e. Bold's) without the added stressor, lines would have
544 been furthest away from the fitness peak in Na_2SO_4 and NaCl, and closest in Herbicides
545 at the start of the experiment. This most likely explains the greater increase in fitness in
546 these two former environments, as more beneficial mutations would have been available.
547 Na_2SO_4 and NaCl are also likely to have the most complex genetic basis of adaptation as
548 both impose osmotic and oxidative stresses that have been shown to require changes in
549 many genes in *C. reinhardtii* (Perrineau et al. 2014). It is therefore unsurprising that
550 sexual populations were at an advantage over asexual populations in these environments
551 as recombination would be helpful in combining the many mutations together instead of
552 waiting for each mutation to fix one after the other. On the other hand, the Herbicides
553 environment contained two herbicides with only two primary targets for selection,
554 photosystem II and very-long-chain fatty acid synthesis, reducing the potential for
555 advantages to sex.
556
557 Aside from differences in the genetic basis of adaptation, differences among sexual and
558 asexual populations in their levels of convergence or divergence could have arisen from

559 temporal effects. For example, divergence of adapting populations can be temporary
560 when different populations follow different paths up the same fitness peak. It is therefore
561 possible that given a few 1000s generations more, variance among populations that have
562 diverged during the first 300 generations would be reduced to zero. While theory
563 suggests that, in general, sexual populations will climb a peak faster than asexual
564 populations (Weismann 1889; Fisher 1930; Muller 1932; Hill and Robertson 1966;
565 Felsenstein 1974; Peck 1994; although see Kondrashov and Kondrashov 2001; Watson
566 and Wakeley 2005), it is unclear what effect recombination will have on the diversity of
567 paths followed by different populations on the same peak.
568
569 Finally, another factor that could have affected the amount of divergence among sexual
570 populations is the frequency of sexual events. In our experimental populations, sexual
571 events occurred about every 50 asexual generations. While such a rate of sexual events is
572 representative of some organisms, others reproduce sexually at much more frequent
573 intervals and this could have an effect on the importance of selection, chance, and
574 ancestral constraints during evolution. A larger interval between sexual cycles leaves
575 time for selection to lead to the increase in frequency of new beneficial combinations. We
576 would expect in theory that this would increase the repeatability of evolution, as it
577 prevents 'escape' genotypes (i.e. genotypes that fall on another peak than the one
578 currently occupied by the population) from being constantly being broken down. A
579 proper investigation of this effect is needed.
580
581 *The evolution of slower growth rates in Bold's*

582 Evolution in the Bold's environment led to lower growth rates than that of the fastest
583 growing ancestral spore. Bold's medium is a benign environment where growth rates are
584 high, and beneficial mutations are likely to be rare. The lower growth rates could be
585 attributable to a lack of relevant variation, inefficient sorting of the standing genetic
586 variation, a failure to remove deleterious mutations, or a trait other than maximum growth
587 rate being under selection. The response of a population to selection should be
588 proportional to the variance in fitness (Fisher 1930). Variance in fitness is initially high in
589 both the asexual and sexual lines in Bold's. As a rough estimate, for a selective advantage
590 of 0.1 (based on the variance present initially in the lines), and an initial frequency of 1/8,
591 we expect the fittest spore to rise to 99% frequency within 45 generations. Diversity of
592 growth rates was almost completely lost within both the asexual and sexual lines, which
593 is further evidence that sorting did occur. It is therefore unlikely that lack of variation or
594 inefficient sorting in the asexual and sexual lines is responsible for their lower mean
595 fitness. It is also unlikely that deleterious mutations fixed (either singly or through
596 hitchhiking) given the short evolutionary timescale (300 generations) and the relatively
597 large deleterious effect size that would be needed to produce such drop in growth rate.
598 Maximum growth rate has generally been found to be the most important component of
599 selection in microbial experimental evolution studies like the one described here where
600 populations are maintained by serial dilutions (Dykhuizen 1990; Vasi et al. 1994; Lenski
601 et al. 1998). Even when we looked at a different estimate of fitness, yield, we found a
602 decrease in yield after evolution (Supplementary Figures 1 and 2), indicating that this
603 result is not an artefact of our choice of estimate for fitness. Ultimately, we cannot
604 exclude the possibility that slower growth rates arose both in asexual and sexual lines as

605 an indirect result of selection on another trait with antagonistic effects on growth rates, or
606 that selection in Bold's selects for slower growth rates instead of faster growth rates as a
607 means to maintain cell health in favourable environments (Schaum and Collins 2014).
608
609
610 **Conclusion**
611 We found that sexual populations converged or diverged to a significantly different
612 degree than asexual populations during evolution, reflecting differences in the importance
613 of chance and ancestral constraints. The effects of sex on evolution are highly dependent
614 on the genetic background and the environment, and we therefore cannot assume that
615 results from experiments with a single genotype or environment will generalise to other
616 environments. More rigorous tests are needed to determine the exact mechanisms by
617 which population and environmental attributes mediate the effect of recombination.
618 While the effects of sex on rates of adaptation and variance within populations are well
619 appreciated, by focussing on changes in variance among populations, we have found that
620 sex also has important downstream consequences on diversity among populations and on
621 the predictability of evolution.
622
623
624 **Acknowledgements**
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629

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781

782

783 **Tables**

784 Table 1. The effect of recombination on the efficiency of selection at increasing
785 maximum growth rates in each of the four selection environments. The parameter
786 estimates for the fixed effect are shown, where 'Selection' has two levels (ancestral and
787 evolved) and 'Reproduction' has two levels (asexual and sexual).

Environment	Effect	Estimate	SE
Bold's	Intercept	4.9	0.22

	Selection (evolved)	-1.4	0.26
	Reproduction (sexual)	-0.63	0.26
	Selection (evolved) : Reproduction (sexual)	0.60	0.37
Herbicides	Intercept	2.7	0.23
	Selection (evolved)	0.16	0.20
	Reproduction (sexual)	-0.11	0.20
	Selection (evolved) : Reproduction (sexual)	-0.11	0.29
Na_2SO_4	Intercept	1.2	0.11
	Selection (evolved)	0.56	0.12
	Reproduction (sexual)	-0.096	0.12
	Selection (evolved) : Reproduction (sexual)	0.71	0.17
NaCl	Intercept	1.6	0.24
	Selection (evolved)	0.85	0.14
	Reproduction (sexual)	0.068	0.14
	Selection (evolved) : Reproduction (sexual)	0.51	0.19

788
789
790 Table 2. The effect of sex on the contribution of selection, the constraints of ancestry, and
791 the importance of chance to evolution; and the effect of sex on the amount of diversity
792 within lines. An upward pointing arrow indicates that the component is significantly

greater in sexual populations than in asexual populations (e.g. the constraints of ancestry are greater in sexual populations than in asexual ones in the Na_2SO_4 environment), a downward pointing arrow indicates that the component is significantly lower in sexual populations than in asexual populations (e.g. there is a greater reduction of diversity in sexual populations than in asexual ones in the Bold's environment), an equal sign indicates that there are no significant differences between sexual and asexual populations for that component.

Environment	Selection	Ancestry	Chance	Diversity
Na_2SO_4	↑	↓	↑	↑
NaCl	↑	=	=	=
Herbicides	=	↑	↓	=
Bold's	=	=	↑	↓

Figure legends

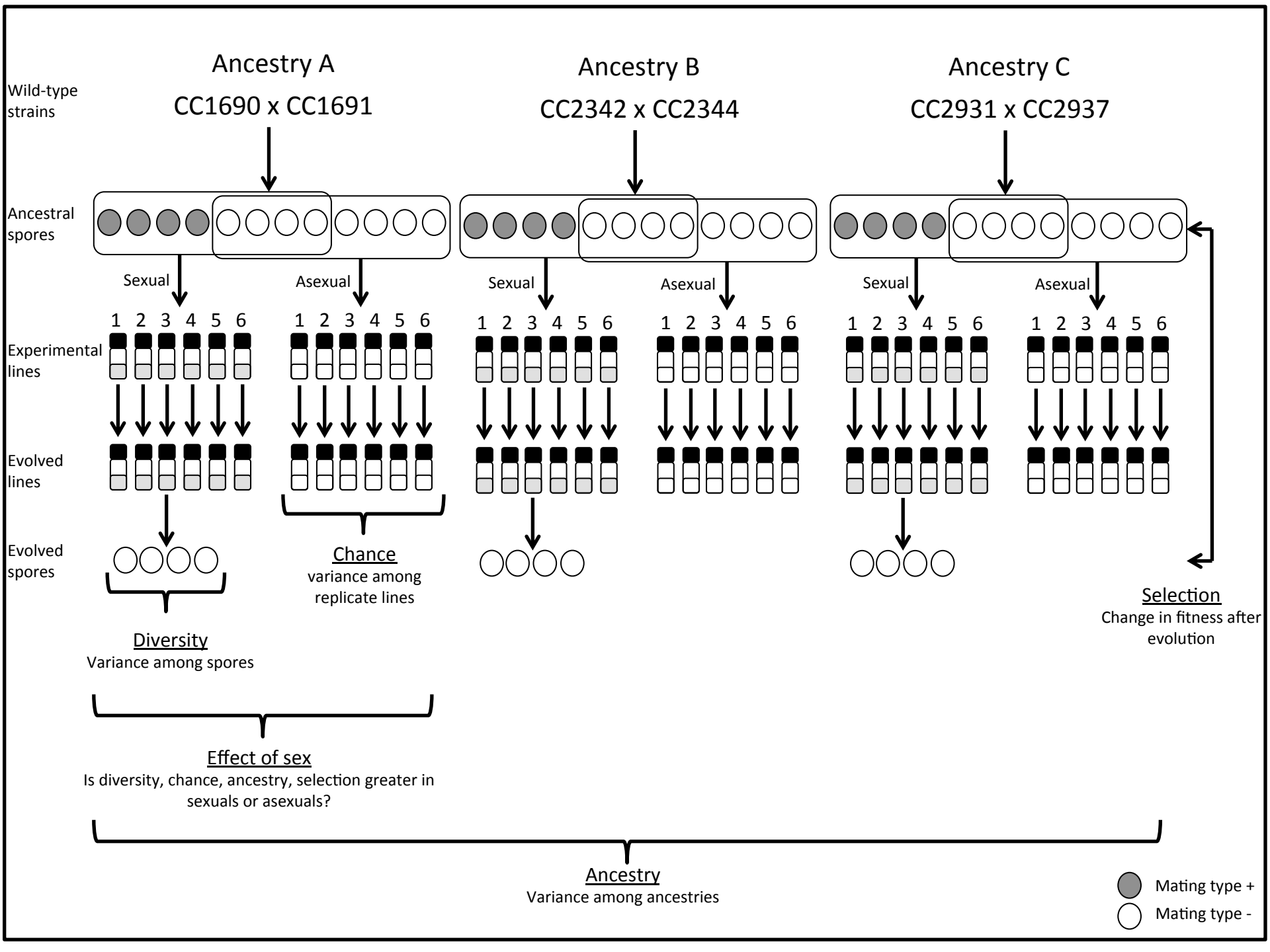
Figure 1. Schematic of the experimental design showing the original crosses that yielded the ancestral spores for each of the different ancestries, the replicate experimental lines, and the sexual treatment. The setup was replicated four times in four different environments: Bold's minimal medium, Herbicides, Na_2SO_4, and NaCl. Four spores from each of the evolved lines were assayed, but only one set for each treatment is shown in this schematic.

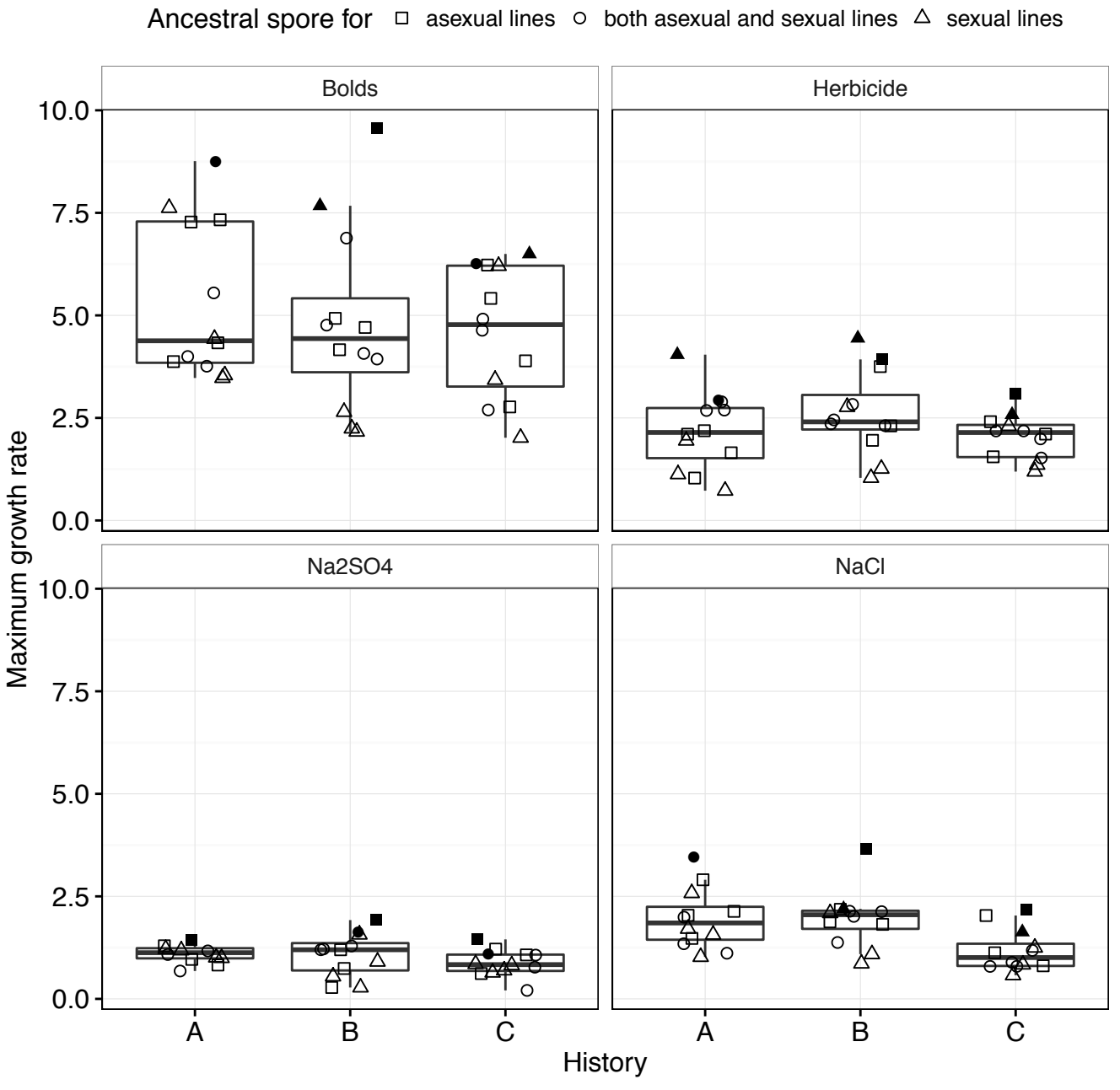
Figure 2. Maximum growth rate of the twelve ancestral spores from each ancestry, in each of the four selection environments. Each point represents the average of the three

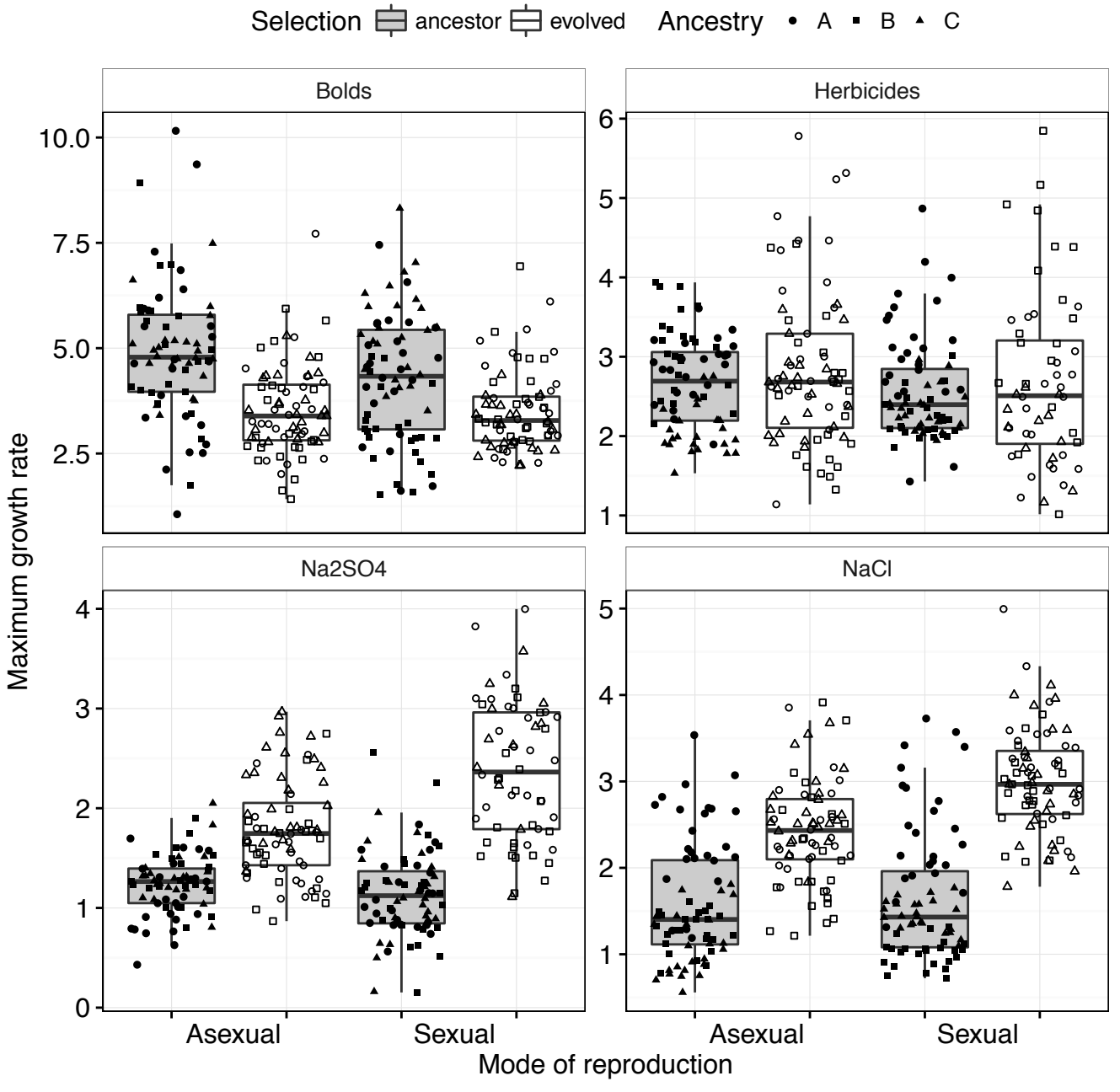
assay replicates. The shape of the points indicates whether the spore was used to found the asexual lines, sexual lines, or both. Filled points indicate the fastest growing ancestral spores used in the evolved fitness assays.

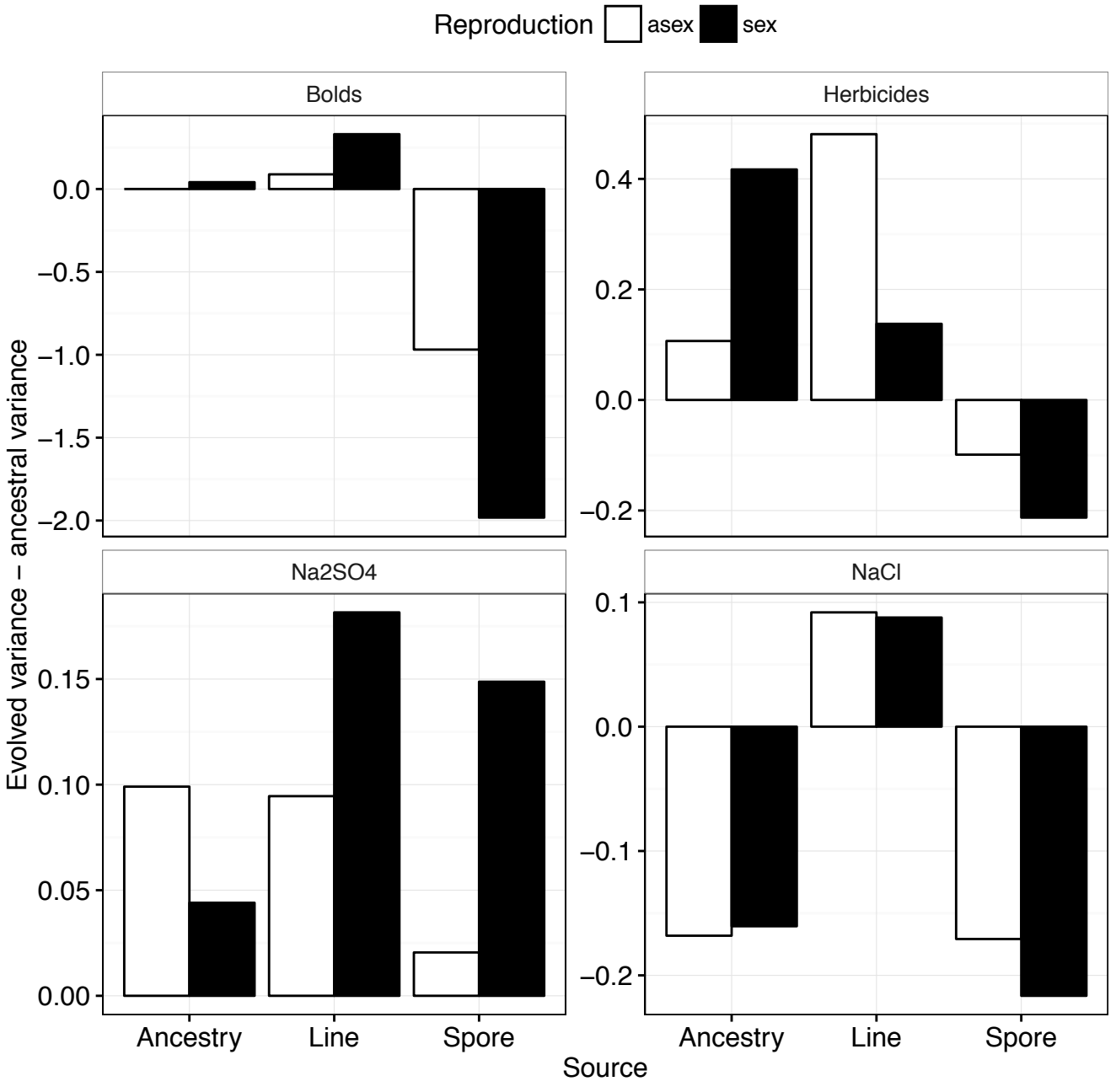
Figure 3. The effect of sex on selection. This plot shows the maximum growth rate of the fastest growing ancestral spores and the evolved spores in their corresponding selection environment. The difference in maximum growth rate between evolved and ancestral indicates the effect of selection. A difference in the magnitude of this change indicates the effect of sex. Each point represents the average of the three assay replicates. There are 4 spores for each of 36 evolved lines (except in Herbicides where there are 32 lines and in Na_2SO_4 where there are 33 lines). The shape of the points indicates from which ancestry the spore comes from.

Figure 4. Change in variance in maximum growth rate after evolution in each selection environment in asexual and sexual populations. Ancestry represents variance among ancestries, Line represents variance among replicate lines within ancestries, and Spore represents variance among spores within lines within ancestries.

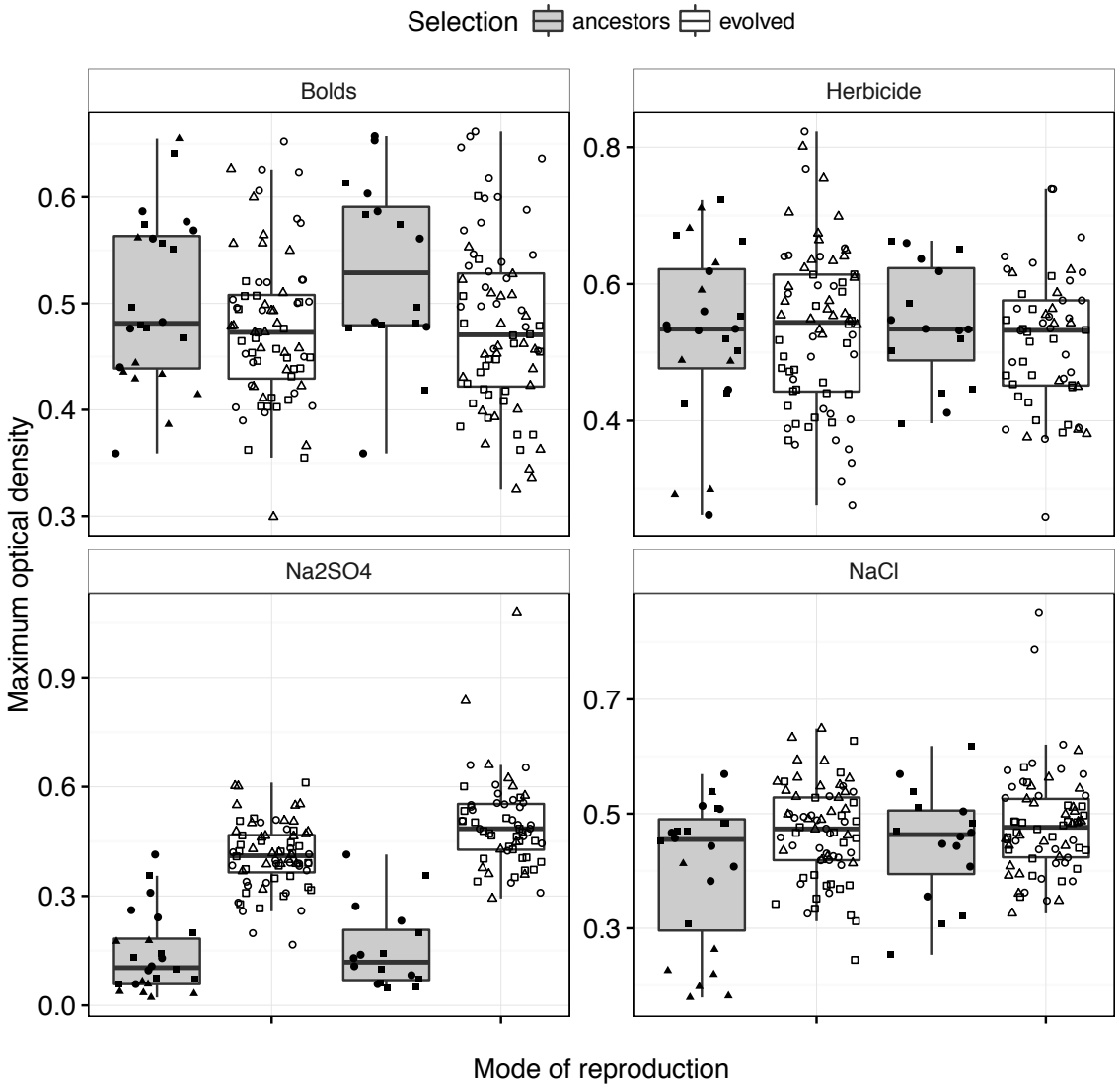

Ancestry A
CC1690 x CC1691
Ancestry B
CC2342 x CC2344
Ancestry C
CC2931 x CC2937
Wild-type strains
Ancestral spores
Sexual
Asexual
Experimental lines
Evolved lines
Evolved spores
1 2 3 4 5 6
Chance
variance among replicate lines
Diversity
Variance among spores
Selection
Change in fitness after evolution
Effect of sex
Is diversity, chance, ancestry, selection greater in sexuals or asexuals?
Ancestry
Variance among ancestries
Mating type +
Mating type -


Ancestral spore for
asexual lines
both asexual and sexual lines
sexual lines
Bolds
Herbicide
Na2SO4
NaCl
Maximum growth rate
10.0
7.5
5.0
2.5
0.0
A
B
C
History


Selection
ancestor
evolved
Ancestry
A
B
C
Bolds
Herbicides
Na2SO4
NaCl
Maximum growth rate
Asexual
Sexual
Mode of reproduction

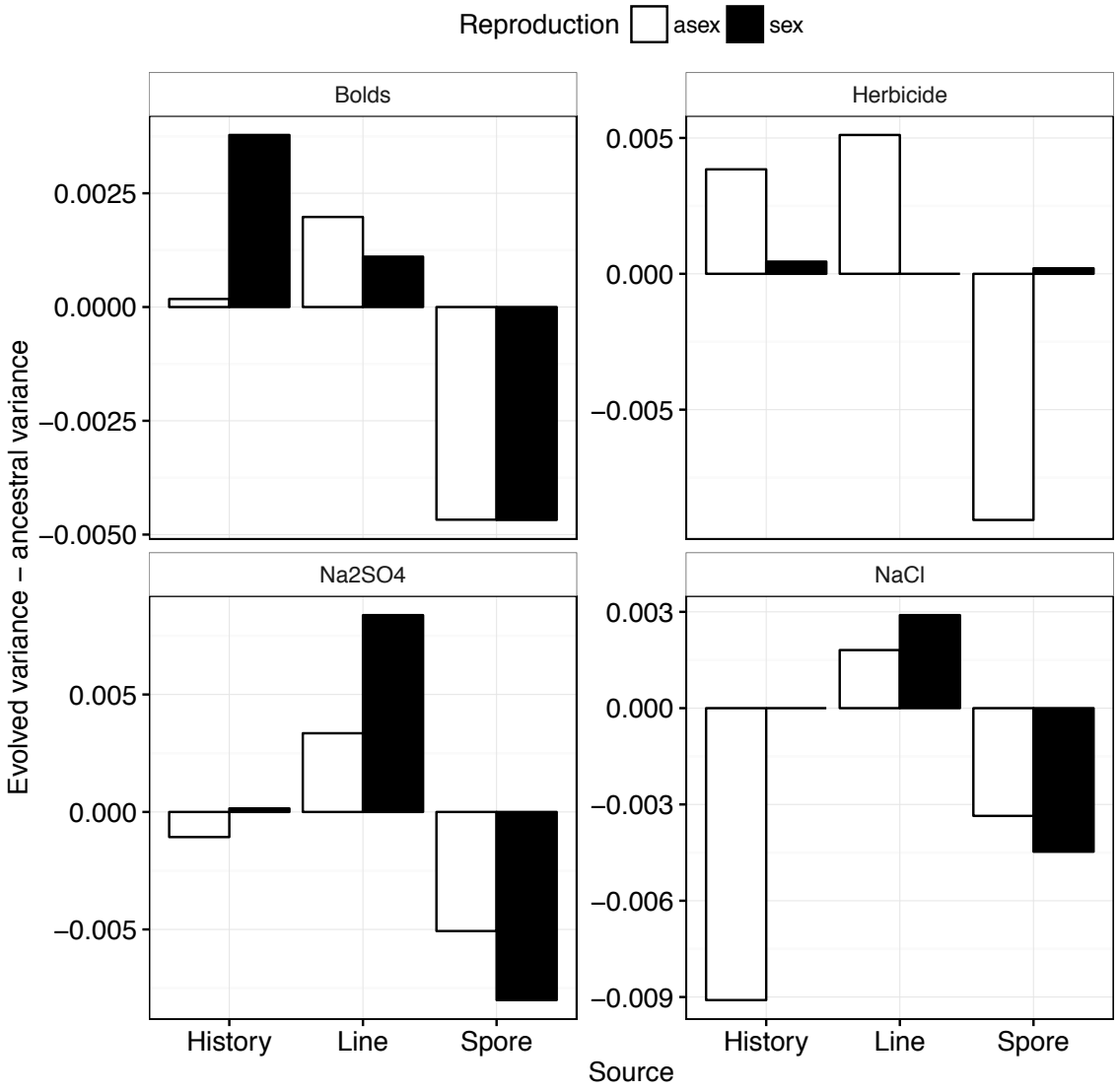

Reproduction
asex
sex
Bolds
Herbicides
Na2SO4
NaCl
Evolved variance – ancestral variance
0.0
−0.5
−1.0
−1.5
−2.0
0.4
0.2
0.0
−0.2
0.15
0.10
0.05
0.00
0.1
0.0
−0.1
−0.2
Ancestry
Line
Spore
Source

Supplementary Figures



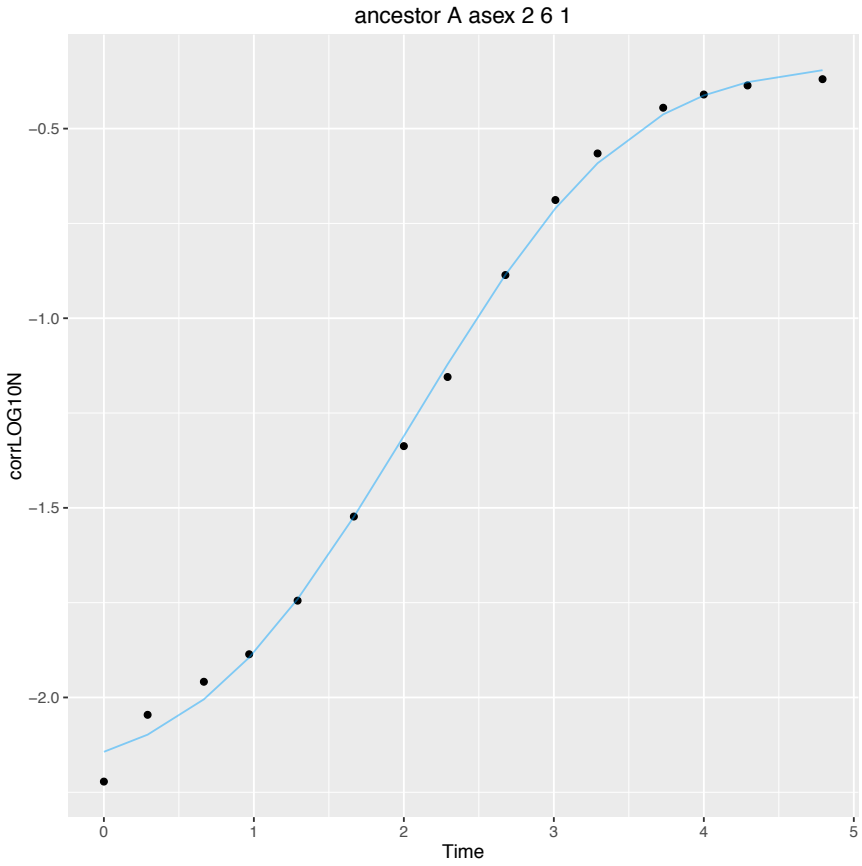
Supplementary Figure 1. The effect of sex on selection. This plot shows the yield of the ancestral spores and the evolved spores in their corresponding selection environment. The difference in yield between evolved and ancestral indicates the effect of selection. A difference in the magnitude of this change indicates the effect of sex. Each point represents the average of the three assay replicates. There are 4 spores for each of 36 evolved lines (except in Herbicides where there are 32 lines and in Na_2SO_4 where there

are 33 lines). The shape of the points indicates from which ancestry the spore comes from.

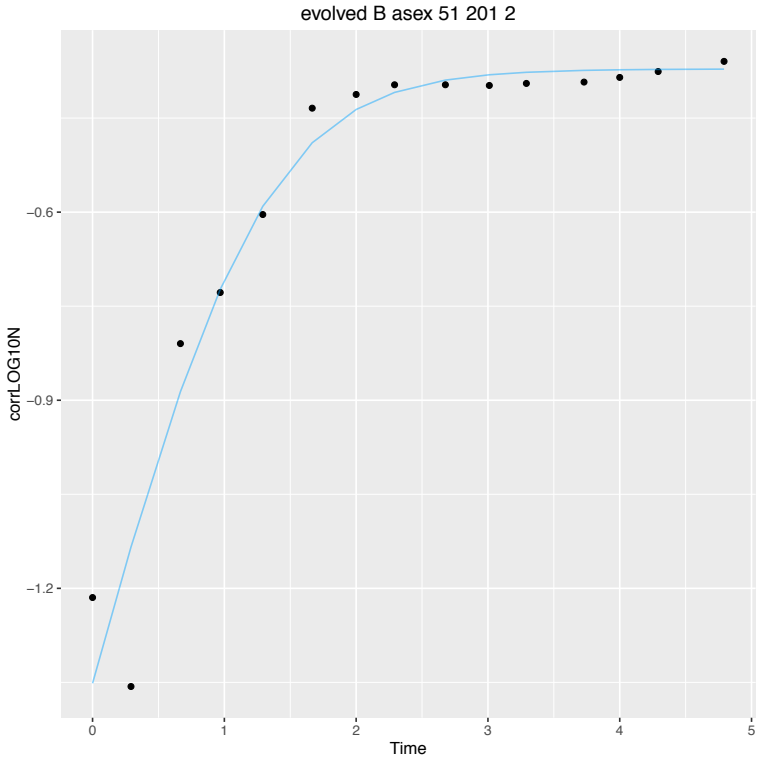


Supplementary Figure 2. Change in variance in yield after evolution in each selection environment in asexual and sexual populations. Ancestry represents variance among ancestries, Line represents variance among replicate lines within ancestries, and Spore represents variance among spores within lines within ancestries.

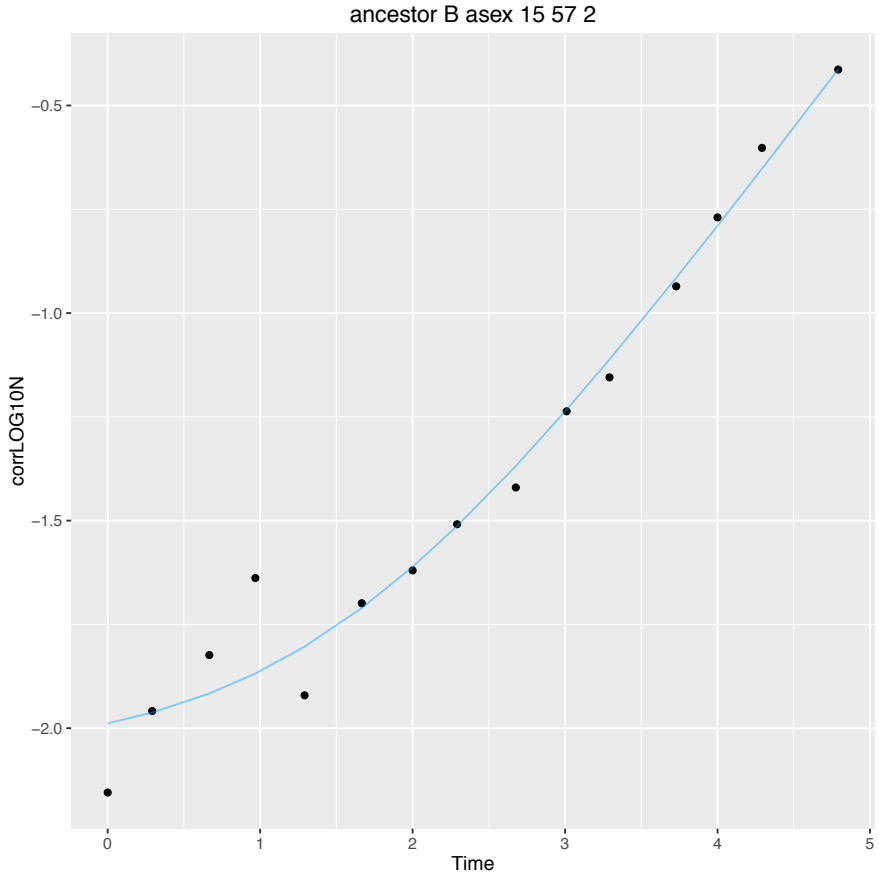
A. Baranyi model



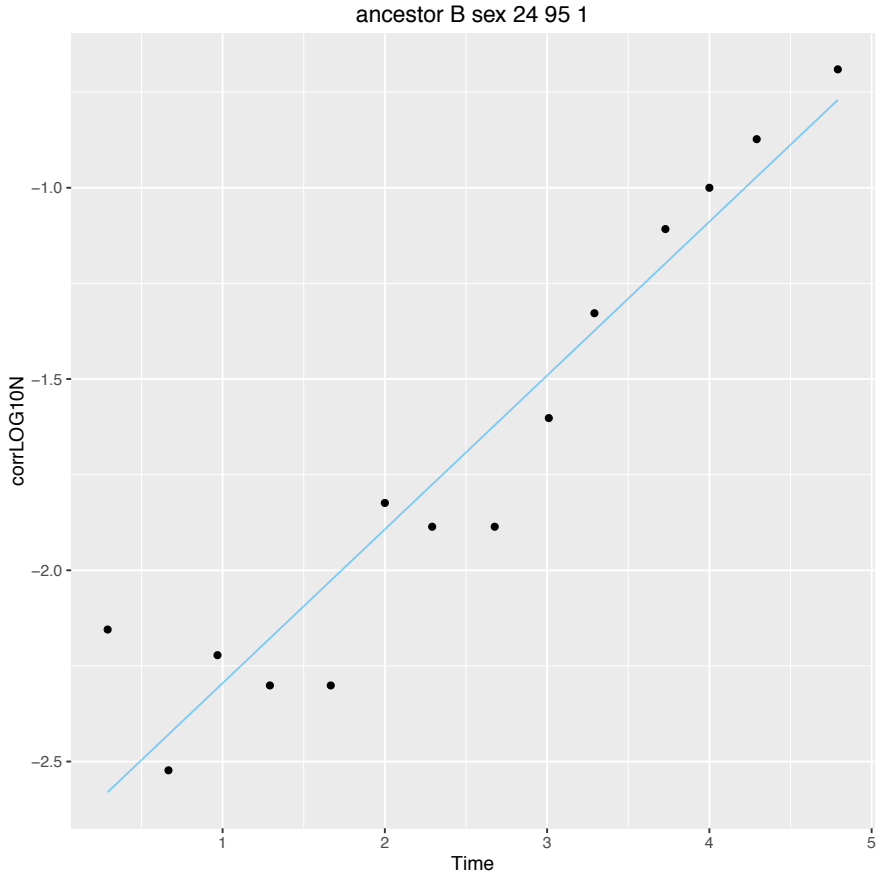
B. Baranyi model without lag



C. Baranyi model without Nmax



D. Linear model



Supplementary Figure 3. Examples of spores grown in 5 gL^{-1} NaCl and fitted with the (A) Baranyi model, (B) Baranyi model without lag, (C) Baranyi model without Nmax, and (D) linear model. The points represent the actual data, and the blue line represents the model fit.