

SELECTIVE PRESSURES THAT DRIVE THE EVOLUTION AND MAINTENANCE
OF OUTCROSSING

by

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Although outcrossing is the most widespread mating system among animals and plants, the reason for this prevalence is not fully understood. Evolutionary theory has classified the potential selective pressures driving the evolution and maintenance of outcrossing into two broad categories: deleterious mutations and changing ecological conditions. Despite the inherent advantages of self-fertilization, exposure to either or both of these selective pressures is predicted to favor outcrossing over self-fertilization.

I tested these predictions using experimental evolution in populations of *Caenorhabditis elegans* with genetically modified rates of outcrossing and selfing. I found that outcrossing reduces the fixation of deleterious mutations under mutation influx and that outcrossing expedites adaptation to a bacterial pathogen. Further, I identified facultative outcrossing, a novel life history characteristic, in specific *C. elegans* strains that predominantly reproduce by selfing but engage in outcrossing when stressed. The shift from a primarily selfing mating system to a predominantly outcrossing system is similar to the environmentally induced facultative sex observed in asexual species, which

is thought to enable more rapid adaptation. Facultative outcrossing, although not previously documented, may play a major role in the life histories of many highly selfing species.

Finally, most mutations are deleterious and therefore elevated mutation rates are generally thought to produce progressively larger reductions in fitness. Using the chemical mutagen ethylmethanesulfonate, I found the surprising result that populations exposed to a mutation rate at least fifty times greater than natural rates exhibited significantly greater fitness than populations exposed to substantially lower mutation rates. This unexpected fitness optimum may be the result of a volatile balance between the influx of deleterious mutations and compensatory mutations.

This work confirms the predictions of several long-standing evolutionary theories by identifying both deleterious mutations and changing ecological conditions as selective pressures capable of driving the evolution and maintenance of outcrossing. These selective pressures, which are ubiquitous in nature, may explain the prevalence of outcrossing relative to selfing.

This dissertation includes previously published and co-authored materials.

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CHAPTER I

INTRODUCTION

Reproduction is the most basic and essential requirement for the maintenance of life. It is the means through which organisms generate new life and transmit genetic material from generation to generation. Because evolution is dictated by the transmission of genes through lineages, reproduction ultimately shapes the evolutionary trajectory of every population and thus influences the genetic architecture of all genomes. Given the incredible significance of reproduction, it is surprising to find that an abundance of diverse reproductive modes, or mating systems, are utilized in nature. Even more surprising, or perhaps disturbing, is the failure of modern evolutionary biology to fully explain the evolution and maintenance of the most prominent mating systems in nature despite several decades of research.

The topic of mating systems had been previously discussed by prominent biologists like Charles Darwin (Darwin 1876), RA Fisher (Fisher 1941), and Hermann Muller (Muller 1964), but it was John Maynard Smith's work on the "two-fold cost of sex" that brought the issue to the forefront of modern biology (Maynard Smith 1978). Maynard Smith exposed a potentially major hole in evolutionary biology by mathematically demonstrating that "all else equal" asexual organisms should have as

much as a two-fold reproductive advantage over sexual organisms in a population at carrying capacity. The premise of this model is that sexual females invest in the production of male offspring, meaning as much as half of their progeny that will not directly bear offspring. In contrast, each offspring produced by an asexual organism is capable of bearing offspring. This essentially means that the lineage of an asexual organism can grow much more rapidly than that of a sexual female simply because asexual lineages can produce more progeny. This revelation by Maynard Smith would not have presented an evolutionary enigma if asexual reproduction were the only mating system in nature. However, sexual mating systems are quite prevalent, thereby causing great concern and debate as to the evolutionary explanation for the widespread existence of sex.

Given the prevalence of sexual mating systems, selection should at least conditionally favor sexual reproduction. Maynard Smith's cost of sex is predicated on the assumption that sexual and asexual offspring have comparable fitness (Maynard Smith 1978). So, the potential key to explaining the evolution and maintenance of sex must be in the fitness of offspring produced through sex versus those produced asexually. Specifically, if sexually produced offspring have a pronounced fitness advantage over asexually produced offspring under certain conditions then the inherent numerical advantage of asexual reproduction may be negated.

The search for the specific conditions facilitating the evolution and maintenance of sexual reproduction has inspired three decades of research, primarily theoretical, on the evolutionary costs and benefits of mating systems. Much of this work has focused on

either deleterious mutations (Felsenstein 1974; Kondrashov 1984; Gabriel et al. 1993; Lynch et al. 1993; Kondrashov 1994; Charlesworth and Charlesworth 1998; Lande 1998) or changing ecological conditions (Maynard Smith 1978; Bell 1982; Lively 1987; Crow 1992; West et al. 1999; Lively and Dybdahl 2000) as the selective pressures favoring sexual over asexual reproduction. Both of these selective pressures capitalize on the ability of sexual organisms to shuffle genetic variation between lineages through recombination. Such shuffling can prevent deleterious mutations from being fixed in the genome by incorporating genes from diverse lineages (Muller 1964), and has the potential to accelerate the rate of adaptation to changing environmental conditions by unifying beneficial alleles with different genetic origins into the same genome (Crow 1992). Empirical work has demonstrated the value of recombination in increasing the rate of adaptation to a novel environment (Colegrave 2002; Goddard et al. 2005, Baltrus et al. 2008) . However, this work was done in organisms that do not produce males. Therefore those organisms that undergo recombination are not doing so at the cost of producing males. This work emphasizes the importance of genetic exchange, but cannot assess the importance of genetic exchange relative to the cost of male production.

Curt Lively assessed the selective benefits of sexual versus asexual reproduction, while accounting for the cost of males, by studying both sexual and asexual populations of the snail *Potamopyrgus antipodarum*. His work shows strong positive correlations between the occurrence of sex and male production in the snail populations with the occurrence of highly virulent parasites that continually facilitate changing ecological conditions (Lively 1987). In addition, the snail and parasite populations exhibit signs of

coevolution indicative of rapid adaptation in the snail population, potentially as a means of avoiding the parasite (Lively and Dybdahl 2000). This work strongly supports the hypothesis that sex facilitates adaptation to changing ecological conditions, but is not a direct test. No studies to date have directly and empirically measured the value of sex and male production in preventing the fixation of deleterious mutations and facilitating rapid adaptation to changing environmental conditions. Therefore the specific selective pressures that drive the evolution and maintenance of sex remain largely unidentified from an empirical standpoint.

A clear understanding and identification of these selective pressures is further complicated by the existence of two forms of sexual reproduction. Although the common usage of the term “sex” generally refers to outcrossing, self-fertilization is also a form of sexual reproduction. Maynard Smith’s model contrasted outcrossing lineages versus asexual lineages (Maynard Smith 1978). However, the model also applies within sexual mating systems. Self-fertilizing hermaphrodites enjoy the same inherent numerical advantage over outcrossing organisms (Uyenoyama 1984; Lively and Lloyd 1990). But again, outcrossing is the most prevalent sexual mating system. Therefore not only is the evolution and maintenance of sex an open question, but within sexual reproduction, the selective pressures driving the evolution and maintenance of outcrossing remain unidentified. Several potential genetic advantages of self-fertilization further complicate the evolution and maintenance of outcrossing, beyond the simple cost of male production. So, in many ways the prevalence of outcrossing relative to selfing is more difficult to justify than the prevalence of outcrossing relative to asexual reproduction.

Again, the key to understanding the evolution and maintenance of outcrossing is finding the selective pressures that permit outcrossing to overcome the relevant two-fold cost relative to self-fertilization. And again, the conditions predicted to favor outcrossing over selfing generally involve either deleterious mutations or changing environmental conditions as the primary mechanisms driving selection. However, the situation is complicated by the fact that self-fertilization is a sexual mating systems and therefore allows recombination. Whereas recombination was the primary factor separating outcrossing and asexual reproduction, the selective advantages of outcrossing over selfing cannot be reduced to merely the presence or absence of recombination.

Although outcrossing and selfing are both sexual mating systems and permit recombination, the genetic consequences of each are quite different. Self-fertilization is the most extreme form of inbreeding. Both sperm and egg originate from the same individual; so there is no opportunity for genetic exchange between lineages in obligate selfing populations. Consequently selfing lineages lose genetic variation over time as alleles at heterozygous loci segregate out to generate homozygous loci, which effectively fixes the allele in that specific lineage because the only source of novel genetic input is mutation (Lande and Schemske 1985; Charlesworth and Charlesworth 1987). As selfing genomes tend towards greater frequencies of homozygous loci, the efficacy of recombination is compromised (Charlesworth et al. 1993). Recombination is an effective means of shuffling genetic variation when there is genetic variation between chromosomes. However, recombination between nearly identical chromosomes has no effect on genetic variation. As a result large portions of selfing genomes become tightly

linked (Hastings 1984). Conversely, outcrossing has the potential to permit genetic exchange between diverse lineages (Lande and Schemske 1985; Charlesworth and Charlesworth 1987), thereby increasing the amount of genetic variation within a lineage. The presence of genetic variation within the genome then permits recombination to generate novel combinations of alleles and greatly reduces genetic linkage (Felsenstein 1974; Barton 1995).

The tendency for selfing to permit the expression of recessive alleles via the production of homozygous loci is thought to be an effective means of purging deleterious mutations from populations (Lande and Schemske 1985; Charlesworth and Charlesworth 1987; Charlesworth et al. 1993; Byers and Waller 1999; Crnokrak and Barrett 2002). As deleterious mutations arise in the genome selection must immediately remove them, or they accumulate in the genome. A novel recessive deleterious mutation arising in a selfing lineage has a fifty percent chance of being lost, or purged, each generation, either through segregation or through expression and subsequent selection. Therefore, selfing has a high probability of purging mutant alleles at a single locus relative to outcrossing. But, purging as a strategy for preventing mutation accumulation may be compromised when deleterious mutations arise at multiple loci. The probability of self-fertilization producing an offspring that is homozygous for a newly arisen mutation at any given locus is fifty percent. But, as more loci are mutated the probability of producing offspring free of mutation rapidly declines (Heller and Maynard Smith 1979). Once accumulated, recessive deleterious mutations that are not expressed, or those with moderate or small effects on fitness, have the potential to drift to fixation within a population (Lande and

Schemske 1985; Charlesworth and Charlesworth 1987; Charlesworth et al. 1993). This process is largely governed by population level properties like effective population size and the overall strength of selection. Should deleterious mutations become fixed in a population they reduce the overall mean fitness of that population, and as more mutations become fixed the population can suffer major fitness consequences (Kondrashov 1984; Lynch et al. 1995; Schultz and Lynch 1997; Charlesworth and Charlesworth 1998; Lande 1998).

Outcrossing, although perhaps more prone to mutation accumulation, is predicted to fix fewer deleterious mutations under the conditions expected to push selfing populations toward mutation meltdown (Charlesworth et al. 1993). Because outcrossing individuals have the potential to generate offspring harboring heterozygous loci by mating with individuals from a diverse lineage, then recessive deleterious mutations are much less likely to be expressed in outcrossed offspring. This aspect of outcrossing results in very inefficient purging, but makes outcrossing populations much less likely to fix deleterious mutations once they have accumulated. In addition, deleterious mutations are less likely to remain fixed in an outcrossing population because recombination between lineages can progressively separate specific alleles from their genetic background (Felsenstein 1974; Barton 1995). Therefore, outcrossing populations are predicted to be a much lower risk of fixing deleterious mutations under conditions in which self-fertilization is unable to effectively purge the arising mutation load (Kondrashov 1984; Charlesworth et al. 1993; Lynch et al. 1995; Schultz and Lynch 1997; Lande 1998). Under these conditions outcrossed offspring should maintain greater fitness

than selfed offspring, so the influx of deleterious mutations may be a selective pressure driving the evolution and maintenance of outcrossing.

Outcrossing is also predicted to facilitate more rapid adaption to changing environmental conditions than selfing (Stebbins 1957; Nagylaki 1976; Maynard Smith 1978; Bell 1982; Crow 1992). Changing environmental conditions can apply selective pressure favoring an adaptive response. An adaptive response requires genetic variation. As selfing lineages tend to lose genetic variation within a lineage, whereas outcrossing can maintain and potentially generate variation within a lineage, outcrossing populations are thought to be better prepared to respond to selection. However, outcrossing may also confer a selective benefit in terms of genetic linkage. Because outcrossing facilitates recombination between lineages and creates novel genetic combinations, potentially beneficial alleles can be liberated from their original genetic background and unified into a common genome (Felsenstein 1974; Barton 1995). This accumulation of beneficial alleles, as driven by selection, is adaptation. Such adaptation can occur in relatively rapid fashion because the alleles may already be present in the population (Fisher 1930; Muller 1932). Adaptation via beneficial alleles in selfing populations requires multiple beneficial mutations to occur in the same lineage, as alleles are not shared between lineages. This process may be significantly slower than that facilitated by outcrossing. Therefore, outcrossing populations are predicted to be capable of adapting more rapidly to changing ecological conditions, and thus changing ecological conditions may favor the evolution and maintenance of outcrossing relative to selfing.

Sex, specifically outcrossing, is the most prevalent mating system among animals and plants. This prevalence is unexpected in light of Maynard Smith's model that detailed an explicit cost to outcrossing relative to asexual reproduction, and further work that demonstrated a cost to outcrossing relative to self-fertilization. However, subsequent research predicted deleterious mutations and changing ecological conditions as two factors that may drive the evolution and maintenance of outcrossing, albeit for slightly different reasons relative to either asexual reproduction or selfing. However, no selective pressures explicitly favoring both outcrossing and male production have yet been empirically identified. Nevertheless, these selective pressures must exist and operate quite frequently because outcrossing is widespread across many diverse species. Empirical identification of these selective pressures is a key to developing a comprehensive understanding of mating systems, their influence on evolutionary change, and to resolving a major puzzle in evolutionary biology.

Dissertation Research

The primary objective of my dissertation research was to empirically identify the selective pressures driving the evolution and maintenance of outcrossing. More specifically my work focused on the maintenance of males and outcrossing in the primarily self-fertilizing nematode *Caenorhabditis elegans* as a model for understanding the forces influencing the maintenance of outcrossing on a much broader scale. To address this objective, I tested specific selective pressures predicted by evolutionary theory to favor the evolution and maintenance of outcrossing and identified a novel mating strategy in mixed mating (capable of outcrossing or selfing) populations. In the

following section I briefly summarize the aims and results for each chapter in the remainder of my dissertation.

Chapter II is entitled “Mutation load and rapid adaptation favour outcrossing over self-fertilization.” This chapter is co-authored by Michelle Parmenter and Patrick Phillips and is published in *Nature*, which is copyrighted by Nature Publishing Group, a division of Macmillan Publishers Limited. The objective of this work was to empirically test selective pressures predicted to favor outcrossing over self-fertilization, thus accounting for the prevalence of outcrossing despite the inherent advantages of self-fertilization. Evolutionary theory predicts that outcrossing may better impede the fixation of deleterious mutations and facilitate more rapid adaptation to changing ecological conditions than self-fertilization. We tested these predictions using experimental evolution, by exposing obligate selfing, mixed mating, and obligate outcrossing populations of *C. elegans* to elevated mutation rates and rearing them in a rugged novel environment. After fifty generations of mutation and selection, outcrossing populations fixed significantly fewer deleterious mutations than selfing populations and exhibited significantly greater rates of adaptation under natural mutation rates. To further test outcrossing’s ability to promote rapid adaptation and determine the role of standing generation variation in facilitating adaptation, we used a similar experimental evolution approach to test the rate at which obligate selfing, mixed mating, and obligate outcrossing populations adapt to the virulent bacterial pathogen *Serratia marcescens*. Prior to selection the populations were infused with genetic variation via EMS mutagenesis. Again, after forty generations of selection, obligate outcrossing populations exhibited the

most rapid and substantial levels of adaptation, while stronger selection imposed by the pathogen and standing genetic variation permitted a stronger response to selection than that observed in our previous experiment. This work is the first empirical test of theory regarding the evolution and maintenance of outcrossing and demonstrates that, as predicted, outcrossing populations have greater fitness than selfing populations under specific conditions. The conditional value of outcrossing may be quite significant as most organisms are presumably subject to deleterious mutations and/or environmental conditions favoring rapid adaptation. Both of these factors likely explain the prevalence of outcrossing as a sexual mating strategy.

Chapter III is entitled “Sexual partners for the stressed: facultative outcrossing in the predominantly self-fertilizing nematode *C. elegans*.” This work, co-authored with Brian Cappy, Jennifer Anderson, and Patrick Phillips, is published in *Evolution*, which is copyrighted by Wiley Blackwell Publishing. We identified and characterized a novel mating strategy, facultative outcrossing, in *C. elegans*. When exposed to starvation stress, specific strains of *C. elegans* respond by elevating outcrossing rates after emerging from their stress response state. This phenomenon rapidly and substantially increases male frequencies, and is the first mechanism promoting robust male maintenance identified in *C. elegans*. Importantly periods of starvation stress are common in natural populations. Stress-induced facultative outcrossing in highly self-fertilizing populations closely resembles stress-induced sexual reproduction exhibited by asexual species. Both highly selfing and asexual organisms suffer from the same genetic complications, low within-lineage genetic variation. Facultative outcrossing has the potential to increase

levels of within lineage genetic variation and enable a more rapid adaptive response to stressful conditions, therefore fulfilling the same role as facultative sex in asexual organisms. This work demonstrates that *C. elegans* populations respond to changing ecological conditions by elevating outcrossing rates, therefore indicating that outcrossing may be more favorable than selfing within that context. Though currently unidentified in other taxa, facultative outcrossing is likely a strategy employed by other highly selfing organisms to avoid the genetic consequences of obligate self-fertilization under conditions in which outcrossing is favorable.

Chapter IV is entitled “Paradoxical increase in fitness with increasing mutation rate in *Caenorhabditis elegans*.” This chapter is co-authored by Aki Ohdera and Patrick Phillips and is in preparation for *PLoS One*. The objective of this work was to test the ability of obligate self-fertilizing populations to purge deleterious mutations across a range of different mutation rates. Most mutations with fitness effects are deleterious to some degree. Increases in mutation rate should therefore result in more deleterious mutations arising in the genome, and those that are not removed by selection accumulate in the genome. Selfing is thought to be an efficient means of purging deleterious mutations, thus preventing mutation accumulation. However, selfing also generates tight genetic linkage between large portions of the genome, which is counterproductive for purging deleterious mutations at multiple loci. Elevated mutation rates are capable of inducing deleterious mutations at multiple loci in the genome, potentially overwhelming purging due to genetic linkage. To understand the dynamics between the two genetic

phenomena we tested the strength of purging and genetic linkage in obligate selfing populations exposed to a range of mutation rates.

Populations of *C. elegans* were exposed to different concentrations of the chemical mutagen ethylmethanesulfonate (EMS) for several generations before we measured the mean fitness of each population. After mutagenesis, we found that elevated mutation rates monotonically decreased population mean fitness, thus indicating that purging was overwhelmed at all of the mutation rates we measured. However, exposure to highly elevated mutation rates produced a fitness increase that was greater than the fitness exhibited by populations exposed to moderately elevated mutation rates. The fitness increase is potentially generated by a delicate balance of interactions between induced deleterious mutations and compensatory mutations. Further, the genetic linkage inherent in selfing may facilitate this interaction.

In Chapter V, I summarize the results from chapters II through IV and conclude with a discussion of this work and its broader impacts on mating system evolution and maintenance.

CHAPTER II

MUTATION LOAD AND RAPID ADAPTATION FAVOUR OUTCROSSING OVER
SELF-FERTILIZATION

A paper published in *Nature* and co-authored with Michelle D. Parmenter and Patrick C.
Phillips

The tendency of organisms to reproduce by cross-fertilization despite numerous disadvantages relative to self-fertilization is one of the oldest puzzles in evolutionary biology. For many species, the primary obstacle to the evolution of outcrossing is the cost of producing of males (Maynard Smith 1978), individuals that do not directly contribute offspring and thus diminish the long-term reproductive output of a lineage. Self-fertilizing organisms do not incur the cost of males and therefore should possess at least a two-fold numerical advantage over most outcrossing organisms (Lively and Lloyd 1990). Two competing explanations for the widespread prevalence of outcrossing in nature despite this inherent disadvantage are the avoidance of inbreeding depression generated by selfing (Heller and Maynard Smith 1979; Lande and Schemske 1985; Charlesworth and Charlesworth 1987) and the ability of outcrossing populations to more rapidly adapt to environmental change (Stebbins 1957; Maynard Smith 1978; Crow 1992). Here we

show that outcrossing is favored in populations of *C. elegans* subject to experimental evolution both under conditions of increased mutation rate and during adaptation to a novel environment. In general, fitness increased with increasing rates of outcrossing. Thus, each of the standard explanations for the maintenance of outcrossing are correct, and it is likely that outcrossing is the predominate mode of reproduction in most species because it is favored under ecological conditions that are ubiquitous in natural environments.

The vast majority of animals and plants reproduce by outcrossing, as opposed to self-fertilization. This observation is puzzling because theory suggests selfing enjoys several substantial fitness advantages over outcrossing (Fisher 1941; Williams 1975). For example, selfing results in the production of offspring that are each capable of bearing offspring, whereas many outcrossing species produce males that do not bear offspring. This halving of the number of offspring-bearing progeny an individual can produce is known as the “two-fold cost of males” and generates a large gap between the mating systems in numerical contribution, and thus fitness, over time (Maynard Smith 1978). In addition to this inherent numerical advantage, selfing also efficiently reduces the mutation load over time by eliminating or “purging” new harmful mutations by exposing them to natural selection via the production of homozygous offspring (Lande and Schemske 1985; Charlesworth and Charlesworth 1987). However, if mutations are too numerous or have effect sizes that allow them to slip below the selection threshold, then deleterious mutations can accumulate unchecked within selfing lineages; something that should not happen in outcrossing populations of sufficient size (Heller and Maynard

Smith 1979; Kondrashov 1984; Schultz and Lynch 1997). Further, any new adaptive mutations will tend to become trapped within different selfing lineages because the lack of outcrossing means that any mutations that arise within separate selfing individuals can not be incorporated into the same lineage or genome (Felsenstein 1974; Barton 1995). In this way, selfing mimics the problems associated with asexual reproduction, with outcrossing providing a more effective means of recombination and thereby generating the genetic variation necessary to adapt to a novel environment (Crow 1992). In order to critically evaluate these theoretical predictions, it is necessary to both experimentally manipulate the mating system of a given species and to recapitulate the evolutionary process under the specific conditions predicted to favor either selfing or outcrossing.

Here, we utilize experimental evolution in populations of *Caenorhabditis elegans* to test the benefits of outcrossing relative to selfing under conditions predicted to favor outcrossing. *C. elegans* populations are composed of males and hermaphrodites. Hermaphrodites reproduce through either self-fertilization or by outcrossing with males. Despite the potential for outcrossing with males, most *C. elegans* populations reproduce predominantly via selfing (“wildtype” outcrossing rates are generally less than 5%) (Chasnov and Chow 2002; Stewart and Phillips 2002; Sivasundar and Hey 2003; Barriere and Felix 2005; Haber et al. 2005; Teotónio et al. 2006). However, by incorporating one of two mating system altering mutations (*xol-1* (Miller et al. 1988) and *fog-2* (Schedl and Kimble 1988)), we generated both obligate selfing and obligate outcrossing populations, yielding three different outcrossing levels (obligate selfing, wildtype, obligate outcrossing) within the same genetic background. These mutations were independently

crossed into two separate genetic backgrounds (N2 and CB4856) with known differences in wildtype outcrossing rates (Teotónio et al. 2006). Exposing these populations to two different novel selection environments, (1) elevated mutation rates coupled with a migratory barrier (Figure A2.1a) and (2) a virulent bacterial pathogen (Figure A2.1b), allowed us to directly test theories advocating either deleterious mutations or adaptation to ecological conditions as the primary selective forces contributing to the prevalence of outcrossing as a means of sexual reproduction.

Selfing populations are thought to be able to purge new deleterious mutations as long as the mutations are not too frequent and their effect sizes are large enough to be exposed to selection (Heller and Maynard Smith 1979; Lande and Schemske 1985; Charlesworth and Charlesworth 1987). Indeed, even relatively small *C. elegans* populations have been shown to escape the most serious consequences of mutation accumulation, even when their mutation rate is increased ten-fold (Estes et al. 2004). However, outcrossing is predicted to slow the fixation of deleterious mutations with weak to moderate effect sizes. To explore these contrasting expectations, we subjected populations to the chemical mutagen ethyl methanesulfonate (EMS) every other generation at a level that increases individual mutation rate by approximately four times the natural rate. Populations exposed to the mutagen and populations maintained at natural mutation rates were reared and passaged within a novel environment, a Petri dish transected by a vermiculite barrier separating populations from their food source upon introduction to the dish, to impose strong selection and thereby facilitate the potential to purge deleterious mutations. We then tracked the subsequent evolution of 60 different

populations for 50 generations under different combinations of mutation, mating system, and genetic background.

Despite strong selection against deleterious mutations, obligate selfing populations fixed significantly more mutations than did the obligate outcrossing populations, as evidenced by the fact that the latter populations maintained fitness over the course of the experiment in spite of elevated mutation rates, whereas the selfing populations displayed a substantial decline in fitness (Figure 2.1a; $F_{1,481} = 456.15$, $P < 0.001$). The purging of deleterious mutations within selfing populations is easily overwhelmed by slight increases in mutation rate. In contrast, while outcrossing populations are more likely to accumulate segregating deleterious mutations (Schultz and Lynch 1997), these mutations do not lead to an overall decline in mean fitness (Figure 2.1a). The value of outcrossing is particularly evident in the wildtype populations, where outcrossing rates are free to vary as dictated by selection. The wildtype populations subject to elevated mutation rates exhibit increased levels of outcrossing (Figure 2.1b; $F_{1,8} = 55.7$, $P < 0.001$), thus indicating that increased levels of outcrossing are favored under these conditions.

While fitness loss due to selfing is offset to a large extent by the intermediate amounts of outcrossing exhibited in the wildtype populations, obligate selfing CB4856 populations lose fitness over time even when maintained at their natural mutation rate (Figure 2.1a; $F_{1,481} = 17.5$, $P < 0.001$). We replicated the deterministic loss of fitness in obligate selfing CB4856 populations under long term maintenance in more permissive laboratory conditions as well (20% fitness loss over thirty generations; $F_{3,71} = 9.85$, $P <$

0.001). Indeed, obligate selfing *C. elegans* populations would in general be expected to go extinct over the course of a few hundred generations (Loewe and Cutter 2008).

Several other studies have investigated the role that elevated mutation rates may play in maintaining males within partially selfing *C. elegans* populations, finding that increases in mutation can prolong the maintenance of males in the population, but at levels that are only slightly greater than wildtype (Cutter 2005; Manoel et al. 2007). Therefore, even partial outcrossing is a valuable, if not always sufficient, means of managing the influx of deleterious mutations.

As predicted, outcrossing ameliorates the fixation of deleterious mutations.

However, alternative theories emphasize that outcrossing should enable a stronger and more rapid adaptive response to ecological conditions than selfing (Stebbins 1957; Felsenstein; Maynard Smith 1978; Crow 1992; Barton 1995). Here, outcrossing (wildtype and obligate outcrossing) populations maintained at natural mutation rates exhibited a significantly greater amount of adaptation than the obligate selfing populations after fifty generations of selection, regardless of genetic background (Figure 2.1a; $F_{1,481} = 51.98$, $P < 0.001$). The observed rate of adaptation in the obligate outcrossing populations (0.34% increase in fitness per generation) is particularly impressive because this adaptation occurred in near-isogenic lines over a span of only fifty generations. Thus, the majority of the adaptive response is likely to have been due to novel mutations.

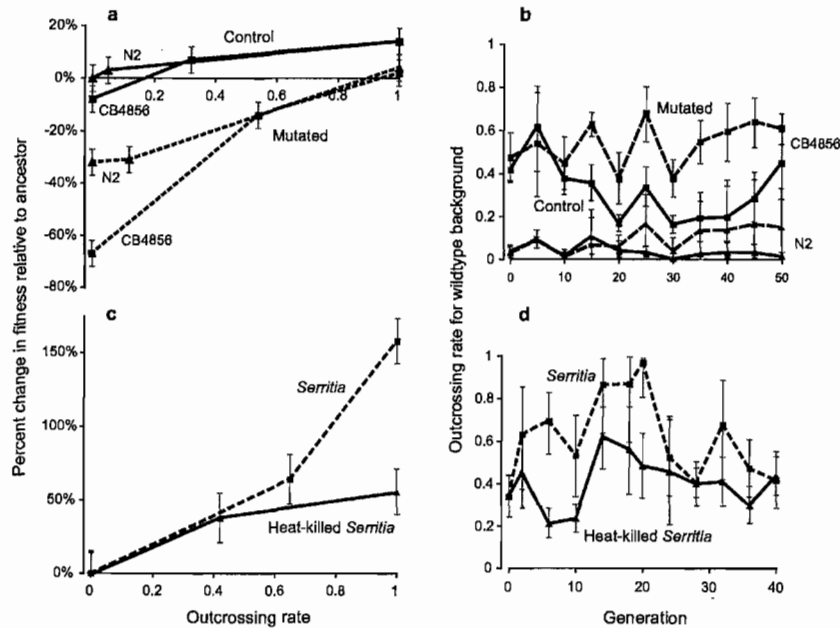


Figure 2.1 | Experimental test of the major theories of the evolution of outcrossing. **a**, Experimental populations (N2, triangles; CB4856, squares) with different outcrossing rates were exposed to a novel, challenging environment at either natural (solid lines) or elevated (4X; dashed lines) mutation rates for 50 generations. Percent change in population mean fitness over time was assessed by comparing the competitive fitness of the ancestral population to that of the evolved population. Obligately selfing populations showed pronounced fitness decline in the face of elevated mutation rates (or even natural mutation rates in the case of CB4856). Both the rate of adaptation and resistance to mutational degradation increased with increasing levels of outcrossing. **b**, Within the wildtype outcrossing treatments, populations exposed to elevated mutation rates evolved higher outcrossing rates. **c**, Experimental populations with a CB4856 background were mutated to generate genetic variation and then exposed to either the bacterial pathogen *S. marcescens* (dashed lines) or heat-killed *S. marcescens* control (solid lines) for forty generations, then percent change in mean fitness measured for each population. The outcrossing populations exhibited both rapid and substantial adaptation to the pathogen, however, the obligate selfing populations failed to adapt. **d**, Populations exposed to *S. marcescens* evolved higher outcrossing rates within the wildtype outcrossing treatment. Thus, in keeping with theory, both the influx of deleterious mutations and adaptation to a novel environment favor outcrossing over selfing. Error bars represent two standard errors of the mean (errors calculated on arcsine-square-root transformed data for **b** and **d**).

To further test the ability of outcrossing to facilitate rapid adaptation, we exposed obligate outcrossing, wildtype, and obligate selfing populations within a common CB4856 background to the bacterial pathogen *Serratia marcescens*. Several strains of *S. marcescens* elicit a pathogen avoidance behavior from *C. elegans* (Pradel et al. 2007), in addition to inducing the expression of a specific set of pathogen resistance genes following ingestion (Mallo et al. 2002). *S. marcescens* 2170 is highly virulent when consumed by *C. elegans*, initially inducing an 80% mortality rate in our experimental regime (Figure A2.1b). Repeated exposures to *S. marcescens* therefore imposes strong selection for either pathogen avoidance or resistance, or a combination of both responses. As a control, replicate populations were passaged on heat-killed *S. marcescens*. Prior to selection on *S. marcescens* the experimental populations were mutagenized with EMS to generate standing genetic variation into the previously inbred experimental populations.

After forty generations of exposure to *S. marcescens*, outcrossing populations adapted to the novel pathogenic conditions whereas the obligate selfing populations did not (Figure 2.1c; $F_{1,80} = 245.79$, $P < 0.001$). The obligate outcrossing populations exhibited very rapid and substantial increases in fitness when exposed to *S. marcescens* (Figure 2.1c; $F_{1,80} = 160.18$, $P < 0.001$). In addition, wildtype mating populations exposed to *S. marcescens* exhibited elevated outcrossing rates (Figure 2.1d; $F_{1,5} = 27.2$, $P = 0.003$) and significantly greater fitness (Figure 2.1c; $F_{1,80} = 9.29$, $P = 0.003$) than wildtype populations maintained on heat-killed *S. marcescens*, indicating that selection favored outcrossing over selfing. In general, outcrossing first increased and then declined over the course of the experiment (approaching its maximum value of 1.0 after 20 generations),

indicating that the change in male frequency is an evolved rather than facultative response (Figure 2.1d). Stronger selection imposed by *S. marcescens* and initial standing genetic variation enabled a much stronger evolutionary response (3.8% increase in fitness per generation) (Figure 2.1c), than that observed in the first experiment (Figure 2.1a). Overall, then, outcrossing enables more rapid adaptation to changing ecological conditions than does selfing.

The prevalence of outcrossing is something of an evolutionary puzzle given the inherent advantages of self-fertilization. This work provides the first experimental tests of the selective pressures favoring the evolution and maintenance of outcrossing. We have demonstrated that outcrossing impedes the fixation of deleterious mutations and facilitates rapid adaptation relative to selfing, such that outcrossing is at the least conditionally favored by selection. Similar results have been observed in accelerated rates of evolutionary change in sexual versus asexual populations (Colegrave 2002; Goddard et al. 2005). While we cannot directly address the question of the origin of selfing and outcrossing in our experiments, overall levels of outcrossing increased in our wildtype treatments in which selfed and outcrossed offspring were competing within the same population (Figures 2.1b,d). These results support the idea that obligate selfing may often be an evolutionary dead-end, in which species that evolve obligate selfing are ultimately doomed to extinction due to an inability to respond to changing environmental conditions (Stebbins 1957).

The fact that obligate outcrossing yielded a much larger response than natural outcrossing rates is something of a surprise, because it is thought that moderate amounts

of outcrossing are sufficient to escape the problems associated with obligate selfing (Schultz and Lynch 1997). One additional feature of this system that has not been previously considered, however, is that an increase in the frequency of males within a population also increases the opportunity for sexual selection, which has been shown to reduce the overall genetic load within a population (Whitlock and Agrawal 2009). Males therefore play multiple roles within these populations, both for enhancing genetic exchange across generations and increasing the efficacy of natural selection within generations. Mutation, changing environmental conditions, and pathogens are nearly ubiquitous selective pressures for many organisms, which likely explains outcrossing's relative prevalence in nature.

Methods Summary

We conducted two large-scale experimental evolution studies. First, we exposed obligate outcrossing, wildtype mating, and obligate selfing populations with approximately five hundred individuals apiece to 0.5 mM of the chemical mutagen EMS every other generation for fifty generations. These mutated populations, in addition to replicate populations maintained at natural mutation rates, were passaged each generation in a selective novel environment (Figure A2.1a). Second, we exposed obligate outcrossing, wildtype mating, and obligate selfing populations composed of approximately five hundred individuals to *S. marcescens* (Figure A2.1b) for forty generations while exposing replicate populations to heat-killed *S. marcescens* as a control. These populations were exposed to 10mM of EMS for four generations prior to selection as a means of inducing genetic variation. We used a competitive fitness assay to

measure the change in fitness for each experimental population relative to its ancestor prior to selection. The competitive fitness assays were conducted within the context of the selective environment and the assay was conducted simultaneously on the experimental population and the previously frozen ancestral population. Fitness was determined by mixing each population (experimental and ancestral) with a GFP-marked tester strain at a 50:50 ratio. After passaging the worms in the relevant selective environment, the GFP ratio of the offspring was calculated and used to estimate fitness. More detailed methods are available in Appendix A.

Bridge to Chapter III

In chapter II we used experimental evolution to test the relative fitness of outcrossing and selfing under conditions of mutation accumulation, exposure to a novel environment, and exposure to a pathogenic bacteria as a means to identify the selective pressures that favor outcrossing, and to reveal the adaptive value of outcrossing. In chapter III we identified facultative outcrossing, a previously unidentified mating strategy, in the primarily self-fertilizing nematode *C. elegans*. Facultative outcrossing is induced in *C. elegans* populations under shifting ecological conditions. Our results from chapter II demonstrate that outcrossing allows more rapid adaptation to changing ecological conditions. Therefore, facultative outcrossing in *C. elegans* may enable more rapid adaptation to stressful environments encountered by natural populations through timely outcrossing.

CHAPTER III

SEXUAL PARTNERS FOR THE STRESSED: FACULTATIVE OUTCROSSING IN
THE SELF-FERTILIZING NEMATODE *CAENORHABDITIS ELEGANS*

A paper published in *Evolution* and co-authored with Brian J. Cappy, Jennifer L. Anderson, and Patrick C. Phillips

Introduction

Sex, although widespread, is theoretically disadvantageous as a consistent reproductive strategy (Maynard Smith 1978). However, facultative sexual reproduction and increased recombination during periods of compromised fitness is predicted to be an evolutionarily stable strategy that could potentially invade both sexual and asexual populations (Hadany and Otto 2007). Environmental stress is known to initiate sexual reproduction in a broad range of species that normally undergo asexual reproduction (Bell 1982; Harris 1989; Dubnau 1991; Kleiven 1992; Gemmill et al. 1997; Dacks and Roger 1999; Mai 2000). Asexual species are subject to deleterious mutation accumulation through Muller's Ratchet as well as a decline in genetic variation due to a lack of recombination (Muller 1964; Gabriel et al. 1993; Lynch et al. 1993). Sex in predominantly asexual organisms is thought to enhance fitness through the infusion of

genetic variation and removal of deleterious mutations, thereby promoting survival and facilitating adaptation under stressful conditions (Muller 1964; Bell 1982; Hoffman 1997; Peck et al. 1999; Colegrave et al. 2002; Kaltz and Bell 2002).

The long-term genetic consequences of obligate self-fertilization closely resemble many of the genetic hazards associated with asexual reproduction, largely because of the systematic loss of genetic variation within lineages due to continuous inbreeding (Stebbins 1957; Heller and Maynard Smith 1979; Kondrashov 1985; Lande and Schemske 1985; Charlesworth et al. 1993; Charlesworth and Charlesworth 1995; Lynch et al. 1995). Extended periods of obligate self-fertilization result in the production of offspring harboring predominantly homozygous loci, which limits the effectiveness of recombination within any given lineage (Heller and Maynard Smith 1979). Extreme inbreeding and the lack of recombination coupled with natural selection and genetic drift result in the consistent loss of population-level genetic diversity within selfing populations. Further, these characteristics of obligate self-fertilization are predicted to reduce the mean time to extinction for selfing populations relative to outcrossing populations (Lynch et al. 1995; Schultz and Lynch 1997). Although currently unexplored, facultative outcrossing may enhance the adaptive response of high selfing populations in the face of environmental stress. Here we explore this possibility using the nematode *Caenorhabditis elegans* as a model system.

A useful study system for examining the potential of stress-induced outcrossing is one that is predominantly self-fertilizing (but is capable of outcrossing), that provides a means of consistently and accurately determining the outcrossing rates within a

population, and that displays a distinct response to environmental stress. The mostly selfing soil nematode *C. elegans* is an ideal system for addressing these questions. *C. elegans* populations are composed of self-fertile hermaphrodites that harbor two copies of the X-chromosome and males with a single X-chromosome as their only sex chromosome (Brenner 1974). Hermaphrodites cannot mate with other hermaphrodites and so outcrossing can only occur via mating with males. Although males facilitate outcrossing, they are rare and tend to be quickly driven out of laboratory populations by their hermaphrodite counterparts (Stewart and Phillips 2002; Cutter 2005; Teotónio et al. 2006). Males and outcrossing also appear to be rare within natural populations (Barriere and Felix 2005; Sivasundar and Hey 2005). Therefore, *C. elegans* populations seem to be predominantly self-fertilizing, but capable of outcrossing in the presence of males. Outcrossing rates within *C. elegans* populations are relatively straightforward to measure. Outcrossing events, the fertilization of eggs (X) by male sperm (X or \emptyset), result in the production of 50% male offspring and 50% hermaphroditic offspring (Nigon 1949). Self-fertilization produces 99.9% hermaphrodites, with rare X-chromosome nondisjunction events resulting in the production of males (0.1%) (Ward and Carrel 1979). After correcting for the number of males produced through X-chromosome nondisjunction, male frequency thus functions as an indicator of the outcrossing rate (Stewart and Phillips 2002).

Early in development and prior to sexual maturation, *C. elegans* larvae that encounter environmental stress (starvation, overcrowding, desiccation, high temperatures) enter a stage of developmental arrest, known as the dauer stage (Cassada

and Russell 1975). This is a migratory non-feeding stage that is common in natural populations and centrally important across most nematode groups (Barriere and Felix 2005). Once the worms reach a new food source in the absence of dauer pheromone (an indicator of overcrowding), they resume normal development. *C. elegans* strains exhibit natural variation for sensitivity to dauer-inducing conditions (Viney et al. 2003), and the genetic basis of the signaling pathway is well characterized (Vowels 1992; Kenyon et al. 1993; Thomas 1993; Gottlieb 1994). Larva can survive in the dauer stage for greater than twice their regular lifespan under normal conditions (Klass and Hirsh 1976; Kenyon et al. 1993). The dauer stage therefore provides a rich ecological and functional context within which to explore the influence of environmental stress on mating system dynamics in populations of *C. elegans*.

Here, we test for stress induced facultative outcrossing directly by repeatedly passing several different natural isolates of *C. elegans* through the dauer stage and observing the subsequent strain specific increases in male frequency. We determine that male frequency can increase both during dauer exposure and in the generation following dauer. We find that an enhanced male presence after dauer coupled with greater outcrossing rates results in a facultative shift in *C. elegans* reproductive strategy from predominantly selfing to primarily outcrossing.

Methods

Population maintenance and dauer induction

C. elegans strains are stock populations originally derived from a single individual isolated from a natural population. Two of these isolates, N2 and CB4856

(originally from Bristol, England and Hawaii, USA, respectively), were obtained from the Caenorhabditis Genetics Center (University of Minnesota, Minneapolis, MN) and one, JU440 (originally obtained from Beauchene, France), was obtained from the laboratory of H. Teotónio (Instituto Gulbenkian de Ciencia, Portugal). The N2 strain maintains males at very low frequencies and has been in a laboratory setting for thousands of generations (Brenner 1974; Teotónio et al. 2006), whereas the JU440 strain is a more recent natural isolate but maintains males at similarly low levels (Teotónio et al. 2006). Like JU440, CB4856 is a relatively recent natural isolate, but maintains males at much higher rates than either N2 or JU440 (Teotónio et al. 2006). These strains were chosen because they are some of the most distinct genotypes that have yet been collected (Haber 2005; M. Rockman, pers. comm.), thus allowing a good sampling of available genetic and phenotypic diversity. All strains were inbred for ten generations before use to minimize within strain genetic variation. Replicate populations were maintained at 20°C on 10cm agar (Nematode Growth Medium Lite, US Biological, Swampscott, MA) plates seeded with OP50 *Escherichia coli* to serve as their bacterial food source. Populations were chunk transferred (approximately five hundred individuals), predominantly as young (L1 or L2) larva to freshly seeded plates each generation (Stiernagle 2006).

Upon transfer, a starting density of approximately 500 nematodes per plate permitted the populations to initially experience standard laboratory conditions but subjected the next generation to dauer-inducing conditions via starvation and overcrowding. The starvation status of populations was determined by assessing the ratio of dauer larvae (measured phenotypically; (Cassada and Russell 1975)) to adults in a

plate transect representing approximately 20% of the total plate area. When the ratio of dauers to adults (L1 and L2 larvae were not counted) was at least 19:1 the population was determined to be “starved.” The dauer ratio of each population was measured daily after initial transfer to a new food source until the population was determined to be starved. Most populations were deemed starved approximately one week after transfer. The populations remained on the same depleted plate for twenty-one days after being identified as starved, and chunk transferred to a fresh food source allowing them to resume development and reproduce.

Effects of starvation stress on male frequency

Populations of each strain (N2, CB4856, and JU440) were subjected to two different starting conditions (no initial males and 10% initial males) and three different treatments (control, single dauer exposure, and successive dauer exposure) within each starting condition. The two starting conditions were chosen to test the response in male frequency based on the initial presence or absence of males. The single dauer exposure treatment was utilized to determine the immediate and long-term effects of a single dauer exposure on male frequency, whereas the successive dauer exposure treatment was used to investigate the compounded effects of multiple dauer exposures. Four replicate populations were run for each combination of strain, starting condition, and treatment. All replicate populations were maintained separately throughout the experiment. Each population was maintained for ten generations.

Populations in the “no initial male” starting condition were composed of approximately five hundred hermaphrodites. Populations that started in the “10% male”

starting condition were composed of approximately fifty males and four hundred and fifty hermaphrodites. The control treatment consisted of populations that were maintained under standard laboratory conditions (not starved). “Single dauer exposure” populations were initially starved for a single generation and then continuously maintained under standard laboratory conditions for the duration of the experiment. The “successive dauer exposure” populations were alternately starved one generation then maintained under standard laboratory conditions the next generation for eight generations, and then maintained under standard laboratory conditions for the final two generations. The first dauer exposure was imposed upon individuals in the second generation of the experiment (Figure 3.1), allowing us to measure male frequency prior to starvation.

Male frequency was assessed each generation for ten generations by sexing worms across a transect representing ~20% of the total plate area and dividing the total number of males counted by the total number of individuals counted (Stewart and Phillips 2002). Male frequency counts were taken three days after transfer to a fresh plate. Only adult and L4 (the latest larval stage) worms were assayed, as only these life-stages exhibit phenotypic sexual differentiation.

The data was analyzed using both a repeated measures categorical data analysis (CATMOD procedure in SAS 9.1, Cary, NC) and a repeated measures MANOVA (JMP-IN 5.1, Cary, NC) testing the effects of strain, initial male presence, dauer exposure, and replicate. The results of both approaches were consistent with one another, so we only report the MANOVA results.

CHANGES IN MALE FREQUENCY DURING DAUER

To examine any possible changes in male frequency while in dauer, we tested male vs. hermaphrodite survival during dauer and hermaphrodite to male sexual conversion during dauer. To assess relative male survival during dauer, replicate populations were chunk transferred to two different freshly seeded plates and were allowed to reproduce and populate the plates. One population was subsequently chunk transferred to a freshly seeded plate and scored for male frequency, while the other population was subjected to dauer exposure for a specific period of time (0, 1, 21, or 42 days), and then chunked to a freshly seeded plate and scored for male frequency. Five replicate population pairs were maintained for each period of time. Changes in relative survival were tested by performing a one-way ANOVA on the log transformed differences in frequency between the treatment and control plates.

Migration

Sex-specific migration in dauer could potentially influence male frequency during dauer. We measured the male frequencies of migrants vs. the source population in dauer using modified “white traps” (Bashey et al. 2007). White traps use liquid to trap migratory individuals by maintaining the source population above but surrounded by liquid so that individuals crawling away from the source population go into the liquid and are unable to return to the source population. To construct the modified white traps, we placed the lid of a 35 x 10mm Petri dish in the center of a 10cm x 15mm Petri dish and placed a piece of 70mm Whatman # 2 filter paper on top of the lid. We then filled the 10cm x 15mm Petri dish with enough S. Basal buffer to fill the dish but not engulf the

filter paper or the lid (approximately 3-4mL). A 60 x 5mm circle of agar (NGM Lite) was placed on top of the filter paper and seeded with OP50 to serve as the base for the source population.

Thirty replicate populations of CB4856 were chunk transferred to the OP50 spots on individual white traps. Approximately ten days after transfer and three days after dauer induction (determined as previously described) individuals were separately removed from the agar (source population) and the buffer (migrants) and transferred to seeded plates permitting sexual maturation. Male frequency was then scored for the source population and the migrants. Only previously dauer individuals were scored, some adult migrants were harvested but not scored. The difference in migrant and source population male frequency was analyzed using a one-way ANOVA.

Sexual conversion

The JK2735 strain, derived from an N2 background, possesses a constitutively expressed GFP-marker on its X-chromosome that is inherited by only hermaphrodite progeny in a cross between a male that carries the marker and an unmarked hermaphrodite. This pattern of inheritance and subsequent expression can serve as an early indicator of sex in the F1 progeny, thus permitting a test of sexual conversion during the dauer stage (Prahlad et al. 2003). The JK2735 GFP-marker was backcrossed into the CB4856 background for 5 generations and inbred for 10 generations to produce the PX360 strain. Ten individual PX360 males, harboring a single green fluorescent protein (GFP)-marked X-chromosome, were mated with approximately two hundred CB4856 hermaphrodites apiece on 35mm agar plates. The large number of

hermaphrodites ensured that the F1 individuals would encounter dauer-inducing conditions. Once in dauer, GFP-expressing and nonGFP-expressing offspring were separated, maintained in the dauer stage for approximately twenty-one days, and monitored for the loss or gain of GFP expression. The dauer worms were then moved to freshly seeded plates to resume development. Upon reaching sexual maturity they were sexed and monitored for the loss or gain of GFP expression. As a control PX360 hermaphrodites were crossed with CB4856 males demonstrating that PX360 x CB4856 crosses can produce males that express GFP.

CHANGES IN MALE FREQUENCY AFTER DAUER

To test for possible changes in male frequency caused by changes in X-chromosome nondisjunction, two hundred L4 hermaphrodites were transferred to twenty replicate populations for each strain (JU440, CB4856, and N2). By starting populations with only hermaphrodites, any male individuals present in the next generation must be the result of X-chromosome nondisjunction (Rose and Baillie 1979). The replicate populations were split evenly into two groups, one exposed to dauer, the other maintained under standard laboratory conditions. Dauer exposure was approximately 21 days. Male frequency was measured in the populations after one generation under their respective rearing conditions. Possible effects of dauer exposure were analyzed using logistic regression in the CATMOD procedure of SAS.

Outcrossing rates

We compared outcrossing rates between group matings in which the males and hermaphrodites were subject to either dauer or standard conditions prior to mating. The

dauer group was starved and allowed to remain in dauer for three weeks then chunked to fresh plates to resume development and mature to the L4 stage, whereas the control group was maintained under standard laboratory conditions. Four L4 hermaphrodites and one L4 male were picked to a single 35mm agar plate seeded with OP50, allowed to mate, and scored for the production of male offspring. Within each strain four crosses were conducted: dauer male x dauer hermaphrodites, dauer male x fed hermaphrodites, fed male x dauer hermaphrodite, fed male x fed hermaphrodite. Each cross was replicated thirteen times within each strain, and the entire assay was replicated twice. The male frequency of each cross was determined by sexing a sample of the progeny across a transect representing ~20% of the total plate area (Stewart and Phillips 2002).

Outcrossing rates were determined by $2(m - \mu)$, where m is the frequency of the male offspring and μ is the rate of X chromosome nondisjunction (modified from Eq. 3 in (Stewart and Phillips 2002)). The strain-specific X chromosome nondisjunction rates (as measured in this study) were used as estimates of μ . The data was analyzed using an ANOVA to test for possible effects of dauer treatment, strain, sex, and dauer status of mate on outcrossing rate. A Tukey's HSD test, testing the effect of having one or both mates experience dauer versus no dauer exposure for either mate, was conducted post-hoc.

EFFECTS OF DAUER ON HERMAPHRODITE SELF FECUNDITY

To determine if elevated outcrossing rates after dauer exposure were the product of sperm-limitation in hermaphrodites or greater mating success by males, we compared the total fecundity of hermaphrodites that experienced dauer exposure to that of

hermaphrodites maintained under standard laboratory conditions. Six replicate populations were established by chunking from a single source population. Three of those replicate populations were exposed to dauer for approximately 21 days, while three were maintained under standard laboratory conditions. Approximately thirty L4 hermaphrodites were sampled from each population (after the dauer populations had resumed development after dauer exposure) and total fecundity calculated for each hermaphrodite. The data was analyzed using a one-way ANOVA.

EFFECTS OF MALE FREQUENCY ON OUTCROSSING RATES

We compared the outcrossing rates in populations started with a broad range of male frequencies. Populations were established by picking twenty L4 worms to a single 5cm agar plate seeded with OP50. Specific numbers of male and hermaphrodites were placed on each plate to generate the desired initial male frequencies (0%, 10%, 20%, 30%, 40%, and 50%). Three replicate plates were established at each initial male frequency. The worms were allowed to mate (hermaphrodites were permitted to self-fertilize in addition to outcrossing with males) and reproduce. Then, the offspring were transferred to a seeded 10cm plate, allowed to reach sexual maturation, and sexed. The outcrossing rates were determined as previously stated. The data was analyzed using regression analysis in JMP-IN 5.1. The outcrossing rate was regressed on the initial male frequency and a stepwise polynomial regression was used to assess the best fitting model.

Results

EFFECTS OF STARVATION STRESS ON MALE FREQUENCY

Successive exposures to the dauer stage permitted males to sweep into populations of the CB4856 and JU440 natural isolates, even into populations in which males were initially absent (Figure 3.1; $F_{1,51} = 124.42$, $P < 0.0001$). Repeated exposure to dauer-inducing conditions was especially effective at generating prolonged maintenance of high male frequencies, with the overall increase dependent on whether males were initially present in the population or not (CB4856 $F_{1,51} = 219.48$, $P < 0.0001$; JU440 $F_{1,51} = 13.47$, $P = 0.0006$). In the most extreme case, replicates of CB4856 moved from 10% males to close to the theoretical maximum of 50% after just two or three exposures to dauer (Figure 3.1a). These increases were sustained as long as the populations continued to experience periodic starvation. In contrast, a single exposure to the dauer stage raised levels of male frequency in the 10% initial male treatments in both the CB4856 and JU440 strains (CB4856 $F_{2,50} = 74.44$, $P < 0.0001$; JU440 $F_{2,50} = 3.44$, $P = 0.0398$), but failed to exhibit prolonged male maintenance (Figure 3.1a,c). The male frequency in all treatments of the N2 strain was unaffected by exposure to the dauer stage ($F_{1,51} = 0.21$, $P = 0.6508$; Figure 3.1e,f). All populations maintained under standard laboratory conditions failed to exhibit a significant increase in male frequency ($F_{9,43} = 1.80$, $P = 0.0965$), while the JU440 and N2 populations rapidly lost males in the 10% initial male treatment (Figure 3.1c,e).

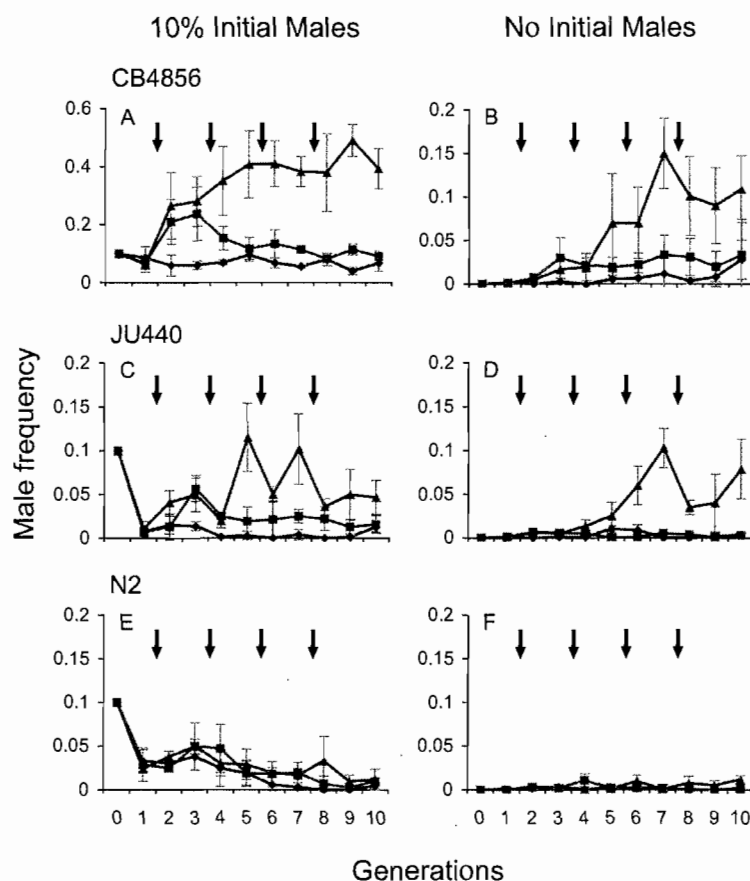


Figure 3.1. Dauer exposure generates strain-specific increases in male frequency. Nematodes were either not exposed to dauer inducing starvation conditions (diamonds), were exposed to a single episode of dauer (squares), or were repeatedly exposed to dauer (triangles). Arrows represent periods of dauer exposure (single dauer exposure occurred at the first arrow). The first point after the arrow represents the frequency of males in populations that have directly experienced dauer. The second point after the arrow represents the frequency of males in the offspring of the individuals that have gone through dauer. (a) CB4856 10% initial male populations, (b) CB4856 no initial male populations, (c) JU440 10% initial male populations, (d) JU440 no initial male populations, (e) N2 10% initial male populations, (f) N2 no initial male populations. The *C. elegans* strains CB4856 and JU440 both exhibit increases in male frequency over time following starvation-induced dauer exposure ($P < 0.0001$). The CB4856 strain approaches a mean male frequency of 50%, which is the theoretical maximum male frequency for *C. elegans* populations. Male frequencies were elevated in these strains regardless of the presence or absence of males upon first dauer exposure. The N2 strain, however, exhibited no male frequency response after dauer exposure ($P = 0.6508$). Data points indicated the mean male frequency (± 2 s.e.) of replicate populations measured over 10 generations.

Causes of elevated male frequency

The elevated male frequencies resulting from dauer exposure can be generated from two possible sources: a change in the male to hermaphrodite ratio during dauer and/or a shift in the reproductive dynamics after exposure to the dauer stage. In the first case, any increase in male frequency immediately following dauer exposure is generated by factors acting directly on individuals experiencing dauer, since reproduction has yet to occur. In the second case, changes in male frequency occur in the generation following dauer exposure and therefore result directly from mating and/or reproduction. We will examine each possible cause of the increase in male frequency in turn.

INCREASE IN MALE FREQUENCY DURING DAUER

The frequency of males within CB4856 populations steadily increases over time while in dauer, indicating either the addition of males or the loss of hermaphrodites ($F_{3,26} = 6.09$, $P = 0.0028$; Figure 3.2). The other strains do not exhibit this effect (instead they tend to lose males during dauer), and so we focus our initial analysis on the CB4856 strain.

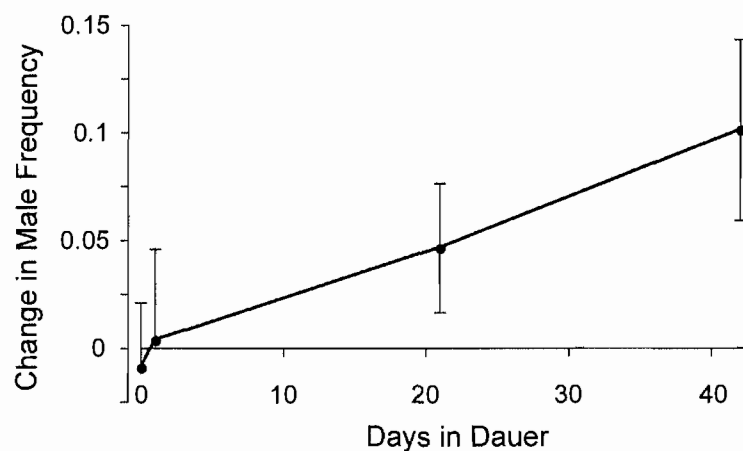


Figure 3.2. CB4856 males survive dauer at rates greater than hermaphrodites. Male frequency increases with time spent in dauer ($P = 0.0028$). The increase in male frequency is a direct result of a greater proportion of males living through dauer, as compared to the proportion of hermaphrodites that survive dauer exposure. The data points represent the change in mean male frequency (± 2 s.e.) of replicate populations exposed to dauer for varying lengths of time.

Sexual conversion

Dauer-induced sexual conversion, the transformation of hermaphrodites into sexually functional males, is a possible source for the increase in male frequency during dauer exposure (Prahlad et al. 2003). Using a GFP-marker to determine sex prior to dauer exposure we found no instances of dauer-induced hermaphrodite to male sexual conversion (0% sexual conversion, power = 80% to determine a 1% conversion rate). A sexual conversion rate of approximately 20% would be required to solely account for the increase in male frequency during dauer. Therefore the increase in male frequency during dauer is not due to sexual conversion.

Migration

The increase in male frequency during dauer is the result of hermaphrodite loss rather than a gain in males. Hermaphrodites could be lost to differential migration rates in dauer, leaving behind greater male frequencies in dauer populations. Using a modified white trap to ensnare migrants from dauer CB4856 populations, we found that the male frequency of migrants was greater than the male frequency of the source populations (migrant mean male frequency = 12.2%, source population mean male frequency = 1.5%; $F_{1,62} = 43.07$, $P < 0.0001$). Therefore, differential migration in dauer decreases, rather than increases, male frequency during dauer exposure.

Differential survival during dauer

If hermaphrodites are not sexually converting into males and differential migration is not driving the increase in male frequency, then the increase in male frequency during dauer is the result of male survival and hermaphrodite mortality while in the dauer stage. We observe a 10% increase in male frequency over a period of 42 days in dauer (Figure 3.2). Male survival coupled with hermaphrodite mortality therefore accounts for the increase in male frequency exhibited in populations that have directly experienced dauer (Figure 3.1).

INCREASE IN MALE FREQUENCY FOLLOWING DAUER

Differential survival of males and hermaphrodites cannot explain the observed subsequent increase in males that occurs in the generation following dauer exposure (Figure 3.1). This delayed response is especially clear in JU440, but is also present in

CB4856. An increase in either the X-chromosome nondisjunction rate or the outcrossing rate is required to explain the increase in male frequency in the generation following exposure to dauer because these individuals did not directly experience the dauer stage.

X-chromosome nondisjunction

Dauer-induced increases in X-chromosome nondisjunction rates could elevate male frequencies in the offspring of dauer-exposed hermaphrodites by increasing the number of spontaneously produced males. However, passage through the dauer stage did not increase the rate of X-chromosome nondisjunction in hermaphrodites ($F_{1,2} = 0.26$, $P = 0.8759$; Figure 3.3). Therefore the elevated male frequencies must be the product of altered mating dynamics after dauer exposure.

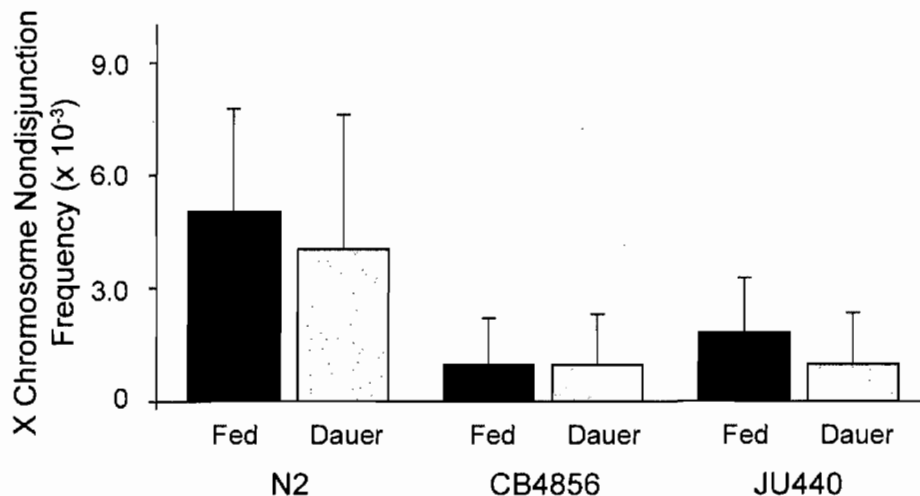


Figure 3.3. X-chromosome nondisjunction events are not responsible for the increase in male frequency after dauer exposure. Exposure to dauer does not increase the X-chromosome nondisjunction rate ($P = 0.8759$). Nondisjunction frequencies were measured in all-hermaphrodite populations after undergoing twenty-one days in dauer. Each bar represents the mean nondisjunction rate (± 2 s.e.) of replicate populations.

Facultative outcrossing

The outcrossing rate in both the CB4856 and JU440 strains increases dramatically following dauer ($F_{1,187} = 58.22$, $P < 0.001$; Figure 3.4). Thus, the environmental stress generated by starvation leads directly to an increase in outcrossing within these two strains. Indeed, outcrossing rates are elevated when at least one of the partners has experienced dauer (Figure 3.4). The increased outcrossing rates are not a consequence of sperm-limitation in dauer-exposed hermaphrodites, as hermaphrodite self-fecundity is not reduced by dauer exposure (CB4856: control mean = 198.8, dauer mean = 211.3; $F_{1,174} = 3.93$, $P = 0.049$; JU440: control mean = 205.1, dauer mean = 216.9; $F_{1,86} = 1.24$, $P = 0.261$). Therefore the increase in outcrossing rate must be the product of more frequent fertilization by males. The effects are nearly additive for the CB4856 strain (i.e., outcrossing rates increase significantly when both sexes have gone through dauer), but saturate in the JU440 strain. These results clearly show that the elevated male frequencies following dauer are the result of an increase in outcrossing.

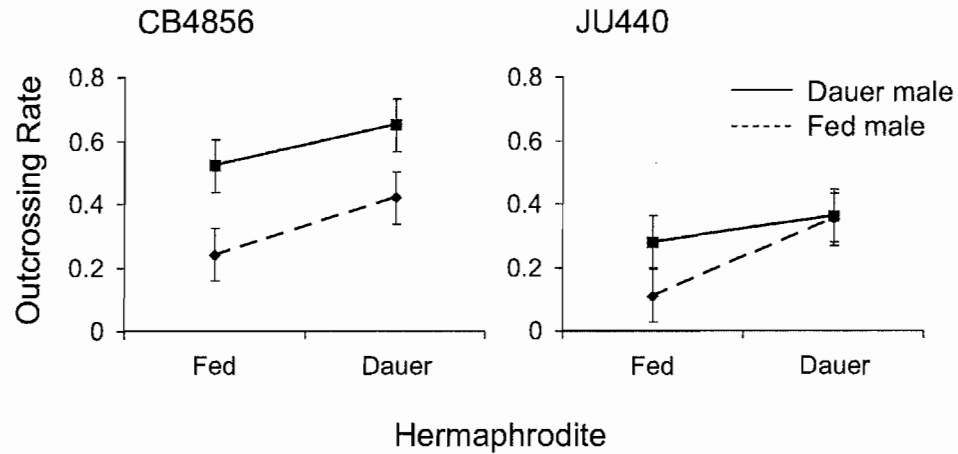


Figure 3.4. Dauer exposure induces facultative outcrossing. Outcrossing rates are elevated when individuals mate with others that have been previously exposed to dauer ($P < 0.001$). The increase in outcrossing occurs whether the male or the hermaphrodite is the partner exposed to dauer. Exposure of both partners further increases outcrossing in CB4856 but yields the same outcrossing rate as single partner exposure in JU440. Data points represent mean outcrossing rates (± 2 s.e.) of replicates for each category of mating.

For the CB4856 strain, greater male survivorship through dauer and the effect of dauer on mating interact synergistically, as the positive correlation between male frequency and outcrossing ($r^2 = 0.91$; Figure 3.5) demonstrates that increases in male survivorship translate directly into heightened outcrossing rates. Therefore, the elevated male frequencies generated through environmental stress via exposure to the dauer stage are the combined result of differential male survival while in the dauer stage and increased outcrossing rates after dauer exposure.

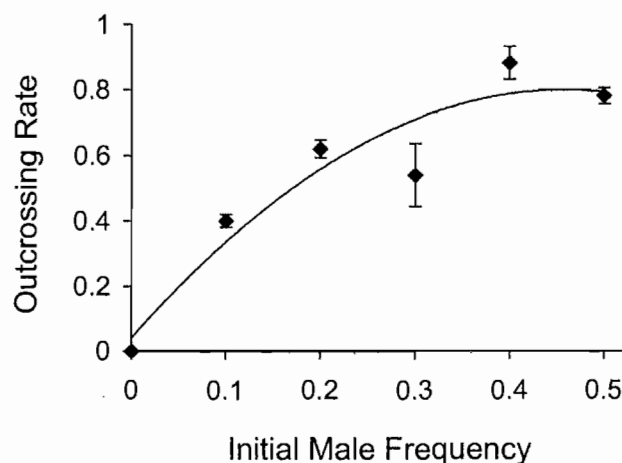


Figure 3.5. Male maintenance function. Population-level outcrossing rates rapidly increase with increasing initial male frequency in CB4856 ($r^2 = 0.91$). Each point shows the outcrossing rate (± 2 s.e.) of replicate populations started at different initial male frequencies. The line shows the best quadratic fit to the data, given by the line outcrossing rate = $0.13 + 0.79 m - 2.04 (m - 0.24)^2$, where m is the initial male frequency. The overall model is highly significant ($F_{2,13} = 70.80$, $P < 0.0001$), as is each individual coefficient ($P < 0.001$).

Discussion

Laboratory populations of *C. elegans* exhibit little or no outcrossing and therefore maintain males poorly (Stewart and Phillips 2002; Cutter 2005; Teotónio et al. 2006). This observation is the basis for the view that *C. elegans* males are evolutionary relics and not functional genetic contributors (Chasnov and Chow 2002). Here, however, we demonstrate that exposure to the dauer stage not only increases male frequency but also elevates outcrossing rates independent of the initial male frequency in two natural isolates of *C. elegans* (Figures 3.1 and 3.5). This shift in mating system dynamics, from predominantly selfing to at least partially outcrossing, is ultimately induced by environmental stress.

FACULTATIVE OUTCROSSING AS EMPLOYED BY *C. ELEGANS*

The shift from asexual to sexual reproduction in most facultative sexual species occurs prior to or during environmental stress. Facultative outcrossing in *C. elegans* occurs after direct environmental stress as the nematodes emerge from dauer and sexually mature. Starvation and overcrowding, stresses that induce dauer formation, signal a need for migration. *C. elegans* populations are predominantly ephemeral in nature (Barriere and Felix 2007), consistently migrating to fresh bacterial blooms and novel locations. Therefore, dauer induction is an indicator of an impending migration, a new food source, and potentially many different environmental conditions that could be encountered in a different location. The dauer stage has adaptive value during stress, whereas facultative outcrossing would presumably generate its value upon colonization, providing the potential to expedite adaption to a novel environment encountered following emergence from dauer.

CAUSES OF FACULTATIVE OUTCROSSING

The increase in outcrossing rates following exposure to dauer can be generated by sex-specific differences in survival during dauer and by dauer-induced changes in mating patterns following dauer exposure. Ailion and Thomas (2000) found that males are more sensitive to the dauer pheromone, entering the dauer stage more readily than hermaphrodites, which should further contribute to the increase in male frequency resulting from dauer exposure apart from male survival. Here, we show that this sex difference is amplified by greater male survival through dauer (Figure 3.2). Although

sex-specific, the disparity between the male and hermaphroditic response and survival in dauer occurs before sexual maturation.

The CB4856 and JU440 strains also exhibited stress-induced increases in outcrossing rates (Figure 3.4). Interestingly, elevated outcrossing rates were not sex-specific, indicating altered mating dynamics in both hermaphrodites and males after dauer exposure (Figure 3.4). The increases in hermaphrodite outcrossing rates are not due to sperm limitation, but rather are driven by interactions between hermaphrodites and males.

Srinivasan et al. (2008) established a potential link between the dauer stage and mating dynamics by demonstrating that a *C. elegans* male attractant was composed of a blend of several dauer-inducing glycolipids. Dauer may induce changes in hermaphrodite mate signaling, receptivity to mating, or sperm preference that enables males to sire a greater proportion of offspring.

The presence of males is required for facultative outcrossing, because males are required for outcrossing. Populations initiated with males experienced a rapid increase in outcrossing rates, exhibiting facultative outcrossing and the subsequent increase in male frequency even after a single exposure to dauer (Figure 3.1). Populations that were established without males were originally dependent upon male production through nondisjunction, requiring more time to generate facultative outcrossing (Figure 3.1). These populations required successive exposures to the dauer stage before males could become established and thereby enhance outcrossing rates. A large proportion of *C. elegans* natural isolates are hermaphrodites, and therefore multiple exposures to dauer

would be required for most natural populations to experience high levels of outcrossing in response to stress (Barriere and Felix 2005; Sivasundar and Hey 2005; Barriere and Felix 2007).

OUTCROSSING WITHIN *C. ELEGANS*

The strains that displayed a reproductive response to dauer, CB4856 and JU440, are more recent natural isolates than the N2 strain, which failed to exhibit facultative outcrossing (Figure 3.1) and is known to suffer developmental defects resulting from dauer exposure (Kim and Paik 2008). An overwhelming proportion of soil natural isolates are found in the dauer stage, indicating that dauer inducing conditions are a consistent selective pressure in natural populations (Barriere and Felix 2005).

Recent studies investigating natural *C. elegans* populations have concluded that outcrossing is usually, but not always, rare (Sivasundar and Hey 2003; Barriere and Felix 2005; Haber et al. 2005; 2005; Barriere and Felix 2007). Natural isolates have been recovered with signatures of periodic outcrossing (Haber et al. 2005; Sivasundar and Hey 2005; Barriere and Felix 2007), leading to speculation that outcrossing may occur intermittently as conditions dictate (Fitch 2005). Barriere and Felix (2007) suggest that outcrossed offspring may be selected against because they observe the stable maintenance of selfing lineages within established populations (see also Dolgin et al. 2007). Although this result is consistent with a disadvantage of outcrossing, it may instead be reflective of a conditional value to outcrossing. Outcrossing may be more beneficial in transient populations or upon colonization than in established populations.

The genetic background of natural isolates may also dictate outcrossing rates. Populations sampled in California (USA) appear to exhibit relatively high levels of outcrossing (Sivasundar and Hey 2005) as compared to populations sampled in France (Barriere and Felix 2005, 2007). These differences could simply reflect strain-specific differences in facultative outcrossing such as those observed in this study (Figure 3.1). Strain-specific differences can alter both dauer induction and life history after dauer (Harvey et al. 2008), in addition to dictating the number of males readily available.

It is also possible that physical outcrossing events fail to leave a genetic signal. Outcrossing events will only appear in microsatellite data if there is sufficient genetic variation present within a population to be shuffled by segregation and recombination. Outcrossing in highly inbred and isolated populations will result in widespread biparental inbreeding and have no effect on the pattern of genetic variation within that population, self-fertilization and outcrossing events would therefore be indistinguishable in sequence data, potentially resulting in downwardly biased estimates of natural outcrossing rates. Recent analysis of a genetic incompatibility system within this species (Seidel et al. 2008) indicates that there has been extensive recombination among strains, even in genomic regions very close to the incompatibility loci. Given the observed strain differences and the temporal nature of dauer-induced facultative outcrossing (Figure 3.1), one would expect that some natural populations would exhibit signs of outcrossing while others may appear as obligate selfers.

EVOLUTIONARY CONSEQUENCES OF FACULTATIVE OUTCROSSING

Through stress-induced facultative sex, normally asexual species utilize sexual reproduction as a novel reproductive strategy to overcome the genetic limitations of asexual reproduction. Theoretical models have demonstrated that alleles that modify the rate of recombination in response to stress can readily invade sexual (Agrawal et al. 2005) and asexual (Hadany and Otto 2007) populations. Selection on induced recombination in diploid populations is weaker because heterozygosity decreases the association between the modifier response and the effects on recombination (Agrawal et al. 2005). Although more theory on this is needed, close inbreeding generates the needed tight linkage between the recombination modifiers and the affected loci, which functionally mimics the asexual situation, and should therefore generate strong selection on stress-induced recombination via outcrossing. Obligate selfers share the same genetic predicament as asexual individuals: a lack of genetic variation within lineages and the potential to accumulate slightly deleterious mutations due to perpetual inbreeding (Heller and Maynard Smith 1979; Kondrashov 1985; Lande and Schemske 1985; Charlesworth et al. 1993). Outcrossing has the potential to introduce genetic variation and allow for the production of offspring harboring fewer deleterious mutations than the parental generation (Heller and Maynard Smith 1979; Bell 1982; Charlesworth et al. 1993; Peck et al. 1999). In this way the genetic consequences of self-fertilization parallel those of asexual reproduction (with the additional complication of homozygosity), and therefore self-fertilizing organisms should also benefit from stress-induced facultative outcrossing.

The long-term evolutionary stability of obligate self-fertilization as a reproductive strategy has long been suspect (Stebbins 1957). In addition to a large body of theoretical work, the phylogenetic positioning of several obligatory selfing species indicates that selfing may be an evolutionary dead-end (Takebayashi and Morrell 2001). Ultimately, all obligate self-fertilizing populations may be at risk of mutation accumulation due to the systematic loss of lineage-specific genetic variation through perpetual inbreeding (Charlesworth et al. 1993; Lynch et al. 1995). The threat of extinction is likely elevated under stressful conditions, as the potential lack of genetic variation at the population level may inhibit adaptation to novel environments. Continued self-fertilization under stress will perpetuate these risks, but timely outcrossing may increase the efficacy of recombination thus providing relief from mutation accumulation and facilitate rapid adaptation. Many plant species once thought to rely solely upon obligate self-fertilization as a reproductive strategy have been found to utilize a broad range of mixed mating strategies by incorporating differing degrees of outcrossing with self-fertilization (Goodwillie et al. 2005). We would therefore predict that stress-induced facultative outcrossing might be a common, but currently unexplored, feature that many partial selfers utilize to periodically generate genetic variation under stressful conditions.

Bridge to Chapter IV

In chapter II we identified deleterious mutations as one potential selective pressure capable of driving the maintenance of outcrossing despite the inherent fitness advantages of self-fertilization. Obligate selfing and mixed mating populations exposed to marginally elevated mutation rates exhibited substantial reductions in fitness. In chapter IV we test the fitness effects of a range of mutation rates in obligate selfing populations. We find that the purging capabilities of self-fertilization are overwhelmed by elevated mutation rates and identify a previously undocumented non-monotonic fitness increase at a mutation rate at least fifteen times greater than the induced mutation rates used in chapter II.

CHAPTER IV

PARADOXICAL INCREASE IN FITNESS WITH INCREASING MUTATION RATE
IN *CAENORHABDITIS ELEGANS*

An unpublished paper co-authored with Aki H. Ohdera and Patrick C. Phillips

Introduction

Although mutations are an essential component of adaptive evolution, most mutations that affect fitness are deleterious (Drake et al. 1998; Keightley and Eyre-Walker 1999). As mutations arise in the genome, selection acts to remove deleterious mutations segregating within natural populations. However, if selection is weak or the expression of a mutation masked by a dominant allele, then deleterious mutations can accumulate in the genome over time (Mukai 1964; Muller 1964). Despite their negative effects on fitness, deleterious mutations are capable of drifting to fixation under certain conditions (Wright 1931; Crow and Kimura 1970; Schultz and Lynch 1997; Charlesworth and Charlesworth 1998; Lande 1998). Fixation of deleterious mutations reduces the mean fitness of a population. The collective effect of fixing multiple deleterious mutations can drastically reduce the mean fitness of a population, particularly if the mutations interact in a negatively synergistic fashion. Although extreme, the

process of fixing deleterious mutations and subsequent fitness decline can perpetuate itself and eventually drive extinction (Gabriel et al. 1993; Schultz and Lynch 1997; Lande 1998; Vassilieva et al. 2000; Morran et al. 2009b). Therefore the ability to curb the accumulation of deleterious mutations is essential for long-term population viability.

Mating systems dictate the way in which mutations can be partitioned among offspring and therefore can have a profound influence on mutation accumulation from generation to generation. Organisms that reproduce through self-fertilization are thought to be at a lower risk of accumulating mutations as compared to outcrossing or asexual organisms, particularly because selfing promotes the expression of recessive alleles (Lande and Schemske 1985; Charlesworth and Charlesworth 1987; Charlesworth et al. 1993). Self-fertilization is the most extreme form of inbreeding, which consequently increases the probability that selfing lineages will harbor homozygous loci relative to either outcrossing or asexual lineages (Stebbins 1957; Lande and Schemske 1985; Charlesworth and Charlesworth 1987). Therefore selfing lineages are more likely to produce offspring that express recessive deleterious mutations and expose deleterious mutations to selection that would otherwise be masked by dominant alleles (Lande and Schemske 1985; Charlesworth and Charlesworth 1987). As these individuals are removed by selection, offspring that did not inherit the mutation are left to propagate the lineage. Such selection facilitates the removal, or purging, of recessive deleterious mutations from lineages and ultimately the population as a whole, assuming that segregation permits the production of offspring that do not carry the mutations.

If selfing facilitates efficient purging, then selfing organisms may be capable of incurring increased mutation rates with few fitness consequences. Conversely, if the influx of deleterious mutations were to overwhelm the purging process by preventing the production of offspring free of newly arisen deleterious mutations, then selfing organisms would be at risk of fixing increasing amounts of deleterious mutations and become subject to a mutation meltdown (Heller and Maynard Smith 1979; Lynch et al. 1995). In general, elevated mutation rates lead to reductions in fitness (Schultz and Lynch 1997). If most mutations are deleterious, then increasing the number of mutations incurred by an individual should lead to the transmission and inheritance of more deleterious mutations. Therefore, successive increases in mutation rate should generate progressively larger reductions in fitness, assuming that the mutations accumulate in the genome and that the effects of the mutations are additive or act synergistically (Schultz and Lynch 1997). These predictions are generally upheld by most studies that have examined the fitness effects of elevated mutation rates (Rosenbluth 1983; Drake et al. 1998; Davies 1999; Manoel et al. 2007; Morran et al. 2009b).

Although individual mutations may be deleterious, genetic linkage has the potential to alter the epistatic interactions between mutations arising within a lineage and thus ultimately influence the fitness effects of those mutations (Heller and Maynard Smith 1979; Hastings 1984). Self-fertilization has a profound influence on the efficacy of recombination, which dictates the scope of genetic linkage within a lineage. As obligate selfing increases the frequency of homozygous loci within a genome, the efficacy of recombination within a lineage becomes limited due to the loss of allelic variants (Heller

and Maynard Smith 1979; Hastings 1984). Therefore selfing lineages generally maintain large portions of their genome in linkage disequilibrium (Charlesworth and Wright 2001). This aspect of selfing should be beneficial when epistatic interactions between mutations reduce their collective effect on fitness, as is the case with compensatory mutations (Phillips et al. 2000). However, such tight linkage may greatly limit the effectiveness of purging via selfing should deleterious mutations at multiple loci be too numerous to segregate out to only a fraction of offspring (Heller and Maynard Smith 1979). If accumulated mutations have reduced the fitness of all genomes in a population, then genetic hitchhiking, facilitated by linkage, could potentially reduce population mean fitness (Hill and Robertson 1966; Lynch et al. 1995; Charlesworth and Charlesworth 1998; Lande 1998). Deleterious mutations that escape purging can be carried to fixation simply due to their association with genomes that have high relative fitness in the population.

We used the predominantly selfing nematode *Caenorhabditis elegans* to test the efficacy of purging and the role of linkage in populations exposed to a range of elevated mutation rates. *C. elegans* is an androdioecious soil nematode with hermaphrodites that reproduce through self-fertilization or outcross with males (Brenner 1974). Importantly, hermaphrodites are incapable of outcrossing with one another. The *xol-1* mutation activates X-chromosome dosage compensation in *C. elegans*, which is necessary to reduce X-chromosome expression in hermaphrodites as they possess two copies of the X-chromosome, but lethal in males because they possess only one copy (Miller et al. 1988). Therefore, *C. elegans* populations harboring the *xol-1* mutation are composed solely of

hermaphrodites and as a result selfing is the only means of reproduction (Morran et al. 2009b).

To elevate mutation rates, we exposed obligate selfing populations of *C. elegans* to increasing concentrations of the chemical mutagen ethyl methanesulfonate (EMS). EMS is commonly used to elevate mutation rates in a wide variety of organisms due to its limited toxicity (relative to other mutagens) and its tendency to induce point mutations, particularly A/T to G/C transitions (Anderson 1995). Another valuable aspect of EMS mutagenesis is that induced mutation rates are positively correlated with increasing EMS concentration, therefore mutation rates can be titrated through EMS exposure (Rosenbluth 1983).

We found that while increases in mutation rates easily overcame any presumed benefits of purging, very high mutation rates yielded an unexpected increase in fitness. This unexpected fitness increase may be the direct result of a previously undocumented interaction between genetic linkage maintained by obligate self-fertilization and increasing rates of beneficial or compensatory mutations at very high mutation rates.

Results

Mutation rate

A study by Rosenbluth and colleagues (Rosenbluth 1983) demonstrated that mutation rates increase exponentially with increasing EMS concentrations at low concentrations (0mM to 30mM) and then increase linearly at greater concentrations (up to 60mM). We tested this relationship at higher concentrations by calculating the relative EMS induced mutation rates at 0mM, 40mM, 80mM, and 100mM by measuring the

reversion rate of *C. elegans* mutants exposed to each EMS concentration. Calculating the reversion rate of a known point mutation with clear phenotypic effects after exposure to EMS is a means of estimating the induced mutation rate of the EMS concentration in question. Based on reversion rate measurements, we found that the mutation rate increased approximately linearly with increasing EMS concentration (Figure 4.1). The induced mutation rate at 100mM EMS was significantly greater than the induced mutation rate at 80mM (Figure 4.1; $F_{1,194} = 26.19$, $P < 0.001$), while the induced rate at 80mM was also significantly greater than that induced by 40mM (Figure 4.1; $F_{1,194} = 7.15$, $P = 0.008$).

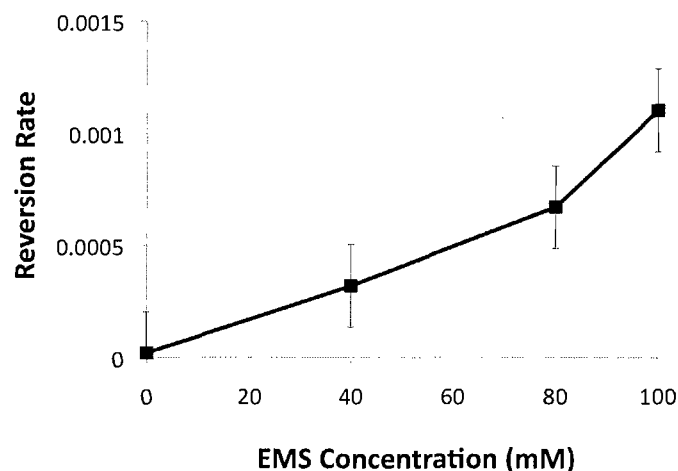


Figure 4.1. EMS induced mutation rates.

Replicate populations of the uncoordinated mutant strain CB665 were mutagenized with a range of EMS concentrations, the F1 generation scored for reversion of the uncoordinated phenotype, and reversion rates calculated for each EMS concentration. The reversion rate scaled with the EMS concentration. The highest EMS concentration, 100mM, induced the highest reversion rate among the concentrations assayed. Error bars represent two standard errors of the mean.

Toxicity

The toxicity of EMS also increases with increasing EMS concentration (Figure 4.2). Although generally considered among the most benign mutagens, EMS is quite toxic at high concentrations, inducing greater than 50% mortality rates (Figure 4.2). The mortality rate induced by exposure to 80mM is significantly greater than that induced by exposure to 40mM (Figure 4.2; $F_{1,36} = 132.84$, $P < 0.001$), however, exposure to 100mM does not induce significantly greater mortality than 80mM (Figure 4.2; $F_{1,36} = 0.49$, $P > 0.05$).

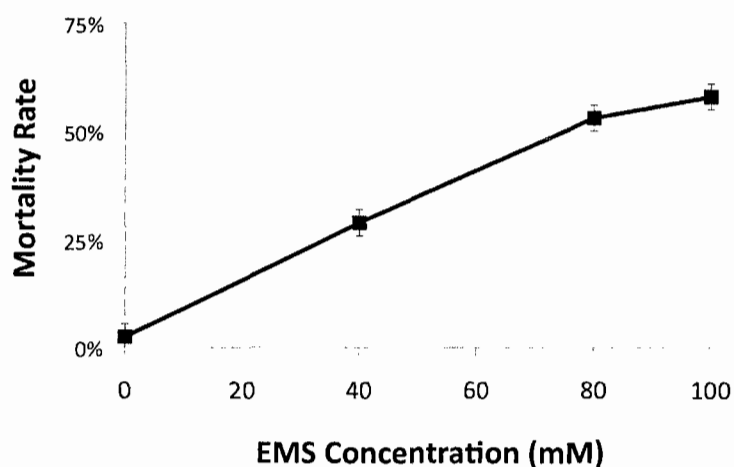


Figure 4.2. EMS induced mortality rates.

Replicate populations of CB665 were exposed to different EMS concentrations. Following mutagenesis the populations were scored for live and dead worms and the mean mortality rate calculated for each EMS concentration. Overall, the EMS induced mean mortality rate, or toxicity, increased with increasing EMS concentration. The 100mM EMS exhibited the greatest level of toxicity. Error bars represent two standard errors of the mean.

Dose response curves

When exposed to increasing EMS concentrations up to 80mM for five generations of mutagenesis, obligate selfing *C. elegans* populations exhibit progressively lower fecundity (Figure 4.3). However, populations exposed to greater EMS concentrations exhibit surprisingly high fecundity under the same experimental regime (Figure 4.3). Fecundity after exposure to 100mM of EMS is comparable to fecundity after exposure to 5mM and 10mM (Figure 4.1; $F_{1,486} = 0.02$, $P > 0.05$) and significantly greater than fecundity after exposure to 20mM, 40mM, and 80mM (Figure 4.1; $F_{1,486} = 28.8$, $P < 0.001$).

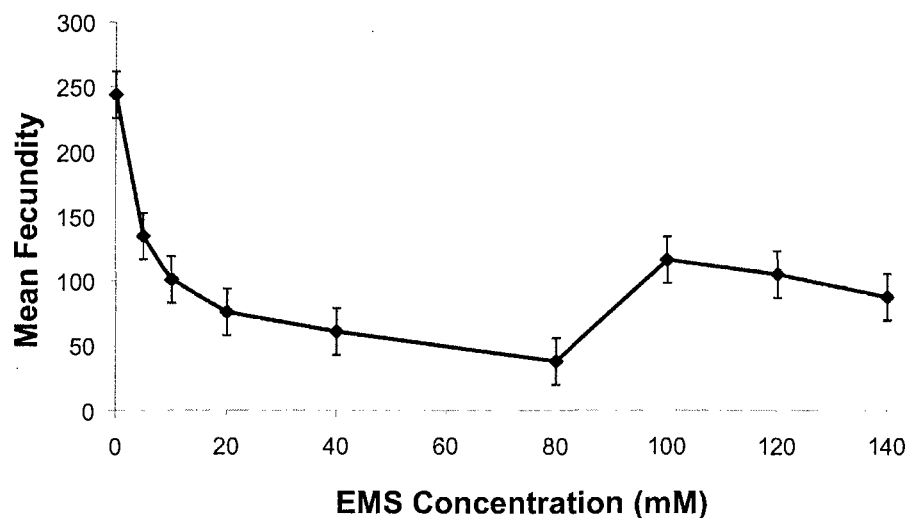


Figure 4.3. EMS dose response curve.

Replicate populations of PX384 were exposed to five generations of mutagenesis across a range of different EMS concentrations. Mean fecundity generally decreased with increasing EMS concentration, however, exposure to 100mM significantly elevated fecundity relative to much lesser concentrations of EMS. Error bars represent two standard errors of the mean.

To test the possibility of pleiotropic effects of *xol-1* driving our results (Figure 4.3), we generated another EMS dose response curve using highly selfing wildtype N2 populations, the same genetic background as was used to generate the previous curve (Figure 4.3) but without the *xol-1* mutation. Although males were produced in these populations, we manually removed them before mating, limiting outcrossing rates to less than 1%. We exposed them to a subset of the EMS concentrations that were investigated in the initial dose response curve, but otherwise maintained identical experimental design. Again, we found that purging was overwhelmed by elevated mutation rates and again we observed the fitness increase at 100mM of EMS (mean fecundity 0mM = 113, mean fecundity 40mM = 41, mean fecundity 100mM = 102; $F_{1,203} = 112.79$, $P < 0.001$).

Next, we tested the role of genetic background in our previous dose response curves. To do this, we consistently exposed populations with a genetic background from a different and highly divergent natural isolate (CB4856 from Hawaii) carrying the *xol-1* mutation to a range of EMS concentrations. After three generations of mutation accumulation we found that purging was again overwhelmed at all concentrations (Figure 4.4). However, prolonging the experiment to five generations of exposure to EMS, as in our previous dose response curves, generated a fitness increase at 100mM (Figure 4.4; $F_{1,225} = 154.67$, $P < 0.001$), while all other concentrations continued to decline in fitness (Figure 4.4). Additionally, the obligate selfing CB4856 populations maintained at natural mutation rates lost fitness over time (Figure 4.4; $F_{2,48} = 9.56$, $P < 0.001$). Therefore, failure to purge deleterious mutations and the ability to recapitulate the unexpected fitness increase at high mutation rates were not dependent upon genetic background.

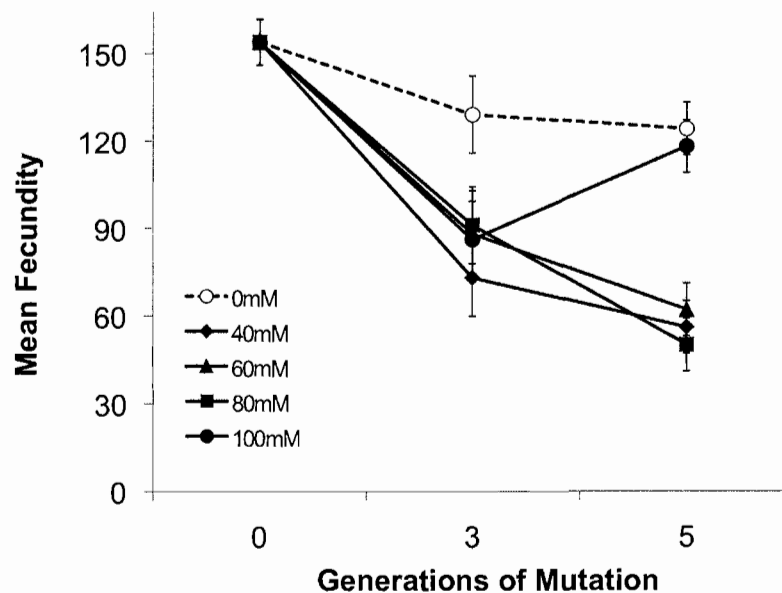


Figure 4.4. Time series EMS dose response curve.

Replicate populations of PX385 were exposed to five generations of mutagenesis across a range of different EMS concentrations. Mean fecundity was assessed prior to mutagenesis, after three generations of mutation, and after five generations of mutation. The mutated populations (solid lines) exhibit reduced mean fecundity, relative to the control populations (dashed line), after three generations. Then, after five generations, the populations exposed to 100mM exhibit a substantial increase in mean fecundity while all of the other mutated populations exhibit further reductions in mean fecundity. Error bars represent two standard errors of the mean.

A similar response is observed when the mutation accumulation process is continued until populations were driven extinct. Populations exposed to 100mM EMS endured significantly more generations of mutation before going extinct as compared to populations exposed to lesser concentrations of EMS (Figure 4.5; $F_{1,16} = 48.78$, $P < 0.001$). The survival time of populations exposed to 100mM EMS was twice that of populations exposed to 80mM EMS (Figure 4.5). Thus the fitness increase at 100mM is not transient, but is persists over time.

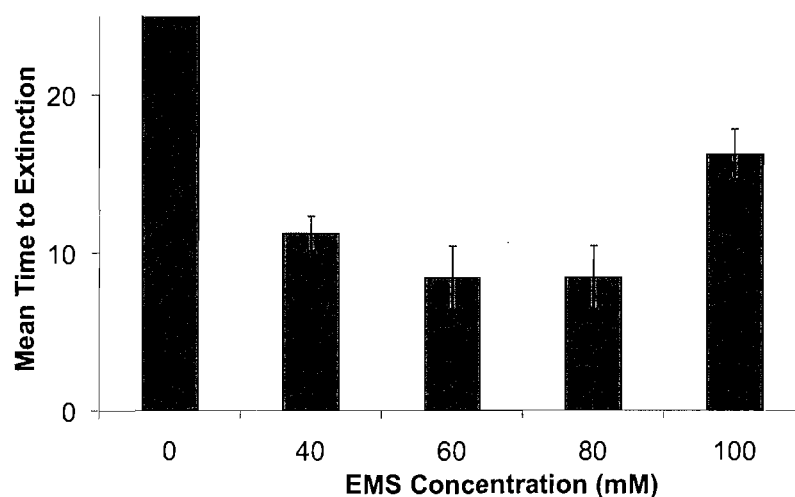


Figure 4.5. EMS induced extinction rates.

Replicate populations of PX385 were continually exposed to a range of different EMS concentrations and driven to extinction. We calculated the mean time to extinction for each EMS concentration. Treatment with 100mM EMS required more generations of exposure to induce extinction than all other EMS treatments. Control populations, with no EMS exposure, did not go extinct during the course of the experiment. Error bars represent two standard errors of the mean.

EMS selection

Might the populations exposed to high concentrations of EMS have simply evolved resistance to EMS itself? We tested this hypothesis by exposing CB4856 populations that had previously exhibited the fitness increase at 100mM to a range of different EMS concentrations for five generations of mutagenesis. If resistance had evolved then we would expect a decreased influence of EMS at all concentrations. Instead, the dose response curve we generated from “pre-adapted” populations (Figure 4.6) closely resembled our original dose response curve (Figure 4.3). The populations exposed to 100mM EMS exhibited a fitness increase relative to the other mutagenized populations (Figure 4.6; $F_{1,275} = 84.68$, $P < 0.001$), and had a higher, but not significantly

different, mean fitness than populations that were not mutagenized for the second dose response curve (Figure 4.6; $F_{1,275} = 1.17$, $P > 0.05$). In other words, further exposure to 100mM EMS generated greater mean fitness than no further exposure to EMS. Therefore, the fitness increase at 100mM is not the product of evolved EMS resistance in populations exposed to 100mM EMS.

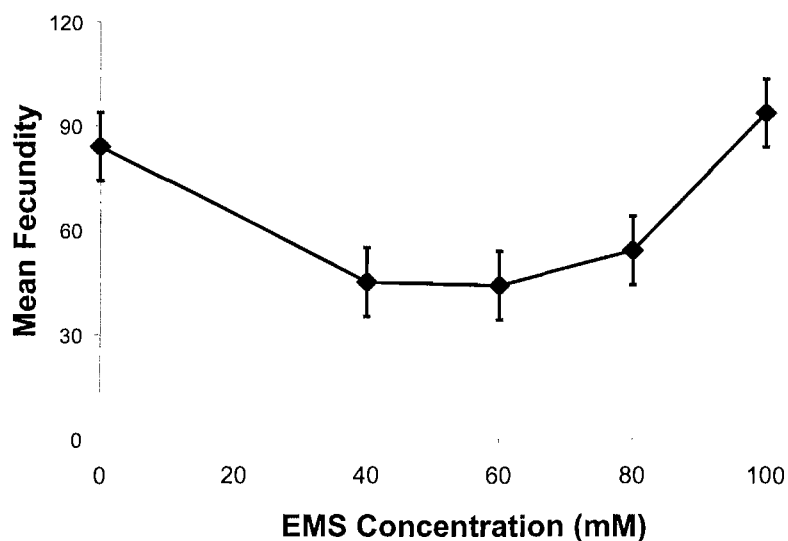


Figure 4.6. Recapitulated EMS dose response curve.

Populations of PX385 that were previously exposed to 100mM for five generations of mutation, and exhibited a fitness increase, were split into replicate populations and exposed to another five generations of mutation at a range of different EMS concentrations. After the second mutagenesis regime the populations exposed to 100mM again exhibited increased fitness relative the other mutagenized populations. Error bars represent two standard errors of the mean.

Discussion

Mutations can interact with mating systems in multiple ways. Most of the work in mating system theory has focused on single locus effects and the fact that potential inbreeding depression can be “purged” from selfing populations by exposing these mutations to natural selection at higher frequency than would be expected in outcrossing

populations (Lande and Schemske 1985; Charlesworth and Charlesworth 1987; Charlesworth et al. 1993). Purging can prevent fitness loss only if all of the newly arisen deleterious mutations are removed from a lineage. Mutations at multiple loci within a lineage have the potential to severely compromise purging and permit mutation accumulation (Heller and Maynard Smith 1979). Mutation can also interact with mating system through linkage. Linkage can permit mutations that accumulate to sweep to fixation through genetic hitchhiking, potentially reducing population mean fitness in selfers due to particularly tight and widespread linkage relative to outcrossing (Hill and Robertson 1966; Hastings 1984; Charlesworth et al. 1993). However, the high levels of linkage disequilibrium generated by selfing may act to increase population mean fitness in the case of compensatory mutations. The epistatic interactions required to maintain the fitness benefits of compensatory mutation are more likely to be maintained in selfing populations relative to outcrossing populations due to linkage.

Purging in obligate selfing populations

We exposed obligate selfing populations to a range of different mutation rates to test the efficacy of purging at multiple loci. As exposure to increasing EMS concentrations increased the mutation rate (Figure 4.1) (Rosenbluth 1983) and thus the number of mutations arising in the genome, we see that purging in obligate selfing *C. elegans* populations is overwhelmed by elevated mutation rates as evidenced by the loss of fitness at all EMS concentrations (Figure 4.3). In fact, even marginal increases in mutation rate are capable of overwhelming purging in obligate selfing populations under strong selection against mutation accumulation (Morran et al. 2009b). Therefore, the

efficacy of purging as a mechanism for preventing mutation accumulation may be quite limited, particularly when dealing with mutations of small to moderate effect size at multiple loci.

Obligate selfing *C. elegans* populations with an N2 background maintain fitness over time when under selection (Morran et al. 2009b). So, it seems that purging at natural mutation rates may be sufficient to prevent mutation accumulation in N2. However, both this study (Figure 4.4) and our previous work (Morran et al. 2009b) demonstrate that obligate selfing populations with a CB4856 background gradually lose fitness over time. Many different factors may contribute to this fitness loss, but it could be that purging in the CB4856 background is an insufficient means of avoiding mutation accumulation. This insufficiency may be the result of a greater natural mutation rate or reduced mutational robustness in the CB4856 strain relative to N2. Such differences in mutational decay have previously been identified in *C. elegans* and among several other nematode species (Baer et al. 2005). Interestingly, the CB4856 strain naturally maintains much greater outcrossing rates than the N2 strain (Teotónio et al. 2006) and does not lose fitness under conditions permitting outcrossing (Morran et al. 2009b). Contrary to selfing, outcrossing is capable of breaking apart groups of linked genes, thus reducing the probability of fixing accumulated mutations (Felsenstein 1974; Barton 1995). It may be that once purging is overwhelmed by mutations at multiple loci, the genetic linkage facilitated by selfing traps populations at a level of reduced fitness.

Fitness increase at high mutation rate

As expected, given that purging was overwhelmed, we found that substantial increases in mutation rate (Figure 4.1) generally led to significantly larger reductions fitness (Figure 4.3). However, contrary to expectation, we identified a non-monotonic fitness response generated by a remarkably high mutation rate (Figure 4.3). By measuring fitness in four separate dose response curve experiments (Figures 4.3, 4.4, 4.6) and measuring extinction rates in populations with prolonged EMS exposure (Figure 4.5), we find that populations in each experiment exhibit a relative increase in fitness after regular exposure to 100mM EMS.

The increase in fitness is a cumulative result of several exposures to 100mM (Figure 4.4), as three generations of mutagenesis were insufficient to drive the fitness increase. Therefore the increase must be driven by a mechanism with a cumulative basis, like mutation accumulation. We tested several aspects of our experimental design to identify a mechanism driving the increase. We see that 100mM induces greater mutation rates (Figure 4.1) and equal or greater toxicity than lesser concentrations of EMS (Figure 4.2). Therefore, the fitness increase is not a direct product of altered mutagenic properties of EMS at 100mM. The fitness increase occurs in N2 populations both with (Figure 4.3) and without the *xol-1* mutation, ruling out the possibility of pleiotropic effects of the *xol-1* mutation. Further, the fitness increase is present in populations with either an N2 (Figure 4.3) or CB4856 (Figure 4.4) genetic background. Finally we found that the fitness increase is not the product of selection during the course of the experiment, as exposure to 100mM did not make the populations more resistant to EMS (Figure 4.6). It may be

that the fitness increase generated by exposure to 100mM EMS is the product of unexpected genomic consequences of high mutation rates coupled with the genomic effects of self-fertilization.

The idea that large increases in mutation rate cause major reductions in fitness is based on the belief that most mutations with fitness effects are deleterious and that the effects of these deleterious mutations are either additive or negatively synergistic. However, compensatory mutations increase fitness by interacting epistatically with deleterious mutations in the genome (Phillips et al. 2000). Therefore, a compensatory mutation itself may have little or perhaps negative fitness effects, but that same mutation has positive fitness effects when expressed in a specific genetic background. Silander et al. (2007) demonstrated that deleterious mutations are context-dependent: as fitness declines, the ratio of beneficial to deleterious mutations increases. Therefore, the classification of mutations as deleterious depends greatly upon the genetic background into which that mutation is incorporated, and fewer mutations have deleterious effects in genetic backgrounds with poor fitness. So, elevating the mutation rate may reduce fitness through the influx of deleterious mutations. However, as fitness declines, the ratio of beneficial to deleterious mutations may shift to a point at which a significant proportion of new mutations are beneficial, or compensatory, and their collective effect begins to elevate fitness, which could be most pronounced at high mutation rates and would likely only materialize after several generations of mutation accumulation.

Compensatory mutation has also been shown to facilitate substantial fitness recovery in mutation accumulation lines and natural populations previously overwhelmed

by accumulated deleterious mutations (Estes and Lynch 2003; Howe and Denver 2008). Such fitness recovery via compensatory mutation may operate at high mutation rates at which the sheer volume of mutations being incorporated into the genome increases the probability of compensatory mutation. The mutation rate induced by 100mM EMS may represent a point at which the dynamics between deleterious mutations and compensatory mutations are somehow more evenly balanced than at other points along the spectrum of mutation rates.

Self-fertilization would play a critical role under this scenario, driving the fitness increase in conjunction with the dynamics of mutation. All of the experimental populations utilized in this study reproduced either predominantly or solely through self-fertilization. The widespread homozygosity resulting from prolonged periods of selfing is a very effective means of maintaining linkage groups (Charlesworth and Wright 2001), especially those favored by selection. If exposure to 100mM were to increase the rate of compensatory mutation relative to lesser EMS concentrations, then selfing would likely permit the epistatic interactions between loci to be maintained for many generations (Hill and Robertson 1966; Heller and Maynard Smith 1979; Felsenstein 1974; Hastings 1984; Barton 1995). Such a phenomenon occurring throughout the genome could potentially lead to increased fitness despite high mutation rates.

Regardless of the mechanism driving the fitness increase exhibited by populations exposed to 100mM EMS, the result is a testament to the resiliency of the genome. Consistent exposure to such high mutation rates should wreak havoc on the genome, and with a mean extinction rate of eight generations of mutation (Figure 4.5) it is fair to say

that exposure to 80mM EMS does just that. However, the genome is able to recover a large proportion of the fitness lost at 80mM when exposed to 100mM EMS (Figure 4.3). This result is quite surprising and challenges the long-held beliefs concerning the relationship between mutation rates and fitness.

Materials and Methods

C. elegans strains are stock populations originally derived from a single individual isolated from a natural population. The N2 and CB665 strains were obtained from the Caenorhabditis Genetics Center (University of Minnesota, Minneapolis, MN). The PX384 and PX385 strains are described in Morran et al. (Morran et al. 2009b).

All populations were reared on agar plates constructed by pouring 24 mL of autoclaved NGM Lite (US Biological, Swampscott, MA) into a 10 cm Petri dish. Each plate was seeded with 5 μ L of OP50 *Escherichia coli*, and all populations maintained at 20°C.

Dose response curves

All dose response curve experiments (Table 4.1) were conducted by exposing approximately one thousand individuals (or the entire population if the census size dropped below one thousand individuals) from each replicate population (Table 4.1) to a specific concentration of EMS (cat. #M0880, Sigma-Aldrich, St. Louis, MO), as described by Anderson (1995), every other generation for ten generations or five mutagenesis events. Control or “0” mM populations were subject to the same buffer and mixing procedures as mutated populations, however no EMS was added to the control populations during mixing. All populations were chunk transferred to freshly seeded

plates every other generation opposite the generations of mutagenesis treatment (Stiernagle 2006).

Table 4.1. Dose response curve logistics.

Dose Response Curve	<i>C. elegans</i> Strain	Genetic Background	<i>xol-1</i>	EMS Concentrations (mM)	Replicate Populations per Concentration
1	PX384	N2	Yes	0, 5, 10, 20, 40, 80, 100, 120, 140	4
2	N2	N2	No	0, 40, 100	3
3	PX385	CB4856	Yes	0, 40, 60, 80, 100	5
4	PX385 Previously mutated at 100mM	CB4856	Yes	0, 40, 60, 80, 100	5

After five generations of mutagenesis (or three generations (Table 4.1)) and a recovery period of at least two generations, we measured the mean lifetime self-fecundity of fifteen to twenty arbitrarily sampled L4 (late stage larval) individuals from each replicate concentration at each EMS concentration. Mean lifetime fecundity can serve as a proxy for fitness in *C. elegans* (Baer et al. 2005). To assess mean lifetime fecundity, single worms were picked to 35 x 10mm plates seeded with OP50 and allowed to self. We counted the total number of offspring per worm four days after picking, allowing time for the offspring to mature to the L3 larval stage, therefore incorporating offspring survival from egg to L3 into our fecundity counts. Individuals that did not produce offspring were counted as having a mean fecundity of zero if the presence of worm tracks indicated that the worm was not killed during transfer. Otherwise worms with no

offspring or tracks were excluded from our analysis. An ANOVA was performed in JMP-IN 5.1 (SAS Institute, Cary, NC) testing the effects of EMS concentration on mean fecundity. Additionally, Tukey's HSD tests were performed post-hoc for specific comparisons between EMS concentrations.

Extinction rate

Four replicate PX385 replicate populations were exposed to 0mM, 40mM, 60mM, 80mM, and 100mM EMS using the same experimental regime employed for running the previously described dose response curves. However, instead of ending the experimental regime after five generations of mutagenesis, we extended the duration of the experiment indefinitely and measured the time to extinction for each population. If one thousand worms were not available we transferred the maximum number possible. The presence of fewer than five worms on a plate was counted as extinction because such low numbers can not be sustained through the buffer and mixing procedures required for EMS mutagenesis (Anderson 1995). An ANOVA was performed in JMP-IN 5.1 testing the effects of EMS concentration on the mean time to extinction. Tukey's HSD tests were performed post-hoc for specific comparisons between EMS concentrations.

Toxicity

Ten replicate populations of PX385 with approximately one thousand L4 stage individuals apiece were given a single exposure to 0mM, 40mM, 80mM, or 100mM EMS. After mutagenesis the worms were transferred to a freshly seeded plate and allowed to mature. Mortality was measured by counting a total number of 200 individuals across a transect representing approximately 20% of the plate and scoring individuals as

either living or dead (by poking the worms and assessing movement). Mortality rates were calculated by determining the frequency of dead worms relative to the total counted. We performed an ANOVA in JMP-IN 5.1 testing the effects of EMS concentration on mortality rate. Tukey's HSD tests were performed post-hoc for specific comparisons between EMS concentrations. The data significantly deviated from normality, so we conducted the nonparametric Wilcoxon signed-rank test, which was in agreement with the ANOVA, but lacks the capability to conduct specific comparisons.

Mutation rate

The mean relative mutation rates of 0mM, 40mM, 80mM, and 100mM EMS were measured with a mutator assay using the CB665 strain, which possess the *unc-58 (e665)* allele. These worms exhibit a dominant uncoordinated phenotype that greatly impairs movement (Park and Horvitz 1986). Reversion of the uncoordinated phenotype is caused by intragenic and extragenic suppressor mutations that restore normal movement (Park and Horvitz 1986). Fifty replicate populations of approximately two thousand L4 individuals apiece were mutated for one generation at each designated EMS concentration. Mutagenesis was conducted as described in Anderson (1995). The populations were transferred to freshly seeded plates after mutagenesis and allowed to self-fertilize. Their adult offspring were then scored for the presence or absence of individuals with restored movement, thus indicating reversion. Then we calculated the mean mutation rate for each EMS concentration.

The total number of mutagenized worms that produced offspring in each population was calculated as:

$$t = x(1 - m),$$

where t is the number of mutagenized worms that produced offspring, m is the mortality rate specific to each EMS concentration, and x is the number of mutagenized individuals.

The estimated number of revertants in each population was calculated as:

$$r = y(z \times t) + y,$$

where r is the estimated number of revertants, y is the measured value of revertants in each population (measured binomially where a value of one indicated the presence of revertants and a value of zero indicated no revertants), and z is probability of multiple reversions occurring in the same population as calculated for each EMS concentration with fifty populations per concentration:

$$z = \frac{\sum_{i=1}^{50} (y/t)}{50},$$

The mutation rate for each population was calculated as:

$$\mu = r/t,$$

where μ is the mutation rate for each population. We then calculated the mean mutation rate for each EMS concentration. We performed an ANOVA in JMP-IN 5.1 testing the effects of EMS concentration on reversion rate. Tukey's HSD tests were performed post-hoc for specific comparisons between EMS concentrations. The data significantly deviated from normality, so we conducted the nonparametric Wilcoxon signed-rank test,

which was in agreement with the ANOVA, but lacks the capability to conduct specific comparisons.

CHAPTER V

CONCLUSION

The unexpected prevalence of outcrossing has been a troubling issue in evolutionary biology for several decades. Many species reproduce via outcrossing despite the numerical cost of producing male offspring relative to self-fertilization and asexual reproduction. Although evolutionary theory had previously identified several potential conditions that could favor outcrossing and male production over selfing and asexual reproduction, no empirical studies had explicitly tested those theories and identified selective pressures capable of facilitating the evolution and maintenance of outcrossing.

We used *C. elegans* as a model system to test both deleterious mutation accumulation and changes in ecological conditions as selective pressures capable of favoring outcrossing over self-fertilization. By genetically manipulating *C. elegans* mating system, we exposed populations with common genetic backgrounds, but different mating systems, to the conditions predicted to favor outcrossing. We found that ability of obligate selfing populations to purge deleterious mutations was compromised with little or no increase in mutation rate. Whereas obligate selfing populations with elevated mutation rates fixed deleterious mutations and lost fitness over time, mutated obligate outcrossing populations gained fitness despite the influx of deleterious mutations.

Exposed to novel rugged terrain or a virulent pathogen, obligate outcrossing populations adapted to the new selective pressure, but the obligate selfing populations failed to exhibit an adaptive response. We further demonstrated the value of outcrossing in preventing the fixation of deleterious mutations and facilitating adaptation by studying populations with mixed mating systems under both conditions. The mixed mating populations were capable of either self-fertilization or outcrossing, and predominantly reproduce via selfing under standard conditions. However, when selective pressure was applied to the mixed mating populations, we found that the populations evolved greater outcrossing rates in response. Therefore, we identified both deleterious mutations and changing ecological or environmental conditions as selective pressures capable of favoring the evolution and maintenance of outcrossing. This is the first work that empirically demonstrates the evolutionary benefits of producing males.

We also identified facultative outcrossing as a novel life history characteristic of *C. elegans*. Although *C. elegans* populations predominantly reproduce through selfing, specific strains of *C. elegans* outcross much more readily after exposure to stressful environmental conditions. Because changing environmental conditions favor outcrossing over selfing, the environmentally induced shift in mating system may confer substantial fitness benefits given the foraging ecology of *C. elegans*. However, facultative outcrossing may not be limited to just *C. elegans*. The ability to recognize specific environmental cues and shift from a highly selfing to outcrossing mating system may be a major life history component of many highly selfing species.

C. elegans males and outcrossing have been the subject of debate in the mating system literature. Because males are generally rare and selfing is the predominant mating system utilized by *C. elegans*, males and outcrossing have been deemed evolutionary relics, still remaining from *C. elegans* dioecious ancestor. Our work refutes these claims and clearly demonstrates the conditional value of males in several strains and necessity of outcrossing in at least one strain.

Given the conditional value of males and outcrossing, facultative outcrossing, rather than obligate outcrossing, may be the most effective means of utilizing males in *C. elegans* populations. It is clear that maintaining high frequencies of males is costly from a reproductive standpoint. However, moderate amounts of outcrossing are sufficient to curb the fixation of deleterious mutations at natural mutation rates and sufficient to generate an adaptive response, though a lesser response than obligate outcrossing. Therefore, facultative outcrossing may be the most effective means of avoiding the cost of consistent or excessive male production and gaining the benefits of periodic bursts of moderate to high levels of outcrossing.

The selective pressures that favor outcrossing over selfing, deleterious mutations and changing ecological conditions, are ubiquitous obstacles which all species encounter. These problems likely plague asexual species as well as selfing species, due to the value of effective recombination in selecting for outcrossing. As asexual lineages do not undergo recombination, outcrossing would likely be favored over asexual reproduction for the given conditions as well. Although selfing and asexual reproduction enjoy an inherent numerical advantage over outcrossing, the prevalence of deleterious mutations

and changing ecological conditions make the prevalence of outcrossing much less troubling for the field of evolutionary biology. Therefore, the initial threat to the theory of evolution by natural selection posed by Maynard Smith's cost of sex model and the prevalence of outcrossing may now be much less imposing. Nonetheless, much work remains in order to develop a fully comprehensive understanding of the evolution and maintenance of mating systems.

APPENDIX

SUPPLEMENTARY INFORMATION: CHAPTER II

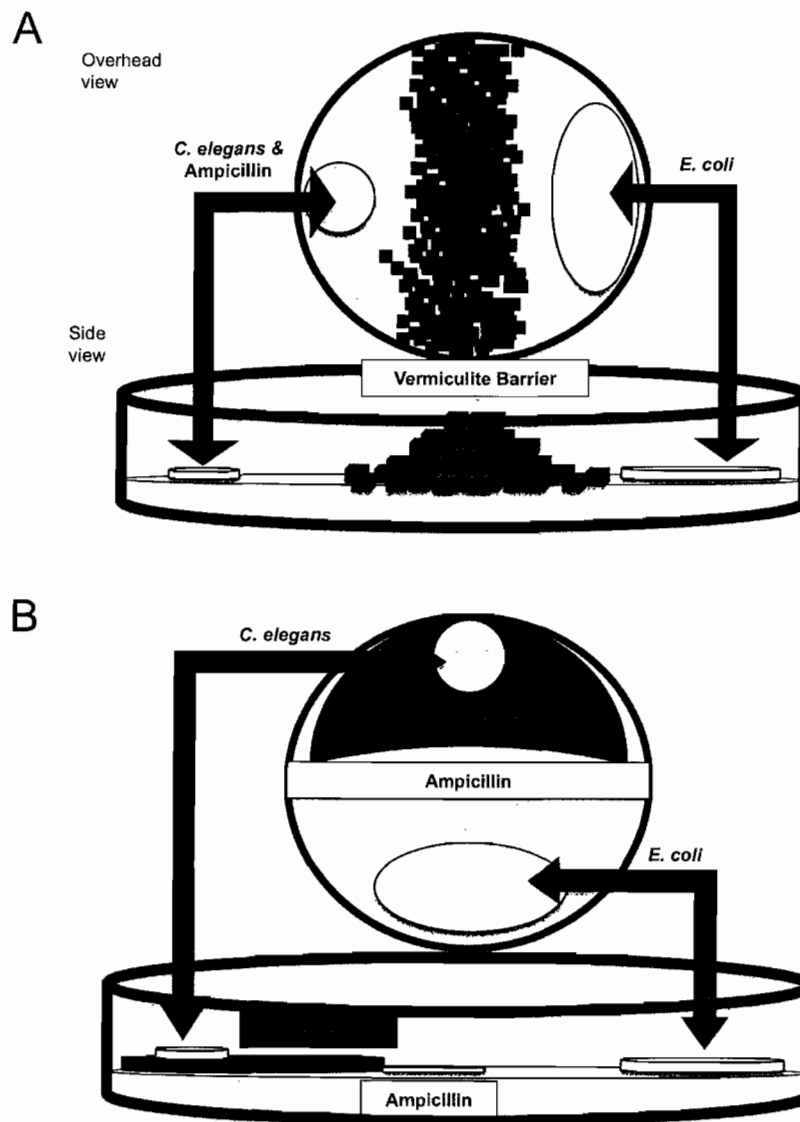


Figure A2.1 | Selective environments for experimental evolution. a. Mountain range plate.**b.** *Serratia* selection plate

Methods

C. elegans strains are stock populations originally derived from a single individual isolated from a natural population. N2 and CB4856 (originally from Bristol, England and Hawaii, USA, respectively) and the GFP marked strain JK2735, were obtained from the Caenorhabditis Genetics Center (University of Minnesota, Minneapolis, MN).

C. elegans is an androdioecious soil nematode with self-fertile hermaphrodites and males that facilitate outcrossing, no outcrossing occurs between hermaphrodites (Brenner 1974). Sex determination in *C. elegans* is genetically based where hermaphrodites harbor two copies of the X-chromosome and males possess a single X-chromosome as their only sex chromosome. *C. elegans* mating system can be manipulated using two specific mutations, *xol-1* and *fog-2*. The *xol-1* mutation induces X chromosome dosage compensation resulting in reduced expression of the X chromosomes (Miller et al. 1988). The *xol-1* mutation is lethal in males because they possess only a single copy of the X chromosome, but is relatively benign in hermaphrodites as they require X chromosome dosage compensation. The removal of males from populations leaves only selfing hermaphrodites, thus producing obligate selfing populations. In contrast the *fog-2* mutation disables sperm production in hermaphrodites generating “female” *C. elegans* (Schedl and Kimble 1988) and obligate outcrossing populations. The *C. elegans* “wildtype” outcrossing rate varies among different strains, although self-fertilization is the prominent means of reproduction in all known strains (Teotónio et al. 2006). In the canonical strain, N2, the outcrossing rate is

substantially less than 1% (Teotónio et al. 2006). However, the CB4856 strain maintains an outcrossing rate between 20% and 40% (Teotónio et al. 2006). By backcrossing the mating system altering mutations separately into both the N2 and CB4856 genetic backgrounds, we generated congenic strains with three different levels of outcrossing (obligate selfing, wildtype, and obligate outcrossing) and identical genetic backgrounds shared between those levels of outcrossing.

Strain Construction

CB4856 and N2 individuals were adapted to the MRP for twenty generations, and then inbred for ten generations via single-worm transfer. The *xol-1* and *fog-2* mutations were separately backcrossed into each strain for five generations and subsequently inbred for ten generations.

MOUNTAIN RANGE PLATE

C. elegans laboratory environment generally exerts very little selective pressure. Individuals with severe disabilities are capable of feeding and reproducing as the worms are essentially surrounded by their bacterial food source. We developed the Mountain Range Plate (MRP) (Figure A2.1a) to apply stronger selective pressure on populations. The MRP environment requires individuals to locate their food and possess the ability to scale steep and uneven terrain to reach their food, mature, and reproduce. This added selective pressure provides the context to observe an adaptive response and facilitates more efficient purging of deleterious mutations in populations subjected to elevated mutation rates.

Table A2.1 | Strain designations, genetic backgrounds, and outcrossing rates of experimental populations generated and used in this study.

Genetic Background	Outcrossing Level		
	Obligate Selfing	Wildtype	Obligate Outcrossing
CB4856	PX384	PX382	PX386
N2	PX385	PX383	PX387

Plate Construction

Mountain range plates (MRP) were constructed by pouring 24 mL of autoclaved NGM Lite (US Biological, Swampscott, MA) into a 10 cm Petri dish. Once the NGM Lite was poured into the dish and still warm, two 5 mL scoops of vermiculite (autoclaved and filtered through a 1mm sieve using only pieces larger than 1mm²) were poured across the agar in a straight line (dividing the dish in half) and maintaining roughly the same height across the vermiculite line. The vermiculite line serves as a barrier across the plate to provide a more complex environment than a standard *C. elegans* Petri dish. One side of the MRP (as divided by the vermiculite) was seeded with 5 μ L of OP50 *Escherichia coli*, requiring the worms to crawl over the vermiculite to reach their food source. All populations maintained on MRP's were stored at 20°C. Passage of a healthy population across the MRP reduces the number of breeding individuals by approximately 60% relative to passage on a plate with no vermiculite barrier ($F_{2,16} = 148.76$, $P < 0.001$).

SERRATIA SELECTION PLATE

We developed the Serratia Selection Plate (SSP) (Figure A2.1b) to ensure that *C. elegans* were exposed to a bacterial pathogen but also to provide us with the opportunity to select those individuals that survived and reproduced on the *E. coli* bacterial lawn. The SSP requires that the worms either resist the *S. marcescens* they consume or avoid consuming *S. marcescens* and then navigate to the *E. coli* and reproduce.

Plate Construction

Serratia selection plates (SSP) were constructed by pouring 24 mL of autoclaved NGM Lite into a 10 cm Petri dish. One side of the plate was seeded with 10 μ L of *S. marcescens* strain 2170 taken from a culture grown overnight in LB at 37°C (Pujol et al. 2001). The control plates were seeded with heat-killed *S. marcescens* that was heated at 80°C for 4 hours and concentrated in a 10mL:1mL ratio prior to seeding (Kurz et al. 2003). The opposite side of the SSP was seeded with 5 μ L of OP50 *Escherichia coli*. After allowing the bacteria to grow at room temperature (~20°C) overnight, 20 μ L of ampicillin (1g/mL) was streaked across the plate between the two bacterial lawns. This setup required the worms to crawl through *S. marcescens* (live or heat-killed) and over ampicillin to reach their food source. All populations maintained on SSP's were stored at 20°C. Passage of a healthy population on the live *S. marcescens* lawn reduces the number of breeding individuals by approximately 80% relative to passage on a plate with heat-killed *S. marcescens* ($F_{1,114} = 1340.7$, $P < 0.001$).

EXPERIMENTAL EVOLUTION ON MOUNTAIN RANGE PLATES

Experimental Design

Five replicate populations of PX382, PX383, PX384, PX385, PX386, and PX387 were exposed to 0.5 mM EMS, every other generation for fifty generations while being passaged on the MRP. Five replicate populations of each strain were also maintained on the MRP for fifty generations with no EMS exposure as a control.

Transfer on MRP's

Approximately five hundred L1-L4 (larval) individuals were transferred in 150 μ L of M9 buffer to the MRP, on the side of the vermiculite that did not contain OP50 bacteria. Populations were transferred to a fresh MRP each generation. To ensure that bacteria transported with the worms did not grow on the transfer side of the vermiculite 20 μ L of ampicillin (1g/mL) was also added to the transfer side in combination with the worms. Approximately 40% of the worms transferred in healthy non-adapted populations reach the bacteria, feed, mature and reproduce, therefore roughly two hundred individuals were successfully transferred each generation. Only the offspring of individuals that reached the bacteria were transferred to a fresh MRP to begin the next generation and repeat the preceding procedure. As mutations accumulated and fitness declined in some populations the rate of successful transfer decreased over time resulting in fewer than five hundred individuals per transfer and fewer than two hundred individuals reaching the bacterial food source. Drift must be partially responsible for the fitness decline in the obligate selfing and wildtype populations once population size was significantly reduced due to mutation accumulation. However, it is unlikely that the initial decline in mean

fitness was the product of drift because the effects of drift should be minimal at the initial population sizes used in our experiment (Schultz and Lynch 1997).

EMS Mutagenesis

We used the chemical mutagen ethyl methanesulfonate (EMS) as a means to elevate mutation rates, therefore allowing us to titrate the influx of mutations into *C. elegans* populations. Mutated populations were exposed to 0.5 mM EMS (cat. # M0880, Sigma-Aldrich, St. Louis, MO), as described in Anderson (1995), every-other generation during transfer between MRP's. Control populations were subject to the same mixing procedures as mutated populations, however no EMS was added to the control populations during mixing.

Calculation of mutation rates

Approximately 92% of the mutations induced by EMS are G/C to A/T transitions (Anderson 1995). Accounting for the number of G/C bases in the haploid genome of *C. elegans* (3.5×10^7 bases) (*C. elegans* Genome Consortium 1998) and the mutation rate at 0.5mM EMS (8.8×10^{-8} mutations per site per generation as estimated from Rosenbluth et al. (1983) our mutagenesis induced approximately 3.1 G/C to A/T transitions per haploid genome per generation as calculated in Davies et al. (1999). *C. elegans* natural mutation rate, 2.1×10^{-8} mutations per site per generation (Denver et al. 2004) in the N2 strain, produces approximately 0.74 G/C to A/T transitions per haploid genome per generation. The EMS concentration of 0.5mM thus elevated the mutation rate to a level that produced G/C to A/T transitions at a rate approximately four times greater than the natural mutation rate.

Competitive fitness assay

Stock populations of the strains PX382, PX383, PX384, PX385, PX386, and PX387 were frozen at generation zero, before mutagenesis. The replicate populations of each strain were frozen after exposure to the MRP for fifty generations (twenty-five generations of exposure to elevated mutation rates in the mutated populations). A sample from all populations at generation zero and generation fifty was thawed and permitted two generations of standard laboratory maintenance (10cm Petri dishes filled with NGM Lite seed with 10 μ L of OP50 stored at 20°C) to recover from thawing.

Three replicate competitive fitness assays were conducted for each replicate population (both control and mutagenized populations) at generation fifty and six replicate competitive fitness assays were conducted for each stock strain at generation zero. Approximately one hundred worms from an individual replicate population were liquid transferred in M9 buffer to an MRP. Approximately one hundred worms from a GFP-marked tester strain, JK2735, were simultaneously transferred to the same location on the same MRP, and 20 μ L of ampicillin also added to the spot (to kill any residual transferred *E. coli*). After allowing the worms to cross the vermiculite barrier and reproduce, the offspring were collected as L4 larva and liquid transferred in M9 buffer to an unseeded 10cm Petri dish. Approximately two hundred individuals were assessed for GFP expression and counted along a cross-section of the Petri dish. The percent change in mean fitness was calculated for each replicate population as $(x - y)/y$, where x is the ratio of replicate population worms from generation fifty to tester strain worms and y is the ratio of replicate populations from generation zero relative to the same tester strain.

A positive percent change in fitness is indicative of fitness gained during the experiment; as the experimental population out-competed the GFP tester strain relative to the generation zero stock performance, thus decreasing the GFP ratio at generation fifty. However, a negative percent change in fitness is indicative of fitness lost during the experiment.

All generation zero populations had comparable fitness as measured relative to the tester strain. One replicate population of the mutagenized PX384 strain went extinct after forty-seven generations and was counted as having a GFP ratio of 1. Another replicate population of the mutagenized PX384 strain failed to cross the vermiculite barrier in any of the replicate competitive fitness assays, therefore it was also counted as having a GFP ratio of 1, as only the tester strain reproduced. The tester strain possessed a dominant marker, therefore cross-progeny between experimental strains and the tester strain would be counted as tester strain progeny in the competitive fitness assay. Because the tester strain possessed few (.01%) males, only the wildtype and obligate outcrossing populations were likely subject to this error. Therefore our measures of competitive fitness in the wildtype and obligate outcrossing populations are potentially underestimates of their fitness. An ANOVA was performed in JMP-IN 5.1 testing the effects of strain, mutagenesis treatment, outcrossing level, all possible interactions, and population nested within strain, mutagenesis treatment, and outcrossing level on the percent change in fitness. Additionally, Tukey's HSD tests were performed post-hoc for specific comparisons.

We present the change in fitness exhibited by the experimental strains relative to the ancestral fitness in Figure 2.1a as a means of reporting evolutionary change. Although the ancestral populations do not differ from one another substantially in terms of mean fitness, we also report the unnormalized fitness of the experimental strains compared directly to the uniform tester strain that was used for every fitness assay (Figure A2.2). Fitness here was calculated $p'/(1-p')$, where p' was the frequency of the experimental strain at the end of the competitive fitness assay, with each strain beginning at an initial frequency of 50%. Subject to the caveat on the competitive fitness assay discussed above, this value provides a lower bound on the fitness measure W_E/W_T , where W_E is the fitness of a given experimental treatment line and W_T is the fitness of the tester strain. Changes in fitness on this scale are large and are qualitatively similar to evolutionary change measured relative to each ancestor (Figure A2.2a), demonstrating that obligate outcrossing populations exhibited significantly greater absolute, as well as relative, fitness than obligate selfing populations after selection.

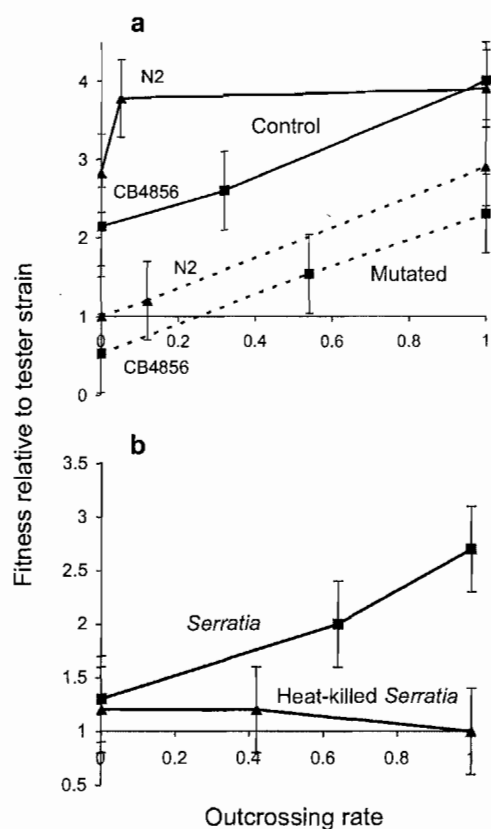


Figure A2.2 | Competitive fitness. Absolute competitive fitness measurements of experimental populations relative to the tester strain fitness. **a**, Experimental populations were exposed to a novel, challenging environment at either natural or elevated (4X) mutation rates for 50 generations. Populations were competed against a marked tester strain to assess fitness in the selective environment after 50 generations of selection. Obligate outcrossing populations exhibited significantly greater fitness than obligate selfing populations (CB4856 $F_{1,481} = 26.41$, $P < 0.001$; N2 $F_{1,481} = 15.95$, $P < 0.001$) when maintained under elevated mutation rates. The N2 obligate outcrossing populations also maintained greater fitness than N2 wildtype populations ($F_{1,481} = 12.73$, $P < 0.001$), however the CB4856 populations were not significantly different. **b**, Experimental populations with a CB4856 background were mutated to generate genetic variation and then exposed to either the bacterial pathogen *S. marcescens* or heat-killed *S. marcescens* for forty generations. Again,

populations were competed against a marked tester strain in the selective environment after selection. The outcrossing populations exhibited both rapid and substantial adaptation to the pathogen, however, the obligate selfing populations failed to adapt ($F_{1,80} = 19.98$, $P < 0.001$). The fitness of the obligate outcrossing populations was not significantly greater than the wildtype populations. Overall, obligate outcrossing not only facilitates greater changes in mean fitness (Figures 2.1a,c), but also permits the maintenance or evolution of greater competitive fitness relative to obligate selfing under the conditions tested. The values presented for the wildtype and obligate outcrossing populations are lower bound estimates of competitive fitness (see “Competitive fitness assay” in Supplementary Information Methods). Error bars represent two standard errors of the mean.

EXPERIMENTAL EVOLUTION ON SERRATIA SELECTION PLATES

EMS Mutagenesis

Seven near-isogenic replicate populations of PX382, PX384, and PX386 were independently mutagenized at 10mM of EMS for 4 hours during four consecutive generations. The populations were maintained separately and received random and independent mutation loads. One population of PX382 suffered major fitness losses as a result of EMS mutagenesis and was discarded before beginning selection on SSP's.

Experimental Design

Seven populations of PX384 and PX386, and six populations of PX382 were passaged on SSP's for forty generations approximately 3 generations after mutagenesis. Replicates of these populations were also maintained on the SSP 's but with heat-killed *S. marcescens* as a control.

Transfer on SSP's

Approximately five hundred L3-L4 (larval) individuals were chunk transferred to the SSP (Figure A2.1). Populations were transferred to a fresh SSP each generation. Only the offspring of individuals that reached the bacteria were transferred to a fresh SSP to begin the next generation and repeat the preceding procedure. On separate occasions, two PX382 and two PX384 populations experienced reductions in population size and were maintained under standard lab conditions for one to three generations to restore the population size before resuming selection. These populations still experienced the full forty generations of selection on the SSP.

Measuring outcrossing in wildtype populations

Outcrossing rates were measured in the same manner the experimental evolution conducted on the MRP's.

Competitive fitness assay

Competitive fitness assays were conducted in the same manner as those run on the MRP's, except that these assays were run on SSP's. The ancestral strains used in these competitive fitness assays were the progenitor strains after mutagenesis, therefore the ancestral strain possessed all of the induced mutations that went into the selection experiment. All generation zero populations had comparable fitness relative to the tester strain. An ANOVA was performed in JMP-IN 5.1 testing the effects of outcrossing level, SSP treatment, population nested within outcrossing level, and all possible interactions on the percent change in mean fitness. Additionally, Tukey's HSD tests were performed post-hoc for specific comparisons.

We present the change in fitness exhibited by the experimental strains relative to the ancestral fitness (Figure 2.1c) as means of reporting the evolutionary change. As for Experiment 1, obligate outcrossing populations exhibited significantly greater absolute fitness than obligate selfing populations after selection (Figure A2.2b).

MEASURING OUTCROSSING RATES IN WILDTYPE POPULATIONS

Male frequency was measured in both the experimental and control replicate populations of PX382 and PX383 every five generations in the EMS mutagenesis experiment and every four generations in the *S. marcescens* experiment. All adult worms were sexed and counted on the MRP or SSP after liquid transfer. *C. elegans* outcrossing

rates were extrapolated from male frequency data. Outcrossing events produce an equal proportion of males and hermaphrodites. EMS is known to elevated outcrossing rates in *C. elegans* populations, this increase in outcrossing is due to the genetic implications of mutagenesis rather than a differential affect on *C. elegans* sexes (Manoel et al. 2007). In addition to outcrossing, males are also spontaneously produced through hermaphroditic self-fertilization if X chromosome nondisjunction occurs during meiosis, however this is a very rare event (Brenner 1974).

EMS exposure increases rates of nondisjunction, therefore strain specific EMS induced nondisjunction rates were used to calculate the outcrossing rate for the mutagenized populations. These rates were determined as previously described (Morran et al. 2009a) with an additional mutagenesis step in which populations of L3 larval individuals were exposed to 0.5 mM of EMS (as described above) the generation prior to scoring for X chromosome nondisjunction. By correcting for the number of males produced through nondisjunction and multiplying the remaining male frequency by two (to account for both males and hermaphrodites produced via outcrossing), we calculated the outcrossing rate (Stewart and Phillips 2002) for each replicate population, we report the mean outcrossing rates of the wildtype populations in Figure 2.1. A repeated measures ANOVA was performed in JMP-IN 5.1 (SAS Institute, Cary, NC) testing the effects of strain, treatment, population (nested within strain), time, and all possible interactions.

LONG-TERM FITNESS OF OBLIGATE SELFING CB4856 POPULATIONS

We assessed the fitness of the PX384 strain maintained for multiple generations under standard lab conditions to determine the consequences of prolonged obligate selfing in the CB4856 background. Ten replicate PX384 populations were maintained at 20°C and stored on 10cm Petri dishes filled with NGM Lite and seeded with 10 μ L of OP50 for thirty generations. Approximately one thousand worms were liquid transferred (1:10 dilution in M9 buffer) every generation to a freshly seeded Petri dish. Samples of each population were frozen every ten generations.

Two samples of each population from each time point, including the stock PX384 at generation zero, were thawed and allowed two generations under standard lab maintenance to recover. After recovery the mean fecundity was determined for each replicate population by measuring the fecundity of twelve randomly sampled individuals from each time point. A repeated measures ANOVA was performed in JMP-IN 5.1 evaluating the effects of generation, freezer sample, population nested within freezer sample, generation by freezer sample, and population [freezer sample] by generation on mean fecundity.

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