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THE ECOLOGY AND GENETICS OF FITNESS IN *CHLAMYDOMONAS*. XII. REPEATED SEXUAL EPISODES INCREASE RATES OF ADAPTATION TO NOVEL ENVIRONMENTS

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Abstract.—We investigated the dynamics of adaptation of the unicellular chlorophyte *Chlamydomonas reinhardtii* to new and hostile conditions of growth provided by novel carbon substrates in the dark. The experiment was designed to contrast perennially asexual lines with lines that had experienced one or more sexual episodes. All lines were capable of adapting to the novel environment. The sexual lines, however, showed greater adaptation over the course of the experiment, especially in more complex environments. Moreover, the effect of sex on adaptation increased with the number of successive sexual episodes. The time-course of adaptation showed that sex initially caused an increase in the standardized variance of fitness and an initial drop in mean fitness, at least after a second or third sexual episode. These short-term effects were followed by a period of recovery during which the fitness of sexual lines eventually exceeded that of asexual lines. The increase in mean fitness was mirrored by a decrease in the standardized variance of fitness relative to asexuals, suggesting that directional selection used up the variation generated by meiotic recombination and thereby conferred a fitness advantage to the sexual lines. These results support the Weismann-Fisher-Muller hypothesis for the maintenance of sex in natural populations.

Key words.—*Chlamydomonas reinhardtii*, cost of sex, epistasis, experimental evolution, maintenance of sex, recombination, recombinational load.

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Why are so many eukaryotic organisms sexual? This question has puzzled evolutionary biologists for many years (Williams 1975; Maynard Smith 1978; Bell 1982), and the causes for the evolutionary maintenance of sexuality are still debated (Michod and Levin 1987; Hurst and Peck 1996; West et al. 1999). Sexuality incurs several kinds of cost, the most general being the breakup of coadapted gene complexes during meiotic recombination. Nevertheless, perennially asexual taxa of multicellular eukaryotes are rare and generally short lived (Bell 1982; Judson and Normark 1996; Burt 2000).

Several advantages of sexuality can be imagined (reviewed in Kondrashov 1993; Otto and Barton 1997; Barton and Charlesworth 1998). The oldest hypothesis supposes that sex provides the variation necessary for adaptation to changing environments (Weismann 1889). This idea was subsequently refined by Fisher (1930) and Muller (1932), who proposed that meiotic recombination following sexual fusion can bring together favorable mutations arising in different lineages into a single individual, thereby reducing the time to fixation of these mutations.

Theoretical models have further formalized the Weismann-Fisher-Muller hypothesis (reviewed in Feldman et al. 1996; Barton and Charlesworth 1998; Otto and Michalakis 1998; Burt 2000; Peters and Lively 2000). The mechanism underlying the advantage of sex in these models is as follows: When the environment changes, the combinations of alleles that would be best adapted to the new conditions of growth are likely to be underrepresented in the population. This negative linkage disequilibrium may exist either because some combinations of beneficial alleles are likely to be absent by chance if populations are finite (e.g., Crow and Kimura 1965; Felsenstein 1974; Pamilo et al. 1987; Peck 1994; Barton 1995b; Otto and Barton 1997), or because combinations of beneficial alleles were previously less fit (and thus relatively less frequent) than expected from their marginal values (e.g., Maynard Smith 1988; Barton 1995a; Charlesworth and Barton 1996). The crucial effect of sexual recombination is then to reduce this linkage disequilibrium, thereby increasing the additive genetic variance of fitness (Pamilo et al. 1987; Maynard Smith 1988; Charlesworth 1993; Barton 1995a; Burt 2000). This will render the response to directional selection more effective so that sexual populations adapt faster to the novel environment than do asexual ones. When sexual and asexual types are in direct competition, genes promoting sex and recombination can spread in the population by ''hitchhiking'' along with the high-fitness genotypes they generate (Maynard Smith 1988; Barton 1995a; Otto and Barton 1997).

Thus, the most general prediction of Weismann-Fisher-Muller models is that sex increases the variance and, subsequently, the mean fitness in novel environments. This will depend on the genetic basis of evolutionary change. Sex will be particularly advantageous if beneficial mutations are frequent (Otto and Barton 1997; Peck et al. 1997), because they can then be recombined while still at low frequency so that adaptation is not retarded by clonal competition. This advantage will be more pronounced if many loci are involved in adaptation, permitting multilocus genotypes to be assembled (Otto and Barton 1997; Peck et al. 1997). From an ecological point of view, a high rate of novel beneficial mutations is equivalent to rapid change in the optimal phenotype, so sex is most likely to evolve in rapidly changing environments (Griffin 1983; Charlesworth 1993; Charlesworth and Barton 1996). We would further predict that sex is more advantageous in more complex environments, because more loci are likely to contribute to adaptation, and additional sexuality should further increase the fitness advantage of sexual over

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asexual populations, at least for some time during adaptation to a novel environment.

One important reservation is that the effect of sex may change during the course of adaptation to novel environments. For example, if the mutations favored by the environmental change were disadvantageous in the previous environment, only a few copies will be present at very early stages of adaptation. Therefore, recombination may initially be less effective than later on, when more beneficial mutations have become established; conversely, the advantage of sex will level off as populations become more adapted to the novel environment (Maynard Smith 1978). In the same vein, a low level of recombination should provide almost the maximal benefit of sexual reproduction (Pamilo et al. 1987; Peck 1994; Green and Noakes 1995; Burt 2000): Recombining additional mutations into already well-adapted variants may then become less and less rewarding, and the costs of recombination, namely the breakup of well-adapted combinations, would begin to outweigh its benefits.

Experimental tests of these ideas are very rare. Malmberg (1977) found that drug resistance in T4 phage evolved more rapidly in populations with high levels of recombination. In naive phage populations, rates of adaptation began to increase only after several rounds of recombination, and in two of three cases the fitness advantage of high-recombination populations disappeared after 15 growth cycles, perhaps because the phenotypic optimum had been reached. In contrast, increased opportunity for recombination over 250 generations did not accelerate adaptation of bacteriophage ϕ6 to its bacterial host, *Pseudomonas phaseolicola* (Turner and Chao 1998). Unfortunately, high recombination levels in both experiments were achieved by increasing the probability of multiple infection of individual host cells. Thus, effects of recombination are potentially confounded with those of intrahost competition, which is generally thought to influence the evolution of parasite growth or virulence (Bull 1994; Turner and Chao 1998).

Using the budding yeast *Saccharomyces cerevisiae*, Zeyl and Bell (1997) compared evolution of sexual and asexual populations on galactose. After 400–600 generations of mitotic growth, with eight sexual episodes in the sexual populations, adaptation to this novel substrate had occurred, but growth rates did not differ between sexual and asexual populations. Greig et al. (1998) grew mixtures of sexually and asexually produced yeast for 500 generations under high temperature. Asexuals increased in frequency to begin with, indicating a short-term cost of sex. In the long run, however, sexuals outcompeted asexuals, at least in treatments where both sexuals and asexuals had a heterozygote genetic background (Greig et al. 1998). In the green alga *Chlamydomonas reinhardtii*, Colegrave et al. (2002) tested the effect of sex on adaptation to heterotrophic growth on exotic carbon sources. After an initial drop in fitness, sexual populations enjoyed temporarily increased rates of adaptation, but after about 50 generations sexual and asexual populations had reached equal fitness.

Thus, experimental evidence bearing on the Weismann-Fisher-Muller hypothesis is currently ambiguous and incomplete. Sex can lead to the predicted increase in the variance and the subsequent increase in the mean of fitness, but is not necessarily more favorable in more complex novel environments (Colegrave et al. 2002). Furthermore, although a single episode of recombination can generate at least a temporary advantage (Greig et al. 1998; Colegrave et al. 2002), it is far less clear whether continued sex provides a long-term advantage in novel environments (Malmberg 1977; Zeyl and Bell 1997; Turner and Chao 1998).

Here, we investigated effects of three consecutive sexual episodes on adaptation to heterotrophic growth in *C. reinhardtii*. Three carbon sources were used to generate novel environments of varying complexity. We induced a first episode of sex at the beginning of the experiment and a second and third episode after approximately 70 and 120 generations of mitotic replication on the novel substrates. We expected sex to increase the variance and subsequently the mean of fitness. Although Colegrave et al. (2002) did not observe more pronounced benefits of sex in more complex environments, we expected to observe this after additional sexual episodes, when new favorable mutations would have established in the populations. We particularly wanted to know whether such additional sexual episodes would turn the temporary fitness advantage observed by Colegrave et al. (2002) into a permanent fitness advantage of sexual over asexual populations.

Materials and Methods

Life Cycle of Chlamydomonas

The unicellular chlorophyte *C. reinhardtii* is a haplont. In the laboratory, nitrogen starvation induces differentiation into isogametes, followed by fusion of mt− and mt+ gametes to form a diploid zygote that secretes a thick wall and acts as a resting stage (Harris 1989). Laboratory cultures usually mate in bright light and are then transferred to plates containing solid medium, which are placed in the dark to allow zygotes to mature. Immediately after removal from the dark, the plates are exposed to chloroform vapor for 45 sec to kill any unmated vegetative cells, so that only zygotes survive transfer. These germinate within a few hours under bright light.

Chlamydomonas is photoautotrophic, and cultures are normally grown in the light on liquid or solid Bold's minimal medium, a mixture of inorganic salts lacking a carbon source (Harris 1989). However, it can also grow in the dark if provided with acetate as a carbon source (Colegrave et al. 2002). Our laboratory has discovered that cultures can also be maintained on a range of other substrates (unpubl. data), although growth is always much less than on acetate. These substrates can be used to provide chemically defined novel environments to which the experimental lines are at first very poorly adapted.

Preparation of Initial Sexual and Asexual Lines

The base population for our experiment was 20 mt- and 20 mt+ clones isolated at random from a mass mating between 12 mt− and 15 mt+ isolates obtained from a range of localities in eastern North America. These isolates have been cultured in laboratory conditions for up to 10–50 years. Because sex in *Chlamydomonas* requires gametes of opposite

mating type, a culture that comprises cells of a single mating type is asexual. Thus, we employed three types of culture in this experiment: asexual (mt−), asexual (mt+), and sexual (a mixture of mt− and mt+). In March 2000, the founders were expanded in liquid Bold's medium on a shaker under continuous illumination. An aliquot of about 10^8 cells of each clone was transferred to one of two Erlenmeyer flasks, holding either the 20 mt− clones or the 20 mt+ clones. The contents of each flask were then transferred to two plastic tubes, which were centrifuged for 5 min at 2000 rpm. In one mt− and one mt+ tube the pellet was resuspended twice in the same amount of nitrogen-free Bold's medium. Half of the cells from each nitrogen-depleted tube were then mixed in a single tube to allow mating, and all tubes were transferred to bright light for about 8 h. The cultures were then transferred to solid medium on plates; plates with the developing zygotes from the sexual population were placed in the dark to mature, and plates with cells from asexual populations were kept in dim light, the standard condition of long-term storage (Harris 1989). After five days, the zygote plates were chloroformed and transferred to bright light to induce germination and allow several mitotic divisions in the resulting offspring colonies. Asexual populations were also transferred to the light so that both types of populations had undergone photoautotrophic growth before the transfer to the novel environments. This procedure generated a sexual, nitrogen-stressed population; nitrogen-stressed asexual (mt− and mt+) populations; and unstressed asexual (mt− and mt+) populations. We applied the nitrogen-stress treatment to asexual populations because stress can affect both the mean and variance of fitness (Goho and Bell 2000) and thus might be confounded with effects of recombination.

Culture Procedure after the Initial Sexual Episode

To transfer these lines to the novel selection environments, we flooded the plates with liquid Bold's medium and pipetted 2-ml samples onto 24-well plates. An aliquot of about 10^6 cells of each population was then transferred to a plate containing Bold's medium supplemented with one or more carbon sources. This plate constituted one replicate line of a given population type (asexual mt−, asexual mt+, or sexual) in a given selection environment.

We used four selection environments, each supplemented with a low concentration of sodium acetate (0.001 M): (1) no additional carbon source added; (2) fructose (0.01 M); (3) glycerol (0.01 M); and (4) fructose and glycerol (0.005 M each). Acetate was included in all environments to permit a low level of growth during the early stages of adaptation to fructose and glycerol, which appear to have a slightly inhibitory effect in unselected populations (Colegrave et al. 2002).

We set up six replicate lines per selection environment: two mt− and two mt+ asexual lines (one nitrogen-stressed, one unstressed, respectively) and two sexual lines. The 24 plates were kept in the dark at room temperature (∼22°C) for three weeks, then flooded with liquid Bold's medium supplemented with the respective carbon sources. We removed 2-ml samples from each line, measured cell density, and transferred approximately 10^6 cells to fresh plates, which then began a new cycle of growth in the dark.

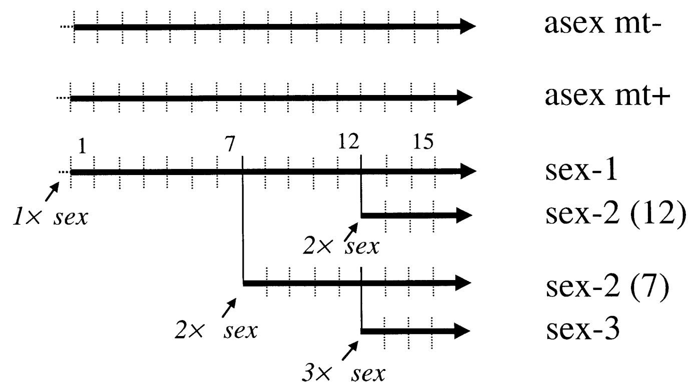

FIG. 1. Overview of the different sexual and asexual (mt- and mt+) population types generated over the course of the experiment (15 growth cycles). Sexual population types went through different numbers of sexual episodes (1, 2, or 3) or went through the same number of sexual episodes but differed in their timing (second episode after seven cycles or after 12 cycles). From cycle 1–7: N = 2 replicate lines per population type and selection environment. Before the second sexual episode (end of cycle 7), lines of same population type and selection environment were pooled and subsequently split in four new replicate lines until the end of the experiment.

Subsequent Sexual Episodes

In October 2000, after growth cycle 7 (i.e., after ∼70 mitotic generations in the dark), a second sexual episode was induced (Fig. 1). Two sexual and two mt+ replicate lines had been lost during previous cycles due to fungal contamination; to balance the experimental design, we pooled the two replicate lines within each population type and selection environment. We then split the (pooled) sexual replicate lines into halves. One-half went through a second sexual cycle, as described above. The resulting offspring lines will be referred to as ''sex-2'' lines hereafter. The other half, referred to as ''sex-1'' lines hereafter, were treated just like an asexual population: It did not go through a sexual cycle and was instead immediately transferred to solid Bold's medium (without carbon sources) and stored in dim light. In a similar manner, the (pooled) asexual lines were also split into one-half that did and one-half that did not go through the nitrogen-depletion procedure before being transferred to dim light.

After germination in the sex-2 lines, all the lines were replated back onto their original selection environment. Replication in each selection environment was increased to four replicate lines per population type (sex-2, sex-1, asexual mt−, asexual mt+). These 64 replicate lines were carried through growth cycles 8–12 (Fig. 1). After cycle 12 (after ∼ 120 generations in the dark), we induced a third episode of sex in the sex-2 lines, and a second episode in the sex-1 lines (Fig. 1). Again, this was done by splitting each replicate line into one-half that did and one-half that did not go through a sexual cycle. Asexual and unmated sexual lines were again kept in dim light while the mated lines went through the sexual cycle. The 96 replicate lines were then replated onto their original selection environment. Aside from the asexual lines, there were now sexual lines that had undergone one episode of sex at the start of the experiment (sex-1), a second sexual episode after the seventh growth cycle (sex-2[7]), a

second sexual episode at the 12th cycle (sex-2[12]), and a third episode of sex at the 12th transfer (sex-3; Fig. 1). These lines were taken through cycles 13–15, until the experiment was terminated in April 2001.

Assay Procedure

After each growth cycle, when replicate lines were transferred to fresh plates, an additional 5×10^4 cells from each replicate line were transferred to a separate plate containing the corresponding selection medium. After three days in an incubator at 22°C in the dark, we fixed these assay plates with Lugol's stain and counted the number of cells in 80 arbitrarily chosen colonies on a plate. Fitness was estimated as the division rate of a colony, from $\log_2$(cell number) divided by the number of days of growth. From these 80 counts we calculated the mean and the standardized variance of fitness (= variance divided by the square of the mean; Burt 1995) for each replicate line.

After growth cycle 15, we determined the response to selection in the novel environments by comparing the fitness of evolved lines and the founder clones from the base populations. The 40 founder clones, stored on solid Bold's medium in dim light throughout the experiment, were grown in liquid Bold's medium in continuous light for four days. To minimize carry-over effects and allow acclimatization to the novel environments, they were then grown in the selection environments in the dark for one week. They were then washed off the plates and equal volumes from each strain were used to reconstruct the mt+ and mt− asexual and sexual (i.e., mixed, but unmated) founder populations for each selection environment in 12 Erlenmeyer flasks. From these flasks, we prepared three assay replicate plates per founder population type and selection environment.

Statistical Analysis

To calculate the response to selection of a replicate selection line after cycle 15, we divided its division rate by that of the corresponding founder population (averaged over the three replicate plates in each selection environment) and subtracted one. We tested whether this response was generally larger than zero, that is, whether adaptation to the selection environments had occurred. Then, a factorial fixed-factor analysis of variance (ANOVA) was used to evaluate variation in response to selection among population types and selection environments.

To analyze differences between sexual and asexual populations over the course of the experiment, we employed factorial ANOVAs to test effects of selection environment and population type on mean (ln-transformed) fitness and on standardized variance of fitness for each growth cycle. We compared asexual with sex-2 lines after the second episode of sex and with sex-3 lines after the third episode of sex. At later cycles, assays were carried out on two consecutive days, accounted for by a block factor in the ANOVAs. Sequential model simplification was used to produce minimal adequate models (Crawley 1993) and sequential Bonferroni correction (Hurlbert 1984) to account for multiple testing over the different growth cycles. Where population type effects were significant, we carried out orthogonal contrasts, specifically testing for a difference between sexual and asexual lines. Nitrogen stress did not significantly influence the mean or standardized variance of fitness in any of the assays (see also Colegrave et al. 2002), and replicate lines from the two nitrogen treatments were therefore pooled for all analyses. Analyses were carried out with JMP statistical package (SAS Institute 1994).

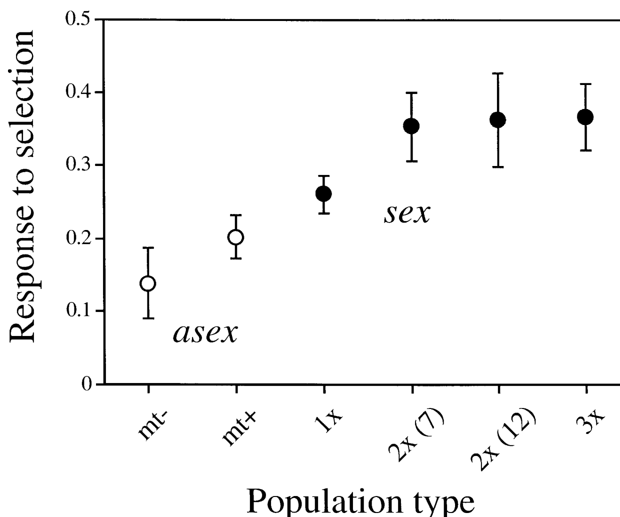

FIG. 2. Overall response to selection of asexual and sexual population types, measured after 15 growth cycles in the selection environments. Response to selection calculated from comparison of division rates between selection lines and the founder populations. Means and standard errors were calculated from the mean response in the four selection environments. For notation of population types, see Figure 1.

RESULTS

Adaptation to Novel Environments

The final assay revealed a significant overall difference in division rates between evolved lines and the founders, with an average response to selection of more than 25% (mean = 0.278 ± 0.020 SE; $t_{92} = 14.13$, $P < 0.0001$; Fig. 2). However, response to selection varied across selection environments ($F_{3,68} = 20.91$, $P < 0.0001$; Fig. 3). Least progress had occurred on acetate alone (A; mean = 0.17 ± 0.03), whereas progress in environments supplemented with additional substrates ranged from 0.24 ± 0.03 (A + G) to 0.45 ± 0.03 (A + F).

Effect of Sex on Adaptation

Population types differed significantly in their response to selection ($F_{5,68} = 8.46$, $P < 0.0001$; Fig. 2). Orthogonal contrasts showed that, overall, sexual population types had responded more than both mt+ and mt− asexual populations ($F_{1,68} = 33.48$, $P < 0.0001$). Sexual lines that had gone through one (sex-2) or two additional (sex-3) sexual episodes had responded more to selection than had sex-1 lines derived from a single sexual episode at the beginning of the experiment ($F_{1,68} = 6.19$, $P = 0.0153$). There was no significant difference between sex-2 lines that had undergone their second sexual episodes after seven and 12 transfers, respectively.

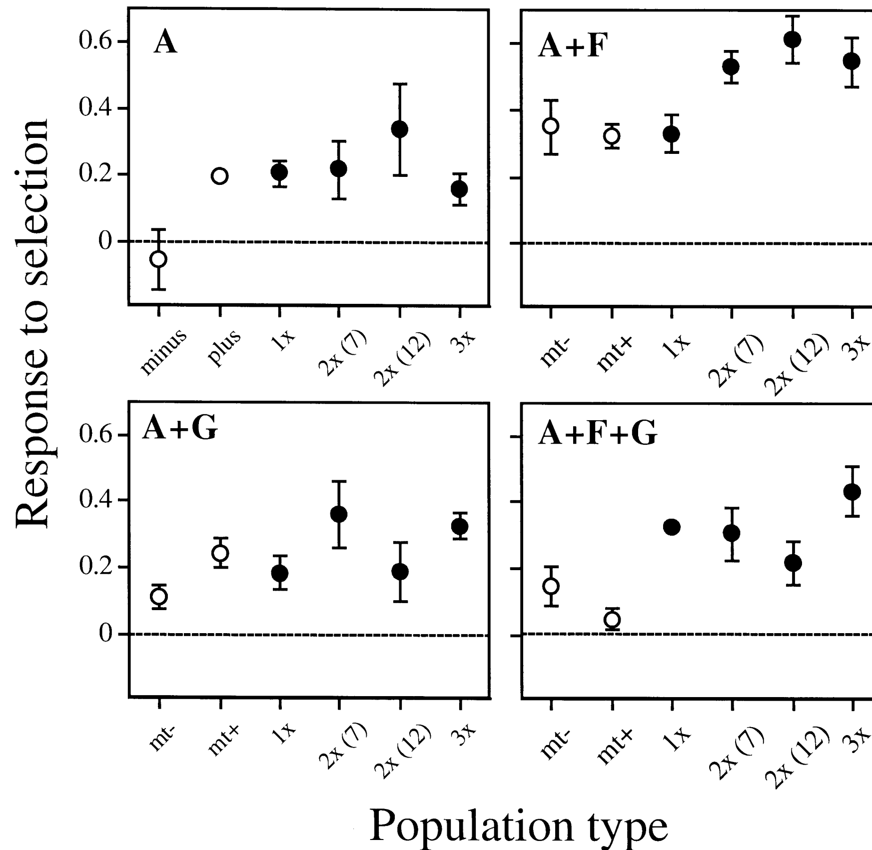

FIG. 3. Mean response to selection (± SE) of asexual and sexual population types in the four selection environments. $N = 4$ for each population type. For further details, see Figure 1.

Sex-3 lines showed the highest response of all, but did not differ significantly from lines that had sex only twice.

The significant selection environment × population type interaction ($F_{15,68} = 2.08$, $P = 0.02$) revealed some variation around these general patterns (Fig. 3). On acetate, the response in the mt+ asexuals did not differ much from that in sexual lines, and there were no clear differences among sexual population types. On A + F and A + G, additional sex generally increased the response to selection, whereas sex-1 lines were very similar to the asexual populations. In the most complex selection environment (A + F + G), there was a clear general benefit of sex, with the highest response in the sex-3 lines (Fig. 3).

Time Course of the Effects of Sex

In most of the assays, the three population types differed consistently in both mean fitness and standardized variance of fitness (ANOVAs summarized in Tables 1, 2); in some assays, these differences depended on the identity of the selection environment (significant population type × selection environment interactions). For both response variables, orthogonal contrasts often revealed consistent differences between sexual and asexual lines, which occasionally were differently pronounced across selection environments.

The overall dynamics of the differences between sexual versus asexual lines over the course of the experiment are

TABLE 1. Significance values in ANOVAs testing effects of block, population type (mt− asexual, mt+ asexual, and sexual), and selection environment on mean division rate for each of 15 growth cycles, after the first (sex-2), second (sex-2[7]), and third (sex-3) sexual episode.

Source	Cycle df	Sex-1							Sex-2(7)					Sex-3		
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Block[1]	1								—	—	***	—	—	—	***	***
Population type	2	(†)	***	(*)	**	(*)	(*)	(*)	(†)	(*)	***	***	***	***	***	***
Sexual vs. asexual	1	(*)	ns	ns	ns	(*)	**	(*)	(*)	ns	***	***	***	***	***	***
Selection environment	3	—	—	***	—	(†)	(*)	ns	***	***	***	***	***	***	***	***
Type × environment	6	—	—	—	—	—	—	—	—	***	(**)	***	(*)	(*)	—	(*)
Sexual vs. asexual × env	3									***	ns	(*)	ns	ns		ns

$N = 24$ (cycle 1); 22 (2–3); 21 (4); 20 (5–7); 60 (8); 63 (9); 62 (10); 64 (11–15). ns, not significant; —, ns and removed from full model; † $P < 0.075$; * $P < 0.05$; ** $P < 0.001$; *** $P < 0.005$; in parentheses, ns after correction for multiple testing.

[1] Only for cycles 8–15.

TABLE 2. Significance values in ANOVAs testing effects of block, population type (mt− asexual, mt+ asexual, and sexual), and selection environment on the standardized variance of division rate for each of 15 growth cycles, after the first (sex-2), second (sex-2[7]), and third (sex-3) sexual episode. For details, see Table 1.

Source	Cycle df	Sex-1							Sex-2(7)					Sex-3		
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Block	1								—	—	—	—	—	(†)	—	***
Population type	2	(†)	—	(*)	(*)	(*)	***	(*)	***	***	ns	(†)	ns	***	(*)	(*)
Sexual vs. asexual	1	(†)		ns	ns	(†)	(*)	ns	***	(†)		ns		***	ns	(*)
Selection environment	3	—	—	(*)	—	ns	***	(*)	(†)	***	**	***	(*)	(*)	ns	***
Type × environment	6	—	—	—	—	(*)	—	—	—	***	***	(*)	(†)	**	(***)	***
Sexual vs. asexual × env	3					ns				***	(*)	ns	ns	(*)	ns	ns

shown in Figure 4. After a slight initial fitness advantage following the first sexual episode, the mean fitness of sexual (sex-1) lines was intermediate between that of mt− and mt+ asexual lines for the first four growth cycles. However, the fitness disadvantage relative to mt+ asexuals gradually diminished, and by the end of the fifth cycle sexual lines had reached a consistent fitness advantage, with division rates being 15–20% higher than in the asexuals (Fig. 4a). In contrast, the standardized variance of fitness of sexual lines tended to be higher than those of asexual ones during the first few cycles; then the difference diminished, and after the seventh cycle sexual and asexual lines had reached almost parity (Fig. 4b).

After the second sexual episode (growth cycles 8–12), the fitness advantage of sexual lines had diminished, and initially, the sex-2 lines resulting from this round of sex were only marginally superior to asexual lines (cycles 8 and 9). Subsequently, the relative fitness of sexual lines increased again, and by the end of the 12th cycle, they had reached a fitness advantage comparable to that at cycle 7. The standardized variance of fitness in sex-2 lines initially exceeded that of asexual lines by more than 25% (cycle 8). This excess variation then declined monotonically until sexual and asexual lines had roughly equal standardized variances (cycles 9–12; Fig. 4b).

The dynamics after the third sexual episode (growth cycles

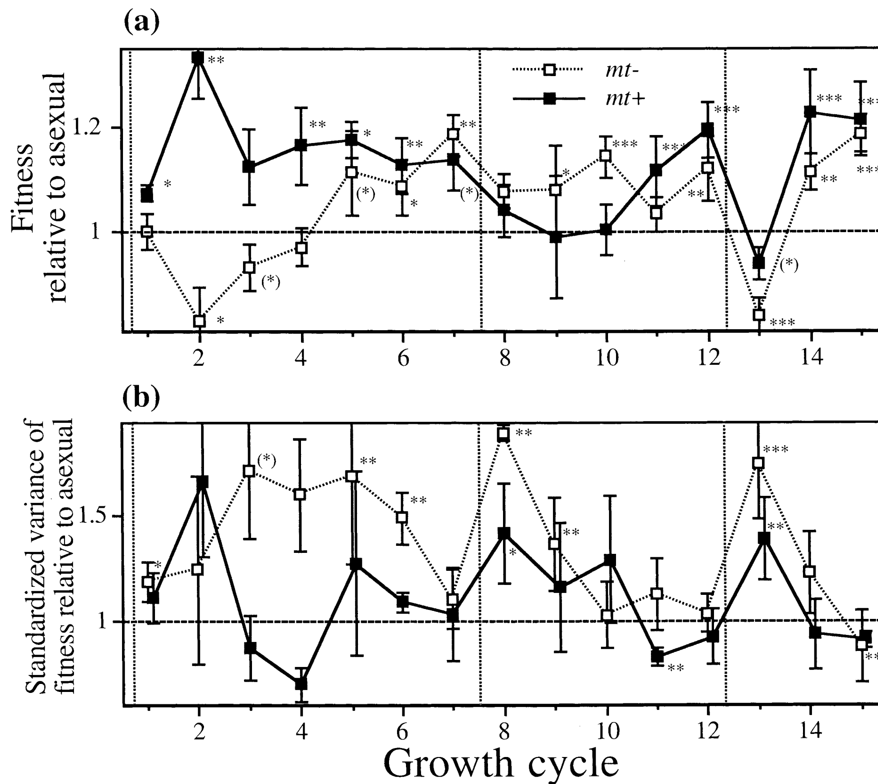

FIG. 4. Overall mean (a) fitness and (b) standardized variance of sexual lines relative to asexual (mating type mt− and mt+) lines over the course of 15 cycles of vegetative growth. Ratios were calculated by dividing the value of sexual lines by the mean of mt− or mt+ asexual lines in a given environment. Overall means and standard errors were calculated by averaging ratios over the four selection environments. Vertical lines indicate the three sexual episodes; sexual population types used for comparison: sex-1 (cycles 1–7), sex-2(7) (cycles 8–12), sex-3 (cycles 13–15); for notation see Figure 1. Asterisks represent significance levels of multiple contrasts (see Tables 1, 2).

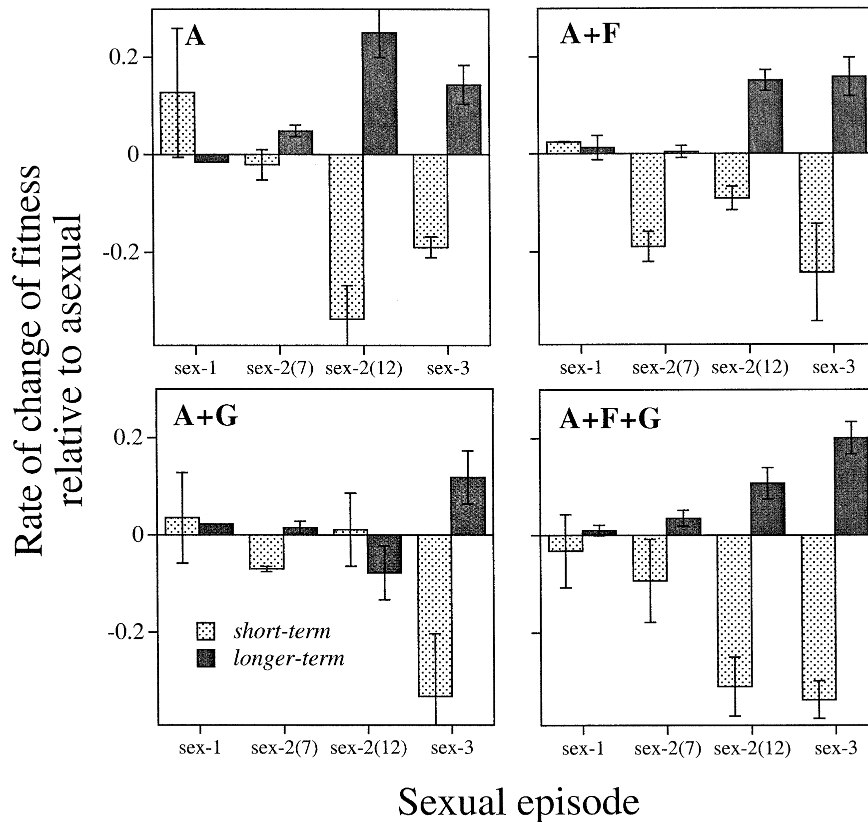

FIG. 5. Short-term and longer-term rates of change of fitness of sexual lines relative to asexual lines after the different sexual episodes. Short-term: relative change in fitness before versus immediately after sexual episode; longer-term: relative rate of change of fitness during growth cycles following a sexual episode (until the next sexual episode or the end of experiment). For additional details, see text. Means and standard errors were calculated from $N = 2$ (sex-1) and $N = 4$ sexual lines. For notation of sexual episodes, see Figure 1.

13–15) resembled those after the second episode. Initially, the mean fitness of sex-3 lines even dropped below that of the asexual lines (cycle 13). However, one growth cycle later the sexual lines had fully recovered their fitness advantage, and at the end of the experiment (cycle 15), division rates of sex-3 lines exceeded those of asexual lines by 20%, on average (Fig. 4a). Conversely, sex-3 lines initially showed a substantially increased standardized variance of fitness relative to asexual lines (cycle 13). This difference diminished rapidly, and after cycle 15 the sexual lines even had a lower standardized variance than asexual lines (Fig. 4b). Altogether, these patterns indicated quite different short-term and longer-term effects of sex on the mean and the variance of fitness, which are investigated in more detail below.

Short-term versus Longer-term Effects of Sex

For each selection line, we calculated the change in mean (ln-transformed) fitness between the last assay before and the first assay after a sexual episode (short-term change). We also calculated the rate of change of mean fitness between the first and the last assay before the next sexual episode, represented by the slopes from a regression of mean fitness on the number of growth cycles (longer-term change). For each of the different periods (cycle 1–7, 7–8, and so on), we then subtracted the mean rate of change of asexual lines from that of each sexual line. Thus, positive values indicate a more rapid increase in fitness relative to asexuals, whereas negative values indicate a more rapid loss of fitness relative to asexuals. In the same way, we calculated the relative changes in the standardized variance of fitness. The short-term change after the first sexual episode was simply taken as the difference in fitness or standardized variance between sexual and asexual lines. Short- and longer-term effects were also calculated for sexual lines derived from a second sexual episode after 12 growth cycles (sex-2[12] lines).

Two main patterns can be seen in the changes of fitness of sexual relative to asexual lines (Fig. 5). First, whereas fitness slightly increased relative to asexuals after the initial sexual episode, it generally decreased directly after additional sexual episodes. This short-term fitness reduction varied with the timing and the number of sexual episodes. A second or third round of sex after 12 growth cycles was likely to produce a stronger reduction than the second round after seven cycles; in three environments, the drop in fitness relative to asexuals was strongest after the third round of sex. Second, in the longer term, the fitness of sexuals increased more rapidly than those of asexual lines. Additional sexual episodes generally lead to faster increases of fitness relative to asexuals than the initial episode of sex. After 12 growth cycles sex produced a faster increase in relative fitness than sex at earlier stages.

Overall, the short-term effects of sex were correlated with

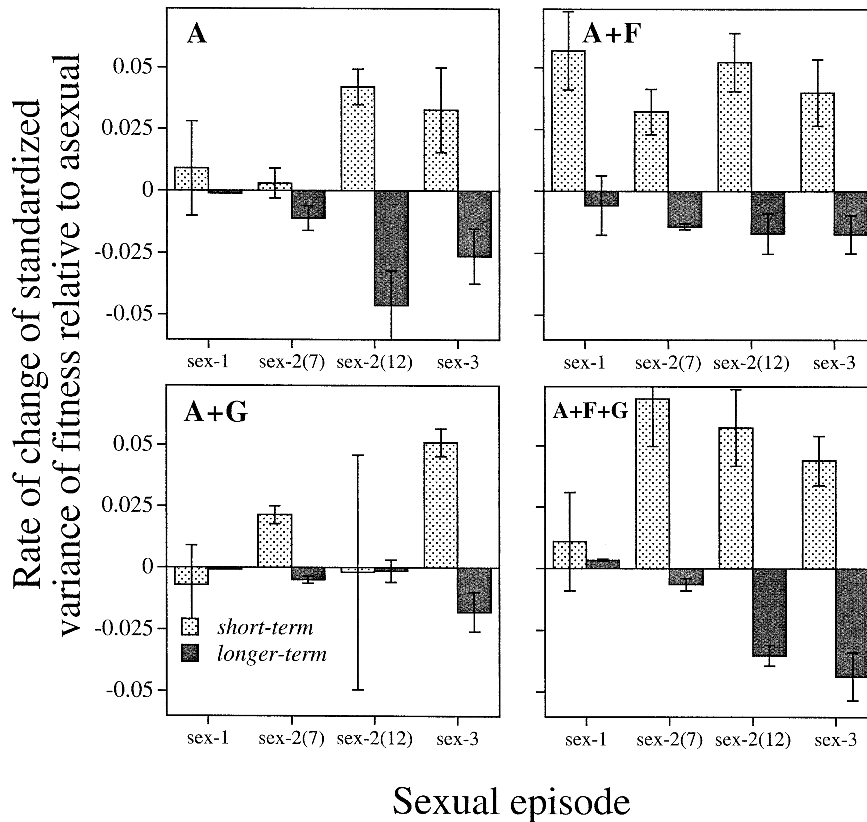

FIG. 6. Short-term and longer-term rates of change of the standardized variance of fitness of sexual lines relative to asexual lines after the different sexual episodes. For further details, see Figure 5.

longer-term effects ($r = -0.79$, $P = 0.0003$, $N = 16$; based on the mean changes per environment and sexual episode), such that a stronger decrease in fitness relative to sexual lines was followed by a more rapid increase. The correlation between short-term and longer-term effects of sex as well as the increase of the magnitude of both effects with additional sexual episodes was most pronounced on A + F + G (Fig. 5).

In almost all cases, a sexual episode resulted in a short-term increase in the standardized variance of fitness of sexual relative to asexual lines (Fig. 6). This increase tended to become more pronounced after an additional second or third sexual episode. Conversely, in the longer-term, the standardized variance of sexual lines generally diminished relative to asexual lines, and the relative decay of variance appeared to accelerate with additional sexual episodes.

Overall, there was a positive relationship between the short-term effects of sex on the variance and the long-term effect on the mean of fitness. That is, the more the standardized variance of fitness had been increased relative to asexuals immediately after sex, the faster sexual lines increased in fitness relative to asexuals during subsequent growth cycles ($r = 0.51$, $P = 0.0421$, $N = 16$; based on the mean changes per environment and sexual episode).

DISCUSSION

Effect of Sex on Adaptation

This experiment showed that *C. reinhardtii* was able to adapt to the hostile environmental conditions provided by growth on novel carbon and energy sources in the dark, with a mean direct response to selection of nearly 30%. Sexually derived lines showed a higher overall response to selection than did either type (mt− or mt+) of asexual population (Fig. 2). At the same time, we found that sexual populations had a higher standardized variance of fitness than asexual populations, immediately after a sexual episode. This difference gradually diminished during subsequent (vegetative) growth cycles, while the relative mean fitness of sexual lines increased (Figs. 5, 6). Furthermore, a larger increase in the standardized variance of fitness led to a faster increase in fitness relative to asexual lines. Such a relationship between the amount of (additive) genetic variance and an increase in mean fitness is predicted by Fisher's fundamental theorem of natural selection (Fisher 1958; Burt 1995). Thus, the complementary changes in relative mean and variance of fitness suggest that directional selection used up the variation generated by meiotic recombination and in doing so conferred a fitness advantage on sexual lines. Our result is thus consistent with the basic Weismann-Fisher-Muller mechanism, that recombination breaks up negative linkage disequilibria during meiosis and thereby increases additive genetic variation in fitness, upon which directional selection can act more effectively (Maynard Smith 1988; Barton 1995a; Burt 1995).

Effects of Repeated Sexual Episodes

We found evidence that additional sex, induced at later stages of the experiment, increased adaptation further (Fig.

2). A possible interpretation is that alleles conferring a benefit in these completely novel environments were initially rare (or of little effect) in the founder populations, so that the first round of recombination had only a modest effect on fitness. At later stages of adaptation, already existing beneficial alleles and possibly new beneficial mutations may have increased to higher frequencies and thus recombination may have generated a larger fitness differential between sexual and asexual populations. This interpretation is not strongly supported by the results of the final assay, because the additional benefits from having a third round of sex as compared to having only two rounds of sex were not formally significant. We note, however, that sex-3 lines attained levels of relative fitness in only three growth cycles comparable to those attained by sex-2 lines in five cycles (see also below).

Obviously, populations will only profit from ongoing sex as long as adaptation is incomplete. This may explain the difference between our results and those of Malmberg's (1977) and Zeyl and Bell's (1997) experiments, where sexual and asexual populations had equal fitness after several hundreds of generations in the novel environments and several episodes of recombination. For example, in the yeast study (Zeyl and Bell 1997), fitness was assayed after 400–600 generations in the novel environment. If adaptation to galactose had already reached a plateau at this point, asexual populations may have had caught up with sexual ones and effects of sex after earlier sexual episodes would have remained undetected.

Short-term Costs versus Longer-term Benefits of Sex

A consistent general pattern emerging from this experiment was that sex provided little or no immediate benefit. Instead, the relative fitness of sexual lines increased only gradually over time spans of up to 50 generations of vegetative growth. Moreover, sex even tended to produce a short-term reduction of relative fitness, especially after the last round of sex. We have repeatedly observed this initial drop in fitness after mating during related experiments in our laboratory (unpubl. data).

Some versions of the Weismann-Fisher-Muller model predict such short-term costs of recombination (Barton 1995a; Charlesworth and Barton 1996). With negative synergistic epistasis among beneficial mutations, recombination generates asymmetric variation around mean offspring fitness. The average fitness gain from offspring above the mean may not compensate for the average fitness loss from offspring below the mean, generating an overall drop in fitness immediately after recombination. Nonetheless, some offspring genotypes may be extremely well adapted and subsequently increase in frequency, resulting in a longer-term net benefit of sex. This pattern would be observed in organisms like *Chlamydomonas*, where repeated asexual reproduction is occasionally interrupted by a sexual episode. Under these conditions, we might expect a sawtooth pattern of changes in the fitness of sexual populations relative to that of parallel, perennially asexual populations.

We have found some evidence for such a sawtooth pattern (Fig. 5). The fitness of sexual relative to asexual lines dropped after the first growth cycle following a second or third sexual episode, whereas it subsequently increased in almost all cases. Furthermore, both short-term costs and longer-term benefits appeared to become more pronounced with additional rounds of sex, such that higher costs were associated with greater subsequent benefits. It will be noted that these estimates were rather crudely based on the number of growth cycles (rather than the actual number of cell divisions) following a sexual episode, and this may have inflated the rates of change after the second and third sexual episodes. With this reservation, it is possible to interpret the dynamics of fitness in terms of models of epistasis. Over time, beneficial mutations will have accumulated in our selection lines. The negative effects of epistasis are expected to increase with the number of loci involved, and a simple model of sex and epistasis (N. Colegrave, pers. comm.) shows that serial recombination can then lead to ever stronger short-term negative and longer-term positive effects, that is, to a more and more pronounced sawtooth pattern.

The costs and benefits of recombination under nonequilibrium conditions may thus depend not only on the frequency, but also on the timing of sexual episodes. In three of the four selection environments, a second round of sex after 12 growth cycles (~120 generations) produced stronger short- and longer-term changes in relative fitness than a second sexual episode after seven cycles (~70 generations).

A combination of short-term cost and long-term net benefits were also observed in experiments with yeast (Greig et al. 1998) and *Chlamydomonas* (Colegrave et al. 2002). Greig et al. grew sexual and asexual lines in mixed populations, and some sexual lines that finally eliminated asexual ones were initially nearly driven to extinction. As in our case, a correlation existed between the magnitude of cost and benefit of recombination (Greig et al. 1998). This suggests that the period immediately after sex can be critical: Competition with asexuals may produce strong selection for new, well-adapted recombinants; but sexual populations may be driven to low densities and go extinct before they can benefit from recombination.

Effect of Sex in Complex Environments

We had predicted that sex would be more profitable in more complex environments because more loci are likely to be involved in adaptation, particularly if there is negative epistasis among these loci. This hypothesis receives general support from our data (Figs. 3, 4). In the most complex environment, A + F + G, we observed the clearest difference in response to selection between the four sexual and the asexual population types, whereas sex only marginally increased rates of adaptation to acetate only (A), the least complex environment (Fig. 3). Moreover, the increase of short-term costs and longer-term benefits of sex with the number and timing of sexual episodes was most obvious on A + F + G: Lines derived from the third sexual episode paid the highest short-term costs, experienced the highest longer-term rate of increase of relative fitness, and were better adapted to this environment than the other sexual population types.

Even a single, initial episode of sex appeared to generate a long-term fitness advantage on A + F + G (Fig. 3). We often observed ungerminated zygotes in our sexual replicate

lines at the end of the three-week growth cycles, however. Lacking a light stimulus, the germination rate of these casual zygotes was low—certainly less than 10% (data not shown)—and we never observed the transfer of such ungerminated zygotes to the next growth cycle. Nevertheless, we suspect that there may have been a constant, although low, background level of recombination in our sexual replicate lines throughout the experiment. This is not fatal to our conclusions, because it will have happened in lines from all sexual population types. It may, however, explain the increased response to selection of sex-1 lines on A + F + G, in a manner consistent with models predicting a benefit from a little recombination in an otherwise asexual population (e.g., Peck 1994; Burt 2000). Any background recombination did not seem to be enough, however, to produce responses to selection as high as those in lines derived from our induced additional sexual episodes.

A recent theoretical model has suggested that sex slows down rather than accelerates adaptation under multidimensional epistasis, that is, if beneficial alleles can only accumulate in a particular order (Kondrashov and Kondrashov 2001). So far, experimental tests of the effect of sex, including ours, have considered relatively simple environmental changes (e.g., carbon acquisition or temperature). This may involve a unidimensional genetic architecture of adaptation (and epistasis) and therefore not relate to the scenario of this model.

Alternative Explanations

Replicate lines may have accumulated deleterious mutations during vegetative growth, and sexual recombination may then have cleared these deleterious mutations rather than assembled beneficial ones. Indeed, mutation assembly and mutation clearance can be considered as complementary processes that have the same underlying mechanism. There is nonetheless a clear conceptual difference between them: Mutation clearance applies to sex in a constant environment, with populations at or near the phenotypic optimum, whereas mutation assembly applies to sex in changing environments, with populations being distant from the optimum (Kondrashov 1993). Our populations were at first weakly adapted to the conditions of growth, and it is straightforward to argue that sex increased rates of adaptation primarily by bringing together beneficial mutations. Our experiment was not explicitly designed to distinguish these possibilities, but there are two reasons to doubt that mutation clearance was primarily responsible for our results. First, given the large population sizes (about 10^6 cells) and the relatively short period of vegetative growth ($\sim$50–70 generations) between sexual episodes, it seems unlikely that substantial mutation loads accumulated. Second, unlike beneficial mutations, deleterious mutations would not be expected to accumulate through selection over the course of this experiment. The sawtooth oscillations of the costs and benefits of recombination should therefore remain constant, which was not what we observed.

Conclusions

Our experiment has supported four major generalizations about the effect of sex on the process of adaptation. First, sexual populations adapted more rapidly to novel environments. This greater rate of adaptation was associated with a greater variance of fitness. Second, in a previous experiment (Colegrave et al. 2002), the advantage of an initial sexual episode gradually decayed over time. The present experiment showed that a superior response to selection can be sustained by repeated sexual episodes. Third, there is a short-term cost to sexuality, in the form of a drop in fitness in the few generations immediately after sex. This leads to a sawtooth pattern of decrease and increase of fitness through time in populations where asexual reproduction is punctuated by sexual episodes. Finally, the effects of sex varied among environments. In this case, the effects were greatest in the environment with the greatest number of novel factors.

These conclusions are all consistent with the Weismann-Fisher-Muller theory of the evolution of sex. Thus, our experiment illustrates how a genetically diverse population may evolve in a new and hostile environment, where sex is maintained through its ability to generate, and remain associated with, genotypes of high fitness. As it stands, however, the experiments are inadequate in two respects. In the first place, the relative fitness of the sexual population is inferred from its mean rate of increase in pure culture. It would be preferable to bring sexual and asexual types into direct competition and observe a correlated increase in the frequency of the sexuals as adaptation progresses. Second, the potential degree of adaptation to the two novel substrates we used is presumably limited, and once this limit has been attained sex would lose its advantage, allowing the asexual lineages to catch up. The indefinite maintenance of sex therefore requires some ecological engine of indefinitely continued change, such as that provided by parasite-host interactions (Jaenike 1978; Hamilton 1980; Bell 1982; Peters and Lively 1999). These two shortcomings point the way to the next steps in the experimental investigation of the evolution of sex.

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