PROPERTIES OF MUTATIONS AFFECTING LIFE HISTORY TRAITS IN Caenorhabditis elegans

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DECLARATION

I declare that this thesis has been composed by me and that the work described in it is my own. Specific contributions by others are acknowledged.
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PUBLICATIONS

Papers arising from this thesis are:


ABSTRACT

Properties of new mutations, including the rate at which they arise and the distribution of their effects, are important for several issues in evolutionary biology. Direct evidence for these properties, however, is sparse, and has predominantly come from mutation-accumulation experiments, involving fitness assays of lines of organisms that have been allowed to accumulate spontaneous mutations in the absence of selection. The results of such experiments have varied greatly: some studies concluded that large numbers of mutations with small effects on fitness (2-3%) accumulate each generation, while others indicated that the detectable mutation rate is up to two orders of magnitude lower, with most mutations having larger effects (~20%).

I have studied a possible cause of this variation, using the nematode worm *Caenorhabditis elegans* as a model system. Life history assays performed under harsh experimental conditions may reveal greater fitness differences between lines than those performed in benign conditions. Life history assays were performed on lines of *C. elegans* in which spontaneous mutations had been allowed to accumulate for 60 generations (Keightley and Caballero, 1997), using a range of harsh conditions. However, no significant degree of genotype by environment interaction was observed. Under some harsher experimental conditions, estimates of mutation rates increased, but the difference was not significant and these results do not lend support to the theory that differences in estimates of mutational parameters are due to variation in assay conditions.

Even harsh conditions may not reveal the effects of all mutations that have accumulated and are deleterious in natural conditions, and the extent to which this may result in underestimation of the mutation rate has been unknown. I have measured the effects of EMS-induced mutations on a series of life history traits in *C. elegans*, in a situation where the number of mutational events induced has been calibrated from studies of suppressor-induced reversion mutations and forward mutations. None of the assays revealed the effects of more than ~10% of the deleterious mutations induced, indicating that standard mutation-accumulation experiments may have dramatically underestimated the rate at which mutations
accumulate. Such mutations, although cryptic, may nonetheless be significant for evolutionary biology. Two of the lines were further analysed using an inbred-backcross approach, which provided confirmation that only a very small proportion of the induced mutations could be detected.

The joint effects of induced mutations on longevity and productivity were also considered. Mortality curves were observed to flatten with age, and this was more notable among the EMS than control lines. One explanation for this was greater heterogeneity among the EMS lines, although other possible causes are discussed. Overall, bivariate analysis revealed strong, positive correlations between longevity and productivity traits, although one line showed a significant increase in longevity, and a correlated decline in early (but not total) productivity.
Chapter 1: INTRODUCTION
1.1 Importance of the Properties of New Mutations

Mutations are changes in the amount or structure of DNA, which may occur spontaneously or be experimentally induced by a variety of means, including exposure to chemical mutagens or radiation. Mutations supply the source of all genetic variation, without which evolution could not progress, as genetic variation among individuals is required for natural selection to have an impact. Knowledge of the properties of mutations affecting life history traits is therefore important for an understanding of evolutionary processes. However, mutations can arise in different ways, and have a variety of different effects, with the result that the study of mutations and their evolutionary consequences can be a complicated task.

In animals, the germline is separated from the somatic tissue early in development. Mutations may arise in somatic tissues, potentially having a dramatic effect on the organism in which they occur. Such mutations will not, however, be passed on to the next generation. Mutations that occur in the germline, however, may be transmitted to future generations, and such mutations are of most interest to evolutionary biologists. Within these two classes, mutations can occur in several different ways. Single base-pair changes in the DNA may take the form of transitions or transversions. Alternatively, regions of the genome ranging from one base-pair to an entire chromosome or set of chromosomes may be deleted, duplicated, inverted or translocated from one region to another. A mutation that changes a wild-type allele into a mutant allele is known as a forward mutation; any change that recreates the wild-type is called a reverse mutation.

The effects of mutations can also vary greatly, on a continuous scale. They may have beneficial effects, although it is expected that such mutations will only arise occasionally. The remainder vary from those with neutral effects to lethal effects. The degree of dominance can also vary, from complete dominance (when the effects of another allele are completely masked in heterozygous form) to complete recessivity; incomplete dominance is expressed when the effects of another allele are not completely masked. The effects of mutations may vary depending on the situation in which they arise – either the genetic background (epistasis) or the environmental background (genotype by environment interaction). They may also affect more than
one trait (pleiotropy). In addition to varying in the way in which they are expressed, the timing of the expression can also vary.

This variation can complicate the study of mutations and their effects. However, such studies have continued because knowledge of the properties of mutations – the frequency with which they occur, the distribution of their effects, and the ways in which they interact with each other and the environment – can help to explain an impressive range of evolutionary phenomena. A number of these are outlined below.

1.1.1 EVOLUTION OF SEX

The evolution of sexual forms of reproduction is one such issue. All else being equal, asexual organisms would be expected to have a two-fold reproductive advantage over sexual organisms. Two models can be used to describe this cost of sex: firstly, there is the ‘cost of male allocation’, as an individual that does not produce male offspring (or gametes) can produce female offspring more efficiently (Maynard Smith, 1971). Secondly, the cost of sex can be viewed as being the ‘cost of meiosis’, whereby asexual organisms benefit from increasing their genetic contribution to offspring (Williams, 1971). These two models are not interchangeable, and apply to different situations (Lively and Lloyd, 1990).

Despite the cost of sex, sexual forms of reproduction persist in a wide variety of organisms studied (Bell, 1982), indicating that there must be a significant advantage to sex in order to compensate for this. A number of theories have been proposed to explain the advantage of sex, one of which is the deterministic mutation hypothesis (Kondrashov, 1988), which makes specific predictions about mutational parameters. This theory states that if mutations interact synergistically, such that increasing numbers of mutations reduce fitness more than would be expected if they interacted in a multiplicative manner, organisms that reproduce sexually may be better able to remove deleterious mutations, thus conferring an advantage to sex. This is because the genetic load, $L$, which is the proportion of individuals that fail to reproduce as a result of selection, is constant in asexual organisms under mutation-selection balance. In diploid asexual species, $L$ is equal to $1 - e^{2U}$, where $U$ is the haploid genomic mutation rate. If mutations act multiplicatively, the genetic load is equal in sexual and
asexual organisms. However, under the condition of synergistic epistasis, where each new mutation leads to a larger decline in fitness, sexual reproduction can increase fitness by allowing recombination, which enables deleterious mutations to be removed more efficiently. In order for the deterministic mutation hypothesis to satisfactorily explain the evolution of sex, however, there must be a high input of deleterious mutations, such that at least one deleterious mutation arises per diploid genome, per generation, regardless of the size of the effects of such mutations (Kondrashov, 1988).

1.1.2 EVOLUTION OF PLOIDY LEVEL
As a consequence of sexual reproduction, organisms undergo both haploid and diploid stages during the life cycle. The relative lengths of these phases vary, but in higher organisms, the diploid phase dominates. One explanation for the predominance of diploidy is that under certain conditions, including quasi-truncation selection, the diploid genetic load may be lower than the haploid load. The result of this will be that the diploid phase will have higher fitness, and thus selection is likely to act to increase its length, relative to that of haploids (Kondrashov and Crow, 1991; Perrot et al., 1991). This model is similar to the deterministic mutation theory of sex, described above, and also requires there to be a high input of deleterious mutations, such that the genomic mutation rate is at least one per generation (Kondrashov and Crow, 1991).

1.1.3 DEGENERATION OF NON-RECOMBINING REGIONS OF THE GENOME
If deleterious mutations can be removed more efficiently in sexual, rather than asexual organisms, they may also accumulate more rapidly in ‘asexual’, non-recombining regions of the genome, such as the Y chromosome or organelle genomes, than in the remainder of the genome. This could therefore explain the degradation of such regions, relative to recombining regions of the genome (Charlesworth, 1991; Lynch and Blanchard, 1998).
1.1.4 **INBREEDING DEPRESSION**

The rate at which mutations accumulate could be a factor responsible for observed levels of inbreeding depression. Dominance coefficients and levels of self-fertilisation can influence levels of inbreeding depression, and an increase in the genomic mutation rate is likely to increase the degree of inbreeding depression (Charlesworth *et al.*, 1990). The magnitude of inbreeding depression can influence the evolution of self-incompatibility and other mechanisms for inbreeding avoidance (Charlesworth and Charlesworth, 1987).

1.1.5 **SURVIVAL OF SMALL POPULATIONS**

If spontaneous mutations accumulate at a significantly high rate, this could have fitness consequences for populations in which the efficiency of natural selection at removing deleterious mutations is lower than it would otherwise be, e.g., when the effective population size is small (Lande, 1994, 1995; Lynch *et al.*, 1995). This could pose a threat to the survival of small populations, though the extent of this risk will depend on both the rate at which mutations accumulate and the magnitude of their fitness effects. As population size decreases, the number of mutations that will accumulate is expected to increase, as the effect of genetic drift, rather than selection, dominates their fate. Mutations with homozygous effects less than $1/N_e$ are effectively neutral, where $N_e$ is the effective population size. Such mutations will not, therefore, be removed by natural selection. Of these, mutations with effects close to $1/N_e$ are expected to have the most serious fitness consequences, as they are small enough to accumulate but may be large enough to be damaging, allowing the gradual erosion of fitness with time. Thus, three parameters are important when evaluating the threat to survival posed by the accumulation of deleterious mutations: the genomic mutation rate ($U$), the magnitude of the mutational effects ($s$) and the effective population size ($N_e$).

It has also been argued that mutations may be accumulating more rapidly than they are being removed in our own species (Muller, 1950; Crow, 1997). Small population size is not responsible in this case, but instead natural selection may be reduced due to a greatly improved probability of surviving and reproducing. Natural selection may still be as efficient as ever in many areas of the world; for the majority of babies born
world-wide, survival to reproductive age is by no means guaranteed. In addition, high levels of infertility and miscarriages even in richer areas indicate that there is ample opportunity for pre-natal selection to occur. However, in richer countries, most babies born have a greatly improved probability of surviving to successfully raise offspring. Natural selection for the effective removal of mutations is therefore likely to have been reduced, and so population fitness may be slowly eroded by the accumulation of mildly deleterious mutations. The rate at which such mutations are arising will affect the consequences of this. However, it is less easy to envision a humane, ethical and effective way of reducing such mutation accumulation.

1.1.6 EVOLUTION OF AGEING
Despite all our efforts at improving the quality of life, and vastly improved health care, ageing and death occur universally in human populations, as well as in other species. Evolutionary theory has proposed that this decline in fitness with age is the result of lower selection later in life, as more individuals will have died or become infertile due to extrinsic causes (Medawar, 1946, 1952; Williams, 1957; Hamilton, 1966). Ageing could evolve through the accumulation of age-specific mutations that only reduce late-life components of fitness, when the impact of selection is too weak to remove them (the mutation-accumulation theory (Medawar, 1952)). Alternatively, it may be the result of mutations with harmful effects late in life, but beneficial effects earlier (the optimality, or antagonistic pleiotropy theory (Williams, 1957)). To distinguish between these two theories, knowledge of the way in which mutations act, as well as the rate at which they accumulate, is important. The mutation-accumulation theory predicts that large numbers of mutations should arise that display age-specific effects, such that they may affect late-life fitness but have no correlated effects early in life. The optimality theory predicts that mutations occur that have correlated responses across ages, and it is for this reason that mutations that could, for instance, increase lifespan, have not reached fixation. However, such mutations need not arise as frequently, as they would be expected to reach a higher equilibrium frequency than those with no beneficial effects.
1.1.7 GENOTYPE BY ENVIRONMENT INTERACTION

Mutations may also vary in the effects they have in different environments, rather than at different ages, a process known as genotype by environment interaction. Such mutations may explain the evolution of ecological specialisation and niche breadth, if mutations that have opposing effects in different environments accumulate, restricting the number of niches that an organism can successfully occupy. Alternatively, ecological generalists may experience weaker selection to remove deleterious mutations, if the harmful effects are only expressed in a subset of environments (Kawecki, 1994; Fry et al., 1996; Kawecki et al., 1997). This may allow such mutations to accumulate, reducing overall fitness relative to that of a specialist.

The occurrence of mutations exhibiting genotype by environment interaction may also explain the maintenance of genetic variation for fitness-related traits. Selection is expected to reduce genetic variation, and yet such variation is observed almost universally. There are several explanations for the maintenance of genetic variation; one of these suggests that variation may be maintained if different genotypes are favoured in different conditions (Levene, 1953; Via and Lande, 1987; Gillespie and Turelli, 1989).

1.1.8 BACKGROUND SELECTION

In the absence of recombination, deleterious mutations will have the effect of reducing genetic polymorphism within a population, as selection acts to reduce the frequency of chromosomes carrying such mutations, thus removing neutral mutations that are linked to deleterious mutations. The process is known as background selection, and its effect is equivalent to that of a reduction in population size, although it applies to tightly-linked regions of the genome, rather than the whole genome (Charlesworth et al., 1993).

As the recombination frequency increases, the effect of background selection decreases as a neutral region associated with a deleterious mutation can recombine with a mutation-free chromosome, thus escaping the fate of the deleterious mutation. Thus, the relative nucleotide diversity can decrease substantially with tighter linkage.
and increasing $U$, as greater background selection eliminates a greater number of chromosomes.

1.1.9 EVOLUTION OF MATE CHOICE

The 'good genes' mechanism is one theory that has been put forward to explain the evolution of female mate choice. This mechanism operates when females select mates on the basis of male traits that are genetically correlated with total fitness, which is related to the number of deleterious mutations a male carries, and their effects. A genetic correlation can then develop between the female preference and male trait, causing each to become exaggerated (Fisher, 1958; Pomiankowski, 1988).

A requirement of the good genes model is that there must be sufficient heritable variation in total fitness between males (e.g. Maynard Smith, 1978). As stated earlier, selection is expected to reduce genetic variation for fitness-related traits. However, if $U$ is high enough, this may create enough genetic variation among males for the good genes mechanism to be able to operate (Rice, 1988).

So, we have established that the rate at which mutations accumulate, and the effects that they have, are important for many areas of evolutionary biology. What, then, has been done to estimate such parameters?

1.2 PREVIOUS WORK ESTIMATING THE PARAMETERS OF MUTATIONS AFFECTING LIFE HISTORY TRAITS

Until recently, the only direct evidence concerning haploid genomic mutation rates ($U$) and their effects ($s$) came from a few experiments carried out in the 1960s and 1970s by Terumi Mukai and collaborators (Mukai, 1964; Mukai et al., 1972; Ohnishi, 1977). An outline of the style of experiment used, known as a mutation-accumulation (MA) experiment, is shown in

Figure 1.1. A number of sublines are generated from a base population, and propagated in parallel for a number of generations in conditions in which selection is minimised, in order to allow mutations to accumulate without being selectively
removed. Life history assays of the MA lines and a non-mutagenised control population are performed, and from the differences between the two, it is possible to obtain estimates of $U$ and $s$.

Figure 1.1: Standard mutation-accumulation experiment

In the experiments of Mukai and Ohnishi, spontaneous mutations were allowed to accumulate on the second chromosome of *Drosophila*, protected from selection by a balancer chromosome. After a number of generations of mutation accumulation, the viability of flies homozygous for the MA second chromosome was compared to those that were heterozygous. By developing the formulae of Bateman (1959), it was possible to obtain estimates of the number of mutations that had accumulated on the
second chromosome and their average effects. This was done by observing the difference in mean and variance for fitness-related traits between the control and MA lines, and from this to extrapolate to the genomic mutation rate per generation. This method is now known as the Bateman-Mukai approach (Bateman, 1959; Mukai, 1964; Mukai et al., 1972; see also Lynch and Walsh, 1998), and makes the assumption of equal effects of mutations. Using this approach, it is possible to obtain a minimum estimate of \( U \geq (\Delta M)^2 / \Delta V_m \), and a maximum estimate of \( \bar{\Delta} V \leq \Delta V_m / \Delta M \), where \( \Delta M \) is the change in mean and \( \Delta V_m \) is the change in mutational variance. This method is described in more detail in Chapter 2 (Materials and Methods). The results of these experiments indicated that the haploid genomic mutation rate was approximately 0.3 per generation, mainly due to the accumulation of mutations with relatively small fitness effects, averaging \( \sim 3\% \).

Due to a decline in interest and the fact that MA experiments are extremely time-consuming, few such experiments were performed for many years, with the exception of some Drosophila studies performed by Houle et al. (1992, 1994a), which produced similar results to those of Mukai and collaborators. Contamination of the control lines, however, had the result that it was not possible to obtain valid estimates of \( U \) and \( s \) from these studies (Houle et al., 1994b).

Some recent work, however, has cast doubt on the results of Mukai and Ohnishi's experiments. The original estimates of \( U \) and \( s \) derived from these experiments were obtained using the Bateman-Mukai approach. However, recent reanalyses of these data have been performed, using more sophisticated maximum likelihood (Keightley, 1996) and minimum distance (García-Dorado, 1997) analysis. Keightley (1996) noted that if a non-mutational effect was fitted, the likelihood improved significantly. He suggested that this might be the result of an increase in the fitness of the balancer chromosomes, which would have the result of increasing the apparent decline in fitness of the MA chromosomes, relative to the balancers. Thus, the spontaneous mutation rate would be lower than previously thought. García-Dorado (1997) obtained estimates that are approximately two orders of magnitude lower than those obtained by Mukai and Ohnishi. Her minimum distance estimation did not make use of data from the balancer chromosomes, and she again suggested that a change in
viability among the balancer chromosomes might be responsible for the variation in results from the different forms of analysis

Given these doubts about the validity of the results of Mukai and Ohnishi, it is valuable to consider the results of a number of new MA experiments that have been performed using both *Drosophila* and other species.

Fernández and López-Fanjul (1996) performed a large MA experiment, which did not involve the balancer chromosome system used by Mukai and Ohnishi. They accumulated mutations over the entire genome in 176 inbred lines of *Drosophila*, derived from an isogenic stock, for over 100 generations. Instead of using balancer chromosomes to reduce the impact of selection, they maintained the lines by full-sib matings, with the flies kept at low density in benign conditions in order to minimise the effects of selection. Although selection is more likely to remove mutations with large effects in an experiment of this design, mildly deleterious mutations should accumulate, as selection is inefficient under such conditions. The decline in viability was smaller than that observed during Mukai and Ohnishi's experiments, and estimates of $U$ from a Bateman-Mukai analysis were significantly lower at 0.02 per haploid genome, with higher average effects, $\bar{\bar{\delta}}$, of $\sim 10\%$ (summarised by Keightley and Eyre-Walker (1999)). However, some doubt has been cast on the validity of these results due to the controls used. As a control, a large population of the same original line was maintained during the course of the MA process, as deleterious mutations would not be expected to accumulate as rapidly in a population of large effective size. However, as this control population was maintained for over 100 generations, it is possible that some evolution occurred in this line (Lynch et al., 1999). In addition, as most new mutations are expected to have small, partially heterozygous effects, they may persist in such a population for a number of generations (Drake et al., 1998). This could have the result of increasing the variance of the controls, leading to an apparent underestimation of the difference in variance between the MA and control lines. Lynch et al. (1999) also argue that the lower values of $U$ may have resulted from selection on the MA lines, due to the large number of generations over which the experiment occurred, and the fact that 12% of the lines were lost during the course of the MA experiment. The loss of so many lines indicates that a large number of backup lines were used, again allowing the possibility for selection to act. However,
if selection had occurred, it would have been most likely to remove mutations with large effects, unless lines with many small mutations were removed. The average effect of the mutations observed was much larger than in Mukai and Ohnishi’s experiments, which suggests that this is perhaps a less convincing explanation for the differences observed.

Two further experiments have been carried out, allowing mutations to accumulate in lines of *Drosophila* protected from selection by favourable environmental conditions, rather than balancer chromosomes (Gilligan *et al.*, 1997; Shabalina *et al.*, 1997). They assayed the productivity of the control and MA lines in both benign and competitive conditions, as it is possible that competitive conditions may reveal larger fitness differences, and are likely to be more relevant to the natural environment. Gilligan *et al.* (1997) measured the genetic load in MA lines, as well as in flies captured from the original wild population, and those from the base population that were maintained in cold conditions, undergoing few generations of MA. They did not observe increased genetic load in smaller populations, nor did they detect an increase in the frequency of chromosomes with deleterious effects.

The results of Shabalina *et al.* (1997) appear to contrast sharply with these results. When the MA lines were compared to a control maintained in cold conditions, and another that had been cryopreserved, they found that productivity decreased dramatically under competitive conditions, though the effect was less apparent when the assays were performed in benign conditions. The decrease in fitness of ~1% per haploid genome, per generation, was very similar to that observed by Mukai and collaborators, indicating a substantial decline in fitness due to the accumulation of spontaneous mutations.

Criticisms have, however, been made of both these experiments. The most serious of these relates to the choice of outbred lines of *Drosophila* as a base population. The reason for this choice was that the authors were particularly interested in the relevance of mutation accumulation to the survival of small natural populations, and their response to captive conditions. Shabalina *et al.* (1997) argue that by using a wild-caught population, they avoid using genetically manipulated populations, such as highly inbred lines or those with balancer chromosomes, which may respond in a
different way from natural populations. A disadvantage of this approach is that it is difficult to be certain that any change in the fitness of the population is only due to the accumulation of new mutations, rather than, for example, inbreeding depression, recombination of gene complexes, or adaptation to a laboratory environment (Keightley et al., 1998; Lynch et al., 1999). Such adaptation is more likely to be a problem when outbred lines are used, because the large amounts of genetic variation present allow more opportunity for selection to act. As MA experiments tend to involve analysis of minor fitness differences between lines, even small changes could swamp the effects of spontaneous mutations. In addition, there could have been a problem with the controls – the recovery rate of the cryopreserved lines used in the Shabalina et al. (1997) experiment dropped from 18% to 8%. As these flies were genetically heterogeneous, it is possible that selective mortality occurred. There may also have been evolution in the wild-caught control population used by Gilligan et al. (Lynch et al., 1999). These problems make it difficult to draw firm conclusions from these two experiments.

One further MA experiment has been carried out using inbred lines of Drosophila, which appears to be free from some of the problems that could be associated with the work described above. Fry et al. (1999) carried out an experiment similar in design to those of Mukai and Ohnishi, but with an additional three isogenic control populations, maintained at large population size. Bateman-Mukai estimates of $U$ were significantly lower than those obtained by Mukai and Ohnishi, with $\hat{U} = 0.02$ mutations per haploid genome and $\hat{s} = 11\%$. Although there remains a possibility of evolution occurring among the control lines, this appears to be unlikely, as they did not observe significant variation among the control lines, and the viability of the controls did not alter significantly over the course of the experiment.

Estimates of $U$ and $s$ obtained from Drosophila experiments therefore appear to vary a great deal, and a number of doubts have been raised about the validity of some of them. Of course, mutational parameters may vary between species, and so it is informative to consider MA experiments that have been carried out in species other than Drosophila.
The only other arthropod in which estimates of $U$ and $s$ have been obtained is *Daphnia pulex* (Lynch *et al.*, 1998). The progenitors of the MA lines were ten clones sampled from the wild. As *D. pulex* reproduces by ameiotic parthenogenesis in the laboratory, problems of inbreeding depression and adaptation to the laboratory environment are unlikely to affect this work. The 100 lines were propagated by the transfer of single offspring for 30 to 35 generations. In addition, control lines could be maintained in a frozen state. Estimates of $U$ of $\sim 0.3$ were obtained, with small mean mutational effects of $\sim 2\%$. Life history assays were performed at generation 7, 16 and 32. However, in the third assay the control lines performed poorly, and the reason for this has not been established. These data were excluded from the analysis, but it seems likely that some uncontrolled environmental effect was influencing the results, and as the reason for this is not known, it remains possible that the other results were affected in a similar way. Such an effect may also have impacted on the MA stage of the experiment; 70% of the MA lines were lost by generation 32.

Two experiments have been performed, allowing inbred, genetically homogeneous populations of the nematode *Caenorhabditis elegans* to accumulate mutations for 60 generations (Keightley and Caballero, 1997) and 50 generations (Vassilieva and Lynch, 1999). These experiments were similar in design, with lines derived from the wild-type N2 strain being propagated by the transfer of single self-fertilising hermaphrodites, maintained in benign conditions with no overcrowding and an excess of food. In both experiments, the isogenic base population was frozen and used as a control, which could be assayed contemporaneously with the MA lines.

The results of the two experiments were qualitatively similar. Both observed small, non-significant changes in mean between the control and MA lines, and an increase in between-line variance for productivity, intrinsic population growth rate, and some other life history traits. Values of $U$ were low, e.g. for intrinsic growth rate Keightley and Caballero obtained an estimate of 0.0035 mutations per haploid genome, compared to Vassilieva and Lynch’s estimate of 0.008. Values of $\bar{s}$ were also quite high, at 10% and 20% respectively. There are a few differences in protocol between the two experiments; for example, Keightley and Caballero’s MA experiment was performed at 25°C, rather than 20°C, though in both cases the life history assay were
performed at 20°C. The way in which the life history assays were carried out also differed between the experiments: Keightley and Caballero allowed adult worms to lay eggs on petri-dishes for four hours, then transferred these offspring onto individual plates 48 hours later, when they were at the L3 larval stage. Vassilieva and Lynch, on the other hand, transferred the assay worms as soon as possible after hatching, when they were at the L1 stage. Worms were transferred with platinum wire picks, a process that is more difficult with smaller worms, and they are more likely to be damaged during this process. Indeed, the productivity of these worms was lower – they produced an average of 175, rather than 250 offspring, and the variance was greater, even among the controls. Several of Vassilieva and Lynch’s control lines, averaged over five worms, produced no offspring at all. In contrast, the lowest number of offspring produced by a control line in Keightley and Caballero’s study was 217, averaged over eight worms. As the control and MA lines were treated in the same way within an experiment, and the fitness of the MA lines was compared to that of the controls, it is probable that these large differences have not affected the interpretation of the results.

An MA experiment involving a self-fertilising plant, *Arabidopsis thaliana*, has recently been reported (Schultz et al., 1999). This involved ten generations of MA in 1,000 inbred lines, propagated by single-seed transfers. Plants grown from seeds maintained in dark, dry conditions at room temperature during the MA process were used as controls. Fitness assays performed in non-competitive conditions were used to determine the number of seeds produced per seed sown. From this, an estimate of $U$ of 0.05 was obtained, with an average effect of 23%. The 95% confidence intervals for $\hat{U}$ of 0.002 to 0.4, and 2% to 90% for $\hat{s}$, are large, probably due to high levels of environmental variance and the small number of generations of MA that occurred.

Kibota and Lynch (1996) have performed an MA experiment involving 50 lines of the bacterium *Escherichia coli*. Again, they started from a genetically identical base population, with selection minimised by putting the lines through repeated population bottlenecks for 300 days (approximately 7500 generations). Their exponential growth rate was then compared to the cryopreserved base population. The estimate of $U$ of 0.0002 was far lower than that obtained from any other organism studied, with
average effects, $\bar{y}$, of 1.2%. It should perhaps not be surprising if a bacterium has a lower rate of mutation than the other organisms studied, given the large differences in genome size, number of cell divisions, generation interval, and many other factors between them.

No MA experiment has been performed using vertebrates. Such results would be of great interest, especially as vertebrates are more appropriate model systems for some issues that require knowledge of mutational parameters, such as the conservation of small populations and implications of mutational degradation for human fitness. However, MA experiments are typically extremely time-consuming and labour-intensive when invertebrates are used, and would be even more so if a vertebrate system was used. There is also the problem of obtaining a suitable control.

Charlesworth et al. (1990) have suggested a method for estimating deleterious mutational parameters that does not rely on such labour-intensive MA experiments. This approach can be used to estimate mutational parameters in highly inbred natural populations. The degree of inbreeding depression can be inferred from the change in fitness-related traits of individuals produced by crossing two inbred strains. Highly inbred populations would be expected to rapidly purge recessive lethal mutations, and few polymorphisms should be maintained by overdominance. Therefore, it is reasonable to assume that this change in fitness is due to mutational load, and thus it is possible to obtain an estimate of the genomic deleterious mutation rate. This technique requires estimation of the average degree of dominance of deleterious mutations. Suitable estimates of inbreeding depression are rare, but data from several plant species suggested that the haploid genomic mutation rate is between 0.06 and 0.43 (summarised by Drake et al., 1998).

Deng and Lynch (1996) have further developed this approach to apply to the change in fitness upon inbreeding in naturally outbreeding populations. This method has the advantage that it can be more widely applied than that of Charlesworth et al. (1990), and estimates of $U$ for Drosophila and Daphnia obtained using this approach have been similar to those obtained from inbreeding plants (Drake et al., 1998; Table 7). However, while both approaches make the assumption that genetic variation for fitness is solely a consequence of mutation-selection balance, this may be less
applicable to outbreeding populations. In such populations, there is a greater probability of selection maintaining variation to some extent, e.g. via overdominant loci. This could result in $U$ being overestimated by the Deng and Lynch approach, to an unknown degree (Drake et al., 1998).

Kondrashov and Crow (1993) have suggested an alternative, molecular approach to estimating mutation rates. If mutations have neutral effects, the substitution rate for new mutations is expected to be equal to the mutation rate (Kimura, 1983; Section 3.5). Deleterious mutations, however, will be eliminated. By comparing the rates of substitution in homologous regions of the genome from two related species, it should be possible to observe the difference in substitution rate between neutral regions of the genome (such as pseudogenes) and the overall rate of substitution. From this, it is possible to obtain estimates of the total number of substitutions that have occurred, and the proportion of these that are selectively deleterious. Eyre-Walker and Keightley (1999) used a modification of this procedure to estimate the deleterious mutation rate for protein coding sequences in hominids. Assuming that synonymous substitutions are neutral, they counted the synonymous and non-synonymous substitutions between protein-coding sequences of humans and chimpanzees, using another primate as an outgroup. By using estimates of the time since divergence of humans and chimpanzees, the average generation time and the number of genes, they estimated the mutation rate per generation in protein-coding sequences to be 2.1 per haploid genome, 0.8 of which are expected to be deleterious. This is likely to be an underestimate, due to the fact that several of their estimates, including generation time, were conservative, insertions and deletions were not included, and these figures only apply to protein-coding regions of the genome. In addition, their sample of genes may not be representative (Eyre-Walker and Keightley, 1999). Therefore, their results indicate that the mutation rate in hominids appears to be remarkably high. A disadvantage of this molecular approach is that it provides no information concerning the distribution of the effects of such mutations.

These estimates of mutation rates show a large degree of diversity, even within a single species such as Drosophila. In addition to criticisms of the experimental design, including potential problems with the controls and other factors that may have
lead to non-mutational changes in the lines being studied, several suggestions have been put forward to explain these differences.

Many traits that differ between flies, worms, plants and bacteria could influence the genomic mutation rate. One suggestion is the number of germline cell divisions per generation (e.g. Lynch et al., 1999), which appears to influence the difference in male and female mutation rates in humans (Crow, 1997). The number of cell divisions varies among the species studied: from one in E. coli, eight or nine in C. elegans (Wilkins, 1993), to 26 in Drosophila (Drost and Lee, 1995). Because plants lack a reserved germline, gametes are formed from somatic cells, which may have undergone many cell divisions. The number of germline cell divisions is therefore likely to vary. These differences in the number of germline cell divisions between species are not enough to explain the variation in estimates of mutation rates, but they may contribute towards the variation observed. The generation interval may also influence the mutation rate, although these two factors are likely to be correlated with each other and other traits, making it difficult to determine the causal mechanisms.

The genome size also differs between species. For example, the Drosophila genome is larger than that of C. elegans, though they appear to have similar numbers of genes (Ashburner, 1989; The C. elegans Sequencing Consortium, 1999). Therefore, there would have to be a large impact of mutations in intergenic regions of the genome for this to account for the difference in mutation rate observed.

It is also possible that different classes of mutational event, such as transposable element insertions, are responsible for the differences observed. Active transposition is negligible in the N2 strain of C. elegans and the E. coli and A. thaliana strains used in the MA studies. Lines of Drosophila, however, show high levels of transposition, and these vary greatly between strains. Taking into account all these differences, it is possible to obtain similar estimates of U in the different species studied (e.g., Lynch et al., 1999), but in order for these explanations to be valid, predictions about mutational parameters in different species need to be upheld.

Finally, it is possible that the assays have not detected all of the mutations that accumulated, and the proportion that was missed may have varied. The ideal would
be to assess the impact of all mutations that affect fitness, but fitness is a complicated trait, and different groups have used different assays, usually based around productivity, to assess the impact of mutations on fitness-related traits. It is possible, though, that mutations have occurred that have not been detected, because they affect different traits, act in different environments, or are simply too small to be detected in laboratory assays of the scale used.

1.3 AIMS OF THIS STUDY
An aim of this study is to assess the degree to which MA experiments may have underestimated mutation rates, through two approaches. Firstly, the MA lines of *C. elegans* generated by Keightley and Caballero (1997) were assayed in a range of harsher conditions, in order to determine whether such conditions would reveal more or less extreme fitness differences than benign conditions (Chapter 3). This approach may help us to understand the very different estimates of mutational parameters derived from different MA experiments, and will provide information concerning the extent to which the effects of spontaneous mutations depend on the environment in which they are expressed (genotype by environment interaction).

It remains possible that no laboratory-based fitness assay will detect the effects of all the mutations that have accumulated. For some of the issues discussed at the beginning of this chapter, such as the evolution of sex, the total number of mutations accumulating is important, regardless of the size of their effects. Thus, even very slightly deleterious mutations are important, though they are less likely to have been detected in these assays. In order to estimate the proportion of mutations that are likely to be detected in such experiments, life history assays were carried out using lines of *C. elegans* in which the number of mutations induced could be inferred. From the proportion of such mutations that are detected, it should be possible to evaluate the extent to which MA experiments may underestimate the rate at which spontaneous mutations accumulate (Chapter 4).

In addition to the number of mutations detected (*U*) and their average effect (*s*), it will be possible to obtain additional information concerning the properties of such mutations. In particular, it is possible to measure the joint effect of mutations on
more than one trait, which is important for understanding correlated responses to selection, and the constraints that influence evolutionary change (Falconer and Mackay, 1997). The correlation between mutational effects on longevity and productivity are of particular interest as they relate to the evolution of ageing (Chapter 4). The two theories put forward to describe the evolution of ageing, outlined earlier, make different predictions about such effects. The mutation accumulation theory predicts a high input of mutations with deleterious effects late in life, but no correlated effects early in life. The optimality, or antagonistic pleiotropy theory, predicts that mutations are likely to reach fixation if they have deleterious effects late in life, but advantageous effects on early components of fitness. There may, therefore, be trade-offs, such that any mutations that increase one trait are likely to do so at the expense of another trait. Studying the effects of any mutations identified that increase either productivity or longevity, in order to identify possible trade-offs, may throw light on this matter (Chapter 5).

In addition, it is possible to determine the extent to which new mutations have correlated effects on more than one trait, and the effects of new mutations on mortality rates (Chapter 4). It was originally observed that mortality rates increase exponentially with age (Gompertz, 1825), and although this has been widely confirmed, some recent data have indicated that mortality rates may actually decelerate with increasing age in some species, including humans (Charlesworth and Partridge, 1997; Vaupel et al., 1998; Partridge and Mangel, 1999). The effects of new mutations on mortality curves can provide information on age-specific properties of mutations, and differences in mutational target sizes at different ages.

1.4 THE STUDY ORGANISM *CAENORHABDITIS ELEGANS*

The model organism used throughout the course of this study is the free-living nematode worm, *Caenorhabditis elegans*. In the natural environment these worms live in the soil, feeding on microorganisms, and are geographically widespread; they have been isolated from samples taken in Europe, North America and Australia (Fitch and Thomas, 1997). They are of no economic importance to humans.
This organism has many features that make it particularly suitable for biological study (Brenner, 1974). It is small; adults are approximately 1mm long, and can be easily maintained in the laboratory, either in liquid culture or on solid agar, with *Escherichia coli* as a food source. It has a short life-cycle of approximately three and a half days at 20°C, will reproduce over a period of three to four days in optimal conditions, and lives for two to three weeks. As with other nematodes, the worms develop through a series of four larval stages, L1 to L4, which are separated by moults. Under conditions of overcrowding and starvation, *C. elegans* can form an alternative L3 stage, known as the dauer larva (Riddle and Albert, 1997). Dauer larvae have a sealed mouth and toughened cuticle, and worms can survive for several months in this arrested state until food is reintroduced, at which point they progress to the L4 larval stage, and continue to develop in the normal manner.

*C. elegans* is a diploid organism, with five pairs of autosomes (I to V) and an X chromosome. There are two sexes, a self-fertilising hermaphrodite and a male. Hermaphrodites cannot mate with each other, but will produce around 250 offspring via self-fertilisation. In contrast with most organisms, the number of sperm, rather than eggs, limits the number of offspring produced. Therefore, when they mate with males, hermaphrodites can produce many more offspring, and sperm from the male participate in fertilisation preferentially. Hermaphrodites have two X chromosomes, and offspring produced by self-fertilisation will be predominantly hermaphrodite, with a low frequency of males (<0.05%) being produced by non-disjunction of the X chromosome (Meyer, 1997). Males have just one X chromosome, and offspring produced via cross-fertilisation are expected to show a 50:50 sex ratio (Emmons and Sternberg, 1997). Neither inbreeding depression nor heterosis has been observed in *C. elegans*, a feature that facilitates the study of life history traits, which are dependent on the overall health of the organism (Johnson and Wood, 1982; Johnson and Hutchinson, 1993).

*C. elegans* has a transparent body wall, and all of the 959 somatic cells in the hermaphrodite are visible with a microscope. A picture of an adult hermaphrodite is shown in Figure 1.2. The genome size is small enough to be genetically tractable and the entire genome has now been sequenced (The *C. elegans* Sequencing Consortium, 21
The standard ‘wild-type’ laboratory strain of *C. elegans* is the N2 strain, isolated by Sidney Brenner (1974) from a strain originally obtained from mushroom compost near Bristol, England.

One final feature that makes *C. elegans* particularly suitable for laboratory work is the fact that it can be cryopreserved almost indefinitely at -80°C, which is useful for maintaining control populations, and for preserving stocks for future use.

Figure 1.2: Adult *C. elegans* hermaphrodite
Chapter 2: MATERIALS AND METHODS
2.1 CULTURE TECHNIQUES

The standard experimental and statistical techniques used throughout the course of this work are described here. Recipes for the solutions used are outlined in Section 2.3. Where necessary, any modifications to the standard technique are outlined in the appropriate results chapters. Methods that are specific to certain chapters are covered in the appropriate chapter.

2.1.1 STRAINS AND CULTURE CONDITIONS

A wild-type N2 strain of C. elegans, which was used as the basis for all the experiments, was obtained from the Caenorhabditis Genetics Center, cryopreserved (see Section 2.1.3) and maintained in a frozen state until required. Live cultures of worms are maintained using the standard techniques employed in the culture of C. elegans (Sulston and Hodgkin, 1988), as follows: worms are fed on a lawn of Escherichia coli strain OP50, which has been allowed to grow overnight on MYOB agar plates, and are kept in incubators at 20°C, unless otherwise stated. Three sizes of agar plate are used throughout the work: small (3.5cm plates seeded with a 30μl suspension of E. coli), medium (5.5cm plates seeded with 90μl of E. coli), or large (9cm plates seeded with 240μl). For the majority of the work described here, small agar plates are used. Individual worms are transferred between plates manually, using a flattened platinum wire pick. Platinum wire is used, as it cools rapidly after sterilisation in a bunsen burner flame.

Dr. Peter Keightley's laboratory strain designation is KL. The names of all strains of worms described here, with the exception of N2, will thus begin with this designation, e.g., the first line derived from the EMS mutagenesis experiment (Chapter 4) will be named KLE1, hereafter called E1 for brevity.

2.1.2 CLEANING AND SYNCHRONISING WORM CULTURES - ALKALINE HYPOCHLORITE TREATMENT

Alkaline hypochlorite treatment (Sulston and Hodgkin, 1988) can be used both to decontaminate worms in order to remove any fungal or bacterial contaminants, and to
synchronise worm cultures. The treatment works by killing larval and adult *C. elegans* and any contaminants, leaving just the worms' eggs, which are resistant to the treatment. Therefore, it has the effect of harvesting the eggs of gravid adults, and thus both decontaminating and synchronising the worm cultures. The procedure can be modified for use with large or small (<10) numbers of worms, as outlined below.

### 2.1.2.1 Alkaline hypochlorite treatment for large numbers of worms

This method can be used for cleaning/synchronising entire platefuls of worms - cultures consisting of hundreds of thousands of worms may be obtained, if required. The plates used should ideally contain large numbers of gravid adult worms and eggs – only fully-formed eggs survive the process. Worms are washed off the plates using 3ml of sterile M9 buffer, by gently swirling the buffer over the surface of the agar and removing it with a pipette while tilting the plate to one side. The worm suspensions are then placed in sterile 15ml screw-top centrifuge tubes, M9 buffer is added to 10ml, and the worms are rinsed by centrifugation to remove bacteria and other debris. Tubes are spun at 2000rpm (200g) for 2 minutes in a table-top centrifuge and then placed on ice to reduce worm movement, while the supernatant is removed. 10ml of M9 is then added to each tube, the contents are mixed, the tubes centrifuged again, and the supernatant removed. To harvest the eggs, 7ml of freshly-made alkaline hypochlorite solution is added to each tube, which is then allowed to stand for 15 minutes at room temperature, with brief and careful vortexing every few minutes. The tubes are centrifuged to collect the eggs, and the pellet rinsed twice by resuspension in 10ml of M9 buffer, as above. The final pellets are resuspended in a small amount of M9 buffer (<0.25ml) for transfer by pipette to agar plates, to allow the eggs to hatch and grow.

### 2.1.2.2 Alkaline hypochlorite treatment for small numbers of worms

This method is more suitable when synchronised cultures consisting of tens, rather than thousands, of worms are required. 7μl of freshly-made alkaline hypochlorite solution is pipetted onto a seeded agar plate, between the bacterial lawn and the edge of the plate. Four gravid adult hermaphrodites are then immersed in the solution. This process kills the worms and any contaminants, but the solution is absorbed into
the agar before the resistant eggs hatch, producing a decontaminated, synchronous culture of worms. The larvae crawl away from the bodies of the adults, towards the food, thus reducing the chances of any bacterial carry-over. Where possible, the young worms are transferred onto a fresh plate, on which no contamination is present.

2.1.3 CRYOPRESERVATION OF LINES

Wild-type and mutant strains of *C. elegans* can be stored indefinitely at -80°C (Suiston and Hodgkin, 1988). This allows cultures to be conveniently stored when not required, permits experiments to be performed on the same population of worms at different times, and is particularly useful for maintaining control populations, enabling experimental lines of worms to be measured contemporaneously with the population from which they were derived.

In order to obtain a culture of worms in a suitable condition for freezing, five adult worms are placed onto each of two medium agar plates. After five or six days (or longer if a strain is slow-growing), the plates contain predominantly just-starving L1 and L2 larvae (one day after food is exhausted). Worms are collected from each plate in 1ml of M9 buffer and pipetted into a sterile 2ml tube, and equal volumes of freezing solution are added. The solution is then mixed and aliquoted into four sterile 0.5ml cryogenic tubes. The tubes are inserted into Styrofoam freezing boxes and immediately placed at -80°C. The following day, the first and last vials are test thawed; the remainder are stored at -80°C. For thawing, tubes are warmed in the hand until the suspension of worms melts, and the contents are mixed and tipped onto the edge of a seeded agar plate (Suiston and Hodgkin, 1988).

2.2 LIFE HISTORY ASSAYS

In order to obtain measures of the fitness of lines of worms, daily reproductive output and lifespan are measured. This involves transferring the adults onto new plates during each day of the reproductive period, and then counting the number of surviving progeny three days after they are produced. The day on which each parental worm dies is also recorded. From these data, it is possible to analyse several traits: longevity, total productivity, early productivity (offspring produced during the first
two days of the reproductive period) and late productivity (offspring produced during
the remainder of the reproductive period, usually three to four days). As the worms
are counted three days after hatching, the productivity assays include the viability of
the parents, their fertility and the viability of offspring until the L3 to young adult
stage.

After thawing, individual replicates of each line are maintained for at least three
generations prior to an assay in order to minimise any effects of the freezing and
thawing processes. Because the results of life history assays can vary between
experiments (Kenyon, 1997), assays of lines that are to be compared are carried out
contemporaneously. At the start of the assay, each line is assigned a random code,
which ensures randomisation with respect to the position of the plates within the
incubator and the order in which transfers and counts are performed. This also
ensures that the measurers are unaware of line identity while they are performing the
life history assays, thus eliminating the possibility of biases being introduced.

To perform life history assays on one line, four gravid adults are placed onto a small
agar plate and allowed to lay eggs for approximately three hours before being
removed. After 48 hours, a single L3 worm is transferred onto a new plate, and then
transferred 48 hours later and at 24-hour intervals for the entire reproductive period in
order for the daily production of viable offspring to be recorded. The N2 strain of C.
elegans, when kept at 20°C, will normally reproduce over a four to five day period.
Worms that are less fit or kept in harsher conditions may exhibit delayed
reproduction, and produce fewer offspring over a longer period. The life cycle of the
worms occurs more rapidly, over a shorter period, as the temperature is increased.
Progeny are counted ~72 hours after the parental transfer, when they are at the L3 to
young adult stages. Plates are cooled to 4°C to inhibit worm movement, and counting
is performed manually. Agar plates are placed on a transparent acetate sheet, on
which thin parallel lines have been drawn at 5mm intervals. This allows worms to be
examined at high magnification under a zoom stereo binocular microscope. Because
the worms on each plate are age-synchronous, stationary, and examined at high
magnification, manually counting the worms is a straightforward process.
The lifespan of the worms assayed in the productivity experiments can also be recorded. Parental worms are scored as being dead or alive every day during the reproductive period; afterwards, they are scored six to seven times per week, and transferred onto fresh agar plates one to two times. The ageing process is easy to observe in *C. elegans* (Kenyon, 1997). Young, healthy worms move and feed continuously, and defecate regularly. As the worms age, these processes become slower and less regular, until they cease altogether. During the final stages of life, worms show visible signs of ageing: a loss of turgor and signs of decay become apparent. They may cease spontaneous movement altogether. Worms are therefore scored as dead if they show such signs of decay and fail to respond to a light touch with a platinum wire pick. Once a worm has died, its body will decay rapidly, allowing the death to be easily confirmed the following day if any doubt remains.

### 2.3 SOLUTIONS

#### 2.3.1 MYOB AGAR

- 7.4g pre-mix (see below)
- 20g agar

Add water to 1 litre. Autoclave; cool to \(-55^\circ C\) before pouring.

- **Pre-mix:**
  - 27g NaCl
  - 7.4 g Tris HCl
  - 3.2g Tris OH
  - 62.2g Bacto-tryptone
  - 0.108g Cholesterol

After pouring, plates can be stored at \(<8^\circ C\), either before or they have been spotted with *E. coli*, but they must be allowed to reach room temperature before worms are added.

#### 2.3.2 LB (LURIA-BERTANI) MEDIUM FOR *E. coli*

- 10g Bacto-tryptone
- 5g Bacto-yeast extract
- 10g NaCl

Use an autoclaved bottle; add deionised water to 1 litre, autoclave and then store at 4°C.

10ml LB medium is seeded with one colony of *Escherichia coli* strain OP50 and grown overnight at 37°C. The solution is aliquoted into sterile 2ml tubes and stored at 4°C to restrict further growth. This bacterial solution can be spotted onto agar plates and grown overnight at room temperature to provide a food source for *C. elegans*.

### 2.3.3 M9 BUFFER
- 6g Na₂HPO₄
- 3g KH₂PO₄
- 5g NaCl

Add water to 1 litre. Autoclave; then, using sterile technique, add 1ml 1M MgSO₄.

### 2.3.4 ALKALINE HYPOCHLORITE SOLUTION
- 2ml fresh Clorax bleach or equivalent (4-6% sodium hypochlorite (NaOCl))
- 5ml 1M NaOH

Alkaline hypochlorite solution should be made freshly for each use.

### 2.3.5 FREEZING SOLUTION
- 5.85g NaCl
- 6.8g KH₂PO₄
- 300g Glycerol
- 5.6ml 1M NaOH

Add water to 1 litre. Autoclave; then, using sterile technique, add 3ml 0.1M MgSO₄.
2.3.6 EMS INACTIVATION SOLUTION

- 0.1M NaOH
- 20% w/v Na₂S₂O₃

Contaminated equipment should be soaked for at least 24 hours in inactivation solution, prior to disposal.

2.4 STATISTICAL TECHNIQUES

2.4.1 VARIANCE COMPONENTS AND CORRELATIONS BETWEEN TRAITS

The phenotypic values of traits measured on the same individual are often found to be correlated. This may be the result of environmental correlation, e.g., harsher conditions may reduce both productivity and survival, or genetic correlations where a single gene may influence several traits (pleiotropy), or several genes with effects on two characters may be associated in the same individuals. Knowledge of the relationship between traits is important for understanding correlated responses to selection (Falconer and Mackay, 1997). The traits studied here are likely to be correlated with each other, for example if generally deleterious mutations affect all the traits. Therefore, the correlation between the traits, and the degree to which a change in one trait can be affected by the other, are of interest.

Analysis of variance (ANOVA) is used to determine the degree of correlation between the traits. An advantage of C. elegans as a model system is the ease with which highly inbred, genetically identical populations may be obtained and studied. Because the lines are highly inbred, there is not expected to be any genetic variance between individuals within a line, and thus the residual component of variance must be purely environmental. Therefore, by subtracting the environmental components of variance and covariance from the phenotypic components, it is possible to obtain an estimate of the genetic components. Combining the covariance with estimates of the variance components for the two traits then allows estimation of the degree of correlation (\( r \)) between the traits (Lynch and Walsh, 1998).

In order to study the degree of covariance between traits, lines in which only one trait had been measured are removed from the data before analysis. For example, if a
worm is accidentally killed on its eighth day of life, it may have completed its reproductive period, so data would be available for productivity traits, but not lifespan. Thus, for all the individuals used in bivariate analysis, measures of both traits must be available. Analysis of variance is carried out for each of the individual data sets, and for the sums of each pair of traits.

The genetic component of variance for a trait, $V_G$, can determined by subtracting the residual mean-squares value (environmental, or within-line; $MSW$) from the between-line mean-squares (phenotypic; $MSB$) and dividing by the number of replicates assayed, $n$:

$$V_G = \frac{MSB - MSW}{n} \quad (2.1)$$

The phenotypic and environmental variance components ($V_P$ and $V_E$ respectively) can be determined in a similar manner, using just the expected between-line ($MSB$) or within-line ($MSW$) mean-squares values, divided by the number of replicates assayed.

In order to determine the correlation between traits, it is necessary to determine the variance components for the two traits, as outlined above, and their covariance. The covariance can be calculated using knowledge of the variance components of the individual traits, and the variance of the sum of the two traits, as follows. The variance of the sum of two traits, $A$ and $B$, is $V(A + B) = V(A) + V(B) + 2Cov(A + B)$. Therefore, the covariance between two traits, $Cov(A + B)$, can be determined by subtracting the sum of the variance components for both the traits from the variance of the sum of the two traits, and dividing by two:

$$Cov(A + B) = \frac{V(A + B) - (V(A) + V(B))}{2} \quad (2.2)$$

The correlation between the traits, $r$, can then be calculated from the covariance and variances of the traits. This is a dimensionless value, which is suitable for comparison with other traits:

$$r = \frac{Cov(A + B)}{\sqrt{V(A) \times V(B)}} \quad (2.3)$$

Using this approach, the phenotypic, environmental and genetic correlations can be determined, by using the appropriate mean-squares values from analysis of variance.
2.4.2 Mutational Heritability

The rate at which new quantitative variation is produced via mutation, $V_M$, can be determined for each trait assayed. According to Lynch and Hill (1986), in mutation accumulation experiments, the mutational variance, $V_M$, can be estimated from the genetic, or between-line, component of variance, $V_G$, obtained by analysis of variance of the mutagenised lines, such that:

$$V_M = \frac{V_G}{t},$$

where

$$V_G = \frac{MSB - MSW}{n},$$

$t$ is the number of generations of mutation accumulation, and $n$ is the number of replicate lines. The environmental variance, $V_E$, is equivalent to the within-line (residual) variance component for the control lines, $MSW(controls)$.

Such mutational variance, $V_M$, can then be scaled relative to the environmental variance, $V_E$, to obtain the mutational heritability, $h^2_m$, a dimensionless value that can be compared with values obtained from other traits:

$$h^2_m = \frac{V_M}{V_E}.$$

2.4.3 Estimates of Mutational Parameters I: The Bateman-Mukai Approach

When mutation accumulation experiments are carried out, the number of mutations induced at the DNA level is not usually known. Instead, mutational parameters are derived from fitness assays that measure the difference in the mean and variance of mutation-accumulation and control lines. One way of estimating mutational parameters is to use the Bateman-Mukai (BM) approach (Bateman, 1959, Mukai, 1964, Mukai et al., 1972, see also Lynch and Walsh, 1998), which makes the assumption that all mutations have equal effects.
The number of new mutations occurring in a line per generation is $2UN$, where $2U$ is the diploid genomic mutation rate and $N$ is the population size. When selection is minimised as a result of small population size and good conditions, all new mutations with selection coefficients $<1/2N$ (i.e., 0.5, if the population size is 1), are effectively neutral. Thus, the number of new mutations that are expected to become fixed is equal to the mutation rate multiplied by their initial frequency, $1/(2N)$, i.e., $U$, the haploid mutation rate. The mean decline in fitness, $\Delta M$, should therefore be proportional to the haploid mutation rate, $U$, and the proportion by which fitness is reduced as a result of a homozygous mutation, $s$. (In the absence of dominance, the effect of a heterozygous mutation is $s/2$). This relationship can be described by the equation

$$\Delta M = US^2.$$  \hfill (2.7)

$\Delta M$ can be obtained from a regression of the line means on time, or as the total difference between the control population and the final, mutated population, which is the method used during the course of this work, as information was not available from intermediate populations.

The variance is simply the sum of the mean squared effects per locus multiplied by the rate of mutation. Summing over all loci, and assuming that mutations have equal and additive effects, the mutational variance (the genetic variation produced by mutation) becomes:

$$\Delta V_M = US^2$$ \hfill (2.8)

(Lynch and Walsh, 1998, p.329). The mutational variance, $V_M$, can be estimated from the between-line component of variance, $V_G$, which is obtained by analysis of variance:

$$V_G = tV_M$$ \hfill (2.9)

where $t$ is the number of generations of mutation accumulation, as described in Section 2.4.2.

Using these equations, it is possible to obtain estimates of $U$ and $s$:  

33
\[(\Delta M)^2 / (\Delta V_G / t) = (U\bar{s})^2 / U(\bar{s}^2) = \hat{U};\]  
(2.10)

\[(\Delta V_G / t) / \Delta M = U\bar{s}^2 / US = \hat{s}.\]  
(2.11)

These results assume that \(\bar{s}^2 = \bar{s}^2\), ignoring any variance of the individual \(s\)'s, which would inflate the genetic variance relative to the change in mean. Therefore, in order to relax the assumption of equal mutational effects, these equations are inequalities, providing a minimum estimate for \(U\) and a maximum estimate for \(s\).

Using the data obtained from life history assays of control and mutagenised lines, it is thus possible to use this method to obtain estimates of \(U\) and \(s\). The values required are \(\Delta M\) and \(\Delta V_G\). \(\Delta M\) can be described as \((M_{MA} - M_{Con})/M_{Con}\), where \(M_{MA}\) is the mean fitness of the mutation-accumulation (MA) lines and \(M_{Con}\) is the mean of the control lines. \(\Delta V_G\) is the difference in the between-line variance of the MA and control lines, as determined from analysis of variance, i.e.,

\[\Delta V_G = \frac{MSB_{MA} - MSW_{MA}}{n} - \frac{MSB_{Con} - MSW_{Con}}{n},\]  
(2.12)

where \(MSB\) and \(MSW\) refer to the between-line and within-line mean squares values, respectively, the subscripts \(MA\) and \(Con\) refer to the MA and control lines, and \(n\) is the number of replicates.

### 2.4.4 Estimates of Mutational Parameters II: The Maximum Likelihood Approach

A second method, developed by Keightley (1994), is available to infer mutational parameters from the phenotypic divergence of replicated inbred lines containing a number of independently accumulated mutations. This requires using maximum likelihood (ML) analysis, an approach used to determine the parameter values that produce the best fit to the data, assuming a Poisson distribution of mutation numbers. This has the advantage that it is not necessary to make the assumption that mutations have equal effects. It is possible to use ML analysis, making the assumption of equal mutation effects (equivalent to BM analysis), and thereby estimate \(U\) and \(s\). Alternatively, it may be assumed that mutations follow a specific distribution, such as the gamma distribution, and estimate \(U\), \(s\), and the gamma distribution parameters. It
is also possible to fix $\hat{U}$, for instance by inferring the minimum number of deleterious point mutations that have been induced, and then estimate $\hat{s}$ or the distribution parameters.

A gamma distribution was chosen because it can take a wide variety of shapes, from L-shaped to approximately normal. Examples of gamma distributions are shown in Figure 2.1. The distribution is continuous with two parameters, $\alpha$ and $\beta$, which specify scale and shape, respectively. The mean mutational effect, $\hat{s} = \beta/\alpha$. By using ML, the fit to the data of different distributions of mutational effects can be compared. As $\beta \to 0$, the distribution becomes more leptokurtic, and $\beta \to \infty$ in the case of equal mutation effects, equivalent to the BM model. Varying $\alpha$ will change the unit of measurement, but will not affect the shape of the density. Evidence from studies in which mutation parameters were estimated from simulated experiments indicate that ML produces more accurate estimates than the BM approach, and is more effective if the data do not correspond closely with the model assumed (Keightley, 1998).

Figure 2.1: Gamma distributions ($\alpha = 0.5$)
Chapter 3: Genotype by Environment Interactions for Spontaneous Mutations Affecting Life History Traits in C. Elegans
3.1 INTRODUCTION

3.1.1 GENOTYPE BY ENVIRONMENT INTERACTIONS

The properties of mutations affecting life history traits are important for many evolutionary theories, but mutational effects can vary in a number of different ways (see Chapter 1). An example of such variation occurs when the effect of a mutation depends on the environment in which it is expressed, a process known as genotype by environment interaction (GEI).

The idea that mutational effects can vary depending on the environment in which they are expressed, and that the same genotype may not be optimal in all environments, is central to a number of evolutionary and ecological ideas. These include the maintenance of genetic variation, and explanations of the extent of biodiversity and the evolution of ecological specialisation, as outlined below. GEI is defined as differences in the response to the environment of different genotypes, which may occur in different ways, as shown in Figure 3.1. The 'norms of reaction' are shown for the means of three lines reared in two different environments. If the norms of reaction of the lines are not parallel, beyond any deviations expected due to sampling error, GEI can be said to occur. Figure 3.1(a) shows no evidence of GEI; the three lines do not vary in their response to the two environments. In Figure 3.1(b), some evidence for GEI can be observed: the lines vary in their response to the environments, though the ranking of the three lines does not change. In Figure 3.1(c), there is evidence of extensive GEI, with different lines having optimal fitness in the two environments (i.e., there is crossing of the reaction norms).

Selection that favours different genotypes in different environments could be responsible for the maintenance of quantitative genetic variation. Natural selection and random genetic drift are normally expected to reduce genetic variation for quantitative traits, and yet genetic variation can be observed for virtually all traits in natural populations. How then is such variation maintained? One possibility is that GEI could maintain quantitative genetic variation if individuals within a population encounter more than one environment, and the optimal phenotypes differ among environments (Levene, 1953; Via and Lande, 1987). This is more likely to be the
case if no one genotype has the highest relative fitness in all environments (Gillespie and Turelli, 1989). Thus, selection would act to increase different allele frequencies in different environments, maintaining variability at such loci.

Figure 3.1: Different forms of genotype by environment interaction (GEI)

The evolution of ecological specialisation is also somewhat puzzling. The advantages of being a generalist would appear to be obvious, and selection would be expected to favour broader, rather than narrower, niche breadths (Futuyma and Moreno, 1988). Yet, in order to explain the existence of specialist species, there must be some costs to being a generalist. One set of theories assumes that there must be a trade-off between adaptation to different environments, such that adaptation to one environment may result in a correlated decline in fitness in another. An alternative view suggests that, when generalists experience a number of different environments, if deleterious mutations accumulate that show GEI, such that they are only expressed over part of the species' range, selection to remove them would be weaker than if they occurred in a specialist, in which they would always be expressed (Kawecki, 1994; Fry et al., 1996; Kawecki et al., 1997). Because of this weaker selection, deleterious mutations that are environment-specific would be more likely to accumulate in ecological generalists, reducing their overall fitness, relative to specialists (Kawecki, 1994). Such mutations need not be involved in trade-offs – there does not need to be a corresponding benefit in a subset of environments - although this could also occur.

There is also the question of how GEI could be maintained in natural populations. Again, this could be due either to opposing selection in different environments (antagonistic pleiotropy), or to mutation-selection balance, caused by a high input of mutations deleterious effect in some, but not all, environments (Fry et al., 1996). If a
high input of mutations with varying effect is the case, GEI should be observable in lines in which spontaneous mutations have accumulated. If, on the other hand, GEI is maintained by selection favouring different alleles in different environments, such alleles would be expected to reach higher equilibrium frequencies, and thus despite their significant impact they need only arise occasionally. If this is the case, the majority of new mutations would not be expected to show evidence of GEI, and so studies of the effects of newly-arisen mutations in different environments may be able to distinguish between the two theories.

3.1.2 Mutation-Accumulation Experiments

If mutations express significant amounts of GEI, this could throw light on some apparently contradictory results derived from experiments that have aimed to measure the rate at which deleterious mutations arise. Such experiments have been designed to allow spontaneous mutations to accumulate in replicated sub-lines, protected from selection, which are then measured for fitness-related traits after a number of generations, relative to control populations. The results of such experiments, carried out using multicellular organisms, have a shown remarkable degree of variation, with haploid genomic mutation rates ($U$) ranging from 0.003 in *C. elegans* (Keightley and Caballero, 1997) to approximately 0.3 in *Drosophila melanogaster* (Mukai, 1964; Mukai *et al.*, 1972; Ohnishi, 1977). Even estimates obtained from *D. melanogaster* vary greatly (see Chapter 1).

A number of explanations have been proposed to explain these differences in estimates of mutational parameters. Firstly, there may be true differences between mutation rates in different organisms. However, despite the fact that the *C. elegans* genome is approximately half the size of that of *Drosophila*, they have similar numbers of genes, and the lethal mutation rates in *C. elegans* and *Drosophila* appear to differ by only a factor of three (Crow and Simmons, 1983; Rosenbluth *et al.*, 1983). Therefore, this would appear to only provide a partial explanation, unless mutations in intragenic regions of the genome play an important role.

Secondly, it is possible that *Drosophila* could suffer from a different class of mutational events, for example a form of transposable element insertion.
Transposition does not occur in the N2 strain of *C. elegans*, which was used in Keightley and Caballero's experiments (Anderson, 1995). However, transposable elements are known to be active in *Drosophila*. Some further evidence to support this hypothesis comes from *Drosophila* experiments carried out by Ohnishi (1977), where the effects of EMS-induced mutations were studied, rather than spontaneous mutations (Keightley, 1996). Despite showing a high proportion of lethal and semi-lethal mutations, there did not appear to be a large class of mutations with detectable minor viability effects, as seen in the *Drosophila* MA experiments. Thus, if transposable elements are causing the mildly deleterious mutations seen in Mukai's experiments, this could explain why such a distribution of effects was not observed in the EMS and *C. elegans* experiments. If it is true that differences in transposition rates cause very different overall distributions of mutational effects, this implies that estimates of the number of mutational events occurring per generation cannot be generally applied, as transposition rates vary widely (Pasyukova and Nuzhdin, 1993, Charlesworth *et al.*, 1994).

A third explanation is that Mukai's spontaneous MA experiments were flawed due to lack of a proper control (Keightley, 1996). Mukai's experiments involved accumulating mutations on wild-type (+) second chromosomes, protected from selection by a balancer chromosome (Cy). The relative frequencies of flies homozygous for the wild-type chromosome (+/+) were compared with those of the heterozygotes (Cy/+) but it is possible that changes in the viability of the Cy balancer chromosome occurred, which could change the apparent viability of the wild-type chromosomes. This could invalidate the results of the experiments. The EMS experiments of Ohnishi (1977) would not have suffered from the same problem as the control and EMS-treated lines were assayed contemporaneously. Thus, the replicates could be back-crossed to the same Cy stock to extract the mutagenised (or non-mutagenised) second chromosomes, so there would have been no potential for changes in the balancer chromosomes to occur. Similarly, the *C. elegans* controls were cryopreserved, so the control and MA lines could be assayed together, but the controls would not have evolved during the course of the experiment. A *Drosophila* MA experiment carried out by reducing selection by using small population sizes and benign rearing conditions, rather than using balancer chromosomes, also failed to reveal such a large decrease in fitness (Fernández and López-Fanjul, 1996).
However, it has been argued that the control used in this experiment was flawed (Drake et al., 1998; Lynch et al., 1999), as discussed in Chapter 1, throwing some doubt on these conclusions. An MA experiment similar in design to that of Mukai and collaborators, but with large homozygous control populations, obtained much lower estimates of the genomic mutation rate (Fry et al., 1999).

Finally, although the *Drosophila* and *C. elegans* MA experiments both involved maintaining the lines in good conditions in an attempt to minimise selection, during the fitness assays, the flies were studied in a competitive environment, whereas the worms were assayed under benign conditions. If the mutations accumulated showed a significant degree of GEI, it is possible that a harsh environment could reveal greater fitness differences than an optimal environment. This could explain the higher number of mutations detected in the *Drosophila* experiments. Repeated assays of the *C. elegans* MA lines, using harsh rather than benign assay conditions, may help clarify this issue.

3.1.3 \textbf{Previous work measuring the contribution of new mutations to GEI}

Several mutation-accumulation experiments have been analysed to determine the extent to which new mutations exhibit GEI. Kondrashov and Houle (1994) and Fry et al. (1996) found evidence for significant GEI when inbred lines of *Drosophila*, in which mutations had been allowed to accumulate, were assayed for productivity in different environments. In a separate study, Fry et al. (1999) measured the viability of lines of *Drosophila* that had accumulated mutations during the course of an experiment similar in design to that of Mukai. They detected significant amounts of GEI when the flies were assayed at different temperatures, but not when the ethanol concentration or the density of the flies was varied. Two further experiments involving *Drosophila* produced apparently contradictory results. An experiment performed by Shabalina et al. (1997) indicated that the mutation pressure depended strongly on the conditions in which life history trait assays were performed, whereas work by Gilligan et al. (1997) found no evidence for a greater mutational load in competitive, rather than benign, conditions. These last two experiments, however, involved mutation-accumulation in outbred flies, freshly sampled from the wild. This makes interpretation of the results more complicated, as fitness changes may result
from factors other than mutation accumulation, such as adaptation to the laboratory environment, or inbreeding depression (Keightley et al., 1998). Further work, preferably studying the effect of mutations in initially inbred lines, would help to clarify the issue.

3.1.4 EXPERIMENTAL OUTLINE

The aim of this experiment was to carry out further studies of the surviving 48 lines of *C. elegans* in which spontaneous mutations had been allowed to accumulate for 60 generations (Keightley and Caballero, 1997), measuring fitness-related traits in a range of conditions. These lines had been cryopreserved at -80°C, so it was possible to thaw the worms and assay their fitness in more stressful conditions, and so to obtain more information concerning the extent to which spontaneous mutations exhibit GEI. This may in turn help to clarify one of the possible explanations for the difference of over two orders of magnitude between estimates of mutation rates from experiments using flies, by Mukai and collaborators, and worms, by Keightley and Caballero. A second MA experiment that involved *C. elegans*, which was similar in design to that of Keightley and Caballero (1997), produced qualitatively similar results to their study (Vassilieva and Lynch, 1999).

3.2 MATERIALS AND METHODS

3.2.1 STRAINS

The strains used during the course of these studies were the wild-type strain of *C. elegans*, N2, and the 48 surviving mutation-accumulation (MA) lines, derived from the N2 strain by Keightley and Caballero (1997). The details of the MA procedures are described in full in their paper. Briefly, 50 lines of worms were derived from a single inbred progenitor worm, and propagated in parallel by the transfer of single, self-fertilising hermaphrodites for 60 generations. During this process, selection was minimised by keeping the worms in good rearing conditions and reducing the population size to one, in order to allow new (non-lethal) mutations to accumulate. Life history traits of the MA lines could then be measured relative to the cryopreserved base population, which had not been able to evolve during the course
of the experiment. From these results, they were able to obtain estimates of the average number of mutations accumulated per genome, per generation ($U$), and their effects ($s$).

3.2.2 CULTURE CONDITIONS

The standard culture conditions in which *C. elegans* worms are maintained, described in Chapter 2, are relatively simple. The worms feed on bacteria (*Escherichia coli*, strain OP50) cultured on agar and are maintained in incubators at a constant temperature (usually $20^\circ C$). The conditions that would be most suitable for measuring GEI would be those that have significant fitness effects, for example reducing the productivity of the wild-type strain by approximately half. However, it was also important that conditions were not so severe that the worms failed to reproduce. Pilot studies, varying the salinity of the agar by altering the concentration of NaCl, and varying the temperature of the incubators in which the worms were maintained, were performed. We measured the effects of these conditions on the productivity of the N2 strain and a mutant strain derived from N2, SP471 *dpy-17 unc-32*, provided by Dr. Mark Blaxter (University of Edinburgh), using the methods outlined in Chapter 2. The mutant strain showed both Dumpy and Uncoordinated phenotypes; by using two strains, it was possible to identify conditions in which GEI could be expressed. The results are presented in Figure 3.2. From these results, it was decided to use three sets of assay conditions: reduced temperature ($14^\circ C$), increased temperature ($26^\circ C$) and increased salinity (0.2M NaCl, compared to the standard concentration of 0.034M NaCl). These conditions would be expected to roughly halve reproductive output, providing appropriately stressful conditions in which to measure life history traits, but they would be relatively straightforward to control.
3.2.3 LIFE HISTORY ASSAYS

Life history assays were performed on the 48 MA lines and 40 control lines, in each of the three conditions described above: reduced temperature (14°C), increased temperature (26°C) and increased salinity (0.2M NaCl). Daily reproductive output and longevity were assayed using the procedures described in Chapter 2, with the modification that two worms were assayed per plate. In total, four worms from each
MA line were assayed in each of the three conditions; i.e., life history assays were performed on 12 worms from each MA line over the course of the three experiments. An average of 43,000 worms were counted in each assay.

The 40 control lines were derived from two frozen stocks; during the first assay (low temperature) these lines were separated into 40 sublines at the start of the assay. However, the results of this assay, described below, indicated that this design might have led to maternal effects influencing the results. In order to reduce this possibility, for the remaining assays the lines were separated into 40 sublines immediately after they were thawed. They were then maintained as individual sublines for three generations prior to the fitness assays, as was the case with the MA lines.

An increase in temperature can cause an increase in non-disjunction of the X-chromosome, resulting in a higher frequency of male ( XO) rather than hermaphrodite ( XX) worms being produced. When the assay was performed at 26°C, a few plates therefore contained male worms. Such plates had to be removed from the data before analysis of the results could be carried out, as the productivity and longevity of hermaphrodites are not comparable to that of males or hermaphrodites that are maintained in the presence of males (Gems and Riddle, 1996). Although male production is a component of fitness, assessing its contribution to total fitness is a complicated task.

Further assays, using the condition of increased salinity, were subsequently performed on seven of the MA lines, including those that showed the most extreme response in this condition, in order to determine whether these effects were replicable. Larger sample sizes were used, with twenty worms being assayed per line, in addition to equal numbers of control worms.

3.2.4 STATISTICAL ANALYSIS

Two methods can be used to determine whether significant GEI can be detected. When data are available from more than one environment, as is the case here, it is possible to perform two-way analysis of variance (ANOVA), fitting the following random effects: genotype, environment and the interaction between these two effects,
and testing for significance of the interaction term (Freeman, 1973). The interaction term provides information concerning the variation among genotypes in response to the environment, $V_{GEI}$.

The second approach, the genetic correlation between environments, is suitable for comparing pairs of environments. This approach involves treating one character measured in two environments as two different traits, from which GEI can be detected from the genetic correlation between them, $r_G$ (Falconer, 1952). A correlation that is significantly different from +1 indicates significant GEI. The relationship between the ANOVA and correlation approaches can be described by the following equation:

$$V_{GEI} = V_G(1 - r_G^2),$$

where $V_G$ is the genetic, or between-line variance component (Via, 1987). This assumes that the data set is balanced and the genetic variance does not differ significantly between the environments. Standard errors obtained, for example, by bootstrapping the data, can be used to determine whether the correlation is significantly different from +1, indicating the presence of GEI. The correlation approach has also been developed to apply to more than two discrete environments (Robertson, 1959; Via, 1987).

Although the two measures of GEI are related, the information they provide differs slightly. The interaction variance will increase as the genetic correlation decreases from 1 to −1. However, although a correlation approaching −1 indicates very strong GEI, the potential for evolutionary change in responsiveness to differing environments is greatest when $r_G = 0$, as this indicates the least constraint on the phenotypic values in different environments. Therefore, this approach can provide a better picture of the potential for evolutionary adaptation (Via, 1987), if a significant amount of GEI is detected. Significant amounts of GEI detected by ANOVA could either indicate changes in the ranking of different lines in different environments, or differences in the variance between lines in different environments. $r_G$, however, will only be significantly less than one if the rankings change, making it possible to distinguish between the two (Fry et al., 1996).
3.3 RESULTS AND ANALYSIS

The distribution of line means for productivity and longevity in each of the conditions used are shown in Figure 3.3 and Figure 3.4. Summary statistics, showing the difference in mean and variance between the control lines (generation 0) and the MA lines (generation 60) are shown in Table 3.1 and Table 3.2. The change in variance is presented as a proportion of the environmental variance (the mutational heritability, or $h_m^2$), and as a proportion of the mean value of the controls (the coefficient of variation, or $CV_m = \sqrt{V_m}/\bar{x}$, where $\bar{x}$ is the mean of the controls). The procedures for determining these values are given in Chapter 2, with one slight modification: because two worms were assayed per plate rather than one, the environmental variance, $V_e$, for productivity is equivalent to $2MSW(controls)$, rather than $MSW$, which affects the estimation of mutational heritability. Standard errors for $h_m^2$ were obtained by bootstrapping the data, by line, 100 times. This method had to be modified to obtain the standard errors for $CV_m$, as resampling would sometimes result in a negative value being obtained for the change in variance attributable to mutation, $V_m$, and it is not possible to take the square root of a negative number. In such cases, $CV_m$ was set to zero.
Figure 3.3: Productivity of mutation-accumulation lines

(a) Standard conditions

(b) Low temperature

(c) High temperature

(d) High salinity
Figure 3.4: Longevity of mutation-accumulation lines

(a) Standard conditions

Proportion of lines

0 0.2 0.4 0.6 0.8
Lifespan (days)

(b) Low temperature

Proportion of lines

0 0.2 0.4 0.6 0.8
Lifespan (days)

(c) High temperature

Proportion of lines

0 0.2 0.4 0.6 0.8
Lifespan (days)

(d) High salinity

Proportion of lines

0 0.2 0.4 0.6 0.8
Lifespan (days)
Table 3.1: Changes in mean and variance for lifetime productivity, per generation. Standard errors are shown in brackets (K&C = Kightley and Caballero)

<table>
<thead>
<tr>
<th>Condition</th>
<th>Mean (Gen 0)</th>
<th>Mean (Gen 60)</th>
<th>ΔMean, %</th>
<th>$V_e$ (Gen 0)</th>
<th>$h_m^2$</th>
<th>$CV_m$, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low temp. (14°C)</td>
<td>120.5</td>
<td>134.0</td>
<td>+0.19</td>
<td>5060</td>
<td>-0.97 x 10^{-3}</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>(6.2)</td>
<td>(5.3)</td>
<td>(0.11)</td>
<td></td>
<td>(0.81 x 10^{-3})</td>
<td></td>
</tr>
<tr>
<td>High temp. (26°C)</td>
<td>84.9</td>
<td>75.0</td>
<td>-0.19</td>
<td>3300</td>
<td>0.40 x 10^{-3}</td>
<td>1.22</td>
</tr>
<tr>
<td></td>
<td>(4.7)</td>
<td>(4.3)</td>
<td>(0.13)</td>
<td></td>
<td>(0.57 x 10^{-3})</td>
<td>(1.80)</td>
</tr>
<tr>
<td>High salinity (0.2M)</td>
<td>165.9</td>
<td>154.2</td>
<td>-0.12</td>
<td>2138</td>
<td>1.88 x 10^{-3}</td>
<td>1.21</td>
</tr>
<tr>
<td></td>
<td>(4.1)</td>
<td>(4.7)</td>
<td>(0.064)</td>
<td></td>
<td>(2.70 x 10^{-3})</td>
<td>(1.28)</td>
</tr>
<tr>
<td>Standard (K&amp;C, 1997)</td>
<td>255.6</td>
<td>251.1</td>
<td>-0.03</td>
<td>1802</td>
<td>1.20 x 10^{-3}</td>
<td>0.55</td>
</tr>
<tr>
<td></td>
<td>(2.5)</td>
<td>(3.2)</td>
<td>(0.03)</td>
<td></td>
<td>(0.8 x 10^{-3})</td>
<td>(0.19)</td>
</tr>
</tbody>
</table>

Table 3.2: Changes in means and variance for longevity, per generation, with standard errors shown in brackets

<table>
<thead>
<tr>
<th>Condition</th>
<th>Mean (Gen 0)</th>
<th>Mean (Gen 60)</th>
<th>ΔMean, %</th>
<th>$V_e$ (Gen 0)</th>
<th>$h_m^2$</th>
<th>$CV_m$, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low temp. (14°C)</td>
<td>16.94</td>
<td>18.58</td>
<td>+0.16</td>
<td>24.5</td>
<td>-9.86 x 10^{-4}</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>(0.53)</td>
<td>(0.44)</td>
<td>(0.067)</td>
<td></td>
<td>(7.95 x 10^{-4})</td>
<td></td>
</tr>
<tr>
<td>High temp. (26°C)</td>
<td>7.15</td>
<td>7.30</td>
<td>+0.035</td>
<td>4.48</td>
<td>-1.05 x 10^{-3}</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>(0.17)</td>
<td>(0.13)</td>
<td>(0.048)</td>
<td></td>
<td>(2.97 x 10^{-4})</td>
<td></td>
</tr>
<tr>
<td>High salinity (0.2M)</td>
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<td>14.03</td>
<td>-0.035</td>
<td>4.63</td>
<td>7.87 x 10^{-4}</td>
<td>0.41</td>
</tr>
<tr>
<td></td>
<td>(0.17)</td>
<td>(0.16)</td>
<td>(0.028)</td>
<td></td>
<td>(7.87 x 10^{-4})</td>
<td>(0.17)</td>
</tr>
<tr>
<td>Standard (K&amp;C, 1997)</td>
<td>14.04</td>
<td>13.40</td>
<td>-0.076</td>
<td>2.57</td>
<td>1.69 x 10^{-4}</td>
<td>0.43</td>
</tr>
<tr>
<td></td>
<td>(0.18)</td>
<td>(0.19)</td>
<td>(0.031)</td>
<td></td>
<td>(1.83 x 10^{-4})</td>
<td>(0.18)</td>
</tr>
</tbody>
</table>

Each of the three harsh conditions reduced mean productivity substantially, relative to benign conditions, although the degree to which it was reduced varied. Increased temperature (26°C) had the harshest effect, but as *C. elegans* has maximal fitness in the laboratory between 20°C and 25°C, a slightly lower temperature than this would not have had the desired effect of making the conditions more stressful for the worms. In addition, the environmental variance increased in harsher conditions.

Perhaps the most surprising result was that 60 generations of mutation accumulation appeared to actually increase the mean fitness of the worms under some conditions, and decrease the variance – the opposite of what would have been expected. This result was observed in the productivity assay at low temperature, and the longevity assays at low and high temperatures, though only the change in variance for
productivity and the change in mean for longevity at low temperature were statistically significantly different from zero. These results contrast with Keightley and Caballero's (1997) results where they measured the same traits in optimal conditions. They found a small (non-significant) decrease in lifetime productivity and lifespan, and a large increase in between-line variance. There are several possible explanations for these results.

One possibility is that the mutations accumulated were mainly advantageous, but this seems unlikely as it is widely accepted that the vast majority of new mutations are either neutral or deleterious (Crow and Simmons, 1983). A second possibility is that selection occurred during the MA experiment, for example because larger worms were transferred, thus generating selection for a more rapid growth rate, which could lead to increased productivity in harsh conditions. However, as the population size was one, selection pressures would be negligible unless the selection coefficients approached one (Keightley and Caballero, 1997). Life history trade-offs may also have caused these results (Stearns, 1992). These could act in several ways, for example though a trade-off between total productivity or longevity and another component of fitness. However, the fact that this result was not observed in the majority of conditions reduces the plausibility of these suggestions.

Maternal effects affecting the lines, for example due to influences from freezing, thawing or contamination, could provide one final explanation for this result. The control lines were derived from just two frozen stocks, and when the life history assays were performed at low temperature, these stocks were not separated into 40 lines until the start of the assay. Any maternal effects from one of these two lines could therefore have a significant effect on the performance of the generation 0 worms in the life history assays, potentially reducing the mean fitness of these worms and increasing the between-line variance. Because the difference in fitness components between the generation 0 and 60 worms is slight, such maternal influences could be enough to cause an apparent increase in productivity and decrease in variance among the generation 60 lines, relative to the controls. Because of this possibility, for the remaining assays, all lines of worms were maintained independently for three generations prior to the assay, to minimise any such effects. A significant increase in mean or decrease in variance was not observed again,
providing support for the hypothesis that this result was simply an artefact of the experimental design.

In cases such as this, where the change in variance was negative, the coefficient of variance, $CV_m$, could not be determined, as this would require taking the square root of a negative number, $V_m$.

In general, the productivity results revealed greater effects of mutation-accumulation than the longevity results, indicating a larger mutational target for productivity. Harsher conditions appeared to cause a greater change in mean and variance between the MA and control lines. In the most extreme case, the change in mean productivity observed at 26°C was more than six times greater than that observed at 20°C, but in no case was the difference statistically significant from zero at the 5% level.

From the change in mean and variance of the life history traits measured, it was possible to obtain estimates of the haploid genomic mutation rate, $U$, and the average effect of such a mutation, $\bar{s}$, using the Bateman-Mukai approach, as described in Chapter 2. The results are presented in Table 3.3. Standard errors were obtained by bootstrapping.

Because the standard errors of all the estimates are so large, a maximum likelihood approach to estimating $\hat{U}$ and $\hat{s}$ would not be expected to be superior to this approach. In such circumstances, a very flattened likelihood was observed, indicating that the results would not be very precise, having large confidence intervals.

Table 3.3: Estimates of $U$ and $s$, using the Bateman-Mukai approach

<table>
<thead>
<tr>
<th>Trait</th>
<th>Condition</th>
<th>$\hat{U}$ (SE)</th>
<th>$\hat{s}$, % (SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Productivity</td>
<td>Low temperature</td>
<td>-0.0052 (0.071)</td>
<td>36.27 (655)</td>
</tr>
<tr>
<td></td>
<td>High temperature</td>
<td>0.013 (0.026)</td>
<td>15.29 (470)</td>
</tr>
<tr>
<td></td>
<td>High salinity</td>
<td>0.0047 (0.055)</td>
<td>24.86 (208)</td>
</tr>
<tr>
<td></td>
<td>Standard</td>
<td>0.0014 (0.016)</td>
<td>20.67 (109)</td>
</tr>
<tr>
<td>Longevity</td>
<td>Low temperature</td>
<td>-0.015 (0.0082)</td>
<td>10.45 (53)</td>
</tr>
<tr>
<td></td>
<td>High temperature</td>
<td>-0.0073 (0.059)</td>
<td>4.80 (250)</td>
</tr>
<tr>
<td></td>
<td>High salinity</td>
<td>0.0037 (0.016)</td>
<td>9.50 (614)</td>
</tr>
<tr>
<td></td>
<td>Standard</td>
<td>0.015 (0.037)</td>
<td>5.04 (24)</td>
</tr>
</tbody>
</table>
The cases where $\hat{U}$ is negative can be explained by the increase in the relative fitness of the generation 60 lines, as discussed above. With the exception of low temperature, where the result is probably an artefact of experimental design, the estimates of $\hat{U}$ for productivity are higher under harsher experimental conditions. However, although estimates of $\hat{U}$ are as much as nine times greater at high temperature, the values of $\hat{U}$ and $\hat{s}$ are not significantly different from those obtained under standard conditions. These results do not, therefore, provide support to the hypothesis that the low values of $\hat{U}$ obtained by Keightley and Caballero (1997) from their MA experiments were the result of using benign, rather than harsh conditions, when assaying the fitness of the MA and control lines.

3.3.1 GENOTYPE BY ENVIRONMENT INTERACTIONS

The mean lifetime productivity of each of the MA lines in each of the assay environments is shown in Figure 3.5.

Figure 3.5: Genotype by environment interaction for mutation-accumulation lines (Generation 60)
In order to determine whether there was significant GEI, analysis of variance (ANOVA) was performed on the productivity data from the generation 60 worms in each of the four assay environments (low and high temperature, high salinity and standard conditions). Figure 3.3 appears to indicate that the variances of the MA lines did not differ greatly across environments. To confirm this, a Bartlett's test was performed to detect any significant heterogeneity of variance, as ANOVA makes the assumption of equal variances. However, no significant effect was detected for heterogeneity of variance (Test Statistic: 4.73; \( P = 0.192 \)). ANOVA was used to analyse the data, with effects fitted for line, environment and the interaction between these two effects. Highly significant effects were detected for line \( (P = 0.001) \) and environment \( (P < 0.001) \). However, the genotype by environment interaction was not significant at the 5% level \( (P = 0.18) \). These results remained valid if the assays performed at low temperature, or in standard conditions, were removed from the analysis. The probability of rejecting a true null hypothesis (a Type I error) would increase if there was heterogeneity of variance. Therefore, as no significant effect was detected under the assumption of homogeneity of variance, if there as in fact such heterogeneity, the GEI would remain non-significant, and thus it would not affect this result.

The genetic correlations between pairs of environments could provide further information concerning the type of GEI present, if significant GEI had been detected. For example, this could provide more information concerning whether the GEI was the result of changes in variance or ranking of the lines, and the potential for evolutionary changes in response to different environments (See Section 3.2.4). However, as this was not the case, such analysis would not be informative.

3.3.2 FURTHER ASSAY OF A SAMPLE OF THE LINES AT INCREASED SALINITY

The lack of significant GEI provides further evidence concerning genetic variation between the generation 60 lines. If no genetic variation were present, no relationship would be detected between the performance of the different lines on separate occasions, giving the appearance of GEI.

Further assays of the most extreme lines in one condition were carried out, with larger
sample sizes, in order to ensure that the results of the assays of these lines were replicable, and not simply the result of some form of environmental effect. The lines with the most extreme productivity results at high salinity were chosen, as changes in productivity were more informative than changes in longevity (Figure 3.3 and Figure 3.4), and high temperatures were less suitable as they result in an increased frequency of male worms being produced. The fitness of males is not directly comparable with that of hermaphrodites.

Seven lines were chosen for further assay: the three lines with the lowest mean fitness in conditions of increased salinity, the two lines with the highest fitness, and two lines chosen from those with productivity values that were not significantly different from the controls. Life history assays were performed on twenty worms per line, with two worms per plate, and equal numbers of control (generation 0) worms were assayed. Over 11,000 worms were counted during the course of the life history assay.

The MA worms assayed were divided into three classes according to their productivity in the original assay under saline conditions: low, medium and high. Highly significant class effects were detected by analysis of variance (F = 8.9; P ≤ 0.001). No significant measurer effect was detected (F = 0.11; P = 0.75). The mean productivity of each of the seven lines in benign conditions and each of the assays at increased salinity are shown in Figure 3.6.

From this, it can be observed that the significant class effect appears to be primarily caused by the two lines that consistently exhibited the lowest performance in saline conditions; indeed, if these two lines are removed from the data, the class effect becomes non-significant (F = 1.1; P = 0.34). One of these lines revealed a slightly lower mean fitness than the average when assayed in benign conditions; the other did not. In addition, one of the lines appeared to reveal consistently high productivity in saline conditions, although this was not significantly higher than the productivity of the control lines, at the 5% level (t-test: t = 1.87; P = 0.084).

These results reveal the presence of some lines that showed a low, replicable productivity result when measured in harsh conditions, indicating the presence of
mutations that decrease fitness in this condition, though not necessarily in benign conditions, although this result was not revealed in the majority of lines studied.

Figure 3.6: Repeated assays of a sample of MA lines at increased salinity

![Graph showing standardized lifetime productivity for different treatments.]

3.4 CONCLUSION AND DISCUSSION
The results presented here do not reveal any significant degree of GEI in the mutation-accumulation lines of *C. elegans* produced by Keightley and Caballero (1997). Performing life history assays in harsh, rather than benign, conditions did not significantly alter the change in mean and variance between the generation 0 (control) lines and generation 60 (MA) lines. Similarly, estimates of the genomic mutation rate, $\hat{U}$, and the average mutational effect, $\hat{s}$, did not differ significantly under different assay conditions. Analysis of variance, fitting genotype, environment and the genotype by environment interaction, did not detect a significant interaction term. Overall, it can be concluded that there was no evidence for GEI for the mutations accumulated over 60 generations. It therefore appears unlikely that the benign assay conditions used were responsible for the low estimates of $U$ obtained by Keightley and Caballero (1997). Some other explanation is required to account for the large differences in mutational parameters observed between their results and those of Mukai and collaborators (Mukai, 1964; Mukai *et al.*, 1972; Ohnishi, 1977).

These results can also throw light on the maintenance of genetic variation at loci contributing to GEI in natural populations. This could either be maintained by a high
input of mutations with different effects in different environments (Fry et al, 1996), or opposing selection in different environments, such that different alleles are favoured. (Levene, 1953). Such mutations would be expected to reach a higher equilibrium frequency than unconditionally deleterious mutations, and so need not arise frequently. If the first explanation is correct, significant levels of GEI should be observed among newly arisen, spontaneous mutations. However, this was not observed in this experiment. These results do not, therefore, lend support to the theory that GEI in natural populations of *C. elegans* is maintained by recurrent mutation.

It remains possible, of course, that significant levels of GEI may be detected in conditions other than those used during the course of this work. The ideal situation may be to perform fitness assays in the natural environment. As *C. elegans* is a millimetre-long, transparent soil-dwelling nematode, such assays would not be possible, and even laboratory conditions designed to closely mimic conditions that they may be expected to meet in the wild would appear to be unfeasible. The fact that very little is known about these worms in their natural habitat makes this even more difficult (Hodgkin and Doniach, 1997). Such assay conditions may only be feasible when plants are used as a model system, and even then, such assays would be challenging, to say the least.

Given that modifications of standard laboratory conditions are the only feasible option when performing life history assays involving *C. elegans* in harsher conditions, the range of conditions in which they can be performed is still large, and could be extended well beyond those considered here. Life history assays such as these are very labour intensive, and become more time-consuming under harsher conditions as the productivity of the worms is often delayed, and they reproduce more slowly over a longer period of time, especially at low temperatures. Constraints of time, money and energy limit the number of such assays that can be performed, and so the benefit of any new assay must be carefully considered. In addition, the environmental variance increases in harsh conditions, making interpretation of the results more difficult. One form of assay that may be more likely to exaggerate the fitness differences between mutant and control lines, however, would be an assay performed under competitive conditions.
When Mukai and collaborators (Mukai, 1964; Mukai et al., 1972; Ohnishi, 1977) assayed the fitness of MA lines of *Drosophila*, the conditions used were harsh and competitive. Fry et al. (1996) and Shabalina et al. (1997) found evidence for GEI when MA lines of *Drosophila* were assayed in a range of competitive conditions, in comparison to benign conditions, though harsh, non-competitive assays were not performed. Gilligan et al. (1997) found no evidence of increased genetic load in a range of conditions that were competitive to different degrees, relative to benign conditions. Kondrashov and Houle (1994), however, found that although the fitness of MA lines of *Drosophila* decreased in harsh conditions, relative to a control, this effect was most extreme under competitive conditions (increased fly density), indicating that competition may reveal fitness differences that harsh but non-competitive conditions do not. Conversely, Fry et al. (1999) found evidence of GEI when temperature was varied, but not when fly density was varied, conditions under which competition would be expected to occur. Thus, the effect of competition on increasing the fitness differences between MA and control lines is not conclusive, but remains a possibility.

All of the competitive life history assays described above were performed with *Drosophila*, in which flies can be maintained in crowded conditions and food restricted, in order to induce competition. Controlled competitive fitness assays have not, to the best of my knowledge, been performed using *C. elegans*. The most likely way in which competition could be induced between hermaphrodite worms would be by restricting their food supply, in order to force them to compete for food. However, doing so would not be a simple task. *C. elegans* is a bacteriavore, and worms are typically maintained on agar plates, with an excess of *E. coli* as a food source. As *E. coli* is a living source of food, limiting it in a controlled manner would not be easy. The bacteria would have to be killed, and antibiotics included in the agar medium, in order to prevent its further growth, or that of any other form of bacteria that the worms may consume. With so little food, worms may approach starvation, with the result that reproduction is likely to cease, making life history assays difficult. Alternatively, the worms may form resistant dauer larvae, which do not feed or reproduce and have extended lifespans, which would again make life history assays very challenging. In addition, worms are normally transferred from plate to plate by a
flattened, metal wire pick. In order to avoid damaging the worms, they are cushioned on a thick layer of bacteria. This would not be possible if the bacteria were restricted, resulting in an increased risk of environmentally induced damage to the worms, which could obviously affect their performance in life history assays. Worms will often temporarily cease to reproduce in the absence of food, so even obtaining offspring on which life history assays could be performed may not be easy.

One way of circumventing some of these problems would be to cultivate the worms in axenic media, in which the food source is synthetic and bacteria are absent. Such axenic media have been described for growth of *C. elegans* (for a review, see Vanfleteren, 1980). However, worms grown on such media grow less well (Sulston and Hodgkin, 1988; Lewis and Fleming, 1995). In addition, the axenic media described involve cultivating *C. elegans* in liquid cultures, with the result that observation and manipulation of individual worms is more difficult, and life history assays would need to be redesigned in order to be appropriate for such conditions. The risk of contamination with foreign microorganisms remains a problem, as nematode growth media will support the growth of many microorganisms that could reduce worm fitness or provide an additional, uncontrolled food source for *C. elegans* (Vanfleteren, 1980). Even if these problems can be dealt with, it is possible that competition would not occur under conditions of restricted food.

For these reasons, competitive fitness assays have not been performed on *C. elegans*. If such problems could be circumvented, however, competitive conditions could provide interesting and informative assay conditions, which may reveal more extreme fitness differences between lines of worms than assays on benign or harsh (but non-competitive) conditions would.

Even harsh, competitive conditions, however, may not reveal the effects of all the mutations that have accumulated. The mutational parameters of interest are those that relate to mutations that would be deleterious in natural conditions. Even if it was possible to perform life history assays in the natural environment, it could not be guaranteed that all such deleterious mutations were being detected. The mutations may affect a trait other than those being assayed, for example, or the effects could be too small to be detected, given the sample sizes used. Thus, all mutation-
accumulation experiments are likely to be underestimating the number of mutations occurring (although some may be simultaneously overestimating the mutation rate, for different reasons). An experiment designed to provide information concerning the extent of this underestimation is described in the following chapter.
Chapter 4: Properties of EMS-induced mutations affecting life history traits, and the frequency of cryptic deleterious mutations in *C. elegans*
4.1.1 IMPORTANCE OF THE PROPERTIES OF DELETERIOUS MUTATIONS

The rates at which spontaneous mutations with deleterious effects accumulate, and the distribution of their effects on fitness, are important for a variety of evolutionary issues. These include the evolution of sexual forms of reproduction (Kondrashov, 1988), conservation of small populations (Lande 1994; Lynch et al., 1995), the evolution of ecological generalists vs. specialists (Kawecki et al., 1997), the evolution of ageing (Partridge and Barton, 1993), the maintenance of genetic variation by mutation-selection balance (Kondrashov and Turelli, 1992), the magnitude of inbreeding depression (Charlesworth and Charlesworth, 1987; Charlesworth et al., 1990), degeneration of the Y chromosome (Charlesworth, 1991), background selection (Charlesworth et al., 1993) and female mate choice (Fisher, 1958; Pomiankowski, 1988; Rice, 1988). For more details, see Chapter 1.

Two of these examples, in particular, are worth covering in more detail, as they rely on specific, testable predictions concerning the properties of deleterious mutations. These are the evolution of sex, and mutation load and the conservation of small populations.

The majority of organisms reproduce sexually. Given that there is a twofold 'cost of sex' relative to asexual reproduction, sexual means of reproduction must have a large selective advantage. One of the theories that has been put forward to explain the widespread occurrence of sex predicts that sexual populations are able to purge deleterious mutations more efficiently than asexual populations (Kondrashov, 1988). In asexual populations, each new deleterious mutation can only be removed by a 'genetic death' – one mutation requires one genetic death (Muller, 1950). The proportion of individuals that fail to reproduce as a result of selection against mutations (the mutation load) can be much smaller in sexual populations, however, if there is synergistic epistasis between mutations, such that that each additional new mutation leads to a larger decrease in relative fitness. This is because recombination between genotypes can produce greater variance in the distribution of mutations within a population. Thus, genomes that are eliminated may contain many mutations, reducing the mutation load and providing an advantage to sexual reproduction. This
is known as the deterministic mutation hypothesis for the evolution of sex (Kondrashov, 1988). In order for this theory to satisfactorily explain the evolution of sexual reproduction, two conditions must be met. Firstly, there must be synergistic epistasis between mutations, and secondly, the rate at which deleterious mutations accumulate across the genome must be sufficiently high, such that \(2U\), the diploid genomic mutation rate, is greater than one.

A second issue requiring knowledge of the distribution of mutational effects involves the conservation of small populations. Currently, there are many threats to the survival of species, the majority of which can be directly or indirectly linked to human actions. Increasing numbers of species have become extinct in recent years, and many more exist in populations so greatly reduced in numbers that they are at risk of extinction (World Conservation Union, 1994). The major environmental factors contributing to extinction include habitat loss, overexploitation, pollution and the introduction of foreign species. As population sizes decrease, the risk of extinction becomes greater and stochastic factors, whether environmental or genetic, can increase the probability of extinction (Frankham, 1995). Population that are small as a result of factors unrelated to human activity, e.g., island populations, and those whose habitat will not support large population sizes, are also likely to be at risk from such stochastic factors. Genetic factors that affect the risk of extinction include inbreeding depression, the loss of genetic variation, outbreeding depression, genetic adaptation to captivity and the accumulation of deleterious mutations. The extent to which the last of these factors, the accumulation of deleterious mutations, contributes to the risk of extinction depends largely on mutational parameters, including the rate at which new mutations arise and the distribution of their effects, as well as population size and reproductive rate.

Most mutations that affect fitness have deleterious effects, which can range from being quasi-neutral to lethal. In populations larger than a few individuals, mutations with mildly deleterious effects have the most serious consequences for fitness and population survival. Although mutations with large effects may have devastating consequences for the individual carrying them, they have a high probability of being rapidly removed from the population by selection, and are extremely unlikely to become fixed (Lande, 1995). Lande (1994) was one of the first to study the
consequences of the fixation of new, mildly deleterious mutations in small, sexual populations. He determined that the magnitude of mutational effect with the most serious consequences – those that minimise the time to extinction – have effects that border on neutrality, i.e., $\overline{s} = 1/(N_e)$, where $\overline{s}$ is the mean homozygous fitness effect of a mutation, and $N_e$ is the effective population size. When mutations are effectively neutral, random genetic drift rather than selection largely dominates their fate. Mutations with effects larger than this can be removed by selection, whereas those with smaller effects may become fixed, but do less damage (Lande 1994; Lynch et al., 1995). Thus, if a population has an effective size of 100, any mutations with $\overline{s} < 1\%$ have a high probability of fixation, but of these mutations, those with smaller effects will have less harmful consequences.

Other factors that reduce the time to extinction caused by fixation of new deleterious mutations are a high genomic mutation rate ($U$), variable mutational effects ($s$), small effective population size ($N_e$) and a low reproductive rate (Lynch et al., 1995). Many of the species most at risk from extinction are, by definition, small, and they frequently have low reproductive rates. If they also have a high input of mutations with $\overline{s} = 1/(N_e)$, the prospects for their continued survival would appear to be particularly bleak.

Despite their importance, the majority of the information concerning deleterious mutational parameters comes from mutation accumulation (MA) experiments in which mutations are allowed to accumulate in conditions designed to minimise selection. By comparing the difference in fitness between MA and control lines, it is possible to obtain joint estimates of $U$ and $s$. For recent reviews of such experiments, see Drake et al. (1998), Keightley and Eyre-Walker (1999) and Lynch et al. (1999). One criticism of the MA approach is that the only mutations that can be detected are those that affect the fitness traits measured in the laboratory. Mutations that are not expressed in laboratory conditions, and those with very small effects on fitness may not be detected – the very mutations that could have the most important consequences for conservation biology, and could be crucial for theories concerning the evolution of sex. One way of tackling this problem is by studying the effects of mutations on a number of traits, and measuring fitness in harsher conditions, as discussed in the
previous chapter. This may increase the number of mutational events detected, but the problem still remains that, because we do not know how many mutations have been induced at the DNA level, we are unaware of the extent to which the results of these experiments may be underestimating mutation rates.

A second approach used to estimate $U$, the molecular constraint approach, involves comparing rates of nucleotide substitution in neutral and conserved regions of the genome (Kondrashov and Crow, 1993). However, this approach can only be used to determine the rate of mutations, and does not provide information concerning the mutational effects ($s$), unless the effective population size ($N_e$) is known, in which case some bounds can be placed on plausible estimates of $s$.

4.1.2 CORRELATIONS BETWEEN TRAITS

In addition to understanding the properties of mutations affecting individual traits, the joint effects of mutations on more than one trait are of interest for understanding the consequences of selection, and the constraints that influence evolutionary change (Falconer and Mackay, 1996). Genetic correlations between traits may result from mutations with effects on more than one trait (pleiotropy), or the association of mutations with effects on different traits in the same individuals (linkage). Environmental correlations may also be common as a result of environmental influences with effects on more than one trait. The response to selection by one trait may be strongly constrained by the effects on another trait if there is a significant amount of genetic correlation between the two.

In order to obtain information concerning the extent of mutational correlations, the results of two mutation-accumulation experiments involving Drosophila have been analysed. Houle et al. (1994) found strong positive genetic correlations between a number of life history traits measured in lines that had accumulated mutations on chromosomes protected from selection by balancer chromosomes ($0.4 \leq r \leq 0.8$). Fernández and López-Fanjul (1996), however, reported low values (<0.25) for mutational correlations between viability traits and fecundity.
Experiments that have aimed more specifically at studying the correlations between traits have fallen into two main classes. The first are those that have involved directional selection on one trait while measuring correlated changes on another trait of interest. The results from these experiments have been mixed; some have indicated that there are correlations between life history traits, while others have failed to detect such effects (Zwaan, 1999). The second set of experiments have studied individual mutations, such as the Age mutations that increase lifespan in *C. elegans* (for a review, see Kenyon, 1997; Age mutations are also discussed in more detail in Chapter 5). The joint effects of these mutations on different traits have now been measured. All the lifespan-extending mutations identified in *C. elegans* and the one isolated in *Drosophila* (Lin et al., 1998) have effects on other traits as well. A subset of the *C. elegans* Age mutations (the clock mutants and *spe-26*) show reduced productivity, relative to the wild-type (Wong et al., 1995; Kenyon, 1997). In contrast, no apparent correlations have been identified between increased lifespan and total productivity in the remaining Age mutants (Kenyon, 1997), though this does not exclude the possibility of correlations between longevity and other life-history traits.

### 4.1.3 AGE-SPECIFIC MORTALITY RATES

Related to the issue of correlations between traits expressed early and late in life is that of age-specific mortality rates. It was previously assumed that mortality rates increased exponentially with age, as first observed by Benjamin Gompertz (1825). Although this observation has since been widely confirmed, some recent work involving large studies of natural and experimental populations has suggested that mortality rates actually increase less rapidly or even decelerate with age in humans, yeast, worms, beetles and flies (for reviews, see Charlesworth and Partridge (1997), Vaupel et al. (1998) and Partridge and Mangel (1999)). The way in which mortality rates change with age is important for the prediction of demographic trends. The effect of new mutations on mortality rates can provide information concerning the age-specific properties of such mutations, and the differences between mutational target sizes at different ages. It also raises some interesting question relating to the evolution of ageing. Evolutionary theory states that ageing will evolve due to greater selective pressures affecting alleles that affect early, rather than late, components of
fitness. If survival and fertility are expected to decline with increasing age, why are mortality rates sometimes observed to decline with age?

4.1.4 EXPERIMENTAL OUTLINE

Here, I attempt to circumvent some of the problems associated with estimating mutational parameters, including the reduced ability of mutation-accumulation experiments to detect mutations with very small (though potentially important) effects, and the inability of the molecular constraint approach to provide information concerning the effects of the mutations detected. I have combined the molecular constraint and mutation-accumulation approaches to measure the fitness effects of induced mutations in *C. elegans*, using a system for which the number of point mutations induced in the DNA can be inferred. I mutagenised 60 lines of worms with the chemical mutagen EMS (ethyl methanesulphonate), using a dosage for which the number of mutations induced has been calibrated. A positive control for mutagenesis was carried out by measuring the mutation rate at a single locus, *unc-22*. The lines were inbred for ten generations by single-worm transfers of self-fertilising hermaphrodites, to fix the new mutations. If selection is minimised, the number of mutations fixed is expected to be equal to the number of mutations induced per haploid genome.

Fitness assays of the inbred EMS lines and controls were carried out, measuring daily reproductive output and longevity. Estimates of $U$, the haploid genomic mutation rate and $s$, the average effect of a mutation, were obtained from these results by comparing the distribution parameters of the control and EMS line means and variances for the fitness traits measured. These results could then be compared to the number of point mutations that were inferred to have been induced, and the proportion of these expected to be deleterious in natural conditions. Thus, it was possible to infer the extent to which MA experiments may underestimate mutation rates, due to the presence of cryptic mutations with effects that could not be detected in the laboratory.

I have also been able to focus on the joint distributions of mutational effects on the traits measured, in particular the effects of mutations on longevity and productivity traits. Due to the ease with which highly inbred, genetically homogeneous
populations of \textit{C. elegans} can be obtained, distinguishing between environmental and genetic correlations is relatively straightforward. By inducing large numbers of mutations, it is possible to increase the likelihood of finding mutations with pleiotropic effects, although distinguishing between mutations with effects on more than one trait, and associations between mutations that affect different traits, becomes more difficult. In addition, it has been possible to determine the age-specific mortality rates of mutagenised and control lines.

4.1.5 \textbf{SUMMARY OF THE AIMS OF THE EXPERIMENT}

The aim of this experiment is to measure the effects of a known number of induced mutations on a series of life history traits, and to infer the number of induced mutations that are expected to be deleterious in natural conditions, and the proportion of these that are detectable using fitness assays. In addition, it will be possible to determine the mutational correlations between the traits measured, in particular longevity and productivity traits, and to obtain information concerning the mortality curves of mutagenised and control populations.

4.2 \textbf{MATERIALS AND METHODS}

4.2.1 \textbf{MUTAGENESIS AND GENERATION OF LINES}

The experimental procedures are described below. An outline of the experimental design is shown in Figure 4.1.

The wild-type strain of \textit{C. elegans}, N2, was used as the progenitor strain. Alkaline hypochlorite treatment was used to generate an age-synchronous culture consisting of tens of thousands of worms. To do this, two gravid adult worms were placed on each of 70 large plates, and then removed after they had laid a total of 20-30 eggs (approximately three hours). Six days later, when the plates contained several thousand gravid adults each, alkaline hypochlorite was used to synchronise the worms, as described in Chapter 2 (Materials and Methods). This treatment harvested the eggs of the gravid adult worms; the eggs were then allowed to hatch and grow to the young adult stage (three days later) for mutagenesis.
4.2.1.1 EMS mutagenesis

Mutagenesis was carried out according to the protocol of Anderson (1995). The worms were divided into two sub-populations; one was exposed to EMS and the other provided a control for the mutagenesis, as described below. This work was performed in a fume hood, appropriate protective clothing was worn, and all contaminated equipment was soaked in inactivation solution (0.1M NaOH; 20% w/v Na₂S₂O₃) for at least 24 hours, prior to disposal.

Figure 4.1: EMS mutagenesis and generation of lines

 Worms from each of the 70 plates were collected in 3ml of M9 buffer, divided between 16 sterile 15ml centrifuge tubes, and rinsed once with M9, as described in
Chapter 2. The supernatant was removed and the contents of the tubes combined: worms from 13 tubes were placed into one sterile 50ml screw-top centrifuge for mutagenesis, and the remaining three tubes were combined into a second 50ml tube, as controls. 1.5ml of M9 buffer was added for each tube used, i.e., 19.5ml of M9 was added to the EMS tube and 4.5ml to the control tube. A solution of 0.1M EMS in M9 buffer was made, and gently agitated until the dense oily liquid had dissolved. For the EMS worms, an equal volume of EMS solution was added to the suspension of washed worms (final concentration 0.05M EMS). For the control worms, M9 buffer was added instead. Both tubes of worms were sealed with Parafilm and incubated at 20°C. The tubes were inverted three times every half-hour, to increase aeration. After four hours, the worms were rinsed three times with M9 buffer. The supernatant was removed and the final pellet resuspended in <0.25ml M9 buffer.

From this culture of worms, a number of sublines of the mutagenised worms and controls were generated, which were then inbred in order to fix the new mutations. In addition to this, as a positive control, large numbers of the F1 progeny of the mutagenised worms and controls were assayed in order to estimate the increase in mutation rate at a single locus, unc-22, using a nicotine assay (Section 4.2.1.3). To generate the lines, a small number of mutagenised worms (~20) were transferred by pipette onto each of 70 small agar plates. A similar number of control worms were transferred onto 60 plates. The remaining worms were distributed between 35 medium agar plates (mutagenised worms), and seven plates (controls) for the quantitative estimate of mutation frequency at a single locus (the nicotine assay). Each of these plates contained several thousand worms.

The worms were allowed to recover on agar plates for 24 hours, before alkaline hypochlorite treatment was carried out to remove any possible bacterial or fungal contamination caused by the EMS treatment, and to harvest the eggs to provide a synchronous population of worms for the nicotine assay. Any eggs present during the mutagenesis would be expelled during this 24-hour period, hatch, and be killed by the alkaline hypochlorite. The alkaline hypochlorite treatment was carried out on the worms for the nicotine assay, using the protocol described is Section 2.1.2.1, for cleaning large numbers of worms, with the modification that plates of worms were not combined. Afterwards, worms from each tube were divided between two medium
agar plates. For the 70 EMS and 60 control lines, the procedure described in Section 2.1.2.2, for cleaning small numbers of worms, was used instead.

4.2.1.2 Generation and propagation of lines

The mutant and control lines were then inbred for ten generations, in order to fix new mutations. Lines were propagated in parallel by the transfer of a single self-fertilising hermaphrodite, chosen at random, per line. Two days after the alkaline hypochlorite treatment, the worms were ~L2 larvae. From these plates, 60 EMS lines and 40 control lines were generated from individual larvae. During the inbreeding process, selection was minimised by keeping the worms in favourable conditions, with small population sizes, and transferring worms chosen at random. A dot was drawn on the base of each plate, at the edge of the bacteria. From the first 60 EMS plates, the three worms closest to this dot were picked and each was transferred to one of three new plates. From the first 40 control plates, two worms were transferred to new plates. Fewer backup plates were needed for the control lines, as the worms were healthier, and so were more likely to successfully produce viable offspring. Further transfers were carried out at five-day intervals, on average. For each line, the first plate was used to obtain all the worms, unless the parent failed to reproduce, in which case the back-up plates were used. If the backup plates also failed to produce offspring, worms from the previous generation, which were stored at 14°C to slow growth and reduce overcrowding, were substituted, up to a maximum of three generations, at which point the line was declared extinct. Ten transfers were carried out in total. From transfer eight onwards, an attempt was made to synchronise the lines by transferring worms of the same larval stage; worms which were as close as possible to the L3 stage were chosen. After the 10th transfer, the 56 surviving EMS lines and 40 control lines were cryopreserved at -80°C (Chapter 2).

4.2.1.3 Nicotine assay: estimation of mutation rate at the unc-22 locus

As a positive control for the mutagenesis, the mutation rate was measured at a single locus, *unc-22*. This approach was particularly suitable because the gene involved is large, with the result that the number of mutants produced is also large. In addition, individuals with mutations at this locus have a distinct phenotype, which it is possible
to identify against a background of wild-type worms, when the appropriate screen is used. Worms that are either homozygous or heterozygous for the \textit{unc-22} mutation twitch or vibrate rapidly in 1\% nicotine solution, whereas wild-type worms become rigidly paralysed (Moerman and Waterston, 1984).

Pilot studies were performed in order to assess the reliability of this technique for identifying \textit{unc-22} mutants. A line of \textit{C. elegans} homozygous for the \textit{unc-22} mutation was obtained from the \textit{Caenorhabditis} Genetics Center, and between 0 and 5 Unc worms were placed onto a plate containing thousands of wild-type worms. The plates were randomised, and examined for Unc worms using the nicotine assay, as described below. After practise, among 18 plates examined in this way, the success rate for identifying the correct number of Unc worms was 100\%.

It is possible that mutations producing the ‘twitcher’ phenotype in a nicotine screen could have been caused by mutations in a gene other than \textit{unc-22}. However, previous studies have been performed, studying such EMS-induced mutants in more detail. Map location and complementation tests performed by Moerman and Baillie (1979) confirmed that all the twitcher mutations they had identified were alleles of the \textit{unc-22} locus. In additions, Moerman and Waterston (1984) state that the combination of the twitch and abnormal body wall structure have only been found associated with the \textit{unc-22} mutation. Thus, it appears unlikely that this phenotype was caused by a mutation other that \textit{unc-22}, although the possibility of obtaining previously unidentified mutations with the same phenotype cannot be entirely excluded.

The protocol for measuring the mutation rate at the \textit{unc-22} locus induced by EMS mutagenesis was as follows. Four days after mutagenesis, the plates (which had been synchronised by alkaline hypochlorite treatment) contained large numbers of the adult F1 progeny of the mutagenised and control worms. The nicotine screen was performed over the course of two days. The worms were washed off the plates with 2ml of M9 solution, and each pair of plates - which had been separated after the alkaline hypochlorite - was combined; the worms were then rinsed with M9 by centrifugation. The supernatant was removed, 1.5ml of 1\% nicotine solution added, and the mixture was placed in a clean petri-dish (without any agar). Each plate was examined for worms with mutations at the \textit{unc-22} locus, those that twitched rapidly in
nicotine solution, rather than becoming paralysed (Moerman and Waterston, 1984). Plates were examined in batches of four or five; any more would require leaving them for too long, with the result that wild-type worms would begin to regain some movement. The number of worms screened was determined by taking an aliquot from each plate and manually counting the number of worms on that plate, after it had been examined for twitchers. All worms were paralysed adults, so counting the worms and obtaining an estimate of the total number of worms assayed was relatively straightforward. Seventy plates of EMS worms and 14 plates of controls were screened; in total, >100,000 control worms were assayed and >700,000 mutagenised worms. Putative mutants were pipetted into a new petri-dish containing 1% nicotine solution to separate them from wild-types which may have been transferred at the same time. They were then individually transferred by pipette onto small agar plates and allowed to self-fertilise, to confirm segregation of the unc-22 locus. In the absence of nicotine, the unc-22 locus is recessive – homozygotes move slowly with constant trembling. Plates that produced such offspring were scored positive; those that did not were scored negative. Immersing the offspring in nicotine solution as before allowed this scoring system to be checked – this proved to be a more sensitive test as heterozygotes could also be detected. Putative mutants that died before their offspring could be assayed in this way, or failed to reproduce (20%), were assumed to show the same frequency of unc-22 mutations as those that could be confirmed.

4.2.1.4 Life history assays

Two life history traits, daily reproductive output and lifespan of the inbred EMS and control lines, were measured, as described in Chapter 2. After thawing, individual replicates of each line were maintained for three generations prior to the assays being carried out. Both traits were measured contemporaneously for all EMS and control lines. One worm from each line was assayed by each of three counters, and the entire assay was performed three times – a total of nine worms per line.
4.3 RESULTS AND ANALYSIS

4.3.1 PHENOTYPIC MUTANTS

Careful visual examinations of the inbred mutant lines revealed a number of obvious phenotypic mutants, listed below.

**Uncoordinated (Unc):** lines E1, E12, E13, E31, and E54. The worms show restricted movement (Brenner, 1974). Lines E1 and E54 are ‘twitchers’ – they exhibit contractions along the body wall, which is more obvious when the worm is gently touched with a wire pick. It appears that either they are caused by different mutations, or else there are other mutations associated with them, as they produce very different numbers of offspring – line E1 produced an average of 86 offspring, compared to line E54 which produced 181. The control worms produced an average of 248 offspring. Lines E12 and E13 exhibit the ‘coiler’ phenotype – they form semi-circles on agar.

**Dumpy (Dpy):** lines E25 and E26. Worms are shorter than wild type, but the same diameter (Brenner, 1974). Again, it appears that they are either not caused by the same mutation, or else other mutations have occurred as their productivity is very different: an average of 244 offspring per worm in line E25, compared to 83 in line E26.

**High incidence of males (Him):** Line E57. Hermaphrodites from this line produce large numbers of male offspring. Of the known Him mutations, this most closely resembles the allele him-8, which produces 37% XO males and 6% 3X hermaphrodites via self-fertilisation, as a result of nondisjunction of the X-chromosomes (Hodgkin et al., 1979). The average productivity for this line was lower than wild type: 88 offspring per worm.

**Long (Lon):** line E60. Worms are ~50% longer than wild type (Brenner, 1974), and productivity is low, with an average of 72 offspring per worm.

**Dauer larva formation abnormal (Daf):** Line E38. The worms form dauer larvae easily and do not revert to normal adults, even in excess food (Riddle and Albert,
The worms assayed revealed extremely low productivity, probably as a result of forming dauers, which do not reproduce. During the life history assays, they produced an average of just over two offspring per worm.

**Ageing abnormal (Age)**: Line E28. Worms have an average lifespan ~30% longer than the controls (average lifespans are 17.7 days for line E28, compared to 13.4 days for the controls). They also exhibit reduced productivity (207 offspring per worm) and a conspicuous post-anal swelling with incomplete penetrance; these phenotypes may or may not be caused by the same mutation. There are a number of known mutants that result in a substantial increase in the lifespan of *C. elegans*, including the Age and Daf mutations (which also affect dauer formation), Clock mutations (which affect the timing of the developmental process) and the *spe-26* mutation, which results in reduced sperm production (*Spe = spermatogenesis abnormal*) (Kenyon, 1997). It is unlikely that this line contains a mutation at *spe-26* as it confers greatly reduced productivity (Van Voorhies, 1992). Further analysis of this line and E1 (Unc) is described in Chapter 5.

### 4.3.2 Estimation of Mutation Rate at the *unc-22* Locus

To provide a positive control for the mutagenesis, I measured the increase in mutation rate at a single locus, *unc-22*. All putative mutants were checked to confirm that they had been correctly identified (Section 4.2.1.3), thus ensuring that I did not overestimate the number of *unc-22* mutants produced. As some of the worms died before they could be checked, it was assumed that the same proportion would have been confirmed as being mutants, as among the survivors. Because of the large numbers of worms involved in the assay, the data were examined in order to determine whether there was any evidence that the number of mutants had been underestimated. This revealed that proportionally fewer *unc-22* mutants were identified on plates containing large numbers of worms, suggesting that when plates were overcrowded, the number of mutants had been underestimated (Figure 4.2). In order to control for this, a quadratic regression line was fitted, with the frequency of *unc-22* mutants, adjusted to account for those that died before they could be checked, as the dependent variable, and the total number of worms per plate as the independent variable. The y-intercept was then taken as the corrected estimate for the mutation rate.
rate at this locus. This indicated a frequency of $4.45 \times 10^{-4}$ per haploid genome (95% CI = $4.1 \times 10^{-4}$ to $4.8 \times 10^{-4}$), an increase of nearly 1500-fold over the spontaneous rate of $3 \times 10^{-7}$ (Eide and Anderson, 1985). As knowledge of the number of mutations induced was particularly important for this study, this positive control for the mutagenesis was performed in order to confirm that the EMS was inducing mutations at the expected frequency. Analysing the results of previous studies that involved a wider range of genes enabled more accurate inference of the number of mutations induced by this dosage of EMS (Section 4.4.1).

Figure 4.2: Frequency of unc-22 mutant worms

\begin{figure}
\centering
\includegraphics[width=\textwidth]{unc-22_frequency.png}
\caption{Frequency of unc-22 mutant worms}
\end{figure}

4.3.3 QUANTITATIVE ASSAYS OF FITNESS TRAITS

Life history trait assays were carried out on the inbred control and EMS lines. Daily reproductive output and lifespan were measured, as described in Materials and Methods (Chapter 2). From these data, several life history traits could be analysed: total productivity, early productivity (number of viable offspring produced during the first two days of the reproductive period), late productivity (offspring produced during the remaining four days) and longevity.

4.3.3.1 Changes in mean and variance

The distributions of line means for each of the traits are shown in Figure 4.3. The change in mean for each trait was determined as a proportion of the value of the
controls: \((M_{Con} - M_{EMS})/M_{Con}\), where \(M_{Con}\) is the mean of the controls and \(M_{EMS}\) is the mean of the EMS lines. The genetic, or between-line variance components \((V_G)\) were obtained from General Linear Models used to partition the variance between and within lines. Additional effects fitted in the analysis were measurer (three people carried out the assay), replicate (the fitness assays were carried out three times), and the interaction between these two effects.

From this, the mutational heritability, \(h_m^2\), could be determined, as described in Chapter 2. This is equivalent to the mutational variance, \(V_M (= V_G/2)\), scaled relative to the environmental variance, \(V_E\), the residual variance component of the control lines. This provides a dimensionless value that can be compared across traits. While the mutational heritability scales the mutational variance relative to the environmental variance, it is also informative to scale the variance relative to the mean, to obtain the mutational coefficient of variance \(CV_M = \sqrt{V_M}/M_{Con}\). Summaries of these statistics showing the effects of the mutagenesis treatment on life history traits are shown in Table 4.1. Standard errors for \(h_m^2\) and \(CV_m\) were obtained from bootstrap analysis, involving resampling the data with replacement, by line, 100 times.
Figure 4.3: Distributions of control and EMS lines for four life history traits

(a) Total Productivity

(b) Early Productivity

(c) Late Productivity

(d) Longevity
Table 4.1: Means and variances for each trait assayed, with standard errors shown in brackets

<table>
<thead>
<tr>
<th>Trait</th>
<th>Mean (Controls)</th>
<th>Mean (EMS)</th>
<th>∆Mean, %</th>
<th>( V_G )</th>
<th>( h^2_m )</th>
<th>( CV_m )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total productivity</td>
<td>248</td>
<td>163</td>
<td>34.3</td>
<td>4931</td>
<td>1.02</td>
<td>0.20</td>
</tr>
<tr>
<td>(2.5)</td>
<td>(9.9)</td>
<td>(3.9)</td>
<td>(0.27)</td>
<td>(0.014)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early productivity</td>
<td>192</td>
<td>97.5</td>
<td>49.2</td>
<td>2633</td>
<td>0.67</td>
<td>0.19</td>
</tr>
<tr>
<td>(2.2)</td>
<td>(7.2)</td>
<td>(4.2)</td>
<td>(0.16)</td>
<td>(0.017)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Late productivity</td>
<td>56.6</td>
<td>65.7</td>
<td>-16.1</td>
<td>1048</td>
<td>0.52</td>
<td>0.40</td>
</tr>
<tr>
<td>(1.5)</td>
<td>(4.7)</td>
<td>(8.9)</td>
<td>(0.13)</td>
<td>(0.045)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Longevity (days)</td>
<td>13.4</td>
<td>12.2</td>
<td>9.0</td>
<td>3.18</td>
<td>0.20</td>
<td>0.094</td>
</tr>
<tr>
<td>(0.16)</td>
<td>(0.29)</td>
<td>(2.3)</td>
<td>(0.091)</td>
<td>(0.022)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The average number of offspring produced by the control lines, 248, is a figure typical of the N2 strain of \( C. \) elegans (Lewis and Fleming, 1995). The mean lifespan of the control lines is also typical. EMS mutagenesis resulted in large, significant changes in the mean and variance of each trait assayed. The overall trends are similar for total productivity, early productivity and lifespan: mutagenesis resulted in a reduction in mean fitness, and an increase in variance between the lines, although in each case there was some overlap between the control and EMS lines. The mutagenesis resulted in an increase in the mean values for late productivity, probably due to delayed reproduction.

The smallest mutational target in each case was longevity. When analysing the change in mean of each trait, it appears that early productivity provides the largest mutational target, though the fact that the mutational heritability is higher for total productivity indicates that there is likely to be greater environmental variance associated with early productivity. The relatively high coefficient of variance for late productivity is likely to be due to low mean values for this trait, as the change in mean and heritability is lower than for the other productivity traits.

None of the EMS lines show greater early or total productivity than the mean for the control lines. As mentioned earlier, the increase in late productivity in a number of EMS lines was probably the result of delayed reproduction. However, at least one line appeared to show longer lifespan than the controls (Figure 4.3d). Line E28 revealed the largest increase in lifespan, and this line was chosen for further study in
an attempt to determine the number of loci responsible for the increase in lifespan, and whether there are pleiotropic effects of the mutation on productivity traits, as might be expected under the optimality theory of ageing. This work is described in Chapter 5.

The remaining 55 EMS lines were analysed to determine whether they also had mean lifespans significantly different from those of the controls. The probability of each line mean falling within a Normal distribution with the same mean and variance as the control lines was determined. Because of the multiple comparisons required, the Dunn-Šidák technique, a form of Bonferroni correction, was used in order to establish the appropriate type I error level ($\alpha$) for each comparison (Sokal and Rohlf, 1995; Section 9.6). If $k$ comparisons are carried out, each with a corresponding type I error level of $\alpha'$, the probability of making no errors is $(1 - \alpha')^k$. Therefore, the probability of making a type I error is $1 - (1 - \alpha')^k$, and so $\alpha' = 1 - (1 - \alpha)^{1/k}$.

The probability that each of the most extreme lines fell within the distribution expected for the controls was determined, and compared to $\alpha'$. Because lines that exhibited both an increase and a decrease in mean lifespan were included, the test was two-tailed, and thus $\alpha = 0.025$. With each progressive line analysed, the number of comparisons remaining, $k$, fell by one, with the result that $\alpha'$ increased progressively. The results for the most extreme lines are given in Table 4.2.

Table 4.2: Probability that the lifespans of the most extreme EMS lines differ significantly from the controls, conditional on the parameter $\alpha'$

<table>
<thead>
<tr>
<th>Line</th>
<th>Mean (days)</th>
<th>Probability, $P$</th>
<th>$k$</th>
<th>$\alpha'$</th>
<th>Significance *</th>
</tr>
</thead>
<tbody>
<tr>
<td>E7</td>
<td>4.50</td>
<td>$&lt;0.000001$</td>
<td>55</td>
<td>0.00047</td>
<td>$P &lt; \alpha' \Rightarrow$ Sig.</td>
</tr>
<tr>
<td>E10</td>
<td>6.94</td>
<td>$&lt;0.000001$</td>
<td>54</td>
<td>0.00048</td>
<td>$P &lt; \alpha' \Rightarrow$ Sig.</td>
</tr>
<tr>
<td>E55</td>
<td>8.86</td>
<td>0.000003</td>
<td>53</td>
<td>0.00049</td>
<td>$P &lt; \alpha' \Rightarrow$ Sig.</td>
</tr>
<tr>
<td>E43</td>
<td>9.81</td>
<td>0.00016</td>
<td>52</td>
<td>0.00050</td>
<td>$P &lt; \alpha' \Rightarrow$ Sig.</td>
</tr>
<tr>
<td>E59</td>
<td>10.11</td>
<td>0.00048</td>
<td>51</td>
<td>0.00051</td>
<td>$P &lt; \alpha' \Rightarrow$ Sig.</td>
</tr>
<tr>
<td>E42</td>
<td>10.25</td>
<td>0.00079</td>
<td>50</td>
<td>0.00052</td>
<td>$P &gt; \alpha' \Rightarrow$ NS</td>
</tr>
</tbody>
</table>

* Sig. = significant, implying that the $P$-value is less than the appropriate Type I error level, $\alpha'$. NS = non-significant ($P > \alpha'$).
From this, it can be seen that five of the lines have mean lifespans significantly lower than the controls (up to and including line E59 in the table). No line other than E28 had a significantly longer lifespan that the controls.

4.3.4 CORRELATION BETWEEN TRAITS

Figure 4.4 shows the line means for each productivity trait plotted against the mean lifespan of that line.

There appear to be strong, positive relationships between the traits. Correlations between the traits studied were determined using the method described in Chapter 2. Standard errors were obtained by performing the analysis on bootstrapped data, 100 times. The results are presented in Table 4.3.

Table 4.3: Estimates of genetic (above the diagonal) and environmental (below) correlation coefficients, and their standard errors

<table>
<thead>
<tr>
<th>Trait</th>
<th>Total Productivity</th>
<th>Early Productivity</th>
<th>Late productivity</th>
<th>Longevity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Productivity</td>
<td>0.90 (0.027)</td>
<td>0.73 (0.075)</td>
<td>0.55 (0.14)</td>
<td></td>
</tr>
<tr>
<td>Early Productivity</td>
<td>0.77 (0.035)</td>
<td>0.37 (0.12)</td>
<td>0.47 (0.11)</td>
<td></td>
</tr>
<tr>
<td>Late productivity</td>
<td>0.65 (0.042)</td>
<td>0.01 (0.078)</td>
<td>0.45 (0.15)</td>
<td></td>
</tr>
<tr>
<td>Longevity</td>
<td>0.37 (0.060)</td>
<td>0.22 (0.055)</td>
<td>0.31 (0.054)</td>
<td></td>
</tr>
</tbody>
</table>

All the traits show strong, positive genetic correlations, which appears to indicate the presence of unconditionally deleterious mutations, reducing the mean values of each trait. The results were checked using restricted maximum likelihood, using the ASREML package (Average Information Restricted Maximum Likelihood, Gilmour et al., 1995), a more computationally intensive approach. This approach has the advantage of being able to account for slight imbalances in the data, caused, for instance, by worms being killed accidentally. The results closely matched those shown above.

The degree of correlation was similar between each of the productivity traits and longevity. Because of this strong correlation between them, the mutational heritabilities for longevity and total productivity were also determined from an analysis of covariance with the variation between the two traits fitted as a covariate.
Thus, it was possible to obtain an estimate of the mutational heritability for both traits, with the variance attributable to the other trait removed, i.e., studying mutations that only affected productivity and not longevity, or vice versa.

Figure 4.4: Correlations between longevity and productivity traits

(a) Total productivity vs. longevity

(b) Early productivity vs. longevity

(c) Late productivity vs. longevity
Analysis of covariance (ANCOVA) was carried out on the control and EMS data for both productivity and longevity, with line, measurer, replicate and the interactions fitted, as well as the covariate (longevity and productivity, respectively). The results are presented in Table 4.4 to Table 4.7 below.

Table 4.4: ANCOVA for control productivity data (longevity fitted as a covariate)

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Longevity (covariate)</td>
<td>1</td>
<td>19170</td>
<td>7.55</td>
<td>0.007</td>
</tr>
<tr>
<td>Line</td>
<td>39</td>
<td>2093</td>
<td>0.82</td>
<td>0.756</td>
</tr>
<tr>
<td>Measurer</td>
<td>2</td>
<td>190</td>
<td>0.08</td>
<td>0.928</td>
</tr>
<tr>
<td>Replicate</td>
<td>2</td>
<td>62414</td>
<td>24.59</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Line*Measurer</td>
<td>78</td>
<td>2205</td>
<td>0.87</td>
<td>0.753</td>
</tr>
<tr>
<td>Line*Replicate</td>
<td>78</td>
<td>2331</td>
<td>0.92</td>
<td>0.658</td>
</tr>
<tr>
<td>Measurer*Replicate</td>
<td>4</td>
<td>3469</td>
<td>1.37</td>
<td>0.248</td>
</tr>
<tr>
<td>Error</td>
<td>151</td>
<td>2538</td>
<td>0.83</td>
<td>0.380</td>
</tr>
</tbody>
</table>

Table 4.5: ANCOVA for EMS productivity data (longevity fitted as a covariate)

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Longevity (covariate)</td>
<td>1</td>
<td>153541</td>
<td>63.44</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Line</td>
<td>55</td>
<td>32183</td>
<td>13.30</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Measurer</td>
<td>2</td>
<td>355</td>
<td>0.15</td>
<td>0.864</td>
</tr>
<tr>
<td>Replicate</td>
<td>2</td>
<td>31002</td>
<td>12.81</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Line*Measurer</td>
<td>110</td>
<td>3760</td>
<td>1.55</td>
<td>0.004</td>
</tr>
<tr>
<td>Line*Replicate</td>
<td>110</td>
<td>3345</td>
<td>1.38</td>
<td>0.024</td>
</tr>
<tr>
<td>Measurer*Replicate</td>
<td>4</td>
<td>6951</td>
<td>2.87</td>
<td>0.024</td>
</tr>
<tr>
<td>Error</td>
<td>201</td>
<td>2420</td>
<td>0.83</td>
<td>0.380</td>
</tr>
</tbody>
</table>

Table 4.6: ANCOVA for control longevity data (productivity fitted as a covariate)

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Productivity (covariate)</td>
<td>1</td>
<td>60.852</td>
<td>7.55</td>
<td>0.007</td>
</tr>
<tr>
<td>Line</td>
<td>39</td>
<td>8.959</td>
<td>1.11</td>
<td>0.319</td>
</tr>
<tr>
<td>Measurer</td>
<td>2</td>
<td>54.288</td>
<td>6.74</td>
<td>0.002</td>
</tr>
<tr>
<td>Replicate</td>
<td>2</td>
<td>161.110</td>
<td>20.00</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Line*Measurer</td>
<td>78</td>
<td>7.393</td>
<td>0.92</td>
<td>0.660</td>
</tr>
<tr>
<td>Line*Replicate</td>
<td>78</td>
<td>7.078</td>
<td>0.88</td>
<td>0.735</td>
</tr>
<tr>
<td>Measurer*Replicate</td>
<td>4</td>
<td>13.223</td>
<td>1.64</td>
<td>0.167</td>
</tr>
<tr>
<td>Error</td>
<td>151</td>
<td>8.057</td>
<td>0.83</td>
<td>0.380</td>
</tr>
</tbody>
</table>
Table 4.7: ANCOVA for EMS longevity data (productivity fitted as a covariate)

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Productivity (covariate)</td>
<td>1</td>
<td>584.595</td>
<td>63.44</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Line</td>
<td>55</td>
<td>33.067</td>
<td>3.59</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Measurer</td>
<td>2</td>
<td>2.335</td>
<td>0.25</td>
<td>0.776</td>
</tr>
<tr>
<td>Replicate</td>
<td>2</td>
<td>89.513</td>
<td>9.71</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Line*Measurer</td>
<td>110</td>
<td>15.711</td>
<td>1.70</td>
<td>0.001</td>
</tr>
<tr>
<td>Line*Replicate</td>
<td>110</td>
<td>11.864</td>
<td>1.29</td>
<td>0.062</td>
</tr>
<tr>
<td>Measurer*Replicate</td>
<td>4</td>
<td>23.269</td>
<td>2.52</td>
<td>0.042</td>
</tr>
<tr>
<td>Error</td>
<td>201</td>
<td>9.215</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

As can be seen, in both the EMS and control lines, the covariance between the two traits is highly significant (p<0.01), providing further evidence that the phenotypic and environmental correlations between these traits are also significant. The mutational heritabilities were determined as before. The heritability values for each trait, both with and without covariates fitted, are presented below:

\[ h_m^2 (\text{total productivity; no covariate}) = 0.86 \]
\[ h_m^2 (\text{total productivity; longevity fitted as a covariate}) = 0.65 \]
\[ h_m^2 (\text{longevity; no covariate}) = 0.21 \]
\[ h_m^2 (\text{longevity; total productivity fitted as a covariate}) = 0.16 \]

In each case, the mutational variance decreases when one trait is studied without factors affecting the other, due to the positive correlations between the traits.

4.3.5 AGE-SPECIFIC MORTALITY RATES

The age-specific mortality rates of the EMS and control lines are shown in Figure 4.5a. Although mortality rates increase with age, this increase is not exponential, as would be predicted by the Gompertzian kinetics (Gompertz, 1825). Instead, after day ~14, mortality rates increase more slowly, and some levelling off can be observed. This is true even in the case of the genetically homogeneous control population, but the trend is far more exaggerated among the EMS worms, to the extent that the lines actually cross over around day 15. The EMS worms show increased early mortality relative to the controls, but reduced mortality later in life.
Figure 4.5: Age-specific mortality rates (EMS and control lines)

(a) Age-specific mortality rates

(b) Ln-ln Age-specific mortality rates

\[ \ln(\text{mortality}) = -11.3 + 3.66(\ln(\text{day})) \]

\[ \ln(\text{mortality}) = -6.14 + 1.65(\ln(\text{day})) \]
4.4 NUMBER OF DELETERIOUS MUTATIONS INDUCED

4.4.1 EMS-INDUCED MUTATION RATES

The number of mutations that are expected to become fixed under conditions of minimal selection, with self-fertilisation and a population size of one, is equal to the number of mutations induced per haploid genome (see Section 2.4.3). Using knowledge of the mutagenic properties of EMS, it is possible to estimate the number of mutations induced, and thus, the number of mutations expected to have become fixed in the lines of worms.

The chemical mutagen EMS was used in these experiments, which involved using a dosage of mutagen - 50mM for four hours - for which the number of base-pair mutations induced has been calibrated. Information obtained from the sequences of 245 EMS-induced mutations indicate that approximately 92% of such mutations are G/C → A/T transitions; the remainder are other kinds of point mutation, plus a low frequency of small deletions and chromosomal rearrangements (Anderson, 1995). The rate at which these mutations are induced has been measured in a number of experiments involving suppressor-induced reversion mutations and forward mutations, in which a single base-pair change will lead to a detectable suppression or reversion event. Thus, using knowledge of the frequency of such mutations, it is possible to obtain a mean estimate for the rate at which such base-pair mutations are induced by a known dosage of EMS.
4.4.1.1 Amber suppressor mutations

Transfer RNA (tRNA) consists of a class of small RNA molecules, each with specificity for a particular amino acid; they carry the amino acids to the ribosome for protein synthesis. A three base-pair site on the tRNA (the anticodon) recognises a codon on the messenger RNA (mRNA); its bases are complementary and antiparallel to the bases of the codon. Stop codons, however, are not recognised by tRNA, which results in the termination of transcription. There are three stop codons: amber (UAG), opal (UGA) and ochre (UAA). A mutation in a gene that creates a stop codon will produce a nonsense mutation, resulting in the premature termination of protein synthesis. Such nonsense mutations can be suppressed by a mutation that alters the tRNA anticodon to produce a tRNA that recognises the stop codon, rather than the appropriate codon. For example, if the tryptophan tRNA (tRNA\textsubscript{Trp}) is mutated in this way (from UGG to UAG), tryptophan will be inserted at that position in the protein, rather than transcription being terminated. This may restore wild-type function, to some extent. Such a mutation is thus known as a nonsense suppressor mutation. These mutations are expected to be dominant and to act on a subset of alleles in a variety of genes. Note: there are many genes encoding each tRNA, so a mutation in one of them will not prevent the others from functioning normally.

There are 12 tRNA\textsubscript{Trp} genes in \textit{C. elegans}, as identified by Southern blotting (Kondo \textit{et al.}, 1988). A single base-pair change must be responsible for a tRNA\textsubscript{Trp} amber suppressor mutation, as a UGG $\rightarrow$ UAG change is the only way of inducing such a mutation from tRNA\textsubscript{Trp}. Thus, by carrying out mutagenesis and then screening large numbers of individuals for such mutations, it is possible to determine the frequency at which these base-pair mutations have been induced.

Waterston (1981) carried out a suppressor screen such as this, through reversion analysis of \textit{unc-13} mutants. A synchronised population of worms homozygous for \textit{unc-13} was treated with 50mM EMS for four hours. Populations of either 150 or 450 worms were then placed near the edge of each plate; \textit{unc-13} animals are paralysed, so revertants could easily be detected by their tracks as they moved. $1.2 \times 10^4$ P\textsubscript{0} animals were distributed over 40 plates and allowed to reproduce (the average brood size was 26). Worms with improved motility were found on six of these. Linkage
analysis was then carried out to identify any unlinked suppressors. If the mutation was the result of a reversion event within the gene (i.e., intragenic), or a closely linked suppressor, crosses would not be expected to reveal any Unc progeny. Alternatively, if the mutation occurred in an unlinked suppressor, the Unc and Sup (suppressor) alleles would segregate independently, and so a number of Unc progeny would be expected. The presence of an extragenic suppressor was subsequently confirmed by mapping the suppressor.

In total, four of the six revertants were found to contain extragenic suppressors; two were mapped to sup-5 and two to sup-7. In a much larger screen in which suppressor frequency was not measured, more than thirty mutations have been identified which act as suppressors of unc-13. All of these mutants map to sup-5 and sup-7, which indicates that these are the only two amber suppressors of this locus that could have been identified (Waterston, 1981). At both of these loci, a single base-pair site at a tRNA<sup>Trp</sup> gene is the mutational target (Bolten et al., 1984, Kondo et al., 1988, Kondo et al., 1990). Thus, the frequency of base-pair mutations induced is \(4/(2 \times 26 \times (1.26 \times 10^4)) = 6.4 \times 10^{-6}\) per worm, or \(3.2 \times 10^{-6}\) per mutagenised gamete.

A larger experiment was also carried out by Waterston (1981), in which the number of worms assayed was estimated with less accuracy. Nineteen extragenic revertants were identified, giving a frequency of approximately \(10^{-6}\).

Hodgkin (1985) and Kondo et al. (1990) have carried out further experiments, screening for amber suppressor mutations in several more tRNA<sup>Trp</sup> genes. Hodgkin (1985) screened for amber suppressors of a sex-determining gene, tra-3. This mutation causes masculinisation of XX (hermaphrodite) animals, resulting in sterility at 20°C, though some self-fertilisation can take place at 15°C. Thus, stocks can be grown at 15°C, but at 20°C only revertants will reproduce, which will be easy to screen for. Stocks of three amber mutants of tra-3 were grown up and mutagenised with 25mM EMS for four hours. Several independent amber suppressors were obtained from each strain, as shown in Table 4.8, below.
Table 4.8: Reversion of *tra-3* mutants (Hodgkin, 1985)

<table>
<thead>
<tr>
<th>Allele</th>
<th>Total F&lt;sub&gt;1&lt;/sub&gt; screened (approx.)</th>
<th>Total no. informational suppressors</th>
</tr>
</thead>
<tbody>
<tr>
<td>e1107</td>
<td>25,000</td>
<td>1</td>
</tr>
<tr>
<td>e1525</td>
<td>100,000</td>
<td>5</td>
</tr>
<tr>
<td>e1903</td>
<td>75,000</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>200,000</td>
<td>9</td>
</tr>
</tbody>
</table>

A total of nine independent revertants were identified at four sites, each with a one base-pair mutational target. Kondo *et al.* (1990) have also carried out reversion experiments with *tra-3(e1525)* mutants, using the same methods as Hodgkin (1985), described above. The results are given in Table 4.9 below.

Table 4.9: Reversion of *tra-3* mutants (Kondo *et al.*, 1990)

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Total F&lt;sub&gt;1&lt;/sub&gt; screened (approx.)</th>
<th>Total no. informational suppressors</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8,000</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>50,000</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td>45,000</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>7,000</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>6,000</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>15,000</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>131,000</td>
<td>9</td>
</tr>
</tbody>
</table>

From these experiments, nine candidate mutations were obtained which were subsequently identified as being tRNA<sup>T<sub>p</sub></sup> genes. Southern analysis was carried out, using specific oligonucleotide probes to detect a change at an anticodon in a tRNA<sup>T<sub>p</sub></sup> gene. Revertants were detected at five genes.

In total, suppressors of *tra-3* were obtained at six genes in the two sets of experiments, indicating that there are six sensitive sites. This implies that the frequency of mutations induced at a single base-pair site in these experiments was \((9/200,000)/6 = 1.1 \times 10^{-5}\) per worm or \(3.75 \times 10^{-6}\) per mutagenised gamete (Hodgkin, 1985) and \((9/131,000)/6 = 1.14 \times 10^{-6}\) per worm or \(5.72 \times 10^{-6}\) per gamete (Kondo *et al.*, 1990).
4.4.1.2  unc-54 null alleles

Estimates of the EMS-induced mutation rate have also been obtained from experiments screening for unc-54(0) (null) alleles (Bejsovec and Anderson, 1988). Such mutations have a much larger mutational target than the amber suppressor mutations. It is possible to estimate the mutation rate per base-pair from the frequency with which unc-54(0) mutations are induced, the proportion of these that are nonsense alleles, and the number of sites at which an EMS-induced transition can produce a nonsense allele.

unc-54 mutations interfere with the assembly of thick myofilaments. unc-54(0) alleles are recessive, loss-of-function alleles; the worms accumulate no detectable myosin heavy-chain B, causing them to be paralysed (uncoordinated). Bejsovec and Anderson (1988) screened for such mutations induced by EMS on a wild-type background. They mutagenised synchronous populations of worms at the L4 stage for 3.5 hours, using 50mM EMS, then placed groups of ten F1 progeny onto separate plates. The F2 progeny were later screened for paralysed unc-54(0) homozygotes and complementation tests were carried out to confirm the genotypes. From 3000 F1 animals screened, eight unc-54(0) mutations were identified, indicating that $1.33 \times 10^{-3}$ mutations occurred per gamete. Dibb et al. (1985) identified five unc-54(0) alleles from EMS screens; sequence analysis revealed that four of these were nonsense mutations and one was the result of a deletion. Bejsovec and Anderson (1990) identified 13 unc-54(0) mutations, 12 of which were nonsense mutations and one was a deletion. Thus, the majority of unc-54(0) alleles (16/18) appear to be the result of nonsense mutations. DNA sequence studies of unc-54 (Karn et al., 1983) show that the gene has 160 G/C base-pairs which will produce a nonsense codon if mutated to A/T (Anderson, 1995). Assuming that all of these sites are mutable, this indicates that the frequency of mutations is $(16/18 \times (1.33 \times 10^{-3})) / 160 = 7.4 \times 10^{-6}$ per haploid genome.
### 4.4.1.3 Summary: EMS-induced mutation rates

The experiments described above are summarised in Table 4.10, below. The total number of sites screened = the number of F₁ animals screened × the number of sensitive sites × 2 (worms are diploid). The results of Bejsovec and Anderson (1988) are adjusted by the fraction of mutations expected to be stop codons (16/18).

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Total no. F₁'s screened</th>
<th>Sensitive sites</th>
<th>Total no. sites screened</th>
<th>No. mutations</th>
<th>Frequency of mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Waterston (1981)</td>
<td>312,000</td>
<td>2</td>
<td>1,248,000</td>
<td>4</td>
<td>3.2 × 10⁻⁶</td>
</tr>
<tr>
<td>Hodgkin (1985)</td>
<td>200,000</td>
<td>6</td>
<td>2,400,000</td>
<td>9</td>
<td>3.75 × 10⁻⁶</td>
</tr>
<tr>
<td>Kondo et al. (1990)</td>
<td>131,000</td>
<td>6</td>
<td>1,572,000</td>
<td>9</td>
<td>5.72 × 10⁻⁶</td>
</tr>
<tr>
<td>Bejsovec &amp; Anderson (1988)</td>
<td>3,000</td>
<td>160</td>
<td>960,000</td>
<td>7.11</td>
<td>7.4 × 10⁻⁶</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td><strong>5.02 × 10⁻⁶</strong></td>
</tr>
</tbody>
</table>

It should be noted, however, that the experiments did not use consistent dosages of EMS. Scaling is required in some cases, in order to compare the mutation rate to that of the standard dosage used (50mM EMS for 4 hours (Anderson, 1995)).

Hodgkin and Kondo et al. used a lower dosage of 25mM EMS for four hours. Rosenbluth et al. (1983) have developed a dose-response curve for EMS by measuring the frequency of recessive lethal mutations induced in a region balanced by the reciprocal translocation, eTI(III;V). This indicates that the rate of mutation induced by 25mM EMS is approximately 5/7 of that induced by 50mM EMS. Hodgkin's results therefore indicate that the frequency of mutations induced by 50mM EMS would be (3.75 × 10⁻⁶) × 7/5 = 5.25 × 10⁻⁶, and the results of Kondo et al. indicate a mutation rate of (5.72 × 10⁻⁶) × 7/5 = 8.02 × 10⁻⁶ induced by 50mM EMS.
Bejsovec and Anderson (1988) used 50mM EMS, but for 3.5 hours, rather than four hours. There is no dose response curve for *C. elegans* that measures the mutation rate induced by exposure to EMS for different lengths of time. Assuming that mutation frequency increases approximately linearly with time, the predicted mutation rate induced by 50mM EMS for four hours would be \((7.4 \times 10^6) \times 4/3.5 = 8.46 \times 10^6\) per haploid genome. These results are presented in Table 4.11 below, adjusted for the dose of mutagen used.

Table 4.11: Summary of EMS-induced mutation rates, adjusted to a standard dosage

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Dose of EMS</th>
<th>Total no. sites screened</th>
<th>No. mutations</th>
<th>Adjusted no. mutations*</th>
<th>Frequency of mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Waterston (1981)</td>
<td>50mM; 4 hours</td>
<td>1,248,000</td>
<td>4</td>
<td>4</td>
<td>(3.2 \times 10^6)</td>
</tr>
<tr>
<td>Hodgkin (1985)</td>
<td>25mM; 4 hours</td>
<td>2,400,000</td>
<td>9</td>
<td>12.6</td>
<td>(5.2 \times 10^6)</td>
</tr>
<tr>
<td>Kondo <em>et al.</em> (1990)</td>
<td>25mM; 4 hours</td>
<td>1,572,000</td>
<td>9</td>
<td>12.6</td>
<td>(8.0 \times 10^5)</td>
</tr>
<tr>
<td>Bejsovec &amp; Anderson (1988)</td>
<td>50mM; 3.5 hours</td>
<td>960,000</td>
<td>7.11</td>
<td>8.1</td>
<td>(8.46 \times 10^6)</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(6.2 \times 10^6)</td>
</tr>
</tbody>
</table>

* Adjusted to a standard dosage of 50mM EMS for 4 hours

Despite the fact that the experiments were carried out by several different people studying a variety of loci, the results are reasonably consistent. These experiments indicate that the frequency of mutations induced in the *C. elegans* haploid genome is approximately \(6.2 \times 10^6\) per mutagenised base-pair, when mutagenised with 50mM EMS for four hours. Using this result, it is possible to obtain upper and lower limits for estimates of the number of deleterious mutations induced.

4.4.2 NUMBER OF DELETERIOUS MUTATIONS INDUCED: UPPER LIMIT

An upper limit for the number of deleterious mutations induced per haploid genome is simply the total number of base-pair (bp) mutations. This can be deduced as follows:
Total number of bp mutations ($U$)  

$$= \text{Genome size} \left(9.7 \times 10^7\right) \times \text{Fraction of susceptible base-pairs} \times \text{Rate of mutation}$$  

$$= \left(9.7 \times 10^7\right) \times 0.36 \times (6.2 \times 10^6)$$  

$$= 229 \text{ bp changes}$$

The average effect of such a mutation, $\bar{\delta}$, is equivalent to the mean proportional reduction in the value of the trait of interest relative to the control, divided by the number of mutations induced. This will depend on the trait assayed; for total productivity, for instance, the mean proportional reduction in fitness is $(248.49 - 163.17) / 248.49 = 0.34$ (Table 4.1). Therefore, the average effect of a mutation on this trait, $\bar{\delta}$, is $0.34 / 229 = 1.5 \times 10^{-3}$, or 0.15%.

These results of $U = 229$ and $\bar{\delta} = 0.15\%$ are upper and lower limits, respectively. A large number of these mutations would not be expected to have an effect on fitness, e.g., if they occur in non-constrained regions of the genome. Therefore, it is informative to determine a lower limit for the deleterious mutation rate: the fraction of amino acid changes induced that are expected to be deleterious in natural conditions.

4.4.3 NUMBER OF DELETERIOUS MUTATIONS INDUCED: LOWER LIMIT

To obtain a lower limit for the number of mutations induced with an effect on fitness, I determined the fraction of amino acid changes induced that are selectively constrained. This was done by examining a sample of genes in which codon usage is known (Nakamura et al., 1999), and working out the fraction of G/C $\rightarrow$ A/T transitions that would produce an amino acid change. From the total number of genes and the rate at which G/C $\rightarrow$ A/T transitions are induced, it was then possible to estimate the total number of mutations induced that would result in an amino acid change. Studies of molecular constraint in *C. elegans* (Stenico et al., 1994) have provided an estimate of the proportion of such mutations that are selectively constrained.
4.4.3.1 Fraction of G/C → A/T transitions that would produce an amino acid change

The CUTG database (Codon Usage Tabulated from GenBank), developed by Nakamura et al. (1999), contains codon usage data for >15,000 C. elegans genes extracted from the GenBank DNA sequence database (Release 108, 15 August 1998), using the ACNUC retrieval system. The frequency of each codon in these genes was then determined. Using this information, I have calculated the number of nucleotides in this sample at which a G/C → A/T transition will result in an amino acid change. An example of this method, performed on a very small number of amino acids, is given below:

Table 4.12: Example of codon usage in C. elegans

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Frequency per thousand</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phe UUU</td>
<td>24.8</td>
<td>No G/C sites ⇒ no mutation.</td>
</tr>
<tr>
<td>UUC</td>
<td>24.4</td>
<td>C → U transition will result in a synonymous change.</td>
</tr>
<tr>
<td>Met AUG</td>
<td>26.2</td>
<td>G → A transition will change the amino acid to Ile.</td>
</tr>
<tr>
<td>Arg CGU</td>
<td>11.2</td>
<td>In each case, a transition at either of the first sites will result in an amino acid change. In the the last two cases, a third site transition will be synonymous.</td>
</tr>
<tr>
<td>CGA</td>
<td>5.0</td>
<td></td>
</tr>
<tr>
<td>CGC</td>
<td>11.5</td>
<td></td>
</tr>
<tr>
<td>CGG</td>
<td>4.3</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>107.4</td>
<td>107.4 codons = 322.2 nucleotides.</td>
</tr>
</tbody>
</table>

The fraction of nucleotides at which an amino acid change would be induced by a G/C → A/T transition, in this sample, is (26.6 + 2(11.2 + 5.0 + 11.5 + 4.3)) / 322.2 = 0.28.

This method was applied to all the amino acids in the genes studied by Nakamura et al. (1999). There are an estimated total of 19,000 genes in C. elegans (The C. elegans Sequencing Consortium, 1998). From a sample of >15,000 genes, the frequency of each codon was determined – a total of >6,000,000 codons. The frequency of nucleotides in these genes at which a G/C → A/T transition will result in an amino acid change is 918.7 per thousand codons. Therefore, the fraction of sites in
genes, at which an amino acid change would be induced, is $918.7 / (3 \times 1,000) = 0.306$.

Sharp and Bradnam (1997) have carried out an analysis of codon usage using similar techniques. However, they present the data in a different manner; it is divided into three subsets, according to the degree of codon bias: the 10% of genes at either extreme and 10% in the middle. They state that codon usage in the middle subset of genes is similar to that observed overall. Using data from the middle subset of genes in this study, the fraction of nucleotide sites at which an amino acid change would be induced by a G/C $\rightarrow$ A/T transition is $103,530 / 327,360 = 0.316$. This figure is similar to that obtained using all the genes in the much larger study by Nakamura et al. (1999). Therefore, although it is possible that selective sampling of genes with high expression occurred amongst those sampled by Nakamura et al. (1999), this has not affected the degree of codon bias.

Information from the complete genome sequence of *C. elegans* reveals that 27% of the 97-Mb genome resides in exons (The *C. elegans* Sequencing Consortium, 1998). This figure was obtained by using GENEFINDER to provide an overview of gene structure from the sequence data. Therefore, the total number of sites at which an amino acid change could be induced by a G/C $\rightarrow$ A/T transition is $0.27 \times 97$-Mb $\times 0.306 = 8,014,140$. The frequency of such mutations following EMS mutagenesis is $6.2 \times 10^6$, indicating that a dosage of EMS will induce an average of $(6.2 \times 10^6) \times 8,564,130 = 50$ amino acid changes per haploid genome.

Stenico et al. (1994) have studied patterns of similarity between 12 homologous genes from *C. elegans* and that of *C. briggsae*, its closest known relative. From differences between the estimated number of nucleotide substitutions per synonymous ($K_s$) and per non-synonymous ($K_A$) site, it is possible to obtain estimates of the fraction of selectively constrained nucleotides. The average selective constraint within these genes is $1 - (K_A/K_s) = 1 - (0.0771/1.0675) = 0.93$, or 93% in *C. elegans*. For the standard dosage of EMS, the number of amino acid changes which are deleterious is therefore $0.93 \times 50 = 45$ per haploid genome.
The average effect of these mutations for total productivity, $\bar{s}$, = Mean reduction in fitness $(0.34) / \text{Number of mutations (45)} = 7.6 \times 10^{-3} = 0.76\%$.

These results of $U$ (45) and $\bar{s}$ (0.76%) are lower and upper limits, respectively, for mutations with a deleterious effect in the natural environment. The actual number of deleterious mutations induced could be substantially higher, since a study of 0.15% of the genome of *C. elegans* and its closest known relative, *C. briggsae*, estimated that approximately 18% of the non-coding DNA is selectively constrained (Shabalina and Kondrashov, 1999). The true values are likely to be somewhere between these values and the inferred upper limit for $U$ (229) and the lower limit for $\bar{s}$ (0.15%).

### 4.4.4 Estimates of mutational parameters I: The Bateman-Mukai approach

Normally, when mutation accumulation experiments are carried out, the number of mutations induced at the DNA level is unknown. Instead, mutational parameters are estimated from the phenotypic divergence of mutation-accumulation and control lines. The haploid genomic mutation rate, $U$, and the average homozygous effect of a mutation, $s$, have traditionally been estimated using the Bateman-Mukai (BM) approach (Bateman, 1959; Mukai, 1963; Mukai et al., 1972; see also Lynch and Walsh, 1998, Chapter 12), which assumes that all mutations have equal effects. For details of the equations used and their derivation, see Chapter 2.

Estimates of $U$ and $s$ are obtained as follows:

\[
\hat{U} \geq \frac{(\Delta M)^2}{\Delta V_G}; \tag{4.1}
\]

\[
\hat{s} \leq \frac{\Delta V_G}{\Delta M}, \tag{4.2}
\]

where $\Delta M$ is the mean change in fitness of the EMS lines, as a proportion of the mean of the controls, and $\Delta V_G$ is the change in genetic variance. These equations were used to obtain a lower estimate of $U$ and an upper estimate of $s$ for each of the traits analysed. The only exception was late productivity, for which mutations had the effect of both increasing and decreasing the value of the trait (Figure 4.3), making it
unsuitable for such analysis. The results are presented in Table 4.13. Standard errors were obtained from bootstrap analysis.

Table 4.13: Bateman-Mukai estimates for $U$ and $s$, with standard errors in brackets

<table>
<thead>
<tr>
<th>Trait</th>
<th>$\hat{U}$ (minimum)</th>
<th>$\hat{s}$, % (maximum)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total productivity</td>
<td>1.44 (0.40)</td>
<td>24 (4.6)</td>
</tr>
<tr>
<td>Early productivity</td>
<td>3.35 (1.02)</td>
<td>15 (2.9)</td>
</tr>
<tr>
<td>Longevity</td>
<td>0.50 (0.74)</td>
<td>19 (16)</td>
</tr>
</tbody>
</table>

Using these equations, I obtain a minimum estimate of $U$ of between 0.5 and 3.35 mutations per haploid genome with a maximum average effect, $\hat{s}$, of 15 to 24% for the traits assayed. These results contrast sharply with the inference that each line contains an average of $>45$ deleterious point mutations. Therefore, it appears that this method is greatly underestimating the number of mutations that have occurred; very large numbers of mutations, which are known to have deleterious effects in natural conditions, have been induced, but only a very small number of these mutations, which have large effects, have been detected. These results indicate that the assumption of equal mutational effects has not been met; many more deleterious mutations than this have been induced, but their effects are evidently smaller than those of the mutations detected.

4.4.5 Estimates of mutational parameters II: The maximum likelihood approach

A second approach used to infer mutational parameters was developed by Keightley (1994), and involves using maximum likelihood (ML) analysis, which uses information concerning the distribution of line means, rather than just the change in mean and variance, and does not make the assumption that mutations have equal effects. Instead, a distribution such as the gamma distribution may be chosen, where the shape parameter $\beta$ can either be fixed at a certain value, or estimated. For further details, see Chapter 2.

Firstly, $\hat{U}$ and $\hat{s}$ were estimated under a distribution assuming equal effects (equivalent to the BM approach; $\beta \rightarrow \infty$), and by assuming a gamma distribution, using
the program mlgenomeu (Keightley, 1998). The results are compared to those of the BM approach in Table 4.14 below.

Table 4.14: Estimates of mutation rates and effects, $\hat{U}$ variable

<table>
<thead>
<tr>
<th>Trait</th>
<th>Method</th>
<th>Model</th>
<th>$\hat{U}$ (95% CI)</th>
<th>$\hat{s}$ % (95% CI)</th>
<th>$\hat{\beta}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total productivity</td>
<td>BM</td>
<td>Equal effects</td>
<td>1.44 (0.64, 2.24)</td>
<td>24 (14.8, 33.2)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>ML</td>
<td>Equal effects</td>
<td>1.61 (1.24, 2.00)</td>
<td>22 (20.4, 23.5)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gamma distribution</td>
<td>&lt;2.5</td>
<td>&gt;15 (20.4, 30.8)</td>
<td>&gt;1.6</td>
</tr>
<tr>
<td>Early productivity</td>
<td>BM</td>
<td>Equal effects</td>
<td>3.35 (1.31, 5.39)</td>
<td>15 (9.2, 20.8)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>ML</td>
<td>Equal effects</td>
<td>2.07 (1.7, 4.5)</td>
<td>29 (22.0, 30.8)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gamma distribution</td>
<td>&lt;5.3</td>
<td>&gt;9.4 (22.0, 30.8)</td>
<td>&gt;1.6</td>
</tr>
<tr>
<td>Longevity</td>
<td>BM</td>
<td>Equal effects</td>
<td>0.50 (-0.98, 1.98)</td>
<td>19 (-13, 51)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>ML</td>
<td>Equal effects</td>
<td>0.84 (0.4, 1.7)</td>
<td>13 (8.1, 19.1)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gamma distribution</td>
<td>$&lt;\infty$</td>
<td>&gt;0 (8.1, 19.1)</td>
<td>&gt;0</td>
</tr>
</tbody>
</table>

Approximate 95% confidence intervals (CIs) for the maximum likelihood estimates correspond to a drop of two in $\Sigma \ln L$ from the maximum of the profile likelihoods. The ML model produces similar results to the BM model under the assumption of equal mutational effects. Under ML, a gamma distribution in which there is a large class of mutations with small effects and a long tail of mutations with larger effects does not provide a better fit to the data than the equal effects model. This suggests that the distribution may be discontinuous, for example there may be one class of mutations with severe effects on fitness and another with far smaller effects, which would mean that a gamma distribution, being continuous, would not provide the best fit to the data.

Models were also fitted in which $\hat{U}$ was fixed at 45, the minimum number of deleterious point mutations that were inferred to have become fixed in the EMS lines. Three models were fitted with different distributions of mutational effects: firstly, one class with equal effects, secondly, a gamma distribution, and thirdly, two classes of mutations, in order to allow for a bimodal distribution of effects. Within a class
mutations had equal effects, though they could vary between classes. The results for total productivity are presented in Table 4.15 below:

Table 4.15: ML analysis of productivity data - estimates of mutation rates and effects, with $\hat{U}$ fixed at 45

<table>
<thead>
<tr>
<th>Model</th>
<th>$\hat{U}_1$ (SE)</th>
<th>$\hat{d}_1$ % (SE)</th>
<th>$\hat{U}_2$ (SE)</th>
<th>$\hat{d}_2$ % (SE)</th>
<th>$\hat{\beta}$ (SE)</th>
<th>LnL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gamma distribution</td>
<td>45*</td>
<td>0.79 (0.05)</td>
<td>-</td>
<td>-</td>
<td>0.029 (0.005)</td>
<td>-3.8</td>
</tr>
<tr>
<td>One class of equal effects</td>
<td>45*</td>
<td>0.99 (0.04)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-160</td>
</tr>
<tr>
<td>Two classes of equal effects</td>
<td>1.6 (0.17)</td>
<td>22 (0.9)</td>
<td>43.4*</td>
<td>&lt;0.074</td>
<td>-</td>
<td>0</td>
</tr>
</tbody>
</table>

*Constrained by the assumption that $U$ is fixed at 45

LnL is the difference in natural likelihood from the model with two classes of equal effects. The higher its value, the better the model fits the data. This distribution has the same likelihood as that with one class, when $\hat{U}$ is allowed to vary (Table 4.14) i.e., allowing a second class of mutations with very small effects does not improve the fit to the data. When this model is used, all of the 45 mutations, with the exception of the 1.6 mutations with effects averaging 22% detected under a univariate distribution, fall into a class with effectively zero effect on productivity. The maximum effect that this second class of mutations could have is just 0.074%.

These results therefore indicate that the distribution of mutational effect is multimodal, with a few, large mutations accounting for the observed change in phenotypes, and a large class of mutations with tiny fitness effects, which are undetectable under the conditions used in these experiments. Though small, these mutations are expected to be deleterious in natural conditions, and thus could have important consequences for evolutionary biology.

### 4.5 CONCLUSION AND DISCUSSION

#### 4.5.1 AGE-SPECIFIC MORTALITY RATES

The results indicate that the rates of mortality differ between the control and EMS lines (Figure 4.5). The EMS lines show higher early mortality rates, but reduced late
mortality. This appears surprising at first: why should mutagenesis reduce mortality rates late in life? There are several possible interpretations of this result.

It is possible that there is simply a larger target size for mutations affecting early, rather than late, fitness. However, although this may explain the levelling-off of mortality rates among the EMS lines, it does not explain why late-life mortality is lower in the EMS lines than the controls. Genetic or environmental heterogeneity provide a second possibility (Vaupel et al., 1979), as frailer individuals may die earlier, producing an increase in early mortality rates, and leaving the healthiest individuals, which being fitter may show reduced mortality. This would explain both the shape of the curves and the difference between the control and EMS lines. Such heterogeneity has been shown to produce similar results in a previous study involving C. elegans (Brooks et al., 1994).

The reduction in mortality rates observed here occurred after reproduction had ceased (~day 14). As the EMS worms had lower productivity than the controls, this could explain the observed trends in mortality rates, if reproductive behaviour has associated mortality costs.

Another potential explanation could be provided by theories concerning the evolution of ageing. Standard evolutionary theory states that selection will be greatest on mutations affecting early fitness traits rather than late, as the number of individuals carrying a mutation will be greatest early in life, with increasing numbers dying or becoming infertile for other reasons with increasing time (Medawar, 1946, 1952; Williams, 1957; Hamilton, 1966). This will lead to a life history in which fertility and other components of fitness decline with age, through one of two possible processes. Firstly, mutations may accumulate that have harmful effects late in life due to reduced selection pressure to remove them - the mutation accumulation hypothesis (Medawar, 1952). Secondly, ageing may be the result of mutations with beneficial effects early in life but harmful effects later – the optimality, or antagonistic pleiotropy theory (Williams, 1957). These two theories are not, of course, mutually exclusive.

According to the mutation-accumulation hypothesis, in natural populations fitness later in life would be reduced by the fixation of late-acting deleterious mutations.
This could explain why the EMS-induced mutations had a greater impact on mortality early in life, when initial components of fitness would have been higher. It is even possible that the mutagenesis caused reversion of some late-acting deleterious mutations, which had become fixed in the population, thus reducing late-life mortality. If this is correct, these results could provide some tentative support for the mutation-accumulation hypothesis of ageing. Alternatively, the results could be caused by a correlation between mortality rates late in life and another trait. For instance, mutations that reduce mortality late in life could also have the effect of increasing mortality rates early in life. This could explain why such mutations, which appear to be beneficial as they reduce mortality, had not become fixed in the control population. Distinguishing between these theories would require more information concerning the correlations of mutational effects at different ages, from a variety of organisms (Partridge and Mangel, 1999).

A slight, post-reproductive decline in mortality rates was observed in the control lines, and in other studies of natural (non-mutagenised) populations of worms (Brooks et al., 1994), fruit flies (Curtsinger et al., 1992), medflies (Carey et al., 1992), beetles (Tartar et al., 1993) and humans (Vaupel et al., 1998). This may be explained by a high input of mutations with correlated effects on more than one trait. If organisms do not display any form of parental care, deleterious mutations that affected only post-reproductive fitness would be expected to accumulate, as there would be no selective pressure to remove them. However, if such mutations also affect components of fitness at other ages, such correlated deleterious effects could prevent these mutations from accumulating. This could explain why adult mortality rates do not always increase as rapidly as expected. The finding of strong positive genetic correlations between the traits assayed provides some support for this hypothesis, although it remains possible that these correlations result from associations in the same individuals of mutations affecting different traits.

This provides one theory to explain why late-life fitness does not decline as rapidly as expected in natural populations - because mutations that act in the post-reproductive period are not neutral if they have correlated effects early in life, and thus are unlikely to spread to fixation (Charlesworth and Partridge, 1997). However, the levelling-off of mortality rates in natural populations could simply be due to a high input of
mutations that cause this levelling-off, such as those observed in this experiment. In other words, rather than having an adaptive function, the effect may be the result of a form of mutation-selection balance. Further information, to identify whether this effect is a common feature of induced or spontaneous mutations, may shed light on whether this is a plausible explanation.

4.5.2 FREQUENCY OF CRYPTOIC DELETERIOUS MUTATIONS

The results presented here clearly indicate that the laboratory assays only detected a very small proportion of the mutations known to have been induced at the DNA level. It was inferred that a minimum of 45 deleterious mutations were induced per haploid genome, and the true figure could be substantially higher, if mutations in non-coding regions of the genome can have deleterious effects, as is likely to be the case (Shabalina and Kondrashov, 1999). However, the laboratory assays performed on the mutagenised and control lines revealed the effects of just a small minority (<8%) of the mutations known to have been induced – values of $U$ ranged from $-0.5$ to $-3.4$, depending on the fitness assay used. For the productivity data, the best fitting model obtained under maximum likelihood, assuming that $U = 45$, is a multimodal distribution of mutational effects, comprising one class consisting of few mutations ($U_1 = 1.4$) with large effects (averaging a 22% reduction in fitness per line), and a second class comprising the majority of the mutations (the remaining 43.6 mutations), each with zero detectable effects. The maximum effect that one of these very mildly deleterious mutations can take is just 0.07%. Thus, it is possible to conclude that fitness assays dramatically underestimated the number of mutations induced, mainly due to the failure to detect over 90% of the induced mutations, which fell into a class with very small effects on the traits measured. These results imply that previous experiments that aimed to obtain estimates of $U$ and $s$ by carrying out laboratory assays to measure the change in fitness of mutation-accumulation lines may also have underestimated values of $U$, by a similar factor, if the same classes of mutation occurred.

The distribution of EMS-induced mutations for productivity is remarkably similar to that of spontaneous mutations, described by Keightley and Caballero (1997). In both cases, an equal effects model fits that data better than a gamma distribution of
mutational effects, and the average effect of the detectable mutations, $\hat{s}$, was 21% (±4%) for spontaneous mutations, compared to 22% (±2%) here. For total productivity, a trait assayed in both experiments, the number of mutations induced by EMS mutagenesis that were detected by the fitness assays was 1.6, a value more than 600 times higher than the spontaneous rate ($\hat{U} = 0.0026$; Keightley and Caballero, 1997). If both experiments underestimated $U$ to a similar degree, this implies that the spontaneous mutation rate is closer to 0.07 (i.e., $0.0026 \times (45/1.4)$).

In order for deleterious mutations to explain the maintenance of sexual reproduction, $2U$ must be greater than one. My adjusted value for $2U$ of ~0.14 is still considerably lower than this, which may imply that the mutation rate is too low by itself to maintain sex in such species. However, since C. elegans reproduces primarily by self-fertilisation, it does not pay the full two-fold cost of sex; it pays the cost of male allocation, but has a reduced cost of meiosis (Lively and Lloyd, 1990). A maximum corrected estimate of $U$ would be 0.82 mutations per diploid genome, a figure much closer to the requirement of the deterministic mutation hypothesis, though this assumes that all 220 EMS-induced nucleotide changes across the genome are deleterious, i.e., $2 \times 0.0026 \times (220/1.4)$.

Some estimates of $2U$, obtained from studies involving Drosophila, already approach one (Mukai, 1964; Mukai et al., 1972; Ohnishi, 1977). Consequently, it seems implausible that $U$ has been underestimated to the same extent as here. However, it has been argued that these studies may have overestimated the true mutation rate, for reasons to do with the experimental controls used (Keightley, 1996; García-Dorado, 1997; Fry et al., 1999). If these arguments are valid, it is possible that the mutation rate has been simultaneously overestimated and underestimated for the reasons discussed here, if the results of this experiment are generally applicable.

The fact that mutation rates appear to have been underestimated in previous studies would, at first sight, appear to have unfavourable consequences for the conservation of small populations. The more deleterious mutations that are accumulating, the more rapidly a population's fitness is expected to become eroded through the fixation of such mutations. However, the magnitude of the effect of such mutations, $s$, is crucial
to such predictions. My results indicate that the class of induced mutations that were not detected during the fitness assays have extremely small fitness effects. In the laboratory assays performed, they had no measurable effects on fitness, although I inferred that they would have deleterious effects in natural conditions. Genotype by environment interactions may increase the fitness effects of such mutations in harsher conditions, as described in the previous chapter, or they may affect traits unrelated to those assayed in these experiments. For example, mutations that inhibited the formation of dauer larvae would not be detected in my assays, but they could have very severe effects in natural conditions. In such circumstances, lack of sufficient food may be a regular occurrence, and so a reduced ability to survive in such conditions could have severe fitness consequences.

The actual values of $s$ that apply to this class of mutations in the natural environment are therefore difficult to determine. Because *C. elegans* is such a small, soil-dwelling nematode, relatively little is known about it in its natural habitat (Hodgkin and Doniach, 1997). It is known to be geographically widespread, with isolates having been obtained from Europe, North America and Australia (Fitch and Thomas, 1997). Because of its small size and rapid rate of reproduction, very large numbers of individuals can exist in relatively small areas. Given the limited range of individual worms, and the large numbers of natural obstacles that could further restrict movement, this large population is likely to be highly subdivided, and thus the effective population size could be enormous. As the magnitude of the selection coefficient of a mutation is inversely proportional to the effective population size, the high proportion of amino acid mutations that are removed by natural selection (90%; Stenico *et al.*, 1994) could have very small deleterious effects. This could explain why the majority of such mutations were undetectable in the laboratory.

As stated in the introduction, the magnitude of mutational effect with the most severe consequences for fitness loss due mutation accumulation is $s = 1/2N_e$. Therefore, the large class of mutations with very small fitness effects may not have significant consequences for the survival of small populations ($N_e < 1000$), because the fitness effects are so small. The class of mutations with large fitness effects (~22%) are also unlikely to have severe consequences for such populations, as these mutations are
large enough to be removed by selection in all but the very smallest of populations. The fact that the distribution of mutational effects appears to be multimodal is encouraging, implying that mutations with the most dangerous effects for small populations may be rare. The size of population expected to suffer the most from this class of small mutations may be reasonably large – large enough that they may not previously have been thought of as at risk. In the United States, a population of animals is only classified as 'endangered' when the population size drops to 1000 individuals, or 100 individuals in the case of plants (Wilcove et al., 1993). In many cases, the population size can be even lower before they are recognised as being endangered. The effective population size is often as little as one tenth to one third of the number of breeding adults, which implies that $N_e$ can be extremely low before a population becomes protected (Lynch, 1996). Populations much larger than this may in fact be more at risk from the accumulation of deleterious mutations, though as the risk of extinction increases with decreasing population size, this may be of less concern than if small populations were most susceptible to such mutations. In particular, the threat of 'mutational meltdown', the synergistic interaction between mutation accumulation and random genetic drift may be reduced. This term describes the process whereby in small populations, deleterious mutations can accumulate that reduce fitness and thus population size, leading to an increase in the importance of random genetic drift when determining the fate of new mutations. This increases the probability of fixation, resulting in an accelerating decline in fitness towards extinction (Lynch and Gabriel, 1990).

Of course, small populations are still very susceptible to extinction due to a number of environmental and genetic factors. However, if the deleterious mutation rate has been underestimated to a large extent, as appears likely, this may not be a critical factor affecting the survival of such populations, if these mutations do indeed have extremely small fitness effects.

To summarise: it is clear that mutation accumulation experiments may substantially underestimate mutation rates due to the presence of a large class of cryptic deleterious mutations with extremely small fitness effects. These are likely to remain undetected unless the number of mutational events induced at the DNA level can be determined, which was possible in this experiment but is a far more difficult task when
spontaneous mutations are being studied. This should be taken into account when predictions are being made about the evolutionary consequences of deleterious mutations.
Chapter 5: Inbred-Backcross Analysis of Two EMS-Derived Mutant Lines
5.1 Biometrical Approaches to the Estimation of Gene Number

Analyses of the fitness differences between the control and mutagenised lines using the Bateman-Mukai (BM) and Maximum Likelihood (ML) techniques have provided estimates of the number of mutations that became fixed in these lines (Chapter 4). It has been deduced that there are, on average, 1.6 mutations per line with large fitness effects (∼22%) on productivity, and a majority of mutations (>90% of those induced) that are undetectable in the conditions used for the life history assays. Therefore, it appears that the observed change in fitness is essentially due to the influence of a few mutations with large effects. Further evidence that would support this hypothesis could be obtained by crossing some of the EMS lines onto a wild-type background. Using one of the several methods outlined below, which involve comparing the differences in phenotypes of sublines produced from a cross between two lines (one of which may be the wild-type), and the parental population, it is possible to obtain an estimate of the number of segregating loci responsible.

Several approaches have been developed in order to estimate the number of loci affecting a trait, \( n \), or, more usually, the effective number of factors, \( n_e \), the number of freely segregating loci that produce the observed differences between two lines. Loci that are very tightly linked are likely to be treated as one effective factor, and loci that could potentially lead to variation in the trait but do not differ between the lines studied obviously will not be detected. There are a number of reasons for this interest: firstly, as a way to obtain information concerning whether the majority of evolutionary change is due to a small number of mutations with large effects or the gradual substitution of many mutations with small effect (Lynch and Walsh, 1998; Chapter 9). Secondly, many quantitative-genetics theories depend on the infinitesimal model, which makes the assumption that traits are determined by a very large number of loci, each with very small effects. When more information is available about the numbers and properties of genes affecting a trait, it may be possible to develop more realistic quantitative genetics models (Falconer and Mackay, 1996; Chapter 21). Finally, if alleles with large effects on a trait of interest can be detected, this can aid their further characterisation, and may allow improvement of the efficiency of
breeding strategies. Identification of alleles of large effect that cause a predisposition towards a certain disease may be of benefit for diagnosis and prevention strategies.

The first approach that can be used to estimate \( n_e \) is the molecular marker approach, in which quantitative-trait loci (QTLs) can be assayed by using linked marker loci (Falconer and Mackay, 1996; Chapter 21)). This approach has become increasingly popular in model organisms and those of economic importance. The second approach, the biometrical method, is based on inferring the number of loci affecting a trait from the statistical properties of the phenotypic distribution of sub-lines derived from a cross between two lines of interest (Lynch and Walsh, 1998; Chapter 9).

5.1.1 **The Castle-Wright Approach**

One of the more widely used methods for estimating \( n_e \) was developed by Castle (1921) with his student Sewall Wright (1968), and further modified by Lande (1981). It involves comparing the phenotypic means and variances of a character in parental, \( F_1 \), \( F_2 \) and backcross lines (Figure 5.1a). However, this approach is not suitable for analysis of the \( C. elegans \) EMS lines for two reasons. Firstly, the results of fitness assay in \( C. elegans \) tend to vary between experiments due to uncontrollable environmental factors (Kenyon, 1997), so it is important where possible that lines that are to be compared are assayed contemporaneously. This would be technically difficult in an experiment such as this, where several generations have to be assayed. More importantly, the Castle-Wright technique makes the assumption that one line contains only alleles that increase the trait of interest, and the other contains alleles that decrease it. Thus, it is usually used in situations where lines have been selected in opposite directions via artificial selection (Lynch and Walsh, 1998; Chapter 9). If this is not the case, a situation known as transgressive segregation can occur, in which the \( F_2 \) generation exhibit phenotypes outside the range of variation displayed by the parental lines. We would expect the majority of new EMS-induced mutations to have detrimental effects on fitness. One exception is line E28, which has longer average lifespan than the wild-type, and thus must have at least one allele that increases lifespan. However, it is likely that the EMS mutagenesis also induced mutations at other loci, which may have the opposite effect of actually reducing lifespan, to some extent. The vast majority of mutations affecting longevity are expected to have
deleterious effects (Lithgow, 1996). Thus, the approach is unlikely to be suitable in this case, as a combination of mutations that increase and decrease lifespan may have been induced.

Figure 5.1: The crossing schemes required for three biometrical approaches to gene number estimation

(a) Castle-Wright technique

\[ P_1 \times P_2 \]

\[ \downarrow \]

\[ F_1 \times F_1 \]

\[ \downarrow \]

\[ F_2 \]

\[ \downarrow \]

\[ F_1 \times P_1 \]

\[ \downarrow \]

\[ B_1 \]

\[ \downarrow \]

\[ F_1 \times P_2 \]

\[ \downarrow \]

\[ B_2 \]

Assay \( F_1, F_2, B_1 \) and \( B_2 \) lines.

(b) Genotype assay

\[ P_1 \times P_2 \]

\[ \downarrow \]

\[ F_1 \]

\[ \downarrow \]

\[ F_1 \]

\[ \downarrow \]

\[ F_1 \]

\[ \ldots \]

\[ k \text{ generations of self-fertilisation} \]

\[ F_k \]

\[ \downarrow \]

\[ F_k \]

\[ \downarrow \]

\[ F_k \]

\[ \downarrow \]

\[ F_k \]

\[ \downarrow \]

\[ F_{k+1} \]

\[ \downarrow \]

\[ F_{k+2} \]

\[ \downarrow \]

\[ F_{k+3} \]

\[ \downarrow \]

\[ F_{k+4} \]

\[ \downarrow \]

\[ F_{k+5} \]

Assay \( F_{k+2} \) lines

(c) Inbred-backcross technique

\[ P_1 \times P_2 \]

\[ \downarrow \]

\[ F_1 \times P_1 \]

\[ \downarrow \]

\[ B_1 \times P_1 \]

\[ \downarrow \]

\[ B_k \]

\[ \ldots \]

\[ k \text{ generations of backcrossing} \]

\[ 5 \text{ generations of self-fertilisation} \]

\[ B_{k+5} \]

\[ B_{k+5} \]

\[ B_{k+5} \]

Assay \( P_1 \) and \( B_{k+5} \) lines
Two approaches have since been developed that avoid the problem of transgressive segregation, and have the added advantage that, unlike the Castle-Wright approach, they do not make the assumption that all mutations have equal effects (Lynch and Walsh, 1998). Both methods – the genotype assay (Jinks and Towey, 1976; Towey and Jinks, 1977) and the inbred-backcross technique (Wehrhahn and Allard, 1965) require the use of species that can be both cross- and self-fertilised. Thus, they are suitable for *C. elegans* experiments, but have mainly been used in studies of plants rather than animals. As they require several generations of crossing or self-fertilisation, they can be quite time-consuming when used in plant studies, but the short life-cycle of *C. elegans* avoids this problem.

5.1.2 GENOTYPE ASSAY

The genotype assay (Jinks and Towey, 1976; Towey and Jinks, 1977), shown in Figure 5.1b, involves crossing two pure-bred lines to produce a heterozygous $F_1$ population, which is then self-fertilised until generation $F_k$, where $k$ is usually two to five. The amount of heterozygosity in the $F_k$ lines is determined by measuring the phenotypes of a number of grand-progeny families (usually two) of these individuals. If the families show significant amounts of variation, it is assumed that one or more alleles were segregating in the $F_k$ generation. A minimum estimate for $n_e$ can be obtained from $k$ and $P$, the observed fraction of families derived from a single grandparent that show significant amounts of variation.

5.1.3 INBRED-BACKCROSS TECHNIQUE

The inbred-backcross technique (Figure 5.1c), developed by Wehrhahn and Allard (1965), is similar to the genotype assay, but involves crossing two inbred parental lines ($P_1$ and $P_2$) to produce an $F_1$, and then backcrossing the $F_1$ individuals onto the recurrent parent ($P_1$) for $k$ generations. The descendants are inbred for several generations to fix any segregating factors, under conditions of minimal selection. By comparing the phenotypic means of these lines with $P_1$, it is possible to determine the fraction of lines that differ significantly from the $P_1$, and from this information to obtain an estimate of $n_e$. 

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Mulitze and Baker (1985a, 1985b) have used simulated data to compare these two techniques for estimating $n_e$. Both procedures require binary classification of the sublines (lines are classified as being parental or non-parental in the inbred-backcross technique; the two families derived from the same $F_k$ grandparent are classified as being alike or different in the genotype assay). It was found that both the type I error ($\alpha$) level used in the classification of lines, and sample size, had a greater impact on biases in estimates of $n_e$ from the genotype assay than the inbred-backcross technique. Thus, they concluded that the inbred-backcross technique is usually preferred. The inbred-backcross technique has the added advantage that lines with single mutations can be obtained, which facilitates further characterisation of mutations in genes of interest, and can also be used to generate lines with pairs of mutations, to test for epistatic interactions between the factors (Lynch and Walsh, 1998; Chapter 9). For these reasons, the inbred-backcross technique was chosen to study a sample of the EMS lines.

The rationale for the breeding scheme outlined above is that as $k$ increases or $n_e$ is small, most of the inbred-backcross lines are expected either to be genetically identical to the parental ($P_1$) line, or to differ from it at a single locus (Wehrhahn and Allard, 1965). The probability that a specific gene from the $P_2$ is incorporated into a specific line after $k$ generations of inbreeding is $p_k = (\frac{1}{2})^k$. The probability that a line contains a certain number ($r$) of $P_2$ alleles can be obtained from the binomial distribution, as follows: the probability, $p$, of obtaining $r$ alleles in a line derived from an inbred population with $n$ effective factors is

$$p(r) = \binom{n}{r} p_k^r (1-p_k)^{n-r}$$

Therefore, the probability that a specific line contains one $P_2$ allele, $p(r = 1)$, is:

$$p(r = 1) = np_k(1-p_k)^{n-1}$$

The probability that a line contains one or more $P_2$ alleles, $p(r \geq 1)$, is:

$$p(r \geq 1) = 1 - p(r = 0) = 1 - (1 - p_k)^n$$

An estimate of the effective number of loci affecting a trait, $\hat{n}_e$, can be obtained from the observed proportion of non-parental lines (those that differ significantly from the recurrent parent ($P_1$), i.e., $r \geq 1$), by rearranging Equation 5.4:
\[ p(r \geq 1) = 1 - (1 - p_k)^n \]
\[ (1 - p_k)^n = 1 - p(r \geq 1) \]
\[ n[\ln(1 - p_k)] = \ln[1 - p(r \geq 1)] \]
\[ \hat{n}_e = \frac{\ln(1 - p(r \geq 1))}{\ln(1 - p_k)} \] (5.4)

Table 5.1 below shows the proportion of non-parental lines that are expected to have a single P_2 allele, for different values of \( n \) and \( k \).

Table 5.1: The proportion of non-parental lines that are expected to contain one parental allele (last column)

<table>
<thead>
<tr>
<th>n</th>
<th>k</th>
<th>( p_k )</th>
<th>( p(r = 1) )</th>
<th>( p(r \geq 1) )</th>
<th>( p(r = 1)/ p(r \geq 1) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>( \frac{1}{2} )</td>
<td>0.50</td>
<td>0.50</td>
<td>1.00</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>( \frac{1}{2} )</td>
<td>0.50</td>
<td>0.75</td>
<td>0.67</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>( \frac{1}{2} )</td>
<td>0.25</td>
<td>0.94</td>
<td>0.27</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>( \frac{1}{2} )</td>
<td>9.8 x 10^{-3}</td>
<td>1.00</td>
<td>9.8 x 10^{-3}</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>( \frac{1}{4} )</td>
<td>0.25</td>
<td>0.25</td>
<td>1.00</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>( \frac{1}{4} )</td>
<td>0.38</td>
<td>0.44</td>
<td>0.86</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>( \frac{1}{4} )</td>
<td>0.42</td>
<td>0.68</td>
<td>0.62</td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>( \frac{1}{4} )</td>
<td>0.19</td>
<td>0.94</td>
<td>0.20</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>( \frac{1}{8} )</td>
<td>0.13</td>
<td>0.13</td>
<td>1.00</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>( \frac{1}{8} )</td>
<td>0.22</td>
<td>0.23</td>
<td>0.94</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>( \frac{1}{8} )</td>
<td>0.33</td>
<td>0.41</td>
<td>0.81</td>
</tr>
<tr>
<td>10</td>
<td>2</td>
<td>( \frac{1}{8} )</td>
<td>0.38</td>
<td>0.74</td>
<td>0.51</td>
</tr>
</tbody>
</table>

As can be seen, with fewer backcrosses (lower values of \( k \)), it is easier to detect difference between small numbers of loci, as the values in the last column change more rapidly. The more loci there are controlling a trait, the more advantageous it is to carry out higher numbers of backcrosses. Using ML and BM methods, it was inferred that there are only 1.6 detectable mutations, on average, that affect productivity. Because so few genes appear to be involved, it appears that the optimum strategy would be to carry out no backcrosses (i.e., \( k = 0 \)), rather than two backcrosses which is a common procedure in inbred-backcross experiments (Mulitze and Baker, 1985). Having a clear idea of whether or not few loci really are involved is more important than having a greater degree of accuracy if large numbers of loci are, in fact, involved. This approach has the added advantage of requiring considerably less time and effort.
5.2 STUDY LINE (A): E28, AN AGE MUTANT

Because of the large number of replicates needed to determine whether the inbred-backcross lines are significantly different from the P₁ line, it was decided to study a small sample of the lines. Of the mutant EMS lines obtained, perhaps the most interesting is line E28, the Age mutant (Ageing abnormal). These worms live ~20% longer than the wild-type strain, and show a reduction in lifetime productivity. Further assays, with larger sample sizes, were performed by Miss Claire Greer (Genetics Honours student, Edinburgh University; unpublished results), which showed that the apparent increase in lifespan was replicable. Furthermore, line E28 was phenotypically indistinguishable from wild-type C. elegans, with the exception of a post-anal deformity exhibited by a fraction of the worms (<20%). This phenotype appeared similar in morphology to that caused by a bacterial infection, as identified by Dr. Jonathan Hodgkin (personal communication). By cleaning the worms with alkaline hypochlorite solution to remove any possible bacterial infection (see Methods section), and then comparing the morphology and lifespan of these worms to the uncleaned population, she inferred that the anal deformity and lifespan extension were caused by genetic, rather than bacterial, factors. Survival curves, showing the results of the longevity assays of the cleaned and uncleaned samples of lines E28, and N2 worms, are shown in . The anal deformity did not appear to influence lifespan, but all the worms from this line were genetically identical, so it remains possible that the anal deformity and increase in lifespan are caused by the same mutation. As genetic factors were found to be responsible for the increase in lifespan, inbred-backcross analysis of this line would identify whether one or more mutations are involved in the lifespan extension.
Figure 5.2: Survival curves of N2 and E28 lines

5.2.1 AGE MUTATIONS IDENTIFIED IN C. ELEGANS

*C. elegans* is one of very few species in which single locus mutants causing significant increases in lifespan have been identified. Others include *Drosophila* (the methuselah gene; Lin *et al.*, 1998) and mice (p66<sup>she</sup>; Migliaccio *et al.*, 1999). *C. elegans* is particularly suited to analysis of lifespan due to its short mean lifespan and lack of inbreeding depression, facilitating the study of mutants on an inbred background, without the reduction in productivity and lifespan that is typical of other organisms. There are a number of mutations known to increase lifespan, though only two of them (*age-1* and *age-2*) have been identified solely by their effect on lifespan (Klass, 1983, Johnson, 1986, Friedman and Johnson, 1988, Yang and Wilson, 1999). The others were first identified by their effect on another trait. The longevity mutants, on the whole, fall into two distinct groups that affect different pathways: group 1 consists of *age-1* (ageing abnormal), *daf-2*, *daf-16* and *daf-18* (dauer larva formation abnormal), and affects the dauer pathway. The dauer stage is an alternative L3 stage that can be induced by conditions of increased crowding and limited food, conditions that severely restrict successful reproduction. Dauer larvae can survive for several months without food, and are resistant to desiccation. When conditions improve, they can revert to normal adult worms, with typical features and post-adult ageing
processes (Riddle and Albert, 1997). The second set of mutants, \textit{clk-1, clk-2, clk-3} (clock abnormal) and \textit{gro-1} (growth rate abnormal), affect the biological clock mechanism in \textit{C. elegans}. They show disruption in the timing of many aspects of growth and behaviour, including the length of the cell cycle, feeding, swimming, defection and egg laying (Kenyon (1997). Other mutations that increase longevity include \textit{spe-26} (spermatogenesis abnormal), which also prevents normal sperm production in both hermaphrodites and males (Kenyon, 1997), and \textit{age-2} (Yang and Wilson, 1999), for which the mechanism that produces an extension in lifespan has not yet been elucidated.

5.2.2 THEORIES CONCERNING THE EVOLUTION OF AGEING

Senescence, the deterioration of fertility and other components of fitness with age, occurs almost universally. This decline with age appears to be the result of a number of biological parameters, rather than being caused by a single factor (Comfort, 1979; Bernstein and Bernstein, 1991). Theories concerning the evolution of ageing were discussed in Chapter 1. Briefly, it is assumed that ageing can evolve due to weaker selection acting on mutations affecting early, rather than late, components of fitness (Medawar, 1946, 1952; Williams, 1957; Hamilton, 1966). This will lead to the evolution of a life history in which fertility and survival chances decrease with increasing age, as the intrinsic ageing processes evolve in response to the extrinsic impact of mortality and sterility.

Theoretical work has identified two possible paths by which this age-specific selection pressure can lead to the evolution of ageing. Firstly, ageing could result from the accumulation of mutations that increase fitness components early in life at the expense of late-life components of fitness. This is known as the optimality, or antagonistic pleiotropy model (Williams, 1957). Secondly, ageing could be the result of an accumulation of deleterious late-acting mutations, as natural selection is less efficient at purging such mutations. This process is known as the mutation accumulation theory (Medawar, 1952).

One of the predictions of the optimality theory of senescence is that there will be a negative correlation between early and late fitness components, such as early
productivity and late life mortality. Inbred-backcross analysis of the EMS line showing increased lifespan will enable the separation of the effects of the mutation(s) affecting lifespan and those affecting other traits. Thus, it should be possible to determine whether the reduction in productivity is a pleiotropic effect of a gene producing an increase in lifespan, or the result of other, independent mutations that reduce fertility. Of course, this is just one Age mutation, and we would require information from other such mutations before being able to make inferences about the generality of such a finding.

5.3 STUDY LINE (B): E1, AN UNC MUTANT
As a second line, E1 was chosen for study. This line is particularly suitable for analysis via the inbred-backcross technique, because it shows a large reduction in fertility (hermaphrodites produce approximately one third as many offspring as the wild-type), and it has a clear, phenotypic mutation which can easily be detected in individual worms. The worms are uncoordinated (Unc), and show a visible twitching along the body wall. The large reduction in fertility indicates that, even with small sample sizes, it should be relatively easy to detect significant differences between parental and non-parental lines. It will also be possible to determine the extent to which this phenotype, which is probably caused by a single mutation, is also responsible for the large reduction in productivity. This is in addition to obtaining an estimate of the number of effective factors \( n_e \) affecting the fitness traits being measured in the two lines, although these two lines were not chosen at random from those produced by EMS mutagenesis, so this does not provide an independent method for judging the precision of the BM and ML analysis described in the previous chapter.

5.4 SUMMARY OF THE AIMS OF THE EXPERIMENT
There are several aims of this experiment. Firstly, to obtain estimates of the number of loci affecting fitness traits in a specific (non-random) sample of the EMS lines. Secondly, to determine whether one or more loci are responsible for the increase in lifespan of the Age line, E28. Thirdly, to determine whether there is a correlation between lifespan and early productivity in lines derived from the Age line. Finally, it should be possible to determine whether there is a significant difference between
fitness among Unc-derived lines, relating to whether they show the Unc phenotype, and if so to determine the extent to which the reduction in fitness is the result of this phenotypic mutation.

5.5 EXPERIMENTAL OUTLINE
Inbred-backcross analysis of a line makes it possible to obtain estimates of the number of loci affecting a trait of interest, and to look for correlations between different traits. The background to the technique is described in more detail in Section 5.1.3. Briefly, the method used here involves crossing hermaphrodites from an inbred mutant line (P1) with N2 males to produce F1 progeny, then inbreeding 20 sublines of the descendants for five generations to fix any segregating factors, under conditions of minimal selection. By carrying out life history assays of these inbred-backcross sublines and P1, it is possible to determine the proportion of these lines that differ significantly from P1, and from this to obtain an estimate of the effective number of loci that account for the differences between the two lines.

5.5.1 OBTAINING MALES FOR CROSSING
Inbred-backcross assays involve setting up crosses between N2 males and mutant hermaphrodites. Appropriate numbers of N2 males are obtained using the following method.

Males of the N2 strain of C. elegans were obtained by placing a few young hermaphrodites at 26°C, and examining the offspring for males produced by an increased rate of non-disjunction of the X chromosome at the higher temperature. Males have one X chromosome (XO) rather than two (XX), and have a distinct phenotype. Once a few males had been obtained, crosses were set up at 20°C, according to the protocol of Lewis and Fleming (1985), in order to produce large numbers of N2 males; the offspring of such a cross are expected to show a 50:50 male:hermaphrodite ratio. These matings consisted of six adult male N2s and one hermaphrodite, in order to maximise mating success. Hermaphrodites were chosen at the L4 (pre-adult) stage, to ensure that their eggs were not fertilised before they were
able to mate. Using this approach, large numbers of males from the N2 strain were produced and used in crosses with hermaphrodites from the mutant lines.

5.5.2 Setting up crosses

Crosses were set up between the N2 and mutant strains, consisting of one L4 mutant hermaphrodite (either line E1 (Unc) or line E28 (Age)) and six adult male N2 worms, placed on a small agar plate at 20°C. The adult worms were transferred onto new plates each day during the reproductive period, in order to reduce overcrowding and allow the offspring to be counted when they reached adulthood, ensuring that males had been produced at the expected frequency. Crosses were initially judged as being successful if the first day's progeny contained males. This could later be verified by counting the number of males and hermaphrodites produced each day, when they reached adulthood. If the crosses had been successful, 50% of the progeny would be expected to be male. From the E28xN2 cross used, 50.5% of the progeny were male; from the E1xN2 cross, 49.6% were male. These percentages are remarkably close to the predicted values, providing strong evidence that the crosses had been successful. From each of one successful E28xN2 cross and one E1xN2 cross, at least 30 of the cross-progeny were transferred onto separate plates. Adult hermaphrodites were not transferred, to avoid the possibility of matings having taken place with their male siblings. Instead, the second day's progeny were chosen, which were at the L4 stage or younger and so did not have a fully-formed vulva, and therefore must have been virgins. Where possible, hermaphrodites were chosen, but as it is more difficult to sex worms at this age, extra replicates were taken to ensure that at least twenty hermaphrodites were among those transferred. In the case of the E1xN2 cross, it was observed that both the male and hermaphrodite offspring of the cross were phenotypically wild-type with respect to the Unc phenotype, indicating that it is caused by an autosomal recessive mutation. Thus, when they reached adulthood, all the offspring of the cross that had been transferred were examined for the presence of the Unc phenotype. If any of them had displayed it, this would have indicated that they were homozygous for this mutation, and were therefore the product of self-fertilisation, rather than cross-fertilisation. This was not the case, however – all the F1 worms displayed the wild-type phenotype for this trait.
5.5.2.1 Generation and propagation of lines

Lines derived from each of the first 20 surviving hermaphrodite F1 progeny of the two crosses were inbred for five generations in conditions designed to minimise selection, in order to fix the segregating mutations. This was done using the same method as that used to inbreed the EMS-generated lines, described in Chapter 4. Transfers were carried out every five days, on average. Lines were propagated in parallel by the transfer of one hermaphrodite, chosen from a random position on each plate. Back-up plates (one per line) were used in cases where the parent failed to produce progeny. If neither replicate produced offspring, a worm was substituted from a plate from the previous generation, which was stored at 14°C to slow the worms' growth and thus reduce the possibility of overcrowding and starvation. Fewer generations of inbreeding were used than in the EMS experiment (Chapter 4) because of the greatly reduced chance of obtaining tightly linked mutations. Under conditions of self-fertilisation, at least 97% of mutations are expected to reach fixation after five generations of inbreeding (Falconer and Mackay, 1996).

After five generations of inbreeding, life history assays were performed on five individuals from each line, and ten individuals from the P1 generation (E1 and E28). All the lines were cryopreserved. For full details of these techniques, see Chapter 2 (Materials and Methods). For each replicate, worms were kept on individual plates for one generation prior to the fitness assays. Lines were randomised and worms maintained individually on small agar plates, and the daily reproductive output and lifespan of each worm was recorded.

5.6 Results and Analysis

Estimation of the number of effective factors \( (n_e) \) affecting productivity was carried out following the approach of Mulitze and Baker (1985a), as explained in Section 5.1.3. Distributions of the line means are shown in Figure 5.3 and Figure 5.4. Each inbred-backcross line was classified as parental if the mean fell within the 95% confidence interval of the P1 line (E1 or E28). Because the phenotypes of individual worms in some of the parental lines showed non-normal distributions, non-parametric confidence intervals were determined, using the Wilcoxon test (Snedecor and
The total proportion of non-parental lines \([p(r \geq 1)]\) was then used to determine \(n_e\), using equation 5.4 (Section 5.1.3), where \(k = 0\) and \(p_k = (1/2)^{k+1} = 1/2\). 95% confidence intervals for \(p(r \geq 1)\) were determined from tables of binomial confidence limits (Steel and Torrie 1980, Table A.14A). These values were then used to determine confidence intervals for \(n_e\), using equation (4). Table 5.2 shows the estimated number of loci for each trait in the two sets of lines.

Figure 5.3: Inbred-backcross lines - distribution of line means for productivity

(a) E1 inbred-backcross lines

(b) E28 inbred-backcross lines
Figure 5.4: Inbred-backcross lines - distribution of line means for longevity

(a) E1 inbred-backcross lines

(b) E28 inbred-backcross lines

Table 5.2: Estimates of the number of loci affecting traits, derived from the inbred-backcross technique

<table>
<thead>
<tr>
<th>Trait</th>
<th>Line</th>
<th>Proportion of non-parental lines ([p(r \geq 1)])</th>
<th>Effective number of loci (n_e)</th>
<th>95% Confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Productivity</td>
<td>E1 (Unc)</td>
<td>19/20</td>
<td>4.3</td>
<td>2.00 - 9.59</td>
</tr>
<tr>
<td></td>
<td>E28 (Age)</td>
<td>13/20</td>
<td>1.0</td>
<td>0.46 - 1.88</td>
</tr>
<tr>
<td>Longevity</td>
<td>E1 (Unc)</td>
<td>10/20</td>
<td>1.5</td>
<td>0.76 - 1.88</td>
</tr>
<tr>
<td></td>
<td>E28 (Age)</td>
<td>8/20</td>
<td>0.7</td>
<td>0.31 - 1.47</td>
</tr>
</tbody>
</table>
Figures 5.3 and 5.4 indicate that the confidence intervals for the parental lines are very large, making it difficult to obtain precise estimates of $n_e$ without using larger sample sizes. Thus, it should be noted that such error could inflate the confidence intervals shown in Table 5.2.

The values presented here can be compared to the maximum likelihood estimates of $U$ ($= n_e$) from the previous chapter: 1.6 for productivity and 0.5 for longevity. The results given in Table 5.2 for line E1 are slightly higher than this but, as this was one of the least fit lines, it is not surprising if more mutations that average have accumulated in it. The results for line E28 are remarkably consistent with those derived from maximum likelihood estimation. In each case, the estimated number of mutations is lower for longevity than for productivity. Previously, I inferred that $\sim 45$ deleterious mutations had been induced on average per haploid genome, indicating that only $\sim 4\%$ of the induced mutations were being detected by the productivity assays. The results presented here appear to confirm that in these lines, at least, small numbers of detectable mutations appear to be responsible for the change in fitness observed. Even taking the most extreme case, the productivity assay for the lines derived from line E1, only 10% of the mutations induced were detected.

Among the E1 (Unc) inbred-backcross lines, there was no significant difference in productivity or longevity between lines that exhibited the Unc phenotype and those that did not (t-tests: productivity $t = -0.25; P = 0.81$, longevity $t = 0.47; P = 0.64$). This indicates that the visible phenotype does not contribute to the change in fitness, which must be the result of independent mutations. Analysis of the lines to determine the Pearson product moment correlation coefficient showed no significant correlation between mean lifespan and productivity in the Unc-derived lines ($r = -0.139, P = 0.558$).

The analysis of the E28 (Age) inbred-backcross sublines indicates that the change in lifespan in this line is predominantly caused by just one mutation. Correlations were measured between longevity and both early and total productivity. The between-line component of variance for lifespan was non-significant ($P>0.05$) in the inbred-backcross sublines, which appeared to contradict the inference that there was a significant difference in lifespan between line E28 and the controls, and that this was
caused by a single mutation. However, the sample sizes used in this study were relatively small, and power analysis, performed using Minitab version 12, indicated that the power of the experiment – the probability of correctly rejecting the null hypothesis when it is false – was low. The power was only 0.5, indicating that there was a 50% chance of making a Type II error. Such an error would explain this apparent contradiction. As power analysis requires knowledge of the variance within the populations, such analysis was not carried out prior to the experiment, although a prediction of the genetic variance could have been made from the differences between parental lines. If the experiment were to be repeated, such analysis would be useful to determine the most appropriate sample sizes required to maximise the power of the experiment.

As a result of the relatively small amount of between-line variance detected, the genetic variance for longevity was negative, with the result that the genetic correlations \((r_G)\) were undefined. However, it was possible to determine the phenotypic \((r_P)\) and environmental \((r_E)\) correlations using the approach described in Chapter 2. No significant relationship was observed between total productivity and lifespan \((r_P = 0.0017, r_E = 0.011)\), but there was a strong negative correlation between early productivity and lifespan \((r_P = -0.54, r_E = -0.084)\). These results are displayed graphically in Figure 5. This provides evidence for a trade-off between early components of fitness and longevity. Similar negative correlations were not observed in the E1 inbred-backcross sublines for longevity and total productivity \((r_G = 0.62)\) or early productivity \((r_G = 0.43)\). Nor were they observed in the means of the EMS and control lines, as noted in the previous chapter (controls - \(r_G\) is undefined, productivity vs. longevity: \(r_P = 0.059, r_E = 0.15\), early productivity vs. longevity: \(r_P = -0.021, r_E = 0.065\). EMS lines - productivity vs. longevity: \(r_G = 0.55\), early productivity vs. longevity: \(r_G = 0.47\).

By carrying out the analysis on a number of separate inbred-backcross sublines, it was possible to avoid the problem of independent mutations causing apparent correlations between traits, unless such mutations were very tightly linked. Correlations caused by closely-linked mutations strongly influenced early interpretations of the action of the \(age-1\) mutation (for a review, see Kenyon, 1997, p.801-2).
Figure 5.5: Reisolates of N2 × longevity mutant (E28) cross (line means)

(a) Longevity vs. total productivity

(b) Longevity vs. early productivity
The Age mutant described here is just one example of a lifespan-expanding mutant; whether a trade-off such as this occurs more generally remains to be seen. The two Age mutants with the most extreme effects on lifespan, *age-1* and *daf-2*, are believed to increase lifespan without reducing brood size (Kenyon, 1997). To the best of my knowledge, no published studies have looked for a link between early (rather than total) productivity and lifespan in the *C. elegans* Age mutants. The optimality theory of ageing predicts a trade-off between traits influencing early and late components of fitness (Williams, 1957). Thus, it may be more appropriate to look for evidence for correlations between age-specific mortality and early, rather than total, productivity. The study described here indicates that there may be a significant negative correlation between early productivity and survival, although there is no significant correlation between total productivity and survival. Thus, it may be advisable to look for such correlations in the other *C. elegans* Age mutants before using the results to conclude that these studies do not provide any evidence to support the optimality theory of ageing.

Evidence for trade-offs between early and late components of fitness in species other than *C. elegans* have mainly come from experiments involving Drosophila. A lifespan-extending mutant, *methuselah*, has been identified in *Drosophila* (Lin et al., 1998). A correlated increase in stress-resistance was observed, as was the case with the *C. elegans* mutations *age-1* and *daf-2*. However, the effect of this mutation on early or total productivity was not reported. *Drosophila* selection experiments involving directional selection on one trait whilst observing correlated changes in another trait have had mixed results: some have found evidence for correlations between life history traits, while other have failed to detect such effects (results summarised by Zwaan, 1999).

5.7 RESULTS SUMMARY
Estimates of the number of effective factors influencing productivity and lifespan obtained from inbred-backcross analysis of a sample of two lines were comparable with results derived from ML and BM analysis of 56 mutagenised lines and controls. The conclusion remains that the change in fitness is caused by few mutations with
large effects, and that the majority of the mutations induced were not detected in the assay conditions used.

The Unc line, E1, showed a significant decline in productivity, relative to the wild-type strain. However, presence or absence of the Unc phenotype in the inbred-backcross isolates of this line was not correlated with the change in productivity, indicating that this phenotypic mutation was not responsible for the decline in fitness; the two traits must have been influenced by independent mutations.

The Age mutant, line E28, showed a consistent 20% increase in lifespan, which appears to be the result of a single mutation. There was no significant correlated change in total productivity associated with this mutation, but a strong negative correlation was observed between early productivity and lifespan among the inbred-backcross sublines, as predicted by the optimality theory of ageing.
Chapter 6: **GENERAL DISCUSSION AND CONCLUSIONS**
6.1 SUMMARY

The aim of this work was to study the properties of mutations affecting life history traits in C. elegans. Properties of particular interest include the rate at which new mutations arise and the distribution of their effects on fitness-related traits, and the way in which these effects may vary in different environments or different traits.

Estimates of mutational parameters derived from mutation accumulation (MA) experiments have varied widely, as discussed in Chapter 1. One possible explanation for these differences is that such experiments, although similar in design, have measured the effects of accumulated mutations on fitness-related traits in different environments, varying from benign conditions with an excess of food and no overcrowding to harsh, competitive conditions with limited food and space. It is possible that there may be significant variation in the effects of mutations when they are expressed in different environments (genotype by environment interaction, GEI), which could in turn influence estimates of the genomic mutation rate, $U$, and the average effects of such mutations, $s$. The extent to which new mutations exhibit GEI is also of interest because it has been suggested that GEI in natural populations may be responsible for the maintenance of quantitative genetic variation (Levene, 1953; Via and Lande, 1987; Gillespie and Turelli, 1989) and the evolution of ecological specialisation (Kawecki, 1994). If a significant amount of GEI is observed among spontaneous mutations, the recurrent input of such mutations may be responsible for the levels of GEI observed in natural populations.

In order to investigate these issues (Chapter 3) lines of C. elegans that had been allowed to accumulate spontaneous mutations for 60 generations were provided by Dr. Peter Keightley and Dr. Armando Caballero. Low estimates of $U$ (0.0026) and relatively high estimates of $s$ (23%) had previously been obtained from productivity assays performed under benign conditions, at 20°C in agar of standard salinity (0.034M NaCl), with an excess of food available (Keightley and Caballero, 1997). The degree to which these mutations exhibited GEI for fitness-related traits was studied here by assaying the same traits in a series of harsher assay conditions: reduced temperature (14°C), increased temperature (26°C) and increased salinity (0.2M NaCl). Significant levels of GEI were not observed, indicating that the assay
conditions used were not responsible for the low estimates of $U$ obtained from these lines. Instead, another explanation for the differences between estimates of mutational parameters obtained from different experiments must be sought. In addition, these results do not provide evidence to support the theory that recurrent input of mutations exhibiting GEI could be responsible for the maintenance of GEI in natural conditions, although these results may not be generally applicable. The limited information available from other species have provided evidence both for and against significant levels of GEI among spontaneous mutations (Kondrashov and Houle 1994; Fry et al., 1996; Gilligan et al., 1997; Shabalina et al., 1997; Fry et al., 1999). It remains possible that conditions other than those used here may reveal significant levels of GEI, with competitive conditions being the most likely. However, because of the difficulty of inducing competition in *C. elegans* (discussed in Chapter 3), such assays have not been performed, though they may provide an interesting avenue for future research.

An aim of this work was to clarify the reasons for the large differences in results between different MA experiments. However, it is possible that all such experiments have been underestimating $U$, as laboratory assays may not be sensitive enough to detect all the mutations that have arisen. This could be because they have effects on traits or in environments other than those considered, or because they are simply too small to be detected, given the sample sizes used. In Chapter 4, I describe a novel method used to determine the frequency of such cryptic mutations. I obtained estimates of mutational parameters using fitness assays of control and mutagenised lines, in a situation where the number of base-pair changes induced by a chemical mutagen (EMS) could be inferred. Only a small percentage of such mutations (<10%) that would be expected to have deleterious effects in natural conditions could be detected by the life history assays used. This indicates that traditional MA experiments may have dramatically underestimated the rate at which mutations arise, due to a large class of mutations with very slightly deleterious effects. Although cryptic, these mutations could be significant for issues in evolutionary biology, including the evolution of sex (Kondrashov, 1988). If different MA experiments have underestimated the mutation rate to different degrees, this could provide a further explanation for the differences in their results, as it could give the impression of genuine differences in the underlying mutational parameters.
Further analysis of two of the EMS-derived lines was described in Chapter 5. A substantially different method, known as the inbred-backcross technique, was used to obtain a further estimate of the proportion of induced mutations that could be detected in these lines. Each mutant line was crossed with the wild-type N2 strain, and a number of sublines were inbred for several generations in conditions of minimal selection, in order to fix the segregating mutations. From the proportion that differed significantly from the parental line, it was possible to obtain an estimate of the effective number of loci responsible for the differences between the wild-type and mutant lines. This provided confirmation that few mutations were responsible for the observed change in fitness-related traits in these lines, despite the fact that large numbers (~45) of deleterious mutations were inferred to have been induced. Again, it can be concluded that the majority of induced mutations could not be detected.

In addition to this, Chapters 4 and 5 considered the joint effects of induced mutations one more than one life history trait, and the age-specific properties of such mutations. There are differences in the mortality curves of the EMS and control lines, with the EMS lines showing higher levels of early mortality, but, surprisingly, lower rates of mortality late in life (Chapter 4). A possible explanation for this is greater heterogeneity among the EMS lines due to genetic, as well as environmental, variance being present. Thus, worms with more severe mutational effects would be likely to die at a younger age, with the result that fitter individuals, showing lower rates of mortality, remain. Studies involving large numbers of worms from each line would be required to determine whether this was the cause of the difference between the EMS lines and controls. If large numbers of genetically identical worms were assayed from one line, the mortality curve for that line would not be affected by genetic heterogeneity, indicating that this could not be the cause of the difference between the EMS and control lines.

Other possible explanations for this crossing-over include trade-offs between lifespan and another trait, such as productivity, as the EMS lines had significantly lower productivity than the controls, and the levelling-off of mortality rates occurred after the reproductive period. Positive correlations between the traits studied were typically observed, although this could be the result of the association of different...
mutations in the same individuals, rather than pleiotropy. Alternatively, the results may provide tentative support for the mutation-accumulation theory of ageing, which predicts that ageing evolved due to the accumulation of mutations affecting fitness late in life, when selection to remove them is weaker. Therefore, new mutations could be more likely to affect mortality early in life, when initial fitness is higher, and may even cause the reversion of some accumulated mutations that had deleterious effects late in life.

Overall, there were no observable trade-offs between longevity and total or early productivity, as may have been expected under the optimality theory of ageing (Chapter 4). However, this theory does not require a high input of mutations causing trade-offs, as such mutations may be expected to reach a higher equilibrium frequency even if they only occur rarely. Mutations that significantly enhance one life history trait may be particularly likely to show evidence of trade-offs, however, as this could explain why they had not previously reached fixation in the natural population. None of the MA lines containing spontaneous mutations, developed by Keightley and Caballero (1997) showed a significant increase in either productivity or longevity. Similarly, none of the EMS lines developed during the course of this work showed an increase in productivity, but one line (E28) showed a significant increase in longevity. In Chapter 5, it was shown by inbred-backcross analysis that this effect was caused by a single mutation, and there was no observable trade-off between longevity and total productivity. However, there was a significant negative correlation between longevity and early productivity, as predicted by the optimality model of ageing. This result is particularly interesting because the other age-enhancing mutations identified in C. elegans have not shown evidence of trade-offs between productivity and longevity, and so it was presumed that they did not provide support for the optimality model. However, early productivity was not considered, and this work suggests that such trade-offs could still occur.

Overall, this work has provided some tentative support for both the mutation accumulation and optimality models of ageing. The two models are not mutually exclusive, and both may apply to some extent. Further work will be required before we can be confident which, if either, of these processes has dominated the evolution of ageing.
6.2 FUTURE DIRECTIONS

The work described here could be developed in a number of possible ways. A limited number of environments were considered during the work on genotype by environment (GEI) interactions, and they do not cover all possibilities. It is possible that other environmental conditions will reveal greater effects, and two possibilities may be particularly interesting: the natural environment and competitive conditions. It is difficult to see how *C. elegans* could be assayed in its natural environment, but this may be feasible (if difficult) if a plant was used as a model system. The effects of mutations in the natural environment are of most interest to evolutionary biologists, although the problem of sample sizes being too small for all deleterious mutations to be detected could remain.

Competitive fitness assays also have the potential to reveal greater differences between lines than benign or harsh, non-competitive conditions. In some species, such as *Drosophila*, such assays have been performed, though for the reasons discussed in Chapter 3, controlled, repeatable competitive assays involving *C. elegans* would be difficult to design. If such a technique could be developed, however, it would be interesting to note whether competitive conditions revealed different mutational effects from non-competitive conditions.

Estimates of mutational parameters have now been obtained from a number of experiments involving *Drosophila*. However, data from other species are sparse, and a focus on species other than *Drosophila* would be useful for comparative purposes; in particular, studies of vertebrate systems would be of interest. Potential explanations for the differences in mutational parameters observed in different species have been proposed (e.g. Lynch *et al.*, 1999), but until such predictions can be tested, their validity cannot be known. However, the results presented here indicate that mutation-accumulation experiments may dramatically underestimate the rate at which mutations accumulate. Therefore, unless the number of events occurring at the DNA level can be estimated, it may be difficult to draw reliable conclusions from such studies. A form of DNA sequence analysis may be one way to circumvent this problem. For example, if it was possible to sequence the entire genome of mutation-
accumulation lines, this could provide information concerning the number of point mutations induced, and from this it should be possible to estimate the proportion of such mutations expected to have deleterious effects in natural conditions. Determining the distribution of the fitness effects of such mutations could remain a problem, however.

In addition to the rates at which mutations arise and their effects, many of the issues described in Chapter 1 rely on other properties of new mutations. The deterministic mutation hypothesis of the evolution of sex, for example, requires not just a high input of mutations, but also that they show synergistic epistasis. Some evidence for synergistic epistasis came from a study by Mukai (1969), in which a non-linear decline in fitness was observed with increasing numbers of mutations in lines of *Drosophila*. However, the validity of such experiments has been called into question (Keightley, 1996). A study by Elena and Lenski (1997) found no overall evidence for epistatic interactions between deleterious mutations, though individual pairs of mutations varied in the magnitude and direction of epistatic interactions.

Some recent evidence for synergistic epistasis has come from studies of *Chlamydomonas moewussi* (de Visser et al., 1996, 1997). This work involved predictions of the effect of variation in mutation numbers on fitness-related traits. Under synergistic epistasis, variance in mutation numbers is expected to decrease mean log fitness, due to the relatively low fitness of organisms with high numbers of deleterious mutations (de Visser et al., 1996). By crossing lines of *C. moewussi* with similar numbers of mutations, they showed that the offspring had lower mean log fitness than their parents. As they must have had the same mean number of mutations, this indicates that the lower log fitness was due to the offspring being more variable than the parents, which they interpreted as evidence for synergistic epistasis. It has been argued, however, that this approach may be unlikely to produce clear results (West et al., 1998). Their main criticism was that if the number of mutations the parents carry differs by even a small number, they are expected to be more, rather than less variable than their offspring. A more appropriate approach would be to use parents with different numbers of mutations, leading to the prediction that they will be more variable, and thus less fit under synergistic epistasis, than their offspring (West et al., 1998).
Another fertile area for future research is the evolution of ageing. A lifespan-extending (Age) allele identified here revealed trade-offs with early, but not total productivity. Previous Age mutants identified in *C. elegans* have not provided evidence for trade-offs with productivity, but correlations between lifespan and early productivity have not been reported, and this may now be worth considering.

It would also be interesting to further characterise this Age mutation, with the aim of discovering more about its nature. Several more generations of backcrossing onto a wild-type background would be required to remove other EMS-induced mutations. Complementation tests with other Age mutations in *C. elegans* would determine whether this was a novel mutation; if so, its location could be mapped. Some of the correlated traits observed in lines of *C. elegans* with other Age mutations could also be assayed, such as altered dauer formation, swimming, feeding and egg-laying rates, motility, developmental time and thermotolerance and resistance to UV light and hydrogen peroxide. Such analyses could reveal more about the genetics of ageing and the genetic control of lifespan.

Several possible explanations are available for the observed difference in mortality rates between the EMS and control lines. Further studies involving large numbers of worms per line could be used to determine whether the levelling-off of mortality rates among the EMS lines was due to greater heterogeneity among those lines. Alternatively, studies of lines in which reproduction did not occur could reveal if it is the result of reproduction increasing the mortality rate. As the N2 strain of *C. elegans* used here as a base population is a self-fertilising hermaphrodite, it is difficult to prevent the worms from reproducing. Performing a similar experiment using a different line, such as one that exhibits temperature-sensitive sterility, may be more appropriate. Alternatively, a species other than *C. elegans*, in which it is easier to prevent reproduction, could be used.

To summarise: the study of mutations is a fascinating field of research, and there are many ways in which future research can enhance our understanding of an area that touches on so many aspects of evolutionary biology.


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High Frequency of Cryptic Deleterious Mutations in Caenorhabditis elegans

Esther K. Davies, Andrew D. Peters, and Peter D. Keightley*
High Frequency of Cryptic Deleterious Mutations in Caenorhabditis elegans

Esther K. Davies, Andrew D. Peters, Peter D. Keightley*

Deleterious mutations with very small phenotypic effects could be important for several evolutionary phenomena, but the extent of their contribution has been unknown. Fitness effects of induced mutations in lines of Caenorhabditis elegans were measured using a system for which the number of deleterious point mutations in the DNA can be estimated. In fitness assays, only about 4 percent of the deleterious mutations fixed in each line were detectable. The remaining 96 percent, though cryptic, are significant for mutation load and, potentially, for the evolution of sex.

The prevalence of sexual reproduction may be explicable by the ability of sexual populations to sustain higher rates of deleterious mutation than asexuals (1). However, it has been argued that the accumulation of deleterious mutations could lead to fitness loss and ultimately to population extinction, even in sexual species, if effective population size is small (2). Mutation accumulation experiments in Drosophila suggest that mutations with harmful effects of the magnitude required for this fitness loss (~1%) occur frequently (3), but joint estimates of the spontaneous genomic deleterious mutation rate, U, and the mean deleterious mutation effect, δ, from these experiments have been questioned (4, 5). An alternative molecular constraint approach to estimate U compares rates of nucleotide substitution in functional and neutral regions of the genome (6), but does not give direct information on the magnitude of mutation effects. Here, we combine the molecular constraint and mutation accumulation approaches to infer the distribution of fitness effects of point mutations in the nematode C. elegans.

We exposed the N2 (wild type) strain of C. elegans to 50 mM ethylmethane sulfonate (EMS) for 4 hours, a dosage for which the number of mutations induced at the DNA level has been calibrated (7). About 92% of the mutations produced by this treatment are G/C → A/T transitions; the remainder are other kinds of point mutations, plus a low frequency of small deletions (7). Data from experiments to measure forward mutation rates (8) and suppressor-induced reversion mutation rates (9–11) under EMS mutagenesis provide a mean estimate for the rate of transitions of 6.2 × 10⁻⁶ (95% confidence interval (CI) of (Table 1) 4.0 × 10⁻⁶ to 8.4 × 10⁻⁶) per G/C base pair. The haploid genome of C. elegans is 9.7 × 10⁷ base pairs, comprising 36% G/C (12), so the mutagenesis generated an expected number of 220 transition mutations per haploid. The fraction of the genome in exons is 27% (12), and we infer from tables of codon usage in >15,000 C. elegans genes (13) that 30.6% of nucleotides in exons are G/C and generate an amino acid change if mutated to A/T. Therefore, we estimate that the EMS treatment generated an average of (6.2 × 10⁻⁶) × (9.7 × 10⁷) × 0.27 × 0.306 = 50 (95% CI of 32 to 68) transition mutations per haploid that changed an amino acid in a protein-coding gene. Protein-coding sequences are under strong selective constraint in C. elegans: Data on synonymous and nonsynonymous substitution rates in C. elegans and its relative C. briggsae (14) suggest that ~90% of amino acid mutations are removed by natural selection. Thus, we estimate that the minimum number of mutations induced per haploid genome that are deleterious in natural conditions is ~45, but the number could be substantially higher because some noncoding DNA is selectively constrained (15). A comparison of the relative changes [using a log-odds (Dayhoff) matrix (16)] arising from G/C → A/T transitions to other types of point mutations (A/T → G/C transitions and transversions) suggests that the severity of individual amino acid changes generated by EMS is similar to that of spontaneous mutations (17).

After the mutagenesis, we bred 60 independent EMS lines toward homozygosity by selfing. In the absence of selection, the number of mutations fixed is expected to be the number of mutations induced per haploid genome. During the inbreeding, conditions were made as favorable as possible to avoid selection. However, some backup cultures were used, and four lines were lost, implying the action of natural selection (Fig. 1) and the selective loss of some mutations (such as recessive lethals). To estimate the magnitude of this loss, we performed computer simulations of lines undergoing multiple generations of selfing, analogous to our experimental design (18). The mean proportion of plates producing at least one progeny (surviving cultures) among the EMS-treated lines in our experiment was 0.74, with a 95% CI of 0.68 to 0.80 (Fig. 1). Under the parameter values simulated, only mutation effects of less than 10⁻³ are predicted to lead to viabilities within this range (Fig. 1). In our simulations, if lines had 45 heterozygous mutations at the start of inbreeding, such mutation effects led to the loss of 6% of mutations or fewer, on average, to selection (Fig. 1). This suggests that at most three mutations, two of which were minor-effect mutations, were selectively lost (on average) per line. Simulations assuming that fitness declines faster than exponentially with increasing numbers of mutations (synergistic epistasis) led to similar or lower estimates of the number of mutations selectively lost (17).

We measured lifetime reproductive output of individual worms from the 56 surviving EMS lines and 40 control lines. This fitness measure includes the viability of the parents.

Table 1. Rates of G/C → A/T transition mutations induced by EMS. The frequency of mutations is adjusted to a dosage of 50 mM EMS for 4 hours [as used here and in (9)] by assuming the EMS dose-response curve of (21) [(10, 11); 25 mM EMS, 4 hours], or assuming mutation rate is linear with time [(8); 50 mM EMS, 3.5 hours].

<table>
<thead>
<tr>
<th>Reference</th>
<th>Sensitive sites</th>
<th>Total sites screened</th>
<th>No. of mutations</th>
<th>Adjusted mutation rate per G/C (x10⁻⁶)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(8)*</td>
<td>160</td>
<td>960,000</td>
<td>7</td>
<td>8.3</td>
</tr>
<tr>
<td>(9)††</td>
<td>2</td>
<td>1,248,000</td>
<td>4</td>
<td>3.2</td>
</tr>
<tr>
<td>(10)‡‡</td>
<td>6</td>
<td>2,400,000</td>
<td>9</td>
<td>5.2</td>
</tr>
<tr>
<td>(11)§§</td>
<td>6</td>
<td>1,572,000</td>
<td>8</td>
<td>8.0</td>
</tr>
</tbody>
</table>

Mean: 6.2 (95% CI 4.0 to 8.4)

*Screened for unc-54 null mutants, the majority of which are nonsense alleles (30). The number of unc-54 sites at which an EMS-induced transition can produce a nonsense allele is 160 (7). †Screened for amber suppressors caused by single base-pair changes at tRNA⁴⁴ genes. ‡Screened for unc-13 extragenic suppressors, all of which mapped to two sites. §Screened for tra-3 extragenic suppressors, and identified mutations at four and five tRNA⁴⁴ genes, respectively, a total of six sites.
Table 2. Estimates of mutation rates and effects with \( U_1 \) variable.

<table>
<thead>
<tr>
<th>Method</th>
<th>Model</th>
<th>( \hat{U}_1 ) (SE)</th>
<th>( \hat{s} ) (SE)</th>
<th>( \hat{\beta} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moments</td>
<td>Equal effects</td>
<td>1.4 (0.36)</td>
<td>24% (3.9)</td>
<td>-</td>
</tr>
<tr>
<td>(3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ML (23)</td>
<td>Equal effects</td>
<td>1.6 (0.21)</td>
<td>22% (1.9)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>( \gamma ) distribution</td>
<td>&lt;2.5</td>
<td>&gt;15%</td>
<td>&gt;1.6</td>
</tr>
</tbody>
</table>

Table 2: Estimates of mutation rates and effects with \( U_1 \) variable.

\[ V_I = U_1 \hat{s} \]

\[ \beta = \frac{\text{fitness change}}{\text{mutation rate}} \]

\[ \hat{U}_1 = \frac{\text{change in mean fitness}}{\hat{s}} \]

\[ \hat{s} = \frac{\text{change in between-line variance from mutation}}{\text{mutation rate}} \]

\[ \hat{\beta} = \frac{\text{fitness change}}{\text{mutation rate}} \]

For the three assays: (A) Mean productivity for the three assays. (B) Distribution of line means, averaged over replicates, for productivity. After mutagenesis, the cryopreserved lines were thawed, then individual replicates within each line were maintained for three generations. Productivity was measured contemporaneously in three replicates from each mutant and control line. In each replicate, four worms were allowed to lay eggs on a plate for \( \sim 3 \) hours, then removed. After \( 48 \) hours, a single randomly picked worm was transferred onto a new plate, then transferred \( 48 \) hours later and at \( 24 \)-hour intervals for the entire reproductive period. The offspring were counted manually from the parental transfer. Each counter assayed one replicate per line, and the entire assay was performed three times. Lines were randomized and counters were unaware of line identity. Analysis of variance was used to estimate the between-line variance for productivity; additional effects fitted were measure, assay number, and their interaction, which was nonsignificant. The between-line variance component was highly significant for the EMS lines (\( p < 0.001 \)) but not the controls (\( p > 0.6 \)). The EMS-induced mutational heritability was \( \frac{V_M}{V_E} \), where \( V_M = V_G/2 \) and \( V_E \) is the environmental variance (2426 worms).

The control and EMS mean productivities were 248.5 and 163.2 worms, respectively.
first accounting for an average of 1.6 mutations per line (these had effects of 22%, as for the single-class equal-effects model under ML), whereas the remaining 43.4 mutations have virtually zero effect (Table 3). The maximum fitness effect that this class of weak deleterious mutations can take is only 0.07% (Table 3). The distribution of mutation effects is therefore multimodal. If a number of deleterious mutations other than 45 is assumed, the estimated number and mean effect of strongly deleterious mutations remains the same (\( \hat{U}_{11} = 1.6, \hat{s}_1 = 22\% \)), whereas the maximum effect of the weak deleterious mutations changes proportionately (for example, \( U = 20, \hat{s}_2 < 0.17\%; U = 80, \hat{s}_2 < 0.04\% \)).

Distributions of EMS-induced and spontaneous (24) mutation effects on productivity therefore appear to be remarkably similar. The model of equal effects fits the data better than a \( \gamma \) distribution model in both cases, and \( \hat{s}_2 \) was 21% (±4%) for spontaneous mutations (24) compared to 22% (±2%) here. Our results imply that the spontaneous genomic deleterious mutation rate \( U \), 0.0026 (±0.0012) per haploid (24), could have been underestimated by a factor of at least 28 (that is, 45/1.6), and that \( U \) is therefore closer to \( \sim 0.07 \). Our estimate for the mean mutation effect under an equal effects model is also nonsignificantly different from the positive mean mutational effect on productivity measured in a more recent spontaneous mutation accumulation experiment in \( C. elegans \) (25), that is, \( s = -24\% \pm 23\% \).

Diploid \( U \) must be greater than 1 for sexual reproduction to be maintained by deleterious mutations (1, 26). Because \( C. elegans \) reproduces primarily as a self-fertilizing hermaphrodite, it does not pay the full twofold cost of sex. However, our corrected estimate for the spontaneous deleterious mutation specific to protein-coding genes (\( \approx 0.14 \) mutations per diploid per generation) leads to the prediction that related sexual species would have higher mutation rates if deleterious mutations explain sex (although a maximum corrected \( U \), assuming all 220 EMS-induced nucleotide changes in the genome are deleterious, is 0.72 per diploid). The high frequency of mutations of very small effect also has implications for the rate of fitness loss due to mutation accumulation.

It has been assumed that mutations with fitness effects on the order of 1% are common (2, 26, 27), but our finding that >96% of mutations are undetectable in the laboratory, and have fitness effects of less than 0.07%, brings the validity of this assumption into question. We have inferred that there is a large class of deleterious mutations with tiny effects in the laboratory. Whether their effects are magnified in harsher natural environments remains to be determined; evidence for strong interactions between deleterious mutation effects and environmental conditions is equivocal (5, 28). It is clear, however, that mutation accumulation experiments may substantially underestimate mutation rates, and this will be undetected unless the number of events at the DNA level can be estimated.

**References and Notes**

8. In *Drosophila*, the rates of detectable mutation for viability and other life history traits under an equal-effects model are also similar to the lethal mutation rate under EMS mutagenesis (29).
9. In the ML inference of the distribution of mutation effects (29), the number of mutations per EMS line was assumed to be a random variable, \( n \), sampled from a Poisson-distribution parameter \( U \). The productivity effects associated with this was \( M = s \hat{M} \), \( s = s \hat{s} \), \( \hat{s} \), \( n \), where \( M \) is the mean distribution effect, \( s \) is the fixed effect of a counter, \( s \) is a normal distribution effect of mean 0 and variance \( \sigma^2 \), and \( s \) is a mutation effect that takes the following values: \( s \) (equal-effects model), \( s \) with probability \( p \) and \( s \) with probability \( 1 - p \) (two equal effects); and a random deviate from a \( \gamma \) distribution with shape and scale parameters \( \beta \) and \( \beta \), respectively. Control lines were included in the analysis with \( n \) assumed to be zero. Models were fitted in which \( U \) was a variable parameter (Table 2) or was fixed at one of a number of values (Table 3). Standard errors of estimates (SIs) for the equal-effects model with \( U \) variable were obtained by bootstrapping the data, by line, 500 times. For models with \( U \) fixed, SIs were obtained from a quadratic approximation to profile likelihoods. Estimates marked **>** or **<** are the lowest or highest values compatible with
asymptotically equivalent to 95% confidence limits. Log \( L \) is the difference in natural log likelihood from that of the model with two classes of equal deleterious effects, which has the same likelihood as the one-equal-effect model with \( U \), variable. In the two-equal-effects model, a positive value for \( S_2 \) gave a higher likelihood than \( S = 0 \), but the difference in log likelihood was nonsignificant.


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