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The effect of sex on adaptation to high temperature in heterozygous and homozygous yeast

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Most explanations for the evolutionary maintenance of sex depend on the assumption that sex produces variation by recombining parental haplotypes in the offspring. Therefore, meiosis is expected to be useful only in heterozygotes. We tested this assumption by competing sexual strains of yeast against constitutive asexuals in a hot (37 °C) culture for 500 generations, in either heterozygous or homozygous genetic backgrounds. We found that there was an initial cost of sex for all the sexual strains, which was indicated by a sharp increase in the proportion of asexuals after the induction of sex. The cost was larger in the heterozygotes than in the homozygotes, probably because of recombinational load. However, in two of the three heterozygote backgrounds, after the initial success of the asexuals, the remaining sexuals eventually drove them out of the population. These two heterozygotes also suffered the largest initial cost of sex. In the other heterozygote and in the three homozygote backgrounds it appeared to be a matter of chance whether sexuals or asexuals won. The average relative fitness increased in all the strains, but the increase was largest in the two strains that showed both the clearest advantage and the largest cost of sex. We conclude that these results are consistent with the traditional view that sex has a short-term cost but a long-term benefit.

Keywords: sex, evolution of; experimental evolution; cost of sex; *Saccharomyces cerevisiae*; heterozygosity; homozygosity

1. INTRODUCTION

There are two classical explanations for the evolutionary advantage of sex. The original view is that a sexual species can evolve more rapidly than an asexual species, because sex brings favourable mutations occurring in separate individuals together into the same individual (Fisher 1930; Muller 1932). The second idea is that a finite asexual population would tend to accumulate deleterious mutations, because random death would occasionally remove the least mutated individual in the population (Muller 1964). Only sexual recombination could restore the lost genotype of this individual to the population.

Both these theories explain why sex might be useful to whole populations, but offer no short-term, individual advantage of sexual reproduction over asexual reproduction (Williams 1975; Maynard Smith 1978; Bell 1982). The two original ideas have therefore evolved into several modern theories that show how sex can be selectively advantageous at an individual level either by accelerating adaptation to an environment that changes generation by generation or by reducing the loss of adaptation in a stable environment (for reviews, see Stearns (1987); Michod & Levin (1988); and Hurst & Peck (1996)). For example, the Red Queen theory says that offspring have to be different from their parents to avoid attack by parasites that would otherwise be able to evolve ways to exploit their hosts ever more efficiently (Hamilton *et al*. 1990). In the other category lies the deterministic mutation hypothesis (Kondrashov 1988), which describes how sex can concentrate deleterious mutations into offspring which are removed by selection. These ideas have aroused much interest and debate about the evolution of sex (for example, see Lyons 1997), but only recently has experimental work begun on this subject.

Saccharomyces cerevisiae is an ideal model organism for experimental evolution. Its short generation time means that it can be used for long-term, real-time adaptation experiments of the type pioneered by Lenski and colleagues (for example, see Lenski & Travisano 1994). It grows mitotically as a diploid when well nourished, but when starved it enters meiosis and each diploid cell forms four haploid gametes. When nutrients are restored, the haploid spores germinate and fuse with other haploids of the opposite mating type to form new diploids that start growing mitotically again. Yeast can be frozen indefinitely for future resurrection, huge populations (up to 3×10^8 cells ml^{-1} of liquid culture) can be propagated and the genome is entirely sequenced, easily manipulated and probably better understood than any other eukaryote.

Two recent studies have used the yeast *S. cerevisiae* to test models for the evolution of sex. Zeyl & Bell (1997) examined the effect of sex on the mean fitness of evolving yeast strains maintained for hundreds of generations on either glucose, on which they grew well, or galactose, on which they grew poorly. Sexual strains were sporulated

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periodically, whereas asexuals were refrigerated to prevent growth or sporulation. All the strains maintained on galactose evolved to grow better, but sex had no effect on this improvement. The asexual strains maintained on glucose showed little change in fitness, but the sexuals grew better, even when they were tested on galactose. The authors state that this shows that the major effect of sex is to remove mutations that are deleterious. This did not occur when the strains were adapting to galactose because selection was acting on a few large-effect alleles conferring high galactose fitness (without the need for sex to produce new combinations), not on many small-effect deleterious mutations that sex can efficiently combine.

Birdsell & Wills (1996) made sexual and asexual strains compete directly. They made diploids that were homozygous at the mating type (*MAT*) locus and therefore unable to sporulate. These asexual diploids were produced from strains that were either homozygous across the rest of the genome, or heterozygous. The asexuals were then made to compete against identical sexual strains, which differed only in being heterozygous at the *MAT* locus. They found that sexual strains always out-competed asexuals, whether or not sex was induced. If sex was induced in heterozygotes, sexuals enjoyed an immediate additional advantage, but in homozygotes sex reduced this advantage. Unfortunately, the results were hard to interpret with respect to the effect of sex because the *MAT* locus controls many haploid and diploid specific genes (Herskowitz *et al*. 1992) and *MAT* heterozygotes were always fitter whether sex occurred or not.

We made diploid (*MAT*a/*MAT*α) *S. cerevisiae* constitutively asexual by deleting the gene *IME1*, a positive regulator of meiosis (Mitchell 1994). The gene was replaced with the genetic marker *KANMX4*, which confers resistance to the drug G418 (Wach *et al*. 1994). *KANMX4* is derived from a bacterial gene with no yeast homologue. It has negligible effects on fitness and is the marker of choice for yeast growth analysis (Baganz *et al*. 1997). We were therefore able to use this genetic marker to distinguish asexual cells from sexual cells. The deletion of *IME1* renders the strains completely asexual so that they can be treated exactly the same as sexuals but are simply unable to sporulate. This also eliminates the possibility of cryptic sex occurring in nominally asexual (but sexually competent) strains that are not exposed to sporulation media. Direct competition gives a more useful and accurate assessment of their relative fitnesses than does measurement of their growth in isolation because differences in all phases of the growth cycle are incorporated into a single measurement.

To determine whether the reassortment of alleles is an important consequence of sex we made sexuals compete against asexuals in six genetic backgrounds. Three were pure homozygotes. The other three were F1 heterozygotes produced by crossing these homozygotes. We created a novel environment for the yeast to adapt to by performing the competitions at 37 °C. The usual temperature for growing laboratory yeast is 30 °C and it is unlikely that the ancestors of these strains were exposed to high temperatures for prolonged periods in the wild. We know that yeast has the potential to adapt to temperatures as high as 42 °C because thermal tolerance is an important virulence factor in clinically isolated pathogenic *S. cerevisiae* strains (McCusker *et al*. 1994).

2. MEDIA

All media were prepared using standard recipes as described in Sherman *et al*. (1986). G418 was prepared by the same method as yeast extract peptone dextrose (YEPD), but with the addition of 0.04% of the drug G418 after autoclaving (GibcoBRL, catalogue number 11811-031). YEPD is a rich medium that permits all the strains to grow; G418 only allows strains to grow if they contain the construct *KANMX4* (Wach *et al*. 1994).

3. METHODS

We used three independently isolated laboratory strain backgrounds for these experiments: SK1, Y55 and YP1. SK1 (Kane & Roth 1974) and Y55 (McCusker & Haber 1988) are both derived from wild isolates from rotting fruit. YP1 was made by crossing a strain to the most popular laboratory strain, S288c and then backcrossing the progeny to S288c at least ten times (P. Hieter, personal communication). S288c was derived from crosses between wild strains and brewing and baking strains, but its major ancestral component (estimated to be 88%) is a wild yeast isolated from rotting figs in California in 1938 (Mortimer & Johnston 1986).

We made a sexual (*IME1*) and an asexual (*ime1::KMX4*) strain from an HO-deleted (therefore heterothallic), *leu2*-deleted haploid of Y55, YP1 and SK1. These genetic markers were inserted by polymerase chain reaction-mediated transplacement (Wach *et al*. 1994) and two-step gene replacement (Orr-Weaver *et al*. 1983), respectively. From these six haploids we made 12 diploids: sexual and asexual versions of three homozygotes and three heterozygotes. We used plasmid HO-12 (from the collection of Ira Herskowitz) to induce mating-type switching in haploids (Herskowitz *et al*. 1992), allowing self-diploidization to produce diploids that were entirely homozygous except at *MAT*. HO-12 is a *LEU2*-marked, 2 μm plasmid containing the *HO* gene. Strains were easily cured of the plasmid by streaking for single colonies.

Frozen stocks of the 12 ancestral strains were streaked for single colonies on YEPD agar, and a single colony of each strain was used to inoculate an overnight culture (5 ml, 30 °C) in liquid YEPD. Samples (0.5 ml) of these cultures were frozen in 25% glycerol at −70 °C. We mixed equal volumes (0.5 ml) of sexual and asexual versions of the three heterozygous and three homozygous strains. A 200 μl sample of each of these mixed cultures was plated onto solid sporulation medium and left for 4 d to allow the sexuals in the mixtures to sporulate.

To demonstrate that there were approximately equal numbers of sexual and asexual strains in each culture at the start of the experiment, 100 μl samples of the six mixtures were serially diluted in sterile water and spread on a single YEPD plate to produce *ca*. 50–200 single colonies. These plates were incubated for a day at 30 °C and then replica-plated using sterile cotton velvet onto solid G418 medium. The YEPD and G418 plates were incubated at *ca*. 25 °C overnight and the colonies were counted, excluding colonies 5 mm or less from the edge of the plates because they might not be transferred efficiently by the velvet. The proportion of asexuals is given by the ratio of the number of colonies on G418 to the number of colonies on YEPD. We used this method throughout the experiment to measure the frequency of asexuals in each population. After 4 d on

sporulation medium, we examined samples of the cells with a microscope to ensure that approximately half of them (the sexuals) had formed tetrad ascospores. All the cells on each plate were washed off with liquid YEPD, and a single drop from a sterile Pasteur pipette was used to inoculate four replicate cultures (labelled A, B, C and D) of 5 ml of fresh YEPD for each of the six genetic backgrounds.

The 24 cultures were grown in liquid YEPD at 37 °C, with shaking at 240 r.p.m., for 56 d or approximately 500 generations. We froze samples from each of the tubes and tested the proportion of the asexuals in the populations periodically.

We performed daily transfers of a single drop (approximately 10 µl) from single-use sterile Pasteur pipettes into 5 ml of fresh YEPD. Sterile, screw-top universal containers (25 ml) were filled with 5 ml of liquid YEPD and pre-incubated at 37 °C for 24 h before use to ensure they were free from microbial contaminants, and so that the cells did not experience any temperature variation. The caps of the tubes were loosened a quarter turn and taped so that gas could escape and so that no aerosol was generated when the tubes were opened. All transfers were performed in a microbial flow cabinet, and we also carried out the transfer regime with a blank, sterile tube of medium. This tube never became contaminated, which shows that there was no migration of cells between the tubes. In addition, the SK1/SK1 homozygote cells and the YP1/SK1 heterozygote cells tended to fall to the bottom of the tube more rapidly than the other strains when the tube was stationary. These differences were still clear at the end of the experiment, again confirming that there was no migration.

We measured the change in fitness over the 500 generations, separating the ancestral strains from the evolved strains by competing cultures derived from single cells at the end of the experiment against cultures derived from frozen stocks of the ancestors. Frozen stocks of each ancestral strain and each replicate evolved culture were streaked-out onto solid YEPD medium and incubated at 30 °C overnight. A single colony was picked at random and used to start an overnight culture in 5 ml of YEPD at 37 °C. Each evolved colony was also picked to G418 medium to confirm whether it was sexual or asexual. We mixed equal volumes of each evolved strain with the appropriate ancestral strain of opposite G418 sensitivity, so that if the evolved strain was sexual it was tested against the asexual ancestor. As a control we also set up four cultures for each strain background in which the sexual and asexual ancestors were competing. These 48 cultures (24 competitions and 24 controls) were grown for 6 d under the regime used for the main experiment. The proportions of G418-resistant cells in the population were measured as before, at the start of the experiment and after 3 and 6 d.

4. ANALYSIS

We calculated the relative fitness of the derived and ancestral strains, and the sexual and asexual ancestral strains (a control), by the ratio of their malthusian parameters (Lenski *et al*. 1991). The Malthusian parameter is a measure of the average rate of increase, and differences between competitors malthusian parameters may reflect differences in all phases of growth. The Malthusian parameter is given by the natural log of (the final number of cells divided by the initial number of cells). Our cells were not grown continuously for six days, but transferred onto fresh medium daily as described above. Therefore, we calculated a virtual final cell number, which is what the count would have been had the cells been grown in a huge culture without transfers.

First, we calculated the number of generations per transfer:

$$\text{generations per transfer} = \frac{\log_{10}\left(\frac{\text{volume transferred}}{\text{total volume}}\right)}{\log_{10}2} = 8.966.$$

Next, we calculated the number of cells in the final tubes by multiplying the number of colonies counted by the ratio of the total plate area to the area counted by the dilution factor. Then we calculated the 'virtual' total cell number from the number of cells in the final tube times 2 raised to the power of the number of generations per transfer times the number of transfers (i.e. number of colonies × plating factor × dilution factor × $2^{8.966}$). Thus, the relative fitness (W) was

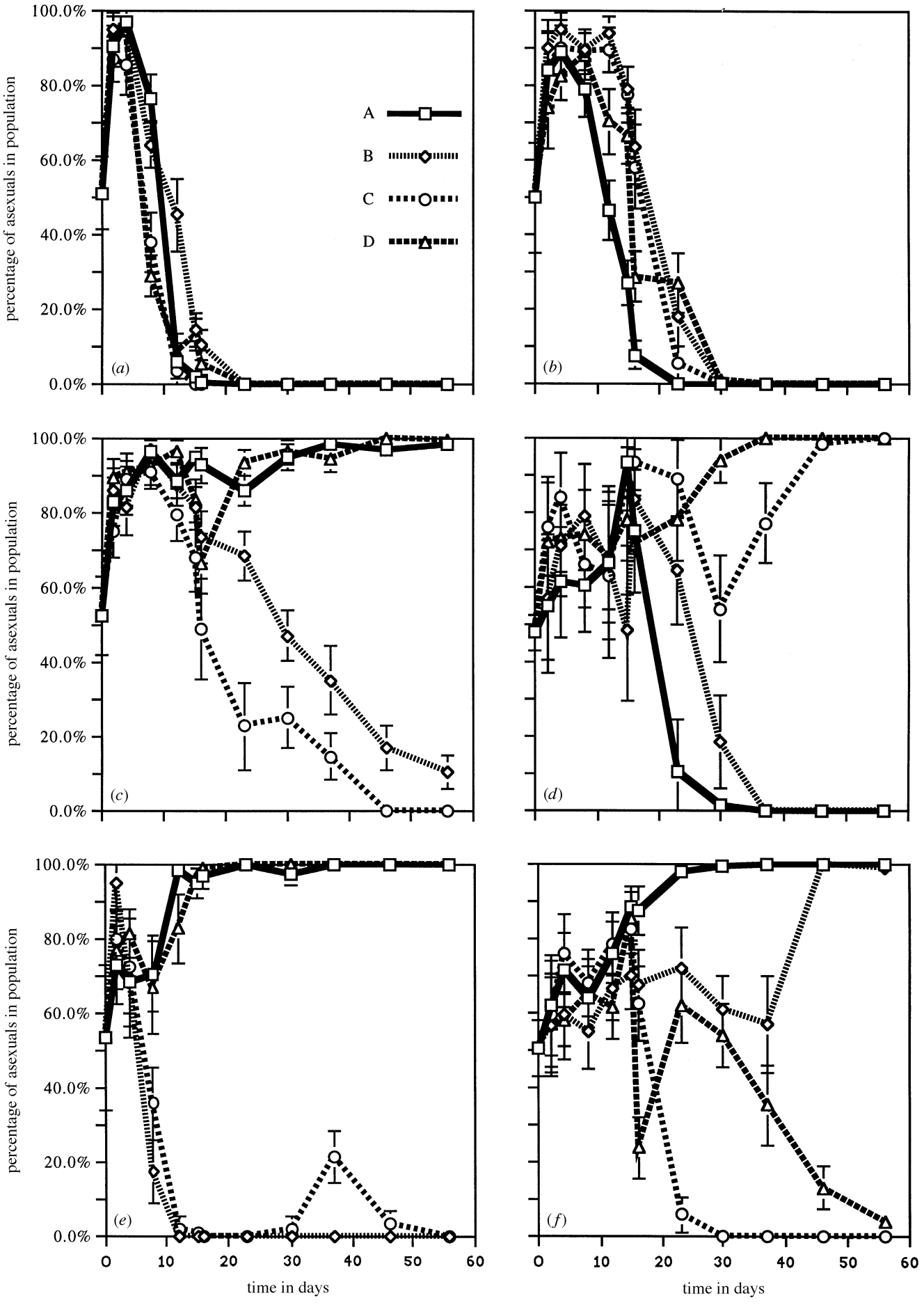
$$W = \frac{\log_e\left(\frac{\text{virtual final evolved cell number}}{\text{actual starting cell number}}\right)}{\log_e\left(\frac{\text{virtual final ancestral cell number}}{\text{actual starting ancestral cell number}}\right)}.$$

The relative fitness of the sexual and asexual ancestors was calculated in a similar way. To find the change in fitness between the ancestral and evolved strains, we took the relative fitness of each evolved strain and subtracted the mean relative fitness of the ancestral control of the same sexuality. Relative fitnesses of ancestors were much nearer to zero than relative fitnesses of the evolved strains. The estimates of the increase in fitness are conservative because in some cases the evolved strain was so much fitter than the ancestral strain that no ancestral colonies were found on the plate. These events were excluded from the analysis. We took the mean of the three-day and six-day fitnesses when both were available.

5. RESULTS AND DISCUSSION

(a) *Cost of sex*

In all six strain backgrounds there is an increase in the number of asexuals, and a corresponding decrease in the number of sexuals, soon after germination (figure 1). Figure 2 shows the difference between the starting frequency of asexuals and the frequency after four days, and represents the mean cost of sex for each strain. Much of this cost is likely to be due to the physical processes of sex. For example, asexuals may be able to grow more on the limited nutrient in the sporulation medium while the sexuals are forming ascospores. Also, the asexuals may get a head start over the sexuals when the nutrients are restored because of the time it takes for the sexual spores to germinate, mate and resume growth. We do not expect such mechanical costs to be greater for heterozygous sexuals than for homozygous sexuals. However, the increase in the number of asexuals at the beginning of the competition is significantly greater in the heterozygotes than in the homozygotes (see figures 1 and 2). A rank order of the increase in frequency of asexuals in every replicate of every strain four days after germination has 11 heterozygotes above the median and one below. If there was no effect of heterozygosity, the expected distribution would be six above and six below the median;


100.0%
80.0%
60.0%
40.0%
20.0%
0.0%
percentage of asexuals in population
A
B
C
D
(a)
(b)
(c)
(d)
(e)
(f)
0
10
20
30
40
50
60
time in days

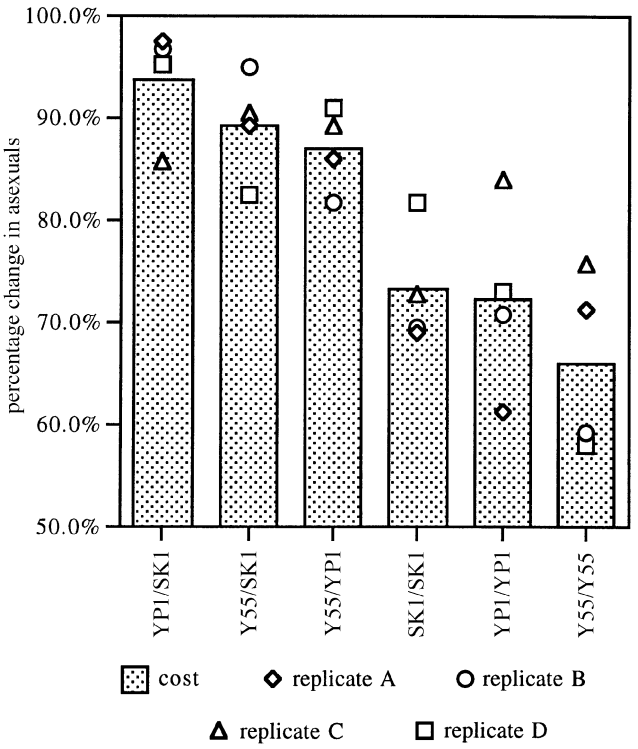


Figure 2. The cost of sex varies between the different strains. The *y*-axis shows the difference between the starting percentage of asexuals and the percentage of asexuals after four days. The data for the four replicate cultures (A, B, C and D) are shown for each strain background and the mean is shown by the shaded bar.

therefore, this deviation is very significant ($p=0.00635$ from binomial distribution). A likely explanation for this effect of heterozygosity on the cost of sex is recombinational load. Many progeny of the sexual heterozygotes are less fit than their parents, because co-adapted gene complexes in the parental haplotypes are disrupted by recombination. Because the asexuals do not undergo meiosis, co-adapted genes are not broken up in the offspring who are therefore just as fit as their parents. Although recombination also occurs in the homozygous sexuals, each allele is exchanged with an identical allele, so co-adapted genes are not split up in the offspring. Therefore, this recombinational cost of sex only occurs in heterozygotes.

Another possible explanation (Borts & Louis 1998) is that heterozygotes are less fertile than homozygotes, producing fewer live spores. However, the difference in fertility between these strains is very small: the heterozygous strains produce about 10% fewer viable haploid spores than the homozygotes. The average frequency of the three homozygote sexuals is 30% after four days and the average for the three heterozygote sexuals is 11%. A 10% reduction of 30%, caused by reduced heterozygote fertility, would still leave 27% sexuals, not 11%. The difference in the cost of sex for homozygotes and heterozygotes cannot therefore be accounted for by differences in fertility.

In their experiment, Birdsell & Wills (1996) found that sexuals enjoyed an immediate increase in fitness after sex, and this increase was larger in heterozygotes than in homozygotes. However, in their experiment sexuals and asexuals were only made to compete after the entire sexual cycle had been completed. The sexuals were sporulated, germinated, mated and then grown to a plateau before being introduced to the asexuals that had been grown to the same density. This eliminates any mechanical costs of sex. The sexuals were allowed to compete against each other before facing the asexuals, so that the sexual population was already enriched with the fittest individuals before the competition started. This would remove any recombinational cost of sex. Therefore, their results are not inconsistent with ours, but their experiment only addressed the benefits, and not the costs, of sex.

(b) *Benefit of sex*

In the heterozygotes, sexuals won 10 out of 12 competitions (figure 1). This is significant ($p=0.0386$ from binomial distribution), whereas in the homozygotes sexuals won only 6 of the 12 competitions, which is exactly what is expected if sexuals and asexuals have an equal chance of winning. This suggests that sex is only advantageous in heterozygotes, and is neutral in homozygotes.

The two strain backgrounds that suffered the largest cost of sex, YP1/SK1 and Y55/SK1 (see figure 2), also enjoyed the clearest long-term advantage to sex, with sexuals winning in eight out of eight replicates (figure 1). This indicates that although most of the progeny of these heterozygotes are less fit than their parents because of the destruction of co-adapted gene complexes, a small proportion are produced which have combinations of genes that are better suited to this unusually hot environment than their parents and the identical asexual competitors.

In the three homozygous strains it appeared to be a matter of chance whether a sexual or an asexual won. In all three strains, two of the replicates were composed primarily of asexuals at the end of the competition and the other two of sexuals (figure 1). We noticed that in the homozygotes the frequency of asexuals sometimes fluctuated up and down. This is probably because new beneficial mutations are selected in either the asexuals or the sexuals, and a clone derived from the cell in which the mutation occurred starts to sweep through the population until halted and reversed by another adaptive mutation occurring in a cell of the opposite sexuality. This supports the idea that in homozygotes there is no difference in the fitness of sexual and asexual offspring and the winner is determined by chance mutations or drift.

Figure 1. (*opposite*) The frequency of asexuals in each of the four replicate cultures (A, B, C and D) changes during the course of the experiment in each genetic background. The *y*-axis shows the percentage of asexuals in the population, the *x*-axis shows the time in days. The error bars show 95% confidence levels for the proportion of asexuals in the population, estimated by counting the colonies on a YEPD plate and its replica G418 plate, as described in § 3. The mean number of colonies on each YEPD plate was 115, and the range was from 19 to 285 colonies. Heterozygotes: (*a*) YP1/SK1, (*b*) Y55/SK1, (*c*) Y55/YP1. Homozygotes: (*d*) YP1/YP1, (*e*) SK1/SK1, (*f*) Y55/Y55.

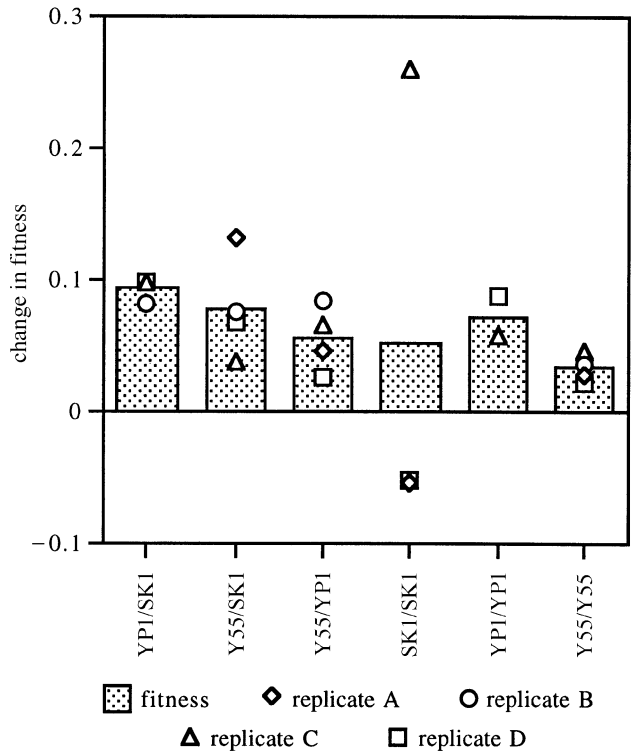


Figure 3. The change in fitness for the different strains (evolved fitness minus ancestral fitness), calculated as described in § 4. The data for the four replicate cultures (A, B, C and D) are shown when available for each of the six strain backgrounds, as well as the mean increase in fitness for each strain (shaded bars).

The other heterozygous strain, YP1/Y55, behaved much more like the homozygotes than the other two heterozygotes. The initial cost of sex was lower in this strain than in the other two heterozygotes and, like the homozygotes, in two replicates sexuals won and in the other two asexuals won. This may be because YP1 and Y55 are more similar to each other than they are to SK1, at least at the loci associated with fitness in this environment.

(c) *Increase in fitness*

The overall fitness increased in the six strains over the 500 generations (18 fitness increases, two fitness decreases, $p=0.000368$ from binomial distribution), showing that the strains had evolved. However, although the increase in fitness of the heterozygotes (0.0768) was larger than that of the homozygotes (0.0534), there was no significant difference ($p=0.435$, two-tailed t-test), and the data appear rather noisy (figure 3). However, it is noticeable that the fitness increase was greatest in the two heterozygotes that showed both the largest cost of sex and the clearest long-term advantage to sex, YP1/SK1 and Y55/SK1 (compare figures 1, 2 and 3). As before, the other heterozygote, YP1/Y55, was more like the three homozygotes than the other two heterozygotes, with a more modest increase in fitness. The large variance in the replicates for SK1/SK1 may be due to sampling error as this strain is much clumpier than the others.

An alternative explanation for the absence of significant differences in fitness increase is that only a small number of loci confer high fitness in this environment. In the sexual heterozygotes, beneficial alleles are combined by recombination. In the homozygotes, new beneficial alleles are acquired by the slower random process of mutation, but perhaps at the end of the experiment all the winning strains have alleles of similar fitness. We may have detected greater differences in the fitness increases had we tested earlier than 500 generations, before the homozygotes had acquired beneficial mutations.

6. CONCLUSIONS

The results show that:

1. Sex is more costly initially in heterozygote populations than in homozygote populations.
2. Sex is more beneficial in the long-term in heterozygote populations than in homozygote populations.
3. The strains suffering the highest cost appear to reap the greatest benefit, but this cannot be proved from the limited data.

Our explanation for this is that heterozygotes with diverged parents have offspring with a low average fitness, because of the destruction of co-adapted gene complexes. However, there is a high variance in fitness, because of the large number of combinations of diverged alleles that are produced. A small proportion of these new combinations will be fitter in a novel environment than the parental combinations. It is these sexuals that drive out the asexuals. Heterozygotes with less diverged parents have offspring with a higher average fitness (less cost) because their alleles are more similar and compatible with other genes in co-adapted complexes. But these heterozygotes cannot produce offspring with enough variation to generate individuals that are much fitter than their heterozygote parents. This conclusion is best summed up by the adage 'you get what you pay for'.

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