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The effects of sex and competition on evolutionary survival of *Chlamydomonas reinhardtii* populations in deteriorating environments

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Declaration

I declare that this thesis has been composed by myself and the research presented is my own original work. Where other individuals have made contributions, this has been clearly stated within the text.

Nikola Petkovic

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Lay summary

Ongoing global change (such as habitat destruction, pollution, climate change) imposes negative impact upon every living thing. This is why during the last couple of decades, many evolutionary biologists have been focusing their research on understanding adaptation to harmful conditions populations are not naturally adapted to and which impose the risk of extinction. This process of adaptation is termed evolutionary rescue. The main aim of these studies is to reveal all the factors positively or negatively associated with the chance of evolutionary rescue, so we could estimate which species will be the most likely to go extinct under global change and potentially prevent extinctions. The impact of many factors influencing the probability of evolutionary rescue is now well-known, but a few still remain insufficiently understood, namely the way that species reproduce and how they interact with one another.

The main goal of my research was to investigate whether and how the way that species reproduce and interact with one another affect the probability of evolutionary rescue. To achieve this goal, I set up a series of experiments, by cultivating the populations of a single cell alga, *Chlamydomonas reinhardtii,* exposed to various stressful conditions, and monitoring their survival and density. To investigate how the way that species reproduce affects survival, I allowed the populations to reproduce either sexually, asexually or both. To investigate how the presence of other species affects survival, I cultivated the populations either in the presence or absence of different competitors.

I first let the environment deteriorate in three different rates by increasing the level of salinity. I found that populations were more likely to survive and adapt if environmental change proceeded in small steps. The influence of mode of reproduction was less prominent, with sex proving to be most beneficial when environment changed in a moderate way.

I then tested whether sex is beneficial if the populations of the focal species compete with another species (*Chlamydomonas moewusii*) while the environment deteriorates. I found that sexual populations were more likely to survive both in the presence and absence of the competitor.

I then tested will the chance of evolutionary rescue change if more related competitors compete. Similarly, I investigated whether species that naturally occur in similar habitats compete more intensely and thus may be more likely to go extinct. I found that the identity of competitor affects the probability of survival, but I found no effects of relatedness or habitat similarity between species on the probability of evolutionary rescue.

Finally, to test under which environmental factors sex could be beneficial enough to be maintained within populations for the longer intervals of time, I subjected experimental populations to environment changing in a directional way or fluctuating over the course of time and monitored whether the frequency of sex increases. The frequency of sex remained approximately the same if environment changed directionally, and dropped if environment fluctuated over the course of time.

My results show that both sex and competition affect the chance of evolutionary rescue. Sex was mostly beneficial and competition was detrimental for the population survival. However, these effects may depend on other factors, such as mode of environmental change or the identity of a competitor species.

Abstract

Ongoing global change has made understanding the factors that affect adaptation and survival of populations in the context of changing environments a central problem in evolutionary biology. Special focus has been given to the probability of survival through genetic adaptation to lethal environments; a process termed evolutionary rescue. Many studies of this process, both theoretical and empirical, have been carried out over the last two decades. As a result, we now understand how a number of factors may affect the probability of population survival. However, two factors that are known to affect evolutionary responses, mode of reproduction and interspecific interaction, have received limited attention.

The main aim of my work was to investigate whether and how mode of reproduction and negative interspecies interactions (competition) affect the probability of evolutionary rescue. To achieve this goal, I set up a series of selection experiments, by propagating populations of unicellular alga *Chlamydomonas reinhardtii* in various stressful conditions, and monitored their survival and fitness. To investigate the effect of sex in these experiments, I manipulated mode of reproduction, by constructing the experimental populations allowed to reproduce either only sexually or asexually or both. To investigate the effect of competition, I manipulated the presence of the competitor(s) in the experimental populations, by cultivating them either in presence or absence of the competitor.

I first tested the effect of rate of environmental deterioration and mode of reproduction on extinction dynamics and evolutionary rescue of the experimental populations. I found positive correlation between the rate of extinctions and the rate of environmental deterioration. The experiment revealed an interaction between mode of reproduction and the rate of deterioration, manifested through significantly reduced extinction rate of sexual populations relative to asexual populations in environment deteriorating at intermediate rate.

I then investigated the effect of sex and competition on the probability of evolutionary rescue, by propagating the experimental populations in environment

deteriorating in a simple way (the change comprising a single abiotic factor) and complex way (the change of both abiotic and biotic factors). I found the negative effect of competition on the probability of evolutionary rescue, and beneficial effect of sex in both types of environmental deterioration, reflected in higher number of rescued populations relative to asexual group.

I then tested whether phylogenetic relatedness between a competitor and the focal species and the extent of their ecological similarity affect the likelihood of evolutionary rescue, by subjecting the experimental populations to the presence of 10 different competitors, isolated from two different types of habitats, and each being positioned on a different branch of the phylogenetic tree of *Chlamydomonas* genus. The probability of evolutionary rescue was contingent on the identity of a competitor species, but the results showed no significant effects of phylogenetic relatedness and ecological similarity.

Finally, I investigated which experimental factors could potentially select for the long-term maintenance of sex, by subjecting the experimental populations to different types of selective environments (directional and fluctuating change of abiotic factors, the presence of the competitor) and monitoring the frequency of sex over the course of time. No selective environment significantly increased the rate of sex in the experimental populations. In contrast, I found reduction in frequency of sex in the populations subjected to fluctuating environmental change.

My results demonstrate that both mode of reproduction and competition affect the probability of evolutionary rescue, which is generally positively affected by sex and negatively affected by competition. However, these general effects may be altered by other factors, namely mode of environmental change and the identity of the competitor species.

Table of Contents

List of Figures

List of Tables

1. General Introduction

1.1 Evolutionary rescue

Adaptation to changing environmental conditions has always attracted attention of evolutionary biologists (for example Darwin, 1859; Weissmann, 1889; Fisher, 1930; Muller, 1932; Wright, 1932; Haldane, 1957). During the recent decades, this general interest has increased due to widely the recognized issue of global change (for example Falkowski et al., 1994; Chivian and Bernstein, 2008; Folger, 2009; Barnosky et al., 2012), which is already moving environmental conditions outside the range of physiological tolerance of many species (Parmesan, 2006). Consequently, the rate of species extinctions has become unprecedented, being as far as 1000 times higher than the background extinction rate for some groups of organisms (May and Lawton, 1995; Chivian and Bernstein, 2008), and recognized by some scientists as "the sixth mass extinction event" (Chivian and Bernstein, 2008). Hence, understanding the patterns of survival and extinctions among species has becoming increasingly important for assessment of vulnerability of the extant species, which could potentially help contribute to conservation efforts, with the main goal of mitigating the detrimental effects of global change.

The response of a species to a changing environment occurs via at least two different mechanisms. Firstly, a species may respond through phenotypic plasticity (without genetic change). Examples of such plastic responses include a change in phenology of European plants, manifested thorough an earlier onset of leafing, flowering and fruiting, due to an increase of mean monthly temperatures preceding these events (Menzel et al., 2006); a response to a single stressful factor of budding yeast may include activation of general stress response mechanism, protecting against other types of stressors (Berry and Gasch, 2008). The second type of response includes migration to less detrimental habitats. For instance, there is a general trend in range shift of species towards the poles (Parmesan and Yohe, 2003) and higher elevations (Telwala et al, 2013), arisen as a consequence of climate change.

If neither of these two mechanisms can provide an adequate response to a changing environment, a species must adapt through genetic change or go extinct (to "adapt or die"; Bell and Collins, 2008). If alleles conferring an advantage exist in the genetic pool of the population, selection can act on standing genetic variation, resulting in the rise in frequency of these alleles (a process termed "sorting"; Bell, 2008). However, if such alleles are absent, a population must adapt through *de novo* mutations. This process of adaptation to conditions that would have otherwise led to extinction of the ancestral population has been termed "evolutionary rescue" by Gomulkiewicz and Holt (1995), who were the first to provide a basic theoretical framework for this phenomenon. The general concept of evolutionary rescue is summarized in Figure 1.1 (see below). A maladapted population has negative growth rate in novel, detrimental conditions, and consequently, mean fitness of the population (population density) decreases as a function of time. Gomulkiewicz and Holt (1995) developed a heuristic concept of a critical (low) population density (assigned as $\mathbf{N_c}$ in Figure 1.1) manifested through high susceptibility of the population to detrimental effects of environmental or demographic stochasticity (e.g. fixation of deleterious alleles due to genetic drift), which increase the risk of extinction. The population reaching $\mathbf{N_c}$ at the time point $\mathbf{t_E}$ is vulnerable to extinction because of negative growth rate. Extinction will occur if all individuals die out before a resistant genotype appears through mutation in the hypothetical time point $\mathbf{t_R}$, and rise in frequency above the critical low density, at hypothetical time point $\mathbf{t_P}$. The period of the highest probability of extinction corresponds to an interval between $\mathbf{t_P}$ and $\mathbf{t_E}$ (Gomulkiewitz and Holt, 1995). As firstly suggested by Maynard Smith (1989), adaptation to a stressful environment could be comprehended as a "race" between (negative) demographic processes and evolution (Gomulkiewitz and Holt, 1995).

To the best of my knowledge, the first experimental evidence of evolutionary rescue event was recorded by Bell and Gonzalez (2009), who subjected the experimental populations of yeast to lethal concentrations of salt. They obtained the "U"-shaped curve of population growth, characterized by the initial decline of maladapted populations and subsequent recovery, which is a hallmark of evolutionary rescue predicted by Gomulkiewicz and Holt (1995). Furthermore, they manipulated population size and found that probability of evolutionary rescue is directly proportional to population size. Numerous experimental studies have been performed after this pioneering study, in order to identify factors

positively or negatively correlated with the probability of evolutionary rescue (for the most recent review of the topic, see Carlson et al., 2014).

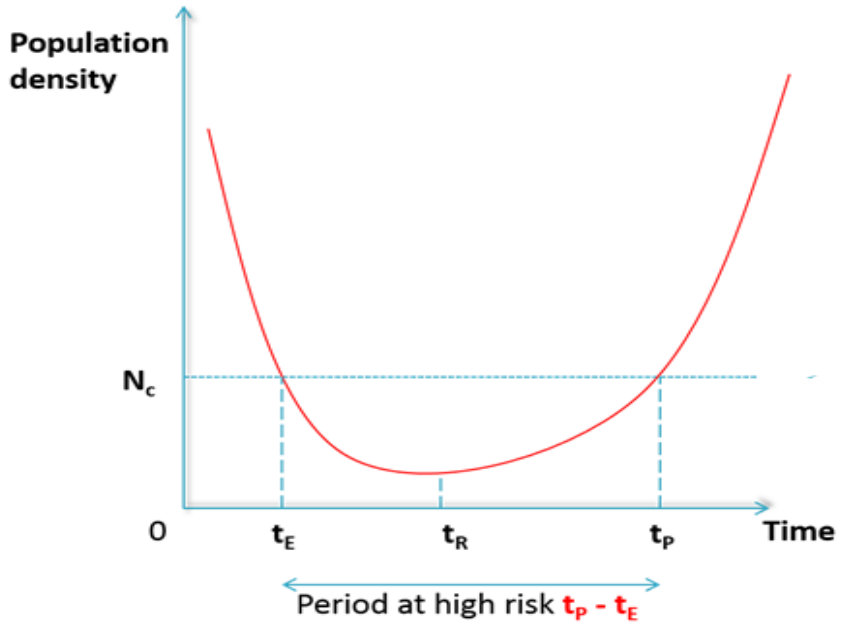

Figure 1.1 – The basic concept of evolutionary rescue (adapted from Gomulkiewicz and Holt, 1995); N_c – threshold of a critical population density; t_E – time point at which population size reaches the critical threshold; t_R - time point at which a resistant genotype potentially arises; t_P - time point at which the genotype conferring resistance increases in frequency above threshold of a critical population density and becomes fixed in a population.

The probability of adaptation (and therefore evolutionary rescue) depends on the rate of environmental deterioration, since higher rates of change impose a proportionally higher demographic cost of adaptation, which in turn, as demonstrated by the classic work of Haldane (1957), limits the rate of evolution (Ridley, 1993). In addition, a high rate of environmental change implies high initial level of maladaptation, which in turn increases the rate of population decline and probability of drop under a threshold of critical population size (Carlson et al., 2014). Lynch and Lande (1993) considered a quantitative trait under stabilizing selection with a moving optimum imposed by a gradual environmental change and found that the maximal rate of environmental change that a population can sustain is governed by the maximal reproductive rate of the population. This is because populations with larger reproductive output could track the moving optimum more efficiently (less burdened by demographic load imposed by selection). Furthermore,

they found that the maximal rate of change that could be withstood by a population increases with mutation supply rate, due to a proportional increase of genetic variance available for selection (Lynch and Lande, 1993).

Several experimental studies provided further evidence that probability of evolutionary rescue is negatively correlated with a rate of environmental change. Perron et al. (2007) manipulated the environmental harshness in *Pseudomonas aeruginosa*, by subjecting the populations to five antibiotic treatments (single antibiotics, alternating exposure to one out of two antibiotics and two antibiotics simultaneously) and found that resistance arose more readily when populations were subjected to a single antibiotic treatment. Furthermore, the probability of resistance of 'sink' populations subjected to antibiotics was proportional to the immigration rate from the source population. Bell and Gonzalez (2011) subjected metapopulations of yeast to three different rates of increasing salinity and manipulated mode of dispersal of component populations (either local or global). They found that a slower rate of environmental deterioration and local dispersal resulted in higher probability of evolutionary rescue. Lindsey et al. (2013) subjected experimental populations of *Escherichia coli* to three different rates of increasing concentration of a single antibiotic and found the highest number of evolutionary rescue events in the treatment group subjected to the most gradual rate of antibiotic increase. In addition, they found that the mutational pathway leading to evolutionary rescue changes with a rate of environmental deterioration. Adaptation to a high rate of increase of the antibiotic concentration occurred through fixation of single mutations in all populations. In contrast, adaptation to a gradual and moderate rate of increasing antibiotic concentration always occurred through fixation of multiple mutations. However, the first mutation arisen during exposure to the gradual and moderate rate of antibiotic increase did not overlap with any mutation conferring advantage to the high rate of antibiotic increase (with the exception of a single population). The authors inferred that mutations conferring advantage to the gradual and moderate rate of environmental change are inaccessible under the high rate of change, which in turn, affects dynamics of adaptation.

The rate of environmental change also affects dynamics of adaptation (Kopp and Hermisson, 2007; Collins and de Meaux, 2009). Adaptation to a high rate of environmental change occurs via fixation of mutations of large fitness effects (Collins and de Meaux, 2009). These mutations arise rarely (Kassen and Bataillon, 2006; Eyre-Walker and Keightley, 2007),

but undergo a rapid sweep through a population once appeared (Collins and de Meaux, 2009). However, once fixed, these mutations may limit the rise of any subsequent mutation due to sign epistasis (a dependence of the fitness effect of a mutation on the genetic background) (Weinreich et al., 2005), thus hampering the adaptive walk of a population. Adaptation to a gradual rate of change occurs via mutations of small effects which are the most frequent in the distribution of mutations (Eyre-Walker and Keightley, 2007). However, due to weaker selection, which operates during the gradual environmental change, these mutations may fail to reach fixation, or otherwise fix in less predictable time (periodic fixation; Collins and de Meaux, 2009). Furthermore, in large asexual populations with higher input of mutations, adaptation may slow down due to competition of multiple beneficial mutations through clonal interference (Gerish and Lenski, 1998). In addition, the gradual rate of environmental change reduces the fitness effect of fixed beneficial mutations (Collins et al., 2007). However, due to availability of more mutational pathways under the gradual rates of environmental change, populations may better explore the fitness landscape (Collins and de Meaux, 2009), and reach the fitter endpoints of adaptive walk. This is experimentally demonstrated by Collins and de Meaux (2009) who subjected the treatment groups of *Chlamydomonas reinhardtii* experimental populations to the same final magnitude of stress (low phosphate - starvation), but at different rates of phosphate decrease. They found that the level of adaptation, measured as growth rate in the final environment, was significantly higher for the populations subjected to more gradual level of phosphate decrease. Hence, these populations reached fitter endpoint of adaptation.

The initial population size is the primary determinant of the rate of change a population can withstand (Carlson et al., 2014), since adaptation to a higher rate of environmental change is proportional to a demographic load (the cost of adaptation). Consequently, smaller populations can withstand comparatively lower rates of environmental change. Furthermore, small populations are more likely to reach the critical low population size and may persist under high risk of extinction for shorter intervals of time relative to larger populations (Gomulkiewicz and Holt, 1995). Population size is also usually positively correlated with the amount of genetic variation (for example, Hague and Routman, 2016). Hence, selection may operate faster in larger populations.

The impact of a population size on the probability of evolutionary rescue has been a subject of several experimental studies. Willi and Hoffmann (2009) maintained populations

of *Drosophila birchii* of different census sizes (20, 100 and 1000 individuals) for 10 generations, before subjecting them to a heat-knockdown selection (for 5 generations). The smallest populations suffered a rapid extinction due to adverse effects of genetic drift, lower growth rate and higher stochasticity in growth rate across generations (Willi and Hoffmann, 2009). In contrast, larger populations had a higher reproductive output and less variation in growth rate. The authors used these fitness parameters to predict the probability of survival under the environmental change in a computer simulation study and found that median survival time (in generations) is directly proportional to a population size. Ramsayer et al. (2013) manipulated the microcosm volume of *Pseudomonas fluorescens* and monitored the incidence of evolutionary rescue events as a response to 5 different doses of antibiotics. The microcosm volume was positively correlated with the probability of evolutionary rescue. Moreover, the advantage of larger population size on the probability of evolutionary rescue was consistent for all doses of antibiotics. In addition to the advantageous effects of increasing the probability of evolutionary rescue, population size is positively correlated with mean fitness of the rescued populations. Samani and Bell (2010) manipulated a culture volume of yeast and measured the fitness of the rescued populations subjected to a deteriorating environment (an increasing salt concentration). They found that adaptation to the deteriorating environment (measured as a doubling rate) was a log-linear function of population size.

Since selection acts more efficiently in populations with higher additive genetic variance for fitness (Fisher, 1930), the probability of evolutionary rescue will depend on standing genetic variation of a population (Burger and Lynch, 1995), unless the advantageous alleles are absent from the genetic pool of the populations, which may impose a genetic limitation on evolutionary rescue (or “genostasis”, as defined by Bradshaw, 1991; Carlson et al., 2014). Standing genetic variation depends on population size, because finite populations may suffer a reduction in genetic variation due to detrimental effects of environmental stochasticity (Burger and Lynch, 1995) or genetic drift. Agashe (2009) manipulated a degree of genetic diversity of populations of flour beetle *Tribolium castaneum* (by manipulating the number of strains per treatment group), and subjected them to a different habitat treatment (ancestral or novel food source and combination of both). She found a strong correlation between genetic diversity and probability of survival and importantly, higher effects of genetic diversity in novel environment. In addition, Agashe et al. (2011) demonstrated that genetically variable

populations of the same model organism adapt faster to novel environment by enhancing the growth rate and maintain a larger population size in the long term. Ramsayer et al. (2013) subjected genetically diverse and clonal populations of *Pseudomonas fluorescens* to different concentrations of streptomycin and recorded a higher number of evolutionary rescue events in genetically diverse populations (60-80% of populations in this treatment group were rescued, contrasting with 40% of the clonal treatment group). In the study by Lachapelle and Bell (2012), genetically diverse populations of *Chlamydomonas reinhardtii* survived for a longer interval of time than asexual clonal populations, when subjected to a gradually deteriorating environment (an increase of salt concentration).

In the absence of alleles conferring an advantage in a changing environment or recombination generating novel (favourable) combinations of alleles on different loci, adaptation occurs solely through fixation of novel beneficial mutations. Hence, the probability of evolutionary rescue of a clonal population depends on mutation supply rate (Bell and Gonzalez, 2009). This can be defined by the equation: $P = 2N_0U\varphi\ (r_0–r_1)/r_0$, which shows that probability of fixation of beneficial mutation depends on population size (N_0), mutation rate (U), proportion of beneficial mutations (φ), and the extent of a change of the current (negative) growth rate of a population (r_0) before the rise of a mutation which beneficially affect growth (r_1) (Orr and Unckless, 2008; Bell, 2008; Bell and Gonzalez, 2009). Furthermore, the probability of fixation of beneficial mutations directly depends on the probability of "avoiding" their stochastic loss by genetic drift (Kirkpatrick and Peischl, 2013). Haldane (1927) found that fixation probability for a beneficial mutation is proportional to a selective advantage it confers: $\Pi = 2s$. However, beneficial mutations are relatively rare events (Eyre-Walker and Keightley, 2007), and most of them confer small fitness advantage (de Visser and Rozen, 2005). The estimate of the rate of beneficial mutations in *Escherichia coli* is 5.9×10^{-8} per genome per generation (Rozen et al., 2002). Since the rate of spontaneous mutations of the same species is estimated to be 0.0025 per genome per generation (Drake et al., 1998), only one mutation in every 10^4 will be beneficial (Rozen et al., 2002). The estimated average fitness effect of beneficial mutations (s) is 0.024, contrasting with 3-4 times higher fitness effects of mutations that ultimately reached fixation (Rozen et al., 2002). Thus, due to a relatively low occurrence and slow fixation rate of beneficial mutations, total mutation supply rate will be primarily driven by population size and mutation rate.

The probability of evolutionary rescue may also increase if exposure to lethal conditions is preceded by exposure to sub-lethal level of the same stressor. In a previously described experiment by Samani and Bell (2010), mutations conferring the advantage in lethal medium started to spread in the population in sub-lethal level of stress. Furthermore, a degree of beneficial effect of these mutations (an increase of a doubling rate) was proportional to effective population size, which was interpreted as genetic correlation between a response to two successive levels of stress (Samani and Bell, 2010). Gonzalez and Bell (2013) subjected experimental populations of two yeast species, *Saccharomyces cerevisiae* and *Saccharomyces paradoxus* to various levels of salt concentration, before subjecting them to lethal salt concentration. They found an interaction between species and salt concentration on the probability of evolutionary rescue. The number of evolutionary rescue events was proportional to the salt concentration experienced prior to exposure to lethal conditions in *S. cerevisiae,* but the pattern was reversed in *S. paradoxus.* Furthermore, for both species, mean fitness of the rescued populations was the highest for large populations with the previous history of exposure to lower level of salt, and small populations with the history of exposure to higher level of salt (Gonzalez and Bell, 2013).

Overall, the probability of evolutionary rescue will be higher for larger populations with a higher genetic variation and mutation supply rate, subjected to lower rates of stress and with the history of exposure to sub-lethal levels of stress. Other factors that affect the probability of evolutionary rescue which received less attention in the experimental studies are mode of reproduction and a type of interspecies interaction. Since the main aim of this Thesis was to investigate the effect of these factors on evolutionary rescue, I will cover them in more details in the following sections.

1.2 The effects of sex on adaptation and evolutionary rescue

Despite the fact that the great majority of extant eukaryotes are sexual, sex still remains one of the most intriguing puzzles in evolutionary biology (Otto and Lenormand, 2002). The reason for this reflects the numerous disadvantages and costs of sex. These costs can be significant, and so the continued existence of sex implies large potential benefits. Unexpected difficulty in providing an explanation for such a widespread phenomenon brought sex the title of “the Queen of problems in evolutionary biology” (Bell, 1982). Since considering the effects of sex on adaptation to deteriorating environments

requires explaining the effects of sex in a more general context, I will review current major hypotheses for the maintenance of sex by natural selection, before explaining its potential influence on the probability of evolutionary rescue.

An inevitable consequence of eukaryotic sexual cycle is a re-assortment of genes as a result of crossing-over and independent assortment of chromosomes. These processes can potentially break up favourable combinations of alleles, accumulated through selection (Barton and Charlesworth, 1998), resulting in a decrease of mean fitness of a population termed recombination load, which is experimentally confirmed (for example Greig et al., 1998; Colegrave et al., 2002). Furthermore, sex imposes direct costs, such as arrested growth due to meiosis (considerably slower than mitotic division) or direct contact between gametes (a prerequisite for fertilization) being associated with several types of risks (parasite transmission, exposure to predation) (Lewis, 1987). Moreover, an obligate sexual female suffers a two-fold reduction in a reproductive output as a cost of producing males (compared to a hypothetical asexual female), termed “the two-fold cost of sex” or “the cost of males" (Maynard Smith 1971; Williams, 1975). Considering all these costs, the question which factor accounts for the maintenance of sex by natural selection could be rephrased: why does amixis not replace sex? However, sex is a prevalent strategy of most organisms (Bell, 1982) despite the costs, which implies that sex must confer a compensatory selective advantage for an individual or a population.

A group of hypotheses, suggested as an explanation for the maintenance of sex, are based on an argument that sex provides a proximate (immediate) benefit on an individual level. The major argument of a ‘DNA repair hypothesis’ is that processes involved in recombination provide a template for repair of double-stranded DNA damage (Bernstein et al., 1988; Michod and Levin, 1988). While these processes could account for the evolution of sex (Barton and Charlesworth, 1998), they are not sufficient to explain its maintenance in extant populations. The reasons lie in the fact that DNA repair does not require the bringing together of homologue chromosomes, except for double-stranded damages which are actively induced during meiosis (Barton and Charlesworth, 1998). The fact that the same type of damage is actively induced during the very process for which the suggested function is to eliminate it, represents a major disadvantage of this hypothesis. Furthermore, there is evidence that double strand breaks can be efficiently repaired even during mitosis (Nassif and Engels, 1993).

Sex might be maintained as a by-product of mechanisms that enable transfer of an infectious genetic element to another host (Redfield, 2001; Otto and Lenormand, 2002). However, under this interpretation, a host does not benefit from sex while still pays the costs, which implies that each asexual mutant, freed from such costs, should rise in frequency and outcompete the sexual individuals. Furthermore, while providing an explanation for the maintenance of outcrossing, this hypothesis does not explain the maintenance of recombination.

Recombination might potentially be beneficial due to immediate reduction of deleterious mutations (Kondrashov, 1993), as a result of the cellular processes activated during (but not directly linked to) meiosis and recombination. However, maintenance of such mechanisms by natural selection requires that deleterious mutations always have a particular molecular nature, which is unlikely (Kondrashov, 1993).

Overall, the hypotheses regarding the maintenance of sex by natural selection based on its potential proximate benefits lack generality and/or experimental evidence. Hence, most contemporary evolutionary biologists argue that advantageous effects of sex are manifested indirectly, through an increase of genetic variability among the progeny, and thus more efficient response of selection. While the original idea stemmed from Weismann (1889), many population-genetic models (hypotheses) have been derived during the last century to provide a theoretical framework for this concept. All of them assume the presence of two mechanisms: a) a mechanism for generating a constant directional selection (a source of additive genetic variance for fitness); b) a mechanism that generates negative associations between beneficial alleles on different loci (negative linkage disequilibrium) (Burt, 2001). Kondrashov (1993) united these hypotheses under a collective term “Variation and Selection hypotheses”.

The Fisher-Muller hypothesis for the maintenance of sex has been mathematically formulised by the classic work of Fisher (1930) and Muller (1932). Generally, this concept assumes a changing environment and presence of mutations conferring an advantage to novel conditions being under negative linkage disequilibrium, generated by genetic drift in finite populations. Because of negative linkage disequilibrium, adaptation is impeded. Furthermore, adaptation may be reduced due to interference of selection acting simultaneously on different (linked) loci, termed Hill-Robertson effect (Hill and Robertson, 1966). The major advantage of sex based on the concept of the Fisher-Muller hypothesis is

a faster assembly of multiple beneficial mutations in a single individual, which in turn increases the response of natural selection. More efficient selection in sexual populations implies faster adaptation to a changing environment than asexual populations. Slower fixation rate of beneficial mutations in asexual populations can ultimately result in competition of genotypes carrying different mutations (Muller, 1932), consequently slowing down the adaptation (the process termed clonal interference; Gerrish and Lenski, 1998). A potential limitation of this hypothesis is an assumption of a relatively low mutation rate, because with a sufficiently high mutation rate, multiple beneficial mutations may appear in asexual populations (Kim and Orr, 2005) and thus the advantage of sex diminishes. Furthermore, the effect of sex might be reduced in infinite populations, because under this condition, all beneficial mutations may appear in the expected frequency (linkage disequilibrium diminishes) (Kim and Orr, 2005).

The Fisher-Muller hypothesis has gained substantial empirical support during the recent two decades. Greig at al. (1998) manipulated the genetic background of sexual populations of yeast (either homozygous or heterozygous) and allowed a direct competition with asexual populations in stressful conditions (an elevated temperature). They found that heterozygous populations outcompeted the asexual counterpart in great majority of mixed populations. Given that homozygous populations did not show the same advantage, the likely explanation for the competitive success of heterozygous sexual populations was accumulation of multiple beneficial mutations. Colegrave et al. (2002) allowed a single sexual episode in populations of *Chlamydomonas reinhardtii* and compared the rate of adaptation in novel environments (heterotrophic growth in all combinations of four different carbon sources) with that of the asexual populations. After the initial decrease (attributable to recombination load), followed by an increase of variance in fitness, mean fitness of sexual populations exceeded that of asexual populations (Colegrave et al., 2002). Despite the fact that mean fitness of both types of populations equalised by the end of the experiment, the results indicate that adaptation to novel environment can be facilitated after only a single episode of sex. However, sex did not provide a significant advantage in complex environments. Kaltz and Bell (2002) performed a similar experiment, with the main difference in induction of three successive episodes of sex during adaptation to novel environments. The results showed that repeated episodes of sex provided a long-term advantage in mean fitness relative to asexual populations. Importantly, the advantage of sex increased with an increase of environmental complexity. Colegrave (2002) subjected

the experimental populations of *C. reinhardtii* to novel growth medium and found that the relative fitness of sexual populations compared to asexual populations was directly proportional to population size prior to induction of sex. This result suggests that sex reduces the constraint of adaptation imposed by the clonal interference in larger populations. Becks and Agrawal (2011) subjected sexual and asexual populations of monogont rotifer *Brachionus calyciflorus* to novel environments (elevated concentration of NaCl and novel food source) and found that sexually derived offspring had higher fitness (measured as lifetime reproduction per female) than asexually produced genotypes in the initial stages of adaptation, but the pattern reversed when adaptation plateaued (Becks and Agrawal, 2011). Bell (2013) found that the populations of *C. reinhardtii* with the history of obligate sexual reproduction had higher probability of survival relative to asexual populations, when subjected to growth in the absence of light.

Another explanation for the maintenance of sex, proposed by Muller (1964), is also based on the effect of random genetic drift in finite populations, which causes a gradual loss of a mutation-free genotype (Kondrashov, 1993). This process proceeds in a fashion analogous to a ratchet, in which a single click corresponds to a loss of a genotype least loaded with deleterious mutations (hence the term of the effect, "the Muller's ratchet"). Thus, in absence of recombination and backward mutations, an asexual population will gradually accumulate deleterious mutations, which may consequently lead to a mutational meltdown (Lynch et al., 1993). In contrast, a sexual population will be able to restore the genotype least loaded with deleterious mutations by means of recombination. However, Muller's ratchet operates slowly in large populations (Judson and Normark, 1996; Eyre-Walker and Keightley, 2007). Furthermore, several factors other than recombination could slow down (or arrest) the ratchet, such as lower mutation rate per locus or lower genome size (Judson and Normark, 1996). Kondrashov (1994) found that sufficiently strong synergistic epistasis between deleterious mutations may also arrest the ratchet. In addition, this hypothesis fails to explain the "ancient asexual" species (e. g. bdelloid rotifers), which had persisted for millions of years without any evidence of meiosis or recombination (Judson and Normark, 1996), and would have likely suffered the negative effects of Muller's ratchet due to substantial accumulation of deleterious mutations.

Deleterious mutations may act synergistically (negative epistasis) (Kimura and Maruyama, 1966), so the potential benefit of sex may still reflect in reduction of mutation

load, as predicted by Muller (1964). By assembling these mutations in some individuals which are, due to a low fitness caused by mutation load, likely to be eliminated from a population, sex potentially makes purging of deleterious mutations more efficient relative to asexual mode of reproduction (Eyre-Walker and Keightley, 2007). This hypothesis, termed "Mutational Deterministic Hypothesis" (Kondrashov, 1993), requires two conditions to be met to be operational: a) deleterious mutation rate (U) >1 (Kondrashov, 1982; Kondrashov, 1988) and b) synergistic epistasis between mutations must be common. If these conditions were met, sexual populations would be more efficient in purging of deleterious mutation than asexual populations, irrespective of population size (Elena and Lenski, 1997; Eyre-Walker and Keightley, 2007). Keightley and Eyre-Walker (2000) found that, while most of the mutations are deleterious, the mutation rate is directly proportional to a generation time, being well under 1 in slower reproducing taxa. The experimental studies investigating the frequency of negative epistasis showed ambiguous results, both supporting (De Visser et al., 1996; Whitlock and Bourget, 2000) and dismissing (Elena and Lenski, 1997) the Mutation Deterministic hypothesis. Zeyl and Bell (1997) provided evidence for higher efficiency of purging of deleterious mutations in sexual populations of yeast relative to asexual populations, manifested through significantly higher relative fitness of sexual populations in the ancestral (benign) medium. However, the authors could not differentiate between two possible fitness effects of deleterious mutations (either synergistic or additive). In contrast, Renaut et al. (2006) found no evidence for mutation clearance in *Chlamydomonas reinhardtii*, given that sexual and asexual populations of this alga had similar mean fitness in benign conditions.

Negative linkage disequilibrium may be established under selection alone (in the absence of random effects such as drift) if there is a negative epistasis between two alleles on different loci, which makes this haplotype disadvantageous in the current environmental conditions. However, this haplotype may become favourable when conditions change, but still remain underrepresented in a population due to the past operation of (negative) selection. This in turn, reduces the additive genetic variance for fitness and, consequently, decreases the rate of adaptation (Barton, 1995). Consider the situation in a hypothetic haploid population with two loci (i.e. A and B) affecting a quantitative trait, each with two alleles (A+ and A-, B+ and B-). Another assumption is that + alleles are advantageous, but under negative epistatic interaction, and all alleles are represented at equal frequencies (hence the expected haplotype frequencies under equilibrium would be 0.25 for each

haplotype). Due to negative interaction between the alleles in the favourable haplotype, the most represented haplotypes would be the ones conferring intermediate fitness (either A+B- or A-B+), which implies a decrease in the additive genetic variance for fitness. This in turns impedes the response of the selection when environmental conditions change. The modifier alleles which increase the rate of recombination and sex could rise in frequency if there is a long-term advantage of sex (a condition to be met here is a change of environmental conditions) compensating for the short-term disadvantage (a rise in frequency of alleles under negative epistasis which implies a decrease of mean fitness of a population). Given that this hypothesis does not rely on a large population size or genetic drift, it is referred to as "Environmental Deterministic Hypothesis" (Kondrashov, 1993). However, the modifier alleles increasing the frequency of recombination and sex could only rise in frequency provided there is negative and weak epistasis (Barton, 1995; Otto and Gerstein, 2006). However, to date, there is no conclusive experimental evidence that either negative and/or weak interactions between alleles on different loci are common in nature, which is why this hypothesis is unlikely candidate to explain the maintenance of sex in nature (Otto and Gerstein, 2006).

A group of hypotheses (in some literature collectively referred to as "ecological hypotheses"; for example, Lively and Morran, 2014) consider spatial heterogeneity as the primary factor that could select for the maintenance of sex. Williams (1975) set out the "Sib-competition model", by assuming a competition among the progeny (and a competition of progeny with the parents) for the limited resources. He argued that if environment comprises different patches of resources, a genetically diverse progeny (generated by sex) would be able to exploit more available patches, which will, in turn, reduce the competition. Furthermore, a sexual offspring could outcompete an asexual (clonal) progeny, due to higher diversity of habitats it could utilise (Williams, 1975; Stearns, 1985). Even though some quantitative-genetic models have provided a support for Williams's hypothesis (for example, Bulmer, 1980), the major critique of the Sib-competition model is its limited applicability (only for organisms with high fecundity) (Stearns, 1985). Williams (1975) also suggested "the Lottery model", by arguing that, if there is a temporal fluctuation of environmental conditions, a generation of genetically variable offspring could increase the chance that some genotypes will match the current environmental conditions. Under this hypothesis, sex is a bet-hedging strategy (Lively and Morran, 2014), because temporal variance in fitness of the progeny is reduced at the

expense of lowered arithmetic mean fitness (definition of bet-hedging *sensu* Ripa et al., 2010). The main prediction that stems from the Lottery model is the prevalence of sex in temporarily variable environments (Lively and Morran, 2014). Bell (1982) suggested that sexual progeny may be advantageous if there is an availability of multiple niches in a spatially structured environment, which could reduce the competition among the progeny ("the Tangled Bank Hypothesis"). Hence, the main prediction of this hypothesis is the prevalence of sex in spatially heterogeneous environments. In addition, Bell performed an extensive analysis of geographical distribution of sexually reproducing species and found that sex is more often associated with constant (predictable) environments, thus dismissing the Lottery model. Despite the fact that the distribution of sexual species was consistent with the Tangled Bank Hypothesis, the major drawback of this hypothesis was reflected in a reliance on the restrictive parameters to operate, such as randomisation of the niches at each generation or one gene involved in specialisation to an individual niche parameter (Gray, PhD Thesis, 2011). For these reasons, the hypothesis was dismissed.

Sex may be maintained by natural selection because of the pressure of the biotic component of an environment. Parasites are selected to exploit the most common genotypes of a host (Van Valen, 1973; Jaenike, 1978; Hamilton et al., 1990; Lively and Jokela, 2002). Hence, rare host genotypes gain a selective advantage and may rise in frequency, thus imposing a selective pressure on the genotypes of parasites that could exploit it. This dynamic of co-evolution has been termed "the Red Queen" by Van Valen (1973), who made a well-known analogy to the Lewis Carroll's Red Queen ("you have to run as fast as you can to stay in the same place"; Carroll, 1872). In the same fashion, in order not to be eliminated from the "arms race", both predator and prey must constantly evolve. Bell (1982) suggested that sex could provide an advantage in this co-evolutionary race, by generating the host's genotypes parasites are not adapted to exploit (since they are rare), thus increasing the probability of survival of individuals which carry them. However, some studies suggested that this model operates on relatively restrictive conditions. Peters and Lively (1999) found that sign epistasis between haplotypes conferring an advantage to the biotic pressure has to fluctuate every 2-5 generations for this mechanism to account for the maintenance of sex. In addition, they found that sex is advantageous only if parasite virulence is moderate or high. Moreover, the Red Queen model assumes strong selection per gene, which requires a high incidence of species interaction and a large effect of these interactions on fitness (Otto and Gernstein, 2006). Nevertheless, many field and

experimental studies provided evidence consistent with The Red Queen hypothesis. Lively (1992) found a positive correlation between the frequency of males in the facultative sexual species *Potamopyrgus antipodarum* and the presence of parasites (trematodes). In addition, in a repeated study, carried out 10 years after the first one, he found similar correlation between sex and infection, which indicates that selection imposed by the parasite remains constant over the longer time scales (Lively and Jokela, 2002). Furthermore, there is evidence that coevolution with pathogen species (bacterium *Serratia marcescens*) selects for outcrossing in a facultative sexual *Caenorhabditis elegans* (Morran et al., 2011).

Most evidence suggests two hypotheses as the most plausible explanations for the maintenance of sex by natural selection: The Fisher-Muller and the Red Queen (Hartfield and Keightley, 2012). Generally, sex is the most beneficial under directional change of the abiotic component of environment, and fluctuating change of the biotic component of environment. The advantage of sex is manifested through the increase of the efficiency of selection, allowing populations to adapt more rapidly and maintain adaptation more effectively. Dynamic environments changing in directional or fluctuating manner are common in nature (Willi and Hoffmann, 2009). Hence, there is an ever-present selective pressure on populations to dynamically adapt to new combinations of the abiotic and biotic factors. Failure to adapt often results in a fitness decline and in the more extreme cases – local extinction (Willi and Hoffmann, 2009).

Given that sex facilitates adaptation to novel environments, we might suggest that it would be beneficial in deteriorating environments, and therefore affect the likelihood of evolutionary rescue. Survival in harsh environments could be facilitated by sex through faster accumulation of beneficial mutations and clearance of deleterious mutations which could reduce population size decline of a maladapted population, and thus increase the probability of the rise of new beneficial mutations which confer advantage to higher levels of stress.

Experimental studies investigating the effects of sex on the probability of evolutionary rescue have started relatively recently. Goddard et al. (2005) tested the rate of adaptation of sexual and asexual yeast populations in benign and deteriorating conditions (an increase of osmolarity and temperature). While mean fitness of both treatment groups was equal in a benign environment, relative fitness of sexual populations (measured as

growth rate) was significantly higher in a deteriorating environment. Lachapelle and Bell (2012) manipulated genetic diversity (low and high) and mode of reproduction (obligate sexual, facultative sexual and asexual) of *Chlamydomonas reinhardtii* populations, and monitored the extinction dynamic while the environment gradually deteriorated (an increase of salinity). They found that a combination of obligate sexuality and high genetic diversity significantly increased the survival rate. Furthermore, obligate sexual and high-diversity populations had significantly higher level of adaptation (measured as population size) relative to other treatment groups. Moreover, some populations (with the history of sexual reproduction), that had become adapted to grow in conditions lethal to the ancestral populations, evolved positive growth in marine conditions in a subsequent continuation of the experiment (Lachapelle, 2015). Since *C. reinhardtii* is normally found in freshwater or terrestrial habitats, but not seawater, sex had not only rescued it from extinction, but also expanded the ecological niche of these experimental populations.

The results of the experimental studies performed to date clearly indicate that sex is beneficial in deteriorating environments, which is reflected in an increase of both survival rate and adaptive rate. However, there are still outstanding questions. While all the experiments have been conducted in environments that deteriorated relatively gradually, it is unclear whether the effect of sex depends on the rate of environmental change. There is evidence that the dynamics of adaptation depend on the rate of environmental change (Collins and de Meaux, 2009), so we might expect variability in the effects of sex as a consequence. Furthermore, while all the experimental studies to date considered environments deteriorating in a relatively simple manner, it is still uncertain whether the effects of sex change in environments deteriorating more complexly. This is plausible, since evidence suggests that relative adaptive rate of sexual populations (Kaltz and Bell, 2002) and frequency of sex (Luijckx et al., 2017) increase with an increase of novel environment complexity.

1.3 Interspecies interactions and evolutionary rescue

Most theoretical and experimental studies of evolutionary rescue have considered a single species (Osmond and de Mazancourt, 2013). More realistically, each species is interconnected with a biotic component of the environment through a multitude of interactions. Hence, we might expect the extinction dynamic to be contingent on the

nature of these interactions (Jones, 2008). Therefore, incorporating interspecies interactions into studies is essential for predicting the outcomes of adaptive evolution in a context of a deteriorating environment. However, only recently evolutionary biologists have started to investigate evolutionary rescue within a multi-species context. While most studies have been focused on relatively simple models of two-species interactions, only a few empirical studies have been conducted.

The probability of adaptation and survival in harsh environments may depend on a type of interspecies interactions (Northfield and Ives, 2013). I will first review the studies considering the +/- interactions between species (fitness of individuals of one species increases at the expense of another species), which comprise parasite-host and predator-prey interactions. Zhang and Buckling (2011) subjected the experimental populations of a parasite (a DNA phage) to a deteriorating environment (an elevated temperature), and monitored the extinction dynamics within a host (*Pseudomonas fluorescence*) which either co-evolved with the phage or remained evolutionary constant (the ancestral type). They found a higher extinction rate and a higher total number of extinctions of the phage populations within co-evolving bacterial populations. The negative effects of coevolution on the probability of survival of the parasite were manifested through reduced population size of the phage and a trade-off between adaptation to the elevated temperature and infectivity. Jones (2008) modelled a predator-prey interaction in a context of a changing environment and tested the extinction probability of both species in isolation and while interacting. He found that mean time of extinction (measured in generations) of both predator and prey significantly increased when interacted. Furthermore, this result was consistent for different rates of environmental change and different strengths of selection. The positive effect of this interspecies interaction on the probability of evolutionary rescue has been interpreted as removal of maladapted individuals in both constituent species (Carlson, 2014). Northfield and Ives (2013) modelled predator and prey co-evolution in a context of climate change which increases the intrinsic rate of increase of one constituent species. They found the negative co-evolutionary feedback: if population density of a predator increases, it will cause a higher investment of a prey in defensive strategies, which in turn reduces an increase of the predator density; similarly, if population density of a prey increases, it will cause higher investment of a predator in a predation rate, which in turn reduces an increase of the prey density. Yamamichi and Miner (2015) modelled a predator-prey interaction in a context of a deteriorating environment and found that evolutionary

rescue of a predator could be facilitated by evolutionary rescue of a prey, if there is a trade-off between investment in a defence against the predator and adaptation to deteriorating conditions. The authors termed this process “indirect evolutionary rescue” (of a predator).

Relatively few studies have been carried out to investigate the +/+ interactions (fitness of individuals of both species increases due to an interaction) in the context of a changing environment. Northfield and Ives (2013) found that climate change may alter mutualistic interactions, by producing conflicting mutualism (for instance, species A may be under selection for increased strength of interspecies interactions, but not species B; Northfield and Ives, 2013). Under this scenario, climate change will be more beneficial for one species (species A) and detrimental to another (species B). Alternatively, an increased investment of one species could be beneficial for the other counterpart, which gives rise to a non-conflicting mutualism, beneficial for both species (Northfield and Ives, 2013). Hom and Murray (2014) performed an experimental study by artificially selecting for obligate mutualism between *Chlamydomonas reinhardtii* and *Saccharomyces cerevisiae.* Two species were subjected to a selective regime lethal for each of them, which both could have been able to survive only if engaged in a mutualistic interaction (*C. reinhardtii* could have obtained CO_2 for photosynthesis only through metabolic processes of *S. cerevisiae;* the yeast could have obtained the source of nitrogen (ammonium) only through metabolic processes of *C. reinhardtii* – converting nitrites to ammonium). Even though the results of this study have not been directly interpreted by the authors as evolutionary rescue, they clearly indicate that survival of conditions lethal to both constituent species could be facilitated by mutualism.

The effects of competitive interactions between species (a decline in fitness of both constituent species, -/-) on the probability of adaptation and survival have been the subject of several theoretical studies. Johansson (2008) modelled a competition between two species in a changing environment and found that the species disfavoured in a competition suffers a decline in effective population size, which in turn reduces the maximal adaptive rate. Furthermore, competition in stressful conditions can result in a trade-off between adaptation to the abiotic and biotic component of environment (Collins, 2011; Lawrence et. al, 2012). Collins (2011) allowed three strains of *Chlamydomonas reinhardtii* to compete in a deteriorating (sub-lethal) environment (increasing concentration of CO_2). The strains exposed to the elevated concentrations of CO_2 that had evolved higher growth rate in the

presence of a competitor were less fit when propagated alone. Furthermore, the experimental evidences indicate that competition increases the likelihood of extinction (e.g. Bengtsson 1989; Bengtsson and Milbrink, 1995). Bengtsson (1989) showed that competition increases the probability of extinction of three species of *Daphnia* cultivated in a benign medium, which was primarily driven by the overlap in the resource use. Bengtsson and Milbrink (1995) corroborated this result, by demonstrating the significant increase of extinction rate of two *Daphnia* species (*D. magna* and *D. longispina)* when propagated together than when grown in isolation.

Given that most theoretical and empirical results indicate the general negative effects of competitive interactions on adaptation to novel conditions and survival in benign conditions, we might predict that competition will generally have a negative impact on the probability of evolutionary rescue. However, to the best of my knowledge, no experimental study testing the effect of competition on evolutionary rescue has been performed. Given that negative ecological interactions potentially have the most serious conservation implications (Jones, 2008), I will focus my research to investigate the effects of competition in the context of evolutionary rescue.

Negative effects of competition are manifested through a reduction of population size (for example, Ayala, 1969), which in turn reduces the rate of adaptation (Johansson, 2008). Based on this concept, we could predict that the probability of evolutionary rescue will be mostly negatively affected by competition. However, there is evidence that competition could affect the adaptive dynamics of species in a less predictable way. The recent models proposed by Jones (2008) and further developed by Osmond and de Mazancourt (2013), are the first to indicate that evolutionary rescue can be promoted by competition. These models assume that competition in optimal conditions usually causes character displacement. For instance, competition of two species of Darwin's finches for seeds of different size caused a divergence in beak size between species (Grant and Grant, 2006). When conditions change, both competitors will likely be maladapted, since environmental change is usually perceived as detrimental by most organisms (Bell and Collins, 2008.) and the average phenotype of both species will lag behind the phenotype optimal in new conditions (Jones, 2008). However, if the environmental change selects for a character frequent in some species, but not in the other, the species will be unequally maladapted (Jones, 2008). The species that starts closer to the optimum could benefit from

competition, if selection for survival in new conditions has the same direction as selection caused by competition (Jones, 2008). I will explain this using an example of Darwin's finches (genus *Geospiza*). A drought of 1977 in Galapagos reduced the availability of small seeds, thus favouring the species with larger beaks (Boag and Grant, 1981). This environmental change has increased selective pressure in populations of *Geospiza fortis* (medium-size beaks) to adapt to the new conditions, and individuals with larger beaks had selective advantage. However, the evolutionary dynamics have been significantly altered by the presence of a competitor species (Grant and Grant, 2006). *G. fortis* failed to adapt to the new conditions if it competed with *Geospiza magnirostris*, which has larger beaks (competition selected in the opposite direction than the environmental change; Osmond and de Mazancourt, 2013). In contrast, the presence of *Geospiza fuliginosa*, a species with smaller beaks, increased the probability of survival of *G. fortis*, by reducing the fitness of *G. fortis* individuals with smaller beaks, which were further away from the moving optimum. Thus, competition and environmental change selected in the same direction, aiding the persistence of *G. fortis*.

The models described above have considered character displacement preceding the environmental change. The effect of competition in lethal conditions not preceded by character displacement is less certain, and may depend on the factors such as differences in the initial maladaptedness caused by an environmental change. For instance, differential thermal sensitivity may affect the adaptive dynamic if the environmental change comprises an increase of temperature). In a model of two-species subjected to environmental change, small differences in mean absolute fitness in new conditions could provide one counterpart with initial advantage. If that species possesses sufficient genetic variability, the augmented section could "push" the favourable genotype towards the moving optimum, thus aiding persistence.

Overall, the effects of competition on evolutionary rescue in deteriorating environments could be both positive and negative, which may depend on the identity of a competitor species and a type of environmental change. However, empirical evidence is required. Furthermore, at least in the initial stages of adaptation, competition will likely negatively affect the population abundance, and thus mean fitness. Hence, it will increase the complexity of environmental deterioration. Given that effects of sex in an environment deteriorating complexly are still uncertain, incorporating a competitor into an environment

with a deteriorating abiotic component would provide an opportunity to investigate the impact of both mode of reproduction and competition, as well as interaction of these factors, on the probability of evolutionary rescue.

1.4 Experimental evolution

The experimental approach used in this Thesis was experimental evolution. The defining feature of this technique is a selection experiment (Bell, 2008), which involves cultivation of different living organisms in a controlled laboratory environment (Buckling et al., 2009). The major unit of experimental evolution is a lineage (experimental line, or simply, line) (Bell, 2008). Experimental lines are individual (independent) populations allowed to undergo through many generations in selective conditions, chosen by an experimenter. Selection is natural, given that the experimenter controls the selective environment, but does not directly impose the artificial selection (Buckling et al., 2009). Furthermore, lineages usually diverge during the selection experiment, because each may accumulate genetic changes caused by rare events, such as the rise of beneficial mutations or fixation of mutations due to genetic drift (Bell, 2008). The major advantage of experimental evolution technique is providing an experimenter with means to record (rare) evolutionary events, thus providing empirical evidence of evolutionary change and study evolution in real time (Buckling et al., 2009).

Various types of species have been used as model organisms in studies based on experimental evolution. Examples include, but are not confined to: plants (Roles and Conner, 2008*), Drosophila melanogaster* (Flexon and Rodell, 1982), *Escherichia coli* (Lenski et al., 1991), *Caenorhabditis elegans* (Morran et al., 2009), yeast (Bell and Gonzalez, 2009) and *Chlamydomonas reinhardtii* (Colegrave, 2002). However, unicellular species stand out as the most suitable potential model organisms. Their advantages include short generation time, large population size and relatively simple requirements for laboratory cultivation. Furthermore, the possibility of sampling and long-term storing of microorganisms offers the potential of assaying experimental populations against the ancestral populations, thus providing an insight into the patterns of adaptation. Moreover, microorganisms tend to have relatively simple, well understood (sequenced) genomes (Buckling et al., 2009), which can contribute to understanding of patterns of evolutionary change at a molecular level.

For all the reasons above, experimental evolution has been widely employed as an experimental technique during the last couple of decades, significantly contributing to general understanding of evolutionary processes such as competitive interactions between species (for example, Gauze, 1934), adaptation to novel environment through *de novo* mutations (for example, Lenski et al., 1991), evolution of sex (for example, de Visser et al., 2005) and many more.

1.5 *Chlamydomonas reinhardtii* as a model organism

Chlamydomonas reinhardtii (described by Dangeard, 1888) is a unicellular haploid (17 pairs of chromosomes) chlorophyte alga (Figure 1.2), normally inhabiting either freshwater or terrestrial habitats. The cell is usually oval (approximately 10 µm), surrounded by cellulose cell wall. The major (distinctive) cell features include two apical flagella, a single cup shaped chloroplast, a nucleus prominent in cross section and an eyespot (Harris, 2001). In optimal growth conditions (temperature: 20°C - 25°C; light intensity: 200– 400 µEinsteins/m^2 sec; minimal medium), the average generation time is approximately 6-8 h (Harris, 2001). Vegetative cells (zoospores) undergo 2-3 successive rounds of mitosis, thus releasing 4-8 daughter cells (zoospores), which differentiate into adult vegetative cells (Harris, 2001). When deprived of a nitrogen source (ammonium or nitrate), this isogamous organism undergoes gametogenesis by producing two types of gametes (mt+ and mt-), which may mate under bright light, thus producing zygotes. A zygote matures in the darkness by acquiring additional layers of a cell wall, thus transforming into a resting stage (zygospore), which can withstand harsh conditions (e.g. low temperature). A zygospore germinates on a solid medium under bright light by dividing meiotically, which results in formation of 4 haploid daughter cells (zoospores), which develop into adult vegetative cells. Details of life cycle and cultivation in the laboratory conditions can be found in “The Chlamydomonas Sourcebook” by Harris (2009).

C. reinhardtii is widely used as a model organism (Harris, 2009) due to its fast growth rate and possibilities of manipulating the life cycle in laboratory conditions (a shift between asexual and sexual cycle). Furthermore, apart from photosynthetic growth, it can grow heterotrophically by utilising acetate as a carbon source (both in the light and dark). Moreover, it readily evolves growth on exotic carbon sources, as previously demonstrated

(for example, Colegrave et al., 2002). The further advantages of this species as a model organism include the sequenced and publicly available genome (http://genome.jgi-psf.org/Chlre3/Chlre3.home.html) and relatively easy isolation of mutants (Harris, 2001).

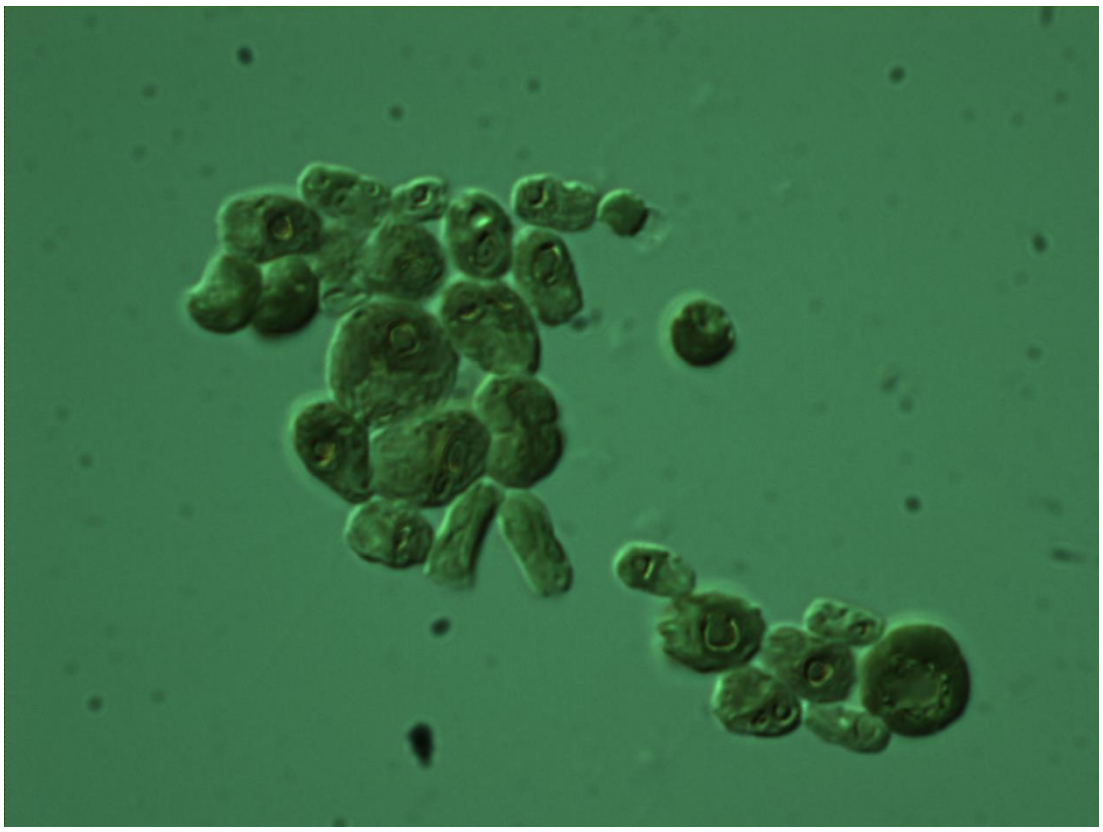

Figure 1.2 - *Chlamydomonas reinhardtii*

1.6 Aims of the Thesis

The main aim of this Thesis was to contribute to understanding of general principles of evolutionary rescue, with a special focus on impact of mode of reproduction and competitive interactions. The model organism chosen for the research was a unicellular chlorophyte alga *Chlamydomonas reinhardtii*, widely used in the experimental study (Harris, 2009), due to short generation time and possibilities of sexual cycle manipulation. I used experimental evolution as a technique, by imposing different selective regimes and monitoring fitness and survival of experimental populations.

In Chapter 2, I focused on investigating the effects of sex in different rates of environmental deterioration. I manipulated mode of reproduction of experimental populations and subjected them to one of three different treatments, each corresponding to the different rate of continual increase of salinity level in the medium.

The main aim of Chapter 3 was to investigate the combined effect of sex and competition on the probability of evolutionary rescue under similar selective regime as in Chapter 2 (increasing concentration of salt). I manipulated mode of reproduction of experimental populations of *Chlamydomonas reinhardtii*, and subjected them to one of two competition treatments: cultivation in the presence or absence of a competitor species (*Chlamydomonas moewusii*).

In Chapter 4, I aimed to test whether the effects of competition on evolutionary rescue are influenced by phylogenetic relatedness of a focal species and a competitor, and a degree of their ecological similarity.

Finally, in Chapter 5, I investigated which experimental factors could potentially select for the long-term maintenance of sex, by subjecting the experimental population to various types of selective environments (directional and fluctuating change of the abiotic factors and the presence of a competitor) and monitoring the frequency of sex over the course of time.

2. The effects of mode of reproduction and a rate of environmental deterioration on extinction dynamics and evolutionary rescue in *Chlamydomonas reinhardtii*

2.1 Introduction

An adverse anthropogenic impact on the Biosphere has produced environmental change at a rate unprecedented in the Cenozoic (Barnosky et al., 2012). Some of these negative effects include but are not confined to: climate change, habitat destruction or fragmentation and pollution. This change has increased the rate of biodiversity loss by two orders of magnitude in comparison with the background extinction rate (Pereira et al., 2010.). A recent study predicted that up to 35% of all species could face extinction by 2050, given the current extinction dynamics (Thomas et al., 2004.). For all these reasons, understanding the patterns of species' response to global change has become increasingly important focus of evolutionary biology during the last couple of decades (Bell and Collins, 2008).

Most organisms are assumed to be reasonably well adapted to the current state of their environment through the past operation of natural selection, so most environmental change is usually perceived as detrimental (Bell and Collins, 2008). Selection acting on standing genetic variation may restore the mean fitness of a maladapted population if genotypes conferring an advantage in new conditions are present in the genetic pool of the population (Bell, 2008). Alternatively, a population can respond through phenotypic plasticity. For example, a single stressor may activate physiological pathways of a general stress response, which confer resistance to various stressors (Samani and Bell, 2016). Organisms can also react to change by dispersal and migration to less stressful habitats (Bell and Collins, 2008). In the absence of these mechanisms, a population has to adapt through *de novo* mutations, via a process termed evolutionary rescue (Gomulkiewicz et al., 1995).

Many previous theoretical studies have investigated the effect of environmental change on the probability of adaptation and evolutionary rescue through quantitative genetic models, by considering a trait under stabilising selection (Orr and Unckless, 2008).

When conditions change, the mean phenotype of the population will lag behind the optimal value, resulting in a decrease of mean fitness of a population (Gomulkiewitz and Holt, 1995), termed as lag load (Maynard Smith, 1976). Lag load (the extent of maladaptation) is directly proportional to the rate of environmental change (Willi and Hoffmann, 2009), since a higher rate of change implies larger difference in mean fitness of a population prior to change and the fitness of the phenotype optimal in new conditions. Thus, the probability of adaptation and survival is also contingent on the rate of environmental change.

A higher rate of environmental change implies large initial maladaptedness and tracking of moving optimum occurs via strong selection, which causes a demographic pressure on a population manifested through higher proportion of selective deaths (Burger and Lynch, 1995). Consequently, a population may be adapting to the changing conditions, but simultaneously going extinct due to demographic load. Under a more realistic scenario, sudden environmental change is followed by the period of stasis (absence of change). As a consequence, the optimal value of the phenotype will remain constant, and despite the decline of a population size, a population may recover the mean fitness by fixing *de novo* mutations of large fitness effects (Collins and de Meaux, 2009). Thus, the persistence of a population becomes a "race" between adaptation and extinction (Maynard Smith, 1989; Gomulkiewicz and Holt, 1995; Kopp and Matuszevski, 2014).

A more gradual rate of environmental change implies lower initial maladaptedness and thus, a lower rate of population size decline. However, it also affects dynamics of adaptive evolution, which proceed at a lower rate, due to weaker selection. In contrast to a high rate of environmental change, gradual change usually occurs over longer intervals of time, during which mean fitness of a population constantly decreases by a small amount and may be recovered by fixation of beneficial mutations of smaller effects (Collins and de Meaux, 2009). Without fixing these beneficial mutations after each step of environmental change, a population may track the moving optimum less efficiently and consequently go extinct (Kopp and Matuszevski, 2014).

Many experimental studies have provided evidence that patterns of adaptations to lethal conditions are contingent on the rate of environmental change. Perron et al. (2007) manipulated the environmental harshness in populations of *Pseudomonas aeruginosa*, and found that exposure to a single antibiotic resulted in more rapid evolution of resistance.

Furthermore, higher immigration rate in combination with the lowest level of environmental harshness maximized the level of resistance. Bell and Gonzalez (2011) manipulated the rate of environmental deterioration (an increase of NaCl level) and mode of dispersal (local and global) in metapopulations of yeast and found that probability of evolutionary rescue was the highest for the populations subjected to a gradual level of deterioration and local dispersal. Lindsey et al. (2013) manipulated the rate of increase of an antibiotic rifampicin concentration (sudden, moderate and gradual) and found that the probability of evolutionary rescue in *Escherichia coli* populations was inversely proportional to the rate of rifampicin increase.

Another factor that affects the likelihood of adaptation in a changing environment is mode of reproduction. Numerous empirical studies have provided evidence that sex enhances adaptation to novel environments relative to that of asexual populations through faster assembly of mutations beneficial in new conditions (Kaltz and Bell, 2002), reduction of clonal interference between beneficial mutations (Colegrave, 2002), an increase in fitness of progeny (Becks and Agrawal, 2011) and an increase of adaptation to the presence of pathogenic species (Morran et al., 2011). Furthermore, sex increases adaptation rate (Goddard et al., 2005) and survival rate (Lachapelle and Bell, 2012) in deteriorating environments relative to that of asexual populations, thus increasing the likelihood of evolutionary rescue. While general beneficial effects of sex in novel and deteriorating environments are relatively well understood, an outstanding question that still lacks empirical evidence is whether the effect of sex depends on the rate of environmental change.

Adaptation to lethal conditions involves an increase in the frequency of *de novo* beneficial mutations. The mutational pathway ("adaptive walk"; Collins et al., 2007) leading to evolutionary rescue changes with a rate of environmental deterioration (Lindsey et al., 2013). Populations adapt to gradual and moderate rate of change by fixing multiple mutations, which are inaccessible under higher rates of change (Lindsey et al., 2013). In contrast, adaptation to higher rates of change may involve single mutations (Lindsey et al., 2013). Based on this concept, we might also expect that the mechanisms of the beneficial effect of sex will depend on a rate of environmental deterioration. A population subjected to a gradual rate of environmental deterioration will likely suffer lower population decline than a population subjected to a high rate of environmental change. As a result, supply of

beneficial mutations will be proportionally higher. Given that at the initial stages of environmental change, selection will be weaker (since the total magnitude of change would still be relatively low), some mutations may not become fixed or alternatively, individuals carrying different beneficial mutations may compete through process termed clonal interference (Muller 1932; Gerrish and Lenski, 1998). Consequently, the rate of adaptation may slow down, hampering a population's tracking of moving optimum. In this context, sex can be beneficial through reduction of clonal interference, as previously demonstrated by Colegrave (2002). Adaptation to a rapid rate of environmental change occurs though fixation of small number of mutations of large effects, as experimentally demonstrated by Lindsey et al. (2013). Mutations of large effect are a rare event (Kassen and Bataillon, 2006) and consequently may arise in a detrimental genetic background, either due to stochastic processes such as random drift and genetic hitchhiking (Barton, 1995) or selection (Otto and Lenormand, 2002). The beneficial effect of sex under these circumstances may occur through "releasing" of these mutations from an inferior genetic background. In contrast, such mutation may fail to reach fixation in a clonal asexual population, or may otherwise become lost through genetic drift.

Despite the fact that mechanisms of the effects of sex may potentially vary under different rates of environmental change, the net effect (faster adaptation and higher probability of evolutionary rescue) should remain the same, irrespective of a rate of change. Hence, there should be no interaction between mode of reproduction and the rate of environmental deterioration. In order to test this hypothesis, I designed a selection experiment by manipulating the rate of environmental deterioration and mode of reproduction of unicellular alga *Chlamydomonas reinhardtii* in a fully factorial design and monitored the extinction dynamics and evolutionary rescue of experimental populations.

2.2 Materials and methods

2.2.1. Base populations of *C. reinhardtii*

In order to establish genetically variable experimental populations of the focal species, C. *reinhardtii,* mass mating of 10 different wild types strains (cc-1952, cc-2935, cc-2344, cc-1690, cc-1691, cc-2937, cc-2343, cc-2938, cc-2932 and cc-2342) was performed.

The mass mating was performed by using the same experimental protocol as described in 'Sexual cycle' (section 2.2.4.) section below, with the only difference in the freezing procedure, being carried out by transferring the cultures with zygotes on agar plates prior to freezing. Due to practical reasons of easier handling of numerous experimental populations, freezing was performed in liquid cultures in the selection experiment. The zygotes obtained from the mass mating were plated on agar and allowed to germinate by producing zoospores. The zoospores differentiated into adult vegetative cells which were allowed to undergo several rounds of mitotic divisions until visible colonies appeared on agar plates. Each colony represents a single (unique) genotype derived from an individual cell. *C. reinhardtii* is an isogamous species and each cell represents one of two mating types (mt): mt+ or mt-. A library of genotypes was established by randomly picking 20 colonies of each mating type from the agar plates by sterile loop. The mating type was determined by crossing the culture derived from each genotype with tester isolates (cc-1690 mt+ and cc-1691 mt-).

The experimental populations were constructed by random selection of 10 different genotypes from the library. Each experimental population represents a unique combination of 10 genotypes. Three sets of facultative sexual populations and three sets of obligate sexual populations (24 populations per each set, 72 populations per each mode of reproduction) were established by combining 5 mt+ and 5 mt- isolates per population; three sets of asexual populations (24 populations per each set, 72 populations in total) were established by combining 10 mt+ and 10 mt- isolates per population, respectively (an equal proportion of populations comprising either mt+ or mt- isolates). However, a single isolate had been incorrectly assigned as a plus mating type which was noted after the experiment had already commenced. As a result, 14 asexual experimental populations comprising this isolate have been withdrawn from the analyses of the experiment: 7 gradual rate populations, 1 moderate rate population and 6 high rate populations.

2.2.2. Selection experiment

The experiment comprised two phases. In the first phase, the experimental populations were subjected to one of three types of deteriorating environments, created by a constant increase of NaCl (hereafter referred to as salt) level in the medium in regular intervals. Salt was chosen as a stressor since previous experiments (e.g. Lachapelle and Bell, 2012; Lachapelle et al., 2015) demonstrated the detrimental effect of elevated salt

concentration on *C. reinhardtii* populations, manifested through osmotic and ionic disbalance in cells (Lachapelle et al., 2015). The experimental populations were allowed 4 growth cycles of asexual reproduction (by undergoing approximately 4-6 rounds of mitotic divisions per growth cycle) during which the salt level remained constant. After completing all 4 growth cycles, a sexual cycle had been initiated in all the populations, but completed only in sexual populations, due to presence of both mating types (see the section 'Sexual cycle' for details). After completion of each sexual cycle, a phase of 4 asexual growth cycles was reinitiated, during which the experimental populations experienced an increased level of salt.

Depending on the rate of salt increase, three treatment groups of experimental populations were established. Each of the three treatment groups experienced a different rate of environmental deterioration: the populations in gradual rate treatment group were subjected to a relatively mild rate of salt increase of 1 g/l after every 4 growth cycles; the populations in high rate treatment group experienced a relatively abrupt change of 3 g/l of salt increase after every 4 growth cycles; the moderate rate populations were subjected to the intermediate level of salt increase of 2 g/l after every 4 growth cycles.

Three treatment groups were established with respect to mode of reproduction. The populations within both obligate sexual and facultative sexual groups were allowed to complete a number of rounds of sexual reproduction during the experiment (see below for details). In the populations of obligate sexual group, gametes which failed to mate were eliminated by freezing. Thus, only sexually derived progeny were transferred to the next phase of asexual growth cycles. In facultative sexual groups, this step was omitted, resulting in mixed progeny derived from both zygotes and unmated gametes. Asexual populations were reproducing entirely mitotically throughout the course of the experiment.

In the second phase, at the point which salt concentration had reached and surpassed the level which could potentially completely stop the growth of ancestral populations (8 g/l; Reynoso and de Gamboa, 1982; Moser and Bell, 2011), thereby increasing the risk of extinctions, I started to run a parallel experiment. The parallel experiment involved sub-sampling of experimental populations and propagating each in a selective medium comprising the same salt concentration as at the time of sub-sampling, by means of serial passaging. During this phase, the salt concentration remained constant. The purpose of this procedure was to evaluate whether evolutionary rescue occurred,

manifested through positive growth in stressful conditions. Simultaneously, I continued to run the main selection experiment by subjecting the main populations to another step of salt increase.

2.2.3. Cultivation and Transfer Procedure

All the experimental populations of C. *reinhardtii* were cultivated in 24-well plates in Bold's basal medium (Bischoff and Bold, 1963), widely used for algal cultivation (Harris, 2009), under standard conditions (26°C, 100 µE illumination, shaking at 180 rpm and covered with sterile breathable membranes to prevent cross-contamination and uneven evaporation across the plates). A serial passage was performed after every 3-4 days by transferring 5 % of each population to the fresh medium. After every second growth cycle and prior to induction of each sexual cycle, each population was sampled (150 µl) and population size estimated spectrophotometrically by measuring optical density (OD_{750}) of the culture.

2.2.4. Sexual Cycle

Prior to each step of salt increase (after completion of asexual growth phase), sexual populations were allowed to undergo a sexual cycle. The first sexual cycle followed the first two asexual growth cycles completed in a benign medium (Bold's without additional salt supplemented). Each subsequent sexual cycle was induced after 4 asexual growth cycles. A sexual cycle was induced under the following protocol. Firstly, all populations were centrifuged at 5000 rpm for 10 minutes, re-suspended in nitrogen-free medium to initiate gametogenesis and incubated in standard conditions under bright light for another 24 hours to allow for mating and formation of zygotes. Immediately following this period, the plates containing experimental populations were wrapped in aluminium foil and incubated in the dark for additional 4-5 days to allow the zygotes to mature. Since sub-lethal stress may affect the mutation rate, the effects of sex may be confounded with effects of mutations (Goho and Bell, 2000; Colegrave et al., 2002). Hence, the nitrogen starvation treatment was also applied to asexual populations, but since they were made up of single mating types, no mating took place. After incubation in darkness, the plates with

mature zygotes were placed in a freezer for 4 hours (-20˚C) in order to eliminate unmated gametes. The zygotes develop the additional layers of cell wall during maturation in the darkness, thereby acquiring resistance to stressful conditions (Harris, 2009). The pilot experiments in our laboratory revealed that this feature enables the zygotes to withstand low temperature. Given the lack of resistance of unmated gametes to freezing, this step was omitted for asexual populations and facultative sexual populations, the latter group being allowed to transfer both the produced zygotes and unmated gametes to the next round of asexual growth cycles. The zygotes of obligate sexual *C. reinhardtii* populations were then transferred to agar plates by sterile loop and incubated in bright light for two days to allow for germination and several rounds of mitotic divisions. The produced zygotes (if any) of facultative sexual populations were transferred the same way as obligate sexual populations, along with additional aliquot (of about 50-100 µl) to prevent a population bottleneck in the case that low number of zygotes were produced. Asexual *C. reinhardtii* populations were transferred to agar plates by pipetting (an aliquot of 200 µl) and incubated for the same period of time as sexual populations. After the given period, all cultures in the agar plates were flooded with 4 ml of Bold's medium (supplemented with salt which concentration increased than prior to induction of the sexual cycle, depending on the rate) for approximately an hour. The population size of each experimental population on agar was estimated spectrophotometrically (OD_{750}) and diluted to the same optical density as prior to induction of sexual cycle. This experimental procedure ensures that effects of sex are not confounded with a variation in population size. All the populations were then returned to the liquid medium by pipetting.

Mating in *C. reinhardtii* requires sufficiently dense populations, to ensure the contact of gametes and thus production of zygotes. Due to different dynamics of a population size decline caused by differences in the rate of environmental deterioration, unequal number of sexual cycles could have been induced for each treatment group. Consequently, gradual, moderate and high rate treatment groups underwent 10, 4 and 3 rounds of sexual reproduction, respectively.

2.2.5. Recording the extinction events

Each experimental population was visually inspected under microscope before each transfer to record the possibility of an extinction event (absence of living cells). If observation under microscope failed to reveal surviving cells, a sample of the population

(200 µl) was transferred to agar plate and incubated for 3-4 days in order to confirm an extinction event. The selection experiment continued until all experimental populations went extinct.

2.2.6. Recording the evolutionary rescue events

After completion of the last (fourth) growth cycle in a given level of salt concentration (starting from 8 g/l), a parallel experiment was initiated. The populations were sub-sampled (by pipetting 5% of the culture), and these samples were used for evolutionary rescue assays. Simultaneously, the main selection experiment continued, with the main experimental populations being subjected to another step of salt increase. Each sampled population was then transferred to 24-well plates containing a medium supplemented with the same salt concentration the populations had been subjected to prior to the transfer. Each subculture was propagated in the corresponding selective medium for 3-4 additional growth cycles during which the salt concentration remained constant. The population size of each population was estimated spectrophotometrically (OD_{750}) after each growth cycle. Furthermore, each population was allowed a single growth cycles in the corresponding selective medium in 96-well plates, during which the population size was recorded spectrophotometrically (OD_{750}) twice a day, until the cultures reached a mid-log/ stationary phase (after approximately 3.5 days). The purpose of the assay in 96-well plates was to estimate the growth rate of the populations, by calculating yield as a function of time. The populations were scored as “rescued” if they scored clear positive growth in both assay environments, manifested through the same or higher population size recorded after both assays. The growth parameters of these populations were compared with those of the ancestral isolates, assayed in the medium with the corresponding concentration of salt prior to commencing of the selection experiment (see the next section for details).

2.2.7. Assay of the ancestral isolates in high salt environments

All 39 ancestral isolates were assayed for growth in Bold's supplemented with an elevated concentration of salt, ranging from 0 g/l (benign medium – ancestral environment) to 10 g/l (detrimental). The assay was performed in 96-well plates using the identical procedure described in the previous section. All populations were allowed two growth cycles (3-4 days per cycle; 5% of a culture passaged), but were assayed only during the second cycle, to avoid possible carry-over effects after plating from slant agar tubes, used for storing the cultures. The fitness parameters of the ancestors used for comparison with the previously described assayed experimental populations were Maximal O.D. of the populations obtained during the assay and growth rate measured as an average yield per time point.

2.3 Data analysis

The effect of elevated salt concentration on fitness of the ancestral isolates was analysed by Kruskal Wallis rank sum test, with 'salt concentration' as a continuous independent variable. The continuous responsive variable was 'maximal O.D.'

The population size dynamics of each treatment group was analysed by fitting Two-way ANOVA with two factors: 'mode of reproduction' and 'the rate of salt increase', both considered fixed factors. The dependent continuous variable was slope of the regression line representing a mean change of a population size as a function of time (growth cycles).

The extinction dynamics of each factor ('mode of reproduction' and 'the rate of salt increase') was analysed by fitting The Kaplan-Meier estimator of survival. The time units used for the analyses were 'growth cycle' and 'salt concentration' (reached when extinction occurred). The survival curves were analysed by Weibull regression model, used to investigate the influence of a factor or a covariate upon timing of an event.

The probability of evolutionary rescue was analysed by fitting Binomial regression models, with two factors, 'mode of reproduction' and 'level of salt increase', both considered independent categorical variables. The binary responsive variable was survival/death.

The mean fitness of the rescued populations with respect to both population size and the growth rate was analysed by fitting one-way or two-way ANOVA. The responsive continuous variables were O.D. (for mean population size) and slope of the regression line representing a mean change of population size as a function of time (for the growth rate).

All the analyses were performed using R (R Core Team, 2017).

2.4 Results

2.4.1. Fitness assay of the ancestral populations in high salt medium

An elevated concentration of salt was detrimental for the ancestral isolates of experimental populations. There was a negative correlation between salt concentration and average maximal O.D. (Kruskal Wallis rank sum test; χ^2= 271.4; df = 10; P < 0.00001) (Figure 2.1). The salt concentration of 5 g/l reduced the maximal population size by 60%, which is 12% higher than previously reported (Reynoso and de Gamboa, 1982). The salt concentration of 8 g/l reduced the maximal population size by 71%. This is inconsistent with the results of previously reported authors, who found that the salt concentration of 8 g/l supresses the growth of *C. reinhardtii* almost completely.

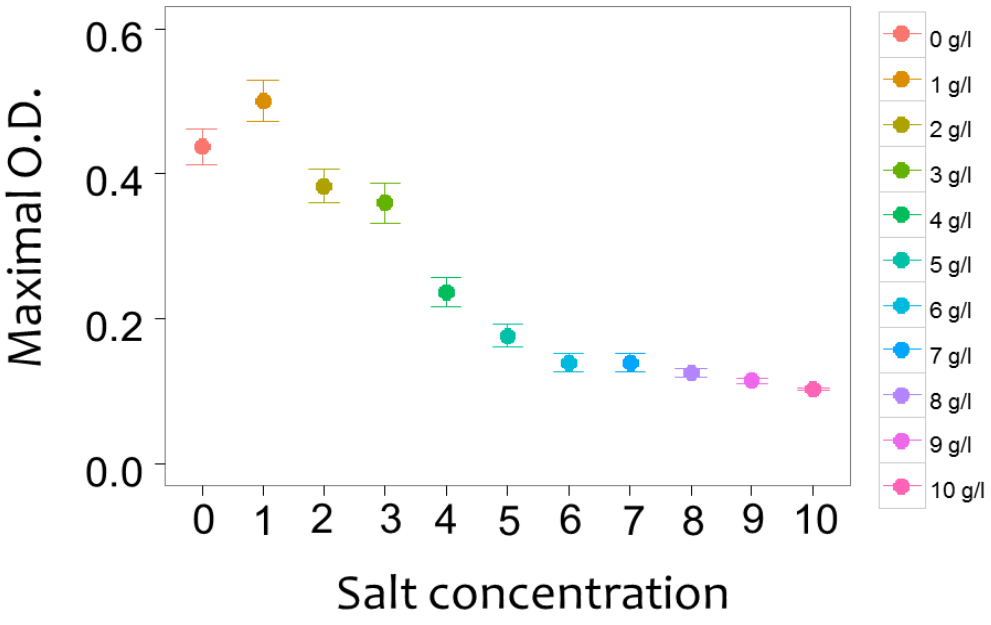

Figure 2.1 – Average maximal population size of the ancestral isolates of experimental populations per each level of salt concentration (g/l); O.D. (750 nm) represents a proxy for population size; the bars represent standard error of the mean;

2.4.2. Selection experiment

2.4.2.1. Population size dynamics

The rate of environmental deterioration affected the rate of mean population size decrease (two-way ANOVA; $F_{2,201} = 102.99$, $P < 0.00001$) (Figure 2.2). Decline in population size was slowest in the populations subjected to the gradual rate of salt increase, and significantly different compared to both moderate ($t = -3.08$, $df = 135$, $P = 0.002$) and high rate of salt increase ($t = -3.92$, $df = 130$, $P = 0.0001$). There was no significant difference between the rate of mean population size decline between moderate rate and high rate groups ($t = 1.08$, $df = 136$, $P = 0.28$) (see Figure 2.4 for the population size dynamics per each mode of reproduction, for each rate of salt increase).

Mode of reproduction affected the rate of mean population size decrease (two-way ANOVA; $F_{2,201} = 102.26$, $P < 0.00001$) (Figure 2.3). Decline in population size was highest in asexual populations for all rates of salt increase, and significantly different compared to both obligate sexual group ($t = -9.82$, $df = 129$, $P < 0.00001$) and facultative sexual group ($t = -9.17$, $df = 129$, $P < 0.00001$). There was no significant difference between sexual groups of populations in this respect ($t = -0.72$, $df = 143$, $P = 0.47$) (see Figure 2.5 for the population size dynamics per each rate of environmental deterioration, for each mode of reproduction).

There was a significant interaction between mode of reproduction and the rate of environmental deterioration (Two-way ANOVA; $F_{4,201} = 10.15$, $P < 0.00001$). Post-hoc Tukey's HSD analysis was conducted on all possible pairwise comparisons and revealed statistically significant difference ($P < 0.005$) between the following pairs of groups: gradual rate - asexual ($M = -0.13$, $SD = 0.002$) and gradual rate - obligate sexual ($M = -0.005$, $SD = 0.003$), gradual rate - asexual and gradual rate - facultative sexual ($M = -0.005$, $SD = 0.003$); moderate rate - asexual ($M = -0.015$, $SD = 0.003$) and moderate rate - obligate sexual ($M = -0.008$, $SD = 0.002$), moderate rate - asexual and moderate rate - facultative sexual ($M = -0.012$, $SD = 0.003$), moderate rate - obligate sexual and moderate rate - facultative sexual; high rate - asexual ($M = -0.016$, $SD = 0.002$) and high rate - obligate sexual ($M = -0.013$, $SD = 0.002$), high rate - asexual and high rate - facultative sexual ($M = -0.012$, $SD = 0.002$). There was no significant difference between the following groups: gradual rate - obligate sexual

and gradual rate - facultative sexual (P = 0.99); high rate - obligate sexual and high rate - facultative sexual (P = 0.49).

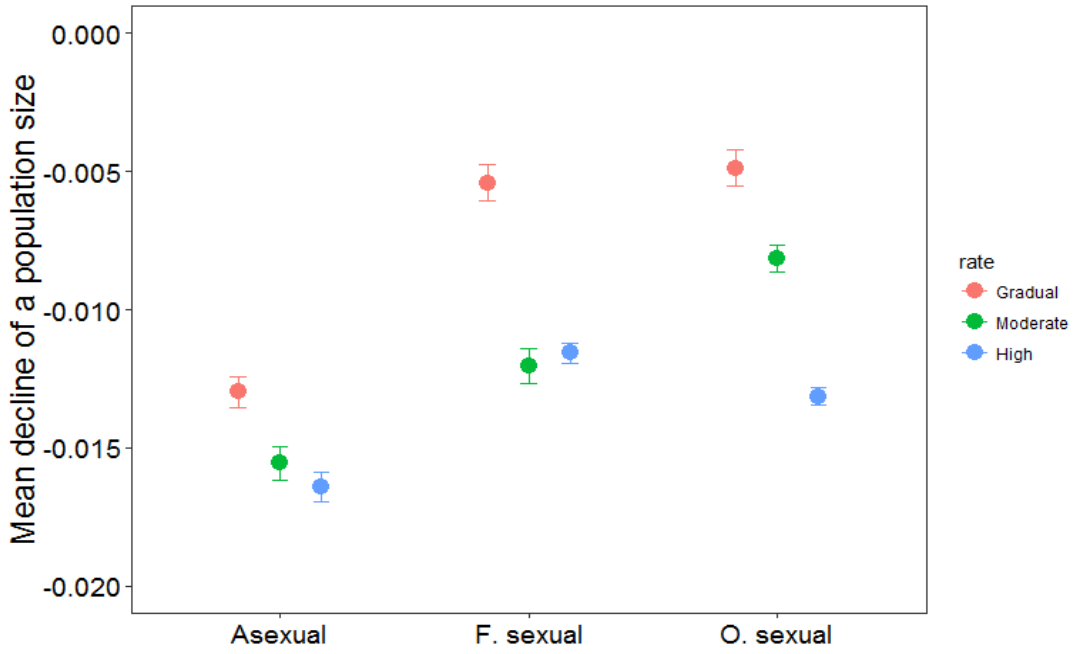

Figure 2.2 – Slope of the regression line representing mean decline of population size as a function of time, for all rates of environmental deterioration; the bars represent standard error of the mean;

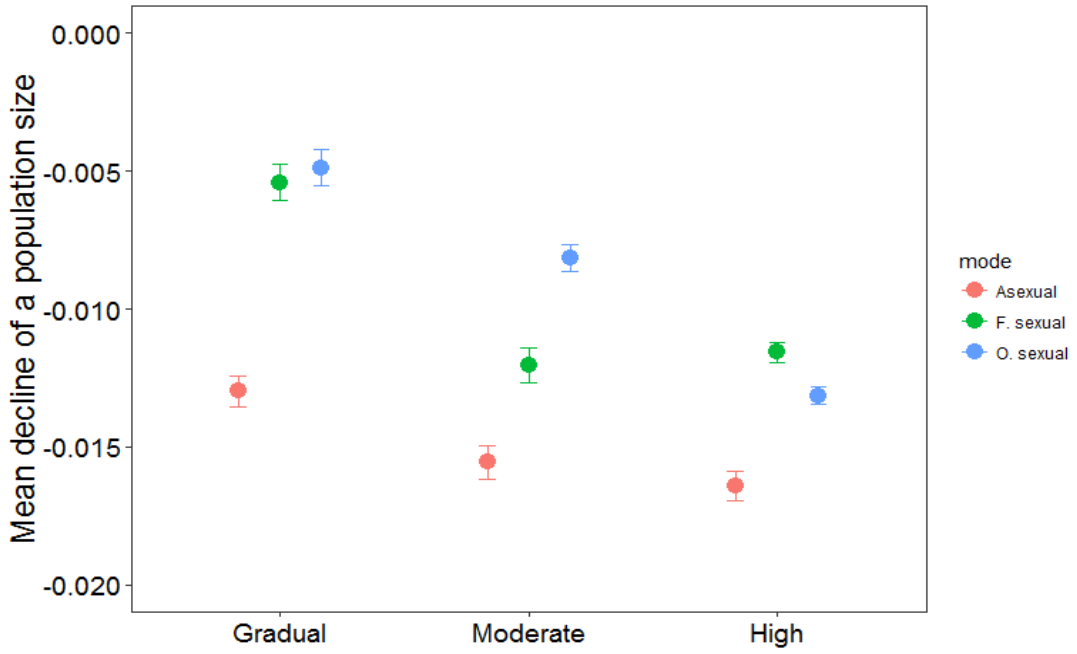

Figure 2.3 – Slope of the regression line representing mean decline of population size as a function of time, per each mode of reproduction; the bars represent standard error of the mean;

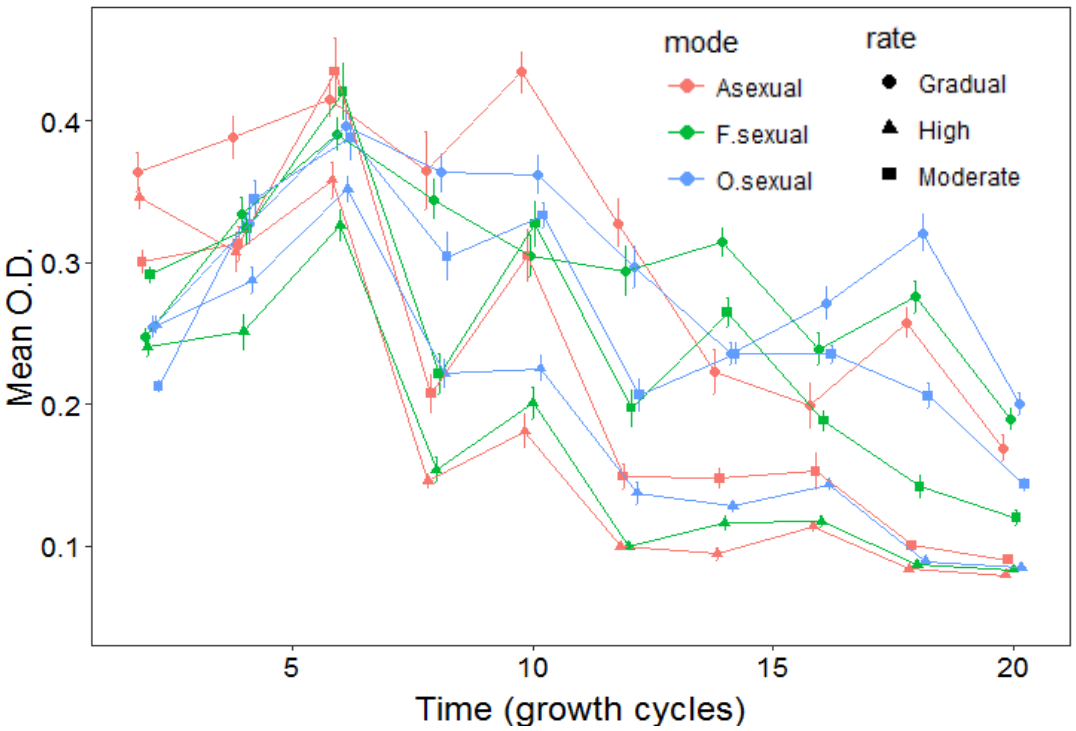

Figure 2.4 – Population size dynamics of treatment groups with respect to both mode of reproduction and the rate of salt increase; the colour of geometric points corresponds to mode of reproduction; the shape corresponds to the rate of salt increase; mean population size corresponds to the average OD_{750}; the bars represent standard error of the mean;

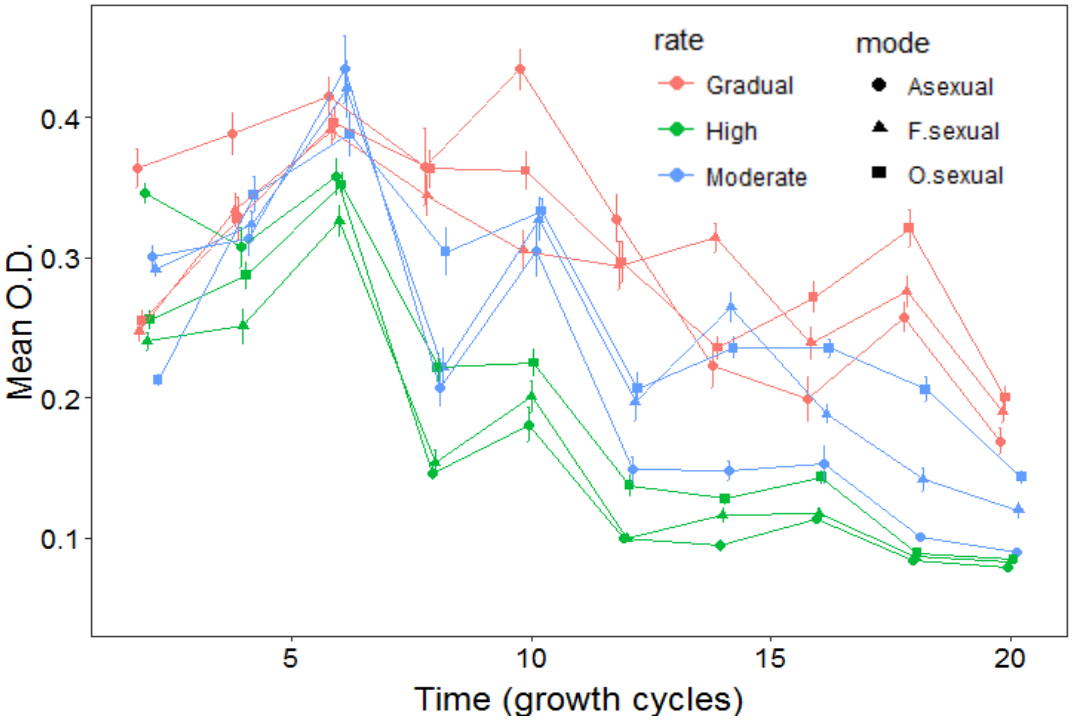

Figure 2.5 – Population size dynamics of treatment groups with respect to both mode of reproduction and the rate of salt increase; the colour of geometric points corresponds to the rate of salt increase; the shape corresponds to mode of reproduction; mean population size corresponds to the average OD_{750}; the bars represent standard error of the mean;

2.4.2.2. *Extinction dynamics*

The rate of salt increase affected the extinction dynamics of experimental populations relative to time in growth cycles (Weibull regression model; $\chi^2 = 1026.24$; df = 2; $P < 0.00001$) (Figure 2.6). The rate of extinction was directly proportional to the rate of salt increase, being the highest in high rate group of populations and the lowest in gradual rate group. The extinction rate within gradual rate group was significantly different than that of both high rate ($z = 57.96$, $P < 0.00001$) and moderate rate group ($z = 9.32$, $P < 0.00001$). Likewise, there was a significant difference between the extinction dynamics between high rate and moderate rate group ($z = 14.72$, $P < 0.00001$).

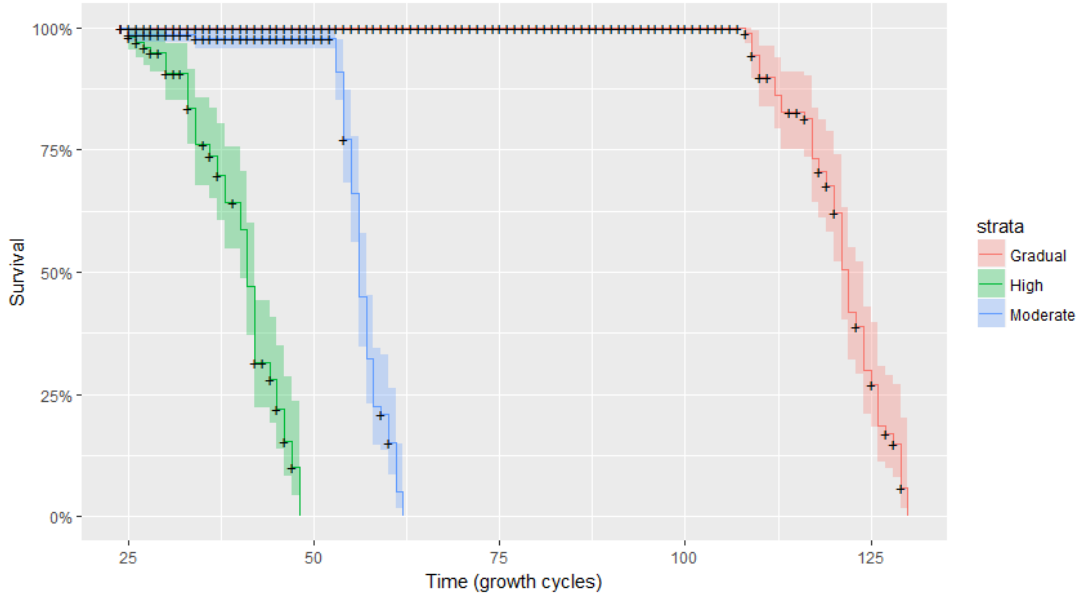

Figure 2.6 – Cumulative extinction dynamics of experimental populations per each rate of salt increase (irrespective of mode of reproduction); extinctions are plotted against time in number of growth cycles which preceded each extinction event; the 95% confidence interval for each survival curve is represented with the shaded area between upper and lower boundary.

The overall effect of mode of reproduction on cumulative extinction dynamics (all rates of salt increase combined) relative to time in growth cycles was not statistically significant (Weibull regression model; $\chi^2 = 0.84$; df = 2; $P = 0.66$) (Figure 2.7). However, the relative advantage of individual levels within 'mode of reproduction' factor was contingent on the rate of salt increase. There was a significant interaction between mode of reproduction and rate of salt increase on the extinction dynamics of experimental

populations relative to time in growth cycles (Weibull regression model; $\chi^2 = 12.32$; df = 4; P = 0.015). While the survival curves of each mode of reproduction group did not differ significantly in gradual and high rate of salt increase (Figures 2.8 and 2.10, respectively), obligate sexual group had significantly different extinction dynamics relative to both asexual group (z = 3.07, P = 0.002) and facultative sexual group (z = -2.15; P = 0.03) when subjected to moderate rate of salt increase (Figure 2.9), reflected in higher number of surviving populations per each time point. There was no significant difference between the extinction dynamics of facultative sexual and asexual groups (z = 1.02, P = 0.31).

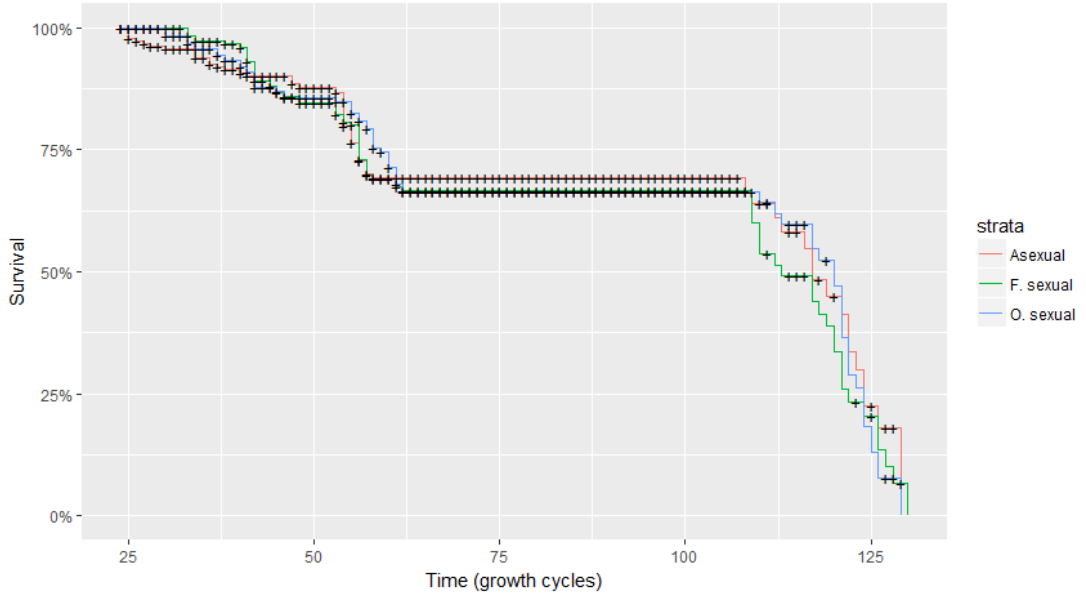

Figure 2.7 – Cumulative extinction dynamics of experimental populations per each mode of reproduction (irrespective of rate of salt increase); extinctions are plotted against time in number of growth cycles which preceded each extinction event.

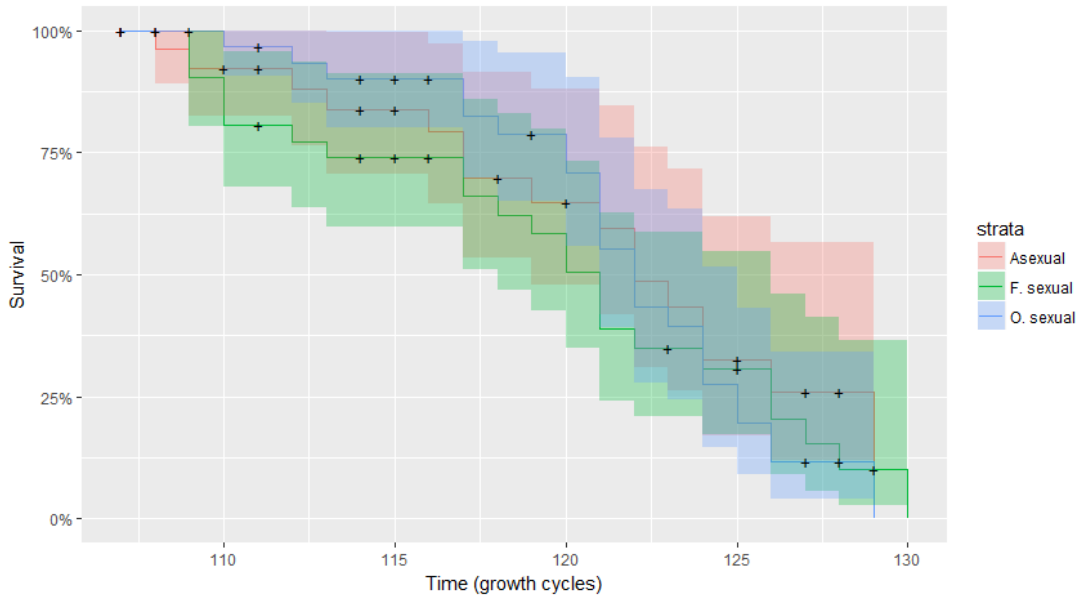

Figure 2.8 – The extinction dynamics per each mode of reproduction in the gradual rate of salt increase; the 95% confidence interval for each survival curve is represented with the shaded area between upper and lower boundary.

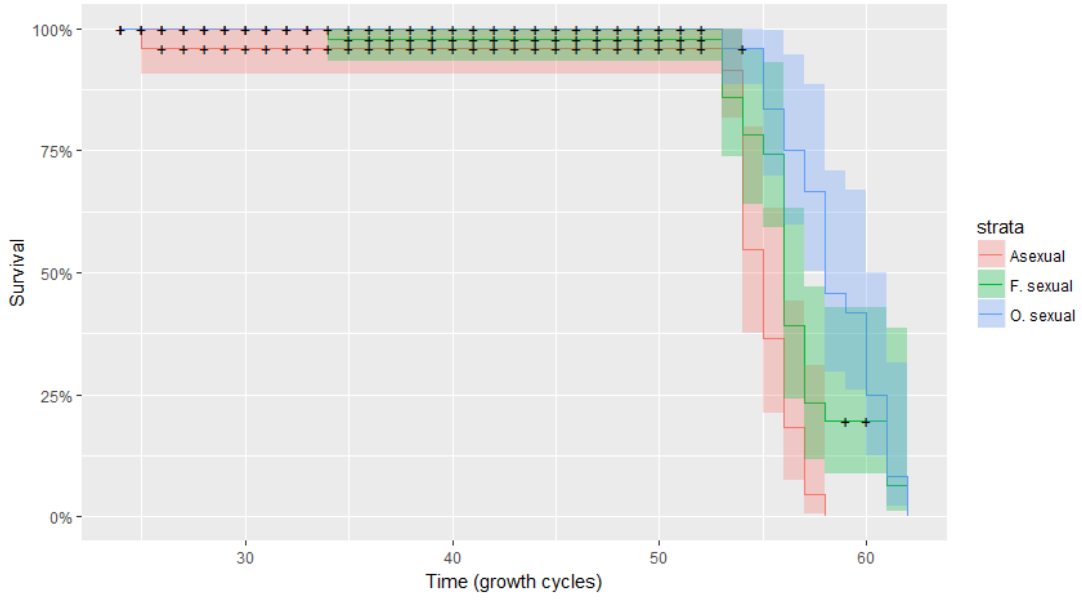

Figure 2.9 – The extinction dynamics per each mode of reproduction in the moderate rate of salt increase; the 95% confidence interval for each survival curve is represented with the shaded area between upper and lower boundary.

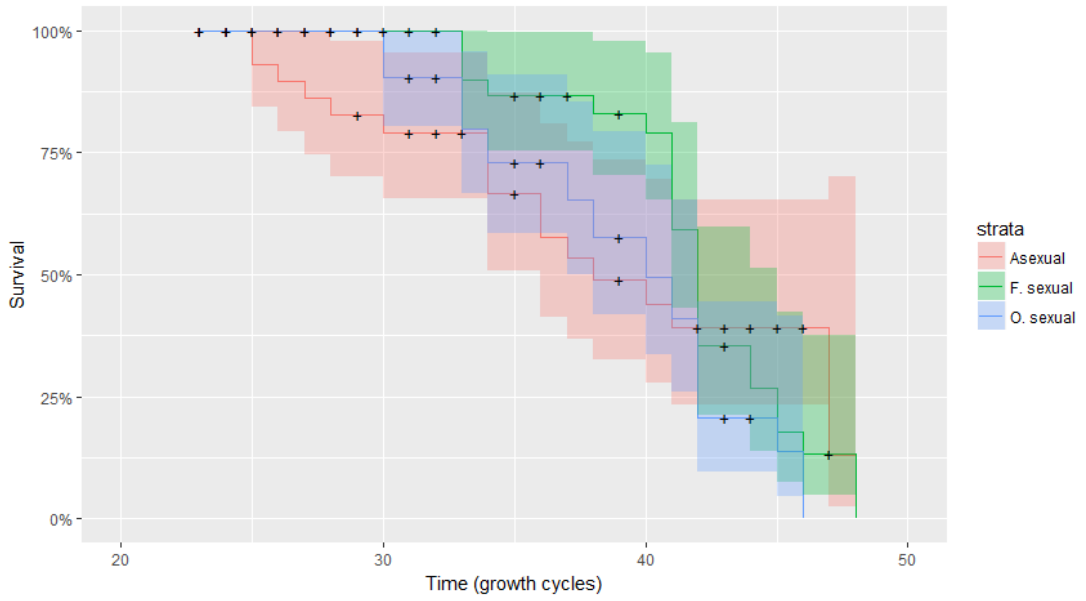

Figure 2.10 – The extinction dynamics per each mode of reproduction in the high rate of salt increase; the 95% confidence interval for each survival curve is represented with the shaded area between upper and lower boundary.

The rate of salt increase affected the extinction dynamics of experimental populations relative to maximal salt concentration reached when each extinction occurred (Weibull regression model; $\chi^2 = 18.9$; df = 2; $P < 0.0001$) (Figure 2.11). Gradual-rate group of populations had the slowest extinction rate with respect to salt concentration. The extinction dynamics of this treatment group was significantly different relative to both high rate ($z = 3.8$; $P = 0.0001$) and moderate rate group ($z = 2.2$; $P = 0.03$). No populations went extinct before salt concentration reached 27 g/l, 60% reached and 26% survived 30 g/l of salt concentration. The last populations went extinct while being subjected to 32 g/l of salt concentration. The extinction dynamics between high rate and moderate rate groups was significantly different ($z = 2.38$; $P = 0.2$). First extinctions for high rate group occurred in salt concentration of 18 g/l, 32% went extinct by the time corresponding to 24 g/l, 45% reached and 20% survived 30 g/l of salt concentration. The last extinctions in this treatment group occurred in salt concentration of 36 g/l. First extinctions for moderate rate group corresponded to the 12 g/l of salt concentration, followed by the long interval during which only a single extinction occurred (between 12 g/l and 26 g/l). The majority of populations (96%) went extinct within a relatively brief interval of time corresponding to salt concentrations between 26g/l and 30 g/l; 20% of populations reached, but none survived 30 g/l of salt concentration.

The overall effect of mode of reproduction on cumulative extinction dynamics (all rates of salt increase combined) with respect to salt concentration was not statistically significant (Weibull regression model; $\chi^2 = 1.67$; df = 2; P = 0.43) (Figure 2.12). However, as in the previous model regarding the extinction dynamics with respect to time in growth cycles, the relative advantage of individual levels within 'mode of reproduction' factor depended on the rate of salt increase. There was a significant interaction between mode of reproduction and rate of salt increase on the extinction dynamics of experimental populations relative to salt concentration (Weibull regression model; $\chi^2 = 9.99$; df = 4; P = 0.04). In the gradual rate of salt increase, the extinction dynamics of facultative sexual group was significantly different than that of both obligate sexual (z = 2.08, P = 0.04) and asexual group (z = -2.85, P = 0.004), due to higher number of extinct populations in comparison to each of these groups during most of the extinction period (27 g/l – 30 g/l) (Figure 2.13). In moderate rate of salt increase, there was a significant difference between the extinction dynamics of facultative sexual and asexual groups (z = -2.51, P = 0.01) (Figure 2.14). Similarly, a significant difference was recorded between facultative sexual and asexual groups in high rate of salt increase (z = -2.51, P = 0.01) (Figure 2.15).

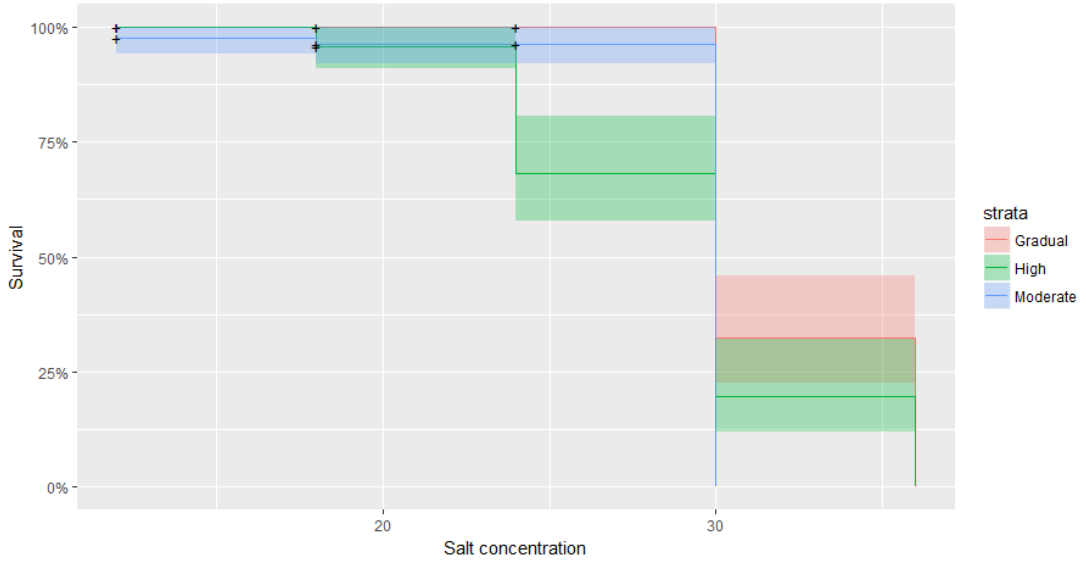

Figure 2.11 – Cumulative extinction dynamics of experimental populations per each rate of salt increase (irrespective of mode of reproduction); extinctions are plotted against salt concentration reached when extinction occurred; the 95% confidence interval for each survival curve is represented with the shaded area between upper and lower boundary.

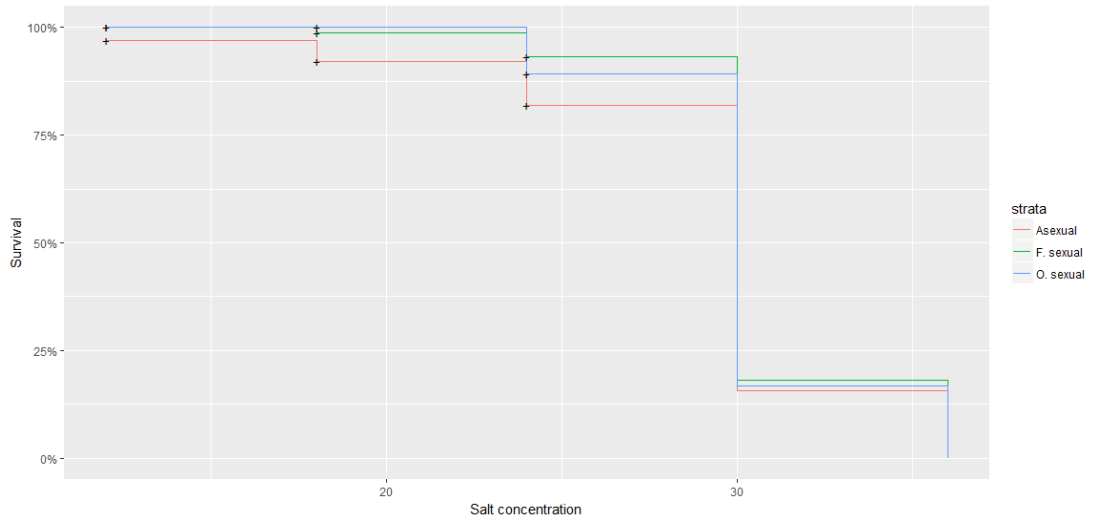

Figure 2.12 – Cumulative extinction dynamics of experimental populations per each mode of reproduction (irrespective of rate of salt increase); extinctions are plotted against salt concentration reached when extinction occurred;

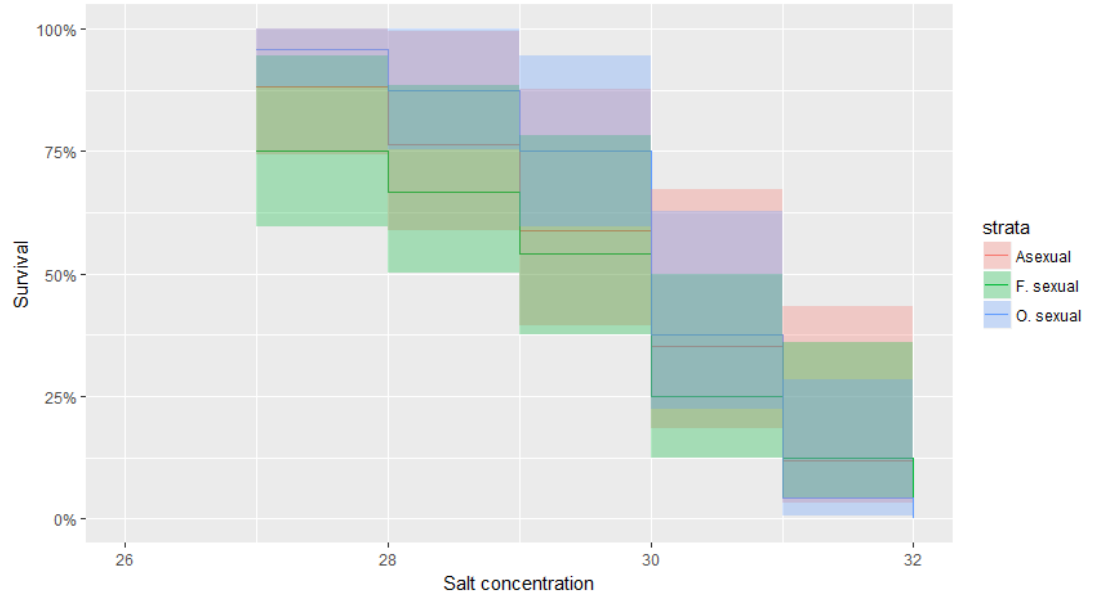

Figure 2.13 - The extinction dynamics per each mode of reproduction, in the gradual rate of salt increase; extinctions are plotted against salt concentration reached when extinction occurred; the 95% confidence interval for each survival curve is represented with the shaded area between upper and lower boundary.

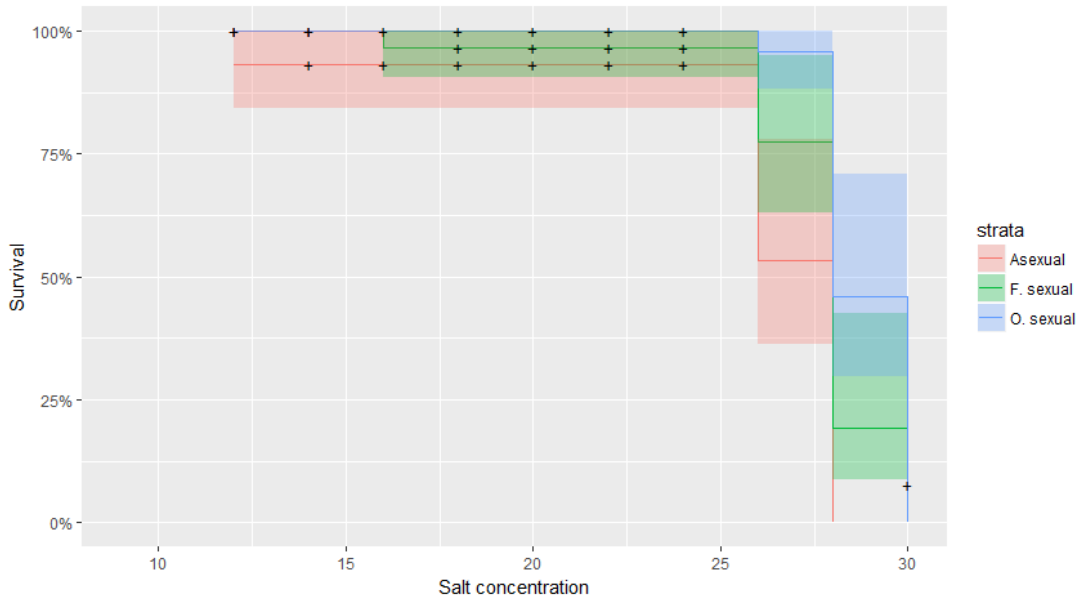

Figure 2.14 - The extinction dynamics per each mode of reproduction, in the moderate rate of salt increase; extinctions are plotted against salt concentration reached when extinction occurred; the 95% confidence interval for each survival curve is represented with the shaded area between upper and lower boundary.

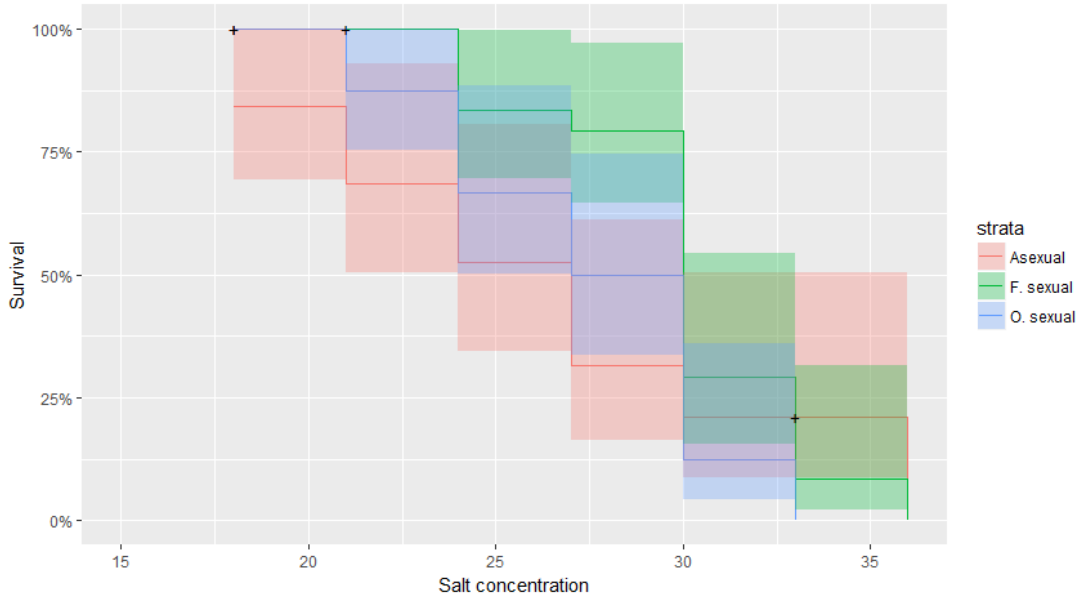

Figure 2.15 - The extinction dynamics per each mode of reproduction, in the high rate of salt increase; extinctions are plotted against salt concentration reached when extinction occurred; the 95% confidence interval for each survival curve is represented with the shaded area between upper and lower boundary.

2.4.2.3. *Examination of evolutionary rescue events in the treatment* groups

In parallel with propagating the main experimental populations in increasing concentration of salt, another experiment was carried out. Before being subjected to the next step of salt increase, each experimental population was sub-sampled and assayed for growth in the same level of salt as at the time of sub-sampling. The purpose of the assays was investigating whether evolutionary rescue had occurred during the main selection experiment.

I recorded 119 evolutionary rescue events in total, out of 475 assayed populations (25%). The probability of evolutionary rescue was significantly affected by the rate of environmental deterioration (Binomial regression; $\chi^2 = 24.77$; df = 2; $P < 0.00001$). Of 130 gradual rate populations tested, 45 were rescued from extinction (35%); of 213 moderate rate populations tested, 60 survived (28%); of 132 high rate populations tested, 14 were rescued from extinction (11%). A significant difference in the number of extinction events was detected between the high rate treatment group and both the gradual rate ($z = -2.75$; $P = 0.006$) and moderate rate ($z = -2.3$; $P = 0.02$) treatment groups. However, no significant difference between the gradual rate and moderate rate treatment groups was recorded ($z = -0.96$; $P = 0.34$). Within the gradual rate treatment group, 17 g/l was the maximal salt concentration for which evolutionary rescue had been recorded, while the maximal salt concentration reached by both the moderate rate and high rate treatment groups is 12 g/l.

Mode of reproduction did not significantly affect the probability of evolutionary rescue (Binomial regression; $\chi^2 = 3.94$; df = 2; $P = 0.14$); of 139 asexual population tested, 32 survived (23%); of 168 obligate sexual populations tested, 51 were rescued from extinction (30%); of 168 facultative sexual populations tested, 36 survived (21%). No significant interaction was detected between the rate of salt increase and mode of reproduction (Binomial regression; $\chi^2 = 1.39$; df = 4; $P = 0.85$).

2.4.2.3.1 Gradual rate

I recorded 45 evolutionary rescue events in total for treatment groups subjected to the gradual rate of salt increase, occurring during exposure to salt concentrations of 16 g/l and 17 g/l. Due to technical difficulties with a culture maintenance, I could not obtain a reliable number of surviving populations for lower salt concentrations. The probability of

evolutionary rescue was contingent on the level of salt increase (Binomial regression; χ^2 = 4.14; df = 1; P = 0.04). Seventeen evolutionary rescue events were recorded for salt concentration of 16 g/l, while 28 populations survived salt concentration of 17 g/l (Figure 2.16). Mode of reproduction did not affect the probability of evolutionary rescue (Binomial regression; χ^2 = 2.31; df = 2; P = 0.31). Twenty evolutionary rescue events were recorded in the obligate sexual group, 13 in the facultative sexual group and 12 in the asexual group (Figure 2.17). No significant interaction between the level of salt increase and mode of reproduction was recorded (Binomial regression; χ^2 = 2.01; df = 2; P = 0.37). The number of evolutionary rescue events occurring during exposure to salt concentration of 16 g/l was approximately equal in all treatment groups (6 in each sexual group and 5 in asexual group). Most evolutionary rescue events during exposure to salt concentration of 17 g/l were recorded in obligate sexual group (14) (Figure 2.18).

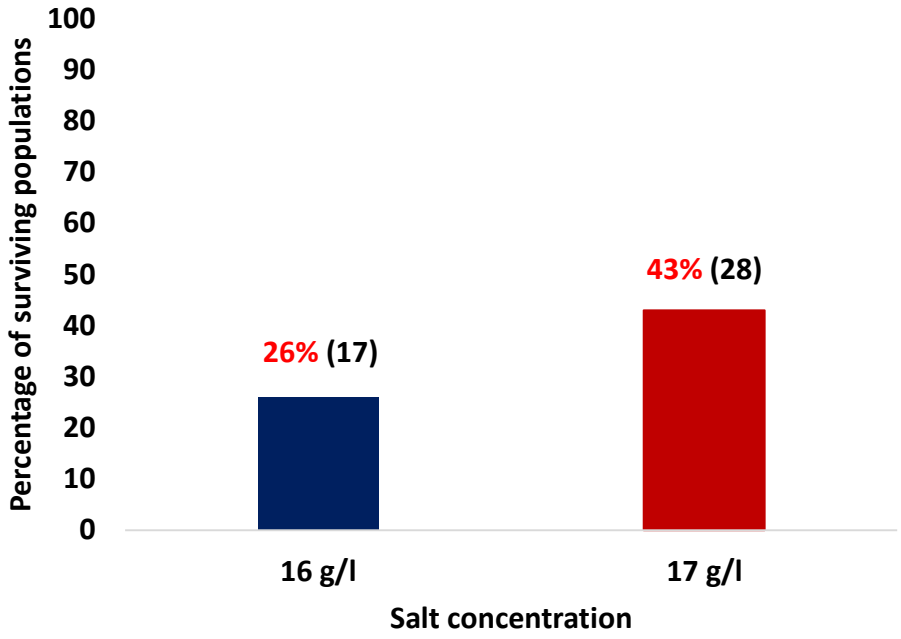

Figure 2.16 - Percentage of evolutionary rescue events relative to total number of populations of gradual rate treatment group (65), with respect to both levels of salt increase; the numbers in brackets correspond to the number of evolutionary rescue events.

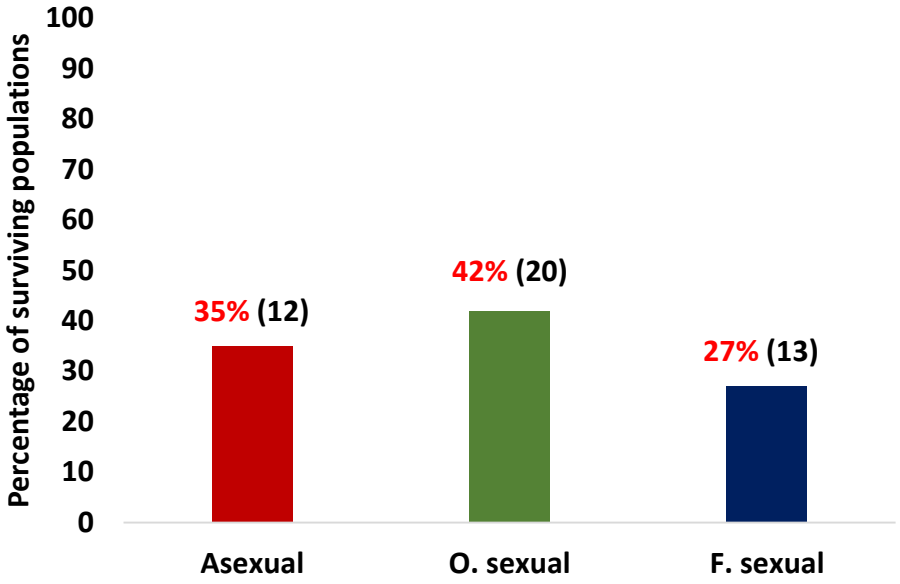

Figure 2.17 – Percentage of evolutionary rescue events per each mode of reproduction of gradual rate treatment group; the numbers in brackets correspond to the number of evolutionary rescue events.

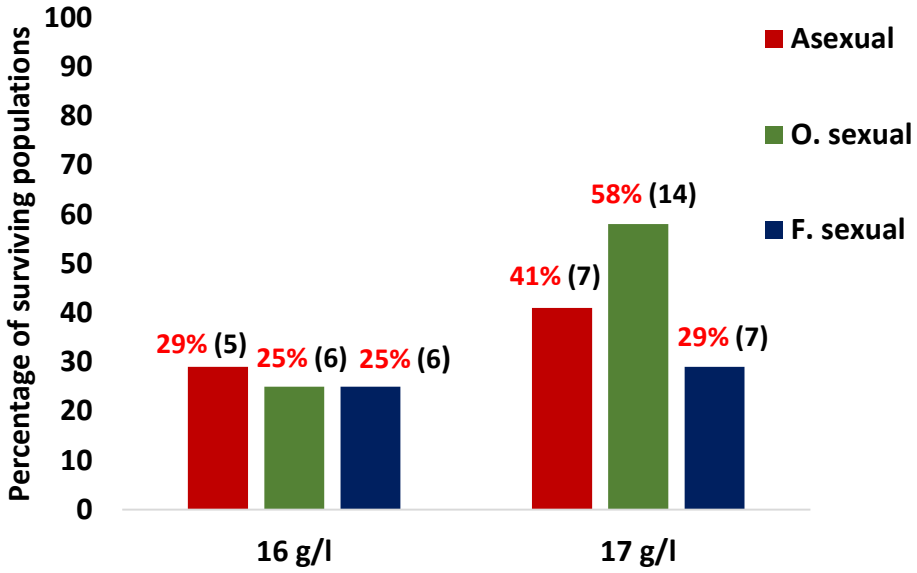

Figure 2.18 - Percentage of evolutionary rescue events per each mode of reproduction of gradual rate treatment group, with respect to each level of salt increase; the numbers in brackets correspond to the number of evolutionary rescue events.

Mode of reproduction did not affect mean fitness of the rescued populations (Two-way ANOVA; $F_{2,44} = 2.19$; $P = 0.13$). Similarly, there was no significant effect of the level of

salt increase on mean fitness of the rescued populations (Two-way ANOVA; $F_{1,44} = 0.005$; P = 0.94) (Figure 2.19).

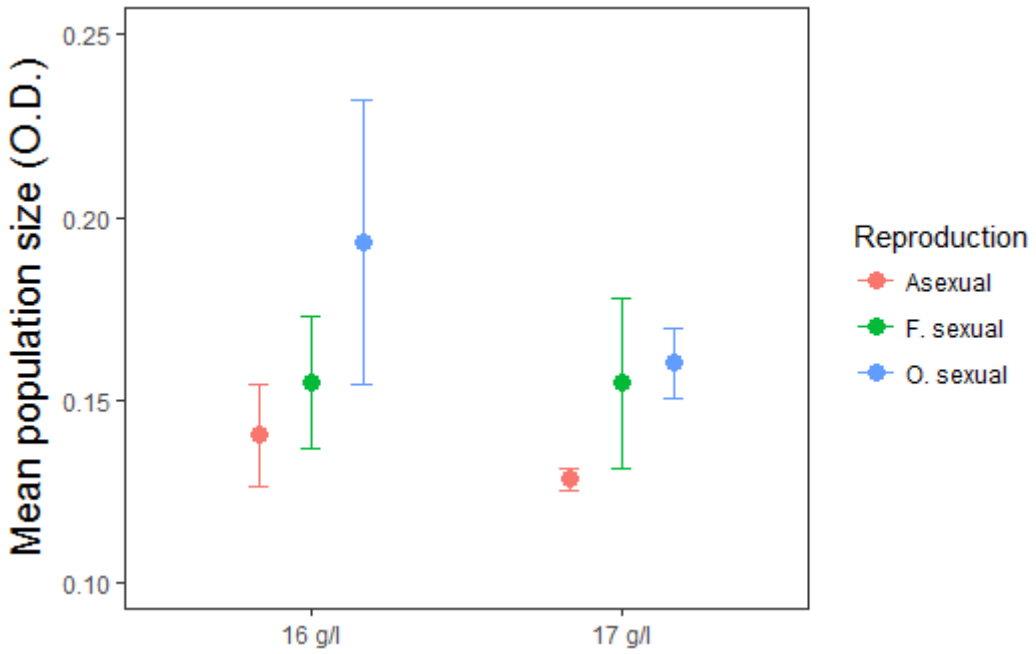

Figure 2.19 – Mean fitness of the rescued populations per each mode of reproduction of gradual rate treatment group, with respect to each level of salt increase; the bars represent standard error of the mean.

In order to rule out the possibility that the observed response of the rescued populations to detrimental environments had occurred through phenotypic plasticity rather than adaptive evolution, I compared the growth parameters (mean population size and growth rate) of these populations (with respect to mode of reproduction) with those of the ancestral isolates, assayed in the corresponding salt concentration prior to commencing of the selection experiment. Since the assay was not performed for salt concentrations higher than 10 g/l, the ancestors were subsequently tested in additional assay, by monitoring their growth rate in the range of salt concentrations between 11-17 g/l.

The mean population size of populations rescued in 16 g/l of salt concentration was significantly different among treatment groups (one-way ANOVA; $F_{3,55} = 30.65$; $P < 0.00001$) (Figure 2.20). The rescued populations of each treatment group had significantly higher mean population size than the ancestral isolates ($t = 5.53$, $df = 43$, $P < 0.00001$ for the asexual group; $t = 6.35$, $df = 44$, $P < 0.00001$ for the facultative sexual group; $t = 6.65$, $df = 44$, $P < 0.00001$ for the obligate sexual group). There was no significant difference between

each sexual treatment group and asexual treatment group (t = -0.26, df = 10, P = 0.79, for the difference between the asexual and facultative sexual group; t = -0.48, df = 10, P = 0.63, for the difference between the asexual and obligate sexual group). Likewise, no significant difference was detected between sexual groups (t = -0.23, df = 11, P = 0.82).

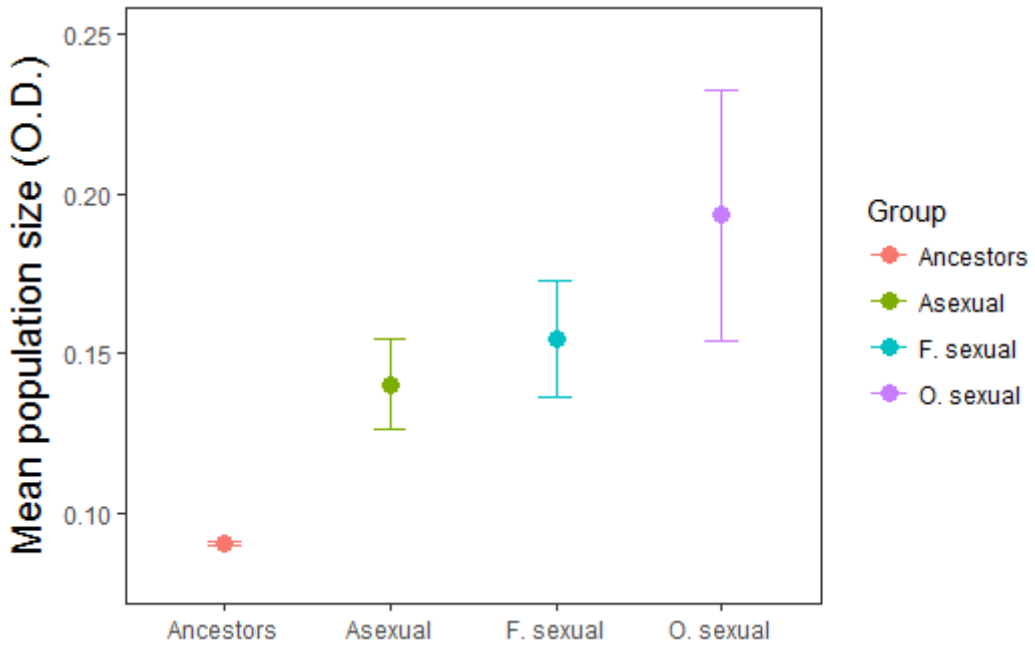

Figure 2.20 – Comparison of mean population size of gradual rate rescued populations with that of the ancestors assayed in the same conditions (16 g/l); the bars represent standard error of the mean.

The growth rate in the selective environment (16 g/l), measured as slope of the regression line representing mean change of population size as a function of time was contingent on the treatment group (one-way ANOVA; $F_{3, 55} = 30.32$; $P < 0.00001$) (Figure 2.21). A significant difference was detected between the growth rate of the ancestors and the rescued populations within all three treatment groups (t = 5.49, df = 43, P < 0.00001, for the difference between the ancestors and asexual group; t = 6.27, df = 44, P < 0.00001, for the difference between the ancestors and facultative sexual group; t = 6.65, df = 44, P < 0.00001, for the difference between the ancestors and obligate sexual group). The differences among other treatment groups were not statistically significant.

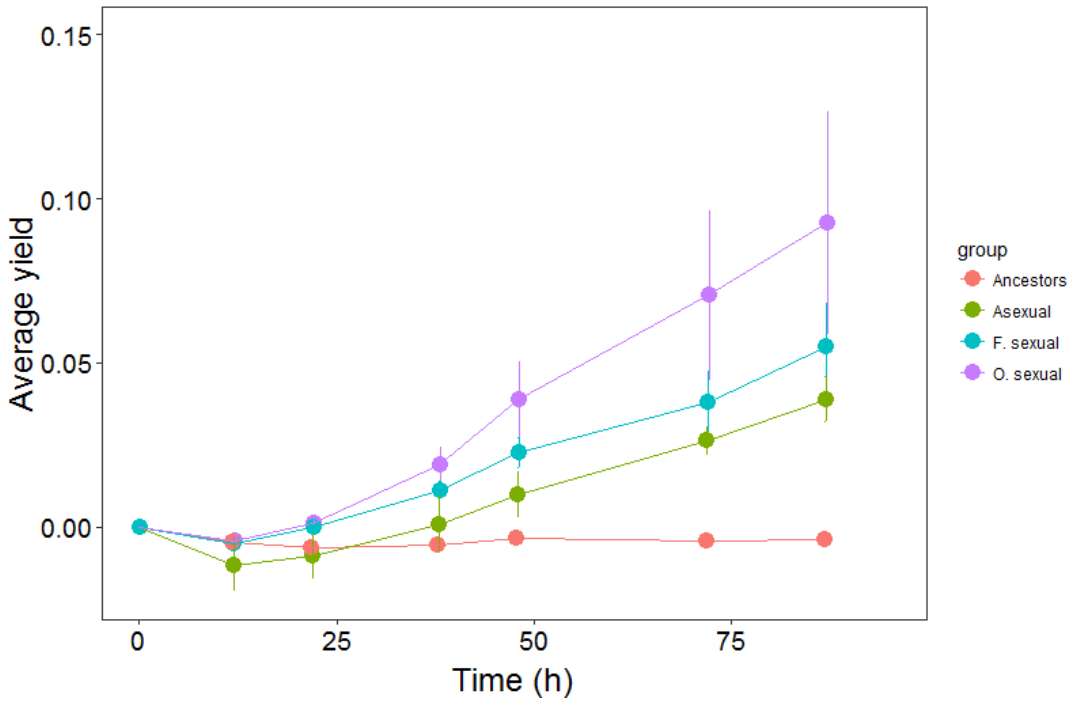

Figure 2.21 - Comparison of growth rate of gradual rate rescued populations (with respect to mode of reproduction) with that of the ancestors assayed in the same conditions (16 g/l); the bars represent standard error of the mean.

The mean population size of populations rescued in 17 g/l of salt concentration was significantly different among treatment groups (one-way ANOVA; $F_{3,66} = 60.72$; $P < 0.00001$) (Figure 2.22). The rescued populations of each treatment group had significantly higher mean population size than the ancestral isolates ($t = 6.91$, $df = 45$, $P < 0.00001$ for the asexual group; $t = 7.88$, $df = 45$, $P < 0.00001$ for the facultative sexual group; $t = 11.56$, $df = 52$, $P < 0.00001$ for the obligate sexual group). There was no significant difference between each sexual treatment group and asexual treatment group ($t = -0.74$, $df = 13$, $P = 0.46$, for the difference between the asexual and facultative sexual group; $t = -1.65$, $df = 20$, $P = 0.1$, for the difference between the asexual and obligate sexual group). Likewise, no significant difference was detected between sexual groups ($t = 0.8$, $df = 20$, $P = 0.43$).

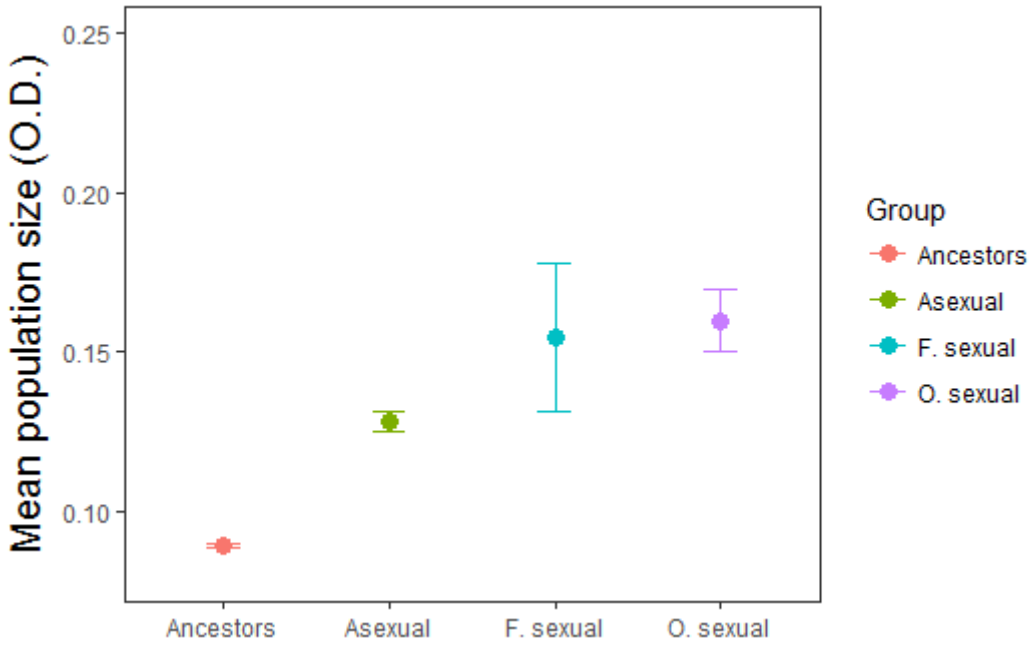

Figure 2.22 – Comparison of mean population size of gradual rate rescued populations with that of the ancestors assayed in the same conditions (17 g/l); the bars represent standard error of the mean.

The growth rate in the selective environment (17 g/l), measured as slope of the regression line representing mean change of population size as a function of time was contingent on the treatment group (one-way ANOVA; $F_{3, 66} = 59.02$; $P < 0.00001$) (Figure 2.23). A significant difference was detected between the growth rate of the ancestors and the rescued populations within all three treatment groups ($t = 7.08$, $df = 45$, $P < 0.00001$, for the difference of the ancestors and asexual group; $t = 7.73$, $df = 45$, $P < 0.00001$, for the difference of the ancestors and facultative sexual group; $t = 11.29$, $df = 52$, $P < 0.00001$, for the difference of the ancestors and obligate sexual group). The differences among other treatment groups were not statistically significant.

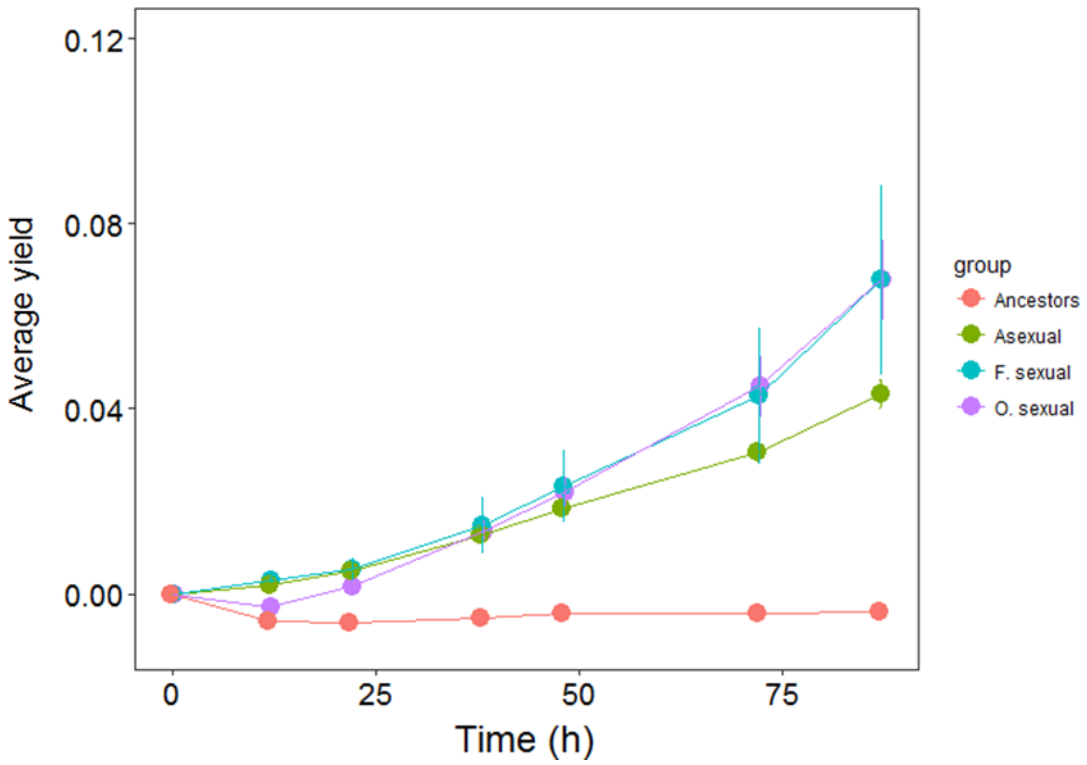

Figure 2.23 - Comparison of growth rate of gradual rate rescued populations (with respect to mode of reproduction) with that of the ancestors assayed in the same conditions (17 g/l); the bars represent standard error of the mean.

2.4.2.3.2 Moderate rate

I recorded 60 evolutionary rescue events in total for treatment groups subjected to the moderate rate of salt increase. All evolutionary rescue events occurred during exposure to salt concentrations of 8 g/l, 10 g/l, and 12 g/l. The probability of evolutionary rescue was contingent on the level of salt increase (Binomial regression; $\chi^2 = 6.71$; df = 2; P = 0.03) (Figure 2.24); 28 evolutionary rescue events were recorded for the salt level of 8 g/l, 15 populations survived in 10 g/l and 17 evolutionary rescue events occurred in 12 g/l of salt concentration. Mode of reproduction did not significantly affect the probability of evolutionary rescue (Binomial regression; $\chi^2 = 1.43$; df = 2; P = 0.49). Twenty-four evolutionary rescue events were recorded in the obligate sexual group and 18 in the facultative sexual and asexual group, respectively (Figure 2.25). There was a significant interaction between the level of salt increase and mode of reproduction (Binomial regression; $\chi^2 = 23.7$; df = 4; P < 0.001) (Figure 2.26). The highest number of evolutionary rescue events during exposure to salt levels of 8 g/l and 12 g/l was recorded in the obligate

sexual group (11 and 12, respectively). Most evolutionary rescue events for salt level of 10 g/l occurred in asexual populations (9).

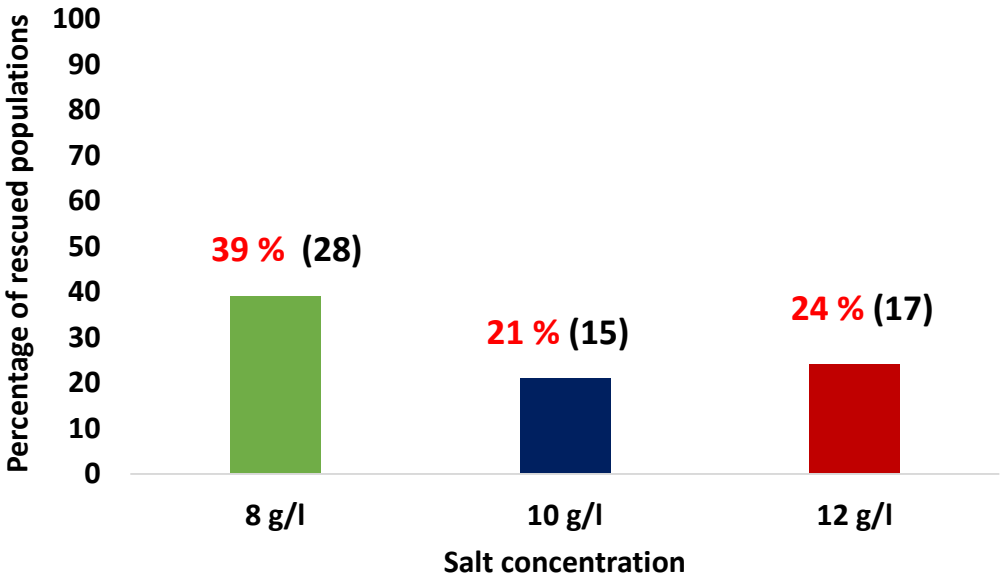

Figure 2.24 – Percentage of evolutionary rescue events relative to total number of populations of moderate-rate treatment group (71), with respect to each level of salt increase; the numbers in brackets correspond to the number of evolutionary rescue events.

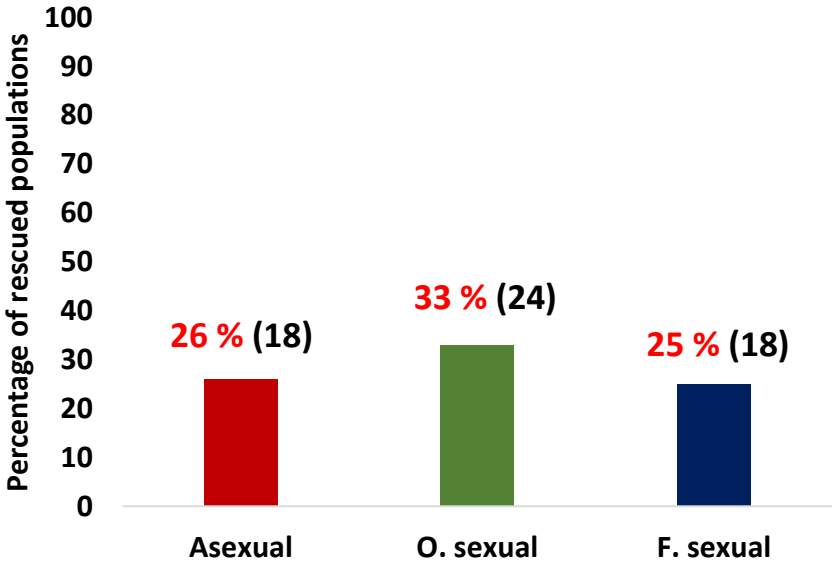

Figure 2.25 - Percentage of evolutionary rescue events per each mode of reproduction of moderate-rate treatment group; the numbers in brackets correspond to the number of evolutionary rescue events.

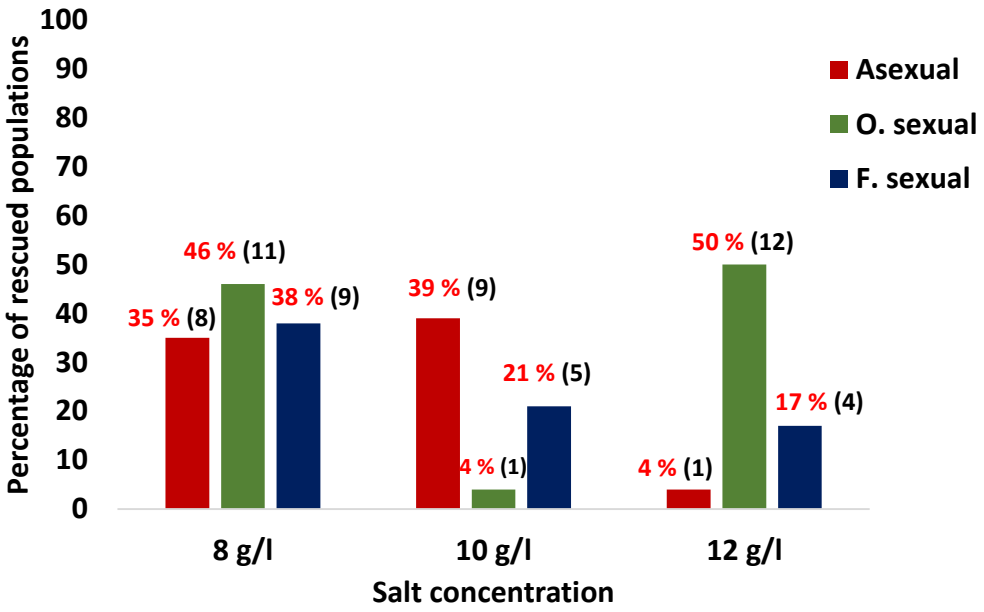

Figure 2.26 - Percentage of evolutionary rescue events per each mode of reproduction of moderate-rate treatment group, with respect to each level of salt increase; the numbers in brackets correspond to the number of evolutionary rescue events.

Mode of reproduction significantly affected mean fitness of the rescued populations (two-way ANOVA; $F_{2,59} = 4.0$; $P = 0.024$) (Figure 2.27). This part of the results requires cautious interpretation, since some treatment groups comprise only a single rescued population. Generally, both sexual groups had higher mean fitness in salt concentrations of 8 g/l and 10 g/l. A single rescued asexual population had the highest fitness in salt concentration of 12 g/l. Mean fitness of the rescued populations was contingent on salt concentration (two-way ANOVA; $F_{2,59} = 67.7$; $P < 0.00001$). The average population size was the highest in salt concentration of 8 g/l, irrespective of mode of reproduction of the rescued populations. No significant interaction between mode of reproduction and salt concentration was detected (two-way ANOVA; $F_{4,59} = 2.09$; $P = 0.1$).

As in the previous section, the growth parameters of the rescued populations (mean population size and the growth rate) were compared with those of the ancestral isolates, assayed in the corresponding salt concentration prior to commencing of the selection experiment.

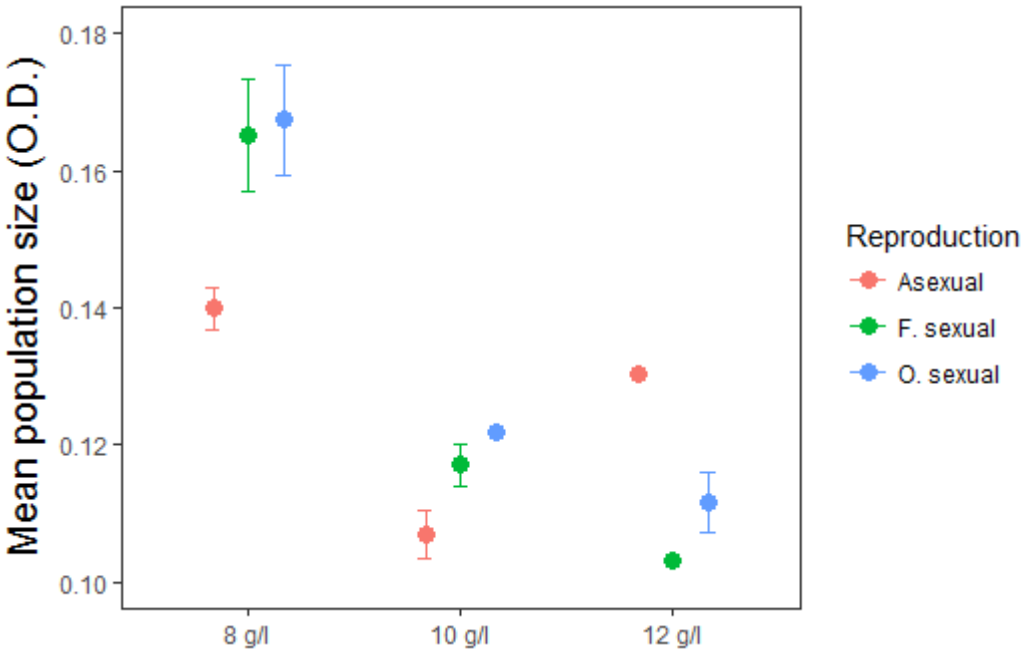

Figure 2.27 – Mean fitness of the rescued populations per each mode of reproduction of moderate rate treatment group, with respect to each level of salt increase; the bars represent standard error of the mean (absent in treatment groups comprising a single rescued population).

The mean population size of the populations rescued in salt concentration of 8 g/l was contingent on treatment group (one-way ANOVA; $F_{3,66} = 18.71$; $P < 0.00001$) (Figure 2.28). The rescued populations of each treatment group had significantly higher mean population size than the ancestral isolates ($t = -2.86$, $df = 46$, $P = 0.006$ for asexual group; $t = 5.45$, $df = 47$, $P < 0.00001$ for the facultative sexual group; $t = -6.02$, $df = 49$, $P < 0.00001$ for the obligate sexual group). A significant difference was recorded between the rescued populations in obligate sexual treatment group and asexual treatment group ($t = -2.03$, $df = 18$, $P = 0.047$). A marginal difference was recorded between the asexual and facultative sexual group ($t = -1.86$, $df = 16$, $P = 0.07$). No significant difference was detected between sexual groups ($t = 0.084$, $df = 19$, $P = 0.93$).

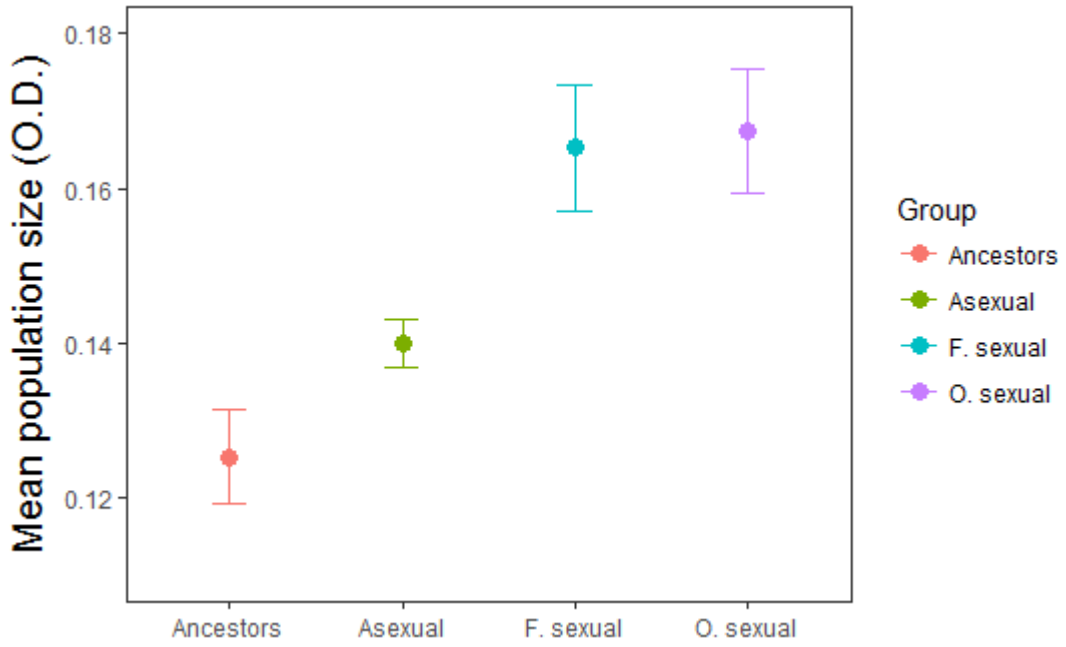

Figure 2.28 – Comparison of mean population size of moderate rate rescued populations with that of the ancestors assayed in the same conditions (8 g/l); the bars represent standard error of the mean.

The growth rate in the selective environment (8 g/l), measured as slope of the regression line representing mean change of population size as a function of time, was contingent on the treatment group (one-way ANOVA; $F_{3, 66} = 47.81$; $P < 0.00001$) (Figure 2.29). A significant difference was detected between the growth rate of the ancestors relative to that of the asexual group ($t = 7.22$, $df = 46$, $P < 0.00001$), obligate sexual group ($t = 9.04$, $df = 49$, $P < 0.00001$) and facultative sexual group ($t = 7.98$, $df = 47$, $P < 0.00001$). No significant differences were detected among the treatment groups of the rescued populations.

There was a marginal difference between mean population size of populations rescued in 10 g/l of salt concentration (one-way ANOVA; $F_{3,53} = 2.68$; $P = 0.057$) (Figure 2.30). The difference between the facultative sexual group and the ancestors was significant ($t = 2.4$, $df = 43$, $P = 0.02$). There was no significant difference in mean fitness between sexual groups ($t = -0.39$, $df = 5$, $P = 0.7$). The difference between the obligate sexual group and the ancestors was undetected by the model, likely due to presence of a single rescued population in the former treatment group ($t = 1.57$, $df = 8$, $P = 0.12$). The difference in mean population size between the asexual group and the ancestors was not significant ($t = 0.69$; $df = 46$, $P = 0.49$).

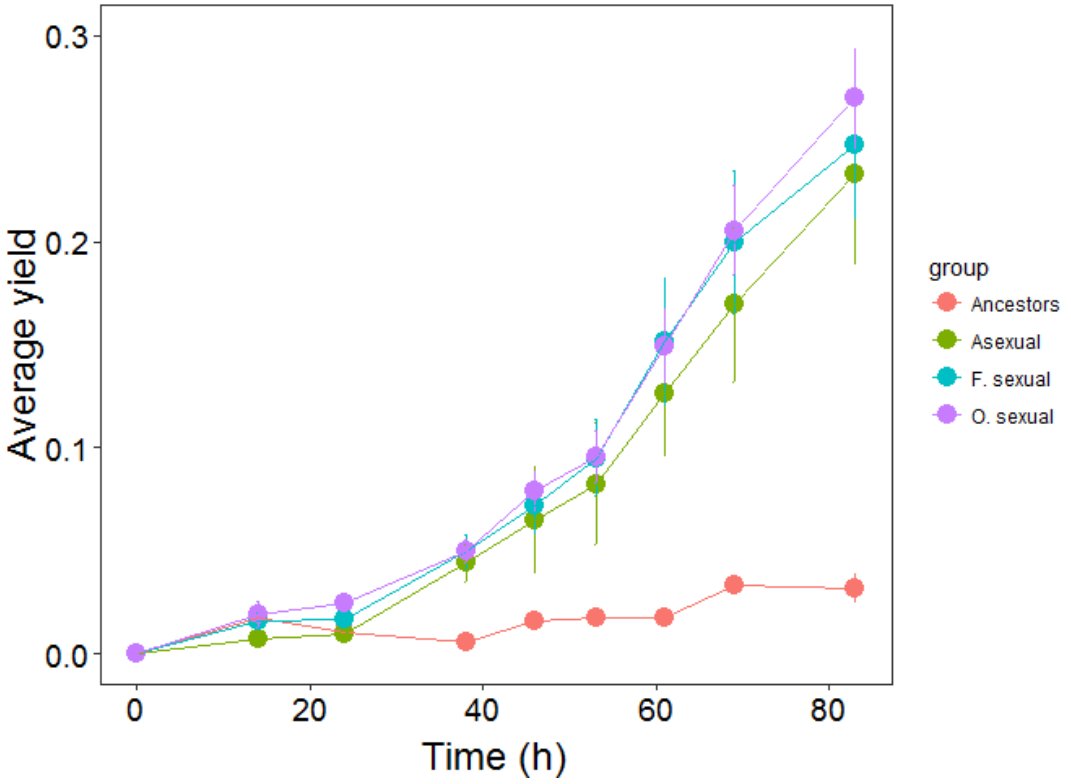

Figure 2.29 – Comparison of growth rate of moderate rate rescued populations (with respect to mode of reproduction) with that of the ancestors assayed in the same conditions (8 g/l); the bars represent standard error of the mean.

The growth rate in the selective environment (10 g/l), measured as slope of the regression line representing mean change of population size as a function of time, was contingent on the treatment group (one-way ANOVA; $F_{3, 53} = 4.16$; P = 0.01) (Figure 2.31). A significant difference was detected between the growth rate of the ancestors relative to that of the asexual group (t = 2.52, df = 46, P = 0.02) and a single rescued obligate sexual population (t = 2.08, df = 8, P = 0.04). A marginal difference was detected between the ancestors and the facultative sexual group (t = 2.0, df = 43, P = 0.05). No significant differences were detected among the treatment groups of the rescued populations.

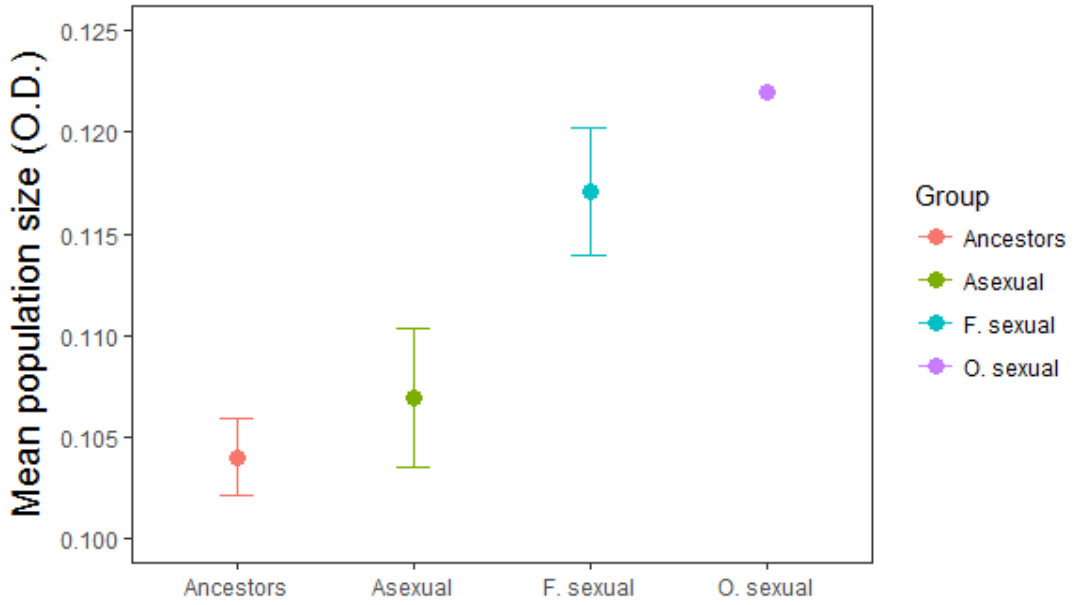

Figure 2.30 – Comparison of mean population size of moderate rate rescued populations with that of the ancestors assayed in the same conditions (10 g/l); the bars represent standard error of the mean.

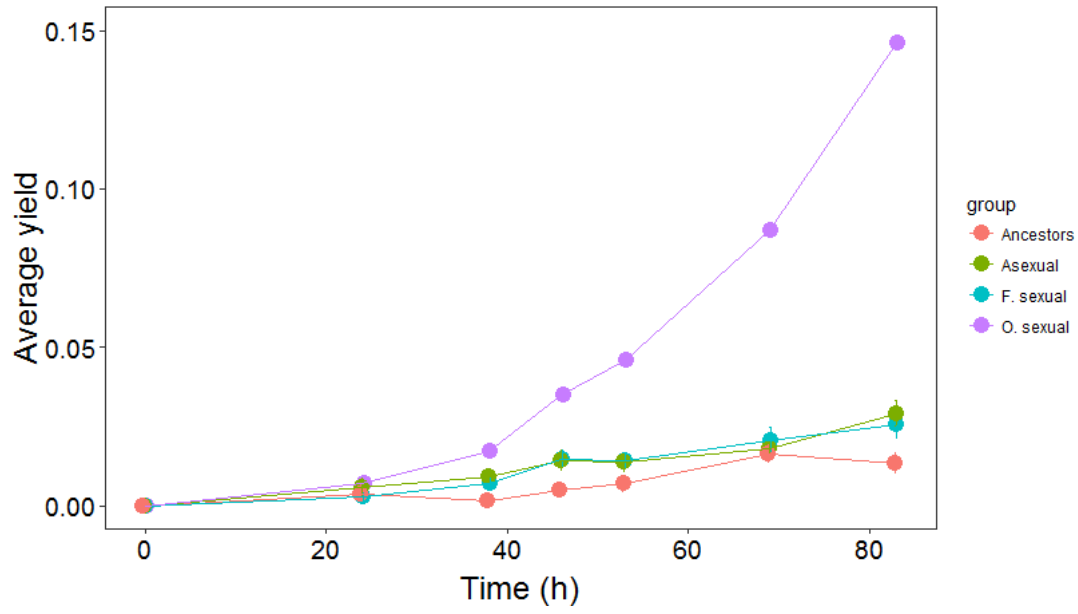

Figure 2.31 – Comparison of growth rate of moderate rate rescued populations (with respect to mode of reproduction) with that of the ancestors assayed in the same conditions (10 g/l); the bars represent standard error of the mean.

There was a significant difference between mean population size of populations rescued in 12 g/l of salt concentration among treatment groups (One-way ANOVA; $F_{3,55}$ = 5.47; P = 0.002) (Figure 2.32). There was a significant difference between mean population

size of the ancestors relative to the asexual (t = 2.06; df = 38; P = 0.04) and obligate sexual groups (t = 3.55, df = 50, P = 0.0008). However, mean population size of the rescued facultative sexual populations did not significantly differ from that of the ancestors (t = 1.35, df = 42, P = 0.18). The difference between asexual and both sexual groups was not detected, nor the difference between sexual groups.

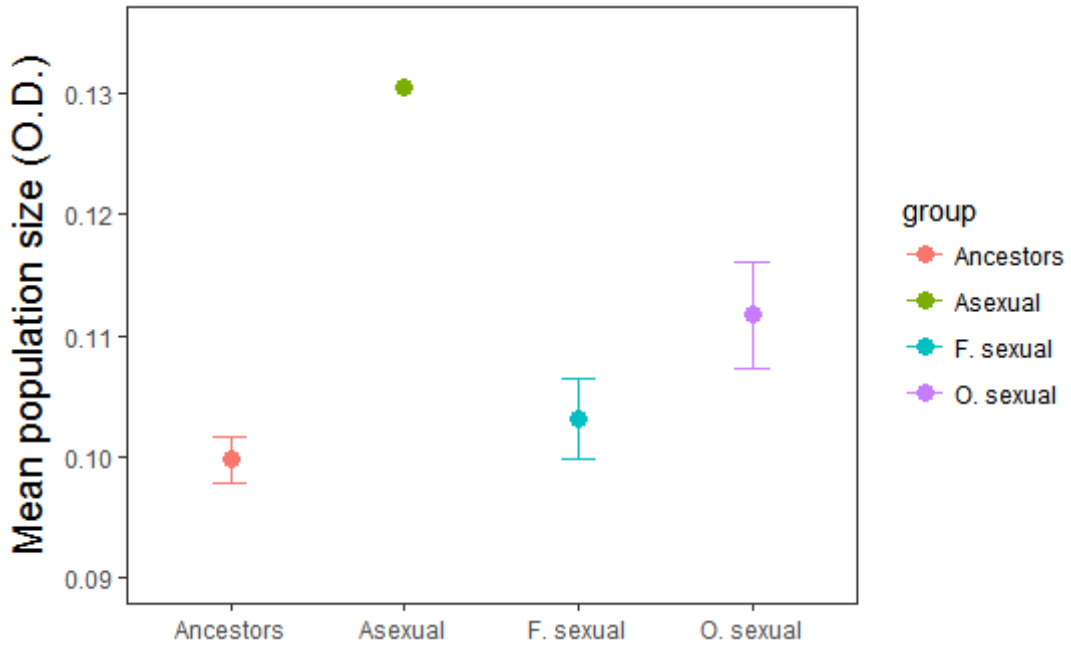

Figure 2.32 – Comparison of mean population size of moderate rate rescued populations with that of the ancestors assayed in the same conditions (12 g/l); the bars represent standard error of the mean (absent in treatment groups comprising a single rescued population).

The growth rate in the selective environment (12 g/l), measured as slope of the regression line representing mean change of population size as a function of time was contingent on the treatment group (one-way ANOVA; $F_{3,55} = 26.65$; $P < 0.00001$) (Figure 2.33). A significant difference was detected between the growth rate of the ancestors relative to that of the asexual group (a single population) (t = 3.35, df = 38, P = 0.001), obligate sexual group (t =8.13, df = 50, P = < 0.00001), and facultative sexual group (t = 3.79, df = 42, P < 0.00001). No significant differences were detected among the treatment groups of the rescued populations.

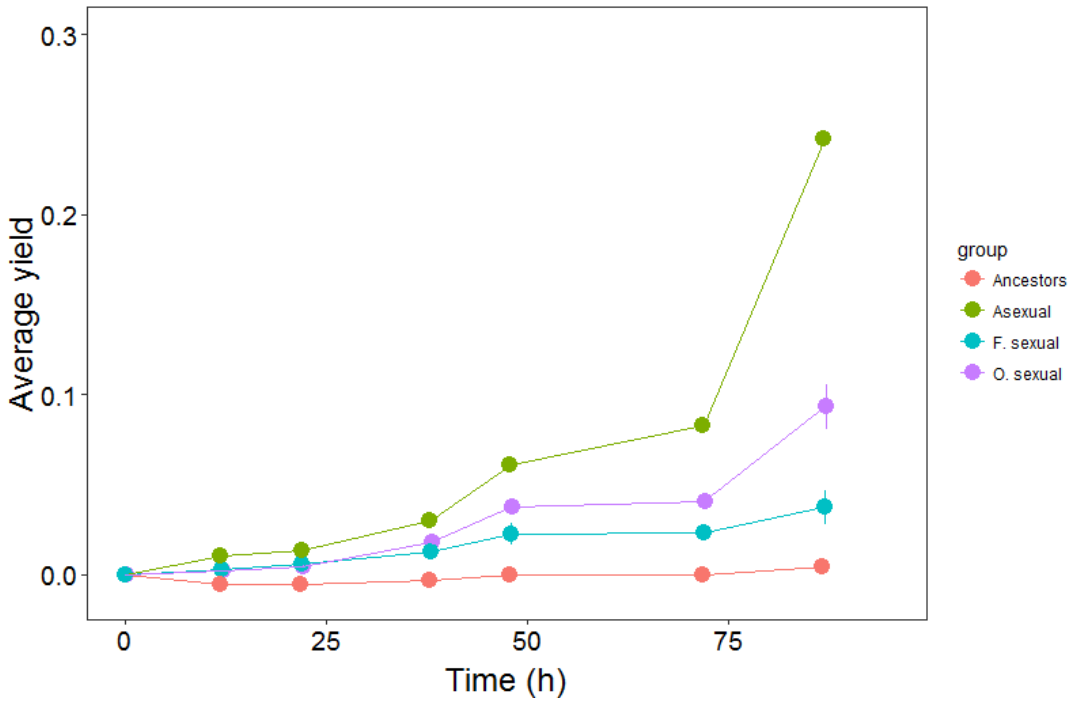

Figure 2.33 - Comparison of growth rate of moderate rate rescued populations (with respect to mode of reproduction) with that of the ancestors assayed in the same conditions (12 g/l); the bars represent standard error of the mean (absent in treatment group comprising a single rescued population).

2.4.2.3.3 High rate

I recorded 14 evolutionary rescue events in total for treatment groups subjected to the high rate of salt increase. All evolutionary rescue events occurred during exposure to salt concentrations of 9 g/l and 12 g/l. The probability of evolutionary rescue did not depend on the rate of salt increase (Binomial regression; χ^2 = 3.0; df = 1; P = 0.08; 4 evolutionary rescue events were recorded for the salt level of 9 g/l and 10 populations survived in 12 g/l (Figure 2.34). Likewise, mode of reproduction did not significantly affect the probability of evolutionary rescue (Binomial regression; χ^2 = 1.88; df = 2; P = 0.39). Seven evolutionary rescue events were recorded in obligate sexual group, 5 in facultative sexual group and 1 in asexual group (Figure 2.35). No significant interaction between the rate of salt increase and mode of reproduction was detected (Binomial regression; χ^2 = 1.8; df = 2; P = 0.4). The highest number of evolutionary rescue events during exposure to salt level of 9 g/l was recorded in facultative sexual group (2). Most evolutionary rescue events for salt level of 12 g/l occurred in obligate sexual populations (6) (Figure 2.36).

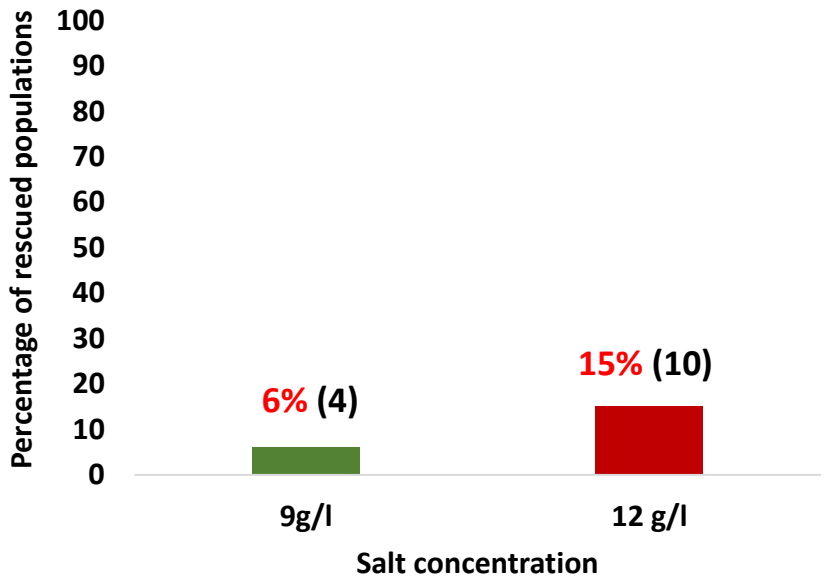

Figure 2.34 – Percentage of evolutionary rescue events relative to total number of populations of high rate treatment group (66), with respect to both levels of salt increase; the numbers in brackets correspond to the number of evolutionary rescue events;

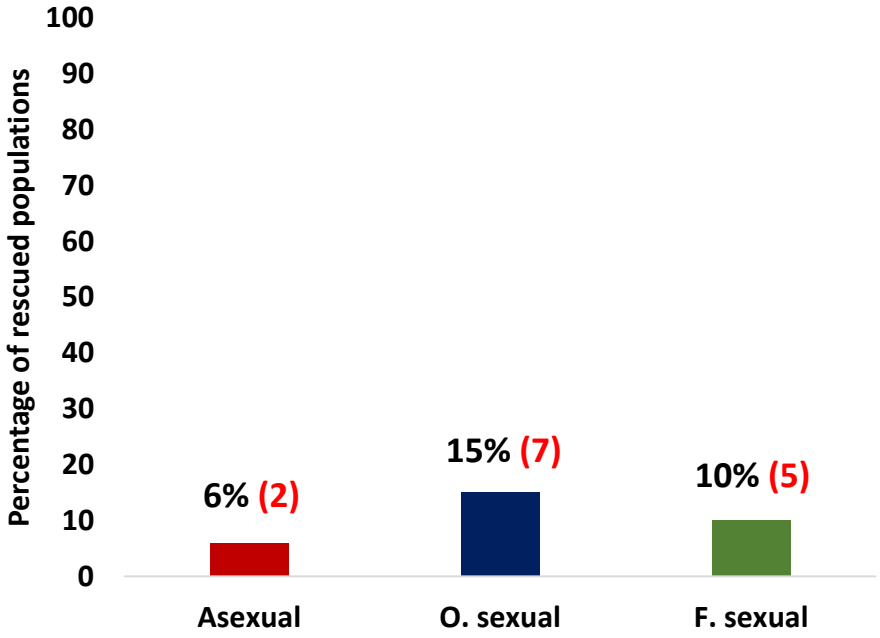

Figure 2.35 - Percentage of evolutionary rescue events per each mode of reproduction of high rate treatment group; the numbers in brackets correspond to the number of evolutionary rescue events;

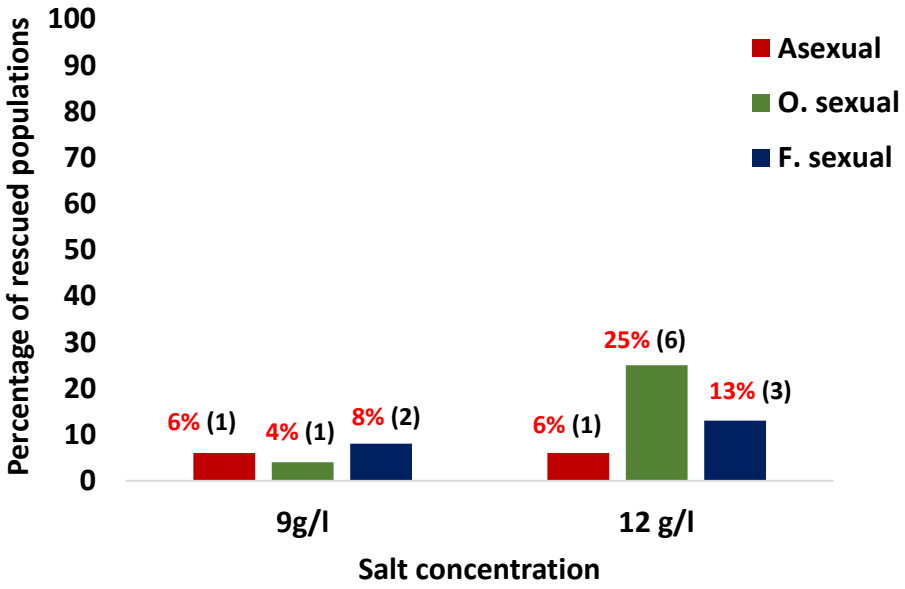

Figure 2.36 - Percentage of evolutionary rescue events per each mode of reproduction of high rate treatment group, with respect to each level of salt increase; the numbers in brackets correspond to the number of evolutionary rescue events;

Mode of reproduction marginally affected mean fitness of the rescued populations (two-way ANOVA; $F_{2,13} = 4.2$; $P = 0.06$) (Figure 2.37). Mean fitness of the rescued populations depended on salt concentration (two-way ANOVA; $F_{1,13} = 20.34$; $P = 0.002$). The average population size was higher in salt concentration of 9 g/l for both sexual groups of the rescued populations; the rescued asexual populations had similar population size recorded for both salt concentrations. A marginally significant interaction between mode of reproduction and salt concentration was detected (Two-way ANOVA; $F_{2,13} = 3.64$; $P = 0.08$).

As in the previous section, the growth parameters of the rescued populations (mean population size and the growth rate) were compared with those of the ancestral isolates, assayed in the corresponding salt concentration prior to commencing of the selection experiment.

A marginal difference in mean population size was detected among the treatment groups rescued in 9 g/l of salt concentration and the ancestors (One-way ANOVA; $F_{3,42} = 2.44$; $P = 0.08$) (Figure 2.38).

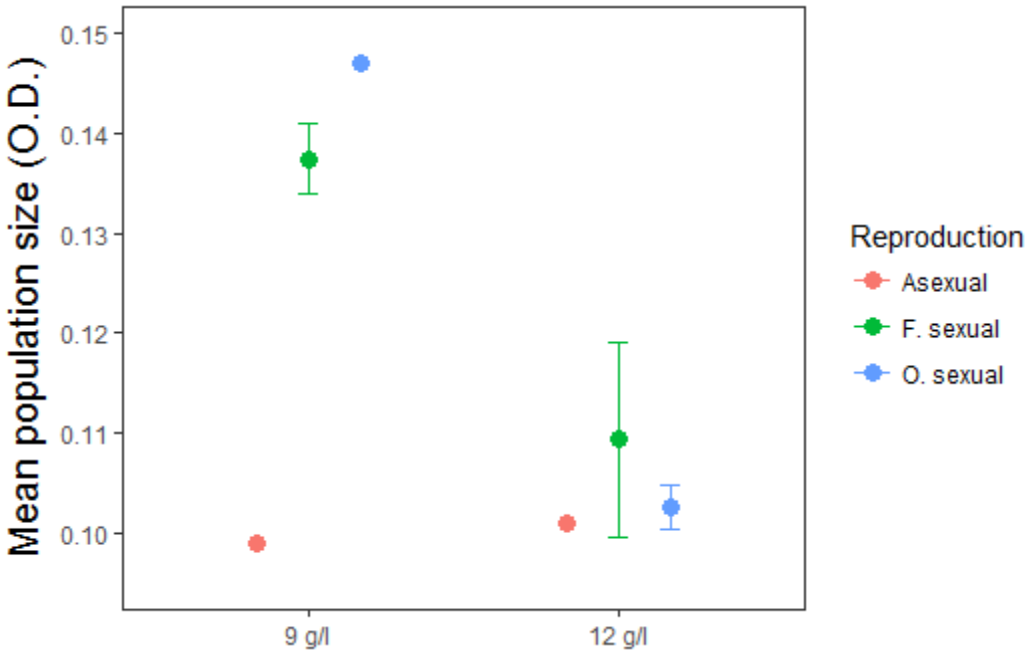

Figure 2.37 – Mean fitness of the rescued populations per each mode of reproduction of high rate treatment group, with respect to both levels of salt increase; the bars represent standard error of the mean (absent in treatment groups comprising a single rescued population).

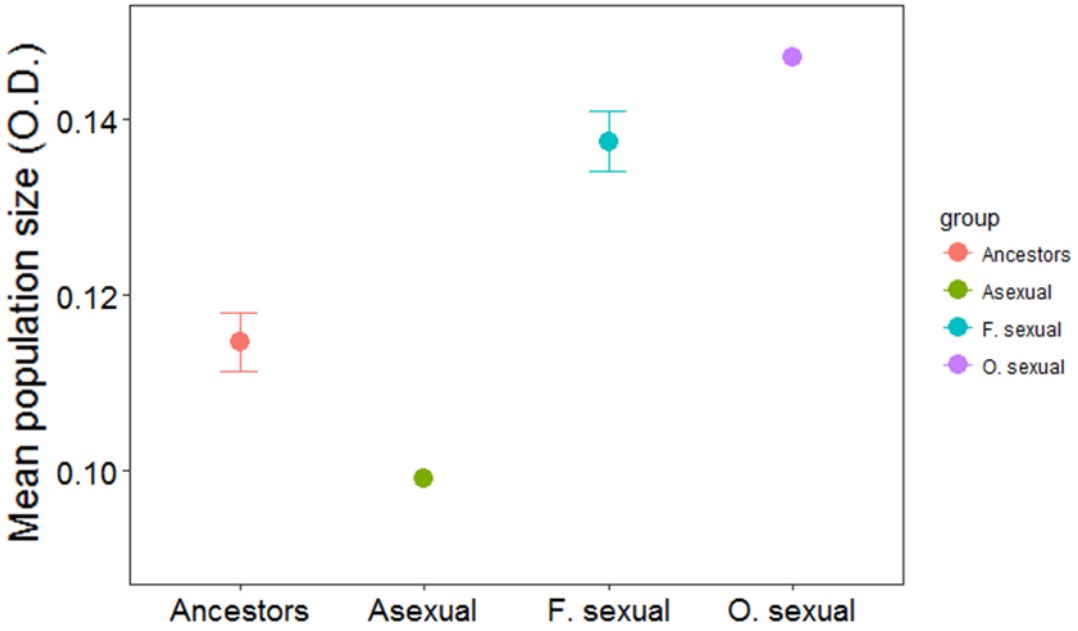

Figure 2.38 – Comparison of mean population size of high rate rescued populations with that of the ancestors assayed in the same conditions (9 g/l); the bars represent standard error of the mean (absent in treatment groups comprising a single rescued population).

The growth rate in the selective environment (9 g/l), measured as slope of the regression line representing mean change of population size as a function of time, was

contingent on the treatment group (one-way ANOVA; $F_{3, 42} = 4.42$; P = 0.009) (Figure 2.39). The facultative sexual populations rescued after propagation in 9 g/l of salt concentration had significantly higher growth rate than that of the ancestral populations, (t = 2.64, df = 40, P = 0.01). A marginal difference was detected between the ancestors and obligate sexual (t = 2.02, df = 39, P = 0.05) and asexual (t = 1.76, df = 39, P = 0.09) groups. No significant differences in the growth rate among the treatment groups of the rescued populations were recorded.

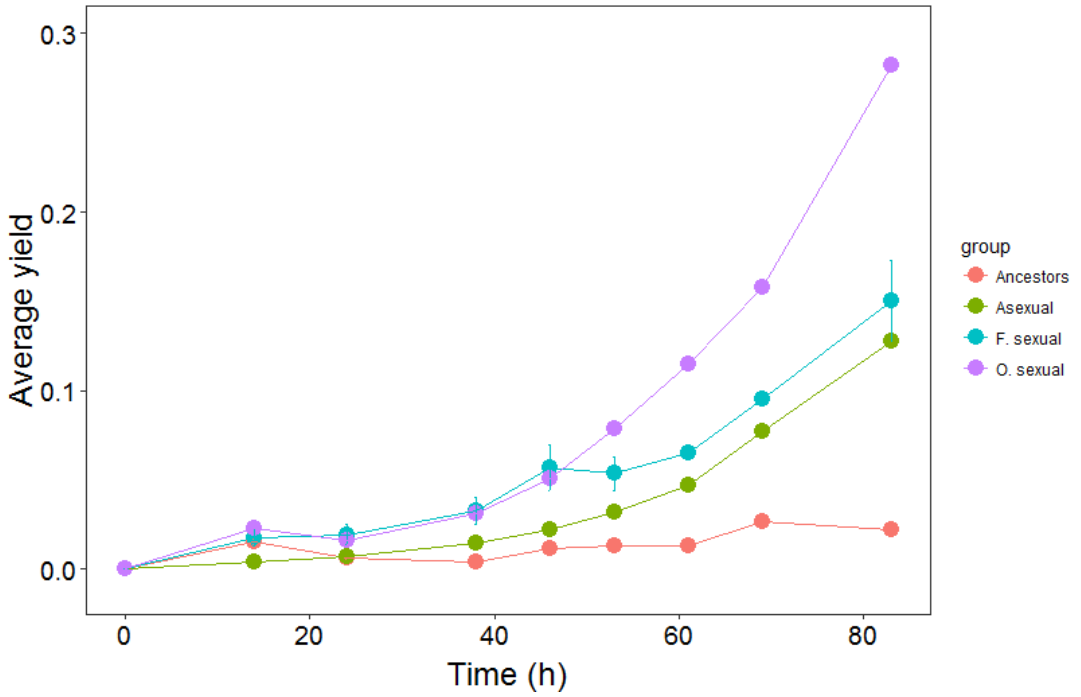

Figure 2.39 – Comparison of growth rate of high rate rescued populations (with respect to mode of reproduction) with that of the ancestors assayed in the same conditions (9 g/l); the bars represent standard error of the mean.

No significant difference in mean population size between the ancestors and the populations rescued in 12 g/l of salt concentration was detected (one-way ANOVA; $F_{3,48} = 1.42$; P = 0.25) (Figure 2.40).

The growth rate in the selective environment (12 g/l), measured as slope of the regression line representing mean change of population size as a function of time was contingent on the treatment group (one-way ANOVA; $F_{3, 48} = 13.99$; P < 0.00001) (Figure 2.41). A significant difference was detected between the growth rate of the ancestors relative to that of the asexual group (t = 2.32, df = 39, P = 0.025), obligate sexual group (t =

5.19, df = 44, P < 0.00001), and facultative sexual group (t = 3.87, df = 41, P = 0.0004). No significant differences were detected among the treatment groups of the rescued populations.

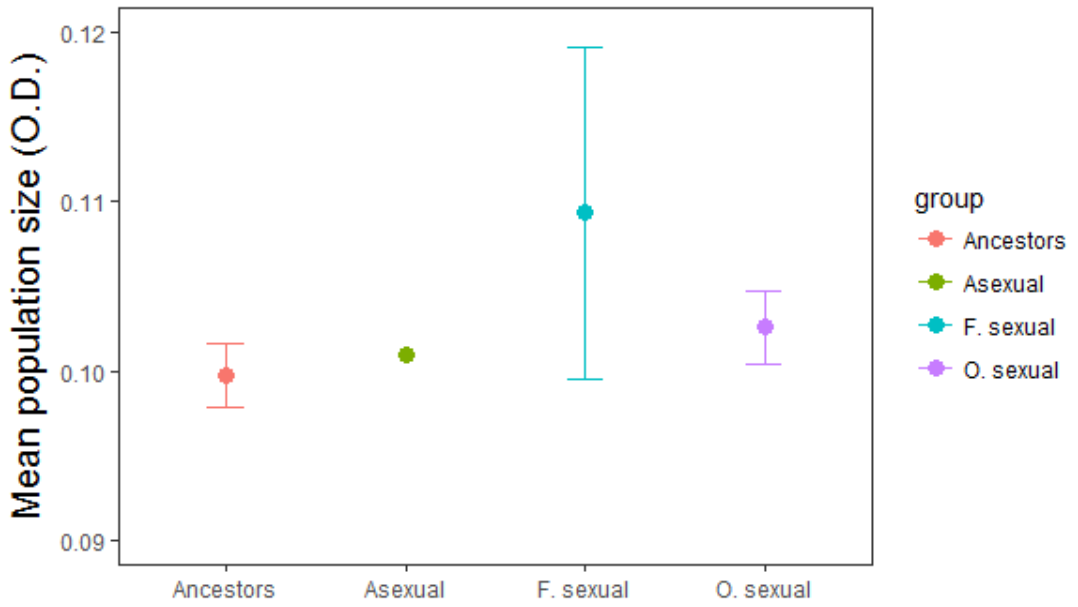

Figure 2.40 – Comparison of mean population size of high rate rescued populations with that of the ancestors assayed in the same conditions (12 g/l); the bars represent standard error of the mean (absent in treatment group comprising a single rescued population).

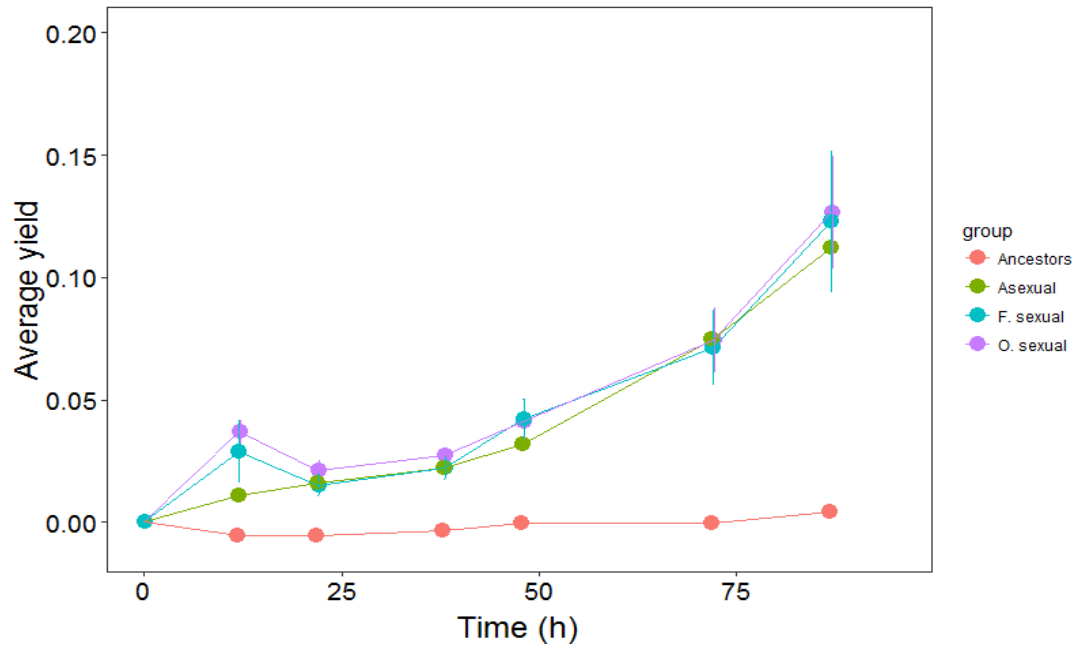

Figure 2.41 – Comparison of growth rate of high rate rescued populations (with respect to mode of reproduction) with that of the ancestors assayed in the same conditions (12 g/l); the bars represent standard error of the mean.

2.5 Discussion

The aim of this study was to investigate the effect of mode of reproduction on the extinction dynamics and the probability of evolutionary rescue of experimental populations of *C. reinhardtii*, in environments which were deteriorating at different rates. The main prediction of the experiment was the relative advantage of sexual populations reflected in lower extinction rate and higher number of evolutionary rescue events for all rates of environmental deterioration (no interaction between mode of reproduction and the rate of environmental deterioration).

The extinction rate among treatment groups was positively correlated with the rate of environmental deterioration. Populations subjected to a gradual rate of deterioration showed the lowest rate of extinction. The earliest extinctions within gradual rate treatment group commenced after all the populations in other two groups had already gone extinct. The advantage held by this treatment group is also reflected in lower extinction dynamics with respect to salt concentration. No extinctions occurred prior to and during the exposure to 24 g/l of salt concentration, contrasting with the high rate group, which had lost almost a third of populations by the time of exposure to this concentration. The advantage relative to the moderate rate group was less prominent, since only 4% of moderate rate populations had gone extinct up to this time point. After exposure to 30 g/l of salt concentration, the gradual rate group had a slight advantage of 6% in number of survived populations relative to the high rate group, while moderate rate populations had gone extinct entirely. The advantage of the gradual rate group could be attributed to the lowest population size decline, which likely resulted in higher supply of beneficial mutations compared to other two groups, and consequently more efficient tracking of the moving optimum.

The difference in the extinction dynamics between the high rate and moderate rate groups was statistically significant, in respect to both time in growth cycles and salt level reached prior to extinction. The majority of moderate rate populations survived the period after extinction of last high rate populations. As in the case of the gradual rate group, slower decline of a population size of moderate rate group relative to high rate group of

populations could have likely influenced these differences in extinction rate. However, a noticeable fluctuation in the extinction dynamics of these groups relative to salt concentration is less straightforward to explain. Moderate rate groups had advantage of 28% in the number of surviving populations relative to high rate group up to and including the level of stress corresponding to 24 g/l of salt concentration. However, this advantage started to decline very abruptly when salt concentration reached and surpassed 26 g/l, which culminated in extinction of all the populations by the time salt concentration had reached 30 g/l/. In contrast, 30% of high rate populations survived this salt concentration.

This sudden increase in the likelihood of extinction of moderate rate populations requires an explanation. It is possible that salt concentration of 26 g/l is near the limit of physiological tolerance for *C. reinhardtii*. In the study of Lachapelle and Bell (2012), 35% of all the experimental populations of *C. reinhardtii* went extinct during the exposure to salt concentrations between 26g/l and 30g/l. This is consistent with the result of this experiment. Immediately after the salt concentration had surpassed 26 g/l, gradual rate populations started to go extinct (first 14% of the populations went extinct) and moderate rate groups lost 55% of the populations.

Why have only the high rate and gradual rate groups survived this "mass-extinction" (Lachapelle and Bell, 2012) period? One of the possible explanations is that survival of the upper limits of physiological tolerance of this species involves mutational pathways available only under high rate of change. Mutational pathways are contingent on the rate of environmental deterioration (Lindsey et al., 2013), so there is a possibility that only stronger selection operating in the high rate of environmental deterioration could have led to fixation of mutations of large effects that conferred advantage under high magnitude of stress. The response of gradual rate populations could have been realized through slow, but progressive fixation of beneficial mutations available due to less prominent decline of mean population size. Consequently, in the absence of strong selection or more stable influx of beneficial mutations, moderate rate populations responded the least efficiently when the magnitude of stress had reached the level of upper physiological tolerance.

Contrary to my prediction, the advantage of sex was not consistent for all the rates of environmental deterioration and significant interaction between mode of reproduction and the rate of salt increase was detected, both with respect to time in growth cycles and

salt concentration reached before the extinction event. The only difference in extinction rate among treatment groups with respect to mode of reproductions was recorded during moderate rate of environmental deterioration. Notably, obligate sexual group had the slowest extinction rate, significantly different than that of both facultative sexual and asexual groups. This advantage was the highest during the interval of "mass extinctions" (26-30 g/l) which suggests that the beneficial effect of sex might have been realized through release of beneficial mutations of large effect from inferior genetic background, crucial for survival of the interval of high extinction probability. The supply of these mutations could have likely been very limited due to a large drop in population size caused by the severe stress. However, moderate rate obligate sexual populations underwent only 4 sexual cycles, the last of which was induced during exposure to 8 g/l of salt concentration – long before the period of mass extinctions. Hence, the beneficial effect of sex must have occurred prior to the interval corresponding to the increased likelihood of extinctions. It is possible that sex increased genetic variance for fitness which enabled populations to track the moving optimum and survive for longer interval of time than asexual populations, but that was not sufficient to overcome higher magnitude of environmental stress.

The probability of evolutionary rescue was contingent on the rate of deterioration. Gradual rate treatment groups reached the maximal salt concentration for which evolutionary rescue had been recorded and had the highest percentage of rescued populations than either of two other groups. The lowest number of extinction events occurred in high rate treatment group. These findings are consistent with previously shown results (Bell and Gonzalez, 2011; Lindsey et al., 2013), which demonstrated the inverse relationship between the occurrence of evolutionary rescue events and the rate of environmental deterioration.

Mode of reproduction did not significantly affect the probability of evolutionary rescue. The obligate sexual group had only a marginal advantage over other two treatment groups in the number of extinction events, and the rescued populations did not have significantly higher fitness both with respect to the growth rate and population size. The only advantage of sex was recorded within the moderate rate treatment groups: higher mean fitness of the populations of both sexual groups rescued in 8 g/l of salt concentration relative to asexual group and higher number of rescued events (obligate sexual group) in 12 g/l of salt concentration.

The results of this experiment indicate a large effect of rate of environmental deterioration on both the extinction dynamics and evolutionary rescue and comparatively modest effect of mode of reproduction, mostly reflected in mitigation of negative effects of stress, manifested through reduction of population size decline. The populations subjected to the gradual and moderate rate of environmental deterioration tracked the moving optimum more efficiently than the populations within high rate group, which is reflected in lower rate of population size decline, lower extinction rate and higher probability of evolutionary rescue. However, probability of survival when exposed to the highest level of stress was lower for the moderate rate group, relative to other treatment groups. Notably, sex provided no significant effect in the groups which were more likely to survive the period of increased chance of extinction, but delayed extinctions within a moderate rate group. This indicates that, in the context of survival, sex might be the most beneficial strategy when the probability of extinction starts to increase rapidly.

3. Sex promotes evolutionary rescue in *Chlamydomonas reinhardtii* in an environment deteriorating in a complex manner

3.1 Introduction

There is a general consensus that the current pace of environmental change is unprecedented in recent human history (Thomas et al., 2004; Bell and Collins, 2008; Bell and Gonzalez, 2009; Barnosky et al., 2012). Facing this global change, many species are likely to be exposed to conditions outside of the range of their ecological niche. If the rate of change is too severe, maladapted species could potentially face extinction.

A population can respond to a detrimental environmental change via two different mechanisms: migration to less detrimental habitats or phenotypic plasticity. The only possible alternative, if neither of these mechanisms is available, is a genetic change through adaptive evolution (Bell and Collins, 2008). This process of adaptation of a population so that it can persist in conditions that would have ultimately caused extinction of the ancestral population has been termed evolutionary rescue (Gomulkiewicz and Holt, 1995). It is manifested through the rise in frequency of alleles advantageous in harmful conditions through the operation of natural selection acting on standing genetic variation, or *de novo* beneficial mutations. Consequently, the population may restore mean fitness and ultimately avoid extinction.

The growing awareness of the adversity of an ongoing global change has stimulated various types of experimental studies of evolutionary rescue during the last two decades (recently reviewed by Carlson et al., 2014). These studies have identified numerous factors positively or negatively correlated with the likelihood of evolutionary rescue. For instance, the probability of evolutionary rescue is higher in large populations due to a proportionally higher influx of beneficial mutations (Bell and Gonzales, 2009; Ramsayer et al., 2013) and reduced detrimental effect of genetic drift (Willi and Hoffmann, 2009). Furthermore, higher standing genetic variation in a population enhances the response to selection and thereby increases the likelihood of survival (Agashe, 2009; Agashe et al., 2011; Lachapelle and Bell, 2012; Ramsayer et al., 2013). The probability of evolutionary rescue is inversely proportional to the rate of environmental deterioration (Perron et al., 2007; Bell and

Gonzales, 2011; Lindsey et al., 2013) given that rapid change implies large initial maladaptation and thus a greater decline of a population size (Carlson et al., 2014).

Mode of reproduction is another factor that affects the likelihood of evolutionary rescue (Lachapelle and Bell, 2012.). Sex can increase genetic variance available for selection and thereby enhances adaptation to a changing environment (for example: Weismann, 1889; Fisher, 1930; Muller, 1932; Burt, 2000). Beneficial effects of sex on adaptation include but are not confined to: faster assembly of beneficial mutations (Fisher, 1930), clearance of deleterious mutations (Muller, 1964) and breaking of negative linkage disequilibrium (Barton, 1995). A large body of data provides a support for hypothesis that sex enhances adaptation to both novel environment (Colegrave et al., 2002; Kaltz and Bell, 2002; Colegrave, 2002; Morran et al., 2009; Becks and Agrawal, 2011; Bell, 2013) and a deteriorating environment (Greig et al., 1998; Goddard et al., 2005; Lachapelle and Bell, 2012; Lachapelle et al., 2015).

To date, all experiments looking at the effect of sex on evolutionary rescue in deteriorating environments have used relatively simple environments. The study conducted by Lachapelle and Bell (2012) demonstrated the advantage of sex in an environment deteriorating in a simple way, manifested through lower extinction rate of sexual populations than asexual populations. This study investigated the effect of change in a single abiotic component of environment (increasing salinity). It is still uncertain whether sex provides an advantage in an environment deteriorating in a more complex fashion. Complex environmental deterioration implies the change in two or more components of environment, which both negatively affect the mean fitness of a population. Kaltz and Bell (2002) investigated the effect of repeated episodes of sex on adaptation to both simple and complex novel environments. They found that sexual populations maintain higher mean fitness than asexual populations in most environments, but the advantage increases with the increase in environmental complexity. Since similar factors determine the adaptation to both novel and a deteriorating environment, it is likely that sex will be more advantageous in a complexly deteriorating environment.

The complex environmental deterioration may comprise the simultaneous change in abiotic and biotic component of an environment. The Biotic component of an environment is a factor that often affects the mean fitness of a population (Begon et al., 2006) and thus may produce a significant impact on chances for population survival when

conditions change. Most of the studies of evolutionary rescue have considered a single species. In nature, the ability to adapt depends on interactions between coexisting species (Jones, 2008; Osmond and de Mazancourt, 2013.). Incorporating these interactions into studies is essential for predicting the outcomes of adaptive evolution in the context of a deteriorating environment and investigating the potential benefits of sex in a complexly deteriorating environment.

In this study, I focus on the competitive (negative) interactions among species, since recent studies (Johansson 2008; Collins 2011) had demonstrated that competition restricts adaptation. Johansson (2008) modelled a competition between two species in a changing environment and found that the species disfavoured in a competition suffers a decline in effective population size, which in turn reduces the maximal adaptive rate. Furthermore, competition in stressful conditions can result in a trade-off between adaptation to abiotic and biotic component of environment (Collins, 2011; Lawrence et. al, 2012). Collins (2011) allowed three strains of *Chlamydomonas reinhardtii* to compete in a deteriorating environment (increased concentration of CO_2). The strains exposed to the elevated concentrations of CO_2 that had evolved higher growth rate in the presence of a competitor were less fit when propagated alone. Based on the restrictive effect of negative interspecies interactions on adaptation, we might predict that competition should also reduce the probability of evolutionary rescue in conditions that would initially drive a population extinct. However, to my knowledge, no experimental study has been performed in environment lethal to the constituent species, so the question whether and how competition can affect evolutionary rescue still remains unresolved.

Despite the fact that both competition and sex independently affect the likelihood of evolutionary rescue, there is still no experimental evidence whether the interaction of these factors alters the probability for survival. If a competitor is incorporated into a deteriorating environment of a focal species, it will increase the complexity of environmental deterioration and intensify the decline of the mean fitness. However, if there is a difference in initial maladaptedness between competitors, one species (more maladapted counterpart) could increase the probability of survival of other species (less maladapted counterpart) because selection imposed by moving optimum would be augmented by competition selecting in the same direction (for instance, if the change involves a resource one species can exploit more efficiently than the other species) (Jones

2008; Osmond and de Mazancourt, 2013). An asexual (clonal) population will likely be limited in a diversity of genotypes available for selection. In contrast, sex could generate such genotypes and, coupled with competition, restore the mean fitness of the population.

In this study, I attempt to contribute to a growing body of experimental studies investigating the effect of sex in deteriorating environments. I test the hypothesis that sex remains beneficial in a complexly deteriorating environment, which both components, abiotic and biotic, negatively affect the mean fitness of a population, thereby reducing the chance for survival. The effect of competition on evolutionary rescue is less predictable and current studies suggest two alternative possibilities. I test the hypothesis that competition will maximize the likelihood for evolutionary rescue of the focal species, if coupled with sex. In contrast, the isolated focal species will have less chance for survival irrespective of mode of reproduction.

To test these hypotheses, I allowed populations of the unicellular green algae (*Chlamydomonas reinhardtii*) to evolve in an environment to which they were initially poorly adapted, such that in the absence of evolutionary change the populations would go extinct. I manipulated both their mode of reproduction (either entirely asexual, or with history of sexual reproduction) and the presence of a competitor in a fully factorial design.

Two predictions arise from my hypotheses:

I. If sex increases the likelihood for evolutionary rescue in a complexly deteriorating environment, sexual populations will have higher number of rescue events than asexual populations in the presence of the competitor.
II. If combination of sex and competition maximizes the likelihood for evolutionary rescue, the number of rescue events will be the highest in sexual populations propagated with the competitor;

3.2 Materials and Methods

3.2.1. The species used in experiment

3.2.1.1. *Base Populations of C. reinhardtii*

Each experimental population (96 in total) was established by random selection of 10 isolates from the library of *C. reinhardtii* isolates, built for the experiment described in Chapter 2, thus representing a unique combination of 10 genotypes: 48 sexual populations were established by combining 5 mt+ and 5 mt- isolates; two sets of 24 asexual populations were established by combining 10 mt+ and 10 mt- isolates, respectively.

3.2.1.2. The Competitor Species (Chlamydomonas moewusii)

The competitor species used in this study was another unicellular chlorophyte alga, *C. moewusii.* This freshwater species was selected for the experiment due to diversity of strains isolated from natural habitats and similar growth requirements as *C. reinhardtii*. I obtained 4 wild type strains (cc-1419, cc-1420, cc-1480 and cc-1481) from the University of Minnesota algae collection (http://www.chlamycollection.org/strains/).

3.2.2 Assay of the experimental species for growth in high salt

The probability of evolutionary rescue of the focal species might be affected by the potential differences in high salt sensitivity between *C. reinhardtii* and *C. moewusii*. To investigate this possibility, prior to commencing of the selection experiment, all 39 ancestral isolates used for constructing the experimental *C. reinhardtii* populations (obtained from the library of *C. reinhardtii* isolates; see Chapter 2 for details) and all four *C. moewusii* wild type isolates were assayed for growth in a selective medium (Bold's supplemented with 15 g/l of salt) and a benign medium (Bold's) as a control. The populations of both species were allowed two growth cycles (3-4 days; 5% of culture passaged), but were assayed only during the second cycle, to avoid the possible carry-over effects after plating from agar slant tubes, used for storing the cultures. The assay was performed in 96-well plates and growth estimated spectrophotometrically, by measuring

OD_{750} twice a day (approximately every 12 hours) until the cultures reached the stationary phase (after approximately 4 days). The fitness for growth in each salt concentration was estimated by calculating the maximal yield of each species (Maximal OD - Initial OD). The maximal yield was selected as a measure of fitness, since it does not depend on the initial population size (Lachapelle and Bell, 2012).

3.2.3. Selection experiment

Experimental evolution was carried out in two phases. In the first phase, populations of the focal species experienced a deteriorating environment in which salt concentration increased to lethal level. In the second phase, their ability to adapt to these conditions was monitored in both the presence and absence of a competing species.

3.2.3.1. Cultivation and Transfer Procedure

All the experimental populations of C. *reinhardtii* were cultivated in 24-well plates in Bold's broth medium under standard conditions (26˚C, 3200 lux illumination, shaking at 180 rpm and covered with sterile breathable membranes to prevent cross-contamination and uneven evaporation across the plates). A serial passage was performed after every 3-4 days by transferring 5 % of each population to the fresh medium. After every second growth cycle, each population was sampled (150 µl) and population size estimated spectrophotometrically by measuring optical density (OD_{750}) of the culture. The experimental populations were propagated in a medium supplemented with NaCl (hereafter referred to as salt), in which the concentration gradually reached 15 g/l in three equal steps of increase (5 g/l). The salt concentration of 15 g/l was chosen as an endpoint because it completely inhibits the growth of C. *reinhardtii* (Reynoso and de Gamboa, 1982). The stepwise increase in salt level was selected as an experimental procedure since the immediate exposure to the lethal stress would have likely resulted in a too abrupt decline of the population density. The likelihood of contact between two opposite gametes decreases in a sparse population which can consequently hamper the completion of the sexual cycle (production of zygotes).

3.2.3.2. Sexual Cycle

Prior to each step of salt increase (after every two growth cycles), sexual populations were allowed to undergo a sexual cycle. I allowed three sexual cycles in total,

since Kaltz and Bell (2002) had demonstrated that three successive episodes of sex provide a long-term increase of the adaptive rate of sexual populations. Each sexual cycle was induced by using the same experimental procedure as described in Chapter 2 (see Section 'Sexual Cycle' for details). Sexual populations in this experiment were not allowed to transfer unmated gametes to the next phase of growth cycles (eliminated by freezing) Hence, mode of reproduction of these populations was obligate sexual.

3.2.3.3. Establishing and Cultivation of Mixed Populations

To examine the interaction between mode of reproduction and competition, during the second phase of the experiment I manipulated the presence or absence of a competitor (summarized in Figure 3.1, see below). In this phase of the experiment, at the point which the environment had reached its maximum salt concentration, the mixed population were assembled by combining each wild type of *C. moewusii* (cultivated separately in standard conditions prior to assembling with the focal species) with six sexual populations and six asexual populations of *C. reinhardtii*. The control asexual and sexual C. *reinhardtii* populations were not combined with the competitor.

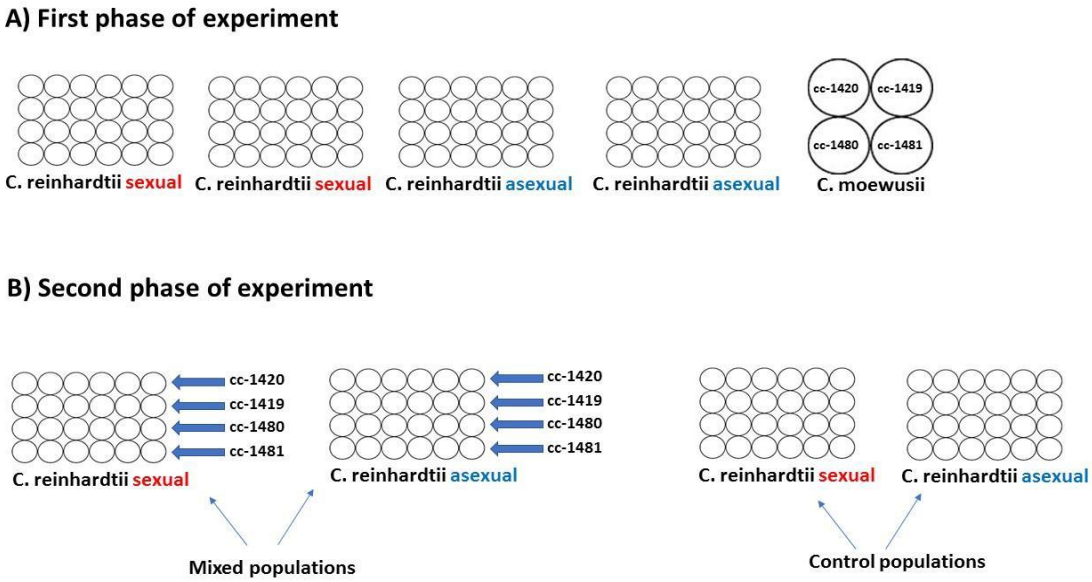

Figure 3.1 – Two phases of the experiment. In the first phase of experiment, which lasted until salt concentration reached 15 g/l, both species were cultivated separately: two groups of sexual populations of *C. reinhardtii*, two groups of asexual populations of *C. reinhardtii* and a single population of each wild type of *C. moewusii*; each circle represents a single population. During this phase, populations in sexual groups of *C. reinhardtii* underwent three sexual cycles. In the second phase, each wild type of *C. moewusii* was combined with six populations of one sexual *C. reinhardtii* group and six populations of asexual *C. reinhardtii* group. The remaining sexual and asexual groups comprised the control populations, propagated without the competitor.

Both counterparts of the mixed populations started this phase of the experiment with equal cell densities, estimated the following way. Nine randomly chosen populations of C. *reinhardtii* and the population of each wild type of C. *moewusii* were sampled (150 µl) and population size estimated by cell counting with a haemocytometer. Each of the *C. moewusii* wild type populations were then diluted to the culture density corresponding to the average cell number of the sampled *C. reinhardtii* populations.

All the mixed populations and control C. *reinhardtii* populations were cultivated in the Bold's broth medium supplemented with 15 g/l of salt throughout the rest of experiment, maintained by serial passage (5 % of each population) performed after every 3-4 days. Prior to each passage to the fresh medium, a sample (200 µl) of each population was serially diluted (10^{-1} or 10^{-2} - fold dilution) and transferred to the corresponding Petri dish with Bold's agar medium supplemented with sodium-acetate. Each sampled population was incubated until colonies appeared (approximately 4 days) and the cell number estimated by colony counting. The cell number of the population sample was converted to the cell number (per ml) of the whole population by using the equation: cell number per ml = number of colonies / dilution factor X sample size (0.1 ml). After given interval of time, all the mixed populations were wrapped in aluminium foil and incubated in dark for 3-4 days to determine the population size of each species.

3.2.3.4. Distinguishing the two species

To distinguish the two species, I grew them under conditions in which they would show different growth characteristics on agar plates. I made use of the fact that *C. reinhardtii* grows well in the dark on acetate supplemented agar plates, whilst *C. moewusii* does not.

Both species are facultative heterotrophs, capable of utilizing sodium-acetate while incubated in light. However, *C. moewusii* cannot metabolize sodium acetate in the dark or otherwise shows poor growth (Harris, 2009.). In contrast, *C. reinhardtii* cells utilize sodium acetate and continue dividing in the dark, although with a reduced growth rate than under light conditions. This biological feature had been exploited in this experiment, given that *C. reinhardtii* colonies clearly increased in size after incubation in darkness, thus becoming distinguishable in a mixed culture. Furthermore, colonies could have been differentiated

based on the individual cell features. The cells of *C. reinhardtii* are oval or spherical, while the moewusii colonies comprise the elongated and ellipsoid cells (personal observation).

3.2.3.5. Recording the evolutionary rescue events

The experiment continued until one or both species of all the mixed populations and all the control populations went extinct or showed clear positive growth. The species-constituent of a mixed population had been scored as 'rescued' if it repeatedly scored the same or higher number of cells/ml each time sampled, thus showing the positive growth in lethal conditions. Each species had been scored as 'extinct' if no cells were detected after visual inspection of liquid culture or plating on agar.

3.3 Data analysis

The probability of evolutionary rescue was estimated by fitting Generalised Linear Model (binomial logistic regression) with two factors as categorical independent variables ('mode of reproduction' and 'competition'), and survival/extinction as a binary responsive variable. 'Mode of reproduction' comprises two levels (sexual or asexual populations) while 'competition' comprises five levels: absence of the competitor and each of the four wild types being considered as a different level. The factors were regarded as fixed effects. The interaction between mode of reproduction and competition was incorporated into the model.

The mean fitness of the surviving populations was analysed by fitting two-Way ANOVA, with two factors as categorical independent variables ('mode of reproduction' and 'competition') and 'mean population size per growth cycle' as a continuous responsive variable. The levels within each factor were analysed with unpaired t-test.

All the analyses were performed using R (R Core Team, 2017).

3.4 Results

3.4.1. Assay of the experimental species for growth in high salt

In a benign medium, the average maximal yield of *C. reinhardtii* isolates was 35% higher than that of *C. moewusii* wild type isolates (Figure 3.2). The average growth of *C. moewusii* wild type isolates in a selective medium was positive, despite considerable reduction in maximal yield in comparison to a benign medium (by 89%) (Figure 3.3). In contrast, the average growth of *C. reinhardtii* isolates was negative. Wild type strains of *C. moewusii* showed variation for growth in a selective medium (Figure 3.4). The wild type strain cc-1480 scored negative growth, while the other wild type strains showed positive growth, reaching different maximal yields.

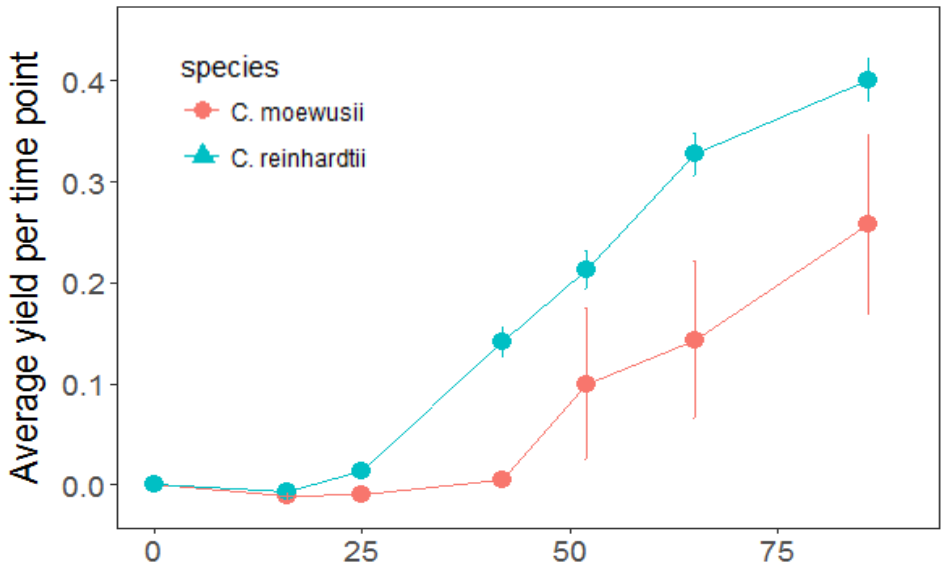

Figure 3.2 – **The average yield of *C. reinhardtii* ancestral isolates (blue) and *C. moewusii* wild type isolates (red) per each time point (h), measured in a benign medium (Bold's)**; the error-bars represent standard error of the mean.

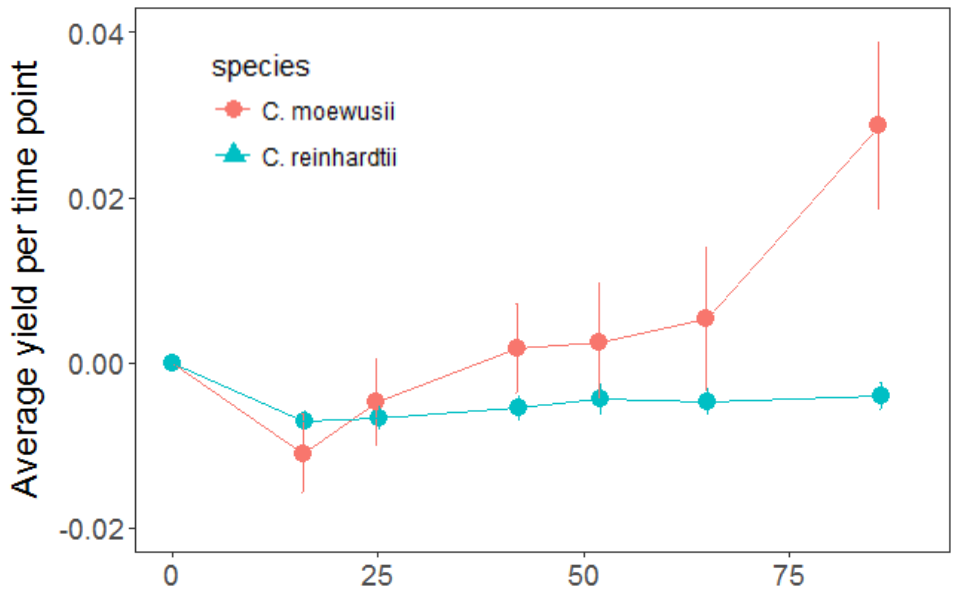

Figure 3.3 - The average yield of *C. reinhardtii* ancestral isolates (blue) and *C. moewusii* wild type isolates (red) per each time point (h), measured in a selective medium (Bold's supplemented with 15 g/l of salt); note the difference in scale in comparison to Figure 3.2; the error-bars represent standard error of the mean.

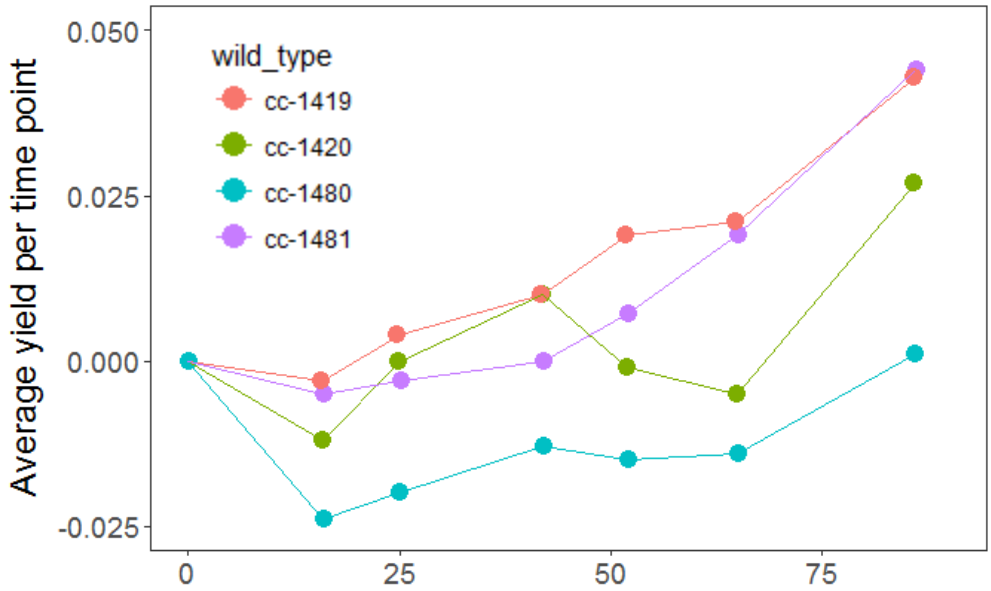

Figure 3.4 – The average yield of four *C. moewusii* wild types per each time point (h), measured in a selective medium (Bold's supplemented with 15 g/l of salt); no error-bars are present since the experimental populations were not replicated.

3.4.2. Selection experiment

3.4.2.1. *Evolutionary rescue*

Sex affected the probability of evolutionary rescue (Binary logistic regression; χ^2 = 5.75; df = 1; P = 0.016). Of the initial 48 populations for each mode of reproduction, 19 sexual populations survived the lethal treatment (40%); in contrast, only 9 asexual populations (19%) avoided extinction (Figure 3.5). Competition treatment also affected the probability of evolutionary rescue (Binary logistic regression; χ^2 = 15.04; df = 4; P = 0.005). Of the initial 48 populations propagated without the competitor, 20 populations survived the lethal conditions (42%). Of the initial 48 populations propagated with one of the four different wild types, 8 populations (17%) in total were rescued (Figure 3.6). Post-hoc Tukey's HSD analysis was conducted on all possible pairwise comparisons and revealed statistically significant difference (P = 0.02) between survival in presence of cc-1419 wild type (no population survived) and the absence of the competitor. There was no significant difference in survival between the populations propagated without the competitor and the populations propagated with cc-1420 wild type (4 populations survived), cc-1481 wild type (3 populations survived) and cc-1480 wild type (a single population survived), despite the fact that 33%, 25% and 8% of the initial 12 populations, respectively, survived when these wild types were present.

In the absence of the competitor, 14 sexual populations (58%) and 6 asexual populations (25%) survived the lethal conditions. When the competitor was present, the probability of evolutionary rescue declined for both modes of reproduction: 5 sexual populations (21%) and 3 asexual populations (12%) survived the lethal treatment (Figure 3.7). When cc-1480 and cc-1481 wild types were present, evolutionary rescue was recorded only in sexual populations (1 and 3 populations survived, respectively). Sex was disadvantageous when cc-1420 wild type was present (1 population survived, contrasted with 3 rescued asexual populations). No mode of reproduction provided advantage when cc-1419 wild type was present, given the extinction of all the populations (Figure 3.8). Despite differential survival in the presence of different wild types, no significant

interaction between mode of reproduction and competition treatment was detected (Binary logistic regression; $\chi^2 = 8.06$; df = 4; P = 0.09).

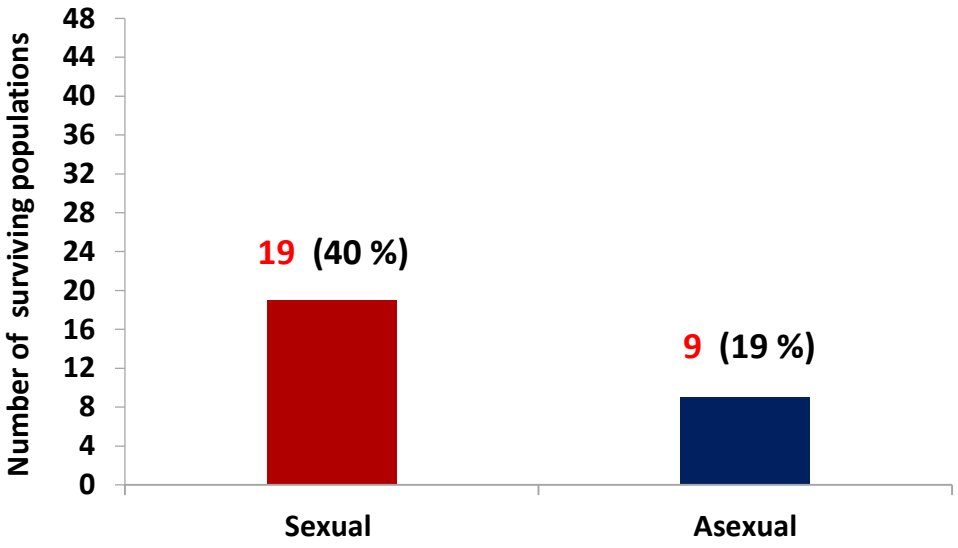

Figure 3.5 – Total probability of evolutionary rescue for sexual and asexual populations irrespective of the type of environment; the number of rescue events per each mode of reproduction is presented above each bar plot, with a percentage of surviving populations out of the initial 48 populations in brackets.

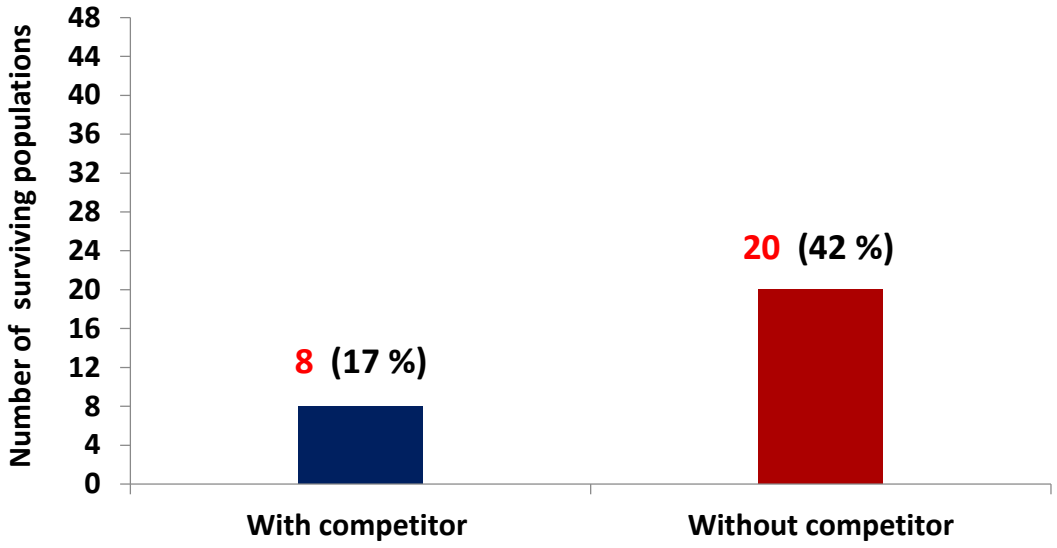

Figure 3.6 – Total probability of evolutionary rescue of the focal species in both environments, irrespective of mode of reproduction; the number of rescue events per each type of environment is presented above each bar plot, with a percentage of surviving populations out of the initial 48 populations in brackets.

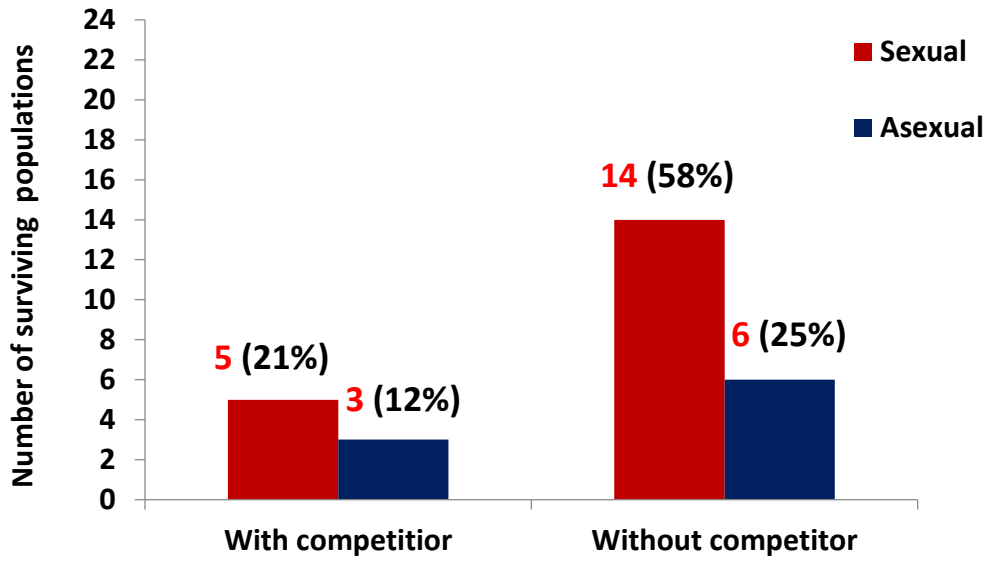

Figure 3.7 – Probability of evolutionary rescue for the focal species when propagated with and without the competitor. The number of surviving populations per each treatment group is presented above each bar plot, with a percentage of surviving populations out of the initial 24 populations in brackets.

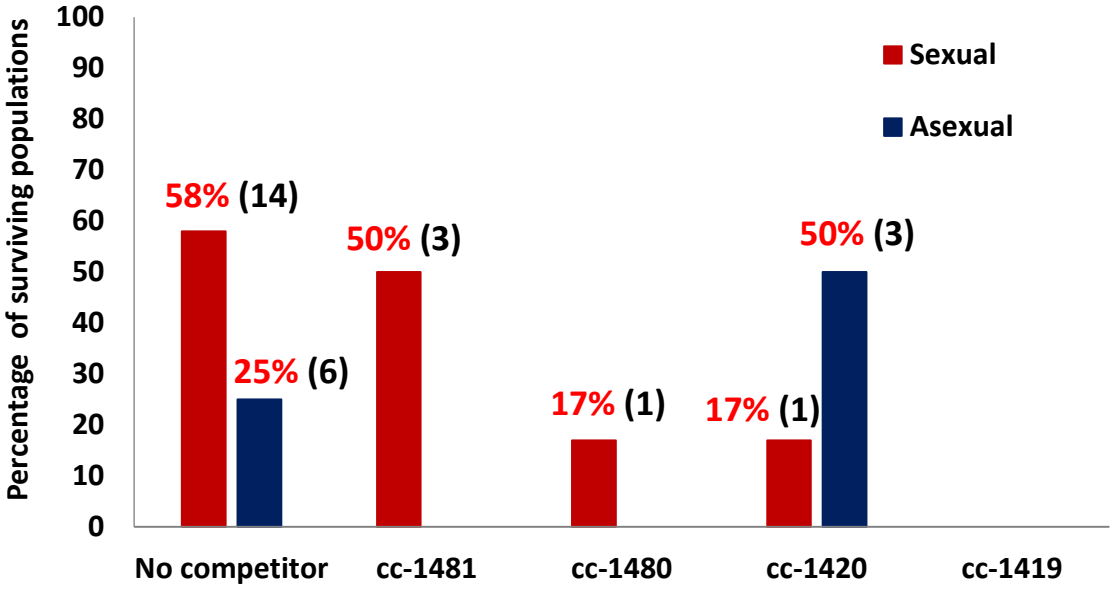

Figure 3.8 – Probability of evolutionary rescue for both modes of reproduction of the focal species, per each level of competition treatment; the probability of evolutionary rescue is presented as a percentage of surviving populations per each treatment group, with a number of surviving populations out of the initial 24 populations (for 'no competitor' treatment) and 6 populations (for each of the wild type strain of the competitor) in brackets; no population of the focal species survived when propagated with cc-1419 wild type.

3.4.2.2. Mean fitness of the rescued populations

In order to determine the effect of competition on fitness of the rescued experimental populations, I estimated the population size of the focal species after each growth cycle by counting the colonies appeared on agar plates after 4 days. Mean fitness of each treatment group (sexual/asexual and presence/absence of the competitor) corresponds to the average number of cells / ml per each growth cycle. Sex significantly affected mean fitness of the rescued experimental populations (two-way ANOVA; $F = 4.97$; $df = 1$; $P = 0.03$). Sexual populations had more than two-fold higher mean populations size (Figure 3.9). The effect of competition on mean fitness of the rescued experimental populations was not significant (two-way ANOVA; $F = 0.24$; $df = 1$; $P = 0.63$), (Figure 3.10). There was no significant interaction between mode of reproduction and competition on the mean fitness of the rescued populations (two-way ANOVA; $F = 0.88$; $df = 1$; $P = 0.36$), since the rescued sexual populations had higher mean population size than asexual populations irrespectively of the presence of the competitor. However, the difference in the population size was significant only in the absence of the competitor (unpaired two-sample t-test; $t = 2.13$, $df = 18$, $P = 0.047$) (Figure 3.11; two rightmost interval plots). In contrast, there was no difference in mean fitness of sexual and asexual rescued populations propagated in the presence of the competitor (unpaired two-sample t-test; $t = 0.72$; $df = 6$; $P = 0.5$) (Figure 3.11; two leftmost interval plots). There was no significant difference in mean fitness between the rescued sexual populations propagated with and without the competitor (unpaired two-sample t-test; $t = 0.84$; $df = 17$; $P = 0.41$). Likewise, the difference in mean fitness between the rescued asexual populations propagated in the presence and absence of the competitor was not significant (unpaired two-sample t-test; $t = 0.85$; $df = 7$; $P = 0.42$).

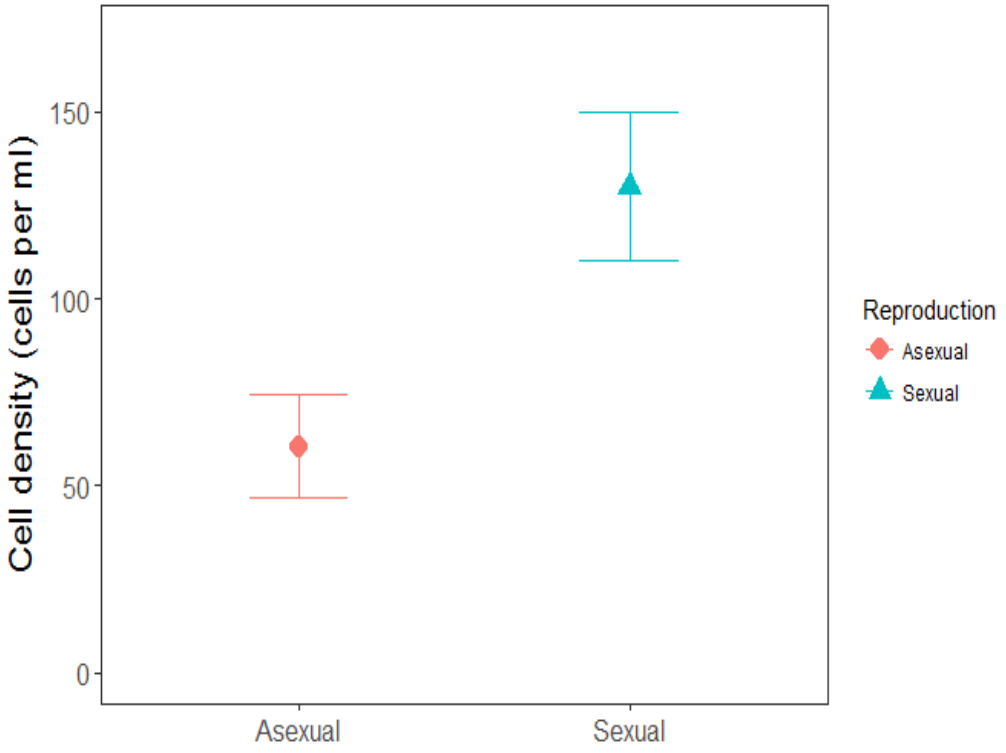

Figure 3.9 – Mean population size of the rescued populations of the focal species per each mode of reproduction, irrespective of the presence of the competitor; the bars represent standard error of the mean; the sqrt-transformed mean population size is plotted on the y axis.

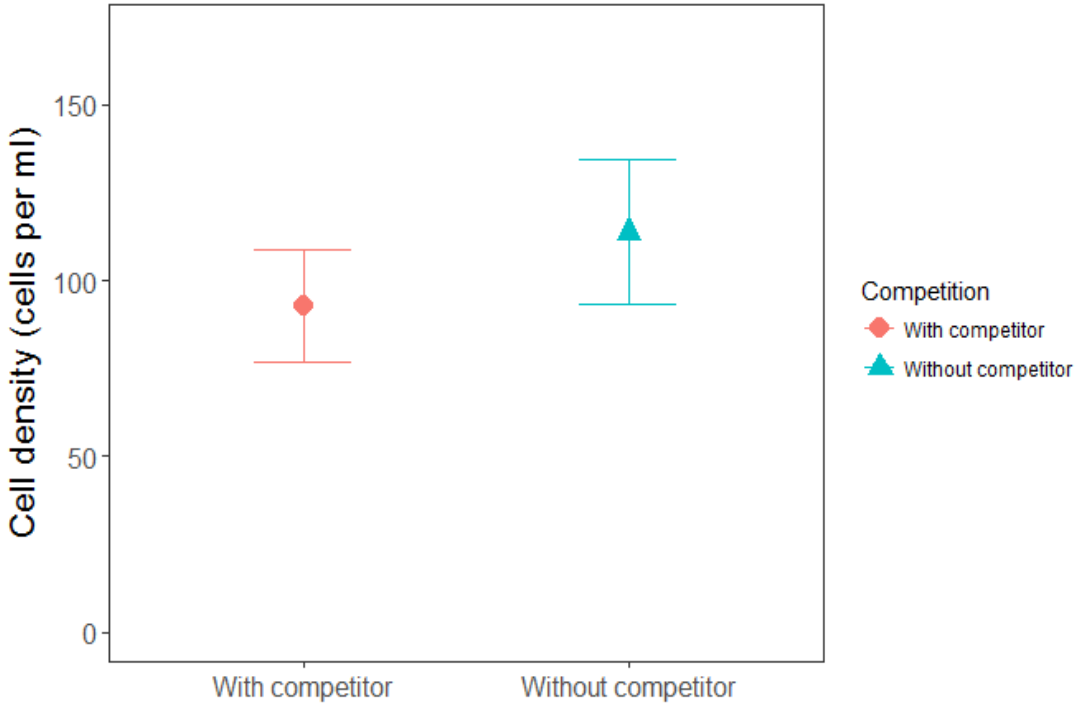

Figure 3.10 – Mean population size of the rescued populations of the focal species in both environments, irrespective of mode of reproduction; the bars represent standard error of the mean; the sqrt-transformed mean population size is plotted on the y axis.

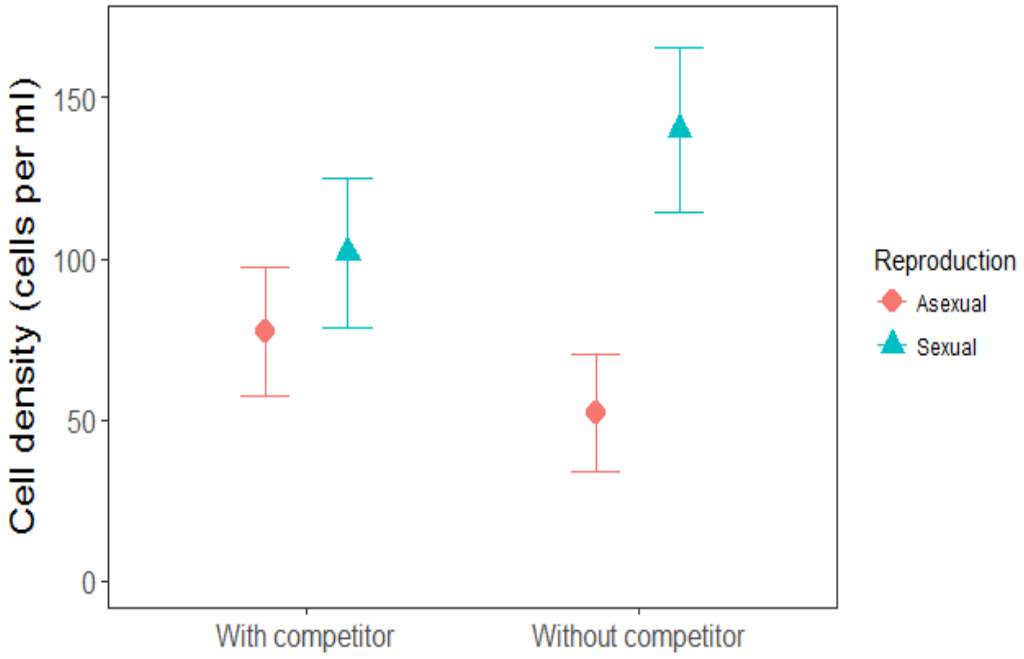

Figure 3.11 – Mean population size of the rescued populations of the focal species; the bars represent standard error of the mean; the sqrt-transformed mean population size is plotted on the y axis; the colour corresponds to the mode of reproduction;

3.5 Discussion

Sex is beneficial when environment deteriorates in a simple way, as shown by previous studies (Lachapelle and Bell, 2012; Bell, 2013; Lachapelle et al., 2015). This is consistent with my results, given that probability of evolutionary rescue was more than two-fold higher for sexual populations than that of asexual populations. Furthermore, the rescued sexual populations have higher mean fitness, given the two-fold larger mean population size.

To the best of my knowledge, this is the first study that demonstrates the advantageous effect of sex in a complexly deteriorating environment. Whilst evolutionary rescue was less likely if a competitor was present, sexual populations maintained the advantage in number of rescue events when the competitor was present (40% higher than that of asexual populations). The consistency of higher number of rescue events in both environments explains the lack of significant interaction between sex and competition treatment. However, sex was not advantageous in the presence of cc-1420 wild type, whilst

the populations of both modes of reproduction failed to survive while propagated with cc-1419 wild type. This suggests that the beneficial effects of sex may be constrained by the genotype of the competitor.

The difference in mean fitness of the rescued sexual and asexual populations propagated with the competitor was not significant. Notably, the rescued sexual populations suffered a 40% reduction in mean population size in comparison to the populations propagated in the absence of the competitor. Despite the fact that the difference in mean fitness between these two treatment groups was not statistically significant, this indicates that the main negative effect of competition in my experiment is a reduction in the population abundance of the focal species, as previously shown (Ayala 1969; Bengtsson 1989; Martin and Martin, 2001). Reduced population size can in turn negatively affect the supply of beneficial mutations, thus hampering the adaptive walk in the lethal environment, which could have been completed in the absence of the competitor. This may explain a remarkable difference in fitness of rescued sexual and asexual populations propagated without the competitor, but no difference in the presence of the competitor.

Competition significantly reduced the likelihood of evolutionary rescue. The focal species was driven to extinction in 83% of all the mixed populations. The average extinction of the focal species per wild type of the competitor was 25% higher than in the absence of the competitor. The high salt assays revealed lower sensitivity of *C. moewusii* to high salt than *C. reinhardtii*, which is a plausible explanation for the elimination of the focal species in the majority of mixed populations. I found general differences in survival of the focal species when propagated among different wild types. The proportion of extinct populations varied from 66% for cc-1420 to 100% for cc-1419 wild type. This is not surprising, given that the pilot experiment performed prior to commencing of the selection experiment revealed the differences in absolute fitness of the wild types when grown in high concentration of salt. However, there is no evidence that presence of different wild types systematically affected the general negative effect of competition, given that the focal species suffered the reduction in number of rescued populations when propagated along any of the four wild types.

The probability of evolutionary rescue was reduced irrespectively of modes of reproduction when the competitor was present. Despite the relative advantage of sexual

populations over asexual populations in total survival, sex could not compensate for the negative effect of competition. This is reflected in 37% reduction in survival of sexual populations than in the absence of the competitor, which refutes my hypothesis that interplay of sex and competition maximizes the chance for evolutionary rescue. A decline in population abundance caused by competition likely affected the availability of favourable genotypes and thus increased the probability of extinction.

This experiment provided clear evidence that sex promotes and competition impedes evolutionary rescue. An outstanding question is: will the effects of sex remain the same if degree of complexity of environmental deterioration increases? Sex increases the rate of adaptation in more complex novel environment (Kaltz et al., 2002). Similar pattern can be expected for a deteriorating environment, but experimental evidences are required. Will competition always lead to a decrease in mean fitness? The negative effect of competition is proportional to the niche overlap between competitors (Osmond and de Mazancourt, 2013). Two ecologically separated competitors would potentially suffer lower population decline than the ones characterized by similar patterns of resource use. Similarly, the phylogenetic distance between competitors may indirectly influence the likelihood of evolutionary rescue, under the hypothesis that the level of competition is directly proportional to relatedness between competitors (Naughton et al., 2015). However, C. *moewusii* and C. *reinhardtii* occupy different ecological niches, the former being a freshwater organism and the latter terrestrial. Moreover, both species are on the opposite ends on the phylogenetic tree of *Chlamydomonas* genus, thus being distantly related. This indicates that the effect of competition on evolutionary rescue will remain negative, regardless of the type of the competitor. Whether this is the widespread pattern in nature yet remains to be investigated.

4. Investigating the effect of an ecological similarity and phylogenetic relatedness between competitors on the probability of evolutionary rescue of the focal species

4.1 Introduction

Global change will impose an inevitable negative impact upon many species. Due to the lack of dispersal abilities or response by phenotypic plasticity, many populations will face extinction unless they adapt *in situ* (Eizaguirre and Baltazar-Soares, 2014) via the process termed evolutionary rescue (Gomulkiewicz and Holt, 1995). Understanding the factors that affect the probability of evolutionary rescue is important for assessing the vulnerability of species and can contribute to conservation effort aimed at preventing potential extinctions.

Most experimental studies of evolutionary rescue have been performed using a single species. However, in natural conditions, a species rarely exists in isolation. More realistically, species are interconnected with the biotic component of the environment through a multitude of interactions. Hence, incorporating ecological realism into the study of evolutionary rescue is important for assessing the likelihood of evolutionary rescue of a focal species.

Competitive interactions among species generally negatively affect the adaptation rate of a population subjected to a novel environment, as shown by both theoretical (Johansson, 2008) and experimental studies (Collins, 2011). This negative effect is reflected in a reduction of population abundance in the presence of a competitor (Johansson, 2008) or a trade-off between adaptation to the abiotic and biotic component of environment (Collins, 2011). The experimental evidence indicates that competition increases the probability of extinction (e.g. Bengtsson 1989; Bengtsson and Milbrink, 1995). Bengtsson (1989) showed that competition among three species of *Daphnia* cultivated in a benign medium increased their likelihood of extinction, primarily due to their overlapping resource use. Bengtsson and Milbrink (1995) corroborated this result by demonstrating that the

extinction rate of two *Daphnia* species (*D. magna* and *D. longispina*) increased significantly when they were propagated together rather than in isolation.

Based on the negative effects of competition, manifested through a reduced rate of adaptation and an increased extinction rate, we might predict that competition should reduce the probability of evolutionary rescue. In Chapter 3, I showed that the presence of a competitor reduced the chance of evolutionary rescue of *Chlamydomonas reinhardtii* populations, which suffered a 25% reduction in their survival rate when propagated in the presence of the competitor. However, this study only investigated the effect of a single species of competitor, and thus the general conclusion regarding the negative effect of competition on evolutionary rescue may not be generalizable to more natural conditions.

Competitors often differ in various ecological and evolutionary traits which may affect the mean fitness of the focal population and thus, indirectly, its survival. If two species use similar resources (high niche overlap), we might predict that competition will reduce the abundance of one or both species, resulting in reduced rate of adaptation to a changing environment. The model by Osmond and de Mazancourt (2013) indicates that the probability of evolutionary rescue in the presence of a competitor is negatively correlated with the amount of niche overlap between competitors. Competition will impede evolutionary rescue if the focal population is forced to adapt to a niche occupied by a competitor, which implies high niche overlap (Osmond and de Mazancourt, 2013). Conversely, if the focal population is forced to adapt to a niche partially occupied by the competitor, competition could facilitate adaptation by increasing the selective pressure on phenotypes closer to the new niche (Osmond and de Mazancourt, 2013).

Thus, the degree of ecological similarity or divergence between competitors can be an important determinant of the probability of evolutionary rescue. However, to the best of my knowledge, no experimental study investigating the effect of competition on evolutionary rescue has considered the ecological traits of the competitors.

Ecological similarity between species is often assumed to be directly proportional to their degree of phylogenetic relatedness (Naughton et al., 2015). This assumption, dating back to the observation of Darwin (1859) that closely related species rarely coexist in nature (Naughton et al., 2015), has led to the Competition-relatedness hypothesis (CRH) which predicts higher level of competition between closer relatives (Cahill et al., 2008). This

hypothesis has been both supported (Maherali and Klironomos, 2006; Strauss et al., 2006; Valiente-Banuet and Verdú, 2008; Castillo et al., 2010; Jiang et al., 2010; Burns and Strauss, 2011; Violle et al., 2011; Peay et al., 2012) and refuted (Cahill et al., 2008; Best et al., 2012; Kunstler et al., 2012; Narwani et al., 2013; Venail et al., 2014; Godoy et al., 2014; Fritschie et al., 2014; Alexandrou et al., 2015; Naughton et al., 2015) by various experimental and meta-analytic studies.

The explanation for the lack of conclusive evidence for or against the CRH may lie in the fact that competitive success can depend on the hierarchical distances in species' competitive abilities (for instance, unequal abilities to exploit limiting resources, differential susceptibility to predation and variation in reproductive output per parent; Mayfield and Levine, 2010), rather than ecological or phylogenetic similarities between species (Kunstler et al., 2012). Moreover, relevant ecological traits are not always phylogenetically conserved (Best and Stachowicz, 2013), which implies that phylogenetic distance between species cannot be considered a priori as a proxy for ecological similarity (and therefore for the strength of competition). However, some important attributes of species' niches do show phylogenetic signal, such as germination time in plants (Burns and Strauss, 2011) and body mass and fecundity in Seagrass Amphipods (Best and Stachowicz, 2013), which implies that the strength of competition could be positively correlated with the degree of relatedness at least in some taxa, as demonstrated by Burns and Strauss (2011). This suggests that the probability of evolutionary rescue of some species may depend on the phylogenetic relatedness to their competitors. However, I am unaware of any experimental studies testing the effect of competition on evolutionary rescue which have directly manipulated phylogenetic relatedness between competitors.

In this study, I aimed to expand our understanding of the effect of competition on evolutionary rescue, by manipulating the level of ecological similarity and degree of phylogenetic relatedness between competitors. I allowed competition under lethal conditions between experimental populations of a focal species (*C. reinhardtii*) and one of each of 10 competitor Chlorophyte species. Each of the competitors differed in ecological traits, being isolated either from a freshwater or a terrestrial habitat, and phylogenetic distance to the focal species, being positioned on a different branch of the *Chlamydomonas* phylogenetic tree.

If the probability of evolutionary rescue is negatively correlated with the degree of niche overlap between competitors (higher ecological similarity), I predict a higher extinction rate in experimental populations of *C. reinhardtii* (isolated from terrestrial habitats) propagated with a terrestrial competitor. If the probability of evolutionary rescue is positively correlated to phylogenetic distance between competitors, I predict a higher extinction rate in experimental populations of the focal species in the presence of more closely related competitors.

4.2 Methods

4.2.1. The species used in experiment

4.2.1.1. Base populations of C. reinhardtii

Each experimental population of the focal species (240 in total) was constructed by assembling 10 isolates randomly chosen from the library of *C. reinhardtii* isolates, established for the selection experiment described in Chapter 2. Two equal sets of populations comprising either mt+ or mt- were established (120 populations for each mating type). The populations remained entirely asexual throughout the course of experiment, given that each comprised the isolates of a single mating type.

4.2.1.2. The Competitor Species

The competitive species chosen for this study were 10 unicellular Chlorophyte algae, currently or previously classified within the genus *Chlamydomonas* (Table 4.1). I obtained 8 species (*Lobochlamys segnis, Lobochlamys culleus, Microglena monadina, Chlorogonium capillatum, Haematococcus pluvialis, Chlamydomonas sphaeroides, Chlamydomonas applanata* and *Chlamydomonas leiostraca*) from the algae collection of the University of Gottingen (The SAG Culture Collection of algae; http://www.uni-goettingen.de/en/45175.html). Two species (*C. globosa* and *C. moewusii*) were obtained

from the algae collection of the University of Minnesota (http://www.chlamycollection.org/strains/).

Two criteria were considered regarding the selection of competitors for the experiment. The first criterion was phylogenetic relatedness to the focal species. The phylogenetic tree of the genus *Chlamydomonas* (which includes other genera previously classified as *Chlamydomonas*) comprise the 8 main branches (clades) (the most recent revision of *Chlamydomonas* phylogenetic tree is by Yumoto et al., 2013). I chose two species from each of 5 clades. Thus, each selected pair of species is characterized by a different degree of phylogenetic relatedness with *C. reinhardtii*. The second criterion for the choice of competitors for the experiment was the natural habitat from which the species were isolated. Since the majority of *Chlamydomonas* strains have been isolated from either freshwater (e.g. marshes, lakes and ponds) or terrestrial habitats (e.g. agricultural habitats and gardens), I selected an equal number of species isolated from each habitat (5). Hence, the selected pair of species from each clade comprised a single freshwater and a single terrestrial natural isolate.

Microorganisms stored in laboratory conditions are subjected to different selective pressures than in their natural environments, which may result in adaptive evolution (Collins and de Meaux, 2009). Consequently, the ecological traits of an ancestral population may change in a population cultivated in a laboratory. In order to examine whether the experimental species preserved the ancestral ecological features, I performed an assay for growth in the media simulating their original natural environment (see the section 'Examination of the ecological features of the experimental species')**.**

In order to quantify phylogenetic relatedness between *C. reinhardtii* and each competitor species, I compared the 18S rDNA sequence of each species (obtained from the supplier or Yumoto et al., 2013) using the Basic Local Alignment Search Tool (BLAST) (http://www.uniprot.org/blast/) (Table 4.2).

Clade	Freshwater species	Terrestrial species
1st Reinhardiinia	*Chlamydomonas globosa (cc-3349)*	*Chlamydomonas sphaeroides (4.83)*
2nd Oogamochlamydinia	*Lobochlamys segnis (1.79)*	*Lobochlamys culleus (53.72)*
3rd Monadinia / Polytominia	*Microglena monadina (31.72)*	*Chlamydomonas applanata (11-9)*
4th Chlorogonia	*Haematococcus pluvialis (34-1b)*	*Chlorogonium capillatum (12-2b)*
5th Moewusiinia	*Chlamydomonas moewusii (cc-2684)*	*Chlamydomonas leiostraca (11-49)*

Table 4.1 – List of the competitors used for the experiment (the isolate code in brackets); selection of competitors was based on: **a) relatedness with the focal species**; 5 pairs of species were chosen with respect to the clade within the *Chlamydomonas* phylogenetic tree; the clades were ranked with numbers corresponding to a degree of relatedness between each pair of competitors and *Chlamydomonas reinhardtii*, which forms the upward gradient of relatedness between the 1st pair (the most closely related to the focal species) and the 5th pair (the most distantly related to the focal species); **b) original habitat**; each clade comprise a single species isolated from a freshwater habitat and a single species isolated from a terrestrial habitat.

Species	Branch length	*BLAST similarity (%)*
C. reinhardtii	0	100
C. globosa	0.0028	99.674
C. sphaeroides	*0.0071*	*99.348*
L. culleus	*0.0595*	*95.313*
L. segnis	*0.0659*	*94.922*
C. leiostraca	*0.0787*	*91.656*
C. applanata	*0.0841*	*95.055*
M. monadina	*0.0890*	*92.283*
C. capillatum	*0.1014*	*94.867*
H. pluvialis	*0.113*	*93.819*
C. moewusii	*0.1729*	*91.451*

Table 4.2 – Phylogenetic relatedness between *C. reinhardtii* and each of the competitors; the estimation was obtained by comparing the 18S rDNA sequences of species using the BLAST.

4.2.2. Assay of the experimental species for growth in high salt

If the competitor species differ in sensitivity to the elevated concentration of salt, the effect of ecology and relatedness might be confounded with the effect of this factor (hereafter referred to as the initial maladaptedness). To investigate this possibility, prior to commencing of the selection experiment, all the competitors were assayed for growth in a gradient of salt concentration, ranging from 1 g/l (permissive) to 17 g/l (lethal). All the species were allowed two growth cycles (3-4 days; 5% of culture passaged), but were assayed only during the second cycle, to avoid the possible carry-over effects after plating from agar, used for storing the cultures. The assay was performed in 96-well plates and growth estimated spectrophotometrically, by measuring OD_{750} twice a day (approximately every 12 hours) until the cultures reached the stationary phase (after approximately 4 days). The fitness for growth in each salt concentration was estimated by calculating the maximal yield of each species (Maximal OD - Initial OD). The maximal yield was selected as a measure of fitness, since it does not depend on the initial population size (Lachapelle and Bell, 2012).

4.2.3. Examination of the ecological traits of the experimental species

Prior to commencing the selection experiment, I examined the ecological similarity between the competitor species and the focal species, by performing an assay in four types of media. Two types comprised: Bold's broth (simulating a freshwater habitat) and Bold's broth supplemented with soil-extract (simulating a terrestrial habitat), hereafter referred to as 'Bold's' and 'Soil-extract Bold's', respectively. The other two comprised spent media from each of these treatments, obtained from stationary phase cultures of *C. reinhardtii* propagated in Bold's and Soil-extract Bold's, respectively.

The spent media were obtained as follows. Thirty populations of the focal species, randomly chosen from the library of *C. reinhardtii* isolates, were propagated for 5 days in Bold's and Soil-extract Bold's, respectively, until reaching the stationary phase. After a given period, the cultures were centrifuged at 5000 rpm for 10 minutes, and the collected mixed supernatant injected through a syringe filter (0.22 µm diameter) to eliminate cells.

All the species were allowed two growth cycles and assayed during the second growth cycle, using the identical experimental procedure as in the previous section (see 'Assay of the experimental species for growth in high salt' for details).

4.2.4. Selection experiment

The experiment consisted of two phases. In the first phase, populations of the focal species were allowed to undergo two growth cycles in a benign environment. In the second phase, each population was subsampled and divided into two parts, which resulted in establishing of two sets of 240 populations. Ten groups of twenty-four populations within one set were assembled with one of the competitors, while the populations comprising the second set were cultivated without the presence of the competitor. During this phase, both sets of populations were subjected to lethal level of salt, and their ability to survive with or without a competing species was monitored.

4.2.5. Establishment and Cultivation of Mixed Populations

The focal species and the competitors were cultivated separately in 24 well-plates in standard conditions (26^{o}C, shaking at 180 rpm and enclosed with sterile breathable membranes). Half of the populations were propagated in Bold's medium, while the other half was cultivated in Soil-extract Bold's medium. Two types of media were used to investigate whether the chance of survival is affected by the similarity of the selective environment to the original habitat of the focal species (soil). The competitor species were maintained in Bold's medium during this stage of the experiment. All the populations were allowed two growth cycles (3-4 days, with a serial passage of 5% of each population). After a given period, the population size of each *C. reinhardtii* population was estimated spectrophotometrically (OD_{750}) and by cell counting with a haemocytometer. The recorded OD_{750} of each population was converted to the cell number using the equation obtained from an OD_{750} - cell number calibration curve (Figure 4.1). The population size of the competitor species was estimated with a haemocytometer. All the base populations were

then diluted to the same population density (approximately 50 000 cells per ml). Two subsets of each experimental population of *C. reinhardtii* were then created, each containing approximately 5000 cells inoculated from the base populations (for the summary of experimental design, see Figure 4.2). The populations within one subset were then assembled with the corresponding competitor species in order to form mixed populations. The mixed populations were established by combining each of the 10 competitor species (using inoculums of approximately 5000 cells) with 24 populations of the focal species. The other subset of each experimental population was propagated without the presence of the competitor throughout the course of the experiment. Both subsets of 240 populations were maintained in the ancestral medium (Bold's or Soil-extract Bold's) supplemented with 15 g/l of salt, by serial passaging after every 3-4 days.

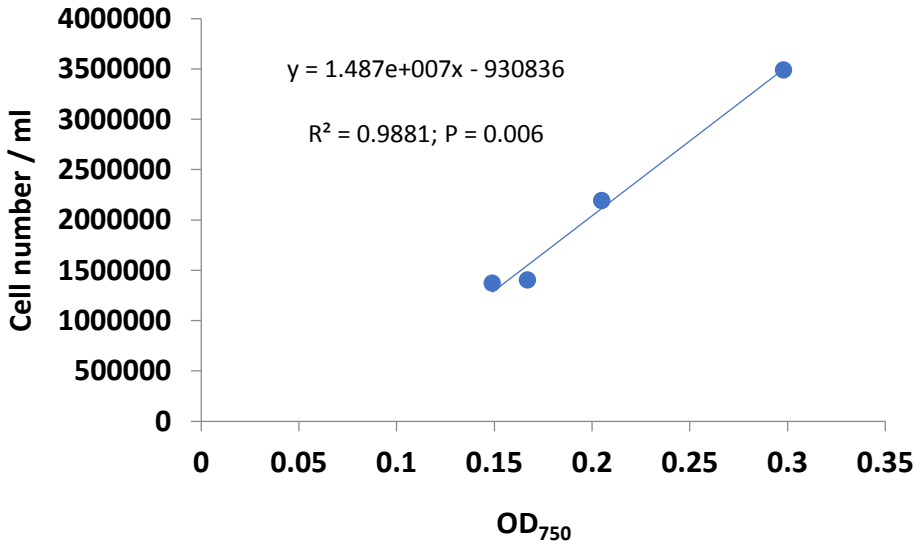

Figure 4.1 – OD_{750} – cell number calibration curve for *C. reinhardtii* experimental populations.

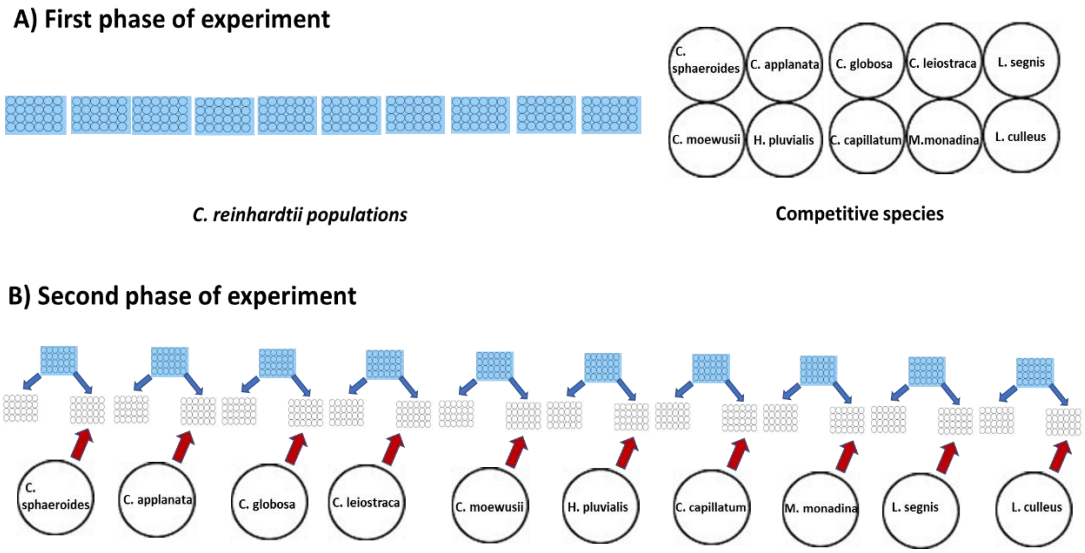

Figure 4.2 – Two phases of the experiment; In the first phase of experiment, populations of all the species were propagated separately in benign conditions; each circle represents a single population. In the second phase, each experimental population of *C. reinhardtii* was subsampled and divided into two subpopulations. As a result, two sets of 240 populations of the focal species were formed; each competitor species was combined with 24 populations of one set; the populations comprising the other set were propagated without the competitor.

4.2.6. The method for distinguishing pairs of species grown on a solid medium

The population size of each experimental population was monitored by plating a sample (100 µl) of each culture on a solid medium (agar) after the completion of each growth cycle. A sample was plated directly from the culture (low density populations) or serially diluted (10^{-1} or 10^{-2} - fold dilution; high density populations) prior to transfer to the corresponding Petri dish. Prior to commencing the selection experiment, a series of pilot experiments were performed in order to determine the combination of growth factors that maximize differentiation in growth of species after propagation on a solid medium. The results of these experiments, summarised in Table 4.3, provide a rationale for utilizing a specific combination of growth factors for each pair of species while plated on agar.

Each sampled population was incubated on 26°C under bright light for 4-7 days until colonies appeared; the mixed populations comprising *L. segnis*, *C. moewusii* or *C. capillatum* were wrapped in aluminium foil and propagated for 3 additional days in the darkness. The population size of each species in a mixed culture was estimated by

converting the number of colonies obtained after plating on a solid medium to the cell number, using the equation: cell number per ml = number of colonies / dilution factor X sample size (0.1 ml).

Competitor in a mixed population	Intervals of growth in the light/dark	Additional carbon or nitrogen source
L. segnis	4 days in light / 3 days in dark	Sodium-acetate
C. moewusii	4 days in light / 3 days in dark	Sodium-acetate
C. capillatum	4 days in light / 3 days in dark	Sodium-acetate
H. pluvialis	7 days in light	Sodium-acetate and Proteose-Pepton
M. monadina	7 days in light	Sodium-acetate and Proteose-Pepton
C. globosa	7 days in light	Sodium-acetate and Proteose-Pepton
L. culleus	7 days in light	Sodium-acetate
C. leiostraca	7 days in light	Sodium-acetate
C. sphaeroides	7 days in light	Acetamide (0.01 mM)
C. applanata	7 days in light	Acetamide (0.01 mM)

Table 4.3 – The combination of growth factors utilised to differentiate *C. reinhardtii* from each competitor within the corresponding mixed population;

4.2.7. Recording the evolutionary rescue events

The experiment continued until one or both species of all the mixed populations and all the populations propagated without the competitor either went extinct or showed clear positive growth. The evolutionary rescue of the focal species was assayed during the

last three growth cycles of survived mixed populations, by visual inspection of liquid cultures and plating of 100 µl sample of each culture on agar plates. The number of colonies obtained after the incubation was converted to the number of cells per ml as described in the previous section. The mean fitness of the rescued populations was estimated by calculating the average number of cells /ml scored during the assay.

The species-constituent of a mixed population was recorded as 'rescued' if it had repeatedly scored the same or a higher number of cells/ml each time it was sampled, thus showing positive growth in lethal conditions. Each species was recorded as 'extinct' if no cells were detected after visual inspection of liquid culture or plating on agar.

Despite the fact that no colonies of the competitor species were detected in the majority of agar plates during the assay (see the section 'Results' for details), I cannot rule out the possibility that the competitors were present in the mixed populations (e.g. some individual cells of the competitor may have remained in the liquid culture after sampling). Therefore, the evolutionary rescue assay of *C. reinhardtii* populations may have been carried out in the presence of the competitors.

4.3 Data analysis

The effects of competition, the competitor species and the environment on the probability of evolutionary rescue were analysed by fitting Generalised Linear Mixed Models. One model was fitted per each of these independent categorical variables, all being considered fixed factors. The random factor incorporated in all the models was 'population', being nested within the factor 'population group' (two subsets of populations propagated either in the presence or absence of a single competitor), nested within the factor 'species'. The factor 'competition' comprises two levels (presence/absence of the competitor); 'the competitor species' includes 10 levels, each species being considered a single level; 'environment' comprises two levels, corresponding to the two types of media used in the selection experiment.

The effects of the ecological traits of the competitors and their relatedness to the focal species on the probability of evolutionary rescue were analysed by fitting a Generalised Linear Mixed Model, with both independent categorical variables being

considered fixed factors. The random factor incorporated in the model was identical as in the previous two models. The factor 'phylogenetic relatedness' was considered a continuous variable; 'ecology' includes two levels: 'freshwater' and 'terrestrial'. The covariate 'initial maladaptedness' was introduced due to results of the pilot experiment (see the 'Results' section).

The mean fitness of the rescued populations, which corresponds to the average population size estimated after counting of colonies obtained after the last three growth cycles, was analysed by fitting a General linear model. The model incorporates the following factors (all being considered categorical variables): 'population subset' comprising two levels, corresponding to either presence or absence of the competitor; 'group', comprising 10 levels, each level corresponding to the competitor allocated to the population group; 'environment', comprising two levels, corresponding to two types of selective media.

The binary responsive variable in all the models estimating the probability of evolutionary rescue was survival/extinction. The continuous responsive variable in the model estimating the mean fitness of the surviving populations was 'mean population size'.

All the analyses were performed using R (R core team, 2017).

4.4 Results

4.4.1. Assay of the experimental species for growth in high salt

An elevated concentration of salt was detrimental for all the competitor species, which differed with respect to the level of high salt sensitivity (Figure 4.3). The most sensitive species (*M. monadina*, *H. pluvialis, C. capillatum* and *C. leiostraca*) showed negative yield for all the levels of salt concentration. The yield of *L. culleus, C. sphaeroides*, *C. globosa*, *L. segnis* and *C. applanata* decreased with an increase of the level of salt, and dropped to zero in salt concentration ranging from 3 g/l – 11 g/l. The yield of *C. moewusii* showed the opposite trend, gradually increasing in salt concentration ranging from 1 g/l – 9 g/l and decreasing in salt concentration higher than 9 g/l, without dropping to zero.

The competitor species were assayed in the salt concentration chosen for the selection experiment (15 g/l), by propagating the cultures until the stationary phase of growth (approximately 4 days) and recording the yield twice a day as a measure of fitness. The yield of all the species was negative (dropping to zero or below zero), except for *C. moewusii,* which showed slow, but positive growth (Figure 4.4).

The results of these assays provide evidence of different abilities of the competitors for growth in high salt conditions. Notably, *C. moewusii* stands out as the only competitor that can sustain positive growth in the selective environment. This difference in the initial maladaptedness to the selective environment between the competitor species will be considered in the analysis of the evolutionary rescue of the focal species, by introducing the factor 'initial maladaptedness' with two levels: 'low initial maladaptedness' (*C. moewusii*) and 'high initial maladaptedness' (other competitors).

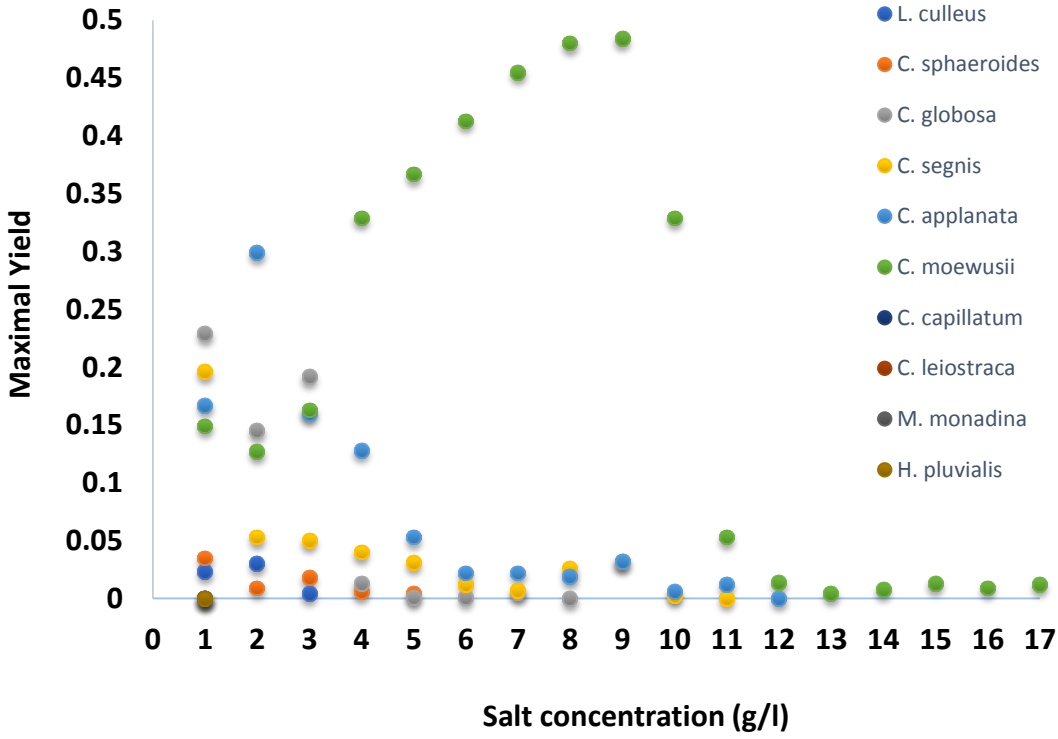

Figure 4.3 – Maximal yield of each competitor species in a gradient of salt concentration ranging from 1 g/l to 17 g/l; no error-bars are present since the experimental populations were not replicated**;** the yield of *M. monadina*, *H. pluvialis, C. capillatum* and *C. leiostraca* was negative for all the levels of salt concentration; *L. culleus, C. sphaeroides*, *C. globosa*, *L. segnis* and *C. applanata* showed negative yield for salt concentration higher than 3 g/l, 5 g/l, 7 g/l, 10 g/l and 11 g/l, respectively; The yield of *C. moewusii* was positive for all the levels of salt concentration.

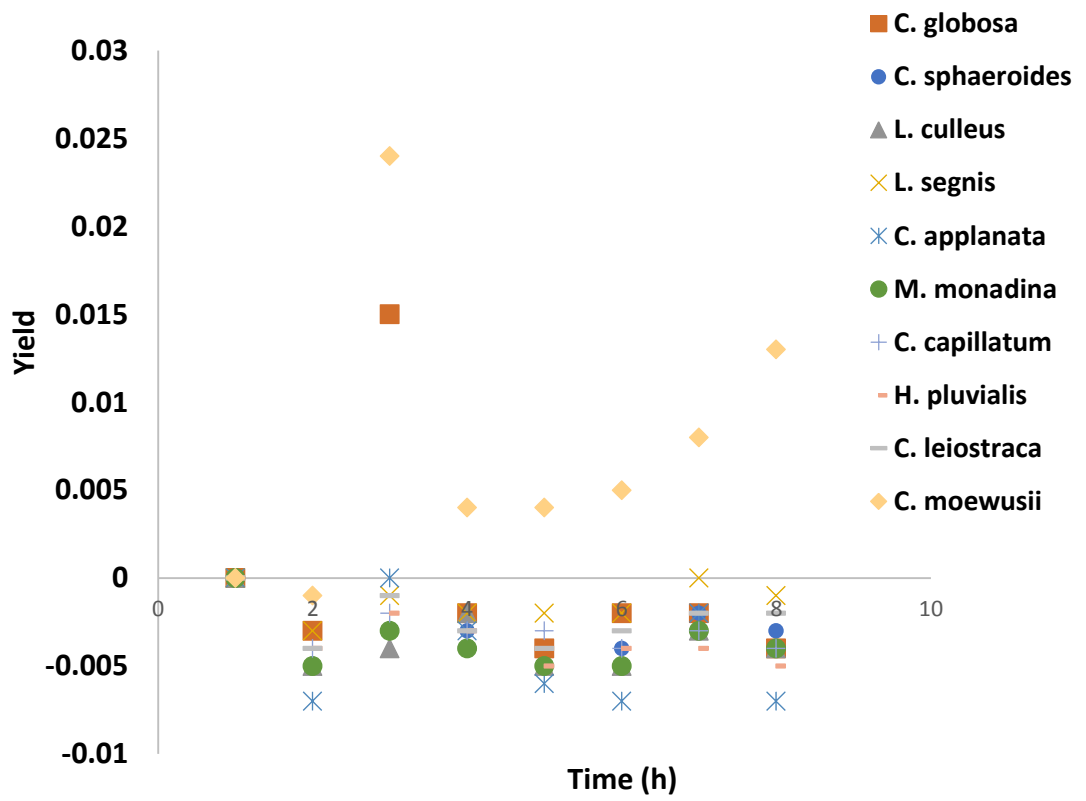

Figure 4.4 – Yield of each competitor species per time unit (h) in salt concentration of 15 g/l; yield of all the competitor species (except for *C. moewusii*) dropped to zero or below zero by the end of the assay; no error-bars are present since the experimental populations were not replicated**;**

4.4.2. Examination of the ecological traits of the experimental species

When tested in both types of fresh media, each competitor species scored higher maximal yield in a different medium (Figure 4.5). *L. segnis*, *C. applanata*, *C. globosa* and *C. moewusii* scored higher maximal yield in Soil-extract medium. *H. pluvialis*, *C. capillatum, L. culleus*, *C. sphaeroides* and *C. reinhardtii* showed higher maximal yield in Bold's medium. *M. monadina* and *C. leiostraca* failed to grow in both media during the pilot experiment. The average maximal yield per species was approximately two-fold higher in soil-extract spent medium, except for *L. segnis* which showed minimal growth in Bold's spent medium and negative growth in Soil-extract spent medium (Figure 4.6).

The reduction of growth of the species in each spent medium relative to the growth in the corresponding fresh medium was calculated by dividing the maximal yield obtained in the spent medium by the maximal yield obtained in the corresponding fresh medium. C.

reinhardtii showed an ecological similarity with *H. pluvialis*, *C. capillatum*, *L. culleus*, *C. sphaeroides*, *C. applanata* and *C. moewusii*, given that each of these species scored higher growth reduction in Bold's spent medium. *C. globosa* and *L. segnis* showed higher reduction in Soil extract spent medium, which indicates ecological divergence from *C. reinhardtii* (Figure 4.7). These differences in ecological similarity between *C. reinhardtii* and the competitors will be considered in the analysis of the evolutionary rescue of the focal species, by introducing the factor 'ecological similarity to *C. reinhardtii*' with two levels: 'ecologically divergent species' (*C. globosa*, *C. segnis* and *C. leiostraca*) and 'ecologically similar species' (the other seven species). *C. leiostraca* and *M. monadina* were allocated to the former and the latter level, respectively, based on their original habitat, given the failure of growth of these species in the pilot experiment.

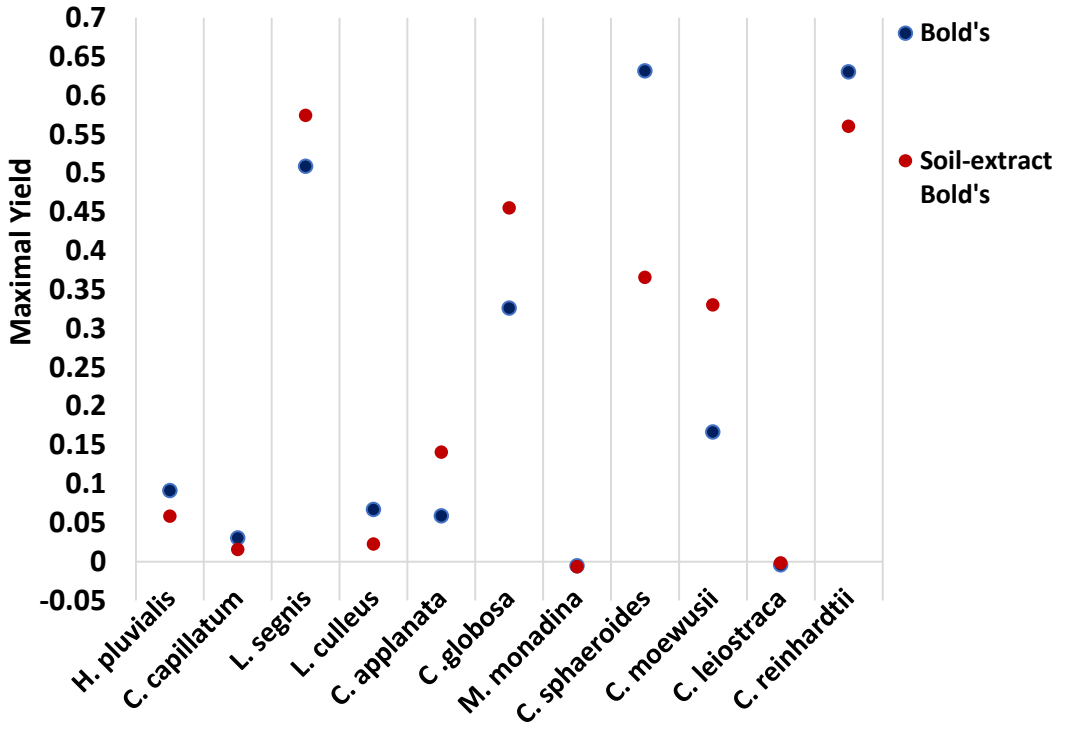

Figure 4.5 – Maximal yield of the focal species and each of the competitor species in both types of fresh media;

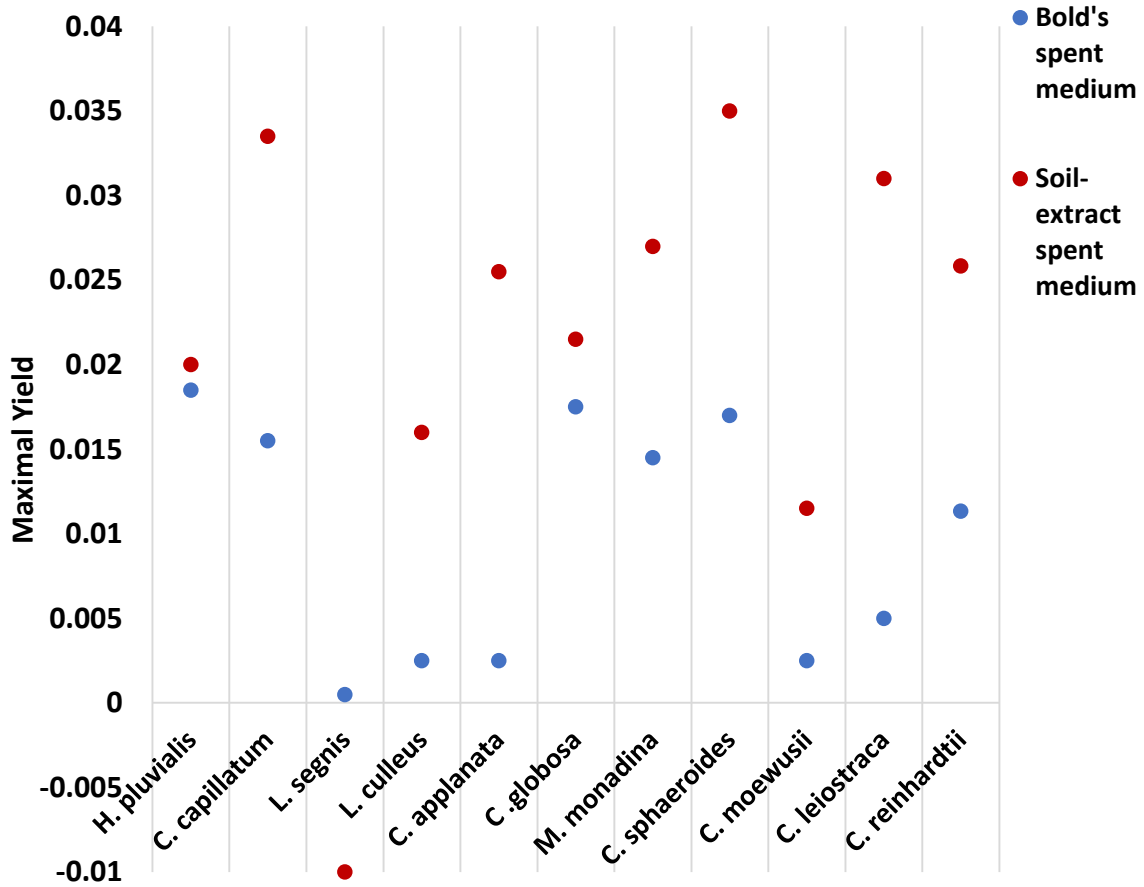

Figure 4.6 - Maximal yield of the focal species and each of the competitor species in both types of spent media;

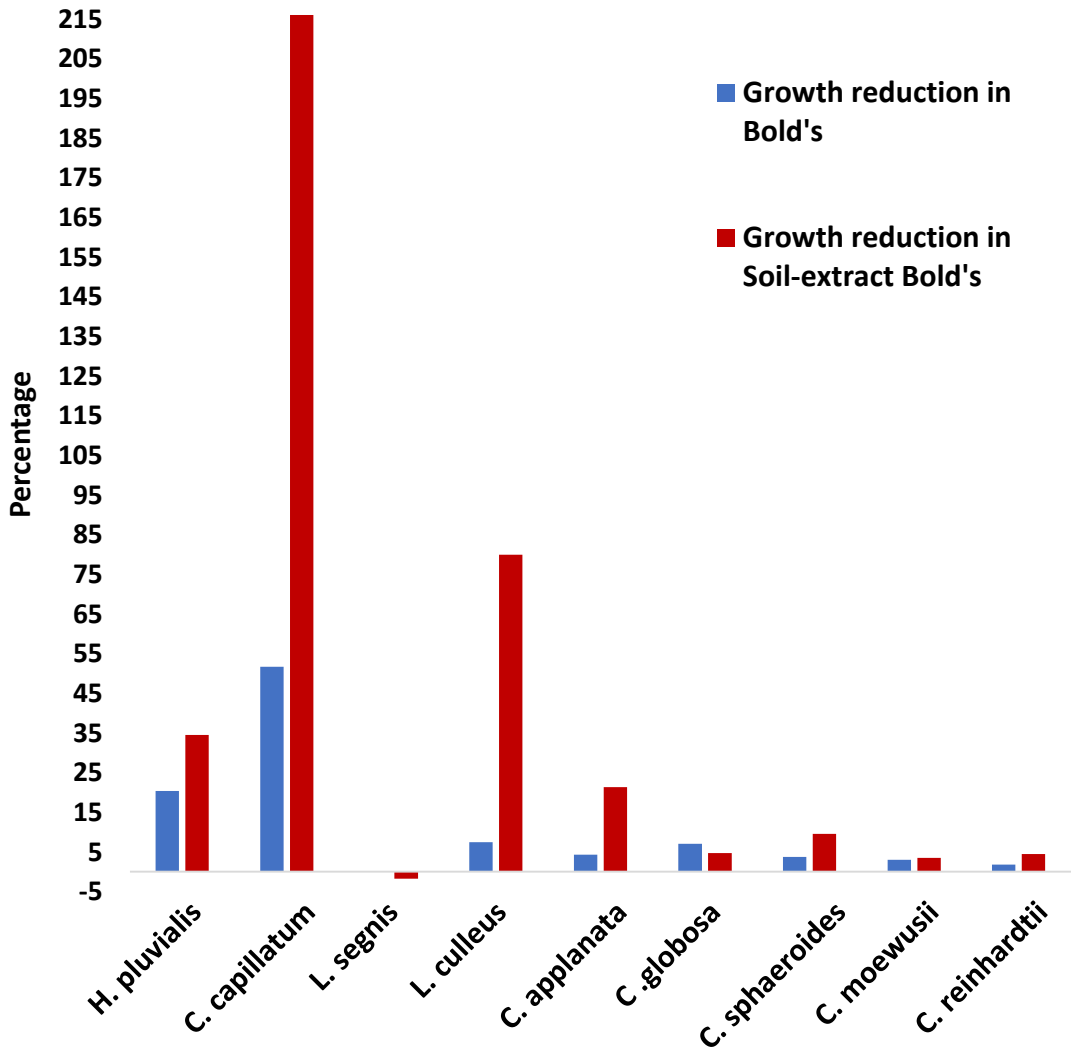

Figure 4.7 – Reduction of growth of each species in Bold's and Soil-extract Bold's spent media, respectively, relative to growth in Bold's and Soil extract Bold's fresh media, respectively; *C. reinhardtii* showed higher reduction of growth in Bold's spent medium (last species from the left). Thus, higher reduction of growth of a competitor species in this spent medium was considered as a proxy for higher niche overlap between *C. reinhardtii* and other species; *C. leiostraca* and *M. monadina* failed to grow in the fresh media during the pilot experiment and were thus not incorporated into the analysis; reduction of growth of all the species (except for *C. globosa* and *L. segnis*) was higher in Bold's spent medium, thus indicating the ecological similarity between *C. reinhardtii* and these species. *C. globosa* and *L. segnis* showed higher reduction in Soil extract spent medium, which indicates the ecological divergence from *C. reinhardtii*.

4.4.3. Selection experiment

4.4.3.1. Evolutionary rescue

The total probability of evolutionary rescue of *C. reinhardtii* was 85%, given the survival of 203 out of 240 populations. Competition significantly affected the probability of evolutionary rescue (Generalised Linear Mixed Model; $\chi^2 = 6.01$; df = 1; P = 0.01). Within a set of 240 populations propagated with one of 10 competitors, 208 populations survived (87%). Within a set of 240 populations propagated in the absence of the competitor, 229 populations (95%) survived. Of the 37 extinct populations of the focal species, 26 populations (70%) went extinct in the presence of the competitor, while surviving when propagated alone; 5 populations (14%) went extinct in the absence of the competitor while surviving in the presence of the competitor; 6 populations (16%) went extinct under both treatments.

The competitor species significantly affected the probability of evolutionary rescue (Generalised Linear Mixed Model; $\chi^2 = 47.62$; df = 9; P < 0.00001) (Figure 4.8). All the populations survived when propagated along with *C. globosa*, *C. sphaeroides* and *C. segnis*. One extinction was recorded for the populations competed with *M. monadina*, *C. leiostraca*, *C. applanata* and *H. pluvialis* (4% of the initial 24 populations). Two extinctions were recorded for the populations propagated with *L. culleus* and *C. capillatum* (8% of the initial 24 populations). Twenty-four extinctions occurred in the presence of *C. moewusii* (100% of the initial 24 populations). Despite the differences in survival of the focal species when propagated with different competitors, only *C. moewusii* significantly affected the probability of survival (accounts for 65 % of all extinctions) (Generalised Linear Mixed Model; z = -2.84; P = 0.005).

Phylogenetic relatedness between the focal species and each competitor did not affect the probability of evolutionary rescue (Generalised Linear Mixed Model; $\chi^2 = 0.1$; df = 1; P = 0.75) (Figure 4.8). The ecology of competitors (with respect to the original habitat) did not affect the probability of evolutionary rescue of *C. reinhardtii* (Generalised Linear Mixed Model; $\chi^2 = 0.51$; df = 1; P = 0.48) (Figure 4.9). Likewise, the probability of

evolutionary rescue was not significantly affected by ecological similarity to *C. reinhardtii* (Generalised Linear Mixed Model; $\chi^2 = 0.54$; df = 1; P = 0.46).

The covariate 'the initial maladaptedness' explained the variation in the number of rescue events (Generalised Linear Mixed Model; $\chi^2 = 40.51$; df = 1; P < 0.00001). The competitor with low initial maladaptedness drove 24 populations to extinctions. The other competitors, characterised by higher initial maladaptedness, drove 8 populations to extinctions.

The environment significantly affected the probability of evolutionary rescue (Generalised Linear Mixed Model; $\chi^2 = 14.38$; df = 1; P = 0.0001). Of 37 extinct population of the focal species, 24 populations (65%) went extinct in Bold's medium, while 13 populations (35%) went extinct while propagated in Soil-extract Bold's medium.

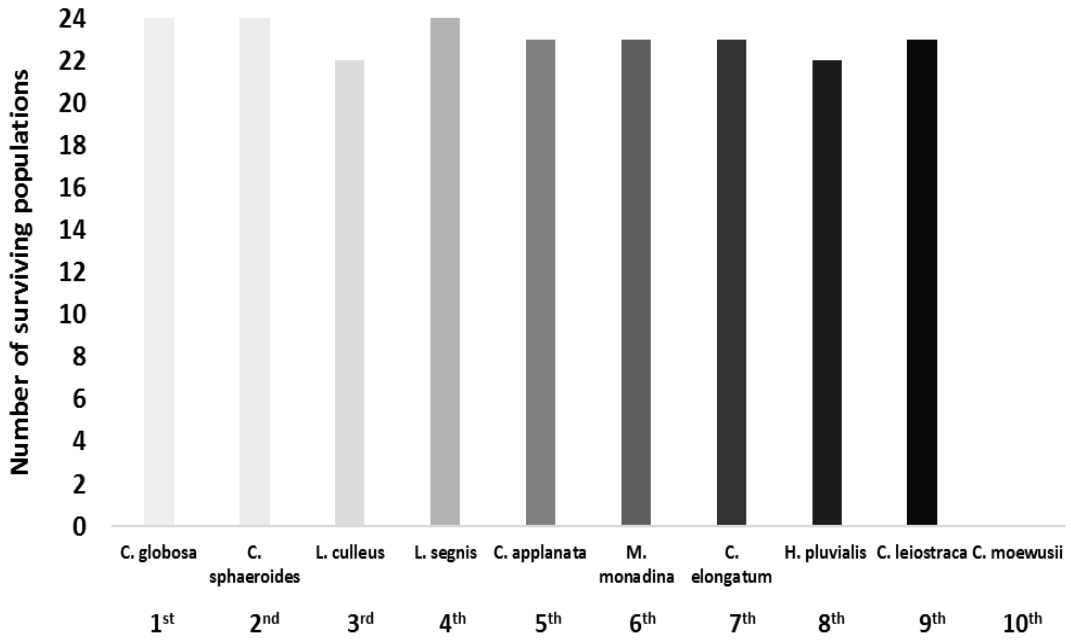

Figure 4.8 – The probability of evolutionary rescue for experimental populations of *C. reinhardtii* with respect to: a) the competitor species; the number of evolutionary rescue events ranged from 24 (100% of the initial 24 populations – e.g. C. *globosa*) to 0 (0% of the initial 24 populations – e.g. C. *moewusii*), depending on the competitor species; **b) degree of phylogenetic distance of each competitor species from the focal species**; the number below each species corresponds to a degree of relatedness between each competitor and *Chlamydomonas reinhardtii*, which forms the upward gradient of relatedness between the 1st competitor (the most closely related to the focal species) and the 10th competitor (the most distantly related to the focal species).

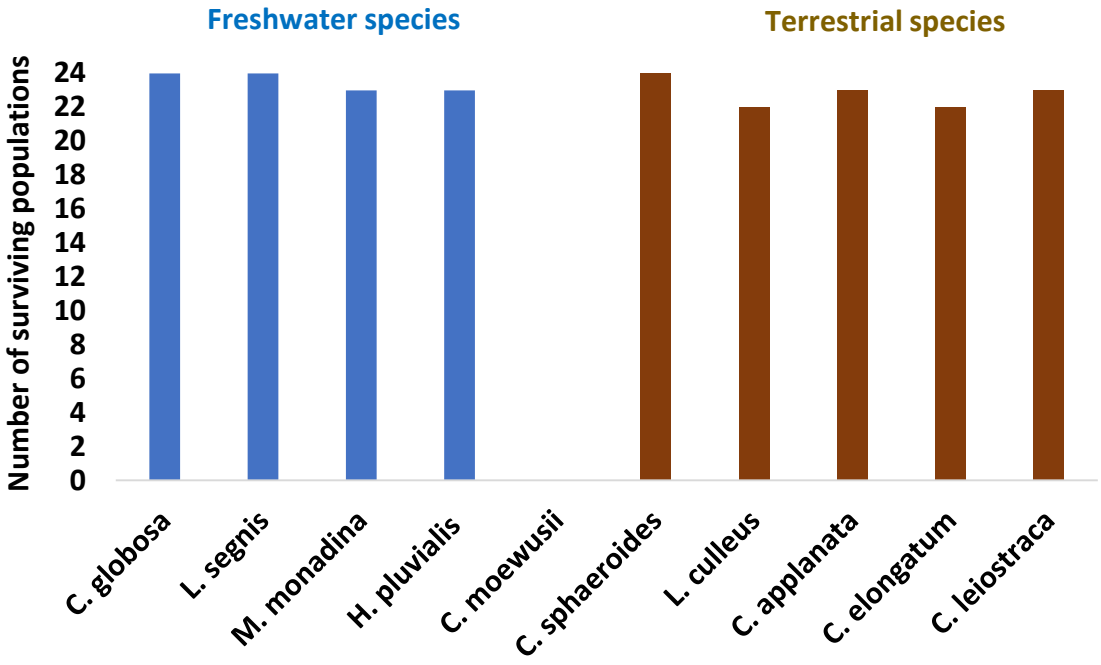

Figure 4.9 – The probability of evolutionary rescue of *C. reinhardtii* with respect to the ecology of the competitors (the original habitat); 4 leftmost bar plots represent freshwater species (blue colour) and 5 rightmost bar plots represent terrestrial species (brown colour); 26 extinctions occurred in the presence of a freshwater competitor (24 extinctions when *C. moewusii* was present); 6 extinctions occurred in the presence of a terrestrial competitor.

4.4.3.2. Mean fitness of the rescued populations

The mean fitness of the rescued populations within 10 different groups of *C. reinhardtii* populations was significantly different (General linear model; $F = 9.11$; $df = 9$; $P < 0.001$) (Figure 4.10) and varied from the most fit group ('*C. applanata*' with approximately 12000 cells per ml/growth cycle) to the least fit group ('*H. pluvialis*' with approximately 3500 cells per ml/growth cycle).

The mean fitness of the rescued populations within two subsets of populations (propagated with and without the presence of the competitor) was significantly different (General linear model; $F = 78.24$; $df = 1$; $P < 0.001$) (Figure 4.11). The subset of experimental populations propagated in the absence of the competitor had a more than two-fold higher average population size than the subset propagated in the presence of the competitor. The

mean fitness of the rescued *C. reinhardtii* populations propagated with *C. globosa* could not be estimated due to high similarity between the colonies formed by some *C. reinhardtii* genotypes and *C. globosa* colonies, which made the differentiation of these species insufficiently reliable.

There was a significant interaction between the factors 'group of populations' and 'the population subset' (General linear model; F = 5.76; df = 7; P < 0.001) (Figure 4.13), despite the fact that for all the groups of populations, the subset propagated with the competitor showed consistently lower average population size than the subset propagated without the corresponding competitor (except for *C. sphaeroides*).

The mean fitness of the rescued populations was contingent on the type of environment used for cultivation of the experimental populations (General linear model; F = 6.76; df = 1; P = 0.01) (Figure 4.12). The average population size of all the experimental populations was 23% higher in Soil-extract Bold's medium.

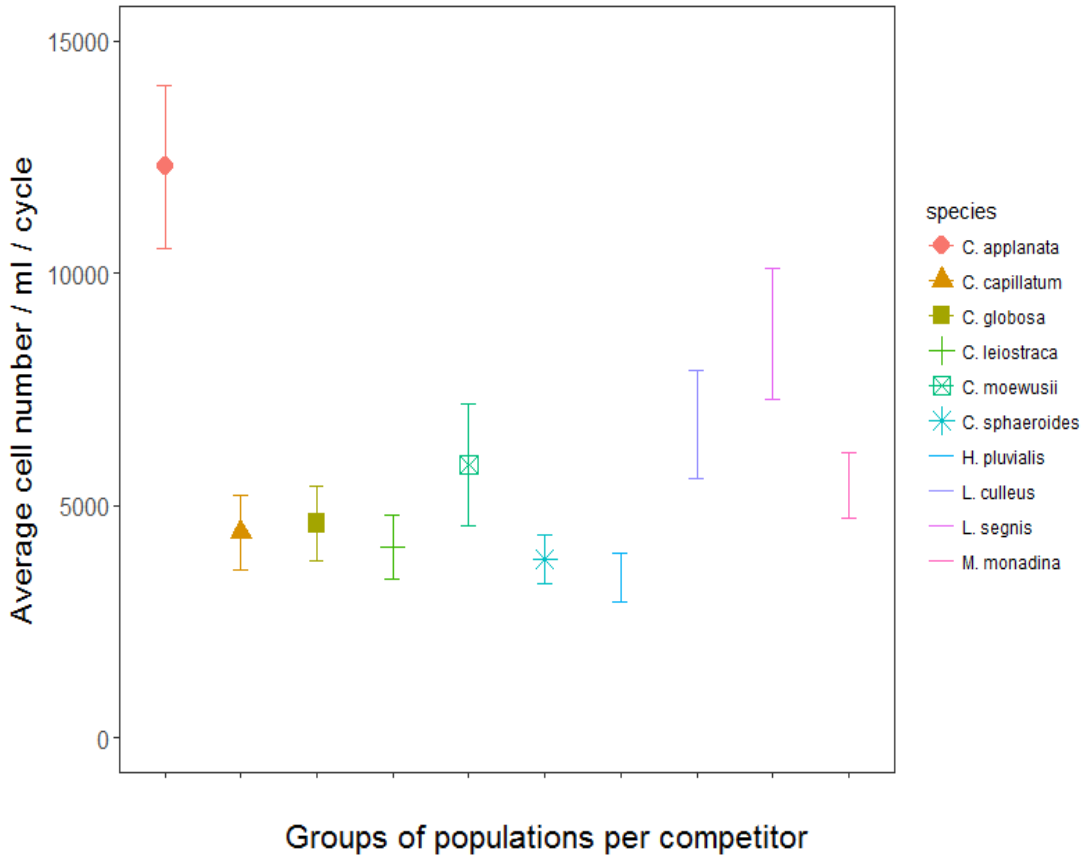

Figure 4.10 - Mean fitness of the rescued populations within each group of populations; each group was assembled with one of the competitor species.

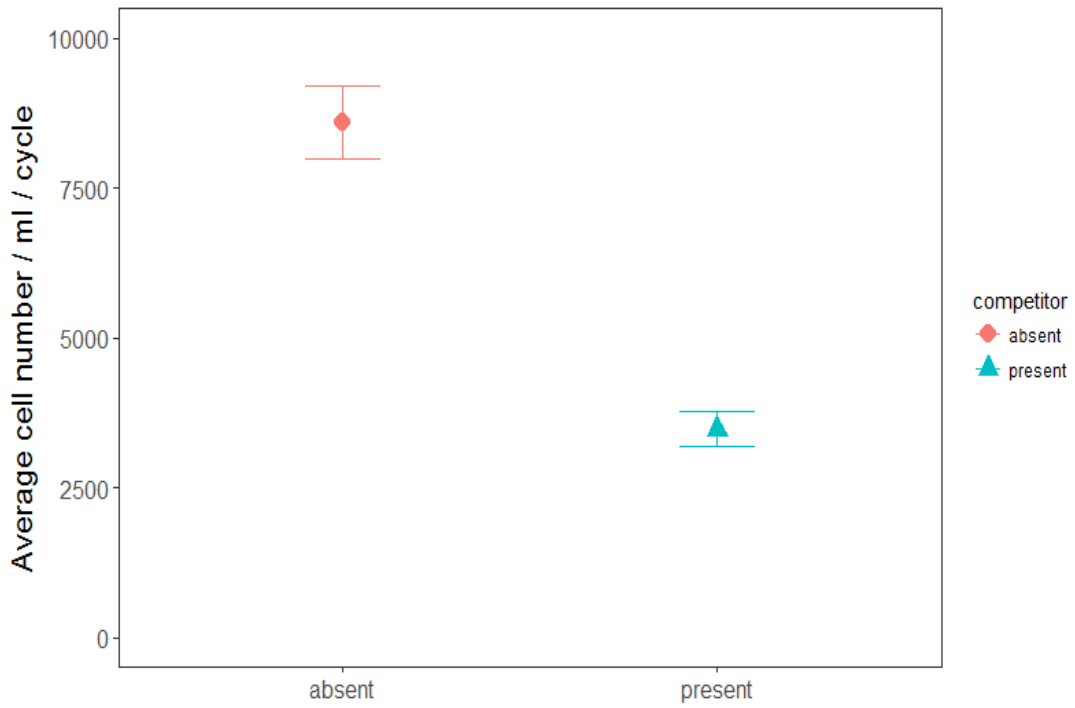

Figure 4.11 – Mean fitness of the rescued populations within both sets of populations (propagated either in the presence or absence of the corresponding competitor).

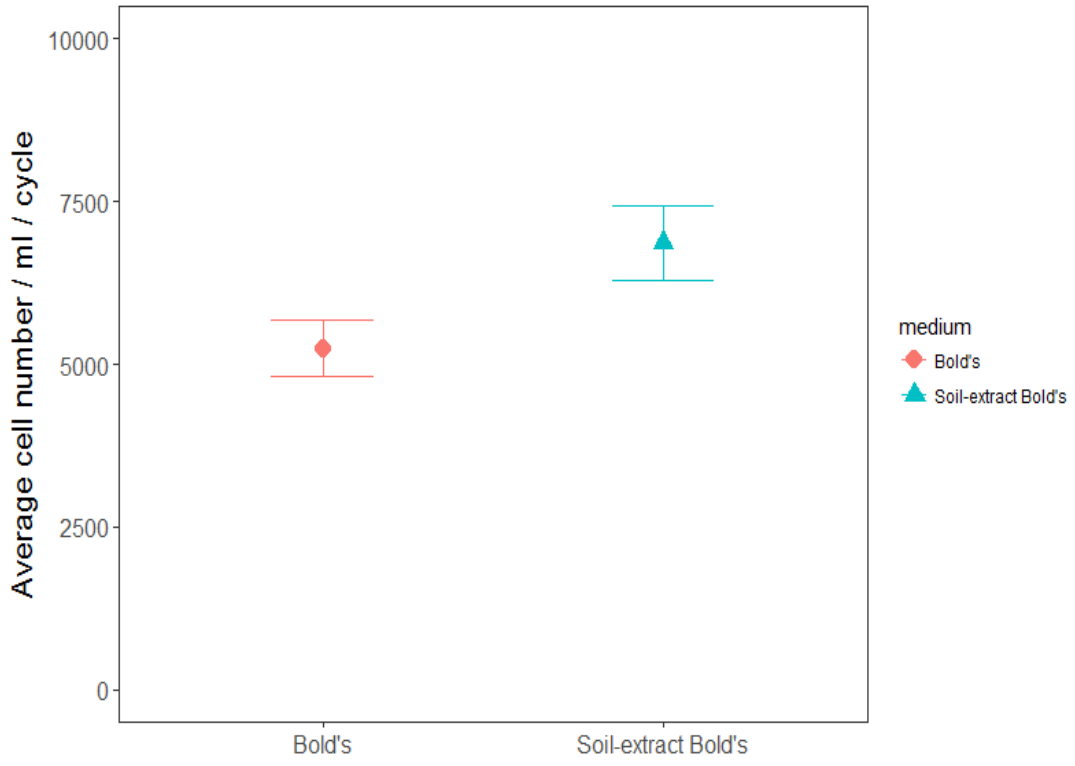

Figure 4.12 – Mean fitness of the rescued populations with respect to the environment.

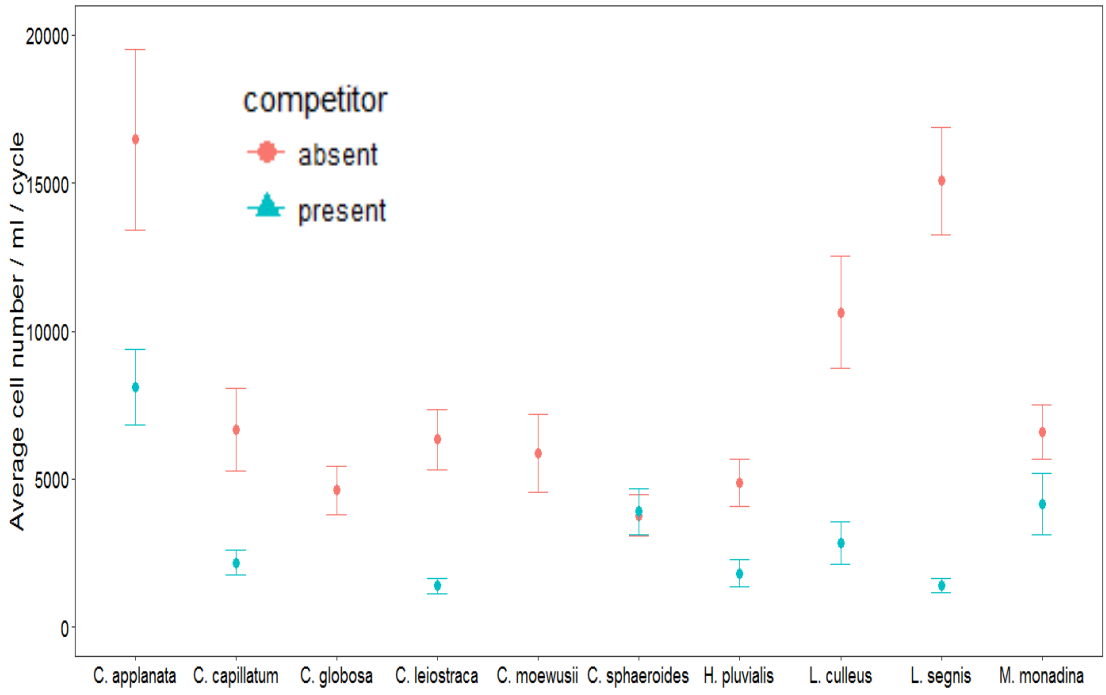

Figure 4.13 – Mean fitness of the rescued populations per each group of populations (defined by the competitor species), per each set of populations (propagated either in the presence or absence of the corresponding competitor); no population of '*C. moewusii*' subset propagated in the presence of the competitor survived; '*C. globosa*' subset propagated in the presence of the competitor was not analysed as explained in the main body of the text.

4.5 Discussion

The probability of evolutionary rescue was affected by competition, which corroborates the findings described in Chapter 3. The results indicate that the presence of different competitor species alters the probability of evolutionary rescue. However, of all the species, only the presence of *C. moewusii* showed a significant effect, while the effect of other species on evolutionary rescue was either neutral or positive/negative, but not statistically significant. Thus, despite the statistical significance of the presence of different competitor species affecting the probability of evolutionary rescue, a cautious interpretation is required. The results indicate a relatively simple pattern of influence of a competitor on evolutionary rescue (strong negative or weak/insignificant effect) which may not be the general pattern in nature.

The variation in the effect among competitors could not have been explained by the extent of ecological and phylogenetic similarity to *C. reinhardtii*. A plausible explanation is that the effects of ecology and phylogenetic relatedness of the competitors on evolutionary rescue could not have been detected due to a relatively low and equal number of extinctions of *C. reinhardtii* per competitor species (except for the subset propagated with *C. moewusii*).

Another explanation is reflected in the fact that most competitors went extinct in the early stage of experiment. I observed faster reduction in population size of most competitors in comparison with *C. reinhardtii* (except for *C. moewusii*), regardless of their ecological and phylogenetic traits, ultimately resulting in the extinction of the great majority of competitor species in the mixed populations. For instance, I did not observe any individual cells of *H. pluvialis*, *M. monadina* and *C. capillatum* in any mixed population after only three growth cycles. In contrast, *C. reinhardtii* showed positive growth in 96% of the mixed populations which comprised these three competitors. This suggests that unequal survival between pairs of competitors was mainly governed by variation in their initial maladaptedness to high salt concentration (lower sensitivity of *C. reinhardtii* to high salt compared to most competitors).

C. moewusii drove 100% of the initial 24 populations to extinction (56% of all extinctions, both in the presence and absence of the competitor). A likely explanation for this disproportionally high number of caused extinctions in comparison with other competitors is the lowest initial maladaptedness of this species to the selective environment. *C. moewusii* is the only competitor capable of positive (albeit very slow) growth in 15 g/l of salt concentration, as shown in the results of the pilot experiment. Furthermore, this is the only competitor species which survived in all the mixed populations. In contrast, of all the other competitors, a single *C. applanata* population survived (although survival of *C. globosa* could not be determined in a reliable way, due to high morphological similarity with *C. reinhardtii*). This suggests that initial maladaptedness might be a factor that generally affects the probability of evolutionary rescue. However, this interpretation should be taken with caution, given that the maladaptedness of species was not directly manipulated in this experiment. Incorporating a proportional number of competitors with respect to their degree of maladaptedness is necessary to test whether this is a common pattern in nature.

The total probability of evolutionary rescue was unexpectedly high (85%) in comparison with a selection experiment described in Chapter 3 (29%), performed using an identical type of stressor. There are three possible explanations for this result, which are not mutually exclusive. Firstly, half of the experimental populations were cultivated in Soil-extract Bold's medium, which supports larger populations. Secondly, the experimental populations were not subjected to a sub-lethal level of stress prior to commencing the selection experiment, which would likely have resulted in lower initial average population size. Higher initial population size implies higher input of beneficial mutations and thus higher probability of survival (Bell and Gonzalez, 2009). Finally, the average light intensity that the experimental populations were subjected to was two-fold higher than in the previous selection experiment (due to technical reasons caused by the change of an incubator), which could have stimulated growth, resulting in a slower decline of population size.

The type of environment significantly affected the probability of evolutionary rescue. Soil-extract Bold's was less hostile environment, which is reflected both in the number of rescue events and the mean fitness of the rescued populations. This result might suggest that the environment more closely related to the original habitat of the focal species (soil) increases the chance of survival. However, this explanation is disputable due to the discrepancy between the expected results based on the pilot experiments, which showed lower population size of *C. reinhardtii* in a benign environment simulating a terrestrial habitat, and the results obtained after the selection experiment. The general features of Soil-extract Bold's (enriched medium, in comparison with Bold's, which is a minimal medium) is a more plausible explanation for the difference in survival, given the experimental evidences that this medium stimulates bacterial growth (Taylor, 1951), which indicates that similar effects might be expected for other microorganisms.

The mean fitness of the rescued populations with the history of exposure to a competitor was lower than that of the rescued populations propagated without a competitor. In addition, this result was consistent for all the groups of populations, except for the ones assembled with *C. sphaeroides*. This result can be interpreted as a trade-off between adaptation to the abiotic and biotic component of environment, as previously shown by Collins (2011).

Taken together, these results provide no evidence that evolutionary rescue is influenced by the ecological characteristics and phylogenetic relatedness of a competitor to the focal species. However, the results also indicate that the probability of evolutionary rescue might be altered by a different competitor species and suggest that the initial maladaptedness of a competitor species is potentially an important factor affecting the probability of survival. However, I cannot rule out the possibility that the effect of ecology and relatedness were confounded with the effect of the initial maladaptedness. Thus, additional experiments are necessary in which the initial maladaptedness of the competitors is controlled. Likewise, to test the generality of conclusions regarding the effects of the initial maladaptedness, a direct experimental manipulation of this factor is required.

5. Investigating which environmental factors select for the long-term maintenance of sex

5.1 Introduction

The great majority of extant eukaryotic species are sexual (Bell, 1982). Despite its ubiquity, sex still remains an unresolved challenge for evolutionary biology due to the lack of conclusive evidence that unequivocally could explain how this phenomenon is maintained by natural selection. This is why sex has been a subject of numerous theoretical and experimental studies for more than a century (Kondrashov, 1983).

The paradox of sex is reflected in the fact that it entails various types of costs that are disadvantageous for an individual and a population. For example, sex often results in a decrease of mean population fitness as result of a break-up of favourable combinations of alleles, termed recombination load (Barton and Charlesworth, 1998), as experimentally confirmed (for example: Greig et al., 1998; Colegrave et al., 2002; Kaltz and Bell, 2002; Becks and Agrawal, 2011). In addition to such genetic costs, sex can impose direct costs as well. For instance, sex is associated with meiosis, which is several orders of magnitudes slower than mitosis, resulting in diminishing of synthetic processes within a cell and temporarily arrested growth (Lewis, 1987). Sex requires a physical contact between organisms or their gametes, which generates other direct costs associated with several groups of risks. These include increased likelihood of transmission of pathogens and parasites between individuals or their gametes, or reduction of motility, which increases vulnerability to predation (Lewis, 1987). Moreover, sex entails the two-fold reduction of reproductive output per female as a cost of producing males, which has been termed “the two-fold cost of sex” or “the cost of males" (Maynard Smith, 1971; Williams, 1975; Maynard Smith, 1978).

Many hypotheses have been proposed as an explanation for the maintenance of sex by natural selection (Kondrashov, 1993). Most of them gravitate towards the idea that sex generates variation within a population available for selection and thus enhances adaptation to a changing environment (Weismann, 1889; Fisher, 1930; Muller, 1932; Felsenstein, 1974; Burt, 2000). While the initial concept dates back to Weismann (1889), the complete theoretical framework has been formalised by Fisher (1930) and Muller

(1932). The major argument of this concept is the occurrence of multiple beneficial mutations, independently arising in different individuals within a population, which could be assembled faster within a single individual by means of sex and recombination. As a result, natural selection would operate faster in sexual populations. In contrast, the fixation of beneficial mutations would proceed at slower rate (one at a time) in asexual populations, which would ultimately result in a competition of genotypes carrying different mutations (Muller, 1932), consequently slowing down the adaptation (the process termed clonal interference; Gerrish and Lenski, 1998).

The variation-selection hypotheses have been the subject of various types of experimental studies (Hartfield and Keightley, 2012), which provided evidence for the beneficial effect of sex in both novel and deteriorating environments. Malmberg (1977) manipulated the rate of recombination in populations of T4 phage and found that the rate of adaptation to the novel environment (proflavine) is directly proportional to the rate of recombination. Greig at al. (1998) directly competed sexually and asexually reproducing individuals in mixed populations of yeast in stressful conditions (elevated temperature). They found that sexual individuals with a heterozygous genetic background outcompeted the asexual individuals in great majority of mixed populations, despite the initial disadvantage caused by the recombination load. Kaltz and Bell (2002) showed that sexual populations of *C. reinhardtii* maintain consistently higher adaptive rate in comparison with asexual populations. In addition, they demonstrated this advantage was directly proportional to the degree of environmental complexity. Colegrave (2002) subjected the experimental populations of *C. reinhardtii* to novel growth medium and found that the relative fitness of sexual populations compared to asexual populations was directly proportional to the population size prior to induction of sex. This result suggests that sex reduces the constraint of adaptation imposed by the clonal interference in larger populations. Bell (2013) found that the populations of *C. reinhardtii* with the history of obligate sexual reproduction had higher probability of survival than asexual populations when subjected to growth in the absence of light. Lachapelle and Bell (2012) demonstrated that sexual populations of *C. reinhardtii* had lower extinction rate than that of asexual populations when subjected to a deteriorating environment.

These results demonstrate that sex is beneficial in changing environments, which is manifested through the enhanced adaptation rate of sexual populations. However, all

aforementioned studies provided evidence for the group (population) advantage of sex. If the costs of sex on the individual level (short-term disadvantage) outweigh the benefits, sex could be selected against despite the long-term advantage. Thus, in order to provide an explanation for the maintenance of sex, it is crucial to identify the environmental factors that provide a constant selective advantage for sex.

Several experimental studies investigated the factors which could potentially select for the long-term maintenance of sex. Morran et al. (2009) provided experimental evidence for increased level of outcrossing in the wild-type population of *Caenorhabditis elegans*, when subjected to elevated mutation rate and a novel environment (the presence of bacterial pathogen). A later study by Becks and Agrawal (2011) demonstrated that frequency of sex increases during adaptation to novel environment (elevated concentration of NaCl and novel food source) of monogont rotifer *Brachionus calyciflorus* (measured as proportion of fertilized mictic eggs). Furthermore, sexually derived offspring had higher fitness (measured as lifetime reproduction per female) than asexually produced genotypes, but this advantage was constrained during the early phase of adaptation. Most recently, Luijckx et al. (2017) manipulated a degree of complexity of environmental change, by exposing the populations of *Brachionus calyciflorus* to different combinations of stressful abiotic factors (increasing salinity and heavy metal concentration – $CuSO_4$, decreasing temperature). They found that the rate of sex proportionally increases with the increase of environmental change complexity.

These studies indicate that frequency of sex increases in the populations exposed to both novel environment (Morran et al., 2009; Becks and Agrawal, 2011) and deteriorating environment (Luijckx et al., 2017). However, several other factors that could potentially increase the propensity for sex within a population have still not been experimentally tested. I will discuss these factors in the following review.

The benefits of sex may include generation of phenotypes optimal in conditions of a changing biotic component of environment (the Red Queen dynamics in a co-evolving system of parasite-host or predator-prey) (Bell, 1982). As a result, we should expect the maintenance of sex in the populations under constant selective pressure imposed by the biotic component of the environment. Lively (1992) found a positive correlation between the frequency of males in the facultatively sexual species *Potamopyrgus antipodarum* with the presence of parasites (trematodes). Furthermore, there is evidence that coevolution

with pathogenic species (bacterium *Serratia marcescens*) selects for outcrossing in a facultatively sexual *Caenorhabditis elegans* (Morran et al., 2011). However, to the best of my knowledge, there is no experimental study testing the effect of the presence of a competitor species on the rate of sex in the focal species. In Chapter 3, I demonstrated the beneficial effect of sex on survival of the focal species in the presence of the competitor. It is still uncertain whether a competitor could select for the long-term maintenance of sex within a population of the focal species.

Deteriorating environments may increase the rate of sex, as previously shown (Luijckx et al., 2017). However, it has still not been experimentally tested whether the rate of environmental deterioration affects the rate of sex. In Chapter 2, I showed that the effect of sex on extinction dynamics of experimental populations of *C. reinhardtii* is altered by the rate of environmental deterioration. This is manifested through the increased survival rate of sexual populations relative to that of asexual populations with an increase of the rate of environmental deterioration. It remains yet to be investigated whether higher rate of environmental change could select for sex in a facultatively sexual species such as *C. reinhardtii*.

The direction of natural selection is likely to vary over the course of time in natural populations (Bell, 2010). Consequently, the haplotypes that have selective advantage in one environment may have lower fitness when conditions temporarily change. Fluctuating selection has been observed both in the field (for example, Gibbs and Grant, 1987; Grant and Grant, 1995) and experimental studies (for example, Hall at all, 2011). While there is evidence that sex is beneficial when fluctuating environmental change involves a biotic component (which corresponds to Red Queen dynamics), there is still the lack of experimental evidence for the benefits of sex in the fluctuating change of abiotic components of an environment.

In order to test which of the described environmental factors could select for the long-term maintenance of sex, I designed a selection experiment by creating the selective environments which simulate these factors (the presence of the competitor, different rates of environmental deterioration, fluctuating stressful environment). I subjected the facultatively sexual experimental populations of *Chlamydomonas reinhardtii* to these environments and monitored the change in the rate of sex over the course of time.

5.2 Materials and methods

5.2.1. Base populations of *C. reinhardtii*

Each experimental population of *C. reinhardtii* (120 in total) was constructed by assembling 10 isolates randomly chosen from the library of C. *reinhardtii* isolates, established for the selection experiment described in Chapter 2. Each population represents a unique combination of an equal number of isolates of both mating types (5mt+ and 5 mt-).

5.2.2. Selection experiment

Four sets of 24 experimental populations were allowed two growth cycles in 4 different selective environments (see the section 'Selective Environments' for details). The fifth set of 24 experimental populations was cultivated in a benign medium (Bold's broth medium; hereafter referred to as Bold's) as a control. After every second growth cycle, a sexual cycle was induced and completed in all populations. After completion of sexual cycle, the experimental populations of each set were allowed another two growth cycles in the corresponding selective environment, except for the populations subjected to the competition treatment, which were allowed two growth cycles in isolation and one growth cycle in the presence of the competitor (see the section 'Selective Environments' for details).

5.2.2.1. Selective Environments

The selective environment was defined by a type of change imposed on the experimental populations, resulting in the establishment of three treatments. In the first treatment, two equal sets of experimental populations were subjected to the constant directional change in salt concentration. One set of populations was subjected to relatively low rate of salt increase (1 g/l after each sexual cycle); the other set of populations was subjected to high rate of salt increase (3 g/l after each sexual cycle).

The second treatment group comprised the set of populations subjected to a fluctuating change of environment, by alternating two types of stressors after every sexual cycle (5 g/l of salt and 50 µM Copper (II) sulphate pentahydrate, $CuSO_4$ X 5 H_20). I aimed to select the fixed concentration of both stressors which will be perceived as stressful by experimental populations, without causing a substantial decline in population size which would have likely prevented a mating reaction. Hence, I selected the concentration of both stressors which reduce the maximal growth of *C. reinhardtii* by 50 %, as previously shown by Reynoso and de Gamboa (1982), Moser and Bell (2011) for salt, and Prasad et al., (1998) for Copper (II) sulphate.

The third treatment group comprised the set of populations propagated in a benign environment which were allowed one additional growth cycle in the presence of the competitor (*Chlamydomonas sphaeroides*) prior to induction of each sexual cycle. *C. sphaeroides* was chosen as the competitor species, because pilot experiments revealed that this species shows slower growth in benign environments compared to *C. reinhardtii* (both in liquid and solid medium). A species less competitive than *C. reinhardtii* was used in the experiment because a strong competitor could have considerably reduced the population size resulting in a low mating reaction or even eliminated *C. reinhardtii* from the mixed populations prior to commencing of the sexual cycle. Given that both species could not have been separated once assembled on agar plates (a procedure performed during sexual cycle, see 'Sexual cycle' section for details), it is likely that at least some proportion of *C. sphaeroides* populations were transferred to liquid selective media once the asexual growth cycles were resumed.

One set of the experimental populations was propagated in a benign environment (Bold's) until the completion of experiment and served as a control for the populations propagated in selective environments.

5.2.2.2 *Cultivation of Populations and Selection Experiment Procedure*

All the experimental populations of *C. reinhardtii* were cultivated in 24-well plates in Bold's medium under standard conditions (26˚C, 6000 lux illumination, shaking at 180 rpm and covered with sterile breathable membranes to prevent cross-contamination and uneven evaporation across the plates). All the populations were allowed two growth cycles

(3-4 days) in the corresponding selective medium by transferring 5 % of each population to the fresh medium after each growth cycle. Each population subjected to the competitor prior to induction of the sexual cycle was mixed with an inoculum of *C. sphaeroides* containing an equal number of cells as mean population size of the whole population set. The population size of each *C. reinhardtii* population was estimated spectrophotometrically (OD_{750}). The average OD_{750} of the treatment group was converted to average cell number using the equation obtained from an OD_{750} - cell number curve calibration, described in Chapter 4 (see Section 4.2.5 for details). The average cell number of *C. sphaeroides* population was estimated with a haemocytometer.

5.2.2.3. Sexual cycle

After every second growth cycle, a sexual cycle was induced by using the identical experimental procedure as in Chapter 2 and Chapter 3 (see 'Materials and Methods' section of any of these chapters for details). The differences in the experimental procedure included resuspending the experimental populations in two times lower volume of nitrogen-free medium (0.5 ml per culture, instead of 1 ml used for previous experiments) to stimulate the contact of gametes by increasing the cell density. The experimental procedure of freezing the cultures in liquid medium was omitted, to avoid direct selection on forming zygotes and consequently for sex. As an alternative procedure, I transferred the whole populations (0.4 ml) to agar plates, which included both zygotes and unmated gametes.

5.2.3. Assaying the rate of sex

After initiating the sexual cycle, all the populations were sampled and fixed on microscope slides. The images of each culture sample were used for estimation of the number of zygotes and unmated gametes for each population. The ratio of zygote number to the number of unmated gametes per population of each treatment group per time point (after each sexual cycle) was considered an estimate of rate of sex.

5.2.3.1. Fixation of cultures on microscope slides

The populations re-suspended in nitrogen-free medium were incubated under bright light for four hours, to allow for gametogenesis and mating. The chosen period of four hours is a minimal interval of time required for a differentiation of gametes which acquire the mating competence (Abe et al., 2004; Lin and Goodenough, 2007). After given interval of time, each population was sampled by pipetting (20% of a culture - 100 µl) and incubated in Lugol's Iodine solution (Scientific Laboratory Supplies) for 10 minutes to kill cells. In addition, this solution acts as a preservative, helps fixation on microscope slides and easier visualisation under microscope. The cultures were then centrifuged at 5000 rpm (minicentrifuge) for 5 minutes, and re-suspended in 100 µl of sterile double distilled water. A sample of each culture (50 µl) was then plated on the corresponding microscope slide (Poly-L-Lysine coated glass slides; 25 x 75 mm; Sigma-Aldrich) and dried in sterile conditions for approximately one hour. After given period, the cultures fixed on microscope slides were enclosed with cover slips (22 X 22 mm; Scientific Laboratory Supplies) by adding approximately 10 µl of Mounting medium (Sigma-Aldrich) and dried for at least one hour.

5.2.3.2. Bright field microscopy and image analysis

The cultures were observed on a ZEISS Axio Imager.Z1 microscope (Carl Zeiss MicroImaging) with ZEISS α Plan-Apochromat 100x 1.46 objective (oil immersion). For each culture, five images were taken by random selection of five microscope fields of view. The images were taken with a Hamamatsu digital CMOS Orca-Flash 4.0 camera and controlled by the Micro-Manage 1.4.23 software (the average exposition time of 500 ms). Image analysis was performed using ImageJ software, in the following way. Twenty zygotes with clear morphological distinction from unmated gametes were selected (a single zygote per treatment group per each sexual cycle; see Figures 5.1 – 5.5). The pixel area of each zygote was calculated to obtain the threshold parameters used for differentiation of zygotes from unmated gametes (an average pixel area, 46731; minimal pixel area, 29424; maximal pixel area 64038; the resolution of each image, 2048 X 1536 pixels). The images were visually inspected by manually measuring the parameter of all the cells by using the 'Freehand selections' tool. The estimated parameter was used for the software calculation of the pixel area of each cell. The cells which pixel area corresponded to the values below the minimal

zygote pixel area (29424) were considered unmated gametes; all the cells above that threshold were considered zygotes. Five populations per treatment were selected for cell counting, carried out for the samples taken after the initial and the last (fourth) sexual cycle. The rationale for the small population set used for cell counting was the technical difficulty in quantifying the number of cells for all of the populations for each time point, due to considerable clumping or multiple-layer aggregation of gametes, which was frequently observed during imaging.

The frequency of sex of each treatment group per time point (sexual cycle) was estimated by calculating the ratio of zygotes: unmated gametes. I did not choose the absolute number of zygotes as a measure of frequency of sex, given that cultivation in different environments directly affects the cell density, which in turn influences the number of zygotes produced.

During the subsequent analysis of the images, I had technical difficulties in distinguishing between *C. reinhardtii* and *C. sphaeroides*, which do not differ in any recognizable morphological feature. Given that the number of zygotes produced could have been confounded with the relative frequency of both species, I concluded that I cannot estimate the frequency of sex in a reliable way for this treatment group. Hence, I withdrew the competition treatment group from the statistical analyses.

5.3 Data analysis

The effects of treatment (each type of selective environment) on the frequency of sex was analysed as a change in proportion of produced zygotes (relative to the cell density of the whole population) between the initial and the last sexual cycle. The results were analysed by fitting one-Way ANOVA. 'Treatment' was considered a fixed factor with five levels, each level representing a different treatment group. The continuous responsive variable in the model was 'change in sex frequency'.

All the analyses have been performed using R (R Core Team, 2017).

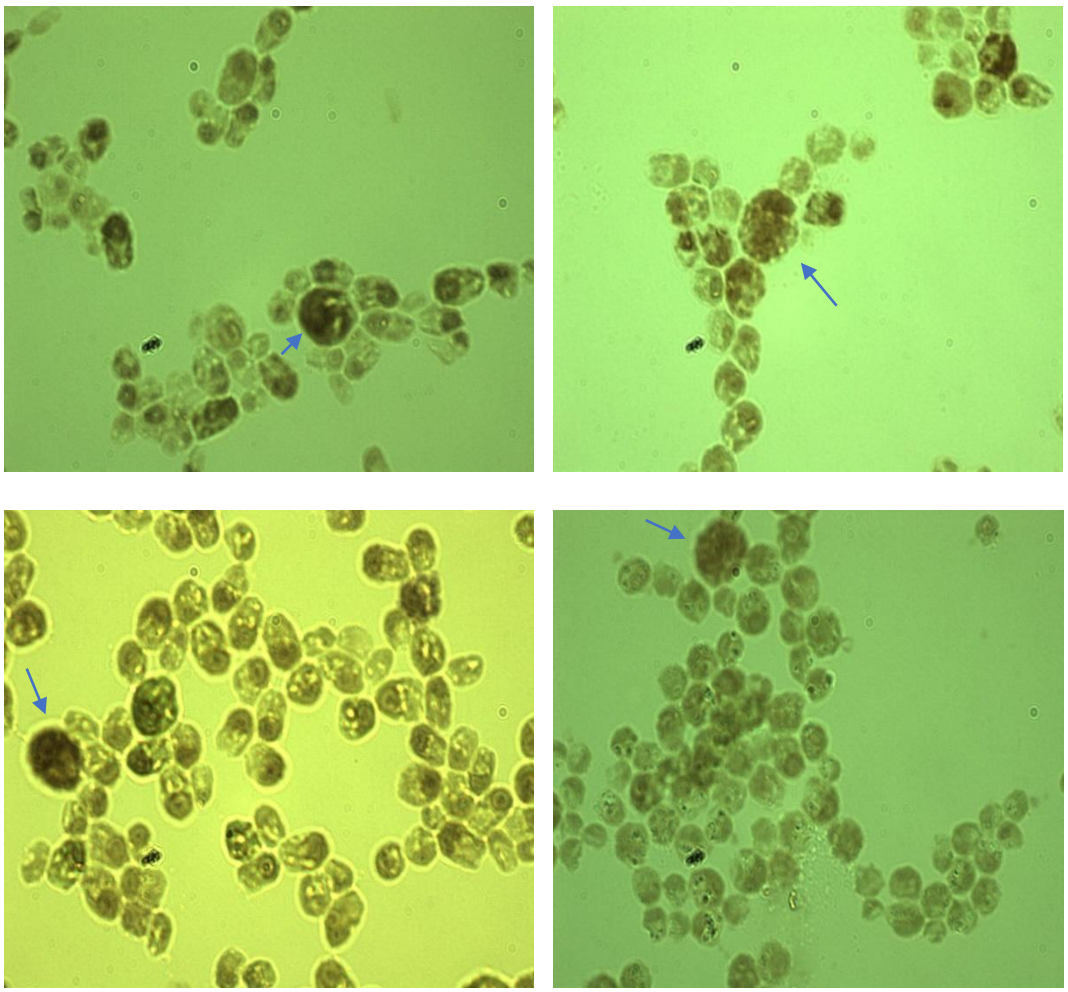

Figure 5.1 – Images of four different control populations chosen due to the presence of distinct zygote(s) and selected after one of the four sexual cycles; top left – 1st sexual cycle; top right – 2nd sexual cycle; bottom left – 3rd sexual cycle; bottom right – 4th sexual cycle. The zygotes chosen for the estimation of the average zygote pixel area are marked with an arrow.

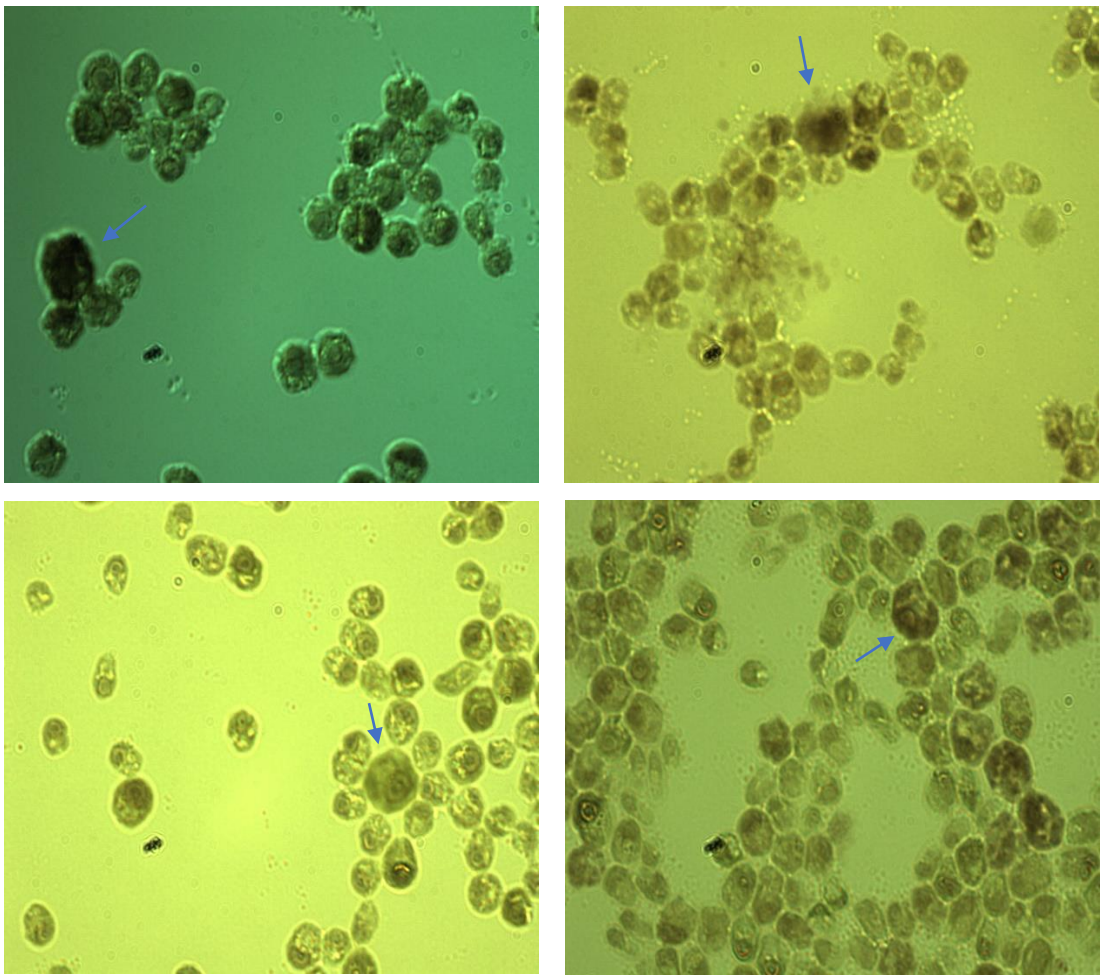

Figure 5.2 – Images of four different populations subjected to the fluctuating environment chosen due to the presence of distinct zygote(s) and selected after one of the four sexual cycles; top left – 1st sexual cycle; top right – 2nd sexual cycle; bottom left – 3rd sexual cycle; bottom right – 4th sexual cycle. The zygotes chosen for the estimation of the average zygote pixel area are marked with an arrow.

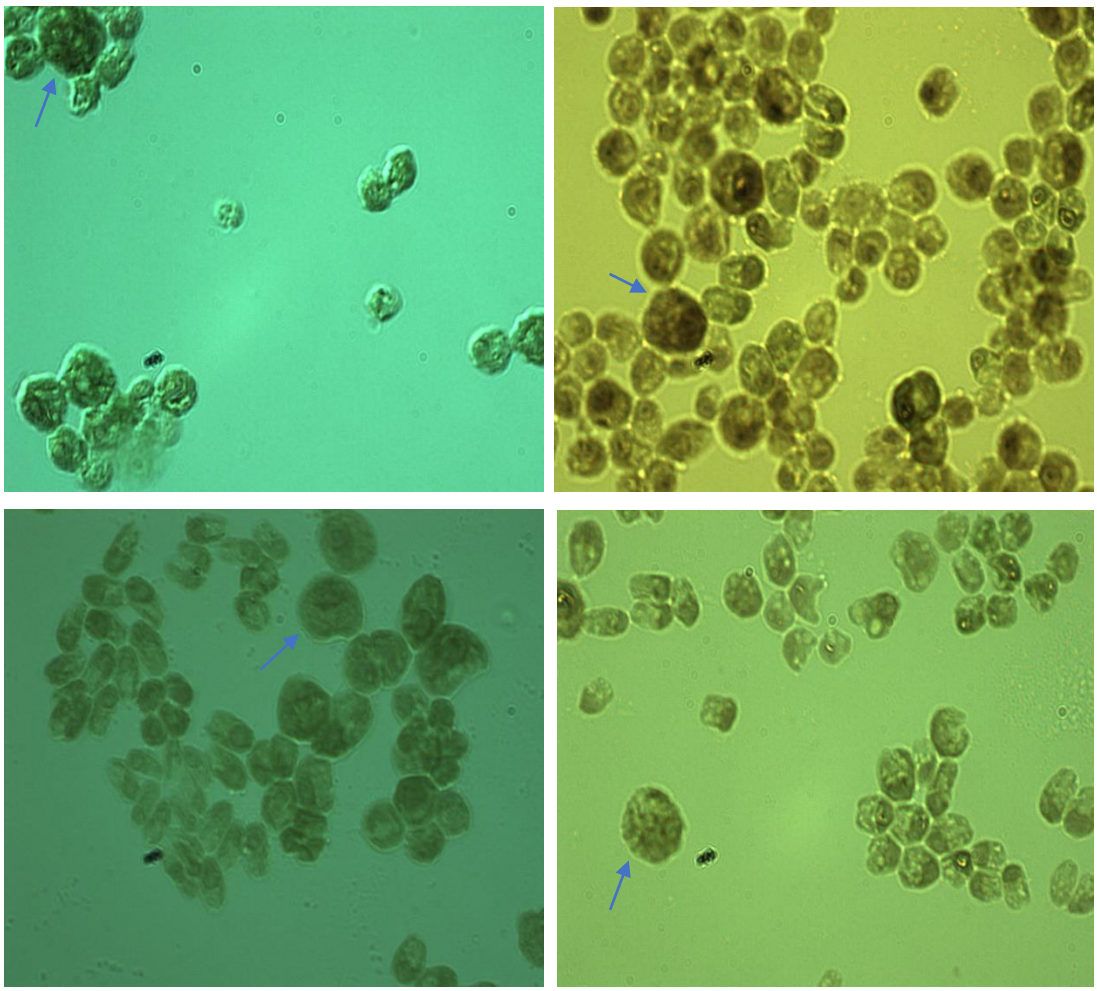

Figure 5.3 – Images of four different populations subjected to a directional environmental change (high rate) chosen due to the presence of distinct zygote(s) and selected after one of the four sexual cycles; top left – 1st sexual cycle; top right – 2nd sexual cycle; bottom left – 3rd sexual cycle; bottom right – 4th sexual cycle. The zygotes chosen for the estimation of the average zygote pixel area are marked with an arrow.

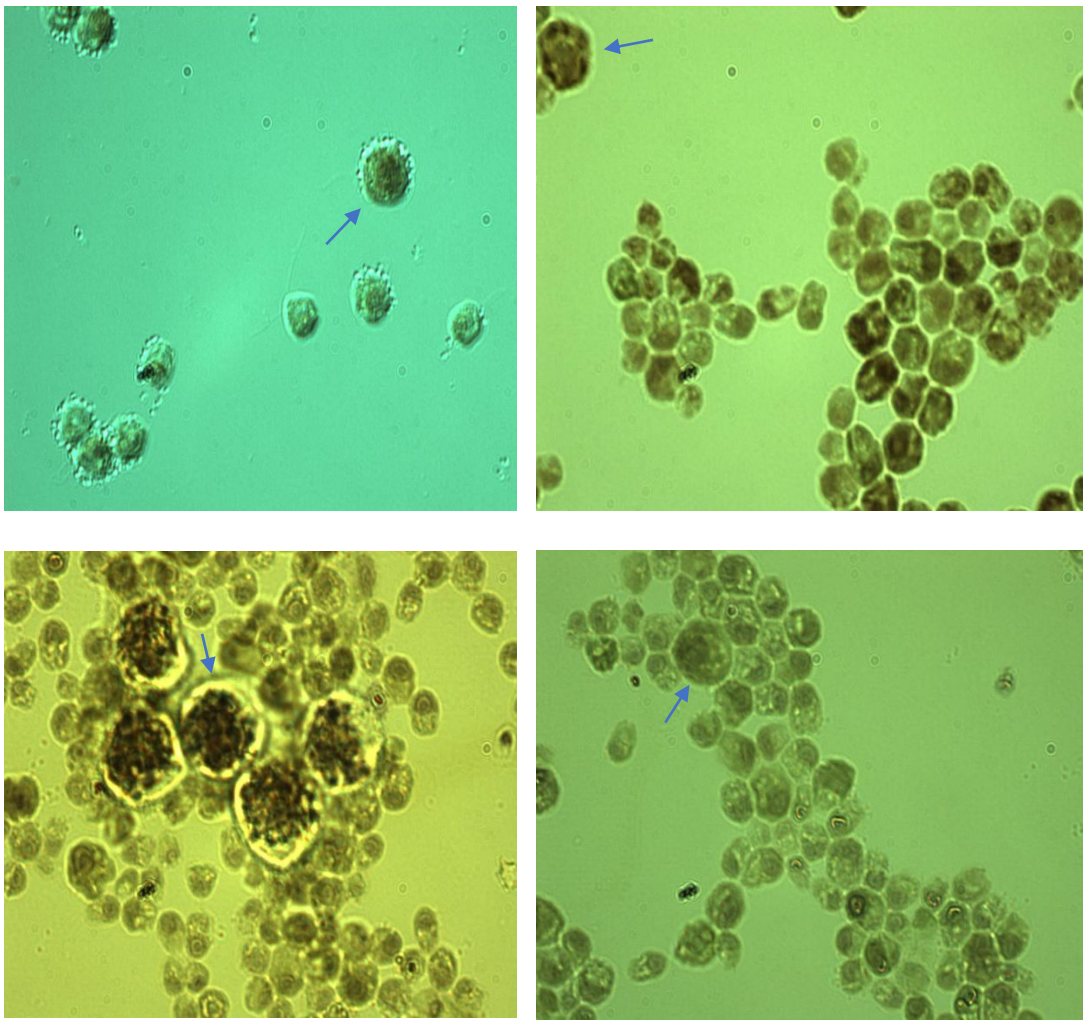

Figure 5.4 – Images of four different populations subjected to a directional environmental change (low rate) chosen due to the presence of distinct zygote(s) and selected after one of the four sexual cycles; top left – 1st sexual cycle; top right – 2nd sexual cycle; bottom left – 3rd sexual cycle; bottom right – 4th sexual cycle. The zygotes chosen for the estimation of the average zygote pixel area are marked with an arrow.

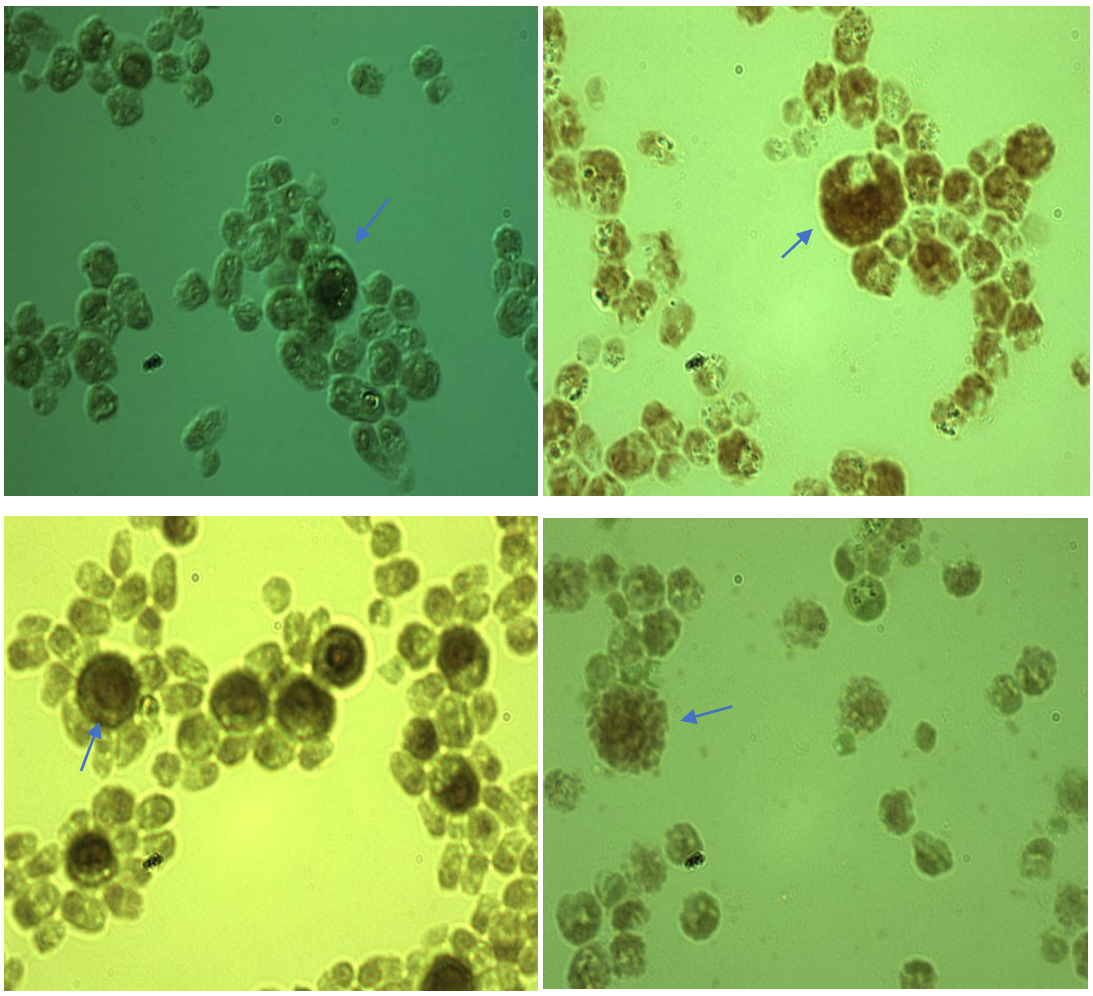

Figure 5.5 – Images of four different populations subjected to the presence of the competitor prior to induction of each sexual cycle, chosen due to the presence of distinct zygote(s) and selected after one of the four sexual cycles; top left – 1st sexual cycle; top right – 2nd sexual cycle; bottom left – 3rd sexual cycle; bottom right – 4th sexual cycle. The zygotes chosen for the estimation of the average zygote pixel area are marked with an arrow.

5.4 Results

The factor 'treatment' affected the change in frequency of sex (one-way ANOVA; $F_{3,19} = 4.58$; P = 0.017). The significant difference was detected between the levels 'control' (benign environment) and 'fluctuating environment' (t = -2.66, df = 9, P = 0.02) (Figure 5.6). The average ratio of zygotes to unmated gametes decreased for treatment group subjected to fluctuating environmental change. No significant differences between other levels of the factor 'treatment' were detected.

The total number of cells of a population sample served as an estimate of the cell density of the whole population, used to estimate the change in average population density per each treatment group. For all treatment groups, the population density increased between the initial and the last sexual cycle (Figure 5.7). However, the change in average cell density was only marginally contingent on 'treatment' factor (one-way ANOVA; $F_{3,19} = 2.79$; P = 0.07).

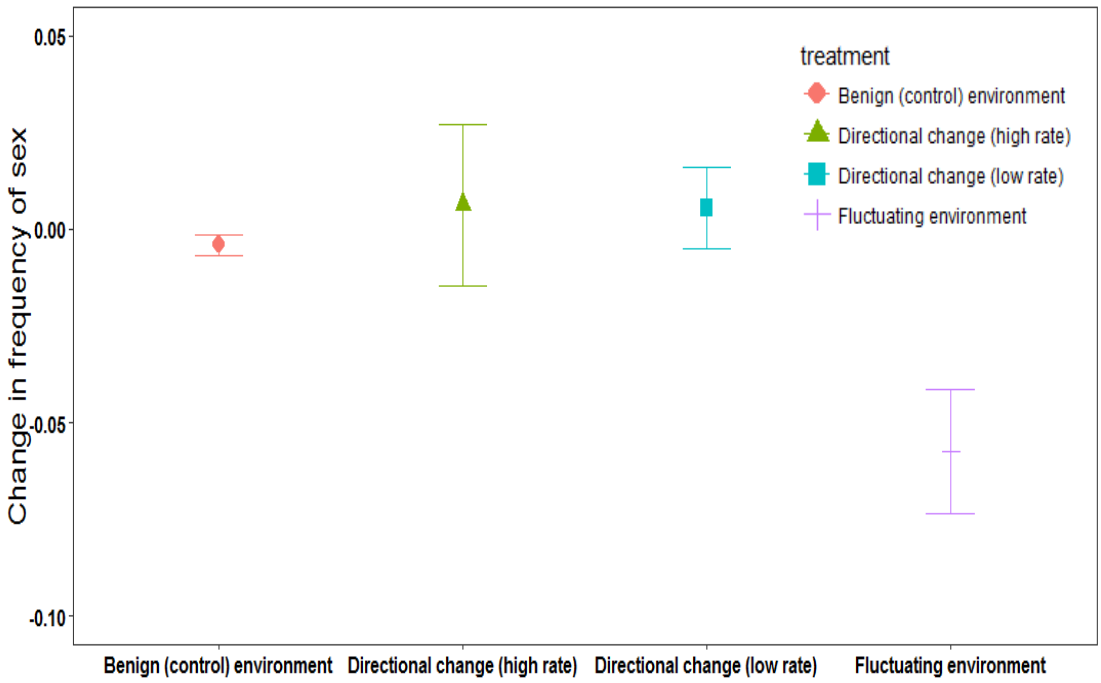

Figure 5.6 – The average change in frequency of sex for all treatment groups, measured as a difference in ratio of zygotes to unmated gametes between the initial and the last sexual cycle; the bars represent standard error of the mean.

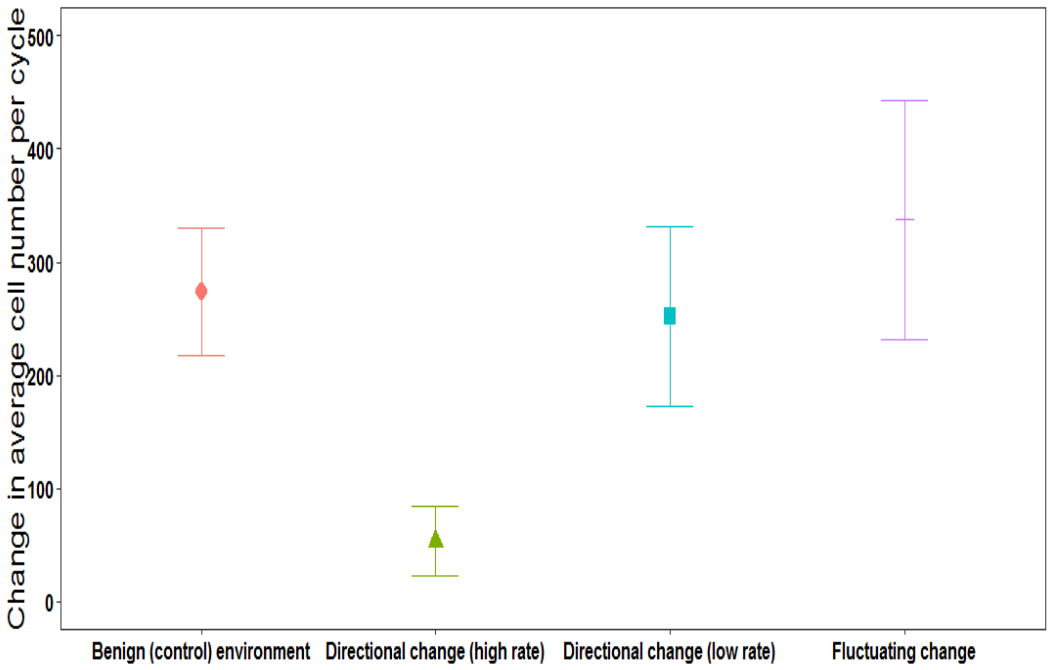

Figure 5.7 – The average change in total number of cells (both zygotes and unmated gametes) for all treatment groups between the initial and the last sexual cycle; the bars represent standard error of the mean.

5.5 Discussion

Out of four selective environments tested, only a single environment significantly affected the change in rate of sex, measured with respect to the initial sexual cycle. The fluctuating environment selected against sex (a twelve-fold decrease in frequency of sex relative to Grand mean of all treatment groups). This cannot be attributed to the negative effect of stress on the population size which could have reduced the total amount of zygotes. The results indicate the opposite pattern, reflected in the increase of average population size of this treatment group compared to the initial sexual cycle. A possible explanation for the reduced frequency of sex might be an interference between selection acting on traits that could directly mitigate the negative effect of stress (for example, smaller cells might be favoured because of comparatively smaller surface area exposed to a stressor) and maintenance for the rate of sex. However, this result should be taken with caution, given the error in the experimental design which lacks the appropriate control for the fluctuating environment. The adequate control would correspond to simpler environments with a single type of stressor (either elevated salt concentration or copper (II)

sulphate). Consequently, it cannot be precisely determined whether the observed effects of sex are due to the treatment itself (alternation of two stressors) or the effect of one particular stressor.

Despite the increase in average cell density compared to the initial sexual cycle, the treatment groups subjected to both types of deteriorating environments did not show significant change in zygote: unmated gametes ratio. Unlike in the group subjected to the fluctuating change, the rate of sex remained relatively constant, which may indicate that the cost of sex was lower than in the group subjected to the fluctuating environment, but high enough to hamper the increase in ratio of zygotes to unmated gametes. Furthermore, the production of gametes may have ceased once the magnitude of stress reached a high value. Namely, the directional change high-rate populations had been subjected to 12 g/l of salt concentration (sub-lethal stress) prior to induction of the last sexual cycle, which may have affected the total amount of gametes produced or kinetics of gametogenesis. The results of Chapter 2 suggest that beneficial effect of sex will increase in higher rates of environmental change. Based on these results, we might predict an increase in frequency in the rate of sex under such mode of environmental change. However, the results described here do not provide a support for this prediction. Nevertheless, populations under high rate of directional change show higher variance in zygote: unmated gametes ratio. Based on this, we might argue that there is a higher genetic variation for the propensity of sex selection can act on in these populations. Potentially, selection would have increased zygote: unmated gametes ratio had the experiment continued after the fourth sexual cycle.

A non-significant change in the ratio of zygotes to unmated gametes in a benign environment is not unexpected. In the study of Becks and Agrawal (2011), the rate of sex of *Brachionus calyciflorus* declined after adaptation to novel environment plateaued. Renaut et al. (2006) did not detect the difference between mean fitness of the sexual and asexual populations of *Chlamydomonas reinhardtii* when cultivated in a benign environment. These results suggest that once a population becomes adapted (in the absence of a continuous environmental change), sex provides no further advantage and it will likely be selected against (the frequency of sex will decline).

The results of this experiment indicate that at least some stressful environments may affect the frequency of sex. However, this experiment had a limited power to detect any significant differences between treatment groups, given that only 5 populations (20%

of total number of populations) could have been used for the analysis. Hence, the results may not reflect the real patterns in nature and further evidence is required.

6. General discussion and future directions

The aim of this Thesis was to investigate the impact of mode of reproduction and competitive interactions on the probability of evolutionary rescue, with a goal to contribute to our previous understanding of adaptation of organisms to global change.

6.1 The effects of sex

Previous empirical work has shown an advantage of sex for populations adapting to novel environment. The results presented in this Thesis indicate that the advantage of sexual populations, relative to asexual populations, depends on the context of environmental change. In environments deteriorating in a simple way, the effect of sex was contingent on the rate of environmental change. Sex was beneficial only when environment deteriorated in an intermediate way, but not under a gradual or high rate of change. Asexual populations can track the environmental change equally efficiently as sexual populations when environment deteriorates gradually, hence the relative advantage of sex diminishes. On the other hand, the high rate of environmental deterioration may be highly detrimental for all the populations, which will result in a significant lag behind the moving optimum for all the populations, irrespective of mode of reproduction, and consequently, an equal probability of extinction. In Chapter 2, exposure to the gradual and high rate of environmental deterioration resulted in similar extinction dynamics of all treatment groups, irrespective of mode of reproduction, despite the initial advantage of sexual groups which maintained higher average population size during the initial phase of experiment (sublethal level of stress).

However, under moderate strength of selection, sex may be the most efficient strategy. In Chapter 2, obligate sexual populations had clear advantage relative to both facultative sexual and asexual populations, manifested through the lowest population size decline and the slowest extinction rate. The possible underlying explanation is that sexual populations likely suffer lower demographic costs of adaptation than in the high rate of environmental change and asexual populations keep up with the environmental change less efficiently than in the gradual rate of change, hence the benefit of sex is maximal.

However, sex may be beneficial even under the higher rates of environmental change, if the change does not continue indefinitely. This was demonstrated in Chapter 3, which considered even the higher rate of environmental change (5 g/l after each sexual cycle) than in the experiment described in Chapter 2 (3 g/l after each sexual cycle for the high rate treatment groups), with the main difference in lower total number of steps of change. Furthermore, sex conferred the advantage, reflected in higher probability of evolutionary rescue and larger population size, to even higher final magnitude of stress (15 g/l of salt concentration) than in Chapter 2. Moreover, this advantage is maintained in higher complexity of environmental deterioration, which adds to previous finding by Kaltz and Bell (2002) that sex is more advantageous in more complex novel environments.

In Chapter 3, obligate sexual mode of reproduction conferred the advantage by significantly increasing the probability of evolutionary rescue relative to asexual mode of reproduction (by 25%). Contrary to this result, I found no evidence of such advantage of obligate sexual populations in Chapter 2, despite the marginal advantage in the number of rescue events relative to both asexual (by 7%) and facultative sexual groups (by 9%). The discrepancy in the results of these chapters requires an additional explanation. In Chapter 3, the rescued populations were subjected to three rounds of salt increase. In contrast, the great majority of the populations rescued during the selection experiment described in Chapter 2 were subjected to the environmental deterioration occurring via more rounds of salt increase (between 4 and 17). Consequently, the longer interval of environmental stasis may have reduced the population size decline of the populations in Chapter 3, which may have increased the likelihood of rise of beneficial mutations and thus, evolutionary rescue. In contrast, prolonged environmental deterioration in Chapter 2 may have imposed the constant lag load to the adapting populations, which increased the probability of extinction.

Taken together, the results of this Thesis corroborate the results of previous studies - the advantageous effect of sex in a changing environment. The new findings described here expand the range of beneficial effects of sex. In sub-lethal level of stress, sexual populations will be fitter than asexual populations regardless of the rate of change. If the environmental change occurs in a complex manner, sex will remain the most efficient strategy. However, this advantage will be limited if a change reaches the lethal level, and the effect of sex on the probability of survival will likely be influenced by the strength of selection.

In nature, many organisms are characterized by obligate sexual reproduction (for example birds and mammals). The fact that the species used in the experiments described here mainly reproduces asexually, with intermittent sexual episodes, raises a logical question how common the observed patterns of adaptation in natural conditions are. Obligate sexual organisms tend to have longer generation time and thus slower reproductive output. Hence, without sufficient standing genetic variation, we could predict slower adaptive rate and potentially higher extinction risk for these species. Sex could increase the response of selection through well understood mechanisms, such as faster assembly of beneficial mutations, clearance of deleterious mutations and the release of mutations from detrimental genetic background, thus prolonging a population persistence, if population density drops under a threshold of critical low population size. However, given that primary response to a long-term environmental change involves adaptation through *de novo* beneficial mutations, which are unlikely to arise fast enough in slower reproducing organisms, we could predict relatively modest long-term effects of sex, and phenotypic plasticity (for example migration or behavioural change) as the main adaptive response of a population. Hence, the applicability of the results of this Thesis will be the most relevant for fast reproducing, small organisms, with larger population size and sufficient beneficial mutation supply rate (e.g. microorganisms).

6.2 The effect of competition

Competition may negatively affect the probability of evolutionary rescue, as demonstrated in the experiments described in Chapter 3 and Chapter 4, which is consistent with evidence of negative impact of competition on extinction in benign environments (Bengtsson 1993; Bengtsson and Milbrink, 1995) and the results of evolutionary experiments demonstrating the negative impact of competition on adaptive rate (Collins, 2011). However, the general applicability of this result in natural conditions can be questioned. The reason reflects in the fact that negative effects of competition were mainly attributable to the presence of a single species (*C. moewusii*), being a strong competitor probably due to higher ability to tolerate the stressor chosen for the selection experiment. I detected the significant effect of the identity of the competitor on the probability of evolutionary rescue. The other nine competitors have produced neutral, positive or

negative effects. Despite the fact that their impact was not conventionally significant, this result demonstrates that the effects of competition may be altered, depending on the competitor.

I found no significant impact of either phylogenetic relatedness of a competitor to the focal species or ecological similarities between competitors to the probability of evolutionary recue, which is consistent with results of the group of experimental studies which indicate that these factors do not predict the outcome of competition (for example, Naughton et al., 2015). This result should be interpreted cautiously, given the potential confounding effect of the initial maladaptedness of the competitors to the elevated salt concentration. To investigate the general applicability of this result in nature, further experiments are required (potentially with a similar design as employed for the experiment described in Chapter 4), with control of the initial maladaptedness of the competitors. This could be achieved through selection of the stressor each competitor will be equally sensitive to. Furthermore, the initial maladaptedness of competitors could be experimentally manipulated, to test whether the probability of evolutionary rescue is altered by differences in initial maladaptedness of competitors. Other potential approaches to the research of this topic may include: allowing competition in benign conditions which precedes the environmental deterioration, or manipulating the degree of complexity of competitive interaction, by allowing multiple-species competition.

6.3 Concluding remarks

In a globally changing environment, sex is likely to be the important factor mitigating the negative effect of stress and prolonging survival of declining populations, though this effect will depend on the mode of environmental change. This suggests that mode of reproduction will be the important factor determining the probability of survival and should be considered during the assessment of extinction probability of extant species. However, the rate and complexity of environmental change remain the predominant factors of survival of extant populations. Whether the current environmental change will proceed in a pace the organisms will be able to keep up with - yet remains to be seen.

7. References

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