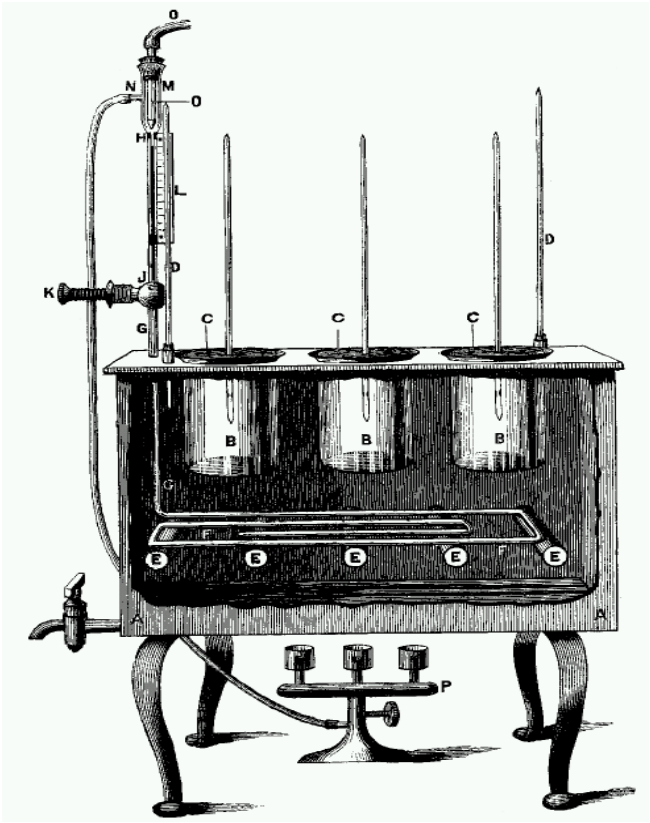
Applied evolution: An integrated approach to studying life history traits in response to drug selection



An example of a very early selection experiment apparatus used by Rev W. H. Dallinger to select for heat tolerance in populations of protists (described in The President's Address 1887).

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General abstract

The use of chemical control measures to reduce the impact of parasite and pest species has frequently resulted in the development of resistance. Thus, resistance management has become a key concern in human and veterinary medicine, and in agricultural production. Although it is known that factors such as gene flow between susceptible and resistant populations, drug type, application methods, and costs of resistance can affect the rate of resistance evolution, less is known about the impacts of density-dependent eco-evolutionary processes that could be altered by drug-induced mortality. The overall aim of this thesis was to take an experimental evolution approach to assess how life history traits respond to drug selection, using a free-living dioecious worm (*Caenorhabditis remanei*) as a model. In Chapter 2, I defined the relationship between *C. remanei* survival and Ivermectin dose over a range of concentrations, in order to control the intensity of selection used in the selection experiment described in Chapter 4. The dose-response data were also used to appraise curve-fitting methods, using Akaike Information Criterion (AIC) model selection to compare a series of nonlinear models. The type of model fitted to the dose response data had a significant effect on the estimates of LD_{50} and LD_{99}, suggesting that failure to fit an appropriate model could give misleading estimates of resistance status. In addition, simulated data were used to establish that a potential cost of resistance could be predicted by comparing survival at the upper asymptote of dose-response curves for resistant and susceptible populations, even when differences were as low as 4%. This approach to dose-response modeling ensures that the maximum amount of useful information relating to resistance is gathered in one study. In Chapter 3, I asked how simulations could be used to inform important design choices used in selection experiments. Specifically, I focused on the effects of both within- and between-line variation on estimated power, when detecting small, medium and large effect sizes. Using mixed-effect models on simulated data, I demonstrated that commonly used designs with realistic levels of variation could be underpowered for substantial effect sizes. Thus, use of simulation-based power analysis provides an effective way to avoid under or overpowering a study designs incorporating variation due to random effects. In Chapter 4, I

investigated how Ivermectin dosage and changes in population density affect the rate of resistance evolution. I exposed replicate lines of *C. remanei* to two doses of Ivermectin (high and low) to assess relative survival of lines selected in drug-treated environments compared to untreated controls over 10 generations. Additionally, I maintained lines where mortality was imposed randomly to control for differences in density between drug treatments and to distinguish between the evolutionary consequences of drug treatment versus ecological processes affected by changes in density-dependent feedback. Intriguingly, both drug-selected and random-mortality lines showed an increase in survivorship when challenged with Ivermectin; the magnitude of this increase varied with the intensity of selection and life-history stage. The results suggest that interactions between density-dependent processes and life history may mediate evolved changes in susceptibility to control measures, which could result in misleading conclusions about the evolution of heritable resistance following drug treatment. In Chapter 5, I investigated whether the apparent changes in drug susceptibility found in Chapter 4 were related to evolved changes in life-history of *C. remanei* populations after selection in drug-treated and random-mortality environments. Rapid passage of lines in the drug-free environment had no effect on the measured life-history traits. In the drug-free environment, adult size and fecundity of drug-selected lines increased compared to the controls but drug selection did not affect lifespan. In the treated environment, drug-selected lines showed increased lifespan and fecundity relative to controls. Adult size of randomly culled lines responded in a similar way to drug-selected lines in the drug-free environment, but no change in fecundity or lifespan was observed in either environment. The results suggest that life histories of nematodes can respond to selection as a result of the application of control measures. Failure to take these responses into account when applying control measures could result in adverse outcomes, such as larger and more fecund parasites, as well as over-estimation of the development of genetically controlled resistance. In conclusion, my thesis shows that there may be a complex relationship between drug selection, density-dependent regulatory processes and life history of populations challenged with control measures. This relationship could have implications for how resistance is monitored and managed if life histories of parasitic species show such eco-evolutionary responses to drug application.

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List of Abbreviations

AIC	Akaike Information Criterion
CRISPR	Clustered regularly interspaced short palindromic repeats
GLM	Generalised Linear Model
GLMM	Generalised Linear Mixed Model
DMSO	Dimethyl sulphoxide
HD	High dose
HR	High random
LD	Low dose
LR	Low random
LD_{50}	the lethal dose representing 50% of a target population
LD_{99}	the dose that represents the maximum dose a given population can tolerate
LDT	Larval Development Test
LNS	Labotatory natural selection
NGM	Nematode Growth Medium
REX	Resistance to Xenobiotics
WHO	World Health Organistaion
Z	Zero dose

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Author's Declaration

I declare that the work recorded in this thesis is entirely my own, except where otherwise stated, and that it is also of my own composition. Chapter 4 has been published in *Evolutionary Applications* (DOI: 10.1111/eva.12376) and that version is presented in this thesis. The manuscript was produced in co-authorship with my supervisors, in press as: Reynolds, A., Lindström J., Johnson, P.C.D., Mable, B.K. Evolution of drug-tolerant nematode populations in response to density reduction (2016).

The simulation-based power analysis in Chapter 3 was developed in conjunction with my supervisor Paul Johnson.

The life history assays in Chapter 5 were conducted with the assistance of Robert Cook and Daniel Crabtree.

Chapter 1: General introduction

1.1 Resistance evolution and communication between disciplines

Parasitic diseases caused more than one million human deaths in 2013 (GBD collaborators 2015), while pests and diseases account for around one third of crop losses annually (Oerke 2006), and are a serious concern in the production of livestock (Oxford Analytica 2012). The financial cost of controlling parasites and pests is considerable: for example, 39.4 billion dollars were spent on pesticides globally in 2007 (Grube et al. 2011). Control agents are designed to reduce target populations but massive global application has led to extensive development of resistance (Kaplan and Vidyashankar 2011; zur Wiesch et al. 2011). Overcoming the problem of resistance requires finding methods of drug use such that parasite and pest populations are kept at low numbers and the evolution of resistance is minimised. Several factors are known to affect the rate at which parasites can evolve resistance, including the type of drug, dosage, timing of application, migration rates between susceptible and resistant populations, the standing frequency of resistance alleles in the population and the specific mechanisms of resistance (Committee on Strategies for the Management of Pesticide Resistant Pest Populations 1986; James, Hudson, and Davey 2009; Gilleard and Beech 2007; REX Consortium 2013; Barnes, Dobson, and Barger 1995). In addition, life history characteristics of parasites and pests, as well as their reproductive strategies could influence the rate at which resistance develops (Kliot and Ghanim 2012; Galvani and Gupta 1998). Current research to date has considered many of these factors in isolation but there has been little attempt to explore interactions between life-history traits and the rate of resistance evolution.

Intensive use of pesticides and drugs has generally been followed by the rapid evolution of resistance in pathogens, pests and disease vectors (Palumbi 2001). Models of resistance evolution take into account the evolutionary forces that shape adaptive responses of populations subject to strong directional selection (Barnes, Dobson, and Barger 1995; Leathwick 2013). Evolutionary forces such as

selection, drift, mutation and migration are common to many pest and pathogen groups including viruses (Bull et al. 1997), bacteria (Lenski and Travisano 1994), fungi (Anderson et al. 2003) and invertebrates (Leathwick et al. 2012). They are therefore likely to play a role in the adaptation of these groups to intense perturbations such as drug treatment (Greene et al. 2012). A recent study by the REX Consortium (2007) found that research in the field of resistance evolution has become divided into two main scientific groups; one group focuses an interest in agricultural pests and pathogens while the other focuses on human pathogens and their vectors (Rex Consortium 2007). Both groups work in parallel, with little exchange of methodology or theory. Within these two groups further subdivisions were found; network analysis of citation and authorship revealed groups working independently on antibiotic, antiviral, anthelmintic and antimalarial drug resistance as well as insecticide, herbicide and fungicide resistance. It would seem logical that the scientific community studying the evolution of resistance in these organisms should collaborate and quote the same references, use similar approaches and strategies to manage resistance evolution. However, communication between different disciplines still remains a persistent problem. For example, the REX Consortium paper entitled 'Structure of the Scientific community modeling the evolution of resistance' (2007), highlighting the lack of interdisciplinary work in the field, had only 13 citations at the time of writing this thesis.

The flow of information between the scientific community and how that information is applied in the field is also an area of concern in resistance management. It has been suggested that resistance management practices are in need of review and that in some circumstances current practices may even promote the evolution of resistance (Leathwick et al. 2009; Greene et al. 2012). However, in many cases these warnings have gone unheeded. For instance, the use of anthelmintics in managing sheep parasites has largely remained the same since the 1980's (Leathwick et al. 2009). Over this same period the resistance of parasitic worms has gone from rare to being commonplace (James, Hudson, and Davey 2009). Management strategies used to control parasites during this period have clearly applied significant selection pressure for the development of resistance to anthelmintics. This highlights the need for the implementation of more effective control measures to prevent the evolution of resistance (Hendry

et al. 2011; Greene et al. 2012). In the case of agricultural systems, resistance management practices need to be implemented that reduce the level of resistance while maintaining an acceptable level of productivity (Committee on Strategies for the Management of Pesticide Resistant Pest Populations 1986; Leathwick et al. 2009). A thorough knowledge of the factors that affect the rate of resistance evolution is therefore required if resistance is to be managed effectively (Georghiou and Taylor 1977; Committee on Strategies for the Management of Pesticide Resistant Pest Populations 1986; Greene et al. 2012).

1.2 Rates of resistance evolution

Research has shown that rapid or 'contemporary' evolution, in the sense of a novel trait spreading through a population, is possible over a short time period, as few as 10-20 generations or less in some circumstances (Stockwell, Hendry, and Kinnison 2003). Contemporary evolution was thought to be a relatively rare event, restricted to a few cases such as industrial melanism in the peppered moth *Biston betularia* (Cook, Sutton, and Crawford 2005). However, since scientists have begun measuring the strength of natural selection in the wild they have found that it is stronger than expected, which suggests that contemporary evolution may be more commonplace than previously expected (Stockwell, Hendry, and Kinnison 2003). For example, studies of wild guppy (*Poecilia reticulate*) populations have shown that their life-histories show rapid evolutionary responses when exposed to a predation (Reznick 1997; Reznick et al. 2004; Reznick and Ghalambor 2005). Contemporary evolution may be promoted by factors such as invasion of new habitats, predation, resource availability, competition and environmental perturbations (Hairston et al. 2005; Ellner, Geber, and Hairston 2011). Evolutionary responses of populations are expected to be high where there is high trait heritability and following an increase in the intensity of directional selection (Reznick and Ghalambor 2001). Other factors that influence the rate of evolution include population size, gene flow and initial allele frequencies within populations (Stockwell, Hendry, and Kinnison 2003). Resistance has been shown to spread rapidly through populations challenged with xenobiotics (Taylor, Quaglia, and Georghiou 1983; Lopes et al.

2008; James, Hudson, and Davey 2009), but how factors such as density dependent competition in target populations interact with the evolution of resistance is unclear.

Anthropogenic factors such as dramatic environmental changes impose particularly strong selection on organisms (Hendry et al. 2011) . The evolution of resistance provides many examples of strong selection pressure resulting in reduced efficacy of treatments. Bacteria, viruses, insect vectors and invertebrate crop pests have all quickly evolved resistance to control measures across many different clades (Palumbi and Mu 2001; Whalon, Mota-Sanchez, and Hollingworth 2008; Reece et al. 2010; Greene et al. 2012). For instance, in the case of pest invertebrates, Whalen et al (2008) summarises 7747 cases of resistance to 331 compounds in 553 species. Thus, the rapid evolution of resistance as a result of human activities is widespread and understanding how species rapidly adapt to efforts to reduce their numbers is of critical importance to disease control. In this thesis I will concentrate on resistance of parasitic nematodes to anthelmintics as an example of how resistance is managed and how rapidly resistance can evolve.

Anthelmintic resistance of parasitic nematodes is now a global problem that affects human health as well as threatening the production and welfare of agricultural livestock (Sangster and Gill 1999). The treatment of parasitic nematodes with anthelmintic drugs has invariably led to the appearance and spread of anthelmintic resistance. Application of new anthelmintic drug treatments such as benzimidazoles, imodothiazoles and macrocyclic lactones has been followed by reports of widespread resistance within three to nine years of their introduction (James, Hudson, and Davey 2009). Agricultural practices such as treatment, followed by movement of livestock into low contamination pasture have been implicated as high-risk strategies that are likely to select for resistance, because any worms surviving the treatment become the major source of subsequent infection (Leathwick et al. 2009). These measures select for highly resistant parasites, which then go on to re-infect hosts as there is no dilution of resistance from free-living susceptible parasites within the population. Martin et al (1981) have shown that exposing a high proportion of parasites that contribute to the next generation will accelerate the rate of

resistance evolution. They conducted a study in which populations of *Haemonchus contortus* were passaged through lambs for six generations and exposed different proportions of populations to Thiabendazole. Resistance was slower to develop in populations with higher proportions of untreated worms. However, due to logistical constraints, such studies do not select parasite populations beyond a few generations so it is not possible to assess the rate of resistance evolution over the long-term.

1.3 Dosage and resistance

The evolution of resistance is determined by the extent to which survivors of drug treatments contribute genes to future generations; timing of treatments and drug efficacy moderate this contribution (Barnes, Dobson, and Barger 1995). Experimentation and monitoring of complicated host-parasite systems is technically difficult, expensive and time-consuming (Leathwick et al. 2009). Because many variables interact to determine the size and genetic background of parasite populations, mathematical modeling has often been used to predict evolutionary responses under different selection regimes (Barnes, Dobson, and Barger 1995; REX Consortium 2013). For example, Barnes et al (1995) investigated the effects of under-dosing on the evolution of resistance when using a single control agent (Barnes, Dobson, and Barger 1995). They found that with an initial resistance allele frequency of 0.01% and allowing 10% of susceptible worms to survive due to under dosing slowed the rate of resistance evolution relative to scenarios where treatment of susceptible worms had 99% efficacy. They suggested that under-dosing can slow the rate of resistance evolution if homozygous susceptible individuals survive and contribute to the next generation. However, they also suggested that if under-dosing occurs at a level where heterozygous resistant individuals are more likely to survive then this might speed the development of resistance.

A series of empirical studies on rye grass investigating under-dosing have also suggested that lower doses may promote the evolution of resistance (Neve and Powles 2005; Busi and Powles 2009; Manalil et al. 2011), and that varying the

level of under-dosing may affect the rate at which resistance evolves (Busi and Powles 2009). For example, Busi and Powles (2009) found that *Lolium rigidum* selected for three generations under both low and high dose treatments of glyphosate caused a rapid increase in survival over three generations, and higher doses were associated with a greater magnitude of resistance. However, assessment of resistance was conducted on first generation offspring at the end of selection, meaning that any observed response in survival could have been a result of maternal effects. Ideally, to explore the role under-dosing plays in the evolution of resistance, selection should occur over more generations than maternal effects can influence responses to treatment. In addition, at the end of selection treated lines should be passaged through a further three generations without treatment to ensure any observed responses to selection are due to treatment. Thus, selection over multiple generations at different sublethal doses would aid in elucidating the relationship between the strength of selection (dose) and the rate of resistance evolution.

Resistance to anthelmintics in nematodes has been shown to evolve in laboratory studies (Lopes et al. 2008), though to date much of this work has concentrated on mechanisms of resistance (James, Hudson, and Davey 2009), rather than focusing on how rates of resistance evolution interact with drug dose. For example, James and Davey (2009) exposed a single population of *Caenorhabditis elegans* to gradually increasing doses of Ivermectin for 44 weeks to investigate the role of transport proteins in resistance evolution. The study found the expression of some transport proteins increased after drug selection and showed that they played an important role in resistance. However, using a gradually increasing dose does not reflect how treatment is applied in the field. Long-term laboratory studies using replicate populations exposed to a consistent level of under-dosing would provide a greater insight into what affect dosage has on the rate of resistance evolution.

1.4 Life history traits and resistance

Life history theory addresses the birth and death schedule of an organism in the context of its environment and attempts to explain how natural selection is expected to shape an organism's reproduction, fecundity and survival (Roff 1992; Stearns 1992). The extent to which individual organisms can adjust different combinations of traits is mediated by resource allocation trade-offs. This results in organisms splitting finite resources between different processes such as growth, survival and reproduction (Roff 1992; Stearns 1992). Fitness costs could occur in the production and maintenance of drug resistance in parasitic or pest species that divert resources from other fitness-enhancing traits. Identifying such costs associated with resistance may be of importance in developing strategies that limit the spread of resistant populations (Kliot and Ghanim 2012). If fitness costs are high then it is likely to take longer for resistance to spread through a population because susceptible individuals may have higher reproductive rates or grow faster even though they may have reduced survival in a drug-treated environment (Kochin, Bull, and Antia 2010).

Fitness costs associated with insecticide resistance have been extensively documented in agricultural systems; see Kliot and Ghanim (2012) for a review. For example, Carriere et al (1994) investigated the effects of selection for insecticide resistance on the oblique-banded leaf-roller, *Choristoneura rosaceana* (Carriere et al. 1994). Insects were collected from pesticide-free and treated orchards; these were then reared in a common garden in the laboratory. Resistant insects from treated orchards were found to suffer considerable life history costs; they had lower larval and pupal masses, and longer development times than susceptible individuals. Also, the degree of resistance was strongly correlated with these costs; resistance levels were negatively correlated with larval growth rates and pupal mass, and positively correlated with development time. Such studies have revealed life history costs to resistant individuals; however, they have not documented whether these costs remain constant over a period of continued exposure. It is possible that continuous drug exposure may promote the selection of modifier genes that reduce the fitness costs associated with resistance (Kliot and Ghanim 2012). Lopes et al (2008) found that replicated

populations of *C. elegans* exposed to anthelmintic treatment over 20 generations at first incurred a cost to resistance in terms of survival, fecundity and a decrease in the proportion of males. However, after 20 generations survival and fecundity returned to previous levels, and male frequency had begun to increase. This suggests that populations can indeed adapt to continued exposure to drug treatment; however, these studies have only begun to explore the relationship between resistance and life history traits. For example, it would be interesting to establish whether there are eco-evolutionary feedbacks between resistance and life history, and whether other ecological factors such as changes in mortality rates and competitive interactions influence resistance evolution.

Differences in mortality rate and population density between treated and untreated populations could result in differential selection due to density-dependent processes such as competition (Gilleard and Beech 2007). Studies of resistance evolution can impose strong directional selection on life history traits such as development time, size at maturity and investment in and timing of reproduction (Chehresa, Beech, and Scott 1997). Because life history traits can respond to selection in many types of environments as a result of changes in mortality and density (Reznick and Ghalambor 2001), adequate controls are needed to assess the effects of changes in traits not directly associated with the application of control measures (Gilleard and Beech 2007). Laboratory-based selection experiments typically assess resistance evolution by comparing survival in treated and untreated control populations (Ranjan et al. 2002; Coles, Rhodes, and Wolstenholme 2005; Lopes et al. 2008). However, this methodology does not account for differences in mortality and density between treatments. Inclusion of treatments that mimic the rate of mortality and density caused by control measures would allow the assessment of any responses in life history traits not due directly to chemical exposure (Fuller, Baer, and Travis 2005).

1.5 Study system

1.5.1 *Selection experiments*

Selection experiments study evolution as it happens, in a controlled and replicated manner, in contrast to observational studies made in the wild where replication and strict control of environmental variables is difficult (Fuller, Baer, and Travis 2005; Garland and Rose 2009; Kawecki et al. 2012). In essence, by replicating the number of populations exposed to a novel environment, it can be established whether there is a consistent evolutionary response to selection. Experimental evolution studies allow the observation of phenotypic and genetic change in populations and communities caused by experimentally imposed selective pressures. Because other environmental factors not of direct interest to a study can be closely controlled, their impact can be reduced to noise, and any changes occurring as a result of those intentionally imposed by the researcher can be attributed to the selection regime (Fuller, Baer, and Travis 2005; Buckling et al. 2009; Garland and Rose 2009). Experimental evolution does not directly attempt to reproduce or predict evolution in the wild; the main purpose is to test evolutionary theory and hypotheses. Selection experiments have been classified into two different categories: artificial selection, where individuals are selected to propagate the next generation on the basis of a specific trait value, and laboratory natural selection (LNS), where the investigator decides on the source and intensity of selection but does not control which traits respond to selection (Fuller, Baer, and Travis 2005). Other categories have been suggested, but these can usually be designated as sub categories, such as culling experiments, a subcategory of LNS experiments, where populations are exposed to a stressful environment causing some mortality, and the offspring of survivors go on to form the next generation.

Culling experiments have been particularly useful in exploring factors that affect the rate of resistance evolution. For example, cage studies of replicate populations of flies exposed to insecticide have been used to assess the effect of dose and decay rate of a control agent, and immigration rate of susceptibles on the rate of resistance evolution (Taylor, Quaglia, and Georghiou 1983). Taylor et

al (1983) found that higher insecticide doses with a slow decay rate and little or no immigration promoted the rapid evolution of resistance, whereas, with a rapidly decaying insecticide and some immigration of susceptible flies, the rate of resistance evolution was slower. In addition to exploring what factors affect the rate of resistance evolution, culling experiments have been used to assess the effectiveness of control strategies used to delay the rate of resistance evolution. For instance, the effectiveness of drug combination treatments in conjunction with maintenance of refugia for susceptible parasites has been investigated in parasitic nematodes (Leathwick et al. 2012). At the end of the selection experiment, drug efficacy was highest when using a combined strategy of drug combinations and refugia, but maintaining a refuge out performed a combination drug treatment when implemented separately, and the use of a single drug with no refugia resulted in the highest rate of resistance evolution. This experiment illustrates how experimental evolution can be exploited to assess the effects of different drug dosage regimes on the rate of resistance evolution.

Culling experiments have also been used to test predictions of life-history theory, with much of this work being conducted on fruit flies (Joshi 2000; Prasad and Joshi 2003). For example, high rates of extrinsic mortality imposed randomly on replicate populations of *Drosophila melanogaster* resulted in shorter lifespan, decreased age and size at maturity, and a shift in peak fecundity to earlier in life, relative to populations exposed to a low rate of extrinsic mortality (Stearns, Ackermann, and Doebeli 1998; Stearns et al. 2000). The results confirmed predictions about how growth, maturation, reproduction, and ageing respond to selection in environments varying in the risk of mortality. The application of control measures reduces population density and imposes a source of high extrinsic morality on target populations, which could both influence life-history evolution of pests and parasites. Selection experiments implementing controls for differences in density between drug-treated and drug-free environments would allow the effects of density and high risk of mortality to be separated from those of direct adaptation to a control agent.

1.5.2 *Caenorhabditis remanei as a model of resistance evolution*

Studies of adaptation have mainly been conducted on small organisms, which are easy to maintain and have a short generation time. The choice of the study system becomes largely a matter of suitability to the question being addressed by a study. For instance, phage-bacteria and Daphnia-pathogen systems have been used to study questions relating to coevolution (Buckling and Rainey 2003; Ebert 2008). For investigating long-term evolution over tractable periods of time, using microbes has obvious advantages. However, metazoan systems are required to answer evolutionary questions involving complex processes such as sexual selection, development and behaviour. Therefore, model systems have expanded to include fruit flies, fish, mice and plants, amongst others (Kawecki et al. 2012). Recently, the nematode worm *Caenorhabditis elegans* and closely related species have been adopted as powerful models for addressing ecological and evolutionary questions using selection experiments (Gray and Cutter 2014). The genus contains 26 species, many of which have had their whole genome sequenced (Kiontke et al. 2011). In addition, many species within the group have differing reproductive modes, making them an ideal model for studying the evolution of sex. *Caenorhabditis* species have numerous other qualities that make them an ideal candidate for use in experimental evolution. Under laboratory conditions, the *Caenorhabditis* life cycle is around two days from egg to adult at 20°C; therefore, selection experiments can be run over many generations within a few weeks. Early larval stages of the genus can survive cryopreservation (Hope 2001), meaning that samples of evolving populations can be stored and preserved indefinitely, and traits of interest can be compared between ancestral and evolved populations. Populations can be reared in liquid culture or on a solid agar substrate in petri dishes, and only require a diet of bacteria as a food source, which means that large numbers of individuals can be cultured quickly and cheaply (Hope 2001). The average lifespan of worms is two weeks under standard laboratory conditions and makes them a tractable model for studying life-history characters such as lifespan, fecundity, and age and size at maturity in replicate populations subjected to different environments (Chen and Maklakov 2012). The development of automation, image processing and microfluidic techniques complementing the *Caenorhabditis* system is now

making it possible for high-throughput studies of many traits to be conducted (Husson 2012). The range of molecular methods open to evolutionary biologists using *Caenorhabditis* species is unrivalled. For instance, reversible gene knockout using RNAi is relatively simple to perform in *Caenorhabditis* by inserting or removing a plasmid into the *E. coli* food source, and can be used to assess gene function in different environments (Timmons and Fire 1998). In addition, strains with specific gene deletions are available publically, and recent work on targeted genome editing using CRISPR/Cas9 has made it possible to engineer specific alleles in *Caenorhabditis* (Frokjaer-Jensen 2013).

Treatment of nematode infections provides a well-documented area of research in which problems relating to the evolution of resistance have been explored using *C. elegans* (Driscoll 1989; Coles et al. 1988; Geary and Thompson 2001; Lopes et al. 2008; James and Davey 2009). Because parasitic helminths are difficult to culture, research into anthelmintic resistance has used *C. elegans* in screening potentially new candidate drugs and identifying resistance loci (Simpkina and Coles 1981; James, Hudson, and Davey 2009; Ghosh et al. 2012). However, *C. elegans* is an androdioecious nematode and reproduces mainly by self fertilisation, although low levels of outcrossing do occur due to a small proportion of males (Brenner 1974; Barrière and Félix 2007). In contrast, most parasitic nematodes are obligately outcrossing; therefore a free-living, dioecious nematode species such as *C. remanei* would provide a more realistic model system to explore resistance evolution. Additionally, *C. remanei* populations, like parasitic species, have abundant standing genetic variation and high levels of recombination due to their reliance on sexual reproduction (Cutter, Baird, and Charlesworth 2006). Both of these attributes should facilitate a rapid response to selection in the face of drug treatment. Thus, selection experiments using *C. remanei* as a model organism afford a tractable model system in which to explore the factors affecting resistance evolution in commercial drugs.

1.6 Thesis aims and objectives

Eco-evolutionary processes such as feedbacks between density-dependence and life history could occur as a result of the application of xenobiotics, but this type of interaction is difficult to observe in parasitic species. Free-living species from clades related to parasitic species can be kept in microcosms allowing population density to be manipulated, either by chemical application or artificially, over generations as part of a controlled selection experiment. This type of experiment allows all other environmental factors to be kept constant during selection, and provides an explicit test of what changes in life history occur as a result of drug application, due either directly to long-term exposure to a control agent or indirectly as a consequence of density-dependent processes. *C. remanei* is a free-living, short-lived nematode worm, and has been previously used as a model to study rapid adaptation of life history to changing environments. As a model system it offers the opportunity to assess how changes in life history, population density and resistance interact. The overall aim of this thesis was to take an experimental evolution approach to assessing how life history traits respond to drug selection, using a free-living dioecious worm (*C. remanei*) as a model. The principal objectives of each chapter were:

1) **Chapter 2:** To establish the relationship between *C. remanei* survival and Ivermectin dose over a range of concentrations within a single generation, in order to control the magnitude of selection during the selection experiments described in **Chapter 4**.
2) **Chapter 3:** To perform simulation-based power analyses aimed at optimising the number of replicate lines and the intensity of subsampling from those lines after selection, for selection experiments similar to that used in **Chapter 4**.
3) **Chapter 4:** To assess whether there is an increase in survival across generations of populations selected in drug-treated environments, and whether this varied with the intensity of selection (i.e. drug dosage). In addition, I asked whether density-dependent selection affects the apparent evolution of resistance by measuring survival of randomly culled

lines, which controlled for differences in mortality and density between drug-treated and drug free populations.

4) **Chapter 5:** To establish whether rapidly passaged control lines, drug selected lines or randomly culled lines from the selection experiment conducted in **Chapter 4** showed any evolved changes in life history.

In **Chapter 2**, I investigated the relationship between *C. remanei* survival and Ivermectin dose. Two doses were chosen that differed in the intensity of selection that would be imposed during the selection experiment in **Chapter 4**. The dose-response data were also used to appraise curve-fitting methods, which could be used to improve model fitting of dose-response studies in parasitology. In addition, I showed how dose-response modeling that incorporates background mortality into the analysis could be used to test for a cost of resistance in terms of survival. In **Chapter 3**, I asked how simulations can be used to inform important design choices used in selection experiments; specifically, the number of replicate selection lines, the number of samples taken from within each replicate line, and variation due to repeated measures used to assess traits at the end of selection. In **Chapter 4**, I investigated how Ivermectin dosage and changes in population density affect the rate of resistance evolution. I created replicate lines of *C. remanei* exposed to Ivermectin at high and low doses to assess whether survival of lines selected in drug-treated environments increased, and if this varied with the intensity of selection. Additionally, I maintained lines where mortality mimicked that of treated lines to control for differences in density between drug-treatments and to distinguish between the evolutionary consequences of drug-treatment vs changes in density-dependent feedback on life history traits. Both adult and juvenile survival was measured to explore relationships between life-history stage, selection regime and survival. In **Chapter 5**, I investigated whether there were any evolved changes in life-history of *C. remanei* populations after selection in a drug-treated environment. I measured larval and adult size, female fecundity and female lifespan in drug-free and treated environments, asking: 1) Does rapid passage of lines in the drug-free (control) environment result in selection for smaller size, lower fecundity or shorter lifespan? 2) Does drug selection affect life-history traits? 3) Does density-dependent selection affect the life history of randomly culled lines? Finally, in **Chapter 6**, I discuss the broader implications of my work. Specifically:

1) how improved methods of analysing dose-response data would benefit parasitology studies monitoring resistance; 2) how *a priori* simulation-based power analysis can help to optimise the experimental design of selection experiments; 3) how ecological change such as changes in density or extrinsic mortality occurring as a result of the application of control measures interacts with life history and susceptibility to control measures.

Chapter 2: Detecting a cost of resistance using dose-response modeling

2.1 Abstract

Dose response studies are an import diagnostic tool for measuring resistance in target populations. Current dose-response models of parasite and vector survival often fit simple sigmoidal curves and do not consider alternative models, which could affect estimates of the metrics used to assess levels of resistance. Standard pre-analysis practices remove valuable data on background mortality, which could be used to test for costs associated with resistance. Using survival data from a larval development test (LDT) following treatment with Ivermectin applied at varying concentrations to *Caenorhabditis remanei*, this study used Akaike Information Criterion (AIC) model selection to compare a range of potential dose-response curves, and used maximum likelihood ratios to test for a cost of resistance at sublethal drug doses. Specifically, comparisons were made between LD_{50} and LD_{99} estimates obtained using log-logistic, Weibull and hormetic dose-response models. Using the best-fitting model, the upper asymptote of the dose-response curve was then used to test whether a cost of resistance in survival would be detectable, based on lower survival at these sublethal lethal doses (i.e. the background mortality that is often removed prior to data analysis) of a simulated resistant population compared to a fully susceptible population. The type of model fit to the dose-response data from the LDT had a significant effect on the estimates of LD_{50} and LD_{99}, suggesting that failure to fit an appropriate model could give misleading estimates of resistance status. A test of difference in asymptotes detected a cost of resistance of 4% survival in the simulated resistant population, meaning that even a small cost of resistance would be detectable using this approach. The results suggested that widening the range of models considered can give more accurate predictions of dose-response curve parameters, and that incorporating background mortality into the analysis can provide an effective method for assessing potential costs to resistance.

2.2 Introduction

Drug resistance in parasites, parasite vectors and pest species poses serious challenges to disease control and agriculture around the world (Oerke 2006; GBD collaborators 2015), and the development of new xenobiotics that combat such species tends to be slow (Livermore et al. 2011). Diagnostic methods that monitor drug efficacy play a key role in preserving the effectiveness of existing control measures, and numerous methods are used to detect and measure resistance in different disciplines (Coles et al. 2006; Bagi et al. 2015). One common approach is to establish the relationship between survival and drug dose using dose-response assays (Coles et al. 1988; Michaela Dolinská, Königová, and Várady 2012). Samples of resistant populations can then be compared either temporally or geographically with known susceptible populations to assess differences in their resistance profile. In addition, it is important to establish whether there is a cost to resistance; i.e. if resistant individuals have lower fitness than susceptible individuals in drug-free environments. If susceptible members of the population have higher fitness than resistant individuals this could slow the spread of resistance alleles, depending on factors such as the frequency of application of control measures (Barnes, Dobson, and Barger 1995; REX Consortium 2013). Costs to resistance in terms of survival can be measured by exposing susceptible and resistant isolates to untreated environments and contrasting survival between the isolates. Current methods of analysis used to detect resistance employing a dose-response approach generally do not incorporate a measure of the cost of resistance, because survival in treated environments is scaled against survival in drug-free environments prior to statistical analysis; i.e. mortality occurring in drug-free controls is deducted from mortality occurring at each dose used in the dose-response assay. This rescaling of survival data means that valuable information about potential costs of resistance is lost during the analysis of dose-response data. Measuring costs of resistance can be easily incorporated into dose-response analyses by including sources of mortality not directly due to a control agent.

Dose-response studies measure the relationship between the quantity of a substance or time of exposure and the effect it has on an organism. The

response variable may be continuous, as in the case of data measuring growth; alternatively, it may be binary (e.g. alive/dead), such as data collected when measuring drug efficacy. Dose-response studies are important in numerous fields, including: ecotoxicology (Ritz 2010), parasitology (Coles et al. 2006), pest control (Bagi et al. 2015) and weed control (Busi and Powles 2009). In addition, dose-response studies are used to establish and control the intensity of selection during experimental evolution studies, with the aim of applying an environmental stressor consistently over the duration of an experiment (Busi and Powles 2009). Dose-response models assume a given response variable *y* is explained in terms of a model function *f* that depends on the dose *x* (Equation 1):

$$y = f(x, \beta) \tag{1}$$

The form of the function *f* is known as it reflects the assumed relationship between *x* and *y*, except for the values of the model parameters *β*, which are estimated from the data (Ritz and Streibig 2007). Dose-response curves are usually sigmoidal and can be defined by four parameters (Equation 2). The first two parameters are *d* and *c* are the upper and lower asymptotes and define the upper and lower limits of mean survival, respectively. In other words, *d* is the expected survival before increasing dose has any effect (background mortality), and *c* is the expected survival at doses where increasing dose has no further effect. The third parameter *e*, is the inflection point, which is the dose where the curve changes from concave to convex and is located at or near the LD_{50} (dose causing 50% mortality) for the drug. The fourth parameter *b*, is the slope of the curve at the LD_{50}. Log-logistic models are often used to describe the dose-response relationship between survival and dose, and generally the 4-parameter model is applied, corresponding to the model function:

$$f\big(x, (b, c, d, e)\big) = c + \frac{d - c}{1 + \exp(b(\log(x) - \log(e)))} \tag{2}$$

Log-logistic models assume that the dose-response relationship is symmetrical around the inflection point *e* (Fig 2.1 A). However, a range of asymmetric

models, such as Weibull and hormetic curves can be applied to dose-response data (Fig 2.1). Fitting a range of potentially suitable models that can accommodate asymmetry in the dose response relationship, in addition to log-logistic dose response models, should ensure that the most appropriate model is applied to a data set to improve the accuracy of estimates.

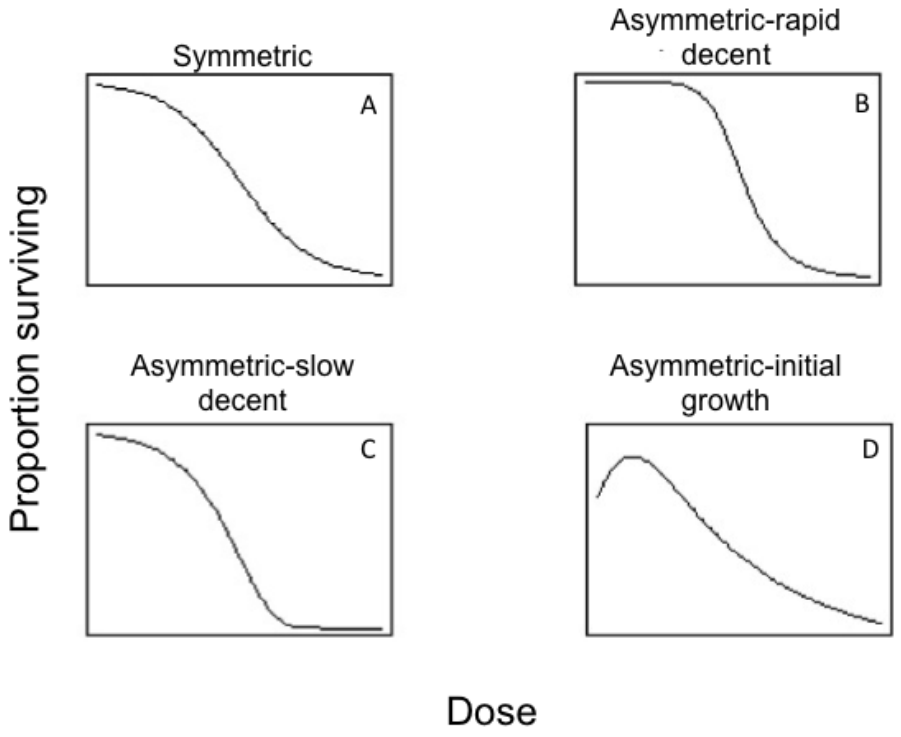


Fig 2.1 Symmetric and asymmetric dose-response curves: A) Asymmetric dose-response curve (logistic or log-logistic); B) An asymmetric curve where the decrease in survival from the upper limit is rapid, but the approach towards the lower limit is slow (Weibull-2); C) An asymmetric curve where the decrease in survival from the upper limit is slow, but the approach towards the lower limit is rapid (Weibull-1); D) An asymmetric curve suitable for modeling an initial increase in survival (Hormetic). Figure adapted from Ritz et al (2007).

Studies interested in the control of pest and parasite populations often summarise estimates from dose response curves of different populations to assess any differences in resistance (Amarante et al. 1997; Busi and Powles 2009). If these estimates are based on inappropriate dose-response models then any attempt to evaluate differences between resistant and susceptible populations could result in inaccurate conclusions. Two frequently used metrics in dose response studies are the LD_{50} and LD_{99}; the lethal doses representing 50% and 99% of a target population. The LD_{50} is useful when comparing resistant and

susceptible isolates; for example, the degree of resistance (resistance factor) can be calculated by dividing the LD_{50} of an isolate of interest by the LD_{50} of a known susceptible isolate (Amarante et al. 1997). The resistance factors of several isolates can then be compared with one another and help in assessment of how resistance changes in time or space (Busi and Powles 2009; Michaela Dolinská, Königová, and Várady 2012). The discriminating dose is the dose (LD_{99}) that represents the maximum dose a given population can tolerate; any individuals surviving are considered to be resistant (Coles et al. 2006). Both the LD_{50} and LD_{99} are estimated from the dose-response model fitted to survival ~ concentration data. Thus, fitting an inappropriate model could result in inaccurate estimates of LD_{50} and LD_{99} and erroneous inferences about the comparisons of resistance between populations. Comparing a range of dose-response models and selecting the most appropriate model could improve the accuracy of estimates of the degree of resistance both within and between populations.

Investigators using a dose-response approach as a diagnostic method to quantify drug resistance usually measure survival over a range of doses but adjustments for background mortality prior to analysis could affect model estimates. During a dose-response assay survival is measured over a range of doses. Counts of the initial number of individuals and the final number of survivors are then used to calculate the proportion of mortality due to drug treatment as a function of concentration. Data about background mortality can be accounted for by subtracting mortality in control (untreated) conditions from that in drug-treated environments (Abbott 1925). It is difficult to establish how prevalent this type of *a priori* data manipulation is within the field of dose-response studies associated with documenting resistance, but it could be extensive. For example, WHO guidelines on conducting dose-response assays on mosquitoes specifically incorporate the use of Abbott's correction prior to data analyses (World Health Organization 2013). In addition, the method of adjusting survival data described by Abbott (1925) has over 11,000 citations (Google Scholar), a search within citied articles for 'vector control' found 2420 citations, and a search of 'parasite' found 918 citations. Modifying the data collected from dose response assays in this way results in two unwanted outcomes. Firstly, manipulating data prior to analysis is not necessary for survival data with a binomial distribution,

and can result in incorrect estimates of variance in adjusted data (Rosenheim and Hoy 1989). Secondly, fitness costs could occur in relation to drug resistance if parasitic species divert resources from other fitness-enhancing traits including survival. If resistant populations have lower survival than susceptible populations at sublethal doses then a fitness cost can be measured. This information about differences in background mortality between resistant and susceptible populations is forfeited in the process of adjusting dose response data. Identifying fitness costs associated with resistance may be of importance in limiting the spread of resistant populations (Kliot and Ghanim 2012). If fitness costs are high then it is likely to take longer for resistance to spread through a population because susceptible individuals may have higher reproductive rates or grow faster even though they may have reduced survival in drug-treated environments (Carriere et al. 1994). Testing for a cost of resistance could be achieved simply by comparison of susceptible and resistant populations in a drug-free environment, but estimating a cost of resistance based on differences in survival at sublethal doses across the upper asymptote of the dose-response curve will provide a more powerful test given the greater sampling effort. The upper asymptote of the dose response curve represents concentrations of drug that have no direct effect on survival. Incorporating background mortality into the analyses of dose-response data would allow direct comparisons of survival to be made between resistant and susceptible isolates across a range of sublethal doses, which could be used to quantify costs to resistance in terms of survival. Thus, lower survival at the asymptote of a resistant isolate relative to a susceptible isolate would provide evidence of a fitness cost. However, to my knowledge, there are no studies that have implemented a test of a cost to resistance at sublethal doses to infer such costs. In addition, I am unaware of any dose response studies assessing drug resistance that have published the original survival data along with the study, so a test of upper asymptotes between resistant and susceptible cannot be conducted on previously collected data. However, simulations can be used to explore the patterns that emerge from biological models, and examine whether models based on mathematical functions with associated probability distributions provide realistic representations of a study system (Bolker 2007). Simulated data with higher survival of resistant individuals over the range of doses associated with normal drug efficacy and lower survival at sublethal doses, relative to susceptible

individuals, would allow the assessment of a test of differences in asymptote. A method of modeling survival data, which assesses a range of suitable dose-response models and incorporates background mortality would provide an effective means of increasing the accuracy of estimates of resistance, and provide a test of a cost of resistance.

Treatment of parasitic nematodes often involves the application of broad-spectrum anthelmintics from one of three major classes: benzimidazoles, imodothiazoles and macrocyclic lactones. Ivermectin is a macrocyclic lactone and has been used commercially since 1981 (James, Hudson, and Davey 2009), with the first reports of resistance in 1988 (Kaplan 2004). Ivermectin causes paralysis in larvae and adult nematodes and inhibits feeding (Sangster and Gill 1999) but also has a repellent effect at sub-lethal doses (Ardelli et al. 2009). Ardelli et al (2009) found that replicated measures of *C. elegans* movement exposed to 2.5 nM and 5 nM Ivermectin resulted in increased traveling behaviour (long-range roaming) relative to zero dose controls, but worms exposed to 10 nM showed no increase in movement. Thus, at high doses where worms are paralysed, repellency does not occur, but at low doses Ivermectin has a repellent effect resulting in hyperactivity and is likely to be an additional source of mortality, due to worms attempting to evade the effects of the drug. This additional source of mortality should be taken into consideration when assessing the relationship between survival and drug dose. Because parasitic helminths are difficult to culture, studies of nematode disease have often used the model organism *Caenorhabditis elegans* in both drug screening and identifying candidate resistance loci (Simpkin and Coles 1981; James, Hudson, and Davey 2009; Ghosh et al. 2012). However, *C. elegans* reproduces mainly by self-fertilisation, and although low levels of outcrossing do occur, genetic variation within the species does not reflect that observed in parasitic species, which are usually obligately outcrossing (Geary and Thompson 2001). Other free-living dioecious nematodes such as *C. remanei* may thus provide a more realistic model system to explore resistance. *C. remanei* populations have abundant standing genetic variation and high levels of recombination, due to their reliance on sexual reproduction (Cutter, Baird, and Charlesworth 2006), which also makes them a good candidate for studying resistance.

The overall aim of this chapter was to fit the most appropriate non-linear regression model to dose-response data, which could then be used to test for evidence of a cost of resistance at sublethal doses. Specifically, I asked:

1) Which dose-response curve model best describes the relationship between Ivermectin dose and *C. remanei* survival?
2) Can background mortality at sublethal doses be used to detect a cost of drug resistance?

2.3 Methods

2.3.1 *Origin and maintenance of worm populations*

In order to perform a LDT on a population with abundant standing genetic variation, I obtained a genetically diverse strain of *C. remanei* (SP8) from N. Timmermeyer in the Department of Biology, University of Tübingen, Germany. This strain was originally created by a fully factorial crossing of three wild-type strains isolated from geographically distant locations (SP146 from Freiburg, Germany; MY31 from Tübingen, Germany; PB206 Ohio, US). Offspring of the crosses had been tested to ensure they were fertile then pooled, and maintained for eight generations to create recombinant genotypes and allow adaptation to standard laboratory conditions (Fritzsche et al. 2014). Upon arrival in Glasgow, strain SP8 was cultured for four generations to acclimate to the laboratory conditions, which were standard for *Caenorhabditis* species: 20˚C and 60% humidity on Nematode Growth Medium (NGM) petri dishes and fed on a lawn of *Escherichia coli* (OP50) (Hope 2001).

2.3.2 *Larval development test*

To quantify the relationship between drug dosage and survival for strain SP8 replicate samples of the population were exposed to a range of doses of

Ivermectin. A stock solution of 2 mg/ml Ivermectin (22,23-Dihydroavermectin B1; Sigma-Aldrich) dissolved in DMSO was decanted into 1-ml aliquots and frozen to provide a standardised drug dose. I used a modified version of the dose response approach taken by Coles et al. (1988), exposing replicate samples of *C. remanei* over a range of 15 doses (0, 0.1, 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 7, 8, 9 and 10 ng/ml); chosen because this range has been previously used in studies of *C. elegans* (James and Davey 2009). Dilutions of Ivermectin were added to 100 ml liquid NGM (50°C) and mixed with a magnetic stirrer before pouring 7 ml aliquots into 5.5 cm plastic petri dishes. These were left to dry, seeded with *E. coli* (OP50) *ad libitum* to minimise indirect mortality resulting from worms leaving the agar surface of the petri dishes in search of food, and incubated at 20°C overnight. Concurrently to preparing dosed plates, age-synchronised eggs were harvested from stock populations of *C. remanei* by bleaching using standard protocols (Hope 2001). This process kills adults and juveniles but leaves unhatched developing embryos unharmed. Eggs were moved to fresh drug and food-free 9-cm petri dishes and incubated overnight to provide a source of L1-arrested larvae for drug screening. After 12 hours incubation, larvae were suspended in M9 buffer solution (3g KH_2PO_4, 6g Na_2HPO_4, 5g NaCl, 1 ml M $MgSO_4$, H_20 to 1 litre and sterilised by autoclaving) and 5 µl aliquots of this suspension were added to Ivermectin-dosed plates, with the aim of applying approximately 60 larvae per plate. Larvae added to petri dishes were counted as they were set up. Survival data were obtained by counting the number of adults present per plate at 75 hours, which is long enough to ensure the adult stage was exposed to the drug but was not long enough for any offspring of the screened population to develop to adulthood and confound accurate survival counts. Twenty replicate plates were established for each Ivermectin dose (ten replicates in each of two different batches, tested one month apart using separately prepared plates).

2.3.3 *Statistical analysis*

Preliminary analysis performed by visual assessment of a range of dose-response curves found evidence of an indirect source of mortality at low doses. Models using all the survival data resulted in overestimates of mortality at very low

doses, and under estimates of survival at the upper asymptote compared to a reduced dataset excluding the values at 0.5 and 1 ng/ml Ivermectin. Because indirect mortality has been previously observed in *C. elegans* (Ardelli et al. 2009), a sister species to *C. remanei* and because I was interested in modeling mortality directly associated with the action of the drug, I used a reduced dataset. The reduced dataset excluded survival data at two doses (0.5 and 1 ng/ml) of Ivermectin where indirect morality was apparent. An assessment and justification for removing these data is given in Appendix A. All further analysis was conduct on the reduced dataset, which included survival data for 13 doses (0, 0.1, 1.5, 2, 2.5, 3, 4, 5, 6, 7, 8, 9 and 10 ng/ml Ivermectin).

All statistical analyses were performed using the *drc* package (Ritz and Streibig 2007) in the statistical environment *R* v 3.1.2 (R Core Team 2014), and I defined a significance threshold of P = 0.05 for all tests. In order to establish the best fitting model of survival as a function of the concentration of Ivermectin, dose-response log-logistic and Weibull and hormetic models were fitted to the reduced dataset collected in the LDT, assuming *X* is a binomially distributed random variable, *n* is the total number of worms, and *p* is the probability of survival at a particular dose determined by the dose response model (Fig 2.1, Equation 3).

$$X \sim B(n, p) \tag{3}$$

Three parameter Log-logistic, Weibull-1, Weibull-2 and Hormetic models with the lower asymptote of the dose-response curve set to zero were used to prevent negative predictions of survival in the fitted dose response curves. Because 4-parameter Log-logistic models are commonly applied to survival ~ concentration data, this model was also used in the analysis. The relationship between survival and dose for each of these models is described in Table 2.1. Model choice was made using AIC, assuming that the lowest AIC value identified the best-fitting model of the dose-response data (Aho, Derryberry, and Peterson 2014). Estimates of the LD_{50} and LD_{99} were calculated for each model to assess differences in predictions between models.

Table 2.1 List of dose-response models and their functions for describing the relationship between survival and dose.

Model **(parameters)**	Model function
Log-logistic (4)	$c + \frac{d - c}{1 + \exp\big(b(\log(x) - \log(e))\big)}$
Log-logistic (3)	$\frac{d}{1 + \exp(b(\log(x) - \log(e)))}$
Weibull-1 (3)	$\exp(-\exp(b(\log(x) - \log e)))$
Weibull-2 (3)	$d(1 - \exp\big(-\exp\big(b(\log(x) - \log(e))\big)\big))$
Hormetic (4)	$c + \frac{c + fx}{1 + \exp(b(\log(x) - \log(e)))}$

NB: The hormetic model has an additional linear parameter (f) to model increases in the response variable at low doses (Fig 2.1D).

To test for a difference in background mortality (a cost of resistance) between susceptible and resistant populations, survival at the upper asymptote of the dose-response curve was compared. Survival data for the susceptible population was the same data collected in batch one of the LDT. Survival data for a simulated resistant population with a cost of resistance in terms of survival was generated by adjusting the data from batch two of the LDT. The data set from batch two was adjusted to decrease survival by 4% for 0 and 0.1 ng/ml Ivermectin to simulate a cost of resistance. In addition, survival across the range of doses where survival dropped in batch two was increased by 5% to simulate resistance, doses 1.5, 2, 2.5, 3 and 4 ng/ml Ivermectin. Adjustments in survival were achieved by changing the number of survivors in each replicate plate of batch two. For instance, to simulate a decrease in survival at the asymptote, survival data for each replicate plate at 0 and 0.1 ng/ml Ivermectin was adjusted by increasing the number of deaths that occurred in each replicate plate by 4%. To confirm that the simulated resistant population had significantly higher survival than the susceptible population over the range of doses where mortality due to drug treatment increases from zero to 100%, a Weibull-1 model of the survival-dose relationship was applied to the simulated data with a further explanatory variable for population, and contrasted with a null model where population was removed. To test for a cost of resistance between the simulated susceptible and resistant populations, a Weibull-1 model of the survival-dose relationship was applied to the simulated dataset, which assumed

different upper asymptotes between populations, compared to a null model assuming a common asymptote. In addition, the survival data from a drug-free environment (zero dose) was used to test for a cost of resistance in the simulated populations using a GLM, assuming a binomial distribution. This test was conducted to assess differences in using a comparison of asymptotes as part of dose response modeling, with a standard test of a cost of resistance in a drug-free environment. Differences in survival between susceptible and resistant populations were assessed by comparing a full model with population as an explanatory variable against a null model assuming no difference in survival due to population.

2.4 Results

2.4.1 *The relationship between C. remanei survival and Ivermectin dose over a range of concentrations*

The 3-parameter Weibull-1 model was identified as the best-fitting model to the survival ~ concentration data and had a AIC value 30 points lower than the next best model (Fig 2.2 and 2.3, Table 2.2). The predicted LD_{50} of the 4-parameter log-logistic model was similar to that of the Weibull-1 model but no estimate of the LD_{99} or confidence intervals could be made due to the negative predictions of survival at high doses (Fig 2.2, Table 2.2). There was no difference between the 3-parameter Log-logistic and hormetic models, indicating no initial increase in survival due to hormesis at low concentrations of Ivermectin (Fig 2.2, Table 2.2); both models gave the same predicted LD_{50} as the Weibull-1 model but the predicted LD_{99} was 20% higher than the Weibull-1 model. The Weibull-2 model, which predicts a sharp descent in survival at low doses, gave the worst fit to the dose response data; the predicted LD_{50} was 4% lower than the Weibull-1 model, whilst the LD_{99} was 35% higher (Fig 2.2, table 2.2).

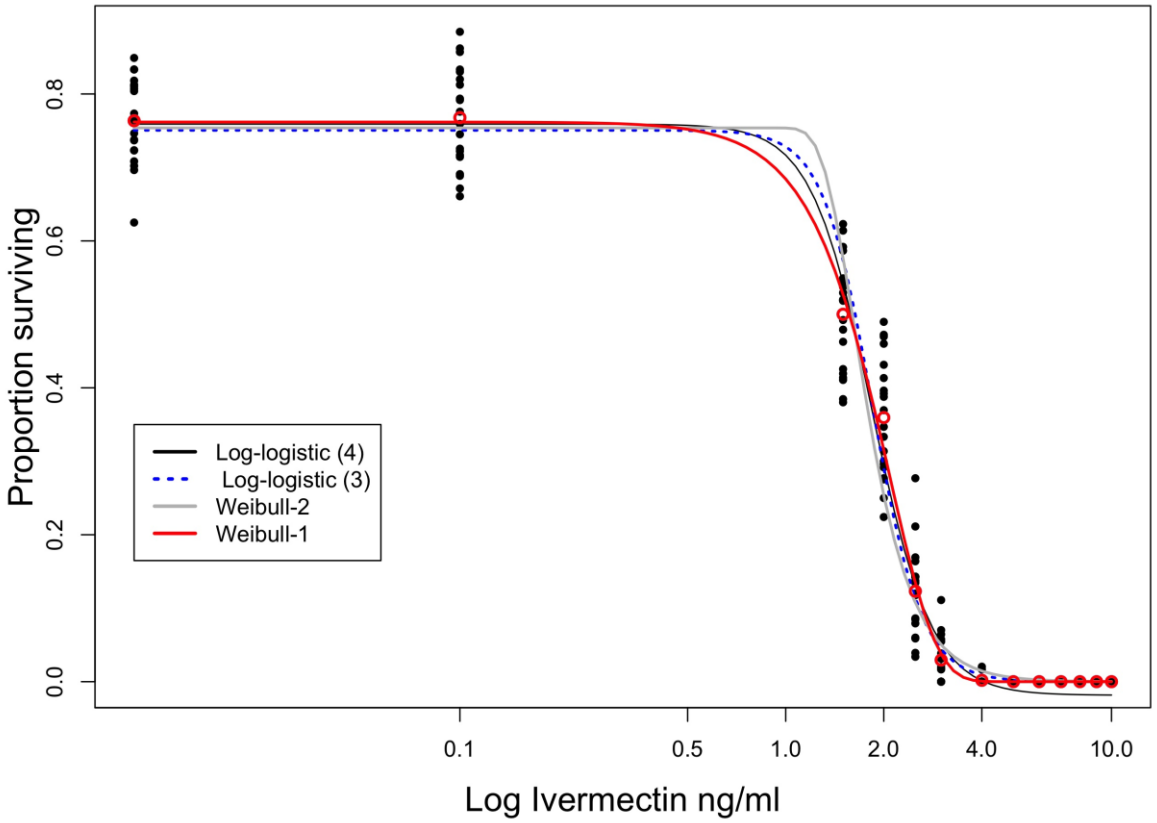


Fig 2.2 Comparison of 3-parameter log-logistic, 4-parameter log-logistic, Weibull-1 and Weibull-2 models for the relationship between *C. remanei* survival and Ivermectin concentration. A hormetic model was indistinguishable from the 3-parameter log-logistic model. Black circles show raw data for each replicate plate of the larval development assay, and red circles show mean survival at each concentration of Ivermectin used in the larval development test.

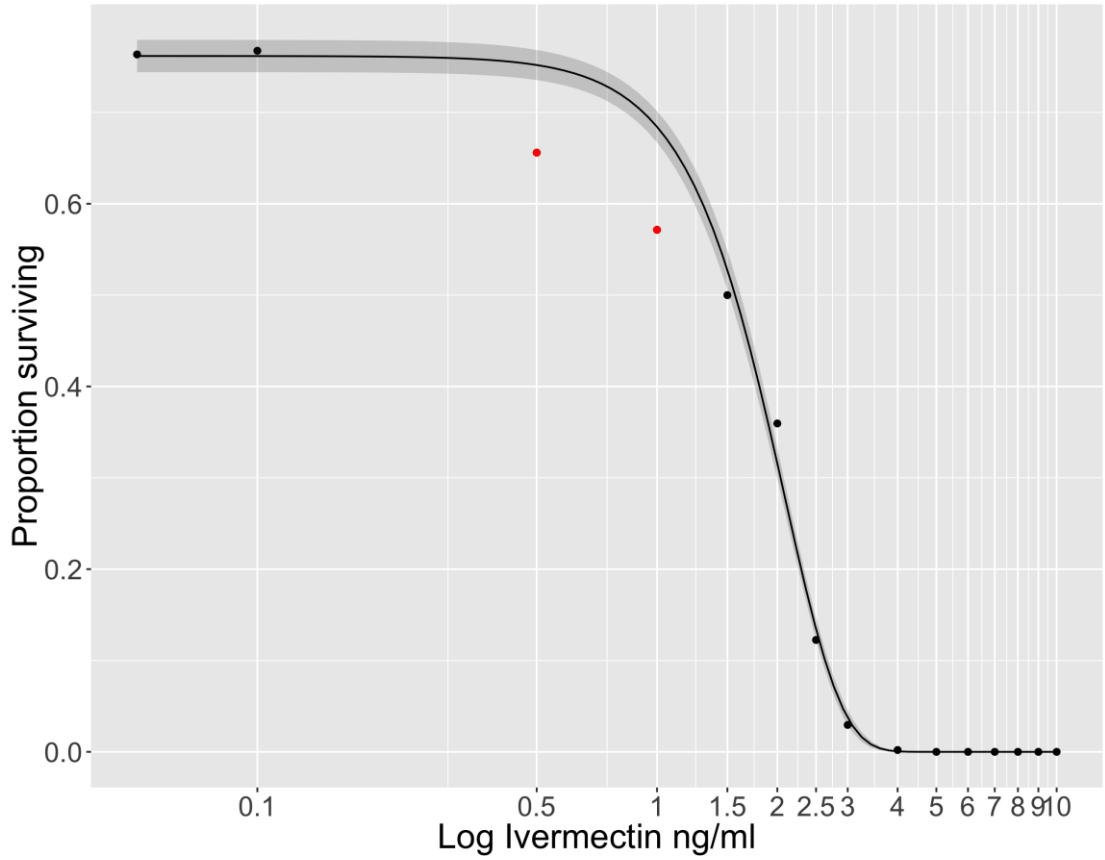


Fig 2.3 Observed data (black circles) and fitted Wiebull-1 model of the relationship between survival of *C. remanei* and concentration of Ivermectin with survival data at 0.5 and 1 ng/ml (red circles) removed form analysis. Circles show observed mean survival at each concentration of Ivermectin used in the larval development test. Dark grey bands show 95% confidence intervals for mean survival based on the model.

Table 2.2 Estimates of the slope, LD_{50} and LD_{99} (ng/ml Ivermectin) for the dose response models fitted to the larval development assay reduced data set (CI: 95% confidence interval for LD_{50} and LD_{99}). AIC (Akaike Information Criterion) is given for each model fit to the full dataset from the larval development test. SE is the standard error of the slope.

Model (no. parameters)	**AIC**	**Slope**	**SE**	**LD_{50} (CI: 95%)**	**LD_{99} (95% CI)**
Weibull-1 (3)	666.63	3.04	0.116	1.85 (1.80, 1.90)	3.45 (3.33, 3.56)
Log-logistic (4)	696.86	4.69	-	1.84	-
Log-logistic (3)	732.83	5.70	0.210	1.85 (1.80, 1.89)	4.13 (3.94, 4.33)
Hormetic (3)	732.83	5.70	0.210	1.85 (1.80, 1.89)	4.13 (3.94, 4.33)
Weibull-2 (4)	805.71	4.39	0.13	1.77 (1.74, 1.81)	4.65 (4.41, 4.89)

No estimates could be extracted for the standard error or the LD_{99} of the 4-parameter log-logistic mode, because of the negative model predictions.

2.4.2 *Detecting a cost of resistance in simulated survival data*

The Weibull-1 model applied to the simulated survival data showed a significant difference in the relationship between survival and Ivermectin dose between the simulated resistant and susceptible populations (x2 = 84.60, d.f. = 3, P < 0.0001; Fig 2.4), and a difference in slopes of the curves (x2 = 33.68, d.f. = 1, P < 0.0001; Fig 2.4). The predicted LD_{50} for susceptibles of 1.80 (95% CI: 1.73, 1.87) was 12% lower than the simulated resistant population: 2.07 (95% CI: 1.99, 2.15; x2 = 74.01, d.f. = 1, P < 0.0001). The test of a difference in background mortality, as indicated by a difference in asymptotes between curves, showed significantly higher mean survival in susceptibles: 78% (95% CI: 75, 80%) than the resistant population: 74% (95% CI: 72, 77%; x2 =4.33, d.f. = 1, P = 0.033; Fig 2.4, Table 2.2). A GLM assuming a binomial distribution found no difference in mean survival when applied to data from a drug-free environment (x2 = 3.08, d.f. = 1, P = 0.079; Table 2.2); estimated mean survival for the GLM was 77% (95% CI: 73, 80%) for susceptibles and 73% (95% CI: 70, 76%) for the simulated resistant population. Thus, the test of a difference in asymptote suggested a cost of resistance, whilst the GLM failed to detect any cost of resistance.

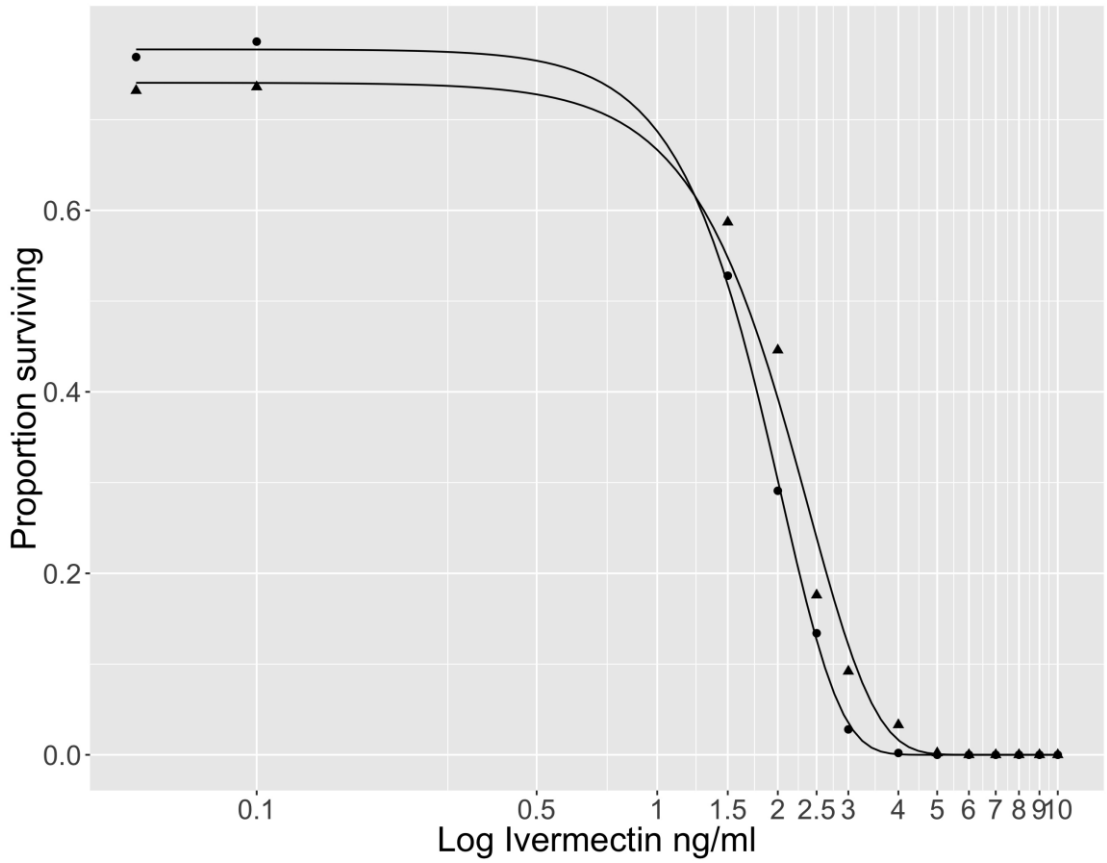


Fig 2.4 Comparison of dose response curves for simulated susceptible (circles) and resistant (triangles) populations.

Table 2.2 Predictions of mean survival at the upper asymptote of the dose response curve for simulated susceptible and resistant populations. The GLM gives predictions of mean survival using only survival data from a drug-free environment.

Model	X_2	**df**	**P**	**Mean survival susceptible (CI: 95%)**	**Mean survival resistant (CI: 95%)**
Weibull-1	4.33	1	0.033	78% (75, 80)	74% (72, 77)
GLM	3.08	1	0.079	77% (73, 80)	73% (70, 76)

2.5 Discussion

In order to establish the relationship between survival and drug dose it is necessary to fit the most appropriate dose response model and recognize other biological sources of mortality, which could affect any estimates derived from the final model. This study shows that failure to assess a range of potentially suitable dose-response models can result in inaccurate predictions of the level of resistance. The best model fit was achieved with the 3-parameter Weibull-1 model (Fig 2.2 and 2.3). In addition, because the survival data were not manipulated prior to analysis, this allowed for a test of a cost of resistance in terms of comparing survival at the upper asymptote of the dose response curves.

2.5.1 *The relationship between C. remanei survival and Ivermectin dose over a range of concentrations*

The principal challenge facing diagnostic measures of resistance is their ability to provide early detection during the development of resistance when the alleles for resistance are rare in the parasite population (Dolinská et al. 2013). Few studies concerned with the detection of resistance consider fitting a range of dose response curves when estimating parameters such as $LD_{50'S}$ and $LD_{99'S}$, with most implementing log-logistic models (Amarante et al. 1997; Tandon and Kaplan 2004; Michaela Dolinská, Königová, and Várady 2012; Dolinská et al. 2013). My analyses suggest that failing to take into consideration other possible dose-response models could result in poorly fitting models with erroneous estimates of parameters of interest. For instance, in my study, predictions of the LD_{50} and LD_{99} differed with respect to the type of dose response curve fitted to the data. In particular, the log-logistic model gave higher predictions of the LD_{99} than the best-fitting Weibull-1 model, meaning that estimates of the maximum dose the population can tolerate were 20% higher than the best-fitting model.

Reduced efficacy of a drug could lead to other strategies being implemented to control parasite populations, and prevent the spread of resistance (Leathwick and Besier 2014). If this decision is made based on incorrect estimates of drug efficacy then this could mean that a more expensive control measure is applied erroneously. Implementing a range of potentially suitable models that can accommodate asymmetry in the dose response relationship, in addition to log-logistic dose response models, should ensure that the most appropriate model is applied to a data set to improve the accuracy of estimates of LD_{50} and LD_{99} values. Studies focusing on the detection of resistance may sample a large number of parasite populations over a number of years (Martin et al. 1984; Michaela Dolinská et al. 2014), assess resistance to a range of drugs (Demeler, Küttler, and von Samson-Himmelstjerna 2010), and perform a variety of in-vivo or in-vitro diagnostic tests (Taylor, Hunt, and Goodyear 2002), all of which can be analysed using a dose-response approach. It seems unlikely that a log-logistic dose response model would be the best fitting under all these circumstances but this requires further testing. My study shows that a range of dose response models, not commonly applied in parasitology, can be easily implemented and assessed when quantifying resistance of parasitic populations. Accuracy, reliability and sensitivity of early detection methods aimed at assessing the resistance status of parasite populations are all likely to be improved by choosing the most appropriate model of dose response data.

2.5.2 *Detecting a cost of resistance in simulated survival data*

The test of a difference in asymptotes found a 4% difference in background survival between the simulated susceptible and resistant population, suggesting that this approach provides a sensitive test to detect a cost of resistance in survival. This test was more sensitive than a test of differences in survival in a drug-free environment because modeling the asymptote assesses differences in a range of doses that cause no drug-induced mortality. However, the difference in survival between resistant and susceptible populations at 0.1 ng/ml was greater than the difference at dose zero so the GLM used only zero dose data and assessed a smaller effect size estimate than the *drc* model. This does not

invalidate the approach of testing for a difference in asymptotes because the greater sampling effort used in a test of asymptotes will be more powerful than the GLM using less data from a drug-free environment. If dose-response analysis was routinely conducted on data that was not standardised for background mortality, then a test of the cost of resistance in survival of resistant populations could be implemented in addition to comparing LD_{50} and LD_{99} derived resistance ratios. Costs of resistance in other traits measured using dose response approaches in relation to resistance could also provide useful data on a suite of traits for a parasite population. Fitness-defining traits such as development time, fertility and size at maturity can all be measured using a dose-response approach, meaning that any of these traits could be assessed for a cost of resistance (Ritz et al. 2015). Studies focused on costs of resistance often measure life-history traits in environments where control measures are not applied (Kliot and Ghanim 2012). However, these investigations could be performed as part of a dose response study and would provide informative data on both the resistance ratio relative to control populations, and a measure of any cost to resistance, for a given trait.

2.5.3 *Conclusions*

My study suggests that fitting the most appropriate model without removing data on background mortality will give more accurate predictions of dose-response curve parameters, compared to methods that manipulate data prior to analysis. Incorporating background mortality into the analysis also provides an effective method for assessing potential costs to resistance. Current software available for analysing dose-response data makes it easy to implement a range of models and select the most appropriate model (Ritz et al. 2015). This software also allows predictions of measures of resistance (LD_{50} and LD_{99}) to be easily extracted, and provides a test for a cost of resistance. To ensure that the amount of useful biological information relating to resistance is maximised when conducting dose-response studies I recommend: 1) not rescaling data to untreated controls; 2) fitting a range of models and testing which provides the best fit to the data to ensure accurate predictions; and 3) a test for potential costs of resistance by

comparison of asymptotes from dose-response curves from susceptible and resistant populations.

Chapter 3: Will my selection experiment actually be informative?

3.1 Abstract

Estimating sample size and statistical power for a study is an important part of experimental design. Formulae are available to calculate power only for simple studies; for more complex designs, simulation provides a potent alternative approach. For example, selection experiments to study evolutionary patterns often include multiple sources of variation with potentially complex interactions. The purpose of this study was to examine how power to detect differences in survival among replicate populations exposed to one of two different treatments is affected by experimental design. Specifically, I focused on the effects of both within- and between-line variation on estimated power, when detecting small, medium and large effect sizes? Using mixed-effect models to analyse simulated data, I demonstrated that commonly used designs with realistic levels of variation could be underpowered for substantial effect sizes. Thus, use of simulation-based power analysis prior to initiation of selection experiments provides an effective tool to avoid under- or over-powering study designs incorporating variation at multiple levels.

3.2 Introduction

Selection experiments are an indispensable tool for asking specific questions about changes in the evolution of traits. They can provide valuable evidence as to whether a given selection pressure is capable of causing a long-term change in either single or multiple traits, and the extent to which genetic correlations in traits might constrain evolution (Hill and Caballero 1992; Prasad and Joshi 2003; Fuller, Baer, and Travis 2005; Hill and Robertson 2007; Garland and Rose 2009). The design of an experiment plays a fundamental role in whether a selection experiment is likely to provide a robust answer to a given research question. In an early selection experiment, Rev Dallinger (1887) cultivated protists in an incubator, gradually increasing the temperature from an initial 60°F up to 158°F, over seven years. Dallinger noted that the ancestral protists were unable to tolerate temperatures above 73°F, whilst protists from the derived 158°F culture could not withstand a temperature lower than 150°F. He concluded that the change in the environment over many generations had resulted in adaptation of the protist culture to increased temperature. However, Dallinger omitted several features of selection experiments considered desirable when conducting experimental evolution studies, including: use of control populations (lines), replication of selected lines, and a method of statistical inference used to assess evolved changes. Dallinger's work is without doubt a brilliant example of pioneering research in experimental evolution. To judge his work in the context of today's research practices would be unfair, but his experiment provides an opportunity to ask a fundamental question facing all researchers when designing a selection experiment, 'Will my study design answer my research question?' or in statistical terms 'What is the power of my study?' (Johnson et al. 2015).

Power is defined as the probability of correctly rejecting a null hypothesis when it is false (Cohen 1962). In other words, power is the chance of detecting an effect given that it exists. In simple scenarios, power depends on four other parameters: sample size, the size and variability of the effect to be detected and the significance level, which is typically set at 5%. Although any of these five parameters can be the focus of a power analysis, the usual role of power

analysis in experimental design is to determine the sample size required to achieve sufficient power (typically 80%, but see Di Stefano 2003). Selection experiments often seek to address questions within the constraints imposed by limited resources. Failure to adequately consider power prior to conducting an experiment, by adopting sampling schemes based solely on constraints or educated guess work using previous research, can result in under or over-powered studies, which are wasteful of resources (Taborsky 2010). Thus, using power analysis in devising a sampling scheme that will optimise the efficiency of the experimental design can result in more robust experiments (Kain, Bolker, and McCoy 2015).

Power analysis has been used in the design of selection experiments but published methods focus on evolve resequence studies (Kim and Stephan 1999; Baldwin-Brown, Long, and Thornton 2014; Kessner and Novembre 2015). For example, in a study focused on detecting divergence in single nucleotide polymorphisms of lines selected during evolve re-sequence experiments, it was found that differentiation between populations was affected by selection coefficient, population size, number of replicate populations, and initial standing genetic variation (Baldwin-Brown, Long, and Thornton 2014). However, use of power analysis to detect divergence in phenotypic traits during selection experiments has been less common in methods publications. The importance of replication, both at the population level, and sampling within replicate lines is well appreciated (Fuller, Baer, and Travis 2005; Garland and Rose 2009c), but power analyses are not always reported. For example, of the studies involving selection experiments published in BMC Evolutionary biology (n = 6) and Evolution (n = 12) in the 12 months from April 2015, none explicitly mention power analysis being involved in experimental design to justify study design choices in terms of numbers of replicate lines or the sampling of those lines. Retrospective power analysis using the observed effect size to estimate the power to reject the null hypothesis of no evolutionary change in traits of interest between selection regimes has been reported (e.g. Sikkink et al. 2015). However, this approach has been frowned upon because calculating power retrospectively will not change interpretation of the result, high power always corresponds with a significant p-value (Hoenig and Heisey 2001). It would be

more useful to address the optimal design of an experiment prior to implementation. Conducting an *a priori* power analysis to optimise the sampling design of the experiment for a given research question and publishing it along with the findings from a study will lend weight to the credibility of the research, whereas retrospective power analysis does not aid the design of a study.

A potential obstacle to the use of power analysis is that standard methods are too simple to deal with the more complex design of many selection experiments (Fuller, Baer, and Travis 2005; Johnson et al. 2015). Selection experiments often seek to measure evidence of selection on traits in environments that differ with respect to some abiotic, biotic or demographic condition. During the course of selection, new genetic variants are produced through recombination, mutation or sampling of alleles, meaning that stochastic processes can make the outcome of selection unpredictable (Garland and Rose 2009). In addition, initial population size, pre-existing genetic variation and the strength of selection are all likely to influence change in any trait. Selection experiments usually aim to manipulate one or two environmental or genetic conditions and keep all other factors fixed, but the magnitude of change in a trait will always be subject to sampling error due to stochastic differences in recombination, mutation or any other factor which affects the response to selection occurring between selected lines. While some of these variables will be of primary interest, others may be included as random effects that could affect interpretation of variance associated with the focal fixed effects. Experimental units in selection experiments are populations, which are replicated within treatments. This type of design introduces random variation at two levels, within and between populations, and can be dealt with by the use of simulation. The type of trait measured during and at the end of a selection experiment will define what type of distribution the response variable takes (Gaussian, binomial, Poisson or negative binomial). Simulation-based power analysis with non-normal responses such as binomial responses need to take account of within- and between-line variation because of the potential for large amounts of variation in the response due to binomial sampling variation. Binomial sampling could be accounted for by assuming approximation to a normal distribution with standard methods, but simulations allow sampling variation in the response to be incorporated into

power studies. In addition, by performing multiple hypothetical experiments, simulations allow the user think about how data will be analysed before it is collected, and identify poor models, which would not be possible when only real data is analysed.

One common type of selection experiment, sometimes referred to as laboratory culling (Juliano 2002), demonstrates the type of complexity that can pose a challenge for standard power analyses. This type of study involves imposing an environmental stress that is lethal to some proportion of a population and the offspring of survivors are carried through to the next generation under selection. Environmental stressors could take the form of temperature, pathogens, predators or chemical control agents and often the strength and consistency of selection can be controlled by the investigator (Busi and Powles 2009; Morran et al. 2011; Chen and Maklakov 2014). Adaptation in the form of increased survival or other traits of lines exposed to the environmental stressor can then be assessed by comparisons between evolved treated and control lines. One way to assess the effect of selection is to test the null hypothesis that there is no difference between treated and control populations. Generalised linear mixed models (GLMMs) are often used in null hypothesis testing of selection experiments, to assess differences in phenotypes between treatments and deal with random variation occurring from multiple sources, such as variation in the response between replicate lines (Bolker et al. 2009). Calculating power analytically for GLMMs is difficult, but an alternative is to use simulations, which can incorporate complex experimental designs with multiple sources of variation at different levels like those occurring in selection experiments (Bolker 2007; Johnson et al. 2015; Kain, Bolker, and McCoy 2015).

The aim of this chapter is to illustrate how simulations could be used to inform important design choices used in selection experiments. Specifically, I asked, how do multiple sources of variation resulting from within and between-line variation affect power estimates of study designs aimed at detecting small, medium and large effect sizes?

3.3 Methods

3.3.1 *Power analysis simulations*

Estimating the power of a range of study designs using a test of a null hypothesis by simulation requires the following steps (Bolker 2007):

1. Identify a suitable range of study designs and establish estimates of sources of additional variation.
2. Simulate many data sets over a range of study designs assuming that the null hypothesis is false; i.e., that the effect of interest is not zero. Typically, 1000 simulations for a given scenario are adequate to ensure confidence intervals of power estimates are acceptable (Johnson et al. 2015). With 1000 simulations and 80% power, 95% of power estimates should fall between 77.5–82.4%.
3. Perform a statistical test of the null hypothesis that the effect size is zero for each simulated data set.
4. The power estimate for a given scenario can then be calculated as the proportion of simulated data sets where the null hypothesis was rejected.

3.3.2 *Selection experiment scenario*

Here I consider the effects that a realistic range of study designs have on the power to detect a difference in survival between treatments in a hypothetical study assessing adaptation to a control agent. Replicate populations were selected in each environment (drug and control) and at the end of selection two replicate samples of each line were exposed to the drug treated environment during a resistance assay, and survival data collected. Survival data from this type of experimental design were analysed using a GLMM assuming a binomial error distribution with a logit link (Zuur et al. 2009). In our hypothetical study, treatment was fitted as a fixed effect, the evolutionary replicate (line) was

fitted as a random effect to account for additional variation in survival due to differences in the response to selection between lines. Additionally, variation in survival due to repeated sampling from lines after selection (within-line variation) was also fitted as a random effect. The GLMM fitted to describe the selection experiment models the number of survivors exposed to the control agent in the *j*th repeated sample from the *i*th line across the number of individuals initially exposed, where survivors y_{ij} from n_{ij} individuals are binomially distributed $y_{ij} \sim Binom(n_{ij}, p_{ij})$. The log odds of survival, $s_{ij} = \text{logit}(p_{ij})$, is modeled by equation 1. β_0 is the log odds of survival when $x = 0$, β_1 is the difference between the control and treatment groups in log odds of survival (or, equivalently, β_1 is the log of the treatment effect odds ratio [OR]), and x_{ij} defines treatment during selection. Where the odds ratio (OR) equals one, there is no difference between treatments x_{ij} ($\beta_1 = 0$); as the OR increases, treatments become increasingly different in terms of the proportion of individuals surviving. Thus, the odds ratio is a measure of effect size. The replicate line random effect, b_i and the repeated sampling random effect w_{ij} are normally distributed with zero means and variances σ_b^2 and σ_w^2, respectively, i.e. $b_i \sim N(0, \sigma_b^2)$ and $w_{ij} \sim N(0, \sigma_w^2)$.

$$s_{ij} = \beta_0 + \beta_1 x_{ij} + b_i + w_{ij} \qquad (1)$$

A likelihood ratio test was used to determine whether survival was affected by treatment, by comparing the full model (equation 1) and a null model where treatment had no effect. The effect size (difference in survival between treatments), total sample size (total number of individuals in the resistance assay), number of replicate selection lines, and the number of samples taken from within each replicate line, will all have an influence on whether the null hypothesis was rejected.

3.3.3 *Simulation methods*

To assess the effect of between-line and within-line variation on power

estimates of a range of experimental designs, I simulated survival data. This was achieved by estimating power across a realistic range of study designs incorporating assumptions about sources of additional variation (Table 1), and then identifying those that gave adequate (≥80%) power. For the simulated selection experiments the number of replicate lines used for each treatment was varied over the range; 2, 4, 6, 8 and 10. To incorporate an estimate of the effect of increasing sampling effort in resistance assays used at the end of the selection experiment into the simulation, the total number of individuals assayed across both treatments was simulated at n = 200, 400, 800, 1600, 3200 and 6400. To explore the effect of within-line variation on power estimates, realistic variance ($\sigma_w^2 = 0.1$) in survival was simulated. This level of variation in survival was consistent with that found during analysis of dose-response data in Chapter 2. To explore the effect of not accounting for within-line variation, $\sigma_w^2 = 0$ and $\sigma_w^2 = 0.1$, were simulated only for large effects, with a sampling effort of n = 400. Differences in effect size, the absolute difference in survival between control and treated lines, were set at 5%, 10% and 20%, and correspond to Cohen's definition of small, medium and large effect sizes (Cohen 1962). In addition, to estimate the chances of making a type I error, the simulations included a scenario with no difference between treatments; i.e. survival in the control lines equaled survival in treated lines. Survival of drug naive populations in a treated environment was fixed at 13%; this information was used to establish the predicted log odds of survival in control lines. To explore the effect that between-line variation (σ_b^2) had on power estimates, differences in survival due to variation in the response to selection were simulated at three levels ($\sigma_b^2 = 0$, 0.1, and 0.2) for both treated and control lines: $\sigma_b^2 = 0$, as a means of assessing the impact of not incorporating realistic assumptions about variation in between-line responses to selection; $\sigma_b^2 = 0.1$, similar to the variation in response to selection observed in Chapter 3; and $\sigma_b^2 = 0.2$, a pessimistic assumption of strong between-line variance.

Table 3.1 Values for the parameters used in the study designs to assess power in the simulation-based power analysis.

Study variable	**Simulated values**
Number of lines used per treatment group	2, 4, 6, 8, 10
Between-line variance σ_b^2	0, 0.1, 0.2
Total number of individuals assayed at the end of selection across both treatments	200, 400, 800, 1600, 3200, 6400
Number of drug resistance assay replicates conducted for each line at the end of selection	2
Within-line variance σ_w^2	0[a], 0.1
Initial survival as a result of control agent	13%
Absolute increase in survival between drug-treated and untreated lines (Odds ratio)	0% (1), 5% (1.5), 10% (2), 20% (3.3)

a: Within-line variance was only simulated at two levels for large effect size (20% difference in survival) with a sampling effort of 400 individuals. For simulations at medium (10%) and small (5%) effect size, within-line variance was fixed at 0.1.

Survival data was then simulated over the range of study designs and sources of additional variation. All combinations of the parameter values from table 1 were used in the simulations, resulting in 360 scenarios. For each scenario 1000 datasets were simulated from binomial distributions using the statistical environment R (R Core Team 2014). Power was then calculated for each scenario by analysing each dataset with a linear mixed effects model (LMM) using Wald t tests to assess significance under the null hypothesis that there is no difference between treated and control lines, with a significance threshold of P = 0.05. We fitted a LMM to the binomial response data rather than a GLMM to test for significance because GLMMs have been found to suffer from high type I errors as a result of variance in random effects being misspecified (Ives 2015), and high

type I errors were observed with GLMMs in preliminary simulations. Where samples sizes are large, as was the case in the simulations, the distribution of binomial data approaches that of a normal distribution and can be modeled with a LMM. The LMMs were implemented using the lme4 package (Bates, Maechler 2014), and p–values calculated using lmerTest (Kuznetsova, Brockhoff, and Christensen 2016). In the final step of the power analysis, the power to detect a difference in survival between drug-treated and control populations for each scenario was estimated as the proportion of 1000 simulated data sets where the null hypothesis was rejected. The simulated survival datasets were then used to assess how within- and between-line variation affect power estimates when aiming to detect small, medium and large differences in survival between treatments.

3.4 Results

The simulation-based power analysis showed that the effect size, the number of replicate lines and subsampling effort all affected the power of the experiment (Fig 3.1). In terms of trade-offs between the number of lines and total sampling effort, power estimates benefitted from greater numbers of lines, but only when a certain level of sampling effort was achieved, and higher levels were required for smaller effect sizes. In addition, power decreased with increasing variance in the between-line response to selection (Fig 3.1; dashed and dotted lines). The false positive (Type I error) rate for null hypothesis testing remained at an acceptable level (5% ±2%) under all scenarios (Fig 3.1A-F; black lines). Not accounting for within-line variation in survival resulting from repeated subsampling from lines led to higher power estimates than where this source of variation was included, but the magnitude of difference in power estimates due to within-line variation decreased with the number of replicate lines (Fig 3.2).

3.4.1 *Large effect size*

In scenarios with a large effect size (20% difference in absolute survival), adequate power (≥80%) was not achieved with only two replicate lines per treatment group, even when using the highest level of sampling effort, and with no variation in the response to selection (Fig 3.1F; blues lines). Where four replicate lines per treatment were used in simulations, the 80% power threshold was achieved when total sampling effort in the resistance assays was 400 and 800 individuals, for scenarios where the variance in the response to selection was zero and 0.1, respectively (Fig 3.1B; blue solid line, and Fig 1C; blue dashed line, respectively). Six replicate lines per treatment and a sampling effort of 400 individuals ensured ≥80% power when variance in the response to selection was at its highest (Fig 3.1B; blue dotted line).

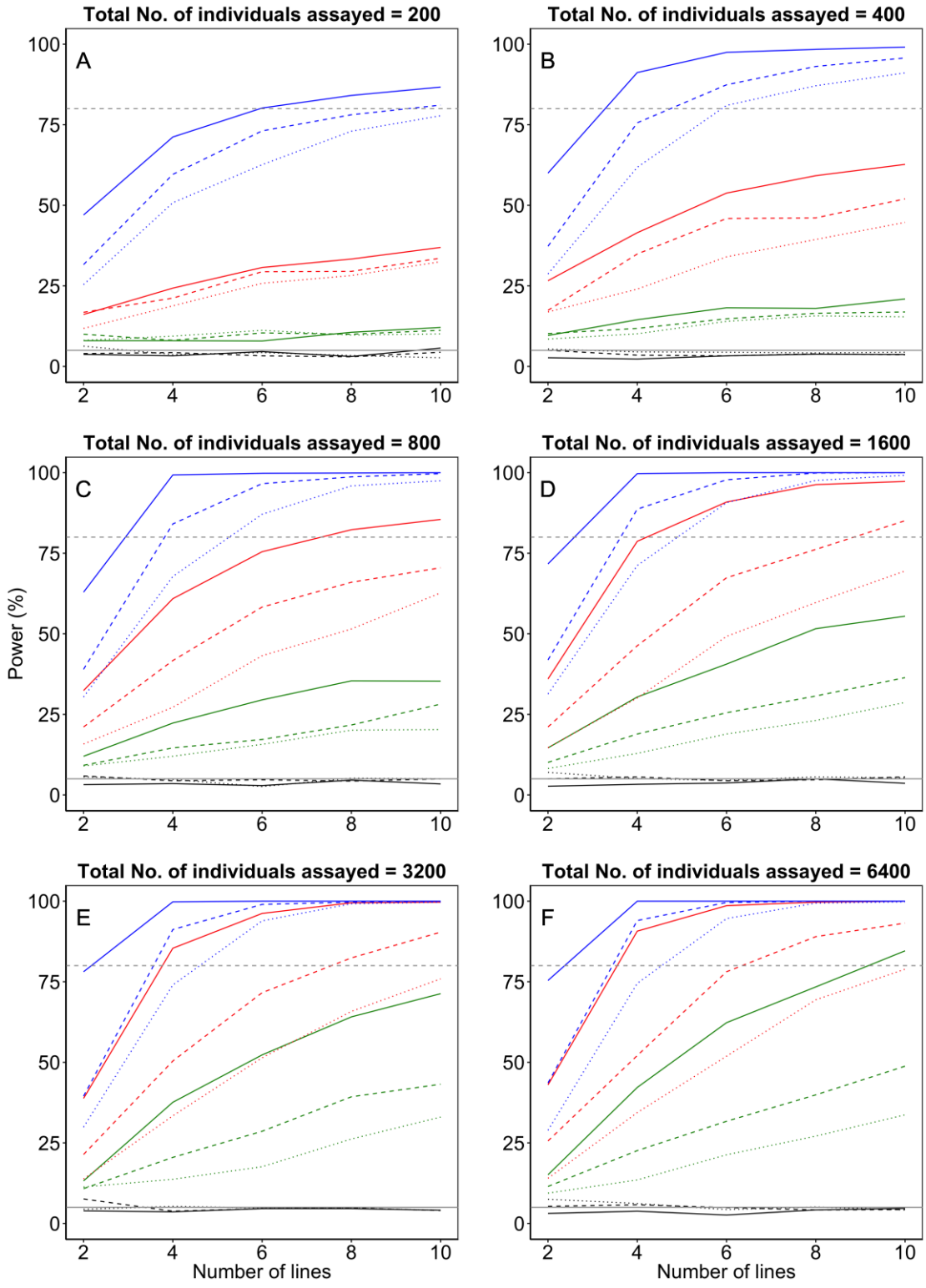
3.4.2 *Medium effect size*

To detect an absolute difference in survival between treatments of 10%, eight replicate lines and a total sampling effort of 800 individuals were required to meet the 80% power threshold under scenarios with no variation in the response to selection (Fig 3.1C; solid red line). Where $\sigma^2 = 0.1$ for the between-line response to selection, scenarios with eight replicate lines and a sampling effort of 3200 individuals were required to meet a target power of 80% (Fig 3.1E; broken red line). Under simulations at the highest variance ($\sigma^2 = 0.2$) scenario for between-line variation in the response to selection, 10 replicate lines and a sampling effort of 6400 individuals achieved 79% power.

3.4.3 *Small effect size*

Power estimates from simulations with a small difference in survival (5%)

between treatments at the end of selection remained well below the 80% threshold under all scenarios, except in the case where sampling effort was at it greatest (n = 6400), with 10 replicate lines per treatment, and no variation in the between-line response to selection (Fig 3.1F; solid green line).



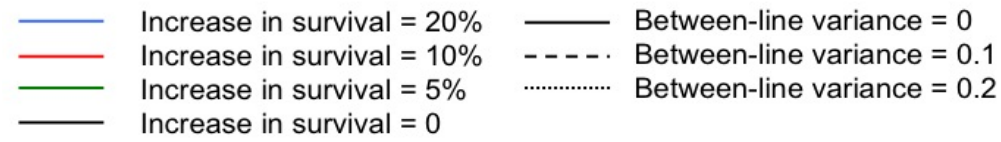
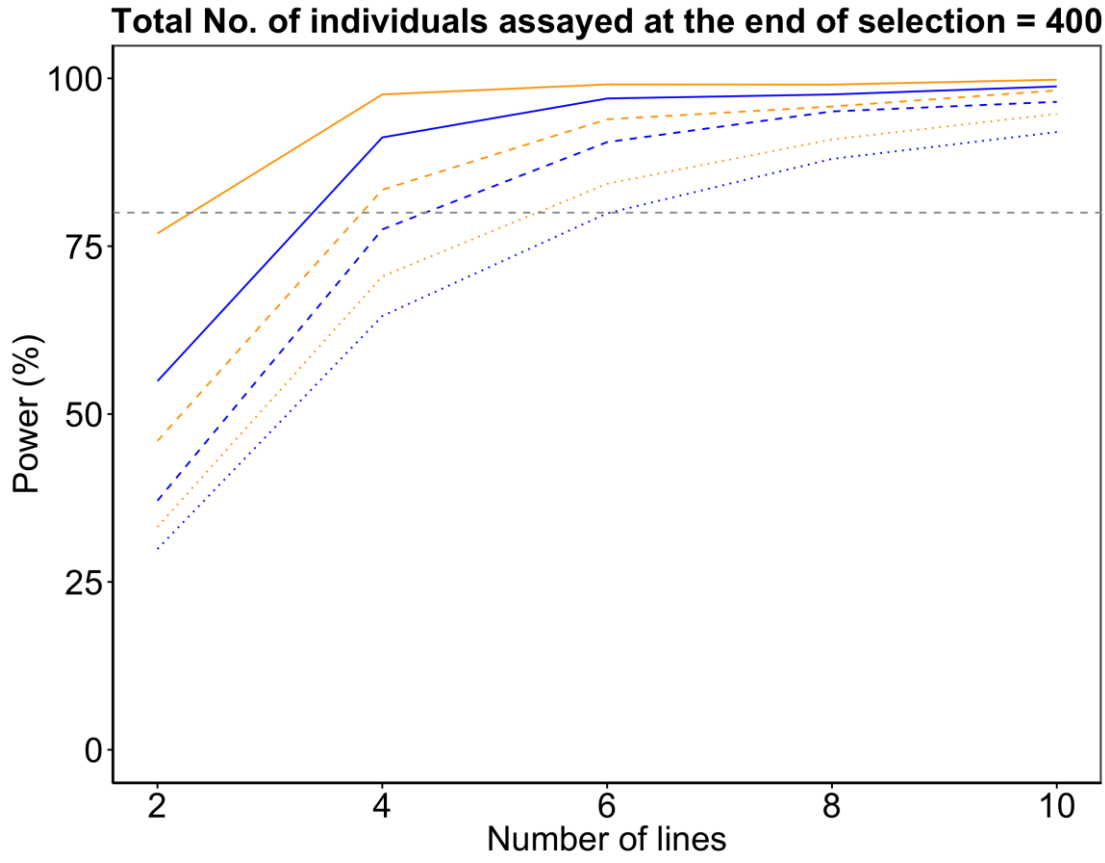


Fig 3.1 The relationship between the power to detect a difference in survival between drug-treated and control lines, and the number of replicate lines within a treatment after a hypothetical selection experiment. Each power estimate was derived from 1000 simulated data sets, generated under scenarios that varied effect size (increase in survival relative to control treatment), and variance in the response to selection between lines (variance). Panels A through F show the effect of increasing sampling effort in resistance assays after selection. The broken grey line shows the target power of 80%, and the solid grey line shows the 5% expected type I error rate when there is no survival difference between control and treatment. Within-line variance was set at $\sigma_w^2 = 0.1$ for all scenarios.



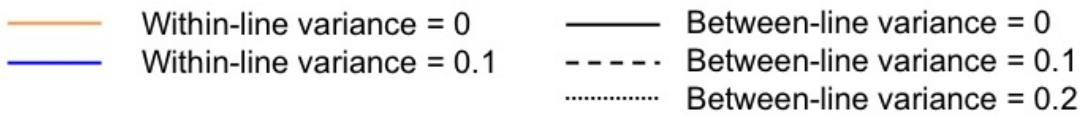


Fig 3.2 The effect of increasing variance in survival due to subsampling of replicate lines over a range of replicate line numbers, where a large difference in absolute survival (20%) between treatments was simulated. Within-line variance is variation due to differences in survival between resistance assay replicates. Between-line variance represents variation in the response to selection between replicated evolutionary lines.

3.5 Discussion

The results of the simulations presented here show that power increases with replication of lines and increasing sampling effort from lines. Study designs simulating large differences in absolute survival (≥20%) between treatments showed adequate power to reject the null hypothesis even when variation in the response to selection was high, so long as line replication was above two. Even where there was no variation in the response to selection (i.e. no variation between replicate lines), detection of medium effects (10%, difference in survival between treatments) required four times the total sampling effort needed to detect a large effect. When between-line variation was included in simulations, a substantial investment in line numbers and total sampling effort was necessary to achieve sufficient power; 10 lines and 1600 individuals in total, respectively. All but the most intensive sampling strategy in terms of line numbers and total sampling effort was insufficient to achieve target power for small effects, and only where there was no variation in the response to selection. Collectively, the results of these simulations indicate the importance of clearly designating the sources and possible magnitude of variation when determining an appropriate experimental design.

Perhaps the most influential constraint facing investigators when planning a selection experiment is the number of replicate selection lines to use. Often this is constrained by the study organism. Line replication is generally higher in systems using viruses and microorganisms (Lenski and Travisano 1994), than in the case of invertebrates (Stearns, Ackermann, and Doebeli 1998) and

vertebrates (Baer, Travis, and Higgins 2000), but is also influenced by the number of treatments used in the experiment. Typical line replication per treatment can be in the range of two to ten lines for many of these study systems. For example, experimental evolution studies using nematodes all use line replication within this range (Lopes et al. 2008; Morran, Parmenter, and Phillips 2009; Anderson et al. 2011;Chen and Maklakov 2012; Dutilleul et al. 2014; Fritzsche et al. 2014; Morran et al. 2014; Savory et al. 2014; Dutilleul et al. 2015). My results suggest that replication within this range is prone to low power to detect an effect for the specific example of divergence in survival during culling selection experiments, under the conditions presented here. The low power estimates are of particular concern when detecting smaller effects where variation in the response to selection and variation affecting estimates of the response to selection is large. It is difficult to assess whether selection experiments often have underpowered designs, as such studies are less likely to be published, but if the results of my simulations generalise to measures of divergence in other traits, as well as survival, then simulation-based power studies would do much to ensure the design of new selection experiments is optimised. The number of samples taken from within each replicate line at the end of selection used to measure divergence in a trait can also be a key constraint when designing a selection experiment. Increasing sampling effort from within lines will improve the precision of line estimates for the trait of interest. Decisions about within-line sampling effort, like replication of lines themselves, are not generally justified by investigators other than the suggestion that greater replication is better (Fuller, Baer, and Travis 2005; Garland and Rose 2009). My study suggests that incorporating both the intensity of repeated sampling and variation that occurs as a result of repeated measures into power analysis, has significant implications for experimental design. Generally, greater sampling effort improves power, but specifically conducting power analysis allows the trade-off between replication of lines and the intensity of sampling effort from those lines to be explored.

Simulation-based power analysis can be extended beyond the binomial response variable modeled in this study, to Gaussian (Arnold et al. 2011), Poisson (Johnson et al. 2015), proportional hazards models (Feiveson 2002), and to detecting differences in among treatment variation (Kain, Bolker, and McCoy

2015). Simulation-based methods of *a priori* power analysis provide a flexible framework in which to test a study design's ability to answer a particular research question. Given the utility of simulation-based power analysis, it seems surprising that it is underused or underreported within the field of experimental evolution. Researchers within the field are aware of sources of random variation that might affect inferences drawn, and take appropriate measures to deal with these effects in statistical analyses (Hurlbert 1984; Bolker et al. 2009). Why then is power analysis infrequently used? One reason is that investigators are unaware, or aware and lack the know-how involved in testing complicated study designs using power simulations. Alternatively, researchers are aware but unconvinced of the value of simulation-based power analysis for their study, and instead rely on experience or duplicating designs used by others. My study shows that selection experiment designs incorporating multiple sources of variation, which would be difficult to deal with using standard analytical techniques, can be assessed using simulation-based methods comparable in complexity to the methods used to analyse real experimental data. Thus, any barrier in adopting simulation-based methods due to lack of know-how can be easily addressed. The results of the power simulations also show the danger in adopting a strategy of copying other study designs. For example, if the expected effect size differs greatly between two studies then a copying strategy to experimental design risks over- or under-powering the study, which are both wasteful of resources. Instead, power simulations allow a study to be designed, which is aimed at detecting the smallest effect judged to be biologically meaningful in the context of the question being asked.

My study shows that commonly used designs with realistic levels of variation can be underpowered, even for substantial effect sizes. The use of simulation to estimate study design power extends conventional power calculations to accommodate complex designs that often arise in practice. Researchers can use simulations to estimate power for virtually any realistic experimental design; meaning that study designs are constructed using a formal statistical approach, and thus, more likely to yield informative results. Promoting the use of a simulation-based approach would improve the standard of study design in evolutionary biology by providing a convenient means to identify and avoid under or overpowered designs.

Chapter 4: Evolution of drug-tolerant nematode populations in response to density reduction

4.1 Abstract

Resistance to xenobiotics remains a pressing issue in parasite treatment and global agriculture. Multiple factors may affect the evolution of resistance, including interactions between life-history traits and the strength of selection imposed by different drug-doses. We experimentally created replicate selection lines of free-living *Caenorhabditis remanei* exposed to Ivermectin at high and low doses to assess whether survivorship of lines selected in drug-treated environments increased, and if this varied with dose. Additionally, we maintained lines where mortality was imposed randomly to control for differences in density between drug-treatments and to distinguish between the evolutionary consequences of drug-treatment vs ecological processes due to changes in density-dependent feedback. After 10 generations we exposed all of the selected lines to high-dose, low-dose and drug-free environments to evaluate evolutionary changes in survivorship as well as any costs to adaptation. Both adult and juvenile survival was measured to explore relationships between life-history stage, selection regime and survival. Intriguingly, both drug-selected and random-mortality lines showed an increase in survivorship when challenged with Ivermectin; the magnitude of this increase varied with the intensity of selection and life-history stage. Our results suggest that interactions between density-dependent processes and life-history may mediate evolved changes in susceptibility to control measures.

4.2 Introduction

Pesticide and drug treatments are designed to suppress populations of parasites, pests and disease vectors. This makes them strong selective factors; as a result, adaptation consistently occurs in natural populations exposed to xenobiotics (Jackson 1993; Carriere et al. 1994; Wolstenholme et al. 2004; Sparks et al. 2012). Resistance can evolve quickly (Lopes et al. 2008; Brausch and Smith 2009; Tabashnik et al. 2014), and the development of resistance is becoming an important theme in applied evolutionary biology due to the risk of reduced efficacy of chemical applications to control parasite and pest species (Palumbi and Mu 2001; REX Consortium 2010; REX Consortium 2013; Hendry et al. 2011). However, evolutionary strategies which could curtail the rate of resistance evolution have yet to be adopted universally (Greene et al. 2012). Several factors are known to affect the rate at which parasites can evolve resistance, including the type of drug, dosage, timing of application, migration rates between susceptible and resistant populations, the standing frequency of resistance alleles in the population and the specific mechanisms of resistance (Committee on Strategies for the Management of Pesticide Resistant Pest Populations 1986; James, Hudson, and Davey 2009; Gilleard and Beech 2007; REX Consortium 2013; Barnes, Dobson, and Barger 1995). Low population densities in drug-treated environments may also have some influence on susceptibility if there are interactions between susceptibility and competition for resources or any other density-dependent processes. However, it is difficult to tease apart the effects of mortality caused by the drug from those caused by density-dependence (Gilleard and Beech 2007). In addition, life history characteristics and reproductive strategies of parasites and pests could influence the rate at which resistance develops (Galvani and Gupta 1998; Lynch, Grimm, and Read 2008; Kliot and Ghanim 2012). The influence of such factors, and their interactions, on resistance evolution has been considered theoretically but there has been little attempt to show that these factors are of practical significance in the laboratory or field.

Experimentation and monitoring of complicated host-parasite systems is technically difficult, expensive and time-consuming (Leathwick et al. 2009) and

thus resistance evolution is often predicted by simulations. For example, Barnes et al (1995) used mathematical modeling to investigate the effects of under-dosing on the evolution of resistance. They suggested that the outcome of under-dosing in terms of the rate of resistance evolution would depend on the genetic mechanism underlying resistance. An alternative to allow specific testing of factors associated with resistance while maintaining more biological complexity, is to use laboratory models to simulate the evolutionary process (Taylor, Quaglia, and Georghiou 1983; Lopes et al. 2008; Busi and Powles 2009). Previous experimental evolution studies have reported rapid evolution of drug resistance in a variety of organisms; including insects, nematodes and other invertebrates (Barros et al. 2001; Lopes et al. 2008; Jansen et al. 2011). These studies often employ one of two strategies in generating resistance: 1) impose a continuous drug or pesticide dose on a population and monitor adaptation over a number of generations; or 2) increase drug dose at regular intervals, often every generation, to track the dose of drug required to cause a target mortality level (e.g. 50% mortality; LD50) in the population under selection. Few studies have specifically looked at the effect of dosage on the rate of resistance evolution, although Busi and Powles (2009) found that selection under exposure to both low and high doses of glyphosate caused a rapid increase in survival of rye grass over three generations and that higher doses promoted a greater magnitude of resistance. However, resistance screens were performed on the first generation offspring of selected plants therefore any response could have been due to maternal effects. Experimental selection over multiple generations at different sublethal doses would help to further elucidate the relationship between dose and the rate of resistance evolution.

In addition to dosage, differences in population density between treated lines of parasites and pests could result in differential selection due to density-dependent processes such as competition (Gilleard and Beech 2007). Laboratory-based selection experiments often impose strong selection on generation time or timing of reproduction when reproductive strategies are influenced by density-dependent effects (Chehresa, Beech, and Scott 1997). Since the application of a drug or pesticide treatment reduces population size, this will create differences in population density between treatments, which could alter apparent evolution of resistance due to changes in traits that are not directly associated with

responding to the chemical exposure (Gilleard and Beech 2007). Selection experiments investigating the rate of resistance evolution typically involve comparisons of survival and/or life history in a drug treatment compared to a control treatment with no drug applied (Ranjan et al. 2002; Coles, Rhodes, and Wolstenholme 2005; Lopes et al. 2008). However, this methodology does not account for differences in population density resulting from differences in mortality between the treatments. If studies are to be biologically realistic and drug treatments involve the bottlenecking of populations then the experimental design must separate the indirect effects of reduced density from the direct effects of the drug (Fuller, Baer, and Travis 2005).

The treatment of helminth diseases provides a well-documented field of research in which to explore problems related to resistance evolution using an experimental approach (Driscoll 1989; Kaplan et al. 2011; Sangster and Gill 1999). Ivermectin is a broad-spectrum antiparasitic drug and has been used commercially since 1981 (James, Hudson, and Davey 2009), with the first reports of resistance in 1988 (Kaplan 2004). Ivermectin causes paralysis in larvae and adult nematodes and inhibits feeding (Sangster and Gill 1999) but also has a repellent effect at sub-lethal doses (Ardelli et al. 2009). Because parasitic helminths are difficult to culture, research into anthelmintic resistance has a long history of using the model organism *Caenorhabditis elegans* in both drug screening and identifying candidate resistance loci (Simpkin and Coles 1981; James, Hudson, and Davey 2009; Ghosh et al. 2012). However, *C. elegans* is an androdioecious nematode species that reproduces mainly by sef-fertilisation, although low levels of outcrossing do occur as a result of the small proportion of males present in a population (Brenner 1974; Barrière and Félix 2007). Since most parasitic nematodes are dioecious and obligately outcrossing, other free-living dioecious nematodes such as *C. remanei* may provide a more realistic model system to explore resistance evolution. *C. remanei* populations have abundant standing genetic variation and high levels of recombination due to their reliance on sexual reproduction (Cutter, Baird, and Charlesworth 2006). Both of these attributes should facilitate a rapid response to selection. Additionally, *Caenorhabditis* species provide an effective microcosm system, which has been used to answer a broad range of evolutionary questions related to rapid evolutionary change (Lopes et al. 2008; Morran et al. 2011; Gray and

Cutter 2014). Manipulating drug dosage, as well as controlling for differences in population density between treated lines in simple microcosm systems, may provide us with a better understanding of how natural populations of parasites and pests adapt to control measures.

The terms resistance and tolerance are often used inter-changeably when defining reduced susceptibility to xenobiotics and has led to much confusion on their relative importance in the evolution of reduced susceptibility. Tabashnik et al. (2014) define resistance as a genetically based decrease in susceptibility as a result of exposure to a control agent; this definition emphasizes a heritable change in susceptibility of a target population due to previous exposure to a control measure. In other words, the spread of resistance through a population is the result of an increase in frequency of pre-existing alleles conferring reduced susceptibility, novel or spontaneous mutations or migration of resistance alleles between populations during a period of time where the population is exposed to a drug (Gilleard and Beech 2007). By this definition a population cannot be resistant prior to exposure to a control agent and resistance results as an evolved response, specifically due to drug application. Tolerance, on the other hand, is due to natural variation in susceptibility already pre-existing within or between populations rather than a result of selection pressure imposed by control measures (Scott 1995). Tolerance may also be used to describe pre-existing differences in susceptibility between different species or between life-history stages of organisms (Coles and Dryden 2014). For example, sensitivity to Ivermectin has been shown to vary substantially among species of sepsid dung flies (Puniamoorthy et al. 2014). Puniamoorthy et al (2014) found that tolerance was explained by phylogenetic relationships; more closely related species had similar levels of susceptibility to Ivermectin on naïve exposure. However, they could not rule out the possibility of rapid adaptation of species to Ivermectin but suggested that this was unlikely as they found more variation in Ivermectin sensitivity between species within sample sites than variation within species between sample sites. Additionally, some of the least susceptible species were known to be drug naïve as they were sampled from locations where anthelmintics have not been used. This suggests that tolerance may occur due to pleiotropic effects and selection on some other unknown trait may result in pre-adaptation in the form of reduced susceptibility. If the frequency and magnitude

of tolerance within a population is affected by selection on unknown traits, the factors which effect selection on those traits will play an important role in governing susceptibility to control agents prior to exposure. In addition, drug-treated populations could evolve tolerance in parallel to resistance if evolved decreases in susceptibility are associated with density-dependent selection, and affect the apparent rate of resistance evolution (Gilleard and Beech 2007). It is difficult to separate tolerance from resistance unless this is explicitly incorporated into the experimental design but this also requires knowledge about which traits confer differences in tolerance to a particular xenobiotic.

The overall aim of this study was to assess how Ivermectin dosage, and changes in population density affect the rate of resistance evolution in replicate lines of *C. remanei*. Specifically, we asked: 1) What is the relationship between *C. remanei* survival and Ivermectin dose over a range of concentrations within a single generation? 2) Is there an increase in survivorship across generations of populations selected in drug-treated environments, and does this vary with dosage? 3) Does density-dependent selection affect the apparent evolution of resistance in selected lines? 4) Is there a cost of adaptation to drug-treated environments in terms of survival in drug-free environments? We also explored the relationship between life-history and drug selection, asking: 5) Does survival of different life-history stages (juvenile and adult) respond to drug-selection in the same way?

4.3 Methods

4.3.1 *Origin and maintenance of experimental lines*

In order to maximise the degree of standing genetic variation available to select for resistance we obtained a genetically diverse strain of *C. remanei* (SP8) from N. Timmermeyer in the Department of Biology, University of Tübingen, Germany. This strain was originally created by a fully factorial crossing of three wild-type strains isolated from geographically distant locations (SP146 from

Freiburg, Germany; MY31 from Tübingen, Germany; PB206 Ohio, US). Crosses had been tested for fertility, offspring pooled, and maintained for eight generations to create recombinant genotypes and allow adaptation to standard laboratory conditions (Fritzsche et al. 2014). Upon arrival in Glasgow, strain SP8 spent a further four generations adapting to any differences in conditions between laboratories and was maintained under standard laboratory conditions for *Caenorhabditis* species: 20˚C and 60% humidity on NGM (Nematode growth medium) petri dishes and fed on a lawn of *Escherichia coli* (OP50) (Hope 2001).

4.3.2 *Dose response assay*

In order to choose two distinct doses that differ in the intensity of selection imposed during the selection experiment, it was first necessary to quantify the relationship between drug dosage and survivorship for strain SP8. A stock solution of 2 mg/ml Ivermectin (22,23-Dihydroavermectin B1; Sigma-Aldrich) dissolved in DMSO was decanted into 1 ml aliquots and frozen to provide a standardised drug dose. We used a modified version of the dose response approach taken by Rufener et al. (2010) to quantify survivorship of *C. remanei* over a range of doses (0, 0.1, 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 7, 8, 9 and 10 ng/ml). Appropriate dilutions of Ivermectin were administered to 100 ml liquid NGM (50˚C) and mixed with a magnetic stirrer before pouring 7 ml aliquots into 5.5 cm plastic petri dishes. These were left to dry, seeded with *E. coli* (OP50) *ad libitum* to minimise indirect mortality resulting from repellence at low doses and incubated at 20˚C overnight. Concurrently to preparing dosed plates, age-synchronised eggs were harvested from stock populations of *C. remanei* by bleaching using standard protocols. This process kills adults and juveniles but leaves developing embryos unharmed (Hope 2001). Eggs were moved to fresh 9 cm drug and food-free petri dishes and incubated overnight to provide a source of L1-arrested larvae for drug screening. After 12 hours incubation, larvae were suspended in M9 buffer solution (3g KH_2PO_4, 6g Na_2HPO_4, 5g NaCl, 1 ml M $MgSO_4$, H_20 to 1 litre and sterilised by autoclaving) and 5 µl aliquots of this suspension were added to Ivermectin-dosed plates with the aim of applying approximately 60 larvae per plate. Larvae added to petri dishes were counted as they were set

up; survival data were obtained by counting the number of adults present per plate at 75 hours. *C. remanei* become reproductively active two days after hatching (Diaz, Lindström, and Haydon 2008) so survivorship was measured at 75 hours after L1 larvae were exposed to the relevant dose of Ivermectin. Twenty replicate plates were established for each Ivermectin dose (ten replicates in each of two different batches conducted at different times).

4.3.3 *Selection experiment*

Two Ivermectin doses were chosen as drug treatments for experimental evolution (Fig B1: Appendix B) a high dose that corresponded to 80% mortality at 75 hours in naïve populations; and 2) a low dose that corresponded to 40% mortality. These two doses were combined with a control of no drug application (zero = Z, low drug = LD, and high drug = HD, Fig 1A). In addition, a random mortality treatment was included for the low and high dosages to account for differences in density between drug treatments (low random = LR, and high random = HR) by randomly removing the same number of individuals from these plates as had died in response to the corresponding drug treatment. For instance, if two females and six males had died in a drug-treated line, a sister random mortality line had the same number of each sex removed. All lines were exposed to high (HD and HR) and low mortality environments (LD and LR), with three replicates per experimental line per treatment, with the exception of the controls, which were replicated six times.

Experimental lines were cultured for 10 generations. The ancestral stock strain (generation 0) as well as samples of larval worms from each line at generations 5 and 10 were cryogenically frozen at -80˚C Fig. B1: Appendix B), at a density of approximately 2000 L1 larvae in liquid freezing solution as described in Hope (2001). Generation 1 (18 lines overall) was initiated using standard bleaching methods from the ancestral stock strain of SP8 cultured in the lab for four generations after thawing and represents the ancestral condition (generation 0; Fig 4.1B). L1-arrested larvae were suspended in M9 buffer and worm density of the suspension obtained by counting worms from five replicates of 5-µl aliquots.

A volume of the suspension corresponding to 400 L1-larvae was then added to *E. coli* seeded NGM plates (9 cm) with the appropriate dose of Ivermectin. Establishing populations with 400 larvae prevented density-dependent competition but still contains sufficient numbers of individuals to ensue a substantial proportion of standing genetic variation (Allendorf 1986). After 48 hours of development worms reach the 4^{th} larval stage (L4) at which point the sex can be determined. At this time, 25 pairs of male and female L4 larvae were transferred to fresh agar plates of the appropriate dose for each replicate. These 50 adults constituted generation one, day one. After 24 hours adults were counted and census data were used to impose an equivalent mortality on the random mortality lines for the respective treatments. After 48 hours of drug exposure the same process of adult census and compensatory-induced mortality was repeated. By 72 hours larvae from the next generation had developed to L4 larvae: 25 pairs were selected to continue the next generation and transferred to fresh petri dishes. This was continued for 10 generations. Census data were gathered each generation to assess whether there was an increase in survivorship of lines selected in drug-treated environments and whether this increase varied with dosage. In addition to adult census, a juvenile census was performed after 48 hours to provide an estimate of juvenile population densities. L2 and L3 larval stages were counted along a 1cm transect covering the center of the petri dish; L1 juveniles were too small and numerous to gather reliable counts.

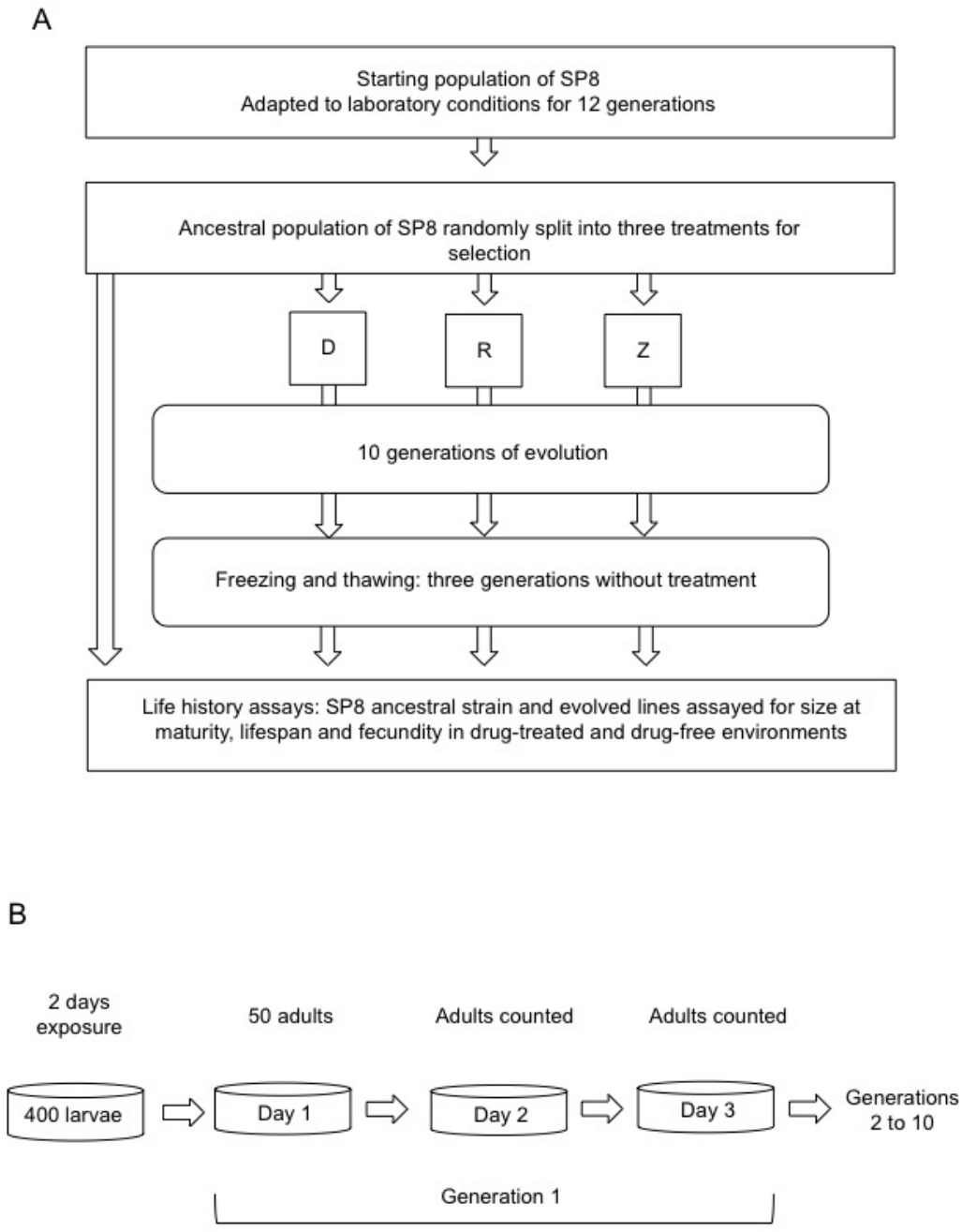


Fig 4.1 A) Schematic representation of dose-response assay, selection experiment and resistance bioassay. The starting population of SP8 was adapted to lab conditions. The lab-adapted strain was then assayed for variation in susceptibility to Ivermectin over a range of 15 doses, to select an appropriate high and low dose for the selection experiment. The lab-adapted strain was then randomly divided into five treatments with three replicates each for HD, LD, HR, and LR lines, and six replicates for Z lines. After 10 generations of selection, lines were frozen and later thawed, before being challenged with the three doses of Ivermectin used during the original selection experiments. B) Schematic representation of selection experiment showing initial population set up and one generation. Initially, lines were established with 400 larvae exposed to the relevant dose of Ivermectin; 50 adults were then selected to begin generation one on day one. After 24 hours lines were counted and compensatory mortality imposed on random lines; this was repeated at 48 hours. After 72 hours sub-

adults from the next generation were transferred to new plates. Generations 2 to 10 proceeded as for generation 1. HD: High dose treatment; HR: High random treatment; LD: Low dose treatment; LR: Low random treatment; Z: Zero dose treatment.

4.3.4 *Drug resistance bioassays*

In order to formally assess whether heritable increases in survivorship were imposed by selection with Ivermectin, ancestral stocks (generation 0) as well as each of the selected lines from generation 5 and 10 were exposed to the same high and low doses of Ivermectin used during selection and raised in a drug-free environment. Firstly, to test the effects of drug dosage on survival, revived samples of HD, and LD lines were exposed to a dose of Ivermectin corresponding to that used during selection. Survival of these lines was then contrasted with survival of Z lines to assess whether there was a change in evolved lines. Secondly, to test for effects of differences in population density on survival of selected lines we exposed HR and LR lines to a high and low dose of Ivermectin, respectively. Survival of HR and LR lines were contrasted with Z lines, with any significant differences in survival between random mortality and Z lines indicating an effect of population density on relative survival. Thirdly, we tested for any cost to adaptation to selection regime in terms of survival by raising evolved lines in a drug-free environment, with the hypothesis that if there is a cost to adaptation then experimentally treated lines should show significantly lower survival than control (Z) lines.

Preserved samples of lines from the selection experiment at generations 0, 5 and 10 were thawed and raised for three generations in a drug-free environment to ensure that any observed responses in survival were due to genetic differences among populations and not maternal or environmental effects due to freezing. Larvae were thawed at room temperature and maintained at a density of approximately 1000 individuals per 9cm agar plate over the three generations from thawing to age synchronization with *ad libitum* lawns of *E. coli* OP50. Transfers between generations were achieved by cutting out sufficient agar from plates already containing samples and transferring these to fresh *E. coli* seeded

plates ensuring the density remained as constant as possible. Agar plates, synchronisation of experimental lines and set-up of larvae were conducted with the same protocol used in the dose response assay. Mortality due to drug application may differ between life history stages; in order to gain some measure of this difference we measured survival both at 52 hours, encompassing juvenile development and 75 hours, during the first day of reproduction. Generations 5 and 10 of each experimental line were replicated four times, as was the ancestral line (generation 0).

4.3.5 *Statistical analyses*

All statistical analyses were performed using R v 3.1.2 (R Core Team 2014) and we defined a significance threshold of P = 0.05 for all tests. A more detailed description of the rationale for the statistical approaches used is provided in the Supplementary information. The doses required to cause 40% and 80% mortality of the ancestral SP8 strain were estimated, with 95% CI's, using the drc package (Ritz and Streibig 2007). In order to calculate estimates of these two doses we constructed a dose-response curve of the relationship between worm survival and concentration of Ivermectin. We fitted a range of dose-response models (log-logistic, Weibull-1 and Weibull-2) with the lower asymptote of the curve fixed at 0% survival and used maximum likelihood to select the most appropriate model of survival data. Ivermectin concentration and batch were fitted as fixed effects in our full model. To assess whether the relationship between survivorship and Ivermectin concentration remained the same between batches performed at different times (i.e. repeatability), batch was removed from the model and compared against the full model using a likelihood ratio test. Estimates of the required doses, with 95% CIs, were then derived from model predictions.

Our experimental design incorporated a power analysis, which specifically adjusted for the effects of the number of lines, interline variation, the potential observable difference in survival between treatments (effect size), and bioassay replicate (Johnson et al. 2015). We estimated that our experimental design gave

93% power to detect an absolute difference in survivorship of 10% in the high dose environment between the control Z lines and both HD and HR lines. To assess whether survivorship changed over the course of the selection experiment, data from the resistance bioassay were analysed using generalised linear mixed models using the glmer function in the lme4 package assuming a binomial error distribution with a logit link function (Bates et al 2014; see Appendix B). Treatment and generation and the interaction between them were fitted as fixed effects. The evolutionary replicate (line) was fitted as a random effect. An observation-level random effect was fitted to account for any overdispersion between replicate lines in the selection experiment and repeated sampling of populations in the drug resistance bioassay (Browne et al. 2005). Treatment effects in the selection experiment were tested using likelihood ratio tests. The null hypothesis of no difference in survival between the three treatments (H_0: Drug = Random = Zero) was tested independently for high and low mortality selection regimes by comparing the full model with a null model with no fixed effect of treatment or interaction terms. Generation was kept in the null model to account for any drift in survivorship. Three post-hoc tests comparing treatment pairs were then conducted to assess the effects of individual treatments. This general approach was used to answer each of our research questions.

4.4 Results

4.4.1 *What is the relationship between C. remanei survival and Ivermectin dose over a range of concentrations within a single generation?*

Two Ivermectin doses were chosen as drug treatments for experimental evolution (Fig B1: Appendix B): 1) a high dose that corresponded to 80% mortality in the stock strain at a concentration of 2.46 ng/ml Ivermectin (95% CI: 2.41, 2.50); and 2) a low dose that corresponded to 40% mortality at 75 hours at a concentration of 1.61 ng/ml Ivermectin, (95% CI: 1.55, 1.68). Analysis using comparisons of log likelihood found that a three-parameter Weibull-1 model with

the lower asymptote fixed at zero gave the best fitting model of survival as a function of the concentration of Ivermectin (Fig B1: Appendix B) and there was no difference between the two survival curves for data collected in the two batches (x2 = 6.821, d.f. = 3, P = 0.0778; Fig B1: Appendix B).

4.4.2 *Is there an increase in survivorship of populations across generations selected in a drug-treated environment, and does this vary with dosage?*

In the selection experiments (Fig 4.2), survival in zero-dose populations remained constant over generations; the mean adult survival in generation 1 was 94% (CI: 90%, 99%), at generation 5 survival was 94% (CI: 90%, 98%) and at generation 10 survival was 94% (CI: 91%, 97%). Larval offspring densities of zero dose lines also remained relatively constant over the course of 10 generations; mean larval density at generations 0, 5 and 10 was 2079, 2051 and 1878 respectively (Fig B2: Appendix B). In lines treated with the lower dose of Ivermectin, survival increased gradually over 10 generations, from 47% in generation 1 (CI: 36%, 57%) to 73% (CI: 45%, 100%) at generation 5 and 75% (CI: 62%, 87%) in generation 10. Larval offspring numbers remained low in LD lines throughout the course of the selection experiment; the mean number of offspring at generation 1 was 1088 at generations 5 and 10 it was 1132 and 1248 respectively (Fig B2: Appendix B). Survival in high-dose treated populations increased more dramatically, from 30% (CI: 20%, 39%) at generation 1, to 65% (CI: 54%, 76%) at generation 5 and 77% (CI: 49%, 100%) at generation 10. Offspring numbers of HD lines increased during the selection experiment; the mean number of offspring was 394, 1166 and 1435 at generations 0, 5 and 10 respectively (Fig B2: Appendix B).

In our formal test of changes in susceptibility of evolved lines, challenge with the dose used during selection, HD lines exposed to a high dose of Ivermectin for 75 hours exhibited an increase in mean survival of 19% and 10%, at generations 5 and 10 respectively, relative to Z lines (H_0: HD = Z: $P < 0.0001$; Fig 4.3A, Table 4.1). Survival was relatively consistent between lines within a treatment (Fig B3: Appendix B). Mean survivorship of the three HD lines remained between 59% and

66% at both generations 5 and 10, except in the case of one line in generation 10 where survivorship dropped to 48%. Variation in the mean survivorship of the six Z lines ranged between 37% and 51% at both generations 5 and 10. At 52 hours of exposure to Ivermectin the HD lines showed a similar increase in mean survival to data collected at 75 hours (Fig B4A, Table B1: Appendix B). Thus, both juveniles and adults exhibited a comparable response to selection in terms of increased survival in the high dose environment. LD lines exposed to a low dose of Ivermectin for 75 hours showed no increase in survival relative to control lines (H_0: LD = Z: P = 0.11; Figure 4.3D, Table 1), but at the earlier observation time of 52 hours LD lines exhibited increased survival relative to Z lines at both generations 5 and 10 (H_0: LD = Z: P = 0.022; Fig B4D, Table B1: Appendix B). Therefore, selection at the low dose of Ivermectin resulted in higher survivorship of juveniles but not adults when re-exposed to a low drug dose.

Exposing drug-selected lines to a dose other than that imposed during selection tested the effects of how evolved responses in survivorship might vary with drug-dosage. After 75 hours of drug exposure HD had significantly higher survivorship than Z lines; the mean difference in survival between treatments was 9% at generation 5 and 15% at generation 10 (H_0: HD = Z: P < 0.0001; Fig 4.3C, Table 1). In addition, there was an interaction between generation and treatment ($x^2 = 7.07$, df = 2; P = 0.029), accounted for by the increase in survival of HD lines between generation 5 and 10 (Fig 4.3C, Table 1). At 52 hours of exposure to a low Ivermectin dose, experimental lines from the HD treatment exhibited an increase in mean survival across generations relative to Z lines (H_0: HD = Z: P = 0.0005; Figure B4C, Table 1). Mean survival of HD populations was 10% higher than Z lines at both generation 5 and 10. There was no interaction between generation and treatment ($x^2 = 3.20$, df = 2, P = 0.20). Thus, the observed evolutionary response in juvenile survivorship of HD lines remained of a similar magnitude from generation 5 to 10. When exposed to a high Ivermectin dose LD lines showed no significant change in survivorship relative to control (Z) lines across generations when challenged with a high drug dose environment at both 52 and 75 hours (H_0: LD = LR = Z: P = 0.43, P = 0.38, respectively for 52 and 75 hours; Figures 4.3B and B4B, Table 1). Therefore, selection in a low drug-dose environment conferred no advantage on survivorship in a high-dose environment.

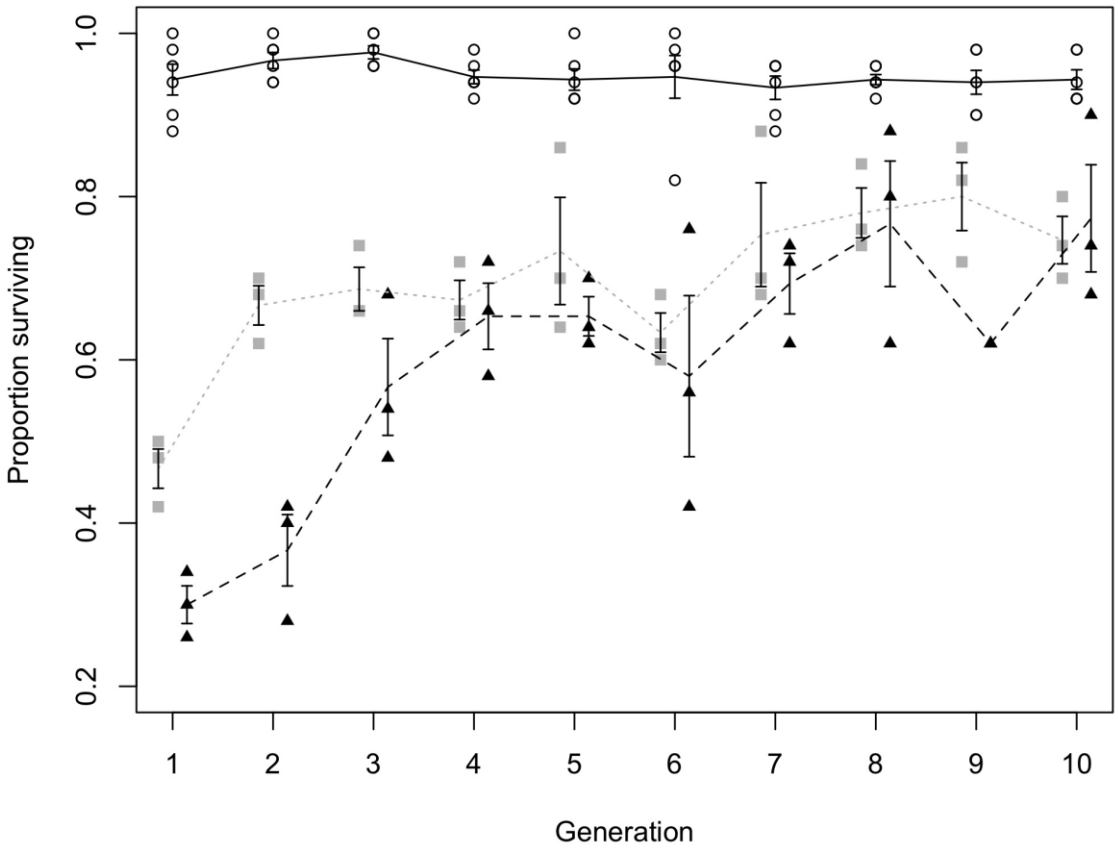


Fig 4.2 Survivorship during original selection experiments. Lines represent mean survival for each treatment; points are the proportion of adults surviving on day two of each generation for each replicate line within a treatment. Circles, solid line = zero dose; squares, dotted line = low dose; triangles, dashed line = high dose. Error bars; standard error for mean survival.

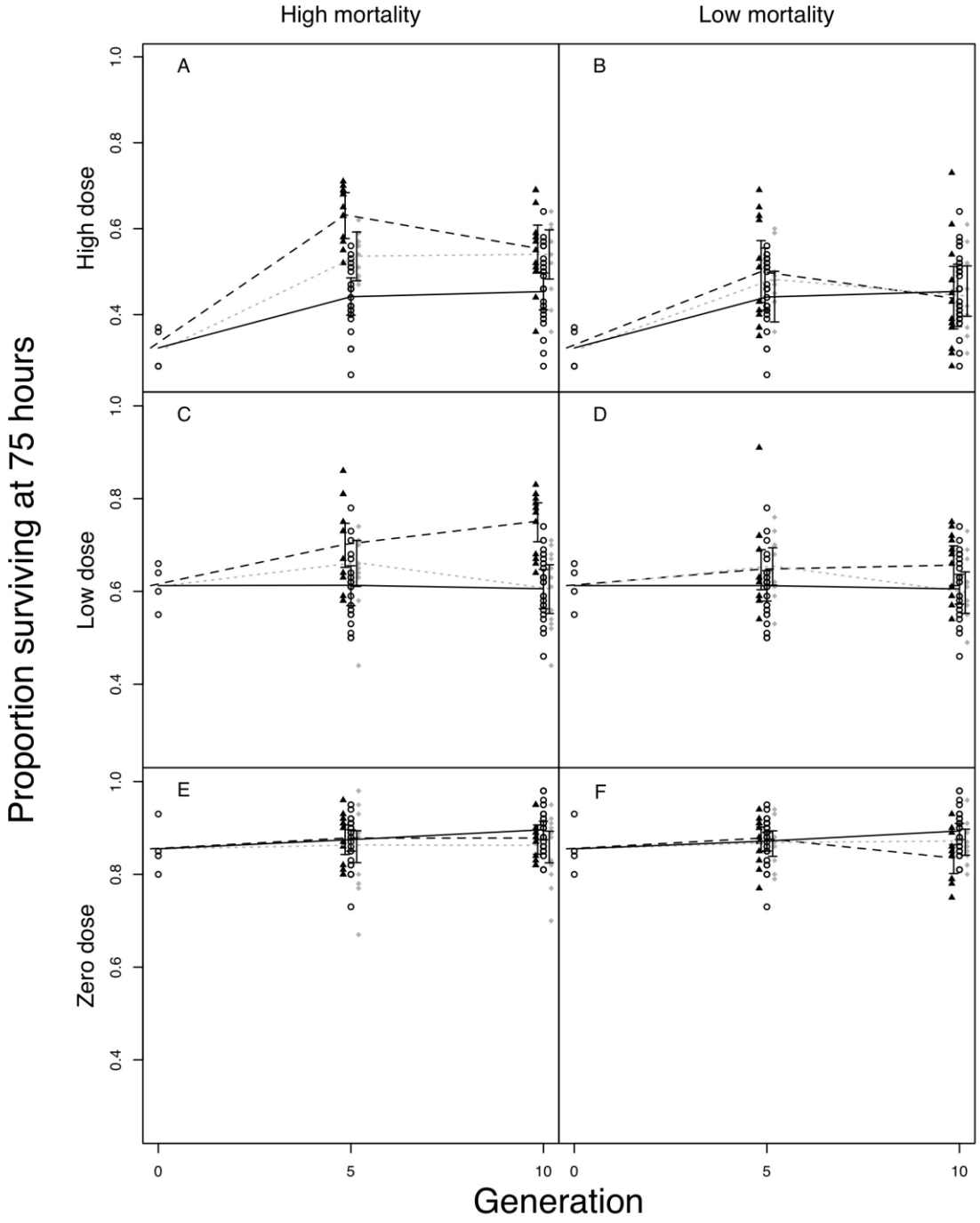


Fig 4.3 Seventy-five hour survival when exposed to the three drug doses used during selection (A and B = high; C and D = low: E and F = zero) of samples taken from generations 0, 5 and 10 during selection. Panels A, C and E show survivorship of high mortality lines: HD and HR. Panels B, D and F show survivorship of low mortality lines: LD and LR. Points are raw survival data from resistance bioassays, lines represent predictions of maximal models (generation + treatment + generation*treatment) for each treatment: circles, solid line = zero dose; triangles, dashed line = drug treatment; diamonds, dotted line = random mortality. Error bars; 95% confidence intervals for mean survival.

Table 4.1 Effect of treatment during selection (mortality treatment) on survivorship (Surv.diff), after 75 hours in drug-treated environments (dose); assessed by null models (see Appendix), using likelihood ratio tests, where survival is constrained to be equal across treatments, and dependent upon the best fitting model. d.f.: Degrees of freedom. Surv.diff: Absolute difference in mean survival between the highlighted treatments (first minus second).

Mortality treatment	Dose	Best fitting model	Null models	x2 (d.f.)	P-value	Surv.diff	
						Gen 5	Gen 10
High	High	G + T + GxT	1) HD = HR = Z	22.26 (4)	0.00018		
			2) HD = Z	21.11 (2)	< 0.0001	0.19	0.10
			3) HR = Z	8.56 (2)	0.014	0.09	0.09
			4) HD = HR	6.56 (2)	0.038	0.10	0.01
	Low	G + T + GxT	1) HD = HR = Z	29.13 (4)	< 0.0001		
			2) HD = Z	25.95 (2)	< 0.0001	0.09	0.15
			3) HR = Z	3.69 (2)	0.16	0.05	0.00
			4) HD = HR	22.52 (2)	< 0.0001	0.04	0.15
	Zero	G	1) HD = HR = Z	3.59 (2)	0.47		
Low	High	G	1) LD = LR = Z	4.20 (4)	0.38		
	Low	G	1) LD = LR = Z	7.67 (2)	0.11		
	Zero	G + T + GxT	1) LD = LR = Z	11.47 (4)	0.022		
			2) LD = Z	11.33 (2)	0.0035	-0.01	-0.06
			3) LR = Z	1.84 (2)	0.40	0.00	-0.02
			4) LD = LR	3.25 (2)	0.20	-0.01	-0.04

G: Generation; T: Treatment; GxT: Generation x Treatment interaction; HD: High dose treatment; HR: High random treatment; LD: Low dose treatment; LR: Low random treatment; Z: Zero dose treatment.

4.4.3 *Does density-dependent selection affect the apparent evolution of resistance in selected lines?*

In the selection experiment, survival in both random mortality treated lines remained of a similar magnitude to zero dose lines prior to random removal of worms; the mean adult survival of LR lines at generation 1 was 95% (CI: 92%, 98%), at generation 5 survival was 97% (CI: 86%, 100%) and at generation 10 survival was 93% (CI: 90%, 96%). Larval densities of LR lines remained similar to those of LD lines during the selection experiment; the mean number of larvae was 1088, 1132 and 1248 at generations 0, 5 and 10 respectively (Fig B2: Appendix B). Mean adult survival of HR lines at generation 1 was 96% (CI: 91%, 100%), at generation 5 survival was 96% (CI: 87%, 100%) and at generation 10 survival was 95% (CI: 87%, 100%). Offspring numbers of HR lines during the selection experiment remained lower than zero dose controls; the mean number of offspring was 1083, 1203 and 1172 at generations 0, 5 and 10 respectively (Fig B2: Appendix B).

Surprisingly, in the resistance bioassays, high random mortality (HR lines) showed an increase in mean survival when populations were challenged with a high dose of Ivermectin. Mean survival of HR lines was 9% for both generations 5 and 10 after 75 hours (H_0: HR = Z: P = 0.014; Fig 4.3A, Table 2). Therefore, reducing density by removing individuals randomly had a similar effect to drug treatment in HD lines. However, there was a difference between HD and HR treatments; HD lines showed higher survival at generation 5 but not 10 (H_0: HD = HR: P = 0.038; Fig 4.3A, Table 1). Variation in mean survivorship of the three HR lines remained consistently between 50% and 56% at both generations 5 and 10; smaller than the between-line variation observed in both HR and Z lines (Fig B3: Appendix B). At 52 hours of drug exposure, the increase in survival of HR lines relative to Z lines was comparable to that of data collected at 75 hours (Fig B4A, Table B1: Appendix B). Thus, when exposed to the high dose of Ivermectin, survival of both juveniles and adults from HR lines responded to selection in a similar manner. Survivorship of lines selected in the LR environment showed no response to selection when exposed to a low dose of Ivermectin for 75 hours; survivorship remained comparable to that of Z lines at both generations 5 and 10

(H_0: LD = LR = Z: P = 0.11; Fig 4.3D, Table 1). However, when survival of LD lines was observed at 52 hours of exposure to a low drug dose, survival was similar to LD lines, relative to Z lines (H_0: LR = Z: P = 0.035; Fig B4D, Table B1: Appendix B). As was the case with LD lines, increased survivorship of LR lines in the low dose environment was only observed for juveniles at 52 hours, and not adults at 75 hours.

When exposed to the low dose of Ivermectin, there no was evidence of a difference between survival and treatment in HR lines relative to Z lines at both generation 5 and 10 (H_0: HR = Z: P = 0.098; P = 0.16, respectively for 52 and 75 hours; Fig 4.3C and B4C, Table 1). In addition, mean survival of HR lines was lower than HD lines in the low-dose environment at both generations 5 and 10 (H_0: HD = HR: P = 0.005, P < 0.0001, respectively for 52 and 75 hours; Fig 4.3C and B4C, Table 1). When exposed to a high Ivermectin dose LR lines showed no change in survivorship relative to control (Z) lines across generations at both 52 and 75 hours (H_0: LD = LR = Z: P = 0.43, P = 0.38; Figures 4.3B and B4B, Table 1).

4.4.4 *Is there a cost of adaptation to drug-treated environments in terms of survival in drug-free environments?*

In an environment where no drug was administered, HD and HR lines performed equally as well as Z lines in terms of survival over 75 hours (H_0: x2 = 3.95, df = 2, P = 0.47; Figure 4.3E, Table 1). In contrast, LD lines had significantly lower survivorship than Z lines in the drug-free environment. However, this was only apparent at generation 10 and the magnitude of the effect was relatively small (H_0: LD = Z: P = 0.0035; Figure 4.3F, Table 1). LR lines also maintained a similar response in survivorship as Z lines at both generation 5 and 10 (H_0: LR = Z: P = 0.40; Figure 4.3F, Table 1), and there was no significant difference between LR and LD lines with respect to survival (H_0: LD = LR: P = 0.20; Figure 4.3F, Table 1). The relationship in survival measurements taken at 52 hours for the evolved lines remained similar to survival measured at 75 hours for all treatments (Table A1, Fig B4E and B4F: Appendix B).

4.4.5 *Does survival of different life-history stages (juvenile and adult) respond to drug-selection in the same way?*

Mortality due to drug challenge continued between 52 hours and 75 hours in HD and LD selected lines when challenged with Ivermectin and was of a greater magnitude than observed in a drug-free environment (Fig 4.3 and B4: Appendix B). When exposed to the dose used during selection, HD lines showed no interaction between generation and selection regime at 52 hours (x^2 = 1.33, df = 2, P = 0.51) but at 75 hours an interaction was apparent (x^2 = 5.96, df = 2; P = 0.05). The change in significance of treatment and generation interactions indicates a change in the way juvenile and adult survival responded to drug selection in HD lines; juvenile survival remained similar between generations 5 and 10, whilst adult survival declined (Fig 4.3A and B4A: Appendix B). When worms were exposed to a low dose of Ivermectin, we observed differential survival between LD and control (Z) lines at 52 hours but not at 75 hours (Fig 4.3D and B4D: Appendix B, Table 1); suggesting that juvenile survival responded to drug selection but adult survival remained unaffected by drug treatment.

In our pooled data sets, we found no evidence of a three-way interaction between selection experiment treatment, bioassay dose and life-history stage at generations 5 or 10 (x2 = 2.77, df = 4, P = 0.60, x^2 = 0.47, df = 4, P = 0.98, respectively). However, there was a significant two-way interaction between selection experiment treatment and bioassay dose at both generations 5 and 10 (x^2 = 28.98, df = 4, P < 0.0001, x^2 = 38.96, df = 4, P < 0.0001, respectively); suggesting that survival in drug-treated environments was dependent on selection regime. In addition, there was an interaction between bioassay dose and life-history stage at generation 5 but not generation 10 (x^2 = 6.07, df = 2, P = 0.048, x^2 = 3.82, df = 2, P = 0.15, respectively). There was no evidence of an interaction between selection experiment treatment and life-history stage for generations 5 or 10 (x^2 = 0.77, df = 2, P = 0.68, x^2 = 4.40, df = 2, P = 0.11, respectively).

4.5 Discussion

4.5.1 *What is the relationship between C. remanei survival and Ivermectin dose over a range of concentrations within a single generation?*

The dose-response curve of the survival of the drug-naive ancestral strain of *C. remanei* (SP8) was similar to those previously reported for drug-naive *C. elegans* when challenged with a range of Ivermectin concentrations (James and Davey 2009). The confidence intervals of the two Ivermectin doses used in the selection experiment differed; the high dose had narrower intervals than the low dose. This suggests that the intensity of selection applied to the first generation of the selection experiment was more variable in lines exposed to low doses of Ivermectin, though even at low doses this would translate into no more than ± 3.25% variation in survival.

4.5.2 *Do drug-treated lines show an increase in survivorship across generations in drug-treated environments, and does this vary with dose?*

Census data from the selection experiment indicated that populations of *C. remanei* exposed to low and high doses of Ivermectin showed a response to selection in terms of increased survival over 10 generations (Fig 4.2, Table 1). Furthermore, the increase in survivorship in HD lines was of a greater magnitude than LD lines, suggesting that evolution was more rapid in populations exposed to a higher drug dose. The data from resistance bioassays support the responses observed in the selection experiment in terms of the greater magnitude of response in survivorship of HD lines relative to LD lines. In both dosage regimes, the increase in survivorship during the selection experiment slowed over the course of the experiment, suggesting a rapid response of populations to drug treatment that reached a peak for a given drug dose. Rapid responses to drug

selection and peaking of the response have been previously observed in Levamisole-selected strains of *C. elegans* (Lopes et al. 2008).

Previous research focused on under-dosing has suggested that lower doses (doses below recommended use) may promote the evolution of resistance, especially where the basis of resistance is polygenic (Manalil et al. 2011; Shi et al. 2013), and that varying the level of under-dosing may affect the rate at which resistance evolves (Busi and Powles 2009). Our data suggest that selection at a low dose of Ivermectin conferred no advantage on LD lines when re-exposed to the low-dose environment for 75 hours. However, HD-selected lines showed higher survivorship relative to Z lines on exposure to the high drug dose. Thus, the intensity of selection played a role in how selected populations responded to Ivermectin treatment. The lack of a response in survival of LD lines exposed to the low dose for 75 hours conforms to models of resistance evolution in nematodes where under-dosing retards the development of resistance (Barnes, Dobson, and Barger 1995). Under such models under-dosing may reduce the evolution of resistance by allowing more susceptible worms to survive.

4.5.3 *Does density-dependent selection affect the apparent evolution of resistance in selected lines?*

Intriguingly, survival of lines selected in random mortality environments showed a similar trend, but of a lower magnitude, to drug-selected lines, and in contrast to zero-dose lines, suggesting that density-dependent effects on life history traits might be affecting the apparent rate of resistance evolution. Random culling of adults reduced larval densities in random-mortality treated lines; meaning that larval densities remained comparable to drug-treated lines and lower than control (Z) lines. Density-dependent natural selection has been shown to affect the competitive abilities of selected lines; Mueller (Mueller 1988) showed that the feeding efficiency of *K*-selected (high density) lines was 58% greater than *r*-selected (low density) lines of *Drosophila melanogaster* after 128 generations of density-dependent selection. Though our selection experiment design aimed to provide an abundant bacterial food source, at the

time lines were transferred to new plates, bacterial lawns were patchy and no doubt some competition for resources is likely to have occurred. Life-history traits such as development time, size at maturity and reproduction may all be influenced by density-dependent selection (Joshi, Prasad, and Shakarad 2001; Prasad and Joshi 2003; Dey, Bose, and Joshi 2012). If traits associated with selection in a low-density environment confer an advantage in a novel drug-treated environment then this may explain the observed increase in survivorship of random mortality lines relative to control (Z) lines. Thus, much of the observed response in survivorship in drug-treated and random-mortality lines when challenged with Ivermectin could be due to increased tolerance as a result of density-dependent processes, rather than resistance evolution *per se*. Put another way, if the response in survival of HR lines is attributable to the evolution of tolerance then perhaps a large part of the response in survival of HD lines, which would have faced similar density-dependent processes to HR lines, is also due to selection for tolerance rather than resistance.

Alternatively, the increase in survivorship of drug-treated and random-mortality lines when exposed to drug treatment could be a result of loss of genetic variation due to drift. This hypothesis would require all lines to drift in the same direction, which could have occurred during bottlenecking of drug treated and random mortality lines, particularly in the early generations of selection. However, the loss of diversity may not have been severe relative to the control zero dose lines (see Appendix: drift and loss of diversity). Our theoretical predictions of the loss of genetic diversity in HR and Z lines suggest that both treatments went through similar losses of genetic diversity. Predicted heterozygosity and the total number of alleles decreased more rapidly in HR lines relative to Z lines but the difference between the two treatments was small. In the case of rare alleles, it is likely that any rare allele would have been lost from populations in both HR and Z lines. Thus, it seems likely that any evolved increase in survivorship of HR and potentially drug-treated lines, was due to ecological processes occurring as a consequence of density-dependent selection and not loss of genetic variation due to drift.

Differentiating between the effects of drug selection and traits not directly associated with resistance has been a long-standing problem in studies of

resistance evolution (Chehresa, Beech, and Scott 1997; Gilleard and Beech 2007). The increase in survival of HR lines over generations when challenged with both low and high drug doses was of a lower magnitude than HD lines; this difference in absolute survival could represent the effects of selection solely due to drug treatment. If this is the case, then our experimental design provides a means of partitioning the evolved response in survival due to drug application and responses due to the effects of population size, density and the risk of mortality. Increased parasite densities generally have a negative effect on traits such as survival and fecundity (Churcher, Filipe, and Basáñez 2006); however, how density-dependence interacts with drug treatment remains unclear and may depend upon which life history stage is most severely affected by the drug (Churcher and Basáñez 2008). It is also possible that the difference in survivorship between HR and HD lines was due to the experimental protocol during selection. Random mortality populations were culled once every 24 hours to simulate the same level of mortality as 'sister' drug-treated populations, but drug-treated populations are likely to have suffered additional mortality over the course of this 24 hour period. This would have resulted in a lag between drug-induced mortality and culling between 'sister' populations. If HR lines had tracked the rate of mortality in HD lines more closely, maintaining similar densities between HD and HR treatments, potentially the same magnitude of response could have been observed in both high mortality treatments, regardless of mortality source. A more synchronised method of tracking drug-dependent mortality and imposing compensatory mortality on random mortality lines would reveal whether the lag in random culling is responsible for the difference in survivorship between HR and HD lines.

4.5.4 *Is there a cost of adaptation in drug-treated environments in terms of survival in drug-free environments?*

When random mortality and drug-treated lines were exposed to a drug-free environment, no differences were observed in survivorship relative to Z lines. Therefore, bottlenecking and small population size of random mortality lines resulted in no beneficial or detrimental effects on survival in an environment

where no extrinsic mortality was imposed. It has been suggested that the evolution of reduced susceptibility may lead to fitness costs in life-history traits if resistance is costly (Roush and McKenzie 1987). In order to asses the fitness costs that might result from reduced susceptibility one could either measure gene frequencies of susceptible alleles over a number of generations in the absence of the drug (Roush and McKenzie 1987) or estimate fitness based on measures of life-history traits such as fecundity, development time, fertility and mating competitiveness (Carriere et al. 1994; Gassmann, Carrière, and Tabashnik 2009) in the presence and absence of the drug. In this study we looked solely at differences in survival in drug-free and drug-treated environments; it would be interesting to assess a suite of traits associated with fitness and explore their relationship with apparent susceptibility to Ivermectin.

4.5.5 *Do different life-history stages respond to drug selection in the same way?*

Mathematical models have suggested that the life history of parasites may evolve in response to drug-treatment as a result of altering parasite survival and reproduction (Lynch, Grimm, and Read 2008). The differing responses of life history stage (juveniles and adults) in HD and LD lines at low and high dosages suggest that age-related effects and interactions with selection intensity may be important to consider in predicting resistance or tolerance evolution. We observed a significant interaction between resistance bioassay dose and life history. In addition, resistance bioassay data from 75 hours showed a response in survivorship of HD lines but not LD lines; i.e. adults of HD lines were less susceptible than Z lines whereas LD lines remained of a similar susceptibility to Z lines, across selected generations. However, 52-hour bioassay data showed that both HD and LD lines responded to drug selection in terms of increased survivorship. Therefore, at the high dose of Ivermectin, both juveniles and adults responded to drug selection, whereas at low doses only juveniles responded to selection.

Body size is often used as a predictor of fecundity across a range of nematode species (Morand 1996). Under standard life history theory, interventions that

reduce adult life expectancy should select for parasites that mature earlier at a reduced size and produce fewer offspring (Roff 1992; Stearns 1992; Skorping and Read 1998). However, Lynch *et al*. (2008) used mathematical models to demonstrate that interventions that affect mortality rates of mature parasitic nematodes could have complicated effects on optimal age to maturity, regardless of whether mortality is size dependent or independent. They argued that where an intervention measure is continuously applied, the optimum age at maturity may be longer relative to a situation with no intervention and that parasites should benefit from a greater reproductive life span. Field experiments studying the evolutionary effects of anthelmintics on *Teladorsagia circumcincta* showed that worm size was consistently larger in resistant isolates when compared to susceptible isolates (Leignel and Cabaret 2001). Worryingly, if drug selection favours increased size at maturity then resistant worms may be more fecund than susceptibles. It would be interesting to measure size at maturity as well as other life history traits of our evolved lines and investigate whether any responses in such traits correlate with apparently reduced susceptibility to Ivermectin.

4.5.6 *Conclusions*

Our inclusion of a novel treatment that controls for both the increased risk of mortality and changes in population size of drug-treated populations raises the question of whether previous studies that have not incorporated such controls should be re-evaluated. For example, Lopes *et al* (2008) report the rapid evolution of resistance to Levamisole within 10 generations of exposure under very similar experimental conditions to this study. Levamisole was administered at a concentration lethal for 75% of the ancestral population. A resistance bioassay was then performed on samples from generations 10 and 20, which showed a 25% increase in survival of populations under drug selection at generations 10 and 20. However, as there was no control for mortality between drug-treated and control populations, it is difficult to assess whether there were effects of differences in density and mortality between treatments. We recommend that future work on resistance should incorporate adequate controls

for parasite/pest density when assessing drug resistance evolution. In addition, controlling for differences in population size and rate of mortality could be implemented in any experimental evolution study where the selective agent induces greater mortality than control treatments.

Standing genetic variation in the form of susceptibility to chemical applications is important in the study of resistance evolution (Gilleard and Beech 2007). This study suggests there may be a complex relationship between the intensity of selection and, density-dependent regulatory processes and life history of populations challenged with control measures. How these factors interact and affect characteristics such as tolerance and resistance could result in significant impacts on the evolution of susceptibility. For instance, studies of drug susceptibility in nematodes have shown that environments where conditions are inhospitable to free-living larvae, which reduces larval densities, promote the evolution of resistance (Besier and Love 2003; Lawrence et al. 2007; Leathwick and Besier 2014). What proportion of this reported resistance is due to drug application or tolerance, and how it interacts with life history, is difficult to establish in the field. In order to understand how drug tolerance and resistance evolution may interact, future research should aim to identify precisely which traits are associated with tolerance and what influence they may have on resistance. The *Caenorhabditis* system allows a range of traits to be assessed over the course of selection experiments (Gray and Cutter 2014), and therefore should provide an invaluable model to explore factors which may affect the evolution of resistance and tolerance.

Chapter 5: The evolution of life-history traits of nematodes in response to drug selection

5.1 Abstract

Resistance management is a key concern in human and veterinary medicine and in agricultural production systems. Studies of resistance evolution consider the influence of factors such as gene flow, drug type, application method and costs of resistance, on the rate of resistance evolution. However, how life history traits of susceptible parasites interact with control measures remains unclear. This study used a free-living soil nematode to investigate the evolutionary effects of drug treatment on life-history traits. I experimentally evolved replicate populations (derived from the same ancestral line) of *Caenorhabditis remanei* in three environments: drug-treated (Ivermectin), drug-free, and drug-free with random-mortality to match the mortality in the drug-treated population. This last group was included to distinguish effects of drug treatment and population density on life history evolution. The effect of these treatments on larval and adult size, female lifespan and fecundity was assayed in both drug-treated and drug-free environments after 10 generations. Adult size was larger for both drug-selected and random-mortality lines compared to control lines, but only when assayed in drug-free environments. In contrast, lifespan was longer for drug-selected lines in drug-treated environments and was not affected by the random-mortality treatment. Higher fecundity was found in drug-selected lines relative to control lines in both treated and drug-free environments while that of random mortality lines was intermediate to drug-selected and control lines in drug-free environments but similar to controls in the treated environments. Our results suggest that life histories of nematodes may respond to selection, acting via ecological processes due to mortality and density-dependence. Failing to take these responses into account when applying control measures could result in adverse outcomes, such as larger and more fecund parasites.

5.2 Introduction

Parasitic diseases caused more than one million human deaths in 2013 (GBD collaborators 2015), while pests and diseases account for around one third of crop losses annually (Oerke 2006). The financial cost of controlling parasites and pests is considerable: for example, 39.4 billion dollars were spent on pesticides globally in 2007 (Grube et al. 2011). Control agents are designed to reduce target populations but massive global application has led to extensive development of resistance (Kaplan and Vidyashankar 2011; zur Wiesch et al. 2011). Standard models of resistance evolution consider the influence of factors such as gene flow, drug type, application method and costs of resistance (Barnes, Dobson, and Barger 1995; James, Hudson, and Davey 2009; Consortium 2013). Evolved responses in life-history in the form of fitness costs associated with insecticide resistance have been extensively documented in agricultural systems (reviewed by, Kliot and Ghanim 2012). Tolerance, natural variation in susceptibility already existing within a population, which is not a result of selection pressures imposed by control measures (Scott 1995; Coles and Dryden 2014), could also influence life history responses to drug-selection via feedbacks between ecological and evolutionary processes. For instance, changes in density as a result of application of control measures may lead to evolutionary responses in life history traits, which in turn affect susceptibility to the control agent. The potential influence of eco-evolutionary interactions between pathogen or pest life history traits and control measures on the evolution of susceptibility (tolerance and resistance) has been paid little attention beyond theoretical consideration (Skorping and Read 1998; Lynch, Grimm, and Read 2008; Ferguson et al. 2012), and it remains unclear how such traits will respond to drug selection. The consequences of responses in life history traits to drug selection may be alarming given that epidemiological properties such as pathogenicity and virulence are often linked to the life cycle of parasites (Anderson and May 1982; Frank 1996; Gandon et al. 2001).

Life history theory predicts how the birth and death schedules of an organism are shaped by its environment. The theory relies on optimality models, which assume trade-offs between fitness-related traits such as growth, survival and

reproduction when making predictions about how populations will respond to selection (Medawar 1952; Roff 1992; Stearns 1992). Human interventions have imposed intense selection on life history traits of commercially exploited species and have resulted in strong responses in such traits. For instance, meta-analysis of commercial fisheries showed that age and size at maturity have been significantly reduced by intense size-dependent harvesting (Sharpe and Hendry 2009). The application of control measures to pest and pathogen populations dramatically changes their environment and will affect both birth and death schedules as a result of high extrinsic mortality caused by the treatments. Thus, drug treatment is likely to induce a change in the optimal allocation of resources between growth, lifespan and reproduction (Skorping and Read 1998).

Some theoretical studies have predicted that the evolution of life history traits of parasitic nematodes in response to drug treatment could be beneficial from the perspective of disease control (Gemmill, Skorping, and Read 1999; Lynch, Grimm, and Read 2008), if increased extrinsic mortality of adult parasites due to drug treatment selects for early maturation and lower investment in growth, resulting in smaller less fecund worms; a situation often found in fisheries-induced evolution (Sharpe and Hendry 2009). A reduction in fecundity could be beneficial in terms of disease control because it may reduce transmission of the parasite (Walker et al. 2009; Reece et al. 2010). Selection experiments imposing high extrinsic mortality on adults have been shown to cause this type of predicted response in life history. For instance, a long-term laboratory study using *Drosophila melanogaster* exposed replicate populations of flies to different extrinsic mortality regimes in early adult life (high and low) over a period of five years (Stearns, Ackermann, and Doebeli 1998; Stearns et al. 2000); they found that higher extrinsic mortality rates led to the evolution of shorter lifespans, earlier maturity at a smaller size, and a shift in peak fecundity to earlier in adulthood. However, divergence in all life history traits between the two treatments did not occur until after the first year of the experiment, when larval food quality was lowered and larval density was increased; suggesting density-dependent competition modulating selection on life history. If parasites respond to drug selection in the same way as invertebrate models like *Drosophila* then the application of control measures could be beneficial from a disease control perspective. That is, if parasites respond to drug selection by

evolving earlier maturation and lower investment in growth, resulting in smaller less fecund worms, then the number of offspring available to transmit infections will be reduced. In addition, life history traits of parasites could respond differently in drug-free and treated environments as a result of adaptive changes in phenotypic plasticity, which occur during drug selection (Reece et al. 2010; Kochin, Bull, and Antia 2010). Therefore, to quantify any evolved changes in life history due to drug-selection, measures of traits should be performed in both drug-free and treated environments (Carriere et al. 1994; Lopes et al. 2008).

Mathematical models have predicted that drug-induced mortality could select for larger more fecund pathogens (Skorping and Read 1998), for example, if the risk of mortality differs between life history stages (Lynch, Grimm, and Read 2008). Where the risk of mortality is higher throughout adult life relative to juvenile mortality (e.g. because adults are more susceptible to the drug treatment), selection may favour delayed maturity, larger size and greater fecundity. In a 20-generation study of the effects of harvesting on the life history in replicate populations of soil mites, populations exposed to adult harvesting showed delayed maturation and had a larger adult size than un-harvested controls (Cameron et al. 2013). This contradicts studies of fisheries induced evolution of life history, where harvesting results in earlier maturation at a smaller size (Sharpe and Hendry 2009). The difference in outcome on life history between the two studies is due to the timing of extrinsic mortality (Stearns et al. 2000). High extrinsic mortality imposed early in adulthood favours selection for early maturation, but if mortality is imposed at the time of maturation and for the whole of the adult lifespan, then selection favours remaining in a juvenile stage for longer. Distinguishing between these two possible evolutionary outcomes in terms of the effects of drug application on life history traits could be achieved by measuring fitness-defining traits (development time, size, lifespan and fecundity) of populations challenged with drug treatment. Previous studies have focused on evolved changes in size (Leignel and Cabaret 2001) or fecundity (Kelly et al. 1978; Maingi, Scott, and Prichard 1990) of treated populations of parasites: a study focusing on a range of life history traits would provide a clearer picture of how life history responds to drug selection.

Differences in population density between drug-treated and untreated populations of parasites and pests could result in differential selection due to density-dependent processes such as competition (Gilleard and Beech 2007), and these processes can also result in evolutionary changes in growth, survival and reproduction (Reznick et al. 2012). For example, a 20-generation experiment selecting replicated populations of *D. melanogaster* in high or low-density environments found that low-density lines evolved longer development times in both growth environments (Roper, Pignatelli, and Partridge 1996). Longer development time was also associated with larger adult size and higher fecundity, but only in low-density growth environments, suggesting environment-mediated evolved differences in size and fecundity. In addition, the same study measured lifespan of high and low-density selected lines and found no differences in lifespan in either growth environment. Thus, long-term differences in population density can be influential on the evolution of life history traits. In order to understand the effects of drug treatment on evolutionary responses in life history it is necessary to separate indirect effects, such as reduced density, from the direct effects of drug application. Adequate controls that compensate for differences in density between treatments are often overlooked in selection experiments but can be achieved with an additional treatment, which mimics mortality and density patterns imposed on drug-selected lines (Fuller, Baer, and Travis 2005).

Measuring life-history traits of parasites within a host is technically difficult and time consuming (Leathwick and Hosking 2009). Studying how model organisms respond to selection pressures causing high extrinsic rates of mortality, and differences in population density, may provide insights into how parasite life history responds to drug selection. *C. elegans* has been used as a model organism to study both evolutionary processes (Manoel et al. 2007; Morran, Parmenter, and Phillips 2009; Fritzsche et al. 2014) and mechanisms of drug resistance (Simpkina and Coles 1981; James, Hudson, and Davey 2009; Ghosh et al. 2012). However, *C. elegans* is a hermaphroditic species and most reproduction is due to self-fertilisation (Brenner 1974), whereas most parasitic nematodes are dioecious and obligately outcrossing. Therefore, dioecious nematodes such as *C. remanei* can provide a more realistic model system to study potential evolutionary changes in life history-traits in response to drug selection. The

basic demography of *C. remanei* has been previously studied under laboratory conditions; the species shows high variation in development time, lifespan and fecundity (Diaz, Lindström, and Haydon 2008; Reynolds and Phillips 2013). Lifespan and fecundity have also been previously studied in *C. remanei* in relation to selection for condition-dependent mortality when subjected to high temperature (Chen and Maklakov 2012). Furthermore, Lopes et al (2008) explored the relationship between survival and fecundity in Levamisole-selected populations of *C. elegans* but did not measure traits such as lifespan and size at maturity. However, to date there are no studies of how life-history traits respond to drug-selection in a free-living dioecious species of nematode. Laboratory-based selection experiments can often impose strong selection on the timing of reproduction and development time due to the desire of researchers to achieve rapid progress through numerous generations (Chehresa, Beech, and Scott 1997; Gilleard and Beech 2007). Caenorhabditis nematodes can be cryogenically frozen and revived, making them an ideal model to test whether control treatments are effected by the design of a selection experiment. By reviving ancestral and selected control lines and measuring a trait of interest, any differences in control lines relative to ancestral lines can be detected (Gray and Cutter 2014; Sikkink et al. 2015). Thus, if there is a no response to selection in control lines relative to ancestral lines, and a response to selection in treated lines relative to controls, then any change associated with phenotypes between treated and control lines must be due to treatment during selection and not rapid passage during experimental evolution.

In a previous study (Chapter 4; Reynolds et al. 2016), I explored the relationship between drug selection, population density and the evolution of resistance to Ivermectin. Replicate lines of *C. remanei* exposed to the anthelmintic Ivermectin, and drug-free control lines, which had mortality imposed randomly at the same rate as drug-treated lines, evolved an increase in survival relative to standard drug-free control lines over 10 generations. The results suggested that similarities in the population density and mortality between drug-treated and random mortality lines could be responsible for the increased apparent drug tolerance observed in both treatments. In this study I asked if the evolutionary changes in survival of these selected lines were associated with changes in other life history traits (larval and adult size, female fecundity and female lifespan),

whether there is a trade-off between adaptation to the drug-treated environment and fitness, and does any trade-off differ in drug-free and treated environments. I specifically asked: 1) Does rapid passage of control lines through numerous generations have an effect on life-history traits; 2) Does drug application have an effect on life-history traits; 3) Do conditions with high extrinsic mortality and density-dependent selection have an effect on life history traits?

5.3 Methods

5.3.1 *Origin of experimental lines and selection experiment*

The construction of the ancestral strain and the details of the selection experiment have been described previously (Chapter 4). Briefly, a genetically diverse starting population of *C. remanei* (SP8) created by crossing three strains (SB146, PB206 and MY31) was provided by N. Timmermeyer in the Department of Biology, University of Tübingen, Germany. After the crosses were performed, strain SP8 was maintained for eight generations to create recombinant genotypes and allow adaptation to standard laboratory conditions (Fritzsche et al. 2014). Upon arrival in Glasgow, strain SP8 spent a further four generations adapting to any differences in conditions between laboratories and was maintained under standard laboratory conditions for *Caenorhabditis* species: 20˚C and 60% humidity on NGM (Nematode Growth Medium) petri dishes and fed on a lawn of *Escherichia coli* (OP50) (Hope 2001). Experimental lines derived from the ancestral SP8 stock strain underwent three selection treatments (Fig 5.1): exposure to Ivermectin at a dose calibrated to cause 80% mortality in a non-resistant population (drug-treated, D); a drug-free control (zero-dose, Z); and a second zero-dose control where the population size was reduced by randomly removing adult worms to match the level of mortality in the drug-treated lines (zero-dose + random mortality, R). The random mortality control was included to control for effects of differences in density between treated and untreated lines. Experimental lines were cultured for 10 generations with three

replicate lines for the D treatment and R control lines and six for the Z controls, making a total of 12 lines. The ancestral (A-line) stock strain as well as samples of larval worms from generation 10 of each selected line were cryogenically frozen at -80˚C), at a density of approximately 2000 L1 larvae in liquid freezing solution as described in Hope (2001).

Prior to each life history assay, preserved samples of the ancestral line and lines from the selection experiment at generation 10 were thawed and raised for three generations in a drug-free environment to ensure that any observed responses in survival were due to genetic differences among populations and not maternal or environmental effects due to freezing. Larvae were thawed at room temperature and maintained at a density of approximately 1000 individuals per 9cm agar plate over the three generations from thawing to age synchronization with *ad libitum* lawns of *E. coli* OP50. Transfers between generations were achieved by cutting out sufficient agar from plates already containing samples and transferring these to fresh *E. coli* seeded plates ensuring the density remained as constant as possible. L1 synchronised larvae from revived lines were generated using a standard bleaching protocol for the beginning of each life history assay (Hope 2001).

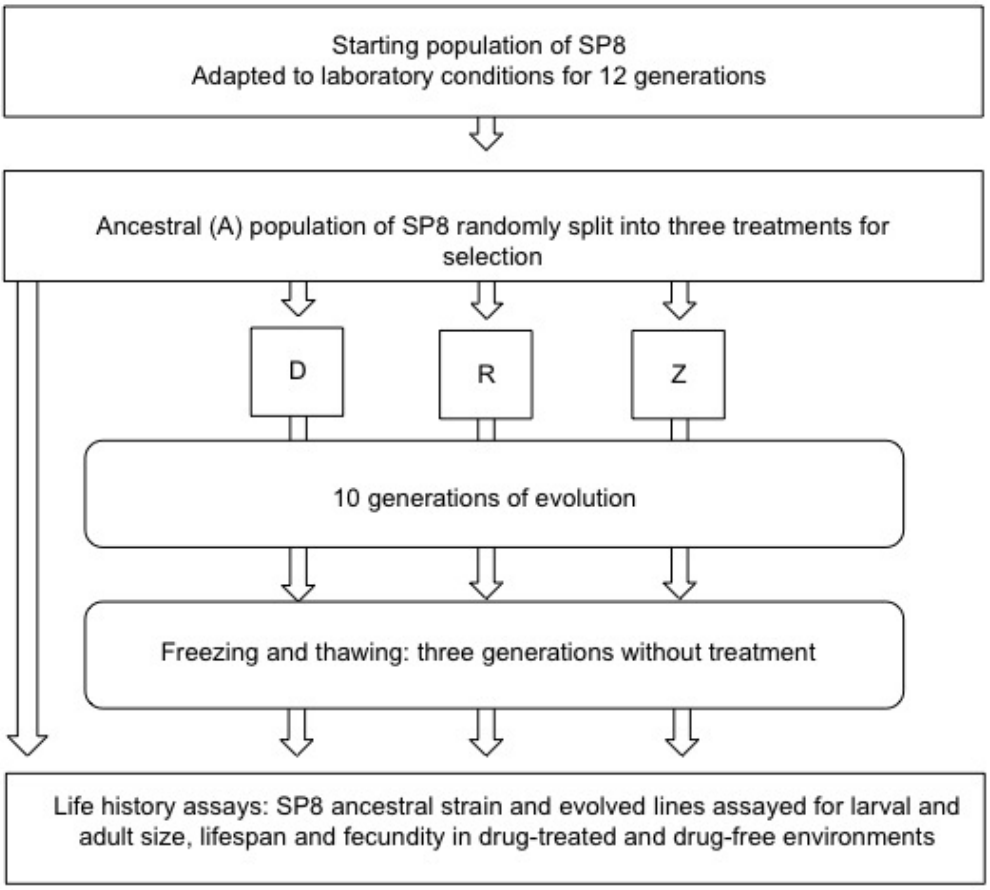


Fig 5.1 Schematic representation of selection experiment and life-history assays. The starting population of SP8 was adapted to lab conditions. The lab-adapted strain was then randomly divided into three treatments with three replicates each for drug-selected (D), and random mortality (R) treatments, and six replicates of zero dose (Z) lines. After 10 generations of selection, lines were frozen and later thawed, before being exposed to drug-free and treated environments and data gathered on life-history traits.

5.3.2 *Larval and adult size*

Size estimates for larvae, females and males were calculated by collecting digital images of worms and then estimating their volume from these images. Images of 50-100 developmentally arrested larvae from the ancestral line and each selected line were collected three hours after hatching on 9 cm NGM plates in the drug-free environment. Larval sizes were not taken in the drug treated environment because they could not be exposed to Ivermectin over the same period of time, as size measures taken for adults. To collect images of adults in

the drug-treated environment 9 cm plates were prepared by adding 2.46 ng/ml Ivermectin dissolved in the solvent DMSO (0.02%) to NGM media: the media was kept at 50˚C and mixed thoroughly using a magnetic stirrer prior to pouring. The dry plates were seeded with *E. Coli* and approximately 1000 L1 synchronised larvae per line were added to the drug-treated plates and incubated for 75 hours. After 75 hours adults were washed onto clean (i.e. no *E. coli*) plates and images were collected with a Zeiss Stemi 200-C stereomicroscope using a Celestron digital microscope camera (Model: 44421) at x 30 magnification. Depending on how many adults had survived to 75 hrs, 50 to 100 images of females and males were collected for each of the 12 selected lines + 1 ancestral line. To collect images of adults in the drug-free environment the same procedure was conducted as for the drug-treated environment but without the addition of Ivermectin to the solvent. The volume of worms in picolitres was estimated from digitised images using wormSizer software (Moore, Jordan, and Baugh 2013); volume calculations assume worms are made up of a series of frustums (cones with the tip removed) and the sum of the volume of all frustums equals total volume for an individual worm.

5.3.3 *Female lifespan and fecundity*

Lifespan was assessed only for females so that it could be compared with differences in reproductive output based on fecundity. I assessed only three out of the six control lines for comparison with the three replicates of the drug-treated and random-mortality lines, to maximise the number of replicates per experimental line. I chose control lines on the basis of the results from the size assay, choosing three lines that had the largest, smallest and intermediate volumes of the six control lines to prevent any bias due to size. Lifespan was measured by counting the number of females alive every two days during the course of the experiment. To measure lifespan in a drug-treated environment L1 synchronised larvae from each line were cultured in drug-free conditions at a density of approximately a 1000 until developing till the L4 stage. At the L4 stage sex can be distinguished but mating does not occur. L4 females were taken from these cultures and transferred to drug-treated 2.5 cm *E. coli* seeded agar

plates, maintained with male worms from their source population, of which the number of individuals was adjusted throughout the experiment to maintain a 1:1 sex ratio on all plates at all times. Females were transferred to fresh drug-treated plates every 48 hours to prevent larval offspring development confounding counts of females. Census data were collected whilst transferring replicates to new plates; census continued until the last female died. Measuring lifespan in the drug-free environment followed the method used for the treated environment except Ivermectin was not added to agar plates. I measured the lifespan of 20 females per line (four replicates of five females) in both the drug-free and treated environments. Lifespan measurements were taken for 400 females in total: 10 lines (3 treatments x 3 replicates + 1 ancestral) x 4 plates per line x 5 females per plate x 2 environments).

Fecundity of selected and ancestral lines was evaluated by census of the number of eggs produced by females from each line during the lifespan assay. The number of eggs laid by females on each replicate plate was counted for a 3-hour period every 48 hours: this was done at the beginning of the experiment and three hours after each transfer, and repeated until females either died or stopped producing offspring. I recorded two measures of fecundity: the total number of eggs counted, which provided an index of total lifetime reproductive output; and the number of eggs counted on day two when fecundity reached a maximum in all lines, which was used as an index of peak fecundity.

5.3.4 *Statistical analysis*

My three main questions were concerned with assessing the effects of treatment during selection on life history traits (larval and adult size, female fecundity and female lifespan). The analysis separately compared each life history trait of lines from the original selection experiment (drug-treated, random mortality and zero dose), as well as the ancestral line, in both drug-free and treated environments, using generalised linear mixed models. I used the same approach to test for differences between treatments for each trait, but models varied for traits depending on the distribution of the response variable and the random effects

included in the model. The first step of the analysis tested for the significance of an interaction between treatment during selection and environment, and provided a test of whether responses of life history traits were the same in drug-free and treated environments; a full model with fixed effects for treatment, environment and their interaction was compared with a null model with no interaction term. Because of differences in sample sizes and variance in the response variable between environments, further tests of differences between treatments were performed on datasets for each environment (drug-free and drug) separately. The second step of the analysis involved testing for a difference in the specified trait between the four treatments, by comparing the full model with a null model with no fixed effect of treatment (H_0: Ancestral = Zero = Drug = Random). A further four post-hoc measures were then used to address my main questions, with the tests performed on discrete datasets for specific treatment pairs addressing each question. The effect of passage of control lines through 10 generations of the selection experiment was assessed for each trait individually by comparing a full model with treatment as a fixed effect to a null model without treatment (H_0: Ancestral = Zero). A difference in a trait due to drug selection was assessed by using the same model but for the zero-dose and drug selected lines (H_0: Drug = Zero). To answer the question: Do conditions with high extrinsic mortality and density-dependent selection have an effect on life history traits? Differences in the response of traits to the random mortality treatment (H_0: Random = Zero) and density-dependence in response to selection (H_0: Drug treatment = Random treatment) were tested in the same way, comparing a full model with a fixed effect of treatment against a null model without treatment. The second step was repeated for each trait in each environment providing an assessment of how traits responded to selection in terms of their phenotype when challenged with each environment.

All statistical analyses were performed using R v 3.1.2 (R Core Team 2014) and I defined a significance threshold of P = 0.05 for all tests. I tested for differences in life-history traits between experimental treatments using generalized linear mixed effects models (GLMMs) fitted using the lme4 package (Bates, Maechler 2014). I fitted a GLMM with a normal error distribution to assess differences in larval and adult size between lines. Treatment during selection and assay environment was fitted as a fixed effects and evolutionary replicate (line) as a

random effect. The interaction between treatment and environment was performed for females and males only, because data on larval size was only collected in the drug-free environment. Furthermore, no interactions between sex and selection regime were explored, because female size was twice that of males in both drug-free and treated environments. Differences in lifespan were assessed with the same model structure as size but included a further random effect for replicate plate in the lifespan assay. In addition, a parametric survival model with a Weibull distribution and a fixed effect of treatment during selection was fitted to the lifespan data to provide a plot of survival over time. Differences in egg count data were evaluated using a GLMM with a Poisson error distribution. Treatment during selection and assay environment were fitted as fixed effects and random effects were fitted for evolutionary line and repeated sampling of lines during the fecundity assay. An additional random effect was added for each observation to account for the overdispersion in the response variable (Browne et al. 2005).

5.4 Results

5.4.1 *Size*

In the drug-free environment, there were no differences in larval size due to treatment during selection ($P = 0.54$; Fig 5.2E, Tables 5.1 and 5.2). There was a significant interaction between assay environment (drug-free or drug-treated) and treatment during selection in terms of adult size for both females and males (females: $x2 = 169.57$, $df = 3$, $P < 0.0001$; males: $x2 = 47.51$, $df = 3$, $P < 0.0001$). In the drug-free environment there were significant differences in mean size accounted for by treatment during selection for both females and males (females: $P = 0.0023$; males: $P = 0.0072$; Fig 5.2A and C, Tables 5.1 and 5.2). There was no significant difference in mean size between ancestral and zero-dose lines for either females or males (females: $P = 0.08$; males: $P = 0.66$; Fig 5.2A and C, Tables 5.1 and 5.2). Thus, rapid passage of control lines had no effect on adult size when comparing the ancestral and zero-dose lines. Females

and males from drug-selected lines were significantly larger (14% and 15%, respectively) than those from zero-dose lines (females: $P < 0.0001$; males: $P = 0.00022$; Fig 5.2A and C, Tables 5.1 and 5.2). Females and males from random mortality lines were also significantly larger (9% and 8%, respectively) than those from zero-dose lines (females: $P = 0.016$; males: $P = 0.035$; Fig 5.2A and C, Tables 5.1 and 5.2). Therefore, density-dependent selection had an effect on size in the drug-free environment. There was no significant difference in size between drug-treated and random mortality lines for either females or males (females: $P = 0.30$ males: $P = 0.35$; Fig 5.2A and C, Tables 5.1 and 5.2). In the drug-treated environment there were no differences in mean adult size of either females or males with respect to selection regime (Females: $P = 0.13$; Males: $P = 0.20$; Fig 5.2 B and D, Table 1 and 2).

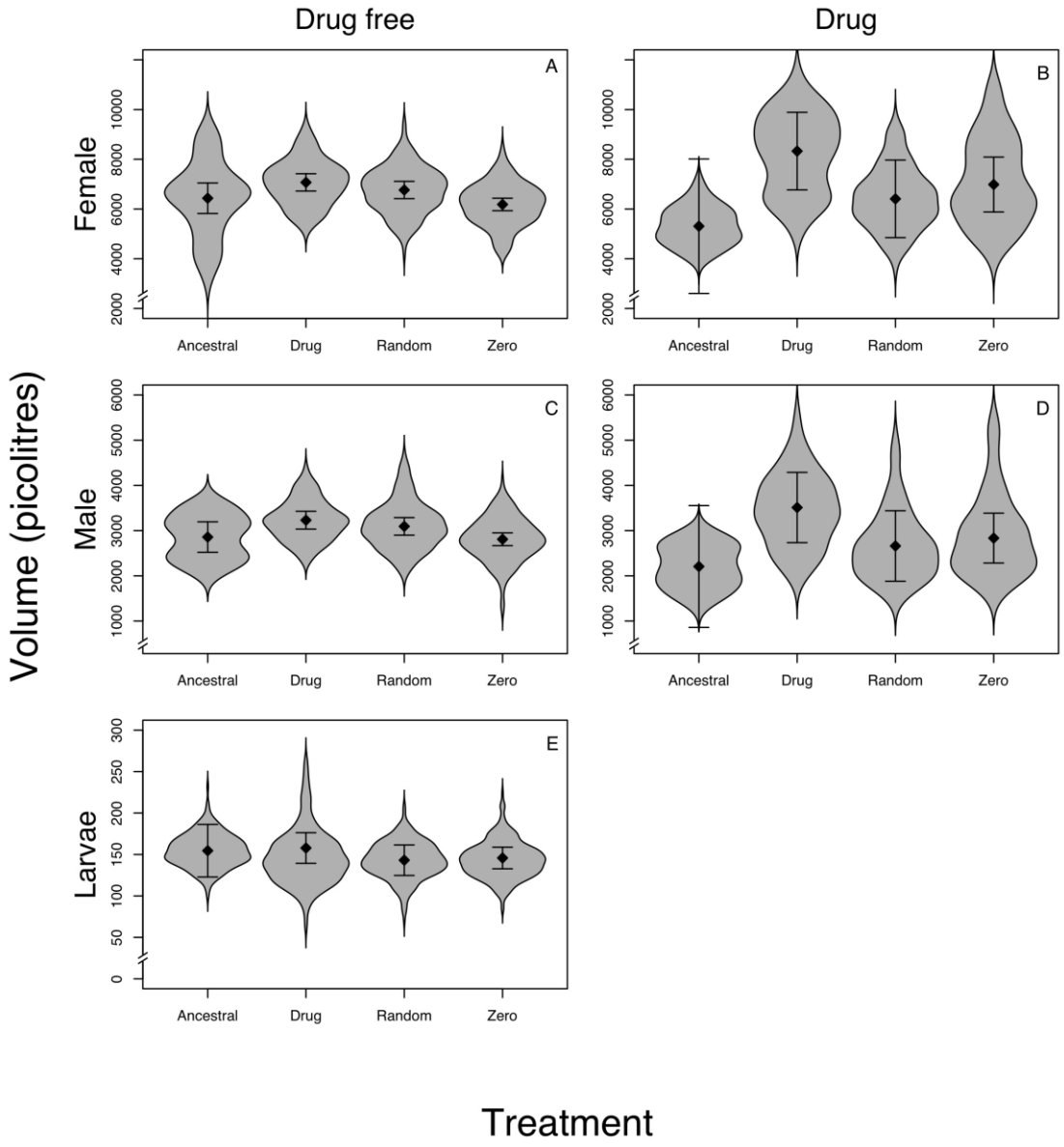


Fig 5.2 Violin plots showing the kernel density estimation of the distribution of size at maturity for selected (Drug, Random and Zero) and Ancestral lines. The grey areas show how size data were distributed within a given treatment. Diamonds represent estimated mean volume for each treatment (estimated from the maximal model – see methods). Females (panel A and B) and males (panel C and D) when exposed to drug-free (panel A and C) and drug-treated (panel B and D) environments. Panel E shows larval size in a drug-free environment. Error bars indicate 95% confidence intervals for mean volume.

5.4.2 *Female lifespan*

There was a significant interaction between assay environment and treatment during selection in terms of female lifespan (x2 = 9.52, df = 3, P = < 0.023). No differences were observed in lifespan between females from different selection regimes in the drug-free environment (P = 0.76; Figs 5.3 and 4, Tables 5.1 and 5.2); mean (95% CI) lifespan was 8.7 (6.6, 10.8), 7.8 (6.6, 9.0), 7.9 (6.7, 9.1), 7.6 (6.4, 8.8) days for ancestral, drug-selected, random mortality and zero-dose lines, respectively (Fig 5.3A and 5.4A, Tables 5.1 and 5.2). Treatment during selection had an effect on female lifespan in the drug-treated environment (P = 0.017, Figs 5.3B and 5.4B, Tables 5.1 and 5.2). There was no difference between ancestral and zero-dose lines in terms of lifespan (P = 0.23; Figs 5.3B and 5.4B, Tables 5.1 and 5.2), suggesting the selection experiment had no effect on lifespan in the control treatment. In the drug-treated environment, females from drug-selected lines had a 42% longer lifespan than the zero-dose lines (P = 0.015; Figs 5.3B and 5.4B, Tables 5.1 and 5.2). There was no difference in female lifespan between random mortality and zero-dose lines in the treated environment (P = 0.86; Figs 5.3B and 5.4B, Tables 5.1 and 5.2); and female lifespan of random mortality lines was significantly shorter than that of drug-selected lines. Therefore, differences in density between random mortality and zero-dose lines during selection had no effect on lifespan when the selected lines were exposed to Ivermectin.

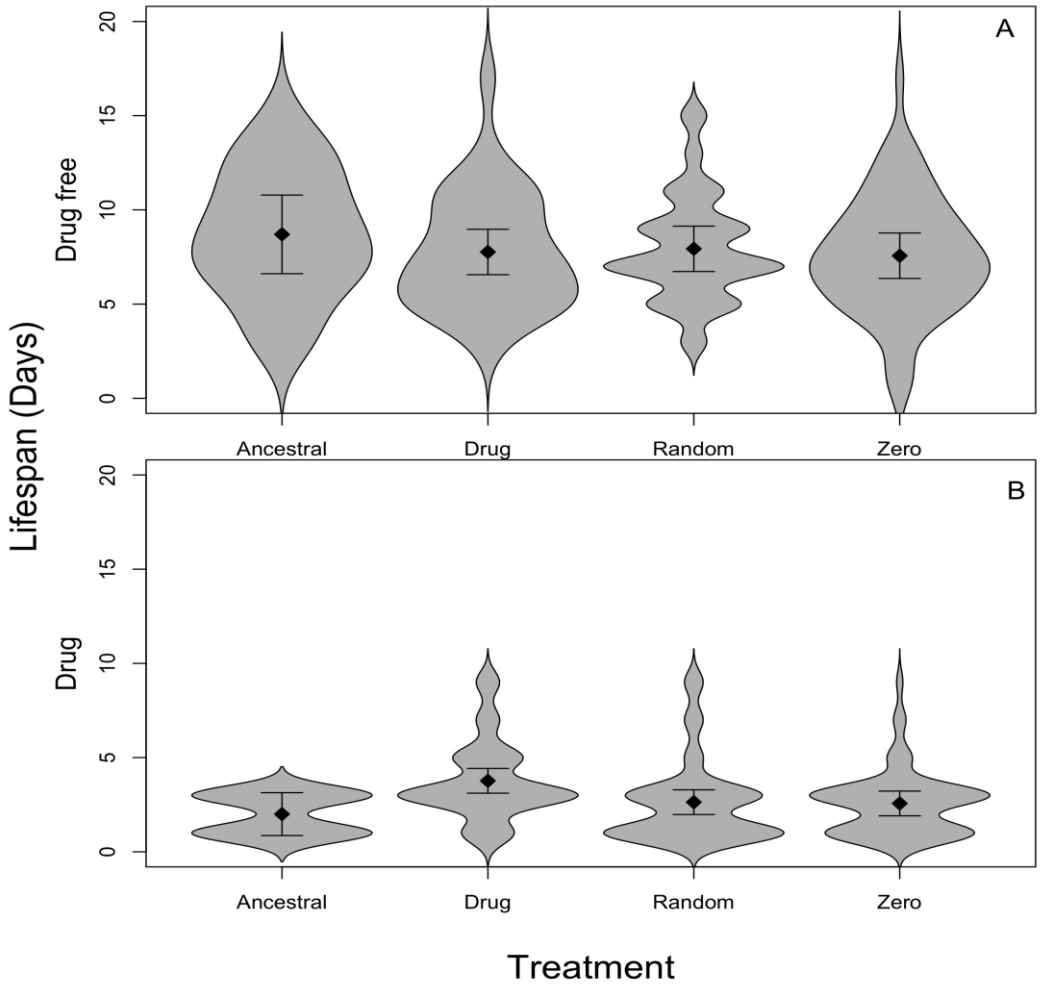


Fig 5.3 Violin plots of mean lifespan for selected (Drug, Random and Zero) and Ancestral lines, grey areas show how lifespan data were distributed within treatments. Diamonds represent estimated mean lifespan in drug-free (panel A) and treated (panel B) environments for each selection regime (estimated from the maximal model – see methods). 95% confidence intervals for mean volume.

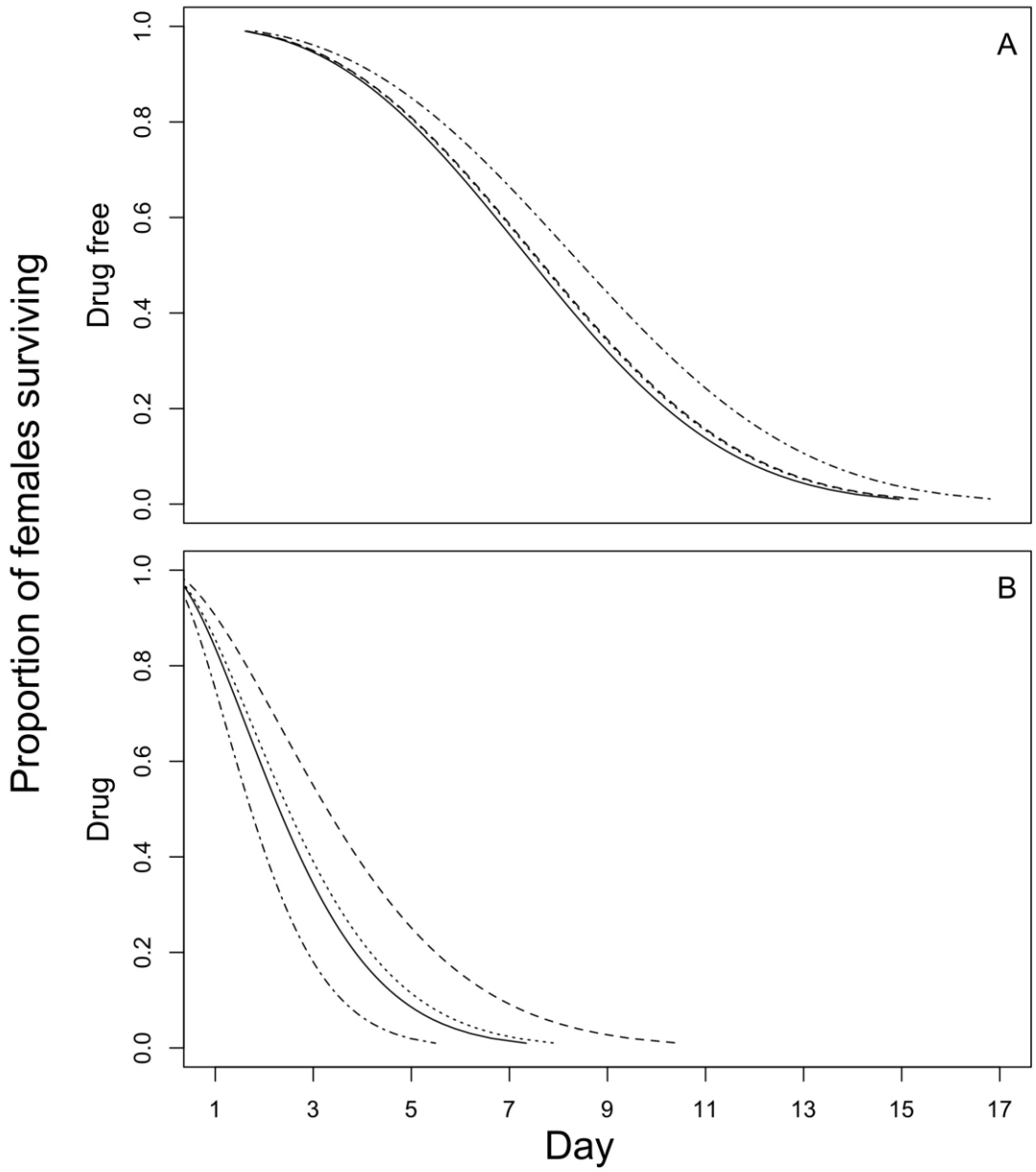


Fig 5.4 Survival curves of females from selected and ancestral lines when exposed to drug-free (A) and drug-treated (B) environments. Solid line = zero dose; dashed line = drug treatment; dotted line = random mortality: dot-dashed line = ancestral population. On plot A, the random mortality curve (dotted) is difficult to see because it is very similar to the drug treatment curve (dashed).

Table 5.1 Effect of treatment during selection on size, female life span and fecundity in treated and drug-free environments, assessed by null models, using likelihood ratio tests. Maximal models assumed a difference between the selection regimes. For each trait and environment, null model 1 assumed no difference between treatments and was tested against a maximal model assuming treatment had an effect. Null models 2-5 are post hoc tests and assumed no difference between the specified pair of selection regimes; they were tested against a model assuming a difference between paired treatments. D: Drug-treated; R: Random mortality; Z: Zero dose; A: Ancestral line.

Trait	Environment	Null models	x2 (d.f.)	P-value
Female size	Drug free	1) A = Z = D = R	14.47 (3)	**0.0023**
		2) A = Z	2.96 (1)	0.08
		3) D = Z	18.9 (1)	**< 0.0001**
		4) R = Z	5.78 (1)	**0.016**
		5) D = R	1.06 (1)	0.30
	Drug	1) D = R = Z = A	5.59 (3)	0.13
Male size	Drug free	1) A = Z = D = R	12.07 (3)	0.007
		2) A = Z	0.19 (3)	0.66
		3) D = Z	13.65 (1)	**0.00022**
		4) R = Z	4.44 (1)	**0.035**
		5) D = R	0.088 (1)	0.35
	Drug	1) A = Z = D = R	4.59 (3)	0.20
Larval size	Drug-free	1) A = Z = D = R	2.14 (3)	0.54
Female lifespan	Drug-free	1) D = R = Z = A	1.38 (3)	0.71
	Drug	1) A = Z = D = R	10.25 (3)	**0.017**
		2) A = Z	1.46 (1)	0.23
		3) D = Z	5.95 (1)	**0.015**
		4) R = Z	0.03 (1)	0.86
		5) D = R	4.97 (1)	**0.026**
Lifetime fecundity	Drug-free	1) A = Z = D = R	9.68 (3)	**0.022**
		2) A = Z	1.62 (1)	0.20
		3) D = Z	6.55 (1)	**0.011**
		4) R = Z	1.44 (1)	0.23
		5) D = R	1.50 (1)	0.22
	Drug	1) D = R = Z = A	22.03 (3)	**< 0.0001**
		2) A = Z	6.87 (1)	**0.0088**
		3) D = Z	13.47 (1)	**0.00024**
		4) R = Z	1.21 (1)	0.27
		5) D = R	10.81 (1)	**0.0010**
Peak fecundity	Drug-free	1) A = Z = D = R	11.06 (3)	**0.011**
		2) A = Z	2.21 (1)	0.14
		3) D = Z	6.96 (1)	**0.0083**
		4) R = Z	0.20 (1)	0.66
		5) D = R	4.83 (1)	**0.028**

Trait	Environment	Null models	x2 (d.f.)	P-value
Peak fecundity	Drug	1) A = Z = D = R	11.50 (3)	**0.0093**
		2) A = Z	4.09 (1)	**0.043**
		3) D = Z	5.69 (1)	**0.017**
		4) R = Z	0.56 (1)	0.81
		5) D = R	4.68 (1)	**0.031**

Table 5.1 Continued.

Table 5.2 Effect of treatment during selection on adult and larval size (volume; nanolitres), female lifespan (days) and fecundity (predicted egg numbers) in drug-free and drug-treated environments, as assessed by predicted trait means of the maximal model with selection treatment as a fixed effect (CI: 95% confidence interval for estimated trait means). Estimates significantly different from Zero dose lines are highlighted in bold.

Trait	Environment	Selection treatment	Trait mean (95% CI)
Female size	Drug-free	Ancestral	6.4 (5.8, 7.0)
		Drug	**7.1 (6.7, 74)**
		Random	**6.8 (6.4, 71)**
		Zero	6.2 (5.9, 6.4)
	Drug	Ancestral	5.3 (2.6, 8.0)
		Drug	8.3 (6.8, 9.9)
		Random	6.4 (4.8, 8.0)
		Zero	7.0 (5.9, 8.1)
Male size	Drug-free	Ancestral	2.9 (2.5, 3.2)
		Drug	**3.2 (3.0, 3.4**)
		Random	**3.0 (2.9, 3.3)**
		Zero	2.8 (2.7, 3.0)
	Drug	Ancestral	2.2 (0.9, 3.6)
		Drug	3.5 (2.7, 4.3)
		Random	2.7 (1.9, 3.4)
		Zero	3.4 (2.2, 3.9)
Larval size	Drug-free	Ancestral	155 (123, 186)
		Drug	158 (139, 176)
		Random	143 (124, 161)
		Zero	145 (132, 158)
Female lifespan	Drug-free	Ancestral	8.7 (6.6, 10.8)
		Drug	7.8 (6.6, 9.0)
		Random	7.9 (6.7, 9.1)
		Zero	7.6 (6.4, 8.8)
	Drug	Ancestral	2.0 (0.9, 3.1)
		Drug	**3.8 (3.1, 4.4)**
		Random	2.6 (2.0, 3.3)
		Zero	2.6 (1.9, 3.2)
Lifetime fecundity	Drug-free	Ancestral	38.1 (36.0, 40.3)
		Drug	**52.8 (50.7, 54.7)**
		Random	48.0 (45.9, 50.0)
		Zero	43.2 (41.1, 45.3)
	Drug	Ancestral	1.9 (0, 4.5)
		Drug	**13.6 (11.3, 15.9)**
		Random	3.6 (1.3, 5.9)
		Zero	4.7 (2.4, 7.0)

Trait	Environment	Selection treatment	Trait mean (95% CI)
Peak fecundity	Drug-free	Ancestral	23.9 (21.7, 26.1)
		Drug	**37.5 (35.4, 39.6)**
		Random	31.5 (29.4, 33.6)
		Zero	30.0 (27.9, 32.1)
	Drug	Ancestral	4.0 (1.3, 6.7)
		Drug	**15.9 (13.6, 18.3)**
		Random	8.0 (5.6, 10.3)
		Zero	8.3 (6.0, 10.7)

Table 2.2 Continued.

5.4.3 *Fecundity*

5.4.3.1 Lifetime fecundity

There was a significant interaction between assay environment and treatment during selection in terms of lifetime fecundity (x2 = 57.34, df = 3, P = 0.0001). In the drug-free environment there was a significant effect of selection regime on lifetime fecundity (P = 0.022; Figs5.5A, Tables 5.1 and 5.2). Independent contrasts of ancestral and zero-dose lines showed no difference in lifetime fecundity in the drug-free environment (P = 0.20; Figs 5.5A, Tables 5.1 and 5.2), suggesting the zero-dose selection regime had no effect on lifetime fecundity relative to the ancestral population. Drug-selected lines had higher lifetime fecundity than zero-dose lines in the drug-free environment (P = 0.011; Figs 5.5A, Tables 5.1 and 5.2); the mean number of eggs produced by females from drug-selected lines was 22% higher than from zero-dose lines. Thus, selection in a drug-treated environment led to higher reproductive output in the drug-free environment relative to the zero-dose lines. Mean lifetime fecundity of random mortality lines was intermediate to drug-treated and zero-dose lines and was not significantly different from either (P = 0.23; P = 0.22, respectively for drug-treated and zero-dose lines, Figs 5A, Tables 5.1 and 5.2). Estimates of lifetime fecundity of females from random mortality lines were 9% lower than drug-

treated lines but 11% higher than zero-dose lines. Selection regime also had a significant effect on lifetime fecundity in the drug-treated environment (P = 0.0001; Figs 5B, Tables 5.1 and 5.2). Lifetime fecundity in zero-dose lines was significantly higher than in the ancestral lines (P = 0.0093; Figs 5B, Tables 5.1 and 5.2). Drug-selected lines had significantly higher lifetime fecundity than zero-dose lines (P = 0.00024; Figs 5B, Tables 5.1 and 5.2); mean lifetime fecundity of drug-selected lines was three times that of zero-dose lines. Lifetime fecundity of random mortality lines was not significantly different from zero-dose lines but was lower than that of drug-treated lines (P = 0.27; P = 0.0010, respectively; Figs 5B, Tables 5.1 and 5.2); mean fecundity of random mortality lines was 23% less than zero-dose lines but 74% less than drug-treated lines in the treated environment. Thus, differences in density between random mortality and zero-dose lines during selection had no effect on lifetime fecundity when the selected lines were exposed to Ivermectin.

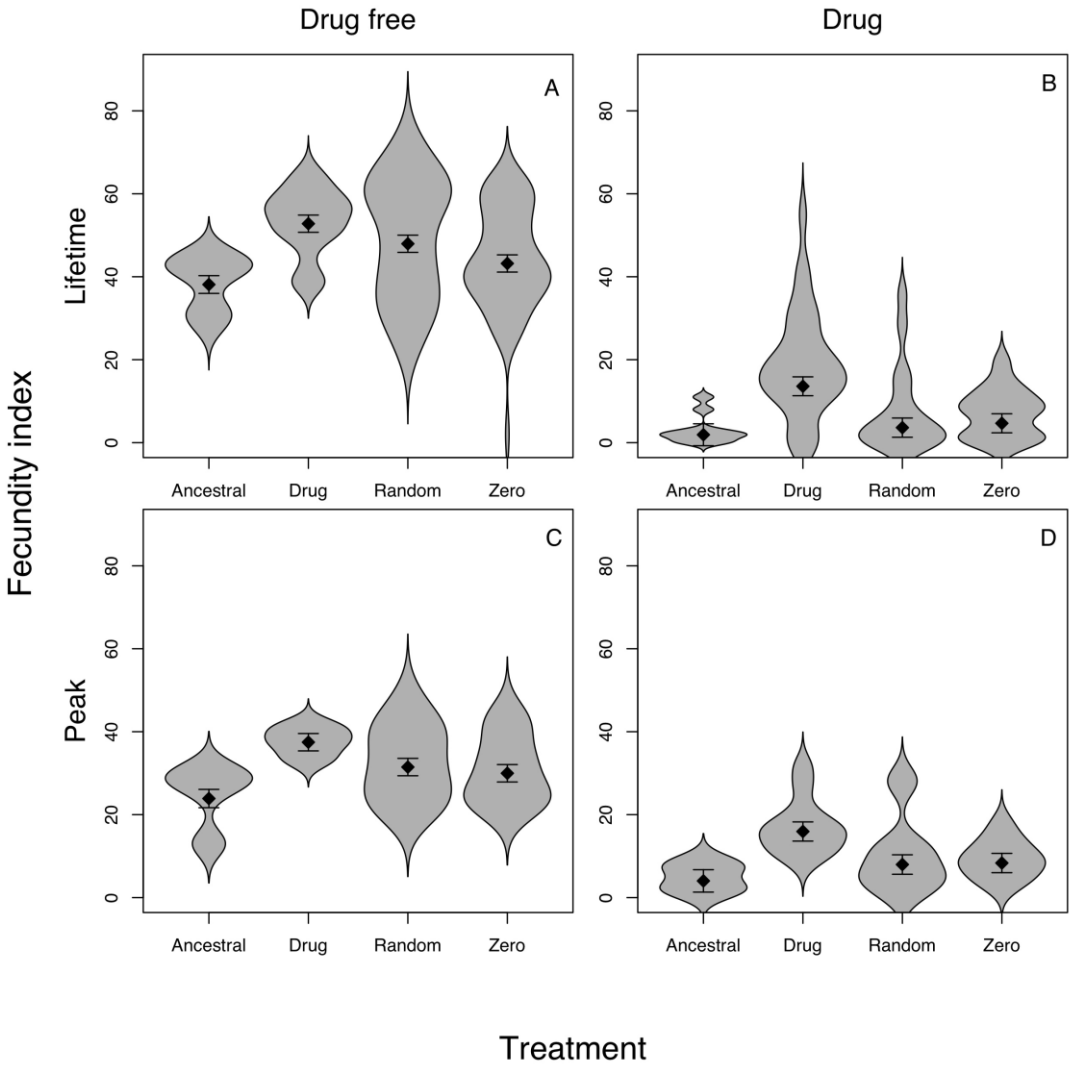


Fig 5.5 Violin plots showing the kernel density estimation of the distribution of fecundity (number of eggs). The grey areas indicate how fecundity data were distributed within a given treatment. Diamonds represent estimated mean number of eggs for each treatment (estimated from the maximal model – see methods). Lifetime fecundity (panels A and B) and peak fecundity (panels C and D) when exposed to drug-free (panels A and C) and drug-treated (panels B and D) environments. Error bars denote 95% confidence intervals for mean fecundity.

5.4.3.2 *Peak fecundity*

There was no interaction between assay environment and treatment during selection in terms of fecundity at the peak time of two days (x2 = 1.30, df = 3, P = 0.73). In the drug-free environment, peak fecundity was significantly different between selection regimes (P = 0.011; Fig 5.5C, Table 5.1 and 5.2). Independent contrasts of peak fecundity for ancestral and zero-dose lines indicated no

difference in fecundity in the drug-free environment (P = 0.14; Figs 5.5C, Tables 5.1 and 5.2) suggesting that selection in the control environment had no effect on peak fecundity when comparing the ancestral line to zero-dose lines. Predictions of peak fecundity from drug-selected lines were 25% higher than zero-dose lines (P = 0.0083; Figs 5C, Tables 5.1 and 5.2) indicating selection in a drug-treated environment resulted in increased reproductive output under drug-free conditions. Peak fecundity of random mortality lines was not different from zero-dose lines (P = 0.66), but was 16% lower than in drug-treated lines (P = 0.028; Figs 5.5C, Tables 5.1 and 5.2). In contrast to lifetime fecundity, peak fecundity in random mortality lines remained similar to that of the zero-dose lines suggesting differences in density between these lines had no effect on peak fecundity in the drug-free environment.

In the drug-treated environment, selection regime also significantly affected the mean number of eggs produced per female (P = 0.0093; Figs 5D, Tables 5.1 and 5.2). Peak fecundity of zero-dose lines was two-fold greater than that of ancestral lines when exposed to the drug-treated environment (P = 0.043; Figs 5D, Tables 5.1 and 5.2) indicating that experimental evolution increased peak fecundity in zero-dose lines relative to the ancestral line. In the drug-treated environment, peak fecundity of drug-selected lines was 92% higher than zero dose lines (P = 0.017; Figs 5D, Tables 5.1 and 5.2). Peak fecundity of random mortality lines was similar to that of zero-dose lines (P = 0.81), but 50% lower than drug-selected lines (P = 0.031; Figs 5D, Tables 5.1 and 5.2). Thus, there was no evidence that density had an effect on peak fecundity in the drug-treated environment.

5.5 Discussion

My study sought to test what effects drug treatment has on life history traits, and at the same time assess the effects of high extrinsic mortality and differences in density-dependent effects, which occur when control measures are applied. In addition, I aimed to test whether life history traits of control

lines were affected by rapid passage through the 10 generations of the selection experiment.

5.5.1 *Does rapid passage of control lines through numerous generations have an effect on life-history traits?*

Control zero-dose lines showed no response to selection relative to the ancestral line in either size or lifespan. However, zero-dose lines did show increased fecundity relative to ancestral lines but only in the drug-treated environment, and though significant, the absolute difference in numbers of eggs was small. Chehresa et al (1997) performed an experiment focused on measuring changes in life history traits of replicate lines of the parasitic nematode *Heligmosomoides polygyrus bakeri* selected for rapid passage using a mouse host over eight generations. By generation eight, development of the parasite was faster and fecundity was higher early in life, suggesting rapid passage of the parasite was associated with evolved changes in life history. In my study, I did not measure development time but changes in worm size can be used to infer changes in development time, as selection for early maturity is associated with small size at maturity (Roff 1992; Stearns 1992). Also, higher peak fecundity in my study would be suggestive of earlier investment in reproduction. I found no evidence of smaller size in either sex for zero-dose lines and a reduction in fecundity was only observed in the drug-treated environment, indicating that my experiment did not select for rapid development and early reproduction in drug-free environments. The lack of response to selection of zero-dose lines relative to the ancestral line means that any differences in life history between the zero-dose lines and drug-selected or random mortality lines must be associated with treatment during selection, and not with rapid passage during experimental evolution.

5.5.2 *Effects of drug-selection on life history*

In drug-free environments, there were no differences in lifespan between drug-selected and zero dose lines but adults of drug-selected lines were larger than zero-dose lines, suggesting a greater investment in growth of the former. In addition, females from drug-selected lines showed greater reproductive output than zero dose lines. In drug-treated environments, longer lifespan and higher fecundity were apparent in drug-selected lines relative to zero dose lines, but there was no difference in size at maturity. Body size has been associated with drug tolerance; i.e. larger individuals require a higher dose of drug to achieve the same effect (Anderson and Weber 1975; Lavadinho 1975; Maggi et al. 2012), and evolved differences in size have been observed in susceptible populations of parasitic nematodes exposed to drug treatment (Leignel and Cabaret 2001). For example, Liegnel and Cabaret (2001) found that both susceptible and resistant genotypes of an isolate of *Teladorsagia circumcincta* exposed to Benzimidazole, increased in size over the course of a two-year experimental infection study. If larger size is advantageous in treated environments then drug-treatment could favour selection on investment in growth (Lynch, Grimm, and Read 2008). Lynch et al (2008) used mathematical models to show that later development and larger size at maturity could be favoured in drug-treated environments but was dependent on the difference in the rate of mortality experienced by juveniles and adults in treated and drug-free environments, on whether survival was size dependent in treated environments, and on how the drug was applied (continuously or in pulses). Their results showed that size-dependent mortality to the drug, in particular, favoured delayed maturation and a larger adult size. I found no differences in juvenile size in our study and therefore attribute the larger size of adults from drug-treated lines in the drug-free environment as evidence of greater investment in growth. I suggest that exposure of populations to a high rate of mortality in adults may favour late maturation or greater investment in growth during the juvenile stage to achieve a larger size, as has been observed in some studies of the effects of harvesting (Conover et al. 2005; Cameron et al. 2013). Similar observations have also been noted in experimental life-history evolution of wild guppies in streams driven by predation by large or small predators specialising on adult or juvenile guppies (Reznick, Bryga, and

Endler 1990; Reznick et al. 1996). Spending a longer period as a juvenile in these circumstances could be beneficial as it lowers the risk of mortality over the same time period, and could result in higher fecundity due to more time to invest in juvenile growth. Such a scenario might explain the observed increase in size at maturity of drug-selected lines in drug-free environments, if drug-induced mortality of adults was high relative to juveniles then selection would favour spending a greater period as a juvenile and investing in growth.

The lack of any difference in female lifespan between drug selected and zero-dose lines in the drug-free environment suggests that any adaptation to the treated environment incurred no cost in the untreated environment. A previous study using the same species found that where mortality was related to survival in heat stress, lifespan increased for populations selected in a high mortality heat-stressed environment (Chen and Maklakov 2012b). Replicate lines of *C. remanei* were selected for 12 generations in heat-shocked environments (high and low mortality), while other lines were selected in random culling environments (high and low mortality) before measuring lifespan of evolved populations in a benign environment. Mean lifespan increased in high mortality heat-shocked lines relative to both low mortality treatments and where mortality was imposed at random (Chen and Maklakov 2012b). In addition, the authors found that high-mortality random-culling of lines led to a shorter lifespan relative to low-mortality controls. Their results confirmed predictions that selection of populations in high mortality environments can result in reduced lifespan (Medawar 1952; Gadgil and Bossert 1970); however, if mortality was condition-dependent longer lifespan was favoured. In my study, selection in a drug-treated environment could also be view as selection based on condition in a similar way to heat stress, but drug-selected lines showed no increase in female lifespan. However, In Chen and Maklakov's study (2012) selection for heat tolerance (condition-dependent) was achieved by exposure to acute heat-shock as opposed to the drug-selection conducted in this experiment, which involved chronic, constant exposure of lines to drug treatment. Thus, the length of exposure to an environmental stress may be of importance in determining the evolutionary outcome of condition-dependent mortality.

The observed longer lifespan of females from drug-selected lines in the drug-treated compared to the drug-free environment suggests that females from drug-treated lines had adapted to the drug-treated environment. Increases in female lifespan in treated environments could have important implications for transmission dynamics of offspring in parasitic nematodes, if females are able to continue reproduction throughout their lifetimes (Skorping, Read, and Keymer 1991; Walker et al. 2009). Furthermore, if my drug-selected lines represent a larger drug-tolerant but still susceptible phenotype due to size-dependent selection, rather than a resistant phenotype, this could have significant consequences for managing the rate of resistance evolution. Maintaining susceptible individuals in a population is thought to play a critical role in slowing the rate of resistance evolution (Leathwick and Hosking 2009): if drug-tolerant and resistant phenotypes compete in terms of the number and duration of offspring they produce, this could potentially affect the rate of resistance evolution. Potentially, larger more fecund drug-tolerant susceptible phenotypes could have higher fitness than resistant phenotypes, especially if there is a cost to resistance, and act to slow the rate of resistance evolution. However, it may be difficult to differentiate between tolerant and resistant phenotypes.

My observation that fecundity (lifetime and peak) of females from drug-selected lines was greater in both drug-free and treated environments relative to control lines has significant implications for the treatment of nematode infections. Several previous studies on parasitic nematodes have compared reproductive fitness of resistant and susceptible strains, but the results have been contradictory. For instance, a study of Benzimidazole-resistant strains of *Haemonchus contortus* found that their fecundity was higher than that of susceptible strains (Kelly et al. 1978) but a similar study using different strains of the same species obtained the opposite result (Maingi, Scott, and Prichard 1990). However, both of these studies used resistant and susceptible isolates from different locations and therefore the contrasting results could be due to the different environmental background of isolates. When resistant and susceptible isolates from the same strain have been compared, no difference was observed in fecundity (Elard, Sauve, and Humbert 1998). Humbert et al (1998) compared female nematodes collected from slaughtered sheep infected with a strain containing resistant and susceptible worms. The number of

offspring produced by these females and the development time of the offspring did not differ with respect to genotype. Collectively, these studies show that differences in life history traits have been observed following field and artificial selection for anthelmintic resistance but in other cases no response in life history occurred. This could be due to life histories of worms responding to differently to different classes of drug. Alternatively, life histories of different species of nematode could respond differently to individual drugs. However, responses of life histories to drug selection have shown to be inconsistent even in situations where a single species has been selected for resistance to one drug (Bartley et al. 2015). Bartley et al (2015) selected three different isolates of *T. circumcincta in vivo* for resistance to Monepantel over 9 to 13 generations and measured responses in life history relative to ancestral isolates. Shorter development time, larger adult size and higher fecundity was observed in only one of the selected isolates. Interestingly, this isolate was also resistant to four other classes of anthelmintic prior to selection. Thus, the previous history of populations, as well as species and drug type could play a role in how life histories of parasites respond to drug selection. The larger size of drug-selected lines in my study is most likely responsible for the observed increase in fecundity. Body size has been found to be a good predictor of fecundity across a range of nematode species (Morand 1996); therefore, evolved increases in size are likely to be associated with greater fecundity. Worryingly, if drug selection favours increased size then resistant or tolerant parasites may be more fecund than drug naive susceptible parasites.

5.5.3 *Effects of high mortality and density-dependant selection on life history traits*

The random-mortality lines showed a similar increase in size as drug-selected lines in the drug-free environment relative to zero-dose lines but not in the drug-treated environment. In the drug-free environment, lifespan of random-mortality lines remained similar to drug-treated and zero-dose lines. Lifetime fecundity of random-mortality lines was intermediate to drug-treated and zero-dose lines, but peak fecundity of random-mortality lines was similar to zero-dose

lines. In contrast, in the drug-treated environments all measured life history traits of random-mortality lines were similar to zero-dose lines. In my previous study (Chapter 4; Reynolds et al. 2016), random mortality lines showed higher survival relative to the zero dose lines when challenged with Ivermectin. The drug resistance assay used to assess susceptibility in that study measured juvenile survival and one day of adult survival, whereas, the drug-treated environment in the lifespan assay exposed females to the drug throughout their lives. A response in survival of juveniles and not adults, suggests that the response is stage specific, as only juveniles from random mortality lines show a decrease in susceptibility. A stage specific reduction in drug sensitivity has been previously observed in juveniles of *Haemoncus contortus* (Sarai et al. 2015). In Sarai et al 2015, juvenile stages of the parasite were treated with Levamisole for nine generations, but adults remained unexposed during selection; drug resistance assays on both juveniles an adults showed only juveniles had evolved resistance. However, in my study, the drug-selected lines showed an increase in lifespan when exposed to a treated environment, suggesting adaptation of drug-treated lines beyond that observed in random–mortality lines. During the selection experiment worms from the drug-treated regime were exposed to Ivermectin throughout their life, and both juvenile survival and adult lifespan in the treated environment showed an increase after drug selection. Thus, drug-selected lines showed adaptation beyond that observed in random–mortality lines in treated environments, and selection of random mortality lines in a low-density drug-free environment, only conferred an advantage in survival for the juvenile stage when challenged with a novel drug treated environment.

The similarities between drug-treated and random mortality lines in terms of size and fecundity in a drug-free environment could be due to similarities in mortality and density between the two treatments during selection. Roper et al (1996) found that populations of *D. melanogaster* selected at low densities evolved larger size when maintained in a low-density environment, and larger size was associated with greater fecundity. Low-density environments can select for larger size if selection favours delayed maturation because of benefits in fecundity to larger adults (Bassar et al. 2010). However, where low density occurs as a result of high extrinsic mortality, a different response of life history might be expected. For example, early maturation and reduced fecundity will be

selected for where the timing of extrinsic mortality on adults is imposed relatively early in adult life (Roff 1992; Stearns 1992; Stearns, Ackermann, and Doebeli 1998). In contrast, if adult mortality is high immediately after maturation then selection will favour remaining in the juvenile stage longer and achieving a larger adult size and greater fecundity (Stearns et al. 2000; Cameron et al. 2013). During the selection experiment, adult worms were culled early in their lives to simulate the high mortality occurring in drug-treated regimes. Thus, high mortality in both drug-treated and random mortality lines resulted in a greater investment in growth and higher fecundity.

5.5.4 *Conclusions*

This study is the first to my knowledge that explores how a range of life history traits of nematodes changes in response to drug-treatment. I have shown that size at maturity, fecundity and lifespan are all affected in lines previously selected in a drug-treated environment, but this depends on which environment populations are exposed to. If these results generalise to parasitic nematodes, this may have important consequences for their pathogenicity and transmission, especially if individuals from drug-treated populations become larger, and thus could cause more host damage as well as producing greater numbers of offspring, increasing the chances of transmission (Skorping 2007; Skorping and Read 1998). In addition, the immune response of hosts is another complicating factor that makes the study of life-history responses to drug selection complicated, as immune responses of the host may vary with differences in life-history of parasites (Leignel and Cabaret 2001). Experimental studies using free-living nematodes provide an opportunity to explore how life-history traits respond to drug-treatment in isolation of host immune responses and allow control of density-dependent effects. Developing such studies to encompass effects including host immune responses would be a logical next step and bring us closer to making accurate predictions of how parasite life histories will respond to drugs and vaccines.

Chapter 6: General discussion

6.1 *Summary*

The overall aim of this thesis was to assess how life-history traits change in response to drug selection, and whether changes in population density and the risk of mortality resulting from drug application are involved in the observed evolved responses in these traits. In Chapter 2, I established the relationship between *C. remanei* survival and Ivermectin dose so that the strength of selection imposed in the experimental evolution study in Chapter 4 could be chosen and applied at a consistent dose. Additionally, the survival data from the dose response assay were used to show that current methods of fitting dose response data in parasitology studies can be improved upon and provide a test of a cost of resistance by not removing background mortality from survival data prior to analysis. Using the best-fitting dose response curve was shown to improve the accuracy of estimates of summary statistics used to measure resistance, such as LD_{50} and LD_{99}, and incorporating a cost of resistance into the analysis ensured that the maximum amount of useful information on resistance could be extracted from dose response data.

In Chapter 3, I used simulation-based power analyses to explore how design choices used in selection experiments, such as the number of replicate selection lines, the number of samples assayed within each replicate line, and variation due to repeated measures when assessing traits at the end of selection, can affect the power of a study. My study demonstrated that selection experiment designs incorporating multiple sources of variation can be assessed using simulation-based methods comparable in complexity to those used to analyse experimental data. This forces researchers to think about the analytical tools they will use prior to conducting a study. My results showed that the number of replicated experimental lines, and within-line sampling effort that have typically been used in published studies to measure responses in survival in culling selection experiments, were prone to low power to detect a difference between treatments, when all potential sources of variation were considered.

Chapter 4 explored the relationship between dose and the rate of resistance evolution in replicated lines of *Caenorhabditis remanei*, asking whether survival of lines selected in drug-treated environments increased, and if this varied with dose. In addition, I maintained lines where mortality was imposed randomly to control for differences in density between drug-treatments to distinguish between the evolutionary consequences of drug-treatment vs ecological processes due to differences in density and mortality between drug-treated and control lines. After 10 generations, both drug-selected and random-mortality lines showed an increase in survival when challenged with Ivermectin, and the magnitude of this increase varied with the intensity of selection and life-history stage. My results suggest that interactions between density-dependent processes, and life-history traits may mediate evolved changes in susceptibility to control measures.

Finally, in Chapter 5, I investigated the effects of selection in the high mortality environments (drug-treated and random mortality; Chapter 4) on life history traits, by measuring larval and adult size, female lifespan and fecundity in both drug-free and treated environments, compared to control populations. In addition, life history traits of controls were compared to the ancestral population to distinguish effects on life history due to rapid passage through 10 generations of selection. Adult size was larger for both drug-selected and random-mortality lines compared to control lines, but only when assayed in drug-free environments. In contrast, lifespan was longer for drug-selected lines in drug-treated environments and was not affected by the random-mortality treatment, when compared to controls. Drug-selected lines had higher fecundity in both drug-free and treated environments, whereas fecundity of random mortality lines was intermediate to drug-selected and control lines in the drug-free environment but similar to controls in the treated environment. These results suggest that life histories of nematodes respond to selection in drug-treated environments, which acts via ecological processes due to the timing of mortality and density-dependence. Failure to take responses of life history traits into consideration when applying control measures could lead to unfavourable outcomes, such as larger, more fecund parasites and over-estimation of evolution of genetically controlled resistance.

6.2 *Dose response modelling and costs of resistance*

Drug efficacy can be preserved with the help of effective monitoring practices, which measure changes in resistance of populations treated with control measures (Coles et al. 2006; Bagi et al. 2015). An approach common to assessment of resistance in many parasite and pest species is to use a dose-response assay to characterise the relationship between survival and dose of the control agent. My results suggest that a risk for such studies is that they will fail to fit the most appropriate dose curve simply because only a single model is assessed; fitting the wrong curve could result in incorrect estimates of summary statistics used to quantify resistance. My study focused on the relationship between dose and survival in only one species and using a single drug treatment. Comparing a range of dose-response curves fit to data collected in dose-response field studies of parasites or disease vectors would give a clearer picture of what effect model selection has on the accuracy of resistance estimates.

The common practice of removing background mortality from datasets prior to analysis means that an opportunity to measure a cost of resistance is lost. I have shown that by incorporating background survival into the analysis of dose response data, a test of a difference in the upper asymptote of the survival curve could be used to detect a relatively small cost of resistance. Fitting a range of dose response curves, which allow the most appropriate model to be selected and including a test of the cost of resistance maximises the amount of useful data extracted from a study. This information could play an important role in decisions about management strategies employed to control target species, such as whether or not to replace an existing treatment with another where the costs of resistance are greater and could slow the rate of resistance evolution. In this case, using a treatment with a higher cost of resistance would slow the rate of resistance evolution, because susceptible individuals with higher survival are likely to produce more offspring, resulting in a smaller proportion of resistant individuals in the next generation. My study used simulated data to test for a cost of resistance in terms of a difference in the upper asymptote of the

dose-response curve. Conducting the same analysis on field data across a number of different species and control agents would provide a more effective test of how model estimates are affected by choice of dose-response curve, and whether incorporating a test of a cost of resistance into analysis would be useful.

6.3 *Powering future selection experiments*

Research areas such as animal behaviour (Taborsky 2010; Smith, Hardy, and Gammell 2011) and biomedical science (Ioannidis 2005; Button et al. 2013) have expressed concern that the statistical power of reported studies is often below the recommended minimum of 80%. This could be attributed to a lack of awareness and/or understanding of the concept of power analysis. Alternatively, researchers may lack the technical know-how needed to conduct power analysis for studies that have complex designs incorporating multiple sources of variation. Within the field of experimental evolution, specific guidance on using simulation-based power studies to optimise the design of selection experiments has recently become available, and has been used to optimise designs of evolve re-sequence studies (Baldwin-Brown, Long, and Thornton 2014; Schlötterer et al. 2015). However, little attention has been paid to optimising the design of selection experiments measuring changes in phenotypes, in terms of publications about power analysis, but it is difficult to demonstrate evidence of a problem with underpowered studies without conducting a meta-analysis. Funnel plots can be used to detect bias in meta-analyses, and could give an indication whether there is any evidence of a publication bias against non-significant results (Egger et al. 1997; Sterne and Egger 2001).

My results, concerning the number of replicate selection lines and the number of samples taken from within each replicate line, conform to those of power simulations of evolve re-sequence studies: increasing the number of selection lines and sampling effort from those lines resulted in higher power (Baldwin-Brown, Long, and Thornton 2014). Furthermore, my results suggest that besides tradeoffs between these types of logistical constraints, additional sources of

variation such as those that occur during measurement of phenotypes have a negative effect on study power. I have demonstrated with a practical example how to use simulation-based power analysis methods to optimise the study design of selection experiments, based on simulating data for the actual response and explanatory variables that would be used in the analysis of the real data. These simulations can be extended to accommodate most complex designs, and to incorporate a range of response variable distributions (Bolker 2007). My study focused on a response variable with a binomial error distribution, conducing similar power studies for other types of response variable would given an indication of what kind of experimental design is appropriate for traits with different error distributions. In addition, my study did not incorporate many other factors, which are important in experimental evolution studies e.g. initial population size, standing genetic variation, strength of selection and the number of generations under selection. Simulation allows all these factors to be included in power studies in addition to accounting for multiple sources of variation. Collectively, my results indicate the importance of clearly defining a biological question and designating sources of variation before performing simulations aimed at detecting evidence of a biologically meaningful effect for a given study design scenario.

6.4 *Drug dosage and the rate of resistance evolution*

Previous research on under-dosing has suggested that low doses may promote the evolution of resistance, especially where the basis of resistance is polygenic (Manalil et al. 2011; Shi et al. 2013), and that varying the level of under dosing could affect the rate at which resistance evolves (Busi and Powles 2009). The mathematical modeling study of Barnes et al (1995) used to explore the effects of under dosing suggested that the outcome in terms of the rate of resistance evolution would depend on the genetic mechanism underlying resistance. My study supports the view that lower doses slow the rate of resistance evolution because lines selected at the lower drug dose showed less response to selection than high dose lines. This conforms to the view that under-dosing may reduce the evolution of resistance by allowing more susceptible individuals to survive

(Barnes, Dobson, and Barger 1995). My results also add to the current body of work on dosage and resistance evolution (Barnes, Dobson, and Barger 1995; Busi and Powles 2009; Manalil et al. 2011), with the finding that life history stage interacts with drug dose. Both juveniles and adults from high-dose lines evolved resistance to Ivermectin, but only juveniles from low-dose lines showed increased survival in the treated environment. My experiment used only two doses of Ivermectin, but it would be interesting to select populations over a range of doses and explore the interaction between life history stage, dose and susceptibility.

6.5 *The evolution of tolerance*

Resistance to a control agent is defined as a genetically based decrease in susceptibility occurring as a direct result of exposure to a control agent (Tabashnik et al. 2014). This definition of resistance emphasizes heritable change in susceptibility due to long-term exposure. Thus, the spread of resistance through a population occurs because of an increase in the frequency of pre-existing alleles conferring reduced susceptibility, novel mutations or migration of resistance alleles between populations, when populations are exposed to a control agent for an extended period of time (Gilleard and Beech 2007). By this definition, resistance evolves because of an increase in the frequency of resistance alleles due to drug application. In Chapter 3, I showed that reduced susceptibility to drug treatment in random mortality lines evolved in populations exposed to an environment that mimicked the mortality and population density observed in the drug-treated environment. I defined this reduced susceptibility as tolerance rather than resistance, because reduced susceptibility evolved in drug-naive populations as a result of selection on traits conferring a benefit in a random mortality environment, which also conferred an advantage in terms of survival in the drug-treated environment. Although tolerance has many definitions, one version is natural variation in susceptibility already pre-existing within or between populations rather than a result of selection pressure imposed by control measures (Scott 1995). The evolution of tolerance is therefore distinct from that of resistance in that tolerance evolves

due to pleiotropic effects and selection on some other unknown trait that results in pre-adaptation in the form of reduced susceptibility or reduced efficacy of the drug (Puniamoorthy et al. 2014).

How the factors that affect resistance and tolerance interact could have significant impacts on the evolution of susceptibility. For example, environments which are inhospitable to free-living larval stages of parasitic nematodes reduce density, and promote the evolution of resistance (Besier and Love 2003; Lawrence et al. 2007; Leathwick and Besier 2014); although, what proportion of resistance is due to drug application or tolerance in this case is difficult to establish. A further example of the potential of tolerance evolution to influence susceptibility can be seen in increasing cuticle thickness of bed bugs (Lilly et al. 2016). Resistance to pyrethroid insecticides was found to be associated with thicker cuticles of bed bugs collected in Sydney, Australia. However, resistance to pyrethroids could have occurred as a result of tolerance, as thicker cuticles could be selected for in drier environments (Benoit et al. 2007), and increased resistance to pyrethroid insecticides by bed bugs coincides with increasing use of air-conditioning since the turn of the century. In addition, tolerance evolution may not be confined to metazoans; drug naive soil bacteria have been shown to exhibit reduced susceptibility to commercial antibiotics, due to selection on efflux pumps, which overcome the effects of natural toxins, but are also effective against synthetic drugs (Walsh and Duffy 2013).

It is difficult to separate resistance from tolerance unless this is explicitly incorporated into experimental design but this also requires knowledge about which traits confer differences in tolerance to a particular control agent. To understand how resistance and tolerance interact, future research should focus on identifying which traits are associated with tolerance and the degree of influence they may have on susceptibility. The *Caenorhabditis* model system allows a range of traits to be assessed over the course of selection experiments (Gray and Cutter 2014), and would provide an invaluable model to explore factors which may affect the evolution of resistance and tolerance. For example, establishing continuous populations with over lapping generations in flasks would allow large populations to be kept, which approximate those of parasitic nematodes in the field. Manipulating population size by drug-treatment and

artificially by random culling in addition to maintaining control lines would allow the population dynamics and age structure of drug-treated lines to be explored in detail. Furthermore, measuring life-history traits of continuous populations at the same intervals as population counts would allow any changes in population density that affect life-history to be identified. This type of selection experiment could potentially provide a clearer picture of the eco-evolutionary responses to drug-selection.

6.6 *Susceptibility and life history evolution*

Differences in population density between drug-treated and drug-free populations of parasites and pests could result in differential selection due to density-dependent processes such as competition (Chehresa, Beech, and Scott 1997; Gilleard and Beech 2007), and these processes can also result in evolutionary changes in growth, survival and reproduction (Reznick et al. 2012). In addition, high extrinsic mortality imposed by application of a control agent could select for either early maturation, small size and low fecundity or delayed maturity, larger size and greater fecundity, depending on the timing of mortality (Stearns et al. 2000). High extrinsic mortality imposed early in adulthood favours selection for early maturation, but if mortality is imposed at the time of maturation and for the whole of the adult lifespan, then selection favours remaining in a juvenile stage for longer. In Chapter 4, I found that lifespan and fecundity of drug-selected lines increased, relative to controls, when evolved lines were challenged with drug treatment, suggesting that the life history of drug-treated lines adapted to the treated environment. In addition, worms from drug-treated lines were larger and more fecund than control lines when exposed to the drug-free environment, suggesting that drug treatment of parasites could result in larger, more fecund parasites, as suggested by Skorping and Read (1998) and Lynch, Grimm, and Read (2008). If parasite life histories adapt to treated environments and drug-selected populations have greater fitness in drug-free environments then this could have serious implications for parasite control and transmission; drug-adapted phenotypes would produce more offspring than susceptible phenotypes, resulting in increased transmission of

drug-adapted populations. However, imposing the same density, and rate and severity of mortality on random mortality lines described in Chapter 4 had no effect on the life history of random mortality lines when exposed to the drug-treated environment, which contradicted the findings of Chapter 3, where selection in the random mortality environment caused higher survival in the treated environment. A response in life history only in juveniles could explain this discrepancy (Sarai et al. 2015); juvenile survival of random mortality lines in the resistance assays responded to selection in low density environments, whilst adult lifespan in the life history assays showed no response to this novel environment. However, in the drug-free environment, similarities between the life history of both drug-treated and random mortality lines in terms of larger size and greater fecundity could be due to similarities in mortality and density between the two treatments during selection. These findings are in agreement with the view that high extrinsic mortality early after maturity or density dependant processes could favour remaining in the juvenile stage longer and achieving a larger adult size and greater fecundity (Stearns et al. 2000; Reznick et al. 2012; Cameron et al. 2013). Measuring development time in addition to lifespan of selected lines in treated environments would give a clearer picture whether juvenile or adult worms suffer greater extrinsic mortality. My thesis suggests that there may be a complex relationship between extrinsic mortality, density-dependent regulatory processes and life history of populations challenged with control measures. I recommend that future work on resistance should incorporate adequate controls for differences in parasite/pest density between treatments when assessing drug resistance evolution.

6.7 *Conclusions*

Understanding life histories and the selection pressures that shape them is a fundamental goal of evolutionary ecology. Ecological change such as changes in density or extrinsic mortality can lead to evolutionary changes in life history traits (Reznick 1982; Roff 1992; Stearns 1992), which in turn can affect density, resulting in a feedback loop (Kokko and López-Sepulcre 2007). These types of eco-evolutionary interactions can result in rapid evolutionary responses in a

variety of traits induced by changes in the environment, mortality rates, and competitive interactions (Reznick and Ghalambor 2001). Rapid adaptive change is often associated with populations subject to human activities such as predation (Darimont et al. 2009), so it is logical to assume that our efforts to control parasitic and pest species will result in similarly rapid adaptive responses. Previous research on the effects that control measures have on life history traits has tended to focus on evidence of costs of resistance (reviewd by, Kliot and Ghanim 2012), but it has been proposed that control measures could have a positive effect on life history (Lynch, Grimm, and Read 2008). My thesis shows that ecological changes occurring as a result of selection in treated environments could result in increased fitness of parasites and pests via interactions with life history. This will have significant implications for control methods if selection in treated environments drives the evolution of larger more fecund parasites and pests. My results suggest that life histories of parasites and pests should be monitored as part of mitigation strategies to control populations in order to prevent unintentional selection of undesirable traits in the target populations. Future work should explore the limits of how much evolutionary change is possible in the life history of populations treated with control measures. For example, parasitic nematode species vary massively in size, development time, longevity and fecundity (Skorping, Read, and Keymer 1991); thus, intense selection in treated environments could drive significant change in life history traits. To explore the limits of evolved responses of life-history to drug selection using experimental evolution, the strength of selection could be gradually increased every generation, to track the dose of drug required to cause a target mortality level. The rate of change in life history traits could then be measured from frozen samples taken at regular intervals during selection.

My use of a novel treatment during the selection experiment, which controls for both the greater mortality and differences in density of drug-treated populations, suggests that a population's tolerance to control measures can evolve independently (i.e. without previous exposure), as a result of pleiotropic effects of selection on traits that confer reduced susceptibility. My results provide experimental evidence of the evolution of tolerance in action. This raises the question of whether previous studies of resistance not incorporating such controls should be re-evaluated, and how influential tolerance evolution is

in efforts to control parasite and pest species. My thesis suggests that eco-evolutionary processes involved in life history adaptation to control measures could be responsible for the evolution of tolerance. Therefore, eco-evolutionary responses of target populations to changes in the environment, mortality rates and competitive interactions should be taken into consideration when implementing control strategies. Research into invertebrate susceptibility to insecticides might provide a good opportunity to establish how important tolerance evolution is in the field, because crop pests have been intensively studied and life history traits, as well as evolutionary processes are much easier to monitor than in parasitic species.

Finally, my thesis shows that both resistance and tolerance can affect susceptibility to xenobiotics, and that the evolution of both should be viewed as distinct processes. Resistance is driven directly by drug selection, tolerance on the other hand is driven by selection on some other unknown trait that results in pre-adaptation, and both result in reduced susceptibility. Thus, possible environmental drivers of tolerance should be considered in resistance management strategies. In addition, both these processes have the potential to interact with life history, which could affect the reproductive schedule of the target populations and their chance transmission.

Appendix A

Assement of indirect mortality at low doses of Iveremctin

To explore whether low doses affects model fitting of the survival-dose relationship, Weibull-1 curves were fitted to the whole LDT dataset including 15 doses (0, 0.1, 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 7, 8, 9 and 10 ng/ml Ivermectin), and a reduced dataset in cases where higher mortality due to indirect mortality was suspected at 0.5 and 1 ng/ml Ivermectin. Weibull-1 models were fitted to the survival data because these are the most accommodating in cases where asymmetry in the dose-response curve occurs, causing survival to decrease more gradually over the initial upper slope of the dose response curve, compared to log-logistic models. Because the datasets for each curve were of different sizes, models could not be compared formally using likelihood ratio tests. Instead, estimates of model parameters (LD_{50}, LD_{99}, and survival at the upper asymptote) were used to assess the goodness-of-fit of the two dose response models.

Does indirect mortality at low doses affect fitting a dose response curve?

The three-parameter Weibull-1 model using all the data collected in the dose response assay estimated the LD_{50} and LD_{99} of Ivermectin at 1.83 (95% CI: 1.78, 1.89), and 3.57 (95% CI: 3.44, 3.71), respectively (Fig A1); predicted mean survival at the upper asymptote of the dose response curve was 72% (95% CI: 71, 74%). The three-parameter Weibull-2 model with data excluded at 0.5 and 1 ng/ml Ivermectin estimated the LD_{50} and LD_{99} of Ivermectin at 1.85 (CI: 95%: 1.80, 1.90; Fig 2.3, Chapter 2), and 3.45 (3.34, 3.56), respectively; predicted mean survival at the upper asymptote was 76% (95% CI: 74, 78%). Thus, the LD_{50} and LD_{99} estimates of the two curves were similar, but at higher survival (>50%) the Weibull-1 model using all the data resulted in an overestimate of mortality at very low doses, and underestimate of survival at the upper asymptote, compared to the reduced dataset excluding the values at 0.5 and 1 ng/ml Ivermectin (Fig A1 and Fig 2.3, Chapter 2).

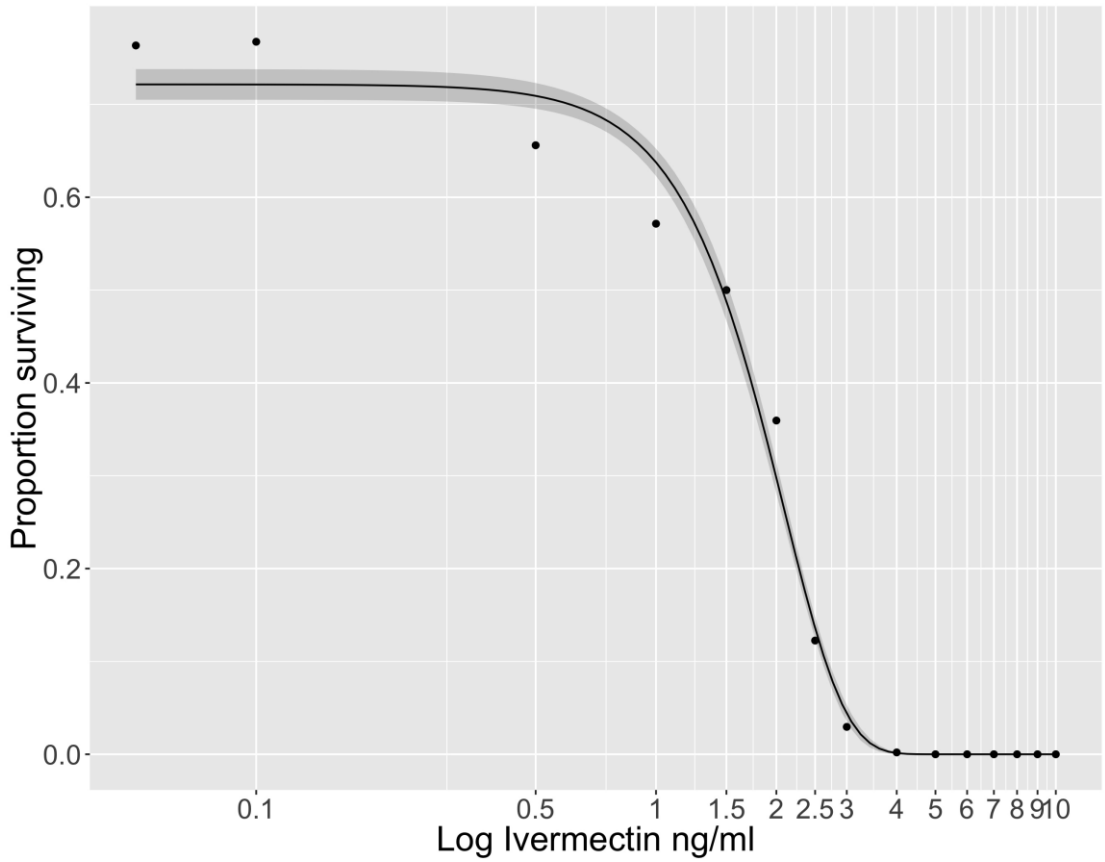


Fig A1 Observed data and fitted Wiebull-1 model of the relationship between survival of *C. remanei* and concentration of Ivermectin using all the data collected in the dose response assay. Black circles show observed mean survival at each concentration of Ivermectin used in the larval development test. Dark grey bands show 95% confidence intervals for mean survival based on the model.

My data suggest that indirect mortality not due to the drug occurred at low doses of Ivermectin and had a significant effect on predictions of survival, both at low doses, where the drug causes little mortality, and in the estimation of background mortality. It is difficult to assess whether indirect mortality at low doses of Ivermectin is common in other studies using LDTs because often only estimates of the LD50 and LD99 are published and the original data are not included. This additional source of mortality could be due to repellency at sub-lethal doses as a result of worms leaving the surface of agar plates in an attempt to evade the effects of the drug. Hyperactivity of worms exposed to low doses of Ivermectin has been shown to occur in *C. elegans* (Ardelli et al. 2009), and the mode of action of the drug is thought to be similar in parasitic species, suggesting that repellency could occur in parasitic species (Cook et al. 2006).

Assays which measure motility over a range of doses could clarify the effect that low doses have on worm movement, with the expectation that the dose-response relationship between motility and concentration would show a hormetic trend if repellency had an effect.

Appendix B

Statistical methods

All statistical analyses were performed using R v 3.1.2 (R Core Team 2014) and we defined a significance threshold of P = 0.05 for all tests. Our first question was concerned with drug dose efficacy and asked 'What is the relationship between *C. remanei* survival and Ivermectin dose over a range of concentrations?' The doses required to cause 40% and 80% mortality were estimated, with 95% CI's, using the drc package (Ritz and Streibig 2007). In order to calculate estimates of these two doses we constructed a dose response curve of the relationship between worm survival and concentration of Ivermectin. Dose response curves are generally sigmoidal and can be defined by four parameters: the first two parameters are the upper and lower asymptote, where changes in concentration have no effect on survival (Ritz 2010). The third parameter is the slope of the curve, which defines the potency of the drug where efficacy increases from zero to its maximum, the more potent a drug is the steeper the curve will be. The fourth parameter is the inflection point, where the curve changes from concave to convex and is located at or near the LD50 (dose causing 50% mortality) for the drug. We fitted a range of dose-response models (log-logistic, Weibull-1 and Weibull-2) with the lower asymptote fixed at 0% survival and used maximum likelihood to select the most appropriate model of survival data. Ivermectin concentration and batch were fitted as fixed effects in our full model. To assess whether the relationship between survivorship and Ivermectin concentration remained the same between batches performed at different times (i.e. repeatability), batch was removed from the model and tested against the full model. Estimates of the required doses, with 95% CIs, were then derived from model predictions.

Our three remaining questions involved potential changes in survival of evolved lines used in the selection experiment, which could result in differential survival between treatments. We addressed these questions with a common general

approach described below. We conducted the analyses on discrete data sets from the resistance bioassays, which specifically addressed our research questions. In order to simplify our analyses, survival of high mortality (HD and HR) and low mortality treatments (LD and LR) were contrasted with control Z lines in separate tests, unless explicitly stated otherwise. The analysis compared survival of lines from the original selection experiment treatment groups (drug-treated, random mortality and zero dose) using generalised linear mixed models with the glmer function in the lme4 package and assuming a binomial error distribution with a logit link function (Bates et al 2014). Treatment and generation, as well as the interaction between them, were fitted as fixed effects. The evolutionary replicate (line) was fitted as a random effect. An observation-level random effect was fitted to account for any overdispersion between replicate lines in the selection experiment and repeated sampling of populations in the drug resistance bioassay (Browne et al. 2005). We refer to this model for the fixed effects as the full model. Treatment effects in the selection experiment were tested using likelihood ratio tests. The null hypothesis of no difference in survival between the three treatments (H_0: Drug treatment = Random mortality = Zero dose) was tested by comparing the full model with a null model with no fixed effect of treatment or treatment x generation interaction. Generation was kept in the null model to account for any drift in survivorship. This first step of the analysis tested for significance of fixed effects and interactions between them. A further three post-hoc measures were then used to assess the effects of individual treatments; differences in survival in the drug treatment relative to the zero dose treatment were tested by comparing the full model to a null model where survival in drug and zero dose treatments were constrained to be equal (H_0: Drug = Zero). Differential survival in response to the random mortality treatment (H_0: Random = Zero) and density-dependence in response to selection (H_0: Drug treatment = Random treatment) were tested in the same way. This general approach was used throughout the analyses to answer our research questions by establishing any evidence of differential survival between treatments challenged with different drug doses and at different life-history stages.

Firstly we asked: ‘Is there an increase in survivorship across generations of populations selected in drug-treated environments, and does this vary with

dosage?' The formal assessment of whether heritable increases in survivorship occurred in drug-selected lines was tested by challenging drug-selected and zero dose lines to the same high and low doses of Ivermectin used during selection. Survival data of HD lines exposed to a high dose of Ivermectin was assessed by the null hypothesis of no difference in survival between the three treatments (H_0: HD = HR = Z) using the general approach discussed. After establishing a significant effect of treatment on survival a post-hoc test was performed with the null hypothesis of no difference in survivorship between HD and Z lines (H_0: HD = Z). Any evolved increase in the survivorship of LD lines relative to Z lines was assessed under the null hypothesis (H_0: LD = LR = Z) and if treatment was significant, a post-hoc test was applied to assess differential survival between drug-treated and control lines (H_0: LD = Z).

Secondly we asked 'Does density-dependent selection affect the apparent evolution of resistance in selected lines?' We challenged random mortality (HR and LR) treated lines to both high and low doses of Ivermectin and again used our general approach to assess differential survival between selection experiment treatments. Differences in survivorship between high mortality treatments where the null model (H_0: HD = HR = Z) was rejected were tested with two post-hoc tests. The first tested for a difference in survival between HR and Z lines, and the second, differential survival between HD and Z lines. Assessment of low mortality lines was conducted using exactly the same approach but with the null model (H_0: LD = LR = Z).

Thirdly we asked 'Is there a cost of adaptation to drug-treated environments in terms of survival in drug-free environments?' We answered this question by exposing evolved lines to a drug-free environment and used our general approach to assess differences in survival between treatments, with the assumption that if there were a cost to adaptation then drug-treated lines would show lower survival than control (Z) lines.

Finally, we asked 'Does survival of different life-history stages (juvenile and adult) respond to drug-selection in the same way? To answer this question we conducted the analyses used in questions two to four separately on survival data collected at 52 and 75 hours and informally compared differences of model

predictions from the two time points. In addition, we pooled our whole datasets from the resistance bioassays using survival data from all drug-treated environments at both 52 hours (juvenile survival) and 75 hours (adult survival) and asked whether there were any interactions between selection experiment treatment, bioassay dosage and life-history stage. This was done for generations 5 and 10 independently to account for any changes in survival over generations. If there was no evidence of a three-way interaction we explored two-way interactions. We assumed that any evidence of interactions of life-history stage with treatment, dosage or both suggests that survival responded differently in juveniles and adults. We used a full model with fixed effects of treatment, bioassay dosage and life-history stage, as well as all possible two-way interactions between them. Random effects were: selection line, bioassay replicate and an observation-level random intercept. The full model was compared with a null model without the interaction of interest using a likelihood ratio test to assess the significance of interactions.

Drift and loss of genetic diversity

The evolutionary potential of small populations may be affected by drift due to the loss of genetic variation (Allendorf 1986). During the course of the selection experiment, population sizes varied both between lines within a treatment, and between treatments, due to bottlenecks imposed by drug application or random mortality. These bottlenecks could have resulted in a reduction in genetic diversity. Although we have no data on levels of genetic diversity it is worth considering the risk and extent of the loss of genetic variation in the selected lines used in this study. Allendorf (1986) outlines three useful measures of genetic variation and its loss: 1) changes in heterozygosity; 2) loss of multiple equally frequent alleles and 3) loss of rare alleles. Using census data from the selection experiment each measure of genetic variation was estimated for the HR and zero dose treatments at each generation (Table B2). Predicted changes in heterozygosity were calculated using equation (1) where N is population size. The expected proportion of the original heterozygosity remaining after each generation is:

$$1 - \frac{1}{2N} \quad (1)$$

This expectation is only valid if there are no selective differences between alleles; all other genetic diversity calculations in this paper also make this assumption of allelic neutrality. Predicted changes in the loss of multiple equally frequent alleles were calculated using equation (2).

$$E(n') = n - \sum_{j=1}^{n} (1 - p_j)^{2N} \quad (2)$$

Where *n* equals the number of alleles in the population, and *p* the frequency of an allele. We calculated the predicted number of alleles present at each generation of the selection experiment assuming the initial number of alleles for a given locus was 10. This potentially overestimates genetic diversity within our study species but *C. remanei* have been shown to be a particularly genetically diverse species (Cutter, Baird, and Charlesworth 2006; A. Dey et al. 2013). The greater the numbers of alleles are at a given locus, the more likely it is an allele will be lost where alleles are considered to have equal frequency. Therefore, our high estimate of initial allele numbers may exaggerate the risk of allele loss; lower estimates of initial allele numbers would result in allele loss being less likely. Rare alleles ($P < 0.01$) are especially susceptible to loss during a bottleneck. The probability of losing a rare allele with frequency $p = 0.01$ is given by equation (3).

$$(1 - p_j)^{2N} \quad (3)$$

An assumption was made that rare alleles were present at a frequency of 0.01, as this should provide some measure of the potential for rare allele loss in both HR and Z lines during the course of the selection experiment.

In HR lines, predicted heterozygosity decreased by 10% and 15% at generations 5 and 10, respectively (Table B2) whereas estimated heterozygosity of Z lines decreased by 5% and 10% (Table B2). The estimated number of alleles present in HR lines fell from 10 to 9.35 and 9.24 at generations 5 and 10, respectively,

whereas in Z lines there was no decrease over 10 generations (Table B2). The high cumulative risk of loss of rare alleles by generation 5 (Table B2), suggests that any rare alleles would be unlikely to persist within both HR and Z lines. Heterozygosity and the number of alleles remaining after a bottleneck are expected to be important in terms of a population's ability to respond to selection. Populations subjected to bottlenecks such as the HD and HR treatments in this study could be subject to considerable drift as a result of bottlenecking events. The increase in survivorship of High-dose and random-mortality lines could be a result of loss of genetic variation due to drift, and would require that all populations drifted in the same direction. However, our theoretical predictions of the loss of genetic diversity in HR and Z lines suggest that both treatments went through similar losses of genetic diversity. Predicted heterozygosity and the total number of alleles did decrease more rapidly in HR lines relative to Z lines but the difference between the two treatments was small. In the case of rare alleles, it is likely that any rare allele would have been lost from populations in both HR and Z lines. Thus, it seems reasonable that any evolved increase in survivorship of random-mortality and potentially drug-treated lines, was due to ecological processes occurring as a consequence of density-dependent selection and not loss of genetic variation due to drift.

Table B1 Effect of treatment during selection (mortality treatment) on survivorship (Surv.diff) at 52 hours, in drug-treated environments (dose); assessed by null models where survival is constrained to be equal across treatments (see Appendix), using likelihood ratio tests of best the fitting model. d.f.: Degrees of freedom. Surv.diff: Absolute difference in mean survival between the highlighted null model treatments (first minus second).

Mortality treatment	Dose	Best fitting model	Null models	x2 (d.f.)	P-value	Surv.diff	
						Gen 5	Gen 10
High	High	G + T	1) HD = HR = Z	16.21 (4)	0.0003		
			2) HD = Z	15.73 (2)	< 0.0001	0.16	0.14
			3) HR = Z	7.05 (2)	0.0079	0.09	0.10
			4) HD = HR	4.18 (2)	0.041	0.07	0.04
	Low	G + T	1) HD = HR = Z	15.19 (4)	0.0005		
			2) HD = Z	15.18 (2)	< 0.0001	0.10	0.10
			3) HR = Z	2.75 (2)	0.098	0.04	0.04
			4) HD = HR	7.83 (2)	0.0051	0.06	0.06
	Zero	G	1) HD = HR = Z	3.91 (4)	0.14		
Low	High	G	1) LD = LR = Z	1.71 (4)	0.43		
	Low	G + T	1) LD = LR = Z	6.72 (2)	0.034		
			2) LD = Z	5.21 (2)	0.022	0.05	0.05
			3) LR = Z	4.43 (2)	0.035	0.04	0.04
			4) LD = LR	0.06 (2)	0.80	0.01	0.01
	Zero	G + T	1) LD = LR = Z	6.29 (4)	0.043		
			2) LD = Z	5.97 (2)	0.015		
			3) LR = Z	0.062 (2)	0.80	-0.02	-0.02
			4) LD = LR	3.90 (2)	0.14	0.00	0.00
			4) LD = LR	3.25 (2)	0.20	-0.02	-0.02

G: Generation; T: Treatment; GxT: Generation x Treatment interaction; HD: High dose treatment; HR: High random treatment; LD: Low dose treatment; LR: Low random treatment; Z: Zero dose treatment.

Table B2 Predicted theoretical loss of genetic diversity based on a simple population genetic model during the course of selection (generation) in HR (High random) and Z (Zero dose) lines. Predictions of heterozygosity, the number of equally frequent alleles and risk of loss of rare alleles were made using mean population sizes for each treatment at each generation, using methods described by Allendorf (1986). Bold values show predicted diversity at generations 5 and 10, which correspond to when resistance bioassays were performed.

Treatment	Generation	Population size	Heterozygosity*	Number of Alleles	Risk of loss of rare alleles (%)
HR	1	15	0.97	9.58	74
HR	2	18	0.94	9.4	33
HR	3	28	0.92	9.38	18
HR	4	33	0.91	9.37	13
HR	**5**	**33**	**0.9**	**9.35**	**13**
HR	6	29	0.89	9.31	17
HR	7	35	0.88	9.29	12
HR	8	38	0.87	9.28	10
HR	9	31	0.86	9.25	15
HR	**10**	**39**	**0.85**	**9.24**	**9**
Z	1	47	0.99	10	39
Z	2	48	0.98	10	38
Z	3	49	0.97	10	37
Z	4	47	0.96	10	39
Z	**5**	**46**	**0.95**	**10**	**40**
Z	6	47	0.94	10	39
Z	7	47	0.93	10	39
Z	8	47	0.92	10	39
Z	9	47	0.91	10	39
Z	**10**	**47**	**0.9**	**10**	**39**

Number of alleles: an assumption of 10 alleles for a given locus was made; loss of rare alleles: rare alleles were assumed to at an initial frequency of 0.01. * Heterozygosity is the proportion of original heterozygosity remaining in the populations in each generation.

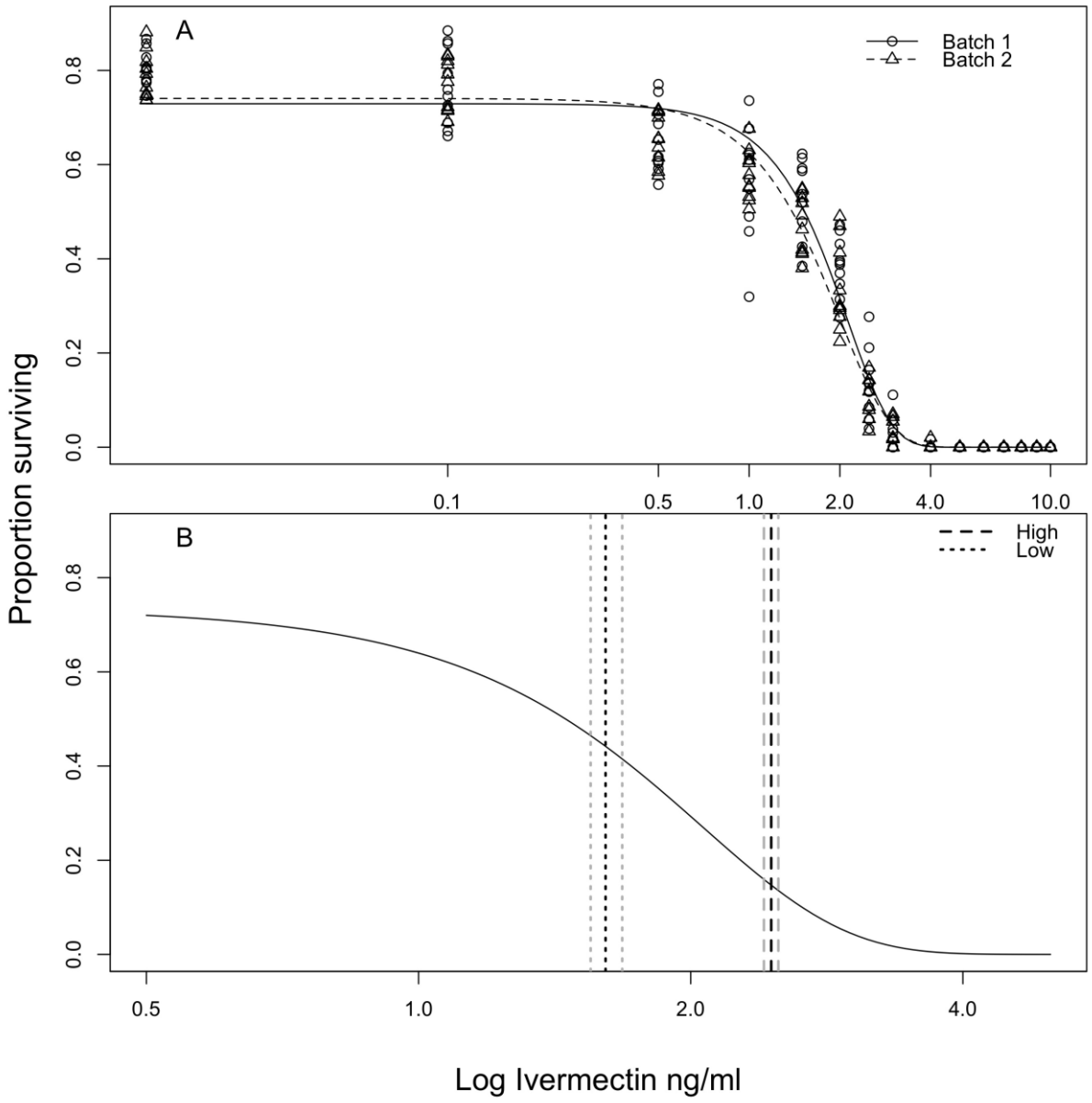


Fig B1 Relationship between survival and dose of Ivermectin for the SP8 strain of *C. remanei*. Panel A shows the relationship between survival and dose for two repeated assays (batches) accounting for differences in survival between dose-response assays performed on different dates. Points are individual replicates for each batch (triangles and circles). Panel B shows the doses used in the selection experiment (black broken lines) estimated to cause 40% and 80% mortality after taking into account any background mortality not due to the drug (LD40 and LD80). Grey broken lines show 95% confidence intervals around the estimated doses.

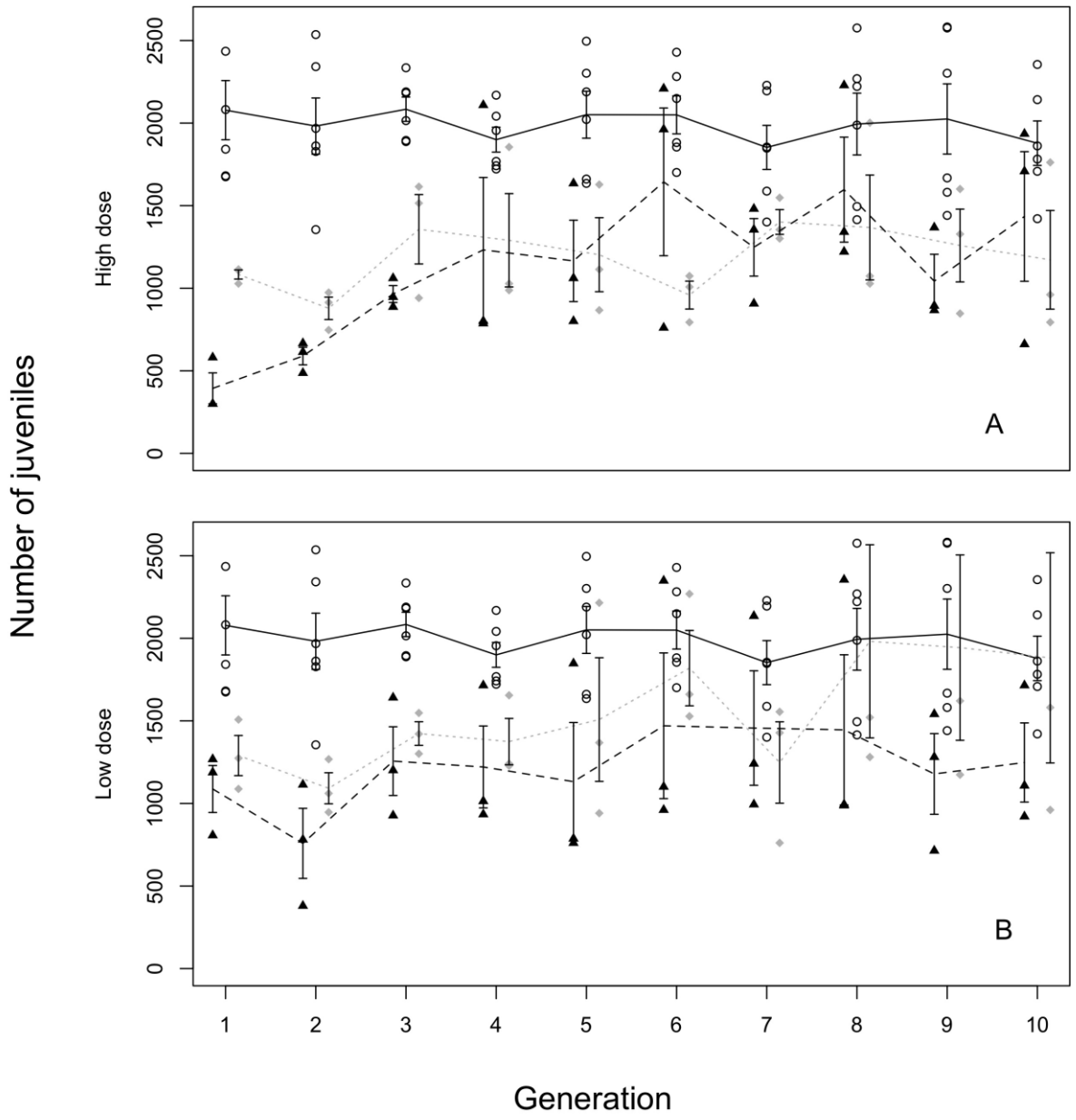


Fig B2 Larval density over the course of the original selection experiment. Lines represent mean number of juveniles for each treatment; points are the number of juveniles on day two of each generation for each replicate line within a treatment. Panel A shows density of high mortality lines: HD and HR with Z lines. Panel B shows density of low mortality lines: LD and LR with Z lines; circles, solid line = zero dose; triangles, dashed line = drug treatment; diamonds, dotted line = random mortality. Error bars; standard error for the mean number of juveniles.

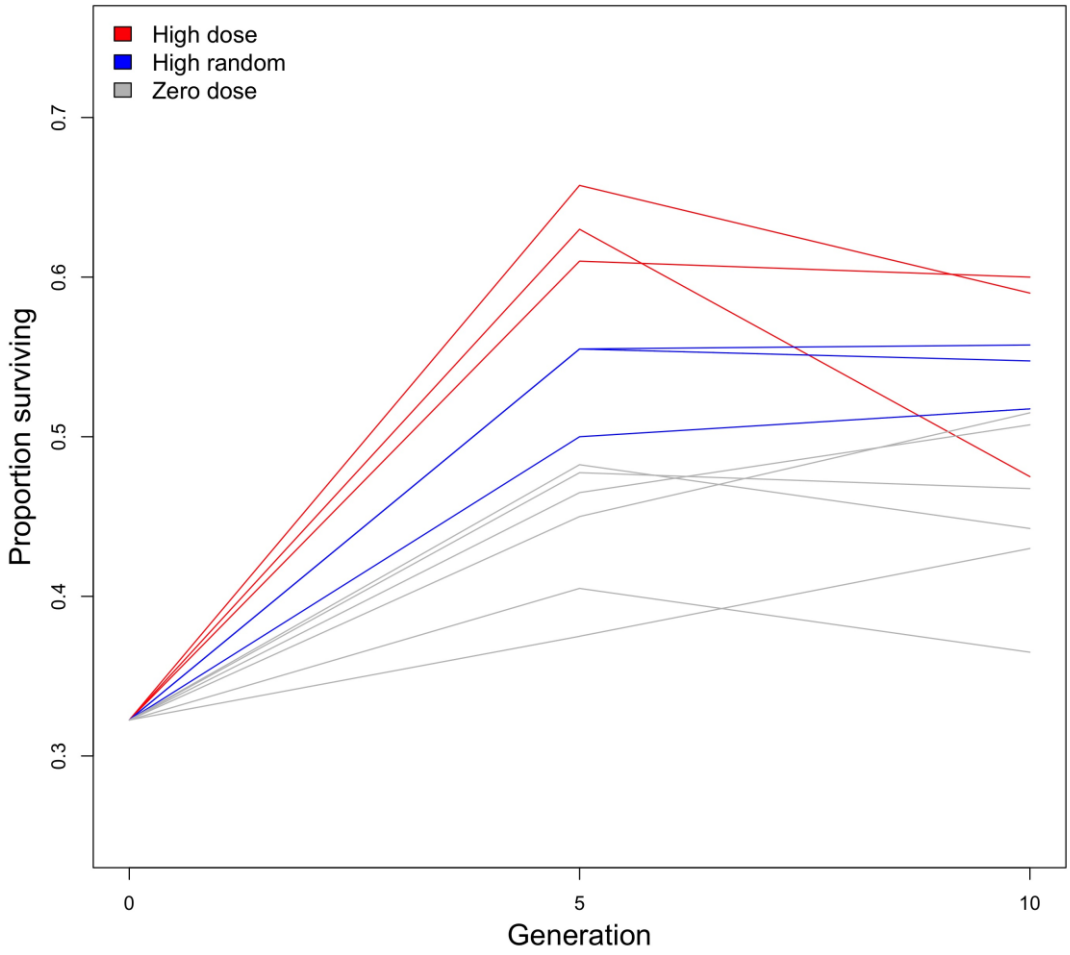


Fig B3 Seventy-five hour survival of High dose, High random and Zero dose lines when exposed to the high dose of Ivermectin used during selection. Red, blue and grey lines show survival of replicate populations for each treatment during selection.

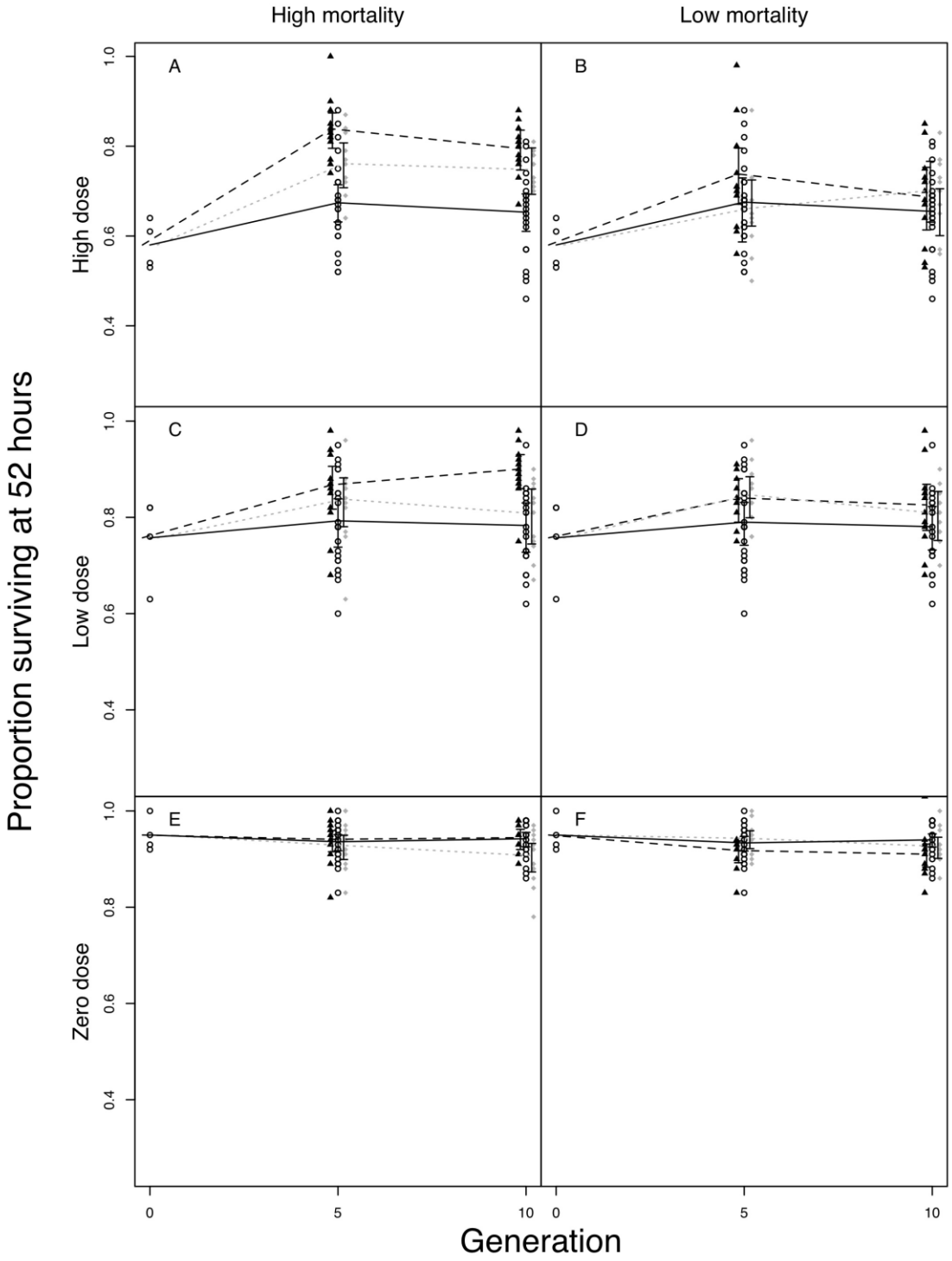


Fig B4 Fifty-two hour survival when exposed to the three drug doses used during selection (A and B = high; C and D = low: E and F = zero) of samples taken from generations 0, 5 and 10 during selection. Panels A, C and E show survivorship of high mortality lines: HD and HR. Panels B, D and F show survivorship of low mortality lines: LD and LR. Points are raw survival data from resistance bioassays, lines represent predictions of maximal models (generation + treatment + generation*treatment) for each treatment: circles, solid line = zero dose; triangles, dashed line = drug treatment; diamonds, dotted line = random mortality. Error bars; 95% confidence intervals for mean survival.

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