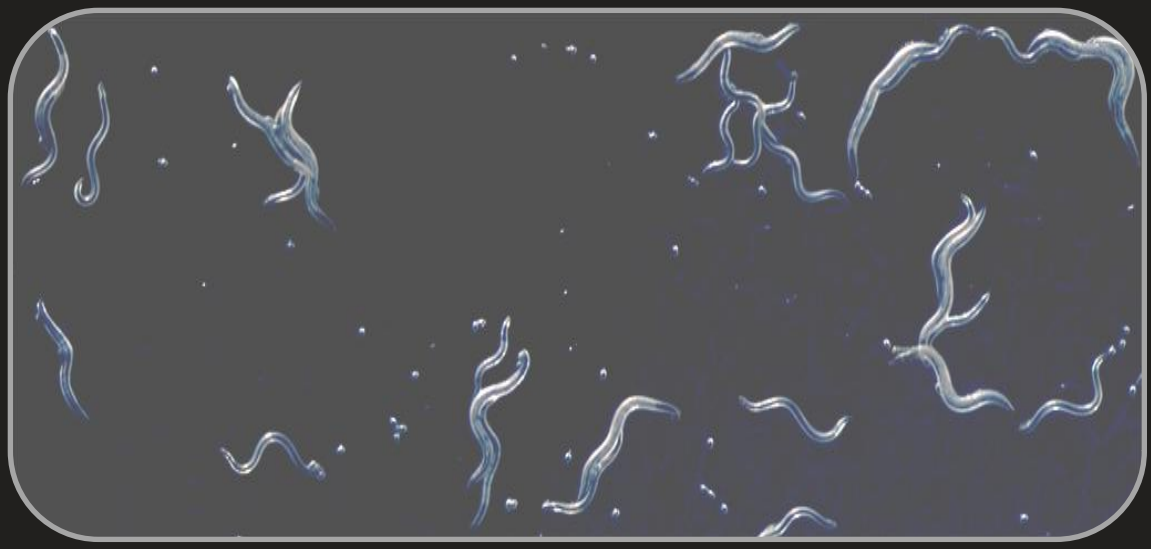
Adaptation from standing genetic variation and from mutation

Experimental evolution of populations of *Caenorhabditis elegans*

Sara Carvalho



Dissertation presented to obtain the Ph.D degree in Biology

Instituto de Tecnologia Química e Biológica | Universidade Nova de Lisboa

Oeiras,
Janeiro, 2012

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Knowledge Creation

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Knowledge Creation

To all the people I love.

Table of contents

List of Figures

List of Tables

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Abstract

Understanding the genetic basis of adaptation is crucial to explain the emergence and maintenance of the multitude of life forms we find on Earth today. Perhaps even more importantly, gaining knowledge about how fast organisms can cope with environmental changes may prove crucial in a world being altered at increasing speed due to the human actions. The study of adaptive evolution may therefore have major implications (and applications) in Agriculture, Conservation of endangered species and even Human Health.

Natural selection has long been appreciated as one of the predominant evolutionary mechanisms and it enjoys a solid theoretical framework regarding its requirements, its effects and its limitations. Empirically, however, it has proved quite challenging to study.

In wild populations natural selection is particularly difficult to characterize and measure since in these settings other evolutionary mechanisms (such as genetic drift or gene flow) often occur simultaneously. In addition to this, the different evolutionary mechanisms may vary greatly in time and in space with respect to their relative influences on the evolutionary dynamics of populations.

The work presented in this thesis constitutes an attempt to describe and characterize adaptive evolution in populations of multicellular organisms at the genotypic and phenotypic levels. Experimentally replicated evolution under controlled conditions may allow us to gain insights into some of the fundamental aspects of adaptation at relatively small evolutionary time scales. Specifically, we investigated the potential contribution of mutation and of pre-existing variation to adaptation. Given the variety of modes of reproduction that can be found among living organisms and the impact these may have on the adaptive potential of populations, the relative roles of mutation and of standing genetic variation to adaptation were investigated in the context of different mating systems.

We performed experimental evolution on a set of populations of the nematode *Caenorhabditis elegans*. Populations differed among themselves in their

initial levels of genetic variation (nearly isogenic or highly polymorphic) and in their mating system - androdioecy or dioecy. Dioecious systems are composed of males and females that reproduce by cross-fertilization. In androdioecious systems self-fertilizing hermaphrodites co-occur with males, with whom they can also cross-fertilize. The differences in initial levels of genetic variation allowed us to characterize adaptation from pre-existing variation or from mutational input exclusively. The two mating systems differ in the levels of homozygosity that are generated. Androdioecious populations are highly homozygous and thus experience reduced effective genetic recombination; this greatly decreases the opportunity for new genotypes to be created even if reasonable levels of genetic variation are present. Dioecious populations exhibit higher heterozygosity and thus represent the conditions where genotypic diversity can be maximized. We started our study by confirming the success of our experimental design through characterization of levels of genetic variation and heterozygosity at nine microsatellite loci in ancestral experimental populations (**Chapter 2**). The genetic differences measured closely matched our expectations. We then compared the fitness of ancestral and evolved populations to infer adaptation. Fitness increased with experimental evolution and, interestingly, adaptation correlated with rates of cross-fertilization (outcrossing) of experimental populations. Because of this, we compared the evolution of components of fitness of populations with different rates of outcrossing, that is, dioecious and androdioecious populations (**Chapter 3**). Our results show extensive response of most phenotypes measured. They further reveal that selection favoring outcrossing may generate a conflict between selective pressures acting on male and female components of fitness if both types of components are expressed in the same individual, as is the case of hermaphrodites. Having performed the characterization of experimental populations at the genetic, phenotypic and fitness level and under different mating systems, we investigated the evolution of mixed mating systems themselves by describing the dynamics of invasion of selfing or obligate outcrossing alleles into our experimental populations (**Chapter 4**). The results confirm theoretical

expectations that the spread of an allele increasing self-fertilization is contingent on the genetic background in which it arises. Finally, we measured the extent of fitness increase of populations relying on mutation as unique source of genetic variation upon which selection could act (**Chapter 5**). We find that beneficial mutations may contribute significantly to adaptation in relatively short timescales. Since patterns of phenotypic evolution of reproduction and survival traits closely match those produced under conditions of abundant genetic variation, we conclude that different sources and amounts of genetic variation may influence rates of adaptation but may not necessarily lead to different patterns of phenotypic evolution.

Altogether, our results show that outcrossing facilitates adaptation independently of the source of genetic variation. In the context of our experiment, this phenotype determines effective rates of recombination between alleles from different loci. We therefore argue that allelic interactions between loci (epistasis) determine to a large extent the levels of additive genetic variance for fitness. The quantification and characterization of these interactions is thus required for a deeper understanding of the adaptive process.

Resumo

Compreender a base genética de adaptação dos seres vivos a novos ambientes é fundamental para explicar a génese e persistência da variedade de formas de vida que podemos encontrar hoje na Terra. Num mundo em mudança a um ritmo cada vez maior - devido em grande parte às acções humanas - o conhecimento acerca da capacidade dos organisms de reagirem e lidarem com estas alterações reveste-se de um carácter crucial. O estudo de evolução adaptativa poderá, pois, ter enormes implicações (e aplicações) em áreas como a Agricultura, a Conservação de espécies ameaçadas ou mesmo a Saúde.

A selecção natural é desde há muito reconhecida como um dos mecanismos evolutivos predominantes e apresenta actualmente um sólido suporte teórico no que diz respeito aos seus requisitos, aos seus efeitos e às suas limitações. Contudo, revela-se extremamente difícil de estudar empiricamente.

A caracterização e quantificação do processo de selecção natural em populações selvagens é particularmente desafiante, uma vez que nesse contexto outros mecanismos evolutivos (tais como deriva e fluxo genético) ocorrem com frequência em simultâneo. A este facto acresce ainda a agravante de todos estes mecanismos poderem variar no tempo e no espaço nas suas contribuições para a dinâmica evolutiva das populações.

O trabalho aqui apresentado sob a forma de tese traduz uma tentativa de descrever e caracterizar a evolução adaptativa em populações de organismos multicelulares ao nível genético e fenotípico. A evolução experimental, onde as condições ambientais podem ser controladas e monitorizadas e onde a replicação é possível, pode constituir uma ferramenta excepcional na busca de respostas sobre alguns dos aspectos mais fundamentais do processo adaptativo a escalas temporais relativamente curtas. Mais especificamente, ao longo deste trabalho investigamos quais as potenciais contribuições de a) mutação e de b) variação genética já existente para a adaptação. Dada ainda a diversidade de formas sob as quais a reprodução pode ocorrer entre organismos e os efeitos que estas

podem ter no potencial adaptativo das populações, os contributos da mutação e da variação genética pré-existente para a adaptação foram investigados no contexto de diferentes sistemas de reprodução.

Deixámos um conjunto de populações do nemátode *Caenorhabditis elegans* evoluir em condições de exposição a um ambiente novo (em laboratório). As populações utilizadas diferiam entre si nos níveis de diversidade genética que exibiam (praticamente isogénicas ou altamente polimórficas) e também no tipo de sistema de reprodução – androdioécia ou dioécia. Os sistemas dióicos são constituídos por machos e fêmeas que se reproduzem por fertilização cruzada. Os sistemas androdióicos exibem a co-segregação de hermafroditas capazes de se auto-fertilizar e de machos, com os quais pode ocorrer fertilização cruzada. Os diferentes níveis iniciais de diversidade genética exibidos pelas populações experimentais permitiram-nos caracterizar a adaptação no contexto de segregação de diversidade genética ou no contexto de evolução exclusivamente por mutação. Por outro lado, os diferentes sistemas de reprodução têem consequências directas nos níveis de homozigotia das populações. As populações androdióicas exibem elevados níveis de homozigotia, facto pelo qual os efeitos da recombinação genética são severamente limitados; como consequência, a possibilidade de gerar novos genótipos é grandemente afectada, mesmo que as populações exibam níveis de variação genética consideráveis. Nas populações dióicas os níveis de homozigotia são baixos, pelo que apresentam as condições sob as quais os a diversidade genética pode ser maximizada.

Para confirmar o êxito do desenho experimental em gerar as populações com as características desejadas, iniciámos o nosso estudo com a quantificação dos níveis de variação genética e dos níveis de heterozigotia em 9 microsatélites nas populações experimentais ancestrais (Capítulo 2). As medidas obtidas corresponderam às esperadas. Seguidamente, comparámos a aptidão (*fitness*, na terminologia anglo-saxónica) das populações ancestrais e das populações evoluídas para confirmar a ocorrência de adaptação. Não só verificámos que a aptidão aumentou com o decorrer da evolução experimental, como encontramos

ainda uma forte correlação entre os níveis de adaptação das populações experimentais e as suas taxas de fertilização cruzada. Perante estas observações, comparámos os padrões de evolução de vários componentes de aptidão de populações com diferentes taxas de fertilização cruzada, ou seja, os padrões exibidos pelas populações dióicas e pelas populações androdióicas (Capítulo 3).

Os nossos resultados demonstram que a resposta da maior parte dos fenótipos medidos foi elevada. Os dados sugerem ainda que a acção positiva da selecção no sentido de aumentar as taxas de fertilização cruzada pode gerar um conflito entre forças selectivas actuando em níves de organização mais baixos. Mais concretamente, se as forças selectivas que actuam sobre o componente masculino de aptidão não forem compatíveis com as forças selectivas que actuam sobre o componente feminino de aptidão, pode gerar-se antagonismo se ambos os componentes forem expressos num mesmo indivíduo, como é o caso de hermafroditas.

Uma vez concluída a caracterização das populações experimentais ao nível genético, fenotípico e de aptidão no contexto dos diferentes sistemas de reprodução, resolvemos testar a evolução dos próprios sistemas de reprodução. Mais especicamente, testámos algumas hipóteses evolutivas para a emergência e estabilidade de sistemas de reprodução mistos, ou seja, nos quais ocorrem simultaneamente auto fertilização e fertilização cruzada. Para tal, descrevemos a dinâmica de invasão de um alelo que confere a possibilidade de auto-fertilização ou de um alelo que impõe fertilização cruzada nas nossas populações experimentais (Capítulo 4). Os resultados confirmam as previsões teóricas de que a capacidade de invasão de um alelo que confira a capacidade para auto-fertilização é determinada pelo contexto genético em que esse alelo surge. Por fim, quantificámos também o grau de aumento de aptidão nas populações cuja eficiência da selecção dependia da diversidade genética que fosse criada por mutação (Capítulo 5). Verificámos que as mutações benéficas podem contribuir de forma significativa para a adaptação em escalas temporais relativamente

curtas. Os padrões de evolução fenotípica de características relacionadas com sobrevivência e reprodução que encontrámos foram surpreendemente semelhantes aos previamente descritos para as populações genéticamente diversas. Desta forma, concluimos que as diversas fontes de variação genética podem determinar as taxas de adaptação mas não conduzem necessariamente a padrões de evolução fenotípica distintos.

Os nossos resultados demonstram, na sua totalidade, que a fertilização cruzada facilita a adaptação, independemente da fonte de variação genética. No contexto da nossas condições experimentais, as taxas de fertilização cruzada determinam a extensão dos efeitos da recombinação genética antre alelos em diferentes *loci*. Sugerimos, pois, que as interacções entre alelos de *loci* diferentes (epistasia) determinam em grande parte os níveis de variância genética aditiva para aptidão. A quantificação e caracterização destas interacções é assim crucial para permitir uma compreensão mais profunda do processo adaptativo.

Chapter 1

Introduction

“Happy is the person who can learn the nature of things”

Virgil (70-19 B. C.)

Contemporary society has lost much of the curiosity of observing the natural world, an old tradition kept from Ancient Greece to the 20th century. However, thoughts such as “Is *that* an animal?“ or “Why would a living creature do *that*?” have surely crossed your mind while watching documentaries about nature on TV. The fact is that it is almost inevitable to be amazed by the number of different living creatures on Earth, as well as by their colors, shapes and habits, many times bizarre or simply beautiful. How were they generated? And why did they become these life forms we see today and not others?

1.1 And yet it…changes

For many centuries, the only explanations for the origin of the Earth and the living beings in it relied in the intervention of supernatural entities. As such, the natural world was believed to be perfect and immutable and Man the highest manifestation of this perfection. The discoveries made in the 17th century in the fields of Physics, Astronomy (on the origin of stars and planets) and Geology (that Earth underwent massive changes since its origin), lead to a progressive loss of the view of the world a static entity. Alternative explanations of natural phenomena (other than by supernatural agents) were then put forward, supported by arguments provided by Natural Sciences. Biological thought was extremely influenced by these ideological revolutions, especially those debated among geologists. Of particular importance was the notion that the geological processes that shaped Earth in the past were the same acting in the present: the changes that had occurred millions of years before should therefore be explained by the same phenomena that could be observed in the present. This idea expanded to Biological Sciences where naturalists - whose work until then consisted mostly of cataloguing and ordering the diversity of the natural world – started developing the idea that living beings could *change* and that singular modifications occurred in different species because they were exposed to different environmental challenges.

“In one of the most breathtaking ideas in the history of science” (Futuyma, 2005), Charles Darwin proposed his theory of evolution. Darwin hypothesized that different organisms arose from common ancestors and that evolution proceeded by means of natural selection. Darwinian Theory advocated that all living (and extinct) species descended from a few or a single form of life (the principle of common descent), being very similar among themselves in the beginning but then progressively accumulating differences over long spans of time (across generations), ultimately leading to very different forms.

With the unraveling of the mechanisms of inheritance - the particulate nature of genes (Gregor Mendel, 1822-1884), the discovery of the processes of mutation and genetic recombination, the foundation of population genetics and the study of genetic variation in natural populations - Darwin’s Theory of Evolution gave rise to The Modern Synthesis. The work of T. H. Morgan (1866-1945), Sewall Wright (1889-1988), R. A. Fisher (1890–1962), J. B. S. Haldane (1892-1964), T. Dobzhansky (1900-1975) and E. Mayr (1904-2005) among others, reconciled the principles of genetics with the theory of evolution by means of natural selection.

The study of evolution is more than a historical curiosity about the processes that have led to the diversity of life forms we find on Earth today. It provides useful insights into many different fields ranging from Human Health to Agriculture, Conservation of endangered species or even Economy. It can help address questions regarding how bacteria evolve resistance to antibiotics, how a few cells evolve into the severe cancer forms known today, how crops are able to withstand particular environmental conditions and parasites or which type of strategies should be implemented to ensure the long term viability of natural populations.

1.1.2 Evolution and adaptation

Many of the questions just outlined are a matter or adaptation - that is, the need of organisms to cope with novel conditions and challenges. In the light of the exampled provided before: bacteria exposed to new antibiotics, cancer cells fighting different immune systems or populations exposed to new culture fields.

While evolution can be broadly defined as changes in the genetic composition (the allele frequencies) of populations with time, the particular instance in which such changes produce populations able to perform better in their environment is called adaptation or adaptive evolution. Natural selection is the predominant mechanism by which adaptive evolution occurs. Although allele frequencies change over the course of one (or more) generations by the action of natural selection, this mechanism does not operate directly on alleles, since they do not exist as single, independent units in the natural world. Instead, alleles are grouped in the form of genomes and contained within individuals. Therefore, understanding adaptation implies understanding the relationship between genotypes (sets of alleles), phenotypes (sets of traits) and fitness (a particular phenotype).

In single-cell organisms such as bacteria and yeast, in which most or all life cycle occurs in the haploid state (meaning that each chromosome is present in the genome as one single copy), the mapping of genotypes onto phenotypes is relatively simple. In diploid organisms, which possess two copies of each chromosome, the relationship between the genotypes and the phenotypes - the so-called genotype-phenotype map - is not as straightforward since alleles at each locus might interact not only with alleles at other loci (epistasis), but also with the other allele present in the same locus (dominance). Furthermore, most diploid organisms are multicellular, which means that developmental processes are responsible for producing a whole individual from the genetic information contained in one cell, the zygote. Diploidy and multicellularity therefore translate into a many-to-one or one-to-many relationship between genotypes and phenotypes (Figure 1.1). Any quantifiable property of organisms can be defined as a phenotype. As a result, phenotypes can be assigned to different levels of biological complexity: for example, the level of expression of a particular gene during embryonic development or the acoustic repertoire exhibited during mating season. They can also be discrete variables such as the different mating types or sexes (or their frequencies). In the context of adaptive evolution, fitness is a phenotype of particular interest.

Despite its pivotal role in evolutionary theory, the definition of fitness, its mathematical formulation and its measurement are still matter of confusion and debate (Orr 2009). It is therefore important to clarify some aspects related to it, since they are embedded in most of the experimental approaches taken in the present study.

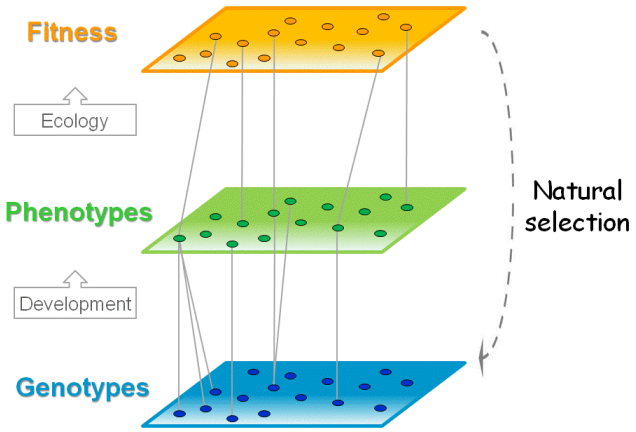

Figure 1.1: Schematic representation of the relationship between genotypes, phenotypes and fitness. Each plane represents a different level of biological organization. Blue circles represent genotypes present in a population. Genotypes give rise to individuals (green circles) occupying a multidimensional phenotypic space (represented here only by two dimensions). Different combinations of phenotypes will differ in their ability to cope with their environment, thus differing in their fitness values (orange circles). Natural selection operates on the distribution of fitness values, shaping the genetic composition of the following generation (adapted from Lewontin, 1974).

As mentioned previously, individuals within populations often differ between themselves in some respects (phenotypes). Such differences make individuals with some particular phenotypic combinations perform better in their environment than others. Fitness is a variable that measures this performance – that is, it embodies the extent to which the different types of individuals match their selective environment. In the absence of other evolutionary forces, the representation of the different types of individuals in the following generation is proportional to their fitnesses. The importance of the concept of fitness stems precisely from its predictive power with regards to evolutionary dynamics: if the

distribution of fitness values among individuals of a population is known, it is in principle possible to project how the relative proportions of the different individuals (and hence alleles) will change over one generation in the absence of other evolutionary forces.

Several aspects should be drawn to attention concerning the definition of fitness just presented. First, and analogously to the *definition* of random variable and of *realization* of a random variable, fitness should be viewed as the *propensity* of individuals with certain phenotypic combinations to be represented to a certain extent in the following generation(s) and not as a certainty (see Mills and Beatty 1979 for the propensity definition of fitness). The point here is that it might be that, by chance, the best individual in a population dies before it reproduces - for example, if it's killed by a meteorite. This doesn't mean that its fitness was zero – *on average*, individuals as good as the one that died would increase in frequency in the population due to their superior performance in their environmental context. Second, the systematically different "propensities" of individuals to be represented in the following generation(s) must necessarily rely on different *intrinsic biological properties* (Maynard Smith, 1978). It should then be possible to assign the *contributions* of each of such properties to fitness. This is to say that fitness embodies the combination of all the phenotypes that can be defined for an individual, but some phenotypes contribute to a larger extent than others. For example, in most cases, phenotypes related to survival (such as egg-to-adult survivorship) and to reproduction (as, for instance, fecundity) are closely associated with fitness. In fact, life-history traits in general are often referred to as fitness components (Stearns 1992). A third aspect about fitness is that its relationship with the intrinsic biological properties is expected to be context-dependent. For example, having a green body in a tropical forest might make individuals of a certain species less likely to be seen by a predator and have better chances of surviving relative to white-colored conspecifics, while the opposite could be expected if the same forms inhabited the North Pole. This context-dependency encompasses both the biotic and abiotic aspects of the environment.

In spite of the apparent complexity of the concept of fitness and the innumerous and inflamed debates it has generated, the truth is that evolutionary biologists have used relatively uniform techniques to measure it, as we will see next. In A. Orr's words: "(…) It is often easier to perform experiments on fitness than to think clearly about it. Our difficulties are, in other words, more often conceptual than empirical" (Orr 2009). Under the action of natural selection, the representation of different individuals is expected to be proportional to their fitnesses. As a result, one of the most widely used methods to infer or quantify fitness is to measure the per capita growth rate, that is, the number of individuals generated by a particular type over the course of one (or more) generation(s). Generally, however, it is of interest to know how the fitness of *populations* (and not of particular types of individuals within them) evolves. Therefore, *mean population fitness* – the fitnesses of the different types of individuals weighted by their relative frequencies – is often the parameter to be estimated. Under most circumstances, population growth rate can be used as a reliable proxy for mean population fitness in both discrete and continuous time models (but see Chevin 2011). In the first case, the mathematical relationship between the two variables is the following: $\bar{w} = \frac{N_{t+1}}{N_t}$, where $\bar{w}$ is the mean population fitness, N_t is the population census size at generation *t* and N_{t+1} is the population census size in the following generation.

Another way to measure population fitness is in a competitive context. For example, pairs of populations can be co-cultured at known frequencies and the change in their relative frequencies over the course of one generation taken as a measure of their relative fitnesses. When many populations are to be measured and fitness values compared between them, it is often easier to establish one *standard* (or *tester*) population and compete all populations against that same reference. The resulting fitness measures are therefore standardized and can be directly compared. In fact, direct competition tests among types have been recently suggested to provide the most reliable quantification of fitness that can be achieved experimentally (Wagner 2009). This methodology was used in early

studies with Drosophila in the 70's and 80's (Ayala 1970,Yamazaki 1984) but it became more broadly employed recently, in studies involving microbial populations (Lenski 1991). In these organisms, cryopreservation and revival of populations is possible, for which ancestral and experimentally evolved populations can be competed directly and the hypothesis that populations adapted can be tested.

Because the differences between individuals must have a genetic basis in order to be inherited by their offspring, adaptive evolution is therefore dependent on the genetic variation for fitness. More specifically, it depends on the portion of such variation that can be transmitted across generations - the *additive genetic variance*. The relative change in the mean fitness of a population over one generation is in fact *proportional* to the additive genetic variance for fitness present in that population. This is the Fundamental Theorem of Natural Selection, derived by R. A. Fisher (Fisher 1930). Mathematically, it is expressed as follows: $\Delta\bar{w} = \frac{V_A(w)}{\bar{w}}$, where $\bar{w}$ represents mean population fitness (as defined above) and $V_A(w)$ represents additive genetic variance for fitness. The additive genetic variance for fitness (or any other phenotype) measures the variance as predicted from a linear (additive) regression of fitness on genotype. As any regression, its intercept and slope depend on the relative proportions of the values of *x* - in this context, the frequency of individuals with each of the genotypes (Figure 1.2).

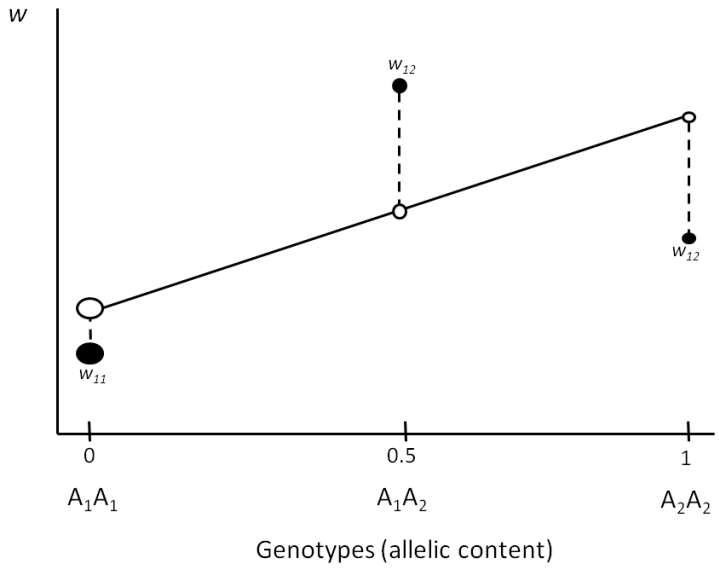

Figure 1.2: Additive genetic variation. Solid dots represent actual fitness of the genotypes, their sizes being proportional to their frequencies in the population. The line is the least square linear regression on these values. Open circles represent the estimates of the genotypic fitnesses based on the regression. Additive genetic variance for fitness is the variance among these values (graphic representation from Rice 2004).

Additive genetic variance therefore depends on the genotypic frequencies and on the variation of (fitness) values among genotypes.

While genetic variation for fitness is required as a substrate for natural selection (and hence adaptation), natural selection tends to erode that same variation by systematically increasing the frequency of some allelic variants (until potential fixation) and decreasing others (until they potentially disappear from the population). It is therefore of the utmost importance to understand how genetic variation is recurrently generated and maintained in populations of organisms.

1.1.3 Mutation and standing genetic variation

Mutation is a process by which new alleles are generated and it therefore constitutes the ultimate source of genetic variation. New alleles can have deleterious or beneficial effects on the fitness of the individuals in which they arise. It can also be the case that novel alleles will not significantly change the fitness of

their carriers. These selectively neutral alleles can persist in populations and segregate at considerable frequencies. Upon environmental change, the selection coefficients of such alleles may change and they might prove themselves significantly beneficial (or deleterious) under the new set of conditions. Adaptation can thus also occur from the standing genetic variation.

Mutation

Mutations result from errors in DNA replication during cell division (mitosis and meiosis). If such errors are not detected and repaired (and occur in the germline, in the case of multicellular organisms), *novel* alleles are generated. The rate of production of mutations varies greatly among organisms. Within microorganisms it can range from 0.003 mutations per genome per replication (bacteriophages, bacteria, yeast), to 1 mutation per genome per replication (lytic RNA viruses) (Drake *et al* 1998). With respect to multicellular organisms, genomic mutation rates can vary at least one order of magnitude: 0.018 mutations per genome per replication in *C. elegans*, 0.058 mutations per genome per replication in *Drosophila melanogaster*, 0.49 mutations per genome per replication in mouse and 0.16 mutations per genome per replication in humans (Drake *et al* 1998). The mutations that are generated spontaneously can be broadly categorized into three different classes regarding their effects on the fitness of the individuals carrying them: deleterious, neutral and beneficial (adaptive).

Deleterious mutations are those which decrease the fitness of the individuals which harbor them. They are thought to constitute the vast majority of mutations that are generated every reproductive cycle. This is partly because most populations have been evolving for a considerable amount of time and they are therefore considered to be reasonably well adapted to their environments. In this case, new mutations are more likely to disrupt some aspect of the organism's biology than to produce even fitter genotypes. This seems indeed to be case, as the effects of novel mutations are, on average, deleterious for a broad range of organisms (Martin and Lenormand 2006). Deleterious mutations are by far the

most studied class (both theoretically and empirically), not only because they seem to constitute the most frequent class but also because of their implications in many evolutionary phenomena such as the origin and maintenance of sexual reproduction (Kondrashov 1988, Barton and Charlesworth 1998, Otto and Marks, 1996), the evolution of recombination (Otto and Feldman 1997), the evolution of self-fertilization (Uyenoyama and Waller 1991), the maintenance of genetic and phenotypic diversity (Barton and Turelli 1989, Charlesworth *et al* 1993), the fate of small populations (Kondrashov 1995, Lande 1998), sexual selection (Burt 1995) and senescence (Partridge and Barton 1993).

Neutral mutations are those who do not exert any effect on the fitness of their carriers. They are therefore impossible to detect phenotypically and their study requires direct analysis of the genome.

Beneficial mutations are those which promote adaptive evolution since they increase the fitness of the genotypes in which they arise. Despite the fact that adaptive mutations might be quite rare - from 0% (Elena *et al* 2007) to 6% (Thatcher 1998) – they *do* occur and they can have a tremendous impact on evolutionary change. For example, adaptive substitutions might compose up to 15% of all nucleotide substitutions in the genome of *Drosophila melanogaster* (Andolfatto 2005). In another example, adaptive mutations were sufficient to restore ancestral fitness values in a few tens of generation in lines of *C. elegans* whose fitness had been reduced by 25% (Denver *et al* 2010). With regards to this class of mutations, theoretical work largely exceeds empirical evidence, composed mostly of studies in microorganisms and hence of limited scope. Fisher's Geometrical model adaptation (Fisher 1930) and further extensions of it (Orr 2000, 2006; Otto and Jones 2000, Rozen *et al* 2002, Martin and Lenormand 2006) seem to provide thus far the most robust predictions about the distributions of effects of adaptive mutations both before and after a bout of adaptation and are thus among the best working hypothesis for the study of adaptive evolution. Fisher's model can be illustrated in a simple, graphical way (Figure 1.3).

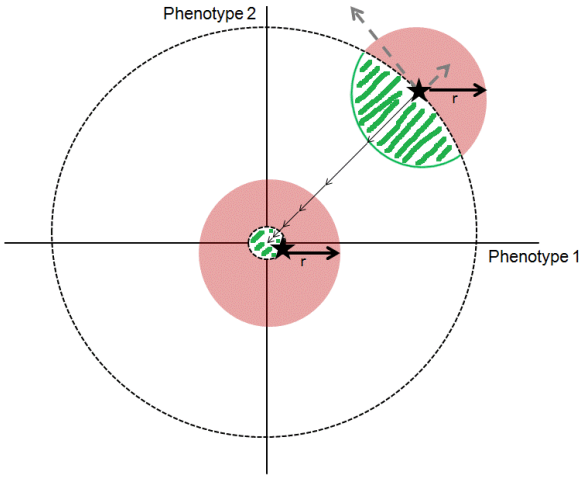

Figure 1.3: Fisher's geometric model of adaptation. The two black stars represent populations at different distances from the highest fitness phenotypic combination (the origin of the graph). Dashed circles represent phenotypic spaces for which fitness does not change. Dashed arrows illustrate mutations with different qualitative (direction) and quantitative (length) effects on fitness. Areas shaded in red represent phenotypic combinations with lower fitness that can be produced by mutations of effect ≤ r; areas shaded in green represent phenotypic combinations of higher fitness that can be produced by mutations of effect ≤ r. Thin arrows illustrate an adaptive walk based on the accumulation of adaptive mutations of successively smaller effects.

Briefly, let's assume the fitness of individuals is determined by only two (independent) phenotypes. The origin of the graph represents the best combination of phenotypic values (that with the highest fitness); distance from the origin therefore reflects the fitness difference relative to the best phenotypic combination. Because mutations are random, they can move the population in any direction (that is, to have different effects on fitness) and by any distance (they can have different magnitude of effects). Adaptation reflects a successive approximation to the phenotypic combination of highest fitness. The first clear prediction of this model is that a larger fraction of mutations will be adaptive when populations are further from the optimum than when they are closer (green-shaded areas in Figure 1.3). The other important prediction of Fisher's model concerns the magnitude of effects of mutations. While adaptive mutations of large phenotypic

effects have some probability of increasing fitness in populations which find themselves far from the optimum, in populations closer to the optimum mutations with similar effects are less likely to contribute to the increase of fitness since they might lead to a phenotypic space that overshoots the optimum. Therefore, the adaptive walk of populations towards the optimum should rely on fixation of mutations of progressively smaller effect on fitness. This result seems to be independent of the shape of the fitness function (that is, of the relationship between the phenotypic states and fitness) (Martin and Lenormand 2006, but see Sanjuan and Elena 2006 and Cowperthwaite *et al* 2005). It's worth pointing out that this model is limited to adaptation from new mutations - it makes no predictions whatsoever with respect to adaptation when several alleles segregate in populations, that is, to adaptation from the standing genetic variation.

The fate of *de novo* mutations does not depend strictly on their effects on fitness. First, because *all* mutations are rare when they appear (they have a frequency of 1/N, where N is the population size) they have a high probability of being lost by chance. If some more copies are produced, their fate – whether elimination or fixation – will still depend on the power of natural selection to detect them relative to other evolutionary forces. With respect to adaptive mutations, natural selection will typically not be able to exert its action if $Ns<<1$, where N is the population size and s is the (hemizygous) effect of the mutation on fitness. This means that if most mutations are of small effects, their fate will be largely governed by random genetic drift. This is one of the reasons why studying adaptive evolution from mutation is so difficult: mutations (in general) are relatively rare and take long to accrue in populations, the vast majority of them might be lost by chance and even among those which remain we are unlikely to have the power to detect mutations whose effects on fitness are below 1% (Eyre-Walker and Keightley 2007). It is therefore not surprising that empirical distributions of effects of mutations have proved difficult to obtain and that most studies come from experimentation on microbial populations. Not only they divide fast and can be maintained at large population sizes, they are also often haploid and asexual

organisms, where mutations are relatively easy to identify and follow - for example with the use of reporter gene constructs (Rozen *et al* 2002). Furthermore, measurement of the fitness of different genotypes is generally quite straightforward (Elena and Lenski 2003). Extant empirical studies involving microorganisms have in general yielded qualitative results compatible with Fisher's Geometrical Model of Adaptation (Burch and Chao 1999, Imhof and Schlotterer 2001, Elena and Lenski 2003, Perfeito *et al* 2007, Schoustra *et al* 2009), although quantitative empirical support is scarcer.

The role of mutation to adaptation in multicellular eukaryotes has rarely been investigated empirically. Rates of mutation and their effects (including beneficial ones) have typically rested on two approaches: DNA-based methods and mutation accumulation (MA) experiments. DNA-based methods rely on the neutral theory of molecular evolution (Kimura 1983) and on the prediction that genomic regions under selection should exhibit lower genetic diversity than neutrally-evolving regions. This is because strongly selected alleles increase rapidly in frequency in populations and will drag along the alleles of the neighboring regions of the genetic background in which they arose; with limited time for recombination to occur between the adaptive alleles and alleles present in other genetic backgrounds, the genetic polymorphisms in the genomic region containing the adaptive allele will be reduced. Therefore, detection of lower levels of polymorphism in certain genomic regions is considered indicative of the presence of an adaptive allele. There are many caveats associated with such methodologies. For example, they require DNA sequence information of pairs of species with known levels of divergence and generation times. Also, they typically require independent estimation of mutation rates, otherwise these must be estimated from levels of divergence of putatively neutral sequences. Mutation accumulation experiments provide an alternative approach to the study of mutation. These experiments consist of first allowing spontaneous mutations to accumulate freely in populations. This is achieved by reducing the effects of selection to a minimum by imposing high inbreeding. In organisms where it is

possible, several lines are maintained by self-fertilization (e.g. plants, the nematode *C.elegans*); in other organisms, such as *Drosophila melanogaster*, brother-sister matings are performed. In such lines, each of them has an extremely small effective population size and all but lethal mutations accumulate. Inferences about the amount of heritable variation that is generated by mutation can then be made by comparing genotypic values of the lines with those of the initial (ancestral) population. Such experiments have been performed in several organisms (Bataillon 2000), invariably showing a reduction in fitness with a rate of decline between 0.1-2% per generation. This again has led to the general belief that the vast majority of mutations are deleterious, since when they are allowed to accumulate freely, only deleterious effects are observed. There are some caveats regarding mutational inferences from MA experiments. First, their power to detect beneficial mutations is probably very low, independently of the statistical approach (Bateman-Mukai or Maximum Likelihood). Second, even if several mutations of large effect are identified, the existence of a large class of mutations of small effect cannot be fully dismissed. Third, such experiments require substantial level of divergence among lines; they therefore involve high levels of replication, making them experimentally difficult and extremely laborious. Fourth, in typically outbreeding species, such as *Drosophila*, the rates and effects of deleterious mutations are likely to be significantly overestimated since manifestations of inbreeding depression, the reduction in fitness due to increased homozygosity of recessive deleterious alleles or to homozygosity at loci with heterozygote advantage, will be expressed simultaneously. Finally, many of the MA experiments performed may have used populations which have been maintained under laboratory conditions for some time. This might be the case for the K-12 strain of *E. coli* (Rasko *et al* 2008), laboratory strains of *Drosophila* (Ruebenbauer *et al* 2008) and the N2 strain of *C. elegans* (Rockman and Krugliak 2009, Weber et al 2010). If this is the case, then according to Fisher's model, mutation accumulation experiments would be expected to provide biased estimates towards deleterious mutations, since for populations close to a fitness optimum (under

laboratory conditions) these are expected to occur at higher frequency. New genotypes, for which laboratory conditions would indeed constitute a *novel* environment, should be tested.

Standing genetic variation

As we have just discussed, new alleles are recurrently produced in populations by mutation. Those which escape stochastic loss and whose effects are small enough for selection not to drive them to fixation or elimination will segregate in populations and constitute (part of) the standing genetic variation.

Since the early studies of molecular genetics, extensive genetic diversity has been found in natural populations, whether by analysis of proteins (Markert and Moller 1959) or DNA (Avise J.C. 1994). In fact, the segregation of considerable amounts of genetic variation in nature has been additionally demonstrated by the significant and sustained responses of natural populations to innumerous experiments of artificial selection and domestication (Hill and Caballero 1992, Falconer and Mackay 1996, Lenski and Travisano 1994, Gilligan and Frankham 2003, Simões *et al* 2006). These experiments further show that evolutionary responses can emerge from the standing genetic variation found in natural populations. The current view of adaptation from pre-existing variation is that selectively neutral or mildly deleterious alleles can be maintained in populations by recurrent mutation and genetic drift. Because fitness has an environmental context, the selective effects of these alleles might change if biotic or abiotic conditions are altered (Gibson and Dworkin 2004, Barrett and Schluter 2008). If some alleles are beneficial in the novel environment, adaptation is likely to occur. In recent years, evidence for the importance of standing genetic variation in facilitating adaptation to novel or changing environments has started to emerge (Pelz *et al* 2005, Colosimo *et al* 2005). Despite the apparent ubiquity of genetic variation in nature and recent support for its potential to drive adaptive evolution, many questions remain unanswered with respect to the dynamics, circumstances and consequences of adaptation from standing genetic variation. First, its

importance relative to the contribution of mutation it is still unclear. Also, there are very few theoretical predictions for the qualitative and quantitative effects of adaptation from pre-existing genetic variation: unlike mutation, there is not a theoretical expectation for the distribution of effects of segregating alleles after environmental change against which to test empirical observations (Barrett and Schluter 2008). This is not very surprising, since mechanisms other than the balance between mutation and genetic drift can also contribute to standing genetic variation in populations. For example, novel alleles may be introduced by migrants from other groups (demes). If demes experience different habitat conditions, particular alleles may segregate in each of them. Migration of individuals between demes can therefore potentially introduce allelic variants that would not otherwise segregate in a particular deme. If the alleles carried by migrants have small effects under the environmental conditions of the new deme (or if the migration rates are high), they can be maintained at appreciable frequencies. Genetic polymorphisms can also be preserved by natural selection itself, as in the case of loci with heterozygote advantage (overdominance). Overdominance describes a genetic relationship among alleles within a locus in which the fitness of heterozygote individuals is higher than the fitness of any of the two homozygotes. Finally, other phenomena such as frequency-dependent selection might further contribute to the segregation of different alleles in populations at any given point in time. Among the most cited examples is the dynamics between parasites and their hosts, which can lead to temporal oscillations in the fitness of different host and parasite genotypes and hence to their maintenance in alternate frequencies over time (Hamilton 1993).

As a result of the diversity of phenomena that may underlie the segregation of different alleles in populations, the understanding of adaptation from standing genetic variation necessarily implies a careful genetic characterization of populations and a sound knowledge about their demographic and selective histories. Because these goals are not trivially achieved – especially in natural populations – experimental evolution might provide the best approach to start

addressing some of the open questions regarding the potential of standing genetic variation to fuel adaptive evolution.

Mutation versus standing genetic variation

Although we are still far from understanding the relative importance of mutation and of standing genetic variation to adaptive evolution, many decades of studies on the genetic basis of adaptation have yielded some indications.

The most general prediction is that if some of the alleles that segregate as polymorphic loci in populations prove to be beneficial upon environmental change (if they were neutral or mildly deleterious prior to the change), they are likely to provide a more immediate substrate for natural selection than the input of mutations with beneficial effects. The reason for this is that alleles are immediately available to natural selection after the environmental change, whereas mutations might take some generations to be produced (depending on the population size). Second, beneficial alleles from standing genetic variation have higher probabilities of becoming fixed in the population because they typically segregate at higher frequencies at the time of the environmental change (relative to mutations, which have only one copy when they arise). Because of their higher initial frequencies, beneficial alleles from standing genetic variation are less likely to be lost by chance. Additionally, average fixation times are smaller and independent of dominance relationships among alleles within loci (Hermisson and Pennings 2005).

It is therefore reasonable to believe that in natural populations the first steps of an adaptive process will rely on standing genetic variation, with mutation exerting a role at later generations: the contribution of mutation to additive genetic variance is expected to be 10^{-3} of the standing genetic variation, and should therefore be of significance only after ~50 generations of selection (Lynch and Walsh 1998, Hill 1982). However, empirical validation of this expectation is virtually inexistent.

Genetic recombination among adaptive alleles

Independently of the relative contributions of pre-existing variation and of mutational input, genetic recombination might greatly enhance both their effects to adaptive evolution. Recombination is a genetic mechanism which allows alleles to be organized in different linear combinations in chromosomes. In bacteria, for example, recombination usually involves uptake of external DNA or extrachromosomal entities such as plasmids (Koonin *et al* 2001). In organisms able to undergo sexual reproduction, genetic recombination occurs during meiosis. Meiotic recombination (crossing-over and gene conversion) consists of the exchange of portions of DNA between homologous chromosomes within a cell (each contributed by a gamete). Because of the molecular and mechanical nature of meiosis, rates of recombination between loci correlate negatively with physical distance. In addition, they also vary across the genome, among individuals and between species (Smukowski and Noor 2010).

Linkage disequilibrium is a statistical measure of the degree of association between alleles at different loci. The association between loci can result from their physical proximity in the chromosomes (and the concomitant decreased rate of recombination between the loci involved) but it can also result from other processes, such as the action of selection itself. For example, some combinations of alleles generate higher fitness than others; natural selection will thus tend to preserve these combinations, leading to non-independence of the allele frequencies at the loci in question. The role of sex and genetic recombination to evolutionary processes and more specifically to adaptation has been the focus of a vast body of theoretical work (Peters and Otto 2003). Again, most of theoretical and empirical studies have focused on the effects of recombination on alleles generated by mutation.

In general terms, the effects of recombination on *de novo* mutations can be considered under two perspectives: that of deleterious mutations and that of beneficial mutations. Deleterious mutations that arise in different individuals can be combined together in one genotype; if they interact additively or synergistically,

more deleterious mutations will be eliminated per selective death. Recombination can therefore potentially attenuate the mutational load of populations (Kimura and Maruyama 1966). Conversely, recombination can bring together adaptive alleles generated by mutation in different individuals into a single genotype (Fisher 1930, Muller 1932, Crow and Kimura 1965). This reduces the competition among beneficial alleles in different lineages (clonal interference), leading to a higher number of fixations and to shorter times for each fixation event (Cooper 2007). Although this phenomenon might be particularly important for populations of microorganisms (in which mutations are produced more abundantly due to the larger population sizes), the extent to which it is relevant to populations of other organisms is still unclear.

In the perspective of recombination from standing genetic variation to adaptation, its effects are much less clear. The systematic breaking up of associations between loci can affect both the mean and the variance of fitness values of a population. Its consequences will depend mostly on the type of genetic associations between loci and on the way in which they interact to determine fitness. Briefly, recombination should facilitate adaptation if it contributes to increased variance for fitness, and hence to higher effectiveness of directional selection. This pattern is expected to occur if negative linkage disequilibrium among deleterious and beneficial alleles is common (that is, if most individuals carry both deleterious and beneficial alleles in similar proportions) and if negative epistasis among beneficial alleles is prevalent (in other words, if the increase in fitness is progressively smaller the more beneficial alleles are found within a genotype). Experimental studies have provided convincing evidence for the generality of the positive effect of recombination on the additive genetic variance for fitness and the concomitant increase in the response to selection (Rice and Chippindale 2001, Colegrave 2002, Goddard *et al* 2005). However, there are still several gaps in the body of empirical work addressing the evolutionary consequences of sex and recombination. First, they have been performed in only a handful of organisms (viruses, *E. coli*, yeast, *Chlamydomonas*, *Daphnia* and

Drosophila) (van der Walt *et al* 2009, Cooper 2007, Goddard *et al* 2005, Colegrave 2002, Presgraves 2005). Second, it is evident that the effects of recombination will be proportional to the amount of genetic variation in populations, since it can only produce variable genotypes if more than one allele is available at each locus. In most of the studies involving microorganisms, recombination is induced by enforcement of sexual reproduction, for example, by changing specific culture conditions. This is likely to impose different selection pressures while generating sexual and asexual experimental populations. The effects of recombination are thus confounded with the effects of different initial levels of genetic variation. Alternatively, empirical studies have relied on the sampling of individuals from natural populations and their measurement under laboratory conditions. As was mentioned previously, expectations regarding the effects of recombination on the mean and variance of fitness are contingent on the genetic associations among alleles. Such associations might change drastically when genotypes are brought to the laboratory conditions. This makes interpretation of the results yielded by such experiments difficult and often ambiguous. Moreover, if similar experimental evolution studies are to be performed at different times, a careful genetic characterization of initial populations is needed to prevent effects other than those at question (as, for example, founder effects) from obscuring conclusions (Matos *et al* 2002). Clear empirical evaluations of the role of recombination from standing genetic variation and from mutation to adaptation are therefore lacking.

1.1.4 Other players in evolution

Evolution has been discussed thus far from the perspective of mutation, recombination and natural selection only. Their role has been discussed assuming ideal conditions, that is, infinitely large and random mating populations. Natural populations, however, are not composed of an infinite number of individuals and reproduction between individuals might not occur in a random fashion either.

The number of individuals that compose a population influences the relative contributions of genetic drift and of natural selection to the changes in

allele frequencies: in larger populations natural selection will dominate, while in smaller populations genetic drift will prevail. But population dynamic parameters other than population census sizes also influence evolutionary dynamics of populations: the age structure of the individuals within populations, survival and reproduction rates or migration patterns of individuals of different groups within populations (gene flow). To summarize all the information concerning a particular population, evolutionary biologists developed the concept of *effective* population size, represented by N_e. Instead of listing all the known parameters about the population, they calculate the size that an unstructured, panmictic (random mating) population would have to have in order to behave similarly (evolutionary speaking) to the population under study. Whether referring to the strength of selection, the strength of genetic drift or the degree of genetic uniformity in populations, the term N_e is usually employed and therefore properties of very different populations of organism can be compared.

The way organisms in populations reproduce has important effects on the genetic properties of populations and therefore in their potential to adapt. The evolutionary consequences of sexual and asexual reproduction have been mentioned throughout this introduction, but even in sexual organisms, several aspects related to reproduction have large effects on the levels and organization of genetic diversity in populations. For example, the evolutionary dynamics of populations which reproduce seasonally or continuously and the extent of mating across individuals of different generations are expected to be very different. The vast majority of evolutionary models assume discrete generation times, and the consequences of relaxing this assumption are not yet fully understood, thus limiting the predictive power of such models. Therefore demographic aspects of populations have to be taken into account if a deep understanding of their evolution patterns is to be achieved. Another important aspect with regards to reproduction is the degree of randomness with which mating occurs between individuals. In some instances, individuals with particular phenotypes mate preferentially with individuals either exhibiting similar phenotypes (positive

assortative mating) or preferentially with individuals with more dissimilar phenotypes (negative assortative mating). The effects of these types of mating preferences will reflect in aspects such as levels of homozygosity of individuals, but their consequences can go far beyond. For example, if such phenotypes and preferences have a genetic basis, then correlations among the alleles at the loci for the preference and for the trait are introduced. Also, such preferences in mating can lead to other forms of selection acting *within* sexes in the populations, a process called sexual selection. Even in the absence of these processes, mating between genetically related individuals can occur to different extents in populations. Inbreeding expresses the correlation between the gametes that unite to form zygotes. Closely related individuals have many alleles in common; if they mate, homozygosity in the progeny is expected to be high. One extreme form of inbreeding is self-fertilization, where both gametes are provided by the same individual. Selfing might be advantageous if parental genotypes have high fitness (e.g. locally adapted genotypes), being that they are preserved in the progeny. It is possible for selfing lineages to have relative high values of fitness, at least in diploid organisms: in such organisms, recessive deleterious mutations of mild effects can be more effectively eliminated by natural selection by being exposed in the homozygote state more frequently, a process called purging (Schoen and Busch 2008). The converse argument can be made regarding adaptive recessive mutations, which increase more rapidly in frequency once they arise and are more efficiently driven to fixation by natural selection once they are found in the homozygote state. However, the significant increase in homozygosity caused by self-fertilization also reduces population effective size - up to one-half in purely selfing populations (Nordborg 2000). This means that the power of natural selection may be severely limited and that deleterious mutations may accumulate in selfing populations (despite their increased exposure in the homozygote state), potentially leading to their extinction. With regards to adaptive evolution, the ability of selfing populations to adapt to novel environments may also be severely reduced. First, because the impact of recombination in generating variance for

fitness is virtually null if a large portion of the genome is in the homozygote state. Second, because adaptive mutations arising in different individuals will only rarely find themselves together in the same genotype; adaptive alleles will also be decoupled from deleterious ones much less efficiently. The study of mixed mating systems, where both selfing and outcrossing can occur at varying rates, might be of particular interest in the context of adaptive evolution and in addressing which of the phenomena mentioned above will prevail over the others.

1.1.5 Evolution in the wild and in the lab

It is clear by now that many processes contribute to the evolution of populations. The main problem is that they might all act simultaneously and trying to measure their effects in natural settings is a cumbersome task. Nevertheless, long-term studies (Grant and Grant 2006) and increased power to study large portions of genomes have granted us some ability to detect and study adaptation of natural populations (Colosimo *et al* 2005, Reznick *et al* 2008). Many of these studies have the goal of *inferring* which evolutionary forces (and to which extent) shaped the genomes of particular populations by directly looking at their DNA sequences. The problem with this approach is that very different evolutionary histories can lead to extremely similar molecular signatures. For example, the effects of adaptation from standing genetic variation may be very difficult to detect from data on neutral polymorphisms (Przeworski *et al* 2005, Hermisson and Pennings 2005). Also, the hallmarks of selection (either positive or purifying) may be extremely similar to those of population bottlenecks in populations of inbred organisms with genomes characterized by high linkage disequilibrium across large genomic regions (Wright *et al* 2008).

Experimental evolution provides one of the most powerful tools to study evolution. The majority of the factors influencing the evolution of populations can be controlled for (at least to some extent) or quantified and their effects disentangled and evaluated in isolation. Indeed, “for the detailed study of adaptation as a process (…) there may be no empirical approach more powerful

than laboratory evolution" (Rose and Lauder 1996). The power of experimental evolution stems not only from the higher control over the many variables that affect populations but mostly from the power of replication, where the unit of evolution – populations – can be evolved under similar conditions over and over again.

Typically, natural populations are sampled and brought to a common, laboratory environment. Under this new environment, populations may experience more benign conditions due to the lack of predators or competitors from other species. Relaxation of previous selection pressures may be revealing of the phenotypes that were being selectively maintained under natural conditions. Simultaneously, laboratory environment imposes new selection regimes and hence allows the study of the degree of uniformity (or heterogeneity) of different populations to similar selective pressures. These populations can also be exposed to particular selective regimes such as different densities, demography or physiological stresses (such as temperature or toxic compounds). In all cases, the potential of natural populations to respond to selection can be assessed. Furthermore, correlated responses may arise for unexpected phenotypes, which can be revealing of patterns of linkage disequilibrium found in the natural populations that were sampled as well as of genetic tradeoffs between life history phenotypes, leading to an overall understanding of the genetic architecture of many traits (see Simões *et al* 2009 for a review of experimental domestication). Although this approach may allow the testing of evolutionary convergence in general, the particular aspects of each species and populations can make comparison between studies difficult. For example, founder effects, varying degrees of inbreeding depression, different mutation rates and life cycles of sampled populations or species may render comparison across studies difficult. Alternatively, many questions regarding the evolutionary and adaptive potential of populations have been approached from the reverse angle – that of divergence among populations. Briefly, this approach relies on the evolution of experimental populations from one ancestor to evolve under different conditions; generally, once sufficient time has elapsed, experimental populations show phenotypic divergence

(Rose 1984). Once diverged, these populations are then returned to the environment of their ancestor and the evolutionary trajectories are followed. This type of experimental design allows to test for the importance of divergent evolution and consequently of evolutionary history to response to selection. An additional advantage stems from the fact that because the ancestral environment and population are known, this provides theoretical expectations regarding the outcomes of reverse evolution to these initial conditions. This advantage is simultaneously the major problem with reverse evolution experiments – inferences rely heavily on the ancestral (control) population. In situations where controls cannot be maintained in a non-evolving state, they should be allowed to attain mutation – selection equilibrium, which may take a long time to be achieved. Despite the questionable reliability of controls in many experiments of the sort, much has been learned with reverse evolution experiments (see Estes and Teotónio 2009 for a review of experimental reverse evolution). The emerging picture from these studies is that convergence may not always be attained. As expected, the features of the adaptive landscape – the relationship between mean population fitness and mean population phenotype – will strongly influence the probability that similar evolutionary trajectories will be exhibited by different populations. In rugged landscapes, where several fitness optima are available, populations are likely to occupy different peaks, according to their proximity to them (Burch and Chao 2000). Such type of landscapes can emerge when genetic variation is limited and evolution is dictated mostly by mutation. Conversely, abundant genetic variation is likely to generate smoother, single-peaked landscapes if the variation at the genotypic level is reflected in the phenotypic distribution of populations (Teotónio *et al* 2009). Another emergent pattern is that evolutionary trajectories of phenotypes more intimately related to fitness are more likely to show convergence, with the evolution of more loosely related phenotypes being mostly governed by chance (Joshi *et al* 2003). Experimental evolution studies have further confirmed the theoretical prediction that genetic interactions

among loci may severely constrain the response of populations (Phillips *et al* 2000, Meffert 2000).

Except for *Drosophila melanogaster*, experimental evolution has been predominantly carried out for viruses, bacteria and other microorganisms. Because of this, there is still a large void with respect to some important evolutionary questions. For example, the effects of recombination in shaping the DNA sequence diversity of populations are still poorly understood. Also, the extent to which the findings obtained with these studies will hold for sexual organisms with more complex genotypes and phenotypes urges the use of other organisms in experimental evolution in addition to *Droshopila* species if a more complete picture of the adaptive constraints and solutions of populations at the genetic and phenotypic levels are to be pursued.

Evidently, a comprehensive study of evolution and of adaptation can never be complete without information from natural populations – after all, it is out there that things *happen* – but experimental evolution can provide a solid framework on which to build the knowledge about these processes.

1.1.6 Objectives

There is a vast body of theoretical work regarding how adaptive evolution should proceed. Empirical evidence, on the other hand, is much scarcer. Moreover, it is dominated by studies involving microorganisms, thus severely limiting the extent to which their findings can be generalized. For example, can adaptive mutations lead to similar increases in the fitness of populations of more complex organisms, where more genes have to work coordinately to produce viable individuals? What effects will higher levels of pleiotropy and epistasis have on adaptive evolution? Levels of genetic variation in natural populations are generally high and lead to strong responses under artificial selection. Standing genetic variation is therefore expected to dominate the earlier steps of adaptive processes relative to mutational input but the time scales at which one and the other might be relevant is largely unknown. Another open question concerns how

different rates of recombination should affect the adaptive dynamics, whether recombination involves preexisting genetic variation or new, beneficial mutations.

In the present study the following questions were addressed:

1. When genetic variation is not limiting, does inbreeding significantly reduce genetic and phenotypic variation and hence the response of populations to selection? (Chapter 2)
2. How does selection operate on components of fitness when they are differently distributed between individuals as in the case of separate sexes versus hermaphrodites? (Chapter 3)
3. How do different life histories affect the evolution of mixed mating systems? (Chapter 4)
4. Can rates and effects of beneficial mutations lead to adaptation in multicellular organisms within relatively short time scales? (Chapter 5)

Finally, the rates and patterns of adaptive evolution from standing genetic variation and from mutation are contrasted and evaluated in the context of different degrees of effective recombination (Discussion).

1.2 *Caenorhabditis elegans* as a model for experimental evolution

Caenorhabditis elegans is a small, free-living nematode. Nematodes are anatomically simple eukaryotes, but which posess some organs and tissues of more complex organisms, such as nervous and digestive systems. Their body is covered by a cuticle which is shed several times during their development. The phylogenetic relationship of nematodes with other groups such as arthropods (which comprises *Drosophila melanogaster*) and mammals is still unclear (Fitch and Thomas 1997). Nematodes are thought to be the most abundant type of animal on Earth (Coghlan 2005); they exhibit extensive intra and inter-specific phenotypic diversity and colonize many different habitats (Andrássy 1976).

C. elegans belongs to a group of bacteriophagous nematodes, the Rhabditids. Rhabditid nematodes are mostly found in nutrient and microorganism-rich environments such as common gardens and compost heaps. They can also be found in association with other organisms. *C. elegans* has been found in millipedes, isopods, gastropods and mites, presumably used by the worm as mean of transportation only. Individuals collected from natural populations often present themselves in the form of dauers. Dauers are non-feeding alternative larval forms which result from a developmental switch in response to harsh conditions (Figure 1.4). Typically, *C. elegans* eggs are laid at the gastrula stage and undergo 4 larval stages until they become young adults; within a short time, they become sexually mature worms. Under benign conditions, the complete life cycle occurs within approximately 3.5 days.

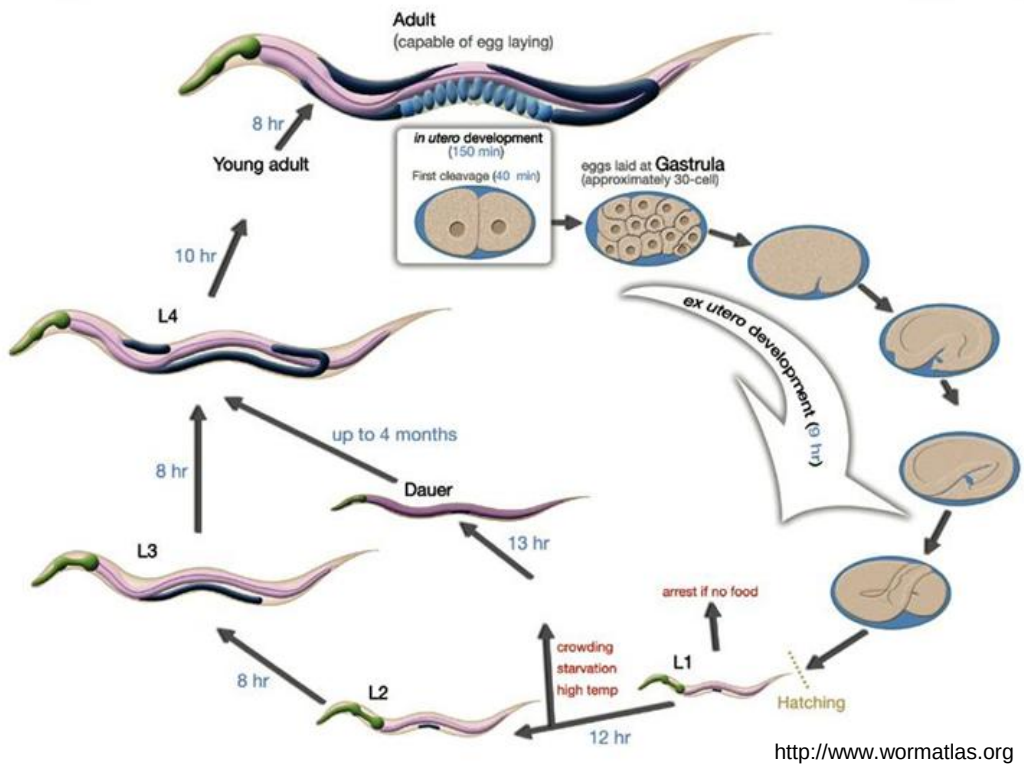

Figure 1.4: C*aenorhabditis elegans* life cycle. Fertilization is represented as time 0 minutes. Numbers in blue along the arrows indicate the length of time the animal spends at a certain stage.

This species exhibits an androdioecious mating system. Androdioecy is a mixed-mating system in which self-fertilizing hermaphrodites co-occur with males. *C. elegans* hermaphrodites have two gonads, each ending in a cavity on either side of the uterus. Each gonad first produces about 150 sperm, which are stored in the cavities flanking the uterus, the spermatheca. Sperm production then ceases and oogenesis begins. As oocytes are produced, they pass through the spermatheca, where they are fertilized by the sperm before proceeding to the uterus and exiting the body via an opening at the mid-body region, the vulva. Because hermaphrodites are able to produce many oocytes (over 1000) compared to sperm (about 300), reproduction by self-fertilization is sperm-limited. Sexual dimorphism of hermaphrodites and males is present in virtually all tissue systems, being more evident in reproduction-related structures (Figure 1.5).

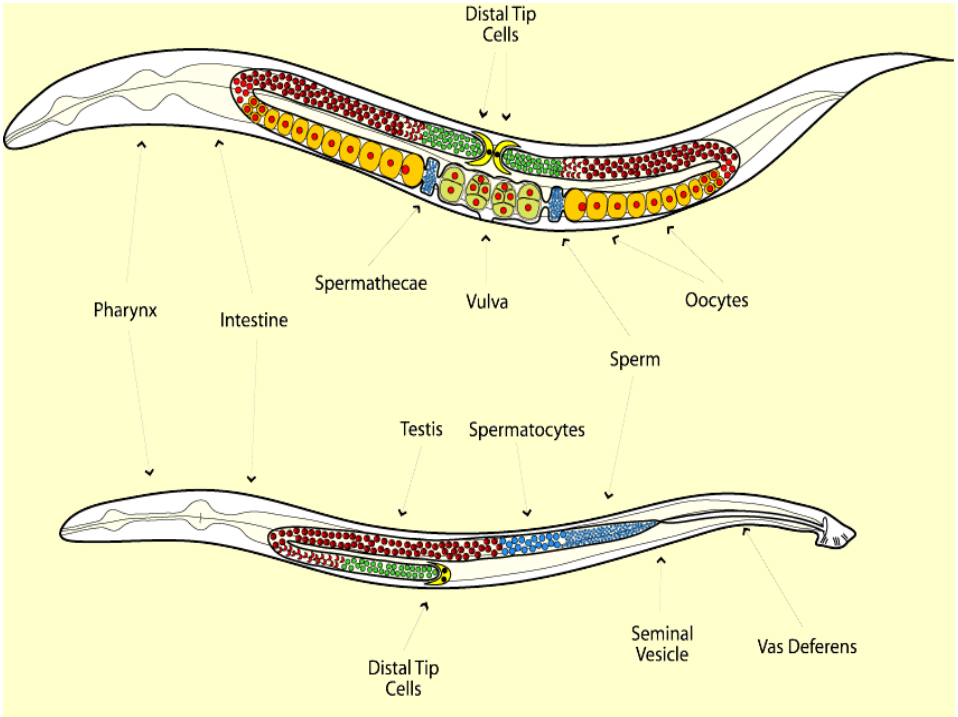

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Figure 1.5: Schematic representation of the morphology of *C. elegans* hermaphrodites and males. The gonads of hermaphrodites (top) and males (bottom) are shown in colors, with different colors representing the different types of germline tissue. Stem cells are shown in green, meiotic tissue in red and sperm in blue. In hermaphrodites, oocytes are also represented (yellow), as well as zygotes (green with red nuclei).

Males possess only one gonad arm in which spermatogenesis occurs throughout the adult life of the individuals. They also exhibit a specialized structure in the posterior end of the body, called the fan, which they use to probe the body of the hermaphrodites to find the vulva. Male-derived sperm are delivered to the uterus and travel to the spermatheca, where they usually outcompete the hermaphrodites' own sperm in the fertilization of the oocytes. Sexual specification is genetically encoded. The *C. elegans genome* is comprised of five autosomes and sexual chromosomes, with hermaphrodites bearing two sexual chromosomes (XX), while males bear only one (XO). It's the different ratio of sex chromosomes to autosomes that determines the developmental pathways to become either a hermaphrodite or a male (Hodgkin 1987). Males can therefore result from

outcrossing events between hermaphrodites and males (generating progeny with a 1:1 sex ratio) as well as from rare events of X chromosome non-disjunction during hermaphrodite gametogenesis. These events of chromosomal non-disjunction are referred to as spontaneous production of males and they are typically quite low (Teotónio *et al* 2006).

The maintenance of males (and outcrossing) in androdioecious populations is paradoxical because such populations have lower growth rates than purely hermaphroditic populations (Hodgkin and Barnes 1991). *C. elegans* males are rarely observed in nature and are typically maintained at frequencies similar to those of meiotic X chromosome non-disjunction under laboratory conditions (Barrière and Félix, 2005, 2007). Yet, male-specific genes (not related to sperm) seem to be among the most conserved in the worm genome (Cutter and Ward, 2005) and substantial genetic variation for the production of males and male-related phenotypes can be found among natural isolates (Teotónio *et al* 2006). Males therefore seem to be more than simple meiotic errors. Additionally, they must confer some evolutionary benefit, otherwise they would be expected to be rapidly driven out of populations. Because males equate with outcrossing in *C. elegans*, most theoretical models for the maintenance of males in androdioecious populations have been framed in terms of mutational arguments and of inbreeding depression, with selfing lineages efficiently purging deleterious mutations but not being able to combine several beneficial mutations in a single genotype and outcrossing lineages successfully avoiding inbreeding depression by bringing mildly deleterious mutations to the heterozygote state while being able to join different beneficial mutations in one individual. However, empirical work has failed to comply with the existing models: estimates of outcrossing in nature based on molecular markers and patterns of linkage disequilibrium have not yielded congruent results (Sivasundar and Hey 2005, Barrière and Félix 2005, Haber *et al* 2005) for which the relevance of males in promoting cross-fertilization in natural settings is still unclear. Also, *C. elegans* does not suffer from inbreeding depression (Dolgin *et al* 2007). Finally, most experimental evolution studies

addressing the maintenance of males (and outcrossing) in androdioecious and trioecious (hermaphrodites, females and males) populations of *C. elegans* have consistently shown decreases of male frequencies with time. Such evolutionary experiments include (artificially) increased initial male frequencies (Stewart and Phillips 2002) and different mutational treatments (Manoel *et al* 2007 and Cutter 2005).

With respect to its geographical distribution, *C. elegans* is a cosmopolitan species. Over 65 natural isolates have been collected throughout North America, Western Europe, Australia, Hawaii, and Madeira. These natural isolates, which are maintained as frozen stocks (strains), are available to the *C. elegans* research community. N2, a natural isolate brought to laboratory in the 1970's, has been used as the canonical strain for this species, although the extent to which it is representative of the species as a whole has been questioned recently (Rockman and Krugliak 2009, Weber *et al* 2010). Globally, the level of genetic polymorphism found among *C. elegans* strains is low (about 20-fold lower than that found in *Drosophila melanogaster*), with the natural isolates collected in Madeira (JU258) and in Hawaii (CB4856) showing the largest genetic distance to the N2 strain (Swan *et al* 2002, Wicks *et al* 2001). However, diversity seems to be high within continents and, more importantly, at finer local scales: within sample diversity can range from virtually null to a level of genetic diversity comparable to the distance between the N2 and Hawaiian strains (Barrière and Félix 2005). Patterns of linkage disequilibrium and frequencies of heterozygote individuals in natural populations are consistent with males occurring seldomly in natural populations. Selfing is therefore though to be the predominant mode of reproduction of *C. elegans* under natural conditions, with local diversity being generated by infrequent cross-fertilization (outcrossing) events with males (~1%) and by migration of individuals between populations. Despite their overall low diversity at the molecular level, natural isolates of *C. elegans* exhibit phenotypic variation for many interesting phenotypes such as rate of spontaneous production of males,

male efficiency in mating, egg-laying, clumping behavior and oxygen and ethanol sensation.

This species of nematodes is easy to rear under laboratory conditions. Although it can be maintained in liquid medium, it is usually cultured in Petri dishes covered with Nematode Growth Medium (NGM) – which consists basically of a matrix of agar supplemented with some chemical compounds such as glycerol. A lawn of *E. coli* over the NGM provides the food source and the Petri dishes are kept in incubators at 20ºC and 80% R.H. (relative humidity). About 1000 worms can be maintained in each plate during one generation without starving, which allows maintenance of populations at large census sizes. Contamination with organisms such as fungi can be prevented by periodically exposing cultures of *C.elegans* to a hypochloride solution (Stiernagle 2006). In addition to being easy to maintain in the lab at considerable population sizes, genetic manipulation is also possible and many transgenic strains are available. Because of its transparent body, transgenic arrays allowing the expression of fluorescent proteins such as GFP (Green Fluorescent Protein) are among the most widely used (Fire *et al* 1998). Its mode of reproduction facilitates the generation of highly inbred lines by imposition of self-fertilization, while cross-fertilization between individuals of different populations allows the introgression of particular alleles into different genetic backgrounds, as well as the construction of highly recombinant inbred lines. This versatility has rendered *C. elegans* a popular model for the study of quantitative phenotypes.

C. elegans was the first metazoan to have its ~100 Mb genome sequenced. The amount of genetic information available nowadays is therefore considerable and includes a fine-scale recombination map of the genome of this organism (Rockman and Krugliak 2009).

1.2.1 Experimental populations of C. elegans

We took advantage of the properties of *C. elegans* described above to produce populations differing a) in their levels of initial standing genetic variation

and b) in their levels of inbreeding. All types of populations generated are represented in Figure 1.6. A description of the methodology employed to generate these experimental populations is provided next.

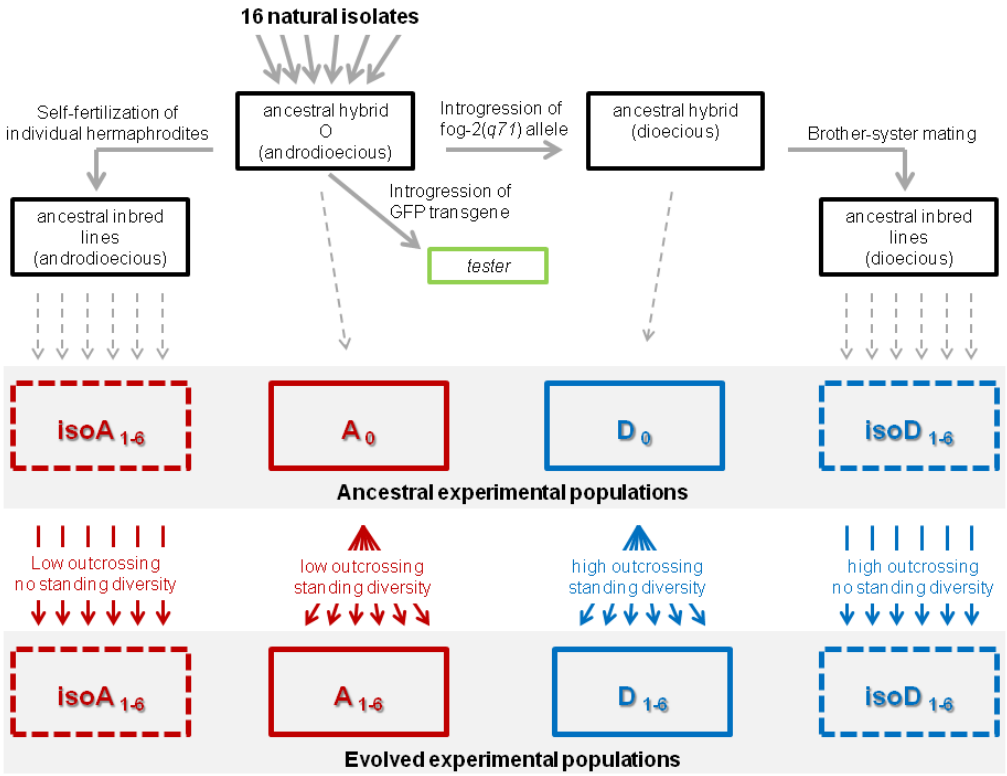

Figure 1.6: Schematic representation of experimental populations used in the present study. Populations and procedures performed during the construction of experimental populations are shown in grey. Experimental populations allowed to evolve under laboratory conditions are represented in colors. For these populations androdioecious mating systems (hermaphrodites and males) are shown in red and dioecious mating systems (males and females) are shown in blue. Dashed boxes represent populations starting experimental evolution from low levels of genetic diversity. Colored arrows symbolize the origin of replicated evolved populations, with replicates being represented by numbers as subscripts.

First, 16 natural isolates presumed to be representative of the global genetic diversity of *C. elegans* were chosen. These were: PB306, AB1, CB4858, CB4855, N2, JU400, MY16, JU319, PX174, MY1, PX179, JU345, CB4856, CB4507, RC301, CB4852. Therefore, the list of strains included the canonical N2, as well as the Hawaiin strain CB4856 (the most divergent from N2). These natural

isolates were inbred by self-fertilization for 10 generations to remove residual heterozygosity. Reciprocal (male-hermaphrodite and hermaphrodite-male) crosses were then performed in a pairwise fashion. Resulting hybrids were reciprocally crossed in a pairwise manner between themselves. Crosses were performed in this way until one hybrid population resulted – the ancestral hybrid population (O). Cycles of population expansion were performed after each round of crosses to minimize stochastic loss of genetic variation. This population is expected to harbor high levels of standing genetic variation and high levels of inbreeding due to its reproduction predominantly by self-fertilization. To generate a population with qualitatively and quantitatively similar levels of genetic variation but reduced inbreeding, we performed introgression of a null allele of the *fog*-2 gene into this ancestral hybrid population. The *fog*-2 gene is involved in hermaphrodite spermatogenesis. The *fog*-2(*q71*) (null) allele is recessive to the wild-type; it differs from the latter in a single nucleotide but it generates a premature stop codon that renders the FOG-2 protein non-functional (Schedl and Kimble 1988). Spermatogenesis is therefore disrupted in *fog*-2(*q71*) homozygote hermaphrodites, rendering them functional females without affecting spermatogenesis in males. Because these homozygote hermaphrodites do not produce sperm, unfertilized eggs accumulate within their body and become highly packed, allowing their phenotypic discrimination. Males from the strain carrying the *fog*-2(*q71*) allele (strain JK574) were mated to hermaphrodites of the ancestral hybrid population. Single heterozygote hermaphrodites from the F2 progeny were allowed to self fertilize (generating different families) and scored for the *fog*-2 (*q71*) homozygote phenotype. This process was repeated 9 times. Finally, homozygote hermaphrodites for the *fog*-2(*q71*) allele were mated with males from different families and their progeny expanded for 2 generations. The resulting population (ancestral hybrid dioecious) should harbor similar levels of genetic variation to the ancestral hybrid population but lower levels of inbreeding, since cross-fertilization between hermaphrodites and males is required for progeny to be produced. This

population thus exhibits a dioecious (male-female) mating system, for which we will refer to its individuals as males and *females* throughout this thesis.

Having two genetically variable populations differing in their level of inbreeding, we then derived several (about 100) inbred lines from each of them. Inbred lines from the ancestral androdioecious hybrid population were obtained by imposition of self-fertilization on single hermaphrodites during 10 generations, while inbred lines from the ancestral dioecious population were generated by 20 generations of brother-sister mating. Six random lines from each of the ancestral androdioecious and dioecious populations were expanded and represent nearly isogenic (that is, genetically uniform) ancestral populations; they are hence called isoA and isoD, respectively. Experimental populations will be referred to by initial capital letter throughout this thesis to distinguish from more general comparisons involving other dioecious and androdioecious populations or species (Androdioecious, Dioecious).

Finally, a *tester* population was also generated. This population resulted from the introgression of a transgenic allele (present in strain PD4251) into the genetic background of the hybrid androdioecious population. The transgenic allele drives the expression of GFP in the muscle cells of the worms, allowing phenotypic discrimination between individuals carrying the transgene and those from other experimental populations under a dissection scope equipped with UV light. The introgression of the transgenic allele proceeded similarly to the introgression of the *fog*-2(*q71*) allele described above.

All experimental populations were kept as frozen stocks prior to experimental evolution. Evolved populations resulted from maintenance under defined laboratory conditions for 100 generations. Experimental evolution conditions are described in detail in Chapter 2.

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Chapter 2

Adaptation from high levels of standing genetic variation under different mating systems

Submitted manuscript

Running title: Recombination load explains the adaptive consequences of sex

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2.1 Summary

The short-term evolutionary response of populations to environmental changes depends on the genetic variation segregating within them. Therefore, the adaptive potential of populations may be constrained by their levels of standing genetic variation and by the extent to which extant genetic variation can be used to produce individuals that will better cope with the new requirements of their habitat. In this context, sexual reproduction is expected to confer great benefits over asexuality by creating novel linear combinations of alleles at different loci (via meiotic recombination). However, its effects can be severely limited by high levels of homozygosity, typical of highly inbred species. We investigated the consequences of inbreeding to adaptation by performing experimental evolution of populations of the nematode *Caenorhabditis elegans* with different mating systems: androdioecy (coexistence of males and selfing hermaphrodites) and dioecy (males and females). Our results demonstrate that outcrossing is selectively favoured in populations where cross-fertilization is also possible. They further argue for a crucial role of interactions within and between loci in determining fitness in a multicellular organism.

2.2 Introduction

Adaptation results from the action of natural selection. The efficiency of this process depends on its strength relative to other evolutionary forces such as genetic drift, but in large populations it is ultimately determined by the amount of genetic variation found within them. Genetic variation seems to be ubiquitous in natural populations of organisms across various taxa. This is supported by 1) the considerable levels of allelic polymorphisms found in genomes, 2) the rapid responses of populations to artificial selection (such as domestication events) (Clarke 1979), which imply a ready supply of segregating alleles, and also by 3) the effects of inbreeding (Darwin 1892, Charlesworth and Willis 2009), which are consistent with the maintenance of heterozygosity at several genetic loci in many natural populations.

Directional selection on pre-existing variation is more likely to dominate adaptation (at least in its earlier steps) than selection on alleles generated by mutation. There are several reasons for this. One is that polymorphisms are readily available to selection while *de novo* creation of genetic variation may take quite a long time (depending on the mutation rate and on the size of populations). In addition to this, the vast majority of new mutations are lost by genetic drift or competition with other mutations (Orr 1998, Gerrish and Lenski 1998). Another reason why standing genetic variation may lead to more immediate responses to selection is that alleles at polymorphic loci which are beneficial in the new selective environment may segregate at relatively high frequencies (if they were neutral or mildly deleterious prior to the change in the environment), while adaptive mutations always arise as single copies and thus have smaller probabilities of fixation and take considerably longer times to become fixed in populations, even if their effect on fitness is significant (Barrett and Schluter 2008, Hermisson and Pennings 2005). The response of a population to an environmental challenge does not, however, depend uniquely on the *number* of alleles segregating at each locus. Because the alleles present in populations at any moment have a demographic and selective history, the potential for adaptation from standing genetic variation further depends on aspects such as the distribution of the different alleles within individuals (which is to say on the degree of inbreeding) or the extent to which allele frequencies at different loci covary with each other, that is, the extent of linkage disequilibrium. Linkage disequilibrium thus translates the fact that some combinations of alleles are found more (or less) often than would be expected if the same alleles were randomly combined in genotypes (Rice 2004). Genetic recombination is a mechanism which can break up the associations between alleles (reduce linkage disequilibrium), combining them in different ways. The creation of novel genotypes by recombination is expected to increase the variance of genotypic fitnesses in populations and hence allow selection to operate more efficiently (Felsenstein 1988). The potential of recombination to enhance adaptive evolution is among the most widely accepted theories for explaining the ubiquity of

sex in nature (Weissman 1889; Barton and Charlesworth 1998). The argument for the adaptive relevance of recombination is thus contingent on the presence of linkage disequilibria among loci in populations experiencing *directional* selection (de Visser and Elena 2007). The beneficial effect of recombination also holds for new adaptive mutations: each mutation arises in a particular genetic background, to which it becomes *linked* in the absence of recombination. With recombination, beneficial mutations in different individuals can be combined and genotypes carrying different numbers of adaptive alleles can be generated (Fisher 1930, Muller 1932).

In the context of adaptation (in the form of directional selection), recombination is thus expected to be beneficial in the presence of genetic disequilibria among loci. It is therefore important to understand what the sources of linkage may be. Linkage disequilibria can be created, for example, by genetic drift (Barton and Otto 2005), by selection that varies in time (Peters and Lively 1999), by sexual antagonism (Ubeda *et al* 2011) or by selection on particular allelic combinations (epistatic natural selection) (Feldman *et al* 1980, Otto and Feldman 1997). With regard to the latter, selection is expected to keep apart deleterious alleles whose combined action enhances the depression of fitness on their carriers; the same is expected for beneficial alleles whose effects are mitigated by their simultaneous presence in a genotype. These two phenomena are both manifestations of *negative* epistasis. Negative epistatic relationships between alleles are therefore required for the adaptive arguments of recombination (de Visser and Elena 2007). However, for recombination to contribute to adaptation, negative epistasis should not be too strong, otherwise the mean fitness of the genotypes produced upon recombination will be severely reduced and will offset the evolutionary advantage of generating variance for fitness upon which natural selection can act (Charlesworth 1996). Empirical studies addressing the role of recombination in populations of *Saccahromyces cerevisiae* (Greig *et al* 1998, Goddard *et al* 2005), *Chlamydomonas reinhardtii* (Colegrave 2002), and bacteriophages (Poon and Chao 2004) have generally provided support for a

positive effect of recombination on adaptation, either by reducing competition among beneficial mutations (clonal interference) or by allowing such mutations to be decoupled from genetic backgrounds carrying deleterious alleles (Rice and Chippindale 2001). In these studies, epistasis need not to be invoked. Experimental work addressing specifically the role of epistatic interactions and recombination to adaptation has yielded a mixed picture, with some studies finding evidence for positive epistasis (deVisser and Hoekstra 1998, Salathé and Ebert 2003), others for negative epistasis (Sanjuan *et al* 2004, Zeyl 2005, Lenski *et al* 1999, Bonhoeffer *et al* 2004, Maisnier-Patin *et al* 2005) and some others the absence of epistatic interactions (Elena and Lenski 1997, Elena 1999).

In diploid organisms, the adaptive effects of sex and recombination might be further limited by the extent of assortative mating in populations. If individuals of a population mate preferentially with genetically dissimilar individuals (negative assortative mating), their offspring will tend to be more heterozygote; conversely, if mating occurs preferentially among genetically similar individuals (positive assortative mating), the progeny will tend to bear high levels of homozygosity. When recombination occurs between two loci in the homozygote state, both recombinant and non-recombinant meiotic products have the same combinations of alleles. In this case, the effect of recombination in increasing genetic variation is obliterated. One form of extreme positive assortative mating is self-fertilization. Self-fertilization inevitably increases homozygosity, which translates into a positive genetic correlation between the alleles present *within* any given locus; this genetic correlation is often called coefficient of inbreeding or homozygosity index.

In summary, theory largely advocates for the relevance or recombination to adaptation. This expectation has been generally met in empirical studies, most of which performed in microbes and from the standpoint of recombination reducing linkage disequilibria between deleterious alleles, between beneficial alleles, and between beneficial alleles that arise in genetic backgrounds segregating high numbers of deleterious alleles. Two important questions regarding the role of recombination to adaptation still remain unclear: a) in sexually reproducing

multicellular organisms, to what extent can increased inbreeding reduce the effects of recombination (do highly inbred populations evolve as asexual organisms)? b) Among the evolutionary processes that can generate linkage disequilibria, what is the relevance of epistatic selection? If populations of organisms with more complex genomes equate with larger extent of epistatic interactions between loci, will this affect the outcome of recombination to adaptation?

The effects of recombination to adaptation thus need to be addressed from the standpoint of standing genetic variation. As we have seen, these effects will strongly depend on many parameters. The study of adaptation from standing genetic variation thus requires a quantitative and qualitative characterization of the population genetic variation. Quantities such as number and frequencies of segregating alleles, the genetic relationships between them and the average and dispersion of genotypic fitnesses before and after the environmental changes will be crucial for understanding how adaptive evolution may proceed (Teotónio et al 2009, Peters and Otto 2003). For the study of adaptation to novel environments from standing genetic variation to be carried out empirically, genetically well characterized populations must be employed and experimental evolution must proceed under simple life-cycles and under conditions in which demography (including aspects related to mating) can be controlled for in order to allow inferences about fitness to be made.

In the work presented here, the effects of recombination and segregation to adaptation were investigated by experimentally evolving populations of the nematode *C. elegans* to a stable, novel environment under a simple life-cycle for 100 generations. Experimental populations exhibited high levels of genetic diversity and were manipulated to differ in their levels of inbreeding by imposing different mating systems – androdioecy (the coexistence of selfing hermaphrodites and males) and dioecy (a mating system composed of males and females) . An evaluation of the competitive performance of ancestral and evolved populations against a *tester* population was carried out to quantify adaptation in the different

experimental populations. Our experimental approach allowed us to test the hypothesis that reduced effective recombination and segregation hamper adaptation by comparing the magnitude of fitness increase of populations of sexually reproducing organisms with known evolutionary histories and different levels of inbreeding. Our results suggest that outcrossing over self-fertilization is adaptive under our experimental conditions.

2.3 Materials and Methods

Experimental populations

Six androdioecious and six dioecious populations with high levels of standing genetic variation (A_{1-6}, D_{1-6}) were initiated by sampling large numbers of individuals (>10^5) from frozen stocks (Figure 1.6; see Introduction for how the different populations were constructed). Similarly, six androdioecious nearly isogenic populations ($isoA_{1-6}$) and six dioecious nearly isogenic populations ($isoD_{1-6}$) were initiated from cryopreserved stocks.

Experimental evolution

All 24 populations (A_{1-6}, D_{1-6}, $isoA_{1-6}$, $isoD_{1-6}$) were cultured in parallel during 100 generations. Large samples were collected every 10 generations and cryopreserved following the Soft Agar Freezing Solution Protocol (Stiernagle 1999). This methodology makes it possible to assay concurrently populations from different time points of the evolutionary trajectory, thus allowing the statistical decoupling of experimental variance (caused by putative differences in laboratory conditions at the time of assays) from the variance components generated by the actual evolutionary forces. Experimental evolution was halted and re-started at generation 60 from large samples. Each generation of experimental evolution was initiated by culturing first larval stage (L1) worms in 9 cm Petri dishes with 25ml of Nematode Growth Medium (NGM) lite agar (US Biological) and supplemented with 1µg of Ampicillin. A lawn of *E. coli* HT115 (Timmons *et al* 2001) grown O/N to a density of 200OD/600nm was used as food source. These plates, with NGM

medium and bacteria, are herein referred to as culture plates. Bacterial colonies were grown from a frozen stock and hence did not evolve during our experiment. One thousand (1000) worms were seeded onto each plate and populations were maintained in 4 days life-cycles (Figure 2.1), for which food was not a limiting resource. Each replicate *C. elegans* population consisted of 10 culture plates, in a total population census size of 10^4 individuals. Worms were incubated for 72h at constant temperature (20ºC) and humidity (80% R.H.). After this period, they were sexually mature. All individuals from the 10 same culture plates were collected into one 15ml polypropylene tube (by washing with M9 solution) and subjected to the *hatch-off* protocol: worms (and eggs) were exposed to fresh hypochloride solution (1M KOH:5%NaOCl, 4ºC) at a 50:50 volumetric ratio relative to M9 solution for 3min50s (± 30s). Worms were then pelleted by centrifugation (1min at 1800rpm) and transferred to new 15ml polypropylene tubes with M9 solution with a Pasteur pipette. They were rinsed 3 more times (pelleted by centrifugation followed by removal of supernatant and addition of M9 solution) to virtually eliminate all hypochloride solution (dilution of ~1:1000). Exposure to this alkaline solution disrupts the body wall of adult worms causing the release of eggs from within hermaphrodites and females. Larvae hatched prior to the *hatch-off* protocol do not survive the exposure to the hypochloride solution. Eggs thus released from individual worms were allowed to hatch in 3-5ml of M9 with aeration (120rpm) at 20ºC for about 24h. Because M9 is a minimal medium, larvae hatched during this period (either released from within the body of hermaphrodites or females or previously laid on the culture plates before the protocol) arrest development until they are in the presence of food. Larval and adult debris were then pelleted by centrifugation (200rpm, 1min) and removed. Densities of L1 synchronized larvae were estimated by counting live L1 larvae in 5 drops of 5µl and appropriate volumes were seeded onto new plates to constitute the following generation. Figure 2.1 represents the life cycle of *C. elegans* and the timings of maintenance procedures of experimental populations (that is, their demography under the conditions of experimental evolution employed in our study).

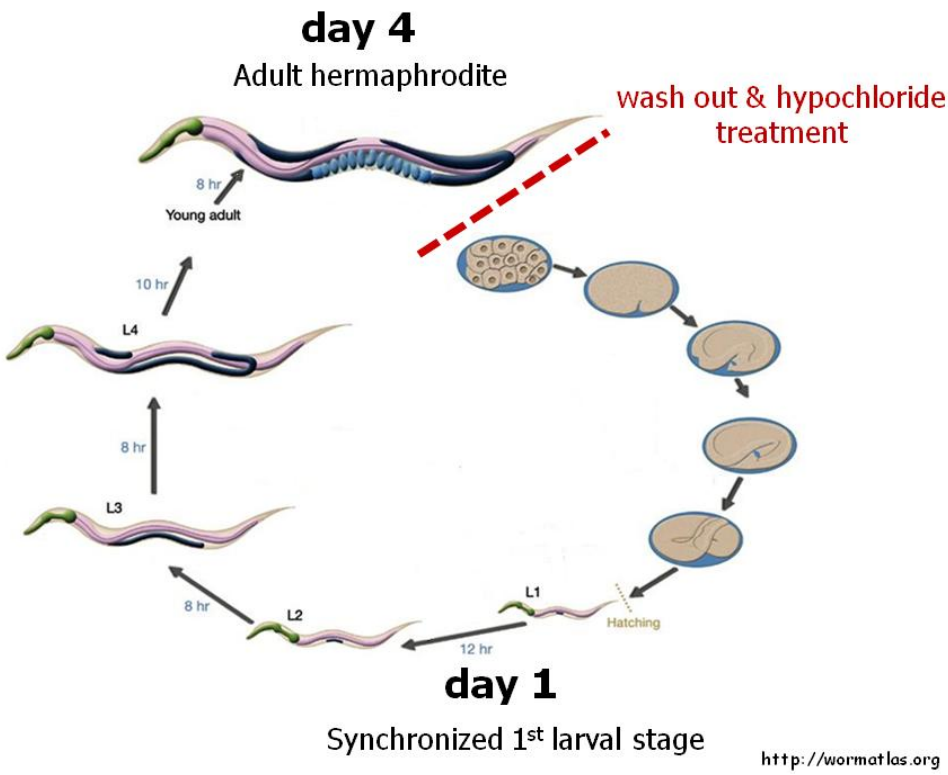

Figure 2.1: Schematic representation of the life cycle of *C. elegans* under laboratory conditions. L1-L4 designates the four larval stages of *C. elegans*; time elapsed between stages (at 20ºC) in expressed in hours (in blue). Each generation was initiated by culturing synchronized larvae after hatching (L1 stage), representing day 1 of the worm life cycle. At age of reproductive maturity (day 4), eggs were collected to constitute the following generation. (see text for details).

The *hatch-off* protocol allows the maintenance of synchronous, non-overlapping, discrete generations and constant census size populations (that is, no population growth occurs). Simultaneously, it removes potential bacterial or yeast contaminants from *C. elegans* culture plates. Furthermore, despite the fact that each individual culture plate constitutes a separate pool of gametes, the zygotes from all the culture plates of each experimental population are pooled during execution of this protocol and emerging larvae are randomly distributed among the 10 new culture plates in the following generation; this design therefore simulates conditions of random mating. Importantly, maintenance of experimental populations under these conditions facilitates the definition of fitness under

laboratory environment: the fitness of individuals is a function of their representation in the pool of zygotes at precisely day 4 of the life cycle. Selection should be able to act on phenotypes exhibited until this day; phenotypes manifested after day 4 of the life cycle are not expressed under our experimental conditions and therefore cannot be acted upon by selection. Due to the large number of culture plates, each generation the protocol was randomized as much as possible across populations, experimenters, hour and incubator. All culture plates were sealed with parafilm after seeding and bench and experimenter hands were washed with ethanol solution (70v/v) between manipulations of different experimental populations to minimize risk of contamination across them.

Experimental populations were thawed and expanded for 2 generations under the laboratory conditions employed during experimental evolution prior to the assays.

Genetic characterization of ancestral experimental populations: genotyping of microsatellite loci

We surveyed genetic variation of ancestral experimental populations in 9 microsatellite loci distributed across autosomes and the sex chromosome of *C. elegans*. Single hermaphrodites or females were randomly sampled from ancestral cryopreserved populations after thawing and expansion for two generations. Sixteen individuals (n=16) were collected from each of the twelve replicate populations which initiated experimental evolution with low levels of genetic variation ($isoA_{1-6}$, $isoD_{1-6}$), whereas a larger number of individuals (n=48) were collected from both ancestral androdioecious and dioecious populations with high levels of standing genetic variation (A_0, D_0). All worms were individually picked from culture plates at day 3 of the life cycle and transferred to 8-strip optical clear flat caps (Sarstedt AG & Co.) containing 5µl of ultrapure water, maintained on ice. Worms at this stage of the life cycle provide enough genomic DNA upon extraction while minimizing the contribution of DNA from eggs. Caps were then inserted in corresponding 96 well Multiply® PCR plates (Sarstedt AG & Co.) containing

genomic DNA extraction Mix (ZyGEM prepGEM™Insect kit, ZyGEM™ Corporation Ltd): 4µl ultrapure water, 1µl 10x Buffer Black and 0.125µl prepGem™ enzyme (for each reaction of extraction). PCR plates were centrifuged briefly to merge the water containing the worms with the DNA extraction mix (30sec, 1800rpm; final volume of 10µl), briefly vortexed and centrifuged once more. The extraction of the genomic DNA proceeded by submitting the samples to 15min at 75ºC followed by 5min at 95ºC in MyCycler Thermal Cycler (Bio-Rad Laboratories, Inc.). Extracted DNA samples were stored at 4ºC until genotyping. Each sample was PCR-amplified for each microsatellite locus separately using 0.6µl of genomic DNA and 14.4µl of GoTaq® DNA polymerase kit (Promega Corporation) as follows: 8.62µl ultrapure water, 3µl of 5x Colorless GoTaq® Reaction Buffer (containing 7.5mM $MgCl_2$), 1.2µl dNTP Set 2.5mM (Fermentas, Thermo Fisher Scientific), 0.75µl forward primer (10µM), 0.75µl reverse primer (10µM) and 0.08µl GoTaq® DNA Polymerase; either the forward of the reverse primer used in each amplification was fluorescently labeled (see Supplementary Table 1 for information about the primer pairs used). PCR amplification proceed in MyCycler Thermal Cycler (Bio-Rad Laboratories, Inc.) under the following conditions: 2min at 95ºC, 35 cycles of 20sec at 95ºC, 30sec at 50ºC and 30sec at 72ºC and a final extension step of 10 min at 72ºC. Annealing temperature of loci II-R, V-L and X-R was 60ºC instead. PCR plates were wrapped in aluminium foil to prevent degradation of fluorescently labelled primers and stored at 4ºC. PCR products from samples were run in a capillary electrophoresis system in a multiplex fashion (due to their different fluorescent labelling and amplicon sizes). The first plex contained 7µl of 0.1% Tween® 20 (Carl Roth GmbH) and 1µl of each PCR product obtained with primers of loci 4001, X004 and II-R; the second plex consisted of 6µl of 0.1% Tween® 20, 1µl of each PCR product obtained with primers of loci 3003 and X-R and 2µl of PCR product obtained with primers of locus V-L; finally, the third plex consisted of 4µl of 0.1% Tween® 20, and 3µl of each PCR product obtained with primers of loci 1003, 4004 and X003. 2µl of each plex were then added to 3µl of MegaBACE™

ET-400 Rox Size Standard (GE Healthcare) diluted in 0.1% Tween® prior to capillary electrophoresis carried out in MegaBACE™1000 Genotyping System.

All electropherograms were visually inspected and sizes of standard peaks were manually assigned when incorrectly done so by the software. Allele calls and quality control of samples were performed based on criteria established for each locus (see Supplementary Table 2). For samples exhibiting 2 peaks, both were considered only if the ratio of the lower height peak to the higher height peak was above 0.65. Samples with more than one source of genomic DNA (as is probably the case of ours, which are likely to contain both genomic DNA of the adult worms and from their eggs) tend to produce multiple peaks; in forensic analysis, only peaks whose ratio relative to the highest intensity peak is above 70% are considered (Butler 2005). We scored alleles with ratios above 65% but the data presented here is conservative in the sense that its analysis did not include alleles whose ratios were below 70%. Two loci (X004 and X003) could not be unambiguously scored and were discarded from analysis. From the remaining dataset, only 2 loci were found on the same chromosome, for which linkage disequelibrium analysis was not performed among genetic markers. Fragment sizes were retrieved from MegaBACE™ Fragment Profiler Version 1.2. Alleles were scored (binned) manually based on the retrieved fragment sizes; the several statistics presented were calculated from custom scripts run in the R software (R Development Core Team 20010). Specifically, the following measures were obtained: N - number of individuals analyzed, A – total number of different alleles found among genotypes, Ae – effective number of alleles, calculated as $1/\sum p_i^2$, where p_i represents the frequency of each allele in the sample, H_{obs} – observed heterozygosity (number of heterozygote individuals/total number of samples corrected by sample size n/(n-1)), F_{is} – coefficient of inbreeding. Fis is a statistic that uses observed heterozygosity (described above) and expected heterozygosity (H_{exp}), calculated as $1- \sum p_i^2$ (where p_i again represents the frequency of each allele in the sample), corrected by sample size as above. F_{is} is thus calculated as $1-(H_{obs}/H_{exp})$.

Frequency of males and rates of outcrossing

Rates of outcrossing are maximal in Dioecious populations, but able to evolve in Androdioecious populations. The frequency of males was measured for all replicate Androdioecious and Dioecious populations with high (A_0, A_{1-6}, D_0, D_{1-6}) and low ($isoA_{1-6}$, $isoD_{1-6}$) levels of initial standing genetic variation at several time points of experimental evolution: generation 0, generation 30, generation 60 and generation 100. After thawing and population expansion, two culture plates were seeded per replicate population and maintained under the standard laboratory condition of temperature and humidity. At day 4 of the life cycle (when the *hatch-off* protocol is performed), the culture plates were stored at 4ºC for at least 48h prior to scoring. Plates were left for 10-15 min at room temperature, then covered with a transparent film (held still with a bit of scotch tape) and placed under a dissection microscope at 10x magnification. All individuals were sexed (males were additionally scored with a mechanical counter) and marked as dots in the transparent film with a marker pen; these films were then computer scanned. Images were imported to *ImageJ* (*rsbweb.nih.gov/ij/*) at a resolution of 600 dpi and grayscale; the number of particles was then obtained under standard conditions of image adjustments (black and white threshold = 120, particle size = 3 -∞) for total number of individuals. *C. elegans* hermaphrodites are not able to transfer sperm or eggs and so cross-fertilization can only occur between hermaphrodites (or females) and males. Resulting progeny have similar proportions of both sexes (Ward and Carrel 1979), while self-fertilization produces hermaphrodites almost exclusively. For that reason, proportions of males (m) in laboratory populations of *C. elegans* provide good estimators of outcrossing rates (r), with r = 2*m. Measurement of male fractions was carried out in 3 blocks, with samples from all time points of experimental evolution being measured in same-numbered replicates in all experimental populations. Ancestral populations with standing genetic variation (A_0, D_0) were measured in all blocks (thus being

pseudoreplicated); in addition to contributing to a more balanced design, this also allowed the quantification of effects due to the different blocks.

Statistical analysis consisted of separate Analysis of Variance (ANOVA) for genetically homogeneous ($isoA_{1-6}$, $isoD_{1-6}$) and genetically heterogeneous populations (A_0 , A_{1-6}, D_0, D_{1-6}). The two plates measured per replicate population were taken as the dependent variable. ANOVA was preceded by Bartlett tests of homogeneity of variances for populations from different mating systems and generation of experimental evolution (regardless of replicate). Because hermaphrodites were found in one of the replicates of inbred Dioecious populations ($isoD_2$), this replicate was excluded from the statistical analysis. Altough this could have resulted from cross-contamination between experimental populations, since they were passaged at the same time, all plates were sealed with parafilm and materials and experimenter hands were washed with ethanol between manipulation of different populations. Also, no other populations showed evidences of cross-contamination at the phenotypic or genetic level, for which we believe the emergence of hermaphrodites in this population most likely resulted from gene conversion, already described for this locus (Katju *et al* 2008). In the case of the genetically variable populations, for which only one ancestral population was available per mating system, ancestral populations (A_0, D_0) were pseudoreplicated in each block and therefore used to investigate the presence of block effects prior to elaboration of the full statistical model: when such effects were not significant at the 5% level, this factor was not included in the analysis. ANOVA took mating system (2 levels), generation (4 levels) and the interaction between both as fixed factors, as well as block (3 levels) and replicate population; the factor replicate has 7 levels in the analysis of genetically heterogeneous populations (one for the ancestral and 6 for the evolved populations), for which is partially collinear with generation. For this reason, no interaction was tested between replicate and the other factors and its significance only reported in the cases when the effects due to generation were not significant. Whenever replicate population and block were non-significant at alpha=0.05, a new analysis was

performed using a reduced model. Normality of model residuals was assessed by Shapiro-Wilk and Kolmogorov-Smirnov tests. Replicate populations were available for ancestral genetically uniform populations ($isoA_{1-6}$, $isoD_{1,3-6}$); the block structure of this assay was collinear with replicate and hence statistically the use of one factor of the other was not relevant. Genetically uniform populations were therefore similarly modelled relative to genetically variable experimental populations. Statistical models for both populations with high and low initial levels of genetic variation assume independence of the populations measured at the different time points. To investigate whether initial frequencies of males in genetically uniform Androdioecious populations ($isoA_{1-6}$) significantly differed from 0 at the beginning of experimental evolution, a linear model with generation, replicate and their interaction was used to test if the intercept of this model significantly differed from 0.

Sex ratio of male-sired progeny

Young (L4) single males from the ancestral (D_0, n=27) and evolved (D_{4-6}, n=35) Dioecious populations were picked at day 3 of the life cycle and allowed to mate with one *fog*-2(*q71*) female (strain JK574) for 24h in 6 cm culture plates previously ringed with 90 µl of palmitic acid (20mg/ml, Sigma-Aldrich) to prevent worms from escaping. The adult worms were removed from the plates and offspring were allowed to grow until adult stage (day 4 of the life cycle), time at which they were sexed under a dissecting scope at 10x magnification.

Statistical analysis: Shapiro Wilk tests of normality and Bartlett test of homocedasticy were performed for ancestral and the three evolved replicate populations. Both conditions were met and hence ANOVA was performed taking generation of experimental evolution (generation 0 in the case of ancestral or generation 100 in the case of evolved populations) and replicate (4 levels – 1 ancestral and 3 evolved) as fixed factors. Because none of the factors was significant at alpha=0.05, a one-sample Student's t-test was performed using all

observations to test the null hypothesis that the mean of the observed sex ratios was equal to 1.

Rates of non-disjunction of the sexual chromosome

Non-disjunction of the X-chromosome during hermaphrodite gametogenesis leads to the production of aneuploid gametes and can therefore also contribute to the (spontaneous) production of males. The rate at which this phenomenon occurs is genetically determined and there is genetic variation among *C. elegans* natural isolates for this trait (Teotónio *et al* 2006). We measured the rate of X chromosome non-disjunction in experimental Androdioecious populations with and without initial standing genetic variation (A_0, A_{1-6}, $isoA_{1-6}$) from generations 0, 30, 60 and 100 to investigate whether spontaneous production of males had evolved with experimental evolution and to which extend they could explain the observed frequencies of males. 10 culture plates with the grand-progeny of ~50 young hermaphrodites were scored for male frequency (see "Frequency of males and Outcrossing" rates) at a density of 800 worms /plate. This assay was carried out in 3 blocks, with ancestral populations being included in all (thus being pseudoreplicated). Only 3 genetically uniform populations were measured at generation 0 (since the phenotypic variance among all of them should be very small).

Statistical analysis: Outlier observations (those outside the interval [Lower quantile - 1.5 Interquantile range, Upper quantile + 1.5 Interquantile range]) in genetically diverse and genetically homogeneous populations at each time point (regardless of replicate) were removed prior to analysis. A Bartlett test of homogeneity of variances revealed heteroscedasticity, confirming the expected differences in variance between Androdioecious populations with high and low levels of standing genetic variation and therefore ANOVA was performed separately for both types of populations. Ancestral populations with high levels of standing genetic variation (A_0) were used to test for block effects; because these

were not significant for alpha=0.05, they were removed from the model, which then took the mean of the fractions of males among the 10 plates measured as response variable, generation (4 levels), replicate (7 levels) and the interaction between both. No significant differences between groups were found when a model taking all observations was analyzed. The ANOVA taking as response the mean of the 10 observations per replicate genetically homogeneous population took generation (4 levels) and replicate (6 levels) as fixed factors; the interaction between both could not be tested due to the insufficient degrees of freedom. The model did not reveal significant differences between the groups. A model involving all individual observations revealed significant differences between replicates, differences between generations (with decreasing frequencies of males relative to ancestral populations) and a significant interaction between generation and replicate population; significance of the generation term was caused by significantly *lower* values of male frequencies at generation 30 and 100 compared to generation 0; the overall model presented an adjusted R^2= 29%. To facilitate comparison between genetically homogeneous and genetically diverse populations, the first model is presented.

Fitness-proxy assay

Fitness was measured as competitive ability of experimental populations against a *tester* population expressing Green Fluorescent Protein (GFP). The *tester* population resulted from the introgression of a transgenic, dominant allele into the genetic background of the ancestral hybrid (androdioecious) population (see Figure 1.6 and chapter 1 for details on the construction of this population). Therefore, the *tester* population has qualitatively and quantitatively similar levels of genetic variation relative to the ancestral androdioecious population, with the exception that is bears the transgenic allele in chromosome I that drives the expression of GFP in the muscle cells of the worms. This allows the phenotypic discrimination between GFP-positive and GFP-negative worms under a dissection scope equipped with UV light, and hence the discrimination between individuals

from experimentally evolved and *tester* populations. Competitive ability was calculated from the relative frequency of GFP-negative (wild-type) individuals after one generation of competition in conditions similar to those of experimental evolution, as described in detail next.

Samples from the tester population were thawed and expanded for 2 generations in parallel with samples from the populations involved in experimental evolution: the ancestral Androdioecious and Dioecious populations with high levels of standing genetic variation (A_0 and D_0), three evolved replicates of both these populations (A_{4-6}, D_{4-6}) and samples from the six genetically uniform Androdioecious populations (iso A_{1-6}) from generations 0, 30, 60 and 100. Competitions were initiated at day 3 of the life cycle (as L4 staged worms), by picking 40 experimental individuals and 60 tester individuals to 6cm Petri dishes with NGM lite agar (US Biological), supplemented with 1µg of Ampicillin and 5µl of bacteria. For these (fixed) proportions of GFP-negative to GFP-positive individuals, numbers of males were manipulated in order for rates of outcrossing within competitions to match those experienced by experimental populations during experimental evolution. Hence, in the competitions involving Androdioecious populations with genetic variation (A_0, A_{4-6}) males were placed at a frequency of 0.2 (8 experimental males, 32 experimental hermaphrodites, 12 GFP-tester males and 48 GFP-tester hermaphrodites for an outcrossing rate of 0.4); at a frequency of 0.4 in competitions involving Dioecious populations with genetic variation (D_0, $D_{4-6,}$ r=0.8), and at increasing frequencies of 0, 0.1, 0.2 and 0.3 in competitions involving the Androdioecious populations without initial genetic variation (isoA_{1-6}) from generations 0, 30, 60 and 100, respectively. Because the number of worms in each competition plate was too small to perform the standard “hatch-off” protocol, an alternative protocol was employed. The latter resembles the “hatch-off” protocol to the greatest extent possible in what concerns the conditions experienced by worms during the procedure, with the exception that is carried out in culture plates instead of liquid medium in polypropylene tubes and that densities of L1 larvae in the following generation are not controlled for. 24h

after set-up, at day 4 of the life-cycle, worms were washed off the plates with 200µl of M9 solution, placed in a new 6cm culture plate without bacteria and exposed to 200µl of fresh hypochloride solution for 5min. Most of the solution was aspirated with a 200ul pipette, the remainder was left to dry at room temperature for additional 5min and the worms were then placed into the incubators at standard conditions of temperature and humidity. 24h later, at day 1 of the following generation, surviving L1 individuals were washed off the plates with ~200µl of M9 and seeded to 9cm plates with bacteria. Three days later assay plates were placed at 4^0C until scoring of adult worms for GFP expression. Although density was not controlled for, it did not exceed 1,000 individuals per plate and hence food was not limiting during this stage of the assay. At the time of scoring, plates were left at room temperature for 15min. A circle with a diameter of 9cm and filled with a grid was printed in a transparent film, and placed under the culture plate during observation under the dissection scope at 30x magnification. This allowed scoring of individuals following standardized transects across the plates. A minimum of 100 individuals were scored per culture plate. Five competitions were performed by replicate population sample in two blocks. The ancestral populations with standing genetic variation were pseudo-replicated 2 times to ensure a balanced design. Scoring of a total of 215 plates was done by two different experimenters with a fairly good concordance rate among them – the Pearson correlation among GFP-positive frequency estimated by both experimenters is of $r = 0.79$ ($p < 0.001$; $n = 38$) . In these plates, the average value among experimenters was taken as another level for statistical analysis. The fitness-proxy (w) was measured as the expected frequency of wild-type (GFP-negative) alleles given the observed proportions of wild-type individuals (P) at the end of the competition. Because the wild-type (*wt*) allele is recessive to the *gfp* allele, wild-type progeny of competitions involving the Dioecious experimental populations (D_0, D_{4-6}) could only be generated by mating among individuals from these populations; hence, the expected frequency of the *wt* allele (and consequently fitness) is $w = \sqrt{P}$. In competitions involving the Androdioecious populations (A_0, A_{4-6}, $isoA_{1-6}$) the

representation of *wt* alleles at the end of the competition was a function of both outcrossing among experimental individuals and self-fertilization of experimental hermaphrodites; therefore, $w = r^*\sqrt{P} + (1-r)^*P$, where r is the outcrossing rate and the first and second terms on the right-hand side of the equality represent the contributions of both cross and self-fertilization events among experimental individuals to the frequency of the *wt* allele in the following generation. The terminology *wt* allele frequency and *gfp* allele frequency was used thus far for the sake of simplicity. In fact, because competitions started with the two homozygote classes – *wt*/*wt* (experimental populations) and *gfp*/*gfp* (tester population) - and because they were carried out for only one generation, no recombinant (haploid) gametes were produced; this is to say that *wt* and *gfp* alleles do not exist as such, since they are inexorably tied to their haploid genetic context. Therefore, to be more precise, what was measured in reality were the frequencies of GFP-negative and GFP-positive *haploid genome complements*, with the fitness-proxy being interpreted as the ratio of the marginal fitness of the GFP-negative haploid complement over the mean populations fitness. Modeling fitness in this fashion assumes that heterozygotes are co-dominant, that under androdioecy unmated hermaphrodites self-fertilize, and also that there are no frequency or density dependent dynamics between experimental and tester populations.

Statistical analysis: Because of the overrepresentation of Androdioecious experimental populations relative to Dioecious populations, two models were used to analyze the data – one including the genetically uniform populations ($isoA_{1-6}$), the other including the genetically variable Dioecious and Androdioecious populations (A_0, D_0, A_{4-6}, D_{4-6}). With respect to the first set of populations, outlier observations (those outside the interval [Lower quantile - 1.5 Interquantile range, Upper quantile + 1.5 Interquantile range]) were removed from groups defined by generation (that is, regardless of replicate population). ANOVA of these populations took the values of fitness (standardized by corresponding rates of outcrossing) as response variable, generation (4 levels), replicate (6 levels), their interaction (15 levels), experimenter at setup (3 levels) and experimenter at

scoring (3 levels) as fixed factors; time of setup was included as covariate; block effects were not modeled due to collinearity with replicate in this dataset. Both experimenter (at setup) and time of setup did not reveal significance at alpha=0.005, for which a reduced model is presented. Normality of data and residuals, and heteroscedasticity among the replicate populations was assessed irrespective of replicate by Kolmogorov-Smirnov tests and Bartlett tests. Adjusted least-square estimates of effects were estimated by generation for each replicate and then used to calculate the linear regression of fitness-proxy on outcrossing rate. Two replicates showed a large standardized residuals (|stRes|>2.5) and were removed from the regression.

Populations with initial standing genetic variation ($A_{0,}$ $A_{4\text{-}6}$, $D_{0,}$ $D_{4\text{-}6}$) were similarly analyzed. The effects of block were first investigated by performing one-way ANOVA of (pseudoreplicated) ancestral Dioecious and Androdioecious populations separately; because none of them revealed significance of block effects at alpha=0.05, block was not incorporated in the full model (due to partial collinearity with replicate). Hence the full model took generation (2 levels), mating system (2 levels), the interaction between them (1 level), replicate (4 levels), experimenter at setup (3 levels) and experimenter at scoring (3 levels) as fixed factors; time of assay setup was taken as covariate but because it was not significant it was removed from the model. Least-square estimates from the ANOVA were obtained for each evolved experimental population and one-sample Student's t tests were performed to test the null hypothesis that their means did not significantly differed from the values predicted by the linear regression of fitness on rates of outcrossing.

Statistical analysis

All statistical analysis was carried out in the R software using custom scripts (R Development Core Team 2010), unless otherwise stated. Whenever Analysis of Variance (ANOVA) was involved, the distribution of residuals was tested for normality by Shapiro-Wilk and Kolmogorov-Smirnov tests; only non-normality is

reported. ANOVA tables are provided as Supplementary Tables. Values plotted in graphical displays refer to raw data, not parameters from the statistical models.

2.4 Results

Evaluation of the levels of segregating variation and levels of inbreeding in ancestral experimental populations

An assessment of the levels of genetic diversity of ancestral experimental populations was carried out by genotyping single individuals from ancestral experimental populations at seven microsatellite loci. The results are presented in Table 1.

Genetic variation	Inbreeding	Population	Statistic	Loci						
				4001	IIR	3003	VL	XR	1003	4004
high	high	A_0	N	45	41	43	43	47	47	39
			A	5	6	7	5	4	4	4
			Ae	2.8	3.2	4.3	2.4	3.6	2.9	2.1
			Hobs	0.32	0.13	0.29	0.21	0.13	0.11	0.11
			Fis	0.51	0.82	0.63	0.64	0.82	0.84	0.8
high	low	D_0	N	44	35	35	29	41	40	33
			A	3	4	7	5	4	4	4
			Ae	2.4	3.0	3.1	2.8	3.0	2.5	2.2
			Hobs	0.49	0.62	0.35	0.25	0.30	0.03	0.31
			Fis	0.17	0.08	0.49	0.62	0.56	0.96	0.43
low	high	$isoA_1$	N	16	15	16	16	13	9	14
			A	2	1	1	1	3	2	1
			Ae	1.1	1.0	1.0	1.0	1.4	1.5	1.0
			Hobs	0.07	0.00	0.00	0.00	0.00	0.00	0.00
			Fis	-0.07	-	-	-	1	1	-
low	high	$isoA_2$	N	16	13	15	15	8	8	13
			A	1	1	1	1	2	2	2
			Ae	1.0	1.1	1.0	1.0	1.3	1.9	1.1
			Hobs	0.00	0.00	0.00	0.00	0.00	0.00	0.08
			Fis	-	-	-	-	1	1	-0.08
low	high	$isoA_3$	N	16	16	16	15	4	7	14
			A	1	2	1	1	1	2	2
			Ae	1.0	1.1	1.0	1.0	1.0	1.3	1.1
			Hobs	0.00	0.07	0.00	0.00	0.00	0.00	0.08
			Fis	-	-0.07	-	-	-	1	-0.08
low	high	$isoA_4$	N	14	14	15	15	16	14	13

			A	3	1	4	2	2	3	3
			Ae	2.3	1.0	2.2	1.5	2.0	2.0	2.1
			Hobs	0.31	0.00	0.36	0.14	0.00	0.00	0.08
			Fis	0.47	-	0.36	0.57	1	1	0.85
low	high	**isoA$_5$**	**N**	16	14	16	16	16	14	14
			A	3	3	4	2	2	1	3
			Ae	1.8	2.1	3.0	2.0	1.3	1.0	2.1
			Hobs	0.27	0.08	0.13	0.00	0.00	0.00	0.15
			Fis	0.40	0.86	0.81	1	1	-	0.72
low	high	**isoA$_6$**	**N**	16	12	15	16	15	15	11
			A	3	4	5	3	2	3	2
			Ae	1.9	2.1	2.8	2.2	2.0	2.2	1.4
			Hobs	0.40	0.27	0.21	0.13	0.00	0.00	0.00
			Fis	0.16	0.49	0.68	0.77	1	1	1
low	low	**isoD$_1$**	**N**	14	13	14	14	14	14	15
			A	3	3	5	4	5	5	4
			Ae	2.2	2.8	3.8	2.4	3.0	2.0	2.0
			Hobs	0.00	0.00	0.08	0.00	0.15	0.31	0.00
			Fis	1.00	1.00	0.90	1.00	0.78	0.42	1.00
low	low	**isoD$_3$**	**N**	12	12	13	13	13	13	14
			A	3	3	5	3	2	2	3
			Ae	2.3	2.9	4.3	2.3	1.6	1.6	1.9
			Hobs	0.00	0.00	0.00	0.00	0.00	0.17	0.08
			Fis	1.00	1.00	1.00	1.00	1.00	0.55	0.85
low	low	**isoD$_4$**	**N**	13	13	12	12	12	14	14
			A	2	3	4	2	3	4	5
			Ae	2.0	2.6	3.6	2.0	1.4	1.8	1.9
			Hobs	0.00	0.00	0.00	0.00	0.09	0.31	0.15
			Fis	1.00	1.00	1.00	1.00	0.70	0.34	0.68
low	low	**isoD$_5$**	**N**	13	13	12	13	12	15	16
			A	2	3	4	2	3	5	5
			Ae	2.0	2.3	3.3	2.0	1.3	2.3	1.7
			Hobs	0.00	0.00	0.00	0.00	0.09	0.14	0.00
			Fis	1.00	1.00	1.00	1.00	0.61	0.75	1.00
low	low	**isoD$_6$**	**N**	14	13	14	13	13	14	15
			A	2	3	4	2	3	4	3
			Ae	2.0	2.6	3.3	2.0	1.6	1.8	1.5
			Hobs	0.00	0.00	0.00	0.00	0.00	0.38	0.07
			Fis	1.00	1.00	1.00	1.00	1.00	0.16	0.79

Table 2.1: Measures of genetic diversity and of inbreeding of ancestral experimental populations calculated from genotyping of microsatellite loci. N – number of individuals analyzed, A – number of observed alleles, Ae – effective number of alleles, Hobs – observed heterozygosity, Fis – coefficient f inbreeding.

Ancestral hybrid populations (A_0, D_0) exhibit a median number of alleles per locus of 5 and 4, respectively, with a maximum number of 7 alleles and a minimum of 3 alleles. The nearly isogenic dioecious and androdioecious populations (isoD$_1$-

$_6$, isoA$_{1-6}$) exhibit medians of 2.5 and 3 alleles per locus, respectively. Although these values may seem higher than expected for the latter populations, they correspond to median *effective* allele numbers of 2.2 and 1.6. With respect to inbreeding, the observed heterozygosity across loci (and replicates) ranged from 0.11 to 0.32 in ancestral hybrid androdioecious populations (A_0); the observed heterozygosity across loci in the ancestral dioecious population (D_0) varied from 0.25 to 0.62 (excluding locus 1003). Mean coefficients of inbreeding (F_{is}) were 0.72±0.02 and 0.39±0.05, respectively. Most loci examined exhibited maximal levels of inbreeding (H_{obs}=0) in genetically uniform Dioecious and Androdioecious populations, although maximum observed heterozygosity ranged from 0.07 to 0.4 across replicate Androdioecious populations (isoA$_{1-6}$) and from 0.14 and 0.38 across the replicate Dioecious populations (isoD$_{1,3-6}$). These data consist only of a modest description of the levels of genetic variation and of inbreeding of ancestral experimental populations. Genotyping of 282 single nucleotide polymorphisms across 1/3 of the genome was also carried out for these populations and its analysis further confirmed significant differences among experimental populations both with respect to levels of standing genetic variation (number of haplotypes) and to levels of inbreeding (Chelo, pers. comm.). In fact, the haplotypic diversity segregating in the ancestral Androdioecious and Dioecious experimental populations was higher than that described for a worldwide collection of natural isolates (Rockman and Krugliak 2009).

Intermediate and stable rates of outcrossing under androdioecy

In Androdioecious populations (A_0, $A_{1-6,}$ isoA$_{1-6}$), rates of selfing and outcrossing can evolve. For that reason, rates of cross-fertilization were monitored in these populations during experimental evolution by measuring the number of males segregating in them (see Methods). Male frequencies were also obtained for Dioecious populations (D_0, $D_{1-6,}$ isoD$_{1-6}$). The results are presented in Figure 2.2 (see Supplementary Table S2.3 for statistical analysis). The 14 genetically diverse populations (A_0, D_0, A_{1-6}, D_{1-6}) revealed different levels of outcrossing

between mating systems (P<0.001) as expected, but not with experimental evolution. Genetically uniform Dioecious and Androdioecious populations ($isoA_{1-6}$, $isoD_{1-6}$) exhibited significant differences due to mating system (P<0.001) as well as some replicate heterogeneity (P=0.034). A significant interaction between mating system and generation of experimental evolution was also found (p=0.026), indicating that levels of outcrossing increased in inbred Androdioecious populations ($isoA_{1-6}$) with time. At the onset of experimental evolution, the rates of outcrossing in these populations did not significantly differ from 0 (regression coefficient = 0.016 ± 0.012; t_{24}=1.40; p=0.17).

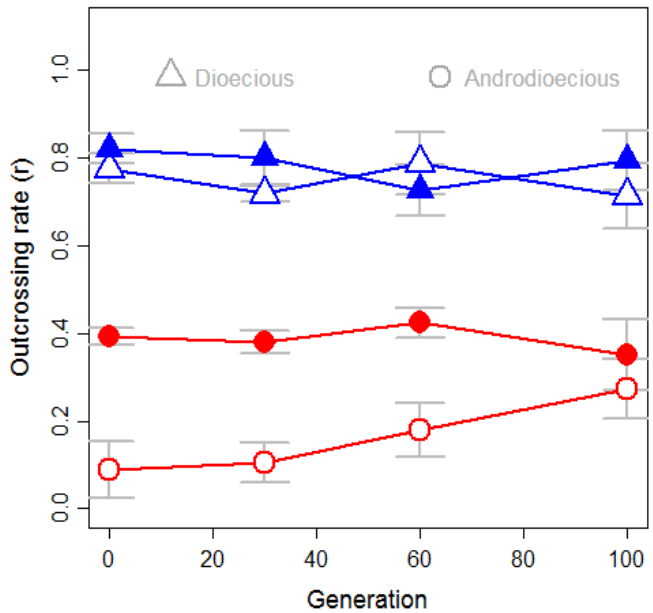

Figure 2.2: Evolution of outcrossing rates. Rates of outcrossing were estimated as twice the observed fraction of males. Red circles represent Androdioecious populations and blue triangles represent Dioecious populations. Experimental populations with high (filled symbols) and low (empty symbols) initial levels of standing genetic variation are plotted. Values are presented as means of pseudoreplicate (ancestral populations with high initial levels of genetic variation, A_0 and D_0) or means of replicate populations (evolved and genetically homogeneous populations, A_{1-6}, D_{1-6}, $isoA_{1-6}$, $isoD_{1-6}$). Error bars (grey) represent the standard error of the mean. See Supplementary Table S2.3 for statistical analysis.

Experimental populations of *C. elegans* under dioecy - with and without genetic variation - maintained high and stable rates of outcrossing (r≈0.8).

Although this pattern was expected (since it was imposed by design), the were somewhat lower than those predicted for populations reproducing exclusively by cross-fertilization (for which the expected rates of cross-fertilization should equal 1). The deviation of frequencies of males in Dioecious populations from the expected values can be explained by either segregation of an excess of females or by segregation of hermaphrodites in Dioecious populations. Regarding the latter possibility, gene conversion allowing back transformation of females into hermaphrodites (Katju *et al* 2008) or cross-contamination among Dioecious and Androdioecious populations could potentially explain segregation of hermaphrodites in Dioecious populations. However, it seems somewhat unlikely that either of these possibilities would occur in *all* replicate populations under dioecy (in a total of 12 populations). The hypothetical presence of hermaphrodites in Dioecious experimental populations could also result from the segregation of the *fog*-2(*wt*) allele at very low levels in ancestral populations. For the observed frequencies of males of Dioecious populations (40%), outcrossing rates should be of 80%, which means that hermaphrodites would have to have segregated at non-trivial frequencies to generate rates of self-fertilization of 20%. Although the differences between observed (0.39±0.01) and expected (0.64±0.003) heterozygosity of the hybrid Dioecious population (D_0) were statistically significant (t_6=-4.1, p=0.005), genetic analysis of Dioecious populations at single-nucleotide polymorphisms (SNPs), failed to reveal significant deviations of heterozygosity from expectations under random mating when measured by several different metrics (Chelo, pers. comm.). It is reasonable to assume that the larger amount of data available from SNP genotyping provides a better quantification of the deviations of allele frequencies from expectations under random mating, especially considering that the quality control performed for microsatellite data might have lead to a bias against heterozygote samples. Patterns produced by different amplification efficiencies of different alleles at a single locus are not always easily distinguishable from the patterns produced by mixed sources of genomic DNA.

Our approach to the analysis of electropherograms was a conservative one and all dubious samples were discarded (see Methods).

An alternative explanation for the discrepancy between expected and the observed rates of outcrossing in Dioecious populations relies on the putative segregation of females at higher frequencies than expected. Both differential viability between female and male zygotes or experimental error could account for this possibility. For example, *C. elegans* males are slightly harder to score than females (or hermaphrodites) under the protocol employed here, which could have led to an underestimation of their frequencies. Furthermore, exploratory behavior in *C. elegans* is well documented, with individuals moving frequently to the sides of the culture plates and dying of starvation or desiccation. This behavior is sex and age specific, being more pronounced in sexually mature males than in females or hermaphrodites (Lipton *et al* 2004). The increased exploratory behavior of males and the concomitant increased probability of being found on the sides of the culture plates (where desiccation is likely to occur) constitute a plausible explanation for the underestimation of the fraction of males in our assay. To address the possibility that our observations could be biased due to higher vagility of males and/or reflect differential survival between female and male zygotes, we performed crosses between ancestral and evolved males and *fog-2*(*q71*) females (strain JK574) in culture plates ringed with palmitic acid. Palmitic acid (or other high osmolarity solutions) (Sawin *et al* 2000, Dong *et al* 2000) forms a precipitate upon evaporation of the solvent, creating a physical barrier to the worms without affecting general worm behavior, fecundity or life span (Miller and Roth 2009, Locke *et al* 2008). Our results show that males from ancestral and evolved dioecious populations with high levels of standing genetic diversity (D_0, $D_{4\text{-}6}$) produced the expected sex ratio of 1 in the progeny when mated with standard females (Figure 2.3) under the conditions employed in the assay.

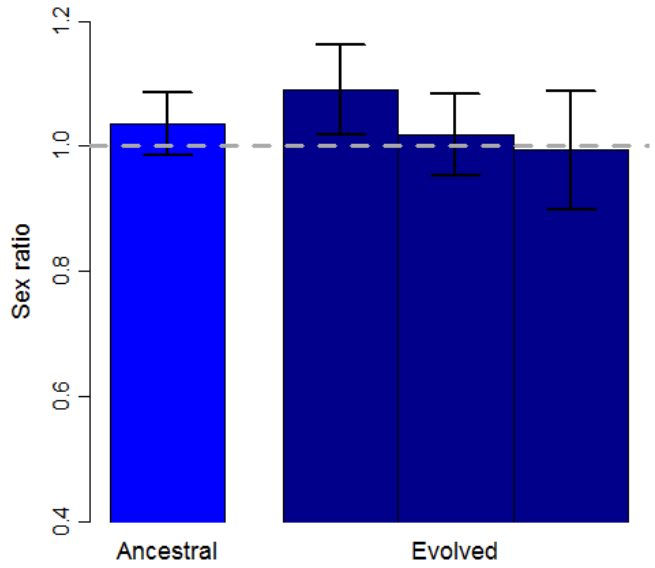

Figure 2.3: Sex ratio of male-sired progeny. The values represent the means of sex ratio (females/males) of progeny sired by males from genetically diverse ancestral (D_0; light blue) and three evolved ($D_{4\text{-}6}$; dark blue) Dioecious populations. Males were mated to *fog*-2(*q71*) females (strain JK574). Error bars (black) represent standard errors of the means. Observed sex ratios do not significantly differ from the expected value of 1 (grey dashed line). See Supplementary Table S2.4 for statistical analysis.

No differences in sex ratios were detected between ancestral and evolved populations or among different evolved replicate populations. Pooling all data, the null hypothesis that the observed sex ratios were equal to 1 could not be rejected statistically (one-sample t-test: t = 1.1002, df = 61, p-value = 0.2755). The fact that we observe the expected sex-ratio in the progeny of females sired by experimental males (from Dioecious populations) when exploratory behavior is spatially restricted, provides strong evidence for the hypothesis that such behavior caused a bias in the estimates of male frequencies in our assay. It also suggests that the deviations of observed male fractions from the expected values should not be due to differential viabilities of male and female zygotes in experimental populations, although this possibility could only be definitely ruled out if a similar assay would be performed including experimentally ancestral and evolved females instead of *fog-2*(*q71*) females from strain JK574.

One of the most surprising results obtained with this assay was that Androdioecious populations which started experimental evolution with high levels of genetic variation (A_0, $A_{1\text{-}6}$) exhibited intermediate levels of outcrossing ($r \approx 0.4$). Given the deviation of outcrossing rates in Dioecious populations from the expected value of 1 and under our explanation that this underestimation resulted from higher vagility of *C. elegans* males, a correction factor (1.25) should be applied to the observed fractions of males of Androdioecious populations with high levels of standing genetic variation. This correction results in estimated fractions of males of 25% and consequently to an outcrossing rate of 0,5. Perhaps even more surprising, these levels were stably maintained throughout 100 generations of experimental evolution. *C. elegans* is a predominantly selfing species with males occurring only seldomly, both in nature (Barrière and Félix 2005) and under laboratory conditions (Chasnov and Chow 2002). The segregation of high proportions of males at the beginning of experimental could have resulted from the crossing scheme among natural isolates to produce the ancestral hybrid populations. One possibility is that the levels of males generated by the enforced cross-fertilization between natural isolates were too high for the rounds of population expansion to efficiently reduce them to their equilibrium frequencies. However, with experimental evolution at large population sizes, frequencies of males could have decreased if their equilibrium frequencies were lower. Another possibility is that the observed rates of outcrossing resulted from genetic differences between the natural isolates for male production and male equilibrium frequencies that were revealed under (common) laboratory conditions. Genetic variation among natural isolates for spontaneous production of males has been reported for *C. elegans* (Teotónio et al 2006). More recently, equilibrium frequencies of males in some of these natural isolates have revealed that some of these populations can maintain males at appreciable frequencies under laboratory environment (see Anderson *et al.* 2010 for a review). In fact, the strain for which the highest fractions of males were found in this latter study (strain AB1) is among the 16 natural isolates used in the construction of our ancestral experimental

populations. Although this can potentially explain the initially high levels of outcrossing of genetically variable Androdioecious populations (A_{1-6}) populations, other explanations must be sought for to justify their maintenance. Perhaps males and cross-fertilization are selectively neutral under the conditions of experimental evolution employed in this study. Under this hypothesis, it is nevertheless odd that none of the six replicate populations significantly decreased their frequency of males over 100 generations, since under neutrality both increases and decreases in male frequencies would be expected among replicates. Another line of evidence further argues against the potential selective neutrality of males: Androdioecious populations without genetic variation ($isoA_{1-6}$), in which virtually no males were segregating at generation 0, significantly increased outcrossing rates with experimental evolution. At generation 100, these populations exhibited outcrossing rates of ~0.3 (Figure 2.2). This increase of the fractions of males in genetically homogeneous populations can only be explained either by evolution of the rate of spontaneous production of males (X chromosome non disjunction) or by positive selection for outcrossing, once some males appeared in these populations. Rates of X-chromosome non-disjunction are genetically determined and there is variation among *C. elegans* natural isolates for this trait (Teotónio 2006). Because of this, we investigated whether rates of spontaneous production of males evolved during experimental evolution in both genetically uniform ($isoA_{1-6}$) and genetically heterogeneous (A_0, A_{1-6}) Androdioecious populations. The results show that this was not the case (Figure 2.3): ANOVA of genetically heterogeneous populations (A_0, A_{1-6}) revealed no overall differences among them; Androdioecious populations without initial standing genetic variation ($isoA_{1-6}$) showed a similar pattern.

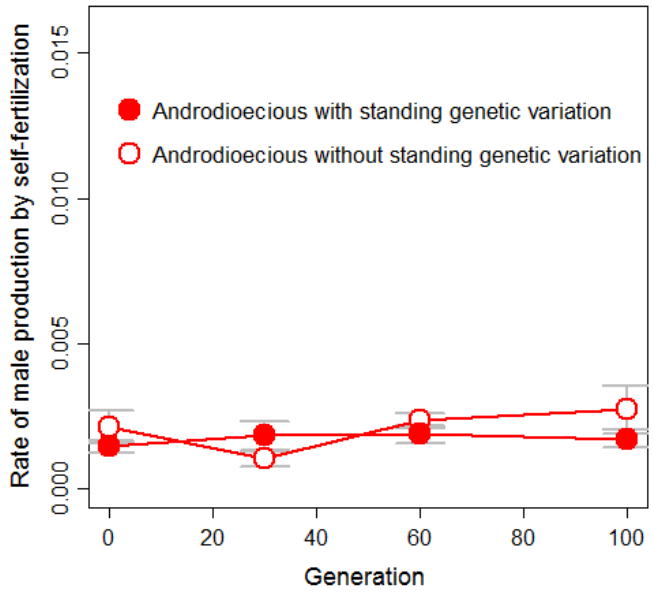

Figure 2.3: Rates of spontaneous production of males in Androdioecious populations. Rates of non-disjunction of the X chromosome were estimated from frequencies of males in the grand-progeny of unmated hermaphrodites of individuals of Androdioecious populations with high (filled circles) and low (empty circles) initial levels of standing genetic variation. Values are presented as means of replicate or pseudoreplicate (ancestral genetically diverse) experimental populations. Error bars (in grey) represent standard error of means. See Supplementary Table S2.5 for statistical analysis.

Furthermore, the frequencies of males produced by non-disjunction of the X chromosome during meiosis were two orders of magnitude lower than the frequencies observed during experimental evolution for the genetically heterogeneous Androdioecious populations. Hence, the rates of spontaneous production of males by self-fertilizing hermaphrodites do not explain the observed outcrossing rates in neither genetically heterogeneous nor genetically uniform Androdioecious populations. Altogether, our results argue for the selective advantage of males and outcrossing in experimental Androdioecious populations, with selection having increased their frequency (in genetically uniform populations) once they appeared spontaneously. In genetically variable populations, several explanations can be proposed to justify the initially high levels of males but if selection was involved in their maintenance (as our results suggest), this begs the question of why their frequencies (and hence rates of cross-fertilization) did not

increase any further. Theoretically, androdioecious populations of *C. elegans* could evolve towards full outcrossing.

Rates of outcrossing and mean population fitness

To investigate the hypothesis of selective advantage of outcrossing under our experimental conditions, we obtained the evolutionary trajectory of fitness of the genetically uniform Androdioecious populations ($isoA_{1-6}$), for which rates of outcrossing evolved with experimental evolution. This was done by measuring their competitive performance against a *tester* population carrying a dominant marker (GFP). In addition, to further test the general hypothesis of adaptation from standing genetic variation to the novel environment, the fitness of ancestral and three evolved experimental populations under dioecy (D_0, D_{4-6}) and androdioecy (A_0, A_{4-6}) was also quantified employing the same methodology. Competitive performance was measured as the change in the relative frequency of experimental and *tester* populations over the course of one generation. Initial frequencies were fixed (60% *tester* individuals, 40% experimental individuals) but relative frequencies of males at setup were manipulated to match those of the experimental populations: 0.4 for the genetically variable Androdioecious populations (A_0, A_{4-6}), 0.8 for the genetically variable Dioecious populations (D_0, D_{4-6}) and variable in the genetically uniform Androdioecious populations ($isoA_{1-6}$), corresponding to the measured frequencies of males at generations 0, 30, 60 and 100 (see Methods).

Evolved Androdioecious populations without initial standing genetic variation ($isoA_{1-6}$) showed a mean population fitness increase of about 20% relative to their ancestral populations. Interestingly, the relationship between the fitness proxy of these populations and the rates of outcrossing exhibited throughout experimental evolution was linear, positive and significant (Figure 2.4).

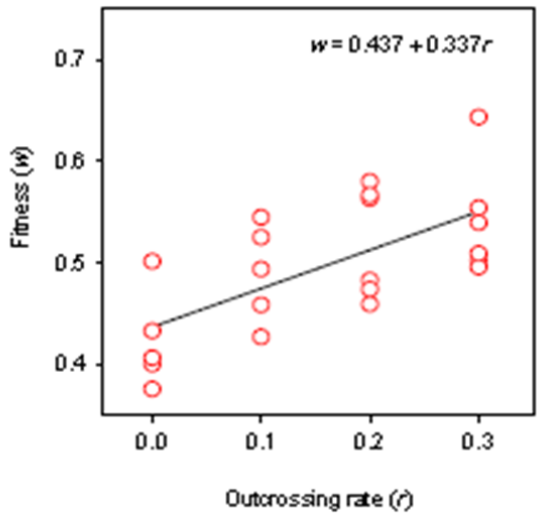

Figure 2.4: Rates of outcrossing and population fitness. Regression of the fitness-proxy on outcrossing rate in the six genetically homogeneous Androdioecious populations ($isoA_{1-6}$). The least-square mean estimates per replicate population obtained from ANOVA are represented by the red circles. Regression of fitness-proxy on outcrossing is shown as a line ($F_{1,20}$=15.2; $p<0.001$; adjusted R^2 = 40.3%; PRESS=0.062; power of 0.93 for $\alpha = 0.05$).

The mathematical description of the expected fitnesses as a function of the values of phenotypic characters is called the individual selection surface (Arnold *et al* 2001). In the context of our experiment, we dealt with only one phenotype (outcrossing rates or the individual production of males), for which this relationship does not describe a surface, but a function (of *x* on *y*) instead. The individual fitness surface is closely associated with the adaptive landscape, which describes the relationship between a (multivariate) phenotypic space and population-level fitness. In fact, the slope of the adaptive landscape can be estimated by the average slope of the individual selection surface weighted by the phenotypic distribution (see Figure 8 in Arnold *et al* 2001), at least at the point close to the trait distribution mean. In the case of the genetically homogeneous Androdioecious populations ($isoA_{1-6}$), their phenotypic distributions should be extremely narrow and therefore they should provide a good approximation of the adaptive landscape. Under our experimental conditions, the selection gradient of rates of outcrossing is 0.337. These results strongly support the hypothesis that

outcrossing is selective advantageous under the environmental conditions employed in this study.

The fitness values of ancestral and evolved genetically diverse Androdioecious and Dioecious populations (after standardization by rates of outcrossing – see Methods) are shown in Figure 2.5.

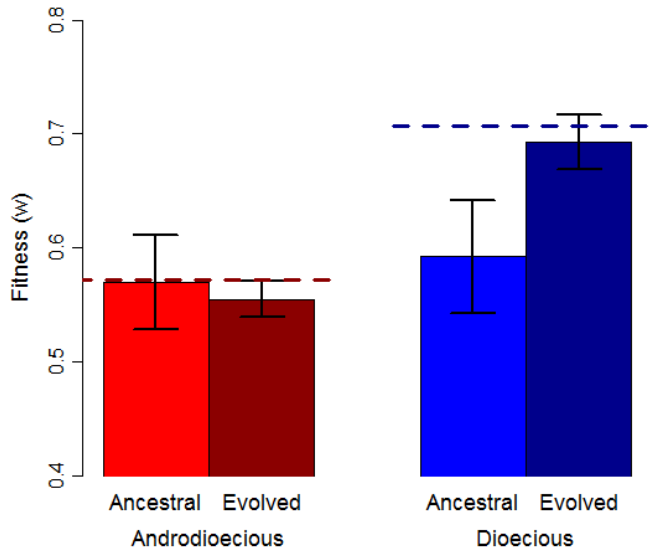

Figure 2.5: Evolution of population fitness of genetically diverse populations. Fitness was measured as competitive performance of experimental populations against a *tester* population expressing GFP. Fitness values represent the expected proportions of wild-type genome complements based on the observed frequency of wild-type (GFP-negative) individuals at the end of the competition. Values are plotted as means of replicate (evolved) or pseudoreplicate (ancestral) experimental populations. Because they are standardized by the rates of outcrossing, these values are comparable between Androdioecious (red) and Dioecious (blue) populations. Error bars (black) represent standard error of the means. Dashed lines indicate the values of fitnesses expected from the linear regression of fitness on rates of outcrossing (Figure 2.4). See Supplementary Table S2.6 for statistical analysis.

Statistical analysis of the fitness of these populations revealed a significant increase under dioecy but not under androdioecy (interaction term between mating system and generation: $F_{1,47}$=7.83, p=0.007), with Dioecious populations having experienced an increase in mean fitness of 17%. The immediate interpretation of these results is that adaptation occurred under Dioecy but not under Androdioecy. Note, however, that both *evolved* Androdioecious ($A_{4\text{-}6}$) and Dioecious ($D_{4\text{-}6}$)

populations exhibit the expected fitness values predicted from the linear regression of fitness on rates of outcrossing presented above (see Figure 2.4). Hence, while Dioecious populations exhibited lower fitness values at the onset of experimental evolution compared to what would be expected for their rates of outcrossing, ancestral Androdioecious populations exhibited values for fitness already matching those expected for rates of outcrossing of 0.5. Therefore, Dioecious populations could maximize their fitness during experimental evolution, whereas Androdioecious populations already exhibited the theoretical maximum value for fitness predicted for their rates of outcrossing at the onset of experimental evolution.

In theory, populations of organisms in which both self and cross fertilization can occur should evolve towards fully selfing or fully outcrossing states. Hence, if outcrossing is advantageous under the experimental conditions employed in our study, why didn't Androdioecious populations increase their rates of outcrossing any further? Mating between individuals comes at a cost. In purely outcrossing species, finding a partner may imply large investments in terms of time and courtship, for example. Also, mating itself may reduce lifespan in a number of organisms, hence compromising future production of progeny; it may also increase the risk of transmission of diseases and, in some cases, the risk of predation (Daly *et al* 1978). Even in the absence of these effects, mating is costly because it requires the production of individuals that do not, on their own, produce progeny (the males) (Maynard Smith 1978, Bell 1982). In other words, a purely hermaphroditic population will have a higher growth rate than a population in which males also segregate. Therefore, for males to be maintained in systems such as androdioecy, they must confer some evolutionary advantage. In light of the results obtained with our experiment, this seems to be the case: males, and consequently increased rates of cross-fertilization, seem to be advantageous under our experimental conditions. However, such benefits are likely not to have been sufficient to completely offset the advantages of self-fertilization, leading to

the maintenance of stable, intermediate frequencies of males in experimental Androdioecious populations.

2.5 Discussion

In this study, we exposed experimental populations of *C. elegans* to a novel (laboratory) environment characterized by a simple life-cycle, controlled demography, stable environmental parameters and high replication. The particular features of this organism, especially those regarding its mode of reproduction, permitted us to generate populations with high or low levels of standing genetic variation and, within these, with different initial levels of inbreeding. This allowed us to investigate the extent to which different mating systems with different levels of inbreeding could influence adaptation to a novel environment.

The first important conclusion that emerges from our results is that despite the relatively narrow range of outcrossing rates (≈0-0.3) exhibited by Androdioecious populations starting experimental evolution with low levels of genetic variation (isoA_{1-6}), they seem to have been sufficient to describe the (univariate) adaptive landscape of our experimental populations across the entire range of possible phenotypic values for rates of outcrossing (0-1).This was shown by the fact that the fitness values (measured as competitive ability against a tester population) of evolved, genetically diverse Androdioecious (A_{4-6}) and of Dioecious populations (D_{4-6}) did not significantly differ from those predicted by the linear regression of fitness on rates of outcrossing. Furthermore, our results demonstrate that adaptation occurred under our experimental conditions and that outcrossing was selectively favored during adaptation to a novel environment.

Genetically variable Androdioecious populations (A_0, A_{4-6}) did not evolve their rates of outcrossing, which were found at presumably optimal levels from the beginning of experimental evolution; because their fitness values also matched the maximum values predicted for the exhibited rates of outcrossing, fitness did not significantly increase in these populations. Nevertheless, this result is extremely interesting since it shows that intermediate and stable rates of cross-fertilization

can be maintained in a population of organisms which reproduce predominantly by selfing. Mating is evolutionary costly, since populations in which single individuals can produce progeny (such as asexuals or selfers), inevitable exhibit higher growth rates (Maynard Smith 1978, Bell 1982). The maintenance of mixed mating systems (where both self and cross fertilization can occur) is therefore evolutionarily puzzling and explanations for the evolution and maintenance of biparental reproduction have been sought for for many decades. The maintenance of males in androdioecious populations such as those of *C. elegans* is an example of the paradox of mixed mating systems. Maintenance of males and of outcrossing has seldomly been reported for populations of *C. elegans* maintained under similar laboratory conditions to those employed in this study (Anderson *et al* 2010). Most theoretical models for the maintenance of males in predominantly selfing populations (Otto *et al* 1993, Chasnov and Chow 2002, Stewart and Phillips 2002, Cutter and Payseur 2003) rely on essentially the same parameters: for males to be selectively maintained in these populations, either 1) viability differs between males and hermaphrodites, 2) inbreeding depression significantly affects the fitness of selfed progeny or 3) males sire twice as many progeny as hermaphrodites. Most studies thus far have failed to detect sex-specific differences in viability (Hodgkin 1987, Gems and Riddle 1996, 2000) or significant depression of fitness components upon inbreeding (Johnson and Wood 1982, Johson and Hutchinson 1993, Chasnov and Chow 2002, Dolgin *et al* 2007) in the canonical strain N2 as well as in natural isolates of *C. elegans*. Similarly, our experimental populations do not show evidence for sex-specific differences in viability (see chapter 3) nor inbreeding depression: ten generations of self-fertilization were imposed in ancestral Androdioecious populations and 21 generations of brother-sister mating were performed in Dioecious populations to generate ancestral populations starting experimental evolution from severely reduced levels of segregating variation. During this process, we did not detect a significant loss of selfed lineages. In effect, only genetically diverse Dioecious populations showed extinction of a significant number of lines (Teotónio, pers.

com.). This pattern was no longer observed when lines were derived from evolved populations, where no significant extinctions occurred during the derivation of neither type of population. With respect to the possibility of males siring twice the number of offspring than hermaphrodites, although there is genetic variation among *C. elegans* strains for male fertilization success (Teotónio et al 2006) and males can sire considerable fractions of progeny in mixed populations (Stewart and Phillips 2002, Cutter and Payseur 2003), these phenotypic values seem to be insufficient to maintain them in androdioecious populations of this organism. To complicate things further, male fertilization success is likely to be frequency-dependent, with low numbers of males being insufficient to lead to a significant fertilization success (compared to hermaphrodites) and higher frequencies also decreasing male fertilization success because of male-male interactions (Stewart and Phillips 2002). Interestingly, this model predicts maximum fertilization success of males for male frequencies of ~0.2 – a very similar value to that observed in our experimental Androdioecious populations. The most successful attempts to investigate the conditions for male maintenance have consisted of experiments designed to increase inbreeding depression by subjecting populations to high mutational input. Although these revealed significant sustenance of males for longer periods, these frequencies still returned to values equivalent to rates of spontaneous production of males (Cutter 2005, Manoel *et al* 2007).

The vast majority of the studies investigating the conditions under which cross-fertilization can be maintained in this androdioecious system have used highly inbred strains. Our results show that in the context of genetic variation (whether pre-existing or generated by mutation) *and* exposure to a novel environment, males and outcrossing can be stably maintained at intermediate levels in mixed-mating systems. But if laboratory environment constitutes a novel selection regime to natural isolates and mutagenesis generates genetic variation at an even higher rate than in our genetically homogeneous populations, then why weren't males maintained in previous experiments? Let's interpret the maintenance of males in genetically diverse populations first. If the maintenance of

males in Androdioecious populations of *C. elegans* is conditional on the presence of genetic variation and selection in novel environments, then their evolutionary value should lie in their ability to increase the genetic variance for fitness (by elevating rates of cross-fertilization) and hence augment the potential to respond to selection. In populations where several alleles are segregating simultaneously, higher outcrossing (or, conversely, reduced inbreeding) equates with elevated heterozygosity and with higher rates of *effective* recombination. The consequence of this is that several alleles with deleterious effects can be combined on the same genotype; simultaneously, beneficial alleles can also be united in the same genotype. In this way, genotypic classes of lower and higher fitness can be created, thus increasing the additive genetic variance for fitness. Cross-fertilization is thus expected to enhance adaptation by rendering natural selection more efficient. However, cross-fertilization is not always advantageous: outcrossed progeny can exhibit greatly reduced fitness compared to inbred progeny. This phenomenon is called outbreeding depression and it can occur upon crosses between individuals from different species (Templeton 1981) but also in crosses between individuals from distant demes within populations (Dobzhansky 1948). Several genetic phenomena can underlie outbreeding depression, among which gene by environment interactions (local adaptation), underdominance (the decreased fitness of heterozygotes relative to either of the homozygotes) and epistatic interactions between loci. *C. elegans* typically exhibits outbreeding depression when different natural isolates are crossed (Dolgin *et al* 2007). In this species, this seems to be caused mostly by the breakdown of gene complexes interacting epistatically and being maintained by selection in natural populations (Dolgin *et al* 2007, Seidel 2008). The ancestral hybrid populations used in our study resulted from enforced crossing among several natural isolates of *C. elegans*. These populations should therefore suffer of depression of fitness due to the exposure of genetic incompatibilities during their derivation. Then, why didn't we observe selection *against* cross-fertilization? Analysis of haplotypes obtained by SNP genotyping suggests selection against recombinant haplotypes generated

during the derivation of the ancestral hybrid androdioecious population. However, it does not seem to have been strong enough to affect overall levels of genetic diversity in this population (Chelo, pers. comm.). Furthermore, deleterious recombinant genotypes were still segregating in the ancestral populations, as shown by the extinction of lines upon inbreeding. Altogether, these results suggest that the enforced cross-fertilization during the derivation of the ancestral populations allowed the elimination of the most deleterious recombinant genotypes, thus overcoming the outbreeding depression barrier typically shown by progeny produced by crossing of different *C. elegans* natural isolates. However, ancestral populations were likely characterized by an abundance of many deleterious recombinant genotypes generated by the breakdown of the gene complexes maintained by selection in the natural isolates. These complexes typically interact epistatically and can lead to strong reductions in the mean fitness of populations with increased recombination (Agrawal 2006). Our results suggest that in our experimental system the reduction in the *mean* fitness of the population caused by the generation of lower fitness classes (recombination load) was outweighed by the increased efficiency of selection on the *variance* for fitness. This interpretation is also valid for genetically homogeneous Androdioecious populations which started experimental evolution with reduced levels of genetic variation. The reason why outcrossing was selectively favored in these populations and not in similar genetically homogeneous populations of *C. elegans* (Stewart and Phillips 2002, Cutter 2005, Manoel *et al* 2007) lies not in overall levels of genetic variation (reduced in both cases) but in the starting genotypes. In our populations, where previously beneficial allelic combinations were disrupted, the ability to generate new combinations is essential. However, because of their genetic uniformity, Androdioecious populations had to rely on the input of mutation to be able to generate novel genotypes and adapt.

We thus find support for the hypothesis that increased rates of recombination (via outcrossing) can enhance adaptation. Importantly, the effects of recombination in generating variation among genotypes seem to prevail even in

the presence of recombination load (which it helps purge). Our findings also argue for a crucial role of genetic relationships within and between loci to the adaptive consequences of increased recombination (Agrawal 2006, Neiman 2006). These relationships may favor recombination even in populations where genetic variation is limited.

2.6 Acknowledgements

Diogo Manoel and Henrique Teotónio generated the experimental populations. Sara Carvalho, D. Manoel and H. Teotonio performed the experimental evolution. Sara Carvalho performed the genotyping of microsatellite loci with the help of Andrei Papkou and Hinrich Schulenburg. All assays were performed by Sara Carvalho (with the exception of the determination of rates of X-chromosome non-disjunction), Miguel Roque, Christine Goy and H. Teotónio. Data presented here was analyzed by Sara Carvalho, with the exception of the fitness function from rates of outcrossing, performed by H. Teotónio, who also wrote the manuscript.

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2.8 Supplementary information

Locus	Chromosome	Repeat type	Size range (bp)	Forward primer (5' to 3')	Reverse primer (5' to 3')	Dye (marked primer)	Ref.
1003	I	AT	150 -180	GCAAAGACTTTTCGACCAACC	AGTTACGGCTATTGGGATCAA	6-FAM (L)	[1]
II-R	II	CCTA	362	TTCTCATTGGAAAGTTGGGC	CAATACCGAGAAACGGATGAA	HEX (R)	[2]
3003	III	TC	190 - 230	TGGTCATACTTCTTCTCGCAA	GGCAAATAGAATGTGAAAGGG	6-FAM (R)	[1]
4001	IV	GA	130 - 140	CCTATTTTCCGCCTCCACTTA	CTCCAATGAAGCTTCCGAAT	HEX (L)	[1]
4004	IV	AG	220 - 250	GCACAATGAGCAACATGCAA	GAAGTCAAAAACCAAAAACCC	HEX (L)	[1]
V-L	V	TAG	318	CGTTGGGACAGGATCTAGTTG	CGCATGTAGCCGAATGTATAG	HEX (R)	[2]
X003	X	CT	220 - 290	CCGACACCGTGATCCATAAAT	AGGATGTGCAGTGATGAAAGG	6-FAM (L)	[1]
X004	X	CA	244	CCCCACATTTTCTGTGTGCTT	TCACTTTTATCCTTCCACCCC	6-FAM(R)	[1]
X-R	X	CCT	394	GCACACGCTTGAATGTCATAA	AAGAGCAGTAGCCGTTGTTGA	6-FAM(R)	[2]

Supplementary Table S2.1: Microsatellite loci examined in the present study.

[1] Sivasunder and Hey (2005)

[2] Haber et al (2005)

	Locus						
	1003	**II-R**	**3003**	**4001**	**4004**	**V-L**	**X-R**
Type of repeat (bp)	2.	4.	2	2	2	3	3
Minimum size (bp)[a]	150	330	180	120	200	220	300
Maximum size (bp)[b]	200	402	260	180	280	320	500
Minimum Height (r.f.u)[c]	100	200	150	100	100	110	100
Minimum height ratio[d]	0.65	0.65	0.65	0.65	0.5	0.65	0.65
Left stutter veto threshold[e]	1.2	-	1.2	-	1.0	1.2	-

[a] The minimum required size of the fragment (in base pairs) to be called as an allele of the locus under analysis.

[b] The maximum allowed size of the fragment (in base pairs) to be called as an allele of the locus under analysis.

[c] The minimum required peak height (in relative fluorescence units).

[d] The ratio between the height of the tallest peak and the other peaks in a given fragment.

[e] The value by which the height of the peak is multiplied; if there are peaks in the adjacent allele classes with heights greater than the returned number the peak under analysis is not considered.

Supplementary Table S2.2: Parameters for allele calls for each microsatellite locus. The parameters were applied to the peaks retrieved from electropherograms obtained by capillary electrophoresis to define true alleles and remove PCR artifacts from analysis.

A.

Model (genetically diverse populations) :
male frequency = Mating system+ Generation+ Replicate+ Mating system x Generation

Source	d.f.	SS	MS	F-value	P
Mating system	1	0.81477	0.81477	169.8886	**< 0.001**
Generation	3	0.00293	0.00098	0.2038	0.895
Replicate	5	0.07492	0.01498	3.1245	**0.013**
Mating system x Generation	3	0.01816	0.00605	1.2625	0.294
Residuals	71	0.34051	0.00480		

$F_{12,71}$=15.83; p-value<0.001; Adjusted R^2=68%

B.

Model (genetically homogeneous populations) :
male frequency = Mating system+ Generation+ Replicate+ Mating system x Generation

Source	d.f.	SS	MS	F-value	P
Mating system	1	1.87642	1.87642	409.1851	**< 0.001**
Generation	3	0.03066	0.01022	2.2287	0.092
Replicate	5	0.05880	0.01176	2.5646	**0.034**
Mating system x Generation	3	0.04486	0.01495	3.2611	**0.026**
Residuals	75	0.34393	0.00459		

$F_{12,75}$=36.54; p-value<0.001; Adjusted R^2=83%

Supplementary Table S2.3: Analysis of variance of outcrossing rates A) genetically diverse Dioecous and Androdioecious populations (D_0, $D_{1\text{-}6}$, A_0, , $A_{1\text{-}6}$) **B)** genetically homogeneous Dioecious and Androdioecious populations ($isoD_{1,3\text{-}6}$, $isoA_{1\text{-}6}$). In the model, X stands for interaction between factors.

Model (genetically diverse Dioecious populations) :
Sex ratio = Generation+Replicate

Source	d.f.	SS	MS	F-value	P
Generation	1	0.0000	0.000004	0.0001	0.994
Replicate	2	0.0564	0.028187	0.4199	0.659
Residuals	58	3.8934	0.067127		

$F_{3,58}$=0.28; p-value=0.84

Supplementary Table S2.4: Analysis of variance of sex ratios of male-sired progeny. Males from genetically diverse Dioecious populations (D_0, $D_{4\text{-}6}$) were allowed to mate with fog-2(*q71*) females (strain JK574); resulting progeny was sexed at the adult stage.

A.

Model (genetically diverse populations) :
male frequency = Generation+ Replicate+ Generation x Replicate

Source	d.f.	SS	MS	F-value	P
Generation	3	$7.7x10^{-07}$	$2.6x10^{-07}$	1.7137	0.389
Replicate	5	$8.5x10^{-06}$	$1.7x10^{-06}$	11.2827	0.083
Generation x Replicate	10	$4.7x10^{-06}$	$4.7x10^{-07}$	3.0947	0.269
Residuals	2	$3.0x10^{-07}$	$1.5x10^{-07}$		

$F_{18,2}$=5.14; p-value=0.1751; Adjusted R^2=79%

B.

Model (genetically homogeneous populations) :
male frequency = Generation+ Replicate+ Generation x Replicate

Source	d.f.	SS	MS	F-value	P
Generation	3	$9.4x10^{-06}$	$3.1x10^{-06}$	1.7658	0.207
Replicate	5	$5.7x10^{-06}$	$1.1x10^{-06}$	0.6429	0.672
Residuals	12	$2.1x10^{-05}$	$1.8x10^{-06}$		

$F_{8,12}$=1.064; p-value=0.4453; Adjusted R^2=2.5%

Supplementary Table S2.5: Analysis of variance of rates of spontaneous production of males of Androdioecious populations A) genetically diverse Androdioecous populations (A_0, A_{1-6}) **B)** genetically homogeneous Androdioecous populations ($isoA_{1-6}$). In the model, X stands for interaction between factors.

A.

Model (genetically homogeneous Androdioecious populations) :
fitness = Generation+ Replicate+ Experimenter at scoring + Generation x Replicate

Source	d.f.	SS	MS	F-value	P
Generation	3	0.23440	0.078134	7.3812	**< 0.001**
Replicate	5	0.53392	0.106783	10.0876	**< 0.001**
Experimenter at scoring	2	0.06107	0.030533	2.8844	0.058
Generation x Replicate	15	0.26788	0.017859	1.6871	0.055
Residuals	210	2.22298	0.010586		

$F_{25,210}$=4.146; p-value<0.001; Adjusted R^2=25%

B.

Model (genetically diverse populations) :
fitness = Mating system + Generation+ Replicate + Experimenter at setup + Experimenter at scoring + Generation x Replicate

Source	d.f.	SS	MS	F-value	P
Mating system	1	0.075498	0.075498	11.3204	**0.002**
Generation	1	0.066913	0.066913	10.0331	**0.003**
Replicate	2	0.002992	0.001496	0.2243	0.800
Experimenter at setup	2	0.080316	0.040158	6.0214	**0.005**
Experimenter at scoring	2	0.049449	0.024725	3.7073	**0.032**
Mating system x Generation	1	0.052215	0.052215	7.8292	**0.007**
Residuals	47	0.313453	0.006669		

$F_{9,47}$=5.454; p-value<0.001; Adjusted R^2=42%

Supplementary Table S2.6: Analysis of variance of population fitness A) genetically homogeneous Androdioecious populations (iso$A_{1\text{-}6}$) and **B)** genetically diverse Dioecious and Androdioecious populations (A_0, $A_{4\text{-}6}$, D_0, $D_{4\text{-}6}$). In the model, X stands for interaction between factors.

Chapter 3

Evolution of life-history phenotypes under dioecy and androdioecy

3.1 Summary

Phenotypes mediate the relationship between genotypes and fitness and therefore constitute the key to adaptive evolution. In many populations of sexual organisms phenotypes are expressed in the form of different mating types – for example, males and females. Understanding how natural selection operates on these different types of individuals and how mating systems determine life-history evolution is crucial to explain phenomena such as sexual dimorphism between the sexes of a species or the evolution of mating systems themselves.

Here we present the patterns of evolution of life-history phenotypes of androdioecious (hermaphrodites and males) and dioecious (females and males) populations of *Caenorhabditis elegans* during adaptation to a novel environment. We describe the evolution of male, female and hermaphrodite components of fitness to unravel the selective pressures that allowed the maintenance of intermediate levels of outcrossing in populations of a predominantly selfing organism. Our results show extensive phenotypic responses of life-history traits. Additionally, they suggest that selection for higher rates of outcrossing may generate a sexual conflict when male and female components of fitness are simultaneously expressed in single individuals, as is the case of self-fertilizing hermaphrodites.

3.2 Introduction

Adaptation is defined as the progressive match between populations of organisms and their selective environment and it is measured by the increase of the mean fitness of populations across generations. This increase results from the action of natural selection on the fitness differences between individuals. These differences arise as a consequence of their various phenotypic combinations. Therefore, studying how phenotypes evolve with time can be revealing of the selective forces that acted upon them (Arnold and Wade 1984).

Individuals are composed of a multitude of phenotypes whose definition can extend from the lowest to the highest levels of biological organization. For

example, phenotypes can consist of the number of copies of a certain transcript at a particular time in the life of the organism or of complex behaviors such as the degree of aggressiveness upon defense of a territory. Different phenotypes vary in their contributions to fitness, for which they can be hierarchically ordered (Falconer and Mackay 1996). This relationship can be mathematically expressed as the covariance between fitness and the phenotype, called the selection differential. However, because of pleiotropy, linkage and assortative mating, this mathematical description embodies the passive response of a trait because of selection on (genetically) correlated traits in addition to the effects of selection acting directly on the trait in question. The isolated *direct* effects of selection on each single phenotype, called the selective gradient, can however be described by the partial regression of fitness on each single character, holding all others constant (Arnold and Wade 1984). The population response to selection on various phenotypes simultaneously, that is, the change in the mean of each phenotype from one generation to the next, is thus a function of the genetic variances and covariances of the phenotypes (the G matrix) and their selective gradients (Lande 1979).

Typically, the effects of selection are evaluated in terms of two main components of fitness: differences in survivorship (viability selection) and in terms of differences in number of offspring per mating (fertility selection) (Walsh and Lynch 2009). Under the simplifying assumptions of discrete and non-overlapping generations, these components of fitness act multiplicatively and the total fitness of an individual (the number of descendants in leaves to the following generation) can therefore be described as the mathematical product of survival and reproduction (Walsh and Lynch 2009). Because of the hierarchical nature of fitness components, these can themselves be further decomposed. For example, fertility in plants, the number of seeds per plant, can be determined by the number of stems per plant, the number of inflorescences per stem, the average number of seed capsules per inflorescence and the average number of seeds per capsule.

Another important point is that in sexual species these fitness components often come in the form of different mating types or sexes. Because the same trait

may have a different relationship with fitness in, for example, males and females, these fitness components have to be defined for each of the sexual forms. For sex-specific selection to contribute to the fitness increase of the population, it has to act in the same direction in males and females. If the sexes experience different selective pressures, this may generate sexual antagonism – “a conflict between the evolutionary interests of individuals of the two sexes” (Parker 1979). For example, some proteins in the seminal fluid of *Drosophila* males increase their fertilization success while simultaneously increasing the mortality of recipient females (Chapman *et al* 1995). It is also possible for alleles to be beneficial for one sex and the same alleles to be deleterious for the other sex (Chippindale *et al* 2001). The particular selective forces that operate on the variance in mating success of males (that emerge from male-male competition and/or female choice for particular phenotypes) and of females are globally termed sexual selection. Sex-specific selection can purge deleterious alleles from populations (Agrawal 2001) and increase the probability of fixation of adaptive mutations (Whitlock 2000), at least in theory. However, many phenotypic traits are subject to opposing selective forces in each sex (Rice and Chippindale 2001, Cox and Calsbeek 2009), with many animal genomes revealing signs of sexual antagonism (Rice and Chippindale 2001, Brommer *et a*l 2007, Prasad *et al* 2007, Bonduriansky and Chenoweth 2009). Sexually antagonistic genetic variation and negative genetic correlations between male and female fitness can have important consequences for the maintenance of genetic variation, the evolution of recombination and rates of adaptation (Delcourt *et al* 2009). Sexual conflict can come in two flavors: it can be mediated by phenotypes with different genetic bases in males and females (inter locus) or by the same genes being subject to conflicting selection pressures between the sexes (intra-locus). An example of inter-locus sexual conflict is provided by the genes coding for seminal fluid proteins in *Drosophila melanogaster* (in males) and those coding for higher resistance of females to the toxicity of these proteins (Rice 1996); intra-locus conflict can emerge, for instance, from different optimal body size between sexes. The extent of the evolutionary

constraints that sexual conflict can impose is indicated by the genetic correlation of fitness components in each sex and it is thus expected to be higher for traits with the same genetic basis (intra-locus). In this case, the limitation of adaptive evolution by sexual conflict will depend on the ease with which sex-limited expression of the trait can evolve, still a debated issue (Rice 1984, Halliday and Arnold 1987, Partridge and Hurst 1998, Rice and Chippindale 2001).

We investigated the response of life history phenotypes to selection in populations of *Caenorhabditis elegans* with different mating systems to determine how these modulate the relationship between life history phenotypes and fitness. Androdioecious and dioecious populations of *C. elegans* were experimentally evolved in a novel environment for 100 generations. Androdioecious systems are composed of selfing hermaphrodites and of males, with whom cross-fertilization can occur, whereas dioecy is characterized by the coexistence of males and females (and therefore self-fertilization does not occur). Experimental evolution was carried out under a simple life-cycle and demography; in particular, non-overlapping and discrete generations were imposed, and only offspring generated at day 4 of the life cycle were propagated (see Figure 2.1, previous chapter). Therefore, fitness under these conditions is determined by how survival and reproduction patterns translate into the production of progeny at this particular day of the life cycle. In other words, phenotypes expressed until day 4 of the life cycle can be acted upon by (laboratory) selection, whereas phenotypes expressed after this period should reflect neutral evolution or reveal potential trade-offs among erly and late life history traits. We have previously demonstrated that adaptation of experimental populations occurred and that it was correlated with rates of outcrossing (chapter 2). Outcrossing is maximal (and fixed) in dioecious systems, but it can evolve in mixed-mating systems such as androdioecy. Our experimental Androdioecious populations maintained stable levels of outcrossing during 100 generations; these levels of outcrossing were significantly higher than the rates reported previously for both laboratory and natural populations of *C. elegans* (Stewart and Phillips 2002, Manoel *et al* 2007, Barrière and Félix 2005),

considered to be a predominantly selfing species. Because outcrossing is adaptive in our experimental system, it posed an excellent opportunity to investigate the evolutionary response of phenotypes related to cross-fertilization, both in a system where only cross-fertilization is possible (dioecy) and in a system where components of fitness additionally involve self-fertilization phenotypes (androdioecy). The advantages of cross-fertilization in our system must have nevertheless been counterbalanced by advantages of self-fertilization, since it is theoretically possible for androdioecy to evolve towards full outcrossing. Many mating-related phenotypes have been characterized in *C. elegans* males, hermaphrodites and *fog-2*(*q71*) females, as well as in closely related dioecious species (such as *C. remanei*). Not only are there differences between the phenotypes of hermaphrodites and females, there is also a strong genetic component underlying those differences (Chasnov *et al* 2007, Teotónio *et al* 2006). Cross-and self-fertilization should thus involve a large number of both male-specific, hermaphrodite/female-specific and sex-unspecific genes. The characterization of reproduction and survival phenotypes of male, female and hermaphrodite in ancestral and evolved experimental populations might shed some light into the evolutionary forces maintaining outcrossing in androdioecious populations not made evident by the evolutionary trajectories of genotypes and fitness alone. Does selection operate similarly in male components of fitness under both mating systems? What is the influence of self-fertilization in the evolution of life history in hermaphrodites compared to females? The patterns of evolution of life-history phenotypes presented here should contribute to an understanding of the evolutionary consequences and limitations of different mating systems to adaptation to novel environments.

3.3 Materials and Methods

Experimental populations

Six androdioecious and six dioecious populations with high levels of standing genetic variation (A_{1-6}, D_{1-6} - see Figure 1.6) were experimentally evolved

for 100 generations under stable environmental conditions and discrete, non-overlapping generations (see chapter 2 for detailed description of population maintenance conditions). Samples from experimental populations were cryopreserved every 10 generations (in addition to samples from ancestral populations). Ancestral and evolved populations were thawed and expanded for 2 generation prior to the assays described here (unless stated otherwise).

Body size measurements

The body width and length of individual worms from genetically diverse experimental populations was measured. About 144 (±44) hermaphrodites or females and 49 (±19) males from ancestral Dioecious (D_0) and Androdioecious (A_0) populations and also from evolved replicates of each (D_{1-6}, A_{1-6}) from generation 50 (G50) were scored. This was achieved by preparing two culture plates in addition to the standard number of culture plates employed during maintenance of experimental populations (both for ancestral experimental populations and then again after fifty generations of experimental evolution). Each of the times, all the plates were maintained concurrently in the incubator until day of culture passage, when two plates per mating system and replicate population were randomly selected and stored at 4ºC. Two to seven days later, photographs were taken on plate sections at 20x magnification encompassing five to ten isolated individuals. Photographs of a thin, glass ruler were also taken whenever culture plates were photographed. The photographs were imported to ImageJ (*rsbweb.nih.gov/ij/*) and measurements of body width (at mid-body vulval region) and length (nose tip to tail tip) were obtained with the ImageJ *straight* line and *measure* functions; distances were expressed as pixels. One milimiter marked in the ruler was similarly measured with ImageJ and all measurements of photographs taken on the same day were standardized by the correspondent value obtained with the ruler to allow for conversion to millimeters and to account for other potential sources of variation; resolution of the system used for body

measurements was of 10^{-3} milimeters. Body size was calculated as V = (body width/2)^2 x pi x body length.

Statistical analysis: Each phenotype was analyzed individually and separately for males and for hermaphrodites and females. Normality of data was assessed for each mating system and generation by Kolmogorov-Smirnov and Shapiro-Wilk statistical tests; for the sets of data for which the hypothesis of normality was rejected, observations lying outside the interval [Lower quantile - 1.5 Interquantile range, Upper quantile + 1.5 Interquantile range] were removed prior to statistical analysis. In general, no datasets failed normality tests upon removal of outliers (as defined by the interval specified above), unless otherwise stated. Final datasets consisted of 30±7 individuals measured per replicate population for body width and body volume and 29±7 individuals for body length; about three times more individuals from ancestral populations were measured to ensure a balanced design. ANOVA was performed separately on each phenotype using the means of replicate populations as response variable; mating system (2 levels), generation of experimental evolution (2 levels) and replicate (7 levels) were taken as fixed factors; the interaction between mating system and generation was also tested.

Viability assay

Viability was estimated as egg-to-adult survivorship. Ancestral Androdioecious and Dioecious (A_0, D_0) populations and six evolved replicates (A_{1-6}, D_{1-6}) from generation 50 were revived and expanded. On day of culture passage (day 4 of the life cycle) samples of 100 eggs were collected from culture plates prior to performance of the *hatch-off* maintenance protocol and transferred to 6-cm culture plates previously provided with Nematode Growth Medium and a spot of *E. coli* HT115. (see Methods in “Fitness-proxy assay”, Chapter 2, for details). Eggs were allowed to hatch and develop in the incubator under the standard conditions of temperature and humidity for 3 days. At day 4 of the life cycle, individuals were counted and sexed. Viability was measured as the fraction of adult progeny

generated from 100 eggs. The assay thus differs from standard maintenance conditions in that eggs were not exposed to hypochloride solution nor maintained overnight in minimal medium. The assay was carried out in two blocks with 3 same-numbered replicates from both mating systems being assayed in each; ancestral populations (A_0, D_0) were assayed in both blocks allowing the quantification of block effects and minimizing the unbalanced design. Three plates were assayed per replicate population in each block.

Statistical analysis: observations of experimental populations (regardless of replicate population) were normally and homocedasticity among experimental groups was fulfilled. Because of this, ANOVA was performed on the dataset, although proportion data are usually analyzed with other statistical methods. Mating system (2 levels) and generation (2 levels) were defined as fixed factors and the interaction between the two was also modeled; possible effects of experimental block were investigated by performing one-way ANOVA on observations from ancestral populations (measured in both blocks) but since they were not statistically significant at the level of alpha = 0.5, replicate population was included as factor in the model instead. Because the adult individuals that hatched from sampled eggs were sexed, male proportions in the progeny that reached maturity were obtained; these were analyzed separately but employing a similar statistical model. No significant differences in male proportions were found between ancestral and evolved populations. Because of this, we tested the hypothesis that the observed fractions of males observed in this assay did not differ significantly from the expected values (0.25 under androdioecy and 0.5 under dioecy) for the rates of outcrossing observed during experimental evolution, using all observations for each mating system regardless of generation or replicate. These hypotheses were tested by performing one-sample Student's t tests for observations from Androdioecious and Dioecious populations separately.

Male competitive performance

The reproductive success of males from genetically variable Androdioecious and Dioecious populations was measured under competitive conditions. Nine young males from experimental populations from generation 0, 30 60 and 100 of experimental evolution were collected at day 3 of the life cycle and transferred to a 6-cm culture plate with 4mm-diameter bacterial lawn. They were allowed to compete against 9 tester (GFP-positive) similarly aged males for fertilization of 22 (isogenic) *fog*-2(*q71*) females (strain JK574) for 24h. After this period, 20 females were transferred to new 6-cm culture plates and killed with 30µl of hypochloride solution (1M KOH:5%NaOCl, 4ºC); plates were maintained in the incubator at standard temperature and humidity. Three days later, a minimum of 50 adult offspring were scored for GFP expression by observing cross-sections of the plates. The proportion of non-GFP individuals relative to the total number scored was taken as male competitive performance. This assay should thus reflect the composite effects of male mating ability, fecundity of males and viability of their offspring. Because all six replicates from evolved populations were assayed (A_{1-6}, D_{1-6}), measurement of male competitive performance was carried out in three experimental blocks with two same-numbered replicate populations each time, as well as ancestral Dioecious (D_0) and Androdioecious (A_0) populations. Four plates were set up per evolved replicate in blocks 1 and 3, while three were prepared in block 2; twice as many plates were prepared for the ancestral populations in each block to ensure a balanced design. Measurements of ancestral populations in each of the three blocks allowed us to test possible statistical effects of assay blocks.

Statistical analysis: The effects of replicate population are partially collinear with the effects of experimental blocks in our assay, for which one-way ANOVA was performed prior to elaboration of the final statistical model using solely the observations from ancestral populations. We failed to detect significant differences among blocks and for that reason replicate population was used as factor in the final model instead. Due to the reduced number of observations within replicate populations, raw data was used in the statistical model after removal of outliers

(defined as in the previously described assays) from groups defined by mating system and generation of experimental evolution (that is, regardless of replicate number). The full statistical (linear) model consisted of mating system (2 levels), generation of experimental evolution (4 levels), replicate population (7 levels), experimenter (2 levels) and all interactions between these factors; non-significant parameters of the model were excluded and the results presented were obtained from the simplified model.

Reproductive Schedule

Hermaphrodites and females from ancestral experimental populations (A_0, D_0) and from 3 evolved replicate populations (A_{4-6}, D_{4-6}) were sampled at day 3 of the life cycle and transferred to 6 cm culture plates with a spot of 5µl of *E. coli*. Hermaphrodites were either kept as single individuals in order to promote self-fertilization or exposed to two *tester*, GFP-positive males; all females were exposed to tester males for the same period of time. Individuals were transferred to new plates every 24h until reproductive cessation; males were only transferred once, therefore hermaphrodites and females from the cross-fertilization treatment where exposed to them for a total time period of 48h. Hermaphrodites and females were maintained until they died (that is, until pharyngeal pumping ceased and they failed to move upon prodding of vulval region – Hard touch assay (Hart 2006). Each day, after transfer of all individuals was accomplished, the number of eggs (fertilized and unfertilized, which can be discriminated phenotypically) left on the plate from which worms had just been removed were counted (under a stereoscope at 40x magnification); the plates were maintained in the incubator for additional 2 days after which they were stored at 4ºC. Five days later, adult worms that had hatched were sexed (and counted) and presence of GFP-positive individuals registered to infer successful cross-fertilization (because the assay was blind, all plates were checked for presence of GFP-positive progeny). Quality control of the observations was performed in two steps: the first, whose description follows, was applied to the whole dataset prior to any statistical analysis; the

second step was carried out specifically for each phenotype (see below). All individuals who failed to produce progeny were discarded; they were equally distributed across experimental populations and treatments and they were therefore not informative. Hermaphrodites from the selfing treatment but for which the treatment could not be unambiguously confirmed were discarded; the selfed hermaphrodites which produced more than 4 males (the expected number assuming a progeny production of 300 offspring and the highest spontaneous production of males measured among the natural isolates - strain PB306 - from which experimental populations were derived) were also discarded from analysis; these individuals were not included in the cross-fertilization treatment because if they were indeed cross-fertilized they were so by males from experimental populations and not by *tester* males and would therefore introduce additional sources of variation to the data. After removal of these observations, selfed hermaphrodites which produced more than 326 offspring (Upper quantile + 1.5 Interquantile range of all observations in this treatment) were also excluded from the dataset, since they were likely to have been previously cross-fertilized by males from their own populations. Finally, under the cross-fertilization treatment, hermaphrodites for which no male or GFP-positive progeny was found were also discarded.

Most of the results presented here describe the patterns exhibited at day 4 of the life cycle of experimental populations. This corresponds to the day of culture passage, where only eggs will survive to constitute the following generation; hence, the relationship between the phenotypes at this particular day and fitness is clear. However, the whole (lifetime) reproductive schedule of hermaphrodites and females during was characterized in this assay. This allowed us to compare the evolutionary responses of phenotypes exhibited until day 4 of the life cycle – which were subject to selection during experimental evolution – to the evolutionary patterns of phenotypes which were never expressed (such as reproduction after day 4 of the life cycle or lifespan); these phenotypes were not under selection and can be revealing of potential genetic trade-offs between early and late life-history

components and of senescence. With respect to this assay we report: a) total progeny produced (lifetime reproductive success, b) number of progeny produced on day 4 of the life cycle (reproductive output), c) number of eggs produced on day 4 of the life cycle (and total number of eggs produced during the lifetime of the individuals), and d) viability (as offspring-to-egg ratio – that is, egg-to-adult-survivorship) of progeny produced on day 4 of the life cycle. All the phenotypes measured specifically on each day accounted for possible residual self-fertilization of hermaphrodites in the sense that hermaphrodites under the cross-fertilization treatment that did not produced male or GFP-positive progeny in the particular day under analysis were scored as belonging to the self-fertilization treatment. Finally, observations for which the number of progeny was higher than the observed number of eggs (indicating underestimation of the latter and overestimation of egg-to-adult survivorship) were discarded in the statistical analysis of viability. Thirty individuals were measured per mating system (Androdioecious or Dioecious), generation (ancestral or generation 100 of experimental evolution), replicate (in the case of evolved populations; replicates 4-6) and treatment (cross-fertilized in the case of females and cross- or self-fertilized in the case of hermaphrodites). In total, and after the first step of quality control, the reproductive schedule of 516 individuals was analyzed. The assay was performed in 3 blocks, with each same-numbered replicate population (from the different mating systems) and ancestral populations being measured in each block and hence, pseudoreplicated. Sample sizes were similar for ancestral and evolved populations but there was total collinearity between replicate number and assay block. Therefore, and similarly to what was performed in the analysis of data from previous assays, a one-way ANOVA was first carried out on data from ancestral populations only to investigate possible effects of block; whenever such effects were not significant, this factor was removed from the statistical model and replicate population was used instead. Also, the estimates of the values of ancestral populations obtained from the 3 experimental blocks were used as pseudoreplicates to calculate standard errors of

means (since for each evolved population measurements from 3 independent replicates were available) to provide error bars in the graphical displays.

Statistical analysis of each phenotype involved two separate models: one comparing hermaphrodites and females mated with males and one comparing selfed and crossed. This means that the dataset of mated hermaphrodites was used in both models. Although no correction was made in the p-values presented, none loses significance upon correction (observed p-value x 2). Because we were not interested in individual variation (but variation experimental populations) the means of the observations per replicate population were used as response variables and hence statistical analysis was performed on 12 data points in each model; because of this, the power to detect interactions among factors was not very high. Whenever a factor (for example, generation of experimental evolution) was significant in one of the models (for example mated hermaphrodites versus mated females) but not in the other (mated hermaphrodites versus selfed hermaphrodites), the significance of this factor was additionally tested for each group defined by mating system and treatment regardless of replicate using all (raw) observations by performing Student's t tests.

Lifespan assay

Experimental hermaphrodites and females from Androdioecious and Dioecious populations (both ancestral – A_0, D_0 – and evolved – $A_{4\text{-}6}$, $D_{4\text{-}6}$) were sampled at day 3 of the life cycle. They were placed in 12-well cell culture plates previously filled with 3,5 ml of NGM lite agar supplemented with ampicillin and a spot of 5µl of *E. coli*. Due to the production of progeny by hermaphrodites and concomitant possibility of overcrowding, individuals were transferred daily to new wells during the reproductive period; the presence of males in the progeny of individual hermaphrodites was recorded. To minimize the effects of differential experimental manipulation, females were transferred as well. Because no offspring were produced by virgin females, these plates were stored at 4ºC and were re-used in the first experimental block to assess the longevity of experimental males.

Females are known to produce diffusible molecules known to be attractive to *C. elegans* males, and we hypothesized that using these plates could prevent their attempts to leave the wells and to dry out of dissecation. Some wells of the re-used plates had not contained females (because they had died in the meanwhile) and this information was incorporated in the dataset. Due to the extremely low numbers of males that remained in the wells until the end of the assay and did not constitute right-censored data, in the following two experimental blocks fresh culture plates were prepared for both hermaphrodites/females and males. In those destined to assay lifespan in males, a single *C. remanei* female was added to each well since females of this species are known to be attractive to *C. elegans* males although viable progeny cannot be produced (Baird *et al* 1994, Chasnov and Chow 2002). Males from experimental populations were sampled in two consecutive days – as young, L4 males (at day 3 of the life cycle) and as adult, mature males (at day of the life cycle). All individuals were observed daily and considered dead if a) pharyngeal pumping ceased and they failed to move upon prodding at vulval region – Hard touch assay b) if they were not observed in the wells for three consecutive days. A total of 720 females, 720 hermaphrodites, 1193 young males and 1087 adult males were sampled. Quality control of the data included assigning females which produced progeny to the "cross-fertilized" treatment, as well as hermaphrodites for which male progeny were found in two consecutive days. It also included right-censoring of individuals who died of dissecation while trying to escape from the wells and right-censoring of individuals whose day of death was inferred by failure to observe them for three consecutive days. The dataset obtained after quality control included 694 females (610 uncensored), 668 hermaphrodites (559 uncensored), 806 young males (343 uncensored) and 1006 adult males (157 uncensored).

Statistical analysis: Males and hermaphrodites/females were analyzed separately using the non-parametric Cox's Proportional Hazards method (*coxph* function in the *survival* package of R software, see below), which allows censoring but does not require the baseline hazard function to be specified. For males, the

statistical model used was the following: (day of death, censoring status) = mating system (2 levels) + generation (2 levels) + age (2 levels) + presence of female (3 levels) + replicate (4 levels; ancestral populations were coded as replicate 0) + all interaction terms. Block was collinear with presence of female so it was not included in the full model. The *step* function (*stats* package of R software, see below) was used for model simplification; this function computes the AIC values of the models produced by removing each term of the full model (above) at a time and it does so iteratively to find the model with the best fit (the lowest AIC value). The final, simplified model for male data was: (day of death, censoring status) = mating system + generation +age + presence of female + mating system:generation + mating system:age. We proceeded similarly with a model in which the factor presence of female was replaced by block. This model did not yield any significant parameters. The likelihood ratio test between the two reduced models did not indicate significant statistical differences between them; however, the model incorporating the effects of block revealed a lower likelihood value despite having more parameters. Keeping in mind these statistical limitations, we opted to provide the results from the full model which included the factor “presence of female”.

Hermaphrodites and females under different treatments (mated and unmated) were analyzed separately due to the large differences in sample sizes between the two. Statistical analysis was carried out as outlined above for male longevity data, with elaboration of a full model ((day of death, censoring status) = mating system + generation + mating system-by-generation interaction +block or replicate) and subsequent simplification. Two full models were tested, one including block and another including replicate. In the analysis of data from the cross-fertilization treatment neither factor was present in the reduced model (which was therefore the same in both cases). With respect to the analysis of data from the unmated treatment, the model including replicate revealed this factor not to be significant, while the model containing experimental block revealed significance of

this factor. We present the latter, since it yielded a lower AIC value (11986.5 versus 12038).

Statistical analysis

All statistical analysis was carried out in the R software using custom scripts (R Development Core Team 2010), unless otherwise stated. Whenever Analysis of Variance (ANOVA) was involved, the distribution of residuals was tested for normality by Shapiro-Wilk and Kolmogorov-Smirnov tests; only non-normality is reported. ANOVA tables and Survival Analysis are provided as Supplementary Tables; values presented in graphical displays represent raw data, not parameters estimated from statistical models.

3.4 Results

Body size of males did not evolve but increased significantly in hermaphrodites and females

The body length and width of experimental males was measured concurrently in individuals from ancestral Dioecious and Androdioecious populations, as well as from populations that underwent fifty generations of experimental evolution. Body volume was then estimated from these measurements assuming a cylindrical shape of the worms (see Methods). We did not detect any significant evolution of body size of males measured as body volume, width or length (Figure 3.1). However, males from Dioecious populations seemed to be larger (wider) than males from Androdioecious populations ($p=0.035$), which contributed to a marginally significant difference in body volume between males from both mating systems ($p=0.064$).

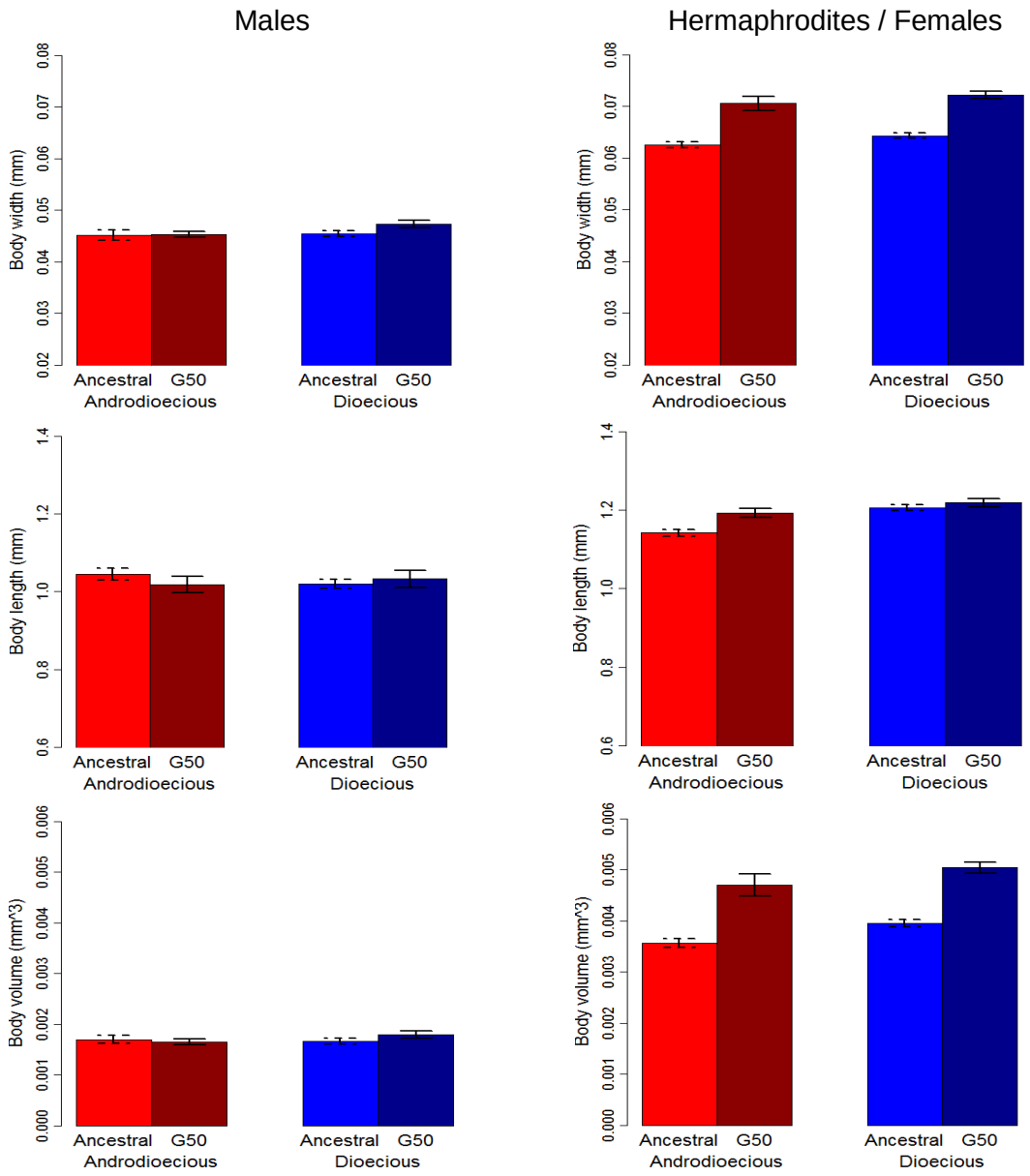

Figure 3.1: Evolution of body size of males and of hermaphrodites and females of genetically diverse populations. Values are presented as replicate means; error bars of evolved populations (solid grey) represent standard errors of means of replicate experimental populations; because no pseudoreplication was performed, error bars of ancestral populations (dashed grey) represent standard error of the mean value among individual measurements, providing graphic illustration of experimental and individual variation. Top graphs: body width; middle graphs: body length; bottom graphs: body volume. See Supplementary Tables S3.1 and S3.2 for statistical analysis.

Differences in the body size of hermaphrodites and females were more evident than those found among males (perhaps because the former contained approximately three-times more observations than male measurements). Both hermaphrodites and females evolved wider bodies ($p=0.002$). Similarly to width, body volume also increased with experimental evolution ($p=0.002$); analysis of the body size measurements further revealed marginally significant differences between hermaphrodites and females ($p=0.047$), with the former exhibiting smaller bodies. However, it should be noted that this observation might have simply resulted from the exacerbation of minor differences in body length found between them ($p=0.032$), since this variable was computed in the calculation of body volume as a power variable. No significant correlation was detected between body width and body length (not shown), which suggests that these two traits may be under different genetic control and/or have different relationships with hermaphrodite and female fitness. Overall, we found evidence for increased body volume with 50 generations of experimental evolution and marginal differences between hermaphrodites and females. Finally, the well known sexual dimorphism for body size between males and hermaphrodites (and between males and females) (Donald 1997) was also evident in the results obtained from this assay.

Sex-independent increase of viability with experimental evolution

Egg-to-adult survivorship cannot easily be estimated independently for *C. elegans* males and hermaphrodites (or females) since phenotypic discrimination of sex is only achievable at larval stages and not during embryonic development. Additionally, under androdioecy, it is impossible to determine whether the sampled zygotes resulted from events of self- or cross-fertilization. Nevertheless, the average egg-to-adult survivorship of experimental populations can be estimated and the sex ratio of the adult individuals that hatch from the sampled zygotes can be assessed and compared to those observed during experimental evolution (Figure 2.2, chapter 2). Overall, egg-to-adult survivorship increased about 25% in

populations evolved for fifty generations under both dioecy and androdioecy (Figure 3.2 A).

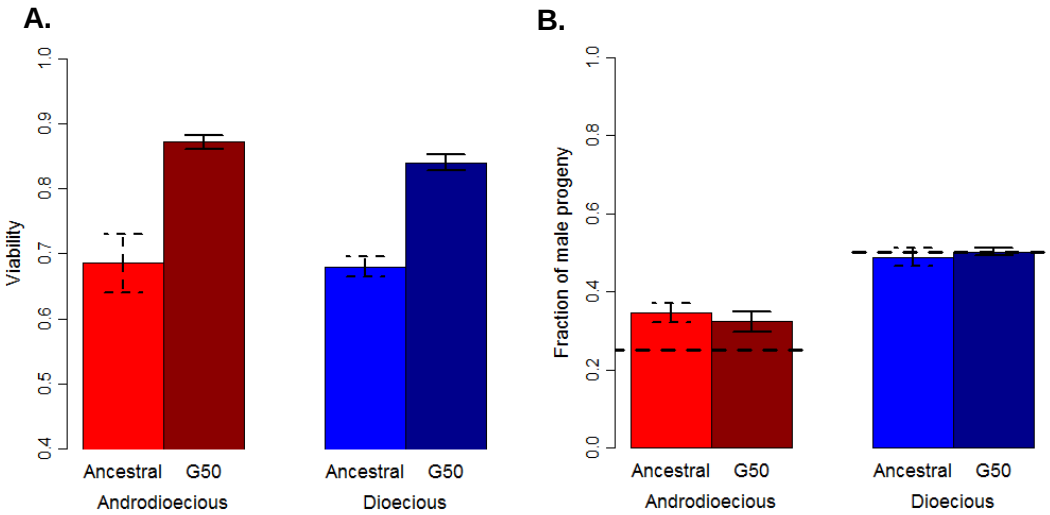

Figure 3.2: **Evolution of viability of genetically diverse populations**. In both graphs values are presented as means across replicate populations. Error bars of evolved populations (solid grey) represent standard errors of means of replicate experimental populations; because no pseudoreplication was performed, error bars of ancestral populations represent standard error of the mean value among the different plate measurements, providing graphic illustration of the experimental variation found for these populations (dashed grey). **A)** Egg-to-adult survivorship **B)** Proportion of male progeny. Dashed, horizontal lines represent expected fractions of males given the rates of outcrossing measured in these populations during experimental evolution. See Supplementary Table S3.3 for statistical analysis.

The observed fractions of males hatched from the sampled eggs were similar between ancestral and evolved populations (Figure 3.2 B). Furthermore, fractions of males of individual observations did not correlate significantly with egg-to-adult survivorship (t_{40} = -0.79, p=0.44). These results suggest that the mean increase in viability observed with experimental evolution was unlikely to be caused by differential selection of viability components between the sexes, but rather it seems to have resulted from the evolution of general, sex-independent components of viability. It is nevertheless interesting that the frequency of males found among adult offspring in this assay did not significantly differ from the expected values for populations experiencing obligate outcrossing, that is, dioecious populations (under the assumption of equal viability among the sexes).

However, the observed fractions of males that emerged from sampled embryos in ancestral (A_0) and evolved (A_{1-6}) Androdioecious populations were consistently and significantly higher than expected based on the rates of outcrossing found during experimental evolution, for which the expected fractions of male progeny were 0.25. Two possible explanations can underlie this observation: greater egg-to-adult survivorship of males relative to hermaphrodites and/or increased viability of outcrossed progeny relative to selfed progeny. The absence of a significant correlation between egg-to-adult survivorship and fractions of males favors the second possibility; however, only an investigation of viability under conditions of selfing and outcrossing separately can tease apart these two possibilities (see below).

Components of fitness

While the relationship between the phenotypes analyzed thus far and fitness is not straightforward, the following assays were designed to specifically investigate the phenotypic response of components of fitness in males, hermaphrodites and females and to allow their comparison across mating systems. The components of fitness are distributed among individuals in both mating systems as follows:

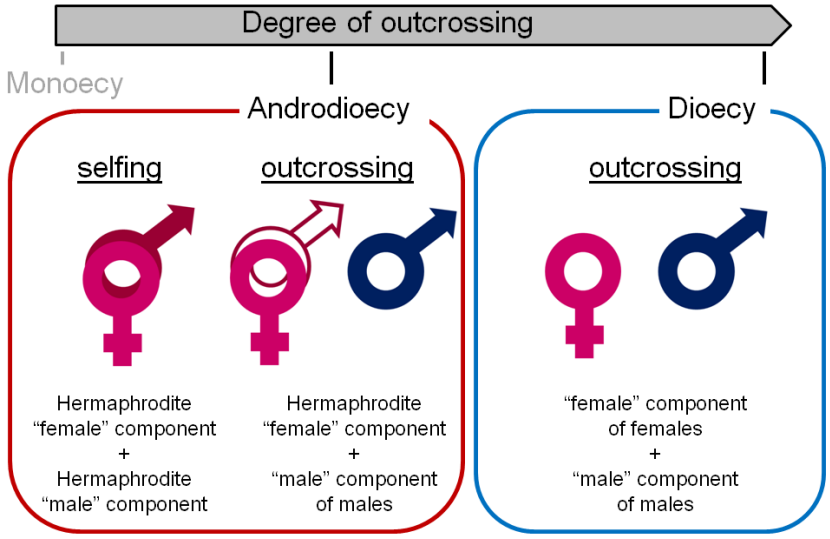

Figure 3.3: Diagram of components of fitness under androdioecy and under dioecy. Female symbols represent phenotypes related to female components of fitness (such as fecundity); male symbols represent male components of fitness (for example, male mating ability). Colors symbolize the different sexes: blue represent males, pink represent hermaphrodites or females. Note that in the androdioecious system, only the "female" component of hermaphrodites is expressed under cross-fertilization (empty "male" component").

Increased male reproductive success under androdioecy and under dioecy

In *C. elegans* the relative production of outcrossed and selfed progeny depends primarily on the number of males generated by meiotic sex chromosome non-disjunction and on the relative efficiency with which males fertilize hermaphrodite eggs compared to hermaphrodite self sperm (Stewart and Phillips 2002). When significant frequencies of males are found in *C. elegans* populations, the reproductive success of individual males is additionally determined by the efficiency with which they fertilize hermaphrodite eggs relative to *other* males. We have shown previously that rates of X-chromosome non-disjunction did not evolve consistently with experimental evolution, thus failing to explain observed patterns of frequencies of males and outcrossing in Androdioecious experimental populations (chapter 2). Here, we investigated the evolution of reproductive success of males from both Androdioecious and Dioecious populations under competition against GFP-positive *tester* males. Experimental and *tester* males

were allowed to compete for *fog*-2(*q71*) females (strain JK574) and the proportion of progeny sired by each was determined by scoring adult progeny for GFP expression. Males of both Dioecious (D_{1-6}) and Androdioecious (A_{1-6}) populations increased their reproductive success (measured as competitive ability for fertilization) in ~23% after 100 generations of experimental evolution (Figure 3.4).

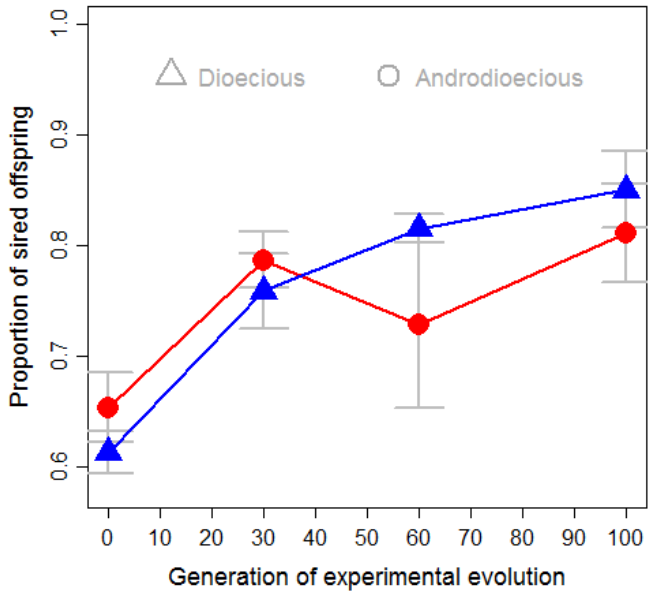

Figure 3.4: Evolution of male competitive performance of genetically diverse populations. Mean proportion of progeny sired by males from Dioecious (blue triangles) and Androdioecious (red circles) populations in competition for fertilization of *fog*-2(*q71*) females (strain JK574) against *tester* males expressing GFP. Error bars (in grey) represent standard errors of the means of pseudo replicate (ancestral) or replicate (evolved) populations. See Supplementary Table S3.4 for statistical analysis.

Interestingly, despite the different numbers of males that segregated in the two mating systems and hence more opportunity for selection of male-related phenotypes under dioecy, the evolutionary trajectories of male reproductive success were similar between the two mating systems. A possible explanation might stem from frequency-dependent fertilization success of males. If the fertilization success of males (relative to hermaphrodites) is contingent upon the frequencies at which they segregate in androdioecious populations, male fertilization success is expected to be maximal at male frequencies of about 20% (Stewart and Phillips 2002): below this value males are too few to successfully

fertilize all hermaphrodites before they use their self-sperm; above this value male-male interactions are expected to decrease the fertilization success of individual males. The male frequencies verified during experimental evolution in Androdioecious populations were stably maintained at values that closely match this frequency. With genetic differences among males being manifested as upper limits of their fertilization success, selection is likely to have proceeded in an extremely efficient way for this component of fitness in Androdioecious populations, allowing a phenotypic response similar to that of males of Dioecious populations.

Components of fitness of hermaphrodites and females

Increased female and hermaphrodite reproductive success

The reproductive success of hermaphrodites and females was investigated. Under standardized conditions of cross-fertilization (that is, when fertilized by males from a same population), the reproduction patterns of hermaphrodites and females can be compared. Contrasting the patterns of selfed and crossed hermaphrodites is revealing of the evolutionary response of self-sperm and fertilization phenotypes (the “male” component of fitness of hermaphrodites – see Figure 3.3), since oogenesis is the same under both conditions. Evolved hermaphrodites and females showed increased lifetime production of progeny in 55% and 39%, respectively, under conditions of cross-fertilization (Figure 3.5):

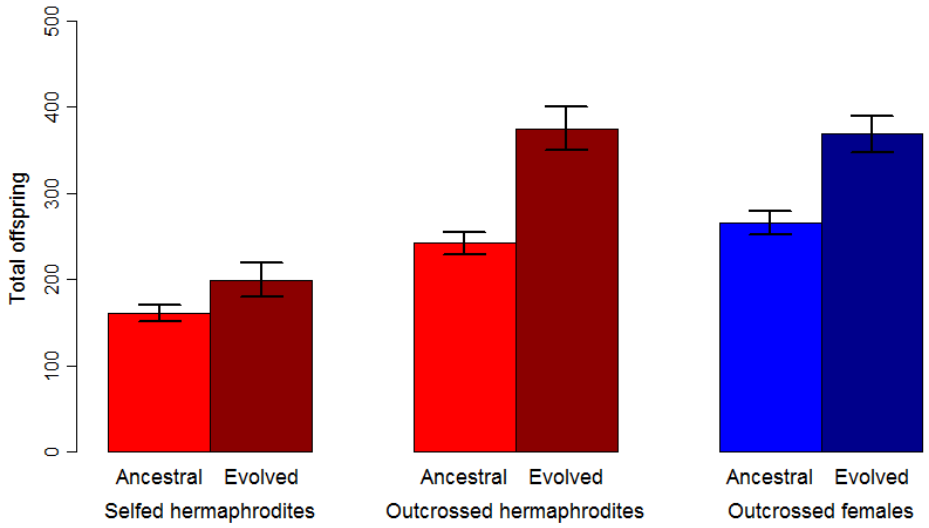

Figure 3.5: Evolution of lifetime reproductive success (LRS) of hermaphrodites and females of genetically diverse populations. Values are presented as mean number of offspring produced at day of culture passage of pseudo replicate (ancestral) or replicate (evolved) populations; error bars represent standard errors of the means. See Supplementary Table S3.5 for statistical analysis.

Self fertilized hermaphrodites showed significantly lower reproductive output compared to conditions of cross-fertilization as expected from their condition of sperm limitation. However, *C. elegans* hermaphrodites are able to produce approximately 300 self progeny (Ward and Carrell 1979), for which an increase in progeny production could have been anticipated given the high levels of genetic variation segregating in these experimental populations and their initially reduced progeny production compared to commonly observed values. There was only a modest increase (24%) of progeny production of self-fertilized hermaphrodites with experimental evolution compared to conditions of cross-fertilization. Although this may still seem a large phenotypic response, it corresponds to an average increase of 38 offspring, whereas under conditions of cross-fertilization the 55% increase translates into an average increase of 132 offspring. Because only early-life reproduction was available for selection under the experimental conditions of our study, late-life reproduction does not contribute to fitness in these populations and the pattern exhibited by self fertilized hermaphrodites could simply express a

negative correlation between early- and late-life reproduction in our system. The reproductive output of hermaphrodites and females on the day of culture passage (hence a component of fitness) was therefore investigated. The observed pattern closely matched that found for lifetime reproductive success (Figure 3.6):

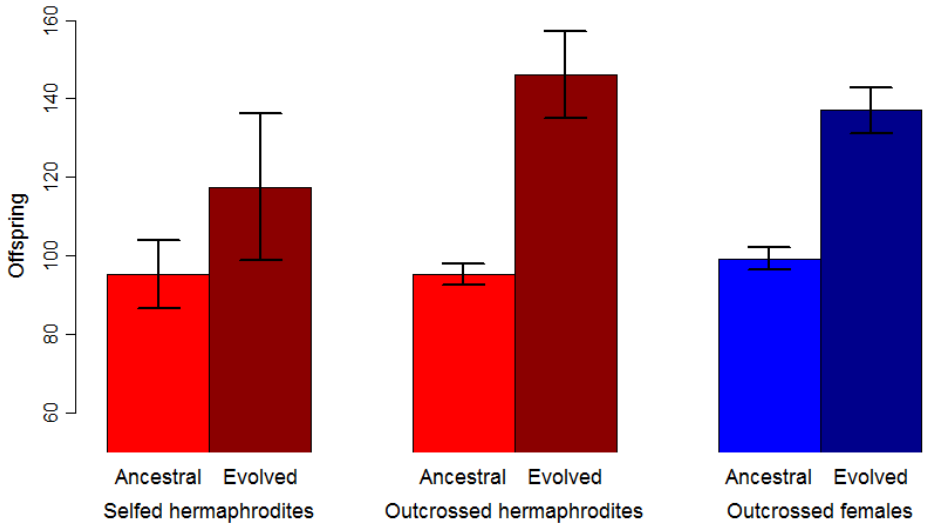

Figure 3.6: Evolution of reproductive output of hermaphrodites and females of genetically diverse populations. Values are presented as mean number of offspring produced at day of culture passage of pseudo replicate (ancestral) or replicate (evolved) populations; error bars represent standard errors of the means. See Supplementary Table S3.6 for statistical analysis.

Both females and hermaphrodites significantly increased progeny production. Under (standardized) conditions of cross-fertilization with *tester* males, the number of offspring produced by ancestral hermaphrodites and females was similar, with both having increased their production of progeny with experimental evolution (53% and 38%, respectively). Hermaphrodites from ancestral populations exhibited similar numbers of progeny production on day 4 of the life cycle under self-fertilization compared to the progeny produced by cross-fertilized hermaphrodites from the same populations. With experimental evolution, however, selfed hermaphrodites again increased progeny production more modestly (23%) compared to outcrossed hermaphrodites (53%). Although the lifetime reproductive success of evolved, selfed hermaphrodites (199±6 progeny) was still lower than those observed in the N2 strain in a similar study (260±5), the observed number of

selfed progeny produced by hermaphrodites from evolved populations at day 4 of the life cycle (120±5) is similar (~118) (Hughes *et al* 2007). Two obvious mechanisms underlying increased reproductive output of hermaphrodites and of females can be hypothesized: increased production of eggs and/or increased viability of the embryos produced.

Evolution of egg production of hermaphrodites but not of females

The number of eggs laid by cross-fertilized females and by selfed or outcrossed hermaphrodites at day of culture passage was measured for individual worms of both ancestral and evolved populations (Figure 3.6).

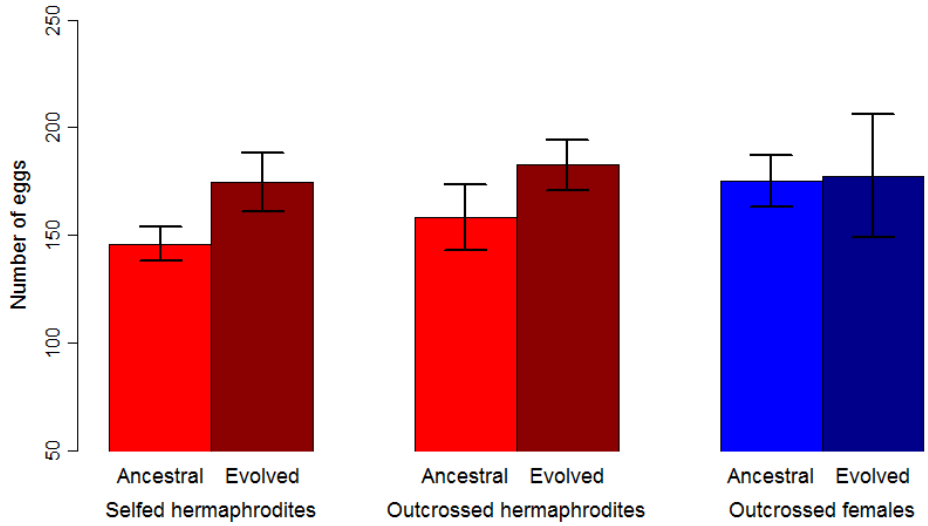

Figure 3.7: Evolution of egg production of hermaphrodites and females of genetically diverse populations. Values are presented as mean number of eggs produced at day of culture passage of pseudo replicate (ancestral) or replicate (evolved) populations; error bars represent standard errors of the means. See Supplementary Table S3.7 for statistical analysis.

Females did not show increased production of eggs. Evolved hermaphrodites, however significantly increased the number of eggs laid relative to ancestral hermaphrodites; interestingly, ancestral hermaphrodites produced less eggs than females, but the increase of egg production with experimental evolution lead them to exhibit similar values by generation 100. For outcrossed

hermaphrodites and females this phenotype should reveal the maximum rate of egg production in the context of unlimited sperm availability since mating for 24 to 48 hours beginning at the L4 stage is usually sufficient to provide hermaphrodites (and females) with enough sperm for the whole reproductive period, that is, until cessation of reproduction (Hughes *et al* 2007). With respect to hermaphrodites, similar patterns of egg production were expected under conditions of selfing and of outcrossing because oogenesis should proceed similarly under the two conditions, unless self and allosperm regulate oocyte maturation and ovulation differently (for which we found no evidence in the literature). This was indeed the pattern we found. In hermaphrodites under conditions of selfing the number of fertilized eggs produced is contingent on the amount of self-sperm. The fact that the patterns of egg production at day of culture passage of selfed hermaphrodites are similar to those of outcrossed hermaphrodites suggests that self-sperm should be depleted only at later stages of the life cycle. In fact, despite being shorter than previously reported in similar studies (Hughes *et al* 2007), the average reproductive span of hermaphrodites under conditions of selfing observed in this assay was of 3.3±0.08 days and showed no evidence of change with experimental evolution (not shown). This indicates that the self-sperm of experimental hermaphrodites is only depleted around day 5 of the life cycle and should therefore not impose a constraint on progeny production by self-fertilization at day of culture passage. This is further confirmed by production of offspring after this day of the life cycle:

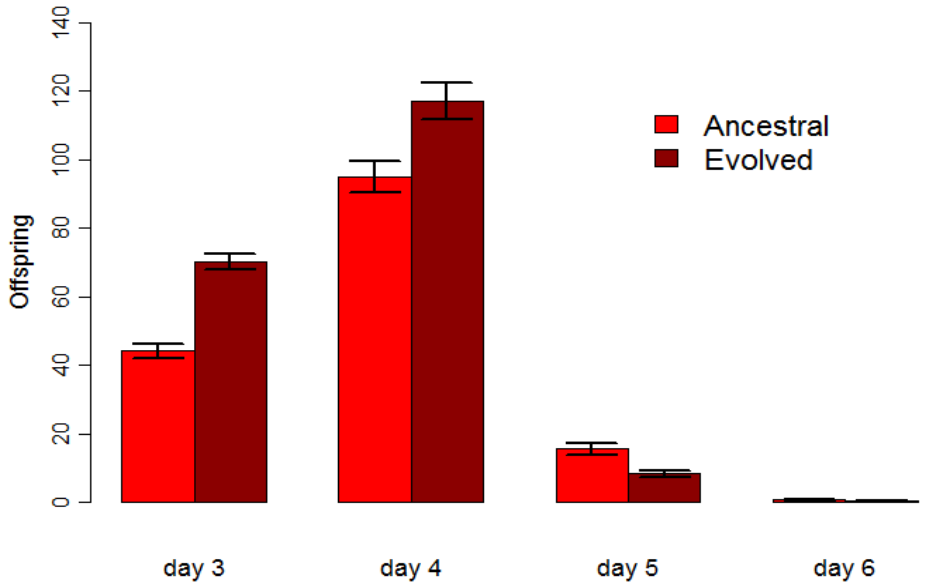

Figure 3.8: Reproductive schedule of self-fertilized hermaphrodites of genetically diverse populations. Values are presented as the mean progeny of individuals from ancestral and evolved populations produced each day of life (since L1); error bars represent standard errors of the means.

Oogenesis in *C. elegans* hermaphrodites is only initiated after cessation of spermatogenesis. Because of this, it is tempting to suggest that the increased ability to produce eggs at day 4 of the life cycle could have resulted from selection for shortened spermatogenesis (that is, for the switch to oogenesis to occur earlier). The timing of the switch from spermatogenesis to oogenesis is genetically determined; several mutations in genes of the germline sex determination pathway in *C. elegans* can affect this timing and lead to lengthened or shortened spermatogenesis (Ahringer and Kimble 1991, Hodgkin and Barnes 1991). Although changes in the timing of the switch from spermatogenesis to oogenesis were not properly investigated, the fact that the total (lifetime) number of eggs laid by ancestral and evolved hermaphrodites under conditions of self-fertilization did not differ significantly suggests this was not the case (Figure 3.9).

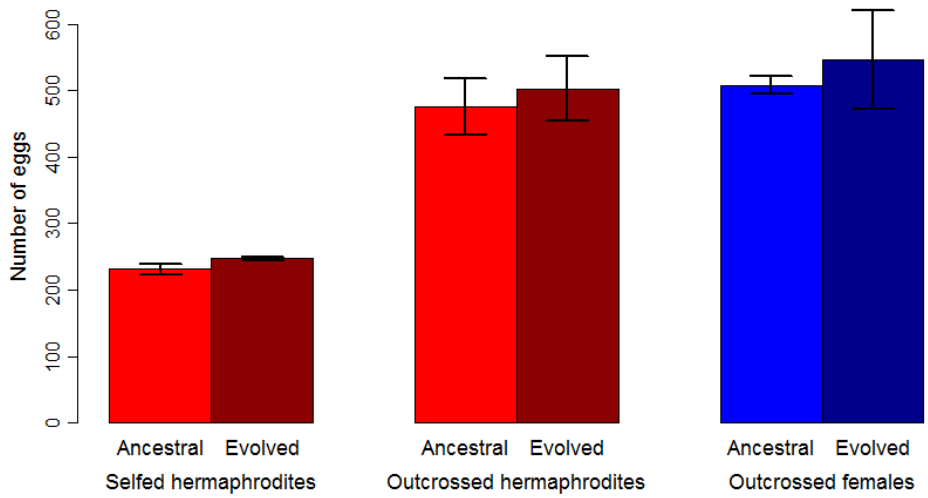

Figure 3.9: Lifetime production of eggs of hermaphrodites and females of genetically diverse populations. Values are presented as the mean number of eggs produced in a lifetime of pseudo replicate (ancestral) or replicate (evolved) populations; error bars represent standard errors of the means. See Supplementary Table S3.8 for statistical analysis.

Other mechanisms such as faster development to reproductive maturity or faster and/or more efficient fertilization could therefore potentially have been involved in the increased egg production at this particular stage of the life cycle. They will have to be investigated if the causes underlying this phenotypic response are to be sought after.

Differential evolution of egg-to-adult survivorship under cross and self-fertilization

The fraction of sexually mature worms that hatched from eggs laid on day 4 of the life cycle (just described) was calculated, providing estimates of egg-to-adult survivorship of progeny of females and of hermaphrodites under self- and cross-fertilization. Outcrossed hermaphrodites and females exhibited similar ancestral values of progeny viability as well as similar increases (13% and 12%, respectively) with experimental evolution (Figure 3.10). As found previously for the number of offspring produced on day 4, ancestral hermaphrodites under selfing revealed similar progeny viability compared to ancestral, cross-fertilized

hermaphrodites but a more modest increase after 100 generations of experimental evolution compared to the phenotypic change observed under conditions of cross-fertilization.

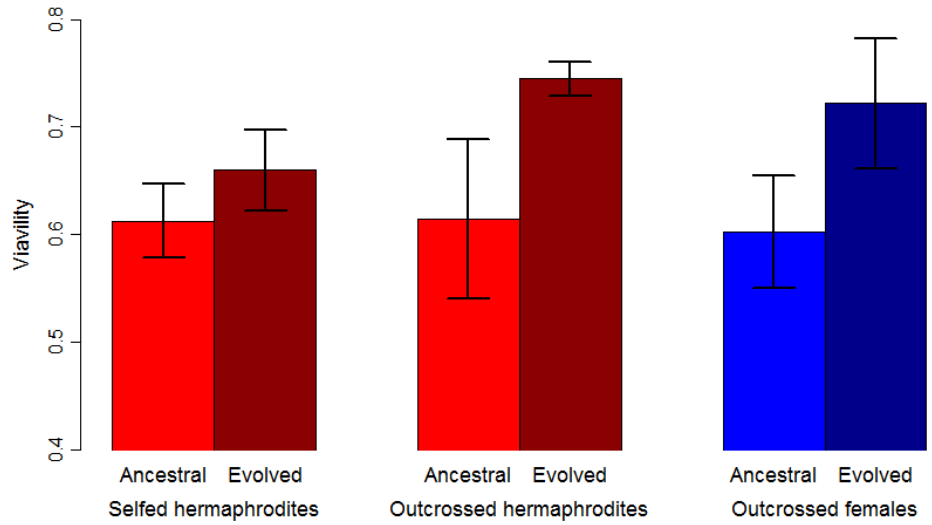

Figure 3.10: Evolution of viability of progeny produced by hermaphrodites and females of genetically diverse populations. Values are presented as the mean egg-to-adult survivorship on day of culture passage of progeny of pseudo replicate (ancestral) or replicate (evolved) populations; error bars represent standard errors of the means. See Supplementary Table S3.9 for statistical analysis.

Because no differences between replicate (evolved) populations were found in our statistical models (see Supplementary Table S3.8) we investigated these evolutionary patterns further, by performing two-sample Student's t tests for each group using all observations (not means), regardless of replicate or block. Increased viability of progeny with experimental evolution was confirmed for females (t_{135}=-3.24, p=0.002), as was for hermaphrodites under conditions of cross-fertilization (t_{136}=-4.49, p<0.001). The viability of progeny produced by ancestral, self-fertilized hermaphrodites did not significantly differ from the viability of progeny produced by evolved hermaphrodites under similar conditions (t_{147}=-1.35, p=0.179). Altogether, the results obtained from this assay not only confirmed the increased egg-to-adult survivorship already observed in previous assays (see Figure 3.2), as they further suggest that increased viability between ancestral and

evolved Androdioecious populations is likely to be explained by a larger relative increase of viability of the progeny produced under conditions of outcrossing compared to the viability of the progeny produced by selfing. These results thus match the prediction of the evolution of cross-fertilization phenotypes underlying the maintenance of outcrossing in Androdioecious populations with experimental evolution.

Evolution of lifespan

Most classical theories for the evolution of aging rely on the idea that natural selection is progressively less efficient in older individuals. In age-structured populations, the number of individuals in consecutive age classes is reduced simply because some individuals in the populations die due to extrinsic factors such as predation or disease. As a result, deleterious mutations that exert their effects later in life may not be purged efficiently from populations (Medawar 1952), including mutations with antagonistic pleiotropic effects, which might greatly increase fitness through early life components while having very strong deleterious effects later in life (Williams 1957). Because of its strong association to fitness, patterns of reproduction and their relationship with lifespan in the context of different extrinsic mortality pressures has been extensively studied in aging research (Reznick *et al* 2004). Although an extensive investigation of evolution of lifespan in our experimental populations was not the main scope of this study, the observation of increased reproduction early in life in our experimental system lead us to test some of the predictions of the classic theories of aging. Traits manifested after day 4 of the life cycle were not expressed under the experimental evolution conditions employed here; therefore, if lifespan results from the phenotypic effects of mutations that act late in life, they could be expected to have accumulated freely in our experimental populations and evolved populations should thus exhibit shortened lifespans. The same pattern is expected if the overall increased reproductive ability of males, hermaphrodites and females early in life (until day 4 of the life cycle) resulted from selection of alleles segregating in

these populations which had deleterious pleiotropic effects on survival late in life (that is, phenotypes expressed after day 4 the life cycle). More interestingly, in the context of phenotypic evolution under androdioecy and dioecy, the questions addressed in this assay focused on the effects of mating and of densities of males (and hence the opportunity for male-male interactions) on male lifespan and also on the differences in survival patterns between mated and unmated hermaphrodites and females.

The effects of mating on male lifespan were investigated by obtaining survival curves of males sampled at the L4 stage (day 3 of the life cycle) and males sampled from the same populations one day later. Adult males are expected to have participated in a higher number of mating events prior to sampling; however, these males were simultaneously exposed to more social interactions with other males, for which the effects of both phenomena cannot be disentangled in our assay. Because young males were sampled one day before adult males, the groups were analyzed separately. No significant differences were found between males from ancestral and evolved populations, neither as young adults nor as fully mature males (Figure 3.11). With respect to young (L4) males, we did not detect significant effects of any of the factors tested; this may in part have resulted from the relatively few uncensored observations available. Adult males from ancestral Dioecious (D_0) populations seemed to exhibit higher risk of death compared to males from Androdioecious populations ($p=0.015$). This difference was no longer apparent after 100 generations of experimental evolution (marginally significant interaction between mating system and generation, p=0,073), suggesting perhaps increased resistance of adult males to deleterious effects of male-male interactions.

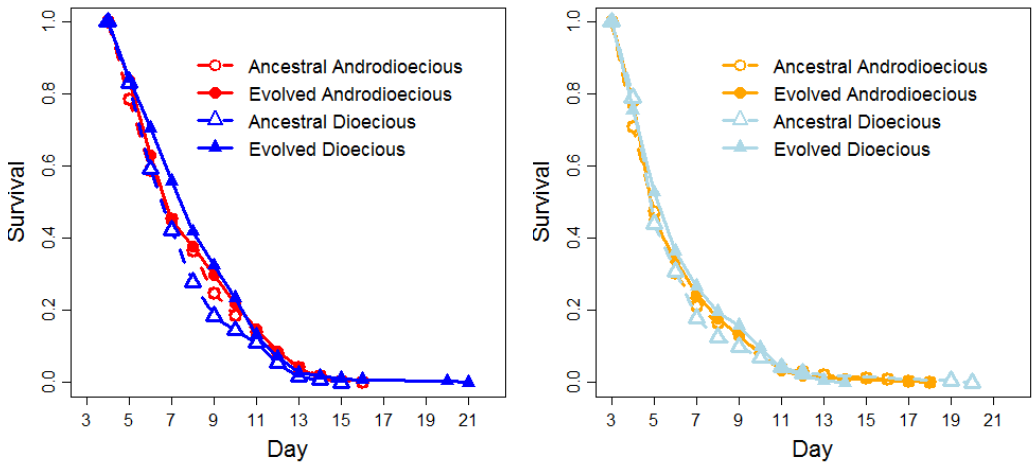

Figure 3.11: Lifespan of males of genetically diverse populations. Survival curves of individuals from genetically diverse populations. The graph shows the proportion of sampled individuals still alive at each day of the life cycle (censored and uncensored observations). **A)** Adult males. Individuals from Dioecious populations are represented in blue and from Androdioecious populations are represented in red; empty symbols and dashed lines illustrate survival curves of ancestral populations, and filled, full lines those of evolved populations. **B)** Young (L4) males. Individuals from Dioecious populations are represented in light blue and from Androdioecious populations are represented in orange; empty symbols and dashed lines illustrate survival curves of ancestral populations, and filled, full lines those of evolved populations. See Supplementary Table S3.10 for statistical analysis.

No differences between experimental groups were found among young (L4) males (Figure 3.11 B) perhaps due to the large numbers of right-censored data. If lack of statistical power was not the cause of homogeneity among groups, biological explanations should be put forward. One possible reason is that, if interactions between males are the factor that most contributes to male lifespan (Gems and Riddle 2000), it might be that such interactions only become relevant when sexual maturity is achieved and competition among males for fertilization of females or hermaphrodites is intensified. The methodology employed in our assay differs from those used in previous studies, namely in that males were not transferred to fresh plates weekly nor were they placed in 6cm Petri dishes but in 24 well cell culture plates instead. These differences may underlie the tremendous difference found for the lifespan of males kept in isolation between our study and

previous reports of male lifespan (Gems and Riddle 2000). In fact, median lifespan under our experimental conditions was even lower than lifespan of males cultured in single-sex monocultures with 40 individuals. Interactions among males can be highly deleterious, since their effects are detected even when males are kept in pairs (Gems and Riddle 2000). In our assay, measuring the lifespan of *C. elegans* males was an extremely hard endeavor: even in the presence of *C. remanei* females, over 62% of the observations were right censored because males attempted to leave the wells, frequently dying out from dissication. Nevertheless, the decreased rate of mortality observed in males assayed in the presence of a female compared to the rate of mortality of males assayed in empty wells or in wells where females had been previously kept for 24h might still reflect some underlying biological factors, since the statistical model accounted for censored data. A previous study reported increased lifespan of groups of mated males compared to groups that had not mated, although such results are more likely to reflect the effects of mating on male-male interactions (Gems and Riddle 1996) than the direct effects of mating in male lifespan.

The survival profiles of hermaphrodites and females were compared in two analyses carried out independently: individuals that had been mated by experimental males and unmated (selfed or virgin) individuals (Figure 3.12).

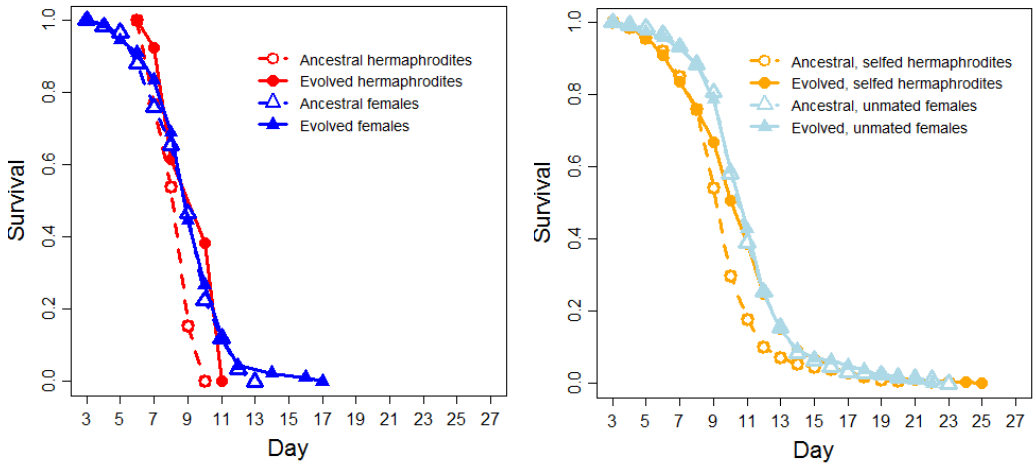

Figure 3.12: Lifespan of mated and unmated hermaphrodites and females of genetically diverse populations. Survival curves of individuals from experimental populations. The graph shows the proportion of sampled individuals still alive at each day of the life cycle (censored and uncensored observations). **A)** Mated hermaphrodites and females. Individuals from Dioecious populations are represented in blue and from Androdioecious populations are represented in red; empty symbols and dashed lines illustrate survival curves of ancestral populations, and filled, full lines those of evolved populations. **B)** Unmated (selfed or virgin) hermaphrodites and females. Individuals from Dioecious populations are represented in light blue and from Androdioecious populations are represented in orange; empty symbols and dashed lines illustrate survival curves of ancestral populations, and filled, full lines those of evolved populations. See Supplementary Table S3.11 for statistical analysis.

Mated females seemed to enjoy reduced risk of death compared to mated hermaphrodites and this pattern did not change significantly with experimental evolution. It is nevertheless interesting that mortality seemed to increase at earlier ages in females than hermaphrodites; this should not be an artifact caused by failure of males to fertilize hermaphrodites since quality control of the data included removal of hermaphrodites with very low numbers of males. It can, however, reflect behavioral differences between hermaphrodites and females since hermaphrodites have been described to avoid mating by males when self-sperm is still available (Garcia *et al* 2007, Kleemann and Basolo 2007, Morsci *et al* 2011), which, in our assay, might have lead to a reduced average number of copulations between males and hermaphrodites than between males and females. It is also possible that females evolved resistance to male-inflicted injury during the derivation of the ancestral dioecious population, which took 22 generations of

enforced mating. Virgin females also exhibited reduced risk of death compared to virgin (selfed) hermaphrodites. Interestingly, however, unmated hermaphrodites evolved patterns of survival similar to those of females.

3.5 Discussion

The aim of the present study was to investigate the evolution of phenotypes related to fitness under conditions known to favor cross-fertilization. The phenotypic characterization was carried out in both dioecious and androdioecious systems, allowing the investigation of how different components underling fitness evolved in each of the cases.

Unclear relationship between body size and fitness in our experimental system

The body size of individuals is a phenotype that correlates with many aspects of organisms, including physiology, life history and ecology. Larger body sizes have been suggested to lead to higher survival (Calder 1983, Peters 1983) and many studies have associated body size with increased mating success and fertility (Andersson 1994). Male reproductive success has been proposed to scale with body size (Perrin 1998, Andersson 1994) and evidence of relationships among these traits have been found among a broad range of animal taxa (e.g. Berrigan and Locke 1991, Reichard *et al* 2005). In *Drosophila hydei*, for example, body size directly correlates with sperm size and siring of offspring (Pitnick and Markow 1994). Although a lot is known about the genetics and physiology of body size in *C. elegans* hermaphrodites (So *et a*l 2011, Salomon *et al* 2009), data on body size of males is virtually inexistent and reduced to reports in which effects of mutations on body size are described to have pleiotropic effects in male tail development (Savage-Dunn *et al* 2003). Although a direct relationship between sperm and body size has yet to be demonstrated, it is known that male sperm is larger and outcompetes hermaphrodite sperm (Ward and Carrell 1979) and that both body size and sperm size of *C. elegans* males can evolve in response to increased sperm competition (LaMunyon and Ward 1999). We did not detect

evolution of body size of males in our experimental populations, although body width significantly differed between males from both mating systems, with males of Dioecious populations exhibiting somewhat wider bodies. Because this phenotype did not significantly evolve, these differences may have arisen during the derivation of the ancestral Dioecious population (which involved additional generations of introgression of the *fog*-2(*q71*) allele compared to the Androdioecious ancestral population A_0). Another possibility is that body size is not a major fitness component of males under our experimental conditions and/or that 50 generations were not sufficient to lead to a phenotypic response; it is also possible that the body volume, as calculated in our study, may have also been an inadequate measure of body size. The study of LaMunyon and Ward reports significant increases of body size of males, measured as body silhouette, with 60 generations of experimental evolution. Although a similar life cycle was employed, in LaMunyon and Ward's study populations were manipulated to always have an excess number of males relative to hermaphrodites (with concomitant intense male-male competition), which may additionally justify their findings compared to ours.

Significant differences were found for the body size of females and hermaphrodites in our experimental populations, with females exhibiting overall larger bodies. However, body size (width and volume) evolved in both. In Rhabditid species including *C. elegans*, a significant correlation has been found for the effects of mutations on body size and on fitness (measured as lifetime reproduction) of hermaphrodite and females (Ostrow *et al* 2007). Because of the reduced number of replicate populations for which both body size and reproductive output were measured, we abstained from testing statistical associations between these two phenotypes. Although the relationship between body size and hermaphrodite/female reproductive fitness in the context of our study could not be investigated, the difference between the body size of hermaphrodites and of females still requires explanation. Similarly to what was evoked for males, it is possible that the additional number of generations elapsed during the derivation of

the ancestral Dioecious population were sufficient to generate the differences in body size observed already among ancestral populations. An alternative, but not mutually exclusive, possibility is that there is some physiological or developmental constraint that prevents hermaphrodites from reaching body sizes as large as those of females. In light of life history evolution theory, one such possibility is that production of self-sperm somehow constrains body size in hermaphrodites. The germline of *C. elegans* hermaphrodites has been shown to repress growth (Patel *et al* 2002), although no specific connection to self sperm was made; hence, hermaphrodites and females would be expected to show similar body sizes. It can also be hypothesized that larger body sizes evolved in response to increased harassment by males, although this possibility remains untested. Despite their differences, both hermaphrodites and females evolved wider bodies, measured at the mid-body region (where embryos are kept during gastrulation). Although purely argumentative, it is tempting to suggest that this phenotypic response resulted from selection for increased retention of embryos within the body of adult worms at day 4 of the life cycle, since these are the eggs that are more likely to survive the *hatch-off* protocol employed in the maintenance of experimental populations. Alternatively, this pattern can simply reflect a more general adaptation to laboratory conditions such as, for example, the food source. In any case, the evolutionary patterns of body size of males and of hermaphrodites/females suggest different relationships of this phenotype with sex-specific components of fitness, although the connection between the two is somewhat unclear.

Extensive evolution of phenotypes related to cross-fertilization

The patterns of evolution of life-history traits (closely related to fitness) were consistent with the maintenance of outcrossing and with its positive correlation with adaptation in our experimental system (see chapter 2). Male components of fitness evolved significantly in dioecious and androdioecious populations. Interestingly, the ability of males to sire progeny evolved to a similar extent in males from both types of populations, despite their different frequencies under the

two mating systems and, consequently, different opportunity for selection on male components of fitness under each of the mating systems. Under dioecy, where males were present at higher frequencies, selection could have been expected to be intensified by stronger male-male competition. It is possible that the effects of increased male-male competition under dioecy were counterbalanced by the fact that the observed frequencies of males in Androdioecious populations correspond to a predicted maximum of male fertilization success as a function of male frequency (Stewart and Phillips 2002). If, on the other hand, male fertilization success is frequency independent, then it may be that competition of sperm provided by males (male-male competition) leads to evolutionary responses as intense as competition between male and self-sperm under androdioecy. This hypothesis finds little empirical support, since male sperm has long been known to be competitively superior to self sperm (LaMunyon and Ward 1998).

The life-history phenotypes of hermaphrodites under conditions of self-and cross-fertilization were also characterized and their evolution contrasted to the evolutionary patterns of life-history phenotypes of females, evolved by definition under conditions of obligate outcrossing. Overall, extensive evolution of life-history traits was observed among hermaphrodites and females, but few differences were found between them under conditions of cross-fertilization. As expected for a component of fitness under our experimental conditions, the mean number of progeny produced at day of culture passage increased with experimental evolution in cross-fertilized hermaphrodites and females. While this pattern may be partially explained by the increased production of eggs by hermaphrodites at day of culture passage (which evolved similar values to those of females), it does not explain the increased production of progeny in females themselves, since their egg production did not increase significantly. Ancestral females exhibited high values of egg production already at the beginning of the experiment, which may indicate that the observed numbers may constitute the physiological maximum that can be produced within this period of time. The fact that the average number of eggs laid at day 4 of the life cycle by individuals from evolved populations (175-183) found in

our study is among the highest values reported for this species – the most similar values found in the literature were 120-150 eggs (Muschiol *et al* 2009, Gruber *et al* 2011) – further supports this hypothesis. In hermaphrodites, increased production of eggs does not seem to have resulted from an earlier switch from spermatogenesis to oogenesis, since this would be expected to lead to decreased numbers of eggs generated in the lifetime of the individuals upon self-fertilization, which was not the case.

Increased lifespan of hermaphrodites under self fertilization

We found differences between the lifespan of mated hermaphrodites and females but no significant evolution of either. These differences may have arisen from the different procedures during derivation of ancestral populations (since it involved additional generations of enforced cross-fertilization in Dioecious populations). Because mating has long been known to shorten lifespan in several organisms (Gems and Riddle 1996, Aigaki and Ohba 1984, Fowler and Partridge 1989), we can hypothesize that females of the ancestral Dioecious population had more time to evolve mechanisms to minimize male-inflicted injury during copulation. In what concerns virgin hermaphrodites, we observed the evolution of lifespan. A previous study performed in similar populations to specifically test the hypothesis that reproduction and longevity are under the action of alleles with antagonistic pleiotropic effects failed to detect evolution of lifespan in selfing hermaphrodites (Anderson *et al* 2011). The authors selected for early fecundity for 47 generations under identical conditions to those employed here, and observed significant responses for the trait in question. Their populations also exhibited intermediate frequencies of males; outcrossing must therefore also have been relevant in their system. Both the study of Anderson and colleagues and ours suggest that the evolution of reproduction and longevity can be decoupled in populations segregating considerable levels of genetic variation and moderate outcrossing rates. The difference in the number of generations of experimental evolution (twice as many in our study) may be sufficient to explain the differences

in the responses of lifespan of hermaphrodites under conditions of selfing. If the survival pattern of virgin females establishes the maximum for these populations, the evolution of lifespan will be quite hard to detect in future studies, since even after 100 generations of experimental evolution and considerable numbers of animals assayed, the median increase in lifespan, although statistically significant, was only of about one day. The lifespan of selfed hermaphrodites was additionally compared to the lifespan of virgin females (of Dioecious populations). Ancestral hermaphrodites showed decreased lifespan compared to females (which did not evolve); however, after experimental evolution the survival pattern of hermaphrodites was extremely similar to the survival curves of virgin females. Genetic variation for lifespan among natural isolates of *C. elegans* had been reported before (Johnson and Wood 1982, McCulloch and Gems 2003), but differences between hermaphrodites and *fog*-2(*q71*) females were not detected in the only study we could find where both were analyzed (Arantes-Oliveira *et al* 2002). Interestingly, “true” females seem to be longer-lived than hermaphrodites in *Caenorhabditis* species (McCulloch and Gems 2003a, Amrit *et al* 2010). It is thus extremely interesting that hermaphrodites evolved under conditions of increased cross-fertilization evolved more “female”-like patterns of survival. Under our experimental conditions, selection could only act on phenotypes expressed until day 4 of the life cycle; whatever patterns found after this period thus evolved neutrally. In the context of life-history theory, they should therefore be revealing of potential constraints between age-related traits such as early and late reproduction and between reproduction and lifespan. In *C. elegans*, the study between reproduction and lifespan has long moved from mere correlative phenotypic studies. Many genes have been identified as lifespan regulators (Kenyon *et al* 1993, McCulloch and Gems 2003b, Curran and Ruvkun 2007). More interestingly, signals from the germline are known to regulate the ageing of this organism by modulating one of the most widely studied signaling pathways, the insulin/IGF-1 pathway (Hsin and Kenyon 1999). In addition to this, there are some evidences linking spermatogenesis to lifespan in *C. elegans* males (van Voohries 1999). This

leads us to hypothesize that the male component of hermaphrodites (sperm) may have underlain the evolution of survival patterns of selfed hermaphrodites, since it's the only component which differs between them and virgin females in the absence of mating. Irrespective of the functional basis of it, the results presented here suggest that age-related phenotypes may be available for selection under self-fertilization but not under conditions of cross-fertilization.

Maintenance of outcrossing generates conflicting selective forces within hermaphrodites

The results shown thus far demonstrate the extensive effects of selection on outcrossing-related phenotypes that accompanied the maintenance of intermediate levels of rates of outcrossing in Androdioecious populations of *C. elegans*. In particular, the reproductive output of crossed hermaphrodites increased significantly and to a larger extent than that of selfed hermaphrodites. The question that emerges is thus "how could selection operate differently on the same individuals"? The answer lies in the investigation of the different components of fitness expressed under conditions of selfing and of outcrossing. As expected, the component of fitness that is expressed in both circumstances – oogenesis – was positively selected, with both hermaphrodites and females showing increased abilities of egg production with experimental evolution. This component cannot therefore underlie the differences in progeny production of hermaphrodites under selfing and outcrossing, and explanations must rely on the male component (sperm) – which is provided by hermaphrodites themselves when selfed but by males under cross-fertilization. A proper investigation of this component was not carried out since traits such as the number of self sperm and their fertilization efficiency were not characterized, but indirect evidence suggest the action of negative selection pressures on the male function of hermaphrodites. While the egg-to-adult survivorship of cross-fertilized progeny increased with experimental evolution, no significant differences were found under self-fertilization. Only an opposing force acting on the male component of hermaphrodites could

counterbalance the positive selection on the female component. Our results thus suggest that the adaptive consequences of outcrossing in populations of *C. elegans* may impose a sexual conflict within the hermaphrodites, in which putative selection against the only component that is uniquely expressed under selfing (the sperm) is constrained by the simultaneous expression of another component of fitness that is positively selected by its expression in cross-fertilization, oogenesis.

The maintenance of a mixed mating system

The maintenance of mixed mating systems necessarily involves a balance of selective forces between outcrossing and selfing. We have demonstrated extensive evolution of outcrossing phenotypes which resulted in increased numbers of progeny produced under these conditions with experimental evolution. As is well known among evolutionary biologists, cross-fertilization comes at an evolutionary cost: the per-capita growth rate of a population composed only of individuals who must cross-fertilize is only half that of a population of selfers. Ancestral hermaphrodites exhibited similar production of progeny under conditions of self- and cross fertilization. Given the advantage of self-fertilization just outlined, selfing rates would therefore be expected to increase in frequency in the absence of any other evolutionary forces. However, evolved hermaphrodites increased their progeny production at day of culture passage under conditions of cross-fertilization to a greater extent compared to conditions of selfing. Thus, selection upon outcrossing phenotypes in our experimental populations led to a decrease in the cost of mating, which generated stability between selfing and outcrossing at intermediate levels of both.

3.6 Acknowledgements

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also measured male competitive performance. S. Carvalho performed the reproductive schedule and lifespan assays as well as the statistical analysis. This work was funded by FCT (SFRH/BD/36726/2007).

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3.8 Supplementary information

A.

Model :
Body width = Mating system+ Generation+ Replicate+ Mating system x Generation

Source	d.f.	SS	MS	F-value	P
Mating system	1	$1.10x10^{-5}$	$1.10x10^{-5}$	8.212	**0.035**
Generation	1	$1.73x10^{-6}$	$1.73x10^{-6}$	1.290	0.308
Replicate	5	$1.75x10^{-5}$	$3.49x10^{-6}$	2.606	0.158
Mating system x Generation	1	$1.25x10^{-6}$	$1.25x10^{-6}$	0.935	0.378
Residuals	5	$6.71x10^{-6}$	$1.34x10^{-6}$		

$F_{8,5}$=2.93; p-value=0.126; Adjusted R^2=54%

B.

Model:
Body length = Mating system+ Generation+ Replicate+ Mating system x Generation

Source	d.f.	SS	MS	F-value	P
Mating system	1	$2,87x10^{-4}$	$2,87x10^{-4}$	0.131	0.732
Generation	1	$9.91x10^{-5}$	$9.91x10^{-5}$	0.045	0.834
Replicate	5	$1.69x10^{-3}$	$3.39x10^{-4}$	1.553	0.321
Mating system x Generation	1	$6.81x10^{-4}$	$6.81x10^{-4}$	0.312	0.600
Residuals	5	$1.09x10^{-2}$	$2.18x10^{-3}$		

$F_{8,5}$=1.03; p-value=0.51; Adjusted R^2=1.9%

C.

Model:
Body volume = Mating system+ Generation+ Replicate+ Mating system x Generation

Source	d.f.	SS	MS	F-value	P
Mating system	1	$5,20x10^{-8}$	$5,20x10^{-8}$	5.6130	0.064
Generation	1	$3.18x10^{-9}$	$3.18x10^{-9}$	0.3431	0.584
Replicate	5	$2.32x10^{-7}$	$4.65x10^{-4}$	5.0186	0.051
Mating system x Generation	1	$1.41x10^{-8}$	$1.41x10^{-8}$	1.5280	0.271
Residuals	5	$4.63x10^{-2}$	$9.26x10^{-9}$		

$F_{8.5}$=4.07; p-value=0.069; Adjusted R^2=65%

Supplementary Table S3.1: Analysis of variance of body size of males of genetically diverse Androdioecious and Dioecious populations. A) body width **B)** body length **C)** calculated body volume. In the models, X stands for interaction between factors.

A.

Model :
Body width = Mating system+ Generation+ Replicate+ Mating system x Generation

Source	d.f.	SS	MS	F-value	P
Mating system	1	$9.63x10^{-6}$	$9.63x10^{-6}$	2.980	0.145
Generation	1	$1.08x10^{-4}$	$1.08x10^{-4}$	33.511	**0.002**
Replicate	5	$5.31x10^{-5}$	$1.06x10^{-5}$	3.283	0.109
Mating system x Generation	1	$1.10x10^{-8}$	$1.10x10^{-8}$	0.003	0.956
Residuals	5	$1.61x10^{-5}$	$3.23x10^{-6}$		

$F_{8,5}$=6.614; p-value=0.02; Adjusted R^2=78%

B.

Model:
Body length = Mating system+ Generation+ Replicate+ Mating system x Generation

Source	d.f.	SS	MS	F-value	P
Mating system	1	0.00343	0.00343	8.607	**0.032**
Generation	1	0.00175	0.00175	4.384	0.090
Replicate	5	0.00580	0.00116	2.911	0.133
Mating system x Generation	1	0.00059	0.00059	1.482	0.277
Residuals	5	0.00199	0.00039		

$F_{8,5}$=3.63; p-value=0.086; Adjusted R^2=62%

C.

Model:
Body volume = Mating system+ Generation+ Replicate+ Mating system x Generation

Source	d.f.	SS	MS	F-value	P
Mating system	1	$5.10x10^{-7}$	$5.10x10^{-7}$	6.819	**0.047**
Generation	1	$2.12x10^{-6}$	$2.12x10^{-6}$	35.200	**0.002**
Replicate	5	$1.38x10^{-6}$	$2.76x10^{-7}$	4.560	0.060
Mating system x Generation	1	$1.03x10^{-9}$	$1.03x10^{-9}$	0.017	0.901
Residuals	5	$3.00x10^{-7}$	$6.01x10^{-8}$		

$F_{8,5}$=8.13; p-value=0.017; Adjusted R^2=81%

Supplementary Table S3.2: Analysis of variance of body size of hermaphrodites and females of genetically diverse Androdioecious and Dioecious populations. A) body width **B)** body length **C)** calculated body volume. In the models, X stands for interaction between factors.

A.

Model :
viability = Mating system+ Generation+ Replicate+ Mating system x Generation

Source	d.f.	SS	MS	F-value	P
Mating system	1	0.00275	0.00275	0.4190	0.522
Generation	1	0.23949	0.23949	36.4569	**<0.001**
Replicate	5	0.01255	0.00251	0.3820	0.858
Mating system x Generation	1	0.00114	0.00114	0.1741	0.679
Residuals	33	0.21678	0.00657		

$F_{8,33}$=4.87; p-value<0.001; Adjusted R^2=43%

B.

Model:
fractions of males = Mating system+ Generation+ Replicate+ Mating system x Generation

Source	d.f.	SS	MS	F-value	P
Mating system	1	0.33624	0.33624	115.3619	**< 0.001**
Generation	1	0.00170	0.00170	0.5845	0.450
Replicate	5	0.02342	0.00468	1.6072	0.186
Mating system x Generation	1	0.00360	0.00360	1.2339	0.275
Residuals	33	0.09618	0.00291		

$F_{8,33}$=15.65; p-value<0.001; Adjusted R^2=74%

Supplementary Table S3.3: Analysis of variance of viability of genetically diverse Androdioecious and Dioecious populations A) proportion of offspring that hatched from eggs and reached the adult stage **B)** fractions of males among adult progeny. In the model, X stands for interaction between factors.

Model :
Male competitive performance = Mating system+ Generation+ Replicate+ Generation x Replicate + Mating system x Generation x Replicate

Source	d.f.	SS	MS	F-value	P
Mating system	1	0.00003	0.000027	0.002	0.961
Generation	3	0.73086	0.243620	22.016	**<0.001**
Replicate	5	0.14236	0.028472	2.841	**0.030**
Mating system x Replicate	10	0.31440	0.031440	2.354	**0.005**
Mating system x Generation x Replicate	18	0.46885	0.026047		
Residuals	111	1.22829	0.011066		

$F_{37.111}$=4.05; p-value<0.001; Adjusted R^2=43%

Supplementary Table S3.4: Analysis of variance of male competitive performance of genetically diverse Androdioecious and Dioecious populations. Analysis was performed in a full model (testing all factors and interactions) but a reduced model is presented (after removal of non significant interactions). In the model, X stands for interaction between factors.

A.

Model (cross fertilized hermaphrodites vs females):
LRS = Group + Generation+ Replicate+ Group x Generation

Source	d.f.	SS	MS	F-value	P
Group	1	217	217	0.600	0.46791
Generation	1	41362	41362	14.186	**<0.001**
Replicate	2	6276	3138	8.663	**0.017**
Group x Generation	1	650	650	1.794	0.229
Residuals	6	2173	362		

$F_{5,6}$=26787; p-value<0.001; Adjusted R^2=92%

B.

Model (cross fertilized vs self fertilized hermaphrodites):
LRS = Group + Generation+ Replicate+ Group x Generation

Source	d.f.	SS	MS	F-value	P
Group	1	49302	49302	83.327	**<0.001**
Generation	1	21879	21879	36.979	**<0.001**
Replicate	2	4024	2012	3.400	0.1029
Group x Generation	1	6554	6554	11.07	0.0158
Residuals	6	3550	592		

$F_{5,6}$=11.87; p-value=0.005; Adjusted R^2=83%

Supplementary Table S3.5: Analysis of variance of lifetime reproductive success (LRS) of hermaphrodites and females of genetically diverse populations. A) Comparison between hermaphrodites and females under outcrossing **B)** Comparison between crossed and selfed hermaphrodites**.** In the model, X stands for interaction between factors.

A.

Model (cross fertilized hermaphrodites vs females):
Progeny at day 4 of life cycle = Group + Generation+ Replicate+ Group x Generation

Source	d.f.	SS	MS	F-value	P
Group	1	19.3	19.3	0.563	0.482
Generation	1	5903.2	5903.2	171.985	**<0.001**
Replicate	2	825.0	412.5	12.018	**0.007**
Group x Generation	1	129.5	129.5	3.773	0.100
Residuals	6	205.9	34.3		

$F_{5,6}$=40.07; p-value<0.001; Adjusted R^2=95%

B.

Model (cross fertilized vs self fertilized hermaphrodites):
Progeny at day 4 of life cycle = Group + Generation+ Replicate+ Group x Generation

Source	d.f.	SS	MS	F-value	P
Group	1	621.5	621.5	4.725	0.073
Generation	1	4019.8	4019.8	30.558	**0.002**
Replicate	2	2548.5	1274.3	9.687	**0.013**
Group x Generation	1	615.5	615.5	4.679	0.074
Residuals	6	789.3	131.5		

$F_{5,6}$=11.87; p-value=0.005; Adjusted R^2=83%

Supplementary Table S3.6: Analysis of variance of reproductive output of hermaphrodites and females of genetically diverse populations. A) Comparison between hermaphrodites and females under outcrossing **B)** Comparison between crossed and selfed hermaphrodites**.** In the model, X stands for interaction between factors.

A.

Model (cross fertilized hermaphrodites vs females):
Number of eggs = Group + Generation+ Block+ Group x Generation

Source	d.f.	SS	MS	F-value	P
Group	1	101.2	101.21	0.372	0.564
Generation	1	538.9	538.92	1.981	0.209
Block	2	6312.3	3156.14	11.599	**0.009**
Group x Generation	1	361.0	360.98	1.327	0.293
Residuals	6	1632.6	272.11		

$F_{5,6}$=5.375; p-value=0.032; Adjusted R^2=67%

B.

Model (cross fertilized vs self fertilized hermaphrodites):
Number of eggs = Group + Generation+ Block + Group x Generation

Source	d.f.	SS	MS	F-value	P
Group	1	309.84	309.84	2.7053	0.151
Generation	1	2111.53	2111.53	18.4361	**0.005**
Block	2	3024.16	1512.08	13.2022	**0.006**
Group x Generation	1	13.97	13.97	13.2022	0.739
Residuals	6	687.19	114.53		

$F_{5,6}$=9.53; p-value<0.008; Adjusted R^2=80%

Supplementary Table S3.7: Analysis of variance of egg production of hermaphrodites and females of genetically diverse populations. A) Comparison between hermaphrodites and females under outcrossing **B)** Comparison between crossed and selfed hermaphrodites**.** In the model, X stands for interaction between factors.

A.

Model (cross fertilized hermaphrodites vs females):
Lifetime number of eggs = Group + Generation+ Replicate+ Group x Generation

Source	d.f.	SS	MS	F-value	P
Group	1	4389	4388.8	1.320	0.294
Generation	1	3278	3278.4	0.986	0.359
Replicate	2	39535	19767.5	5.945	**0.038**
Group x Generation	1	76	76.1	0.023	0.885
Residuals	6	19949	3324.9		

$F_{5,6}$=2.84; p-value=0.1178; Adjusted R^2=46%

B.

Model (cross fertilized vs self fertilized hermaphrodites):
Lifetime number of eggs = Group + Generation+ Block + Group x Generation

Source	d.f.	SS	MS	F-value	P
Group	1	187858	18785	62.7780	**<0.001**
Generation	1	1449	1449	0.4842	0.513
Block	2	7293	3646	1.2185	0.360
Group x Generation	1	110	110	0.0366	0.855
Residuals	6	17954	2992		

$F_{5,6}$=13.15; p-value<0.003; Adjusted R^2=85%

Supplementary Table S3.8: Analysis of variance of lifetime production of eggs of hermaphrodites and females of genetically diverse populations A) Comparison between hermaphrodites and females under outcrossing **B)** Comparison between crossed and selfed hermaphrodites**.** In the model, X stands for interaction between factors.

A.

Model (cross fertilized hermaphrodites vs females):
viability = Group + Generation+ Block+ Group x Generation

Source	d.f.	SS	MS	F-value	P
Group	1	0.00092	0.00092	0.271	0.621
Generation	1	0.04667	0.04662	13.792	**0.010**
Block	2	0.05184	0.02592	7.660	**0.022**
Group x Generation	1	0.00009	0.00009	0.026	0.877
Residuals	6	0.02030	0.00338		

$F_{5,6}$=5.88; p-value=0.026; Adjusted R^2=69%

B.

Model (cross fertilized vs self fertilized hermaphrodites):
viability = Group + Generation+ Block + Group x Generation

Source	d.f.	SS	MS	F-value	P
Group	1	0.0055941	0.0055941	1.3677	0.287
Generation	1	0.0235854	0.0235854	5.7662	0.053
Block	2	0.0249662	0.0124831	3.0519	0.122
Group x Generation	1	0.0051614	0.0051614	1.2619	0.304
Residuals	6	0.0245416	0.0040903		

$F_{5,6}$=2.9; p-value=0.11; Adjusted R^2=46%

Supplementary Table S3.9: Analysis of variance of viability of progeny produced by hermaphrodites and females of genetically diverse populations. A) Comparison between hermaphrodites and females under outcrossing **B)** comparison between crossed and selfed hermaphrodites**.** In the model, X stands for interaction between factors.

A.

Model (young males) :
Survival = Mating system+ Generation+ Presence of female + Mating system x Generation

Parameter estimates	coef	exp(coef)	se(coef)	z	P
Mating system (Dioecy)	-0.168	$8.46x10^{-1}$	$2.21x10^{-1}$	-0.758	0.448
Generation (evolved)	-0.179	$8.36x10^{-1}$	$2.17x10^{-1}$	-0.827	0.408
Presence of female (prior to assay)	13.55	$7.67x10^{+5}$	$1.91x10^{+3}$	0.007	0.994
Presence of female (*C. remanei*)	15.41	$4.93x10^{+6}$	$1.91x10^{+3}$	0.008	0.994
Mating system (D) x Generation (evolved)	-0.031	$3.70x10^{-1}$	$3.25x10^{-1}$	-0.094	0.925
n=806, number of events (uncensored observations) = 157					

B.

Model (adult males) :
Survival = Mating system+ Generation+ Block + Mating system x Generation

Parameter estimates	coef	exp(coef)	se(coef)	z	P
Mating system (Dioecy)	0.3817	1.465	0.157	2.439	**0.015**
Generation (evolved)	0.1157	1.123	0.158	0.731	0.465
Presence of female (prior to assay)	-0.9518	0.386	1.120	-0.850	0.395
Presence of female (*C. remanei*)	1.0015	2.722	1.005	0.997	0.319
Mating system (D) x Generation (evolved)	-0.3918	0.676	0.219	-1.792	0.073
n=1006, number of events (uncensored observations)= 343					

Supplementary Table S3.10: Survival analysis of A) young males (L4) and of B) adult males of genetically diverse populations. coef – estimated coefficients of the model; exp(coef) – exponential of the coefficient, it represents the hazard (instantaneous rate of death) of individuals of the group relative to the hazard of individuals from the group defined by the lower levels of the factor; se(coef) – standard error of the estimate of the coefficient. In the model x denotes interaction between factors.

A.

Model (mated hermaphrodites and females) :
Survival = Mating system+ Generation+ Mating system x Generation

Parameter estimates	coef	exp(coef)	se(coef)	z	P
Mating system (Dioecy)	-0.9749	0.377	0.320	-3.046	**0.002**
Generation (evolved)	-0.7088	0.492	0.398	-1.780	0.07
Mating system (D) x Generation (evolved)	0.6997	2.013	0.437	1.599	0.110

n=174, number of events(uncensored observations) = 157

B.

Model (selfed hermaphrodites and virgin females) :
Survival = Mating system+ Generation+ Block + Mating system x Generation

Parameter estimates	coef	exp(coef)	se(coef)	z	P
Mating system (Dioecy)	-0.5169	0.596	0.089	-5.830	**<0.001**
Generation (evolved)	-0.4642	0.629	0.088	-5.30	**<0.001**
Block (2)	-0.5467	0.579	0.072	-7.635	**<0.001**
Mating system (D) x Generation (evolved)	0.4934	1.638	0.127	3.870	**<0.001**

n=1188, number of events (uncensored observations)= 1012

Supplementary Table S3.11: Survival analysis of A) mated hermaphrodites and females and of B) selfed hermaphrodites and virgin females of genetically diverse populations. coef – estimated coefficients of the model; exp(coef) – exponential of the coefficient, it represents the hazard (instantaneous rate of death) of individuals of the group relative to the hazard of individuals from the group defined by the lower levels of the factor; se(coef) – standard error of the estimate of the coefficient. In the model x denotes interaction between factors.

Chapter 4

A test on evolutionary transitions between mating systems

4.1 Summary

The breeding mode of sexual organisms has important evolutionary consequences. It determines how diversity is structured within and between individuals in populations, and therefore strongly influences the strength and efficiency with which selection can exert its action. Mating systems can range from monoecy (purely selfing organisms) to dioecy (pure outcrossing) with a multitude of mixed-mating systems establishing a continuum between these forms. More importantly, the mating system of species can change over evolutionary time. Understanding how mixed-mating systems are maintained by natural selection and how mating systems evolve has been a quest for evolutionary biologists since the days of Darwin. Despite the wide availability of theoretical models (both genetic and ecologically based), their empirical evaluation is extremely limited, in part because they are hard to test experimentally and thus relies mostly on correlations obtained from studies in which mating systems of phylogenetically diverse taxa are compared.

We used populations of *C. elegans* experimentally evolved under two different mating systems and which had previously been characterized at the genetic and phenotypic levels, to test evolutionary hypotheses concerning the evolution of mating systems. We followed the frequencies of a sex-determining allele and of outcrossing over 8 generations to infer whether selfing hermaphrodites could invade a population composed of males and females and whether a small number of males and females could invade a population solely composed of selfing hermaphrodites.

Our results show that the fate of an allele allowing self-fertilization depends on the genetic background of the individuals in which it is found but does not depend on the genotypic composition of the remainder population. Interestingly, in our experimental system males and outcrossing were maintained even when the selfing allele increased in frequency.

4.2 Introduction

The breeding mode of organisms has important effects on the genetic properties of populations, determining to a large extent levels of homozygosity or linkage disequelibrium. Because of this, they have important consequences for the evolutionary potential of populations. Explaining how different mating systems are brought about and maintained is thus an important question in evolutionary biology. In sexually reproducing organisms, dioecy and monoecy delimit the range of possible mating systems classified as a function of rates of cross-fertilization. Dioecious systems are composed of males and females which must cross-fertilize (outcross) in order to produce offspring; monoecy describes a system in which both gametes required for the production of zygotes are provided by the same individual (hermaphrodites), with reproduction thus occurring by self-fertilization (selfing). Other mating systems where both cross- and self- fertilization take place are called mixed mating systems. Evolutionary transitions between mating systems can occur in both directions (monoecy to dioecy and the reverse) in plants and animals (Pannell 2002), although the evolution of hermaphroditism from dioecy seems to be more common (Pannell 2002, Charlesworth 2006). Several theoretical models have been put forward to determine under which conditions one or the other mating system should emerge and hence gain understanding of the forces underlying their evolution. They can be broadly categorized into genetic and ecological models. Genetic models explore the conditions under which the genetic transmission advantage of selfing can be counterbalanced by its costs, namely in the form of inbreeding depression. Similarly to the arguments in favor of asexuality, the genetic transmission advantage of selfing stems from the fact that an allele conferring the ability to self-fertilize is twice represented in the progeny of selfing individuals (Fisher 1941). In other words, outcrossing is evolutionarily costly since the per-capita production of offspring will be half in outcrossers (which requires two individuals) compared to selfers (assuming similar number of progeny produced in both situations). The cost of self-fertilization, on the other hand, relies on the possible reduced fitness of self-fertilized progeny relative to cross-fertilized progeny (Lande and Schemske 1985, Charlesworth and Charlesworth 1987), a

phenomenon called inbreeding depression. Inbreeding depression can result both from the increased homozygosity of recessive deleterious alleles (which segregate in populations at mutation-selection equilibrium) and from homozygosity at loci with heterozygote advantage (overdominance) (Charlesworth and Willis 2009). These models generally predict the evolution of mating systems towards pure selfing (monoecy) or pure outcrossing (dioecy), which is confirmed by the U-shaped distribution of rates of self-fertilization among plant species (Lande and Schemske 1985, Goodwillie *et al* 2005), in which 60% of 345 species (in the latter study) exhibit either predominant selfing or predominant outcrossing. In any case, the 40% of the species with mixed mating systems still have to be explained. With respect to animals, the mating systems of many species remain unknown but if the presence of hermaphroditism somehow correlates with mixed-mating strategies, it may be much more common than previously thought: hermaphrodites were found in 21 of 32 phyla in one study (Jarne and Charlesworth 1993) and in 33% of the animal species surveyed (insects excluded) (Jarne and Auld 2006). Therefore, the existence of mixed mating systems in nature cannot be ignored. Because the genetic models presented above don't seem to provide all the answers for the existence of mixed mating systems, several ecologically-based models have been proposed. Such models are based on arguments of reproductive assurance in the face of scarcity of potential partners for reproduction (Darwin 1876), of maintenance of locally adapted genotypes (Antonovics 1968) or of reduced expenditure of resources on copulation/pollination (Charlesworth and Charlesworth 1978).

In any case, mixed mating systems will inevitably be generated in the transitions between extreme mating systems (Charlesworth 1984, Charlesworth 2006). Therefore, the main question is whether they *always* represent intermediate stages in evolutionary transitions or if they can, in and of themselves, constitute evolutionarily stable strategies. The answer to this question may lie in the phylogenetic analysis of mating systems: if mixed strategies are only transient, they should be rare and short-lived, thus found mostly at terminal branches of

phylogenetic trees, as recently derived states. Surprisingly, phylogenetic approaches have contributed little to the question of the long-term evolutionary stability of mixed mating systems, partly because they have focused on presence or absence of selfing, neglecting the quantification of rates of outcrossing (Dorken *et al* 2002, Goodwillie *et al* 2005, Charlesworth 2006).

There are three main mixed mating systems that can emerge during evolutionary transitions: trioecy (co-occurrence of males, females and hermaphrodites), gynodioecy (mixtures of females and hermaphrodites) and androdioecy (presence of males and hermaphrodites). Trioecy seems to be a highly unstable system (Lloyd 1975, Charlesworth and Charlesworth 1978), for which most transitions are likely to occur via gynodioecy and androdioecy. Among these, gynodioecy seems to be the most common (Charlesworth 1984, Pannell 2002), although the generality of this pattern is questionable since it results from the study of plant species, which dominate the literature on mating systems and their evolution (Jarne and Charlesworth 1993). In fact, a recent investigation of the presence of androdioecy among plant and animal species has found several examples, although this mating system should still be regarded as rare (Pannell 2002). Luckily, this mode of reproduction is found in one of the most widely used animal laboratory models, *Caenorhabditis elegans*, for which several mutant alleles which change the mating system are available (Anderson *et al* 2010). This species thus provides an excellent system in which to test evolutionary hypothesis concerning the evolution of mating systems.

Androdioecy has evolved independently in the family Rhabditidae and within the genus *Caenorhabditis* from dioecious ancestrals (Fitch and Thomas 1997, Kiontke *et al* 2004). In most of these species, reproduction seems to occur predominantly by self-fertilization of hermaphrodites. In *C. elegans*, naturally occurring rates of cross-fertilization are estimated to lie between 0% and 22%. This disparity partly stems from the use of different methodologies such as frequency of heterozygote genotypes among sampled individuals to estimates based on genome-wide patterns of linkage decay (Barrière and Félix 2005, 2007;

Haber *et al* 2005; Sivasundar and Hey 2005; Rockman and Kruglyak 2009). The presence of males in androdioecious populations where hermaphrodites are perfectly able to maintain populations on their own is paradoxical. Hence, there have been quite a few experimental evolution studies trying to investigate the conditions under which males (and outcrossing) are beneficial and thus unraveling the forces behind the maintenance of this mixed mating system. Because of the central role of inbreeding depression in most genetic models of mating system evolution, most studies investigated the possibility that different outcrossing equilibria would be attained under different levels of inbreeding depression. Since *C. elegans* does not naturally exhibit inbreeding depression (Dolgin *et al* 2007), these studies relied on the creation of recessive, deleterious alleles via mutagenesis and manipulation of initial rates of outcrossing (Manoel *et al* 2006) and/or mating system (Cutter 2005). These studies have shown that males (and outcrossing) can be sustained for longer under these conditions but they are eventually reduced to the levels at which they are spontaneously generated. More recently, two other studies have described the maintenance of intermediate levels of cross-fertilization in populations of *C. elegans*. In one, the results that were obtained can be equally (or better) explained by ecological models, namely temporal environmental heterogeneity (Morran *et al* 2009a). The other study argues for the evolutionary advantage of outcrossing in generating variation among genotypes during adaptation to a novel environment (Morran *et al* 2009b). However, this outcrossing advantage can also be explained by purging of the deleterious alleles resulting from the mutagenesis that was applied to generate initial genetic variation, as well as by inadvertent artificial selection for males caused by the experimental conditions. To create a harsher environment than the apparently benign standard laboratory environment, the authors created a vermiculite barrier the worms had to overcome to have access to food. Only individuals who succeeded in reaching food were allowed to contribute to the following generation. *C. elegans* males explore more their environment than hermaphrodites, even in the presence of food (Lipton *et al* 2004). The possibility

that high rates of outcrossing were caused by these differences in behavior was not accounted for in the study of Morran and colleagues. In summary, the conditions for the evolution and stability of androdioecy in this species are still unclear.

We used populations of *C. elegans* experimentally evolved under two different mating systems and which had previously been characterized at the genetic and phenotypic levels (see Chapters 2 and 2), to test the hypothesis of emergence of androdioecy (male–hermaphrodite) from monoecy (selfing hermaphrodite), as well as the emergence of androdioecy from dioecy (males and females). Experimental populations were generated to harbor high levels of genetic polymorphism without the use of mutagenesis, by crossing genetically diverse natural isolates of the species. Adaptation was shown to occur in our experimental populations and intermediate levels of outcrossing were maintained in Androdioecious populations, revealing stability of this mating system under the conditions imposed. These were characterized by a stable environment, abundant food, constant and large population sizes and discrete, non-overlapping generations. Specifically, to test the hypothesis of evolution of androdioecy from dioecy, we introduced an allele conferring the ability for self-fertilization in a population composed of males and females. This was the wild-type allele found at the *fog*-2 locus in *C. elegans* populations. To test the hypothesis of evolution of androdioecy from monoecy, we introduced and obligate outcrossing allele into populations composed of selfing hermaphrodites. This was achieved by delivering males and females from populations in which an alternative allele at the *fog*-2 locus (*fog*-2(*q71*)), had been introgressed prior to experimental evolution. The *fog*-2(*q71*) allele disrupts hermaphroditic spermatogenesis, rendering hermaphrodites functional females, without affecting spermatogenesis in males. The frequencies of the selfing and obligate outcrossing alleles were monitored in time simulataneously in both situations, as well as frequencies of males. Because Androdioecious and Dioecious experimental populations did not exhibit significant levels of inbreeding depression (and these effects should be only transient in

populations as large and those employed here – N=10.000), and also because the imposed demography rules out ecological explanations for the maintenance of androdioecy, the prediction from genetic models would be that the geometric advantage of selfing would lead to the spread of the wild-type allele of the *fog*-2 during evolution regardless of its initial frequency unless the average fitness of evolved, Dioecious individuals increased to the point of offsetting this advantage.

4.3 Materials and Methods

Experimental populations

Six androdioecious and six dioecious populations with high levels of standing genetic variation (A_{1-6}, D_{1-6} – Figure 1.6) were experimentally evolved for 100 generations under stable environmental conditions and discrete, non-overlapping generations. A tester population was constructed by introgression of a transgenic allele expressing GFP into the genetic background of ancestral Androdioecious populations (see Chapter 1). Samples from ancestral and evolved experimental populations and from the *tester* population were cryopreserved. These populations were thawed and expanded for 2 generation prior to the assays described here.

Competitions among Dioecious and Androdioecious populations

We experimentally tested two contrasting hypothesis concerning the emergence of mixed mating systems and their evolution. The possibility of emergence of a mixed mating (or fully dioecious) system from a population of selfing hermaphrodites (hypothesis A) was tested by investigating the possibility of an allele responsible for obligate outcrossing (the *fog*-2(*q71*) allele) of increasing in frequency when delivered to a population composed of self-fertilizing hermaphrodites. This was achieved by introducing individuals of the ancestral or evolved Dioecious populations (D_0, D_{4-6}) into ancestral or evolved Androdioecious populations (A_0, A_{4-6}). The experimental setup took place at day 3 of the life-cycle (after thawing and population expansion), by picking 190 L4-staged

hermaphrodites, 5 females and 5 males to 6cm Petri dishes with NGM agar and 5µl of bacteria. The evolutionary scenario of emergence of a mixed mating (or fully selfing) system from a dioecious population (hypothesis B) was tested by investigating the possibility of an allele allowing self-fertilization in females (the *fog*-2(*wt*) allele) of increasing in frequency when delivered to a population composed of males and females. To this purpose, we introduced 10 hermaphrodites of the ancestral or evolved Androdioecious populations (A_0, A_{4-6}) into ancestral or evolved Dioecious populations (D_0, D_{4-6}) composed of 95 males and 95 females. The two treatments (testing hypothesis A and hypothesis B) were carried out simultaneously. Overall, the assay encompassed 14 populations (henceforth designated competitions): 7 of them in which the *fog*-2(*q71*) allele started at lower frequencies (0.05) and the remaining ones in which this allele started at higher frequencies (0.95 – and conversely, the *fog*-2(*wt*) allele started at a frequency of 0.05). Although there were only 3 combinations of population states in the competitions within each treatment (ancestral-ancestral, evolved-ancestral or ancestral-evolved), the number of competitions was higher because 3 experimental populations were used (A_{3-6}, D_{3-6}) whenever competitions included evolved states. Because each competition was initiated from 200 individuals (set-up), the *hatch-off* protocol was not performed. Instead, culture plates were incubated for 48h, after which (corresponding to day 1 of the life cycle of the following generation) individuals were washed from culture plates with M9 solution to 15ml polypropylene tubes. Adults were pelleted by centrifugation (1min, 200rpm) and discarded. Larval densities were estimated by scoring of live larvae in 5 drops of 5µl and each competition/population was expanded by seeding 1000 individuals in three culture plates. Worms were incubated for 2 days, after which worms from the same competition plates were subjected to the *hatch-off* protocol. 5 culture plates of each competition were maintenance under the same conditions as those of experimental evolution (see Chapter 2) for a total of 8 generations, except for population census sizes, which were smaller in this competition assay (3000 individuals). During this time, both the frequency of the *fog*-2(*q71*) allele and

the frequencies of males were monitored. At generations 2, 5 and 8, an additional culture plate was seeded per replicate competition. At day 4 of the life cycle, these plates were stored at 4ºC until measurement of the frequency of males (see Frequency of males and Outcrossing rates). Prior to their storage, 16 non-male individuals (virtually all individuals are fertilized at this point, rendering phenotypic distinction of females from hermaphrodites nearly impossible) were collected for DNA extraction and genotyping. These worms were transferred to 5µl of ultrapure water in 8-strip optical clear flat caps (Sarstedt) kept on ice. Genomic DNA extraction Mix was prepared with ZyGEM prepGEM™Insect kit (ZyGEM™ Corporation Ltd) as follows: 4µl ultrapure water, 1µl 10x Buffer Black, 0.125µl prepGem™ enzyme (for each reaction of extraction). 5µl of the DNA extraction Mix were pipetted onto 96 well Multiply PCR plates (Sarstedt); the wells were sealed with the 8-strip caps containing the individual worms in water and the PCR plates were centrifuged briefly to merge both solutions (30sec, 1800rpm) to a final volume of 10µl. Genomic DNA extraction proceeded by exposing the samples to 15min at 75ºC followed by 5min at 95ºC in MyCycler Thermal Cycler (Bio-Rad Laboratories, Inc.); extracted DNA samples were stored at 4ºC until genotyping. The genotyping reaction was performed using one forward and two reverse primers. The two reverse primers differed in their first 5' base, with each one binding specifically to each allele at the *fog*-2 locus and also in that one of them had a random 3' sequence of 31 bases to allow discrimination between the alleles on agarose gel electrophoresis; amplicon sizes were 295bp (*fog*-2(*wt*) allele and 264bp (*fog*-2(*q71*) allele). The DNA was PCR-amplified using the GoTaq Flexi kit (Promega Corporation): 3µl 5x Green GoTaq® Flexi Buffer, 0.9µl $MgCl_2$ 25mM, 0.12µl GoTaq® DNA Polymerase, 1.5µl dNTP 2mM (Fermentas, Thermo Fisher Scientific), 0.6µl forward primer 12.5µM (CTGTCCAGATACGCCTCTCGTCT), 0.3µl reverse primer long (ACGCCTGTGTGAAATTGGGCAAAAGATTAGACTGATTGAGCAATATCGATAA TC), 0.9µl reverse primer short (CTGATTGAGCAATATGCTGAATT), 5.68µl miliQ water, 2µl genomic DNA (~10ug/ul). The PCR reaction was carried out in

MyCycler Thermal Cycler (Bio-Rad Laboratories, Inc.): 3min at 95ºC, 35 cycles of amplification with 30sec at 95ºC, 30sec at 58ºC, 30sec at 72ºC, 3min at 72ºC for final extension. Samples were stored at 4ºC until they were run in gel electrophoresis in 2% agarose stained with 6µl ethidium bromide (10mg/ml).

Statistical analysis: Statistical analysis of changes in relative frequencies of males and of alleles at the *fog*-2 locus was performed separately for each treatment. Because the treatments involved the manipulation of the initial frequencies of males, females and hermaphrodites, only data from generations 2, 5 and 8 were analyzed. We employed a linear mixed-effects model with repeated measures using the *lmer* function of the *nlme4* package (Bates and Maechler 2010) in the R software (R Development Core Team 2010). The types of competitions within each treatment were coded as 1 (ancestral recipient population, ancestral invading population), 2 (ancestral recipient population, evolved invading population) or 3 (evolved recipient population, ancestral invading population). Each model thus took competition type (3 levels) and generation (3 levels) as fixed factors, and incorporated a random term specifying the source of the repeated measures (in this case, each replicate competition). The general form of the model was therefore: Response (male frequency or *fog*-2(*q71*) frequency) = type (fixed effect)+generation (fixed effect)+(generation|competition) (competition as random effect accounting for potential correlation of measurements across generations). Due to the small number of replicate competitions and the unbalanced design between states (ancestral versus evolved), replicate evolved population was not incorporated into the model.

4.4 Results

Evolution of the frequencies of alleles of a sex-altering locus

Several lines of evidence suggest that dioecy is the ancestral state in the Rhabditid phylogeny, with hermaphroditism having arised at least ten times within Rhabditid clades (Kiontke and Fitch 2005). In the genus *Caenorhabditis*, *C. elegans* and *C. briggsae* seem to have evolved hermaphroditism independently

(Kiontke *et al* 2004). We tested the hypothesis of a) the emergence of obligate outcrossing in a population of self-fertile hermaphrodites and, conversely, b) the emergence of self-fertilization in a male-female population, in a scenario of abundant natural genetic variation. We followed the frequency of alleles at a sex-determining locus (*fog*-2) for 8 generations. The results are shown in Figure 4.1.

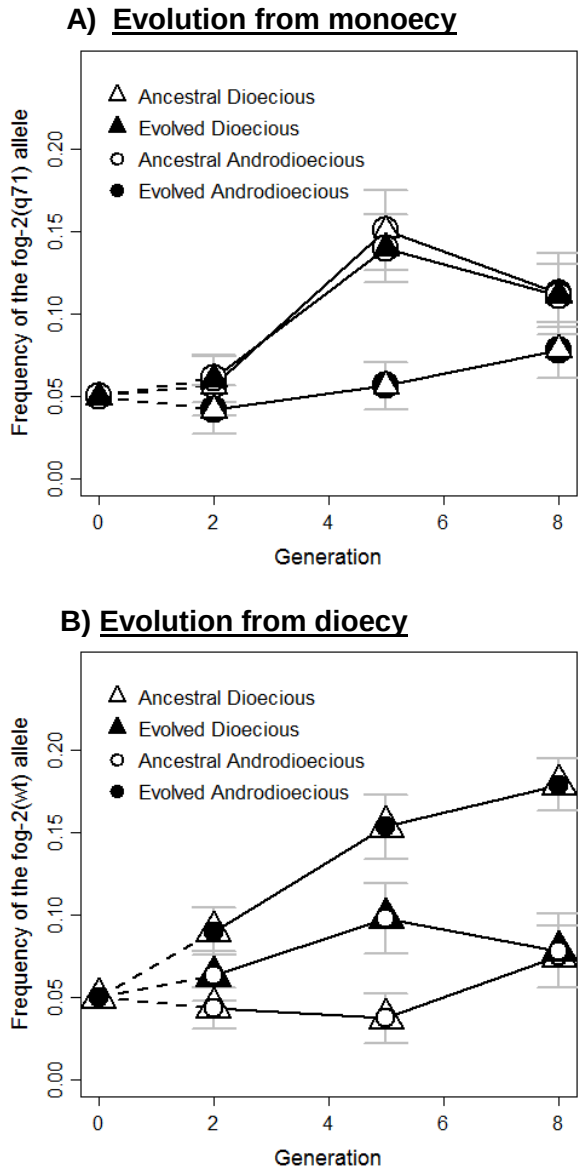

Figure 4.1: **Evolution of allele frequencies at the fog-2 locus under two evolutionary scenarios**. Ancestral and evolved experimental populations with standing genetic variation were used to test **A)** the possibility of spread of an allele responsible for self-sterility (thus making cross-fertilization the only mode of reproduction possible) in a population composed of self-fertile hermaphrodites or **B)** the possibility of spread of an allele conferring females the ability to self-fertilize in a population composed of males and females. Large symbols represent the mating system and state (ancestral or evolved) of the population at the highest frequency at onset of the experiment; smaller (inset) symbols, represent the mating system and state of the least represented (“emerging”) population at the onset of the experiment. The values are presented as the mean expected frequencies of the fog-2(*q71*) and fog-2(*wt*) from sampled genotypes in replicate experimental populations. Error bars (grey) represent standard error of the mean. See Supplementary Table S4.1 for statistical analysis.

We found a significant increase in the frequency of the *fog*-2(*q71*) allele with time when introduced into hermaphroditic populations at initially low frequencies (Figure 4.1 A). This allele disrupts spermatogenesis in hermaphrodites without affecting male spermatogenesis (Schedl and Kimble 1988). The observed increase in frequency of this allele (almost 3-fold) was similar whether it was found on the genetic background of ancestral (D_0 - empty triangles inset in empty circles) or evolved (D_{4-6} - filled triangles inset in empty circles) individuals from the Dioecious populations; however, its spread seems to have been hampered in the presence of evolved hermaphrodites (A_{4-6}, filled circles with empty triangles. Conversely, when a selfing allele (*fog*-2(*wt*)) was introduced in a dioecious population, its fate was the same regardless of the evolutionary state (ancestral or evolved) of the "recipient" population (Figure 4.1 B). However, when introduced via the genetic background of evolved hermaphrodites, its increase in frequency was much faster. It is worthwhile noticing though, that the frequency of males segregating in the male-female-hermaphrodites populations consistently reached appreciable levels by generation 8, regardless of their starting frequencies or competition (Figure 4.2).

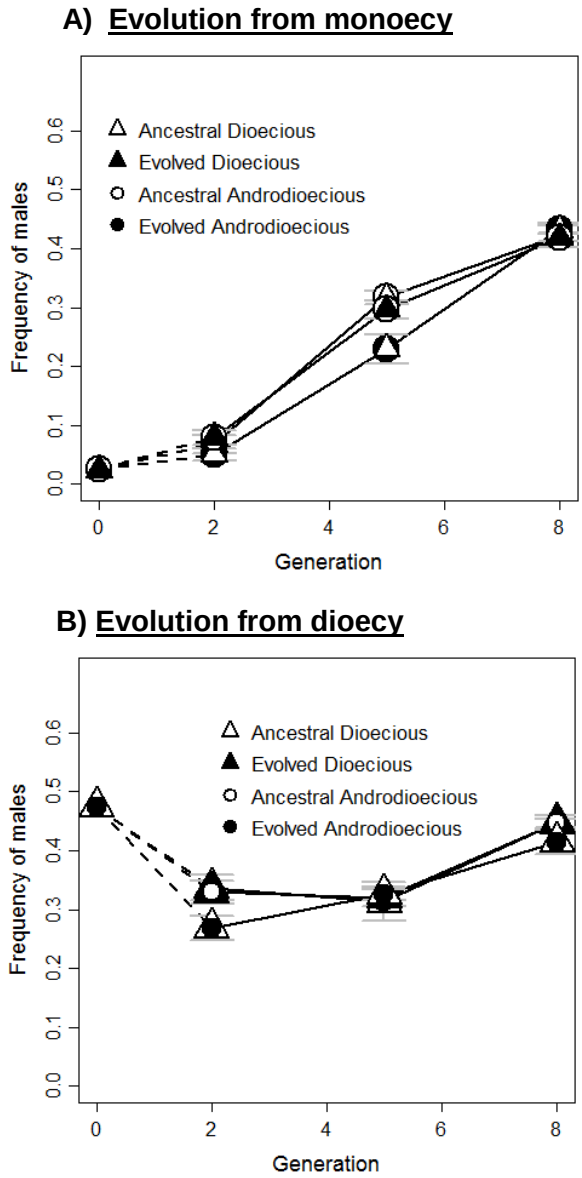

Figure 4.2: **Evolution of male frequencies under two evolutionary scenarios**. Ancestral and evolved experimental populations with standing genetic variation were used to test **A)** the possibility of spread of an allele responsible for self-sterility (thus making cross-fertilization the only mode of reproduction possible) in a population composed of self-fertile hermaphrodites or **B)** the possibility of spread of an allele conferring females the ability for self-fertilize in a population composed of males and females. Large symbols represent the mating system and state (ancestral or evolved) of the population at the highest frequency at onset of the experiment; smaller (inset) symbols represent the mating system and state of the least represented ("emerging") population at the onset of the experiment. The values are presented as the mean frequencies of males in replicate experimental populations. Error bars (grey) represent standard error of the mean. See Supplementary Table S4.2 for statistical analysis.

The increase of male fractions was expected for conditions under which the obligate outcrossing allele would spread. The maintenance of initially appreciable frequencies of males (47.5%) with increased frequency of the selfing allele is, however, more difficult to explain. Interestingly, the frequencies of males under both evolutionary scenarios converged to similar values (40%). In fact, these frequencies correspond to the frequencies of males measured in fully outcrossing populations during adaptation (see Chapter 2). This is not an artifact of this experiment in particular, since a previous experiment similar to the one presented here but with different initial frequencies of the *fog*-2 (*q71*) and *fog*-2(*wt*) alleles held similar results (Figure 4.3). In this first experiment we were not able to follow the frequencies of the alleles at the *fog*-2 locus, but frequencies of males were monitored for 10 generations, starting at frequencies of 2.5% (corresponding to a 5% initial frequency of the *fog*-2 (*q71*) allele) or 5% (corresponding to a 10% initial frequency of the *fog*-2 (*q71*) allele). By generation 8, the frequencies of males in all competitions had increased to values similar to 40% (Figure 4.3).

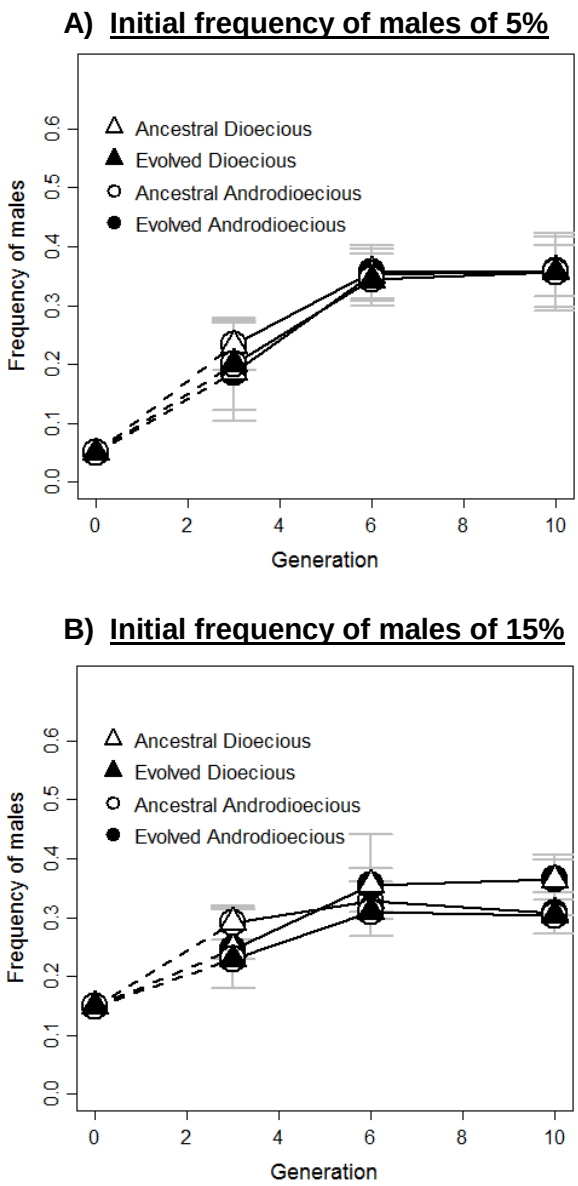

Figure 4.3: **Evolution of male frequencies in populations with different initial frequencies of alleles at a sex-determining locus**. Ancestral and evolved experimental Dioecious and Androdioecious populations with standing genetic variation were used to investigate the evolution of rates of outcrossing. Male frequencies were used as a proxy. Initial populations composed of **A)** 10% individuals or **B)** 30% individuals from Dioecious populations (homozygote for the fog-2(*q71*) allele) and hermaphrodites from Androdioecious populations were allowed to evolve under standard conditions of experimental evolution. Large circles represent the state (ancestral or evolved) of the Androdioecious population; smaller (inset) symbols represent the state of the Dioecious ("invading") population. The values are presented as the mean frequencies of males in replicate experimental populations. Error bars (grey) represent standard error of the mean.

4.5 Discussion

Several attempts have been made to test the predictions of theoretical models for the evolution of (mixed) mating systems. In plants, for example, empirical tests have been carried out mostly in the form of comparisons of genetic profiles (such as rates of nucleotide substitutions) of selfing and closely related outcrossing species to infer rates of fixation of deleterious and adaptive alleles (Wright *et a*l 2008). This is because one of the main consequences of selfing is the significant increase in homozygosity in loci across the whole genome, which means that each individual will have fewer allele types in its genome. Therefore, meiotic recombination will be less effective and levels of linkage disequilibrium (statistical associations of allele frequencies at different loci) increase. In addition to this, hitchhiking of positively selected mutations and background selection against deleterious mutations can further reduce the amount of genetic variation in selfing populations. Altogether, these effects cause strong reductions of effective population sizes and decrease the efficiency of natural selection, facilitating the accumulation of deleterious mutations, a phenomenon which may contribute to increase the probability of extinction of populations (Lynch *et al* 1995). On the other hand, the extent of linkage disequilibrium is also determined by the effective population size ($4N_e r(1-s)$, Nordborg 2000). Therefore, sufficiently large population sizes may allow recombination to compensate for the high rates of selfing. Finally, a higher self-fertilization rate can allow a more efficient purging of deleterious recessive alleles (Crnockrak and Barrett 2002), including selfish genetic elements – those which enhance their own transmission despite null or negative fitness consequences, such as transposable elements or mutations causing cytoplasmic male sterility. Selfing can also enhance the fixation of beneficial alleles in populations if they are mostly recessive (Charlesworth 1992). Analyzing the genome of closely related species with different degrees of self- and cross-fertilization can help to determine the relevance of these processes (Wright *et al* 2008).

In animals, mixed mating systems are rare. In species where hermaphrodites have been found, the extent of outcrossing or even the mating system proper have not yet been determined in many cases (but see Jarne and Auld 2006). There are, however, two well documented cases: one in branchiopod crustaceans (namely the clam shrimp *Eulimnadia texana*) and another one in Rhabditid nematodes. Both exhibit androdioecious mating systems, composed of selfing hermaphrodites and males. However, they differ in the genetics of sex determination and also in what is considered the ancestral state when looking at a broader phylogenetic context. Evidence points to an androdioecious ancestral of the *Eulimnadia* genus (Weeks *et al* 2006) and to preservation of this mating system throughout many speciation events, which suggests stability of androdioecy in this group of animals. *C. elegans*, on the other hand, seems to result from a recent ancestral dioecious state (Kiontke et *al* 2004, Haag and Doty 2005, Nayak *et al* 2005, Loewe and Cutter 2008). In fact, for some time the existence of males in populations of these organisms was thought to be remnant of the dioecious state, until several lines of evidence suggested they are selectively maintained (see Anderson *et al* 2010 for a review on the subject). With respect to *C. elegans*, the paradox of persistence of males in a predominantly selfing species has been dealt with by looking for evidences of inbreeding depression, without much success (Dolgin *et al* 2007). The maintenance of males at higher frequencies under increased rates of mutation and/or artificial generation of trioecious systems has been reported in previous studies (Cutter 2005, Manoel *et al* 2007) but these apparently beneficial effects of higher outcrossing have proved insufficient to prevent the spread of hermaphrodites in populations composed also of males and females (Cutter 2005). These studies were performed in highly inbred strains of *C. elegans*. Recent reports of evolution experiments with populations segregating genetic variation suggest that the segregation of genetic variation is a common denominator for the maintenance of intermediate rates of outcrossing (Morran *et al* 2009b, Anderson *et al* 2010). In these studies, however both genetic and ecological models can explain the

results, but the exact mechanisms underlying the preservation of males and outcrossing under those circumstances remain obscure. In any case, only the study of Cutter (Cutter 2005) involved a trioecious state, which almost certainly occurred in the transition from dioecy.

Here we tested the possibility of spread of an allele conferring the ability to self in a fully outcrossing (dioecious) population and the invasion dynamics of an allele conferring obligate cross-fertilization in a population composed of selfing hermaphrodites. This was achieved by co-culturing individuals of populations which had previously adapted to a novel, laboratory environment for 100 generations. These populations bore high levels of genetic variation and were evolved under androdioecy or dioecy. Dioecious populations were generated by introgressing a mutant allele at a sex-determining locus (*fog*-2(*q71*)). We previously demonstrated that adaptation occurred in our experimental populations under both mating systems and also that it was facilitated by cross-fertilization, with androdioecious populations maintaining intermediate levels of outcrossing throughout experimental evolution (see chapter 2). Given the geometric advantage of self-fertilization over outcrossing (and the negligible levels of inbreeding depression in ancestral androdioecious populations from where hermaphrodites were sampled, see chapter 2), we anticipated the spread of the allele conferring the ability to self-fertilize in all competitions except perhaps when delivered to evolved Dioecious populations. Under these conditions it would be possible for the genotypes of these individuals to exhibit large enough fitnesses that would counterbalance the inherent advantage of self fertilization. Instead, our results showed that the fate of the “selfing allele” was contingent on the genetic background in which it was found at the beginning of the competitions: it was able to “resist invasion” by an allele favoring outcrossing, while more efficiently increasing in frequency when delivered to a dioecious population. They further showed that under conditions where linkage disequilibrium is reduced, as is the case of Dioecious and Androdioecious ancestral populations (Chelo, pers. comm.), the evolutionary fate of a selfing allele is decoupled from rates of cross-

fertilization with males, since these are systematically maintained at high levels regardless of the frequencies of the outcrossing allele *fog*-2(*wt*). In other words, evolved hermaphrodites resisted the invasion of an obligate outcrossing allele and were better at spreading in a dioecious population. However, they did so by cross-fertilizing with the males available.

The argument for the transmission advantage of selfing cannot therefore explain our results. In the context of our experimental system, outcrossing correlates with increased effective recombination, for which our observations may be understood in the light of models of recombination modifier loci. Directional selection on multiple loci and tight linkage are two predicted requirements for substantial selection for recombination in meiotic recombination modifier theoretical models (Otto and Barton 1997). As in most selfing populations, linkage disequilibrium in natural isolates of *C. elegans* is high. Although we know that the novel environment imposed by experimental evolution translated into directional selection for multiple life-history components involving several genetic loci (see Chapter 3), the requirement for high linkage is not met in the ancestral populations, since the crossing scheme employed in their derivation lead to reduced levels of linkage disequilibrium in these populations (Chelo, pers. comm.). This draws attention to the fact that in our experiment the genetic backgrounds of individuals from ancestral and evolved populations should have been mixed after a couple of generations. Explanations for the observed results should therefore focus on the dynamics of the alleles at the sex-determining locus. This locus does not affect the biology of males, which explains the convergence of their frequencies in all the experiments carried out: they fertilize equally females and hermaphrodites, with a 50-50 sex ratio being generated in the progeny; they were thus able to increase (or maintain) their frequencies. As for the *fog*-2 alleles proper, if they were selectively neutral their frequencies would have been expected to evolve accordingly and be maintained at frequencies similar to initial ones. However, we have seen that this is not the case and that the fate of the selfing allele is determined by the genetic background in which is delivered to the

populations (that is, if it is carried by individuals from ancestral or evolved populations). A possible explanation is therefore the building up of linkage disequilibrium of this locus with one or multiple loci affecting fitness, perhaps via epistatic interactions. Such an example has been described for the clam shrimp *Eulimnadia texana* (Weeks *et al* 1999, Weeks *et al* 2000, Weeks *et al* 2006, Weeks *et al* 2009, Weeks *et al* 2010), another animal androdioecious species. In their most recent study, the authors investigated the genetic basis of inbreeding depression found in this organism. They did not find evidence of purging of deleterious alleles over several generations of inbreeding for several natural isolates. This result was then interpreted as inbreeding depression in this species resulting from the segregation of deleterious alleles in linkage with a large group of loci containing both the sex determining loci and also loci contributing largely to fitness (Weeks 2004). Our androdioecious experimental populations (and hence hermaphrodites) do not show signs of strong inbreeding depression, but nevertheless epistatic selection involving specifically the *fog*-2 locus and other loci contributing significantly to fitness may have occurred during adaptation to the novel environment by epistatic selection. Alternative, ecological models, such as those relying on reproductive assurance for species with high rates of colonization and extinction, cannot be ruled out to explain the existence of mixed-mating systems in animal species in nature; however, such arguments hardly explain our results under the population dynamics employed during experimental evolution. With respect to the specific conditions for the emergence of androdioecy from monoecy and from dioecy, our results show that both are possible, although the specific mechanisms underlying it were not clear. Perhaps performing a similar experiment to the one described here using genetically uniform Androdioecious and Dioecious populations subjected to experimental evolution, for which expectations from genetic models are more straightforward, would help elucidate the mechanisms behind the evolution of mixed mating systems.

The experiments reported here argue for a crucial role of allelic interactions within and between loci in determining the fate of mating system modifier alleles.

These interactions are likely to be important because we cannot explain our results with standard models of recombination modifier loci. Although the role of dominance and epistasis has already been acknowledged with respect to the adaptive consequences of increased effective recombination (Agrawal 2006, Neiman and Linksvayer 2006) and to the genetic basis of inbreeding depression (Carr and Dudash 2003), perhaps they should be incorporated more fully in future models for the evolution of outcrossing and mating systems.

4.6 Acknowledgements

Sara Carvalho and Henrique Teotónio performed the experiment. S. Carvalho carried out the genotyping and measurement of frequencies of males, as well as the statistical analysis presented here. This work was funded by FCT (SFRH/BD/36726/2007).

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4.8 Supplementary information

A.

Model (spread of the fog-2(q71) allele):
Allele frequency = Type + Generation + (Generation|competition)

Fixed effects	Estimate	Std. Error	t-value
Intercept	0.0716	0.0186	**3.855**
Evolved donor	-0.0031	0.0192	-0.162
Evolved recipient	-0.0422	0.0192	**-2.197**
Generation 5	0.0553	0.0175	**3.158**
Generation 8	0.0449	0.0149	**3.015**
		AIC=-260.4; logLikelihood=142.2	

B.

Model (spread of the fog-2(wt) allele):
Allele frequency = Type + Generation + (Generation|competition)

Fixed effects	Estimate	Std. Error	t-value
Intercept	0.0217	0.0198	1.099
Evolved donor	0.0874	0.0203	**4.303**
Evolved recipient	0.0300	0.0203	1.471
Generation 5	0.0414	0.0159	**2.604**
Generation 8	0.0493	0.01701	**2.897**
		AIC=-246.4; logLikelihood=135.2	

Supplementary Table S4.1: Statistical model of the evolution of allele frequencies at the *fog*-2 locus under two evolutionary scenarios. A) evolution from monoecy (initial high frequency of the *fog*-2(*wt*) allele) **B)** evolution from dioecy (initial high frequency of the *fog*-2(*q71t*) allele)**.** A linear mixed-effect model was employed, with the terms representing fixed factors; to account for non-independence of observations in time a repeated measures approach was taken, with each competition taking into account the correlation structure between observations (last term of the models).

A.

Model (spread of the fog-2(q71) allele):
Male frequency = Type + Generation + (Generation|competition)

Fixed effects	Estimate	Std. Error	t-value
Intercept	0.0798	0.0150	**5.331**
Evolved donor	-0.0043	0.0153	-0.284
Evolved recipient	-0.0304	0.0153	-1.995
Generation 5	0.2096	0.0170	**12.347**
Generation 8	0.3567	0.0160	**22.263**
		AIC=-298.4; logLikelihood=161.2	

B.

Model (spread of the fog-2(wt) allele):
Male frequency = Type + Generation + (Generation|competition)

Fixed effects	Estimate	Std. Error	t-value
Intercept	0.3172	0.0178	17.802
Evolved donor	-0.0296	0.0181	-1.629
Evolved recipient	-0.00067	0.0181	-0.037
Generation 5	0.01792	0.0145	1.232
Generation 8	0.12867	0.0145	**8.844**
		AIC=-265.8; logLikelihood=144.9	

Supplementary Table S4.2: Statistical model of the evolution of male frequencies at the *fog*-2 locus under two evolutionary scenarios. A) evolution from monoecy (initial high frequency of the *fog*-2(*wt*) allele) **B)** evolution from dioecy (initial high frequency of the *fog*-2(*q71t*) allele)**.** A linear mixed-effect model was employed, with the terms representing fixed factors; to account for non-independence of observations in time a repeated measures approach was taken, with each competition taking into account the correlation structure between observations (last term of the models).

Chapter V

Adaptation from mutation

5.1 Summary

In genetically homogeneous populations of organisms – such as those with high levels of inbreeding or recently subjected to recent episodes of strong selection - the ability to adapt upon environmental changes rests mostly on the emergence of novel, beneficial alleles by mutation. The genetic theory of adaptation is built mainly from this perspective, yet for a long time the vast majority of experiments to determine rates and effects of mutations focused on the emergence of deleterious alleles. Only recently have the dynamics of adaptive mutations been started to be addressed empirically. Not surprisingly, virtually all studies have involved microorganisms and therefore the extent to which mutation may contribute to adaptation in sexual, multicellular organisms is poorly understood.

We experimentally evolved nearly isogenic populations of the nematode *C. elegans* to a novel environment for 100 generations at large population sizes (N=10.000). The effects of *de novo* mutations ton adaptation were investigated by quantifying the extent of phenotypic response of components of fitness in experimental populations. The level of increase of mean population fitness and the degree of phenotypic differentiation after 100 generations of evolution under a novel environment were surprisingly high.

5.2 Introduction

The ability of populations to cope with changes in their environments depends on the amount of additive genetic variation for traits contributing to the fitness of organisms. Although populations can respond initially from segregating variation, long term rates of adaptation may be determined by the creation of novel alleles at single loci by mutation. This is perhaps one of the reasons why theoretical models for the genetics of adaptation have dealt mostly with this source of variation as fueling the adaptive process. Most models stem from R. A. Fisher's geometrical model of adaptation (Fisher 1930). This model acknowledged the fact that mutations are random and, as such, can have different effects on the fitness

of organisms. The probability that a new mutation is beneficial or deleterious is dependent on the degree of conformity of organisms with their selective environment. In other words, if the average fitness of the population is high, a mutation is more likely to disrupt any biological process than to improve it; in a situation where existing genotypes prove themselves unfit, an allele created by mutation may be more likely to improve some feature of the organism than to make things worse. With respect to the magnitude of their effects, deleterious mutations with large phenotypic (and fitness) consequences should be rapidly removed by natural selection, while large effects beneficial mutations should promptly sweep through the population. In the context of adaptation, Fisher's model (and further extensions of it) thus predicts that initial evolutionary steps should rely on the fixation of adaptive mutations of large effects. It further predicts that progressively smaller adaptive mutations will contribute to the adaptive process as population fitness increases. In fact, if the rate of mutation is not high enough to produce double mutants and if the fitness of the ancestral genotype is not too low (meaning that there are relatively few beneficial sequences that can be generated), the distribution of effects of beneficial mutations should follow an exponential function. This distribution should be invariant, that is, independent of the specific fitness value of the ancestral phenotype (Orr 2002, Orr 2003). It is of note that these expectations were derived for a relatively *local* landscape (Orr 2002), which is another way of stating the second assumption that the fitness of the ancestral genotype should not be extremely low. Therefore, existing theory does not necessarily apply to the distribution of effects of adaptive mutations arising in chemically mutagenized populations or in populations in which deleterious mutations were previously allowed to accumulate freely (mutation-accumulation experiments). Most empirical studies attempting to characterize adaptive mutations have employed these methodologies (Elena *et al* 1998, Sanjuan *et al* 2004, Silander *et al* 2007). However, a few studies report the fitness effects of spontaneously produced mutations under conditions more compatible with the assumptions of the models (Rokyta et al 2006, Kassen and Battaillon

2006). In these studies, a general support for Fisher and Orr's theoretical model of adaptation is found (although data are also compatible with alternative distributions of effects of spontaneous mutations). This model further predicts that mutations of a certain magnitude of phenotypic effects are less likely to be beneficial in more complex organisms relative to simpler ones (Orr 2000). This prediction stems from the inverse relationship between the probability of a mutation being beneficial and the number of components interacting at the genotypic and phenotypic levels of an organism. Complexity is mathematically defined as the "number of independent characters or dimensions comprising an organism" (Orr 2000) and can thus have a direct biological interpretation: more characters are likely to involve, for example, more (interacting) units, for example genes. The implications of this may extend even further in that the additive effects of adaptive mutations may become more difficult to measure as organisms become more complex and more epistatic interactions among loci can emerge.

A second body of theory was built to deal with violations of the other assumption in the Fisher-Orr model: that of multiple beneficial alleles being generated in the same population. The segregation of beneficial alleles in different genotypes of asexual organisms may lead to competition between them. The effect of this can be the fixation of only the allele with the strongest effects, thus reducing the number of potential adaptive fixations that could occur in the population (Hill and Robertson 1966). This may be true for populations of asexual organisms (Miralles et al 1999, Rozen *et al* 2002) but also for populations of organisms that reproduce sexually but have high levels of inbreeding (such as predominantly selfing species). In populations of such organisms effective recombination is likely to be reduced (Colegrave 2002). Genetic recombination is therefore thought to have an important role regarding adaptation from *de novo* mutations. In fact, adaptive mutations are at the basis of some of the arguments to explain the ubiquity of sexual reproduction in nature (Muller 1932, 1964). Sexual reproduction requires two individuals to produce progeny while asexuality proceeds from one single individual; therefore, the *per capita* growth rate of

asexual organisms can be twice that of sexuals. However, reshuffling genomes through sex and recombination may allow beneficial mutations that arise in different individuals to be combined in a single genotype. Several studies have addressed empirically the effects of sex (and hence recombination and segregation) to adaptation. Similarly to the studies investigating the distributions of effects of beneficial mutations, most of them have been carried out in microbes (Bolback and Huelsenbeck 2007, Cooper 2007).

The vast majority of studies involving multicellular organisms have relied on mutation-accumulation (MA) experiments in a few (laboratory) model organisms such as *Drosophila melanogaster* (Mukai and Yamazaki 1968, Fry 2001), *Caenorhabditis elegans* (Keightley and Caballero 1997, Vassilieva and Lynch 1999) *Daphnia pulex* (Lynch *et al* 1999), and *Arabidopsis thaliana* (Schultz *et al* 1999, Shaw *et al* 2000). In these experiments, populations are released from the action of natural selection by being maintained at the smaller sizes possible - single individuals (in species where asexual reproduction or self fertilization are possible), or in pairs, in the case of gonochoristic (separate sexes) sexual species. Because the fate of mutations with effects $< 1/2N_e$ is largely governed by genetic drift, they can accumulate neutrally under these experimental settings (except lethal mutations). This methodology should thus be applicable to a wide variety of organisms (from viruses to insects), further allowing comparisons between them. However, due to the replication requirements of such experiments, they are typically performed in only one genotype. This is problematic because even if rates of mutation are accurately measured it is uncertain whether they are representative of the species, considering that mutation rates may vary between species, populations and/or individuals (Drake et al 1998, Baer et al 2007, Lynch 2010). For example, one MA experiment performed in natural isolates of *C. elegans* and closely related species found evidence for varying rates of mutation at both the strain and species levels (Baer *et al* 2005). Perhaps a more serious concern is that if experimental populations consist mostly of laboratory-adapted genotypes, mutations allowed to accumulate freely are likely to be strongly biased

towards deleterious effects. For example, the estimated diploid genomic mutation rates (U_t) obtained from the study of Baer *et al* are quite similar to estimates of the rate of genomic deleterious mutation (U_d) obtained from analysis of synonymous nucleotide substitutions between *C. elegans* and *C. briggsae* (Cutter and Payseur 2003). This pattern would be predicted if most mutations arised in genotypes with high fitness under laboratory conditions, a situation in which most (or all) mutations would be expected to have deleterious effects. In another experiment, populations of the canonical strain of *C. elegans* (N2) maintained at large populations sizes for over 80 generations under stable laboratory conditions failed to show significant phenotypic responses (Estes and Lynch 2003). More recently, genetic studies have supported the hypothesis that this strain has experienced laboratory domestication (Weber *et al* 2010, McGrath *et al* 2011). If, on one hand, laboratory domestication of *C. elegans* may render total mutation rate and deleterious mutation rate indistinguishable, on the other hand it can make us more confident in its estimates, with control populations indeed providing good standards.

More recently, experimental evolution in *C. elegans* has moved beyond estimates of total and deleterious genomic mutation rates and questions regarding adaptive evolution have started being addressed. Conversely to the rationale behind neutral accumulation of mutations in MA experiments, expanding populations with previously depressed fitness (such as MA populations) at large census sizes creates more opportunities for mutations to appear; according to Fisher's model, a large fraction of them should be beneficial. Estes *et al* (2003) found that MA populations maintained at population sizes conducive to efficient selection allowed fitness recovery about 3 times the rate of mutational degradation. They further demonstrated that fitness recovery was not due to back mutation of alleles previously mutated to deleterious forms, but caused mostly by emergence of compensatory epistatic mutations. This and subsequent studies (Denver *et al* 2010, Estes *et al* 2011) have started unraveling some of the dynamics of the adaptive process in a multicellular organism. However, many questions remain unanswered. For example, if levels of fitness depression as

those found among MA populations are seldomly found in nature, then it becomes relevant to know whether similar dynamics of adaptive evolution will be found under less severe scenarios of finess depression. What will be then the fraction of novel mutations with beneficial effects? Will adaptive alleles under these conditions be unconditionally advantageous? Or will their effects be mostly epistatic as MA-recovery experiments suggest (Estes *et al* 2003)? Another question that has yet not been addressed on more complex organisms is whether rates of effective recombination (and segregation) play any role in the adaptive process. In a predominantly selfing species such as *C. elegans*, can competition between adaptive alleles in different lineages play any role? Or are rates of mutation (and population sizes) small enough for recombination to be irrelevant to the adaptive process?

Instead of attempting the daunting task of characterizing the distribution of effects of adaptive mutations during an adaptive process of a non-microbe organism, we focused on assessing their overall contribution instead. In particular, we asked to what extent mutation could lead to adaptation to a novel environment within a relatively short period of time (100 generations) in a multicellular, sexual organism. With expectations that the answer would be “yes”, we simultaneously investigated whether the advantageous effects of recombination could be detected within such a timeframe. To achieve these purposes, we generated highly inbred populations of the nematode *C. elegans* from a hybrid population which resulted from crosses among 16 natural isolates. Our approach differs from previous ones in that starting populations do not necessarily start from very low fitness (since deleterious mutations were not deliberately allowed to accrue); they simply consist of combinations of alleles (genotypes) which are not expected to have ever existed before and have therefore never been exposed to the standard laboratory conditions. The role of recombination in decreasing the potential for interference among adaptive mutations was assessed by imposing different levels of outcrossing. This was achieved by genetic manipulation of the mating system. Experimental evolution thus consisted of exposing Androdioecious (male-

hermaphrodite) and Dioecious (male-female) genetically homogeneous populations of *C. elegans* to a novel environment at large population sizes.

5.3 Materials and Methods

Experimental populations and evolution

Six androdioecious and six dioecious populations with low levels of standing genetic variation ($isoA_{1-6}$, $isoD_{1-6}$ - Figure 1.6 and Chapter 1 for how the different populations were constructed) were initiated by sampling large numbers of individuals (>10^5) from frozen stocks. These populations were allowed to evolve under spatially and temporally stable conditions at large census sizes (N=10.000). A detailed description of the conditions of experimental evolution is given in Chapter 2 (see Methods). Briefly, populations were cultured with abundance of food and controlled densities. At reproductive maturity (day 4 of the life cycle – see Figure 2.1), adults were sacrificed and eggs were allowed to hatch overnight in minimum medium to achieve synchronization among experimental populations. The following day, a new cohort was initiated by random sampling of 10.000 larvae per experimental population. This methodology imposed a demography characterized by non-overlapping and discrete generations. Samples of all populations were cryopreserved during experimental evolution (at several timepoints) to allow them to be assayed concurrently after thawing and two generations of population expansion. A *tester* population was also generated and employed in most of the assays described here. This population should exhibid a genetic background similar to that of ancestral hybrid Androdioecious populations with the exception that it carries a dominant transgenic allele in chromosome I driving the expression of GFP (Green Fluorescent Protein) which allows phenotypic discrimination between experimental and *tester* individuals. Experimental population $isoD_2$ revealed segregation of hermaphrodites while experimental evolution ensued and was therefore discarded from all analysis.

Characterization of genetic diversity of ancestral and evolved populations

Individual worms from all ancestral and evolved populations were genotyped at nine microsatellite locus. A detailed description of the methodologies involved in DNA extraction, genotyping and analysis can be found in Chapter 2 (Methods). The results of ancestral populations have also been previously reported in Chapter 2 (see Results) but are presented again here to allow comparison with those of evolved populations. Briefly, sixteen single hermaphrodites from all twelve ancestral populations ($isoA_{1-6}$, $isoD_{1,3-6}$) were randomly picked from culture plates as young adults (day 3 of the life cycle); hermaphrodites from the twelve evolved populations were similarly picked but at larger sample sizes (n=48). Genomic DNA was extracted from each individual and separately amplified by PCR reaction for each microsatellite loci (see Supplementary Table S2.1 for information about the genotyped loci) using fluorescently labeled primers. The amplification products of each sample were mixed according to fluorescent labeling and fragment sizes (in groups of three) and run by capillary electrophoresis (MegaBACE™1000 Genotyping System) to obtain amplicon sizes. Alleles were then binned manually according to their sizes.

Statistical analysis: estimation of parameters was obtaining by running custom made scripts run in the R software (R Development Core Team 2010). Two loci could not be unambiguously scored for which results are presented for 7 loci only.

Body size measurements

Culture plates were photographed to obtain measurements of the width and length of individuals from ancestral Androdioecious ($isoA_{4-6}$) and Dioecious ($D_{1,2-6}$) populations; this procedure was repeated again at generation 50 of experimental evolution. Two additional culture plates for each replicate population were therefore prepared at generation 0 and generation 50 to be used exclusively for measurement purposes. At day 4 of the life cycle (when the “bleach” maintenance protocol was performed), these additional culture plates were stored at 4ºC for two to seven days. Images were taken randomly across culture plates under

standardized conditions and isolated individuals were measured using the ImageJ software (*rsbweb.nih.gov/ij/*). A more detailed description of this assay can be found in Chapter 3 – Methods. Body size was calculated as V = (body width/2)^2 x pi x body length, therefore assuming a cylindrical shape of the worms

Statistical analysis: the final dataset consisted of 33±8 individuals measured for body width, length and volume per (ancestral and evolved) replicate population. For each phenotype (body width, body length and body size), normality of data was assessed for each mating system and generation by Kolmogorov-Smirnov and Shapiro-Wilk statistical tests after removal of outliers defined as lying outside the interval [Lower quantile - 1.5 Interquantile range, Upper quantile + 1.5 Interquantile range]. Bartlett tests were also performed regardless of replicate to assess homocedasticity of groups. ANOVA was performed separately for each phenotype using the means of replicate populations; the model took mating system (2 levels), generation of experimental evolution (2 levels) and replicate (6 levels) as fixed factors.

Male competitive performance

The evolution of male fitness components was investigated by measuring the competitive performance of ancestral and evolved experimental males against males from the *tester* population expressing GFP. Males from all 12 experimental populations ($isoA_{1\text{-}6}$, $isoD_{1,2\text{-}6}$) were measured at generation 0, 30, 50 and 100 for their competitive performance. The assay consisted of transferring 9 young adult experimental males (day 3 of the life cycle) and 9 similarly aged *tester* males to 6-cm Petri dishes with a spot of food. These plates contained 20 *fog*-2(*q71*) females from strain JK574; both types of males were then allowed to compete for fertilization of females for 24h. The fraction of GFP-negative progeny produced by females was taken as the measure of male competitive performance (see Chapter 3 – Methods, for details). The assay was carried out in 3 blocks with all experimental populations (generation 0, 30, 60 and 100) from two Dioecious and two Androdioecious replicate populations being assayed in each block. Four plates

were set-up per experimental population in blocks 1 and 2, while in block 3 only three plates were set-up. An average of 20±2 plates were measured per mating system and generation.

Statistical analysis: Shapiro-Wilk tests of normality of data and Bartlett tests of homocedasticity were performed for experimental populations regardless of replicate. Also, the response variable in the ANOVA was the average value across plates for each replicate population; the model took generation (4 levels), mating system (2 levels) and replicate (6 levels) as fixed factors. Because two replicates were measured in each block, these are partially collinear variables and should not be employed simultaneously in the model. Separate analysis including one variable or the other revealed none of them to be significant at alpha = 0.5, for which the variable which generated the best model was kept. The interaction between generation and mating system was also incorporated in the model. Normality of model residuals was investigated with Shapiro-Wilk test of normality; the hypothesis of normality of residuals could not be rejected.

Reproductive schedule

Ancestral and evolved hermaphrodites ($isoA_{4-6}$) and females ($isoD_{4-6}$) were assayed for phenotypes related to reproduction by performing the same assay as previously reported for population with standing genetic variation (Chapter 3 – Methods). Young individuals (day 3 of the life cycle) were randomly picked from previously thawed and expanded populations and transferred to 6cm Petri dishes with food; two males from the *tester* population were transferred to each assay plate as well. Experimental and *tester* males were removed after 48h. This constituted the “outcrossing” treatment and its purpose was to provide a characterization of the female component of reproduction of hermaphrodites and of females as well as to allow comparison between them. A second treatment, only applied to hermaphrodites, consisted of keeping individuals in isolation and therefore allowing them to simultaneously express both male and female reproductive components by self-fertilization. Individuals from all treatments were

transferred daily to fresh plates until reproductive cessation. Each day, the number of eggs laid on the plate was counted and, three days later, all adult progeny was scored for GFP expression and for production of males. Several phenotypes were calculated from these data: daily production of eggs, daily production of progeny and viability of progeny. Quality control of the data proceeded in the same way as in the analysis of data from genetically variable populations (Chapter 2).

Statistical analysis: Thirty individuals were measured per mating system (Androdioecious or Dioecious), generation (ancestral or generation 100 of experimental evolution), replicate (4-6) and treatment (cross-fertilized in the case of females and cross- or self-fertilized in the case of hermaphrodites). In total, and after quality control, the reproductive schedule of 515 individuals was analyzed. The assay was performed in 3 blocks, with one replicate population (from the different mating systems and generations) being measured in each block. In this dataset there is total collinearity between replicate number and assay block and the effects of both cannot be decoupled. However, the experimental populations whose analysis we report here were assayed simultaneously with the experimental populations which started evolution with high levels of genetic variation. Ancestral hybrid populations were pseudoreplicated in each assay block, for which the effects of this variable could be tested for significance. They were only found significant at the level of alpha =0.5 for the number of eggs laid on day 4 of the life cycle and their viability. Therefore, block was included in the statistical analysis of these phenotypes; in the remaining phenotypes presented here, replicate population was used instead. Although computationally they are exactly the same, this provides more accurate information to the reader about the source of variation.

Statistical analysis of each phenotype involved two separate models: one including mated hermaphrodites and females and one including data from hermaphrodites only (in an attempt to overcome the unbalanced design caused by the existence of twice the number of observations in the cross-fertilization treatment relative to the self-fertilization treatment, which only occurred in

hermaphrodites). Within each model, the response was modeled by taking group (2 levels), generation (2 levels), their interaction and replicate or block (3 levels; the use of one or the other is explained above). Because we were not interested in the variation among individuals but among experimental populations, the values used in the models were the means of the observations per replicate population and hence statistical analysis was performed with 12 datapoints in each model; because of this, the power to detect interactions among factors was not very high. Whenever a factor (for example, generation of experimental evolution) was significant in one of the models but not in the other (mated hermaphrodites and females versus mated and unmated hermaphrodites), the significance of this factor was additionally investigated by performing two-samples Student's t tests for each group defined by mating system and treatment using all (raw) observations.

Statistical analysis

All statistical analysis was carried out in the R software using custom scripts (R Development Core Team 2010), unless otherwise stated. Whenever Analysis of Variance (ANOVA) was involved, the distribution of residuals was tested for normality by Shapiro-Wilk and Kolmogorov-Smirnov tests; only non-normality is reported. ANOVA tables are provided as Supplementary Tables. Values plotted in graphical displays refer to raw data, not parameters estimated from the statistical models.

5.4 Results

Assessment of levels of genetic diversity in ancestral and in evolved populations

Several individuals from ancestral and evolved Dioecious ($isoD_{1\text{-}6}$) and Androdioecious ($isoA_{1,2\text{-}6}$) populations were genotyped at 7 microsatellite loci. The results are presented in Table 5.1.

Outcrossing	Population	State	Statistic	Locus

				4001	IIR	3003	VL	XR	1003	4004
low	**isoA$_1$**	ancestral	**N**	16	15	16	16	13	9	14
			A	2	1	1	1	3	2	1
			Hobs	0.07	0.00	0.00	0.00	0.00	0.00	0.00
		evolved	**N**	48	48	42	42	44	48	40
			A	2	2	3	2	1	1	2
			Hobs	0.11	0.02	0.32	0.27	0.00	0.00	0.21
low	**isoA$_2$**	ancestral	**N**	16	13	15	15	8	8	13
			A	1	1	1	1	2	2	2
			Hobs	0.00	0.00	0.00	0.00	0.00	0.00	0.08
		evolved	**N**	44	46	47	48	47	47	41
			A	3	1	3	3	1	4	3
			Hobs	0.67	0.00	0.17	0.00	0.00	0.02	0.33
low	**isoA$_3$**	ancestral	**N**	16	16	16	15	4	7	14
			A	1	2	1	1	1	2	2
			Hobs	0.00	0.07	0.00	0.00	0.00	0.00	0.08
		evolved	**N**	47	48	41	45	47	40	37
			A	3	3	3	4	3	1	2
			Hobs	0.33	0.17	0.25	0.16	0.02	0.00	0.06
low	**isoA$_4$**	ancestral	**N**	14	14	15	15	16	14	13
			A	3	1	4	2	2	3	3
			Hobs	0.31	0.00	0.36	0.14	0.00	0.00	0.08
		evolved	**N**	48	48	47	47	47	47	34
			A	1	1	2	2	1	1	2
			Hobs	0.00	0.00	0.02	0.00	0.00	0.00	0.00
low	**isoA$_5$**	ancestral	**N**	16	14	16	16	16	14	14
			A	3	3	4	2	2	1	3
			Hobs	0.27	0.08	0.13	0.00	0.00	0.00	0.15
		evolved	**N**	31	30	43	45	44	42	40
			A	3	1	2	2	5	4	2
			Hobs	0.13	0.00	0.12	0.05	0.02	0.05	0.03
low	**isoA$_6$**	ancestral	**N**	16	12	15	16	15	15	11
			A	3	4	5	3	2	3	2
			Hobs	0.40	0.27	0.21	0.13	0.00	0.00	0.00
		evolved	**N**	48	47	47	45	47	46	32
			A	3	3	4	3	1	4	2
			Hobs	0.32	0.17	0.22	0.36	0.00	0.02	0.06
high	**isoD$_1$**	ancestral	**N**	14	13	14	14	14	14	15
			A	3	3	5	4	5	5	4
			Hobs	0.00	0.00	0.08	0.00	0.15	0.31	0.00

		evolved	**N**	4	48	48	48	47	48	45
			A	2	2	3	1	2	2	2
			Hobs	0.00	0.02	0.04	0.00	0.00	0.02	0.25
high	**isoD$_3$**	ancestral	**N**	12	12	13	13	13	13	14
			A	3	3	5	3	2	2	3
			Hobs	0.00	0.00	0.00	0.00	0.00	0.17	0.08
		evolved	**N**	48	47	46	48	47	47	46
			A	2	4	3	2	4	3	3
			Hobs	0.06	0.20	0.11	0.13	0.07	0.15	0.02
high	**isoD$_4$**	ancestral	**N**	13	13	12	12	12	14	14
			A	2	3	4	2	3	4	5
			Hobs	0.00	0.00	0.00	0.00	0.09	0.31	0.15
		evolved	**N**	47	48	48	48	48	44	0
			A	2	1	2	3	1	3	NA
			Hobs	0.02	0.00	0.04	0.04	0.00	0.12	NA
high	**isoD$_5$**	ancestral	**N**	13	13	12	13	12	15	16
			A	2	3	4	2	3	5	5
			Hobs	0.00	0.00	0.00	0.00	0.09	0.14	0.00
		evolved	**N**	42	41	32	44	44	42	41
			A	3	5	6	4	5	6	2
			Hobs	0.00	0.00	0.00	0.00	0.00	0.15	0.00
high	**isoD$_6$**	ancestral	**N**	14	13	14	13	13	14	15
			A	2	3	4	2	3	5	5
			Hobs	0.00	0.00	0.00	0.00	0.09	0.14	0.00
		evolved	**N**	47	48	43	43	43	38	0
			A	3	5	6	4	5	6	2
			Hobs	0.00	0.00	0.00	0.00	0.00	0.15	0.00

Table 5.1: Measures of genetic diversity and heterozygosity of ancestral and evolved genetically homogeneous experimental populations. N – number of individuals analyzed, A – number of observed alleles, Ae Hobs – observed heterozygosity.

These results indicate that ancestral populations segregated significant levels of polymorphism at these loci. However, when the effective number of alleles (a statistic that takes into account the frequency of the segregating alleles) was calculated, the values lied on the range of 1 to 3 alleles (data not shown). This suggests that the high numbers of alleles were caused by segregation of alleles at very low frequencies. Because we have not yet calculated the error rate of our

genotyping methodology, we are not able to infer the extent to which this reported variation is real or a genotyping artifact. Another description of the levels of genetic variation present in ancestral populations comes from genotyping of individuals at single nucleotide polymorphisms (SNPs) densely distributed across 1/3 of the genome of *C. elegans*. The analysis of these data was revealing of the genetic uniformity of both Dioecious and Androdioecious populations (Chelo, pers. com.). A similar genetic characterization of evolved populations was not carried out. It is apparent from Table 5.1 that there were both gains and losses of allele classes across microsatellite loci with experimental evolution. This pattern is expected since alleles at this type of locus are defined by the number of repeat units, with alleles gaining or losing repeats by mutation and consequently transiting to a different - not necessarily new - allelic class. Unfortunately, however, the heterogeneity of the statistical values obtained for the different loci and replicate populations did not allow us to clearly infer a general increase in the amount of genetic variation segregating in our evolved experimental populations.

Adaptation under Androdioecy

Despite the limitations of the genetic characterization of variation generated by mutation, phenotypic analysis of experimental populations revealed response to the new selective environment. One of the phenotypes which evolved was population competitive performance. Assuming this trait is a good proxy for fitness, adaptation in these populations is demonstrated (Figure 5.1).

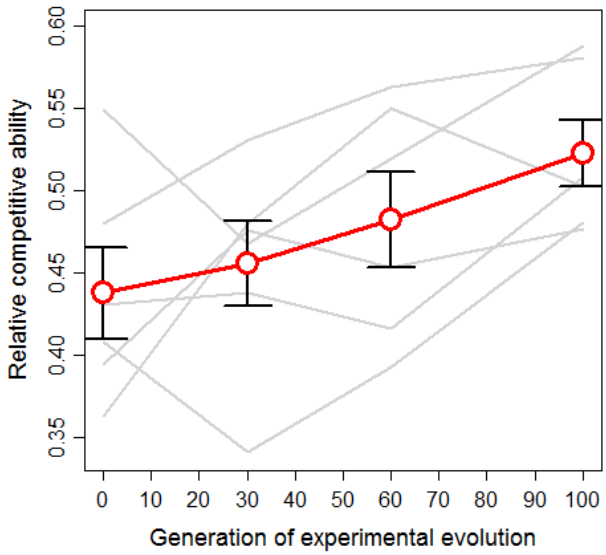

Figure 5.1: Evolution of population fitness of genetically homogeneous Androdioecious populations. Fitness was measured as competitive performance of experimental populations against a *tester* population expressing GFP. Fitness values represent the expected proportions of wild-type genome complements based on the observed frequency of wild-type (GFP-negative) individuals at the end of the competition. Red circles represent mean values of replicate experimental populations.. Error bars (black) represent standard error of the means. Grey lines show the evolutionary trajectories of individual replicate populations. See Supplementary Table S5.1 for statistical analysis.

Over 100 generations of experimental evolution, evolved Androdioecious populations increased their fitness by 15% relative to ancestral populations (standardized for rates of outcrossing). The regression of the fitness values of the six experimental populations on generation of experimental evolution yielded a coefficient of 0.0325 ($F_{1,22}$=5, adjusted R^2 = 15%); this coefficient represents the per-generation change in mean trait value. On average, the rate of fitness increase between the generations at which was measured, seems to increase progressively: 1.8% (between generation 0 and 30), 4.5% (between generation 30 and 60) and 8.5% (between generation 60 and 100). Statistically, only the last two slopes are significant (p=0.09 and p=0.003, respectively), but because of our limited replication, these results should be interpreted with caution. Under the Fisherian model, the populations with the lowest initial fitness would be expected to increase their fitness to a larger extent than those starting experimental

evolution with higher values of fitness. The regression of the fitness values of experimental populations at generation 100 on their initial values yields a coefficient of 0.64 (±0.17); the fact that this coefficient is <1 provides qualitative support for Fisher's model of adaptation.

When investigating the individual evolutionary trajectories of experimental populations, the heterogeneity between them is clear (Figure 5.1). In some populations the increase in fitness is apparent from very early on, whereas in other cases it is only detected after 60 generations of experimental evolution. There is, however, a common feature among the trajectories of our Androdioecious populations: the *increases* in fitness are typically quite large. This result is qualitatively consistent with extant theory supporting the view that adaptation should proceed mostly by fixation of mutations of large phenotypic effects; mutations of smaller effects may be more common but they are also most likely to be lost by genetic drift or by Hill-Robertson effect (Orr 2002, Rozen *et al* 2002).

Evolution of life-history phenotypes from *de novo* mutations

Fitness and outcrossing

Adaptation of Androdioecious populations to the imposed environment was accompanied by an increase of rates of cross-fertilization with time (these results were reported in Chapter 2; Figure 2.2 and Figure 2.4). Rates of outcrossing were estimated from frequencies of males measured in populations at several time points of experimental evolution. With mutation as the only possible explanation for any changes in the phenotypes in these populations, we confirmed that increased outcrossing did not result from higher rates of spontaneous generation of males (see Chapter 2, Figure 2.3). The relative proportions of males and hermaphrodites can evolve under androdioecy; therefore, to invoke adaptive explanations for such evolution it is necessary to investigate the relationship between rates of outcrossing and population fitness. As described in Chapter 2, this relationship seems to be positive and significant in our experimental populations and under the experimental conditions employed in our study.

Altogether, our results indicate that the few males that arose spontaneously and the creation of novel alleles by mutation were sufficient to generate heritable genetic differences among hermaphrodites and males in their ability for cross-fertilization, allowing selection to exert its action.

This explanation is consistent with the evolutionary dynamics shown by rates of outcrossing themselves: until generation 30 of experimental evolution there was a general lack of phenotypic response; presumably, this resulted from the fact that mutations (even adaptive ones) are expected to take time to accrue in populations. After generation 30, a progressive (and apparently linear) increase of rates of outcrossing was observed. Under the adaptive explanation of increased rates of outcrossing, then evolution of reproduction-related traits (especially those involved in cross-fertilization) could be anticipated. The evolution of such phenotypes was investigated in hermaphrodites and in males and compared to those exhibited by obligate outcrossers – males and females of Dioecious populations.

Male competitive performance under androdioecy and under dioecy

We measured the ability of ancestral and evolved experimental males from Androdioecious and Dioecious populations to sire progeny under competitive conditions against *tester*, GFP-positive males for fertilization of *fog*-2 females (JK574 strain). This phenotype was expressed as the average proportion of wild-type adult progeny produced by females. It was clear that the competitive ability of experimental males increased with experimental evolution both under conditions of Dioecy and of Androdioecy (Figure 5.2).

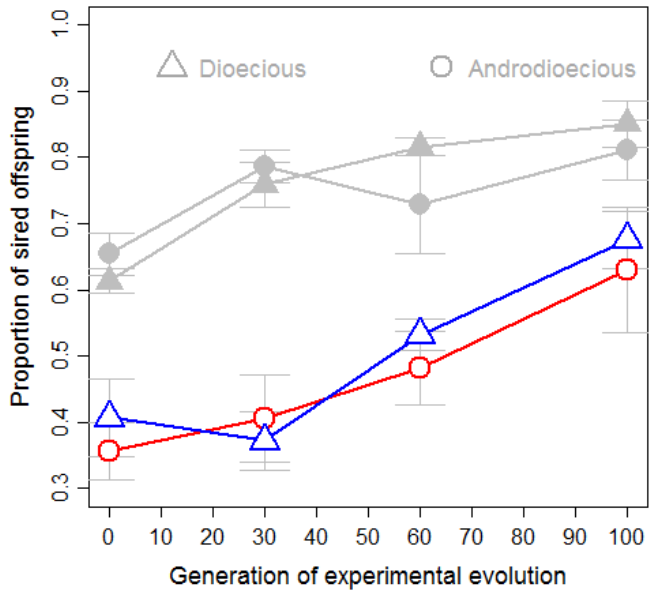

Figure 5.2: Evolution of male competitive performance of genetically homogeneous Androdioecious and Dioecious populations. Mean proportion of progeny sired by males from Dioecious (blue triangles) and Androdioecious (red circles) populations in competition for fertilization of *fog*-2(*q71*) females (strain JK574) against *tester* males expressing GFP. Error bars (in grey) represent standard errors of the means of replicate populations. Values of competitive ability of males from genetically diverse experimental populations are plotted for comparison. See Supplementary Table S5.2 for statistical analysis.

Similarly to what had been observed for fitness, there was a clear absence of phenotypic response in the first 30 generations of exposure to the novel environment; after this period, the increase of the mean phenotype was apparently linear. These dynamics are again consistent with evolution from mutational input, with very few alleles segregating in the populations in the initial steps of the evolutionary trajectory. The lack of phenotypic response in the beginning of experimental evolution in Androdioecious populations could also have resulted from the low initial numbers of males and the concomitant inability of natural selection to act efficiently. However, the same pattern is exhibited by Dioecious populations, which segregated maximal frequencies of males from the start (imposed by design). The average increase in male competitive performance was 77% under androdioecy and 66% under dioecy; however, no statistically significant differences were found between mating systems. Interestingly, male

competitive performance increased to a greater extent in genetically uniform experimental populations relative to those exhibiting genetic variation.

The evolution of body size

Body size has been suggested to correlate with male fitness (Calder 1983, Peters 1983). However, because of the segregation of few males in Androdioecious populations, we could not obtain large enough sample sizes for proper statistical analysis. Sampling of males from Dioecious populations, however, did not constitute a problem and ANOVA of the data indicated significant increases in body width (6%) and body volume (20%) after 50 generations of experimental evolution, compared to ancestral populations (Figure 5.3 A).

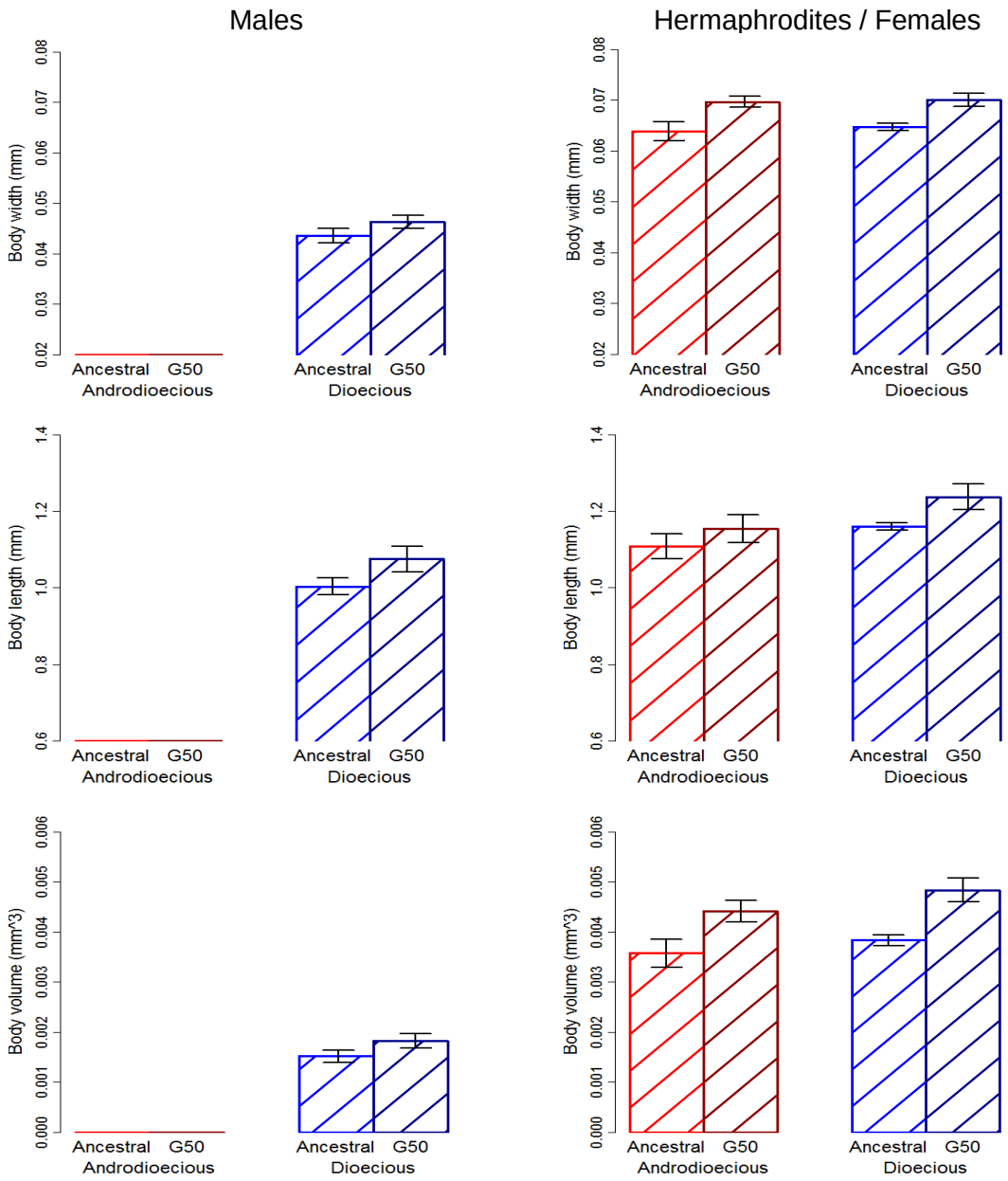

Figure 5.3: Evolution of body size of males and of hermaphrodites and females of genetically homogeneous Androdioecious and Dioecious populations. Values are presented as replicate means; error bars of evolved populations (solid grey) represent standard errors of means of replicate experimental populations; because no pseudoreplication was performed, error bars of ancestral populations (dashed grey) represent standard error of the mean value among individual measurements, providing graphic illustration of experimental and individual variation. Top graphs: body width; middle graphs: body length; bottom graphs: body volume. See Supplementary Table S5.3 and S5.4 for statistical analysis.

Evolution of body size of *C. elegans* males has been previously reported for populations experiencing increased sperm competition (LaMunyon and Ward 2002) under conditions similar to those to which Dioecious populations were subjected (that is, genetic manipulation of mating system leading to obligate outcrossing). LaMunyon and Ward did not report the extent of the phenotypic response of male body size. They did, however, report that no significant statistical associations were found between male body size and sperm size, which they also showed to have increased after 60 generations of experimental evolution. The authors propose that larger males may be able to produce sperm at higher rates (LaMunyon and Ward 1998). The increased fertilization success under competitive conditions verified for the males segregating in our Dioecious experimental populations, leads us to further hypothesize that larger males might also have a direct reproductive advantage because of male-male interactions. For example, it is possible that larger males more efficiently displace other males at the time they are attempting to mate with females/hermaphrodites.

In *C. elegans* hermaphrodites, body size seems to be positively associated with reproductive fitness (measured as lifetime reproduction). Evidence for this comes from mutation accumulation studies, which point to correlated effects of deleterious mutations on both phenotypes (Ostrow *et al* 2007). Experimental hermaphrodites and females evolved larger bodies in 50 generations: we detected significant increases in body width, body length and body volume (Figure 5.3 B). Increased body width can be explained by the potential selective advantage of wider individuals due to retention of a larger number of fertilized eggs at day of culture passage (internal eggs are more likely to survive the population maintenance protocol). Selective explanations for the evolution of longer bodies are somewhat more difficult to conceive, although they could simply reflect a correlated response with body width. However, significant differences were found between hermaphrodites and females, with the latter exhibiting longer silhouettes and a larger phenotypic response (4% increase in evolved hermaphrodites and 7% increase in evolved females). These changes in body width and body length

translated into the evolution of overall larger body sizes, with marginal differences between females and hermaphrodites. In addition to the apparent relationship between body size and reproductive ability, it is possible that the evolution of bigger females and hermaphrodites emerged as a consequence of mating. Male-inflicted damage during copulation is a well known effect in *C. elegans* (Gems and Riddle 1996, Hughes et al 2007); if larger hermaphrodite and female body sizes equate with less damage caused by mating, our results are explained: selection for increased body size would be expected to be stronger under elevated rates of cross-fertilization that is, under dioecy compared to androdioecy. This hypothesis, however, remains speculative. We thus proceeded to investigating the evolution of reproduction components in our experimental populations.

Evolution of reproduction phenotypes in hermaphrodites and in females

Several reproduction-related phenotypes were measured in hermaphrodites under conditions of self or cross-fertilization. The latter were compared to the reproductive patterns of females (which can only outcross). More specifically, we measured daily production of eggs, as well as the number of offspring that reached age of reproductive maturity. Overall, the reproductive ability of hermaphrodites and females evolved significantly (Figure 5.4):

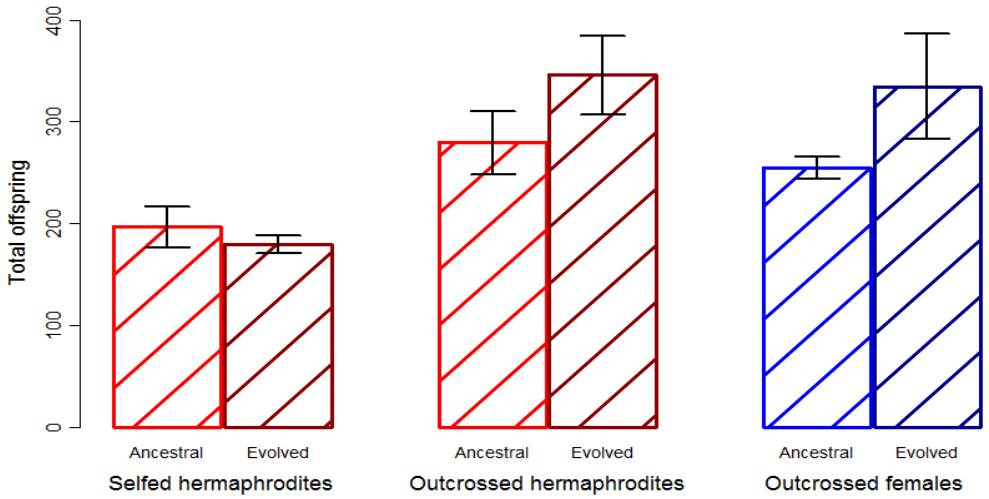

Figure 5.4: Evolution of lifetime reproductive success (LRS) of hermaphrodites and females of genetically homogeneous populations. Values are presented as the mean of total offspring produced by replicate populations; error bars represent standard errors of the means. See Supplementary Table S5.5 for statistical analysis.

Total progeny production of hermaphrodites and females increased 24% and 31%, respectively, under cross-fertilization. Self fertilized hermaphrodites, however, did not increase their progeny production. In fact, despite statistical insignificance, their average production of offspring decreased 9%. This pattern is not expected to be a consequence of self-sperm limitation because C. elegans produces at least ~300 sperm, which are utilized with nearly 100% efficiency (Ward and Carrel 1979). Because of the experimental conditions under which Androdioecious and Dioecious populations were evolved, the reproduction and survival phenotypes expected to contribute the most to fitness are those expressed on day of culture passage (day 4 of the life cycle). Offspring production at this particular day showed similar patterns to those observed for lifetime reproductive success: they increased significantly under conditions of cross-fertilization (34% in hermaphrodites and 39% in females) but not under conditions of selfing (Figure 5.5).

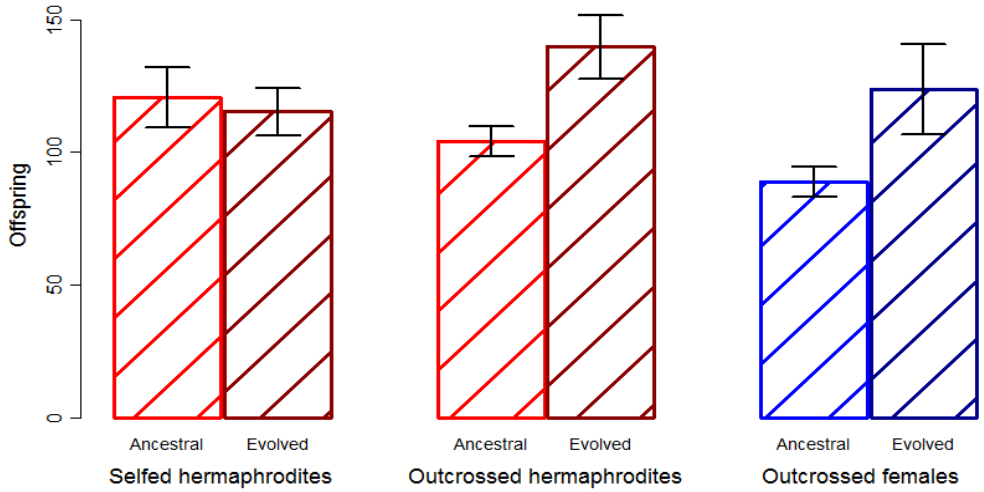

Figure 5.5: Evolution of offspring production of hermaphrodites and females of genetically homogeneous populations. Values are presented as the mean offspring produced at day of culture passage of replicate populations; error bars represent standard errors of the means. See Supplementary Table S5.6 for statistical analysis.

Clearly, self-sperm limitation cannot explain the lack of phenotypic response of hermaphrodites under conditions of selfing at this particular day of the life cycle. This pattern perfectly matches the evolutionary response of genetically diverse populations (Chapter 3) for this phenotype. Underlying the evolution of offspring production can be different abilities to produce eggs (oocytes) and/or different viability of the zygotes; we measured both these phenotypes.

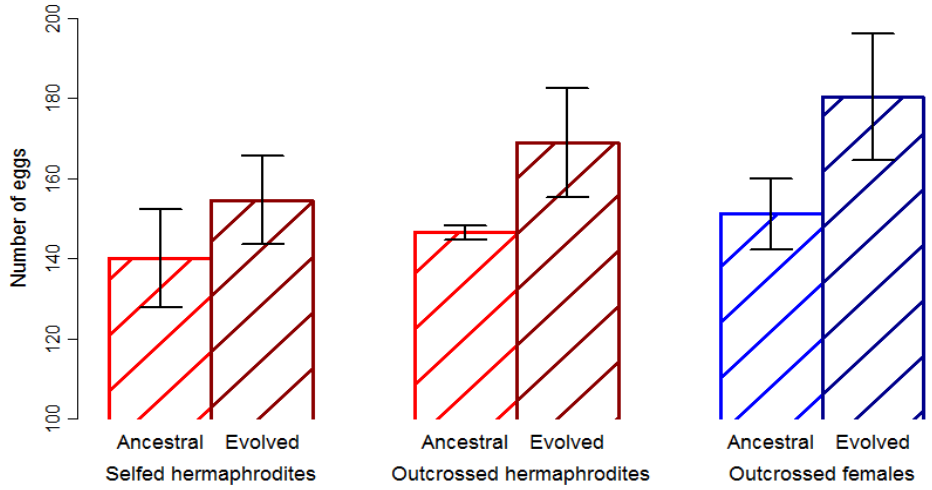

Figure 5.6: Evolution of egg production of hermaphrodites and females of genetically homogeneous populations. Values are presented as mean number of eggs produced at day of culture passage of replicate populations; error bars represent standard errors of the means. See Supplementary Table S5.7 for statistical analysis.

There was an overall trend for increased egg production at day 4 of the life cycle, although differences were only marginally significant (p=0.056 for the dataset of cross-fertilized hermaphrodites and females and p=0.059 for the dataset containing selfed and crossed hermaphrodites). No significant differences were detected between cross-fertilized hermaphrodites and females nor among hermaphrodites under the two treatments. Because of the borderline significance of the model, Student's t-tests were performed for each group using all individual observations (pooled across replicate populations). These tests revealed significant increases in the number of eggs produced at day 4 of the life cycle of selfed hermaphrodites (t_{172}=-2.4, p=0.018), cross-fertilized hermaphrodites (t_{139}=-3.1, p=0.002) and females (t_{162}=-3.8, p<0.001). It is worthwhile noticing, however, the suggestion of decreased ability of evolved hermaphrodites to produce eggs when selfed compared their production upon cross-fertilization by males. This is puzzling because oogenesis itself should proceed exactly in the same way in hermaphrodites regardless of the source of sperm. If this result would hold upon analysis of more replicate populations or the same populations evolved for a longer period of time, it would be suggestive of the evolution of differential abilities

of self sperm to fertilize the eggs (relative to allosperm). Although speculative, this hypothesis finds some support in the patterns of production of unfertilized eggs. In the last two blocks of the reproductive schedule assay, the scoring of the number of eggs involved the discrimination of fertilized and unfertilized eggs. The number of unfertilized eggs produced on day 4 of the life cycle alone was too small to allow comparison between groups, but using the information from the first two days of the reproductive period (those upon which selection could act), the trend for an increasing production of unfertilized eggs under self-fertilization was evident (Figure 5.7). For this period of time, sperm was not limiting, since offspring were still produced in following days during this assay (see below):

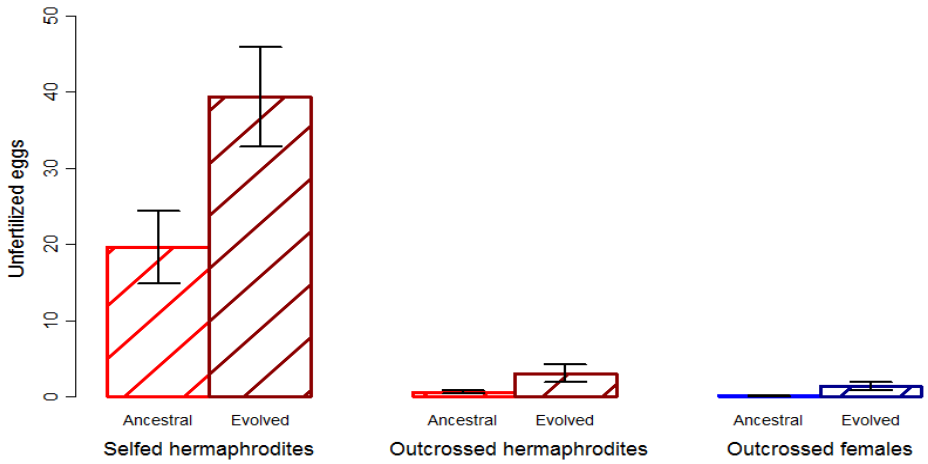

Figure 5.7: Evolution of the production of unfertilized eggs of hermaphrodites and females of genetically homogeneous populations. Values are presented as mean number of unfertilized eggs produced in the first two days of the reproductive period of two replicate populations; error bars represent standard errors of the means.

The differences in the patterns of offspring production cannot be explained by the production of (fertilized) eggs measured in our assy. Therefore, egg-to-adult survivorship (viability) on day 4 of the life cycle was also measured.

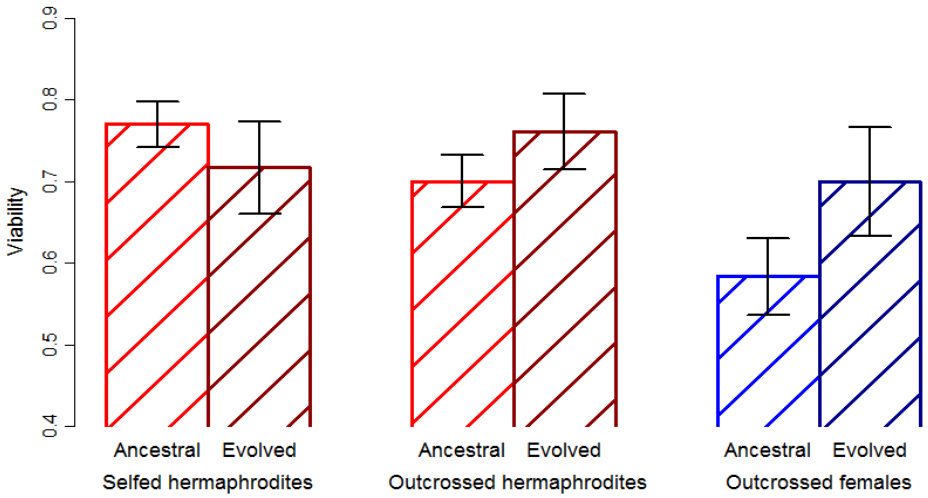

Figure 5.8: Evolution of viability of progeny produced by hermaphrodites and females of genetically homogeneous populations. Values are presented as the mean egg-to-adult-survivorship of progeny of replicate populations; error bars represent standard errors of the means. See Supplementary Table S5.8 for statistical analysis.

Egg-to-adult survivorship under outcrossing showed an increasing trend, while the opposite was found for progeny produced by self-fertilization; however, neither tendency is statistically significant. This pattern was enhanced when the viability of the total progeny produced was analyzed (with the differences becoming statistically significant), but it is more informative to look at the daily patterns of the viability of the offspring produced under each condition. An inspection of the viability of progeny produced each day revealed that the trend for decreased viability under self-fertilization was apparent from day 4 of the life cycle and sustained until late in the reproductive period (Figure 5.9 A). Viability of the offspring of cross-fertilized hermaphrodites and females was apparently higher in evolved populations relative to their ancestors throughout the whole reproductive period (Figure 5.9 B and C).

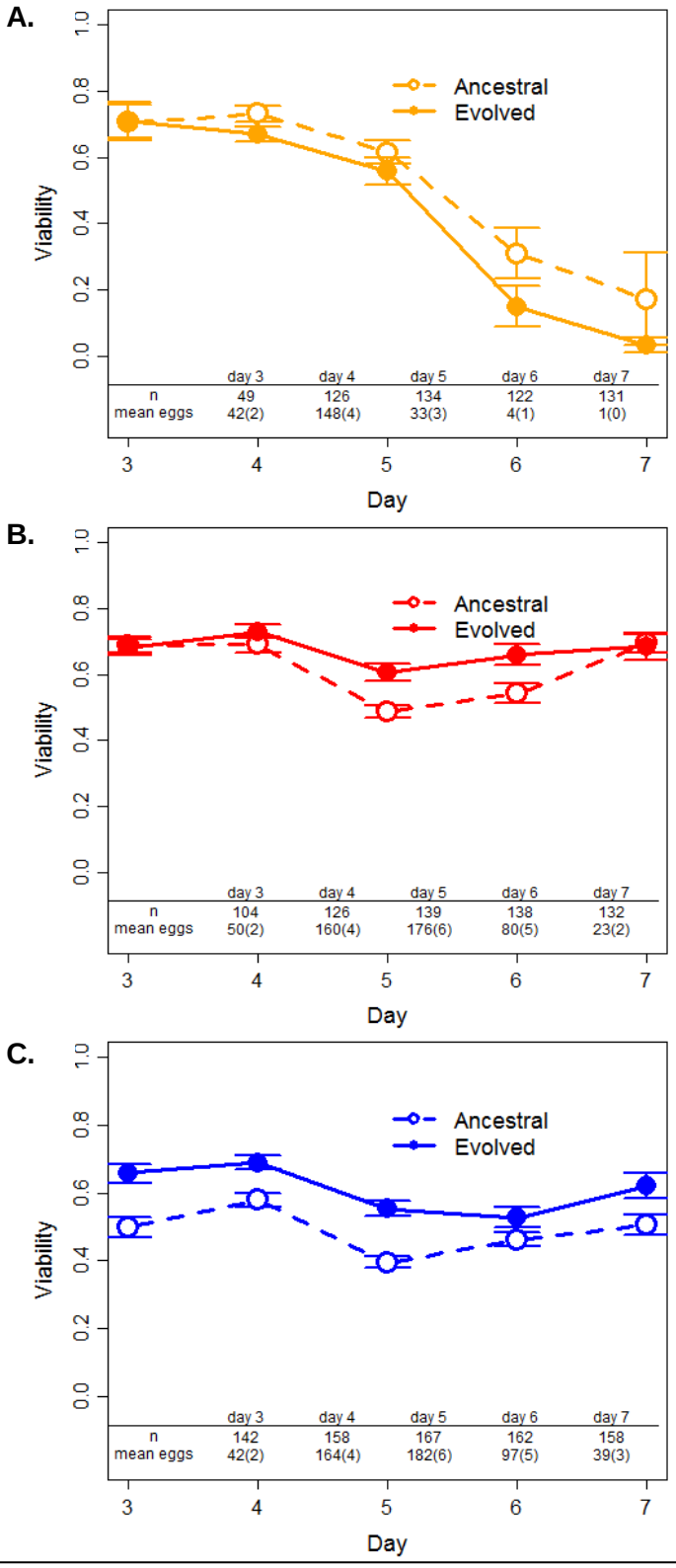

Figure 5.9: Evolution of daily viability of progeny produced by hermaphrodites and females of genetically homogeneous populations. Values are presented as the mean egg-to-adult-survivorship of progeny of replicate populations produced on each day of the reproductive period; error bars represent standard errors of the means. **A)** Self fertilized hermaphrodites **B)** Cross-fertilized hermaphrodites **C)** Cross fertilized females.

It is important to highlight that the sperm donors to mated hermaphrodites and females were males from *tester*, outbred populations. Male sperm is known to outcompete self-sperm in *C. elegans* (Ward and Carrel 1979). It has also been shown that allosperm provided by males of the N2 strain for 24h-48h to hermaphrodites and females does not become unviable during the whole reproductive period (Hughes *et al* 2007).

5.5 Discussion

Adaptation is generally perceived as the approximation of populations to a fitness optimum by the action of natural selection (Fisher 1930). In the case of genetically homogeneous populations, this approximation relies on the generation of genetic variation necessary for natural selection by mutation. However, little is known about rates of adaptive mutations in organisms other than microbes (Eyre-Walker and Keightley 2007). It has been suggested, though, that adaptation in organisms with more complex genomes and phenotypes should proceed more slowly compared to simpler ones, because mutations with beneficial effects are less likely to appear in a context where the number of interactions among genetic and physiological processes is higher (Orr 2000). The work presented here indicates that populations of multicellular organisms can evolve in a relatively short time with mutation as the major source of genetic variation, given the extent of phenotypic response shown for several life-history traits.

It can be argued that such responses could have stemmed from genetic polymorphisms segregating in the (presumably) genetically uniform Androdioecious populations, for example as overdominant loci. With increased outcrossing this could generate sufficient genetic variation to allow phenotypic responses to occur. This possibility could not be completely ruled out from the genetic analysis of microsatellite loci. However, microsatellite loci typically exhibit higher mutation rates than genomic mutations rates because they facilitate slippage of DNA polymerase during replication of the genome. As mentioned previously, analysis of single nucleotide polymorphisms (SNP) in our experimental

populations strongly supported genetic uniformity of inbred Androdioecious populations, which exhibited values of expected heterozygosity of 99% (Chelo, pers. comm.). Even if heterozygosity at the remainder 1% loci would reflect allelic polymorphisms at overdominant loci, these would have to contribute significantly to fitness; furthermore, they would necessarily have to be found in the populations from which these inbred populations were derived: the genetically diverse Androdioecious ancestral (A_0). If overdominance was an important feature of loci determining fitness, then these ancestral hybrid populations would have been expected to show depression of fitness upon inbreeding, a phenomenon that was not observed during the 10 generations of self-fertilization imposed during the derivation of these inbred populations (Teotónio, pers. com.). Finally, in light of current estimates of the diploid genomic mutation rate for this species ($0.01<U<4.1$), it is possible that some mutations emerged during the derivation of these inbred androdioecious populations. If this occurred, only those with very small effects are likely to have been retained in inbred Androdioecious populations following the population expansion carried out prior to cryopreservation of these populations before experimental evolution. Finally, the results obtained in this study are qualitatively (and to some extent quantitatively) consistent with existing empirical data, as will be discussed next.

Despite the fact that the literature is ruled in large part by studies on deleterious mutations, at least another study in *C. elegans* investigated the properties of adaptive mutations. Estes and colleagues allowed mutation accumulation (MA) lines to regain their fitness by propagating them at large population sizes for 80 generations. The estimated per-generation change in mean fitness in these populations was 0.55 (±0.14) (Estes *et al* 2003), a value ~17 times higher than the rate of fitness increase of our experimental populations. This is not surprising, however, because it translates a recovery in fitness which that had been depressed in 25% (Vassilieva *et al* 2000). The authors find support for the theoretical predictions that the increase in fitness should be inversely proportional to the initial fitness value – that is, that populations further away from a fitness

optimum should reveal the larger increase. Despite our limited replication, it is encouraging that we find the same pattern. A direct comparison of the rates of fitness increase between the two studies is difficult because different proxies for fitness were used – competitive performance against a *tester* strain (in ours) and number of progeny produced over the first four days of the life cycle (in the study by Estes and colleagues). However, progeny production was also measured in our experimental populations (in the reproductive schedule assay); our ancestral Androdioecious populations show levels of progeny production which correspond roughly to the upper third of values of those exhibited by the MA lines of Estes *et al*. The fitness values of our experimental populations are therefore similar to those of the best performing MA lines, which exhibited the lowest increase in fitness upon recovery. This being the case, the rate of fitness increase observed in our Androdioecious populations may be at least on the same order of magnitude than the MA lines with the lower initial fitness depression. There are other similarities that emerge from both studies, such as the heterogeneity among the individual evolutionary trajectories of lines and populations and the magnitude of the fitness-enhancing changes. These patterns are consistent with the randomness of the mutational process on one hand, but also with the predictions that adaptation should proceed (at least in its earlier) stages from mutations with large effects on fitness. This is suggestive of considerable pleiotropic or (synergistic) epistatic effects – or perhaps both. However, comparisons between these two studies should be taken with caution, since they assume similar adaptive landscapes. This similarity is not very likely, since starting genotypes were quite different and also because MA-recovery lines did not seem to segregate significant fractions of males, whereas in our experimental system males and outcrossing were abundant and intimately related to population fitness. To quantify the contribution of mutations to the adaptive process we can compare the evolution of populations which started this experiment with extremely reduced levels of genetic variation with those which evolved in conditions of abundant genetic variation. Genetically diverse Androdioecious populations ($A_{4\text{-}6}$) did not

significantly increase in fitness since they presumably started experimental evolution at or near a fitness optimum (see Chapter 2). However, Dioecious ($D_{4\text{-}6}$) populations significantly increased fitness with experimental evolution (20%). The genetically homogeneous Dioecious populations ($isoD_{1\text{-}6}$) were not assayed, for which a direct comparison between the two cannot be made, but genetically homogeneous Androdioecious populations ($isoA_{1\text{-}6}$) increased mean population fitness in 17%. Because these values are standardized by rates of outcrossing, they are still informative, and suggest that rates of adaptation can be similar in genetically diverse and genetically homogeneous populations for the time window considered in this study (100 generations). The rates of adaptation from mutational input observed in our study may be considerably larger than previously thought for more complex organisms. However, they are consistent with recent reports of similar genomic rates of beneficial mutations are between *C. elegans* and *E. coli* (see Denver *et al* 2010 and Perfeito *et al* 2007). If adaptive mutation rates are similar between such different organisms, it would be interesting to investigate if competition among beneficial alleles can also play a role in the adaptive dynamics of populations of multicellular, selfing organisms.

The fact that fitness of genetically diverse populations was measured only at generation 0 and generation 100 may hinder some important aspects concerning the relevance of initial levels of genetic variation to adaptation. Evidently, evolutionary trajectories of all experimental populations would be desirable to unravel different dynamics. Rates of outcrossing correlate with fitness in our experimental system and thus an examination of the phenotypic responses of life-history traits among experimental populations should provide more insights into the effects of different levels of initial genetic variation to adaptation. Evolutionary trajectories were obtained for at least one phenotype, male competitive performance (Figure 5.2). Genetically homogenous populations showed lower values compared to genetically diverse populations, but by generation 100 the differences between them were hardly detected. This pattern can be understood by investigating the dynamics of the phenotypic response:

genetically diverse populations significantly increased male competitive performance during the first 30 generations of experimental evolution, showing relative stability in the subsequent time points that were assayed. Genetically homogeneous populations, on the other hand, did not respond to selection between generation 0 and generation 30, but showed a stable increase in male competitive performance from this point onwards. If the stability of populations segregating high levels of standing genetic variation after generation 30 is interpreted as proximity to the phenotypic optimum, this means that the presence of high levels of additive genetic variance for this phenotype allowed the population to rapidly achieve this optimum. On the other hand, genetically homogeneous populations had to wait for genetic variation to be created by mutation to respond. The rapid increase in male competitive performance from mutational input was nevertheless surprisingly large, which may argue for average effects of novel, beneficial mutations on the same order of magnitude than the estimated effects of deleterious mutations from MA studies, on the order of 10%-20% (Baer *et al* 2005). If most mutations are recessive, their increased homozygosity in Androdioecious populations would have been expected to lead them more rapidly to fixation. It could be hypothesized that the mutations responsible for increased male competitive ability arose in male-specific genes. If this was the case, however, Androdioecious populations would not be expected to show a similar response, given their lower frequencies of males throughout a substantial part of experimental evolution (see Chapter 2); assuming they are thus advantageous in both sexes, it can be suggested that most of the beneficial mutations responsible for increased male competitive performance were dominant. The hypothesis that dominant mutations may be more common among beneficial mutations than deleterious ones is not new (Charlesworth 1992). This could also lead to comparable probabilities of loss of new beneficial alleles under both mating systems (and hence the similar dynamics), since the argument that self-fertilization decreases the probability of loss of adaptive mutations relies either on very small population sizes or recessivity (Haldane 1924).

Overall, genetically homogeneous populations exhibited phenotypic responses similar to populations which initiated experimental evolution with abundant genetic variation. Interestingly, the evolutionary patterns of components of fitness of Androdioecious and Dioecious populations were also remarkably similar. In addition to the evolution of male competitive performance, patterns of reproduction of hermaphrodites and females under conditions of cross-fertilization (with *tester* males) were also similar under conditions of abundant or limited genetic variation. Under conditions of cross-fertilization, all hermaphrodites and females increased offspring numbers at the day of culture passage, as well as their production of eggs and viability (although not significantly in homogeneous populations). Perhaps one of the most interesting evolutionary patterns found among life-history traits was the suggestion of decreased ability of self sperm to fertilize eggs, based on the observation of a decreasing tendency of viability of progeny produced by self- but not cross-fertilization. To our knowledge, this is the first time that the potential reduction of viability of self-sperm with time is reported in *C. elegans* hermaphrodites; moreover, our data suggests this phenotype can evolve. The viability of progeny produced after day 4 of the life cycle is not a component of fitness under our experimental conditions (since it is never expressed). However, the observed decline in viability verified at age of reproductive maturity (day 4) must have necessarily resulted from the action of selection on this phenotype, either directly or indirectly.

In summary, our results suggest that levels of genetic variation may not influence the rate of adaptation of populations at the evolutionary timescale investigated here. If they do, our results suggest that their effects are manifested within even shorter periods of time, on the order of 30 to 60 generations. More evolutionary trajectories of fitness would have to be obtained to investigate this. Even if low levels of segregating variation may limit rates of adaptation, they certainly do not seem to affect adaptive patterns, as show by the remarkable similarity of patterns of phenotypic evolution between genetically homogeneous and genetically diverse populations. Whether this phenotypic convergence reflects

underlying genetic and biological constraints on possible evolutionary pathways remains an open question.

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5.8 Supplementary information

Model :
Population competitive performance = Generation+ Replicate

Source	d.f.	SS	MS	F-value	P
Generation	3	0.025	0.008	4.515	**0.019**
Replicate	5	0.054	0.011	5.857	**0.003**
Residuals	15	0.027	0.002		

$F_{8,15}$=5.35; p-value=0.003; Adjusted R^2=60%

Supplementary Table S5.1: Analysis of variance of population fitness of genetically homogeneous Androdioecious populations.

Model :
Male competitive performance = Mating system+ Generation+ Replicate+ Generation x Mating system

Source	d.f.	SS	MS	F-value	P
Mating system	1	0.008	0.008	0.479	0.494
Generation	3	0.528	0.176	10.023	**<0.001**
Replicate	5	0.162	0.032	1.841	0.134
Generation x Replicate	3	0.014	0.005	0.258	0.855
Residuals	31	0.544	0.018		

$F_{12,31}$=3.38; p-value=0.003; Adjusted R^2=40%

Supplementary Table S5.2: Analysis of variance of male competitive performance of genetically homogeneous populations. Analysis was performed in a full model (testing all factors and interactions) but a reduced model is presented (after removal of non significant interactions). In the model, X stands for interaction between factors.

A.

Model :
Body width = Generation+ Replicate

Source	d.f.	SS	MS	F-value	P
Generation	1	$1.46x10^{-6}$	$1.46x10^{-6}$	38.630	**0.008**
Replicate	3	$4.44x10^{-5}$	$1.48x10^{-5}$	39.084	**0.007**
Residuals	3	$1.14x10^{-6}$	$3.79x10^{-7}$		

$F_{4,3}$=39.97; p-value=0.006; Adjusted R^2=96%

B.

Model :
Body length = Generation+ Replicate

Source	d.f.	SS	MS	F-value	P
Generation	1	0.010	0.010	6.943	0.078
Replicate	3	0.015	0.005	3.476	0.167
Residuals	3	0.004	0.002		

$F_{4,3}$=4.34; p-value=0.129; Adjusted R^2=66%

C.

Model :
Body volume = Generation+ Replicate

Source	d.f.	SS	MS	F-value	P
Generation	1	$1.84x10^{-7}$	$1.84x10^{-7}$	40.707	**0.008**
Replicate	3	$4.09x10^{-7}$	$1.36x10^{-7}$	30.197	**0.010**
Residuals	3	$1.35x10^{-8}$	$4.51x10^{-9}$		

$F_{4,3}$=32.82; p-value=0.008; Adjusted R^2=95%

Supplementary Table S5.3: Analysis of variance of body size of males of genetically homogeneous populations. A) body width **B)** body length **C)** calculated body volume.

A.

Model :
Body width = Mating system+ Generation+ Replicate+ Mating system x Generation

Source	d.f.	SS	MS	F-value	P
Mating system	1	$1.78x10^{-7}$	$1.78x10^{-7}$	0.021	0.888
Generation	1	$1.67x10^{-4}$	$1.67x10^{-4}$	19.333	**<0.001**
Replicate	5	$5.51x10^{-5}$	$1.10x10^{-5}$	1.276	0.328
Mating system x Generation	1	$6.49x10^{-7}$	$6.49x10^{-7}$	0.075	0.788
Residuals	14	$1.21x10^{-4}$	$8.63x10^{-6}$		

$F_{8,14}$=3.23; p-value=0.027; Adjusted R^2=45%

B.

Model:
Body length = Mating system+ Generation+ Replicate+ Mating system x Generation

Source	d.f.	SS	MS	F-value	P
Mating system	1	0.020	0.020	5.892	**0.029**
Generation	1	0.017	0.017	5.126	**0.034**
Replicate	5	0.040	0.008	2.389	0.091
Mating system x Generation	1	0.002	0.001	0.357	0.560
Residuals	14	0.047	0.003		

$F_{8,14}$=2.92; p-value=0.038; Adjusted R^2=41%

C.

Model:
Body volume = Mating system+ Generation+ Replicate+ Mating system x Generation

Source	d.f.	SS	MS	F-value	P
Mating system	1	$2.49x10^{-7}$	$2.49x10^{-7}$	1.495	0.242
Generation	1	$4.14x10^{-6}$	$4.14x10^{-6}$	24.809	**<0.001**
Replicate	5	$2.19x10^{-6}$	$4.37x10^{-7}$	2.623	0.071
Mating system x Generation	1	$1.04x10^{-8}$	$1.04x10^{-8}$	0.062	0.807
Residuals	14	$2.33x10^{-6}$	$1.67x10^{-7}$		

$F_{8.14}$=4.94; p-value=0.005; Adjusted R^2=59%

Supplementary Table S5.4: Analysis of variance of body size of hermaphrodites and females of genetically homogeneous populations. A) body width **B)** body length **C)** calculated body volume. In the models, X stands for interaction between factors.

A.

Model (cross fertilized hermaphrodites vs females):
LRS = Group + Generation+ Replicate+ Group x Generation

Source	d.f.	SS	MS	F-value	P
Group	1	980.8	980.8	0.208	0.665
Generation	1	15980.2	15980.2	3.381	0.116
Replicate	2	2864.4	1432.2	0.303	0.749
Group x Generation	1	124.3	124.3	0.026	0.877
Residuals	6	28355.5	4725.9		

$F_{5,6}$=0.84; p-value=0.564; Adjusted R^2=0%

B.

Model (cross fertilized vs self fertilized hermaphrodites):
LRS = Group + Generation+ Replicate+ Group x Generation

Source	d.f.	SS	MS	F-value	P
Group	1	46336	46336	17.922	**0.006**
Generation	1	1885	1885	0.729	0.426
Replicate	2	1977	989	0.382	0.697
Group x Generation	1	5161	5161	1.996	0.207
Residuals	6	15512	2585		

$F_{5,6}$=4.28; p-value=0.053; Adjusted R^2=60%

Supplementary Table S5.5: Analysis of variance of the lifetime reproductive success (LRS) of hermaphrodites and females of genetically homogeneous populations.. A) Comparison between hermaphrodites and females under outcrossing **B)** Comparison between crossed and selfed hermaphrodites**.** In the model, X stands for interaction between factors.

A.

Model (cross fertilized hermaphrodites vs females):
Progeny at day 4 of life cycle = Group + Generation+ Replicate+ Group x Generation

Source	d.f.	SS	MS	F-value	P
Group	1	634.8	634.8	1.399	0.282
Generation	1	3692.9	3692.9	8.137	**0.029**
Replicate	2	643.0	321.5	0.708	0.529
Group x Generation	1	0.	0.4	0.001	0.978
Residuals	6	2723.0	453.8		

$F_{5,6}$=2.319; p-value=0.1676; Adjusted R^2=38%

B.

Model (cross fertilized vs self fertilized hermaphrodites):
Progeny at day 4 of life cycle = Group + Generation+ Replicate+ Group x Generation

Source	d.f.	SS	MS	F-value	P
Group	1	47.4	47.42	0.273	0.620
Generation	1	680.79	680.79	3.918	0.095
Replicate	2	1271.90	635.95	3.660	0.091
Group x Generation	1	1265.14	1265.14	7.28	**0.036**
Residuals	6	1042.56	173.76		

$F_{5,6}$=11.87; p-value=0.005; Adjusted R^2=83%

Supplementary Table S5.6: Analysis of variance of the reproductive output of hermaphrodites and females of genetically homogeneous populations.. A) Comparison between hermaphrodites and females under outcrossing **B)** Comparison between crossed and selfed hermaphrodites**.** In the model, X stands for interaction between factors.

A.

Model (cross fertilized hermaphrodites vs females):
Number of eggs = Group + Generation+ Replicate+ Group x Generation

Source	d.f.	SS	MS	F-value	P
Group	1	196.86	196.86	0.551	0.486
Generation	1	1995.92	1995.92	5.586	**0.056**
Replicate	2	970.97	485.49	1.359	0.326
Group x Generation	1	35.29	35.29	0.099	0.764
Residuals	6	2143.67	357.28		

$F_{5,6}$=1.79; p-value=0.249; Adjusted R^2=26%

B.

Model (cross fertilized vs self fertilized hermaphrodites):
Number of eggs = Group + Generation+ Replicate+ Group x Generation

Source	d.f.	SS	MS	F-value	P
Group	1	322.08	322.08	1.692	0.241
Generation	1	1024.89	1024.89	5.385	**0.059**
Replicate	2	1615.72	807.86	4.245	0.071
Group x Generation	1	45.18	45.18	0.237	0.643
Residuals	6	1141.88	190.31		

$F_{5,6}$=3.161; p-value=0.097; Adjusted R^2=50%

Supplementary Table S5.7: Analysis of variance of the egg production of hermaphrodites and females of genetically homogeneous Androdioecious and Dioecious populations.. A) Comparison between hermaphrodites and females under outcrossing **B)** Comparison between crossed and selfed hermaphrodites**.** In the model, X stands for interaction between factors.

A.

Model (cross fertilized hermaphrodites vs females):
Viability = Group + Generation+ Replicate+ Group x Generation

Source	d.f.	SS	MS	F-value	P
Group	1	0.024	0.024	2.919	0.138
Generation	1	0.024	0.024	2.889	0.140
Replicate	2	0.011	0.005	0.657	0.552
Group x Generation	1	0.002	0.002	0.281	0.615
Residuals	6	0.049	0.008		

$F_{5,6}$=1.48; p-value=0.32; Adjusted R^2=18%

B.

Model (cross fertilized vs self fertilized hermaphrodites):
Viability = Group + Generation+ Replicate+ Group x Generation

Source	d.f.	SS	MS	F-value	P
Group	1	0.0005	0.0005	0.134	0.727
Generation	1	0.00005	0.00005	0.013	0.913
Replicate	2	0.021	0.010	2.728	0.144
Group x Generation	1	0.010	0.010	2.587	0.159
Residuals	6	0.023	0.004		

$F_{5,6}$=1.64; p-value=0.282; Adjusted R^2=23%

Supplementary Table S5.8: Analysis of variance of viability of progeny of hermaphrodites and females of genetically homogeneous populations.. A) Comparison between hermaphrodites and females under outcrossing **B)** Comparison between crossed and selfed hermaphrodites**.** In the model, X stands for interaction between factors.

Chapter VI

Discussion

Understanding the power and the limitations of natural selection in shaping the evolution of populations is fundamental for our comprehension of the living world. The conceptual framework for the study of adaptive evolution was laid out in the 19th century by Charles Darwin and Alfred Russell Wallace and the many decades that have since past have provided contemporary biologists with a solid theoretical (and quantitative) framework for the study of adaptation.

The development of quantitative genetics theory and of the field of experimental evolution in the 1980's (Orr 2005) laid the foundations for the empirical approach to the study of adaptive evolution. This approach has relied on the manipulation of populations of organisms under laboratory conditions. To investigate the effects of selection on populations of organisms, other evolutionary forces must be taken out of the equation or be controlled for. For example, decreasing the effects of genetic drift can be accomplished by maintaining populations at large census sizes. Also, the use of asexual species simultaneously sets aside sexual selection as driving force of the response of populations. Then, evidently that generation times have to be small enough so that phenotypic evolution can be measured. In the particular instance of adaptation, organisms with simple life cycles should be used to facilitate inferences about fitness. For this reason, microbes have been the organism of choice for studies of experimental evolution. Although extremely informative, these studies have also limited to a significant extent our knowledge about important aspects of adaptation. First, because most microorganisms reproduce clonally, adaptive evolution has been studied from the standpoint of *de novo* mutation as single source of genetic variation. Therefore, the experimental study of adaptation from segregating genetic variation is virtually inexistent. Second, our view of adaptation is largely limited to populations of organisms with relatively small genomes and simple physiology and life cycle. The extent to which current theories of adaptation will hold for species with more complex genotypic and phenotypic interactions is uncertain.

There are, however, important studies involving natural populations of *Drosophila* species (Teotónio and Rose 2000, Matos *et al* 2000). They have shown that observable phenotypic responses can occur in reasonable timeframes under both relaxed selection (Matos 2000) and directional selection (Teotónio 2000). They have also exposed some problems inherent to the study of sexual populations of more complex organisms, such as the need for extensive replication and proper (non-evolving) controls (Matos *et al* 2000), the contingency of evolutionary patterns and rates on initial segregating genotypes (Matos et al 2002) or the importance of inbreeding depression to multivariate phenotypic evolution (Rose *et al* 2005).

The work presented here constitutes an attempt to broaden our understanding of adaptation of multicellular, sexual organisms by overcoming some of these limitations. Replicate populations of *C. elegans* were evolved under stable conditions and simple demography under laboratory environment for 100 generations. Because of the ability of *C. elegans* hermaphrodites to self-fertilize while simultaneously being able to be cross-fertilized by males, a genetically diverse population was constructed by crossing several natural isolates. Also, cryopreservation is possible in this model organism, which allows ancestral and evolved populations to be assayed simultaneously, minimizing experimental error. Our initial experimental populations were genetically manipulated to differ in their mating system, androdioecy (co-existence of selfing hermaphrodites and males) or dioecy (males and females). They therefore differed in their levels of inbreeding and, consequently, in the effects of recombination and segregation in generating genotypic diversity available for selection. Populations with low levels of initial genetic variation were also created to evaluate the contribution of mutation to the adaptive process.

Adaptation and the evolution of life history

Not all evolution is adaptive. Hence, adaptive arguments for evolutionary patterns imply the demonstration that mean population fitness augmented with

time. Adaptation of experimental populations both from mutation and from standing genetic variation was demonstrated by the increase competitive performance of evolved populations against a *tester* (non-evolved) population relative to their corresponding ancestral. Competitions between genotypes as a methodological approach to measure fitness have long been performed in microbes but has seldomly been applied to other organisms. Our methodology seems to have been successful since it provided sufficient power to detect changes in the relative frequencies of experimental and *tester* genome complements over one generation. This assay, however, is not without its flaws. Because of the sexual mode of reproduction and the possibility for cross-fertilization, estimates of relative frequencies of both types of individuals at the phenotypic level are only interpretable if only one generation of competition is performed. Over more generations, the alleles segregating at the locus conferring phenotypic discrimination of experimental and *tester* individuals and their respective genetic backgrounds become dissociated. Even over the course of only one generation, the measurement of fitness obtained from our assay relies on several assumptions. For example, it assumes a) random mating between both types of individuals, b) that the frequency of outcrossing events is twice the number of males at setup and c) full penetrance of the GFP allele. To overcome these limitations, we performed similar assays in which the individuals generated after the competition were not scored phenotypically; instead, they were pooled, genomic DNA was extracted and the frequency of transgenic and wild-type alleles was assessed by quantitative real-time PCR. Despite extensive replication of the competitions, this methodology failed to precisely quantify the two types of alleles in the populations. This could have resulted from the cumulative effects of sampling of individuals and of deficient amplification of the alleles in the system used. In fact, the error associated with this methodology was so large that it failed to detect difference between the canonical strain N2 and mutation accumulation lines derived from it, whose fitness was dramatically reduced. However, improvements of these types of assays should be pursued. With increasing

availability of genotyping and sequencing methods, more powerful measurements of fitness based on competitions between genotypes are at sight.

Nevertheless, our proxy for fitness indicated beyond doubt that adaptation of experimental populations occurred. More interestingly, adaptation positively correlated with rates of cross-fertilization, with androdioecious populations of *C. elegans* having evolved unusually high values for a species which reproduces mostly by self-fertilization. Instances of maintenance of intermediate rates of outcrossing in *C. elegans* have seldomly been reported (Anderson *et al* 2010, Morran *et al* 2009). Several attempts to confirm the adaptive value of males (and hence outcrossing) in populations of *C. elegans* have systematically failed, even under elevated rates of (deleterious) mutations (Chasnov and Chow 2002, Stewart and Phillips 2002, Cutter 2005, Manoel *et al* 2007), one of the theoretical conditions for the evolution of outcrossing (Stewart and Phillips 2002).To our knowledge, our results provide the clearest evidence that adaptation can be facilitated by outcrossing in populations of this species. The fact that outcrossing was favored in genetically diverse, but inbred, populations argues for an important role of (dominance and) epistatic interactions between alleles in generating variance for fitness. It is possible that the crossing scheme employed to generate ancestral populations may have generated such sort of interactions (Charlesworth and Charlesworth 1978, Anderson *et al* 2010). The implications of this to the particular demography and population genetics of *C. elegans* is unclear. However, if epistasis correlates with genomic complexity (Sanjuan and Elena 2006), this can have important consequences for our understanding of the evolution of natural populations of many species. For example, the effects of migration of individuals between populations can be much larger than previously anticipated.

The relationship between the selective environment of experimental populations and outcrossing was further expressed by the extensive phenotypic responses of components of fitness related to cross-fertilization. Components of fitness are those that typically show the largest (and most consistent) responses to selection. Male competitive performance increased dramatically, further supporting

previous findings of significant amounts of genetic variation for male-related traits in this species (Teotónio *et al* 2006). Interestingly, life-history phenotypes of hermaphrodites evolved similarly under conditions of cross-fertilization relative to females (from dioecious populations). This pattern can be caused by the recent dioecious state of *C elegans* (Loewe and Cutter 2008). Nevertheless, it demonstrates that significant levels of genetic variation for the female functions can also be found among natural isolates. Under conditions of selfing, however, the directional response to selection by the "female" component of fitness of hermaphrodites seems to have been hampered by stability of their male function fitness components. In fact, some lines of evidence further suggest *decreased* performance of this component of fitness in hermaphrodites: the evolution of lifespan and the decreased viability of progeny at later reproductive ages of selfed hermaphrodites. With respect to the former, and under life history theory, increased lifespan could result from a negative genetic or functional relationship between sperm and processes required for somatic maintenance. If hermaphroditic sperm was negatively acted upon by selection but the female function of hermaphrodites was positively selected, then a sexual conflict within hermaphrodites is likely to have been generated. Evidently, this hypothesis needs to be evaluated empirically before any definite conclusions can be drawn. One such possibility is to perform artificial insemination of *tester* females with sperm from hermaphrodites, a methodology that is available for this organism (LaMunyon and Ward 1994).

Androdioecious populations evolved in a remarkably similar fashion to Dioecious populations. Because in most cases initial differentiation of populations was not found, we conclude that the rates of cross-fertilization observed in Androdioecious populations during experimental evolution were sufficient to generate similar phenotypic responses compared to Dioecious populations. Therefore, higher inbreeding did not seem to have impacted significantly the rate of adaptive evolution of genetically diverse populations.

Adaptation from mutation

The magnitude of the fitness increase of genetically diverse and of homogeneous experimental populations was similar (15% and 13%, respectively). However, these values should be interpreted with caution since direct comparison of different levels of initial genetic variation within each mating system was not available and hence these values correspond to genetically homogeneous Androdioecious populations ($isoA_{1-6}$) and to genetically diverse Dioecious populations (D_{4-6}). The rate of fitness increase due to mutational input was nevertheless surprisingly high *per se*: it is comparable to rates of fitness increase of some experimentally evolved populations of microbes such as *Pseudomonas cichorii* (15%, Weigand 2011) or *E. coli* (17%, Perfeito et al). Therefore, our results show that mutation can be an important source of genetic variation in sexual, multicellular organism in relatively short periods of time. Furthermore, the phenotypes for which evolutionary trajectories were obtained (male competitive performance) show the predicted dynamics: a) genetically homogeneous populations took more generations to start responding (between 50 and 60), although by the end of the experiment showed phenotypic responses similar to those of genetically diverse populations and b) genetically divese populations responded fast, with phenotypic means changing in the first 30 generations of experimental evolution. Overall, our results indicate that limited genetic variation may limit the rates but not the patterns of multivariate phenotypic evolution. The extent of the response of genetically homogeneous populations further suggests that current estimates of adaptive mutations rates for *C. elegans* are likely to provide lower bounds. Previous estimates of adaptive genomic mutation rates in *C. elegans* obtained from sequencing data ($U_a=3.8x10^{-5}$, Denver *et al* 2010) may still miss a substantial number of mutations, since given this value our experimental populations would be expected to contain only four novel mutations ($Ua \times N_e \times$ generations ≈ 4). Alternatively, pleiotropic effects of each of these mutations could in part explain the extent of the phenotypic response. The observed heterogeneity of evolutionary trajectories among genetically

homogeneous Androdioecious populations further suggests than novel alleles created by mutation are likely to interact epistatically with alleles at other loci.

Epistasis between genetic loci, coupled to potential pleiotropic effects of individual loci may therefore underlie the surprising uniformity of the observed phenotypic responses of experimental Androdioecious populations of *C. elegans* with so disparate levels of initial segregating variation. This sort of genetic mechanisms may therefore prove extremely important not only for adaptive explanations of sex and recombination (Otto and Gerstein 2006), but also for other phenomena such as the evolution or maintenance of mating systems.

Concluding remarks

We described adaptation to a novel environment of populations of sexually-reproducing nematodes. While initial levels of standing genetic variation do seem to significantly affect rates of adaptation at the time scales presented here, they do not necessarily lead to different patterns of life history evolution. Under these conditions, the fitness landcape could be quite smooth.

It is somewhat paradoxical that the same genetic phenomena many times argued to be at the basis of the "cost of complexity" – the delay of adaptation because of higher interdependency of phenotypes – may actually be the ones allowing populations of these so-called more complex organisms to respond so extensively (and intensively) to new environmental challenges and in quite uniform patterns.

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