Experimental Evolution of Herbicide Resistance in *Chlamydomonas reinhardtii*

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DECLARATION

The material presented in this thesis has resulted from the author's individual contribution. The material presented in Chapter 4 has been previously published (Lagator et al. 2012).

SUMMARY

Our understanding of the evolutionary dynamics of selection for herbicide resistance is limited by the time and space required to conduct meaningful selection experiments in higher plants. This constrains the study of the dynamics of resistance evolution predominantly to mathematical models. The primary goal of this thesis was to overcome these limitations, and to study the evolutionary phenomena underpinning several management strategies. To do so, a series of experimental evolution studies were conducted using *Chlamydomonas reinhardtii*, a single-cell green chlorophyte susceptible to a range of commercial herbicides. In particular, this thesis explored the impact of herbicide sequences, rotations and mixtures, as well the impact of herbicide dose, on evolution of resistance. Applying herbicides in sequence allowed the study of the impact of environmental perturbation on the dynamics of resistance and the associated fitness costs, finding more rapid selection for resistance to a second and third mode of action in some populations. Cycling between herbicides creates conditions of temporal environmental heterogeneity, the outcomes of which are not easily predictable as resistance was slowed down in some cycling regimes, while in others it accelerated the evolution of resistance or gave rise to cross-resistance. Herbicide mixtures are a management strategy relying on increases in environmental complexity to provide better control of resistance. The results presented show that mixtures were effective at slowing the evolution of resistance when all mixture components were used at fully effective doses, while low doses of mixtures accelerated resistance evolution and led to more cross-resistance. Finally, modifications of the applied herbicide dose allowed the study of local adaptation along an environmental gradient, where the differences in outcomes based on the specific herbicides used were again evident. Overall, the work presented here uses applied scenarios to study the underlying evolutionary phenomena, in order to feed back into the applied thinking.

CHAPTER 1: INTRODUCTION

1.1 PEST CONTROL IN AGRICULTURE

1.1.1 From the Invention to the Present Day

For over 10,000 years, agriculture has been contributing to the exponential growth of the human population, facilitating the spread and development of human civilization (Pringle 1998). Throughout this time agricultural pests have caused losses to crop yields, but in spite of centuries of utilization of diverse methods of pest control (Harris 1841), it was the development of synthetic pesticides (along with fertilizers) that led to an agricultural revolution (Smith & Kennedy 2002). The commercialization of the first synthetic plant hormone analogue 2,4-diclorophenoxyacetic acid (2,4-D) in the 1940s (Quastel 1950) followed and led to the birth of the 'herbicide age' (Smith & Kennedy 2002), transforming crop protection and pest management practices (Zimdahl 2007) (Table 1).

Table 1. **The Evolution of weed control methods in the United States** (taken from Zimdahl, 2007: *Fundamentals of Weed Science*, publisher: Academic Press, MA)

TABLE 12.1. The Evolution of Weed Control Methods in the United States (Alder et al., 1977).

	% Control by year in US			
Year	Human energy	Animal energy	Mechanical energy (Tractor)	Chemical energy
1920	40	60		
1947	20	10	70	
1975	5	TR[a]	40	55
1990	<1	TR	24	75

[a] tr = trace.

Novel herbicides were being developed rapidly, and today there are 16 herbicide families, distinguished by their mode of action, with over 300 registered active chemicals (Heap 2012). The constant increase in the number of commercially available herbicides is somewhat misleading. Even though novel active chemicals are being discovered regularly, no new mode of action has been found in over 20 years (Ruegg et al. 2007).

1.1.2 The Importance of Pest Control

Pests can reduce crop productivity by competition for resources (weeds), herbivory (invertebrates) or pathogenic activity (microbes, viruses and fungi) (Boote et al. 1983). Losses imposed by agricultural pests can be dramatic, causing as much as 90% reduction of potential crop yield (Oerke 2006). They threaten not only crop productivity and farmer's incomes, but also the overall food supply and regional economies (Zadoks & Schein 1979).

Crop protection refers to management practices that have as their goal the reduction in pest-induced crop losses. These practices include mechanical, cultural, chemical and biological approaches. Throughout most of history, mechanical methods of protection, such as hand pulling, mowing and hoeing were predominant, and still play a crucial role, particularly in weed management (Mohler 2002). Cultural methods (crop rotation, crop row spacing etc.) attempt to exploit specific pest-host associations, as well as the seasonal variations in pest numbers to increase yield (Rajendran 2002). Chemical control relies on a range of chemicals that target specific groups of pests (insecticides for insects, fungicides for pathogens and herbicides for weedy plants) and act to reduce their numbers in the field. Biological control aims to introduce organisms that are natural predators or pathogens of the pest species (Hokkanen 2002), and it comes with highest potential benefits, but greatest risks (Lynch & Hokkanen 1995). Overall, crop protection has a tremendous impact on productivity (Figure 1).

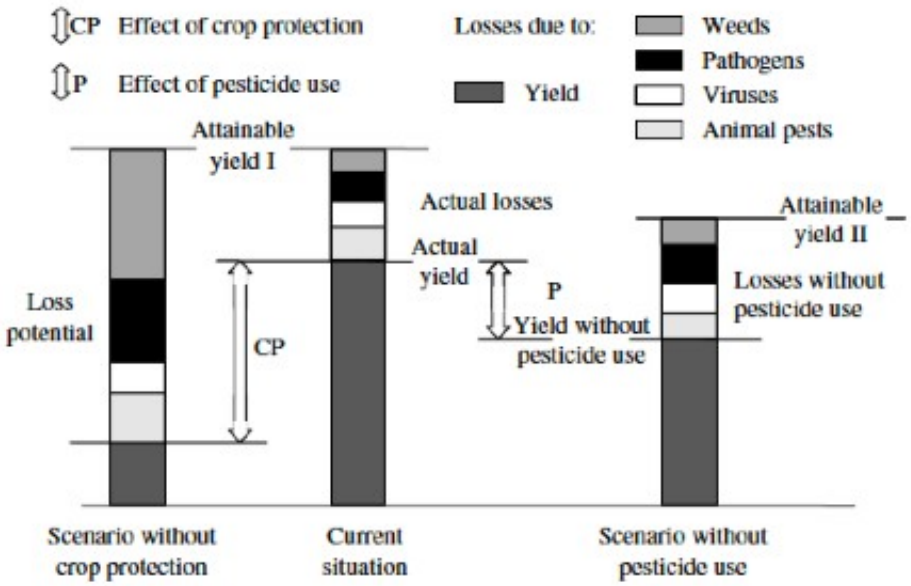


Figure 1. **Crop loses and yield levels.** The bars represent the percentage of the attainable yield lost due to various pests under three scenarios – no crop-protection, the current situation, and the projected scenario without pesticide use. Use of pesticides increases the attainable yield. Taken from (Oerke 2006).

1.1.3 Chemical Methods of Pest Control

Chemical methods of crop protection are the predominant type of pest control today (Lomborg 2001). The relative ease of use and production coupled with a generally high level of control have led to their widespread use, and to development of a vast number of individual molecules. Active molecules are classed according to their mode of action – the manner in which they affect the molecular and cellular mechanisms of the target organism (Smith & Kennedy 2002). Herbicides are broadly classed into ten groups, some with further distinctions into more specific mode of actions: (i) auxin growth regulators; (ii) aromatic amino acid inhibitors; (iii) branched-chain amino acid inhibitors; (iv) chlorophyll pigment inhibitors; (v) meristem destroyers; (vi) cell-membrane disruptors; (vii) inhibitors of photosynthesis; (viii) cell division inhibitors; (ix) root inhibitors; and (x) shoot inhibitors (Heap 2012).

There are many benefits of pesticide use. In terms of crop yield, figure 1 illustrates the differences in the attainable yield with and without pesticide use (Oerke 2006). The economic return on pesticide use has been estimated to be four times the original investment (Peshin 2002). Economically, these benefits have driven the global pesticide industry to be valued at over 30 billion US dollars in 2007 (McDougall 2007). The consequences of pesticide use on the environment and health are less well understood. Their use can lead to the destruction of local environments and natural species (Pimentel & Grainer 1997) and the cumulative cost of negative effects of pesticide use has been estimated at over $8 billion (Peshin 2002). The impact that pesticides can have on the environment, health and quality of life remains under active debate (Cooper & Dobson 2007). Nevertheless, pesticides remain the most widely used method of crop protection and their effective use is essential in establishing future global food security (Parliamentary Office of Science and Technology 2009). So what poses a threat to their effectiveness?

1.2 EVOLUTION OF HERBICIDE RESISTANCE

1.2.1 Defining Herbicide Resistance

The Weed Science Society of America defines herbicide resistance as the "acquired ability of a weed population to survive a herbicide application that previously was known to control the population" (WSSoA 2012). Herbicide resistance should therefore be studied as an evolutionary phenomenon, the definition and characteristics of which are relative to and dependent on the dose of the herbicide applied (Gressel 2009).

1.2.2 History of Herbicide Resistance and the Magnitude of the Problem

The potential for the evolution of resistance to herbicides was hypothesized soon after their introduction (Harper 1956), with the first resistant weed population being found in Canada in 1968 (Ryan 1970). With time, our awareness of the global spread of herbicide resistance and its potential economic and agricultural impact has grown (Duke 1996). In spite of this, it was the emergence of glyphosate resistance, thought to be resistance-proof due to the high fitness costs associated with modifications of the target enzyme (Bradshaw et al. 1997) that concluded a shift in focus from attempts to completely prevent, to understanding how the evolution of resistance could be slowed down (Powles 2008).

Since the commercial introduction of herbicides in the 1940's, resistance has been reported to an increasing number of modes of action, in a rising number of species, with resistance populations covering a growing portion of arable land (Heap 2012) (Fig.2). Further, resistance to one herbicide usually provides resistance to all those that share its mode of action (Gressel 2002). Because of this, and the fact that no new mode of action has been identified in over 20 years (Ruegg et al. 2007), it is becoming apparent that herbicides are a limited resource. The global distribution of resistant populations mirrors the proportional use of herbicides across the globe, suggesting that a major factor in determining where and when resistance will evolve is the frequency and extent of exposure (Fig.3). To ensure future effectiveness of herbicides, and through that enable their contribution to global food security, measures need to be taken to limit the evolution and contain the spread of resistance.

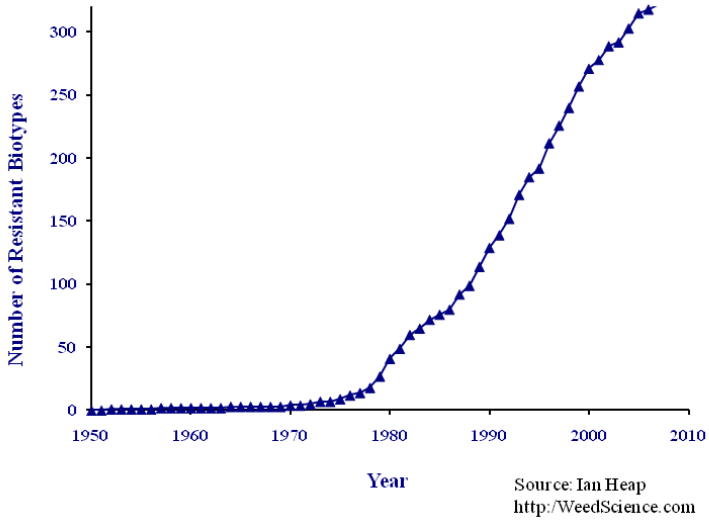


Figure 2. **Number of known resistant populations through time** (taken from Heap, 2012: The International Survey of Herbicide Resistant Weeds, online)

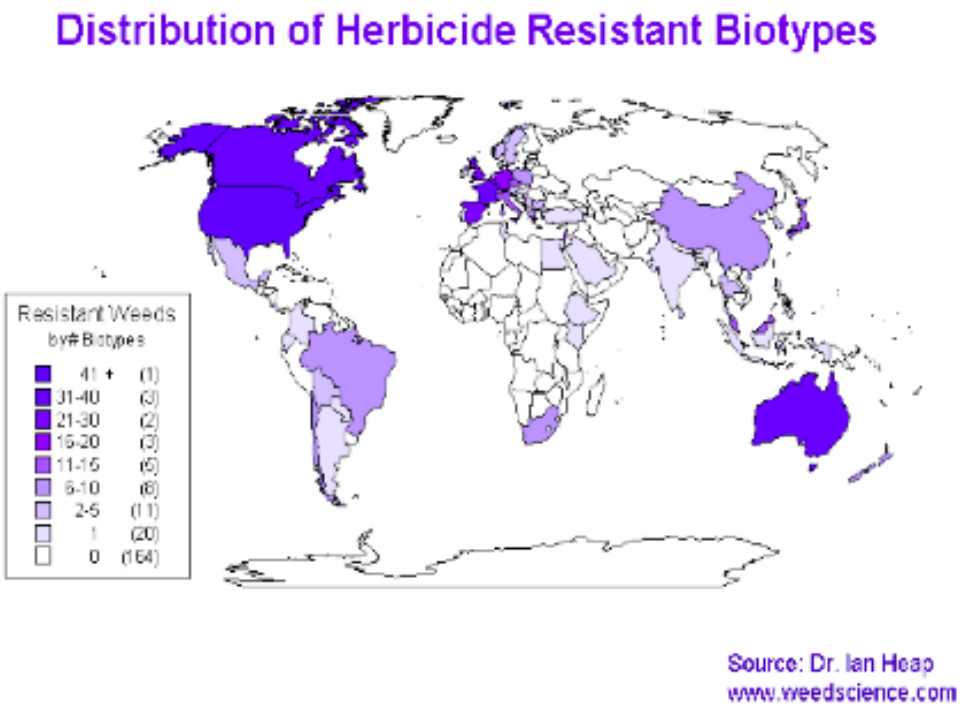


Figure 3. **Global distribution of herbicide resistant populations** (taken from Heap, 2012: The International Survey of Herbicide Resistant Weeds, online)

1.3 MECHANISMS OF HERBICIDE RESISTANCE

Mechanistically, two major types of resistance have been described based on the underlying molecular mechanisms: target-site and non target-site resistance (Powles & Yu 2010).

1.3.1 Target-Site Resistance

Most herbicides act by binding to specific plant enzymes and inhibiting their activity. Target-site resistance occurs when a mutation changes the target site in such a way that, despite the herbicide reaching the target site in full, lethal dose, its impact is reduced or removed (Powles & Yu 2010). Such changes can be achieved by a modification of the herbicide target through one or more point-mutations, reducing the herbicide binding affinity (Powles & Yu 2010). This is the most commonly identified resistance mechanism, implicated in resistance to many major herbicide families (Powles & Shaner 2001). An alternate mechanism potentially giving rise to target-site resistance is the over-production of the target enzyme (Powles & Yu 2010). Target over-production can be achieved either by over-expression or the amplification of the target enzyme gene, and has been observed in populations resistant to glyphosate (Powles 2010). If resistance-endowing mutations alter the functionality of the enzyme or the plant performance, they may be accompanied by a fitness cost (Vila-Aiub et al. 2009). Target-site resistance is often associated with target-site cross-resistance – a correlated response to selection whereby evolution of resistance to one herbicide provides fitness benefits to others that share its mode of action (Gressel 2009). This form of cross-resistance is specific to a herbicide family and can be seen as a specialist response to selection. In addition, target-site resistance can evolve independently to multiple herbicides, giving rise to multiple resistance (Gressel 2009).

1.3.2 Non Target-Site Resistance

Non target-site resistance occurs when the herbicide dose that reaches the target enzyme is reduced to a non-lethal dose as a consequence of i) decreased herbicide penetration into the plant, ii) enhanced rates of, or capacities for, herbicide metabolism, iii) sequestration of the herbicide into metabolically inactive compartments of the plant cell (i.e. the vacuole), iv) decreased rates of translocation of the herbicide or v) combinations of the above mechanisms (Powles & Yu 2010). All of these mechanisms could be associated with a fitness cost (Vila-Aiub et al. 2009). Mechanisms of non target-site herbicide resistance often involve the up-regulation or changes in the specificity of members of large enzyme families involved in stress metabolism and inter- and intracellular transport. Cytochrome P450 monooxygenases and glutathione S-transferases are two such enzyme families that have been implicated in herbicide resistance (Powles & Yu 2010). Often, evolution of resistance to one herbicide mode of action via these mechanisms can have a positive correlated response to selection giving rise to coincidental evolution of resistance to modes of action that the population has never been previously exposed to – resulting in the so-called cross-resistance. Such cross-resistance to novel modes of action can be seen as a generalist response to selection under herbicide exposure (Gressel 2009).

1.4 GENETIC, EVOLUTIONARY AND ECOLOGICAL FACTORS AFFECTING THE EVOLUTION OF RESISTANCE

1.4.1 Standing Variation and Mutation

The frequency of resistant individuals (standing variation) for resistance in a wild-type population not under herbicide exposure remains poorly characterized (Preston & Powles 2002;

Neve et al. 2009), and it depends on the population size, the frequencies of resistance mutations and the costs associated with them (Vila-Aiub et al. 2009). It is therefore herbicide- and resistance mechanism-specific (Gressel 2009). If the standing variation for resistance under herbicide exposure is low, the main source of its variation are novel mutations (Jasieniuk et al. 1996). Novel mutations arise at somewhat steady rates (Crow & Kimura 1970), but this rate can be substantially different between different genes (Orr 2000; Futuyma 2009). In addition, the magnitude of the beneficial effects of mutations has been linked to their frequency (Orr 2010), so that the strength of selection pressure could impact the proportion of mutations that confer positive fitness under those conditions. The lower mutation rates in chloroplast genomes compared to the nuclear genome could have an impact on the frequency of resistance mutations, if the herbicide targets a gene product encoded in the chloroplast (LeBaron & Gressel 1982). The use of herbicides can also affect the mutation rates themselves (Plewa et al. 1984), potentially leading to a local increase in the emergence of resistance (Plewa 1985). The effective population size, which for infesting weed species can be very high, can result in a high number of individuals carrying resistance mutations, even in spite of low mutation rates (Jasieniuk et al. 1996).

1.4.2 Number of Resistance-Bearing Mutations

The number of individual mutation events required to give rise to resistance is another factor determining the likelihood of a resistant individual arising in a population. Unlike many instances of adaptive evolution (Lande & Arnold 1983), majority of described cases of herbicide resistance are due to a single point mutation (Powles & Yu 2010). This phenomenon is likely due to herbicides imposing strong selection, which decreases the likelihood of accumulating mutations of smaller effect (Macnair 1991), assuming the fitness effects of beneficial mutations are exponentially distributed (Orr 2005). At lower doses (weaker selection pressures), polygenic

inheritance could be favored through accumulation of smaller-effect mutations (ffrench-Constant et al. 2004; Manalil et al. 2011), as was found in some experimental studies (Neve & Powles 2005a; Manalil et al. 2011). Lowered doses could also favor non target-site resistance, as it has often been linked to polygenically inherited resistance (Gressel 2002; Powles & Yu 2010). In spite of the consequences it might have on selection for resistance, little is known about the distribution of beneficial effects of novel herbicide resistance mutations (Gressel 2009).

1.4.3 Mode of Inheritance

The mode of inheritance of resistance plays a role in determining the dynamics of evolution (Gressel 2002). The vast majority of characterized herbicide resistance mutations are dominant or semi-dominant (Powles & Yu 2010). The dominance of the resistance allele affects the dynamics of resistance as the rate of spread of a rare mutation through the population is expected to increase with increase in dominance (Charlesworth 1992). The effects of mating type on resistance evolution also depend on the dominance of the mutations, as recessive alleles are fixed more slowly in sexual than in asexual organisms (Haldane 1924). Whether the resistance gene is nuclear or cytoplasmic also affects the mode of inheritance (Clark 1984), as cytoplasmic genes are most commonly maternally inherited (Birky 1995). As such, they often do not undergo recombination and are therefore subject to more rapid loss of allelic polymorphism when the resistance mutation is favored, than the nuclear-inherited traits (Clark 1984). Mutations in the cytoplasmic genome have been linked with triazine resistance (Hirschberg & McIntosh 1983).

1.4.4 Fitness Costs

Fitness costs of resistance are the negative pleiotropic effects of resistance mutations (negative correlated responses to selection), most commonly estimated in relation to the wild-type population in herbicide-free environment (Vila-Aiub et al. 2009). Theoretically, costs may be

associated with resistance mutations for three reasons. First, modifications of the target site could introduce changes to a well-adapted enzyme, which are likely to disturb its function (Cohan et al. 1994; Chevillon et al. 1995). Second, increased resource investment in the resistance, particularly if metabolic, should carry a trade off with other life history traits (Herms & Mattson 1994). Last, resistance mutations can bring about phenotypic changes that affect the organism's ecological interactions (Strauss et al. 2002). For these reasons, existence of fitness costs has often been assumed, in spite of the data suggesting that they are not universal (Vila-Aiub et al. 2009). As discussed above, fitness costs associated with a resistance mutation determine its frequency in a non-exposed population (Jasieniuk et al. 1996). Further, the effectiveness of some proposed management strategies relies on the existence of fitness costs (Beckie 2006). As such, understanding the frequency of their occurrence and their magnitude is necessary in order to make predictions about the dynamics of resistance and the effectiveness of management strategies.

1.4.5 Gene Flow

Gene flow (through seed or pollen movement, or migration) between populations of the same species can affect the evolution of resistance. If the gene flow occurs from a population with resistant individuals into a population with only susceptible plants, it can increase the rates of resistance evolution by providing novel mutations (Jasieniuk et al. 1996). Rates of gene flow are thought to be higher than mutation rates (Ellstrand 2003), but even at low levels, gene flow can stimulate the evolution of herbicide resistance as novel beneficial mutations are very rare in susceptible weed populations (Mulugeta et al. 1992). Gene flow can also act to maintain the otherwise-declining (sink) populations until rescued by resistance evolution (Holt 1996). Immigration into a well-adapted (resistant) population, on the other hand, could be decreasing the relative proportion of resistant individuals or diluting the effects of resistance mutations, in

particular in sexually-reproducing populations (Jasieniuk et al. 1996). For this reasons, the maintenance of a susceptible source population has been advocated as a potential management strategy (Beckie 2006). Formation of soil seed banks (or presence of dormant spores, in fungi and bacteria) provides an additional, temporal source of immigration, by which past genotypes are re-introduced in the population (Maxwell et al. 1990). In addition to maintaining a declining population in the presence of herbicides, temporal immigrants have experienced periods of relaxed selection (depending on the soil persistence of the herbicide) and potentially bring in novel mutants (Gressel 2002). Seed banks also allow a refuge from periods of herbicide exposure, potentially disrupting management strategies that rely on temporal variations in the applied herbicide(s) (Gressel 2002).

1.4.6 Mating Type

Weed species have an unprecedented diversity of breeding systems (Barrett 2002). Where the population lies on the spectrum from highly self-fertilizing to randomly mating has consequences on its response to selection pressure imposed by herbicides (Jasieniuk et al. 1996). Recombination often leads to greater genetic diversity (Agrawal 2006) and can enhance rates of adaptation (Colegrave 2002; Becks & Agrawal 2010). Its effects are dependent on the number of resistance mutations required (Neve & Powles 2005b) and their dominance, with recessive mutations fixed more rapidly in selfing populations, while random mating and outcrossing favor resistance through non-recessive mutations (Charlesworth 1992; Jasieniuk et al. 1996).

1.4.7 Selection Pressure

The strength of selection imposed by herbicides surpasses most common evolutionary pressures occurring in natural plant populations (Jasieniuk et al. 1996). Most herbicides are applied at rates that eliminate over 90% of individuals in the population (Gressel & Segel 1982). At such

extreme selection pressures, a resistance mutation rapidly moves to fixation, almost irrespective of its initial frequency (Gressel & Segel 1990). The likelihood of resistance evolution varies between herbicides (Friesen et al. 2000). These differences can be due to the availability of mutations that reduce the binding to the target or due to the ease of sequestering or metabolizing the herbicide (Gressel 2002).

1.5. MANAGEMENT OF RESISTANCE

As the inevitability of herbicide resistance evolution and its economic and environmental impact became apparent (Hamill et al. 2004), management strategies aimed at prolonging herbicide longevity were devised, although often with the lack of understanding of underlying evolutionary processes involved (Gressel 2009). Among the proposed methods of herbicide application, manipulation of the dose, cycling (rotating) between herbicides and the use of herbicide mixtures have seen widespread adoption in field practices (Beckie 2006). Here I outline the theoretical underpinnings of these methods.

1.5.1 Post-Resistance Herbicide Sequences

A common practice among farmers, and often a necessity rather than a management strategy, is to respond to reduced effectiveness of one herbicide by employing a novel/different mode of action (Beckie 2006). In spite of such practices, there is a tendency to observe every case of evolving resistance independently, and how resistance to one herbicide affects the evolution of resistance to another is poorly understood. Exposure to a sequence of herbicides could slow down evolution of resistance to subsequent herbicides, if independent resistance mechanisms with associated fitness costs were accumulating (Andersson & Hughes 2010; Hall et al. 2010). On the other hand, environmental perturbations could affect the adapting population by allowing

access to previously inaccessible adaptive peaks (Arnold et al. 2001), potentially speeding up resistance evolution or leading to a higher peak where resistance is greater or less costly. With respect to herbicide resistance, a correlated response to selection in the form of cross-resistance after exposure to a herbicide is an example of a mechanism that could provide access to novel peaks and accelerates rates of resistance evolution (Powles & Yu 2010). These mechanisms will be discussed in more detail in Chapter 3, where I present experiments to test these hypotheses. Understanding the effects of evolution of resistance on the rates of resistance in another mode of action is of relevance to other xenobiotics, as similar practices are observed upon the emergence of insecticide (Denholm & Rowland 1992), fungicide (Russell 2005) and antibiotic (Bonhoeffer et al. 1997) resistance.

1.5.2 Herbicide Cycling

A commonly recommended management practice is to cycle herbicides - a temporal rotation between two or more herbicides with different modes of action. Herbicide cycling introduces temporal environmental heterogeneity. This means that over a given time scale fewer generations are exposed to any single herbicide. This can lead to a reduction in the strength of selection for resistance to that herbicide (MacArthur 1964; Futuyma & Moreno 1988). The real potential of strategies based on herbicide cycling to retard evolution of resistance depends on the existence of antagonistic pleiotropy, so that adaptation in one environment (herbicide) incurs a fitness cost in other environments (Lewontin 1974; Whittaker & Levin 1976). If antagonistic pleiotropy is strong enough, it could completely prevent the evolution of resistance in a cycling environment. When the environment varies in time, a generalist strategy may be more likely to evolve (Hedrick 1986) and in terms of herbicide resistance this can often mean a wider-pattern of cross-resistance (see section 1.3). The detailed discussion of these mechanisms can be found in Chapter 4.

There is no clear consensus on the efficacy of herbicide cycling, as some studies report its beneficial effects (Gressel & Segel 1990; Jasieniuk et al. 1996) while others warn against widespread use (Diggle et al. 2003; Neve 2008). The effects of cycling on the level of resistance and fitness of the evolved individuals are even less well understood and the potential of this method of application to lead to cross-resistance has not been empirically tested (Gressel 1995). The consequences of cycling on the evolution of antibiotic (Brown & Nathwani 2005) and insecticide resistance (Caprio 1998) remain equally poorly understood and ambiguous, identifying a clear need for further investigations.

1.5.3 Herbicide Mixtures

Environments that contain mixtures of herbicides expose a population to multiple selection pressures simultaneously. To gain positive fitness, an organism requires either multiple independent mutations conferring resistance to each of the herbicides, or mutations that give rise to broad-range (cross-) resistance. The probability of multiple resistance mutations arising in one individual decreases with each additional chemical in the mixture (Wrubel & Gressel 1994). Therefore, these conditions could favor mutations that give rise to a wider pattern of cross-resistance as a potentially lower number of mutations could provide resistance to all herbicides. The detailed discussion of these evolutionary mechanisms can be found in Chapter 5, where I present the experiments designed to test these hypotheses.

Use of multiple chemicals with different modes of action is generally seen as offering the best level of resistance control (for herbicide resistance: (Naylor 2002); insecticide: (Denholm & Rowland 1992); and antibiotics: (Brown & Nathwani 2005)). The propensity for resistance evolution depends on the dose of the xenobiotics used in the mixture (Yeh et al. 2009; Trindade et al. 2009). For herbicides, environmental concerns increase the need to understand the relationship between the dose, satisfactory control and long-term effectiveness. Lowered doses

in a mixture have been shown to offer comparable levels of short-term control (Blackshaw et al. 2006) but the effects of lowered combined doses on the evolution of resistance are not well understood.

1.5.4 Herbicide Dose Manipulations

It has been suggested that reductions in administered doses could lead to 'creeping' resistance by allowing accumulation of mutations of smaller effect (Gressel 1995; 2009). In fact, several studies have demonstrated that lowered herbicide rates can lead to rapid evolution of polygenic resistance in controlled environments (Neve & Powles 2005a; 2005b; Busi & Powles 2009) and in the field (Manalil et al. 2011), as the propensity for resistance evolution in a single-xenobiotic environment is thought to be equivalent to the frequency of resistant mutations as a function of dose (Drlica 2003). The question remains whether adaptation at lower doses can lead to comparable levels of resistance at the recommended dose. This issue may be viewed from the perspective of local adaptation (Kawecki & Ebert 2004). Varying the applied dose creates an environmental gradient of increasing harshness. If populations are best adapted to their local conditions, resistance to high and commercially relevant doses will only occur when populations are selected at those doses, and therefore selection at lower doses might not pose a great management risk.

1.6 EVOLUTIONARY DYNAMICS OF HERBICIDE RESISTANCE

1.6.1 The Dynamics of Evolving Herbicide Resistance

In spite of their potential to play a fundamental role in the understanding of resistance management (chapter 1.5), there have been very few experimental studies exploring the dynamics of herbicide resistance evolution (Neve et al. 2009). In large part due to the slow

replication times of weedy plants and the large areas required for experimental approaches, the focus of the majority of herbicide resistance studies has been on characterizing resistance mechanisms in already evolved populations (Powles & Yu 2010). Such an approach limits the understanding of resistance to the outcomes of prolonged exposure, ignoring the ecological and evolutionary events that occur early during selection. In fact, the key events that would determine the characteristics of the evolved resistance are likely to have occurred at these early stages of exposure (Neve et al. 2009), and include the standing genetic variation for resistance (Preston & Powles 2002), and the presence of a variety of resistance mutations that differ with respect to the magnitude of the fitness benefit (Gressel 2009) and the associated fitness costs (Andersson 2003; Wijngaarden et al. 2005). These factors could impact the effectiveness of various management strategies and determine the outcomes of selection (Neve et al. 2009). The outcomes of a mixture strategy, for example, depend on the frequency of an individual with multiple resistance mutations relative to the frequency of a generalist with cross-resistance to all mixture components, and which is selected for can have different implications for management. In short, the dynamics determine the outcomes, and to study them, mathematical models and model organisms have been employed.

1.6.2 Models in Herbicide Resistance Research

Mathematical models enable rapid, large-scale comparisons between in-silico populations, allowing the evaluation of the impact that various factors might have on evolutionary dynamics (Neve et al. 2009). A model can break down the biological complexity and look at the impact of individual components, such as mutation frequency, type of resistance and the initial frequency of resistance. (Jasieniuk et al. 1996). In addition, models have been employed to address specific cropping system-related questions. Most commonly, these models evaluate the impact of continuous exposure to a single herbicide, cycling and mixtures strategies on the rates of

resistance evolution (Gressel & Segel 1990; Powles et al. 1997; Diggle et al. 2003; Neve et al. 2011). The relevance of models to field situations can be questioned as they rely on a series of underlying assumptions used to define the properties of the evolving populations. The validity of those assumptions needs to be evaluated in actually evolving populations.

1.6.3 Model Organisms in Herbicide Resistance Research

Certain model organisms, such as *Arabidopsis thaliana*, share many features with agriculturally relevant weeds, while having much shorter life cycles. This makes them suitable for testing of ecological and evolutionary hypotheses. In their own right, these model organisms can be used to develop experimental evolutionary approaches to understanding herbicide resistance. In addition, they can help elucidate the assumptions used in mathematical models, and bridge the gap between *in silico* predictions and actual events in the fields. For example, studies with *A.thaliana* have investigated some important parameters driving resistance evolution, such as the frequency of resistance mutations (Jander et al. 2003) or the costs associated with herbicide resistance (Roux 2004; Roux & Reboud 2005; Roux 2005).

1.7 EXPERIMENTAL EVOLUTION AND *CHLAMYDOMONAS REINHARDTII*

1.7.1 Experimental Evolution with Microorganisms

Conceptually, experimental evolution is simple – expose a series of replicate populations to a novel environment, while maintaining a series of populations in the ancestral environment to serve as a control (Garland & Rose 2009). The researcher can vary one or more biotic, abiotic or demographic factors to create the novel conditions and adaptation is inferred via increased population fitness and growth rates. The fitness of the evolved populations can be measured and compared to that of the control populations in a variety of ways (Elena & Lenski 2003),

allowing the evaluation of the evolutionary changes that occurred. Ultimately, experimental evolution as a method allows monitoring evolution in real time, and through direct comparisons between different stages in the selection process allows characterization of the patterns of change and adaptation.

Certain properties of microbes render them particularly well suited for use in experimental evolution (Elena & Lenski 2003). Single-cell organisms have short generation times, some dividing as often as every 20 minutes. This property allows experiments to run for many generations, making selection experiments manageable in time and thus overcoming the single greatest obstacle to studying evolution in real time in higher organisms. Due to their small size, microorganisms permit large population sizes to be maintained in small spaces, reducing the effects of genetic drift and facilitating experimental replication. In addition, space and resources can be controlled to define the total population size. Another benefit of using microbes for studies in experimental evolution is that they can be frozen and/or stored in suspended animation, and thus preserved in time. Later retrieval of the frozen/stored populations allows comparisons between different time points in the selection experiment, allowing the researcher to trace the progress of adaptation. It is due to these factors, and the simplicity of the system that allows modifications of a single variable between conditions, that experimental evolution has been successful in exploring a wide-variety of questions in evolutionary biology (Buckling et al. 2009; Garland & Rose 2009).

1.7.2 Chlamydomonas reinhardtii as a model organism

Chlamydomonas reinhardtii was first described in 1888 (Proschold et al. 2005). *Chlamydomonas reinhardtii* is a unicellular green chlorophyte, capable of growing both as an autotroph (through photosynthesis) and a heterotroph (by metabolizing acetate). Under most conditions it replicates asexually, while nitrogen starvation induces the formation of spores following a sexual

period between the two mating types (Harris 2008). It has been used as a model system for studying flagellar motion and phototaxis, due to the relative simplicity and ease of system perturbations (Harris 2008). The fact that it is a well-characterized system, with a sequenced genome, large community of researchers and a vast resource database (www.chlamy.org) makes *C.reinhardtii* a good organism to use in experimental studies.

The typical length of the *C.reinhardtii* life cycle is 7-10 hours under laboratory conditions, in full medium (one that meets all nutritional requirements of the cell growing photosynthetically) and in the presence of light. Cells can be stored on agar for up to six months and successfully re-suspended at a future point. The difficulty of reviving *C.reinhardtii* cells after being frozen makes cryopreservation, a method commonly used in experimental evolution studies, very difficult (Harris 2008). *C.reinhardtii* has been adopted for use in experimental evolution studies as an attempt to step away from prokaryotes and explore experimental evolution in a eukaryotic organism (Bell 1990a; 1990b). It has been used to study the properties of single-strain (Bell 1990a; 1991) and mixtures of populations (Bell 1990b); evolution of heterotrophy (Bell 1997; Reboud & Bell 1997); adaptation (Bell & Reboud 1997; Kassen & Bell 2000); the impact of sex on natural selection (Silva & Bell 1996; Colegrave 2002), and other questions. Through such extensive research the behavior of *C.reinhardtii* under laboratory conditions is well characterized, informing its suitability as a model organism for the experiments described in this thesis.

1.7.3 Use of C.reinhardtii in Herbicide Resistance Research

An obstacle limiting much experimental research attempting to understand the evolution of herbicide resistance is the length of weedy plant life cycle, which for many agriculturally relevant species is a year. This has resulted in the majority of studies on herbicide resistance having been conducted after the resistance has evolved, or through mathematical modeling

(Powles & Yu 2010). Difficulties in carrying out direct experimental tests have distanced weed science from evolutionary thinking, resulting in a lack of understanding of herbicide resistance as an evolutionary phenomenon (Neve et al. 2009). *C.reinhardtii* has been suggested as a model system for studying herbicide resistance evolution (Reboud et al. 2007). In addition, *C.renhardtii* is susceptible to a range of commercially available herbicides of different modes of action, due to shared biochemical and metabolic pathways with higher plants (Reboud 2002). The ability of *C.reinhardtiii* to evolve resistance to atrazine, a member of the triazine family of herbicides, has been demonstrated (Reboud et al. 2007), paving the way for its establishment as a model organism for pro-active herbicide resistance research.

1.8 MAJOR STUDY OBJECTIVES

In its essence, this thesis is concerned with the exploration of the evolutionary events occurring under extreme anthropogenic environmental conditions imposed by herbicides. It works within a combined applied-theoretical framework by taking as its starting point an applied principle – a real-life method of herbicide application – and attempts to understand the underlying evolutionary principles. It does so by observing the effects of various application methods on the dynamics of resistance evolution (rates at which resistance evolves), the level of resistance reached by evolving populations, their growth rates in the absence of herbicides, and the extent of cross-resistance they exhibit. In this thesis, I attempt to address questions of importance to the study of evolution and adaptation by examining the effects of:

- Herbicide sequences, investigating how accumulation of resistance mechanisms impacts rates of evolution.
- Cycling between herbicides, assessing the impact of temporal environmental heterogeneity.
- Herbicide mixtures, investigating the effects of environmental complexity.
- Herbicide dose, exploring local adaptation along an environmental gradient.

CHAPTER 2: MATERIALS AND METHODS

This chapter outlines the materials and methods adopted in all or the majority of experiments presented in this thesis. Each experimental chapter contains a separate materials and methods section explaining the experiment-specific methodology, and detailing any possible deviations from the contents of this chapter. Each experiment therefore, unless specifically stated, utilized the methods described in this chapter.

2.1 Culture conditions

The culture media used in all experiments was modified Bold's Medium (subsequently BM) (Harris 2008). For all selection experiments, populations were cultured in disposable 25x150mm borosilicate glass tubes, in 20ml of BM and maintained in an orbital shaker incubator, at 28^{o}C and 180rpm, under continuous light exposure, provided by six fluorescent tubes mounted in the incubator lid (Osram L30 W/21-840, cool white; light intensity measured at the location of the tubes was 161 $\mu molm^{-2}s^{-1}$).

2.2 Founding population

The *Chlamydomonas reinhardtii* strain used in the experiments is Seger's CC-1690 wild type mt+ 21gr, obtained from the *Chlamydomonas* Resource Center's core collection. Prior to selection experiments, the strain had been adapted to the culture conditions (Chapter 2.1) in the absence of herbicides through continuous exposure for over 700 generations. To do this, 200µl of the growing population were transferred into fresh herbicide-free media every seven days for 18 months prior to the start of the first experiment. Every 10 transfer periods (2 months), as well as prior to the start of a selection procedure, a contamination check was performed on the stock

populations. This was done by transferring approximately 15,000 cells onto three agar plates with BM and three agar places with BM and acetate under sterile conditions. Plates were then placed under light for 5 days, when they were checked for contamination. Two weeks before the start of all selection experiments, 20µl of this adapted population (approximately 15,000 cells) was spread on an agar plate. After 7 days of growth, a single colony was picked and used to inoculate a BM liquid culture. This colony was multiplied for two transfer cycles (14 days) and was used to found experimentally evolving populations.

2.3 Measuring OD_{750} and estimating the number of cell divisions

To study how a population responds to herbicide application, the population growth was inferred from the measurements of population density. To do this, the optical density at 750nm (OD_{750}) was measured in a Jenway 6315 benchtop-spectrophotometer, with a 24-25.5mm test tube holder accessory that allowed for 25x150mm glass tubes to be fitted. The machine was sensitive to OD_{750} of 0.001, but the natural variation in glass thickness and residue, as estimated by repeated measurements of OD_{750} of tubes containing only BM, accounted for the lack of sensitivity below OD_{750} of 0.020.

To produce a calibration equation converting OD_{750} into the approximate number of cells in that population, I carried out a series of cell counts and correlated them to a corresponding OD_{750} measurement. 125,000 cells were used to inoculate a BM culture grown for nine days. An OD_{750} measurement and cell counts were taken on a series of dilutions, at 10,20,30,40,50,60,80,90 and 100% of the final volume. The measurements were repeated on 10 independent populations. A sample of 10µl was taken from each population at each measurement point and diluted 100 fold with ddH_2O in 1.5ml microcentrifuge tube. 5µl of Lugol stain was added, and the sample gently hand shaken to minimize cell burst. 10µl of the sample was placed on the haemocytometer plate (Improved neubauer, depth 0.1mm, $1/400mm^2$) and covered with a glass

lid. The plate with the sample was placed under a light microscope, the number of cells counted and converted into the total number of cells per ml of diluted sample by multiplying with 10^4, as each plate surfaces contained 0.1µl of the diluted sample. The optical density-cell count pairs were used to determine the relationship between the two by finding the equation for the curve of best fit and constraining it to 0 on both axes (Fig.4). The resulting function was quadratic and described by the below equation. It is worth noting that populations reached a threshold OD_{750} value at approximately 1.00, and that the relationship is accurately described by the function below only within the presented range of OD_{750} measurements.

$$\text{Number of cells/ml} = 854{,}534*(OD_{750})^2 + 1{,}277{,}248*OD_{750}$$

The cell size of wild type and cells that have evolved resistance was estimated using the grid on the haemocytometer plate, to check whether OD_{750} was a good estimate of population size. No significant variations in cell size were observed between wild type and resistant cells.

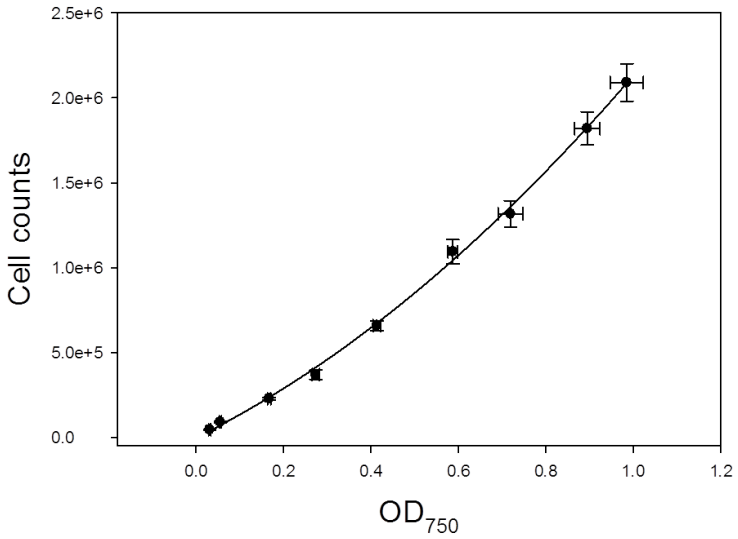


Figure 4. **Optical density-cell count pairs** and the curve of best fit. The measurements were taken one, two, three and four days after inoculation, OD_{750} measured and the cells counted. The pairs were modeled to obtain the curve of best fit, which described the relationship between OD_{750} and the cell count. Each point is the mean OD_{750} and mean cell count for the ten independent measurements at each day. Bars are standard errors.

2.4 Herbicides

A variety of herbicides were utilized in each experiment, some to select for resistance, others to determine if cross-resistance (generalism) was selected for. To determine if the active ingredient inhibited the growth of *C.reinhardtii* populations, 125,000 cells of the ancestral CC-1690 populations were inoculated into BM supplemented with a range of concentrations of a number of herbicides of interest. These populations were placed under culture conditions for seven days. If there was no measurable growth over 7 days (defined as OD_{750} below the sensitivity levels of the apparatus (Chapter 2.3)) at one of the herbicide concentrations within this range, I concluded that *C.reinhardtii* was susceptible to that herbicide. To more accurately determine the minimum inhibitory concentration (MIC) of each active herbicide, I exposed 125,000 cells to a narrower range of concentrations around the previously identified limiting concentration, with three replicates per concentration. The populations were incubated for seven days, at the end of which the OD_{750} was measured. The assay was repeated twice in two consecutive weeks. To test if the relationship between herbicide concentration and growth was sigmoidal and reached 0 value, the data was used to construct a dose response curve by fitting a non-linear 4-parameter regression on the relationship between the concentration of the herbicide (dose) and the growth in OD_{750} after seven days (drm function of the drc package in R2.15.0). Figure 5 shows a sample dose response curve illustrating the desired sigmoidal curve shape. If the relationship was confirmed as sigmoidal, the lowest concentration tested at which there was no measurable growth (OD_{750} below 0.020, the natural variation of the apparatus) was used as the MIC. The tested value as opposed to a value extrapolated from the dose response curve was used, as the fitted model assumed the curve never reached 0 value (complete control was impossible), and was therefore likely to overestimate the minimum inhibitory concentration. New herbicide solutions were made prior to the start of each experiment, their MIC determined

according to the above protocol, and the same solution was used for the duration of that experiment. Table 2 provides a summary of all tested herbicides, their activity in *C.reinhardtii,* and the MIC, when applicable.

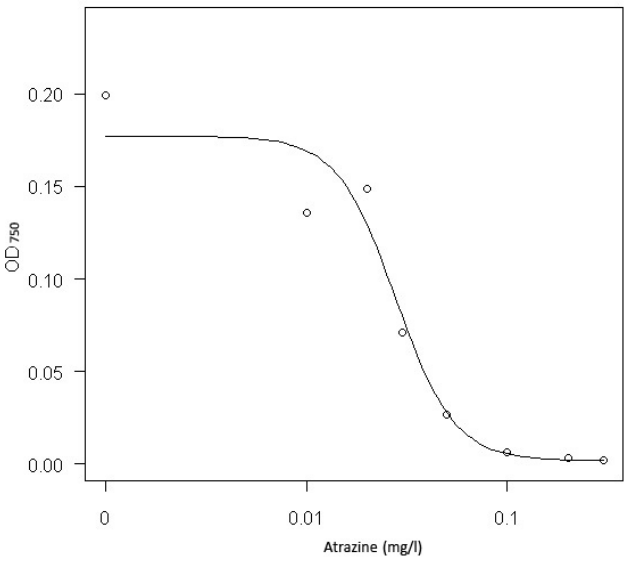


Figure 5. **Sample dose response curve**. Atrazine dose response, relating growth in OD_{750} after seven days to the concentration of atrazine. The points are the mean of three replicate observations.

2.5 Transfer Protocol

Populations were transferred into appropriate fresh media (supplemented with appropriate herbicides) every seven days. In addition to the evolving populations, source populations were also maintained by transferring into herbicide-free media. Each source population corresponded to a single replicate population within each regime, so that if the design called for six replicates of each regime, six source populations were maintained and used as described below. The source populations also served as controls in each experiment. At each transfer, the OD_{750} of the population was estimated and 200µl of the evolving culture was transferred into fresh media. If the number of cells in 200µl of culture medium estimated from OD_{750} was less than 125,000, as would happen until resistance evolved, then the appropriate number of cells from one of the

source populations was added to make the total cell number at the transfer approximately 125,000. Therefore, the minimum number of cells at the beginning of each cycle was 125,000. For each replicate within the experimental regime, the same source population was used for immigration throughout the experiment. According to this protocol, when undergoing sufficient growth (at least 6.64 cell division in seven days), a population is capable of maintaining itself after the weekly bottleneck event. When growth did not reach this number of cell divisions, weekly bottlenecks would drive the population towards extinction, and these populations were maintained by immigration from the corresponding source population.

2.6 Measuring level of resistance and growth rates in absence of herbicides

The number of cell divisions undergone by a population in exposure to a selective dose of an herbicide (MIC) was adopted as the estimate of the level of resistance. After the selection procedure, 125,000 cells from each evolved population were used to inoculate each resistance assay and the final population size was determined by measuring OD_{750} after seven days of growth. This assay was replicated twice for each tested population and the mean used as the level of resistance.

The number of cell divisions in the absence of herbicides was adopted as the estimate of the population's growth rate in the ancestral environment. Whether evolved resistance was associated with a fitness cost was determined by comparing the growth rates in the absence of herbicides of the evolved population to the mean of the source populations. The relative differences in fitness costs between evolved populations were estimated by directly comparing their growth rates in the absence of herbicides. 125,000 cells were used to inoculate each assay and the population size determined after four rather than seven days of growth, in order to more clearly distinguish between populations as they grew more rapidly in the absence of

herbicides. The assay was replicated twice for each tested population and the mean number of cell divisions used as the growth rate in the ancestral environment.

2.7 Cross-resistance assays

Cross-resistance was defined as observable growth after seven days of exposure to a novel herbicide the population has not been in prior exposure to. The number of cell divisions were estimated by inoculating BM containing the MIC of a novel herbicide with 125,000 cells from the evolved population, and the OD_{750} measured after seven days. Each assay was replicated twice, and the population marked as cross-resistant to the tested herbicide if it reached OD_{750} above 0.02, the natural variation of the apparatus (Chapter 2.3).

Table 2. **Tested herbicides**: their mode of action, activity in *C.reinhardtii* and the determined minimum inhibitory concentration. When multiple values are provided for the MIC, they correspond to different experiments/chapters.

Commercial herbicide	Mode of action	Active in *C.reinhardtii?*	**MIC** (mg/l)
Atrazine	Photosystem II inhibitor	Yes	**0.125** (C.3/6);**0.115** (C.4); **0.140** (C.5)
Glyphosate	Aromatic amino acid synthesis inhibitor	Yes	**90** (C.3/4/6); **95** (C.5)
Carbetamide	Mitosis inhibitor	Yes	**2.8**
S-metolachlor	Inhibitor of very long chain fatty acid synthesis	Yes	**1.1**
Iodosulfuron-methyl-sodium	Inhibitor of acetolactate synthase	Yes	**8** (C.3/5); **7.8** (C.4/6)
tembotrione	Inhibitor of 4-hydrohyphenyl-pyruvate-dioxygenase	Yes	**65**
Flurochloridone	Inhibitor of carotenoid synthesis	Yes	**2.25**
Isoproturon	Photosystem II inhibitor	Yes	**0.7**
2,4 dichlorophenoxy-acetic acid	Synthetic auxin	No	n/a
Dicamba	Plant growth inducer	No	n/a
Bentazone	Photosystem II inhibitor	No	n/a
Imazaquin	Plant growth inducer	No	n/a
Metribuzin	Photosystem II inhibitor	No	n/a
Sulcotryone	4-Hydroxyphenylpyruvate oxygenase inhibitor	No	n/a

CHAPTER 3: HERBICIDE SEQUENCES – CONSEQUENCES OF RESISTANCE ACCUMULATION

3.1 Introduction

Exposure to extreme environmental conditions can lead to rapid adaptation (Hardie & Hutchings 2010), such as the evolution of resistance due to widespread use of pesticides and antibiotics (Bergstrom & Feldgarden 2007; Powles & Yu 2010). In the face of emerging resistance and the reduced effectiveness of a xenobiotic, it is often necessary to employ a chemical with a novel mode of action to ensure population control (Beckie 2006; Bergstrom & Feldgarden 2007), in the hope that this chemical will provide sufficient control or even eliminate the resistant individuals. The consequences of such xenobiotic sequences for resistance management depend on a range of genetic and evolutionary factors, and their outcomes are not well understood.

Following evolution of resistance to the primary component of a herbicide sequence, rates of evolution to secondary and subsequent herbicides may be unaffected, or, they may conceivably be accelerated or slowed in comparison to selection for resistance to those herbicides in populations without previous herbicide exposure (wild type populations). Evolution of resistance to secondary herbicides may be slowed where there is a cost of resistance associated with resistance to primary the herbicide (Vila-Aiub et al. 2009; Andersson & Hughes 2010). Assuming a correlation between fitness costs in different environments, costs reduce the competitive ability of the resistant individuals when exposed to a novel xenobiotic, so that wild-type populations were likely to outcompete them. As fitness costs have been associated with both target- and non target-site resistance (Chapter 1.3.2.1), sequential exposure to herbicides could lead to the accumulation of fitness costs associated with resistance (Hall et al. 2010; Lagator et al. 2012), providing a limit to the number of xenobiotics a population can evolve independent resistance to.

Exposure or evolution of resistance to one xenobiotic could enhance the rates of resistance evolution to another, if the outcome of selection to the first involves a positive correlated response to selection in the second. Following a herbicide-induced environmental perturbation, two mechanisms have been shown to provide benefits to the population exposed to a novel herbicide: cross-protection and cross-resistance. Cross-protection is a form of a general stress response (Booth 2002), whereby exposure to a source of environmental stress provides a temporary fitness benefit in other stressful conditions (Hill et al. 2002). That fitness benefit leads to a temporary increase in the number of cell divisions in a novel xenobiotic environment, potentially generating a greater number of mutations and enabling a population to adapt more rapidly, as observed in *C.reinhardtii* (Lagator et al. 2012). Cross-resistance to herbicides, on the other hand, is a long-term generalist evolutionary response, whereby a single underlying mechanism provides resistance to a range of herbicides, some of which the population has not experienced before (Powles & Yu 2010). Changes in the efficiency and selectiveness of efflux pumps to increase the rate and range of chemicals that are being transported out of the cell is an example of a cross-resistance mechanism described in prokaryotes (Van Bambeke et al. 2003a) and eukaryotes (Van Bambeke et al. 2003b). Although often arising in response to environmental heterogeneity (Beckie 2006; Powles & Yu 2010), cross-resistance can develop under stable exposure to a single herbicide (Gressel 2002). When cross-resistance confers a fitness benefit in a novel herbicide that is not sufficient to deem the population immediately resistant, the elevated growth rates in the novel environment could allow the population to persist for longer and to maintain a larger size, increasing the likelihood of generating further resistance mutations. Alternatively, resistance mutations of small effect that would not provide sufficient fitness benefit to allow their fixation in a completely non-resistant population, could get fixed if cross-resistance provides higher starting fitness. Both these mechanisms could lead

to accelerated rates of evolution to subsequent herbicides. In a wider sense, herbicide exposure is a form of environmental perturbation while cross-resistance and cross-protection could lead to a correlated response to selection that could shift the population along the adaptive landscape allowing access to previously inaccessible adaptive peaks (Arnold et al. 2001), in a fashion similar to the observed consequences of environmental heterogeneity (Collins et al. 2007; Morris 2011; Lagator et al. 2012).

In the absence of fitness costs and perturbation-induced shifts across the landscape, rates of resistance evolution when experiencing an herbicide in a sequence would not be altered when compared to the rates in a wild-type population, as the resistant individuals would not be at a selective advantage or disadvantage compared to the wild-type. In such a case, mutation availability, genetic diversity of the population and the strength of selection pressure should be the principal determinants of the rates of resistance evolution (Ricklefs & Miller 2000; Gaston 2003), and the rates of adaptation of an already resistant population would be the same as the rates of a non-resistant one.

C.reinhardtii populations were selected in exposure to three herbicides with different modes of action over a period of 20 weeks, transferring populations to a novel herbicide environment once resistance to the previous herbicide was observed. I investigated if prior selection for resistance to one herbicide mode of action impacted the dynamics of resistance evolution to subsequent herbicides, as well as the effects that switching had on fitness costs.

3.2 Materials and Methods

3.2.1 Selection procedure and dynamics of resistance evolution

The strain used and the culture conditions the populations were grown in were described in Chapter 2. Herbicides used were atrazine, glyphosate and carbetamide. 125,000 cells were

inoculated into six replicate populations at MIC of each of the three herbicides used in the experiment (18 initial populations), as well as six source populations that were propagated in the absence of herbicides. Populations were transferred into fresh media containing the appropriate herbicide every 7 days, according to the protocol described in Chapter 2.5, and weekly OD_{750} measurements used to monitor the dynamics of resistance evolution. A population was considered resistant when it underwent at least three cell divisions in seven days of growth (one transfer cycle). At this point, 125,000 cells from the population were used to inoculate two novel populations to be exposed to MIC of each of the remaining two herbicides (Fig.6). 200µl of the population ('initial resistant population') were also placed on BM with 1.5% agar, grown for seven days in light and then preserved in dark for subsequent measurement of initial growth rates in the absence of herbicides (Fig.6). The resistant population was also maintained and propagated in its original environment in order to remain in exposure to those conditions for the duration of the selection procedure (Fig.6). The secondary populations were propagated in the same manner as described above. When resistance was observed in secondary environments, 125,000 cells were used to found a population to be exposed to MIC of the last remaining herbicide used in the study (tertiary populations), 200µl transferred into BM with 1.5% agar to preserve the initial resistant population, and the resistant population maintained in the secondary herbicide for the duration of the experiment. Upon the evolution of resistance in a tertiary herbicide, 200µl were transferred onto 1.5% agar and the population maintained in the same conditions. The selection procedure was carried out for the total of 20 transfer cycles, from the first inoculation.

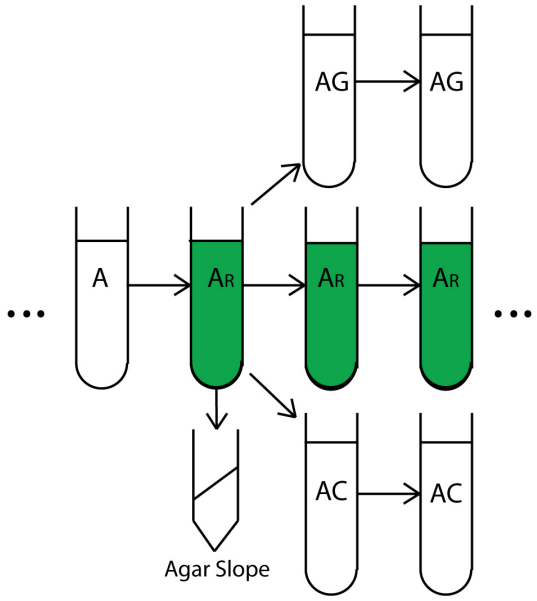


Figure 6. **Schematic of the selection procedure.** A population in primary exposure to atrazine (A) develops resistance (A_R). 125,000 cells were used to inoculate the secondary herbicides glyphosate (AG) and carbetamide (AC), while the population in atrazine was maintained in that herbicide as well. Initial resistant populations were preserved on BM containing 1.5% agar by transferring 200µl of the population upon emergence of resistance.

3.2.2 Growth rates in the absence of herbicides and cross-resistance

Upon the completion of the selection procedures, 125,000 cells of all evolved populations that exhibited resistance were transferred into herbicide-free BM and grown for seven days to eliminate potential carryover effects of herbicides. The initial resistant populations that were preserved on agar slopes were revived by collecting a sample with a sterile loop, transferring it into herbicide-free BM and growing for seven days. The growth rates in the absence of herbicides were measured for the (i) final resistant populations ('final fitness costs'); (ii) initial resistant populations ('initial fitness costs'); and (iii) the source populations (for protocol, see section 2.6). Each final resistant population was tested for growth at MIC of all herbicides it was previously exposed to, by testing for growth after seven days of exposure (see section 2.6). This test confirmed that resistance was not lost in any populations.

Growth rates of each final population after seven days of growth in MIC of tembotrione, iodosulfuron, S-metolachlor and isoproturon, as well as any of the herbicides the population was not in previous exposure to (for example, cross resistance to atrazine was estimated in the population that evolved resistance to glyphosate and carbetamide), were estimated to assess whether populations were cross-resistant (see section 2.7).

3.2.3 Statistical analyses

The first week when a population's OD_{750} upon transfer was above 0.1 was marked as the 'week to resistance'. The week when a secondary or tertiary population was inoculated was considered week 0 for those populations. The rates of resistance were analyzed using a censored parametric survival analysis model (function survreg of 'survival' package in R 2.15.0). Week to resistance and its status (whether resistance was ever observed or not) were used to construct survivorship functions, which were fitted as a response variable. If the population did not evolve resistance, its 'week to resistance' was marked as the last week when measurement was taken, and its status marked to 'non resistant'. The dynamics of resistance to each herbicide were analyzed separately. The response variables were analysed by the population's adaptive past, differentiating between the previous herbicides the population evolved resistance to. As such, when comparing the rates of atrazine resistance, populations experiencing atrazine as a primary herbicide ('A') were compared to those experiencing it as secondary ('GA') as well as tertiary ('GCA') herbicide, taking into account the order of previous herbicides.

Growth rates in the absence of herbicides were analyzed using a pair-wise Dunnett's corrected T-test in Minitab statistical software. In order to analyze the differences in the final fitness costs, the number of cell divisions in the absence of herbicides was compared between populations grouped by the number of herbicides they were resistant to (one, two, three or the source populations). I tested whether compensation (increase in the growth rates in the absence of

herbicide following emergence of resistance) through prolonged exposure to same conditions occurred in experimental populations, as would be evident from the potential decrease in fitness costs over the course of selection procedure. To do so, the differences in herbicide-free growth rates of the same population were compared at two time points: initial and the final resistant populations. To do this, a series of Dunnett's corrected T-tests was performed comparing the mean initial fitness costs to the mean final fitness costs.

3.3 Results

When evolving resistance to atrazine, populations experiencing it as a second herbicide evolved resistance significantly more slowly than the populations experiencing atrazine as the first ($z=2.39$, $P<0.05$) or third ($z=3.18$, $P<0.005$) herbicide (Fig.7a, Appendix A). There were no significant differences in the rates of resistance evolution to glyphosate, irrespective of the adaptive history (Fig.7b, Appendix A). In exposure to carbetamide, populations that experienced it as the first herbicide did not evolve resistance at all. When compared to the populations experiencing carbetamide after evolving resistance to glyphosate (GC), populations experiencing carbetamide as the third herbicide evolved resistance significantly more rapidly – AGC ($z=2.53$, $P<0.05$) and GAC ($z=2.43$, $P<0.05$). No significant differences in rates of adaptation were observed between any other populations that evolved carbetamide resistance (Fig7.c, Appendix A). Loss of resistance was not observed in any of the populations, as all final populations were resistant to all herbicides they were previously exposed to.

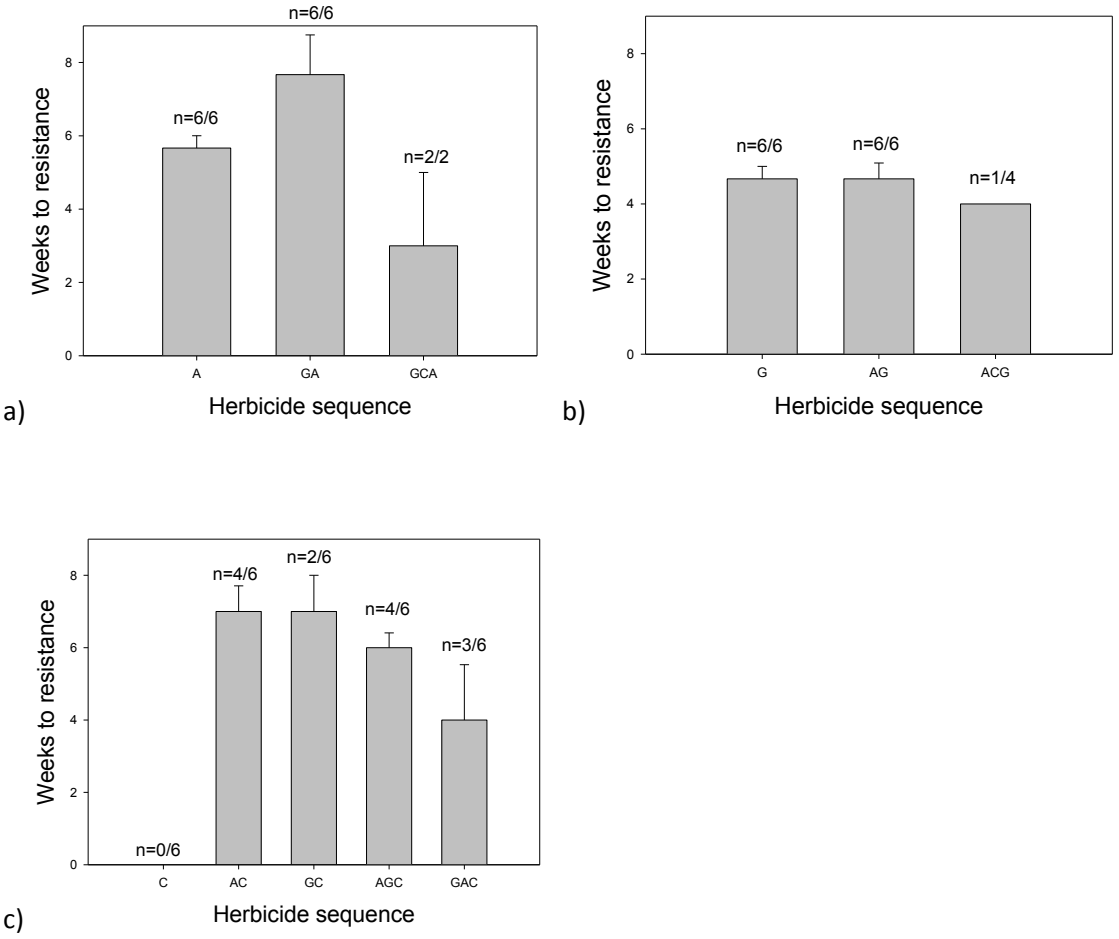


Figure 7. **Rates of resistance evolution**. Bars are mean weeks to resistance of the populations that evolved resistance, n indicates the number of populations that evolved resistance. a) Rates of atrazine resistance ('A' represents primary atrazine populations; 'GA' populations that evolved resistance to glyphosate prior to exposure to atrazine; 'GCA' those that evolved glyphosate followed by carbetamide resistance prior to exposure to atrazine); b) glyphosate; c) carbetamide. Error bars are standard errors of the mean.

A cost associated with resistance was evident when populations resistant to one (T_{16}=8.35, P<0.001), two (T_{22}=4.12, P<0.001) and three (T_{14}=3.42, P<0.001) herbicides were compared to source populations. Final fitness costs were lowest in the populations that evolved resistance to three herbicides - they were significantly lower than in the populations that evolved resistance to one (T_{20}=7.16,P<0.001) and two (T_{26}=3.84,P<0.005) herbicides (Fig.8). Populations that evolved resistance to two herbicides had significantly lower final fitness cost than the ones

exposed to a single herbicide (T_{28}=4.00,P<0.001) (Fig.8). The final and the immediate herbicide-free growth rates were not significantly different between populations in any of the regimes, suggesting no compensation occurred. When tested in herbicide populations' had no previous exposure to ('cross-resistance', see section 2.7), none of the final evolved populations exhibited.

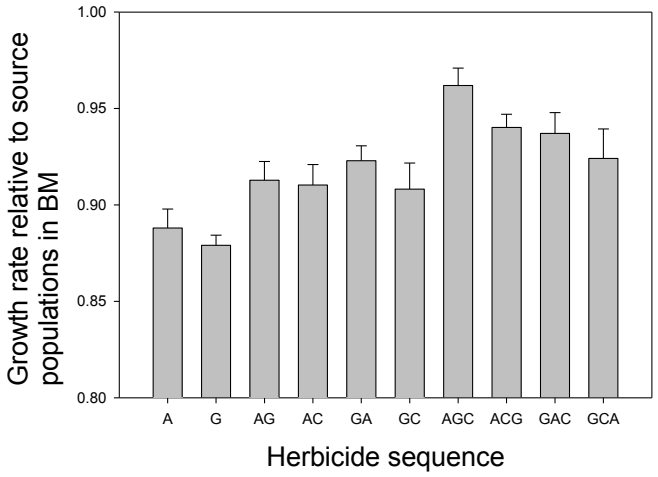


Figure 8. **Final fitness costs** as the number of cell divisions after four days of growth in the absence of herbicides, relative to the source populations. Bars are mean final herbicide-free growth rates; error bars are standard errors of the mean.

3.4 Discussion

3.4.1 Perturbation can allow access to previously inaccessible peaks

Some populations selected in exposure to secondary and tertiary herbicides were identified in which rates of resistance were elevated, indicating that sequential application of herbicides could enhance rates of adaptation (Fig.7). The most outstanding result is that resistance to carbetamide did not emerge in populations initially exposed to it, while it did when carbetamide was the secondary or tertiary herbicide (Fig7.c). As such, environmental perturbation, in the form of exposure to atrazine or glyphosate, enabled access to previously inaccessible adaptive

peaks (carbetamide resistance). Cross-protection (Hill et al. 2002; Lagator et al. 2012) and a correlated response to selection in the form of cross-resistance (Powles & Yu 2010) are known mechanisms providing a fitness benefit in a novel herbicide environment. Even if they do not lead to a fully resistant phenotype, the fitness benefit they impart could ultimately affect the mutation supply rate through the increase in growth rates. Elevated mutation supply could in turn allow access to novel, more rare fitness peaks. Even small, immeasurable benefits to fitness have been suggested to affects rates of adaptation in *C.reinhardtii* (Lagator et al. 2012). Cross-protection provides such small, transient benefit upon transfer into a novel herbicide (Chapter 4) and could therefore affect rates of resistance. The correlated response to selection in the form of cross-resistance in plants (Powles & Yu 2010) and *C.reinhardtii* (Lagator et al. 2012) is often assumed to confer greater fitness benefit in a range of novel herbicide environments, but the breath and the magnitude of fitness benefit can be much more constrained (Gressel 2009). Even though cross-resistance was not identified in any of the evolved populations in this study, the magnitude of the correlated response to selection in a novel herbicide could have been below the sensitivity of the equipment, still providing a fitness benefit allowing access to a novel phenotype.

3.4.2 Fitness costs decrease as resistance accumulates

Resistance is often associated with a fitness cost - a reduction in growth in herbicide-free environment (Vila-Aiub et al. 2009; Andersson & Hughes 2010) – and the accumulation of resistance mechanisms can result in accumulation of costs (Hall et al. 2010). When comparing final herbicide-free growth rates, no evidence was found for accumulation of fitness costs and the opposite pattern was identified - increase in the number of herbicides a population was resistant to was accompanied by a decrease in the population's final fitness costs, in spite of resistance to each individual herbicide carrying a cost (Fig.8). This finding is also surprising as the

resistance to all herbicides a population was previously exposed to was maintained, a finding frequently observed in prokaryotes with multiple drug resistance when resistance is not costly or when compensatory mutations are common (Davies & Davies 2010). For such a pattern to emerge, it could be that strong selection in a novel herbicide magnified the importance of small variations in fitness costs, with competition from other resistant individuals and/or from non-resistant immigrants favouring selection of phenotypes with lower fitness costs, for their higher competitive ability.

If multiple resistant phenotypes with different growth rates in the novel herbicide exist, direct competition between them would select for those with lowest fitness costs (MacLean et al. 2004). In addition, the competition from immigrants would further magnify the selection for lower fitness costs, potentially resulting in the observed reductions in fitness costs after sequential application. The contribution of fitness costs to the differences in the overall fitness is amplified in a novel xenobiotic, where the overall fitness is low (Andersson 2003). As such, the strength of selection for the phenotypes with lower fitness costs is stronger in a novel herbicide, potentially explaining why the same individuals were not selected in continued exposure to one herbicide. A weak correlation between growth rates in the presence and in the absence of herbicides (Coustau et al. 2000) could also explain why the individuals with lower fitness costs were not selected in continued exposure to one herbicide – if selection for higher growth rate in the presence of herbicides is not associated with higher growth rates in the absence of herbicides. Previous studies have reported a positive correlation between fitness in the presence and absence of herbicides (Vogwill et al. 2012), questioning the likelihood of this explanation. Finally, epistatic effects between different resistance mutations could be additive in the herbicide-free environment, so that the fitness is impaired less when multiple mutations are present (Andersson & Hughes, 2010).

Alternatively, competition from immigrants and other resistant individuals in a novel herbicide could select for fixation of compensatory mutations (Wiesch et al. 2010). Compensatory mutations could increase the growth rates in the absence of herbicides to improve the competitive ability of resistant individuals (Lagator et al. 2012). When testing for compensation in populations experiencing continued exposure to the same herbicide, differences between the immediate and final herbicide-free growth rates were not found. This finding suggested that compensation did not occur when a population was already resistant, exhibiting high growth rates and competing only with other resistant individuals. Selection for compensation is stronger when individuals have to compete in a novel environment (Andersson 2003), and could therefore have been favoured in populations experiencing a novel herbicide and stronger competition from non-resistant immigrants.

3.4.3 Applied considerations

Many management strategies rely on the existence of fitness costs to control emerging resistance (Vila-Aiub et al. 2009). Situations in which a xenobiotic is introduced upon the observed reduction in effectiveness of another are common in herbicides (Beckie 2006) and antibiotics (Bergstrom & Feldgarden 2007). The results presented here show such strategies could enhance or not affect the rates of resistance evolution, and even enable otherwise inaccessible resistant phenotypes to emerge. No evidence for accumulation of fitness costs was found, and instead an opposite pattern was identified, where further resistance mechanisms led to a reduction in the population's fitness costs. As such, the results show the dangers of applying herbicides sequentially, as a strategy potentially leading to more rapid selection for resistance of individuals with lower fitness costs, and therefore exacerbating the emerging resistance problem.

CHAPTER 4: HERBICIDE CYCLING – IMPACT OF TEMPORAL ENVIRONMENTAL HETEROGENEITY ON RESISTANCE EVOLUTION

The contents of this chapter were published under the title: 'Herbicide cycling has diverse effects on evolution of resistance in *Chlamydomonas reinhardtii*' by Lagator, Vogwill, Colegrave and Neve in *Evolutionary Applications*, 2012. The content presented here was modified from the published material to fit the purposes of the thesis.

4.1 Introduction

Synthetic herbicides have become the dominant means of controlling weedy plants in agricultural settings (Powles & Shaner 2001) and evolution of resistance to herbicides is widespread (Heap 2012). As discussed in Chapter 1.3.1, there are two modes of herbicide resistance evolution; target-site resistance and non target-site resistance (reviewed in (Powles & Yu 2010)). Target-site resistance confers resistance to a single herbicide mode of action, whereas non target-site resistance may result in complex patterns of cross-resistance rendering populations resistant to multiple modes of action (Powles & Yu 2010). In evolutionary terms, target-site and non target-site resistance represent specialist and generalist modes of herbicide resistance, respectively. As both mechanisms can provide resistance to the same herbicide, specialist and generalist phenotypes can coexist.

A commonly recommended resistance management practice is to cycle chemicals with different modes of action (Beckie 2006). Cycling (often referred to as herbicide rotation) introduces temporal environmental heterogeneity so that consecutive generations are exposed to different selection pressures. This can potentially affect the rate of resistance evolution in a number of ways. First, over a given time scale, fewer generations are exposed to any single environment, leading to reduced selection for resistance to each component environment (MacArthur 1964; Futuyma & Moreno 1988; Whitlock 1996). Second, if adaptation to one environment incurs a fitness cost in others, cycling may retard or even prevent resistance evolution (Leeper et al.

1986; Gressel & Segel 1990). Additionally, environments in which herbicides are cycled are more complex and may require a greater degree of genetic variation for adaptation to occur. However, ecological and evolutionary theory would predict that environments characterised by a greater degree of temporal heterogeneity would result in the evolution of more generalist phenotypes (Chesson 2000; Kassen 2002) and hence it may also be the case that cycling exacerbates the spread of generalist resistance phenotypes (Gomulkiewicz & Kirkpatrick 1992; Tufto 2000). This effect is therefore likely to crucially depend on the frequency of cycling between different modes of action, with more rapid rates of switching more strongly favouring generalist types of resistance.

The difficulties associated with performing selection experiments on large weed populations with slow generation times (one generation per year) have limited testing of these hypotheses mostly to theoretical and simulation models, with only a few experimental studies (Porcher et al. 2004; Roux et al. 2005; Kover et al. 2009; Springate et al. 2011). Models have shown that, in the absence of pleiotropic costs of resistance, cycling may not retard resistance evolution (Diggle et al. 2003; Bergstrom et al. 2004; Roux et al. 2008). It is not possible to generalise on the existence of pleiotropic costs associated with evolved resistance to herbicides, as it seems that fitness costs vary according to the mechanism of resistance (Vila-Aiub et al. 2009). A similar lack of understanding of the dynamics of resistance evolution has led to failed attempts to slow the spread of resistance to antibiotics in clinical settings (Bergstrom & Feldgarden 2007).

In this experiment, populations of *C. reinhardtii* were experimentally evolved with continuous cycling between pairwise combinations of three herbicides with different modes of action: glyphosate, atrazine and carbetamide. The frequency of cycling between herbicides was varied to explore the impacts of the degree of environmental heterogeneity on the dynamics of resistance evolution. In particular, I was interested in investigating if (i) cycling leads to reduced

rates of resistance evolution; (ii) there was a relationship between the frequency of cycling and the rates and outcomes of evolution; (iii) cycling leads to comparable levels of resistance as homogeneous environments, and (iv) cycling could result in the selection of more generalist resistance phenotypes.

4.2 Methods and Materials

4.2.1 Selection regimes

The experimental populations were founded and grown according to the conditions outlined in Chapter 2. Three herbicides were used in this study – atrazine, glyphosate and carbetamide (Chapter 2.4). Three experimental conditions involved continuous exposure to a single herbicide (A0 denoting continuous exposure to atrazine, G0 to glyphosate and C0 to carbetamide). A weekly, bi-weekly and tri-weekly cycling regime was created for all three possible pairwise combinations of herbicides (AG1 denoting the weekly cycle between atrazine and glyphosate; AG2 the bi-weekly cycle, and so on). Each experimental condition (12 in total) was replicated 6 times, giving rise to 72 independently evolving populations. Six populations were propagated by serial transfer in the absence of herbicides and used as source populations for control and immigration (see chapter 2. 5). The experiment was carried out for 12 transfer cycles (12 weeks) according to the transfer protocol described in section 2.5.

4.2.2 Measuring the rates of evolution

OD_{750} was measured on transfer. Resistance was considered to have evolved when detectable population growth was consistently measured (OD_{750} > 0.045, corresponding to at least three cell divisions). The rate of resistance evolution was quantified by measuring the first week when resistance was observed. The rate of resistance evolution to each component herbicide in

cycling regimes was expressed as the number of weeks that the population had been exposed to that herbicide.

4.2.3 Isolation of the evolved populations and assays

In order to ensure that populations used for subsequent resistance and fitness assays contained only herbicide resistant cells, approximately 20,000 cells of each final population were plated on BM agar plates that contained the MIC of a single herbicide. For cycling regimes, 20,000 cells of each final population were plated independently onto two plates, one containing each of the herbicides that the population had been exposed to. After 7 days of growth, 200 colonies from each population were randomly selected and used to inoculate a fresh population in liquid BM. If the population had been exposed to two herbicides, 100 colonies were randomly selected from each of the plates containing those herbicides and used to inoculate a fresh population in liquid BM. These populations were grown for 7 days prior to conducting further assays. In addition, for lines evolving under cycling regimes, 10 single colonies from each BM+herbicide plate were picked and multiplied for 7 days in BM. For all 10 populations, 125,000 cells were then transferred into MIC of the second herbicide from that cycling regime. In all cases, populations derived from single cells were resistant to both herbicides in the cycling regime, indicating that evolved populations always consisted of individuals with resistance to both herbicides cycled, rather than to mixtures of individuals with resistance to individual cycle components. I measured the level of resistance independently at MIC of each herbicide a population was exposed to, and growth rates in the ancestral environment (for protocol see Chapter 2.6). Both level of resistance and growth rates in the absence of herbicides were expressed as a proportion of the growth of source populations in the ancestral (BM only) environment. The degree of generality of each population was also estimated (see Chapter 2.7) by testing for growth in tembotrione, iodosulfuron, isoproturon and S-metolachlor, as well as

whichever of atrazine, glyphosate or carbetamide they had not been exposed to (i.e. cross-resistance to carbetamide was assayed in populations evolved in cycling between atrazine and glyphosate).

4.2.4 Cross-protection assays

To investigate a possible contribution of cross-protection, the phenomenon whereby exposure to one stress provides a degree of physiological acclimation (cross-protection) to subsequent stresses, naïve *C. reinhardtii* populations were grown in the presence of low doses (0.8MIC for atrazine, 0.7MIC for glyphosate and carbetamide) of each of three herbicides. Doses below MIC were used so that detectable population growth was apparent between transfer periods. After seven days in one herbicide 125,000 cells were transferred into below MIC doses of each of the two other herbicides. 125,000 cells without previous herbicide exposure were also transferred into below MIC doses of all three herbicides as a control. Seven days after transfer, growth rates of each population were estimated. Each condition was replicated three times. The time frame used for testing cross-protection was insufficient for significant levels of resistance to be selected.

4.2.5 Statistical analysis

The rate of resistance evolution (weeks to resistance) was analyzed using a Cox regression. The herbicide regime was fitted as a covariate, with the ancestral immigration source as the strata. For cycling regimes, the number of weeks until resistance evolved to individual herbicide components (weeks exposed to that herbicide) were compared to rates of evolution of resistance when continuously exposed to that herbicide. The Cox regressions were performed in SPSS. The level of resistance and growth rates in the ancestral environments of the evolved populations were first analyzed using a General Linear Model with the herbicide cycled with and the cycling frequency as fixed factors, and ancestral immigration source as the random factor.

The interaction between herbicide and cycling frequency was also investigated. When populations under a cycling regime evolved resistance to only one of the herbicides, I only analyzed the effects of the cycling frequency, making it a fixed factor. The level of resistance to individual herbicide in cycling regimes was subsequently compared to resistance in the continuous exposure treatment using a Dunnett's corrected paired T-test, with the herbicide regime fitted as a fixed factor, and the ancestral immigration source as the random factor. When some populations in a regime did not evolve resistance, I compared them to the continuous exposure treatment using a Dunnett's corrected T-test. The level of resistance of the three continuous exposure populations was compared in the same fashion. The growth rate in the ancestral environment of all populations was compared to source populations and to populations that underwent continuous exposure using a Dunnett's corrected paired T-test, except when some of the populations in a regime did not evolve resistance, in which case Dunnett's corrected T-test was used. The growth rates in the ancestral environment of the three continuous exposure regimes was compared in the same fashion. Growth rates from the cross-protection assay were compared between the populations that underwent previous exposure to an herbicide and those that did not in a Dunnett's corrected paired T-test. The previous herbicide the population was exposed to was fitted as a fixed factor, and the replicate population as the random factor.

4.3 Results

4.3.1 Dynamics of herbicide resistance

Evolution of herbicide resistance was observed in many populations, under various continuous exposure and cycling regimes. Resistance evolved in all populations with continuous exposure to atrazine (Fig. 9a) or glyphosate (Fig 9b), and to both herbicides in all populations that underwent cycling between these two herbicides (Fig. 9a, b). Resistance evolved in 2 of 6 populations that underwent continuous carbetamide exposure (Fig 9c), while resistance to both atrazine and carbetamide evolved in 3 of 6 populations that underwent weekly cycling between the two (Fig 9a, c). Atrazine, but not carbetamide resistance, evolved in all populations under a bi- and tri-weekly cycle between the two herbicides (Fig 9a, c). No resistant individuals were observed in the populations cycling between glyphosate and carbetamide (Fig. 9b, c). These results demonstrate that cycling can prevent, accelerate or have no impact on the evolution of resistance to herbicides.

Continuous exposure to glyphosate resulted in significantly more rapid evolution of resistance than continuous exposure to atrazine ($z=6.096$, $P<0.05$) or carbetamide ($z=6.083$, $P<0.05$). Rates of evolution of atrazine and carbetamide resistance were not significantly different.

The number of weeks until resistance evolved to individual herbicides in cycling regimes was compared for each regime to the rate of evolution in populations that underwent continuous exposure to that herbicide. Resistance to atrazine evolved more rapidly in a weekly cycle between atrazine and glyphosate ($z=10.169$, $P=0.001$) (Fig. 9a). Though there was a trend towards more rapid evolution of atrazine resistance in the biweekly ($z=3.381$, $P=0.066$) and tri-weekly cycle with glyphosate ($z=3.369$, $P=0.066$), these differences were not significant (Fig. 9a). A weekly cycle between atrazine and glyphosate yielded faster-evolving resistance to glyphosate

than continuous exposure to glyphosate (z=3.930, P=0.047) (Fig. 9b). Rates of evolution of carbetamide resistance were not significantly different between any of the regimes in which it evolved.

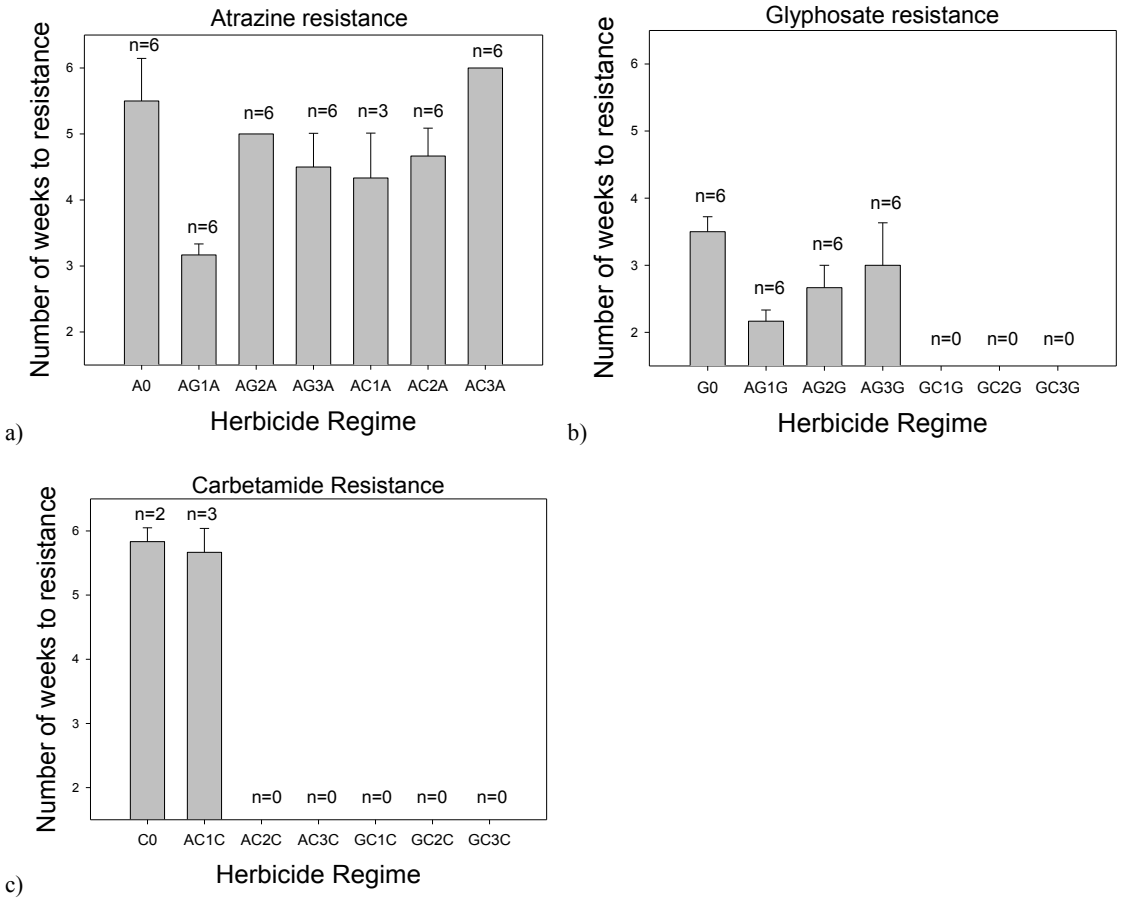


Figure 9. **The dynamics of resistance evolution** measured as number of weeks until resistance evolved. Bars represent the mean weeks to resistance amongst the replicates where resistance was observed; n is the number of replicate populations that evolved resistance. a) atrazine resistance (A0 indicates continuous exposure to atrazine, AG1, AG2, AG3 a weekly, bi-weekly and tri-weekly rotation between atrazine and glyphosate, respectively. AC1, AC2 and AC3 refer to weekly, bi weekly and tri-weekly rotation between atrazine and carbetamide, respectively); b) glyphosate resistance (labelling convention as above); c) carbetamide resistance. Error bars are standard errors of the mean.

4.3.2 Level of resistance

The level of resistance was expressed as the proportion of growth rate retained in populations with evolved resistance in comparison to source populations in herbicide-free environments. In continuous selection regimes, the level of resistance was greater in populations exposed to glyphosate than in atrazine resistant (T_{10}=19.61, P<0.01) and carbetamide resistant populations (T_6=5.963, P<0.005). Carbetamide resistant populations had a higher level of resistance than atrazine resistant populations (T_6=4.854, P<0.01) (Fig.10).

Overall, in cycling regimes, the herbicide that atrazine was cycled with had no significant impact on the level of atrazine resistance. However, the frequency of cycling did significantly affect the level of resistance ($F_{2,16}$=8.10, P<0.005), and there was a significant interaction between the herbicide used and the frequency of cycling ($F_{2,16}$=8.03, P<0.005). As indicated by Dunnett's corrected T-tests, the levels of atrazine resistance that evolved in the AC1 regime were significantly greater than in continuous atrazine exposure regimes (T_7=5.487, P<0.001), as well as all other regimes (Fig. 10a). For glyphosate and carbetamide resistance there were no significant differences in the level of evolved resistance in any of the regimes in which resistance evolved (Fig. 10b and 10c).

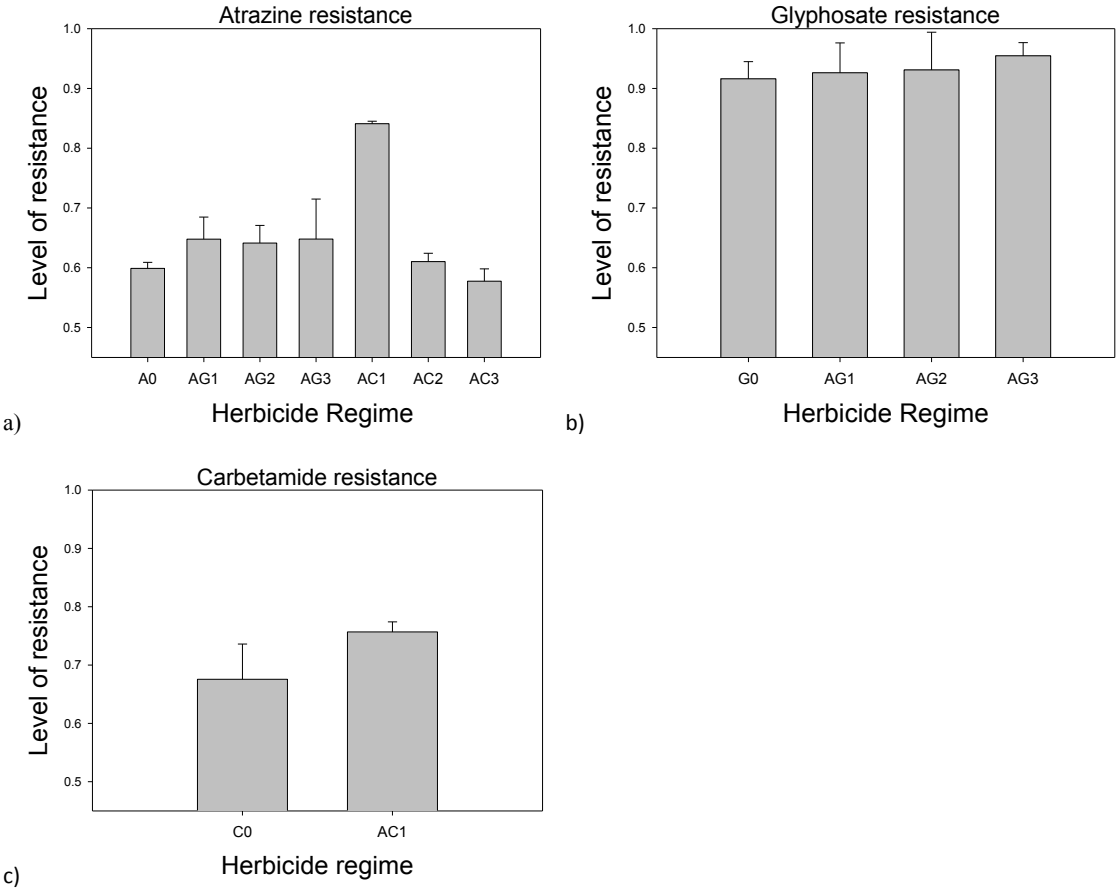


Figure 10. **The level of evolved resistance** expressed as the proportion of growth retained in herbicide environments in comparison with source populations in herbicide-free environments. Bars are mean values of all the evolved replicates in each condition. a) atrazine level of resistance; b) glyphosate level of resistance; c) carbetamide level of resistance. Error bars are standard errors of the mean.

4.3.3 Growth rates in the ancestral environment

Comparing the growth rates in the ancestral environment of evolved populations to the source populations, fitness costs (a significant difference between growth rate in BM of the ancestral and evolved populations) were frequently associated with evolved resistance (Fig. 11). All populations that evolved resistance in continuous exposure to a single herbicide exhibited significant fitness costs – exposure to atrazine (T_{10}=-2.80, P<0.05), glyphosate (T_{10}=-9.76, P<0.001) and carbetamide (T_6=-4.711, P<0.05) (Fig. 11). The growth rates in the ancestral environment of populations evolved under continuous exposure to atrazine were significantly

higher than in the populations evolved in continuous exposure to glyphosate (T_{10}=3.95, P<0.01) or carbetamide (T_6=3.598, P<0.05). Fitness costs were also observed in populations under weekly cycle between atrazine and glyphosate (T_{10}=-5.94, P<0.001) and weekly cycle between atrazine and carbetamide (T_7=-6.034, P<0.001) (Fig. 11). Populations that evolved in a bi- and tri-weekly cycle between atrazine and glyphosate or atrazine and carbetamide did not exhibit significant fitness costs.

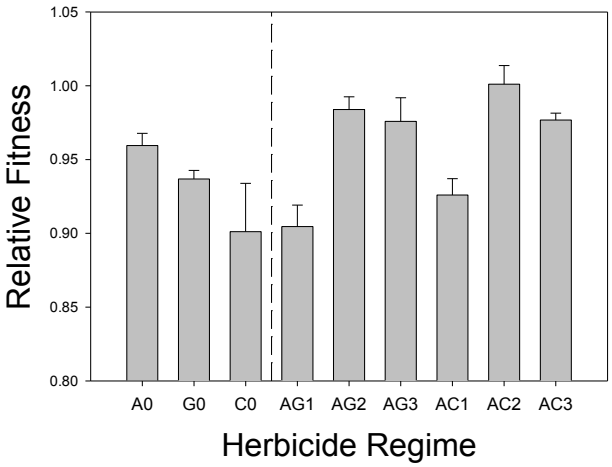


Figure 11: **Growth rates in absence of herbicides** of populations with evolved resistance expressed as the proportion of the source populations' growth rate in herbicide-free environments. Bars are mean values of all the evolved replicates in each condition. Error bars are standard errors of the mean.

4.3.4 Cross-Resistance

For most selection regimes, no cross-resistance was observed (Fig.12). Only the populations selected under a weekly cycle between atrazine and carbetamide and under continuous exposure to carbetamide exhibited cross-resistance to herbicides to which they had never been exposed (Fig.12). All of these populations exhibited growth at the MIC of the herbicide tembotrione. All three populations that evolved resistance to both atrazine and carbetamide under a weekly cycle were also resistant to S-metolachlor and iodosulfuron.

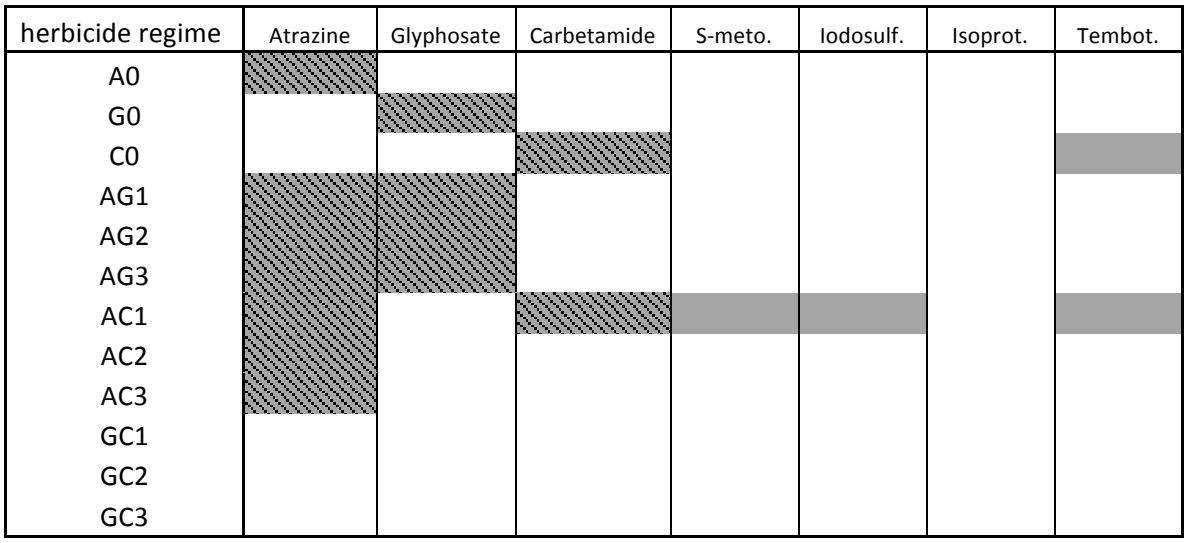


Figure 12: **Cross-resistance profiles for evolved populations**. Hatched shading indicates resistance to herbicides included in corresponding selection regimes. Cross-resistance to herbicides to which populations had no previous exposure is indicated by grey shading.

4.3.5 Cross-Protection

Seven days of exposure to carbetamide significantly increased the growth rates in 0.8MIC of atrazine when compared to the populations that had no previous exposure to any herbicides (Fig. 13) (T_4=7.801, P<0.005). Previous exposure to atrazine significantly increased the growth rates in glyphosate (T_4=7.64, P<0.005), while the exposure to carbetamide decreased subsequent growth rates in glyphosate (T_4=-5.732, P<0.01).

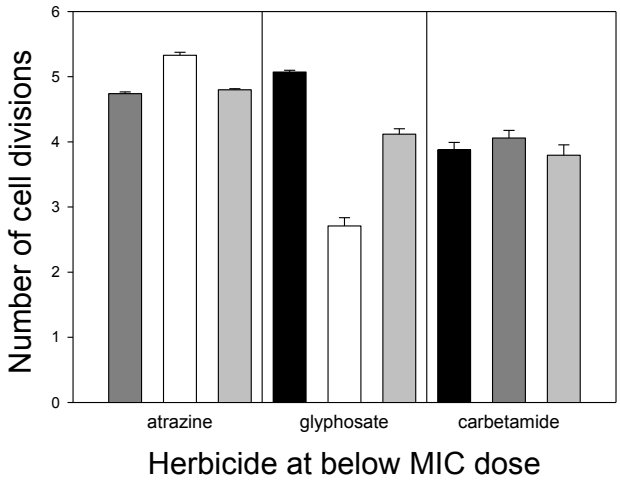


Figure 13. **Cross protection.** Number of cell divisions the populations underwent after four days in below MIC levels of the indicated herbicide. Bars represent mean values. Black bars indicate the populations with previous exposure to atrazine, dark grey bars previous exposure to glyphosate, white bars previous exposure to carbetamide and light grey bars indicate the populations with no previous herbicide exposure. Error bars are standard errors of the mean.

4.4 Discussion

In spite of a lack of evidence for its effectiveness, herbicide cycling has been advocated as a means of slowing or preventing evolution of herbicide resistance (Beckie 2006). A successful cycling strategy must do more than simply extend the chronological time until resistance evolves as this outcome will result simply from the fact that the population is exposed to each component herbicide for less time. A truly effective strategy must increase the time that a population can be exposed (selection-time) to at least one of the cycled herbicides before resistance evolves. In other words, if continuous exposure to herbicide *A* results in evolution of resistance in selection-time *x* and continuous exposure to herbicide *B* results in resistance in selection-time *y*, when *A* and *B* are cycled, the strategy is successful if either *x*, *y* or the sum of *x* and *y* is increased. According to these criteria, in this study, I have shown that cycling between pairwise combinations of three herbicides can slow, accelerate or have no impact on the

dynamics of selection for herbicide resistance. These contrasting outcomes depend on the herbicides being cycled and the frequency of cycling.

4.4.1 Dynamics of resistance under herbicide cycling

Fitness costs associated with resistance are seen as key determinants of the effectiveness of cycling (Leeper et al. 1986; Gressel & Segel 1990; Jasieniuk et al. 1996). In this study, fitness costs (significantly lower growth rates in absence of herbicide) were not universally observed, as found in other studies (McCart et al. 2005; Lopes et al. 2008). Models assuming no fitness costs have predicted that cycling will be ineffective in slowing down the evolution of resistance in selection-time (Diggle et al. 2003; Neve 2008). My results support this general trend, as cycling was most effective when occurring between herbicides where evolved resistance yielded the highest cost (glyphosate and carbetamide), and was much less effective when less costly atrazine resistance evolved (Fig. 9).

It seems somewhat counterintuitive that cycling regimes can, in some instances, increase rates of resistance evolution. I offer two explanations i) cross-protection and ii) population size effects, that can account for increased rates of glyphosate and atrazine resistance evolution, respectively, in the AG regimes. Cross-protection gives rise to a temporary increase in growth rates in one stressful environment after exposure to another (Hill et al. 2002), and a variety of sublethal stresses have been shown to alter antibiotic resistance evolution (McMahon et al. 2006). I have found that exposure to atrazine offers positive cross-protection to glyphosate (Fig.13) and hypothesize that this phenomenon accounts for enhanced rates of glyphosate resistance evolution in the weekly atrazine and glyphosate cycling regime, as it increases the number of non-resistant cells replicating in glyphosate, increasing population size and mutation supply rate. Assuming that cross-protection is a transient effect, this hypothesis is supported by the observation that increased rates of glyphosate resistance evolution are only observed in the

weekly cycle. In relation to increased rates of atrazine resistance evolution in the AG1 regime I conclude that increases in population size, driven by the relatively rapid evolution of glyphosate resistance, are resulting in an increased probability of atrazine resistant mutations arising in the glyphosate resistant background. Once this occurs, atrazine resistance is selected in both phases of the cycling regime and hence evolution of atrazine resistance (measured in selection-time) is accelerated. I predict that this dynamic is likely to occur when rapid cycling occurs between pesticides where the rate of resistance evolution varies substantially.

4.4.2 Impacts of cycling on the evolution of generalists

The frequency of cycling has the potential to change the trajectory of evolution as evidenced by the evolution of a generalist phenotype in the weekly atrazine and carbetamide cycle and no evolution of resistance in bi- and tri-weekly cycles. Even though this generalist phenotype conferred significantly higher levels of atrazine resistance, it was never selected in the continuous atrazine regime. A number of explanations are possible here. It may be that the generalist phenotype requires fixation of more than one mutation and that the initial mutation confers low levels of resistance to atrazine and carbetamide while carrying a high fitness cost. More about the underlying genetics and the number of mutations could have been understood if mating and segregation experiments were carried out. In a weekly cycle, populations are exposed to carbetamide frequently enough that these mutations are maintained whereas in other regimes with more frequent or lengthier periods of exposure to atrazine they are lost due to clonal interference and population bottlenecks. It could also be that the first mutations that get fixed in the population affect the fitness consequences of others, as reported for antibiotic resistance (Yeh et al. 2009; Trindade et al. 2009). Indeed, if the fixation of mutations that confer resistance to atrazine modify the genetic background such that subsequent mutations conferring resistance to carbetamide have a higher selection coefficient (positive epistasis), then

generalists are more likely to evolve in a cyclic environment, compared to a homogeneous one. In general, it appears that the outcomes of herbicide selection regimes are contingent on complex interactions between the level of resistance, costs of resistance, frequencies of different mutations, cross-resistance phenotypes and the scale of temporal heterogeneity.

In the pesticide and antibiotic literature, generalist resistance usually refers to single mechanisms that confer resistance to multiple toxin modes of action (Alekshun & Levy 2007; Delye et al. 2011). The expectation is that generalism will confer lower levels of resistance, often at a higher cost and will therefore only be selected in environments with spatial or temporal variation in selection pressures (Georghiou & Taylor 1986; Futuyma & Moreno 1988; Kassen 2002; Gressel 2009). In this study, broad generalist resistance was selected in the weekly cycle between atrazine and carbetamide, providing some evidence that cycling promotes the evolution of generalist resistance, though in most cycling regimes generalist phenotypes were not observed. Contrary to the major theoretical (Via & Lande 1985; Ravigné et al. 2009), most experimental (Morgan et al. 2009; Hall et al. 2010; Legros & Koella 2010) and the findings in pesticide resistant organisms (Gressel 2002; Jonsson et al. 2010), I found generalists to have a significantly higher resistance than specialists in both of the selective environments, as well as comparable growth rates in absence of herbicides to the specialists (populations that underwent continuous exposure), a result previously reported for other traits (Turner & Elena 2000; Buckling et al. 2006).

4.4.3 Cycling affects fitness costs

The accumulation of multiple, discrete mechanisms of resistance is an alternative means via which a more generalist resistance phenotype may evolve and it seems likely that this accounts for evolved resistance to atrazine and glyphosate in the atrazine and glyphosate cycling regimes. The evolution of this multiple resistance may be constrained by the accumulation of fitness

costs associated with each resistance trait, particularly where these costs are cumulative, or potentially even synergistic. In populations that evolved resistance in a weekly atrazine and glyphosate cycle, the growth rates in absence of herbicide are significantly lower than in continuous exposure to atrazine, and seem to be additive (Fig.11), suggesting there may be a limit to multiple resistance in the absence of compensations (Andersson & Hughes 2010; Hall et al. 2010). Bi- and tri-weekly cycles between atrazine and glyphosate resulted in significantly higher growth rates in absence of herbicides (Fig. 11) than the weekly cycle or continuous exposure to either herbicide. It therefore appears that lower frequencies of cycling favour the compensation of fitness costs as longer periods spent in the non-focal environment will favour selection for reduced costs of resistance. Alternatively, more heterogeneous environments have lower chance of leading to a global optimum (Collins 2011), and as such less rapid rates of cycling could be more effectively selecting for mutations with lower fitness cost.

4.4.4 Herbicide cycling: forward with caution

Herbicide cycling has been advocated for resistance management as it introduces environmental complexity and heterogeneity and thus may slow adaptation. Results from this study illustrate that cycling can result in diverse outcomes, though some caution is advisable in translating results to annual weedy plants. Temporal heterogeneity of environments may impact the direction of evolution (Levins 1968; Kassen & Bell 1998; Jasmin & Kassen 2007), with more fine-grained environments (where environment varies at a rate faster than the generation time) favouring more generalist traits. In my design even the rapid rates of cycling far exceeded the generation time of *C.reinhardtii*, meaning that all the environments were coarse-grained. The herbicide cycling advocated for weed management is fine-grained, generally requiring alternating generations to be exposed to different herbicide modes of action. In addition, the order in which the herbicides are cycled could affect the trajectory of evolution and this was not

explored. *Chlamydomonas* is haploid and reproduction in these experiments was asexual. Higher plants have complex and diverse modes of sexual and asexual reproduction. There may also be gene flow between evolving meta-populations of agricultural weeds. Finally, most annual weedy plants have a soil reservoir of dormant seeds that acts as a temporal refuge from herbicide selection. Notwithstanding these important differences, my results clearly demonstrate that herbicide cycling may not always slow the rate of evolution of resistance and may result in the evolution of generalist resistance phenotypes resistant to a broad range of herbicide modes of action.

CHAPTER 5: HERBICIDE MIXTURES – EFFECTS OF ENVIRONMENTAL HETEROGENEITY ON RESISTANCE EVOLUTION

5.1 Introduction

Mixture strategies that expose weeds to two or more herbicides with different modes of action have been widely advocated for resistance management (Gressel & Segel 1990; Friesen et al. 2000; Powles & Shaner 2001). Similar strategies have been proposed for the prevention of antibiotic resistance (Brown & Nathwani 2005; Powles & Yu 2010) and management of resistance to antiretroviral and anti-cancer drugs (Pastan & Gottesman 1987). Mixture strategies rely on the assumption that mutations conferring resistance to one component of the mixture do not increase fitness in the presence of the second component. Indeed, the most desirable situation arises when there is antagonistic pleiotropy between resistance mechanisms (sometimes referred to as negative cross-resistance (Gressel 2002)). Where the assumptions of independent resistance are met, resistance to the mixture can occurs via spontaneous evolution of resistance mechanisms to both (or all) mixture components (Diggle et al. 2003). The likelihood of this occurring decreases with each additional herbicide in the mixture (Wrubel & Gressel 1994). Alternatively, generalist resistance may be favoured in more complex, multi-herbicide environments (Gressel 2002) and this may compromise the potential efficacy of mixture strategies.

Mathematical models have been used to demonstrate the potential effectiveness of mixtures for herbicide resistance management (Powles et al. 1997; Diggle et al. 2003; Neve 2008). However, these models predominantly focus on the evolution of target-site resistance. Empirical evidence for the efficacy of herbicide mixture strategies is limited and often anecdotal (Beckie 2006), although these studies do tend to confirm the benefits of mixtures over other

management strategies (Manley et al. 2002; Beckie & Reboud 2009). Models exploring the effectiveness of mixtures of insecticides or fungicides for managing resistance provide conflicting evidence for its benefits (Mani 1985; Denholm & Rowland 1992; Russell 2005), as do experimental studies - some supporting mixtures as an effective method of resistance management (McKenzie & Byford 1993; Prabhaker et al. 1998), others cautioning against their widespread use (Immaraju et al. 1990; Blumel & Gross 2001; Castle et al. 2007). It is interesting to compare this to the situation in studies of antibiotic resistance, where clinical trials predominantly report mixtures as effective strategies in slowing resistance evolution (Bergstrom et al. 2004; Brown & Nathwani 2005; Beardmore & Peña-Miller 2010).

Increased economic and environmental costs are a major obstacle to the adoption of herbicide mixtures in agricultural settings (Hart & Pimentel 2002). Short term economic interests favour the use of single herbicides as the level of control achieved prior to the evolution of resistance may often be equivalent, and does not require investment in multiple herbicides (Buttel 2002). From an environmental perspective, herbicide mixtures raise concerns as they increase inputs of pesticides into the environment (Hart & Pimentel 2002). In response to these problems, there have been calls to use synergistic mixtures of herbicides whereby the total combined dose of herbicides in the mixture is reduced (Gressel 1990; Powles & Shaner 2001). The implications of such strategies for resistance evolution are not well understood. In antibiotic resistance it has been shown that synergistic mixtures can exacerbate resistance evolution as appearance of resistance to one of the components leaves a population exposed to an ineffective dose of the other (Hegreness et al. 2008).

Populations of *C.reinhardtii* were experimentally evolved in exposure to mixtures of two or three herbicides with different modes of action (atrazine, glyphosate and carbetamide) at a variety of total combined doses, as well as in single exposure to each of those herbicides. The

objectives of this study were to investigate if (i) mixtures are effective in delaying and/or preventing the evolution of herbicide resistance; (ii) the effectiveness of mixtures is dependent on the total combined dose and the number of herbicides; (iii) increase in the number of herbicides and a reduction in their combined dose increases the likelihood of adaptation towards a generalist optimum.

5.2 Materials and Methods

5.2.1 Herbicides

I selected for resistance to three herbicides – atrazine, glyphosate, and carbetamide (Chapter 2.4). Prior to selection, I determined the minimum inhibitory concentration (MIC) of each herbicide (Chapter 2.4). I also determined the 'MIC equivalent' value when herbicides were used in combination (subsequently MICeq), this being the equal proportion of each herbicide in the mixture that completely inhibited growth of the founding population over seven days. In all pairwise and three-way herbicide mixtures, the growth inhibitory effects of herbicides were synergistic, such that complete growth inhibition was achieved with each herbicide at 45% of its MIC in a two-way, and at 30% of its MIC in the three-way mixture.

5.2.2 Selection regimes

The experimental populations were founded and grown following the conditions outlined in Chapter 2. Three experimental conditions involved continuous exposure to a single herbicide (A0 denoting continuous exposure to atrazine, G0 to glyphosate and C0 to carbetamide). Conditions containing pairwise mixtures of herbicides at MICeq, 50% (MIC), 75% (1.5MIC) and 100% (2MIC) of each herbicide MIC were created (AGeq, AG, AG1.5 and AG2 denoting a mixture between atrazine and glyphosate at MICeq, 50%, 75% and 100% of each herbicide MIC,

respectively). For a three-herbicide mixture, MICeq, 33%, 50% and 66% doses of each herbicide's MIC were used to create selection conditions (AGCeq, AGC, AGC1.5 and AGC2, respectively). Each experimental condition (19 in total) was replicated 6 times, for a total of 114 evolving populations. Six populations were propagated in the absence of herbicides and were used as controls and as source populations to sustain the evolving populations (see chapter 2.5). The evolving populations were transferred as outlined in Chapter 2.5, and the dynamics of resistance evolution monitored by recording the OD_{750} of the population at each transfer. The experiment was carried out for 15 transfer cycles (15 weeks), at which time populations were transferred into BM and allowed to grow for 7 days to multiply evolved populations. Populations were assayed for cross-resistance in tembotrione, iodosulfuron, fluorochloridone, and S-metolachlor, as described in Chapter 2.7. Cross-resistance was studies as a composite measure consisting of the number of novel herbicides the population was resistant to and the level of resistance in those herbicides, and it was used to indicate how generalist the evolved phenotype was.

5.2.3 Statistical analyses

Three questions were addressed – how do (i) the number of herbicides and (ii) the combined dose affect rates of resistance evolution, and (iii) how do rates of resistance evolution compare between dose treatments within herbicide mixture combinations? None of these questions requires a comparison of all treatment groups. Rather than analysing subsets of the data set to address the different questions, the entire data set was analyzed, using appropriate nesting (see below for details of the nesting structure used in each case) to separate treatments of interest from other treatments. This approach ensures that all hypotheses are being tested using the same measure of between-observation variability, and maximises the degrees of freedom (and hence statistical power) associated with the source of variation.

Effect of the number of herbicides. To analyze for the effects of herbicide number, the temporal dynamics of population size were modelled using a linear mixed model within ANOVA (aov function in R 2.15.0). To do so, the regimes that evolved in single herbicide environments were compared to those in mixtures at MICeq doses, as these regimes offered the same initial level of population control and therefore rates of adaptation could meaningfully be compared. Regimes selected in mixtures at MIC, MIC1.5 and MIC2 were not relevant to this question. As discussed above, a nested model was used to allow the hypothesis of interest to be tested based on an analysis of the entire data set. The response variable was population size (measured as OD_{750} at the end of each transfer period). Nested within the entire dataset, an initial fixed term with two levels was fitted; the first level included all treatments relevant to this question (A0, G0, C0, AGeq, ACeq, GCeq, AGCeq), whilst the second level included all other treatments. Within the first level I nested a factor with three levels to allow comparison of the treatments with different numbers of herbicides (one, two or three). Further terms were then nested to account for variation amongst the three single herbicide treatments, and three different herbicide pair treatments. Within the second level of the initial fixed term a nested factor with 12 levels was included to account for variation amongst the 12 treatments that are not directly relevant to this question. The random (error) term consisted of time (weeks, 15 levels) nested within each regime (19 levels), nested within replicate (population, 6 levels). Significance of fixed effects was tested with F-tests.

Effects of combined dose. When investigating the effects of combined dose on the dynamics of resistance, I was only interested in regimes with more then one herbicide as the single herbicide environments had only one dose. Similar approaches to the above were adopted to partition the data within the entire dataset. An initial fixed term was nested within the entire dataset and separated into two levels: the 16 treatments of interest (all of the regimes involving more than

one herbicide) and the remaining three treatments (A0, G0 and C0). Within the first level, two nested factors accounted for the variation due to differences between herbicide mixtures (AG, AC, GC, AGC), and due to differences between doses (4 different levels), and the fixed model also included the interaction between these two factors to account for all variation among the 16 treatments of interest. Within the second level, a nested factor accounted for the variation between the three single herbicide treatments (though not of direct interest for this question). The error term was same as above, and the significance of fixed effects was tested with F-tests.

Comparing the time of resistance evolution in selection regimes. To analyse the dynamics of resistance evolution in herbicide mixtures and single herbicide exposure regimes, OD_{750} as the response was modelled in a further set of linear mixed models using ANOVA in GenStat (13^{th} edition). I separately modelled resistance for regimes associated with each herbicide mixture (AG, AC, GC, AGC), enabling comparison between all four dose regimes for each mixture as well as the two or three relevant single herbicide regimes (i.e. A0 and G0 for the AG mixture, and all three single herbicide conditions for the AGC mixture), following the nesting approach outlined above. An initial term in each model compared the mean for the six or seven regimes of interest with the mean of the remaining treatments, with nested terms accounting for the variation among the treatments not of direct interest. Each model also included the time term, using a series of linear contrasts to identify the time periods over which there were changes in the level of resistance across the six or seven treatments of interest, and the interaction of these contrasts with the treatment terms identified above, to detect where there were differences in the patterns of resistance evolution between conditions. Each linear contrast assessed the slope of the linear regression over four consecutive time points (the first for weeks 1-4, the second for weeks 2-5, and so on), allowing identification of both the first point and last point at which a significant change in resistance was seen for each condition. To illustrate, as all regimes started

with a slope of linear regression that was not significantly different from 0 (no resistance), the point when a slope of one regime started becoming significantly different from the slopes of other regimes indicated when resistance in that regime started evolving. It was in this way that the rates or resistance evolution were analyzed as a comparison between the linear regression slopes at each of 12 contrasts to assess the time when each population started exhibiting measurable growth. These 12 linear contrasts are not independent, so that they do not provide a complete partitioning of the between-time variation, and some care is needed in the interpretation of significant effects for overlapping periods.

Cross-resistance. The differences in the cross-resistance profile of selected populations were analyzed by ANOVA with population growth after seven days (measured as OD_{750}) as the response variable. Fixed factors were genotype (selection regime, 14 levels, as the regimes that did not give rise to any resistant populations were excluded) and environment (novel herbicide environment, 4 levels), while the error term consisted of the source population. I was particularly interested in the genotype x environment interaction as this represents the differences in the range of novel herbicides that a population expressed cross-resistance to. A subsequent analysis was conducted using Tukey's honestly significant pairwise tests between the mean OD_{750} of the populations selected in each regime across all four novel herbicide environments. This test treated cross-resistance as a composite measure that included both the number of herbicides a population was resistant to and the growth rates achieved in each of those herbicides.

5.3 Results

5.3.1 Dynamics of herbicide resistance

Evolution of resistance. Adaptation to the selection regimes occurred in many experimental populations, under various single- and multiple-herbicide conditions. Resistance (defined here as elevated growth rates in herbicide regimes) evolved in all populations under exposure to atrazine and glyphosate, and in two of six populations under carbetamide exposure (Fig. 14). Resistance was observed in all populations exposed to mixtures of atrazine and glyphosate at MICeq, MIC and MIC1.5, as well as in four populations at AG2 (Fig. 14a). Populations exposed to a mixture of atrazine and carbetamide evolved resistance in three populations at ACeq and two populations at AC. Resistance did not evolve in AC regimes at AC1.5 or AC2 (Fig. 14b). Mixtures of glyphosate and carbetamide gave rise to resistance in all populations evolving at GCeq and GC, two populations at GC1.5, and was never observed at GC2 (Fig. 14c). In the three-herbicide regimes, resistance evolved in all populations at AGCeq and AGC, in two populations evolving at AGC1.5 and never at AGC2 (Fig. 14d).

Effects of herbicide number and combined dose. A significant effect of the number of herbicides in the mixtures on the dynamics of resistance evolution (measured as the mean population size at transfer over the 15 week selection regime) was identified, with resistance evolving more slowly with an increase in the herbicide number ($F_{2,90}$=7.85; P<0.001). Increase in the total combined dose slowed resistance evolution, as the interaction between herbicide mixture and overall herbicide dose was significant ($F_{9,90}$=6.49; P<0.001).

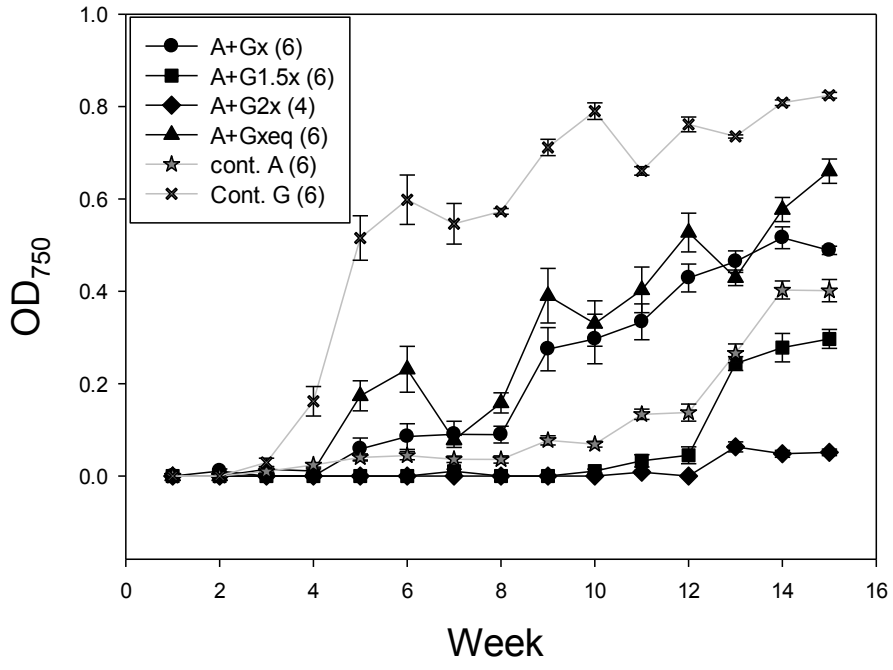
Rates of resistance between regimes. The rates of resistance evolution were analyzed as a comparison between the linear regression slopes at each of 12 contrasts and the F statistic indicating the differences between all 6 or 7 treatments at each time interval is reported (Tables

3-6). Considering comparisons between the AG mixtures and continuous exposure to glyphosate or atrazine (Fig.14a; Table 3), resistance to the continuous glyphosate regime was first observed (between weeks 2-5, $F_{5,90}$=16.50; P<0.001). Resistance in populations exposed to AG and AGeq followed (between weeks 6-9, $F_{5,90}$=2.84; P=0.015), with the populations exposed to atrazine (A0) and AG1.5 evolving resistance subsequently (between weeks 10-13, $F_{5,90}$=2.43; P=0.004). Resistance evolved most slowly in populations selected at AG2, and since it occurred only in four populations near the end of the selection procedure. Growth rates (slopes of regression lines) for AG2 populations never became significantly different from 0.

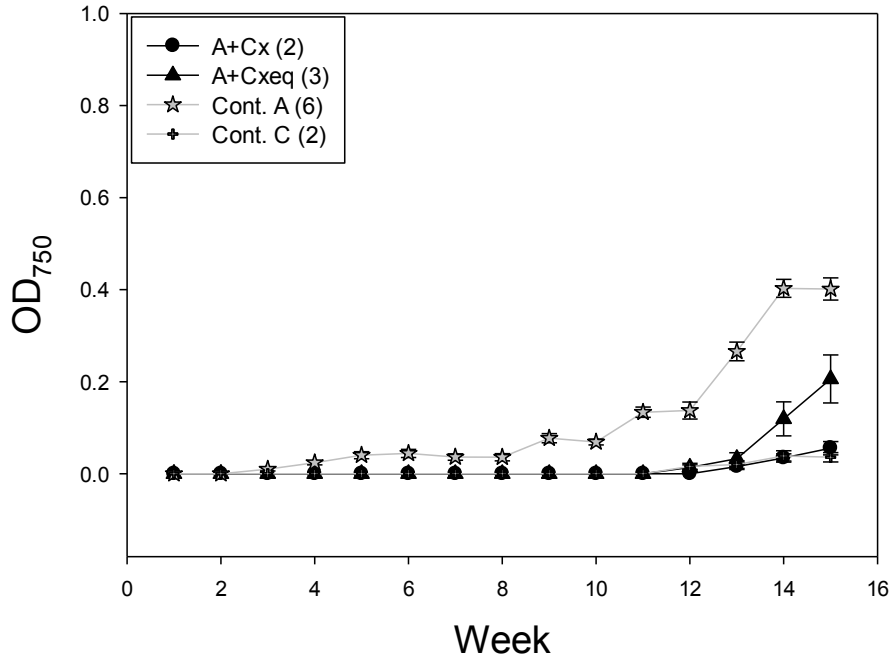
In populations exposed to mixtures of atrazine and carbetamide and the individual component herbicides (Fig.14b, Table 4), the populations exposed to atrazine evolved resistance first (between weeks 10-13, $F_{5,90}$=2.34; P=0.048), closely followed by the populations growing at ACeq (between weeks 11-14, $F_{5,90}$=5.07; P<0.001). The slopes of regression lines for exposure to carbetamide (C0), AC, AC1.5 and AC2 never become significantly different from 0.

In the GC comparisons, resistance evolved most rapidly in the populations exposed to glyphosate only (between weeks 2-5, $F_{5,90}$=16.93; P<0.001). Populations exposed to GCeq were the second to evolve resistance (between weeks 9-12, $F_{5,90}$=5.05; P=0.001), with the populations exposed to GC exhibiting resistance in the subsequent interval (between weeks 10-13, $F_{5,90}$=10.12; P<0.001) (Fig.14c, Table 5).

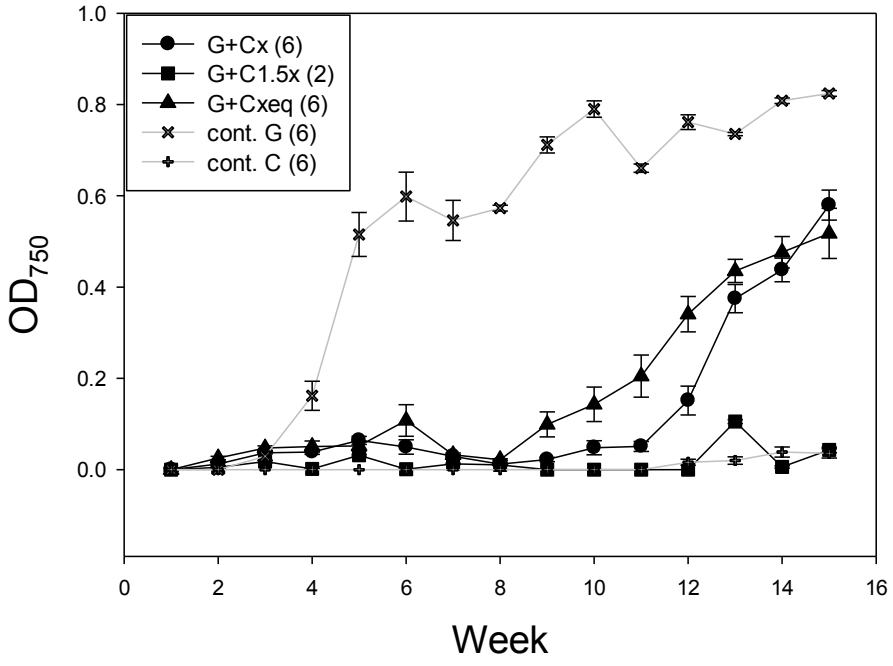
In the AGC comparisons, resistance evolved most rapidly in the G0 regimes ($F_{6,90}$=15.43; P<0.001), followed by the populations selected at AGCeq and in A0 (between weeks 10-13, $F_{6,90}$=6.32; P<0.001). Exposure to AGC of the mixture gave rise to resistance in the subsequent interval (between weeks 11-14, $F_{6,90}$=6.21; P<0.001) (Fig.14d, Table 6).


A+Gx (6)
A+G1.5x (6)
A+G2x (4)
A+Gxeq (6)
cont. A (6)
Cont. G (6)
OD750
Week

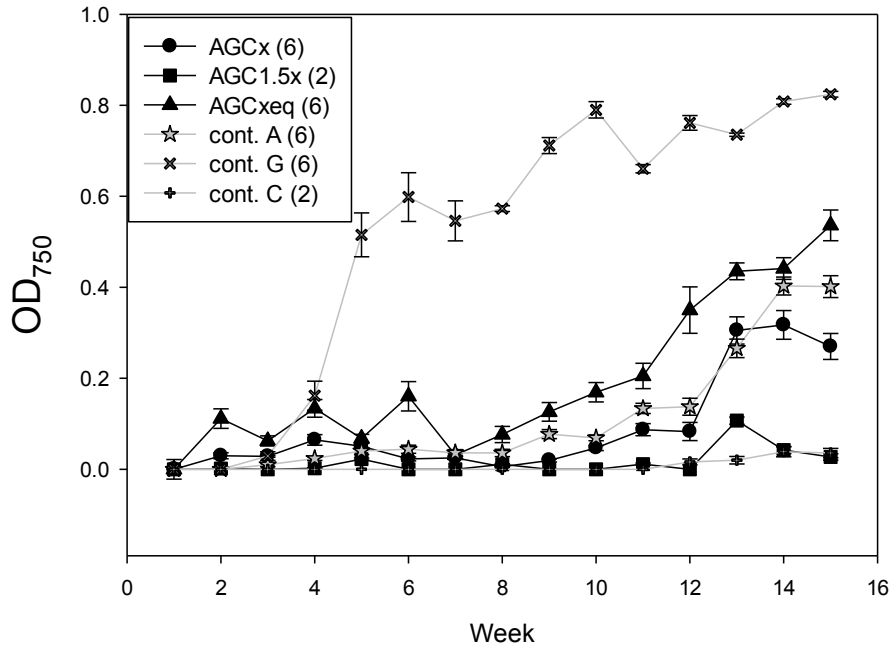
a)


A+Cx (2)
A+Cxeq (3)
Cont. A (6)
Cont. C (2)
OD750
Week

b)



c)



d)

Figure 14. **Mean population size at transfer (measured as OD_{750}) during 15 weeks of selection** to herbicide selection regimes. a) Dynamics of resistance in regimes containing mixtures of atrazine and glyphosate ; b) atrazine and carbetamide; c) glyphosate and carbetamide; d) atrazine, glyphosate and carbetamide. Individual selection regimes are indicated in the legend with the number of replicates (of 6) in which resistance evolved shown in parentheses. Bars are standard errors of the mean.

Table 3: **Comparisons of dynamics of resistance evolution for different dose regimes in atrazine and glyphosate (AG) mixtures**. Values are average slopes of linear regression lines for each consecutive four-week interval between all evolving populations in a regime. The 'comparison F value' gives the value of the F test investigating the effect of the regime on the slope during the particular 4-week interval. * indicates a 4 week interval during which the mean slope was significantly different from all other unmarked regimes. **Bold** values indicate the first week when the differences were significant.

week interval	1to4	2to5	3to6	4to7	5to8	6to9	7to10	8to11	9to12	10to13	11to14	12to15
A0	0.008	0.013	0.012	0.004	0.002	0.010	0.014	0.029	0.025	**0.059***	0.094*	0.093*
G0	0.051	**0.168***	0.206*	0.124*	0.012	0.037	0.087	0.034	0.005	-0.006	0.042	0.026
AGeq	0	0.029	0.073	0.033	0	**0.056***	0.099*	0.067*	0.009	0.042	0.042	0.055
AGx	0	0.014	0.032	0.030	0	**0.057***	0.080*	0.075*	0.031	0.030	0.038	0.023
AG1.5x	0	0	0	0.003	0	0	0	0.011	0	**0.071***	0.093*	0.079*
AG2x	0	0	0	0	0	0	0	0.003	0	0.018	0.018	0.014
$F_{(5,90)}$ value	1.63	16.50	24.85	8.67	0.13	2.84	8.57	3.42	1.85	2.43	3.62	4.15
Significance (P value)	0.150	<0.001	<0.001	<0.001	0.986	0.015	<0.001	0.005	0.101	0.004	0.003	<0.001

Table 4: **Comparisons of dynamics of resistance evolution for different dose regimes in atrazine and carbetamide (AC) mixtures**. Values are average slopes of linear regression lines for each consecutive four-week interval between all evolving populations in a regime. The 'comparison F value' gives the value of the F test investigating the effect of the regime on the slope during the particular 4-week interval. * indicates a 4 week interval during which the mean slope was significantly different from all other unmarked regimes. **Bold** values indicate the first week when the differences were significant.

week interval	1to4	2to5	3to6	4to7	5to8	6to9	7to10	8to11	9to12	10to13	11to14	12to15
A0	0.008	0.013	0.012	0.004	0.002	0.010	0.014	0.029	0.025	**0.059***	0.094*	0.093*
C0	0	0	0	0	0	0	0	0	0.005	0.008	0.012	0.008
ACeq	0	0	0	0	0	0	0	0	0.004	0.011	**0.038***	0.066*
ACx	0	0	0	0	0	0	0	0	0	0.005	0.012	0.019
AC1.5x	0	0	0	0	0	0	0	0	0	0	0	0
AC2x	0	0	0	0	0	0	0	0	0	0	0	0
$F_{(5,90)}$ value	0.04	0.12	0.09	0.01	0.00	0.06	0.13	0.53	0.36	2.04	5.07	6.05
Significance (P value)	0.999	0.988	0.933	1.000	1.000	0.997	0.986	0.752	0.878	0.071	<0.001	<0.001

Table 5: **Comparisons of dynamics of resistance evolution for different dose regimes in glyphosate and carbetamide (GC) mixtures**. Values are average slopes of linear regression lines for each consecutive four-week interval between all evolving populations in a regime. The 'comparison F value' gives the value of the F test investigating the effect of the regime on the slope during the particular 4-week interval. * indicates a 4 week interval during which the mean slope was significantly different from all other unmarked regimes. **Bold** values indicate the first week when the differences were significant.

week interval	1to4	2to5	3to6	4to7	5to8	6to9	7to10	8to11	9to12	10to13	11to14	12to15
G0	0.051	**0.168***	0.206*	0.124*	0.012	0.037	0.087*	0.034	0.002	-0.006	0.042	0.026
C0	0	0	0	0	0	0	0	0	0.005	0.008	0.012	0.008
GCeq	0.017	0.008	0.018	0	0	0	0.041	0.059	**0.079***	0.101*	0.091*	0.057*
GCx	0.014	0.016	0.006	-0.004	0.018	0	0	0.014	0.039	**0.108***	0.138*	0.135*
GC1.5x	0.002	0.006	-0.002	0	0.005	0	-0.005	0	0	0.032	0.012	0.003
GC2x	0	0.008	0.003	0	0.002	0	-0.004	0	0	0.005	0.002	-0.001
$F_{(5,90)}$ value	1.54	16.93	26.70	10.16	0.49	1.07	0.19	2.58	5.05	10.12	11.61	10.66
significance (P value)	0.175	<0.001	<0.001	<0.001	0.785	0.373	<0.001	0.025	0.001	<0.001	<0.001	<0.001

Table 6: **Comparisons of dynamics of resistance evolution for different dose regimes in atrazine, glyphosate and carbetamide (AGC) mixtures**. Values are average slopes of linear regression lines for each consecutive four-week interval between all evolving populations in a regime. The 'comparison F value' gives the value of the F test investigating the effect of the regime on the slope during the particular 4-week interval. * indicates a 4 week interval during which the mean slope was significantly different from all other unmarked regimes. Bold values indicate the first week when the differences were significant.

week interval	1to4	2to5	3to6	4to7	5to8	6to9	7to10	8to11	9to12	10to13	11to14	12to15
A0	0.008	0.013	0.012	0.004	0.002	0.010	0.014	0.029	0.025	0.059*	0.094*	0.093*
G0	0.051	0.168*	0.206*	0.124*	0.012	0.037	0.087*	0.034	0.002	-0.006	0.042	0.026
C0	0	0	0	0	0	0	0	0	0.005	0.008	0.012	0.008
AGCeq	0.035	-0.006	0.023	-0.021	0.010	0.006	0.046	0.043	0.071	0.094*	0.079*	0.056
AGCx	0.019	0.010	-0.003	-0.015	0.013	0.003	0.008	0.027	0.023	0.077	0.091*	0.057
AGC1.5x	0.001	0.006	0	0	0	0	0	0	0	0.031	0.020	0.002
AGC2x	0	0	0	0	0	0	0	0	0	0	0	0
$F_{(6,90)}$ value	1.62	15.43	23.15	9.84	0.28	0.83	4.39	1.44	2.57	6.32	6.21	4.95
significance (P value)	0.137	<0.001	<0.001	<0.001	0.945	0.545	<0.001	0.197	0.018	<0.001	<0.001	<0.001

5.3.2 Patterns of cross-resistance

An overall effect of the regime-by-herbicide (genotype-by-environment) interaction ($F_{42,295}$=3.37, P<0.001) was identified, indicating the emergence of phenotypes with different cross-resistance profiles (Table 7). Populations evolving at MIC and MICeq of a three herbicide mixture were significantly more cross-resistant than all other evolved populations, with the exception of the populations evolved in a mixture of atrazine and glyphosate at MIC (Table 7). There were no significant differences in cross-resistance between populations that evolved in any other regimes.

Table 7. **Patterns of cross-resistance** measured as populations growth (mean OD_{750}) after 4 days of growth in a novel herbicide for each regime/standard error of the mean. F - fluorochloridone; T - tembotrione; I - iodosulfuron-methyl-sodium; and S - s-metolachlor.

Regime (genotype)	F	I	S	T
A	0/0	0.021/0.021	0/0	0/0
C	0/0	0/0	0/0	0.055/0.035
G	0/0	0/0	0/0	0/0
A+Geq	0/0	0.067/0.031	0/0	0.081/0.028
A+Gx	0/0	0.118/0.039	0/0	0.06/0.028
A+G1.5	0/0	0/0	0/0	0/0
A+G2	0/0	0/0	0/0	0/0
A+Ceq	0/0	0/0	0/0	0.232/0.087
A+Cx	0/0	0/0	0/0	0.065/0.041
G+Ceq	0/0	0.048/0.033	0/0	0/0
G+Cx	0/0	0.144/0.028	0/0	0/0
G+C1.5	0/0	0.0532/0.034	0/0	0/0
AGCeq	0.073/0.033	0.084/0.042	0/0	0.186/0.017
AGCx	0.139/0.030	0.127/0.03	0/0	0.096/0.044
AGC1.5	0/0	0/0	0/0	0/0

5.4 Discussion

Results indicate that herbicide mixtures may be successful at preventing or slowing evolution of resistance when all components are used at or close to the MIC. The benefits of increasing the number of herbicides in the mixture depend on the combined dose in the mixture: lower

combined doses of a three-way mixture led to significant levels of cross-resistance, while higher combined doses were successful at preventing adaptation in those regimes.

5.4.1 Lower combined doses of mixtures do not effectively slow resistance evolution

Regardless of herbicide identity, populations exposed to the two lowest combined doses (MICeq and MIC) evolved resistance more rapidly to the mixture than they did when exposed to the least resistance-prone of the mixture components at MIC (Fig.14). At lower combined doses, resistance is likely to evolve rapidly to the more resistance-prone component of the mixture, leaving populations exposed to lower-than-MIC doses of the other herbicide(s). Such dynamics allow populations to rapidly circumvent the effectiveness of mixture strategies as these elevated growth rates enable rapid population growth and this in turn may increase mutation supply rates for rarer mutations that increase population fitness in the presence of the second (and further) herbicide(s) (Drlica 2003; Busi & Powles 2009; Powles & Yu 2010). As such, low dose mixture strategies may facilitate the accumulation of multiple resistance mechanisms in the same individual (Wrubel & Gressel 1994; Busi & Powles 2009; Powles & Yu 2010). Growth assays conducted at the termination of selection procedures indicated that this was likely the case in this study as all populations that had evolved resistance to mixture regimes were individually resistant to all mixture components at MIC. An alternative explanation is that exposure to lower doses selected for generalist mutation(s) that provide resistance to all herbicides in the mixture (Neve & Powles 2005a; Powles & Yu 2010). If the number of mutations required for such a mechanism is low, resistance could emerge as rapidly as was observed. Appearance of such a mechanism would have to be dose specific, as it was not observed at higher combined doses. The findings are in line with some previous studies (Immaraju et al. 1990; Birch & Shaw 1997), indicating that the use of equivalent or lowered MICs poses a significant risk for resistance

management as resistance to these mixtures may evolve more rapidly then to single herbicides at high relative doses (Fig. 14).

5.4.2 Mixtures increase the likelihood of cross-resistance

The requirements for successful mixture strategies (Wrubel & Gressel 1994; Powles et al. 1997; Diggle et al. 2003; Neve 2008) may be overcome if evolution proceeds towards a single generalist phenotype instead of requiring resistance to multiple herbicides through independent mutations (multiple resistance) (Rubin 1991; Elad et al. 1992; Beckie 2006). A significant trend towards cross-resistant phenotypes was observed as the number of herbicides in the mixture was increased (Table 7). Increase in the number of herbicides can lead to a generalist optimum either because the likelihood of acquiring non-target site resistance is greater than the likelihood of acquiring multiple resistance mutations; and/or because the accumulation of fitness costs associated with each independent resistance becomes too large (Manley et al. 2002; Beckie & Reboud 2009; Poisot et al. 2011). From an applied perspective, use of more complex mixtures elevates the risk for management, as wider cross-resistance patterns can reduce the number of available herbicides that could be used for subsequent control.

5.4.3 Mixtures in a wider applied setting

As in medical settings, where high doses of multiple antibiotics have to be balanced against toxicity to patient cells (Mani 1985; Denholm & Rowland 1992; Russell 2005; Gluckman et al. 2011), the use of multiple pesticides in agricultural settings has to be considered in the light of environmental concerns and economic constraints (McKenzie & Byford 1993; Prabhaker et al. 1998; Carroll et al. 2011). The results, in line with previous studies (Immaraju et al. 1990; Gressel 1997; Blumel & Gross 2001; Diggle et al. 2003; Russell 2005; Beckie 2006; Castle et al. 2007), support the use of mixtures at full dose of each component herbicide. This study shows that reductions in the combined dose lead to more rapid resistance and potentially to cross-resistant

phenotypes, questioning the suitability of mixtures for sustainable management unless these can be applied at high doses.

Antibiotics acting synergistically – offering the same control of susceptible populations at lower combined doses (Bergstrom et al. 2004; Brown & Nathwani 2005; Trindade et al. 2009; Beardmore & Peña-Miller 2010) – have been shown to elevate rates of resistance evolution (Hart & Pimentel 2002; Michel et al. 2008), as a lower effective dose is experienced once resistance evolves to one of the components in synergistic mixtures, as opposed to a mixture of non-interacting or antagonistic antibiotics (Buttel 2002; Hegreness et al. 2008). The results support these findings and extend the implications to alterations of the dose of components in a mixture. In line with previous studies (Hart & Pimentel 2002; Manley et al. 2002; Beckie 2006; Neve et al. 2011), the importance of the composition of the xenobiotic mixture is also highlighted, as the rates of evolution in a mixture depend on how resistance-prone individual components are.

CHAPTER 6. EFFECTS OF HERBICIDE DOSE – LOCAL ADAPTATION ALONG AN ENVIRONMENTAL GRADIENT

6.1 Introduction

There is an increasing recognition of, and interest in, the potential for anthropogenic environmental change to illicit rapid evolutionary responses (Palumbi 2001; Carroll et al. 2011). When fitness is compared across environments, local adaptation is evident if populations that evolve under focal conditions (sympatric populations) exhibit higher fitness than populations that adapt in response to other environments (allopatric populations) (Kawecki & Ebert 2004). The majority of studies that experimentally explore local adaptation compare fitness across environments that differ qualitatively - adaptation to novel parasites and hosts (Greischar & Koskella 2007; Eizaguirre & Lenz 2010); adaptation to different food sources (Fraser et al. 2011); soil environments (Belotte et al. 2003), or across different geographic areas (Sanford & Kelly 2011). Less well studied is the long-term evolution of local adaptation on a single-stressor environmental gradient, with previous studies focused on adaptation along a temperature gradient (Bennett et al. 1992). Anthropogenic environmental change results from, amongst other things, climate change, pollution, as well as the use of pesticides in agriculture and antibiotics in medicine. Many of these changes manifest as gradients of environmental change in space and time and so it is becoming increasingly important to understand the nature of adaptation across these gradients to explain current patterns of adaptation and to predict likely future responses in continuously changing environments (Moser & Bell 2010).

Herbicides are used globally to control undesirable weeds in agricultural crops and the evolution of resistance to herbicides is ubiquitous (Powles & Yu 2010). The propensity for different doses of xenobiotics to more or less rapidly select for resistance is of fundamental importance in resistance management (Blackshaw et al. 2006; Isturiz 2010). The economic and environmental

pressures to reduce the utilized dose of herbicides (Doyle & Stypa 2004; O'Donovan et al. 2007) are in opposition to the demonstrated rapid selection for resistance at suboptimal doses for herbicides (Neve & Powles 2005b; Busi & Powles 2009; Manalil et al. 2011), insecticides (Roush & McKenzie 1987), fungicides (Shaw 2006) and antibiotics (Andersson & Hughes 2012).

The question of whether selection along a single stressor gradient leads to local adaptation is about the relationship between the direct (response in the sympatric environment) and correlated responses (in allopatric environments) to selection. Along an environmental gradient, fitness is predicted to decrease as the environmental distance from the adapted conditions increases (Moser & Bell 2010), giving rise to local adaptation. One potential form of genotype-by-environment interaction giving rise to variation between populations selected along a gradient resulting in local adaptation is antagonistic pleiotropy, occurring when mutations exhibit trade-offs in different environments (Hedrick 1986). While theory often requires antagonistic pleiotropy in order to explain local adaptation (Kawecki & Ebert 2004), trade-offs are not always identified in populations adapting to constant environments (Hereford 2009), questioning the frequency of local adaptation in such conditions. Along a single-stressor gradient, antagonistic pleiotropy arises if greater adaptation at the selected dose is accompanied by a greater fitness cost in non-selected environments. If mutations of larger effect are favoured at higher xenobiotic doses (Gressel 2002; Kawecki & Ebert 2004) and if they are associated with higher fitness costs (Sousa et al. 2012), antagonistic pleiotropy would give rise to local adaptation along a gradient. Previous studies of adaptation along a temperature gradient did not identify such trade offs (Bennett et al. 1992). Mutation accumulation is often contrasted to antagonistic pleiotropy as a potential mechanism giving rise to fitness costs in novel environments and therefore local adaptation (Heller & Smith 1978). In prolonged exposure to stable conditions, mutation accumulation arises from accumulation of mutations

that are neutral in that environment, but detrimental in others (Lynch et al. 1995; 1999). The availability and the rate of accumulation of such mutations is instrumental in determining whether such effects will be observed (Nakayama et al. 2012).

A third form of genotype-by-environment interaction that could give rise to local adaptation occurs when the variance of fitness effects of beneficial mutations increases with the harshness of the environment (Chevin et al. 2010). Selection at different points along the gradient could magnify or conceal fitness differences between mutations (Charmantier & Garant 2005), such that mutations with comparable fitness effects at lower xenobiotic doses would differentiate as the dose increased, giving rise to the environment-dependent differential fitness effect of beneficial mutations (Fig.15). In other words, this mechanism can arise when responsiveness – a component of genotype-by-environment interaction due to differences in variances among genotypes (Bell 1990a) - is differential across environments, so that the variance in fitness changes along the gradient. If true, such a mechanism would give rise to local adaptation as certain mutations would provide greater fitness benefit at higher doses, while at lower doses a larger number of mutations could be selected for as the fitness benefits are similar. To illustrate, consider an efflux mechanism secreting the xenobiotic from the cell (Van Bambeke et al. 2003a). Two mutations giving rise to such resistance could differ with respect to the number of molecules they could remove per unit time. At lower doses, their effectiveness would be equivalent, and would only differentiate as the number of xenobiotic molecules present increased. Such a differential response would result in local adaptation in populations selected at higher but not lower xenobiotic doses. Non-parallel reaction norms have been observed in deleterious mutations, with mutation effect on fitness becoming greater (aggravated) (Fernández & López-Fanjul 1997; Remold & Lenski 2001) or alleviated (Kishony & Leibler 2003) under environmental stress.

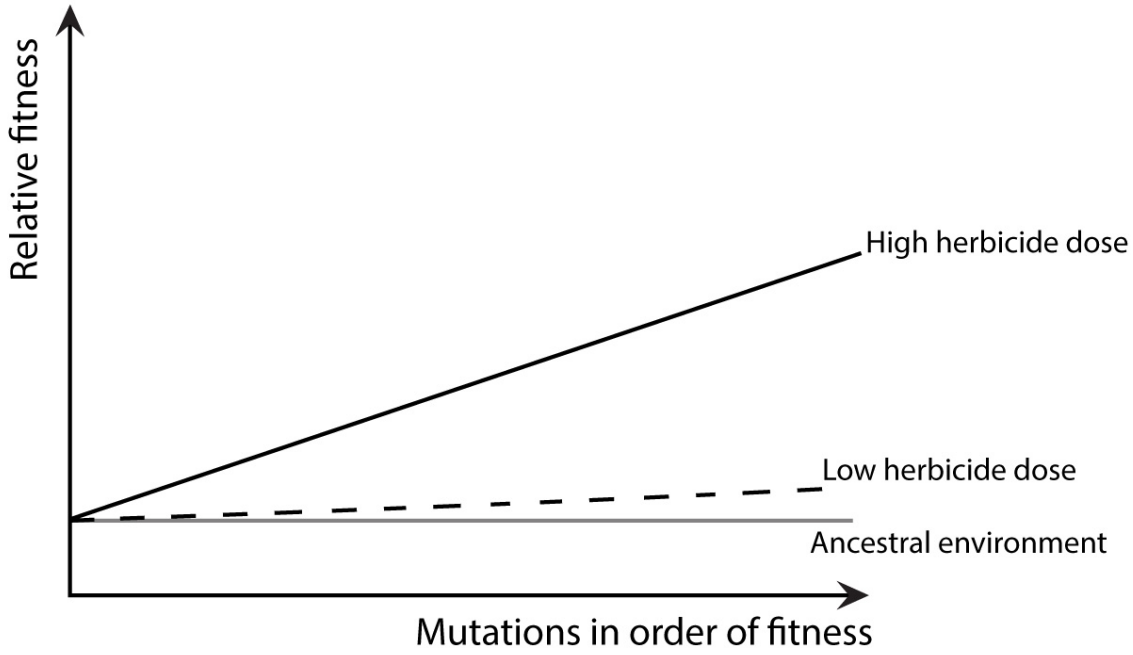


Figure 15. **Differential responsiveness.** Three environments are considered – ancestral (grey), low (broken line) and high herbicide dose (full line). The differences in fitness benefits of mutations (relative fitness) are greater in a more stressful environment, while in the absence of fitness costs they are indistinguishable at lower doses and in the ancestral environment. The rank order of fitness of mutations is assumed to be the same across all environments.

Local adaptation should result in the population being best adapted to its focal environment (Kawecki & Ebert 2004), but the driving mechanisms on a continuous environmental gradient are not well understood. Local adaptation can be studied in two ways. One approach is to compare growth of a population in a range of environments and identify local adaptation if the population exhibits highest growth rates in the environment it was selected in. This approach treats each population independently, and because it compares growth across a range of environments, it assumes comparable quality of different habitats (Kawecki & Ebert 2004). This conjecture is likely not true along a xenobiotic gradient, where higher doses exert greater pressure on a population. In addition, due to the adopted definition of MIC, difference in growth of a source population above MIC remain unknown, preventing the comparison between those

points. An alternative approach avoids making this assumption, and views local adaptation as a comparative property. At each dose, the growth rates of a population selected under that condition (sympatric population) are compared to the growth rates of all other populations when grown at that dose (allopatric populations). A locally adapted population exhibits higher fitness in its selected environment compared to other populations in that same environment (Kawecki & Ebert 2004). Local adaptation was studied in this way in order to address whether selection at below-optimal herbicide doses can give rise to comparable levels of resistance at the administered dose. This applied question is in contrast to the theoretical tested hypotheses: (i) whether the selection along the environmental gradient results in populations that are better adapted to their local (sympatric) environment; and (ii) if increasing the environmental distance from its selected conditions reduces the population's fitness;

6.2 Materials and Methods

6.2.1 Selection procedure

The populations were founded and grown under conditions described in Chapter 2. Five replicate experimental populations were exposed to five doses (0.5MIC, 0.75MIC, MIC, 1.25MIC and 1.5MIC) of each of the four herbicides used – atrazine, glyphosate, iodosulfuron and S-metolachlor (atrazine, for example – A.5, A.75, A1, A1.25 and A1.5). Each experimental condition (20 in total) was replicated 5 times, giving rise to 100 independently evolving populations. Five lines, which were propagated in the absence of herbicides, were also inoculated at this stage, and were used as controls and also as source populations to sustain the evolving populations. A weekly transfer into appropriate media was carried out as outlined in Chapter 2.5.

6.2.2 Local adaptation

Upon completion of selection procedures, all populations exhibiting growth at their selected herbicide dose were transferred into fresh culture containing only BM and multiplied for seven days. Subsequently, growth rates in the presence of herbicide were measured for each population in their sympatric (selected) environment, as well as in all experimental concentrations of that herbicide to which resistance evolved (allopatric environments). Assays were inoculated with 125,000 cells and assays were replicated twice. The OD_{750} was measured after seven days of growth and used to estimate the number of cell divisions that the population underwent. The growth rate of each evolved population in the absence of herbicides was also measured (see Chapter 2.6).

6.2.3 Statistical analyses

To test for local adaptation at each herbicide dose for each of the four herbicides, the mean number of cell divisions undergone by sympatric populations was compared to the combined mean of the allopatric populations, a significantly higher number of divisions for sympatric populations being indicative of local adaptation. This analysis was conducted using a linear mixed effects model (nlme function in lme package in R 2.15.0) with the number of cell divisions after seven days of growth as the response variable. In order to maintain the same underlying estimate of between-observation variability and maximize the degrees of freedom (and therefore increase statistical power), I adopted an approach whereby the entire dataset for each herbicide was analyzed, separating the treatments of interest from other treatments using appropriate nesting, in a manner similar to the analysis described in Chapter 5.2.3. As such, each division of data into sympatric and allopatric populations at each herbicide dose (2 levels at each dose) was fitted as fixed factors nested within the entire dataset for that herbicide, with further nesting of component regimes within the allopatric mean to capture the variability between

them. For example, when analysing local adaptation in populations selected in atrazine, the first factor nested within the entire dataset differentiated between the mean of the sympatric and allopatric populations at 0.5MIC, with a further factor nested within it differentiating between the sympatric populations selected at different doses. The second factor nested within the entire dataset differentiated between the mean of sympatric and allopatric populations at 0.75MIC, and so on. The source population (5 levels) was fitted as a random factor.

To address objective ii) in the introduction, I investigated whether increasing environmental distance between sympatric and allopatric environments was positively correlated with local adaptation. An environmental distance (ED) was calculated as the absolute difference in sympatric and allopatric herbicide doses. For example, the ED for the populations selected at 1.5MIC and whose growth was measured at 0.5MIC was 1. Adaptation was estimated as the difference in the number of cell divisions between the mean of the sympatric populations and each allopatric population, at each dose, a positive value being indicative of local adaptation. To represent this data, two matrices were constructed for each herbicide, with the selected environment (sympatric populations) along the x-axis and the tested environment along the y-axis – one containing the environmental distances, the other containing the differences in growth rates between the mean of the sympatric populations and the allopatric population. To test for the effects of environmental distance on local adaptation, the Mantel test for the correlation of matrices (mantel function in package vegan in R 2.15.0) was performed. For each herbicide, the Kendall rank statistic was estimated for the correlation between the environmental distance matrix and the matrix containing the differences in growth rates, and the significance tested against 999 randomly generated permutations.

Growth rates in the absence of herbicides of the evolved populations were analyzed using a pair-wise Dunnett's corrected T-test. The OD_{750} after four days of growth was compared between the populations selected at each dose of the same herbicide.

6.3 Results

Whether a population was locally adapted was investigated by comparing its growth rates in its selected environment to the mean growth rate of all allopatric populations (selected at different herbicide doses) in the same environment. For example, I compared growth rates at 0.5MIC of atrazine of populations evolved at 0.5MIC of atrazine (sympatric population) with the mean growth rate of populations selected at other atrazine doses (allopatric populations). This was done at each selected dose.

Most sympatric populations in the experiment grew as well as or better than the allopatric populations (Fig.16), indicating a general pattern consistent with local adaptation. Evidence for local adaptation was found in all populations that evolved at 1.5MIC in iodosulfuron (χ_{21}=42.65, P<0.001) and S-metolachlor (χ_{20}=136.02, P<0.001). All populations that evolved at 1.25MIC were also locally adapted in glyphosate (χ_{19}=49.64, P<0.001), iodosulfuron (χ_{21}=93.40, P<0.001) and S-metolachlor (χ_{20}=39.80, P<0.001). At MIC, locally adapted populations were observed in glyphosate (χ_{19}=7.85, P<0.005) and iodosulfuron (χ_{21}=46.06, P<0.001), but not in S-metolachlor (χ_{19}=1.41, P=0.234). At 0.75MIC, local adaptation was found only in S-metolachlor (χ_{20}=9.25, P<0.005), while at 0.5MIC, it was identified in atrazine (χ_{15}=4.30, P<0.05), glyphosate (χ_{20}=9.45, P<0.005) and iodosulfuron (χ_{21}=15.96, P<0.001) (Fig.16).

Some selection regimes gave rise to locally maladapted populations - populations exhibiting lower growth rates in their selected environment than the populations selected at other doses

(Fig.16). This was observed in the populations selected at MIC of atrazine (χ_{15}=24.54, P<0.001) 0.75MIC of glyphosate (χ_{19}=8.22, P<0.005), and 0.5MIC of S-metolachlor (χ_{20}=4.23, P<0.05).

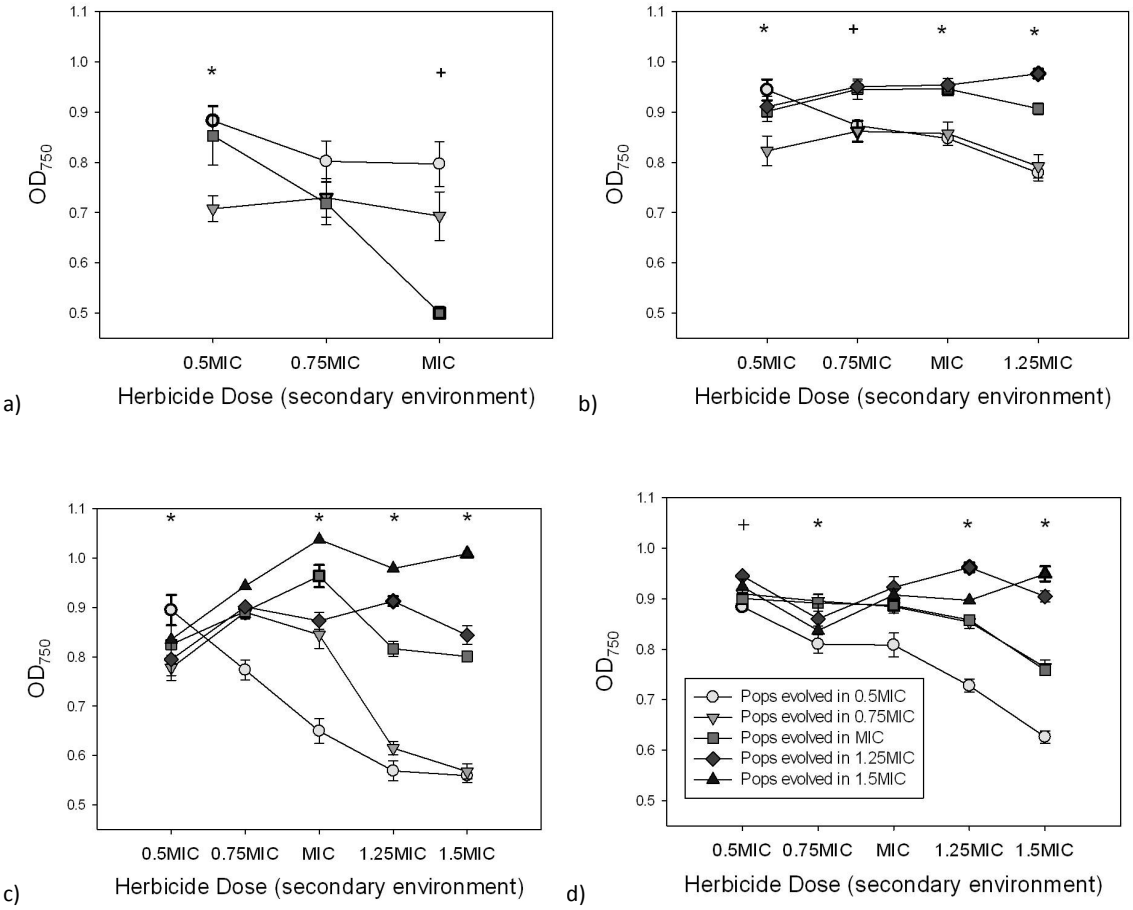


Figure 16. **Mean growth rates as the optical density across all secondary environments (same herbicide) in which resistance was observed.** Bolded edges on data points indicate the sympatric population at that dose. * mark sympatric populations with significantly higher mean growth rate than the allopatric ones; + the sympatric populations with significantly lower mean. The error bars are standard errors. a) Pattern of local adaptation in atrazine resistant populations; b) glyphosate; c) iodosulfuron; d) S-metolachlor.

Increasing environmental distance led to higher comparative fitness of sympatric populations compared to the allopatric populations in all herbicides except atrazine, where the opposite was identified (Fig.17). The effect was significant in all herbicides – atrazine (r=0.24, P<0.01), glyphosate (r=0.69, P<0.001), iodosulfuron (r=0.84, P<0.001) and S-metolachlor (r=0.73, P<0.001).

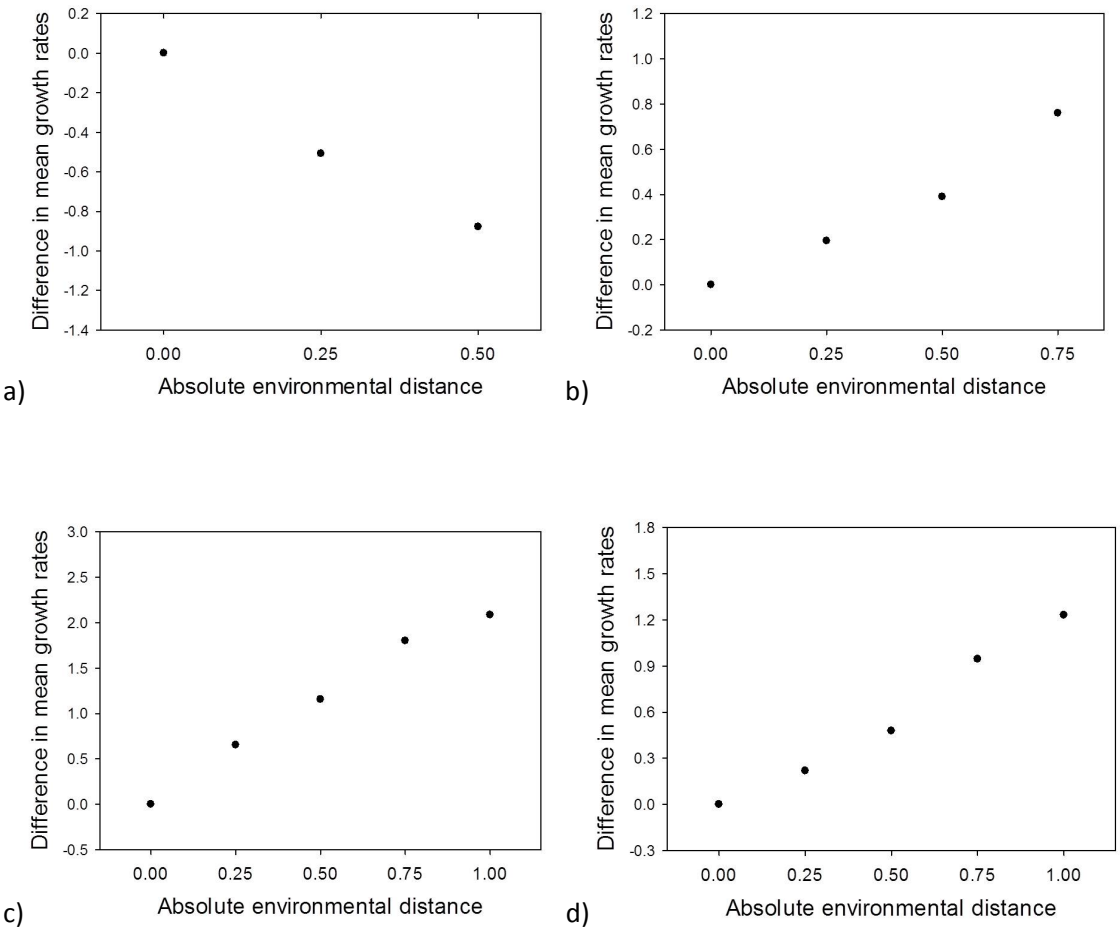


Figure 17. **Mean difference in growth rates between the sympatric and allopatric populations, at each absolute environmental distance**. Bars are standard errors of the mean. a) Fitness-by-absolute environmental distance for populations selected in atrazine; b) glyphosate; c) iodosulfuron; d) S-metolachlor.

Growth rates in the absence of atrazine were significantly lower in populations evolved at 0.5MIC and 0.75MIC when compared to the populations selected in MIC (T_8=-5.512, P=0.001; T_8=-6.755, P<0.0005, respectively) (Fig.18). Similarly, populations evolved under lowest iodosulfuron dose had lower growth rates in the absence of herbicide than the populations selected in 0.75MIC (T_8=-4.308, P<0.01) and at MIC (T_8=-4.906, P<0.005) (Fig.18). In glyphosate and S-metolachlor, growth rates in the absence of herbicides did not significantly differ between populations selected under any dose (Fig.18).

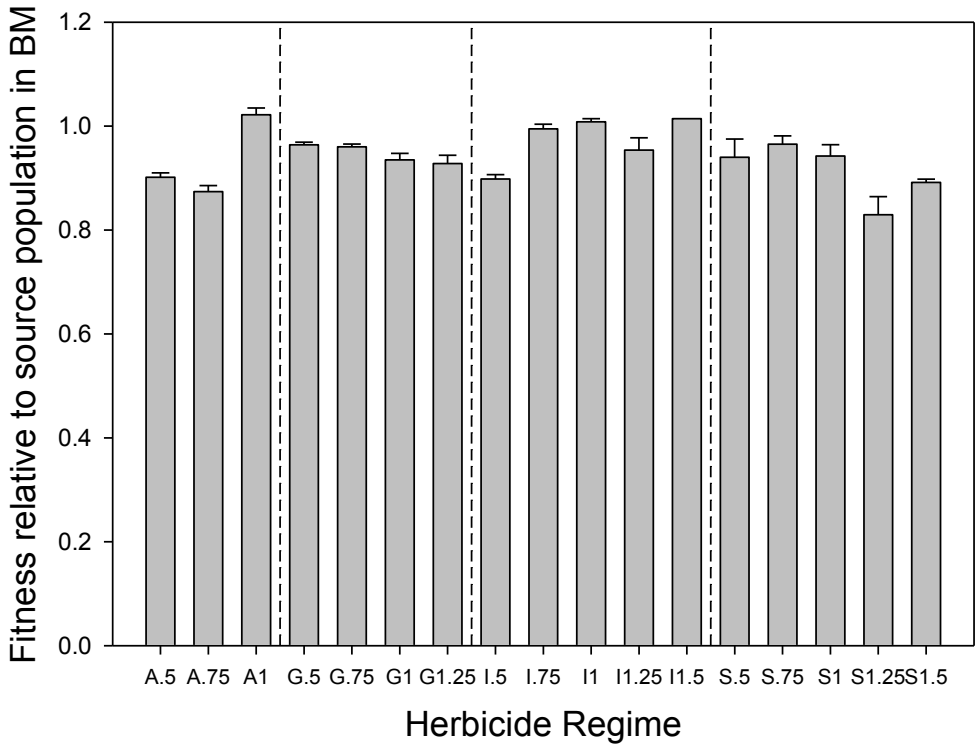


Figure 18. **Growth rates in the absence of herbicides** as a proportion of the total number of cell divisions of the source populations in herbicide free environment in four days. Bars are mean growth rates. Error bars are standard error.

6.4 Discussion

The evolutionary response along an environmental gradient of increasing herbicide dose was investigated, finding evidence for local adaptation in the majority of evolved populations, with local maladaptation observed in only three selection regimes (Fig.16). Local adaptation was more pronounced as the environmental distance between environments increased (Kawecki & Ebert 2004), as a positive correlation between environmental distance and local adaptation was identified in all but the populations selected in atrazine, where the correlation was negative (Fig.17). Differences in relative growth rates in the absence of herbicides were rarely identified between populations evolved at different doses of the same herbicide (Fig.18).

6.4.1 Explaining local adaptation

Antagonistic pleiotropy is often seen as a condition giving rise to local adaptation, resulting from the fitness trade-offs between environments (Hedrick 1986; Kawecki & Ebert 2004). For antagonistic pleiotropy to arise along an herbicide gradient, some, but not all, environments have to favour a trade-off between population's growth rate in the presence and in the absence of herbicides. This relationship between higher level of resistance and differences in fitness costs would have to be progressively stronger as the selected dose is increased, in order to observe a positive relationship between environmental distance and local adaptation (Fig.17). Consequently, the fitness costs should be highest in the populations selected at highest doses. When comparing the growth rates in the ancestral environment, no evidence of such a gradient in relative fitness was identified, as the majority of evolved populations had comparable growth rates in the absence of herbicides (Fig.18). This finding suggests that antagonistic pleiotropy did not give rise to locally adapted populations in this study, and is in line with many other studies failing to find evidence for antagonistic pleiotropy (Roff & Fairbairn 2006).

Mutation accumulation is another mechanism potentially giving rise to local adaptation (Lynch et al. 1995), occurring when neutral mutations in one environment are deleterious in another. As the adaptation along the gradients in this experiment differs only with respect to the concentration of the herbicide, the type of differential response of mutations between environments required for mutation accumulation is unlikely. In addition, the time scale of this study is likely to be short for mutation accumulation to play a major role (Nakayama et al. 2012). As such, under experimental conditions of this study, mutation accumulation is unlikely to have contributed to the observed local adaptation.

In the absence of antagonistic pleiotropy and mutation accumulation, local adaptation can arise when mutations have differential responsiveness (Fig.15). According to this mechanism, at

higher doses, where the variance in fitness effects of mutations is greater, local adaptation simply arises from the selection of more fit phenotypes. Similarly, the observed increase in the magnitude of local adaptation with environmental distance (Fig.17) could result from the differences in fitness effects being dependent on the herbicide gradient, so that increase in dose leads to further differentiation between mutations. Local adaptation was also observed at lower doses (Fig.16). For populations to differentiate under such conditions, small differences in the fitness effects of mutations have to exist even at lower concentrations. In addition, the mutations conferring higher fitness at lower doses could not be the same mutations providing higher fitness at higher doses as well – the rank order of mutations for fitness changes along the gradient. As the differences in fitness effects according to the proposed mechanism (Fig.15) are smaller at lower than at higher doses, smaller magnitude of local adaptation was observe at lower than at higher doses (Fig.16). In the light of the absence of relative differences in growth rates in herbicide-free environments, the results of this study suggest the environment-dependent differential fitness effect of beneficial mutations as the mechanism driving local adaptation along a gradient.

6.4.2 Local maladaptation

Local maladaptation, here defined as a pattern opposite to local adaptation when the fitness of the sympatric population is lower than the fitness of the allopatric populations, was observed in three experimental regimes - in populations selected at lower doses of glyphosate (0.75MIC) and S-metolachlor (0.5MIC), and the populations selected at MIC of atrazine (Fig.16), where an inverse relationship between environmental distance and local adaptation was also observed - increase in distance resulted in less locally adapted populations (Fig.17). Maladaptation can arise as a consequence of the exponential distribution of the beneficial effects of mutations (Orr 2005), where mutations of smaller effect are more common than the mutations of larger effect.

In fact, the nature of the distribution of beneficial effects of novel mutations has been suggested to play a role in evolution of antibiotic resistance (Andersson & Hughes 2012), although some studies do not support such distribution in particular at higher antibiotic doses (MacLean & Buckling 2009). Maladaptation at higher doses implies that mutations of greater fitness benefit were selected at lower doses. If such more beneficial mutations are less common, they are more likely to arise in a population selected at lower dose, where the fitness is higher and the population undergoes more cell divisions, generating more mutations. If the exponential distribution of beneficial effects of mutations was to give rise to maladaptation at lower doses, the more frequent mutations of lower fitness have to modify the genetic background to prevent fixation of less frequent mutations of higher fitness, so that once a small effect mutation had fixed, the initial large effect mutation had a neutral or even negative effect (Trindade et al. 2009; Lagator et al. 2012). At higher doses, the stronger selection pressure could prevent the fixation of more frequent mutations of smaller fitness effect, and therefore allow the fixation of more rare mutations that confer greater fitness benefit.

6.4.3 Herbicide dose – practical considerations

The recommended dose of a herbicide is determined by economic and environmental considerations, and is designed to provide effective control in a range of environments (Doyle & Stypa 2004; O'Donovan et al. 2007). Consequently, it is often possible to reduce the recommended dose with no observable reduction in effectiveness (Gressel 2002; Blackshaw et al. 2006; Sexton et al. 2010). The study provides evidence adding to the growing body of works suggesting the potential hazards of using reduced xenobiotic doses (Neve & Powles 2005b; Busi & Powles 2009; Manalil et al. 2011; Andersson & Hughes 2012), as it was shown that exposure to lower-than-MIC doses can result in phenotypes that are well adapted to the recommended dose (MIC). In a wider context, this study captured the range of outcomes that can arise when

evolving along a gradient, showing the complexity that can emerge in response to gradual environmental change. The results also indicate that the population's fitness along a gradient tends to drop with distance from its local conditions, illuminating the dangers of rapid and drastic anthropogenic environmental changes.

CHAPTER 7: GENERAL DISCUSSION

7.1 Summary of findings

The material presented in this thesis takes an applied evolutionary biology approach. Research questions were formulated with a goal of exploring proposed options for delaying or preventing evolution of herbicide resistance. The potential of management practices was considered in the light of underlying ecological and evolutionary theory, with the applied considerations used as a starting point to address the underlying theoretical issues. For example, sequential herbicide application - a frequent response to emergence of resistance – was used as a model to study the consequences of accumulation of resistance mechanisms on rates of resistance evolution and the associated fitness costs. This study identified the possibility for environmental perturbation to enhance rates of adaptation in subsequent environments when resistance with a positive correlated response to selection in those environments was selected for (Chapter 3). Cycling as a management strategy relies on temporal environmental heterogeneity and the existence of fitness costs to slow down resistance evolution (Gressel & Segel 1990). The results presented in Chapter 4 illustrate how cycling can exacerbate the resistance problem by enhancing rates of adaptation even in the presence of fitness costs, or by selecting for a cross-resistant generalist. Another frequently utilized management strategy (Beckie 2006), mixtures of herbicides, was used in this thesis to formulate research questions for the work presented in Chapter 5. Their hypothesized effectiveness is based on the assumption that multiple resistance mutations are exceedingly rare in one individual, while being required to provide positive fitness in the face of increased environmental complexity (Wrubel & Gressel 1994). While the results presented in Chapter 5 support these predictions when each component herbicide is utilized at or close to the full dose, use of lower doses of each component led to more rapid resistance evolution. In

agricultural fields, the applied dose of a herbicide will not be constant and often economic and environmental considerations may dictate that lower than optimal herbicide doses are used. Lastly, the impacts of a range of herbicide doses on evolution of resistance was investigated (Beckie 2006) was used as a model to study selection along a gradient of selection pressures. Understanding that local adaptation along an environmental gradient is a rule rather than an exception (Chapter 6) contributes to the understanding of whether the selection at lower doses could lead to resistance at the recommended dose. Each chapter contained a discussion section describing the implications of the findings and their contribution to the current knowledge. Here, I will discuss some inferences that arise by looking at the thesis as a whole.

7.2 Dynamics and outcomes of resistance evolution are herbicide-specific

The works presented in this thesis failed to identify many universal resistance management principles, as there was a lack of a uniform response to designed experimental conditions. In particular, populations selected in different herbicides would, not surprisingly, show very different evolutionary dynamics. In Chapter 4, the same rates of cycling resulted in very different outcomes, the only difference being the herbicides cycled - the weekly cycle between atrazine and glyphosate sped up, while the weekly cycle between glyphosate and carbetamide slowed down resistance evolution. Similarly, in Chapter 5, different herbicide mixtures gave rise to very different rates of resistance evolution, irrespective of the combined herbicide dose. In Chapter 6, whether local adaptation was observed or not depended on the herbicide the population was exposed to. As such, the instances where broad generalizations could be made were rare, with a few exceptions such as the mixtures being universally effective at higher combined doses (Chapter 5). This thesis shows that any broad conclusions about the effectiveness of one management practice over another are likely to be inaccurate, as whether

the strategy will be successful depends at least in part on the properties of the specific herbicides used.

7.3 Generalist phenotypes evolve frequently

Understanding the conditions under which generalist phenotypes evolve is critical both from the theoretical (Chevin et al. 2010) and the applied (Gressel 2009; Powles & Yu 2010) standpoint. Increases in environmental complexity are predicted to lead to a generalist peak (Tienderen 1991), although the exact conditions under which this is true are highly dependent on particular circumstances (Futuyma & Moreno 1988; Meeus et al. 1993; Reboud & Bell 1997). Chapters 4 and 5 explore the circumstances under which temporal variability and environmental complexity lead to generalists, and show a variety of cross-resistance profiles selected in response to equivalent application methods that differ only with respect to herbicides present (same rate of cycling, for example, yielded different cross-resistance profiles, Chapter 4). From an applied perspective, cross-resistant generalists pose a greater threat to management. The results presented in this thesis highlight that generalists evolve often in response to environmental heterogeneity, but that the exact conditions that favor their evolution, as well as their fitness in the presence and absence of herbicides, depend on the specific interaction between the target population and the xenobiotic used.

7.4 Application methods affect the magnitude of fitness costs

Fitness costs associated with evolved resistance were commonly observed in the conducted experiments, but their magnitude was variable and unpredictable. As expected, the magnitude of fitness costs depended on the herbicide the population was evolving resistance to (Vila-Aiub et al. 2009), but was also affected by the population's adaptive past – as the resistance mechanisms accumulated in a population the fitness costs were not additive but were actually reduced (Chapter 3). The rate of herbicide cycling also affected fitness costs, with slower rates

selecting for lower costs (Chapter 4). Understanding the conditions that select for highest fitness costs is of importance to management, as (i) the effectiveness of many strategies relies on their existence (Beckie 2006), and (ii) the populations with lower fitness in the absence of herbicides could be more easily controlled through competition with wild-type individuals (Gardner et al. 1998).

The starting fitness of a population in a novel herbicide environment is in part determined by its adaptive past, if negative (fitness costs) and/or positive (cross-resistance) correlated responses to selection in the previous environment exist. Variations in the magnitude of the correlated responses have been demonstrated to impact the dynamics of resistance, with even small differences below the detection levels arising from cross-resistance (Chapter 3) or cross-protection (Chapter 4) potentially enhancing rates of resistance evolution to a novel herbicide. For the impact they could therefore have, understanding the magnitude of fitness costs on a case-by-case basis is of importance to increasing the effectiveness of management practices.

7.5 Experimental evolution with *C.reinhardtii* in a wider context

A fundamental limitation to the experimental testing of the efficacy of various management strategies arises from the length of the weedy-plant life cycle, making long-term evolution experiments difficult to carry out (Reboud et al. 2007). Adopting *C.*reinhardtii and experimental evolution as a method to study herbicide resistance allowed overcoming these difficulties, as *C.reinhardtii* is a fast-replicating single cell chlorophyte capable of 7-10 cell divisions during a single weekly transfer. For this reason, the greatest benefit of using it as a model organism is the ability to conduct long-term experiments where a population is allowed to adapt for tens to hundreds of generations. An additional benefit of adopting microbial experimental evolution is the ease of manipulating the growth conditions in a test tube and measuring the growth rates to assess population fitness (Buckling et al. 2009). The ability to precisely control the growth

conditions enables the isolation of a range of extraneous factors that could not be controlled in the environment and through that allow the study of the effects of a single factor on evolution (Kawecki et al. 2012).

The controlled laboratory conditions in experimental evolution studies create a difficulty when attempting to translate the findings to other organisms and environments (Buckling et al. 2009). This is a particular concern when working within the framework of applied evolutionary biology, as this thesis does in an attempt to inform management practices. Differences between *C.reinhardtii* and weedy plants are many, and some of them are relevant to the considerations of how the findings presented in this thesis could be scaled up. First, higher plants are complex multicellular bodies with distinguished organs, offering a wider range of targets for herbicides and resulting in some herbicides not being effective in *C.reinhardtii* (Table 2). Greater complexity of higher plants allows for a wider range of possible resistance mechanisms than in *C.reinhardtii*, such as tissue-specific sequestration and limited herbicide translocation between plant's organs (Powles & Yu 2010). Second, unlike weedy plants, *C.reinhardtii* is haploid. While the rates of fixation of dominant alleles are not predicted to be different between diploid and haploid organisms, the rates of fixation of recessive alleles through a population are enhanced in haploid organisms (Charlesworth 1992). The more rapid rates of fixation of dominant than of recessive alleles in diploid organisms could have led to the majority of characterized resistance mutations in higher plants being dominant (Powles & Yu 2010), a finding that could be different in the haploid *C.reinhardtii*. Third, while *C.reinhardtii* is capable of sexual reproduction, all selected populations presented in this thesis were reproducing asexually. Recombination is predicted to lead to higher genetic diversity and enhanced rates of adaptation (Colegrave 2002; Agrawal 2006; Hartfield & Keightley 2012), in particular when multiple dominant alleles are required to confer resistance (Neve & Powles 2005b). While some weeds do self-fertilize, most

undergo some form of outcrossing (Barrett 2002) and their dynamics of herbicide resistance evolution could consequently differ from those observed in this thesis. Finally, the demographics of weed populations in natural environments are vastly more complex than the ones created in the described experiments. The initial diversity of *C.reinhardtii* populations in the described studies was limited and controlled by allowing a single colony to grow for 15-20 generations prior to the start of the experiment. This is in contrast to highly diversified naturally-adapting weed populations (Thrall et al. 2011). Adapting weeds experience gene flow from a range of sources and populations (Jasieniuk et al. 1996), including seed banks (Gressel 2002), while gene flow in this thesis was constrained to immigration from the source populations.

These issues contribute to the difficulty of scaling up the microevolution described in this thesis. The presented results should be used as an indication of the outcomes that *could* arise, as opposed to a firm statement on what *will* occur.

7.6 Future research

Phenotypic studies. Due to time constraints, this thesis focused on the most frequently adopted management strategies – sequential application, cycling, mixtures and dose manipulations (Beckie 2006). To strengthen the findings, future research would focus on exploring the impact of other strategies, in addition to cycling and mixtures of herbicides, such as the dose alternation, whereby a rotation of different doses of one herbicide is employed (Gardner et al. 1998). The scope of studies was constricted to a specific definition of each management strategy. For example, in Chapter 4 cycling was defined as a symmetrical rotation between fixed doses of two herbicides. A follow-up study would explore the consequences of increasing the number of herbicides, and of cycling strategies that involve variable herbicide concentrations and patterns. Similarly, the experiment described in Chapter 5 could be expanded by increasing the number of herbicides in the mixtures, as well as uneven herbicide concentrations. In

addition, evolutionary outcomes of cycling regimes that are interrupted by herbicide-free periods could be drastically different and are worth exploring.

Sexual reproduction has been shown to impact the rates of evolution (Colegrave 2002; Goddard et al. 2005). Comparing the response of sexual and asexual populations to more complex and variable environments could provide a more detailed understanding on how weedy plants develop resistance. On a wider scale, involving a sexually reproducing organism in experimental evolution studies that explore adaptation to a changing and variable environment would get a step closer to the 'real world' (Buckling et al. 2009). Due to the ease of reproductive cycle manipulation, I believe that exploring herbicide resistance in *C.reinhardtii* offers a simple yet powerful system that allows for this step to be taken.

Molecular and genotypic studies. The experimental work presented in this thesis focused on exploring the consequences of certain environmental manipulations on evolved phenotypes. Understanding the underlying genetic mechanisms that are associated with the evolved populations would allow explaining the process of adaptation more fully. Exploring the molecular changes in the described experimental designs could provide insights into the relationship between different types of resistance (single-gene vs. polygenic) and their effects on the dynamics of evolution. Tracking the progress of adaptation through time could provide insight into how the two different types of mechanisms develop, and if those differences could be utilized for management purposes (Powles & Yu 2010). Studying the genotypic changes could also allow understanding of the process of fitness compensation (Wiesch et al. 2010), which was likely to have occurred in some of selected populations. Finally, adding the molecular evidence to the phenotypic observations could contribute to the understanding of genotype-phenotype mapping.

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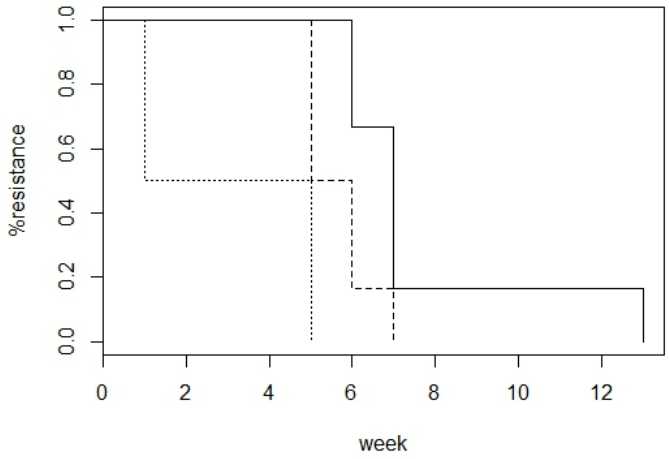
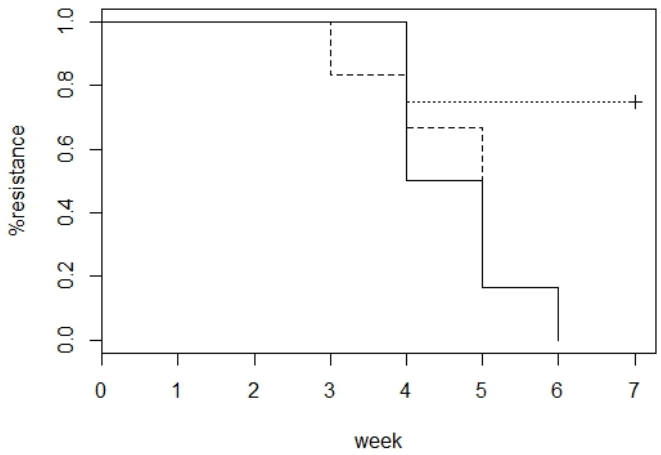
APPENDIX A

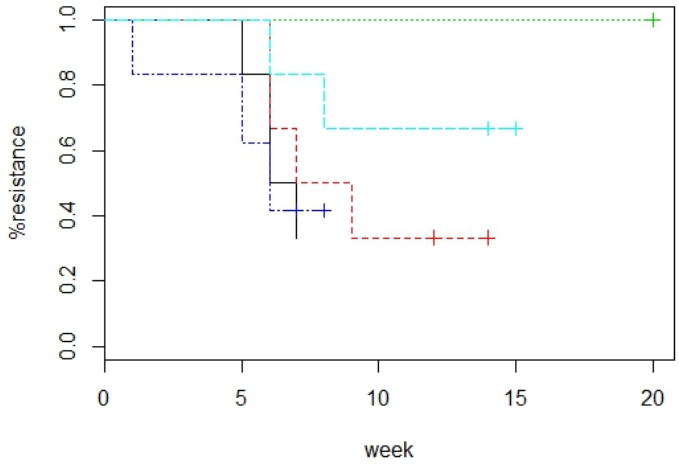
Survivorship functions for the analysis of dynamics of herbicide resistance in Chapter 3.



Appendix A1: Dynamics of atrazine resistance as a survivorship function. Percentage of replicate populations that are susceptible is plotted for each tested week. Fine dotted line is continuous exposure to atrazine (A), broken line (GA) and full line (GCA).



Appendix A2: Dynamics of glyphosate resistance as a survivorship function. Percentage of replicate populations that are susceptible is plotted for each tested week. Fine dotted line is continuous exposure to glyphosate (G), broken line (AG) and full line (ACG).



Appendix A3: Dynamics of carbetamide resistance as a survivorship function. Percentage of replicate populations that are susceptible is plotted for each tested week. Fine dotted line green line is continuous exposure to carbetamide (C), broken turquoise line (AC), broken red line (GC), broken purple line (AGC) and full line (GAC).