Deleterious mutations and the evolution of sex

Nadelige mutaties en de evolutie van sex

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Functionele Ecologie

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STELLINGEN

1. Bij het experimenteel toetsen van hypothesen over de evolutie van sex verdient het toetsen van aannamen de voorkeur boven het toetsen van voorspellingen.

dit proefschrift

2. Dat sexuele soorten kenmerkend zijn voor verzadigde milieu's kan niet uitsluitend verklaard worden door de Parasiet-hypothese.

dit proefschrift

3. De frequentie waarmee nadelige mutaties optreden is een van de belangrijkste onbekenden in de discussie over de evolutie van sex.

dit proefschrift

4. De evolutie van sex houdt menigeen nachtenlang wakker.

5. Het bestaan van de uitdrukking 'als de wijn is in de man, is de waarheid in de kan' naast de oude Latijnse uitdrukking 'in vino veritas' (Plinius), doet vermoeden dat het effect van wijn op de menselijke geest in de loop der jaren is veranderd.

6. De studie naar buitenaards leven is wenselijk voor het toetsen van de algemeenheid van hypothesen over de evolutie van het leven op aarde.

7. Etwas weniger wäre mehr gewesen.

Goethe

8. Omdat de dood thans als iets zeer slechts wordt beschouwd, zal het doodsritueel steeds schraler worden, tot de ideale regeling een ophalen door de gemeentelijke reinigingsdienst zal zijn.

Gerard Reve

9. Waar *Chlamydomonas* licht werpt op de oorzaak van sex, is *Chlamydia* het gevolg.

10. Intuïtie is ook in de wetenschap onmisbaar.

11. Uit de recente publieke ophef over frauduleuze wetenschappers blijkt het maatschappelijke belang dat aan de wetenschap wordt toegedicht.

Stellingen behorend bij het proefschrift 'Deleterious mutations and the evolution of sex' van Arjan de Visser

Wageningen, 8 november 1996

Voor mijn vader

Contents

1

General Introduction

The origin of sexual reproduction is considered to be one of the major transitions in evolution. Other examples of major transitions are the origin of life itself, the appearance of multicellular organisms and the origin of language (Maynard Smith and Szathmáry 1995). The importance attributed to the origin of sex is due to its large consequences for the evolution of life on earth. For instance, sex was the prerequisite for the evolution of phenomena such diverse as gender, the sex ratio, sexual selection, and societies with different roles for males and females. However, despite more than a century of debate and the existence of some twenty theories (Kondrashov 1993), no agreement has been reached so far about its evolutionary causes. This has led some authors to use a rather dramatic terminology: the evolution of sex has been called "the masterpiece of nature", but also "the queen of evolutionary problems" (Bell 1982), as well as "the deepest mystery in all of biology" (Trivers 1985), and the persistence over millions of years of lineages of organisms that never have sex has been considered "an evolutionary scandal" (Maynard Smith 1986).

The problem

Sex and reproduction are two. While all living organisms reproduce, only a fraction does so by means of sex. Many especially lower organisms reproduce by binary fission or parthenogenesis (i.e. development of a new individual from an unfertilised egg). Furthermore, most plants have the ability to reproduce vegetatively (i.e. development of a new individual from a group of somatic cells). In a way, sexual reproduction can even be considered a *contradictio in terminis*: while sex starts with the fusion of two cells into one zygote, reproduction causes one individual to become two or more (Maynard Smith 1978). The functional separation of sex and reproduction has stimulated the evolutionary question "why sex?".

What do I mean with sex in this context? The terms sex and sexual reproduction will be restricted to the typical eukaryotic type of sex observed nowadays, with syngamy (i.e. the fusion of two haploid nuclei into a diploid zygote) and meiosis (i.e. reduction division), usually with recombination (by independent assortment of chromosomes and physical crossing-over), alternating in the life cycle. Furthermore, sex will be used only in the context of outcrossing (i.e. between different individuals), since only then it may lead to genetic recombination, which is generally believed to be the main function of sex. The mechanisms prokaryotes use to exchange DNA between cells are so different that dealing with its evolution requires a different treatment (Maynard Smith 1989).

An evolutionary explanation for the present predominance of sex should tackle both its origin and its persistence on an evolutionary time scale. Finding an evolutionary explanation is equal to finding selective advantages. Modern eukaryotic sex did probably not arise by a single mutation. An evolutionary account of its origin should, therefore, provide selective advantages for all presumed ancestral stages of sex, while an explanation for its maintenance involves selective advantages for the type of sex observed nowadays.

A fundamental problem with explaining the origin as well as the maintenance of sexual reproduction arises from the realisation of a number of short-term disadvantages related to sex. The most prominent of these disadvantages has been called 'the cost of sex' (Maynard Smith 1971) or 'the cost of meiosis' (Williams 1975). Its argument goes as follows. Imagine a sexually reproducing population with a 1:1 sex ratio, where males do not contribute to the offspring, except gametes. If in such a population a mutation occurs that causes a female to reproduce parthenogenetically, this mutant allele would double its frequency every generation. The reason for this is that an asexual female transmits every allele to all her offspring, while a sexual female transmits every allele on average to only half of her offspring. A sexually reproducing population is thus vulnerable to invasion by asexual mutants. Although this cost of sex does not apply to recombination (Charlesworth 1989), recombination involves another cost by breaking up favourable gene combinations (Maynard Smith 1978; Charlesworth 1989). More short-term disadvantages of sex exist, which are reviewed by Crow (1994). In sum, a theory for the maintenance of sex should provide a short-term advantage that is at least

able to compensate its two-fold cost. This has been called 'the balance argument' in the debate on the evolution of sex (Williams 1975).

The origin of sex

Maynard Smith and Szathmáry (1995) distinguish three stages in the evolution of eukaryotic sex. The first stage is the evolution of a haploid-diploid life cycle without syngamy in a haploid organism: a haploid cell becomes diploid by endomitosis and haploidy is restored by a single-step meiosis (i.e. without premeiotic duplication of the chromosomes). In the next stage, endomitosis is replaced by syngamy, but meiosis is still a single step. This is also the stage where crossing-over is thought to have arisen, because only now crossing-over results in genetic recombination. The last stage is similar to the modern sexual life cycle, with syngamy and a two-step meiosis, involving duplication of the chromosomes immediately before meiosis.

The authors propose selective advantages for each of the three stages, but emphasise that these are tentative. The selective advantage of a haploid-diploid cycle over permanent haploidy, they suggest, comprises the combined advantages of the ability to repair double-strand DNA damage in the diploid phase due to the presence of a good template, and limitation of the mutation load in the haploid phase due to selection. However, the return to haploidy seems somewhat more problematic to explain. The reason for syngamy to have replaced endomitosis would have been the shielding of (partially recessive) deleterious mutations. The selective advantage of a two-step meiosis relative to a meiosis without preceding chromosome doubling, might have been the ability to stop the spread of a selfish genetic element called a sister-killer allele, which kills its sister gamete if it will find itself in a heterozygote prior to division (Haigh and Grafen 1991). In a two-step meiosis, the possibilities for killing depend on whether crossing-over between centromere and killer allele has made the first (if crossing-over occurs) or the second (if no crossing-over occurs) meiotic division reductional. Thus, the uncertainty for a killer allele when to be active will limit its spread in a two-step meiosis, and it will increase the fitness of the individual.

This leaves unexplained the origin of crossing-over. However, since crossing-over only increases recombination since syngamy and meiosis (resulting in random assortment and segregation of chromosomes) were already present, most short-term selective advantages to modern eukaryotic sex apply as well to the origin of crossing-over. A selection of the main ideas on the maintenance of sex is given below.

The maintenance of sex

Sex has become the predominant mode of reproduction, either because asexual populations have a lower long-term fitness (i.e. a higher probability to become extinct) or since one or more short-term advantages balance its costs (Williams 1975; Maynard Smith 1978; Charlesworth 1989). Especially the notion that many lower organisms have the ability to reproduce both sexually and asexually has largely stimulated the invention of short-term advantages. At present, some twenty hypotheses exist that claim a short-term advantage of sex that may overcome its two-fold cost (Kondrashov 1993). These ideas have been reviewed in many publications in recent years (e.g. Williams 1975; Maynard Smith 1978; Stearns 1987; Michod and Levin 1988; Charlesworth 1989; Hamilton et al. 1990; Kondrashov 1993; Crow 1994; Hurst and Peck 1996). For this reason, and because all subsequent chapters of the thesis will concentrate on a single theory, I will only very briefly mention the main ideas.

The hypotheses can be classified as providing immediate benefits, or benefits resulting from the generation of genetic variation which subsequently facilitates natural selection (Felsenstein 1974; Kondrashov 1993). An example of an immediate sex benefit is the ability to repair double-strand DNA damage during meiosis (e.g. Bernstein et al. 1988). However, there is some agreement that immediate benefits like the repair function may have been important for the origin of sex, but not for its maintenance (Maynard Smith 1988; Crow 1988).

The variation-and-selection hypotheses emphasise the inability of sex to enhance the fitness of the offspring directly. Instead, they attribute the advantage of sex to the breaking up of gene combinations in the parents and the production of new ones in the offspring, thereby possibly increasing the genetic variation for fitness. If so, natural selection can

more efficiently select good genotypes, which leads to faster adaptation and a higher mean fitness among sexual offspring. However, sex can only increase the genetic variation for fitness if there is negative linkage disequilibrium between the alleles at the different loci affecting fitness, i.e. favourable alleles at certain loci tend to be associated with alleles with a negative effect at other loci (Felsenstein 1985). There are two possible reasons why these individuals may predominate in a population: (1) chance, i.e. genetic drift in a small population, or (2) epistatic selection (Bulmer 1980).

In Table 1 the main variation and selection hypotheses are classified by two criteria (Maynard Smith 1988; Kondrashov 1988): (1) the origin of negative linkage disequilibrium, genetic drift or epistatic selection, and (2) the source of selection pressure, environmental or mutational. The environment may be the major source of natural selection if the optimum genotype varies due to environmental fluctuation, either biotic or physical. Alternatively, recurring deleterious mutations may cause directional selection for genotypes with a low number of mutations, even if the environment does not change.

Table 1. A classification of the main variation and selection hypotheses

	Source of selection pressure	
Origin of linkage disequilibrium	**Environmental**	**Mutational**
Drift	Faster evolution (Fisher 1930; Muller 1932)	Muller's ratchet (Muller 1964)
Selection	Shifting optimum (Maynard Smith 1980) Coevolving parasites (e.g. Hamilton 1980)	Deterministic Mutation Hypothesis (Crow 1970; Feldman et al. 1980; Kondrashov 1982)

The distinction between environmental and mutational hypotheses coincides with the distinction by Maynard Smith (1988) between the function of sex in combining good genes versus eliminating bad genes, respectively. Basically, the hypotheses invoking epistatic selection as cause for the negative linkage disequilibrium are more general than those based on genetic drift, because the latter hypotheses depend on small population

sizes. Similarly, the mutational hypotheses are basically more general, since they are independent of environmental conditions: they are equally valid in a constant environment.

There is some concensus that of all hypotheses two are most plausible in providing a short-term advantage: the Parasite Hypothesis and the Deterministic Mutation Hypothesis (Hamilton et al. 1990; Crow 1994; Maynard Smith and Szathmary 1995; Hurst and Peck 1996). The former hypothesis comprises rather a group of related ideas, all invoking coevolving parasites as the major selection pressure for the evolution of sex, (e.g. Hamilton et al. 1990). The central idea is built on the fact that most parasites have shorter generation times than their hosts. As a consequence, parasites have a greater evolutionary potential to adapt to their hosts than their hosts can coevolve resistance. Sex has a higher probability than asexual reproduction to produce rare genotypes that may escape parasites, which are mostly adapted to the most common host. Thus, a major assumption of the Parasite Hypothesis is that resistance loci are the major focus of selection. Since the optimum genotype of a host may change frequently, old resistance alleles may become advantageous again, and sex may be a means to conserve resistance alleles in the population (Hamilton et al. 1990). Of all sex theories, the Parasite Hypothesis seems to have stimulated most of the experiments that have been performed on the subject. However, much of the supportive evidence is only moderately compelling, because it is based on comparative analyses and not on testing unambiguous predictions (Hamilton et al. 1990; Kondrashov 1993; Hurst and Peck 1996).

The Deterministic Mutation Hypothesis (DMH)

This thesis is dedicated to experimentally testing the DMH. The DMH emphasises the role of sex in reducing the mutation load, which is a property of a population and has been defined as the proportion of individuals that do not reproduce as a result of selection against deleterious mutations (Crow 1970). When deleterious mutations occur with a sufficiently high frequency at many loci each generation, there is a perpetual pressure of selection to reduce their number. Therefore, in the long term a balance is to be expected between recurring deleterious

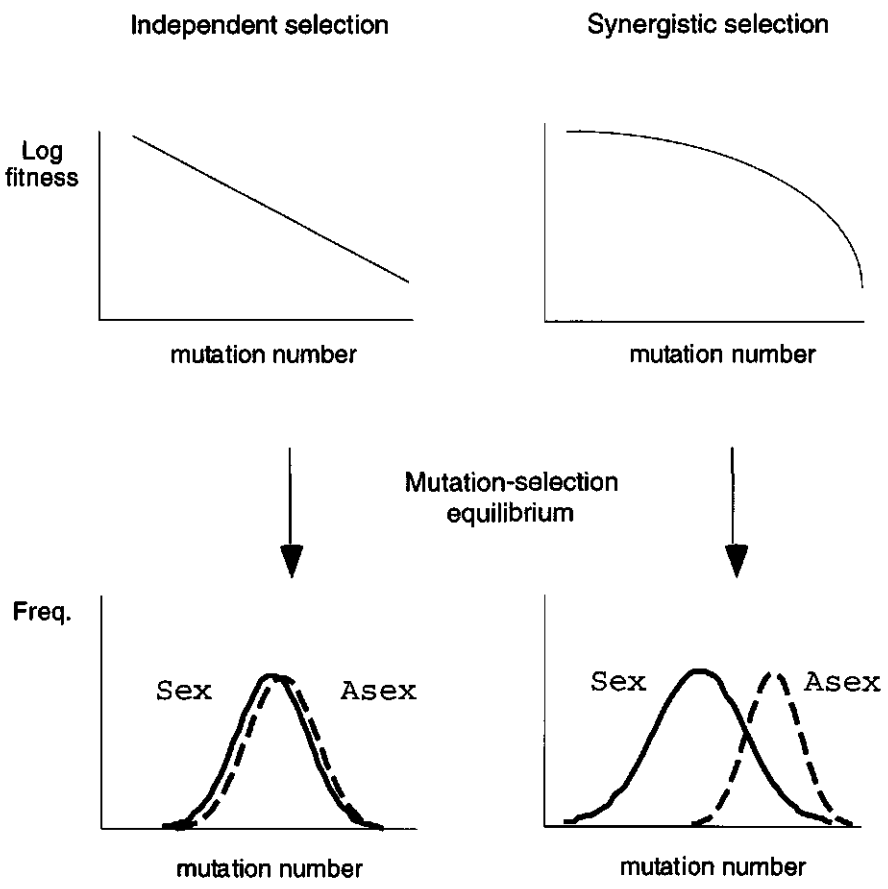

Figure 1. The mechanism of the Deterministic Mutation Hypothesis of the evolution of sex. Depending on the nature of selection against deleterious mutations, sex may facilitate the elimination of mutations from a population in mutation-selection balance. If there is *independent* selection against mutations (left), sex cannot increase the variance in individual mutation number, and the mutation loads of a sexual and asexual population are equal. If selection is *synergistic* (right), then the variance in mutation number decreases under mutation-selection equilibrium and sex facilitates mutation elimination by increasing the variance again. In that case, the resulting mutation load in a sexual population (continuous curve) is lower than in an asexual population (dashed curve).

mutations and selection: the number of newly occurring mutations equals the number selected from the population by death or infertility during the same time interval.

The role of sex in the DMH is to enhance selection against deleterious mutations in a population in mutation-selection equilibrium. Sex can do so by generating variation in the individual mutation number.

Every generation in a sexual population recombination warrants the production of extreme genotypes with either many or few mutations. However, under mutation-selection equilibrium the production of extreme genotypes leads only to increased genetic variation if selection is synergistic, i.e. if fitness decreases faster than log-linearly with the number of deleterious mutations of an individual (Kimura and Maruyama 1966; Crow 1970). Under synergistic selection this sex-related advantage may be as high as to compensate the two-fold cost (Kondrashov 1982; Crow 1983). If mutations are eliminated independently, the mode of reproduction (sexual or asexual) does not influence the efficiency of mutation elimination, because then sex cannot increase the genetic variation. Antagonistic (i.e. diminishing returns) selection against mutations turns the advantage of sex even into a disadvantage, because then sex decreases the genetic variation (Charlesworth 1990). The mechanism of the DMH is shown in Figure 1.

The attraction of the DMH lies in its potentially general validity and in its simple assumptions. These are: (1) the rate of deleterious mutations should be sufficiently high. Usually a value of one per genome per generation is cited (Kondrashov 1988; Charlesworth 1990), although relaxation of the model parameters can lead to a higher necessary mutation rate (Howard 1994; Hurst and Peck 1996). (2) Selection against deleterious mutations should be synergistic. Very small synergistic effects may already provide a large advantage to sex (Charlesworth 1990). Truncation selection, i.e. fitness is unaffected up to a certain mutation number beyond which fitness drops sharply, is the extreme form of synergistic selection. The sex advantage provided by the DMH would be maximal under such selection. Selection may not be able to truncate accurately, but very crude approximations to truncation selection have almost the same mutation-eliminating properties as strict truncation (Crow and Kimura 1979; Crow 1994). However, genetic trade-offs between different fitness components may make simultaneous truncation selection against deleterious mutations problematic (Crow and Kimura 1979).

There is an additional reason for finding synergism between deleterious mutations. Experimental evidence of synergism would not only support the DMH, but it would simultaneously devaluate Muller's ratchet (Muller 1964) as a competing explanation for the maintenance of sex. Muller's ratchet reduces the fitness of a finite asexual population by

the random loss of individuals with the minimum number of deleterious alleles, possibly accelerated by a 'mutational melt down' due to the correlated decline of population size with mutation accumulation (Lynch and Gabriel 1990). This is true if mutations have independent effects. However, if deleterious mutations interact synergistically, the probability of loss of the least mutated class decreases and Muller's ratchet slows down or may even be stopped (Charlesworth et al. 1993; Kondrashov 1994; but see Butcher 1995).

Present support for the DMH

In contrast to the abundance of theoretical solutions to the problem of the evolution of sex, facts to test them are severely lacking. As a result, calls for experimental data have been made on many occasions (e.g. Felsenstein 1988; Charlesworth 1989; Crow 1993; Kondrashov 1993; Kondrashov 1994; Hurst and Peck 1996). The lack of knowledge of genetic processes in natural populations is, however, not a problem that is unique to the evolution of sex, but rather symptomatic for all evolutionary biology (e.g. Felsenstein 1988; Dykhuizen 1990; Lenski 1992; Kondrashov 1993).

Possibly due to its recent origin, the lack of data on the two crucial parameters of the DMH is evident. With respect to synergistic epistasis, the first, and probably best, supportive evidence comes from Mukai's mutation-accumulation study in *Drosophila* (Mukai 1969). It was found that synergism between deleterious alleles affecting viability is rather weak. Willis (1993) has found some evidence for synergism for pollen viability in the monkey flower *Mimulus guttatus*, but not for three other fitness components. Willis (1993) also gives a review of some more inferential evidence for synergism from other studies. Clearly, the present evidence for synergism between deleterious mutations is inconclusive and warrants further study (Charlesworth 1989; Kondrashov 1993; Hurst and Peck 1996).

With respect to the availability of data on the deleterious mutation rate, the situation is not much better (Crow 1993; Hurst and Peck 1996). The best available estimate is again for *Drosophila*, where the mutation rate per haploid genome was estimated to be at least 0.42 for viability (Mukai et al. 1972). Houle et al. (1992) repeated the mutation-accumulation

experiments of Mukai and found a rather similar estimate for the deleterious mutation rate for fitness in *Drosophila*, but they later retracted their conclusions since the control lines they used appeared to have been contaminated with a transposable element. A few more indirect estimates come from plants. Estimates derived from inbreeding depression in a number of highly self-fertilising plants, show that the deleterious mutation rate should be at least 0.5 (Charlesworth et al. 1990; Charlesworth et al. 1994), and may as well be higher than 1 in two species of *Amsinckia* (Johnston and Schoen 1995). A recent estimate of the deleterious mutation rate to overall fitness in the prokaryote *Escherichia coli* of at least 0.0002 per cell division is consistent with the value for *Drosophila*, if relative genome size and number of cell divisions are considered (Kibota and Lynch 1996). In conclusion, estimates of the deleterious mutation rate in eukaryotes seem to be close to the necessary minimum of 1 per genome per generation in eukaryotes, but further data are necessary, especially on organisms other than *Drosophila* and plants.

Aim and outline of the thesis

It is likely that there is more than one reason for the maintenance of sex. Many models seem theoretically sound, but are not mutually exclusive. For instance, the sex-related advantages of eliminating deleterious mutations and escaping parasites have been shown to be cumulative (Howard and Lively 1994). However, progress on this topic is much more helped by direct support to individual hypotheses, since only then the relative value of the various mechanisms can be appreciated (see also Kondrashov 1993).

The aim of the work presented in this thesis is to test the DMH. Basically, a hypothesis can be tested in two ways: by testing its premises or by testing its predictions. The ultimate prediction of the DMH is a higher mean fitness of a sexual relative to an asexual population that are both in mutation-selection equilibrium. However, mutation-selection equilibrium is rather a theoretical population genetic concept than something that can be easily mimicked in experiments. Furthermore, testing a theory by testing its predictions is sensitive to ambiguous interpretation, due to lack of experimental control. An example of the

latter may be the study by Kelley et al. (1988) with the short-lived perennial grass *Anthoxanthum odoratum*. While they attempted to test a presumed advantage to sib competition, and succeeded in demonstrating a substantial fitness advantage to sexually produced offspring over asexuals, this advantage appeared to have little to do with sib competition. Supportive evidence for the crucial premise(s) of a hypothesis is less likely confounded by that for other hypotheses, and may thus provide more direct support. Therefore, the approach of the work presented in this thesis is to test the two assumptions of the DMH: (1) is the rate of deleterious mutations higher than 1 per genome per generation? and (2) is selection against deleterious mutations synergistic?

For the sake of studying the generality of the hypothesis, it would be relevant to test the assumptions in a variety of organisms. However, the choice of experimental organisms is limited by other criteria. The experiments in this thesis involve two species, the unicellular alga *Chlamydomonas moewusii* and the asexual fungus *Aspergillus niger*. The thesis has been supplemented with a literature study involving a number of fungal and plant species. Both micro-organisms have been chosen for their short generation times (10 hours in *Chlamydomonas* and three days in *Aspergillus*) and the ease of experimental manipulation related to that property, for their simple genetics due to their being haploid, as well as for the availability of useful laboratory strains. An additional reason to use *Chlamydomonas* has been the availability of a protocol for measuring the fitness of large numbers of *Chlamydomonas* genotypes by Bell (1990).

Chapters 2 and 3 present two experimental tests for synergistic epistasis between deleterious mutations, one considering the mean fitness of parents and offspring ('means test'), the other considering the skewness of the fitness distribution of the offspring ('skewness test'). In these chapters, two experiments are described using two old strains of *Chlamydomonas*, that are interesting since they presumably accumulated deleterious mutations. Furthermore, in chapter 2 interaction between UV-induced mutations is studied, while in chapter 3 the focus of study concerns interaction between naturally accumulated deleterious mutations. The fitness of *Chlamydomonas* is measured by estimating the logistic parameters r and K in batch culture. The rate of deleterious mutations in *Aspergillus* is addressed with a mutation-accumulation experiment in chapter 4. There, the relative fitness is measured in

competition with a genetically marked reference strain. In chapter 5, the question is tackeled whether mutations interact synergistically in an experiment using marker mutations in *Aspergillus*. Interaction between the markers is studied by measuring the effect of marker-combinations on two components of the fitness: mycelial growth rate and maximum spore production. In chapter 6, the skewness test is applied to literature data on a number of fungal and plant species to reveal the nature of epistasis for a variety of fitness components.

2

The effect of sex and deleterious mutations on fitness in *Chlamydomonas*

ABSTRACT In this paper we present an experimental test of the deterministic mutation hypothesis on the evolution of sex. We studied the direct effect (i.e. before selection) of sex on offspring fitness of two strains of the unicellular alga *Chlamydomonas moewusii*, that had been kept in the laboratory for over sixty years. The logistic parameters r and K of each genotype, estimated in batch culture, were used as a measure of fitness. Strains were treated with UV to cause additional deleterious mutations. By comparing mean log fitness of parents and offspring in relation to the fitness difference of the parents, we tested whether and how deleterious mutations interact. No significant recombinational load was found in the offspring of the untreated strains. However, a significant negative effect of sex on log r and log K was found after crossing UV treated strains. We argue that this negative effect of sex on fitness suggests synergistic interaction, at least between the UV-induced and the naturally accumulated deleterious mutations. The latter result therefore supports the deterministic mutation hypothesis.

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Introduction

Although intensively studied, the evolution of sex is still considered one of the major problems in evolutionary biology. According to the balance argument (Williams 1975), a short-term advantage of sex is needed to compensate for the two-fold cost of sex in anisogamous species that occurs at the same time scale (Maynard Smith 1971). Numerous theoretical short-term advantages of sex have been proposed in the last 30 years (e.g. reviews by Stearns 1987; Michod and Levin 1988; Kondrashov 1993). However, experimental data to test these theories are scarce (reviewed by Bierzychudek 1987).

A theoretical advantage of sex, that is appealing due to its simplicity and general validity, is provided by the deterministic mutation hypothesis (Kondrashov 1988). According to this hypothesis, the elimination rate of deleterious mutations may be higher in a sexual than in an asexual population in mutation-selection equilibrium. The only two restrictions of the model are given by the minimum deleterious mutation rate of one per genome per generation (Kondrashov 1982; Charlesworth 1990) and the mode of selection against deleterious mutations, that should be synergistic, i.e. fitness should decrease faster than multiplicatively with an increasing number of deleterious mutations (Kimura and Maruyama 1966; Crow 1970; Kondrashov 1982; Charlesworth 1990). Evidence, both on deleterious mutation rate (Mukai et al. 1972; Houle et al. 1992) and synergistic interaction between deleterious mutations (Mukai 1969; Malmberg 1977; Willis 1993), is not conclusive, however.

In this paper, we present an experimental test of the deterministic mutation hypothesis with the heterothallic unicellular alga *Chlamydomonas moewusii*. This species was used, since it is able to reproduce both vegetatively and sexually, and its reproductive mode is easily manipulated experimentally (Van den Ende et al. 1988; Bell 1991). Furthermore, its genetics is simple, because individuals of this genus are haploid. Asexual reproduction rate in standard growth medium was used as a measure of fitness (cf. Bell 1990). Since mutation-selection equilibrium is hard to imitate experimentally, it is not feasible to directly test the theoretical consequence, i.e. the higher mean fitness of a sexual population. Therefore, we devised an experimental test for the assumption of the hypothesis that deleterious mutations interact

synergistically. The basic idea of the experiment is to compare the fitness of sexually and asexually produced offspring of two laboratory strains, before and after irradiation with ultraviolet radiation (UV), meant to cause additional mutations.

The two strains of *C. moewusii* we used in the crosses are isolates that have been kept on agar slants in the laboratory probably since 1931 (E.H. Harris of Duke University, personal communication). Since the two strains are of opposite mating type, reproduction has been purely vegetative in the laboratory. Two processes that affect fitness may be expected to have occurred in this period. First, it is likely that Muller's ratchet (Muller 1964) has caused the accumulation of deleterious mutations by the frequent transfer of a small population sample to fresh medium. Second, advantageous mutations that occurred may have caused associated gene combinations with relatively high fitness on the culture medium used. Thus, sex between these two strains might cause the breaking up of associated favourable genes and the distribution of deleterious mutations among the offspring.

Breaking up favourable gene associations will always have a negative effect on offspring fitness, but the redistribution of deleterious mutations can be either neutral, positive or negative. Because *Chlamydomonas* is haploid, this only depends on whether deleterious mutations interact, because without interaction the expected mean fitness of the offspring equals the midparent value (Mather and Jinks 1982; Bell 1991). Since without interaction mutation effects are expected to be multiplicative (e.g. Charlesworth 1990), it is the effect of sex on the logarithm of fitness that matters. Hence, if deleterious mutations have independent effects on fitness, no effect of sex on log fitness is expected: parents and offspring will have equal mean log fitnesses. If, however, deleterious mutations interact, the effect of sex depends on (1) the mode of mutation interaction (synergistic or antagonistic, i.e. mutations weaken each others negative effect) and (2) the difference in mutation load of the parents. If, for example mutations interact synergistically and the two parents carry similar numbers of mutations, the effect of sex on log fitness will be negative, since equal numbers of offspring with higher and lower mutation loads than that of their parents are produced, but the ones with higher loads affect fitness more severely (Figure 1a). If on the other hand the two parents carry different numbers of deleterious mutations, the

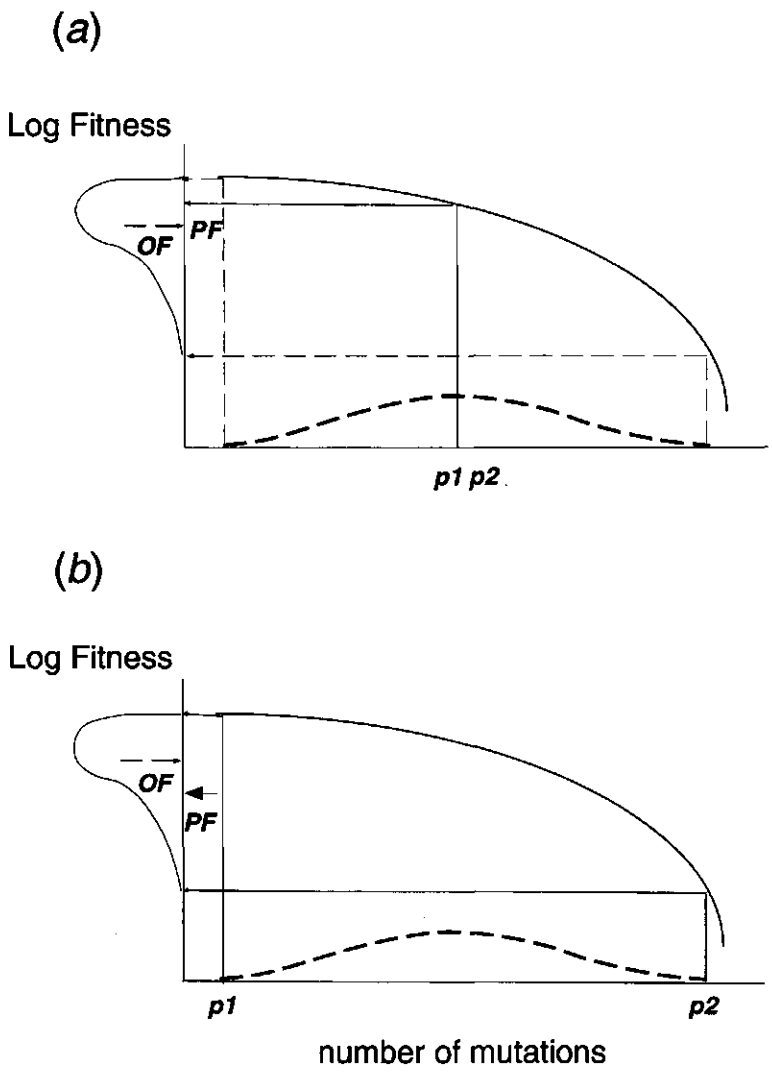

Figure 1. The effect of sex on mean log fitness of the offspring of a cross depends on the mode of mutation interaction and the relative difference in mutation load of the two parents. This is illustrated for the case in which mutations show synergistic interaction. (*a*) If mutations interact synergistically and both parents (*p1* and *p2*) have similar mutation loads, the effect of sex on mean log fitness is negative. Because equal numbers of offspring with higher and lower mutation loads than the mean of the parents are generated by sex, this negative fitness effect is caused by the greater influence of offspring with many mutations. OF = offspring mean log fitness, PF = parental mean log fitness. The dashed bell-shaped curve represents the distribution of the number of mutations per individual among the offspring. (*b*) But, if the parents carry rather different numbers of deleterious mutations (*p1* has a low, *p2* a high number of mutations), most offspring will obtain intermediate mutation numbers and consequently show higher mean log fitness. The reverse argument holds in case of antagonism.

majority of the offspring will obtain intermediate numbers of mutations and the effect of sex on log fitness will be positive (Figure 1b). The reverse argument holds if deleterious mutations show antagonism: a positive effect of sex if parents have similar mutation loads, a negative effect of sex if parents carry different numbers of mutations.

Since no direct information on numbers of mutations carried is available, we will assume an ordinal relationship between fitness and numbers of mutations. Then parental differences in mutation load can be ranked by ranking the fitness differences between parents. To distinguish between the two possible effects of sex by breaking up favourable gene associations and distributing deleterious mutations, we compared the effect of sex before and after the introduction of additional deleterious mutations in the parents of *C. moewusii* by UV irradiation. When additional, UV-induced, mutations are introduced in the parental strains, a change in the effect of sex on mean log fitness, compared to the effect in the untreated cross, depends only on whether and how these new mutations interact with the mutations that were already present.

Materials and methods

Cultures

We used *C. moewusii* strains UTEX 9 (mating-type plus: mt^+) and UTEX 10 (mating-type minus: mt^-) from the Algal Collection at the University of Texas, Austin, USA.

UV treatment

Vegetative cells were treated with ultraviolet radiation (UV) in order to cause deleterious mutations. Liquid log-fase samples (grown in 500 ml Erlenmeyer flasks with 300 ml Bold's Basal medium (BBM, see Harris 1989) under two fluorescent tubes and a 16h:8h L:D regime and 22°C) of the two strains were diluted to $5 \cdot 10^5$ cells$\cdot$ml^{-1}. Six ml aliquots of the diluted suspensions were brought into a petridish (diameter 5 cm) and irradiated with UV (fluence rate at the suspension surface was about 2 J$\cdot$m$^{-2}\cdot$s^{-1}), while continuously being stirred with a magnetic stirrer. Samples of 0.2 ml of both suspensions were taken every 30 sec up to 4 min. They were kept in the dark for 16 h to avoid photo-repair. Then the cells were plated on

solid M1 medium (Mesland 1976) and kept at 21°C and continuous light (fluorescent and incandescent light with fluence rate of 33 $J \cdot m^{-2} \cdot s^{-1}$). The fraction surviving cells was used to match the effect of the UV treatment on the two strains. Three batches of samples were distinguished for each strain: `controls` (no UV irradiation), `low UV` (0.5 min of irradiation, resulting in about 80% survival) and `high UV` (1.5 min for the mt^+ strain and 3 min for the mt^- strain, resulting in about 1.5% survival).

Production of sexual offspring

Three random colonies of each strain-UV dose combination were selected for crossing UTEX 10 (mt^-) with UTEX 9 (mt^+) within each of the three UV treatment levels, resulting in 27 crosses (three treatment levels * nine colony combinations). The protocol of Schuring et al. (1987) was followed to produce zygotes and to isolate the four meiotic segregation products of a number of zygotes of each of the 27 crosses. A variable number of zygotes could be isolated in only a small number of these crosses. Furthermore, only a fraction of the zygotes germinated (26 out of 58 zygotes = 45%) and only a fraction of the isolated offspring formed a colony on agar plates (99 out of 170 spores = 58%). In most zygotes meiosis was immediately followed by one or more mitoses, resulting in more than four meiotic products. In these cases a mating-type test was used to select at most two of the four meiotic products with opposite mating-types.

Only the cross with the highest number of sexual progeny (further referred to as 'sexuals') of each UV treatment was used in the analysis, resulting in: 13 sexuals in the control, nine in the low UV and 18 in the high UV cross, together with six parental genotypes (two parental strains * three UV treatments, further referred to as 'asexuals'). All genotypes, sexuals and asexuals, were kept on agar slants of M1 medium under 16h:8h L:D regime and 22°C before fitness was measured.

Fitness measurement

Fitness of sexuals and asexuals was estimated by measuring clonal growth rate of each genotype in three different media: five times diluted BBM (low-B), BBM with a growth limiting concentration of NO_3 (low-N: 20% of normal concentration) and BBM with a growth limiting concentration of PO_4 (low-P: 20% of normal concentration; cf. Bell 1990). Different media

were used to increase the number of deleterious mutations that will show an effect on fitness.

Fitness was measured as follows. All sexuals and asexuals were transferred to 0.25 ml of BBM in a 96 wells multi-well plate. After four days all suspensions were diluted to equal cell density by measuring the absorbance at 405 nm on a plate reader. 20 µl of each diluted suspension (containing about 2500 cells) was used to inoculate a test tube (1 cm diameter) with a screw-cap with 5.5 ml medium. The screw-cap of each test tube was loosened to permit diffusion of air. Each genotype-medium combination was replicated twice, resulting in 46 (40 sexuals and six asexuals) * three * two = 276 cultures. All test tubes were randomised over 10 racks that were kept under continuous light (fluence rate 40 $J \cdot m^{-2} \cdot s^{-1}$) and a constant temperature of 21°C on the same shelf.

During six weeks each tube was briefly vortexed daily and the absorbance at 660 nm was recorded with a colorimeter (Corning colorimeter, model 257). In a colorimeter absorbance of a culture in a test tube can be measured without removing the screw-cap, so cultures remained axenic during the whole experiment. Cell density ($cells \cdot ml^{-1}$) of the cultures was inferred from the absorbance data in a dilution series by linear regression (Cell density = $1.65 \cdot 10^6$ $cells \cdot ml^{-1}$ * Absorbance at 660 nm). Bell (1990, 1991), when measuring fitness of *Chlamydomonas* in a similar way, used transmittance of the cultures to estimate the clonal growth rate, but in our experiment transmittance showed a curvilinear relationship with cell density, especially at high densities, while absorbance showed a more or less linear relationship (cf. methods of Bell 1991).

A batch culture of an obligate autotrophic species dies some time after the first component in the medium has been depleted. It appeared that some of the cultures showed a typical temporary growth arrest at sub maximal cell density. In order to estimate the intrinsic (= maximum) growth rate (r) of each culture, cell density data were truncated at or one day after reaching the (first) growth plateau and the logistic growth equation was fitted to the truncated data of each culture. The estimates of r were obtained by least-squares minimisation, using the Quasi-Newton numerical procedure of SYSTAT's NONLIN procedure (Wilkinson 1988) and fixing the initial cell number at 430 $cells \cdot ml^{-1}$. Of the 276 cultures ten estimates of r were missing, because the logistic growth equation failed to

fit or showed a bad fit (corrected $r^2 < 0.7$). As carrying capacity (K) of the cultures, the maximum cell density of each culture was used.

Statistical analysis

To test the main effects of UV, parental strain and medium and their interactions, a fixed factor ANOVA was done on parameters r and K of the asexuals. The effect of sex was tested with a one-sample t-test on mean log fitness over media and replicates per offspring against midparent value, i.e. mean log fitness of the parents. To estimate the genetic variance for fitness of the offspring, a mixed model ANOVA per cross was performed on log fitness, using sexual offspring genotype as a random factor and medium as a fixed factor. All ANOVA's were done with the GMLH procedure of SYSTAT (Wilkinson 1988). With only two replicas per treatment group the homogeneity of variances could not be accurately tested. However, a negative correlation between mean and variance of the replicas over all genotypes and media existed for log K (product-moment correlation $\rho = -0.34$, $n = 138$, $p < 0.01$), but not for log r ($\rho = -0.12$, $n = 128$, ns).

Results

The effect of strain, medium and UV on fitness

As a measure of fitness we used the demographic parameters of the logistic growth equation: the maximum asexual reproduction rate, r, and the asymptotic maximum number of individuals in a certain environment, known as the `carrying capacity`, K. Since *Chlamydomonas* normally reproduces asexually, these parameters together seem good predictors of total fitness (Bell 1991). Both are considered as predictors of fitness in a typical ecological setting: r is an estimate of fitness in a recently colonised habitat, where nutrients are *ad libitum*, and K is thought to represent fitness when there is competition over the limited nutrients (MacArthur 1962). *Chlamydomonas* species are known to occur among the first green alga colonisers of artificial ponds, but to disappear soon thereafter when other species take over (Happey-Wood 1988; Williams et al. 1994). For *Chlamydomonas*, therefore, maximum growth rate is likely to be the more relevant fitness parameter. Figure 2 shows the performance

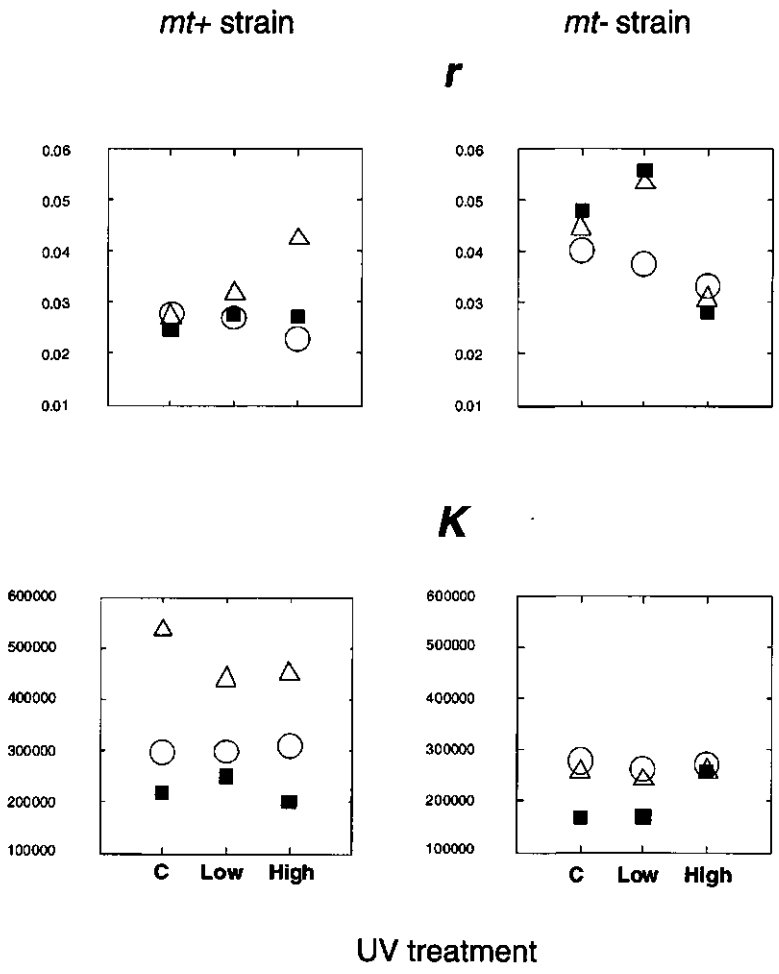

Figure 2. The effect of UV treatment on the fitness (r and K) of both parental strains. Symbols are the means of two replicates. Different symbols are for different media: squares for low-B medium (see *Materials and methods*), circles for low-N medium and triangles for low-P medium. Units for r are h^{-1} and for K are number of cells·ml^{-1}.

of the two strains in the three media and how they respond to UV treatment. We tested the effect of UV, parental genotype and medium on fitness (r and K) with a fixed factor ANOVA. The results of the ANOVA are in Table 1.

As can be seen from Figure 2 and Table 1, the untreated strains show a significantly different r and K, the mt^- strain has the higher r and

Table 1. ANOVA results on fitness (r and K) of the two parental strains. A three-way ANOVA model is used, in which all three factors are considered as fixed factors. Mean Squares (MS) are multiplied by 10^8 for r and 10^{-10} for K. *: $p < .05$, **: $p < .01$, ***: $p < .001$.

	r			K		
Source	df	MS	F	df	MS	F
UV	2	1.67	4.83*	2	0.255	1.31
Control vs. Low + High UV	1	0.871	2.51			
Control + Low UV vs. High UV	1	0.028	0.08			
Strain	1	18.6	53.7***	1	7.14	36.8***
Medium	2	0.115	0.33	2	8.82	45.5***
Low-B vs. Low-N				1	4.19	21.6***
Low-B vs. Low-P				1	17.6	90.9***
Low-N vs. Low-P				1	4.63	23.9*
UV*Strain	2	3.58	10.3***	2	0.414	2.13
UV*Medium	4	0.573	1.65	4	0.252	1.30
Strain*Medium	2	0.592	1.71	2	3.61	18.6***
UV*Strain*Medium	4	0.734	2.12	4	0.379	1.95
Error	27	0.347		30	0.194	

the mt^+ strain the higher K. The performance in different media is significantly different only for K: K is overall higher in low-P medium than in the other two media and still higher in low-N than in low-B medium (shown by medium contrasts, Table 1). Furthermore, both strains show a different reaction of K to the three media, the mt^+ strain performing best in medium in which phosphate is growth limiting (low-P) and the mt^- strain having its highest K in medium in which nitrate is growth limiting (low-N, see Figure 2).

The most important observation is that UV treatment significantly affected r and this reaction was significantly different for both strains (see Table 1 and Figure 2). In the mt^- strain the high UV treatment caused a decrease in r, while in the mt^+ strain the variance in performance over the three media seems to be increased by the high UV treatment, caused by an increase of r in low-P medium and a slight decrease of r in low-N medium. Although the decrease in r seems mainly the result of the high UV treatment (Figure 2), the contrast of this treatment with the other two

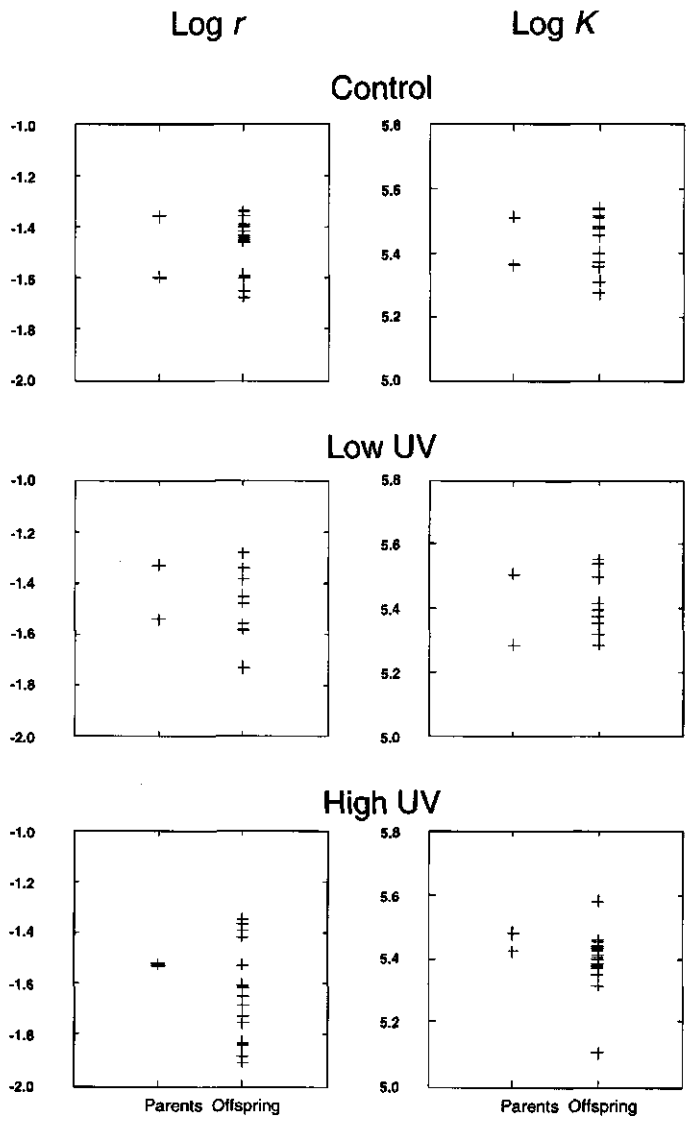

Figure 3. Mean log fitness, log r and log K, of parents and offspring of the three crosses. Each value is the mean value of six estimates (three media and two replicas).

treatments was not significant, probably due to the different reaction of the two strains. Nevertheless, it seems plausible that deleterious mutations with respect to r were introduced in the mt^- strain by the high UV treatment. UV caused a small overall decrease in K of the mt^+ strain and a

small increase in K of the mt^- strain, but the influence of UV on K was not significant.

The effect of sex on log offspring fitness

For our test of mutation interaction it is the effect of sex on mean log fitness that is informative. Therefore, log fitness was used in the analyses. Deleterious mutations that are expressed in specific environments show a cumulative negative effect on the mean value over these environments. To improve expression of the deleterious mutations, therefore, mean log fitness over the three media for each genotype was considered. In Figure 3 mean log fitness of parents and offspring are shown for the three crosses. Clearly, UV caused a decreased difference in parental log fitness in the high UV treatment, both with respect to log r and log K. Since additional deleterious mutations have been introduced with respect to r in (one of) the parents of the high UV cross, the genetic variance for log r is expected to be higher among the sexuals of this cross than among the sexuals of the other two crosses. The genetic variance for log fitness has been calculated with a mixed model ANOVA (see Table 2). Since only two non-random parental strains were used, no accurate estimate of the asexual genetic variance could be calculated. The genetic variance for log r of the sexual offspring is indeed higher among the offspring of the high UV cross than among the control cross, although not significantly (F-test: $F = 2.31$, $df = 15, 12$, $p = 0.075$), but comparable to the genetic variance in the low UV cross. The genetic variance among the sexuals for log K does not show an increase with UV treatment, as expected, because UV did not have a significant effect on K of the parental strains.

Both, the breaking up of gene associations with relative high fitness and interaction between deleterious mutations (see Introduction) can cause a change in mean log fitness among the control and the low UV cross (the latter cross is comparable to the control cross, since no fitness reduction of the parental strains occurred by the low UV treatment). In Table 2 it can be seen that there is no clear effect of sex, neither on log r, nor on log K, in these two crosses. However, the effect of sex on mean log fitness in the high UV cross is negative. Mean log r and log K of the offspring of this cross are significantly lower than the midparent value when tested two-tailed with a one-sample t-test (Table 2). When we assume an experimentwise error rate of $\alpha = 0.05$, to correct for multiple

Table 2. Effect of sex on mean and genetic variance of log fitness. Data are mean performances in three different media. The genetic variance of log fitness was estimated with a mixed model ANOVA of the offspring per cross, in which offspring genotype is a random and medium a fixed factor. The expected Mean Squares for genotype effect in the model is $\sigma_\varepsilon^2 + n\,a\,\sigma_\gamma^2$, where σ_ε^2 = replicate error, n = number of replicates, a = number of treatment levels of the factor medium and σ_γ^2 = genetic variance. T-tests are two-tailed one-sample tests in which midparent value is tested against mean log offspring performance (per offspring genotype averaged over three media).

	mid-parent	distance	*n*	offspring mean	*t*	*p*	σ_γ^2
log *r*							
Control	-1.47	-1.59 to -1.35	13	-1.47	0.003	1.00	0.061
Low UV	-1.44	-1.54 to -1.33	9	-1.47	-0.69	0.51	0.096
High UV	-1.52	-1.53 to -1.52	18	-1.63	-2.48	0.024	0.140
log *K*							
Control	5.44	5.36 to 5.51	13	5.43	-0.29	0.77	0.035
Low UV	5.40	5.29 to 5.51	9	5.42	0.62	0.55	0.046
High UV	5.45	5.42 to 5.48	18	5.40	-2.58	0.020	0.038

comparisons in a conservative way (Sokal & Rohlf 1981), the type I error of each of the three individual tests for log *r* and log *K* becomes 0.017, making our results, both for log *r* and log *K*, in the high UV cross nearly significant. Mean log *r* of the offspring of the high UV cross is even lower than the value of the low fitness parent (t = -2.41, df = 17, p (two-tailed) = 0.03) and also compared with mean log *r* of the offspring of the control cross (two-sample t-test with unequal variances: t = 2.97, df = 12, 17, p (two-tailed) = 0.004). Mean log *K* of the offspring of the high UV cross is not significantly lower than the value of the low fitness parent (t = 1.25, df = 17, p = 0.23), nor than mean log *K* of the offspring of the control cross (two-sample t-test with unequal variances: t = 1.03, df = 12, 17, p (two-tailed) = 0.30).

Discussion

In this paper an experimental test for synergistic interaction between deleterious mutations is presented, as well as a first application of the test

to data on the unicellular alga *Chlamydomonas moewusii*. From the results two interesting observations can be reported. First, no negative effect of sex on fitness ('recombinational load') was found in the control cross. Two explanations are possible for the lack of a recombinational load. The simplest one is that during about sixty years of adaptation of the two parental strains to a standard growth medium (soil extract from Indiana: E.H. Harris personal communication), selection did not cause significant associations of genes with relative high fitness *and* deleterious mutations do not interact. Possible coadapted gene complexes might involve interactions between nuclear genes only, as well as interactions between nuclear genes and cytoplasmic genomes (Beale and Knowles 1978). Perhaps this result is surprising, because a recombinational load was found in similar experiments with *Aspergillus* (Jinks et al. 1966; Butcher 1969) and with *Drosophila* (Charlesworth and Charlesworth 1975). Possibly, in the parental strains we used gene associations exist that are specific adaptations to the soil extract medium on which the strains were kept for about 60 years, that do not cause a relative high fitness on the medium we used.

Another explanation could be that the negative effect of a recombinational load has been cancelled by a positive effect of the redistribution of deleterious mutations. The latter could either be the case if mutations interact synergistically and both parental strains carry rather different numbers of mutations, or if mutations interact antagonistically and parents carry similar numbers of deleterious mutations (see Figure 1). Since most offspring appear to have a log r and log K that is intermediate relative to both parents (see Figure 3), this suggests that Figure 1b is the relevant situation, indicating synergism with respect to both parameters. Because this can also explain the surprising lack of a recombinational load, synergism between accumulated mutations in both strains seems to be the more plausible explanation for the results of the control cross.

The second interesting observation was a significantly negative effect of sex on mean log fitness after the introduction of additional deleterious mutations in the parental strains by ultraviolet irradiation. Since some low fitness offspring that obtained many mutations may not have formed colonies and consequently have not been isolated, this negative effect is a conservative estimate. The different effect of sex in the high UV cross relative to the control cross can only be explained by

interaction between the new UV-induced and the already present deleterious mutations. Again, what matters is the difference in numbers of mutations between both parents. UV caused a decrease in r, together with a slight increase in K, of the mt^- strain in the high UV cross. This resulted in a decreased difference, especially in log r, but also in log K of both parents, indicating that Figure 1a is likely to be the relevant situation here. Together with a clear negative effect of sex on mean log fitness, this suggests that the UV-induced mutations show synergistic interaction with the already present mutations for both fitness parameters, r and K.

The value of the test mainly relies on the assumption of a rankorder relationship between fitness and number of deleterious mutations, that is necessary to decide whether or not parents differ substantially in the number of mutations they carry. Fitness differences can arise not only from different numbers of mutations, but also from variation in individual mutation effects. It is very likely that Muller's ratchet (Muller 1964) will have caused the accumulation of many mutations in the two strains we used, because we have estimated the number of cells transferred to fresh medium every three months during five years in our laboratory to be in the order of 10^5 (during the preceding 55 years similar transfers have been done every six months). The number of cells that actually start dividing upon transfer is still lower, roughly in the order of 10^3 to 10^4. Using conservative estimates of the deleterious mutation rate and the mean selection coefficient of mutations (0.4 and 0.02 respectively for *Drosophila*; however, the deleterious mutation rate of *Chlamydomonas* will probably be lower, due to its smaller genome) and assuming multiplicative mutation effects, these data suggest that Muller's ratchet has caused the accumulation of at least one mutation every transfer (see Haig 1978). This results in an expected total load in each of the two strains of more than 100 mutations. Selection between the still substantial number of viable cells that recurrently have been transferred to fresh medium will have prevented the accumulation of mutations with large effect, putting an upper limit to the variation of individual mutation effects. This strengthens the relationship between fitness and *number* of mutations carried. If, however, mutations interact in a synergistic way, considerable fitness differences may still occur with rather similar mutation numbers. Yet, since the decrease in parental fitness difference in the high UV cross is obvious, we feel justified in our qualitative

conclusion of a decreased difference in parental mutation load in this cross.

An even simpler test under the assumption of a rankorder relation between fitness and the number of deleterious mutations, would be a comparison between mean parental log fitness and mean log fitness of the offspring with intermediate log fitness relative to both parents. A higher 'intermediate offspring' mean log fitness supports synergistic mutation interaction, a lower 'intermediate offspring' mean log fitness antagonistic mutation interaction. Numbers of offspring with intermediate fitness are too low in this study for a meaningful comparison.

Biological arguments exist that make synergistic selection against deleterious mutations plausible. Kondrashov (1988) argues that competition over resources or copulations with one or a few winners implies truncation selection. Since antagonism ('diminishing returns epistasis') is unlikely to persist under mutation-selection balance, Charlesworth (1990) argues that multiplicative fitnesses and synergistic epistasis are likely to prevail. Szathmáry (1993) found theoretical support on metabolic grounds for synergistic mutation interaction between deleterious mutations affecting the carrying capacity. He showed that if mutations affect the activity of the same or different enzymes and selection is for optimum flux through a metabolic pathway or optimum concentration of a metabolic intermediate, deleterious mutations show synergistic interaction. These metabolic strategies are thought to predominate under *K*-selection. However, if selection is for maximum flux, mutations affecting different enzymes appear to interact antagonistically, while mutations affecting the activity of the same enzyme show synergistic interaction (Szathmáry 1993). Since selection for maximum flux is thought to be important under *r*-selection, Szathmáry's results on maximum flux only support our conclusions on the maximum growth rate if the relevant genetic variation for r in our crosses is caused by recombination between deleterious mutations within a single gene affecting the activity of a crucial enzyme.

In conclusion, we think that the method we present is a simple and promising tool for application to other organisms. In a first application of the test to data of *Chlamydomonas*, we found a negative effect of sex on $\log r$ and $\log K$ after the introduction of additional UV-induced deleterious mutations, that, paradoxically, provides support for the idea

that a sexual population has an advantage under mutation-selection equilibrium according to the deterministic mutation hypothesis (Kondrashov 1988). The conclusions from our first application of the test should, however, be considered as tentative. In the first place, because we can not be conclusive about interaction between naturally accumulated mutations and secondly, because numbers of offspring in our crosses were rather limited. Since very weak synergism can have large effects on equilibrium fitness (Charlesworth 1990), also finding of subtle synergistic epistasis is important. For the detection of such subtle synergism large sample sizes are needed. Therefore, at present we are performing comparable experiments with new strains and without the aid of UV to obtain higher numbers of offspring. Since this study is one of the very few, more experiments with simple eukaryotes like *Chlamydomonas* are needed, that provide data for testing the various theories of the evolution of sex.

Acknowledgements

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3

An experimental test for synergistic epistasis and its application in *Chlamydomonas*

ABSTRACT Theoretically, one of the most general benefits of sex is given by its more efficient elimination of deleterious mutations. This theoretical advantage of sex may be deterministic if deleterious mutations affect the fitness of an individual in a synergistic way, i.e. if mutations amplify each others negative fitness effect. We present a new test for synergistic epistasis by considering the skewness of the log fitness distribution of offspring of a cross. Also, we applied this test to data of the unicellular alga Chlamydomonas. The results indicate that mutations that affect the carrying capacity (K) show synergism, while mutations that affect the maximum growth rate (r) seem to have independent effects. These results speculatively suggest an alternative explanation for the general observation that sex is related to constant environments, where selection on K predominates, while asexual reproduction is found in more variable environments, where selection on r is more important.

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Introduction

To explain the ubiquity of sexual reproduction, a short term advantage of sex is needed that may compensate its twofold disadvantage (Maynard Smith 1971). One of the most attractive advantages of sex is provided by the Deterministic Mutation Hypothesis (Crow 1970; Kondrashov 1988). Its attraction is due to its general validity, but also to its clear-cut assumptions: (1) the rate at which deleterious mutations occur should be at least one per genome per generation (Kondrashov 1982; Charlesworth 1990), and (2) deleterious mutations should have synergistic effects on fitness, i.e. they should amplify each others negative effect (Kimura and Maruyama 1966; Crow 1970; Kondrashov 1982; Charlesworth 1990). At present, data both on mutation rate and on epistasis between deleterious mutations are too scarce to be conclusive (Kondrashov 1993).

Here, we present a new way of testing for epistasis between deleterious mutations. Due to random segregation and recombination, a sexual cross between two individuals (who carry an unknown number of deleterious mutations) produces a symmetrical distribution of mutation number among the offspring, with the mean equal to that of the parents. In a population with a symmetrical distribution of mutation number, skewness of the fitness distribution provides information on mutation interaction: a negative skewness reflects synergism, a positive skewness reflects antagonism, i.e. mutations weaken each others' negative fitness effect. The distribution of the *logarithm* of fitness should be considered, because mutations with multiplicative effects, i.e. no interaction (Kondrashov 1988; Charlesworth 1990), will cause the distribution of log offspring fitness to be symmetrical. Deviation from symmetry, i.e. skewness, can then be used as indication for mutation effects other than multiplicative. In Figure 1 the test is illustrated for the simplistic situation of all mutations having equal effect. However, since half of the offspring receive each mutation carried by a parent and the other half does not receive this mutation, non-equal mutation effects will also lead to no skewness if mutations show no interaction.

The relation between synergism and negative skewness can be derived analytically, as follows. Let x be a symmetrically distributed trait (in our case the number of mutations carried by an offspring) and let y be a function of x, $g(x)$ (in our case log fitness). Deviations of x and y from their

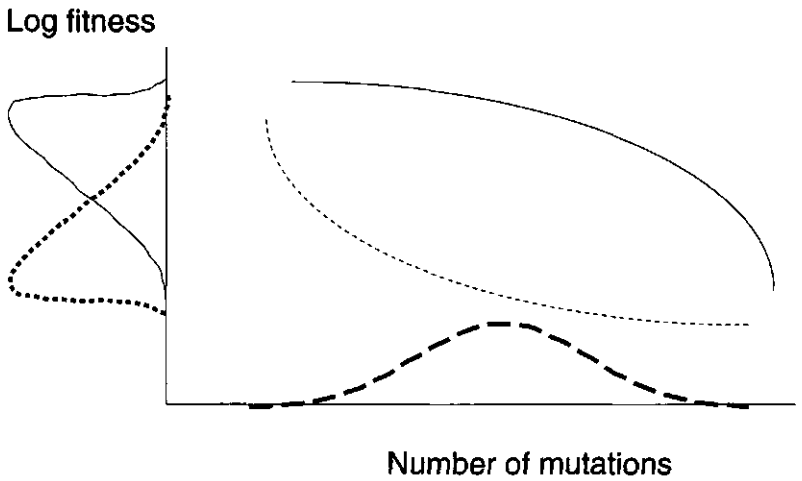

Figure 1. The skewness test for epistasis between deleterious mutations, illustrated in graphical arguments for the situation of mutations having all equal effects. Mapping a symmetrical mutation distribution (among sexual offspring of two parents) on a concave curve (continued line), representing synergism between mutations, results in a negatively skewed log fitness distribution, while mapping this mutation distribution on a convex curve (dashed line), representing antagonism between mutations, results in a positively skewed log fitness distribution.

mean are represented by δx and δy. Then, δy can be approximated with the first two terms of the Taylor expansion:

$$\delta y \;\; (\delta x)\, g' + 0.5\, (\delta x)^2\, g'' \qquad (1)$$

where primes denote derivatives evaluated at the mean. The skewness of y can then be expressed as the expectation of $(\delta y)^3$ in which the odd moments of x are dropped:

$$E\{(\delta y)^3\} = 1.5\, (g')^2\, (g'')\, E\{(\delta x)^4\} + 0.125\, (g'')^3\, E\{(\delta x)^6\} \qquad (2)$$

which is <0 if $g''<0$. The latter condition corresponds to a concave relationship between log fitness and mutation number, i.e. synergism between deleterious mutations.

We applied the skewness test to data of two crosses between strains of the haploid unicellular alga*Chlamydomonas moewusii*. One cross, the 'accumulation cross', was made between strains that have been kept

separate in the laboratory for more than 60 years (E.H. Harris, personal communication). Since the species is heterothallic, no sex occurred during this period. The frequent transfer (every three to six months) of a small sample (containing in the order of 10^4 viable cells) of each strain to fresh medium made it likely that deleterious mutations have accumulated due to Muller's ratchet (Muller 1964; Haigh 1978). Moreover, given this relatively small population size, additional mutations with a very low deleterious effect might have become fixed by genetic drift, since they are effectively neutral (Kimura 1983). Another cross, the 'control cross', was made between two strains that have been isolated from nature only recently (in 1990) and, therefore, had not had the opportunity to accumulate many mutations. The fitness of individual offspring of both crosses was measured by estimating the two parameters of the logistic growth model, maximum growth rate (r) and carrying capacity (K) in batch culture (cf. Bell 1990).

The results suggest synergism between deleterious mutations that affect K, while the absense of skewness for r is most consistent with multiplicative effects between mutations that affect this parameter. If the different results on r and K hold more generally, they support an alternative explanation for the ecological distribution of sex by the Deterministic Mutation Hypothesis.

Materials and methods

Strains

The following strains of *Chlamydomonas moewusii* were used in the two crosses: strains UTEX 9 (mating-type plus: mt^+) and UTEX 10 (mating-type minus: mt^-) (Harris 1989) in the accumulation cross and the recently (in 1990) isolated strains SAG 23.91 (mt^+) and SAG 24.91 (mt^-) (Schloesser 1994) in the control cross.

Crossing protocol

For the isolation of sexual offspring of both crosses, we used the protocol of Schuring et al. (1987). Crossing the two recently isolated strains of *C. moewusii* (SAG 23.91 and SAG 24.91) resulted in more offspring than crossing the two old strains (UTEX 9 and UTEX 10): 176 verus 72 offspring.

The offspring of the control cross were all isolated in one crossing experiment, while the offspring of the accumulation cross were the total of three independent attempts to isolate offspring from these old strains. After isolation, offspring have been kept on agar slants with 3 ml solid M1 medium (Mesland 1976).

Measuring fitness

We used the two parameters for logistic growth, the maximum growth rate (r) and the carrying capacity (K), of each offspring as measure of fitness. The idea of measuring r and K in a test-tube is derived from Bell (1990). However, the logistic growth model is not an appropriate model for a closed system like a batch culture and, therefore, our estimate of K should rather be interpreted as the total yield from a given amount of nutrients (R.E. Lenski, personal communication).

All offspring and parents were transferred to a multi-well plate with M1 liquid medium (i.e. the medium they were kept on for the last five years) for four days, and the cell densities of these cultures were equalised by measuring absorbance at 405 nm on a plate reader. Then 15 µl, containing about 640 cells, were taken to inoculate two test-tubes with 6 ml M1 liquid medium for each offspring, and ten test-tubes for each parent. All 496 test-tubes (72 offspring of the accumulation cross and 176 offspring of the control cross, all replicated twice) were randomised over 11 racks and placed under continuous light (fluence rate 40 $J \cdot m^{-2} \cdot s^{-1}$) at 21°C during 3 months. Test-tubes were briefly vortexed daily (after six weeks: three times a week) and absorbance at 660 nm was scored with a colorimeter (Corning colorimeter, model 257). Cell density ($cells \cdot ml^{-1}$) was inferred from the absorbance data in a dilution series by linear regression. Due to irregular growth of some of the cultures, maximum cell density was used as estimate of K, while r was estimated from the data, that were truncated before the first irregularities occurred. For the estimation of r we used the NONLIN procedure of SYSTAT (Wilkinson 1988) to fit the logistic growth model to the cell density data. Initial cell number was estimated as well, because it improved the fit of the model, reflected by a higher explained variance after correction for estimating the additional parameter from the data.

Statistical analysis

The growth of 18 cultures of the control cross and 11 cultures of the accumulation cross had not stopped at the end of the growth period, so that no accurate estimate of K of these cultures was available. Furthermore, for five cultures of the control cross and five cultures of the accumulation cross, no valid estimate of r could be generated, due to no or bad (corrected $r^2 < 0.8$) fit of the logistic growth model. This resulted in at least one estimate per genotype of K for 173 offspring in the control cross and for 71 offspring in the accumulation cross. Of r, at least one estimate per genotype was obtained for all 176 offspring in the control cross and for 71 offspring in the accumulation cross.

Analyses of variance were performed to estimate and test the significance of the genetic variance for fitness, V_G. For this purpose, only genotypes with two estimates of r or K were considered. To test the significance of the skewness of the log fitness distributions, first the arithmetic mean value of the two replicates was computed (in case of one available replicate, this one value was used) and then the logarithm was taken. Next, the skewness of the log mean values was computed and its significance was tested with a two-tailed t-test, using the exact formula for the standard error of the skewness statistic g_1 (Sokal and Rohlf 1981, p 139). Since we only had two replicates per genotype, we could not accurately test for homogeneity of variances. However, a significantly positive correlation exists between mean and variance per genotype for r (control cross: $\rho = +0.61$, $df = 174$, $p < 0.001$; accumulation cross: $\rho = +0.38$, $df = 67$, $p < 0.01$). For K, the correlation between mean and variance is only slightly positive (control cross: $\rho = +0.15$, $df = 172$, $p < 0.05$; accumulation cross: $\rho = +0.16$, $df = 67$, $p > 0.05$).

Results

Accumulation of mutations in control and accumulation cross

If the parents of the accumulation cross carry a higher number of deleterious mutations than the parents of the control cross, we expect a higher genetic variance for fitness in the accumulation cross. As expected, among the offspring of the accumulation cross the genetic variance for K

Table 1. Mean and genetic variation in fitness (K and r) in the control and accumulation cross.

		Genotypes		Error			
Cross	mean [SE]	*df*	*MS*	*df*	*MS*	*VG*	*p*
K							
Control	1.51E6 [1.56E4]	160	7.62E+10	161	4.69E+11	1.46E+10	0.0011
Accumulation	1.66E6 [5.70E4]	61	4.84E+11	62	7.65E+10	2.04E+11	5.5E-12
r							
Control	0.0253 [5.8E-4]	172	1.41E-4	173	1.52E-4	(0)	0.69
Accumulation	0.0145 [6.6E-4]	67	6.17E-5	68	2.47E-5	1.85E-5	0.00011

is almost 14 times higher than among the offspring of the control cross (Table 1). The genetic variance for r is not significant in the control cross ($V_G = -5.40 \cdot 10^{-6}$, 95% confidence limits are $-2.75 \cdot 10^{-5}$ and $1.54 \cdot 10^{-5}$), due to a high error variance, but is significant in the accumulation cross. These results suggest that the higher genetic variance for fitness in the accumulation cross is due to the redistribution of accumulated deleterious mutations.

Epistasis between deleterious mutations

If deleterious mutations show interaction with respect to the fitness parameters, we expect the effect to be clear only in the accumulation cross, due to the distinct genetic variation in fitness in this cross relative to the control cross. In the control cross the distribution of log K appears to be unskewed (Figure 2: $t_S = +0.33$, $df = 172$, $p = 0.74$), while it is significantly negatively skewed in the accumulation cross ($t_S = -2.11$, $df = 70$, $p = 0.038$). These results suggest synergism between deleterious mutations that affect the carrying capacity. The distribution of log r appears to be unskewed, both in the control cross ($t_S = +0.55$, $df = 175$, $p = 0.58$) and in the accumulation cross ($t_S = -1.04$, $df = 70$, $p = 0.30$). These results suggest independent effects of deleterious mutations that affect the maximum growth rate.

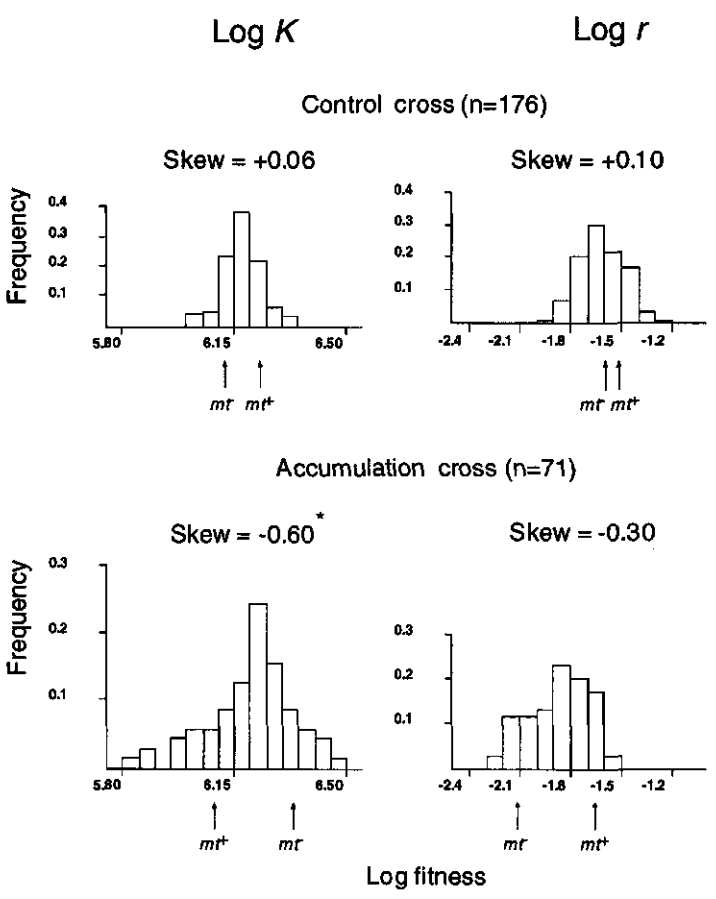

Figure 2. Log fitness distributions of control and accumulation cross. Individual estimates of log r and log K are the mean of two independent estimates of two replicate cultures. The arrows indicate mean log fitness of the parental strains.

Discussion

The aim of this paper is twofold. The first is to present a new test for the mode of interaction between deleterious mutations. The second is to present and discuss the results of a first application of this test to data of the unicellular alga *Chlamydomonas moewusii*.

The Skewness test for Mutation Interaction

The skewness test for epistasis has a simple rationale and, basically, can be applied very generally. It does not lean on the additional assumption of a

rank-order relationship between mutation number and fitness that an earlier test for epistasis by us used (De Visser et al. 1996a). The application of the skewness test is only limited by three conditions. First, it can be applied only to haploid sexual species with large progenies per cross. In diploids, dominance may also affect the skewness. The relative contribution of dominance and non-allelic interaction effects on the overall skewness needs to be studied, before the skewness test can be applied to diploid species. Second, the parents of a cross need to carry sufficient deleterious mutations to warrant a significant genetic contribution to the fitness variation among their offspring. Third, the offspring isolated from a cross should be a random sample, including (a representative fraction of) offspring with many mutations. Missing individuals with many mutations, for instance because they are small or inviable, generates a positive skewness of the log fitness distribution. The third condition implies that finding a negatively skewed log fitness distribution will rather be conservative evidence for synergism, while finding a positive skewness may be ambiguous.

Application to data of *Chlamydomonas*

The results of the application of our test to data of *Chlamydomonas moewusii* are consistent with synergistic effects of deleterious mutations on the carrying capacity and independent effects on the maximum growth rate. The results for K, however, may be somewhat stronger than the results for r for three reasons. In the first place, the genetic variance for K in the accumulation cross has a higher significance than the genetic variance for r, which emphasises the stronger dependence of (the form of) the distribution of K on genetic differences between offspring. In the second place, a negatively skewed log fitness distribution will generally be conservative evidence for synergism, as explained above. In the third place, because the probably rather low statistical power of finding significant skewness makes conclusions drawn from the absense of skewness tentative.

How do the two parameters we estimated, r and K, relate to fitness? The maximum growth rate (r) of a specific offspring genotype estimated in monoculture can be compared to its Malthusian parameter when nutrients are available ad libitum. Under such conditions, growth limitation is mainly density-independent and, therefore, maximum

growth rate is comparable to relative fitness. The carrying capacity (K), as stated in the Methods, basically cannot be measured in batch culture and should rather be interpreted as the total yield of a specific offspring genotype. Moreover, since at carrying capacity density is high, growth limitation is mainly density-dependent and K, estimated in monoculture, is not necessarilly comparable to relative fitness in a compatitive situation. Huisman and Weissing (1994, 1995) and Weissing and Huisman (1994), however, argue on theoretical grounds, that when light is the limiting nutrient, the relative fitness of an algal monoculture is linearly proportional to the fraction of light that is absorbed by the culture. Since our estimate of K is based on the maximum absorbance (at 660 nm) of a monoculture, K is at least comparable to relative fitness under high density conditions when light is limiting growth.

In an earlier paper (De Visser et al. 1996a), where we used a test for epistasis that considered the difference in mean log fitness between parents and offspring of a cross, we found indications for synergism between UV-induced mutations for both fitness parameters, K and r. We think the skewness test presented in this paper is more robust, because it does not lean on the additional assumption of a rank-order relationship between mutation number and fitness that the earlier test made. Consistent with this notion is the fact that we could not unambiguously show synergism for K among accumulated mutations with the former test (De Visser et al. 1996a), while we can with the skewness test. Furthermore, mutations induced by UV irradiation may have a different character than the slightly deleterious ones that are thought to predominate if accumulation is by Muller's ratchet (Haigh 1978).

Speculatively, our results might imply that the advantage of sex provided by the Deterministic Mutation hypothesis (Kondrashov 1988) depends on situations of high population density. Hamilton et al. (1990) predicted the relative importance of the Deterministic Mutation hypothesis in saturated environments, emphasising the significance of truncation-like selection in such situations due to limited space or nutrients. Szathmáry (1993) predicted the prevalence of synergism if fitness is density-dependent on metabolic grounds. Also, sexual species are found relatively more abundantly in constant environments, where populations may reach high densities and selection on K might predominate (Bell 1982; Trivers 1985). Although our results on the

maximum growth rate should be interpreted with care due to the relatively low power of the skewness test, they appear to be consistent with this ecological distribution of sex. Data on deleterious mutation rate and epistasis between deleterious mutations in other species and with respect to other fitness estimates, related to different ecological settings, are needed to test this alternative explanation of the ecology of sex. We think the skewness test presented in this paper provides a valuable tool for this purpose.

Acknowledgements

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4

The deleterious mutation rate in *Aspergillus niger*

ABSTRACT This study is the first report of an estimate of the total rate of deleterious mutations in a lower eukaryote, the filamentous fungus*Aspergillus niger*. Our specific aim is to test a major premise of the Deterministic Mutation Hypothesis (DMH) on the evolution of sex, that the rate of deleterious mutations should be at least one per generation. We have performed mutation-accumulation experiments, in which we maintained 25 mutation-accumulation (MA) lines during 60 generations under conditions of minimal selection by transferring single uninucleate spores. Fitness was measured as the relative number of spores produced in competition with a reference strain, in a high and in a low density situation. Unexpectedly, the ancestral strain showed the lowest fitness. However, the fitness of the MA lines significantly decreased between generation 30 and 60 of mutation-accumulation. From a comparison between generation 30 and 60, we estimated the total deleterious mutation rate to be at least 0.19 per generation, although confidence intervals are large. Due to these uncertainties, our estimate should be considered as tentative. Reasons for the low fitness of the ancestor and why U is likely to be underestimated from mutation-accumulation experiments are discussed. More estimates of the deleterious mutation rate in various organisms are clearly needed to settle the discussion on the general plausibility of the DMH.

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Introduction

The rate at which spontaneous mutations with a deleterious effect on fitness occur is largely unknown, but important for, among other things, understanding the functional significance of sex. The Deterministic Mutation Hypothesis (DMH) of the evolution of sex (Crow and Simmons 1983; Kondrashov 1982, 1988) emphasises the role of sex and recombination in purging the genome from inevitable deleterious mutations. One of the two simple assumptions on which this hypothesis is based, is that the rate of slightly deleterious mutations should be at least one per genome per generation (Kondrashov 1982; Charlesworth 1990). The other assumption is that deleterious mutations interact synergistically (Kimura and Maruyama 1966; Crow 1970; Kondrashov 1982; Charlesworth 1990). However, there are hardly any data to support these assumptions (Kondrashov 1993; Crow 1994; Hurst and Peck 1996; Charlesworth 1996). In an earlier paper (De Visser et al. 1996a), we adressed the assumption of synergistic mutation interaction experimentally. The purpose of this study is to test the second assumption by generating empirical data on the deleterious mutation rate.

Since the 1930's, it was generally assumed that the majority of mutations that are non-neutral have a deleterious effect on fitness (Fisher 1930; Haldane 1937; Muller 1950). Mukai (1964; Mukai et al. 1972) was the first to experimentally study the total deleterious mutation rate in *Drosophila melanogaster*. His focus was on mutations affecting one aspect of total fitness, i.e. viability. Houle et al. (1992) estimated the total rate of mutations with a deleterious effect on fitness in Drosophila, but later retracted their results due to contamination of their control line with a transposon. Indirect estimates, derived from inbreeding effects, were obtained for a number of plant species (Charlesworth et al. 1990; Charlesworth et al. 1994; Johnston and Schoen 1995). The minimum estimates from these studies are about 0.5 deleterious mutation per genome per generation, but may well be higher than one under more realistic assumptions (see discussion of Hurst and Peck 1996).

In this paper we used a method, based on the mutation-accumulation experiments by Mukai (1964), to estimate the total (i.e. per-genome) deleterious mutation rate for fitness in the asexual filamentous fungus *Aspergillus niger*. This approach implies the accumulation of

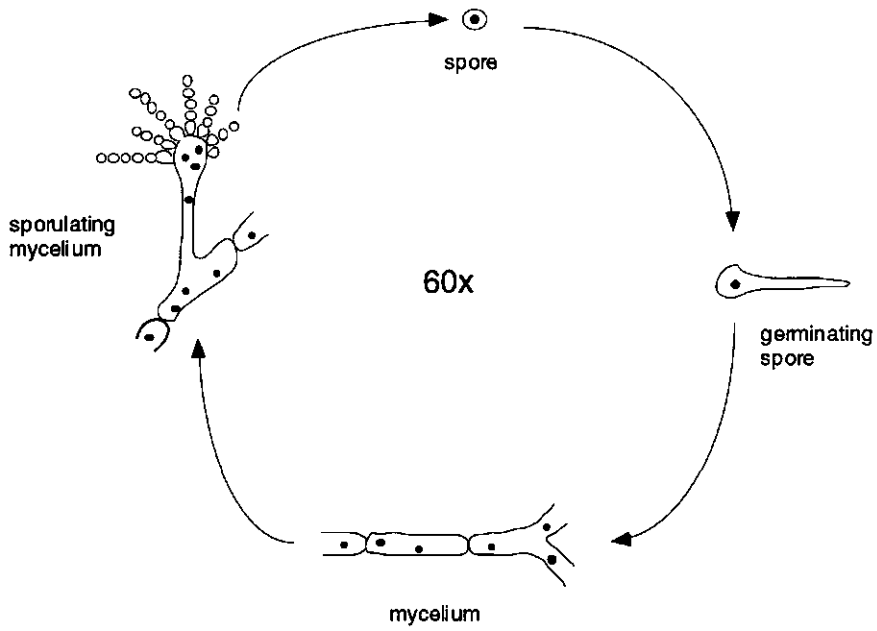

Figure 1. Mutation-accumulation protocol for the asexual fungus*Aspergillus niger*, illustrated for a single mutation-accumulation line. Twenty-five mutation-accumulation lines from a common origin have been maintained under conditions of minimal selection during 60 generations by transferring single spores to fresh medium every generation. One generation takes 3-4 days; mycelium and spores are haploid, but mycelial cells are multinucleate.

deleterious mutations in the absence of selection in a number of independently maintained lines of common origin. Each line is maintained by repeatedly transferring a single spore to fresh medium (see Figure 1). The decrease in mean and increase in variance of fitness among the lines permit a conservative estimate of the total deleterious mutation rate per generation (Bateman 1959; Mukai 1964). We used *A. niger*, because its generation time is short and many individuals can be easily handled. Although spores have a single haploid nucleus, we expected selection to be greatly absent during the vegetative part of the life cycle, since cells in the mycelium are multinucleate. Therefore, shielding of nuclei carrying deleterious mutations is expected by the majority of wildtype nuclei.

As a measure of fitness we used the relative number of spores produced in competition with a single reference strain. Kondrashov and Houle (1994) found support for the existence of conditionally deleterious

mutations, i.e. mutations that are neutral in some ('benign') and deleterious in other ('harsh') environments. Since they found a particularly strong negative effect of crowding on the fitness of lines that accumulated mutations in *Drosophila*, we measured fitness in two environments, wich differed with respect to the density at the start of the competition.

Materials and methods

Strains

In the mutation-accumulation experiments, we used *Aspergillus niger* strain N402 (Bos et al. 1988). This strain is a mutant of wildtype isolate N400 (CBS 120.49), with short conidiophores and normal black spores. In the fitness assay, competition of the mutation-accumulation (MA) lines of strain N402 was studied against wildtype isolate F8A9 of *A. niger* from Indonesia, with normal (long) conidiophores and fawn-coloured spores (kindly provided by A.D. van Diepeningen, Wageningen Agricultural University). Strain F8A9 is vegetatively incompatible with ancestral strain N402 (A.D. van Diepeningen, unpublished results).

Media and Culture Conditions

The proliferation of the MA lines was performed on a solid minimal salt medium (described in Coenen et al. 1994), containing 1% saccharose, in order to avoid the accumulation of auxotrophic mutants. In the fitness assay, the same medium was used. Fitness was also measured on a starch medium, to test whether selection had occurred during mutation-accumulation (see below). The starch medium differed from the minimal medium only with respect to the carbon source used: 1% starch in stead of 1% saccharose. Viable counts were performed on minimal medium, containing 2% of saccharose and 0.05% of Triton X-100. Triton X-100 was used to facilitate counting high numbers of colonies by reducing the size of the colonies. Saline, containing 0.8% sodiumchloride in deionised water, was used for the spore suspensions. During mutation-accumulation as well as in the fitness assay, all strains were kept at 30°C in the dark.

Mutation-accumulation protocol

The experiments started with spreading a thin spore suspension, made from dry spores of strain N402 stored on silica-gel (at 5°C), on a plate with minimal medium. After a few days, a single sporulating colony was randomly chosen and a spore suspension was made from it using a wet inoculation needle. The density of the spore suspension was estimated with a Coulter counter (Coulter ZF6 system, Coulter Electronics Ltd.), diluted to roughly 100 spores per ml and used to inoculate 25 plates by spreading 0.2 ml with a Drigalski spatula, so that individual colonies could originate from single uninucleate spores. The colonies grown on these plates represented the first generation of the MA lines. After a few days, a random sporulating colony was selected from each plate, its spores were harvested and a diluted spore suspension was spread on 25 fresh plates. For each of the 25 MA lines, this procedure was repeated every three to four days, until generation 60.

A sample of spores of the ancestral strain and each of the MA lines at generation 30 and 60 was taken to inoculate a 25 ml bottle with solid minimal medium. These bottles were incubated for one week and then stored at 5°C in the dark. A few weeks after the experiment, once again all cultures were transferred to a 25 ml bottle with fresh medium, incubated for one week, and stored at 5°C in the dark.

Competition experiments

The fitness of the ancestral strain and the MA lines at generations 30 and 60 was assayed in a competition experiment by measuring the relative number of spores produced when grown together with the reference strain (F8A9). One MA line was omitted from this assay, because, as inferred from the size of the spores, it had most likely become diploid before generation 30.

Before competition, a spore suspension of reference strain F8A9, ancestral strain N402 and all MA lines was spread on minimal medium. After three days all plates were brought at 5°C to synchronise spore germination (Debets et al. 1989) and after 16 hours, fresh spores were harvested and spore suspensions of $2 \cdot 10^7$ spores$\cdot$ml^{-1} saline were made. The two competitors of each experiment were then mixed by bringing 0.25 ml of both spore suspensions together, resulting in a High Density mixture. This mixture was diluted in two steps to a Low Density mixture

of $2 \cdot 10^4$ spores·ml^{-1}. Of each High and Low Density mixture, 0.1 ml, containing about $2 \cdot 10^6$ (giving High Density competition) and $2 \cdot 10^3$ (Low Density competition) spores respectively, was spread on three replicate plates with minimal medium. This resulted in 49 (ancestral strain + 24 MA lines at generation 30 and 60) * 2 (competition densities) * 3 (replicas) = 294 competition plates, which were incubated for five days at 30°C before they were put at 5°C and the spores of the new generation were harvested. Initial and final ratio of the spores of the two competitors were estimated by making viable counts on minimal medium with 0.05% Triton X-100. Typically, a few hundred colonies of both competitors together were counted on four plates, the minimum number of one competitor being 15 colonies in one occasion. In total 246,881 colonies were counted.

As measure of fitness we used the difference in the logarithm of final and initial competitor ratio:

$$r_{ij} = \ln[N_i(1)/N_j(1)] - \ln[N_i(0)/N_j(0)]$$

where $N_i(1)$ and $N_j(1)$ are the numbers of colonies of MA line and reference strain respectively after competition, and $N_i(0)$ and $N_j(0)$ are these numbers before competition. Our fitness measure, r_{ij}, can be written slightly differently to represent the selection-rate constant (Lenski et al. 1991; Travisano et al. 1995). We did not use the relative ratio, W_{ij}, of final and initial spore number of both competitors (Lenski et al. 1991), because this implies a model involving cell doublings that is not appropriate for a sporulating fungus.

Control for absence of selection

We minimized selection by starting each generation from a single spore, but could not avoid selection against mutations that for example prolonged germination time. In order to obtain a qualitative indication whether or not selection has restrained mutation accumulation, we also measured fitness of ancestral strain and nine randomly chosen MA lines at generation 60 in a novel environment: minimal medium with starch in stead of saccharose as carbon source (cf. Travisano et al. 1995). If selection has inhibited the accumulation of (a class of) mutations, it would affect mutations that determine fitness in the MA environment, and not necessarily mutations determining fitness in a rather novel environment.

In that case, one would expect a higher genetic variation in fitness in the novel environment. Starch was chosen, because small defects in the enzyme amylase, necessary to hydrolyse starch before saccharose can be taken in, may affect growth on starch medium, but not on saccharose medium.

Procedures for estimating deleterious mutation rate and mean mutation effect

For the two accumulation periods and the two competition densities, the genetic variance for fitness among the MA lines, V_G, was calculated using a single-classification model II ANOVA (Sokal and Rohlf 1981), in which MA lines were a random factor: $V_G = \{MS_{MA\ lines} - MS_{error}\}/3$, where MS = Mean Squares and 3 represents the number of replicate fitness measures.

The analytic procedure is based on a method proposed by Bateman (1959) and adopted and more fully explained by Mukai (1964; Mukai et al. 1972). This method assumes the fitness of MA lines to decline linearly and the numbers of mutations accumulated to be distributed according to a Poisson distribution. Then, the total deleterious mutation rate, U, and the mean selection coefficient against deleterious mutations, s, can be calculated from the following two equations:

$$M = Us \qquad (1)$$

$$V_M = U(s^2 + V_s) \qquad (2)$$

where M is the rate of decline per generation of mean fitness of the MA lines, V_M is the rate of increase of the genetic variance in fitness among MA lines per generation and V_s is the variance of mutation effects. Due to the low fitness of the ancestral strain (see Results), we estimated U and s from a comparison between generation 30 and 60. Therefore, M has been estimated by dividing the difference in mean fitness between generation 30 and 60 by 30 and V_M has been estimated by dividing the difference in genetic variance in fitness between generation 30 and 60 by 30 (cf. Houle et al. 1992). Assuming $V_s = 0$, U and s can be calculated from $U >= M^2/V_M$ and $s <= V_M/M$, leading to a conservative estimate of U. By assuming a probably more realistic (Mukai et al. 1972) exponential distribution of s, giving $V_s = s^2$, U becomes twice as large and s twice as small.

Table 1. Genetic variance for fitness of nine randomly chosen MA lines at generation 60 on Saccharose and Starch. Given are the median and 95% confidence interval from 1000 bootstrap resamplings (as described in the Materials and methods section).

	Saccharose	Starch
High Density	0.262 [0.013 - 0.542]	0.096 [0.023 - 0.200]
Low Density	0.118 [0.033 - 0.214]	0.106 [0.032 - 0.204]

To obtain confidence limits to the estimated genetic variance on saccharose and starch and to the estimated parameters M, V_M, U and s, we performed bootstrap resampling. Bootstrap resampling was done hierarchically, by first sampling MA lines and then sampling three replicate fitness values from a normal distribution with a mean equal to the mean fitness of that MA line and a variance equal to that of the residuals for all MA lines for the generation and competitive density involved. Since the fitness of MA lines at generation 30 and 60 are not independent, we have resampled pairs of fitness for each MA line drawn.

Results

Did selection occur during mutation accumulation?

As a control for possible selection during mutation accumulation, we measured fitness of nine randomly chosen MA lines at generation 60, not only on the standard medium during mutation accumulation with saccharose as carbon source, but also on medium with starch as only carbon source. If selection against newly arisen deleterious mutations had occurred, the genetic variance for fitness is expected to be higher on starch medium. In Table 1, it can be seen that this is not the case. Under High Density (HD) competition, the genetic variance for fitness is even higher on standard saccharose medium, although not significantly (F=2.73, df=8, 8, p=0.09). Under Low Density (LD) competition, the genetic variance for fitness is equally high on saccharose and starch medium. In sum, therefore, we have no direct experimental indication that selection against newly arisen deleterious mutations has played a role during mutation accumulation in our experiment.

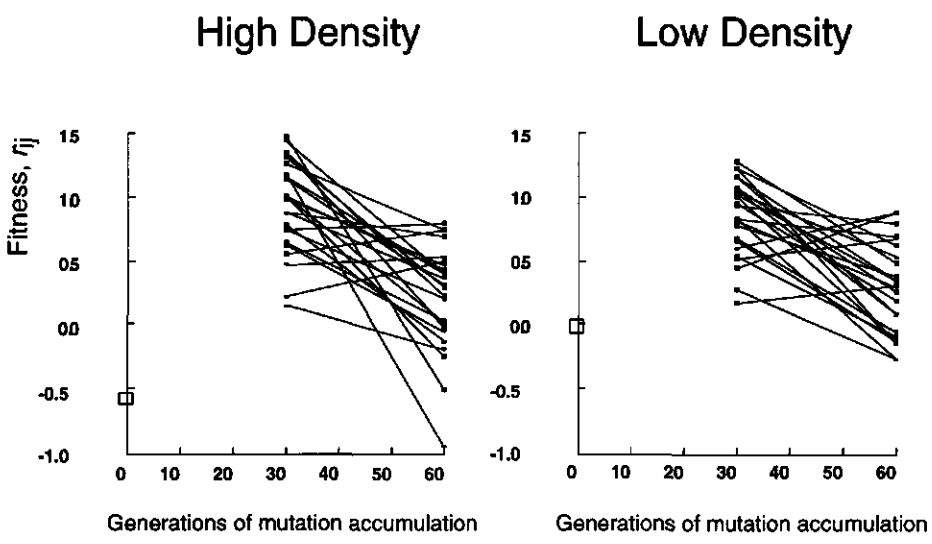

Figure 2. Fitness of ancestral strain and Mutation-Accumulation (MA) lines at generation 30 and 60 in a situation of High and Low Density competition. The open square represents the ancestral strain. Lines are no regression lines, but are only used to connect values of the same MA line. Every value is the mean of three estimates.

Fitness of the ancestral strain

In Figure 2, the mean values of our fitness estimates of ancestral strain and MA lines at both generations and competitive densities are shown. The ancestral strain, surprisingly, shows a lower competitive fitness than the MA lines at generation 30 and at generation 60, especially under HD-competition. The MA lines show a clear decrease in fitness between generation 30 and 60 under both competitive conditions, as expected.

To reject the possibility of a simple experimental error as the cause of the unexpectedly low fitness of the ancestral strain, we repeated the fitness assay of this strain and 10 randomly chosen MA lines, for the HD competitive situation. This time the ancestral strain was represented by 10 independent competition mixtures and every competition mixture was represented by two competition plates. Although fitnesses of individual lines differed somewhat from the original measurement, the overall pattern was similar: the ancestral strain showed the lowest fitness, the MA lines at generation 30 showed the highest and at generation 60 an intermediate mean fitness. Moreover, the fitness estimates of the repeated and the original measurement showed a significantly positive correlation

(Pearson ρ=0.51, n=21, p=0.018). Therefore, the method we used in our fitness assay appears sound, since it is capable of generating reproducible results. A genetic cause for the low ancestral fitness is not plausible, because the transfer of single uninucleate spores during mutation accumulation precludes the possibility of the accumulation of at least one advantageous mutation during 30 generations in every of the 24 MA lines. Possibly, the spores of the ancestral strain were less synchronised with respect to their physiological state than the spores of the MA lines. The high variation between the independent competition mixtures of the ancestral strain in our retrial experiment (results not given) is consistent with a varying physiological state of its spores. We will obtain our estimate of the deleterious mutation rate from a comparison between the fitness of the MA lines at generation 30 and 60.

Fitness at generation 30 and 60

The overall decrease in the mean fitness of MA lines from generation 30 to generation 60 is highly significant (see Table 2), strongly suggesting the accumulation of deleterious mutations. The generation Mean Squares is even significantly larger than the generation * line interaction MS (F=37.5, df=1, 23, p<0.001). Of the 24 MA lines, only four show a slight increase in fitness in this period, and these are the same four lines under HD-competition as under LD-competition. Competition density shows no

Table 2. Analysis of variance of main experimental factors at generation 30 and 60. *: p<0.01, **: p<0.0001.

Source	*df*	*MS*	*F*
Generation	1	29.707	890.6 **
Competition Density	1	0.004	0.127 ns
MA line	23	0.522	15.66**
Generation * Density	1	0.439	13.17 **
Generation * Line	23	0.792	23.74 **
Density * Line	23	0.209	6.27 **
Generation * Density * Line	23	0.101	3.01 **
Error	192	0.033	

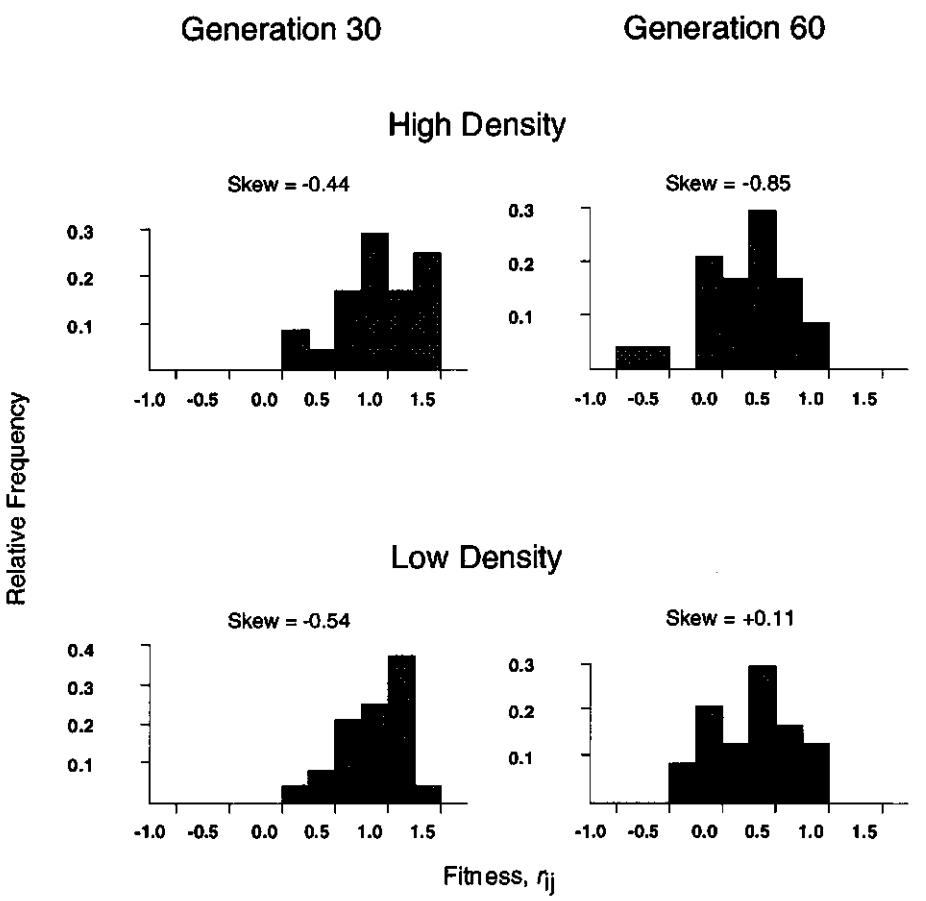

Figure 3. Frequency distribution of the fitness of MA lines at generation 30 and 60, in a situation of High and Low Density competition.

overall effect on fitness, which is due to the different patterns of fitness decline under different competitive densities. The significant interaction between Generation and Density indicates that the fitness decline is significantly more pronounced under HD- than under LD-competition (see also Figure 2). Figure 3 shows that the fitness distributions of the MA lines tend to be negatively skewed in three of the four situations.

The genetic component of the variance for fitness among MA lines is highly significant at generation 30 and 60 at both competition densities, as can be seen in Table 3. The fact that the estimated error variance varies threefold in value over the different competition densities and

Table 3. The results of four separate single-classification ANOVA's on the fitness of the MA lines.

Source	*df*	*MS*	*F*	*p*
High Density				
Generation 30				
Between lines	23	0.406	11.6	1.1E-12
Error	48	0.035		
Generation 60				
Between lines	23	0.537	29.0	8.2E-21
Error	48	0.019		
Low Density				
Generation 30				
Between lines	23	0.285	10.8	4.3E-12
Error	48	0.026		
Generation 60				
Between lines	23	0.398	7.5	2.9E-9
Error	48	0.053		

Table 4. Mutation parameter estimates for fitness. M, V_M, U and s are in units per generation. Given are the median above, and 95% confidence interval below from 1000 bootstrap resamplings, performed as described in the Materials and methods section.

	$Mx10^2$	V_Mx10^3	U	s
High Density	2.42	1.31	0.19	0.056
	[1.66 - 3.21]	[-2.46 - 6.52]	[-4.05 - 2.43]	[-0.128 - 0.235]
Low Density	1.89	1.20	0.19	0.065
	[1.23 - 2.48]	[-1.41 - 3.72]	[-2.66 - 3.72]	[-0.079 - 0.230]

generations, suggests an uncontrolled factor in our fitness assay. If lumped over both generations, the error variance is somewhat higher under LD-competition than HD-competition. This is probably due to the higher impact of sampling variation at the start of competition on competition outcome, if a smaller total number of spores is involved in the competition. The genetic variance for fitness increases between generation

30 and 60 in both HD- and LD-competition, although not significantly (see Table 4; HD: F=1.40, df=23, 23, p=0.21; LD: F=1.33, df=23, 23, p=0.25).

Estimating U and s

In Table 4, the estimates of the decrease in mean, M, and increase in genetic variance, V_m, of line fitness between generation 30 and 60 are given, together with the resulting estimates of U (lower limit) and s (upper limit) and their 95% confidence intervals. The best estimate for the lower limit of U from our data is 0.19 per generation, both under High and Low Density competition. The confidence intervals of the estimates of U are, however, large and include both zero and one. This is mainly due to the large confidence interval of our estimates of V_m. Furthermore, the distribution of bootstrap samples of U shows strong peakedness and is negatively skewed in the situation of HD-competition, and slightly positively skewed under LD-competition (distributions not shown). Contrary to our expectation based on the stronger fitness decline under HD-competition, the data could not show an effect of competitive density on the number of mutations showing effect (U), nor on the estimated mean effect of mutations (s).

Discussion

This paper is the first report of an experimental estimate of the total rate of deleterious mutations in a lower eukaryote. We estimated this rate in the filamentous fungus *Aspergillus niger* to be at least 0.19 per generation. Our estimates of U and s are somewhat lower, but still rather well in agreement with the high estimates that are available from studies on *Drosophila* (Mukai et al. 1972) and a few plant species (Charlesworth et al. 1994; Johnston and Schoen 1995). However, caution should be taken with extrapolating our results for three reasons. First and most importantly, because the confidence intervals for our estimates are large. Secondly, because the unexpectedly low fitness of the ancestral strain might cast some doubt on our methodology of measuring fitness. Thirdly, because we did not measure competitive fitness of the MA lines directly against the common ancestor, but relatively to a genetically marked reference strain. However, complex interactions that decrease the correlation between a

strain's competitive ability versus the reference strain and its competitive ability versus the common ancestor may not be very important, since they did not occur during 2000 generations of selection in *Escherichia coli* (Travisano et al. 1995).

Low fitness of the ancestral strain

The low fitness of the ancestral strain found in two trials is a most remarkable result. A genetic cause for the increased fitness of the MA lines after 30 generations is not plausible, due to the extremely high mutation rate that is necessary to generate at least one favourable mutation in each of the MA lines in this period. Estimates of the mutation rate in the bacterium *Escherichia coli* suggest that the ratio between advantageous and deleterious mutations is very low: advantageous mutations were estimated to occur at a rate of 10^{-9} to 10^{-10} per cell per generation (Lenski et al. 1991), while deleterious mutations appeared to occur with a rate of at least 0.0002 per cell per generation (Kibota and Lynch 1996). Assuming a similar ratio in *A. niger*, even if the deleterious mutation rate would be as high as 1 per generation, on average no more than 0.0015 advantageous mutations would be expected in each MA line after 30 generations. Moreover, no apparent selection for competitive ability has been exerted in our experiment during mutation-accumulation. An uncontrolled environmental factor is not a likely cause either, since all competition experiments have been performed simultaneously in a single randomised-block design.

A variable physiological state of the ancestral spores seems to be most consistent with the data, since that might explain the high variation in fitness found for the 10 replicates of the ancestral strain in our retrial. The possible physiological variability of the ancestral spores may have two reasons: (1) variability may have occurred during the longer maintenance of the ancestral strain than the MA lines at 5°C (i.e. from the start of mutation-accumulation), or (2) the ancestral strain may have been variable already when it was taken from silica-gel, while the rigid transfer-regime of the MA lines has forced their spores to become highly synchronised physiologically. In that case, our attempts to synchronise the spores by transferring them to fresh medium and giving them a cold treatment (Debets et al. 1989) were apparently not sufficient to obtain a

same level of synchrony among the spores from the ancestor and the MA lines.

Reasons why *U* is underestimated

Although we did not find direct empirical evidence that selection has restrained the accumulation of deleterious mutations in our experiment, our estimate of *U* is an underestimate for a number of reasons. First, our comparison between the genetic variance for fitness on the accumulation medium and on medium with a novel carbon source, might have revealed only selection against a small fraction of mutations, i.e. mutations affecting amylase function. Second, the conditions during mutation-accumulation make it likely that the scala of mutations that were allowed to accumulate was limited. For instance, lethal and (leaky) auxotrophic mutations were selected against in this environment, and the regime of transferring spores to fresh medium every 3 to 4 days prevented the accumulation of mutations that decelerate germination. Third, the assumption that all mutations have equal effects is not realistic (e.g. Mukai et al. 1972). Under the more realistic assumption of mutation effects following an exponential distribution (Mukai et al. 1972; Crow 1993), our estimate of *U* becomes twice as high and *s* twice as small.

However, there is also a reason why *U* may be overestimated and *s* underestimated. The methodology we used assumes a linear fitness decline between generation 30 and 60. A departure from linearity would bias our estimates towards a higher *U* and lower *s* (Houle et al. 1992). Mukai (1969) found evidence for synergistic interaction between mutations in *Drosophila* in later generations, i.e. after generation 40. Since we have analysed generations later than 40, synergsitic epistasis may have significantly affected the fitness at generation 60 in our experiment as well. Therefore, we cannot preclude synergism between deleterious mutations in our experiment. In total, however, we think that the total rate of deleterious mutations in *A. niger* may well be higher than the estimated 0.19.

Competition density

Our results show a more pronounced decline of fitness of the MA lines under high density competition. This result is consistent with the finding of a stronger decline in fitness of MA lines with crowding in *Drosophila*

(Kondrashov and Houle 1994). However, our estimates of U and s for high and low competition density are not very different. Therefore, they do not support Kondrashov and Houle's finding of conditionally deleterious mutations, nor the possibility of increased deleterious effects of mutations under high competition density.

This paradoxical combination of results may be confounded by a combination of unconditionally deleterious mutations and increased synergistic epistasis under conditions of high population density. We found empirical support for synergistic interaction between deleterious mutations under conditions of high population density (i.e. *K*-selection), and not under conditions of low population density (i.e. *r*-selection) in *Chlamydomonas* (De Visser et al. 1996b). There, we used a test for epistasis based on the skewness of the log fitness distribution of the offspring from a cross, negative skewness being an indication of synergistic epistasis. The finding of an increase in negative skewness of the log fitness distribution of the MA lines from Low to High Density competition at generation 60, although not significantly (t_s=-1.44, df=46, p(two-tailed)=0.16), may indicate the prevalence of synergistic epistasis under conditions of high population density in *Aspergillus* as well. Since a synergistic fitness decline would bias our estimates towards higher U and lower s, our estimate of U should then be lower and our estimate of s higher in the situation of high density competition. If so, our results do not support Kondrashov and Houle's (1994) finding of conditionally deleterious mutations, but are rather consistent with unconditionally deleterious mutations that show an increased effect under conditions of high density.

Implications for the evolution of sex

Clearly, the estimate for U from this study could not settle the discussion on the plausibility of the empirical evidence obtained so far for the DMH (e.g. Hurst and Peck 1996). For this purpose an accurate estimate of the total rate of deleterious mutations is needed that is clearly higher than one. Recently, the total rate of deleterious mutations in the prokaryote *Escherichia coli* has been estimated to be in excess of 0.0002 per cell division (Kibota and Lynch 1996), which is consistent with the few values available for *Drosophila* (Mukai et al. 1972) and a few plant species (Charlesworth et al. 1990, 1994; Johnston and Schoen 1994), if relative genome size and number of cell divisions is considered. Therefore, the

present data are consistent with a constant per-base-pair (and per-locus) rate of deleterious mutations per nuclear division, and not with a constant per-genome mutation rate found in a wide variety of microbes (Drake 1991). If so, the sex advantage generated by the DMH is probably too small to provide the sole explanation for the maintenance fo sex in lower eukaryotes, due to their smaller genomes and smaller number of cell divisions per generation.

An interesting test of the significance of the reproductive mode for the deleterious mutation rate, would be to compare our estimate of U in the asexual fungus *A. niger* with a similar estimate in the related facultatively sexual species *A. nidulans*. If the DMH bears some importance for protists like *Aspergillus*, one would expect a higher rate of deleterious mutations in *A. nidulans*. However, although obligatory asexual, *A. niger* does have a way to exchange genes. All *fungi imperfecti* have the ability to recombine genetically via heterokaryosis and the parasexual cycle, i.e. the karyogamy and subsequent segregation and recombination of two genetically different nuclei (Caten and Jinks 1966). Although karyogamy in a heterokaryon is known to occur infrequently in *A. niger* (about one in a million spores are diploid: J.A.M. Debets, personal communication), perhaps an occasional round of parasexual recombination may provide most of the benefits due to purging the genome from deleterious mutations (see Hurst and Peck 1996).

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5

Interaction between genetic markers that affect fitness in *Aspergillus niger*

ABSTRACT In this paper we investigate whether and how a number of randomly chosen marker mutations interact in their effect on fitness, which is relevant for our understanding of the evolution of sex. If synergistic interaction prevails, the main function of sex may be to facilitate selection against deleterious mutations. We constructed strains of the filamentous fungus *Aspergillus niger* with variable combinations of marker mutations. The marker mutations included two colour mutations, five auxotrophies and two resistances. Two components of fitness were measured on supplemented medium: mycelial growth rate and maximum number of spores produced. The results show that interactions between markers have a strong but diverse effect on both fitness parameters, because interactions of opposite type, synergistic and antagonistic, were found. However, most marker combinations tend towards synergism with respect to maximum spore production, while no tendency towards a prevailing type of interaction was seen for the mycelial growth rate. The results are consistent with the previous finding that synergism is weak and only apparent if fitness is density-dependent.

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Introduction

Whether or not deleterious mutations interact in affecting fitness can have important consequences for the mutation load of a population (Kimura 1961; Kimura and Maruyama 1966), and the advantage sex can give to reduce this load (Crow 1970; Kondrashov 1982). If the interaction is synergistic, i.e. if the logarithm of fitness declines more rapidly than linearly with the number of mutations, sexual reproduction may be maintained because it reduces the mutation load (Kondrashov 1988; Charlesworth 1990). At present, the empirical evidence for synergistic epistasis is rather sparse (e.g. Hurst and Peck 1996). Furthermore, most experimental studies present indirect tests that produce ambiguous data (Kondrashov 1993).

The simplest way to study experimentally the relationship between the number of deleterious mutations and fitness experimentally would be to measure the fitness of individuals with a varying, but known, mutation load. However, direct information on mutation number of particular individuals is very difficult to obtain. Therefore, experimental studies on epistasis between deleterious mutations have been based on indirect estimates of the individual mutation load, by assuming a relationship between mutation number and some other experimental variable which can be known more accurately. Examples of such assumptions include a linear relationship between mutation number and the period of mutation accumulation (Mukai 1969), a linear relationship between mutation number affecting fitness and inbreeding coefficient, (Willis 1993, and refs. therein), a rank-order relationship between mutation number and fitness (De Visser et al. 1996a), and a symmetrical distribution of individual mutation number among the offspring from a cross (De Visser et al. 1996b). All these assumptions make the conclusions from such studies less certain.

In this paper, we report on direct measurements of fitness interactions between deleterious mutations. We constructed strains of the filamentous fungus *Aspergillus niger* with a common genetic background, that vary only with respect to a number of marker mutations. The marker mutations involved were randomly chosen and include two spore colour mutations, two resistances, and five auxotrophies. We measured the fitness of all strains on supplemented medium to tone down the severe

fitness effects of the auxotrophic mutations. Consequently, a marker mutation that causes a fitness decrease can be interpreted as a deleterious mutation with small effect in the relevant part of the underlying metabolism. For instance, an auxotrophic mutation with decreased fitness on supplemented medium relative to the wild type, can be interpreted as a deleterious mutation in the upstream part of the relevant metabolic pathway, e.g. in a specific permease.

Recently, we have found some experimental support for the notion (Hamilton et al. 1990; Szathmáry 1993) that synergistic epistasis between deleterious mutations is likely to prevail in a situation of density-dependent selection, while it is unimportant or absent under density-independent selection (De Visser et al. 1996b). Here, we aim at further investigating the possible dependence of synergism on ecological conditions. For that purpose, two components of the fitness of *A. niger* were measured: the mycelial growth rate and the maximum number of spores produced on a limited amount of medium. We argue that the former fitness parameter may be important under density-independent selection, while the latter may predict fitness in a density-dependent situation.

Materials and methods

Parental strains

Strain N411 and N890 of *Aspergillus niger* (Bos et al. 1993) were used as parental strains for the construction of the multiple-marker strains. Strain N411 has one marker mutation on chromosome 1, i.e. olive-coloured conidiospores (*olvA1*). Strain N890 carries eight marker mutations, one on each of the eight chromosomes. The eight marker mutations of strain N890 are (on increasing chromosome number): a colour mutation: *fwnA1* (fawn-coloured conidiospores); five auxotrophic markers: *argH12* (arginin deficiency), *pyrA5* (pyrimidin deficiency), *leuA1* (leucin deficiency), *pheA1* (phenyl-alanin deficiency) and *lysD25* (lysin deficiency); and two resistancies *oliC2* (oligomycin resistance) and *crnB12* (chlorate resistance).

Media

The basic medium was a minimal medium (MM), containing 8.5g saccharose, 6g $NaNO_3$, 1.5g KH_2PO_4 and K_2HPO_4, 0.5g $MgSO_4 \cdot 7H_2O$, 0.5g KCl, ±1mg of the spore-elements $FeSO_4$, $ZnSO_4$, $MnCl_2$ and $CuSO_4$ and 15g agar per liter; pH = 5.8. Isolation of the mutants and the fitness assays were performed on this medium, supplemented (MMsup) with 348mg arginin, 244mg uridin, 197mg leucin, 165mg phenyl-analin, 365mg lysin and 200mg ureum per liter. For testing the genotype of haploid segregants, 200mM ClO_3^- and 20mM ureum was added to MM to test for *crnB12*, and 1mM oligomycin to test for *oliC2*.

Construction of multiple marker strains

Although *Aspergillus niger* is an asexual fungus, segregants of the two parental strains can be obtained by forced haploidisation of diploids that arise spontaneously in a heterokaryon (Pontecorvo et al. 1953). In this way, from a diploid of strain N411 and N890, $2^8 = 256$ different haploid segregants can be obtained with all possible combinations of the parental markers. Methods for obtaining heterokaryons, for isolating diploids, haploidising diploids, and isolating haploid segregants have been described by Bos et al. (1988).

Haploid segregants could be distinghuished by their fawn or olive spore colour from the sporulating diploid mycelium, which has the wild-type black colour due to complementation. About 2500 segregants were isolated, purified by transferring spores from single spore heads and tested for marker genotype on eight selective media: MMsup (control), MMsup in which serially arginin, lysin, leucin, phenyl-alanin and uridine were left out, MMsup with ClO_3^- and ureum, and MMsup with oligomycin. It appeared difficult to isolate segregants with many marker mutations, due to poor growth. We isolated 186 of all 256 possible multiple marker strains (73%).

Fitness assay

Two components of fitness were measured: the mycelial growth rate and the maximum number of spores produced on a colony with limited diameter on a limited amount of nutrients. Since the rate of spore production rather than the growth rate of the mycelium is a component of fitness, we estimated the correlation between colony surface area and the

total number of spores of that colony in a pilot experiment. The correlation between colony surface area and total spore number was measured for five multiple-marker strains that vary in marker number from one to eight. Each strain was grown on 10 plates that were placed at 5° after varying periods of growth. All spores were harvested and their total number was estimated from a sample (0.5ml) with the aid of a Coulter counter (Coulter ZF6 system, Coulter Electronics Ltd.). Within strains the correlation between colony surface area and spore number showed a highly significant correlation in all cases (at least ρ=0.95, n=10, p<0.001). The overall correlation for the five strains, by lumping all data, was slightly lower but still highly siginificant (ρ=0.87, n=43, p<0.001). Thus, the mycelial growth rate, expressed as the increase of mycelial surface area per unit time, seems a good predictor of the rate of spore production.

(a) Mycelial growth rate (MGR): The medium for this assay (i.e. MMsup) was prepared in a single batch. Petri dishes (9cm diameter) were filled with 20ml of medium with the aid of a calibrated pump. Each of the 186 multiple-marker strains was brought on two plates by pricking a platinum needle with spores in the middle of the plate. Plates were randomised and put in the dark at 26°C. The colony diameter was measured in two perpendicular directions after three days and again after 12 days. The colony surface area at both points in time was calculated, and the difference divided by the 234h it took to grow it.

(b) Maximum spore production (MSP): The medium for this assay, four times diluted MM with supplements in normal concentrations, was prepared in a single batch. Test-tubes with a diameter of 1cm were filled with 1ml medium with the aid of a dispensette. Two test-tubes were inoculated with one of each of the 186 multiple-marker strains, by bringing spores with a platinum needle in the middle of the test-tube. Test-tubes were semi-closed with a click cap, randomised over eight racks and placed in the dark at 26°C for 10 days. All spores were harvested by bringing 6 ml saline, containing 0.8% sodium chloride and 0.05% Triton-X100, on top of the colony in each test-tube. After 10s of vigourous shaking on a Vortex at maximum speed, the spore density of a 0.5ml sample was counted on a Coulter counter and total number of spores in each of the cultures was calculated.

Statistical analyses

Due to infection or breakage of cultures, four individual estimates of the mycelial growth rate and three estimates of the maximum spore production have been omitted from the analysis. Where the mean fitness of genotypes is used in the analysis, the value of the one remaining replica was used for these genotypes.

To test whether the prevailing type of interaction between the markers in a 'complete subset of strains' (see Results) is significantly synergistic or antagonistic, we used a test that is very similar to one we recently presented for another haploid organism, the alga *Chlamydomonas* (De Visser et al. 1996a). The principle of the test is to compare the mean log fitness of the strain with only the colour marker and the strain with all the markers ('extreme strains'), with the mean log fitness of all strains with an intermediate number of markers ('intermediate strains'). If log fitness would decrease linearly with the number of marker mutations (i.e., if mutations would have independent effects), the mean log fitness of the intermediate strains would coincide with the mean log fitness of the extreme strains. However, under synergism, involving a convex curve relating mutation number and log fitness, mean log fitness of the intermediate strains is expected to be higher than that of the extreme strains, and vice versa for antagonism. Therefore, the prevalent type of interaction, synergism or antagonism, was tested with a one-sample two-tailed t-test (Sokal and Rohlf 1981) comparing the mean log fitness of the intermediate strains with the mean value of the extreme strains.

In principle, a marker mutation can have a negative as well as a positive effect on fitness, if measured on supplemented medium. We wished to include both effects in our analyses, because both are relevant for the study of mutation interaction: synergism between deleterious mutations coincides with antagonism between advantageous alleles. The confounding principle (that is used in our test) is that synergism, irrespective of the sign of the fitness effect, causes the double-mutant to have lower fitness than expected on the basis of the two single mutants, and vice versa in the case of antagonism.

Four-way ANOVA was performed on the complete subsets of strains with four markers to measure the relative contribution of single markers and their interactions to the total variation of a fitness

Table 1. Numbers of expected and isolated multiple-marker strains from parental strains N411 and N890, classified into categories with equal mutation number.

Marker number	Expected number of strains	Isolated number of strains	
1	2	2	(100%)
2	14	13	(93%)
3	42	36	(86%)
4	70	51	(73%)
5	70	46	(66%)
6	42	31	(74%)
7	14	7	(50%)
total	**256**	**186**	**(73%)**

Table 2. The isolated number of strains with a specific marker (in bold on diagonal), or combination of marker mutations.

	fwn	**olv**	**arg**	**pyr**	**leu**	**phe**	**lys**	**oli**	**cm**
fwn	**95**								
olv	*	**91**							
arg	47	43	**90**						
pyr	47	46	45	**93**					
leu	42	46	43	42	**88**				
phe	49	41	45	44	43	**90**			
lys	35	31	31	30	28	33	**66**		
oli	41	41	40	41	40	39	22	**82**	
cm	56	54	53	56	53	52	51	49	**110**

component. The distribution of the signs of interaction, indicating synergism or antagonism, among the subsets was tested with a binomial test against the null-hypothesis that in case of no interaction both signs would occur equally frequently. The difference in the frequency of the sign of interaction for both fitness parameters was tested with a Wilcoxon Signed-Ranks test of the paired one-sample t values of the test described above.

Results

Number and genotype of strains isolated

We isolated 186 of all 256 possible multiple-marker strains (73%). As can be seen in Table 1, the fraction of all strains that was isolated decreased with increasing marker number. This was probably caused by the small size and poor sporulation of these genotypes, which made isolation difficult. Therefore, it is likely that of the strains with a high marker number, we isolated only those with a relatively high fitness. Consequently, the data set as a whole is probably biased towards a concave (i.e., antagonistic) relationship between marker number and log fitness, and does not represent a random sample of all possible haploid segregants of the diploid we used.

In Table 2, the number of strains isolated with a specific marker, and with a specific combination of two markers, is given. A relatively low number of strains carried the *lys*-marker (significantly less than 50%: χ^2=15.7, df=1, p<0.001), while *crn* was found in more than 50% of the strains (χ^2=6.2, df=1, p<0.05). Furthermore, the combination of *lys* and *crn* was found in more strains than expected from the frequency of the individual marker mutations (χ^2=4.7, df=1, p<0.05), while the combination of *oli* and *lys* occurred somewhat less than expected (χ^2=2.1, df=1, 0.1<p<0.2).

Fitness of all strains

Figure 1 shows the natural logarithm of fitness versus number-of-markers plot for both fitness parameters. The mycelial growth rate (MGR) shows a clear, slightly antagonistic, decline with increasing number of markers. However, the fitness decrease of the maximum spore production (MSP) is not so clear. The high fitness of strains with seven markers may either be caused by the occurrence of both types of interaction, or by the isolation bias mentioned above, or by both. If isolation was biased, the difference between the two patterns in Figure 1 suggests that it more strongly affected the MSP than the MGR.

In view of this presumed isolation bias in the data set as a whole, we restrict our analysis of mutation interaction to complete subsets of strains, i.e. groups of strains in which all possible combinations of a particular set of markers are present. The whole data set contains two

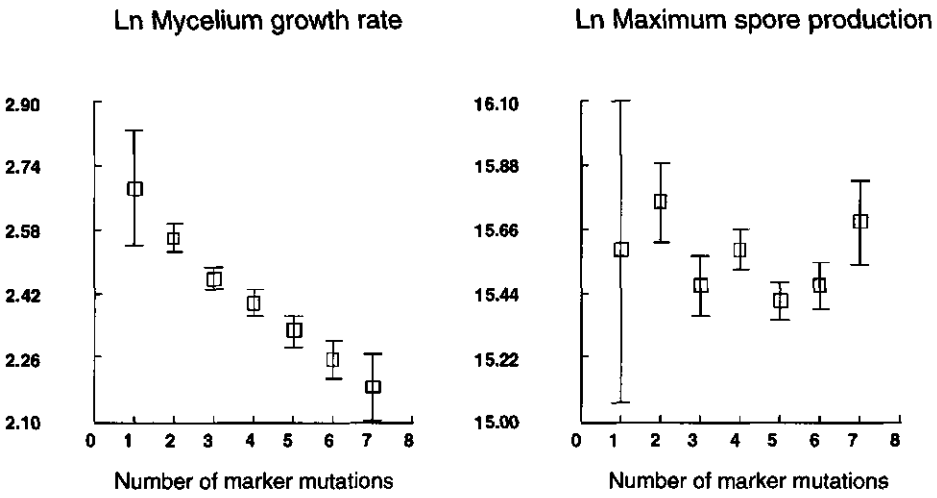

Figure 1. Log fitness for all strains with an increasing number of marker mutations for the two fitness parameters: the mycelial growth rate, and the maximum number of spores produced. Error bars are standard errors, based on the mean value of each strain.

Table 3. Mean ANOVA results of the 16 complete subsets of strains with four markers on both fitness parameters. %Var = percentage of total variance explained by a specific factor. *: p<0.05, **: p<0.01, ***: p<0.0001.

		Ln (MGR)		Ln (MSP)	
Source	*df*	*MS*	% Var	*MS*	% Var
Single marker	4	0.335***	29.3%	0.244*	5.8%
Interactions					
2 markers	6	0.344***		1.374***	
3 markers	4	0.253***		1.553***	
4 markers	1	0.063**		0.625**	
total	11	0.660***	68.6%	3.552***	88.9%
Error	15	0.006	2.1%	0.057	5.3%

complete subsets with five markers, 16 with four markers, 35 with three markers and 32 with two markers.

Analysis of variance on complete subsets of strains

To estimate the relative contribution of single markers and the different orders of interaction between them on the total fitness variance, separate

ANOVA's were performed on each of the 16 complete subsets with four markers. In Table 3, the averaged results of these 16 ANOVA's are given. The error involved in estimating the MSP appears to be somewhat larger than the error in estimating the MGR, but genetic effects can explain most of the variation in both parameters (97.9% for the MGR and 94.7% for the MSP). Individual markers have a much stronger effect on the MGR than on the MSP. However, markers do not have independent effects on either of the two fitness parameters, since on average all orders of interaction (involving two, three or four markers) are significant. Clearly, the interaction between markers has a stronger effect on the MSP than on the MGR, since it can explain a larger fraction of the variance of the former (88.9%) than of the latter parameter (68.6%).

Moreover, interactions show a decreasing effect on the MGR with an increasing number of markers involved. This suggests that lower-order interactions, i.e. between pairs of markers, partly mask each other's effects on the MGR. The MSP is most strongly affected by interactions between three markers, and only interactions between four markers have a decreasing effect. These results do, however, not reveal the nature of the interactions, i.e. whether they are synergistic or antagonistic.

The nature of interaction

Table 4 shows the results of a test of the nature of the interaction between the four or five markers of the corresponding 18 complete subsets only. One subset shows significant antagonism with respect to the MGR at the 5% level (p=0.015). Consequently, the interactions between four or five markers do not result in a significant prevailing type of interaction in any of the subsets for neither fitness parameter, after correction for multiple comparisons.

However, a significantly higher number of subsets show a tendency towards synergism with respect to the MSP (binomial test: p=0.0038). The slighlty higher occurrence of subsets with a tendency towards antagonism for the MGR is not significant (binomial test: p=0.24). Within subsets, a significant tendency towards synergistic interaction exists for the MSP relative to the MGR (Wilcoxon Signed-Ranks test: Z=3.38, n=18, p<0.001). Therefore, although individual subsets do not show significant interaction of one type, there appears to be a tendency towards synergistic interaction for the MSP, while no prevalence of a specific type of interaction can be

Table 4. The nature of interaction between the markers in the 18 complete subsets of strains with four or five markers.

	Ln (MGR)		Ln (MSP)	
Complete subsets[1)]	D/I[2)]	t_S[3)]	D/I	t_S
5 markers				
1. (olv) arg, pyr, leu, oli, crn	D	+0.30	D	-0.05
2. (olv) arg, pyr, leu, phe, oli	D	-0.03	D	+0.03
4 markers				
1. (fwn) arg, pyr, phe, crn	D	-0.95	D	-1.31
2. (fwn) arg, pyr, oli, crn	D	-0.15	D	-0.58
3. (fwn) arg, phe, lys, crn	I	-0.16	D	-0.18
4. (fwn) pyr, leu, oli, crn	D	-0.44	D	-0.44
5. (fwn) pyr, phe, lys, crn	I	+0.67	D	+0.13
6. (fwn) pyr, phe, oli, crn	D	-0.84	D	-0.93
7. (fwn) leu, phe, oli, crn	D	-0.37	D	-0.24
8. (olv) arg, pyr, leu, phe	D	+1.07	D	-0.32
9. (olv) arg, pyr, leu, oli	D	+0.50	D	-0.07
10. (olv) arg, pyr, leu, crn	D	+2.80*	I	+1.43
11. (olv) arg, pyr, phe, oli	D	+0.004	D	-0.23
12. (olv) arg, pyr, oli, crn	D	+0.02	D	-0.50
13. (olv) arg, leu, phe, oli	D	+0.007	I	-0.44
14. (olv) arg, leu, oli, crn	D	+0.31	I	-0.04
15. (olv) pyr, leu, phe, oli	D	+0.11	D	-0.14
16. (olv) pyr, leu, oli, crn	D	+0.25	D	-0.63

1) The marker between brackets is the background colour marker.
2) 'D/I' refers to the decreased (D) or increased fitness (I) of the strain with the maximum number of markers (four or five) relative to the strain with only the colour marker.
3) 't_S' = one-sample *t*-value from a test of the nature of interaction (described in the Materials and methods section); a negative value indicates synergism, a positive value antagonism; * = $P<0.05$.

shown for the MGR. Some caution should be paid with the interpretation of this result, since different subsets may be not fully independent due to the occurrence of the same markers in different subsets.

Table 5. The effect of marker number on the nature of interaction.

Marker number	Number of subsets	Ln (MGR) t_S[1)]	(SE)	Syn/Ant[2)]	Ln (MSP) t_S	(SE)	Syn/Ant
2	32	+0.29	(0.47)	17/15	-31.1	(27.7)	30/2
3	35	+0.21	(0.17)	20/15	-0.05	(0.10)	17/18
4	16	+0.18	(0.22)	6/10	-0.28	(0.14)	14/2
5	2	+0.14	(0.16)	1/1	-0.01	(0.04)	1/1

[1)] t_S: the mean (and SE) of the one-sample *t*-values of the test of the nature of interaction (as described in the Materials and methods section), performed on the individual complete subsets of strains. A positive value indicates antagonism, a negative value synergism.
[2)] 'Syn/Ant': the numbers of subsets in which the sign of the *t*-value indicates synergism or antagonism, respectively.

The effect of marker number on the nature of interaction

The nature of an interaction may not only depend on the fitness parameter affected, but also on the number of markers involved. In order to test this, we have performed the one-sample *t*-tests for the type of interaction also for the complete subsets with two and three markers (see Table 5). Again, also for complete subsets with two and three markers, none of the individual tests were significant (after correction for multiple comparisons). For the MGR, the low mean level of antagonism among pairs of markers decreases with increasing marker number. The ratio of subsets with a tendency towards synergism and antagonism, however, remains roughly equal. For the MSP, an average tendency towards synergism is apparent in subsets with different marker number, which shows some decline with marker number. The high mean negative value of t_S for the MSP found in pairs of markers is mainly due to two subsets in which the two 'intermediate strains' have almost equal fitness (causing the denominator of t_S to become almost zero). Strikingly, however, subsets with three markers show the lowest mean level of synergism and an equal ratio of subsets with prevailing synergism and antagonism.

Discussion

In this paper, we have studied whether and how mutations interact in affecting fitness, which may have important consequences for

understanding the maintenance of sexual reproduction (Kondrashov 1988). Our approach has been to measure two fitness components of strains of *Aspergillus niger*, that have been constructed to obtain a known number of marker mutations from a randomly chosen set of markers. We have assumed that these marker mutations provide a good model for studying epistasis between spontaneously occurring slightly deleterious mutations.

How good is our model?

A good model system for studying the nature of the interactions between slightly deleterious mutations should meet the following premises.

First, the genotype of the mutants should be accurately known. In all other studies on this topic, the number of mutations carried by individuals is derived from some other variable that can be manipulated experimentally (Mukai 1969; Willis 1993; De Visser et al. 1996a; De Visser et al. 1996b). However, in this study individual mutation loads are known precisely from testing on selective media. The error stemming from misinterpreting individual mutation loads, therefore, is negligible.

Second, the fitness of the mutants should be accurately measured. The high fraction of the total variance of both fitness parameters that could be explained by differences between genotypes (97.9% for the MGR and 94.7% for the MSP) in our study, makes us confident that we have met this requirement.

Third, the marker mutations of the model system should relate to those regulatory aspects of the metabolism that are most generally relevant for determining fitness. Only then spontaneously occurring slightly deleterious mutations may show a similar type of interaction. Of all marker mutations that we used, *oli* is the only marker whose negative fitness effect can be directly interpreted in metabolic terms: this mutant suffers a defective energy transduction due to a malfunctioning subunit of the enzyme ATPase (Edwards and Unger 1980). A negative fitness effect of each of the five auxotrophic mutants (*arg*, *pyr*, *leu*, *lys* and *phe*) can be interpreted as a deleterious mutation in the uptake system of the relevant metabolic route. In *Escherichia coli*, transport enzymes of nutrients that are regularly growth limiting are thought to be the major targets for selection (Dykhuizen and Dean 1990; Travisano et al. 1995). Since all active nutrient uptake is organised similarly, interactions between the five

auxotrophic markers may be relevant for the fitness of *Aspergillus* under growth limiting conditions. The *crn* marker of *A. niger* that we used is probably similar to the *crnA* marker in *A. nidulans* (Brownlee and Arst 1983), which causes a nitrate permease with a lower affinity for chlorate, and in young mycelium also for nitrate. Its effect on fitness in our assay was not strong (which is consistent with its high occurrence among the strains we isolated), which is possibly due to the compensatory uptake of ureum. We conclude that all marker mutants are involved in rather unrelated parts of the metabolism. Therefore, the conclusions of our study are relevant for interactions between deleterious mutations that occur in different metabolic routes.

Epistasis between slightly deleterious mutations

Our results warrant three conclusions. First, interactions between mutations appear to be very important for both fitness parameters studied, although their impact on the MSP was stronger. This means that mutations do not have independent effects on either fitness parameter. The effect of interaction apparently decreases with an increasing number of mutations involved, if they affect the MGR. On the MSP, interactions between many mutations still have a relatively strong effect, although their effect also decreases if more than three mutations are involved. In a number of studies on the radial growth rate in fungi, the alleles involved were found to have mainly additive effect, if inherited from parents that are not of too divergent origin (Caten 1979 and refs. therein). Simchen (1966) also found simple additivity between alleles affecting the growth rate of the Hymenomycete *Schizophyllum commune*, but he found indications for interactions between alleles controlling fruiting time and fruit weight. Since all these characters are thought to be under polygenic control (Simchen 1966; Caten 1979), these results are consistent with our finding of a low impact of interactions on growth rate if many mutations are involved, while interactions between many mutations affecting the spore production still have impact. Many models in population genetics assume independent action of the genes involved. Our results apparently do not support this assumption for a small number of genes. However, the results also suggest that the assumption of independent gene action may be reasonable if the effect of many genes is considered, especially if they affect fitness under density-independent conditions. The mutual

masking of interactions between a low number of mutations, especially if they affect the growth rate, is not understood, but seem a quality of the underlying metabolism. Possibly, the higher impact of interactions on the MSP is caused by a higher number of metabolic pathways affecting this parameter than the MGR.

Second, a low level of synergism appears to prevail among mutations that affect the MSP, while mutations that affect the MGR tend to show no prevailing type of interaction, or possibly a low level of antagonism. However, our results may be biased towards antagonism, because our isolation procedure favoured antagonistic combinations, particularly when many markers are involved. This isolation bias might have affected the MSP more severely than the MGR, because only sporulating segregants could be isolated. Therefore, the level of synergism found for the MSP may be underestimated.

Third, both types of interaction, synergism and antagonism, occur simultaneously between different combinations of mutations that affect the two fitness parameters in this study. This conclusion is justified by the combination of our finding of a low level or even absence of a prevailing type of interaction within the complete subsets of markers, while individual marker combinations showed significant interaction. Therefore, the evidence that we found for synergism between mutations that affect the MSP, is not the cumulative result of all interactions showing a low level of synergism, but rather of interactions of opposite type.

Implications for the evolution of sex

Our results show that synergism, where evident, is rather weak. This is consistent with the previous empirical reports of synergism in *Drosophila* (Mukai 1969), *Mimulus* (Willis 1993) and *Chlamydomonas* (De Visser et al. 1996a, 1996b). However, even very weak synergism between deleterious mutations can have a large effect on the equilibrium fitness of a population and the advantage sex may exert by facilitating selection against these mutations (Charlesworth 1990).

The different results for the MGR and the MSP are consistent with our previous finding of synergism between naturally accumulated deleterious mutations that affect the carrying capacity (K) and no prevalent interaction between mutations that affect the maximum growth rate (r) of

Chlamydomonas (De Visser et al. 1996b). We assume with it that the MGR of *Aspergillus* predicts fitness in a density-independent situation (like r) and the MSP in a density-dependent situation (like K). Since the MGR was measured in a situation where the major nutrients were plentiful (A. Coenen, personal communication), it was nearly maximal and equal to the intrinsic maximum growth rate, which is the sole determinant of density-independent growth. The MSP was restricted externally by nutrient concentration and colony diameter (the diameter of the test-tube). Therefore, this parameter reflects the colony's efficiency of producing spores from limited nutrients in a limited space, both limitations being typically for a situation of density-dependent growth.

Hamilton et al. (1990) predicted synergism for fitness in stable environments with high population densities, since there selection would be truncation-like due to a limitation of space and nutrients. Szathmáry (1993) predicted the same dichotomy in the nature of epistasis for mutations affecting fitness in a density-independent and a density-dependent situation on metabolic grounds. He studied interaction between two mutations involved in the same metabolic route (or even the same gene). The results showed that two mutations have antagonistic effects if selection is for maximum flux through the pathway (related to density-independent fitness), and synergistic if selection is for an optimum concentration of an intermediate metabolite (i.e. pool size) or optimum flux (both thought to be related to density-dependent fitness). Since the mutations we studied relate to different metabolic routes, they cannot be explained by the model of Szathmáry (1993). However, we think the use of metabolic control theory to produce simple and testable predictions on the interaction of mutations involved in different metabolic mechanisms will be very useful for making progress on the subject.

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6

Synergistic epistasis between loci affecting fitness: evidence in plants and fungi

We have searched the literature for empirical evidence for the nature of epistasis between loci affecting fitness. Evidence for synergistic epistasis is relevant to the ongoing debate on the function of sex. In an earlier paper, we have argued that negative skewness of fitness distributions of offspring from full-sib crosses may reflect synergism. Here, we have studied the form of the distribution of a variety of quantitative characters related to fitness. Most examples have been found in the plant breeding literature, but also some data on fungi have been obtained. The fitness characters encountered include the mycelial growth rate in fungi, and earliness, resistance against pathogens, seed number, and pollen fitness in plants. Fitness components in plants show almost exclusively negative skewness, which is evidence for synergism, while the epistasis observed in fungi is more ambiguous. The relative importance of other causes of skewness than epistasis is discussed. We argue that the results provide substantial evidence for the hypothesis that sex is maintained because it facilitates the removal of deleterious mutations. The finding of synergism between resistance loci, however, can also be considered as support for the parasite hypothesis of the evolution of sex.

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Introduction

In recent years, studies on the evolution of sex have gradually changed from inventing new theories to developing and performing experimental tests (Hurst and Peck 1996). At present, over 20 theories rival to explain why sexual reproduction is maintained in the presence of asexual competitors (Kondrashov 1993). One very general theory of which the assumptions can be tested experimentally is the Deterministic Mutation Hypothesis (Crow 1970; Kondrashov 1988; Charlesworth 1990). The DMH emphasises the function of sex in facilitating natural selection against unavoidable deleterious mutations. Its attraction for experimentalists is due to the fact that the model has been studied thoroughly (Kimura and Maruyama 1966; Crow 1970; Kondrashov 1982; Charlesworth 1990), which has resulted in two simple and testable premises: (1) a sufficiently high rate of deleterious mutations, and (2) synergistic epistasis between mutations. Finding evidence for synergistic epistasis would furthermore increase the significance of the DMH relative to the hypothesis of Muller's ratchet (Muller 1964), because synergistic epistasis slows down the ratchet (Charlesworth et al. 1993; Kondrashov 1994).

Recently, we proposed a new experimental test for the nature of epistasis between deleterious mutations (De Visser et al. 1996b). This test considers the skewness of the log fitness distribution among the offspring of a sexual cross between two parents. Since every allele affecting fitness in the parents is inherited by half of the offspring, skewness of the log fitness distribution reveals prevailing synergistic (resulting in negative skewness) or antagonistic interaction (resulting in positive skewness) between the segregating alleles involved. The logarithm of fitness should be considered, since the absence of interaction between mutations means multiplicative mutation effects (Kondrashov 1988; Charlesworth 1990; Szathmáry 1993), causing a symmetrical distribution of *log* fitness. The notion that skewness of a phenotypic distribution may provide information on interaction between genes affecting this phenotype appears to be more than half a century old. In the 1930's, several authors interested in quantitative characters of plants like fruit size or age at maturity, found skewed distributions in crosses between different races, which they related to non-additive gene action (Rasmusson 1933; Powers 1936; Sinnot 1937; MacArthur and Butler 1938). For example, genes

affecting fruit weight in *Cucurbita pepo* (Sinnot 1937) and *Lycopersicon* (MacArthur and Butler 1938) appeared to have multiplicative effects, which was concluded from the positively skewed distribution of F1 and F2 fruit weights and the roughly normal distribution of *log* fruit weight.

In this paper, we used the skewness test to search the literature for empirical evidence for the nature of epistasis between loci affecting fitness in various organisms. However, the skewness test was originally developed for haploid organisms. In such organisms, the genetic contribution to the fitness distribution is exclusively determined by allelic effects and by interaction between loci (i.e. non-allelic interaction). In diploid organisms, dominance (i.e. allelic interaction) may affect the form of the distribution as well. Although dominance may not be very important for the form of the phenotypic distribution if many loci are involved (Rasmusson 1933), its effect relative to that of non-allelic interaction needs further study. An interesting opportunity for applying the skewness test to diploids is provided by studies using recombinant-inbred lines or doubled-haploid lines for the construction of genomic maps. Recombinant inbred lines are F1 recombinants that have been inbred during a number of generations (via selfing or sib-mating) to render most loci homozygous, while doubled-haploids have been made homozygous at all loci (e.g. Simpson and Snape 1981). The relative contribution of dominance to the skewness observed is studied by comparing progenies with different levels of homozygosity.

Critical to the skewness test is knowledge of the form of the relationship between fitness components and total fitness. Synergistic epistasis found at the level of the fitness component may result in antagonistic epistasis at the level of total fitness, if for instance total fitness would relate to its component by an exponentially increasing relationship. However, for most fitness components their relation to total fitness is still poorly understood. Furthermore, differences in experimental protocols and scale of measurement obscure the quantitative interpretation of the fitness component involved. Our approach, therefore, was to include all relevant fitness components encountered in our search, by leaving the original scale of measurement intact and assuming a linear relationship between each character and fitness. The effect of deviations from a linear relationship between fitness components and total fitness on our conclusions are discussed.

Methods

The phenotypic distributions used in this study were gathered by searching the literature, with emphasis on the plant breeding literature. The following criteria were used: (1) only full-sib progenies, i.e. offspring from two parents, were used; (2) the character should be quantitatively inherited; studies mentioning the segregation of only one or two genes and studies showing clearly bimodal distributions were excluded; (3) the quantitative character involved should be a fitness component, i.e. should affect survival and/or fecundity; (4) distributions should represent random samples of the offspring.

In order to obtain an estimate of the skewness of a distribution, the length of the columns in the frequency distributions was measured in half millimetres (on photocopies of at least 50% the original size). Next, the logarithm of the mean value of each phenotypic class was calculated, since skewness of the *log* fitness distribution reflects the relevant type of epistasis (see Introduction). However, in some data sets the value zero or negative values were included, which made log-transformation impossible. A transformation such as $\log(x + c)$, where c is a constant, is inappropriate because it would affect the skewness. Therefore, also the skewness of the untransformed data was measured. Negative skewness at the original scale would be even more negative at a log-scale, and thus provides conservative evidence for synergism. The significance of the skewness statistic (g_1) was tested with a two-tailed t-test, using the exact formula for the standard error of g_1 in cases with sample size smaller than 100, otherwise the approximation was used (Sokal and Rohlf 1981, p 139).

We assumed a linear relationship between a character and fitness. Two exceptions were made for different reasons. First, the character *mycelial growth rate* was originally given as a one-dimensional rate of increase (radial or linear growth rate). However, a better estimate of the fitness of fungi is given by the rate of spore production, and spore number has been found to show a highly significant positive correlation with mycelial surface area (De Visser et al. 1996c). Since the logarithm of the rate of increase of mycelial surface area (i.e. log fitness) is proportional to the untransformed rate of increase of the one-dimensional colony diameter, we used this character on the original linear scale as a representation of fitness at a log-scale. Fitness at a linear scale was obtained

for this character by raising the original data to the square. Taking the logarithm of the original one-dimensional growth data would result in more negatively skewed distributions. Second, the characters *earliness* and *resistance* were originally given in *days to flowering* or *days to maturity* and *some index of host infection*, respectively, which are inversely related to fitness. Therefore, a translation into the fitness components *earliness* and *resistance* was needed. In order to leave the original scale intact, *days to flowering* and *amount of infection* were mirrored in the mean value of each data set.

Where possible, an indication of the relative genetic contribution to the total phenotypic variance was given. The higher the genetic contribution, the less effect a possibly skewed error variance can have on the distribution of mean values. As an estimate of the relative genetic contribution, the value of the broad-sense heritability, $h^2 = V_G/V_{tot}$, was given where possible. However, in some studies only an estimate of the fraction of the total variance that could be explained with a number of significant quantitative trait loci (QTLs) was presented. This estimate provided a lower limit for the broad-sense heritability (indicated by ">" in Table 1).

Results

Table 1 presents the nature of epistasis for fitness components encountered in the literature. All data on haploids refer to fungal species, while all data on diploids are exclusively from plant species. Studies involving animals did either not concern fitness components, or did not present distributions (e.g. Shook et al. 1996). In 21 crosses evidence was found for synergistic epistasis at the appropriate log-scale (18 crosses showed synergism at the untransformed scale), while four crosses showed evidence for antagonistic epistasis (six crosses did so at the original scale). Nine crosses did not significantly reveal a prevailing type of epistasis (versus 21 crosses at the original scale).

Haploids

The only fitness component for which accurate data were found in haploids, was the mycelial growth rate for a number of fungal species.

Table 1. The nature of epistasis between loci affecting fitness characters in fungi and plants

Fitness component	Species	*F*	*n*	Skewness[a] log-scale	Skewness untransf.	$h_B^{2b)}$	Reference
Haploids							
Mycelial growth rate	*Aspergillus nidulans*	F1	98	+1.39	+1.80	0.45	Jinks et al. 1966
		F1	60	+2.51*	+3.84***	0.83	idem
		F1	96	+3.36**	+4.30***		Caten 1979[c]
	Schizophyllum	F1	95	-0.85	+0.18	0.93	Simchen 1966
		F1	100	+0.32	+1.59	0.85	idem
	Collybia velutipes	F1	87	-3.19**	+1.06	0.94	Croft & Simchen 1965
		F1	76	-0.18	+0.62	0.90	idem
		F1	76	-3.17**	-1.90	0.94	idem
		F1	68	-2.60*	-0.85	0.96	idem
		F1	95	-1.80	+1.47	0.83	idem
	Neurospora crassa	F1	84	-1.99*	-1.54	0.87	Papa et al. 1967
Diploids							
Earliness (flowering time)	*Hordeum vulgare*	DH[d]	69	-4.30***	-0.09	0.98	Laurie et al. 1995
Earliness (flowering time)	*Oryza sativa*	F8	194	-9.13***	-7.62***	>0.61	Xiao et al. 1996
Earliness (age at maturity)		F8	194	-7.68***	-7.18***	>0.74	idem
Earliness (flowering time)		F4	2418	-13.9***	-10.2***	>0.77	Li et al. 1995a
Earliness (flowering time)	*Arabidopsis thaliana*	F8	64	-4.63***	-3.20***	0.96	C Alonso Blanco[e]
Earliness (flowering time)		F3	50	-7.57***	-2.33*		Clarke et al. 1995

Table 1. *Continued.*

Fitness component	Species	*F*	*n*	Skewness[a] log-scale	Skewness untransf.	$h_B^{2b)}$	Reference
Diploids							
Earliness (flowering time index)	*Brassica oleracea*	F3	92	-0.71	+0.78	>0.54	Camargo & Osborn 1996
Earliness (spring bud flush)	*Populus*	F2	55	-2.84**	-2.05*	0.98	Bradshaw & Stettler 1995
Resistance to *Pyrenophora teres*	*Hordeum vulgare*	DH	150	-13.9***	-6.78***	0.92	Steffenson et al. 1996
Resistance to *Cochliobolus sativus*		DH	150	-5.55***	-0.98	0.91	idem
Resistance to potato virus Y (1,2)	*Capsicum annuum*	DH	94	-0.01	+1.20	0.90	Caranta & Palloix 1996
Resistance to *Pyricularia oryzae*	*Oryza sativa*	F7	281		-3.26**	>0.76	Wang et al. 1994
Resistance to *Rhizoctonia solani*		F4	255	-5.53***	-1.88	>0.70	Li et al. 1995b
Resistance to *Heterodera glycines*	*Glycine max*	F6:7	298	-36.7***	-6.45***	0.97	Webb et al. 1995
Resistance to *Gibberella zeae*	*Zea mais*	F3	112	+0.30	+2.90**	0.37	Pè et al. 1993
Resistance to *Xanthomonas campestris*	*Phaseolus vulgaris*	F3	70	-4.86***	-1.68	>0.75	Nodari et al. 1993
Resistance to *Acanthoscelides obtectus*		F2	±100	+4.64***	+6.44***		Kornegay & Cardona 1991
		F2	±100	+3.25***	+5.00***		idem
Resistance to *Verticillium*	*Solanum tuberosum*	F1	100		-4.71***		Concibido et al. 1994
		F1	100		-12.4***		idem
		F1	100		-7.69***		idem
		F1	100		-8.67***		idem
		F1	100		-2.15*		idem
		F1	100		-4.71***		idem

Table 1. *Continued.*

Fitness component	Species	*F*	*n*	Skewness[a] log-scale	Skewness untransf.	h_B^{2}[b]	Reference
Diploids							
Max. total seed number (long season)[f]	*Arabidopsis thaliana*	F8	64	+1.27	+2.47*	0.90	C Alonso Blanco[e]
Max. total seed number (short season)[f]		F8	64		+0.07	0.92	idem
Grain yield	*Oryza sativa*	F8	194	-4.08***	-1.10	>0.14	Xiao et al. 1996
Relative pollen tube growth at 41°C	*Zea mais*	F8	45	-1.10	-0.43	0.64	Frova & Sari-Gorla 1994
Relative pollen germinability at 41°C		F8	45		-1.61	0.68	idem
Pollen tube growth rate		F8	45		-0.76	0.71	Sari-Gorla et al. 1992
Pollen germinability		F8	45	-2.52*	+0.74	0.77	idem
Flowering synchrony (well watered)[g]		F3	234	-5.49***	-3.34***		Ribaut et al. 1996
Flowering synchrony (drought stress)[g]		F3	234	-11.0***	-8.61***	0.78	idem
Proportion flowering at end of season	*Brassica oleracea*	F3	92	-4.73***	-3.63***		Camargo & Osborn 1996

a) Skewness: t_s-value of the estimate of skewness statistic g_1, at a log-scale or at the untransformed original scale: negative values indicate synergism, positive values antagonsim; *:$p<0.05$, **:$p<0.01$, ***:$p<0.001$.
b) Broad-sense heritability $h_B^2 = V_G/V_{tot}$, where V_G is the genetic variance and V_{tot} is the total phenotypic variance.
c) Only continuous distribution of the three (at 35°C).
d) DH: doubled haploid lines produced from F1 plants.
e) Unpublished results.
f) Max. total seed number was extrapolated from a representative fruit by multiplying the seed number of this fruit with fruit number of the main stem and with the number of side branches and shoots containing fruits; long season: all plants ripened their seeds; short season: maximum total seed number when the slowest plant started seed ripening.
g) Male and female flowering synchrony expressed as the anthesis-silking interval in days.

Both types of epistasis were found for this character: two crosses showed antagonistic, four crosses synergistic, and five crosses no significant epistasis. At the untransformed scale, only significant antagonistic epistasis is found for two *Aspergillus* crosses. Unfortunately, mycelial growth rate in *Aspergillus* was measured on colonies (Jinks et al. 1966; Caten 1979), while in the other three species, it was measured as linear growth in special growth tubes (Croft and Simchen 1965; Simchen 1966; Papa et al. 1967). Therefore, it is unclear whether the difference in epistasis for *Aspergillus* and the other three fungal species is due to experimental method or to a difference in epistasis in different species or crosses.

Diploids

All diploid data concern plant species, for which a relatively large number of examples was found in the plant breeding literature on the characters earliness and resistance against pathogens. For earliness, all crosses that revealed significantly skewed distributions showed negative skewness, indicating synergism. The only study lacking significant negative skewness for earliness was on *Brassica oleracea*, wich presented a flowering time index that included the proportion of plants of a line that flowered at a given time (Camargo and Osborn 1996) instead of 'days to flowering' used in all other studies. In six of the seven crosses involved, significant negative skewness was also present at the untransformed scale, which emphasises the robustness of this result.

For resistance against pathogens, all but two crosses showing significant skewness were negatively skewed. For resistance against *Pyricularia oryzae* in *Oryza* and against *Verticillium* in *Solanum*, only a skewness measure of the untransformed data is available. The significantly negative skewness at this scale suggests that skewness will certainly be negative at the log-scale. The two crosses showing significantly positive skewness were different from the other studies on resistance in at least three respects. First, the resistances concerned in all other studies were against micro-organisms (mostly fungi, a bacterium, and a nematode), while the two crosses showing antagonism concerned resistance against an insect, the bean weevil (*Acanthoscelides obtectus*). Second, the resistance in the two aberrant crosses involved resistance of the seeds (i.e. beans) instead of resistance of the vegetative tissue of the plant that was the subject of all other studies. Third, while in all other

studies resistance was measured by some index of the relative amount of infection of the host plant, in these two crosses it was quantified by measuring the time until the adult weevil came out of the beans. No skewness was observed for resistance against potato virus Y(1,2) in *Capsicum* and for resistance against *Gibberella zeae* in *Zea mais*. At the untransformed scale, nine crosses showed significantly negative skewness versus three crosses that revealed significantly positive skewness.

For the other fitness characters, skewness of the distributions, if significant, was only negative at the log-scale. These fitness characters included grain yield, pollen germinability, male and female flowering synchrony and the proportion of all plants of each F3 family that were still flowering or budding at the end of the season (for a variety of species). No significant skewness was observed at this scale for total seed number in *Arabidopsis*, and for some components of pollen fitness in *Zea mais*. However, in three of these five crosses only a value of the skewness of the untransformed data was available, due to the presence of zero values in the data.

The effect of error variance

A skewed error variance (on a log-scale) may have contributed to the skewness of log mean offspring performance, obscuring the skewness caused by epistasis. The relative contribution of the error variance is inversely related to the broad-sense heritability: $h_B^2 = V_G/(V_G + V_E)$, where V_G and V_E are the genetic and error variance, respectively. Thus, if the negative skewness found in most crosses would (partly) be due to a negatively skewed error variance, a positive correlation between the skewness, t_s, and h_B^2 is to be expected. Figure 1a shows that the correlation between t_s and h_B^2 is negative rather than positive, if calculated for all diploid data that presented an exact estimate of the heritability (ρ=-0.28, n=22, p=0.21; excluding the extreme value of t_s=-36.7: ρ=-0.31, n=21, p=0.18). Therefore, no evidence for a generally significant contribution of the error variance to the negative skewness was found in our data. If skewed, the error variance would be rather positively skewed, thereby partly masking the negative skewness due to epistasis. Sample sizes were too small to test this relationship for the various fitness components individually.

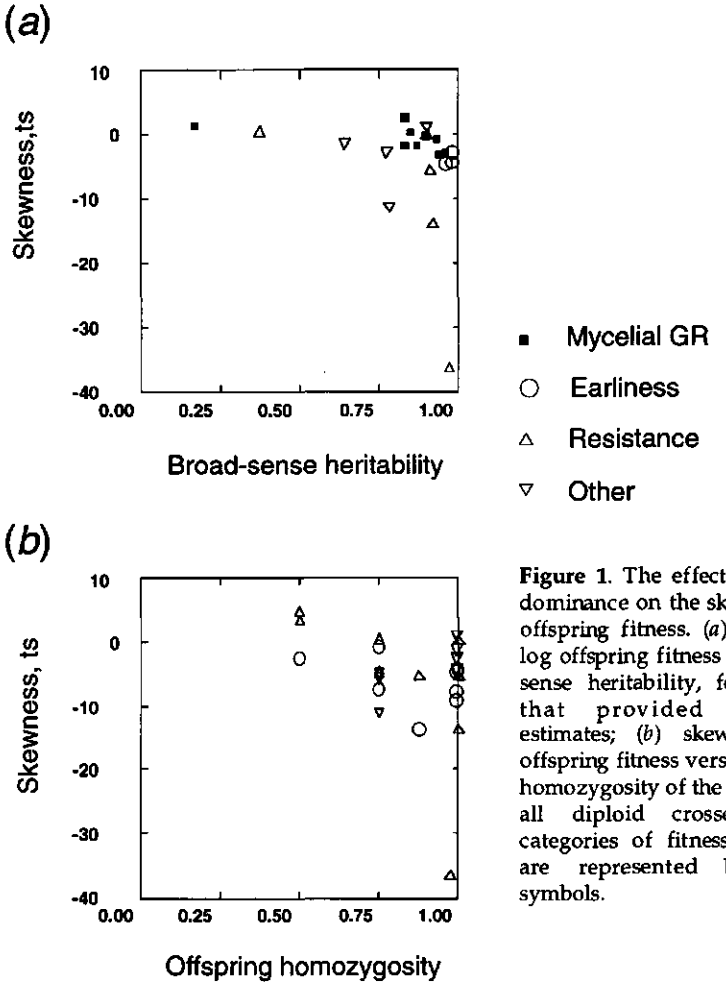

Figure 1. The effect of error and dominance on the skewness of log offspring fitness. (*a*) Skewness of log offspring fitness versus broad-sense heritability, for all crosses that provided heritability estimates; (*b*) skewness of log offspring fitness versus the level of homozygosity of the progenies, for all diploid crosses. Different categories of fitness components are represented by different symbols.

The effect of dominance

Dominance, causing the fitness of heterozygotes to deviate from the (log) mean value of both homozygotes, might have affected the skewness of the early generation distributions (F1 and F2) as well. However, since skewness was significantly negative in the almost entirely homozygous F8 and doubled-haploid progenies as well, dominance was clearly not the only cause of skewness. The relative contribution of dominance to the skewness may be revealed by comparing the skewness of progenies with different mean levels of homozygosity. If the negative skewness found would be partly due to dominance, one would expect the negative

skewness to decrease with increasing homozygosity of the progeny. Figure 1b shows the correlation between t_s and the mean homozygous fraction of progenies, derived from information on the number of generations the different recombinant inbred lines have been selfed. It has been assumed that the parents were homozygous and that the F1 was completely heterozygous. Doubled-haploid progenies were assumed to be completely homozygous. If the various fitness components were lumped for all diploids, an almost significantly negative correlation between skewness and homozygosity was found (ρ=-0.34, n=24, p=0.11). Therefore, we did not find evidence for an important contribution of dominance to the skewness observed. The increase in negative skewness with increasing homozygosity is likely to be due to synergistic epistasis between the segregating loci involved. Again, for individual fitness components sample sizes were too small to test this relationship.

Discussion

We presented the results of a literature search for the nature of epistasis between loci affecting fitness. A broad variety of fitness characters was encountered in a restricted number of organisms: fungi and plants. The results for the fitness component encountered for fungal species were ambiguous, but for plants synergistic epistasis was found to be common for most fitness components encountered.

Skewness and synergistic epistasis

Our conclusions depend on the assumption that the skewness of the log fitness distribution of full-sib offspring is due to epistasis between loci affecting fitness (De Visser et al. 1996b). However, epistasis is not the only possible cause of skewness. We will discuss the relative importance of three alternative sources of skewness.

In the first place, a negatively skewed error variance due to a skewed distribution of some environmental quality or measurement error might have contributed to the negative skewness that was observed for most characters. Since we considered skewness of the *log* fitness distribution, also a normally distributed error could have contributed to the negative skewness on this scale (Sinnot 1937). However, the error

variance should be high relative to the genetic variance to make a significant contribution to the skewness. We used our data to check the relationship between the relative contribution of the error variance and the level of skewness, but found no evidence for a positive correlation with negative skewness. However, our analysis was based on the simultaneous evaluation of the error variances of different fitness components, since we could not accurately measure the relative contribution of the error for individual fitness components. It is conceivable that the error may be skewed differently for different fitness components. The studies on the mycelial growth rate in *Collybia velutipes* (Croft and Simchen 1965) and *Neurospora crassa* (Papa et al. 1967) presented distributions of the parental performance as well, which showed a tendency to be negatively skewed. Croft and Simchen (1965) argued that deleterious mutations may be the reason for this pattern. In the plant studies, no information on the error variance was given, although the application of ANOVA in many studies might suggest that the error was roughly normally distributed. The high heritabilities presented in most studies suggest that the contribution of the (possibly skewed) error variance was relatively insignificant for most crosses of our review.

Secondly, dominance may have caused skewness in diploid progenies that are heterozygous at many loci (e.g. in the F2). For instance, if advantageous alleles are dominant, the heterozygote resembles the advantageous homozygote more than the disadvantageous homozygote, leading to negative skewness. Similarly, recessive advantageous alleles cause positive skewness. If some advantageous alleles are dominant while others are recessive, this may lead to mutual cancelling of the dominance effects, resulting in no skewness. Metabolic arguments exist that support the likelihood of advantageous alleles being dominant (Hoekstra et al. 1985). However, for the fitness components in this study, this appeared not to be generally true. Some studies presented the performance of the F1 and the parents, so that information on dominance could be derived from a comparison between these two generations. This revealed evidence for dominance of earliness alleles in *Populus* (Bradshaw and Stettler 1995), while in *Arabidopsis* (Clarke et al. 1995) and *Brassica* (Camargo and Osborn 1996) alleles appeared to be mainly additive. Resistance alleles were found to be recessive in *Phaseolus*, both for resistance against the bean weevil (Kornegay and Cardona 1991) and against common bacterial blight (Nodari

et al. 1993), and in *Capsicum* (Caranta and Palloix 1996), but dominant in *Oryza* (Wang et al. 1994), and both dominant and recessive in *Glycine* (Webb et al. 1995). However, dominance of earliness alleles did not affect the sign of skewness in the studies we reported, and therefore was at least not a very important factor. For resistance, two studies involving recessive resistance alleles showed positive skewness (Kornegay and Cardona 1991; Caranta and Palloix 1996), possibly indicating some dominance effect, but a third study involving recessive resistance alleles showed negative skewness (Nodari et al. 1993). Moreover, we tested the effect of dominance on skewness by comparing the skewness between different levels of homozygosity for all traits together, and found a slightly negative correlation between the level of homozygosity and skewness. This suggests that a possible contribution of dominance would cause rather positive than negative skewness, resulting from recessiveness of advantageous alleles. However, it is more likely that the increase in negative skewness with increased homozygosity is caused by the presence of synergism that is increasingly revealed by the disappearance of obscuring heterozygosity.

In the third place, skewness may be caused by selection of advantageous alleles during the repeated inbreeding of F1 lines. Each generation of inbreeding, segregants that carry a higher than average number of advantageous alleles for the trait under study might have a higher probability of being selected as parents for the next generation. This would lead to negative skewness in the later generations. A special case of such selection bias could be 'segregation distortion', i.e. deviation from the expected Mendelian segregation ratio due to competition among gametes or abortion of the gamete or zygote (Harushima et al. 1996). The result is a typically over-representation of a certain part of one parental genome in the offspring (e.g. Wang et al. 1994; Xiao et al. 1996). Segregation distortion favours genes with high fitness in the gametophyte and the young zygote, but does not necessarily affect genes that control fitness components later in life. Its effect on most fitness components in our study may, therefore, be limited. However, it cannot be ruled out that favourable alleles for traits like pollen fitness or grain yield have increased in frequency during several generations of inbreeding.

In general, however, negatively skewed log fitness distributions provide conservative support for synergistic epistasis. Selection tends to

cull individuals in the low-fitness tail of the distribution, causing a shift towards positive skewness. This is illustrated by the observation, that in dense monocultures of plants a typical change occurs from a normal distribution of seedling masses to a positively skewed distribution of adult plant masses. This phenomenon has been ascribed to competitive elimination of small individuals (White and Harper 1970; Ford 1975; Bazzaz and Harper 1976), although in grasses this change in the form of the distribution may be rather due to differential growth rates (Turner and Rabinowitz 1983). Individuals that have been eliminated should actually be given a fitness of zero, but these will be missed in many occasions. Measuring fitness in a laboratory environment may increase the chances of finding negative skewness, since selection will be limited under such benign conditions.

Epistasis for total fitness

Probably more important for our conclusions than the alternative sources of skewness mentioned above, is the simplistic assumption of a linear relationship between each component of fitness and total fitness. Since non-linear relationships change the skewness of the resulting total fitness distribution, deviations from linearity may affect our conclusions. One such deviation from linearity that is likely to occur is an optimum relationship between fitness component and total fitness, caused by trade-offs between individual fitness components. There is empirical support for the significance of such trade-offs (Stearns 1992; Shook et al. 1996; Rausher 1996). If the optimum curve has an overall concave shape, its second derivative is negative everywhere, which can be shown analytically to cause a negatively skewed distribution of total fitness if the distribution of the fitness component were Gaussian (De Visser et al. 1996b). Thus, even finding no epistasis at the level of the fitness component may lead to synergistic epistasis at the level of total fitness under optimum relationships. The effect of mapping an already negatively skewed distribution on an optimum curve has not been considered, but it seems very likely that this will also increase the negative skew, especially if the most frequent fitness component coincides with the optimum class. A saturation curve relating fitness component and total fitness will increase the negative skewness at the level of total fitness as well, because its second derivative is negative too. Only an exponentially

increasing (i.e. convex) relationship may affect our conclusions qualitatively. Therefore, it is likely that deviations from a linear relationship between fitness components and total fitness do not affect our conclusion that synergistic epistasis is present for most fitness components in plants.

Relevance for the evolution of sex

Our results demonstrate that loci controlling fitness in plants often show synergistic epistasis. In most studies, loci with relatively large effect were involved (explaining up to 50% of the phenotypic variation), which enhanced the detectability of synergism (De Visser et al. 1996b). Our main goal, however, was to find evidence for synergistic epistasis between slightly deleterious mutations, which are more relevant for the Deterministic Mutation hypothesis of the evolution of sex (Kondrashov 1988), due to their presumably much higher frequency (Kondrashov 1988; Charlesworth 1990; Kibota and Lynch 1996). How relevant are our results to epistasis between slightly deleterious mutations?

Obviously, our conclusions are directly relevant to slightly deleterious mutations that affect the loci with major effect for which we found synergistic epistasis. To what extent mutations affecting other loci will show synergistic epistasis for fitness cannot be answered directly. However, the finding of synergism between loci affecting such various components of fitness, suggests that synergism may be due to very general physiological or metabolic mechanisms. Furthermore, the results obtained for major loci in this study are consistent with the few empirical studies on epistasis between deleterious mutations with smaller effect. In an earlier study, we have found little evidence for epistasis between marker mutations with relatively small effect that affect the mycelial growth rate of *Aspergillus niger* (De Visser et al. 1996c), which is consistent with the ambiguous results obtained in this study for the same parameter in a number of fungal species. Willis (1993) studied epistasis between deleterious mutations affecting a number of fitness components in the monkey flower, *Mimulus guttatus*, by comparing different levels of mutation expression due to inbreeding. He found evidence of synergism only for pollen viability, which is consistent with our finding of synergism for pollen germinability in maize. However, we cannot check his failure to

find synergism for seed germination, flowering and flower production, because these fitness components are not included in our study.

There are other reasons why synergism between deleterious mutations is likely to occur in nature. In the first place, traits with a high impact on fitness are expected to be canalised, i.e. under the control of mechanisms that constrain the trait to be closer to the optimum (Rendel 1967). A trait can be canalised against environmental perturbations (e.g. changes in temperature), or against genetic perturbations (e.g. mutation). Genetic canalisation has been demonstrated to increase with a trait's impact on fitness in *Drosophila melanogaster* (Stearns and Kawecki 1994). If canalisation would be perfect up to a certain mutation load, this would result in truncation selection against mutations, i.e. no effect of mutation accumulation on fitness up to a certain mutation number, beyond which fitness decreases steeply. Less perfect canalisation may lead to more moderate synergistic mutation selection. That the phenotypic suppression of mutational damage to developmental homeostasis may be limited up to a certain mutation load has been hypothesised by Kimura and Maruyama (1966).

Secondly, synergism between deleterious mutations is likely to occur in situations of high population density (Crow 1988; Hamilton et al. 1990). The reason for this would be that limitation of space and nutrients causes truncation-like selection under such conditions. This argument seems very compelling to us, and might explain the prevalence of sexual reproduction in saturated environments (Hamilton et al. 1990; Szathmáry 1993). We have found some support for the prevalence of synergism for fitness under high population density in the unicellular alga *Chlamydomonas* (De Visser et al. 1996b) and the filamentous fungus *Aspergillus* (De Visser et al. 1996c).

The finding of synergism for plant resistance against pathogens is significant support for both main hypotheses on the major function of sex. It provides support to the DMH (Kondrashov 1988), emphasising the role of sex in eliminating recurrent deleterious mutations, but it may also be considered as support to the hypothesis that sex has evolved as a means to resist parasites (Jaenike 1978; Hamilton 1980). The preservation of resistance alleles, which is essential to the latter hypothesis, is largely enhanced by soft truncation selection (Hamilton et al. 1990). Other support to the parasite hypothesis comes from the finding of polygenic in stead of

monogenic ('gene-for gene') control of resistance in all instances cited in this study. Polygenic control of resistance has been shown to enhance selection for sex and recombination in the light of resisting parasites (Hamilton et al. 1990). To compare the significance of both hypotheses for the evolution of sex, only data on the deleterious mutation rate may be decisive.

To summarise, we have provided empirical evidence for a fairly general occurrence of synergistic epistasis between deleterious mutations in a variety of plant species. These results lend substantial support to the significance of the DMH (Kondrashov 1988) to explain the maintenance of sexual reproduction, especially in plants.

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7

Summarizing Discussion

In spite of decades of intense debate, the evolutionary reasons for sex are still unknown. In the light of the 'two-fold cost of sex' (Maynard Smith 1971; Williams 1975), a plausible short-term advantage to sex must be found to explain its maintenance. At present, two hypotheses seem to predominate (Crow 1994; Hurst and Peck 1996): the Parasite Hypothesis (e.g. Hamilton 1980), emphasising the selection pressure created by the presumed abundance of parasites, and the Deterministic Mutation Hypothesis (further referred to as DMH; e.g. Kondrashov 1988), which emphasises the necessity of sex to eliminate recurring deleterious mutations. As the number of theoretical solutions to the problem has risen to about 20 (Kondrashov 1993), the lack of knowledge of the genetics of natural populations that is needed to discriminate between the various ideas, has become inconvenient.

Progress on this topic can be made in two ways: either by testing the unique assumptions of a specific hypothesis, or by finding and testing the discriminating prediction. The latter has proven to be very hard: most predictions made by specific hypotheses are not unique (Hurst and Peck 1996). Especially the predictions of the Parasite Hypothesis resemble those of the DMH (Hamilton et al. 1990). For instance, both hypotheses predict the predominance of sexual reproduction in saturated environments, where selection is truncation-like due to limited space or nutrients (i.e. if selection ranks individuals with respect to fitness, and subsequently truncates the number of individuals by eliminating those with low fitness). Thus, it may be more revealing to test the assumptions that are unique to the various theories.

The present thesis has aimed at testing the DMH. The value of this hypothesis is not only its theoretical generality (deleterious mutations are inextricably bound up with DNA replication), but also its simple basis of only two discriminating assumptions: (1) a sufficiently high rate of deleterious mutations, and (2) synergistic interaction between deleterious mutations. These two assumptions have been addressed by performing

experiments designed to generate estimates for the relevant parameters involved in the assumptions.

Methodology

Four of the five chapters describe attempts to reveal the relationship between deleterious mutation number and fitness. More specifically, the question is raised whether mutations show synergistic interaction, i.e. whether mutations amplify each other's negative fitness effect. However, the lack of direct information on the number of deleterious mutations carried by specific individuals prevents a quick answer. Since a deleterious mutation is defined by its negative effect on fitness, information on the number of deleterious mutations can only be inferred from fitness measurements. In the chapters 2 and 3, two simple tests are proposed to derive relevant information on the relationship between mutation number and fitness from a sexual cross between two parents.

The test proposed in chapter 2 ('means test') is based on comparing the mean log fitness of parents and offspring: the concave relationship expected under synergism predicts a higher mean log fitness of the offspring, if both parents carry a sufficiently different mutation number. (The *logarithm* of fitness is considered, since the absence of mutation interaction means multiplicative mutation effects.) If both parents carry similar mutation numbers, synergism predicts a lower mean log fitness of the sexual offspring. Thus, for the 'means test' information on the difference in parental mutation number is needed. In chapter 3, an even simpler test ('skewness test') is proposed, which only considers the fitness of the offspring from a cross. The skewness of the log fitness distribution of the offspring reflects information on the nature of epistasis (i.e. interaction) between alleles at loci controlling fitness that segregate in the cross. This is so, because genetic recombination between the parental genomes produces a symmetrical distribution of the number of mutations among the offspring from a cross. If these mutations do not interact, they act additively at a log-scale and will produce a symmetrical log fitness distribution as well. Synergistic interaction is reflected by negative skewness at a log-scale.

Application of the means test to *Chlamydomonas* and *Aspergillus*

In **chapter 2**, the means test is applied to a number of crosses between strains of *Chlamydomonas moewusii*. Parental mutation number is manipulated by treating strains with different doses of ultraviolet radiation to cause additional deleterious mutations. Fitness of parents and offspring is measured by measuring the two logistic parameters of the asexual part of the life cycle in batch culture: maximum growth rate (r) and carrying capacity (K). These two parameters are thought to be relevant predictors of the fitness of asexual unicells under different ecological conditions: r in a disturbed, and K in a saturated and constant environment, but together they provide a complete description of the fitness (Bell 1991). The results show a significantly lower mean log fitness of the offspring relative to their parents in the 'high UV cross' for both r and K. Since in the high UV cross the parental fitnesses have become more similar than in the control cross, it is argued that their mutation number has probably become more similar as well. Therefore, the lower mean log offspring fitness in this cross reveals evidence for synergistic interaction between UV-induced mutations with respect to these fitness parameters.

In **chapter 5**, the means test is applied to strains of the asexual filamentous fungus *Aspergillus niger*. In this study, marker mutations are used as a model system to study epistasis between slightly deleterious mutations. Strains carrying different marker combinations are constructed by using the parasexual cycle of *A. niger*. The benefit of using marker mutations is that individual mutation number is accurately known. Here, the means test is applied by comparing the mean log fitness of a strain with for instance four markers and the wild-type strain, with the mean log fitness of all strains with intermediate numbers of these four markers: synergism is reflected by a higher mean log fitness of the 'intermediate strains'. The marker mutations involved are two colour, two resistance, and five auxotrophic mutations. To convert the auxotrophic mutations into slightly deleterious ones, two components of the fitness are measured on supplemented medium: the mycelial growth rate, which correlates strongly with the rate of spore production, and the maximum spore

production when nutrients and space are limited. The results show highly significant interactions between markers for both fitness components, but no prevalent synergism or antagonism. Thus, it is likely that some marker combinations show antagonistic interaction, while other combinations interact synergistically. A tendency towards synergism is demonstrated for the maximum spore production, but not for the mycelial growth rate.

Application of the skewness test to *Chlamydomonas*, fungi and plants

In **chapter 3**, the skewness test is applied to two *Chlamydomonas* crosses. Here, the focus of study is interaction between naturally accumulated instead of UV-induced (chapter 1) mutations. For this purpose, two strains are crossed that have been kept without sex in the laboratory for over 60 years. It is argued that the frequent transfer of samples of cells to fresh medium will have caused the accumulation of numerous deleterious mutations due to the action of Muller's ratchet (i.e. the random loss of the least-mutated cells; Muller 1964). As a control, two strains are crossed that presumably had not accumulated many mutations, since they were isolated only recently. The results show significant negative skewness of the distribution of log K in the 'accumulation cross', together with a highly significant increase of the genetic variance for K in this cross relative to the control cross. These results suggest that deleterious mutations have accumulated in the parents of this cross, and that they show synergistic interaction with respect to K. The lack of significant skewness of the log r distribution suggests no interaction between mutations there, although this result is less robust than the results for K since the power of detecting skewness is rather low.

Inferring conclusions from skewness on the nature of epistasis depends on the absence of other sources of skewness. For example, a skewed error variance that is large relative to the genetic variance may affect the skewness. Furthermore, the skewness test has originally been developed for haploids. In diploid organisms, dominance, causing the heterozygote to have a different fitness than the mean of both homozygotes, may cause skewness as well. In **chapter 6**, these alternative sources of skewness are studied in an analysis of data gathered from the

literature on a number of plant and fungal species. The fitness characters involved include the mycelial growth rate for fungi, and earliness, resistance against various pathogens, seed number and pollen fitness for plants. The results on the mycelial growth rate are ambiguous, but the skewness observed in the plant fitness components is almost exclusively negative, indicating synergistic interaction between the loci controlling these characters. It is argued that, due to the likelihood of a concave relationship (optimum curve) between fitness components and total fitness, the synergism found is likely to apply to total fitness as well. An optimum relationship between a fitness component and total fitness may arise from trade-offs between different fitness components. A special case is the synergism found between resistance loci: this result gives also support to the significance of the Parasite Hypothesis if selection for resistance is the main determinant of fitness (Hamilton et al. 1990). It is demonstrated that the negative skewness observed is probably not due to a skewed error variance or dominance. The latter result justifies a more general application of the skewness test to diploids. Taken together, these results provide substantial empirical support to a fairly general occurrence of synergism between deleterious mutations in plants.

Implications for the ecology of sex

The different results for the fitness parameters r and K in chapter 3 and mycelial growth rate and maximum spore production in chapter 5, provide support for the notion that synergism between deleterious mutations may depend on the ecological control of fitness: synergism may be only significant for fitness components like competitive ability, that are important in saturated environments. The extreme form of synergism is truncation selection, where fitness is unaffected up to a certain mutation number but drops sharply beyond. It has been notified that truncation-like selection is expected in saturated environments, where fitness is determined by competitive ability rather than exponential growth rate, due to limitation of nutrients and space (Crow and Kimura 1979; Crow 1994; Hamilton et al. 1990). Also on metabolic grounds synergism has been predicted to depend on the ecological control of fitness: only if selection favours a constant optimal concentration of some metabolic intermediate,

the effects of mutations on fitness are synergistic (Szathmáry 1993). If selection favours the most rapid synthesis of the end product of the (e.g. growth limiting) pathway, the effects of mutation are predicted to be antagonistic.

The carrying capacity (K) measured in *Chlamydomonas*, and the maximum spore production measured in *Aspergillus* are expected to predict fitness in density-regulated situations, while the maximum growth rate (r) and the mycelial growth rate rather represent fitness if nutrients and space are abundant. The selective finding of synergism for K and (of a tendency towards synergism) for the maximum spore production, but not for r and the mycelial growth rate in these two chapters, suggests that the predictions may be valid. If so, they provide an alternative explanation for the predominance of sex in saturated environments: sex is maintained in these environments since it facilitates selection against deleterious mutations that affect the relevant fitness components there, while sex exerts no advantage by purging the genome from mutations in disturbed environments because the necessary synergism is absent for the relevant fitness components for these environments. So far, the ecological predominance of sex in saturated environments has been explained by the Parasite Hypothesis (Trivers 1985; Hamilton et al. 1990) and by other hypotheses as well (Hurst and Peck 1996).

The rate of deleterious mutations

Chapter 4 addresses the other assumption of the DMH, i.e. whether the per-genome rate of deleterious mutations is sufficiently high. For this purpose a mutation-accumulation experiment, derived from a classical similar experiment with *Drosophila* by Mukai (1964), is performed in the asexual filamentous fungus *Aspergillus niger*. During 60 generations 20 mutation-accumulation (MA) lines with a common ancestor have been maintained under conditions of minimal selection to allow accumulation of mutations. At the end of mutation-accumulation, fitness of ancestral strain and mutation-accumulation lines at generation 30 and 60 have been measured in competition with a reference strain with differently coloured spores. From the expected decrease of mean fitness and increase of the genetic component of the variance in fitness, a lower limit of the

deleterious mutation rate (U) can be calculated. Unexpectedly, however, the ancestral strain appears to have a lower fitness than the MA lines. But, as expected, the fitness of the MA lines decreases significantly between generation 30 and 60 of mutation-accumulation. It is argued that the low fitness of the ancestral strain is probably due to a variable physiological state of the spores of the ancestral strain. The estimate of U obtained from a comparison between generation 30 and 60 is at least 0.19 deleterious mutations per genome per generation. This is the first estimate of the deleterious mutation rate for a lower eukaryote. The result is consistent with the few estimates available for *Drosophila* (Mukai et al. 1972) and a number of plant species (Charlesworth et al. 1990; Charlesworth et al. 1994; Johnston and Schoen 1995). However, the confidence intervals of our estimates are rather wide, which makes interpretation speculative.

Conclusions

The contribution made by the work described in this thesis is two-fold. In the first place, two simple experimental tests are proposed for the assumption of the DMH that deleterious mutations show synergistic interaction. These tests have proven to be simple and widely applicable, and may be valuable tools for further studies on the nature of epistasis (Hurst and Peck 1996).

Secondly, experimental data are generated that are relevant to testing both assumptions of the DMH. The application of the two tests for the nature of epistasis to a variety of experimental data involving the unicellular alga *Chlamydomonas* and the filamentous fungus *Aspergillus*, and to literature data on a number of fungal and plant species, has revealed a fairly general occurrence of synergistic epistasis between deleterious mutations. The results suggest also that synergism may be more pronounced in plants than in lower organisms like unicellular algae and fungi. The finding of the possible dependence of synergism on ecological conditions, which is consistent with theoretical predictions and with the ecology of sex, may lead to a refinement of the applicability of the DMH. Also, a first estimate of the rate of deleterious mutations (relevant to the other assumption of the DMH) in a lower eukaryote (*Aspergillus*) is given, together with the methodology to obtain such estimate. The

estimate obtained is too low to support the DMH as explanation for sex in lower eukaryotes. However, the uncertainty revealed by a wide confidence interval makes this result tentative.

In sum, the results obtained on the nature of epistasis provide conditional support to the DMH: sex may be maintained by facilitating selection against deleterious mutations in plants, and in lower eukaryotes that live in saturated environments. However, conclusions on its plausibility are hindered by the lack of knowledge of the deleterious mutation rate. The few estimates available suggest that the DMH may provide a significant advantage to sex in higher organisms like *Drosophila* and plants. The constant per-base-pair deleterious mutation rate that is consistent with the present data (including the recent estimate for *E. coli*), suggests that its mechanism may not be able to provide the sole explanation for sex in lower eukaryotes. However, also in these organisms the DMH may add to the advantages provided by sex due to other mechanisms, and consequently help its maintenance.

The future of the evolution of sex

Further study of the DMH is necessary and should primarily concentrate on estimating the deleterious mutation rate in various organisms. Such estimates would be especially interesting for lower anisogamous eukaryotes, because there the coexistence of sexual and asexual reproduction urges the finding of a short-term sex advantage most explicitly. Furthermore, data on the deleterious mutation rate may discriminate between the DMH and the Parasite Hypothesis unambiguously, while finding synergism can sometimes be interpreted as support for the Parasite Hypothesis as well (if synergism is for resistance against parasites, see discussion chapter 6). Mutation-accumulation experiments, like the one described in chapter 4, provide the most direct estimates. It is conceivable that various fitness components may be subject to different rates of deleterious mutations. Therefore, measuring various components of the fitness of mutation-accumulation lines may reveal information that is relevant to the ecology of sex, analogous to the finding of a possible ecological dependence of synergism.

However, the search for synergism should not stop. Knowledge of its generality may help to differentiate the applicability of the DMH. Improvements on the detection of synergism should be possible. Metabolic control theory (Kacser and Burns 1973, 1979) has proven to be a useful tool for predicting the nature of interaction between enzyme mutations (Szathmáry 1993). Predictions from such theoretical studies are well-defined and can easily be tested in simple organisms by comparing the relevant fitness components of double and single mutants (in ways similar to that of the experiment described in chapter 5). Better knowledge of the metabolic conditions for synergism can lead to refined predictions of the ecological predominance of sex that may be unique to the DMH.

In general, progress on the problem of the evolution of sex is best helped by finding direct and exclusive support for individual hypotheses. Studying the combined effect of sex advantages provided by different mechanisms might be helpful to reveal possible synergistic or antagonistic interaction between the separate advantages. However, accurate knowledge of the value of individual hypotheses is a prerequisite. The evidence required may either concern the discriminating prediction or the unique assumption(s) of any hypothesis. Since most predictions on the prevalence of sex under specific conditions appear not to be unique to single hypotheses (Hurst and Peck 1996), attempts to find a truly discriminating prediction may be futile. Furthermore, comparing the fitness of sexuals and asexuals in experimental settings meant to test a specific hypothesis is tricky, since it may be difficult to exclude all other possible causes that may generate an advantage to sex. Parameter estimates that are relevant to specific assumptions provide better controlled and more direct support.

References

Bateman, A.J. 1959. The viability of near-normal irradiated chromosomes. *International Journal of Radiation Biology* **1**:170-180.

Bazzaz, F.A. and J.L. Harper. 1976. Relationship between plant weight and numbers in mixed populations of *Sinapis alba* FL. Rabenh. and *Lepidium sativum* L. *Journal of Applied Ecology* **13**:211-216.

Beale, G. and J. Knowles. 1978. *Extranuclear genetics*. Edward Arnold, London.

Bell, G. 1982. *The masterpiece of nature*. University of California Press, Berkeley.

Bell, G. 1990. The ecology and genetics of fitness in *Chlamydomonas*. I. Genotype-by-environment interaction among pure strains. *Proceedings of the Royal Society of London B* **240**:295-321.

Bell, G. 1991. The ecology and genetics of fitness in *Chlamydomonas*. III. Genotype-by-environment interaction within strains. *Evolution* **45**:668-679.

Bernstein, H., F.A. Hopf and R.E. Michod. 1988. Is meiotic recombination an adaptation for repairing DNA, producing genetic variation, or both? 139-160 *in* R. E. Michod and L. B.R., ed. *The evolution of sex: An examination of current ideas*. Sinauer Associates Inc., Sunderland.

Bierzychudek, P. 1987. Resolving the paradox of sexual reproduction: a review of experimental tests. 163-174 *in* S. C. Stearns, ed. *The evolution of sex and its consequences*. Birkhauser Verlag, Basel.

Bos, C.J., S.M. Slakhorst, A.J.M. Debets and K. Swart. 1993. Linkage group analysis in *Aspergillus niger*. *Applied Microbiology and Biotechnology* **38**:742-745.

Bos, C.J., A.J.M. Debets, K. Swart, A. Huybers, G. Kobus and S.M. Slakhorst. 1988. Genetic analysis and the construction of master strains for assignment of genes to six linkage groups in *Aspergillus niger*. *Current Genetics* **14**:437-443.

Bradshaw, H.D. and R.F. Stettler. 1995. Molecular genetics of growth and development in *Populus*. IV. Mapping QTLs with large effects on growth, form, and phenology traits in a forest tree. *Genetics* **139**:963-973.

Brownlee, A.G. and H.N. Arst. 1983. Nitrate uptake in *Aspergillus nidulans* and involvement of the third gene of the nitrate assimilation gene cluster. *Journal of Bacteriology* **55**:1138-1146.

Bulmer, M.G. 1980. *The mathematical theory of quantitative genetics*. Oxford University Press, Oxford.

Butcher, A.C. 1969. Non-allelic interactions and genetic isolation in wild populations of *Aspergillus nidulans*. *Heredity* **24**:621-631.

Butcher, D. 1995. Muller's ratchet, epistasis and mutation effects. *Genetics* **141**:431-437.

Camargo, L.E.A. and T.C. Osborn. 1996. Mapping loci controlling flowering time in *Brassica oleracea*. *Theoretical and Applied Genetics* **92**:610-616.

Caranta, C. and A. Palloix. 1996. Both common and specific genetic factors are involved in polygenic resistance of pepper to several potyviruses. *Theoretical and Applied Genetics* **92**:15-20.

Caten, C.E. 1979. Quantitative genetic variation in fungi. *in* J. N. Thompson and J. M. Thoday, ed. *Quantitative genetic variation*. Academic Press, New York.

Caten, C.E. and J.L. Jinks. 1966. Heterokaryosis: its significance in wild homothallic Ascomycetes and fungi imperfecti. *Transactions of the Brittish Mycological Society* **49**:81-93.

Charlesworth, B. 1989. The evolution of sex and recombination. *Trends in Ecology and Evolution* **4**:264-267.

Charlesworth, B. 1990. Mutation-selection balance and the evolutionary advantage of sex and recombination. *Genetical Research* **55**:199-221.

Charlesworth, B. 1996. The good fairy godmother of evolutionary genetics. *Current Biology* **6**:220.

Charlesworth, B. and D. Charlesworth. 1975. An experiment on recombinational load in *Drosophila melanogaster*. *Genetical Research* **25**:267-274.

Charlesworth, B., D. Charlesworth and M.T. Morgan. 1990. Genetic loads and estimates of mutation rates in highly inbred plant populations. *Nature* **347**:380-382.

Charlesworth, D., E.E. Lyons and L.B. Litchfield. 1994. Inbreeding depression in two highly inbreeding populations of *Leavenworthia*. *Proceedings of the Royal Society of London B* **258**:209-214.

Charlesworth, D., M.T. Morgan and B. Charlesworth. 1993. Mutation accumulation in finite outbreeding and inbreeding populations. *Genetical Research* **61**:39-56.

Clarke, J.H., R. Mithen, J.K.M. Brown and C. Dean. 1995. QTL analysis of flowering time in *Arabidopsis thaliana*. *Molecular and General Genetics* **248**:278-286.

Coenen, A., A.J.M. Debets and R.F. Hoekstra. 1994. Additive action of partial heterokaryon incompatibility (partial-het) genes in *Aspergillus nidulans*. *Current Genetics* **26**:233-237.

Concibido, V.C., G.A. Secor and S.H. Jansky. 1994. Evaluation of resistance to *Verticillium* wilt in diploid, wild potato interspecific hybrids. *Euphytica* **76**:145-152.

Croft, J.H. and G. Simchen. 1965. Natural variation among monokaryons of *Collybia velutipes*. *The American Naturalist* **99**:451-462.

Crow, J.F. 1970. Genetic loads and the cost of natural selection. 128-177 *in* J. I. Kojima, ed. *Mathematical Topics in Population Genetics*. Springer-Verlag, New York.

Crow, J.F. 1983. Disease and evolution. *Evolution* **37**:863-865.

Crow, J.F. 1988. The importance of recombination. *in* R. E. Michod and B. R. Levin, ed. *The evolution of sex: An examination of current ideas.* Sinauer Associates Inc., Sunderland.

Crow, J.F. 1993. Mutation, mean fitness, and genetic load. 3-42 *in* R. Dawkins and M. Ridley, ed. *Oxford Surveys in Evolutionary Biology.* Oxford University Press, Oxford.

Crow, J.F. 1994. Advantages of sexual reproduction. *Developmental Genetics* **15**:205-213.

Crow, J.F. and M. Kimura. 1979. Efficiency of truncation selection. *Proceedings of the National Academy of Sciences USA* **76**:396-399.

Crow, J.F. and M.J. Simmons. 1983. The mutation load in Drosophila. 1-35 *in* M. Ashburner, H. L. Carson and J. N. Thompson, ed. *The Genetics and Biology of Drosophila.* Academic Press, London.

De Visser, J.A.G.M. and R.F. Hoekstra. 1996a. The genomic rate of deleterious mutations in *Aspergillus niger. Genetical Research* (submitted).

De Visser, J.A.G.M., R.F. Hoekstra and H. Van den Ende. 1996a. The effect of sex and deleterious mutations on fitness in *Chlamydomonas. Proceedings of the Royal Society of London B* **263**:193-200.

De Visser, J.A.G.M., R.F. Hoekstra and H. Van den Ende. 1996b. An experimental test for synergistic epistasis and its application to *Chlamydomonas. Genetics* (submitted).

De Visser, J.A.G.M., R.F. Hoekstra and H. Van den Ende. 1996c. Interaction between genetic markers that affect fitness in *Aspergillus niger. Genetical Research* (submitted).

Debets, A.J.M., K. Swart and C.J. Bos. 1989. Mitotic mapping in linkage group V of *Aspergillus niger* based on selection of auxotrophic recombinants by Novozym enrichment. *Canadian Journal of Microbiology* **35**:982-988.

Drake, J.W. 1991. A constant rate of spontaneous mutation in DNA-based microbes. *Proceedings of the National Academy of Sciences USA* **88**:7160-7164.

Dykhuizen, D.E. 1990. Experimental studies of natural selection in bacteria. *Annual Review of Ecology and Systematics* **21**:378-398.

Dykhuizen, D.E. and A.M. Dean. 1990. Enzyme activity and fitness: evolution in solution. *Trends in Ecology and Evolution* 5:257-262.

Edwards, D.L. and B.W. Unger. 1980. Defective mitochondrial energy transduction in an oligomycin-resistant mutant of *Neurospora crassa. Biochemical International* **1**:262-269.

Feldman, M.W., F.B. Christiansen and L.D. Brooks. 1980. Evolution of recombination in a constant environment. *Proceedings of the National Academy of Sciences USA* 77:4824-4827.

Felsenstein, J. 1974. The evolutionary advantage of recombination. *Genetics* 78:737-756.

Felsenstein, J. 1985. Recombination and sex: is Maynard Smith necessary? 209-220 *in* H. Greenwood and M. Slatkin, ed. *Evolution: essays in honor of John Maynard Smith.* Cambridge University Press, Cambridge.

Felsenstein, J. 1988. Sex and the evolution of recombination. *in* R. E. Michod and B. R. Levin, ed. *The evolution of sex: An examination of current ideas.* Sinauer Associates Inc., Sunderland.

Fisher, R.A. 1930. *The genetical theory of natural selection.* Clarendon Press, Oxford.

Ford, E.D. 1975. Competition and stand structure in some even-aged plant monocultures. *Journal of Ecology* **63**:311-333.

Frova, C. and M. Sari-Gorla. 1994. Quantitative trait loci (QTLs) for pollen thermotolerance detected in maize. *Molecular and General Genetics* **245**:424-430.

Haigh, D. and A. Grafen. 1991. Genetic scrambling as a defence against meiotic drive. *Journal of Theoretical Biology* **153**:531-558.

Haigh, J. 1978. The accumulation of deleterious genes in a population - Muller's ratchet. *Theoretical Population Biology* **14**:251-267.

Haldane, J.B.S. 1937. The effect of variation on fitness. *American Naturalist* **37**:337-349.

Hamilton, W.D. 1980. Sex verus non-sex versus parasite. *Oikos* **35**:282-290.

Hamilton, W.D., R. Axelrod and R. Tanese. 1990. Sexual reproduction as an adaptation to resist parasites (a review). *Proceedings of the National Academy of Sciences USA* **87**:3566-3573.

Happey-Wood, C.M. 1988. Ecology of freshwater planktonic green algae. 175-225 *in* C. D. Sandgren, ed. *Growth and reproductive strategies of freshwater phytoplankton.* Cambridge University Press, Cambridge.

Harris, E.H. 1989. *The Chlamydomonas sourcebook. A comprehensive guide to biology and laboratory use.* Academic Press, San Diego.

Harushima, Y., N. Kurata, M. Yano, Y. Nagamura, T. Sasaki, Y. Minobe and M. Nakagahra. 1996. Detection of segregation distortions in an indica-japonica rice cross using a high-resolution molecular map. *Theoretical and Applied Genetics* **92**:145-150.

Hoekstra, R.F., R. Bijlsma and A.J. Dolman. 1985. Polymorphism from environmental heterogeneity: models are only robust if the heterozygote is close in fitness to the favoured homozygote in each environment. *Genetical Research* **45**:299-314.

Houle, D., D.K. Hoffmaster, S. Assimacopoulos and B. Charlesworth. 1992. The genomic mutation rate for fitness in *Drosophila*. *Nature* **359**:58-60.

Howard, R.S. 1994. Selection against deleterious mutations and the maintenance of biparental sex. *Theoretical Population Biology* **45**:313-323.

Howard, R.S. and C.M. Lively. 1994. Parasitism, mutation accumulation and the maintenance of sex. *Nature* **367**:554-557.

Huisman, J. and F.J. Weissing. 1994. Light-limited growth and competition for light in well-mixed aquatic environments: an elementary model. *Ecology* **75**:507-520.

Huisman, J. and F.J. Weissing. 1995. Competition for nutrients and light in a mixed water collumn: a theoretical analysis. *The American Naturalist* **146**:536-564.

Hurst, L.D. and J.R. Peck. 1996. Recent advances in understanding of the evolution and maintenance of sex. *Trends in Ecology and Evolution* **11**:46-52.

Jaenike, J. 1978. An hypothesis to account for the maintenance of sex within populations. *Evolutionary Theory* **3**:191-194.

Jinks, J.L., C.E. Caten, G. Simchen and J.H. Croft. 1966. Heterokaryon incompatibility and variation in wild populations of *Aspergillus nidulans*. *Heredity* **21**:227-239.

Johnston, M.O. and D.J. Schoen. 1995. Mutation-rates and dominance levels of genes affecting total fitness in two angiosperm species. *Science* **267**:226-229.

Kacser, H. and J.A. Burns. 1973. The control of flux. *Symposium of the Society of Experimental Biology* **32**:65-104.

Kacser, H. and J.A. Burns. 1979. Molecular democracy: who shares the controls? *Biochemical Society Transactions* **7**:1149-1160

Kelley, S.E., J. Antonovics and J. Schmitt. 1988. A test of the short term advantage of sexual reproduction. *Nature* **331**:714-716.

Kibota, T.T. and M. Lynch. 1996. Estimate of the genomic mutation rate deleterious to overall fitness in *E. coli*. *Nature* **381**:694-696.

Kimura, M. 1961. Some calculations on the mutation load. *Japanese Journal of Genetics* **36**:179-190.

Kimura, M. 1983. *The neutral theory of molecular evolution*. Cambridge University Press, Cambridge.

Kimura, M. and T. Maruyama. 1966. The mutational load with epistatic gene interactions in fitness. *Genetics* **54**:1337-1351.

Kondrashov, A.S. 1982. Selection against harmful mutation in large sexual and asexual populations. *Genetical Research* **40**:325-332.

Kondrashov, A.S. 1988. Deleterious mutations and the evolution of sexual reproduction. *Nature* **336**:435-440.

Kondrashov, A.S. 1993. Classification of hypotheses on the advantage of amphimixis. *Journal of Heredity* **84**:372-387.

Kondrashov, A.S. 1994. Muller's ratchet under epistatic selection. *Genetics* **136**:1469-1473.

Kondrashov, A.S. 1994. Sex and deleterious mutation. *Nature* **369**:99-100.

Kondrashov, A.S. and J.F. Crow. 1993. A molecular approach to estimating the human deleterious mutation rate. *Human Mutation* **2**:229-234.

Kondrashov, A.S. and D. Houle. 1994. Genotype-environment interactions and the estimation of the genomic mutation rate in Drosophila melanogaster. *Proceedings of the Royal Society of London B* **258**:221-227.

Kornegay, J.L. and C. Cardona. 1991. Inheritance of resistance to *Acanthoscelides obtectus* in a wild common bean accession crossed to commercial bean cultivars. *Euphytica* **52**:103-111.

Laurie, D.A., N. Pratchett, J.H. Bezant and J.W. Snape. 1995. RFLP mapping of five major genes and eight quantitative trait loci controlling flowering time in a winter x spring barley (*Hordeum vulgare* L.) cross. *Genome* **38**:575-585.

Lenski, R.E. 1992. Experimental evolution. 125-140 *in* J. Lederberg, ed. *Encyclopedia of Microbiology*. Academic Press, San Diego.

Lenski, R.E., M.R. Rose, S.C. Simpson and S.C. Tadler. 1991. Long-term experimental evolution in *Escherichia coli*. I. Adaptation and divergence during 2,000 generations. *American Naturalist* **138**:1315-1341.

Li, Z., S.R.M. Pinson, J.W. Stansel and W.D. Park. 1995a. Identification of quantitative trait loci (QTLs) for heading date and plant height in cultivated rice (*Oryza sativa* L.). *Theoretical and Applied Genetics* **91**:374-381.

Li, Z., S.R.M. Pinson, M.A. Marchetti, J.W. Stansel and W.D. Park. 1995b. Characterization of quantitative trait loci (QTLs) in cultivated rice contributing to field resistance to sheath blight (*Rhizoctonia solani*). *Theoretical and Applied Genetics* **91**:382-388.

Lynch, M. and W. Gabriel. 1990. Mutation load and the survival of small populations. *Evolution* **44**:1725-1737.

MacArthur, J.W. and L. Butler. 1938. The processes in the tomato fruit. *Genetics* **23**:253-268.

MacArthur, R.H. 1962. Some generalized theorems of natural selection. *Proceedings of the National Academy of Sciences USA* **48**:1893-1897.

Malmberg, R.L. 1977. The evolution of epistasis and the advantage of recombination in populations of bacteriophage T4. *Genetics* **86**:607-621.

Mather, K. and J.L. Jinks. 1982. *Biometrical genetics*. Cambridge University Press, Cambridge.

Maynard Smith, J. 1971. What use is sex? *Journal of Theoretical Biology* **30**:319-335.

Maynard Smith, J. 1978. *The Evolution of Sex*. Cambridge University Press, Cambridge.

Maynard Smith, J. 1980. Selection for recombination in a polygenic model. *Genetical Research* **35**:269-277.

Maynard Smith, J. 1986. Contemplating life without sex. *Nature* **324**:300-301.

Maynard Smith, J. 1988. The evolution of recombination. 106-125 *in* R. E. Michod and B. R. Levin, ed. *The evolution of sex: An examination of current ideas.* Sinauer Associates Inc., Sunderland.

Maynard Smith, J. 1989. *Evolutionary genetics.* Oxford University Press, Oxford.

Maynard Smith, J. and E. Szathmáry. 1995. *The major transitions in evolution.* Freeman, Oxford.

Mesland, D.A.M. 1976. Mating in *Chlamydomonas moewusii.* A scanning electron microscopical study. *Archives of Microbiology* **109**:31-35.

Michod, R.E. and B.R. Levin 1988 *The evolution of sex: an examination of current ideas.* Sunderland:Sinauer Associates Inc.

Mukai, T. 1964. Spontaneous mutation rate of polygenes controlling viability. *Genetics* **50**:1-19.

Mukai, T. 1969. The genetic structure of natural populations of *Drosophila melanogaster.* VII Synergistic interaction of spontaneous mutant polygenes controlling viability. *Genetics* **61**:749-761.

Mukai, T., S.I. Chigusa, L.E. Mettler and J.F. Crow. 1972. Mutation rate and dominance of genes affecting viability in *Drosophila melanogaster. Genetics* **72**:335-355.

Muller, H.J. 1932. Some genetic aspects of sex. *The American Naturalist* **66**:118-138.

Muller, H.J. 1950. Our load of mutations. *American Journal of Human Genetics* **2**:111-176.

Muller, H.J. 1964. The relation of recombination to mutational advantage. *Mutation Research* **1**:2-9.

Nodari, R.O., S.M. Tsai, P. Guzmán, R.L. Gilbertson and P. Gepts. 1993. Towards an integrated linkage map of common bean. III. Mapping genetic factors controlling host-bacteria interactions. *Genetics* **134**:341-350.

Papa, K.E., A.M. Srb and W.T. Federer. 1967. Inheritance of growth rate in *Neurospora crassa*: reverse selection in an improved strain. *Heredity* **22**:285-296.

Pè, M.E., L. Gianfrancheschi, G. Taramino, R. Tarchini, P. Angelini, M. Dani and G. Binelli. 1993. Mapping quantitative trait loci (QTLs) for resistance to *Gibberella zeae* infection in maize. *Molecular and General Genetics* **241**:11-16.

Pontecorvo, G., J.A. Roper and E. Forbes. 1953. Genetic recombination without sexual reproduction in *Aspergillus niger. Journal of General Microbiology* **8**:198-210.

Powers, L. 1936. The nature of the interaction of genes affecting four quantitative characters in a cross between *Hordeum deficiens* and *Hordeum vulgare. Genetics* **21**:398-420.

Rasmusson, J. 1933. A contribution to the theory of quantitative character inheritance. *Heriditas* **18**:245-261.

Rausher, M.D. 1996. Genetic analysis of coevolution between plants and their natural enemies. *Trends in Genetics* **12**:212-217.

Rendel, J.M. 1967. *Canalisation and gene control.* Logos Press, London.

Ribaut, J.-M., D.A. Hoisington, J.A. Deutsch, C. Jiang and D. Gonzalez-de-Leon. 1996. Identification of quantitative trait loci under drought conditions in tropical maize. 1. Flowering parameters and the anthesis-silking interval. *Theoretical and Applied Genetics* **92**:905-914.

Sari-Gorla, M., M.E. Pé, D.L. Mulcahy and E. Ottaviano. 1992. Genetic dissection of pollen competitive ability in maize. *Heredity* **69**:423-430.

Schloesser, U.G. 1994. Sammlung von Algenkulturen at the University of Goettingen: Catalogue of strains 1994. *Botanica Acta* **107**:113-186.

Schuring, F., J.W. Smeenk, W.L. Homan, A. Musgrave and H. Van den Ende. 1987. Occurrence of O-methylated sugars in surface glycoconjugates in *Chlamydomonas eugametos*. *Planta* **170**:322-327.

Shook, D.R., A. Brooks and T.E. Johnson. 1996. Mapping quantitative trait loci affecting life history traits in the nematode *Caenorhabditis elegans*. *Genetics* **142**:801-817.

Simchen, G. 1966. Monokaryotic variation and haploid selection in *Schizophyllum commune*. *Heredity* **21**:241-263.

Simpson, E. and J.W. Snape 1981 The use of doubled haploids in a winter barley programme. Edinburgh University, Edinburgh: *Proceedings of the 4th International Barley Genetics Symposium.*

Sinnot, E.W. 1937. The relation of the gene to character in quantitative inheritance. *Proceedings of the National Academy of Sciences USA* **23**:224-227.

Sokal, R.R. and F.J. Rohlf. 1981. *Biometry*. Freeman, W.H., San Francisco.

Stearns, S.C. 1987 *The evolution of sex and its consequences*. Basel:Birkhauser Verlag.

Stearns, S.C. 1992. *The evolution of life histories*. Oxford University Press, Oxford.

Stearns, S.C. and T.J. Kawecki. 1994. Fitness sensitivity and the canalization of life-history traits. *Evolution* **48**:1438-1450.

Steffenson, B.J., P.M. Hayes and A. Kleinhofs. 1996. Genetics of seedling and adult plant resistance to net blotch (*Pyrenophora teres* f. teres) and spot blotch (*Cochliobolus sativus*) in barley. *Theoretical and Applied Genetics* **92**:552-558.

Szathmáry, E. 1993. Do deleterious mutations interact synergistically? Metabolic control theory provides a partial answer. *Genetics* **133**:127-132.

Travisano, M., F. Vasi and R.E. Lenski. 1995. Long-term experimental evolution in *Escherichia coli*. III. Variation among replicate populations in correlated responses to novel environments. *Evolution* **49**:189-200.

Trivers, R. 1985. *Social evolution*. The Benjamin/Cummings Publishing Company, Inc., Menlo Park.

Turner, M.D. and D. Rabinowitz. 1983. Factors affecting frequency distributions of plant mass: the absence of dominance and suppression in competing monocultures of *Festuca paradoxa*. *Ecology* **64**:469-475.

Van den Ende, H., F.M. Klis, A. Musgrave and D. Stegwee. 1988. Sexual agglutination in *Chlamydomonas eugametos*. 92-104 *in* G. P. Chapman, C. C. Ainsworth and C. K. Chatham, ed. *Eukaryote cell recognition: concepts and model systems*. Cambridge University Press, Cambridge.

Wang, G.L., D.J. Mackill, J.M. Bonman, S.R. McCouch, M.C. Champoux and R.J. Nelson. 1994. RFLP mapping of genes conferring complete and partial resistance to blast in durable resistant rice cultivar. *Genetics* **136**:1421-1434.

Webb, D.M., B.M. Baltazar, A.P. Rao-Arelli, J. Schupp, K. Clayton, P. Keim and W.D. Beavis. 1995. Genetic mapping of soybean cyst nematode race-3 resistance loci in the soybean PI 437.654. *Theoretical and Applied Genetics* **91**:574-581.

Weissing, F.J. and J. Huisman. 1994. Growth and competition in a light gradient. *Journal of Theoretical Biology* **168**:323-336.

White, J. and J.L. Harper. 1970. Correlated changes in plant size and number in plant populations. *Journal of Ecology* **58**:467-485.

Wilkinson, L. 1988 *SYSTAT: The system for statistics*. Evanston:SYSTAT Inc.

Williams, D.D., A. Travares-Cromar, J.R. Coleman, D.J. Kushner and C.M. Happey-Wood. 1994. Colonization dynamics of algae in small artificial ponds. *Canadian Journal of Botany* **72**:1654-1665.

Williams, G.C. 1975. *Sex and evolution*. Princeton University Press, Princeton.

Willis, J.H. 1993. Effects of different levels of inbreeding on fitness components in *Mimulus guttatus*. *Evolution* **47**:864-876.

Xiao, J., J. Li, L. Yuan and S.D. Tanksley. 1996. Identification of QTLs affecting traits of agronomic importance in a recombinant inbred population derived from a subspecific rice cross. *Theoretical and Applied Genetics* **92**:230-244.

Samenvatting

De vraag naar de evolutionaire redenen voor de tegenwoordige algemeenheid van sexuele voortplanting staat centraal in dit proefschrift. Sex is op het eerste oog niet erg efficiënt en brengt een aantal nadelen met zich mee die ongeslachtelijke voortplanting niet kent. Bij sex in eukaryoten wordt het aantal genen gehalveerd (tijdens de meiose bij de productie van geslachtscellen) alvorens deze worden doorgegeven aan de volgende generatie. Dit heeft tot gevolg, dat elk gen slechts met een kans van 0.5 wordt doorgegeven aan de nakomelingen. Een asexuele mutant, die al haar genen doorgeeft aan elke nakomeling, heeft daarom een tweevoudig verspreidingsvoordeel in een sexuele populatie. In het licht van deze "tweevoudige kosten van sex" is het wijdverbreide voorkomen ervan onbegrijpelijk.

Momenteel bestaan er zo'n 20 theorieën met een evolutionaire verklaring voor sex, waarvan de meesten een voordeel opperen dat verbonden is aan een essentiële consequentie van sex: genetische recombinatie, d.w.z. sexuele nakomelingen zijn het product van willekeurige combinaties van de genen van de ouders. Twee hypothesen worden de laatste jaren als meest veelbelovend gezien: de Parasieten hypothese en de Deterministische Mutatie hypothese (DMH). De eerste hypothese veronderstelt dat parasieten voor de selectiedruk zorgen die sex noodzakelijk maakt. De tweede hypothese veronderstelt dat nadelige mutaties de relevante selectiedruk veroorzaken die sex voordelig maakt. Het werk beschreven in dit proefschrift is geheel gericht op het testen van deze laatste hypothese.

Het voordeel van sexueel (t.o.v. asexueel) voortplanten wordt volgens de DMH geleverd doordat sex, via het vergroten van de variatie in het aantal mutaties per individu, selectie tegen nadelige mutaties efficiënter kan maken. Sex kan door recombinatie nakomelingen produceren met veel en met weinig mutaties. Het gevolg is dat het sterven (of steriel zijn) van een beperkt aantal sexuele nakomelingen voldoende is om een groot aantal mutaties op te ruimen. De hypothese is echter ontwikkeld voor een populatie in mutatie-selectie evenwicht, d.w.z. een populatie waarin per generatie het aantal nieuw ontstane en

weggeselecteerde mutaties met elkaar in evenwicht is. In zo'n situatie blijkt sex alleen de variatie in het aantal mutaties per individu te vergroten als de mutaties elkaars negatieve effect op de fitness versterken. Dit laatste wordt synergisme genoemd. Ook hebben model-studies berekend, dat om een voldoende voordeel voor sex op te leveren, er minstens één nadelige mutatie per genoom per generatie moet optreden.

Het werk beschreven in dit proefschrift richt zich op het experimenteel toetsen van beide genoemde aannamen van de DMH: (1) Is de nadelige mutatie snelheid minstens één per genoom per generatie? en (2) Bestaat er synergistische interactie tussen nadelige mutaties? De empirische kennis omtrent de geldigheid van beide aannamen is op dit moment zeer beperkt.

Vier van de vijf hoofdstukken gaan over de vraag of nadelige mutaties synergistische interactie vertonen. In hoofdstuk 2 wordt een test voor de aard van mutatie-interactie beschreven die gebaseerd is op het vergelijken van de gemiddelde log fitness van ouders en nakomelingen van een kruising. (Het *logaritme* van de fitness wordt gebruikt, omdat het ontbreken van mutatie-interactie inhoudt dat mutaties multiplicatieve effecten hebben.) In geval van synergisme voorspelt de concave vorm van de relatie tussen log fitness en aantal mutaties, dat de gemiddelde log fitness van de nakomelingen hoger is dan die van de ouders, als de ouders aanzienlijk verschillen in het aantal mutaties dat ze bezitten. Als beide ouders ongeveer evenveel mutaties hebben voorspelt synergisme een lagere log fitness van de nakomelingen t.o.v. de ouders. Deze "gemiddelden-test" is in dit hoofdstuk toegepast op kruisingen tussen stammen van de ééncellige groenwier *Chlamydomonas moewusii*. De stammen zijn al dan niet bestraald met UV om het verschil in aantal mutaties van beide ouders te manipuleren. Fitness van ouders en nakomelingen is gemeten door de maximale groeisnelheid (r) en de draagkracht (K) te meten in een reageerbuis. De resultaten laten een significant lagere log fitness van de nakomelingen t.o.v. de ouders zien, als de ouders met een hoge UV-dosis zijn behandeld. Vanwege het geringe verschil in r en K van deze ouders wordt geconcludeerd, dat door UV geïnduceerde mutaties met een nadelig effect op r en K synergisme vertonen.

In hoofdstuk 3 wordt nog een simpeler test voor de aard van mutatie-interactie voorgesteld. Deze is gebaseerd op de scheefheid van de frequentie-verdeling van de log fitness van de nakomelingen van een

kruising: negatieve scheefheid (met staart richting lage fitness) duidt op synergisme, positieve scheefheid (staart richting hoge fitness) geeft antagonisme tussen mutaties aan (d.w.z. mutaties heffen gedeeltelijk elkaars negatieve effect op). Een symmetrische log fitness verdeling duidt op onafhankelijke mutatie-effecten. Deze "scheefheids-test" is hier opnieuw toegepast op *Chlamydomonas* kruisingen. Dit keer zijn twee stammen die meer dan 60 jaar in het laboratorium zijn bewaard met elkaar gekruist, evenals twee stammen die enkele jaren geleden uit de natuur zijn geïsoleerd. De oude lab-stammen hebben zeer waarschijnlijk vele kleine nadelige mutaties opgehoopt door het verwachte herhaaldelijke verlies van de cellen met weinig mutaties tijdens het overenten (Muller's ratchet). De kruising tussen de nieuwe stammen dient als controle, want deze stammen hebben nauwelijks de gelegenheid gekregen mutaties op te hopen. In de controle kruising zijn de verdeling van log r en log K beide niet scheef, terwijl in de mutatie kruising de verdeling van log K negatief scheef is en de verdeling van log r niet. De resultaten wijzen daarom op synergisme tussen mutaties die K beïnvloeden en onafhankelijke effecten van mutaties die effect hebben op r. Omdat r bekend is als voorspeller van de fitness in een variabel milieu en K voor fitness in een stabiele, verzadigde omgeving, wijzen deze resultaten mogelijk op het belang van sex voor de selectie tegen nadelige mutaties in dit laatste type omgeving. Interessant is dat sex inderdaad relatief vaker voorkomt in stabiele milieu's.

Hoofdstuk 4 is het enige hoofdstuk dat de andere aanname van de DMH, een voldoende hoge nadelige mutatiesnelheid, aan de orde stelt. In dit hoofdstuk wordt een mutatie-accumulatie experiment beschreven met de asexuele schimmel *Aspergillus niger*. Gedurende 60 generaties worden 25 mutatie-lijnen met een gemeenschappelijke voorouder instant gehouden, waarbij zoveel mogelijk selectie wordt vermeden. Op deze manier wordt verwacht dat kleine nadelige mutaties ophopen. Uit de verwachte afname van het gemiddelde en toename van de variantie van de fitness van deze lijnen kan een conservatieve schatting van de nadelige mutatiesnelheid worden berekend. De fitness van voorouder en mutatie-lijnen op generatie 30 en 60 is gemeten in competitie met een stam met licht gekleurde sporen (i.p.v. het zwarte wild-type). Onverwacht echter blijkt de ouder de laagste fitness te hebben, terwijl de fitness zoals verwacht afneemt tussen generatie 30 en 60. Waarschijnlijk is het niet synchroon zijn van de fysiologische toestand van de ouder-sporen de

oorzaak van dit onverwachte resultaat. Uit een vergelijking van de mutatie-lijnen op generatie 30 en 60 is berekend dat de ondergrens van de nadelige mutatiesnelheid 0.19 nadelige mutaties per genoom per generatie is, hetgeen goed overeen komt met de paar schattingen voor de fruitvlieg *Drosophila* en enkele planten. De betrouwbaarheidsintervallen van de schattingen zijn echter te ruim voor harde conclusies.

In hoofdstuk 5 worden stammen van *Aspergillus niger* met een aantal marker-mutaties (mutaties met een duidelijk fenotypisch effect) gebruikt als model om interactie tussen nadelige mutaties te bestuderen. De marker-mutaties zijn twee sporenkleur mutaties, twee resistenties en vijf auxotrofe mutaties. Door twee componenten van de fitness, de mycelium groeisnelheid en de maximale sporenproductie (beperkt door ruimte en nutriënten), op gesupplementeerd medium te meten, worden ook van de auxotrofe mutaties kleine nadelige mutaties gemaakt. De resultaten laten zien dat interacties tussen mutaties zeer belangrijk zijn voor beide fitness-componenten, maar dat niet één duidelijk type interactie (synergisme of antagonisme) aantoonbaar is. Er bestaat wel een neiging tot synergisme voor de interacties m.b.t. de maximale sporenproductie, terwijl geen voorkeur kan worden aangetoond voor de interacties m.b.t. de mycelium groeisnelheid. Omdat de mycelium groeisnelheid waarschijnlijk belangrijker is in een variabel milieu en de maximale sporenproductie in een verzadigd milieu, geven deze resultaten steun aan de bevindingen bij *Chlamydomonas* van hoofdstuk 3.

Tenslotte wordt in hoofdstuk 6 de scheefheids-test gebruikt om in de literatuur aanwijzingen te vinden voor de aard van de interactie tussen genen die effect hebben op fitness. Voorbeelden worden gepresenteerd van verdelingen van fitness-componenten van een aantal schimmels, maar vooral van planten. Voor schimmels laten de resultaten aanwijzingen zien voor beide typen interactie voor de mycelium groeisnelheid. Voor planten zijn met name veel voorbeelden gevonden voor de eigenschappen vroegheid en resistentie tegen pathogenen, die bijna zonder uitzondering negatief scheef zijn en dus synergisme suggereren. Het effect op de conclusies van een niet-lineair verband tussen de fitness-component en de totale fitness worden besproken, evenals de mogelijk storende effecten van dominantie en meetfouten op de scheefheids-test. De conclusie is dat, terwijl in lagere organismen als *Chlamydomonas* en *Aspergillus* synergsime afhangt van de gemeten fitness-component en relatief zwak is, in planten synergisme vrij algemeen lijkt.

Nawoord

Het is een bekend verschijnsel dat promovendi aan het eind van hun promotietijd vaak wat sentimenteel worden. Ze overzien dan het onderzoek en denken: "Is dat alles?" Ook word menig promovendus in deze fase geplaagd door het besef hoe klein zijn of haar eigen bijdrage eigenlijk is geweest een hoe groot die van anderen. Deze laatste sentimenten zijn ook mij niet vreemd. Ik wil ze hier proberen te beteugelen door alle mensen die wezenlijk hebben bijgedragen aan het onderzoek te bedanken.

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Professional experience

1989-1991 Energy budget study in the pond snail, Theoretical Biology, Free University Amsterdam (Civil Service).

1990 Teaching introductory course Theoretical Biology (Model making and statistical analysis) to undergraduate biology students.

1995 Teaching courses in population and quantitative genetics and evolutionary biology to undergraduate students in Biology, Phytopathology and Plant Breeding (foreign students).

Publications

Van Schaik, C.P. and J.A.G.M. de Visser. 1990. Fragile sons or harassed daughters? Sex differences in mortality among juvenile primates. *Folia Primatologia* **55**: 10-23.

De Visser, J.A.G.M., A. ter Maat and C. Zonneveld. 1994. Energy budgets and reproductive allocation in the simultaneous hermaphrodite pond snail, *Lymnaea stagnalis* (L.): a trade-off between male and female function. *American Naturalist* **144**: 861-867.

De Visser, J.A.G.M., R.F. Hoekstra and H. van den Ende. 1996. The effect of sex and deleterious mutations on fitness in *Chlamydomonas*. *Proceedings of the Royal Society London B* **263**: 193-200.

De Visser, J.A.G.M. 1996. Deleterious mutations and the evolution of sex. Thesis, Wageningen Agricultural University.

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