Mean fitness of long-term sexual and asexual populations of *Chlamydomonas* in benign environments.

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Contribution of authors to manuscript, acknowledgment.

The assay work for the manuscript "The ecology and fitness in *Chlamydomonas* XIII. Mean fitness of long-term sexual and asexual populations in benign environments." was performed by S. Renaut. The preparation of the manuscript was done by S. Renaut and G. Bell.

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Abstract (English)

Populations of *Chlamydomonas* were maintained in a benign laboratory environment as obligatory sexual or asexual populations for five years. Sexual reproduction is expected to facilitate the elimination of mildly deleterious mutations and thereby increase the mean fitness of a sexual population relative to an asexual population (Kondrashov 1988). Fitness in competition and in pure culture was measured. In neither of the fitness assays, both in solid and liquid cultures of *Chlamydomonas*, was a fitness advantage of sexual reproduction seen, even though the results varied depending on the definition of fitness. I hypothesized that the effect of mutation clearance could be masked by different forces acting on the selection strain (such as an antagonistic relationship between sexual and vegetative fitness).

Résumé (Français)

Des populations de *Chlamydomonas* furent maintenues dans un environnement bénin de laboratoire pendant les cinq dernières années en leur faisant subir un traitement sexué ou asexué. La reproduction sexuelle devrait faciliter l'élimination de mutations néfastes pour l'organisme et donc accroître la valeur sélective des populations sexuées par rapport aux populations asexuées (Kondrashov 1988). La valeur sélective en compétition et en culture pure de populations maintenues dans un environnement solide ou liquide fut testée. Dans aucunes de mes analyses j'ai trouvé un avantage des lignes sexuées par rapport aux lignes asexuées même si les résultats dépendent de la définition de la valeur sélective**.** J'ai lancé comme hypothèse que l'effet d'élimination de mutations néfastes pourrait être masqué par différentes forces actant sur les lignes de sélection (comme par exemple une relation inversement proportionnelle entre la valeur sélective végétative et sexuée).

Introduction to research and objectives.

Sexual reproduction is widespread yet not universal among plants and animals. Evolutionary biologists still view sex as one of the great mysteries of nature. The characteristic sexual processes of gametogenesis, gamete fusion, zygote maturation and meiosis are costly and time-consuming and thus must also have some advantages to be evolutionarily maintained. One of the most widely accepted explanations for the advantage of sex lies in the production of genetic variation (through random gamete fusion and recombination) on which natural selection can act (Weismann 1889).

Recently, this has been divided into two main hypotheses. Firstly, adaptation to new conditions brings together mutations that were previously deleterious but are now beneficial into the same genome. These mutations can then spread by selection through *mutation assembly* (Fisher 1930, Muller 1932). Secondly, current adaptedness is continuously eroded because the genome becomes more corrupt. Adaptedness can then be maintained in a benign environment through *mutation clearance*, with sex acting to bring together currently deleterious mutations to create severely crippled genomes that are then eliminated from the population (Kondrashov 1982). A benign environment therefore refers to an environment where individuals are already well adapted such that new mutations will have a deleterious effect. Although complementary, the two theories rely on different parameters. Mutation clearance will rely on the mutation rate per genome and the distribution of the effect of mutations (further explained in section 7, p10-11). Mutation assembly on the other hand will rely on the rate of change of the environment.

Few experiments have been designed to explicitly look at the effect of sexual reproduction on fitness (Colegrave *et al.* 2002; Colegrave 2002, Kaltz & Bell, 2002, Greig *et al.* 1998, Zeyl & Bell 1997) and none, so far, have found good evidence for mutation clearance. I have used a unicellular alga, *Chlamydomonas reinhardtii,* to demonstrate the fitness effects of mutation clearance in a benign environment. The fact that it has a relatively short generation time and can reproduce both sexually and asexually makes it a perfect candidate for experimental work looking at the evolution of sex.

Populations of *Chlamydomonas* were selected in a benign laboratory environment as obligatory sexual or asexual populations for the last five years. I then proceeded to test the fitness of the different selection lines to look at the predicted effect of sexual reproduction. In order to have a good general idea of the effect of sexuality in the selection lines, different methods to measure both competitive ability and growth rate in pure culture were used. The fitness values obtained can then be compared and contrasted according to the method used. This was my primary objective. The fitness assays performed will also show if there are differences between fitness in pure culture and in competition; between lines selected on solid or liquid media, between the selection lines and the ancestral population and finally between lines transferred by one or many zygotes.

The first chapter will exclusively look at the result of one fitness assay in competition, while in the second chapter I will then proceed to look at the results of the pure culture assays.

Following the fitness assays, an experiment was performed to test an alternative explanation for my findings. There could possibly be an antagonistic relationship between sexual selection and natural selection. According to the antagonistic pleiotropy theory of trade-offs, different components of fitness are negatively correlated (see Rose & Charlesworth 1980, Futuyma & Moreno 1988; Sgrò & Partridge 1999) If a single set of loci controls the expression of two fitness components, the trade-offs among fitness components should be symmetrical such that selection on either component should cause a regress of the other (Cooper & Lenski 2000). Zygote production and asexual spore production can be viewed as two components of fitness and thus might show an antagonistic relationship such that individuals that adapted were better at mating might conversely be worse at dividing asexually. The final chapter of the thesis will test this hypothesis.

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The Ecology and Genetics of Fitness in *Chlamydomonas*.

XIII. Mean fitness of long-term sexual and asexual populations in benign environments.

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Abstract

We maintained populations of *Chlamydomonas* in a benign laboratory environment as obligatory sexual or asexual populations for five years. Sexual reproduction (random gamete fusion and recombination) is expected to facilitate the elimination of mildly deleterious mutations and thereby increase the mean fitness of a sexual population relative to an asexual population. A competitive assay showed that sexual lines propagated by mass transfer maintained a mean fitness comparable to that of a well-adapted ancestor, whereas sexual lines selected by single zygote transfers became less fit. This demonstrates that deleterious mutations must be removed by selection for organisms to remain well adapted. Asexual lines were on average less fit than sexual lines, but there was great variation among lines.

Keywords: *Chlamydomonas reinhardtii*, evolution of sex, sexual selection, experimental evolution, deterministic mutation hypothesis, mutation clearance.

1. Introduction

The sexual cycle usually acts to interrupt reproduction and thereby to reduce the rate of vegetative proliferation. The characteristic sexual processes of gametogenesis, gamete fusion, zygote maturation and meiosis are costly and time-consuming: in well-known eukaryotes such as yeasts, chlorophytes and ciliates a sexual cycle will extend over several or many vegetative cycles, while producing no net population growth. In many multicellular organisms where sex is obligately associated with reproduction this effect is exacerbated by the involvement of individuals (usually males) who contribute few resources to the zygote (Williams 1975, Maynard Smith 1971). Sex is not necessary for regular development, error repair or any other physiological process in eukaryotic microbes, because large asexual populations can be cultured indefinitely with no signs of impairment. It seems likely, therefore, that a cumbersome sexual cycle is maintained despite reducing the number of progeny because it improves the quality of progeny.

An outcrossed sexual cycle, comprising the fusion of two unrelated gametes followed by genetic recombination, brings together mutations that arose independently in different lines of descent. This will increase genotypic diversity, because progeny will inherit different numbers of mutations from their sexual parents through random segregation. Sex may then increase the rate of response to selection, to the extent that it increases the genetic variance of fitness. This argument goes back to Weismann (1889) and has been summarized recently by Burt (2000). It explains the maintenance of sex, provided that the conditions of growth deteriorate fast enough that enhanced adaptation more than compensates for retarded reproduction. This might happen for two reasons. The first is that current adaptedness is eroded because the environment becomes more stressful, the situation first envisaged by Weismann (1889) and subsequently explained in terms of Mendelian genetics by Fisher (1930) and Muller (1932). Adaptation to the new conditions then requires *mutation assembly*, bringing together mutations that were previously deleterious but are now beneficial into the same genome, which then spreads through selection. The second reason is that current adaptedness is eroded because the genome becomes more

corrupt. Adatedness can then be maintained in a benign environment through *mutation clearance*, with sex acting to bring together currently deleterious mutations to create severely crippled genomes that are then eliminated from the population (Kondrashov 1982). Sexual populations are expected to have greater mean fitness than asexual populations through their more efficient process of mutation clearance, provided that the rate of deleterious mutation is high enough to pay for the lower rate of reproduction.

Mutation clearance is an attractive explanation for the maintenance of sex because deleterious mutations degrade adaptedness in all populations at all times, whereas a continual increase in environmental stress requires a special auxiliary explanation such as host-parasite coevolution (reviewed by Hamilton *et al.* 1990). In order for sex to accelerate mutation clearance, however, two necessary conditions must be met. The first is that the rate of deleterious mutation exceeds 1 per genome per generation (although this rate might be lowered, if mutation clearance acts together with other advantages of sex, West *et al.* 1998). The rate estimated from mutation accumulation experiments in microbes is substantially lower, with values of 0.001 – 0.01 being reported in most studies (Drake 1998, Zeyl & de Visser 2001). The magnification of mutational load through the germ line may increase this rate in multicellular organisms, but recent careful estimates fall short of a value of 1 in *D. melanogaster* (Fernández & López-Fanjul 1996, Fry *et al.* 1999, Keightley and Eyre-Walker 2000) and in *C. elegans* (Keightley & Caballero 1997; Vassilieva & Lynch 1999). The second necessary condition is that there should be strong interactions among loci, such that the fitness of the double mutant is much less than expected (Kondrashov 1988, reviewed by Charlesworth 1990). This is plausible on theoretical grounds (Szathmary 1993), yet the experimental detection of epistasis has been difficult, and only a few studies exist on the interaction of deleterious mutations. There is, however, some evidence for positive epistasis between deleterious mutations in *Chlamydomonas moewusii* (de Visser *et al.* 1996), *E. coli* (note however that an equal amount of antagonistic and synergistic epistasis was found, Elena & Lenski 1997) and *Drosophila* (Whitlock & Bourget 2000).

The properties of sexual and asexual populations have attracted a great deal of theoretical and comparative studies (Williams 1975, Maynard Smith 1971, Bell 1982, Stearns 1987, Michod &

Levin 1987), but few experiments have addressed the issue directly. One of the reasons is that they are technically difficult to perform in multicellular organisms. Nevertheless, the manipulation of chromosomes in *Drosophila* has provided some evidence that suppressing recombination reduces the rate of response to selection (McPhee & Robertson 1970, Rice 1994). Even in eukaryotic microbes, where sexual and vegetative cycles are distinct, few experiments have been reported. Selection experiments in *Chlamydomonas* (Colegrave *et al.* 2002; Colegrave 2002, Kaltz & Bell, 2002) and yeast (Greig *et al.* 1998) have shown that sexually reproducing strains are able to adapt more rapidly than their asexual counterparts. Zeyl & Bell (1997) failed to find evidence for mutation assembly in yeast cultures adapting to growth on an exotic medium (galactose), perhaps because adaptation involved very few beneficial mutations of large effect. They suggested instead that performance on a benign medium (glucose) demonstrated mutation clearance. There does not seem to be any clear demonstration that sexual and asexual populations diverge in mean fitness after many generations of culture on a benign medium to which they are well adapted. We have conducted an experiment designed to provide a convincing test of the mutation clearance theory.

2. Material and Methods

(a) Model organism

The unicellular chlorophyte *Chlamydomonas reinhardtii* can be grown in the light on liquid or solid Bold's minimal medium, a mixture of inorganic salts lacking a carbon source (Harris 1989), as a haploid photoautotroph. It can also grow heterotrophically in the dark on medium supplemented with acetate as sole source of carbon and energy. Gametogenesis is induced by nitrogen deprivation, which provokes differentiation into mating type plus (mt^+) or mating type minus (mt^-) gametes, followed by fusion of gametes of opposite gender to form a diploid zygote that secretes a thick wall and acts as a resting stage (Harris 1989). Laboratory cultures are mated in the light, transferred to plates containing solid medium and are then placed in the dark for 4-5 days to allow zygotes to mature. The plates are then exposed to chloroform vapor for 45 sec. to

kill all unmated vegetative cells, while most of the zygotes survive. The plates can then be placed back in the light where the zygotes will germinate within a few hours and the haploid meiospores used to initiate the next cycle of vegetative growth.

(b) Base population

The base population for the experiment was a line constructed by Clifford Zeyl as a complex cross involving three wild types that was propagated through 16 sexual cycles and about 200 vegetative doublings under standard laboratory conditions. It carries a marker that causes cells to form yellow (rather than green) colonies when grown on acetate in the dark; this marker is inherited uniparentally through the mt^+ parent and is therefore presumed to be located on the chloroplast. We have never observed reversion despite examining many thousands of colonies. Several such mutants, involving the deletion of a gene involved in photosynthesis, are known and produce the yellow phenotype by causing the accumulation of a chlorophyll precursor (see Ford & Wang, 1980).

(c) Selection experiment

Lines were extracted as samples of about 10^5 cells from the base population and subsequently propagated in liquid medium. Liquid cultures were grown in 300 ml of Bold's medium with continuous light (4 soft white fluorescent elements at 30 cm) and aeration (sterile-filtered room air) at room temperature(22-26 ^{0}C) for about a week (the period varied somewhat over the experiment), during which they achieve about 10 doublings. Over the course of the selection experiment, the lines went through 1000-1500 mitotic generations. We used four treatments to create a total of 12 lines with different sexual histories.

(1) Sexual Mass-transfer (4 replicates). An obligate sexual cycle was imposed at the end of each period of vegetative growth, and about 200-500 germinated zygotes were used to inoculate fresh medium. These lines went through 115 sexual cycles.

(2) Sexual Single-zygote (4 replicates). An obligate sexual cycle was imposed at the end of each period of vegetative growth, and a single germinated zygote used to inoculate fresh medium. These lines went through 115 sexual cycles.

(3) Unselected (2 replicates). Gametogenesis was induced but zygotes were not selected. Hence, the population transferred was an unspecified mixture of sexual and vegetative products. These lines went through 115 cycles of induced but unselected gametogenesis by transferring 250ul of culture cells (1-4 x 10^5 cells) every time.

(4) Asexual (2 replicates). Lines were transferred directly without either gametogenesis or zygote selection. These lines went through 115 transfers by transferring 250ul of culture cells (1-4 x 10^5 cells) every time.

Zygote production in the Unselected lines soon became rare or absent, indicating that the Unselected and Asexual lines could potentially be grouped for analysis. However, because of an unexpected selective pressure faced by the Unselected lines, the lines should perhaps be kept as separate treatments (this will be further explained in the discussion, p.18-19). The experiment was inoculated in September 1997 and suspended for assay in September 2002.

(d) Competitive assay

We measured the fitness of the ancestor (base population) and the selection lines by competing them as populations against one of two wild-type strains, CC-2931 and CC-3079. These wild types form green colonies on acetate-supplemented medium in the dark. The lines cannot be used directly, because powerful and long-continued selection for mating had caused zygotes to be formed spontaneously in the sexual lines, and zygote formation would reduce the rate of vegetative growth. Consequently, we isolated heterothallic spores of known mating type from each line, and used these to set up populations derived from the selection lines but comprising only mt^- spores. We used 16-18 spores to reconstruct the Sexual Mass-transfer lines; 2-7 for the Sexual Single-zygote lines; 23-31 for the Unselected lines; and 28-39 for the Asexual lines. Each reconstructed population was mixed with a roughly equal quantity of the wild types and propagated in liquid medium for four transfers. At the beginning of the assay, and after each

transfer, a sample was thin-spread on acetate-supplemented plates and incubated in the dark for 2-3 weeks, after which colony colour could be scored. Thus at time 0 and after each transfer, the number of yellow and green colonies could be counted and a ratio (number of yellow colonies / number of green colonies) calculated. The fitness of the selection line relative to the wild type was estimated as the slope of the regression of the ratio (log) of the yellow and green colonies on the number of generations of competition.

3. Results

Competitive fitness assay

The mean fitnesses of Liquid lines in the four Sexual Mass-transfer treatments as measured by competition are shown in Figure 1a,b. The two wild types used as common competitors had markedly different fitness relative to the selection lines, but the correlation of lines between the two assays ($r = 0.65$, $df = 11$, $P = 0.02$) is reasonably high. Analysis of variance with the two replicate assays as blocks, and treatments (Asexual/Unselected grouped together, Sexual Mass-transfer and Sexual Single-zygote) as fixed effects, showed that mean fitness differed significantly among treatments ($F_{2,40} = 25.0$, $P < 0.001$). Post-hoc comparisons showed that this overall effect was largely attributable to the difference between Sexual Mass-transfer and Sexual Single-zygote treatments ($F_{1,25} = 75.5$, $P < 0.001$) rather than to the difference between Sexual Mass-transfer and Asexual ($F_{1,17} = 3.12$, $P = 0.095$).

In both cases, the two Asexual lines and the four Sexual Mass-transfer lines had attained greater mean fitness than the ancestor. On average, Asexual lines exceeded the ancestor by 28.3 % (+/-SE 9.4) and Sexual Mass-transfer by 29.3 % (+/-SE 9.5). The estimates for Unselected and Sexual Single-zygote lines fell short of the ancestor in both cases.

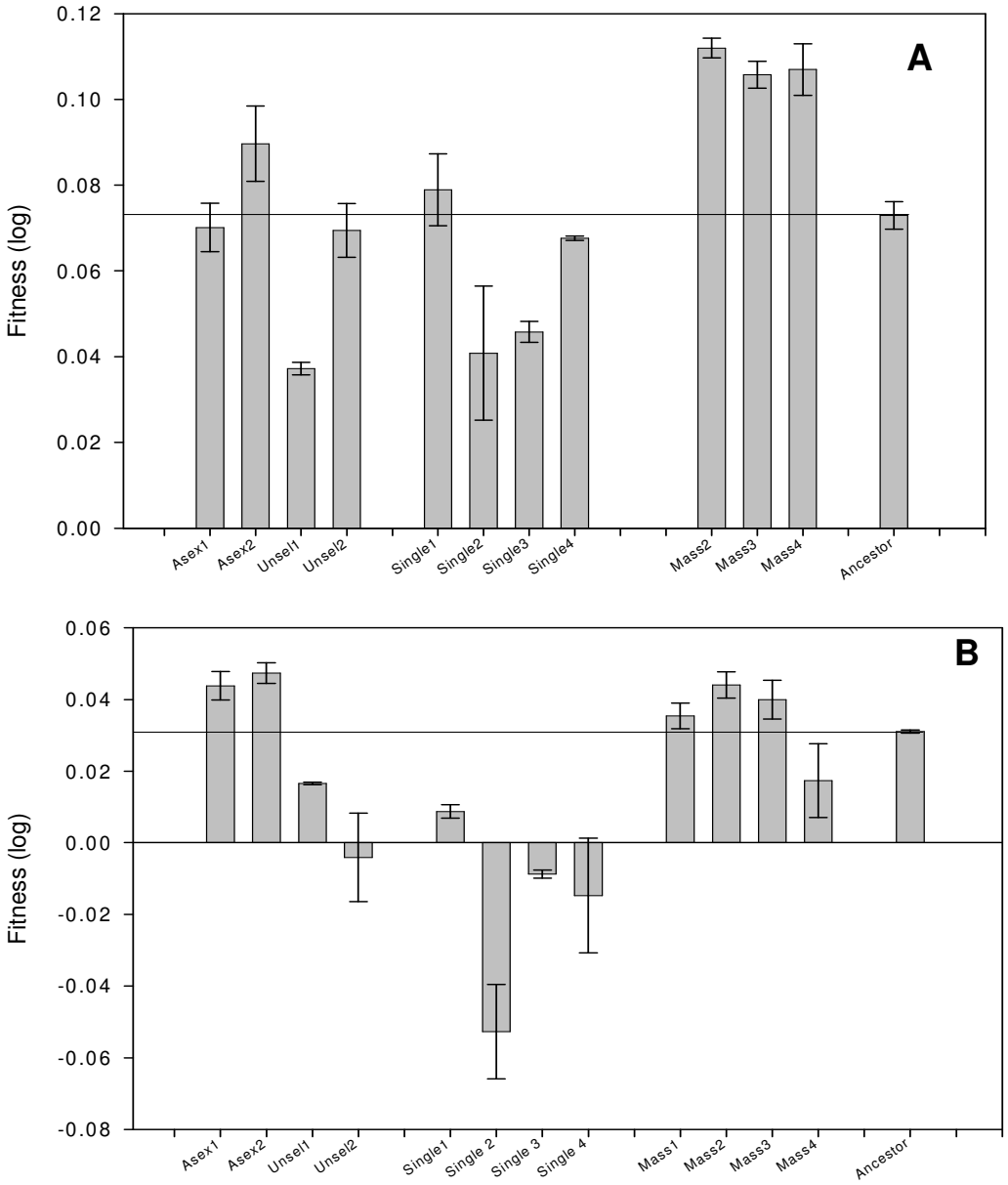

Figure 1 a, b) Mean fitness (+/- SE) of the 12 selection lines and ancestral population relative to the marker strain (CC-3079, figure 1a; CC-2931, figure 1b). Each selection line was replicated twice in order to get a replication error value. Sexual Mass-transfer 1 could not be scored in figure 1a because of fungal contamination.

4. Discussion

(a) Effect of sex

First of all, we found that the base population (ancestor) was competitively superior to both wild types (CC-2931 and CC-3079), showing a modest degree of adaptation to laboratory conditions of growth attributable to about 200 vegetative generations in culture before the start of the experiment. The Sexual Mass-transfer lines all have similar mean fitness that is comparable with that of the ancestral population: they were estimated to be superior to the ancestor relative to one of the testers, and equivalent with respect to the second. They may therefore have attained a somewhat greater degree of adaptation to the laboratory, but any advance seems to be slight, and perhaps sensitive to the precise assay procedure used. It is likely therefore, that directional selection acting on the selection lines was weak, so that any effect of sex can be attributed to mutation clearance rather than to mutation assembly.

The mean fitness of the Sexual Mass-transfer lines exceeded that of the Asexual and Unselected lines combined together. However, the overall effect was only significant when combining the Asexual and Unselected lines as one treatment. At the same time, the Sexual Single-zygote lines were consistently inferior to the Asexual lines. The greater mean fitness of the Sexual Mass-transfer lines therefore cannot stem from some physiological or developmental property of sex, but must be caused by the population consequences of genetic recombination and random gamete fusion. The sexual treatment imposed on the spores for the last five years must then be responsible for the fitness difference seen between the Asexual and Sexual Mass-transfer lines. Thus, when combining the Asexual and Unselected lines, our results seem to corroborate with the findings of Zeyl and Bell (1997) who suggested that the performance of yeast on a benign medium demonstrated mutation clearance. If the fitness difference is solely due to the effect of sex, this would imply synergistic epistasis between different deleterious mutations and a rate of mutation greater than 1 per genome per generation (Kondrashov 1988).

It is also worth mentioning that the population transfer size was smaller in the Sexual Mass-transfer lines (200-500 diploid zygotes) than in the Asexual lines (10^5 haploid cells). There is

evidence that populations transferred in small size can suffer a fitness cost due to the accumulation of deleterious mutations (see Charlesworth & Charlesworth 1998). Thus, this could have reduced the fitness of the Sexual Mass-transfer lines. However, we feel that in this particular environment, the transfer size was large enough to prevent the accumulation of deleterious mutations and its associated fitness cost.

(b) Mutation accumulation in the Sexual Single-zygote lines

The Sexual Single-zygote lines are not only inferior to the other selection lines, but are also inferior to the ancestral population. This demonstrates that deleterious mutations must be removed by selection for organisms to remain well adapted to their environment. The Sexual Single-zygote lines suffered a bottleneck every transfer as their population was reduced to one single zygote and because this transfer is random, mutations are allowed to accumulate in those populations that have been shielded from selective forces (see Lynch & Gabriel 1990). Sex can still have an effect even in small bottlenecked populations. However, the population is more greatly exposed to the effects of genetic drift as the zygote chosen might by chance have been formed by the fusion of gametes both carrying a mutation, which is thereafter fixed in the population. Furthermore, a deleterious mutation that has occurred in the lineage of one gamete, and therefore in one mating type, will be transferred to the other mating type by recombination in the zygote.

Alternatively, mutations in the large chloroplast genome (196 Kb) that have occurred in the lineage of the mt^+ gamete will be transmitted to all meiotic products and thereby fixed in the population immediately. Nuclear and chloroplast mutations have different implication in sexual populations. The load of nuclear mutations may be reduced by mutation clearance, and this process will be obstructed by single zygote transfer. The load of chloroplast mutations, on the other hand, will be increased in sexual populations through uniparental inheritance, so that mutations on a mt^+ nuclear background infect all sexual descendants, and this process will be facilitated by single zygote transfer.

The fitness decrease seen in the Sexual Single-zygote lines is probably dependent on the assay procedure: the fitness effects of mutation accumulation usually being more prevalent when tested under competitive or stress conditions (Kondrashov & Houle 1994, Shabalina *et al.* 1997, Korona 1999). Conditions that cause protein damage or stress can divert certain proteins (e.g. Heat Shock Proteins) from their normal function and enhance the phenotypic effects of mildly deleterious mutations (Rutherford & Lindquist 1998).

(c) Variance among Asexual lines

The average performance of the Asexual lines conceals the wide variance among these lines. Two of the Asexual lines have mean fitness equal to that of the Sexual Mass-transfer lines, whereas the other two (Unselected lines) have a lower fitness and are inferior to the ancestor. In the course of other assays, we have measured the growth of the selection lines in pure culture, and have repeatedly observed that the same two lines have markedly reduced growth, whereas the other two are unimpaired. The two inferior lines are the Unselected lines, and thus have a somewhat different history from the others. They were initially set up as lines where gametogenesis was induced, but zygotes were not selected. For some time, these lines may have been propagated as a mixture of sexually and asexually derived spores. The evidence that they are now asexual is that no zygotes can be detected in the cultures, and that the lines comprise a single mating type (mt^-). The other two Asexual lines also comprise a single mating type (mt^-), perhaps as the result of a selective sweep early in the history of the experiment, as the populations at transfer are too large for drift to be a plausible explanation. All four lines are known to have become fixed for a single mating type within the first 20 transfers. Thus, the two inferior lines have been treated in the same way as the two superior lines for most of the duration of the experiment, and all are perennially asexual.

It seems conceivable however that early in the experiment individuals in the Unselected lines were selected for growth or endurance in low Nitrogen conditions (because spores were subjected to nitrogen starvation every transfer). Spores capable of growing in low Nitrogen might therefore grow slower than high Nitrogen requiring spores. This was not intended when

the selection experiment was set up, yet it could explain why we see a fitness decrease in the Unselected lines but not in the other two Asexual lines.

Another possibility is that the stress suffered due to the Nitrogen starvation induced a higher mutation rate in those lines. Several authors (Echols 1981; Wills 1984; McDonald 1987) have proposed that natural selection might favour some form of stress-induced increase in the mutation rate. There is also some experimental evidence that mild environmental stresses, such as starvation, can be mutagenic (Goho & Bell 2000, Bjedov *et al.* 2003). In this case, the Unselected lines would have a lower fitness than the Asexual lines because they suffered a higher mutation rate. Sexual Single-zygote and Sexual Mass-transfer lines would also have suffered a higher mutation rate which should however be compensated by the effect of sexuality.

With the possibilities that the Asexual lines were selected for growth in low Nitrogen conditions or suffered a higher mutation rate, the evidence for the beneficial effect of sex becomes mitigated. Our results give limited evidence (only significant if the Asexual and Unselected lines are grouped together, see table 1, p.25) that sexual reproduction brings a competitive fitness advantage to well adapted population through genetic recombination and random gamete fusion. This advantage, if any, would be for the greater part a result of clearing the population of mildly deleterious mutations rather than combining beneficial ones.

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Chapter 2: Fitness in pure culture.

1. Introduction

I decided to perform two more fitness assays in order to measure fitness in pure culture in the selection lines. This will give a more complete support for the effect of sexuality. Fitness in pure culture and in competition may be different if they are controlled by different genes or if a single set of genes has pleiotropic effects and is expressed differently according to the conditions of growth.

The fitness assays were conducted on selection lines maintained on solid and liquid media. I can then compare and contrast the values obtained with the different methods. They will show if there are differences in the lines selected on solid or liquid media; differences between the selection lines and the ancestral population and finally the effect of the transfer population size (i.e. Sexual Single-zygote transfer lines compared to Sexual Mass-transfer lines).

2. Materials and Methods

The base population and method of selection for the Liquid lines are described in the previous chapter (page 12-13). The Solid lines were kept on solid agar (in petri dishes) instead of in flasks and transferred with a sterile loop (about 1-4 X 10^5 cells) instead of a pipette. Furthermore, gametogenesis by nitrogen starvation was not induced in the Solid Unselected lines which were treated in a similar way to the Asexual lines.

(a) Mating system in C. reinhardtii

As mentioned previously (p. 11-12), *C. reinhardtii* is a heterothallic and isogamous species. Mating type (+ or -) is permanently fixed in a lineage, behaving as a single Mendelian locus in crosses. Both mating types are also similar in size and appearance (Harris 1989). Our laboratory has discovered that some individuals in the Sexual Mass-transfer lines can also form homothallic spores (unpublished data). This has never been described before in *C. reinhardtii* but is known to

be the possible in many closely related species (Harris 1989). The genetic and mechanistic way by which homothallism in *C. reinhardtii* is possible is still not understood. It is hypothesized that cells might carry the information for both mating types while some control mechanism usually inactivates one set of gene responsible for one of the mating type (Harris 1989).

(b) Pure Culture Assay (Liquid).

Maximal rate of increase (r_{max}) and maximal density (K) of each reconstructed selection line in pure culture (each line was reconstructed with spores of a single mating type as described in the previous chapter) was estimated. This was done in culture tubes rather than in bubbled flasks so as to allow adequate replication and standardized measurement. The populations were first grown in bubbling flasks for 4 days and then used to inoculate screw-top culture tube with 20 mL of Bold's medium. 5 replicates of each line were arranged in tube racks as a single completely randomized block with a single border row. Tubes were scored every day for 3 weeks by vortexing briefly and recording transmittance at 665 nm on a Milton-Roy Spec-20 digital spectrophotometer. Transmittance (T) is related to cell density over a range of roughly 20 to 80% transmittance according to the equation $T = a\ [1\text{-}exp(-bV)]$ where a is the limiting value of T at large cell density (V) and b is the rate at which T approaches this limit. By constructing a dilution series, a and b could be estimated as 86.51 and 0.06155 respectively (data not shown). The cultures are assumed to grow logistically according to the equation $N(t) = K / [1\text{-}exp(i\text{-}r_{max}t)]$, where K is the limiting density and r_{max} is the maximal rate of increase approached as N becomes very large (i is a constant involving the initial population parameter and t is time). Estimates of cell density were used to calculate r_{max} and K for each line. The whole assay was performed twice consecutively.

(c) Pure Culture Assay (Solid).

The principle of the assay of the Solid lines was to pipette a dilute suspension of cells onto agar and fix them with Lugol's solution after 24 h of growth under the same conditions as in the experiment. There was no need to isolate spores of a single mating type because no mating

occurs during this early period of colony expansion; visual inspection was confirmed by chloroforming plates after 24h, without any zygotes being found. The number of cells in 80 random colonies was then counted under a dissecting microscope, and used to estimate the rate of increase as the initial number of doublings per day. In practice, I used four quadrants on each plate to measure samples from three selection lines and a standard wild type (CC-3079). The values for the selection lines were then expressed as fractions of the value for the wild type, in order to reduce variance among plates. The whole assay was performed twice consecutively.

3. Results

(a) Pure culture assay (Liquid).

When populations are grown in culture tubes, the selection lines do not grow faster nor do they attain a greater final density (K) than the ancestor (Figure 2a & b, table 1). Because of the fitness difference between the Asexual and Unselected lines for reasons discussed previously (see p. 17-18), they were both kept as separate treatments for the analysis of variance. Therefore, there is a significant difference in mean fitness among the four treatments (Asexual, Unselected, Sexual Single-zygote and Sexual Mass-transfer) for K ($F_{3, 108}$=16.33; P<0.001), but this is wholly attributable to the anomalous behaviour of the Unselected lines. There is no evidence of any systematic difference between Sexual Mass-transfer and Asexual ($F_{1, 50}$=3.2, P=0.08) nor between Sexual Mass-transfer and Sexual Single-zygote ($F_{1, 50}$=0.027, P=0.87). Once again, it seems like the Unselected lines behaved differently as they have a lower fitness than the Asexual lines ($F_{1, 31}$=38.51, P<0.001). The failure to detect treatment effects may stem in part from the low repeatability of this assay: the correlation between replicates (blocks) was low for K (r = 0.41, df = 11, P=0.165).

For r_{max}, there was no significant difference among the four treatments ($F_{3, 108}$=1.54; P=0.208). Given that there was no positive correlation between the two replicates for r_{max}, it will not be used to calculate fitness.

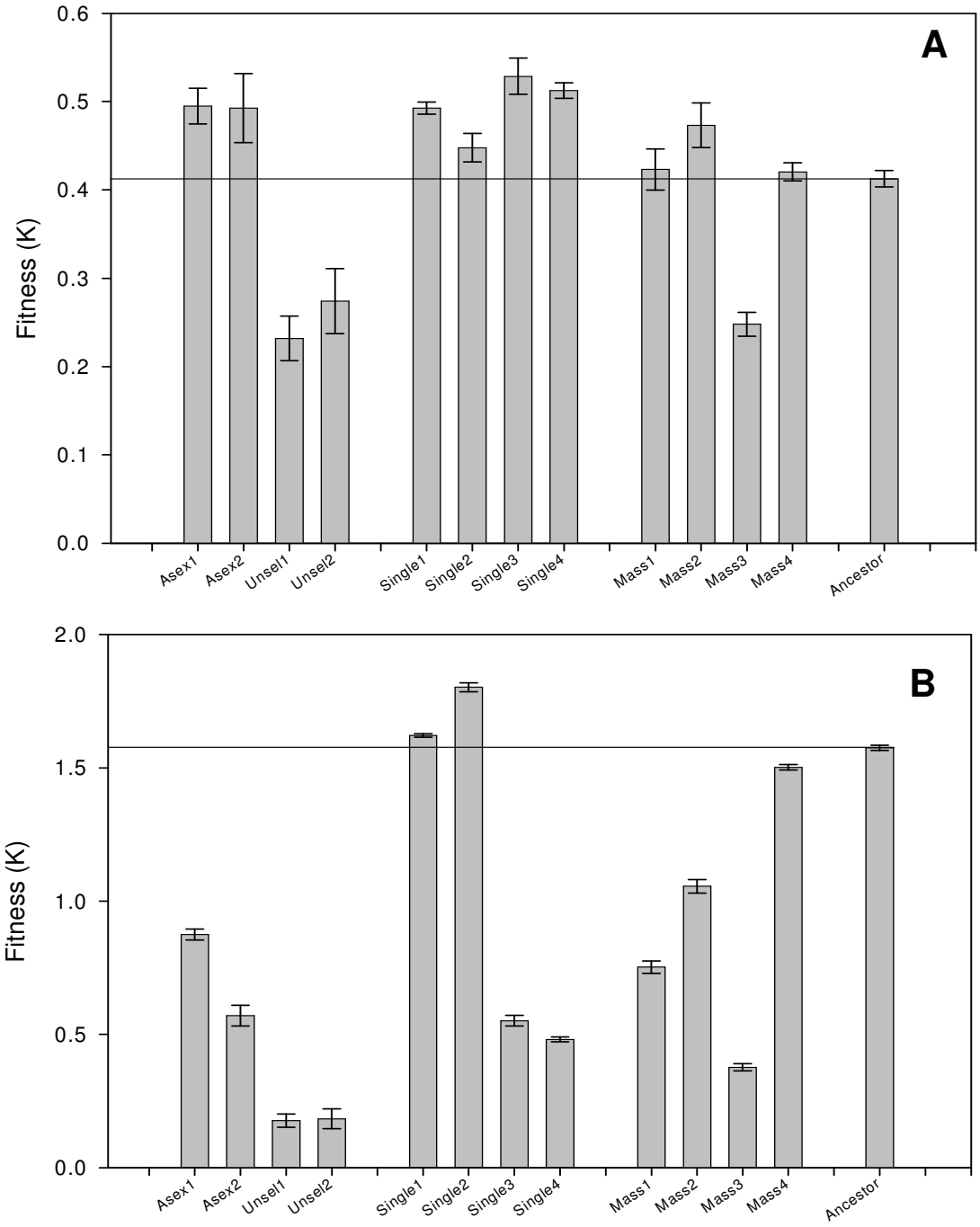

Figure 2 a, b) Mean carrying capacity (+/- SE) of the 12 selection lines and the ancestor population (fig 2a is the first replicate and fig 2b, the second replicate experiment). Each selection line was replicated in 5 culture tubes in order to get a replication error value.

(b) Pure culture assay (Solid).

The assay on solid medium was highly repeatable (r = 0.88, df = 11, P<0.001) and revealed significant variance among treatments (Figure 3, $F_{2, 18}$=16.27, P<0.001). In the Solid selection experiment the Asexual and Unselected lines received the same treatment and in fact have attained, as expected, similar fitness ($F_{1,4}$=1.06, P=0.36). They can therefore be grouped both as Asexual lines (see figure 3, table 1).

It is the Asexual lines that achieved the highest rate of growth: they exceeded the ancestor by 33.5 % (+/- SE 5.5) and are consistently superior to the Sexual Mass-transfer lines ($F_{1,12}$=24.4, P < 0.001). The Sexual Mass-transfer lines did not differ appreciably from the ancestor ($F_{1,6}$=0.99, P = 0.36) or from the Sexual Single-zygote lines ($F_{1,12}$=0.076, P=0.79).

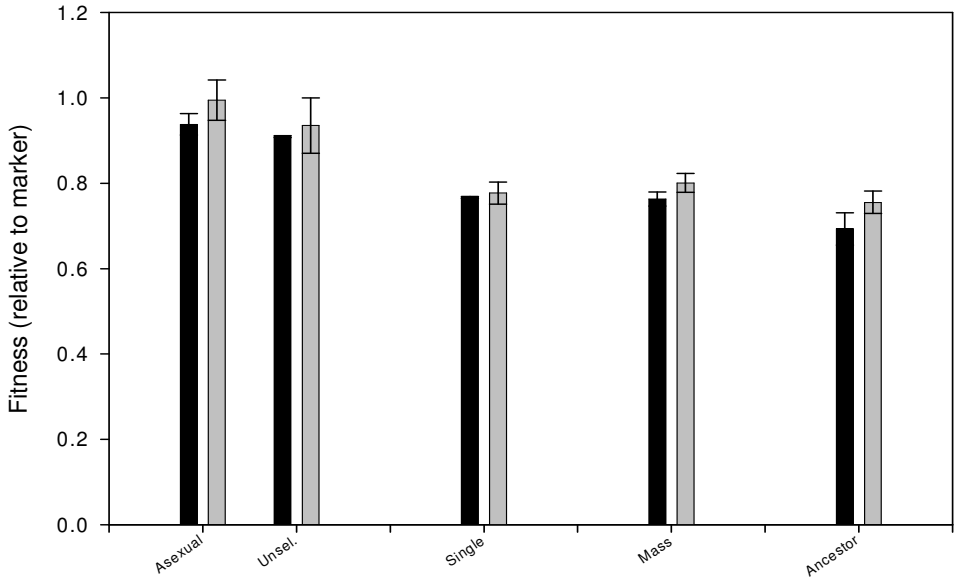

Figure 3 Mean (+/- SE) of fitness (number of generation of selection line relative to marker strain) for two replicate experiments. Each bar represents an average of the fitness of 80 spores for all the selection lines for each treatment (e.g.: Sexual Mass-transfer: 4 selection lines X 80 spores).

ANOVA of the relative fitness of the different treatments.							
	Difference between…						
	4 treatments (A,U,S,M)	**3 treatments (A/U,S,M)**	**A and U**	**A and M**	**U and M**	**A/U and M**	**M and S**
In competition (Liquid)	-	***	***	n.s.	***	***	***
In pure culture (Liquid, r_{max})	n.s.	-	n.s.	*	*	-	n.s.
In pure culture (Liquid, K)	**	-	***	n.s.	***	-	n.s.
In pure culture (solid)	-	***	n.s.	-	-	***	n.s.

Table 1: ANOVA of relative fitness values with the replicate assays as random block effects and the different treatments as fixed effects. Values of F associated with probabilities of less than 0.05, 0.01 and 0.001 are marked with one, two or three asterisks respectively; n.s.: non-significant; dashed line: test not performed. **A, U, S, M** represent Asexual lines, Unselected lines, Sexual Single-zygote lines and Sexual Mass-transfer lines respectively while **A/U** represents both treatments combined as one asexual treatment. Tests between A and U; U and M; A/U and M; and M and S are post hoc comparisons between only two treatments.

4. Discussion

(a) Response of mean fitness to selection treatments.

We have seen in the previous chapter that the predicted fitness advantage due to sexual reproduction is only seen when comparing the Sexual Mass-transfer lines to the Asexual and Unselected lines grouped together (figure 1a, b, table 1). Yet there are reasons to believe Unselected lines should not be grouped with the Asexual lines (see p. 17-18), in which case the fitness advantage of the Sexual Mass-transfer lines is not present. The results of the pure culture assay in Liquid and Solid lines show that sexual selection did not confer a fitness advantage in a benign environment. In fact, in the Solid growth assay, the Asexual lines did better than the Sexual Mass-transfer.

The results of the competitive fitness assay showed a slight fitness gain of both the Asexual and Sexual Mass-transfer lines (figure 1a, b) over their ancestor. This is not seen in the pure culture Liquid assay. The Asexual and Unselected lines have a lower *K* value than the ancestor (figure 2a, b). In the Solid pure culture assay, the Sexual Mass-transfer and the Sexual Single-zygote lines all have similar mean fitness that is comparable with that of the ancestral population while the Asexual and Unselected have attained a higher mean fitness. The selection lines may therefore have attained a somewhat greater degree of adaptation to the laboratory. However, even if directional selection played a role (as seen by the fitness gain of the Asexual lines over their ancestor in the Solid experiment), it was most likely only important during the beginning of the experiment (as this was a long term experiment over the course of 5 years) such that spores became rapidly adapted to their environment. For the greatest part of the selection experiment, any effect of sex can be attributed to mutation clearance rather than to mutation assembly.

(b) Definition of fitness

There is still an open debate as to what is the most relevant method to estimate fitness. The three assays I performed have been used almost exclusively in the past in *Chlamydomonas* (pure cultures in liquid: Bell 1990, de Visser *et al* 1996; pure cultures on solid: Colegrave *et al* 2002, Kaltz & Bell 2002; competitive fitness in liquid: Goho & Bell 2000).

Nevertheless, there are differences based on the definition of fitness (in competition or in pure culture). During the course of our experiment, individuals were constantly selected in highly competitive environments. Only individuals with the smallest deleterious load and the highest fitness would be selected. Consequently, our fitness values from the competitive fitness assay seems the most appropriate ones as it was performed in the same conditions the selection experiment was carried in. Nonetheless, testing spores in pure culture definitively still has value: individuals growing in a competitive environment still need to adapt to have a rapid division rate.

Competitive fitness assays are more labour intensive and consequently are usually performed in less replicates. Fitness in pure culture might be easier to perform technically but could be less ecologically relevant to the conditions that organisms actually face in natural competitive

environment. Fitness in pure culture (Liquid) seems to also have a lower predictive power as shown by the poor correlation between the two replicate (blocks) experiments. For the Solid pure culture assay however, the results are more easily replicable probably due to the fact that generation time for each selection line was measured from 80 replicate colonies compared to only 5 replicate culture tubes in the Liquid pure culture assay.

(c) Direct and indirect response to selection.

There are explanations for the difference between the fitness assays. First of all, because the environment in the growth assay (culture tubes) is slightly different than the selection environment (bubbled flasks), one can argue that I measured an indirect response to selection that is not necessarily correlated to the direct response. In bubbled flasks, the cells are continuously mixed and supplied with a high amount of dissolved air. In test tubes, however, the air-liquid interface is only about 1 cm^2 for a volume of 20ml. Since the tubes are only vortexed once a day, the CO_2 concentration in the media may be much lower than in bubbled flasks.

Secondly, the correlation between fitness in competition and pure culture is pretty low when comparing r_{max} and fitness in competition (r=0.45, df = 11) and even lower when comparing K and fitness in competition (r=0.23, df = 11). This was not expected, although it is consistent with several unpublished observations in our laboratory. It could imply that there are different set of genes involved in pure culture growth and competitive growth or that a single set of genes has pleiotropic effects and responds differently according to the environmental conditions encountered.

(d) Difference between Solid and Liquid experiment

In the Solid selection experiment, there was no difference between the Asexual and Unselected lines, which received the same treatment (no gametogenesis by Nitrogen starvation was induced in the Solid Unselected lines). This strengthens the hypothesis that the low fitness of the Liquid Unselected lines must be a result of a higher mutation rate or selection for low Nitrogen resistant spores (see p.17-18).

This selection experiment was started with different goals in mind: to look at the effect of mutation clearance but also to look at speciation when individuals are segregated in different environments. Although my study did not explicitly look at speciation, the effect of sex in liquid or solid environment can be predicted. Liquid and solid media, although chemically similar, provide very different growth conditions for *Chlamydomonas*. In a liquid environment, spores are not spatially constrained and can mate with virtually any individuals in the flask. In a solid environment however, individuals are constrained to a position on the plate and therefore most of a spore's neighbour are likely to be asexual clones of itself. Consequently, it is more likely that mating will take place between similar clones in a structured environment (many spores in the Sexual Mass-transfer lines can undergo homothallic mating instead of the usual heterothallic mating, unpublished results).

When two clonal, haploid spores mate, their offspring will be similar to the parents. However, when two spores of a different background mate, mutation clearance can act by selecting out the recombinant genotype carrying the greatest mutational load (figure 4). Inbreeding will then prevent mutation clearance from taking place. The greater the inbreeding coefficient, the less efficient sexual reproduction will be in purging the genome of deleterious mutations.

Even in the solid experiment, probably a substantial part of the mating happens between non-clonal parents (partly because not all spores can undergo homothallic mating). However, one can clearly see that there is an increased likeliness of inbreeding in an environment with physical dispersal constraints. This would explain why the fitness of the Sexual Mass-transfer lines is even lower in the Solid experiment than in the Liquid.

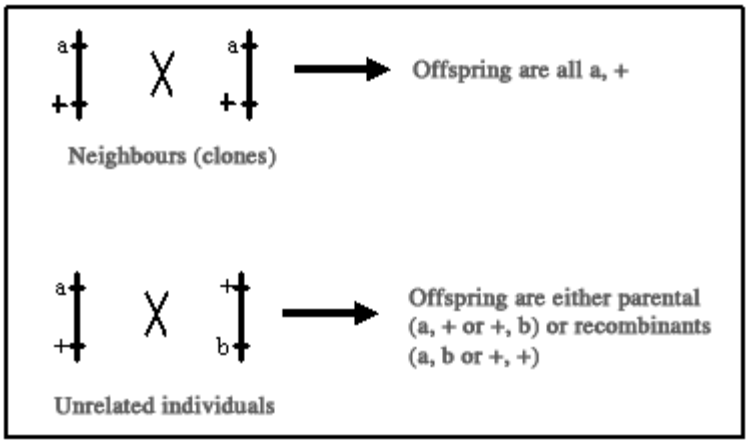

Figure 4 Two different loci carrying either a mutated (a or b) or a wild type allele (+). If the two parents have the same genotype, sex is inefficient to remove deleterious mutations. When the two parents have a different genotype however, sex will be more efficient by purging the population of severely crippled individuals.

(e) Mutation accumulation in the Sexual Single-zygote lines

As we have seen previously, the effect of the mutation accumulation is seen, as predicted, in the competitive fitness assay in the Sexual Single-zygote lines. However, it is absent from pure culture assays in Solid and Liquid (no significant difference between Sexual Mass-transfer and Sexual Single-zygote lines for *K* in Liquid or for fitness on Solid). The fitness decrease seen in the Sexual Single-zygote lines is probably dependent on the assay procedure: the fitness effects of mutation accumulation usually being more prevalent when tested under competitive or stress condition. Kondrashov & Houle (1994) and Shabalina *et al* (1997) found that in *Drosophila*, fitness declines caused by accumulation of deleterious mutations are substantially greater when measured under competitive conditions. Korona (1999) also found that in yeast, the genetic load is greater when organisms are grown in poorer environments. Conditions that cause protein damage or stress can divert certain proteins (e.g. Heat Shock Proteins) from their normal function and enhance the phenotypic effects of mildly deleterious mutations (Rutherford & Lindquist 1998).

(f*) Assumptions of the mutation deterministic hypothesis.*

Overall, my results seem to go against the established theory (Kondrashov, 1988) that sex should be favourable because it clears out populations of mildly deleterious mutations. From the results of the fitness decline in the Sexual Single-zygote lines (at least in competition), it is clear that selection acts to remove deleterious mutations from the genome. What is less clear however is that this clearing process is more efficient in sexual populations. The two main assumptions of the deterministic mutation hypothesis: a rate of mutation greater that one per genome per generation and synergistic epistasis among different deleterious mutations are still under debate in the scientific community (for example, see Kondrashov 2001, Keightley & Eyre-Walker 2001).

Recent evidence from comparison of genomes of related species seems to suggest that the rate of mutation in organisms with a short generation time (*Drosophila, Caenorhabditis*) fall much below the value of 1 (for example see Keightley & Eyre-Walker 2000). Nevertheless, those results are argued by Kondrashov (2001) to be underestimates due to selection at synonymous sites. Similarly, the rate estimated from mutation accumulation experiments in microbes is reported to be << 1 in most studies (Drake 1998, Zeyl & de Visser 2001). Yet again, those experiments are argued to be underestimates since mutations of small effect are missed (Keightley & Eyre-Walker 1999, Lynch *et al*. 1999). Only in longer lived organisms (hominoids) have researchers come to a relative consensus and agreed that mutation rate is most likely above 1 per generation per genome (Nachman & Crowell 2000). *Chlamydomonas*, with a generation time ≈ 8 hours should have a mutation rate closer to the estimates in microbes, *Drosophila* or *Caenorhabditis* than that of hominoids.

Secondly, while gene interaction is definitively important in determining the phenotype of individuals, evidence for synergistic epistasis is scarce and inconclusive. Whether it is the rule rather than the exception is still far from a certainty. The experimental detection of epistasis has been difficult, and only a few studies have directly addressed this question. For example, Mukai (1969), Temin *et al.* (1969) and Whitlock & Bourget (2000) have all suggested and shown with

some degree of significance that in *D. melanogaster* mutations interact synergistically. In *D. magna*, Salathé & Ebert (2003) detected synergistic epistasis, as fitness decreased in a nonlinear way across sets of increasingly inbred clones. Plants have also been examined for fitness components at different levels of inbreeding (Willis, 1993; Dudash *et al.*, 1997; De Visser & Hoekstra, 1998; Koelewijn, 1998; Ouborg *et al.*, 2000). Most of these studies only found limited evidence of epistasis in some fitness components. In *E. coli*, Elena & Lenski (1997) found significant interactions among loci although they are antagonistic as often as synergistic. De Visser *et al.* (1996) also showed some evidence for positive epistasis between ultraviolet-induced and naturally accumulated deleterious mutations in *C. moewusii.*

It then seems that the deterministic mutation hypothesis sits on unstable ground given that its two main assumptions are being criticised in the scientific community as speculative and unsupported. My results would suggest that possibly one or both of those assumptions are erroneous.

Chapter 3: Sexual and Natural Selection

1. Introduction

I have shown in the last chapter that there is no clear evidence for the fitness effect of mutation clearance. One possibility is that the assumptions of the mutation deterministic hypothesis are erroneous. However, there could also be another explanation for my findings. With the results of the different fitness assays in mind, I did a preliminary experiment to see if there was a trade off between vegetative and sexual fitness (zygote production).

Fitness assays in *Chlamydomonas* conventionally rely on the rate of division or generation time of the asexual spore (Bell 1990, de Visser *et al* 1996, Colegrave *et al* 2002, Kaltz & Bell 2002, Goho & Bell, 2000). However, during the selection experiment, a treatment favouring both zygote production and asexual spore division was imposed on the sexual lines. Populations that adapt to be better at mating might conversely be worse at dividing asexually.

One of the most important hypothesis in evolutionary ecology is that trade-offs exist among components of fitness (Futuyma & Moreno 1989). These can be broadly divided into two categories: trade-offs among life-history characters that are expressed at different ages (for example in *Drosophila:* a trade-off between early fecundity and decreased longevity, see Chippindale *et al* 1994; Leroi *et al* 1994) and trade-offs among fitness components that are expressed in different environments (for example, in *E. coli*: a trade-off between rate of adaptation to a novel environment and loss of catabolic functions, see Cooper & Lenski 2000). One of the leading theories that explain the genetic basis for the cost of adaptation is the antagonistic pleiotropy of trade-offs which states that different components of fitness are negatively correlated (see Rose & Charlesworth 1980, Futuyma & Moreno 1988; Sgrò & Partridge 1999). If a single set of loci control the expression of two fitness components, the trade-offs among fitness components should be symmetrical such that selecting on the first component should cause a regress of the second component and vice versa (Cooper & Lenski

2000). A negative genetic correlation between components 1 and 2 would result of such a selection scheme.

The theory of antagonistic pleiotropy would predict that populations of *Chlamydomonas* that have adapted to be better at mating will conversely be worse at dividing asexually (if zygote production and asexual spore production are seen as two components of fitness). In their study, Da Silva and Bell (1992) have looked specifically at this issue in cultures of *Chlamydomonas*. They found a negative genetic correlation between the two components of fitness (vegetative increase and mating success). In another field study, Rispe (1996) measured the intrinsic rate of increase of aphids (*Rhopalosiphum padi*) that vary in their ability to produce sexual individuals. Yet, he showed that life cycle (whether the female would give rise to offspring through sexual or asexual reproduction) had no significant effect on the age at first reproduction, fecundity or longevity of the asexual generation. If, as predicted, there is a negative correlation between the two traits in our experiment, it might be one explanation why the fitness benefit of sexual reproduction was not as prevalent as expected.

2. Materials and methods

(a) Isolating spores and identifying mating type.

20 spores were isolated from one of the Sexual Mass-transfer line (20 in Solid and 20 in Liquid). Those 20 spores had a high degree of homothallism {many spores in the selection lines undergo homothallic mating instead of the usual heterothallic mating (mt^+/mt^-), see p. 20}. I chose lines with the greatest amount of homothallic mating and therefore did not need to add another tester strain for mating to take place.

Homothallism was tested by individually growing isolated spores in a 24 multi-well plate and looking at the formation of a mat at the surface of the well. A thick mat at the surface will indicate the presence of zygotes (Harris 1989). I used the spores isolated by an undergraduate student (G. Perron). From 20 spores isolated in Liquid Sexual Mass-transfer 3 line, 17 were identified as homothallic when tested for mating in liquid. From 20 spores isolated in the solid Sexual Mass-transfer 4 line, all were identified as homothallic when tested for mating in liquid.

(b) Testing vegetative and sexual fitness

Isolated spores were tested for sexual and vegetative fitness. They were tested for vegetative fitness in liquid (Liquid Sexual Mass-transfer spores) and in solid (solid Sexual Mass-transfer spores). The procedure was similar to the pure culture assay in Liquid and in Solid done previously (see page 20-21).

Following this, zygote production for each of the 20 spores was measured. In liquid, I inoculated 20 wells (24 multi-well plate) and grew it for 4 days after which a diluted sample was plated, placed in the dark for 4 days (to allow thickening of cell wall), chloroformed and then grown in the light until the colonies were large enough to be scored. To correct for cell density, another plate was put directly in the light to determine the cell concentration in the wells. Zygote production measurements were then given as: (number of zygotes / ml) / (number of vegetative cells / ml).

On solid, the spores were grown on plates for 10 days until tested for mating on solid. I then sampled a loopfull of cells and re-suspended them in Bold's. One plate was inoculated with this suspension, placed in the dark for 4 days, chloroformed, placed back in the light and grown until colonies could be scored. To correct for cell density, another plate was put directly in the light to determine the cell concentration in the suspension. Zygote production measurements were given as: (number of zygotes / ml of re-suspended solution) / (number of vegetative cells / ml of re-suspended solution). The whole assay was performed twice consecutively.

3. Results

Many of the isolated spores did not show homothallism. In Liquid, three and on Solid, 12 spores did not produce any zygotes from homothallic mating (this was consistent in both replicate experiments in Solid and Liquid). Those spores (hereafter named heterothallic spores) are also the ones that have obtained the highest vegetative fitness value (see figure 4a, b). There is no clear evidence of a general negative correlation between intensity of homothallic mating

and vegetative fitness. Rather there is a negative effect of homothallic behavior on vegetative fitness. Spores that showed homothallic mating had a low vegetative fitness and spores that did not show a homothallic behaviour had a high vegetative fitness both in Liquid ($F_{1,18} = 85.1$, $P<0.001$) and Solid ($F_{1,18} = 14.0$, $P<0.005$) environments (see table 2). Probability values of each separate ANOVA were used to confirm that the difference in fitness was significant for both Solid and Liquid experiment combined ($\chi^2_{(5)} = 25.4$, $P<0.001$, Sokal 1981).

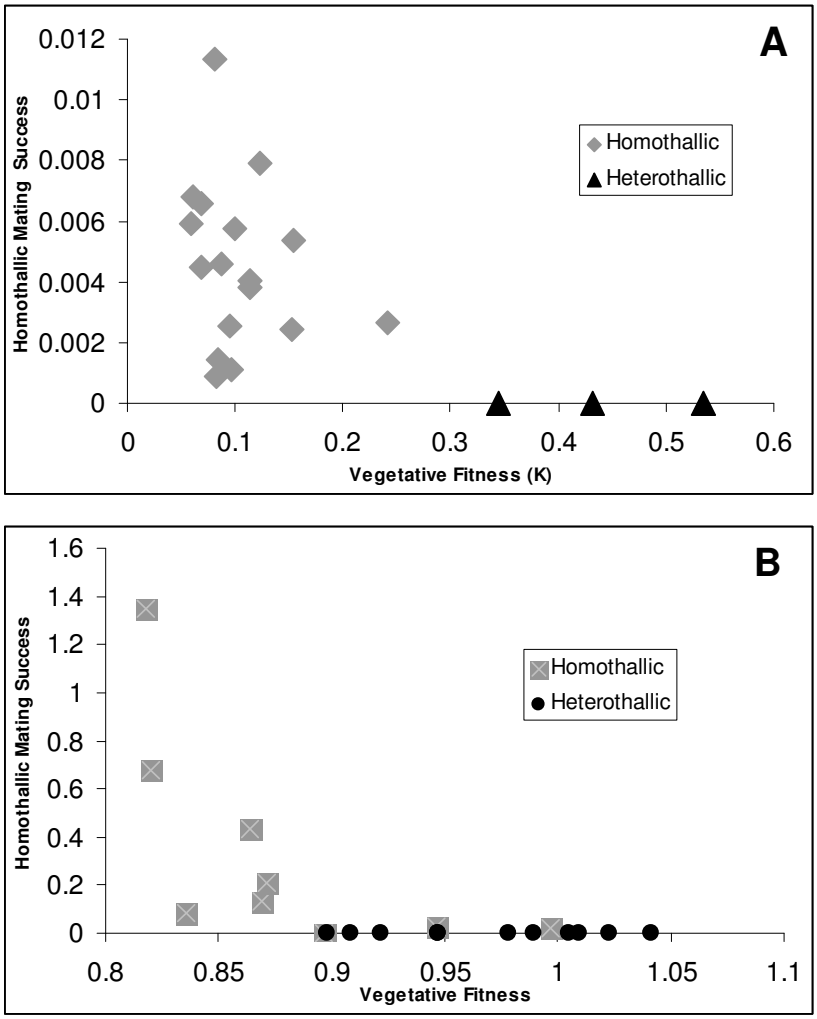

Figure 4 a, b) Homothallic mating (number of zygotes / total number of spores) and vegetative growth for 20 isolated spores. 4a) shows the data for the liquid experiment (Carrying capacity was used as vegetative fitness values) and 4b) for the solid experiment.

ANOVA of relative fitness						
	SOLID			**LIQUID**		
Source	d.f.	Mean Square	F	d.f.	Mean Square	F
Mating	1	0.0402	**14.0***	1	0.038509	**13.0***
Error	18	0.0029		18	0.002956	

Table 2: ANOVA of relative fitness of spores both in Solid and Liquid environments. Spores were classified as either homothallic or heterothallic. Values of F associated with probabilities of less than 0.005 are marked with one asterisk. In Liquid, carrying capacity was used to calculate fitness (but the relationship is similar using r_{max} values)

4. Discussion

(a) Trade-off hypothesis

Spores in the Sexual Mass-transfer lines that have adapted to be homothallic maters have consequently paid an adaptive cost for this behavior in terms of vegetative fitness. This is not exactly what was expected (I expected a continuous negative correlation between vegetative fitness and zygote production), yet it still shows a trade-off between different components of fitness. Unfortunately, the genetic basis behind the evolution of homothallism in the Sexual Mass-transfer selection lines is still unknown and will not be discussed in this paper.

Heterothallic spores should benefit to a greater extent of the predicted effect of sex as compared to homothallic spores that might be forced to mate with clones of themselves (see p. 28). Consequently, heterothallic spores would have a greater vegetative fitness than the

homothallic ones and the heterothallic phenotype would be expected to be fixed in the population. On the other hand, homothallic mating could have evolved because of the high selective pressure for mating (only mated individuals can survive in the Sexual Mass-transfer and Sexual Single-zygote lines). Homothallic spores then have twice as many potential mates than the heterothallic spores. Homothallism can be an adaptive behavior for the particular selection regime individuals were faced with during the experiment.

Individuals are therefore faced with two different strategies they can adopt in order to maintain a high overall fitness. One strategy consists of producing lots of zygotes (through homothallic mating); proportionally get transferred in great numbers and following the transfer divide asexually at a slower rate. The second strategy consists of growing in great number as an asexual spore, produce fewer zygotes (through solely heterothallic mating) and proportionally get transferred in smaller number. Individuals can pay the adaptive cost of the homothallic behavior or maintain their efficiency at dividing asexually.

In the Sexual Mass-transfer lines the optimum genotype in the selection environment would be both high vegetative fitness and high zygote production. However, because of the genetic trade-off between components of fitness, this prevents the spores from attaining the highest vegetative fitness possible. This would explain why I did not see a fitness increase in the Sexual Mass-transfer lines compared to the Asexual lines. The vegetative fitness of the Asexual lines is not brought down by the trade-off as none of the spores in the Asexual or Unselected lines showed a homothallic behavior (unpublished results).

(b) Objections against the trade-off hypothesis

The connection between homothallic mating and low vegetative fitness suggests a possible explanation for the failure to see the fitness effect of mutation clearance in the Sexual Mass-transfer lines; however it might not be sufficient enough to prove it.

Because of practical reasons, I only looked at frequency of homothallic mating. To confirm the trade-off hypothesis, one should probably also look at the frequency of heterothallic mating compared to vegetative fitness. Many (if not most) events of fertilization in the populations will

happen between spores of different mating type (+ or -). Homothallic mating is just a special case that is most likely the exception than the rule. The logical assumption would be that when tested for heterothallic mating, homothallic spores would also show a high efficiency of heterothallic mating and a low vegetative fitness. This would strengthen the hypothesis that there is a trade off between sexual and vegetative fitness. However this has not been proven experimentally yet.

Another argument against the trade-off hypothesis could be that spores that are efficient maters will have a lower vegetative fitness purely because there are physically constrained to spend more time going through a sexual cycle at the expense of asexual growth. This is probably not the case. In the Liquid assay, a population at or near carrying capacity (after 4 days of growth in 24 well plates) will only contain between 0-1% of zygotes (see figure 5, mating success = number of zygotes/ number of spores). Therefore, throughout the growth assay in test tubes, more than 99% of the population was always reproducing vegetatively. In the vegetative growth assay on solid, it is even more conclusive as no zygotes were present during the assay (visual inspection was confirmed by chloroforming plates).

(c) Result of the trade-off in relation to mutation clearance and fitness of the selection lines.

If the conclusions of this trade-off between homothallic behaviour and vegetative fitness are extrapolated, one would predict that this should hold true across all selection lines. Thus, there should be a negative correlation between the sexual and the asexual lines; whereas the Sexual Mass-transfer lines have low vegetative fitness and a homothallic behaviour while the Asexual lines would have a high vegetative fitness and a heterothallic behaviour. This would be the opposite of what was originally predicted when setting up this experiment. It was expected that the Sexual Mass-transfer lines would have a higher vegetative fitness (because of mutation clearance) than the Asexual lines. This is not what was found experimentally. Overall, the three fitness assays showed no significant difference in fitness between the Sexual Mass-transfer lines and the Asexual ones.

The explanation of the results might lie in combining the effect of two opposite forces acting on fitness. On one side, mutation clearance is pulling vegetative fitness up in the sexual lines and down in the asexual lines. The trade-off due to the homothallic behaviour, on the other hand, is pulling the sexual lines down. Thus, for vegetative fitness, the effects of those two forces could be cancelling each other in the Sexual Mass-transfer lines and be the reason why they have a similar fitness to that of the Asexual lines.

Final conclusion

Many theories have been proposed to explain the wide occurrence of sexual reproduction in nature. Most scientists agree that sex can be advantageous through the production of genetic variation on which natural selection can act. This might happen because sex, through *mutation clearance*, brings together currently deleterious mutations to create severely crippled genomes that are then eliminated from the population (Kondrashov 1982). Consequently, in a benign medium, where individuals are well adapted, sexual and asexual populations should diverge in mean fitness after many generations. As there has not been any clear demonstration of this theory, I set out to verify it with experimental evidence.

The results of the competitive fitness assay in the Liquid experiment suggested a possible long-term benefit of sex. The fitness effect of the mutation accumulation in the Sexual Single-zygote lines demonstrated that deleterious mutations must be removed by selection for organisms to remain well adapted. There was an overall advantage of sex, but this masked a large difference in the asexual lines and disappeared when the Unselected lines were removed from the analysis. It is possible that selection for growth in low nitrogen conditions or an elevated mutation rate induced by nitrogen stress were responsible for this anomaly.

In the second chapter, I used two different methods to estimate fitness in *Chlamydomonas*, growth in pure culture and in mixture. There is still no definitive answer as to which method is the more relevant. The results of the fitness assays failed to support the established theory that sex should be favourable because it facilitates the clearance of mildly deleterious mutations from populations. Furthermore, in the pure culture assay, the fitness decrease in the Sexual Single-zygote lines is not present, indicating that it is dependent on whether growth is measured in pure culture or in mixture.

There is a need for a better understanding of the key assumptions behind the deterministic mutation hypothesis. Recent evidence suggests that the rate of mutation in organisms with a short generation time (*Drosophila, Caenorhabditis*) is much less than 1. Secondly, although gene interaction may be important, evidence for synergistic epistasis is scarce and inconclusive.

Whether the mutation rate falls above or below the value of one per genome per generation and what kind of role epistasis plays have tremendous impact on our understanding of the evolution of sex and evolutionary biology in general. My results would imply that mutation rates are lower or epistasis less pronounced than the mutation clearance hypothesis requires (as others have suggested, see Keightley & Eyre-Walker 2000).

In the last chapter of the thesis, I have suggested that zygote production and asexual spore production represent two components of overall fitness; implying that other forces acting on the selection lines could have masked the effect of mutation clearance. According to the theory of antagonistic pleiotropy, populations of *Chlamydomonas* that have adapted to be better at mating will suffer a cost and thus be worse at dividing asexually. I proposed that the evolution of homothallism in the Sexual Mass–transfer lines, which increases the rate of mating, is correlated to costs in terms of vegetative fitness. The explanation of the results might therefore lie in the combination of two opposed effects. Mutation clearance would increase vegetative fitness up in the Sexual Mass-transfer lines relative to the Asexual lines; and the trade-off, attributable to homothallism, would reduce it in the Sexual Mass-transfer lines. Thus, if two forces were cancelling each other in the Sexual Mass-transfer lines, this would explain why a fitness benefit of sexual reproduction in a benign environment was not expressed as expected.

To follow up this work, it would be illuminating to bring sexual and asexual types into direct competition and observe how the frequency of sexual types changed through time. This situation would resemble more closely how sexual reproduction is maintained in nature. There are severe technical difficulties in designing such an experiment, however.

Understanding the evolution of sex requires the synthesis of every important process in evolutionary biology (selection, epistasis, mutation, migration, recombination and drift) and has motivated many theoretical studies. They have shown that the answer to the "paradox of sex" is more elusive than was originally thought. Solving this paradox might require that we integrate more of the complexities of the real world into the experimental designs; performing a selection experiment in a natural environment with coevolving species (predators, competitors, parasites) probably being the next step.

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